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The Reaction of [^{3}H]Norepinephrine with Particulate Fractions of Cells Responsive to Catecholamines

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SUMMARY

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Some previous reports of [^{3}H]norepinephrine binding to *beta* adrenergic receptors were re-examined in cultured glial cells (C6), other clonal cell lines, and canine cardiac microsomes. There was no correlation between [^{3}H]norepinephrine binding to particulate preparations from five clones and the ability of catecholamines to stimulate cyclic 3′,5′-AMP synthesis in intact cells. While the [^{3}H]norepinephrine binding with particulate fractions required hours for completion, cyclic AMP accumulation rates in intact cells were maximal within seconds even at low hormone concentrations. The [^{3}H]norepinephrine binding reaction could only be blocked by those adrenergic drugs with a catechol moiety. There was no apparent pharmacological specificity. Furthermore, *o*- and *p*-dihydroxybenzene derivatives (1–10 μM), but not *meta* derivatives (e.g., catechol and hydroquinone, but not resorcinol), blocked the reaction of [^{3}H]norepinephrine with the particulate fractions. Since effective inhibitors thus appeared to be reducing agents, ascorbic acid and sodium metabisulfite were tested. They were found to be potent inhibitors of the reaction in micromolar concentrations. When tested in intact cells, many compounds able to block binding (dopa, 5-hydroxydopa, pyrogallol, catechol, hydroquinone, ascorbate, metabisulfite) neither stimulated (at 0.1 mM) nor blocked (at 10^5:1 excess) catecholamine-stimulated cyclic AMP synthesis. Inhibition by EDTA of the [^{3}H]norepinephrine binding reaction was reversed by micromolar Cu^{++}, Fe^{++}, and Mn^{++} but not by Ca^{++}, Mg^{++}, and Ba^{++}. Depletion of oxygen in reaction mixtures with N_2 inhibited binding. The reaction of [^{3}H]norepinephrine with cell particulate fractions could not be reversed after prolongee exposure to 1 N HCl, 5% trichloroacetic acid or 1 mM norepinephrine. After incubation little of the ^{3}H in the medium was identifiable as [^{3}H]norepinephrine. We hypothesize that observed [^{3}H]norepinephrine binding to cell fractions involves oxidation and subsequent covalent reaction with macromolecules and, as such, does not represent binding to the *beta* adrenergic receptor. Conditions are discussed under which true binding of catecholamines to *beta* adrenergic receptors might be measured.

INTRODUCTION

The effect of a hormone on a cell is the characteristic final expression of a sequential series of steps initiated by the highly specific interaction of the hormone with its cellular receptor. Until recently, hormonal effects have been studied by observing the specific cellular response. The introduction of radioisotopically labeled, biologically active hor-

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mones of high specific activity has led to numerous recent studies of the first step in hormonal response: the interaction of the hormone with its receptor. Our studies on the expression of hormonal responses in somatic cells and their hybrids would be greatly facilitated by methods for the measurement (quantitatively or qualitatively; see ref. 1) of specific membrane receptors. We have been prompted to re-examine several aspects of reported catecholamine binding to putative *beta* adrenergic receptors because published data (2–9) have raised several questions (1, 10). For example, studies of the binding of catecholamines have failed to demonstrate the stereospecificity observed when the effects of catecholamines are studied, and *beta* adrenergic antagonists do not prevent such hormone binding. In addition, surprisingly large quantities of catecholamine can be bound to membrane preparations with a time course that is, in some cases, much slower than that of the response. As a result of these and other questions, our subsequent investigations have led us to hypothesize that much published data on the binding of catecholamines to putative receptors actually involves the oxidation of catecholamine and the subsequent covalent bonding of the oxidized products to macromolecules.

MATERIALS AND METHODS

Materials. *l*-[7-^{3}H]Norepinephrine was purchased from either New England Nuclear (5.2 Ci/mmole) or Amersham/Searle (7.9 Ci/mmole). [*G*-^{3}H]Adenosine cyclic 3′,5′-monophosphate was obtained from New England Nuclear (22.1 Ci/mmole). [^{3}H]-Norepinephrine was assessed for isotopic purity by paper or column chromatography (see below), and it was stored frozen in 1 mM HCl. More than 95% of the ^{3}H fractionated with authentic norepinephrine, and there was no difference in the reaction of particulate fractions with purified [^{3}H]norepinephrine or with stock [^{3}H]norepinephrine. Unlabeled catecholamines, other biochemicals, and some drugs were obtained from standard sources. Many drugs were the generous gifts of Drs. T. C. Westfall, M. J. Peach, and D. R. H. Gourley. The structural formulae of pertinent compounds are shown in Table 1.

