MOLECULAR PHARMACOLOGY, 10, 582–596

Binding of *dl*-[^{3}H]Epinephrine to Proteins of Rat Ventricular Muscle: Nonidentity with *Beta* Adrenergic Receptors

BARRY B. WOLFE, JOSEPH A. ZIRROLLI, AND PERRY B. MOLINOFF[1]

Department of Pharmacology, University of Colorado Medical Center, Denver, Colorado 80220

(Received April 22, 1974)

SUMMARY

WOLFE, BARRY B., ZIRROLLI, JOSEPH A., AND MOLINOFF, PERRY B.: Binding of *dl*-[^{3}H] epinephrine to proteins of rat ventricular muscle: nonidentity with beta adrenergic receptors. *Mol. Pharmacol.* **10,** 582–596 (1974).

The binding of *dl*-[^{3}H]epinephrine to proteins in a supernatant fraction obtained from rat ventricular muscle has been studied and compared with the properties which would be expected of *beta* adrenergic receptors studied *in vitro*. The binding to heart proteins took place over a time scale of hours and was correlated with the time course of destruction of epinephrine as determined by alumina chromatography. The binding of *dl*-[^{3}H]epinephrine was markedly temperature-dependent. The rate of binding was enhanced if tissues were stored at −22°, homogenates were stored at 4° or −22°, or homogenates were heated to 95°. Binding was not reversed by excess propranolol, nonradioactive epinephrine, or strong acid. Binding activity was distributed uniformly with the distribution of protein on differential centrifugation, while adenylate cyclase activity was associated with rapidly sedimenting membrane fragments. The stimulation of adenylate cyclase activity by *l*-epinephrine was prevented by *dl*-propranolol but was not affected by *d*-epinephrine or catechol. The binding reaction was blocked, on the other hand, by both the *d* and *l* stereoisomers of norepinephrine and epinephrine, by ascorbic acid, and by catechol. It was not affected by propranolol and was only slightly reduced by non-catechol inhibitors of catechol *O*-methyltransferase. Binding that appeared to be quantitatively similar to that seen with heart supernatant proteins was observed when bovine serum albumin was incubated with [^{3}H]epinephrine. The results suggest that the binding of catecholamines to proteins is a nonspecific interaction which is dependent upon the oxidative destruction of the amines. The binding does not appear to reflect interaction with *beta* adrenergic receptors or with any other specifiable constituent of cardiac tissue.

INTRODUCTION

Specific receptors for hormones, neurotransmitters, and drugs exist on the surface of many types of cells. The interaction of these receptors with appropriate agonists elicits specific cellular responses. In the case of the cholinergic neurotransmitter acetylcholine the interaction leads to changes in the membrane conductance to one or more ions. In the case of *beta* adrenergic receptors the interaction with catecholamines induces changes in the activity of the enzyme adenylate cyclase (1–3).

Numerous attempts to study and isolate receptors for neurotransmitters and hor-

This work was supported by Grant-in-Aid 72-887 from the American Heart Association and by Grants NS 10206 and RR 05357 from the United States Public Health Service.

[1] Established Investigator of the American Heart Association.

Copyright © 1974 by Academic Press, Inc.
All rights of reproduction in any form reserved.

mones have been made, and the successful isolation of at least some receptors has recently been achieved (1). The greatest successes have been with the cholinergic receptor (1) and with those for the peptide hormones glucagon (4, 5) and insulin (6).

Attempts to isolate adrenergic receptors have been complicated by the lack of an irreversible ligand of the requisite specificity. Several reports have appeared, however, which describe interactions of catecholamines with putative *beta* receptors in cardiac tissue (7–9), turkey erythrocyte membranes (10–12), and hepatic cell membranes (13).

There are quantitative differences in the properties of catecholamine binding in the several systems thus far examined. The binding to cardiac tissue (7, 9) and to hepatic cell membranes (13) is extremely slow and is essentially irreversible. The binding to turkey erythrocyte membranes (10, 12) is more rapid and is reversible. It is still slow, however, in comparison with the rapidity of the adenylate cyclase response to injected catecholamines (14). In other respects the binding properties seen in the various systems are similar to one another. Binding is inhibited by catechols (7–13, 15, 16), is relatively insensitive to *beta* adrenergic blockers such as propranolol (7–10, 12, 13, 15, 16), and does not show stereospecificity (9, 10, 13, 15–18), which is seen with most *beta*-mediated responses (15–20). In many cases the binding capacity is considerably higher than appears reasonable. In a study utilizing turkey erythrocyte membranes the amount (picomoles) of [^{3}H]isoproterenol bound at saturation actually exceeded cAMP[2] formed during a 10-min incubation (10). This apparently negative amplification factor would imply either that the turnover of adenylate cyclase is unreasonably small or that the number of spare receptors is unreasonably large.

In this report the properties of binding of *dl*-[^{3}H]epinephrine to proteins derived from rat ventricular muscle have been compared with those predicted from studies of *beta* adrenergic receptors carried out with intact preparations. The results suggest that the binding observed has no relationship to *beta* adrenergic receptors but rather represents a nonspecific interaction between oxidized catecholamine derivatives and proteins present in the homogenates.

[2] Abbreviations used are: cAMP, adenosine cyclic 3′,5′-monophosphate; Tris-NaCl, 5 mM Tris and 152 mM NaCl, pH 7.5; phosphate-NaCl, 5 mM sodium phosphate and 152 mM NaCl, pH 7.5.

METHODS

Rat ventricular tissue. Male Sprague-Dawley rats, 110–150 g, were killed by cervical fracture. The hearts were removed and washed with cold 0.9% NaCl. The atria and large vessels were removed, and the ventricles were homogenized in either 5 volumes (for binding studies) or 10 volumes (for adenylate cyclase determinations) of Tris or phosphate-NaCl[2], using a Polytron PT-10-ST apparatus (Brinkmann) at a setting of 6 for 15–20 sec. A homogenate to be used for binding assays was routinely centrifuged at 10,000 × *g* for 10 min. The resulting supernatant fraction was diluted 10-fold with Tris-NaCl or phosphate-NaCl and was designated the heart supernatant.

Canine tissues. A 15-kg dog was killed with pentobarbital, and the heart, liver, and adrenal glands were quickly removed and placed on ice. The atria, large vessels, and adipose tissue were removed from the heart, and the ventricles were sliced and homogenized (Polytron, at setting 6 for 1 min) in 10 volumes of ice-cold Tris-NaCl. Homogenates of liver and of the adrenal cortex, separated from the medulla under a dissecting microscope, were prepared similarly and were used to measure binding and adenylate cyclase activity. A portion of each homogenate was centrifuged at 10,000 × *g* for 10 min, and the supernatant fractions were diluted 13-fold with Tris-NaCl for use in binding assays.

Binding assay. dl-[^{3}H]epinephrine (5–40 nM) was incubated with the heart supernatant fractions at 37°. At appropriate times aliquots (usually 1 ml) were withdrawn and filtered with suction through Millipore filters (HAWP 0025, 0.45 μ). After washing with 5 ml of 0.9% NaCl, the material remaining on the filters was dissolved in 5 ml of a dioxane-based scintillation fluor containing 60 g of naphthalene, 4 g of 2,5-diphenyloxazole, and 0.2 g of *p*-bis[2-(4-methyl-5-phenyloxazolyl)]benzene per liter

of 1,4-dioxane (Fisher). Radioactivity was determined in a Nuclear-Chicago scintillation spectrometer at an efficiency for ^3H of 25–35%. The data plotted in the figures are the means of duplicate determinations except where otherwise noted. Protein determinations were performed by the method of Lowry *et al.* (21), and the amount of ^3H binding is expressed as picomoles of ^3H bound per milligram of protein.