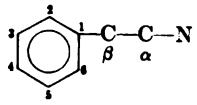
Cell culture. The methods for culture and experimental incubation, as well as the origin of most of the cell lines used, have been described previously (11). VA2 is an SV_{40}-transformed human fibroblast obtained from Dr. J. Minna. Cells used for preparation of the 30,000 × *g* pellet for binding studies were grown in 150-mm culture dishes until confluent.

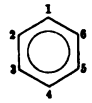
Preparation of particulate fractions. Cultured cells were rinsed free of medium and serum with ice-cold 5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.4, scraped off the plates, and allowed to swell for 60 min at 0° in the same buffer. The cells were then homogenized by 10 strokes of a Dounce homogenizer before being centrifuged for 2 min at 700 × *g*. This supernatant fraction showed no evidence of nuclei or intact cells by phase contract microscopy. The supernatant fraction was then centrifuged at 30,000 × *g* for 60 min, and the pellet was resuspended in the above buffer and frozen in aliquots at −20° until use.

Canine ventricular microsomes were prepared and stored as described by Lefkowitz and Haber (2). For some experiments, as noted, this particulate fraction was washed twice by suspension in 5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.4, and centrifugation at 78,000 × *g* for 60 min.

Assay of norepinephrine binding. Experiments were normally performed in a total volume of 200 μl in 20 mM potassium phosphate buffer, pH 7.4, at 37°. About 50,000 cpm of [^{3}H]norepinephrine were added (final concentration, 50 nM). The reaction was initiated by addition of protein or [^{3}H]norepinephrine. In some experiments the concentration of norepinephrine was 5 nM, as used by Lefkowitz and Haber (2). After incubation for the appropriate time (2 hr unless otherwise indicated), samples were diluted with 1 ml of cold 20 mM potassium phosphate, pH 7.4, and immediately filtered through a Millipore filter (HAWP, 0.45 μm) that had been rinsed with 4 ml of the same buffer. The filter was then washed with an additional 4 ml of buffer. In some experiments larger incubation volumes were used, and 200-μl aliquots were withdrawn from the reaction mixture at appropriate times and

TABLE 1

Compound	Structure					
β-Phenylethylamines 	Position					
	3	4	β	α	N	Other
l-Norepinephrine	—OH	—OH	—OH			
d-Norepinephrine	—OH	—OH	—OH			
l-Epinephrine	—OH	—OH	—OH		$—CH_3$	
dl-Isoproterenol	—OH	—OH	—OH		$—CH—(CH_3)_2$	
Dopamine	—OH	—OH				
Dopa	—OH	—OH		—COOH		
dl-α-Methyldopamine	—OH	—OH		$—CH_3$		
dl-α-Methylnorepinephrine	—OH	—OH	—OH	$—CH_3$		
5-Hydroxydopa	—OH	—OH		—COOH		5—OH
5-Hydroxydopamine	—OH	—OH				5—OH
6-Hydroxydopamine	—OH	—OH				6—OH
l-Tyrosine		—OH		—COOH		
Tyramine		—OH				
Octopamine		—OH	—OH			
dl-α-Methyl-*p*-tyrosine methyl ester		—OH		$—COOCH_3$ $—CH_3$		
Phenylephrine	—OH		—OH		$—CH_3$	
Normetanephrine	$—OCH_3$	—OH	—OH			
dl-Amphetamine				$—CH_3$		
Ephedrine			—OH	$—CH_3$	$—CH_3$	
Dichloroisoproterenol	—Cl	—Cl	—OH		$—CH—(CH_3)_2$	

Polyhydric phenols 	Position					
	1	2	3	4	5	6
Pyrocatechol	—OH	—OH				
Resorcinol	—OH		—OH			
Hydroquinone	—OH			—OH		
Pyrogallol	—OH	—OH	—OH			
2,4-Dihydroxybenzoic acid	—COOH	—OH		—OH		
3,4-Dihydroxybenzoic acid	—COOH		—OH	—OH		
3,5-Dihydroxybenzoic acid	—COOH		—OH		—OH	
dl-*p*-Hydroxymandelic acid	—CH(OH)COOH			—OH		
p-Hydroxyphenylacetic acid	$—CH_2COOH$			—OH		
2,3-Dihydroxynaphthalene		—OH	—OH		—CH=CH—CH=CH—	

filtered without dilution. A sample could be filtered and washed in less than 10 sec. Filters were dissolved in 1.5 ml of methyl Cellosolve before addition of 4.5 ml of toluene containing 4 g/liter of diphenyloxazole. In the absence of added particulate fraction a relatively small number of counts (300–500 cpm) was trapped by the filter. This blank was subtracted from experimental data. Aqueous samples were counted in Aquasol or Triton X-100–toluene (3:1).

Analytical techniques. Cyclic AMP was determined as described previously (12), as was adenylate cyclase (EC 4.6.1.1) activity (13). Protein was measured by the method of Lowry *et al.* (14).

Descending paper chromatographic systems employed were 1-butanol–glacial acetic acid–water (12:3:5 or 4:1:5, upper phase) on Whatman No. 1 paper. Alumina columns (1 g) were washed thoroughly with water before use. Each sample received 0.4 mg/ml of EDTA, pH 7, and 1.2 mg/ml of sodium metabisulfite before adjustment of the pH to 8.3 and application to the column. The column was subsequently washed with 75 ml of water, 15 ml of 0.25 N acetic acid, and 25 ml of 1 N HCl. The acetic acid fraction received EDTA as above and 1 mg/ml of ascorbic acid before the pH adjustment to 6.5 and application to a 0.6 × 5 cm column of Bio-Rad AG 50W-X8 resin in the H^+ form that had been washed extensively with water. The column was then washed with 15 ml of water, 10 ml of 0.2 N HCl, 20 ml of 1 N HCl, 10 ml of 2 N HCl, and 10 ml of 4 N HCl. Norepinephrine was eluted in the 0.25 N acetic acid fraction from alumina columns and in the 1 N HCl fraction from resin columns. High-voltage electrophoresis was carried out on Whatman No. 3MM paper in pyridine–glacial acetic acid–water (5:45:945, pH 3.5) at 3000 V for 30 min.

Experiments were performed in duplicate or triplicate. Replicates usually agreed within 5%. In most experiments control binding to particulate fractions was 5,000–10,000 cpm, with a blank of 300–500 cpm.

RESULTS

Demonstration of beta adrenergic receptors in particulate fractions. Two different particulate fractions were utilized in this study. The rat glioma cultured cell line C6TG1A (11) was examined in detail, since these cells respond dramatically to catecholamines with increased production of cyclic AMP and since many hybrid cell clones involving this line have been characterized (11, 15). Cyclic AMP accumulation in intact cells (11) and adenylate cyclase activity in the 30,000 × *g* particulate fraction can be stimulated by isoproterenol, and these stimulatory responses are inhibited by the *beta* adrenergic antagonist propranolol. The 30,000 × *g* particulate fraction contains, in addition, the vast majority of the basal and isoproterenol-stimulated adenylate cyclase of these cells (data not shown) and thus is assumed to contain most of the *beta* adrenergic receptors present in the homogenate. The magnitude of the catecholamine stimulation in the particulate preparation from C6TG1A is considerably less than that seen with intact cells (11). Similarly reduced levels of stimulation in particulate preparations from glial cells have been reported previously (16, 17).

Adenylate cyclase specific activities and effects of catecholamines have been reported by Lefkowitz *et al.* (3) for the canine cardiac particulate preparation. The subcellular distribution of adenylate cyclase from such canine ventricular preparations has been studied by Wolfe *et al.* (10).

Kinetic studies. As shown in Fig. 1, the reaction of [^{3}H]norepinephrine with the particulate fraction from C6TG1A cells is markedly temperature-dependent and requires at least 2 hr for completion at 37°. At this time a large amount of tritiated material is apparently bound: about 2 pmoles/17 μg of protein. This time course is very similar to data obtained by Lefkowitz *et al.* (2–4) and by us (Fig. 9) with the cardiac particulate fraction.