Catecholamine assay with alumina. Alumina was treated with HCl, washed thoroughly, and oven-dried (22). A sample of the incubation mixture (1 ml) containing *dl*-[^3H]epinephrine was mixed with 0.3 ml of 1.1 M $HClO_4$. Precipitated protein was removed by centrifugation at 12,000 × *g* for 2 min (Eppendorf 3200 centrifuge). The supernatant fluid was decanted into 10 ml of water containing 1 ml of 2% disodium EDTA and 60 mg of $NaHSO_3$. The solution was adjusted to pH 3, using NaOH, and 400 mg of alumina were added with stirring. The suspension was adjusted to pH 8.6, stirred for 5 min, and poured onto a column (6-mm inside diameter) containing an additional 400 mg of alumina which had been washed with 10 ml of 0.2 M sodium acetate, pH 8.6. The column was washed with 10 ml of 0.2 M sodium acetate, pH 8.6, and then with 10 ml of H_2O. Epinephrine was eluted with 4 ml of 1 N HCl. Recovery from this procedure was routinely 86–96%.

Adenylate cyclase assay. The method of Krishna *et al.* (23) was used. The standard reaction mixture included Tris-HCl, pH 7.5, 20 μmoles; theophylline, 5 μmoles; cAMP, 0.35 μmole; phosphoenolpyruvate, 5 μmoles; pyruvate kinase, 100 μg [including 20 μmoles of $(NH_4)_2SO_4$]; $MgSO_4$, 2.5 μmoles; [8-^3H]-ATP, 1.56 μCi, 0.2 μmole; and a tissue sample containing approximately 1 mg of protein. The final reaction volume of 500 μl was incubated at 37° for 10 min and was then placed in a boiling water bath for 5 min. The samples were centrifuged to remove heat-denatured proteins, and the supernatant fluid was quantitatively transferred to Dowex 50W-X8 H^+ columns (0.4 × 4.0 cm) prepared in Pasteur pipettes. Elution of cAMP from the columns was performed as described by Krishna *et al.* (23). Carrier ATP was added to the 3.5-ml cAMP fraction to give a final concentration of 0.62 mM. $MgSO_4$ (5%) and $Ba(OH)_2$ (0.3 N) (0.3 ml each) were then added to precipitate the remaining [^3H]ATP. After centrifugation a portion of the supernatant fraction was analyzed for radioactivity, and another was used to determine the absorbance at 260 nm, which made it possible to correct for losses of cAMP throughout the assay.

Materials. *dl*-[7-^3H]-Epinephrine (New England Nuclear; specific activity, 9–13 Ci/mmole) was purified by chromatography on alumina (22) and used within 6 weeks of purification. [8-^3H]ATP (tetralithium salt, Schwartz/Mann; specific activity, 21 Ci/mmole) was purified on Dowex 50W-X8 (200–400 mesh, H^+ form) (Bio-Rad) on the day of each adenylate cyclase assay. *l*-Epinephrine, *l*-norepinephrine, ATP, and pyruvate kinase (type II, crystalline) were obtained from Sigma Chemical Company; L-ascorbic acid, Tris buffer, and BSA, from J. T. Baker Chemical Company; cAMP, from Schwarz/Mann; theophylline, from Nutritional Biochemicals; and phosphoenolpyruvate tricyclohexylammonium salt, from Boehringer/Mannheim. The *d* stereoisomers of epinephrine and norepinephrine were gifts from Dr. F. C. Nachod, Sterling-Winthrop Research Institute. *dl*-Propranolol (Inderal) was kindly provided by Mr. Henry L. Lemien, Jr., of Ayerst Laboratories, and we are indebted to Dr. C. R. Creveling for the gift of two non-catechol inhibitors of catechol *O*-methyltransferase.

RESULTS

Binding assay. After incubating a radioactive ligand with a tissue preparation it is useful to attempt to decrease the degree of contamination due to occluded radioactivity. Since the rate of dissociation of epinephrine from the *beta* adrenergic receptor is not known (16, 17), it was considered possible that washing the filter would remove amine from the receptor. However, the net amount of epinephrine bound (binding in the presence of tissue minus a blank without tissue) was unchanged by washing with up to 20 ml of 0.9% NaCl. Even in the absence of tissue, however, concentration-dependent binding of [^3H]epinephrine to the filters was observed. Some of this radioactivity could

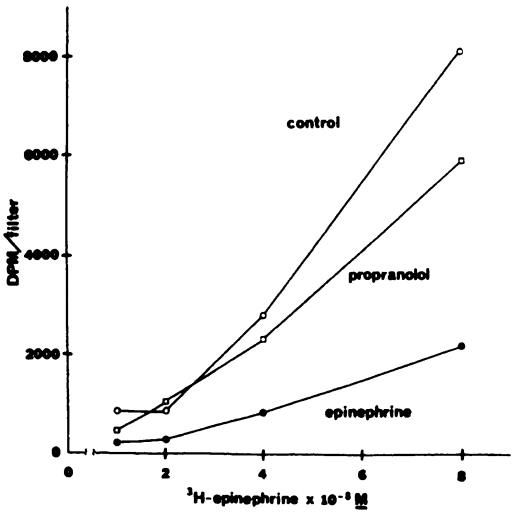

FIG. 1. *Relationship between concentration of [³H]epinephrine and amount of ³H retained on filter*

dl-[³H]Epinephrine was incubated in Tris-NaCl (○), or in Tris-NaCl with 0.2 mM *dl*-propranolol (□) or 0.2 mM *l*-epinephrine (●). Incubations were conducted for 2 min at 37°, and 1-ml aliquots were then filtered and washed with 10 ml of 0.9% NaCl. Results are expressed as disintegrations per minute per filter.

be displaced by propranolol (0.2 mM), and most could be displaced by 0.2 mM epinephrine (Fig. 1). The existence of epinephrine-displaceable binding sites on the Millipore filters implies that duplicate assays containing an excess of nonradioactive epinephrine do not provide an acceptable blank (10). On the other hand, buffer (no tissue) blanks were not useful, since oxidative destruction of the epinephrine occurred during the prolonged incubations employed. The rate of this oxidation was markedly affected by the presence of tissue.

Properties of binding. The binding of epinephrine was proportional to the concentration of protein between 0.2 and 1.5 mg/ml and was markedly dependent on temperature (Fig. 2). In most experiments binding to fresh tissue was relatively slow for as long as 2 hr at 37°. Higher rates of binding were observed over longer periods. The delay in binding was either absent or much shorter at higher temperatures or if the assays were carried out on pooled heart supernatant fractions which had been aged by storage at −22° (Fig. 2). When individual heart supernatant fractions were stored at 4° there was a progressive increase in the amount of binding observed (Fig. 3). When binding assays were carried out using supernatant fractions stored at −22°, there was a marked increase in the amount of binding after only 1 day of storage (Fig. 3). There were no further increases if the frozen supernatant fractions had been stored as long as 9 days. The increases seen with both the refrigerated and frozen supernatant fractions reflected primarily an increase in the rate of binding. Thus, for example, the binding observed with the 2-day refrigerated supernatant fractions increased markedly between 3 and 5 hr, but that seen with the frozen supernatant fractions was complete in less than 3 hr.

The increase in binding observed when heart supernatant fractions were stored may have reflected denaturation of proteins. An attempt to accelerate the rate of protein denaturation was made by heating a heart supernatant fraction in a water bath at 95°. At various times aliquots were removed and their ability to bind [³H]epinephrine was determined. After 5 min at 95° there was a 6-fold increase in the binding seen on incubation for 1 hr (Fig. 3, inset).

Binding and adenylate cyclase. Since the effects of *beta* adrenergic agonists appear to be mediated through activation of adenylate cyclase (2, 3), it is likely that the receptor and the enzyme will be found on the same membrane fragments. Sequential centrifugation of a fresh homogenate of rat ventricular tissue was carried out at 1000, 10,000, and 48,000 × *g* for 10 min in each case. Portions of the initial homogenates and of each of the supernatants were saved and used for the determination of protein, the ability to bind [³H]epinephrine, and the assay of adenylate cyclase activity (Table 1A). There was a progressive but parallel decrease in protein content (milligrams per milliliter) and binding (picomoles per milliliter). Thus the specific activity of binding (picomoles per milligram) remained relatively constant. On the other hand, adenylate cyclase activity and specific activity decreased with increasing force of centrifugation more rapidly than did protein concentration or the ability to

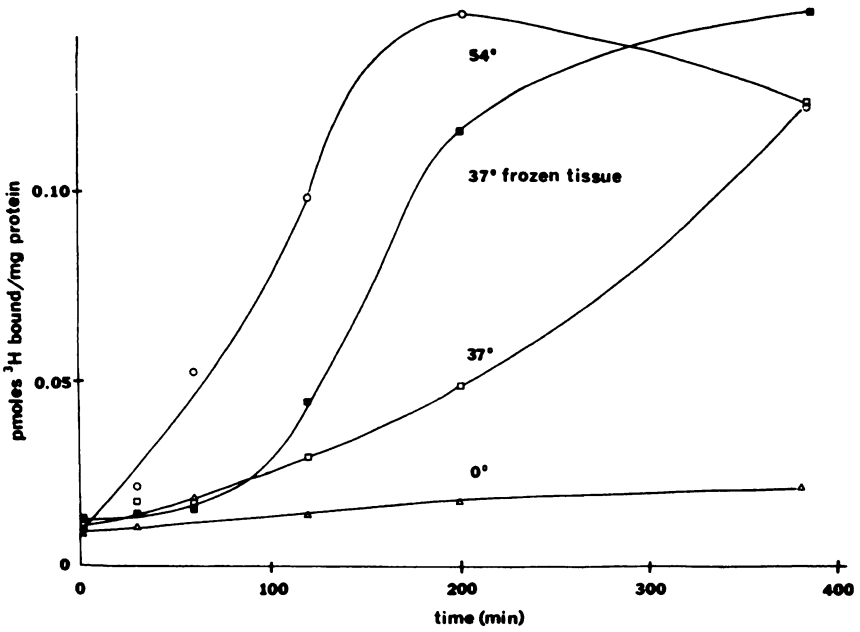

FIG. 2. *Time courses of binding of [³H]epinephrine at various temperatures*

dl-[³H]Epinephrine (17 nM) was incubated at 0° (△), 37° (□), or 54° (○) in Tris-NaCl with fresh heart supernatant fraction (from six rats, 1.53 mg of protein per milliliter). The same concentration of epinephrine was incubated at 37° (■) in Tris-NaCl with a homogenate of tissue stored for 7 days at −22° (from four rats, 2.14 mg of protein per milliliter). Binding data for tissue (picomoles per filter) has been divided by the milligrams of protein in the aliquots to give picomoles per milligram of protein. In the binding assays, the values for the blanks (no tissue) must also be divided by 1.53 and 2.14 to make it possible to compare them directly with the values obtained with tissue. The buffer blanks thus calculated were 0.009 and 0.006 pmole of ³H, respectively, for the two tissue preparations used for this figure.

bind [³H]epinephrine. The adenylate cyclase results were the same with regard to basal, epinephrine-stimulated, and fluoride-stimulated activity, and were also the same whether hearts were homogenized in hypotonic Tris (Table 1A) or in isotonic sucrose (Table 1B).

Reversibility. The binding of catecholamines to *beta* adrenergic receptors apparently is rapidly reversible (24–26). When a high concentration of nonradioactive epinephrine was added to an incubation mixture, the time-dependent increase in ³H binding did not occur (Fig 4). Epinephrine added after a binding reaction had been initiated (Fig. 5) caused a 60% decrease in the binding observed at 6 min, but only a 25% decrease by 62 min. This could imply that specific binding occurred quickly while binding after the first few minutes was not related to *beta* receptors. However, high concentrations of epinephrine decreased the buffer blank (Fig. 1) by an amount sufficient to explain the constant absolute decrease in binding observed with epinephrine. The binding of [³H]epinephrine was neither reversed (Fig. 5) nor prevented (Fig. 4) by the addition of the *beta* antagonist propranolol. When the Millipore filters were washed with 5 ml of 0.4 N perchloric acid there was a decrease of less than 20% in the amount of [³H]epinephrine retained on the filters. In other experiments aliquots of an incubation mixture were filtered and the filters were soaked in 0.4 N perchloric acid for 2 hr before the radioactivity remaining on the filters was determined (Fig. 6). A relatively small percentage of the radioactivity was removed from the filters by the acid, and less than half of that removed could be adsorbed to alumina. The small amount of ³H which could be adsorbed to alumina probably reflected reversible binding of [³H]epinephrine to Millipore filters, since all the radioactivity

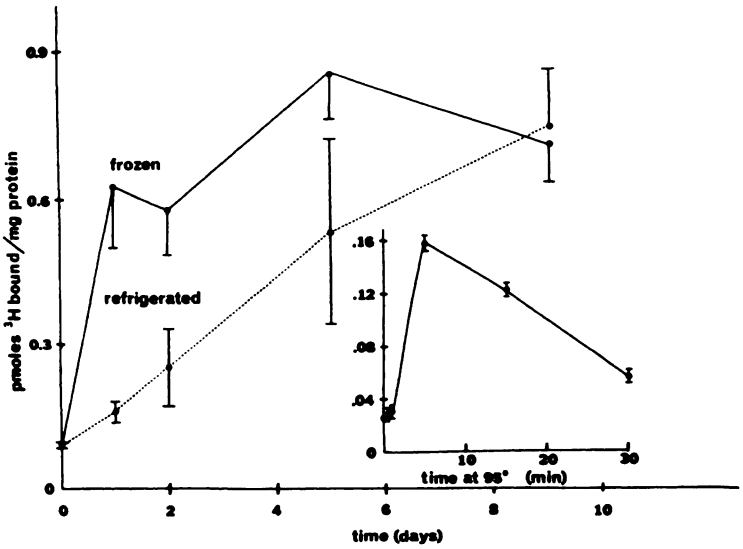

FIG. 3. *Effect of storage of tissue on binding of dl-[³H]epinephrine*

Tissue homogenates were prepared as described in METHODS and stored at either −22° (frozen) or 4° (refrigerated) for up to 9 days. At the indicated times binding studies were performed by incubating the heart supernatant fractions (0.6 mg of protein per milliliter) with *dl*-[³H]epinephrine (15 nM) at 37° for 3 hr. Results show the means ± standard errors of determinations carried out on four heart supernatant fractions. The buffer blanks, corrected as in Fig. 2, were approximately 0.07 pmole of ³H.

Inset: Binding of [³H]epinephrine to heat-denatured protein. A heart supernatant fraction was prepared as described in METHODS, and aliquots (3.5 ml, 0.7 mg of protein per milliliter) were exposed to a temperature of 95° in a boiling water bath in polypropylene tubes from 30 sec to 30 min. Binding assays were then carried out by incubation of the aliquots with *dl*-[³H]epinephrine (5 nM) in Tris-NaCl for 1 hr. Results are the means ± standard errors of assays performed in triplicate. The buffer blanks, corrected as in Fig. 2, were approximately 0.01 pmole of ³H.

bound after filtration of buffer blanks (Fig. 1) was removed by washing the filters with 5 ml of 0.4 N perchloric acid. These results meant that we could not verify the claim (9) that the ³H which bound to the tissue proteins represented unchanged catecholamine.

The phenomena observed with supernatant fractions of rat ventricular muscle were also evident with supernatants of dog heart, liver, and adrenal cortex. Binding was minimal or nonexistent in fresh supernatants but was present in all three cases after the fractions had been stored for 5 days (Fig. 7). The adrenal cortex was studied since it contains adenylate cyclase sensitive to ACTH but not to epinephrine (2). The lack of epinephrine-sensitive adenylate cyclase in adrenal cortex was confirmed (Fig. 8), as was the presence of epinephrine-sensitive enzyme in the dog heart and liver homogenates. Identical results were obtained when binding studies were carried out with the homogenates (not shown) and with the 10,000 × *g* supernatant fractions (Fig. 7).

Stereospecificity and pharmacology. The *d* and *l* stereoisomers of catecholamines have markedly different potencies in eliciting responses in most systems examined (Fig. 9A) (15, 16, 18–20). There was no difference, however, in the abilities of *d*- and *l*-norepinephrine or epinephrine to block the binding of [³H]epinephrine (Fig. 10A) (see also refs. 9, 10, 13, 15, 16, 18). Catechol was only slightly less potent than epinephrine in preventing the binding of [³H]epinephrine, while propranolol was essentially without effect (Fig. 10A). The effects of catechol and *d*-epinephrine were of particular interest, since these compounds had little or no effect on adenylate cyclase activity (Fig. 9A) or on the activation of adenylate cyclase by *l*-epinephrine (Fig. 9B).