In contrast to the relatively slow reaction of [^{3}H]norepinephrine with the particulate fractions, stimulation of cyclic AMP accumulation in intact C6TG1A cells is virtually instantaneous (Fig. 2). Most importantly, a maximal *rate* of accumulation of the nucleotide is seen within seconds with 50 nM or with a maximally effective concentration (10 μM) of norepinephrine or isoproterenol.

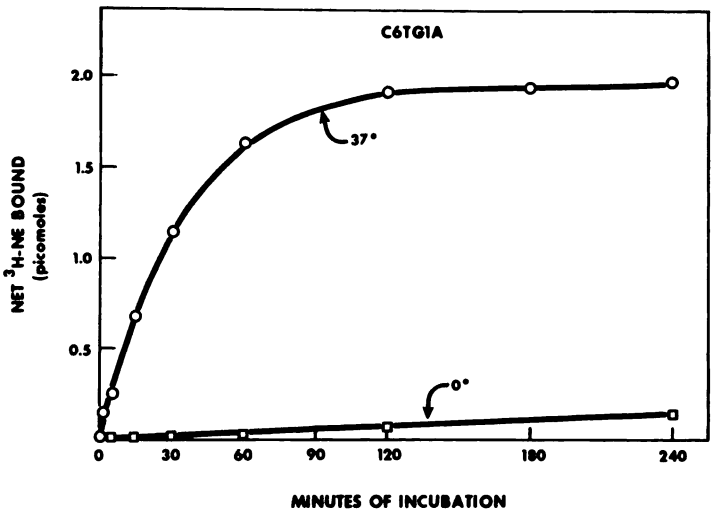


FIG. 1. *Time course and temperature dependence of* [³]*norepinephrine* (³*H-NE*) *reaction with C6TG1A particulate fraction* (*17 μg/200 μl*)

Maximal *accumulation* of cyclic AMP is observed by 15 min (11).

Correlation of [³*H*]*norepinephrine-particulate reaction with effects on cyclic AMP.* The ability of [³H]norepinephrine to react with particulate fractions from a variety of cell lines that differ markedly in their ability to respond to catecholamine with enhanced accumulation of cyclic AMP is shown in Fig. 3. Fractions with high ability to react with [³H]norepinephrine were derived from an unresponsive and from two highly responsive lines, while relatively poor abilities to react with [³H]norepinephrine were exhibited by both a responsive and an unresponsive line. In addition, the observed reaction of [³H]-norepinephrine was never a linear function of protein concentration. Plots deviate from linearity at the lowest protein concentration examined (less than 1 μg/ml).

Structure-activity relationship. Previous studies have indicated a lack of correlation between the ability of compounds to compete for [³H]catecholamine reaction sites and their known pharmacological activity (2–9). Specifically, any compound with a catechol moiety appears to be an extraordinarily good competitor for these reaction sites. We examined this question in considerable detail in the C6TG1A system, since its large magnitude of response to catecholamine facilitates functional comparisons. All

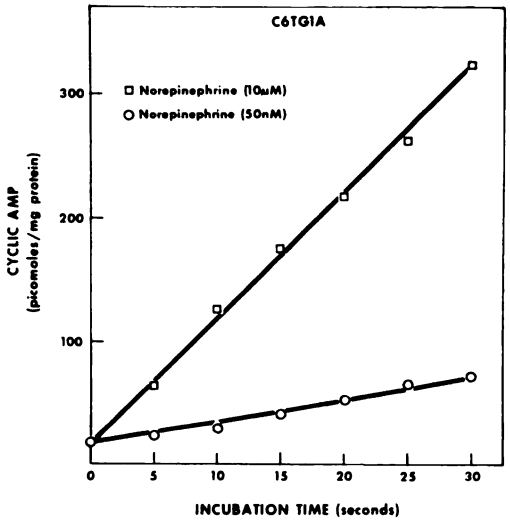


FIG. 2. *Time course of accumulation of cyclic AMP in intact C6TG1A cells exposed to 50 nM and 10 μM l-norepinephrine*

catecholamines tested (Fig. 4) were excellent competitors regardless of additional substitutions. We failed to detect significant differences in this activity between such compounds as *dl*-isoproterenol, *l*-norepinephrine, *d*-norepinephrine, and 5-hydroxydopa, among others. However, a large number of compounds were without activity. These include *beta* adrenergic antagonists that effectively abolish catecholamine-stimulated