Mechanism of catecholamine binding. The

TABLE 1

Effect of differential centrifugation on adenylate cyclase activity and binding of [^{3}H]epinephrine

A. Hearts from five animals were homogenized in 10 volumes of Tris-NaCl. A portion of each homogenate was saved ("total"), and the rest of each homogenate was subjected to sequential centrifugation at 1000, 10,000, and 48,000 × *g* for 10 min at each force. Aliquots of each supernatant fraction were saved and used for the determination of protein, binding activity (diluted so that 1 ml of each supernatant and of the total homogenates contained 0.4–0.8 mg of protein), and adenylate cyclase activity. Basal, epinephrine-sensitive (60 μM), and F^--sensitive (4 mM) adenylate cyclase activities were determined. Binding was carried out with *dl*-[^{3}H]epinephrine (17 nM) at 37° for 2 hr. Results are expressed (means ± standard errors) as milligrams per milliliter for protein, as picomoles of ^{3}H bound per milliliter and per milligram of protein for epinephrine binding, and as picomoles of cAMP formed per milliliter per minute and per milligram of protein per minute for adenylate cyclase activity. In order to compare the relative amounts of protein, binding activity, and adenylate cyclase activity remaining in each supernatant fraction, the percentage of the value obtained with the total homogenate is indicated in parentheses.

B. Hearts from five animals were homogenized in 10 volumes of 0.25 M sucrose–5 mM Tris at pH 7.5. Centrifugations, determination of protein, and assay of adenylate cyclase activity were performed as in A.

A. Tris centrifugation

Fraction	Protein	Binding		Adenylate cyclase activity					
				Basal	Epinephrine	F^-	Basal	Epinephrine	F^-
	mg/ml (%)	*pmoles/ml (%)*	*pmole/mg*	*pmoles/ml/min (%)*			*pmoles/mg protein/min*		
Total	15.8 ± 1.3 (100)	7.39 ± 0.85 (100)	0.468 ± 0.052	72.8 ± 8.9 (100)	149 ± 5.0 (100)	488 ± 29 (100)	4.61 ± 0.56	9.42 ± 0.32	30.9 ± 1.8
1,000 × *g*	6.6 ± 0.1 (42)	3.71 ± 0.13 (50)	0.562 ± 0.021	20.8 ± 3.6 (29)	45.7 ± 5.9 (31)	133 ± 9 (27)	3.15 ± 0.55	6.93 ± 0.89	20.2 ± 1.4
10,000 × *g*	5.0 ± 0.3 (32)	1.66 ± 0.12 (22)	0.331 ± 0.024	0.6 ± 1.2 (0.8)	8.2 ± 1.6 (5)	49.9 ± 5.3 (10)	0.11 ± 0.22	1.63 ± 0.32	9.97 ± 1.06
48,000 × *g*	4.0 ± 0.3 (25)	1.24 ± 0.10 (17)	0.310 ± 0.024	0	8.1 ± 4.9 (5)	11.6 ± 5.0 (2.4)	0	2.02 ± 1.22	2.90 ± 1.25

B. Sucrose centrifugation

Fraction	Protein	Adenylate cyclase activity					
		Basal	Epinephrine	F^-	Basal	EPI	F^-
	mg/ml (%)	*pmoles/ml/min (%)*			*pmoles/mg protein/min*		
Total	11.9 ± 0.4 (100)	113 ± 5 (100)	294 ± 13 (100)	1060 ± 43 (100)	9.52 ± 0.40	24.7 ± 1.1	89.1 ± 3.7
1,000 × *g*	3.25 ± 0.30 (27)	18.1 ± 2.4 (16)	36 ± 3.1 (12)	125 ± 14 (12)	5.56 ± 0.74	11.1 ± 0.9	38.4 ± 4.3
10,000 × *g*	2.88 ± 0.15 (24)	1.2 ± 1.8 (1)	8.7 ± 3.7 (3)	60.8 ± 10.5 (6)	0.41 ± 0.62	3.03 ± 1.30	21.1 ± 3.6
48,000 × *g*	3.11 ± 0.16 (26)	0	0	0	0	0	0

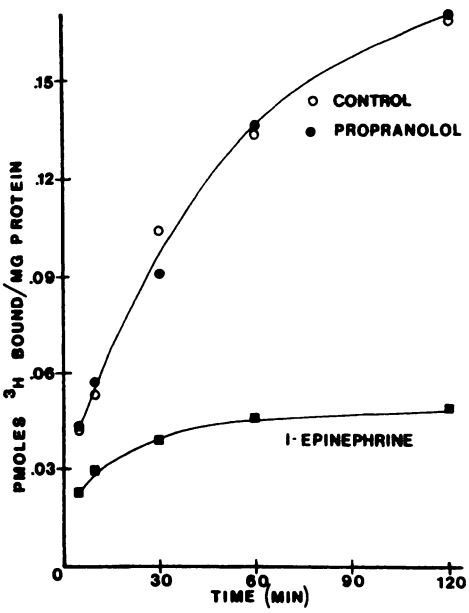

FIG. 4. *Blockade of binding of [³H]epinephrine by propranolol or l-epinephrine*

Heart supernatant fractions (0.7 mg of protein per milliliter) were incubated with *dl*-[³H]epinephrine (5 nM) in Tris-NaCl. The time course of binding was determined in controls (○) and in the presence of 0.1 mM propranolol (●) or 0.1 mM *l*-epinephrine (■). The buffer blanks, corrected as in Fig. 2, were approximately 0.02 pmole of ³H for the controls and in the presence of propranolol, and 0.007 pmole of ³H in the presence of 0.1 mM *l*-epinephrine The results are expressed as the means of triplicate determinations.

ability of catechols (Fig. 10A) (see also refs. 15 and 16) to inhibit binding led to the idea that membrane-bound catechol *O*-methyltransferase may be involved in the binding phenomenon (15).[3] To examine this possibility binding studies were carried out in the presence of two inhibitors of catechol *O*-methyltransferase. Both 3,5-dimethoxy-4-hydroxybenzoic acid and 3,4-dimethoxy-5-hydroxybenzoic acid are noncompetitive inhibitors of this enzyme which appear to act at the site for substrate binding (27).[3] These compounds, as well as the monoamine oxidase inhibitor pargyline, had only a slight effect on the binding of [³H]epinephrine to the heart supernatant fraction (Fig. 10B).

In view of the presence of binding in subcellular fractions which are virtually devoid of hormone-sensitive adenylate cyclase, and the relatively constant specific activity of binding after differential centrifugation (picomoles per milligram of protein, Table 1A), the binding of [³H]epinephrine to BSA was examined. When albumin (0.6 mg/ml) was incubated with [³H]epinephrine (40 nM) a time-dependent increase in binding was observed. Both the time course and amount of binding were comparable to those seen with rat heart supernatant fractions under similar conditions. Furthermore, the binding of [³H]epinephrine to BSA was inhibited by catechol at concentrations similar to those which inhibited binding to heart supernatant fractions (Fig. 11).

Chromatography on alumina columns (22) was used to quantitate destruction of epinephrine during the prolonged incubations employed in these studies. The increase in binding at 37° to a fresh heart supernatant fraction was usually relatively slow for 60–120 min (Figs. 2, 12A and D). The time at which there was a rapid increase in the rate of binding correlated with the time at which relatively rapid destruction of the amine occurred, as measured by alumina column chromatography (Fig. 12A and D). Since the oxidative destruction of catecholamines is a pH-dependent process, a series of assays were carried out to compare the effects of pH on binding and on the stability of epinephrine. The rate of binding was found to correlate with the pH. Both binding and lability of epinephrine increased as the pH was raised from 6.9 to 7.8. In other experiments heart supernatant fractions were first incubated for 90 min at 4°, 20°, or 37°. These preliminary incubations resulted in a progressive increase in the initial rate of binding and in a seemingly identical increase in the rate of destruction of [³H]epinephrine (Fig. 12A–C). Both the tissue-mediated destruction of [³H]epinephrine and its binding to the heart supernatant fraction were inhibited by catechol (Fig. 12D).

DISCUSSION

The first goal and major problem in an attempt to isolate an adrenergic receptor is finding some means of recognizing the re-

[3] C. R. Creveling, personal communication.

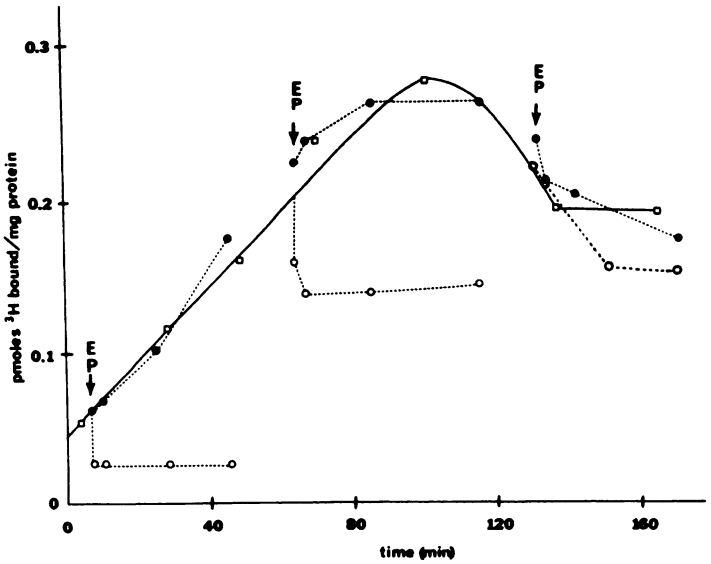

FIG. 5. *Reversibility of binding of [³H]epinephrine with excess nonradioactive epinephrine and propranolol*

A time course of binding of [³H]epinephrine (8 nM) was determined using a heart supernatant fraction (hearts were stored for 12 days at −22°; 0.71 mg of protein per milliliter). After 6 min of incubation epinephrine (E, ○) or propranolol (P, ●) was added to aliquots of the incubation mixture to a final concentration in each case of 0.1 mM. The binding was followed for the next 40 min. The additions of epinephrine and of propranolol to further aliquots of the incubation mixture were repeated after 62 min and 128 min of incubation. The buffer blanks, corrected as in Fig. 2, were approximately 0.05 pmole of ³H for the control, 0.02 pmole of ³H in the presence of 0.1 mM epinephrine, and 0.03 pmoles of ³H in the presence of 0.1 mM propranolol.

ceptor after the tissue has been disrupted (see ref. 17). In addition to the nonspecific binding sites which may complicate an assay *in vitro* there are a wide variety of specifiable but non-receptor binding sites which are certainly present in any adrenergically innervated organ (24).

An early question faced in the design of these studies concerned the selection of a blank. The following possibilities were considered and rejected: (a) buffer (no tissue) blanks, (b) boiled tissue blanks, (c) excess nonradioactive epinephrine blanks, and (d) zero-time blanks. (a) and (c) were discarded because of the existence of epinephrine binding sites on the Millipore filters (Fig. 1). Boiled tissue blanks were unsuitable, since heat denaturation resulted in an increased capacity to bind [³H]epinephrine (Fig. 3, inset). The lability of epinephrine—a function of time, pH, and the presence of tissue—meant that significant changes in amount of this amine would occur during the course of an incubation. Thus neither buffer blanks nor zero-time blanks were entirely satisfactory. In view of the difficulties inherent in any of the usual types of blank, we elected to present the absolute amounts of [³H]epinephrine retained by the filters (per milligram of protein). The values for appropriate buffer blanks are presented in the figure legends, and zero-time blanks can often be deduced from the figures themselves.

Consideration of the responses seen with intact preparations makes it possible to list a set of criteria which should be satisfied if the binding is to physiologically functional *beta* receptors. Thus the binding of an agonist to a *beta* receptor should be extremely rapid. It has been shown that adenylate cyclase activity in rat heart increases to maximal levels within 1 sec following an injection of epinephrine (14). The time course of binding (Fig. 2) (7, 9, 10, 15, 16) is far slower than the responses *in vivo*. It is possible to explain this discrepancy by assuming a large

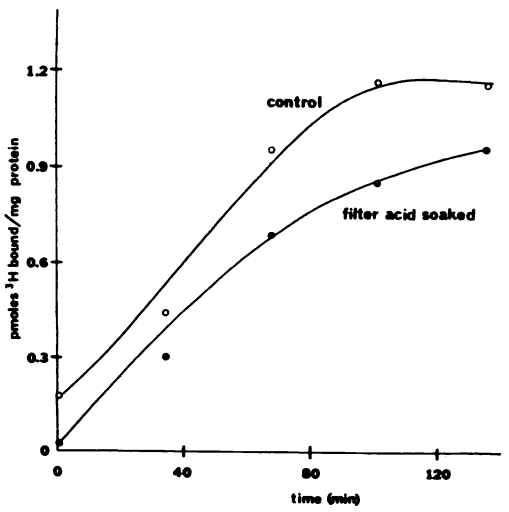

FIG. 6. *Reversibility of binding of [³H]epinephrine with perchloric acid*

A heart supernatant fraction (0.85 mg of protein per milliliter) was incubated with *dl*-[³H]epinephrine (30 nM). The hearts had been stored for 16 days at −22°. Radioactivity was determined with some of the filters (○), while others were soaked in 6 ml of 0.4 M $HClO_4$ for 2 hr and washed with 10 ml of 0.9% NaCl before the amount of ³H was determined (●). The buffer blanks, corrected as in Fig. 2, were approximately 0.15 pmole of ³H for non-acid-soaked filters and 0.01 pmole of ³H for acid-soaked filters.

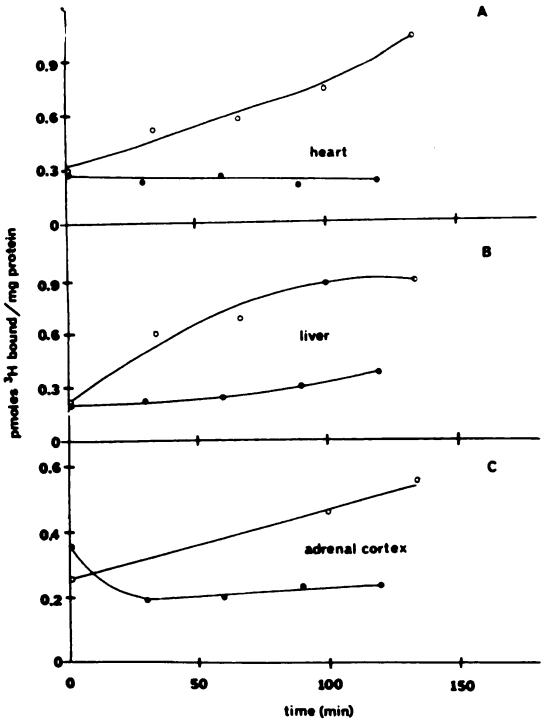

FIG. 7. *Time course of binding of [³H]epinephrine to supernatant fractions of dog heart, liver, and adrenal cortex*

A. A heart supernatant fraction (0.4 mg of protein per milliliter) was incubated with *dl*-[³H]-epinephrine (30 nM) in Tris-NaCl at 37°. The time course of binding was determined for the fresh supernatant (●) and for the same fraction after storage at 4° for 5 days (○). The buffer blank, corrected as in Fig. 2, was 0.25 pmole of ³H.

B. The same experiments were performed as in A, using a liver supernatant fraction (0.9 mg of protein per milliliter). The buffer blank, corrected as in Fig. 2, was approximately 0.12 pmole of ³H.

C. The adrenal cortex was separated from the medulla under a dissecting microscope. The same experiments were performed as in A, using an adrenal cortex supernatant fraction (0.54 mg of protein per milliliter). The buffer blank, corrected as in Fig. 2, was approximately 0.2 pmole of ³H.

number of spare receptors. A modest number of spare receptors is possible and perhaps likely, but the vast excess which would be required to explain the discrepancy between work *in vivo* and binding rates is rather unlikely.

Binding would be predicted to be completely and rapidly reversible. For example, the epinephrine-stimulated increase in adenylate cyclase activity in fat cells (25) and the *beta* receptor-mediated increase in pineal *N*-acetylserotonin transferase (26) are both rapidly reversed by *beta* antagonists. In our experiments no reversal of binding was seen with high concentrations of propranolol, and only relatively slight reversibility could be induced with nonradioactive epinephrine (Fig. 5) or with perchloric acid (Fig. 6) (9, 13, 16). The effects of epinephrine and perchloric acid were probably due to displacement from binding sites on the filters.

Binding would be predicted to be stereospecific. *l*-Norepinephrine is 10–100 times more potent than *d*-norepinephrine when tested on most preparations (15, 18–20). Even the relatively low degree of potency of *d*-epinephrine may of course be due to contamination with small amounts of the *l* isomer. The stereospecific nature of the ability of catecholamines to activate adenyl-

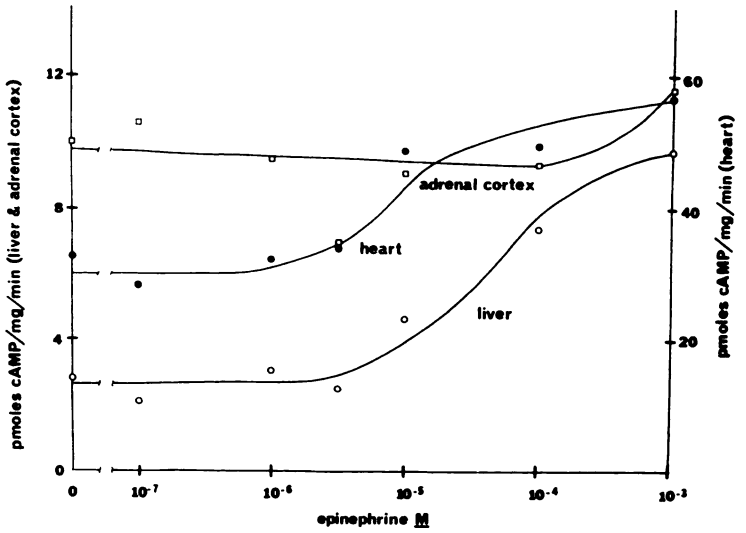

FIG. 8. *Epinephrine sensitivity of adenylate cyclase in dog heart, liver, and adrenal cortex homogenates*

The homogenates were the same as those used for experiments in Fig. 7, except that they had been filtered through gauze and had not been subjected to centrifugation. Adenylate cyclase activity was determined in the absence of epinephrine and in the presence of epinephrine at concentrations ranging from 10^{-7} to 10^{-3} M. Each determination was made in duplicate. The results are expressed as picomoles of cAMP formed per minute per milligram of protein.

ate cyclase is not reflected in the binding of amines to the various membrane preparations studied (Fig. 10A) (9, 10, 15, 16, 18).

It has been suggested that *beta* adrenergic receptor function involves the interaction of catecholamines with two types of sites. One of these sites is reputed to be specific for and to recognize the catechol nucleus, while the second recognizes the ethanolamine (stereospecific) portion of the molecule (10, 13). This hypothesis would predict that *d* stereoisomers, which are able to bind but not activate adenylate cyclase, should inhibit the ability of the *l* isomers to activate the enzyme. This inhibition has not been observed (Fig. 9B) (15, 18, 19).

The pharmacological properties of binding should reflect the ability of *beta* ligands to activate or inhibit the activation of adenylate cyclase. The properties of binding observed in this and other studies differ in several ways from those which would be predicted: (a) propranolol blocked the ability of *l*-epinephrine to activate adenylate cyclase but had little effect on binding; (b) catechol was an excellent inhibitor of binding, but it neither activated adenylate cyclase nor blocked the ability of *l*-epinephrine to activate the enzyme; (c) *d*-epinephrine blocked binding but had no effect on adenylate cyclase.

It has recently been suggested (15) that the binding of [^{3}H]norepinephrine is to a membrane protein possibly related to the enzyme catechol *O*-methyltransferase. It seems unlikely, however, that any significant percentage of the binding occurs to particulate catechol *O*-methyltransferase or to any other of the specifiable constituents of any adrenergically innervated organ (17). Pyrogallol and quercetin, the catechol *O*-methyltransferase inhibitors which showed the most marked effects on binding (15), are themselves catechols, and all compounds of this class have been shown to inhibit the binding of catecholamines (Fig. 10A) (15, 16). Reducing agents, such as ascorbate, are potent inhibitors of binding (Fig. 10B) (16) but would not be expected to affect the enzyme. Two non-catechol inhibitors of catechol *O*-methyltransferase had only a slight effect on the binding of [^{3}H]epinephrine (Fig. 10B).

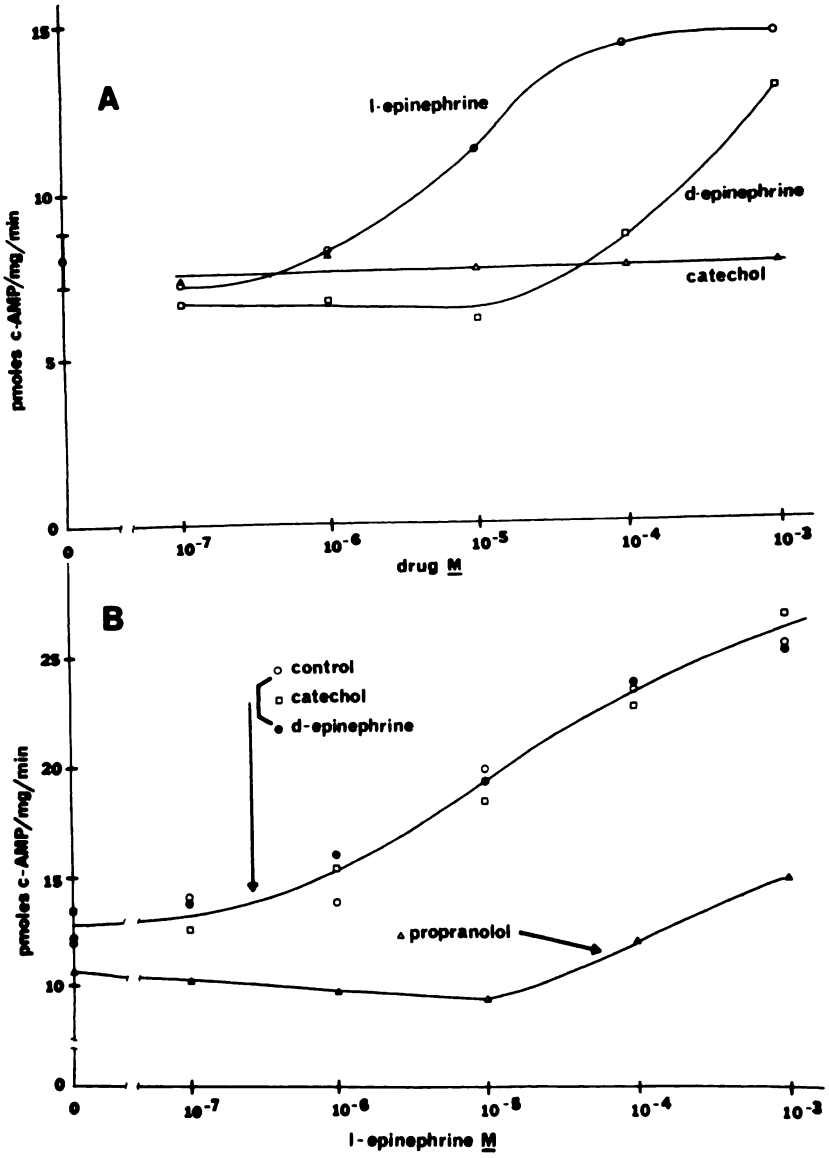

FIG. 9

A. *Activation of adenylate cyclase by l-epinephrine, d-epinephrine, and catechol*

Adenylate cyclase activity was determined on the same fresh homogenates used for the experiment in Fig. 10A. Assays were carried out in the absence of added drug (●) (mean ± standard error of four determinations) and in the presence of *l*-epinephrine (○), *d*-epinephrine (□), or catechol (△) at concentrations ranging from 10^{-7} to 10^{-3} M. The results are expressed as in Fig. 8.

B. *Ability of catechol, d-epinephrine, and propranolol to modify the stimulation of adenylate cyclase by l-epinephrine*

Adenylate cyclase activity was determined on a fresh homogenate from rat ventricular tissue in the absence of epinephrine and in the presence of *l*-epinephrine at concentrations ranging from 10^{-7} to 10^{-3} M. The following sets of assays were performed: ○, control; □, plus catechol (10^{-5} M); ●, plus *d*-epinephrine (10^{-4} M); △, plus propranolol (10^{-5} M). The results are expressed as in Fig. 8.

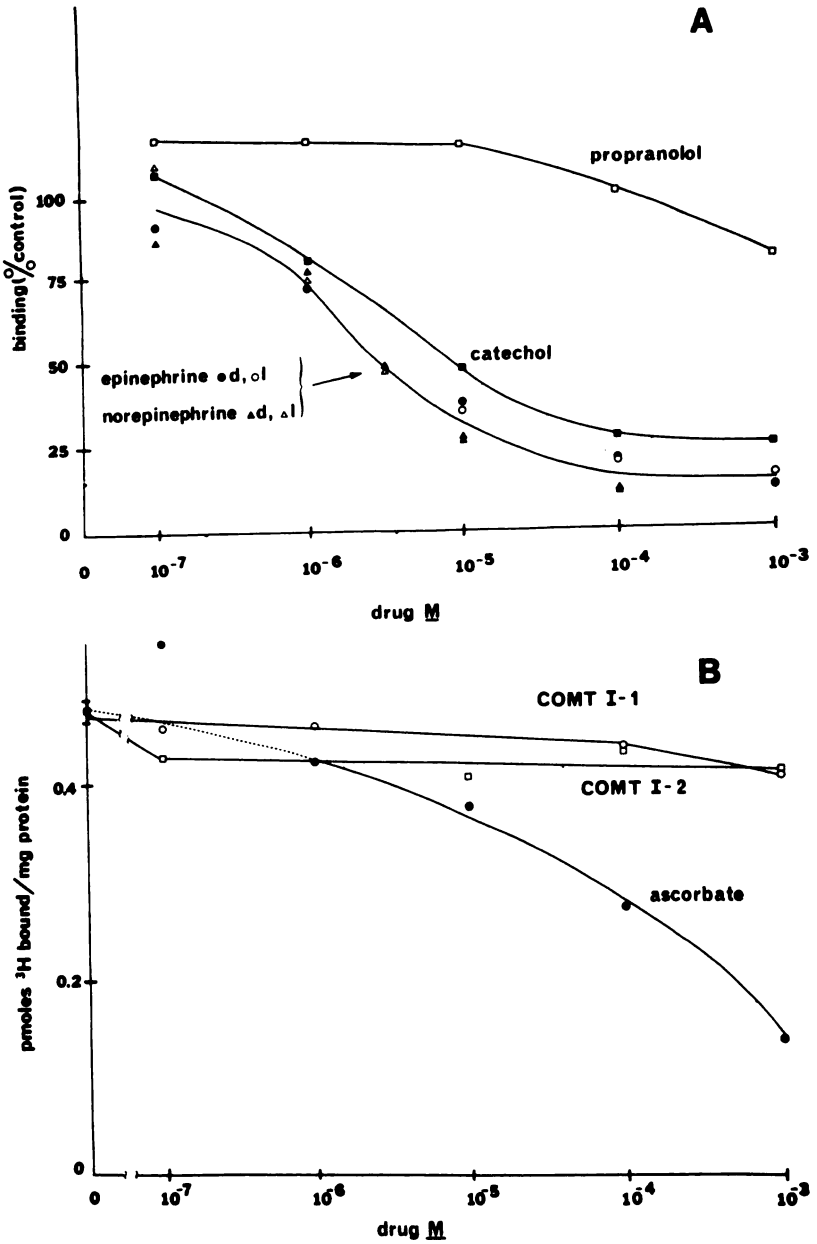

FIG. 10

A. *Inhibition of* [³H]*epinephrine binding by catechol, propranolol, norepinephrine, and epinephrine*

dl-[³H]Epinephrine (10 nM) was incubated with heart homogenate (fresh, homogenized in 300 volumes of Tris-NaCl and filtered through gauze, 0.22 mg/ml) at 37° for 80 min in the presence of catechol (■), propranolol (□), *d*-epinephrine (●), or *l*-epinephrine (○) at the indicated concentrations. In another experiment *dl*-[³H]epinephrine (10 nM) was incubated with a heart supernatant fraction (stored for 7 days at −22°, 0.51 mg/ml) in Tris-NaCl at 37° for 1 hr in the presence of *d*-norepinephrine (▲) or *l*-norepinephrine (△) at the indicated concentrations. Buffer blanks, corrected as in Fig. 2, were approximately 0.075 pmole of ³H in the absence of added drug, 0.035 pmole of ³H with 10^{-3} M catechol, 0.05 pmole of ³H with 10^{-3} M propranolol, 0.02 pmole of ³H with 10^{-3} M *l*-epinephrine, and 0.044 pmole of ³H with 10^{-4} M *d*- or *l*-norepinephrine. Binding in the absence of drug was 0.373 pmole of ³H per milligram of protein in the experiment with norepinephrine, NE and 0.577 pmole of ³H in the experiment with propranolol, catechol, and epinephrine.

B. *Inhibition of* [³H]*epinephrine binding by catechol O-methyltransferase (COMT) inhibitors and ascorbic acid*

dl-[³H]Epinephrine (16 nM) was incubated for 80 min with heart supernatant fraction (0.7 mg/ml, tissue stored for 10 days at −22°) at 37° in Tris-NaCl. The incubations were performed in the presence and absence of the indicated concentrations of sodium ascorbate (●) and catechol *O*-methyltransferase inhibitors [COMT I-1, 3,4-dimethoxy-5-hydroxybenzoic acid (○); COMT I-2, 3,5-dimethoxy-4-hydroxybenzoic acid (□)]. The buffer blank, corrected as in Fig. 2, was 0.15 pmole of ³H.

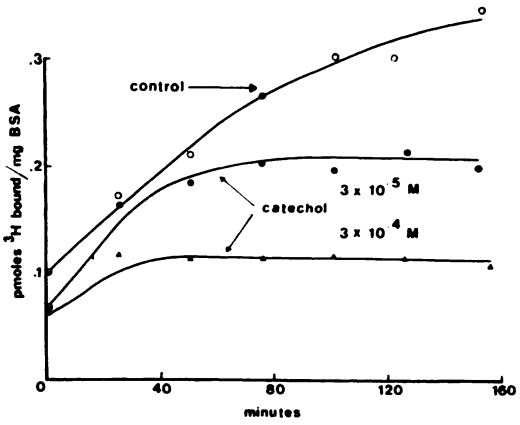

FIG. 11. *Time course of binding of [³H]epinephrine to BSA and its inhibition by catechol*

dl-[³H]Epinephrine (10 nM) was incubated with BSA (0.6 mg/ml) in phosphate-NaCl in the absence (○) and presence (●, 30 μM; ▲, 0.3 mM) of catechol. The buffer blanks, corrected as in Fig. 2, were approximately 0.07 pmole of ³H.

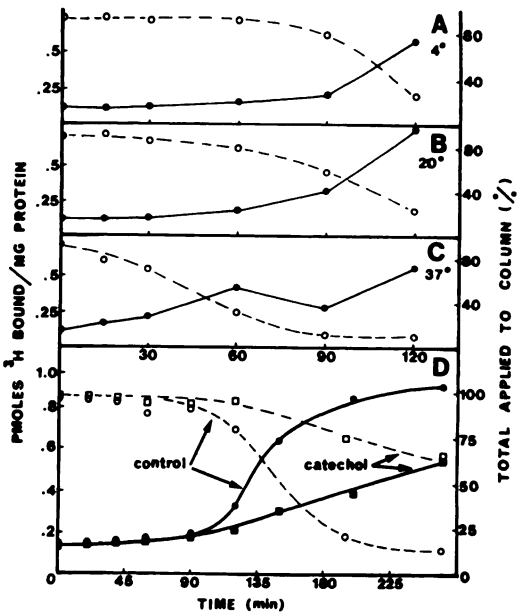

FIG. 12. *Effects of various preliminary treatments and catechol on binding and stability of epinephrine*

A. *dl*[³H]Epinephrine (12.5 nM) was incubated in 0.05 M phosphate-NaCl with a heart supernatant fraction (1.04 mg/ml) which had been stored for 90 min at 4°. Aliquots (0.5 ml) were filtered at the indicated times (●). A duplicate series of aliquots (1.0 ml) was added to tubes containing 0.3 ml of 1.1 M $HClO_4$, and alumina column chromatography was performed (○). The binding blanks, corrected as in Fig. 2, were approximately 0.05 pmole of ³H.

B. Assays identical with those described in A were performed on the same heart supernatant fraction, which had been stored for 90 min at 20°.

C. Assays identical with those described in A were performed on the same heart supernatant fraction, which had been stored for 90 min at 37°.

D. *dl*-[³H]Epinephrine (12.5 nM) was incubated with a heart supernatant fraction (1.32 mg/ml) in the absence (○, ●) and presence (□, ■) of 30 μM catechol. The tissue had been stored at −22° for 1 day. Assays identical with those described in A were performed. The binding blanks, corrected as in Fig. 2, were approximately 0.05 pmole of ³H. In these experiments binding data are shown with solid lines and symbols, and amine retained by alumina is shown by dashed lines and open symbols.

The potencies of these inhibitors are equal to or greater than that of pyrogallol (27), and they appear to act at the substrate site (27).[3] [³H]Epinephrine binds to BSA (28), and this binding is quantitatively and qualitatively similar to that seen with heart supernatant fractions (Fig. 11). Thus it is not necessary to implicate catechol *O*-methyltransferase to explain the binding phenomenon.

The properties of binding observed in this study are similar to or identical with those reported from other laboratories (7–13, 15, 16). A number of results suggest a role for oxidized degradation products of catecholamines in the binding process. These include (a) the ability of reducing agents to block binding (Fig. 10B) (16), (b) the correlation between binding, pH, and rates of degradation of catecholamines (Fig. 12), (c) the finding that divalent cations increase binding while chelating agents inhibit it (16), and (d) the observation that the binding is decreased by replacement of air by nitrogen (16).

The properties of the binding are such that it does not appear to be meaningfully related to *beta* adrenergic receptors. The seemingly specific ability of catechols to inhibit binding (Fig. 10A) (15, 16) and the increases in binding which have been observed as a function of storage or other preliminary treatments (Figs. 2, 3, 7, and 12) (15) suggest, however, that some other po-

tentially specifiable site may be involved in the binding of catecholamines (15). On the other hand, a lack of specificity of the binding is indicated by the observation that catecholamines can bind to crystalline BSA. Furthermore, the ability of catechols to inhibit binding can be explained by their ability to inhibit the tissue-catalyzed oxidation of catecholamines (Fig. 12D), and at least some of the preliminary treatments of tissue cause increased binding by increasing the rate of destruction of [^{3}H]epinephrine (Fig. 12A–C). The increased rate of destruction may be mediated by divalent cations released or exposed during storage or incubation of the tissue. All of the findings can thus be explained in terms of the formation of oxidation products of catecholamines which appear to bind nonspecifically to tissue constituents. Recourse to a specific macromolecular binding site(s) is not required to explain the observed phenomena.

ACKNOWLEDGMENT

We thank Ms. Jane Andrews for excellent technical assistance.

REFERENCES

1. Hammes, G. G., Molinoff, P. B. & Bloom, F. E. (1973) *Neurosci. Res. Program Bull.*, **11**, 155–294.
2. Robison, G. A., Butcher, R. W. & Sutherland, E. W. (1971) *Cyclic AMP*, Academic Press, New York.
3. Perkins, J. P. (1973) *Adv. Cyclic Nucleotide Res.* **3**, 1–64.
4. Rodbell, M., Krans, H. M. J., Pohl, S. L. & Birnbaumer, (1971) *J. Biol. Chem.*, **246**, 1861–1871.
5. Birnbaumer, L. & Pohl, S. L. (1973) *J. Biol. Chem.*, **248**, 2056–2061.
6. Cuatrecasas, P. (1972) *Proc. Natl. Acad. Sci. U. S. A.*, **69**, 318–322.
7. Lefkowitz, R. J. & Haber, E. (1971) *Proc. Natl. Acad. Sci. U. S. A.*, **68**, 1773–1777.
8. Lefkowitz, R. J., Haber, E. & O'Hara, D. (1972) *Proc. Natl. Acad. Sci. U. S. A.*, **69**, 2828–2832.
9. Lefkowitz, R. J., Sharp, G. W. G. & Haber, E. (1973) *J. Biol. Chem.*, **248**, 342–349.
10. Bilezikian, J. P. & Aurbach, G. D. (1973) *J. Biol. Chem.* **248**, 5577–5583.
11. Bilezikian, J. P. & Aurbach, G. D. (1973) *J. Biol. Chem.*, **248**, 5584–5589.
12. Schramm, M., Feinstein, H., Naim, E., Lang, M. & Lasser, M. (1972) *Proc. Natl. Acad. Sci. U. S. A.*, **69**, 523–527.
13. Dunnick, J. K. & Marinetti, G. V. (1971) *Biochim. Biophys. Acta*, **249**, 122–134.
14. Drummond, G. I., Duncan, L. & Hertzmann, E. (1966) *J. Biol. Chem.*, **241**, 5899–5903.
15. Cuatrecasas, P., Tell, G. P. E., Sica, V., Parikh, I. & Chang, K. J. (1974) *Nature New Biol.*, **247**, 92–97.
16. Maguire, M. E., Goldman, P. H. & Gilman, A. G. (1974) *Mol. Pharmacol.*, **10**, 563–581.
17. Molinoff, P. B. (1973) in *Frontiers in Catecholamine Research* (Usdin, E. & Snyder, S., eds.), pp. 357–360, Pergamon Press, New York.
18. Tell, G. P. E. & Cuatrecasas, P. (1974) *Biochem. Biophys. Res. Commun.*, **57**, 793–800.
19. Ariëns, E. J., Simonis, A. M. & Van Rossum, J. M. (1964) in *Molecular Pharmacology*, (Ariëns, E. J., ed.), Vol. 1, p. 237, Academic Press, New York.
20. Rosen, O. M., Erlichman, J. & Rosen, S. M. (1970) *Mol. Pharmacol.*, **6**, 524–531.
21. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 267–275.
22. Anton, A. H. & Sayre, D. F. (1962) *J. Pharmacol. Exp. Ther.*, **138**, 360–375.
23. Krishna, G., Weiss, B. & Brodie, B. B. (1968) *J. Pharmacol. Exp. Ther.*, **163**, 379–385.
24. Molinoff, P. B. & Axelrod, J. (1971) *Annu. Rev. Biochem.*, **40**, 465–500.
25. Manganiello, V. C., Murad, F. & Vaughan, M. (1971) *J. Biol. Chem.*, **246**, 2195–2202.
26. Brownstein, M., Saavedra, J. M. & Axelrod, J. (1973) *Mol. Pharmacol.*, **9**, 605–611.
27. Nikodejevic, B., Senoh, S., Daly, J. W. & Creveling, C. R. (1970) *J. Pharmacol. Exp. Ther.*, **174**, 83–93.
28. Saner, A. & Thoenen, H. (1971) *Mol. Pharmacol.*, **7**, 147–154.