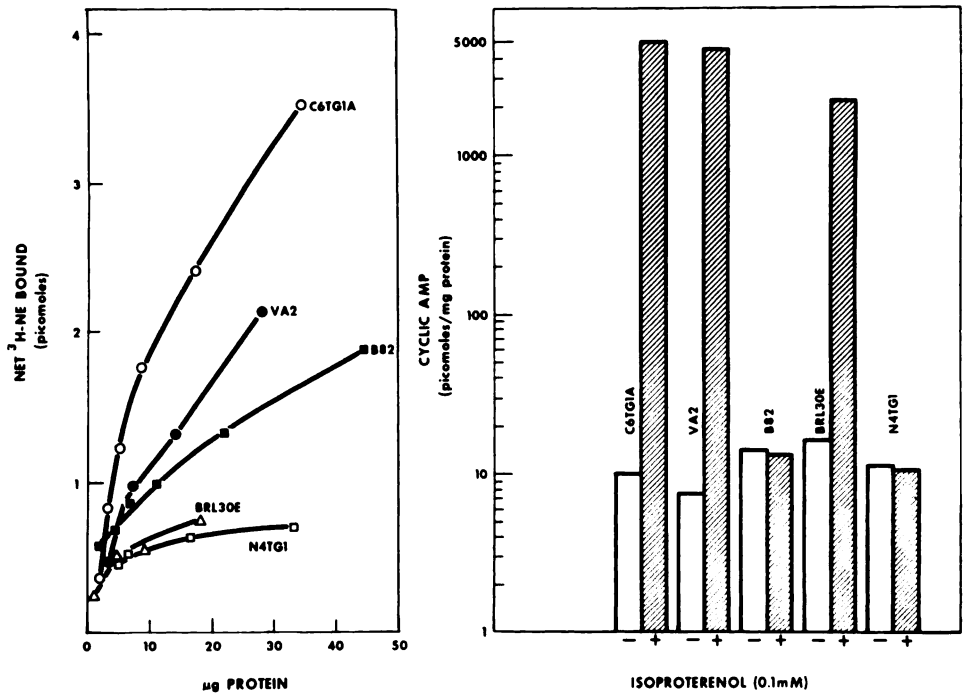


FIG. 3. *Reaction of [³H]norepinephrine (³H-NE) with particulate fractions derived from various cell lines (left) and effect of isoproterenol (100 μM, 10 min) on cyclic AMP concentration in intact cultured cell lines (right)*

All responsive cell lines are stimulated by isoproterenol at about 1 nM, while maximal stimulation is about 1 μM.

accumulation of cyclic AMP in C6TG1A (11), monoamine oxidase inhibitors, inhibitors of catecholamine uptake, and others (Fig. 4).

Essentially identical patterns of inhibition of the [³H]norepinephrine reaction were observed with this series of compounds when canine ventricular microsomes were utilized (not shown).

Compounds that effectively prevented [³H]norepinephrine reaction with the particulate fraction were tested for their ability to stimulate (Fig. 5) or antagonize the stimulation (Fig. 6) of cyclic AMP accumulation by intact C6TG1A cells. This *beta* adrenergic receptor appears able to discriminate between *d*- and *l*-norepinephrine by at least two orders of magnitude (assuming optical purity). Dopa and 5-hydroxydopa were completely without stimulatory effects. In addition, neither they nor dopamine antagonized the stimulatory effects of isoproterenol (Fig. 6). This was the case even when these compounds were present at a molar ratio to isoproterenol of 10^5:1. Ephedrine stimulated cyclic AMP accumulation but had no effect on the [³H]norepinephrine reaction with particulate fractions.

This pattern of activity prompted us to examine the effects of simpler polyhydric phenols. The effects of catechol, pyrogallol, resorcinol, hydroquinone, and polyhydric derivatives of benzoic acid and naphthalene are shown in Fig. 7. It is striking that with both the C6TG1A and cardiac particulate preparations all compounds containing hydroxyl groups in *ortho* or *para* juxtaposition are inhibitors of the reaction. However, *m*-dihydroxy compounds (resorcinol and 2,4- and 3,5-dihydroxybenzoic acid) fail to inhibit. (The fact that resorcinol stimulated the reaction with cardiac microsomes is unexplained.) It is well known that *ortho* and *para* polyhydric phenols oxidize spontaneously to the corresponding quinones and are thus excellent reducing agents. This type of oxida-

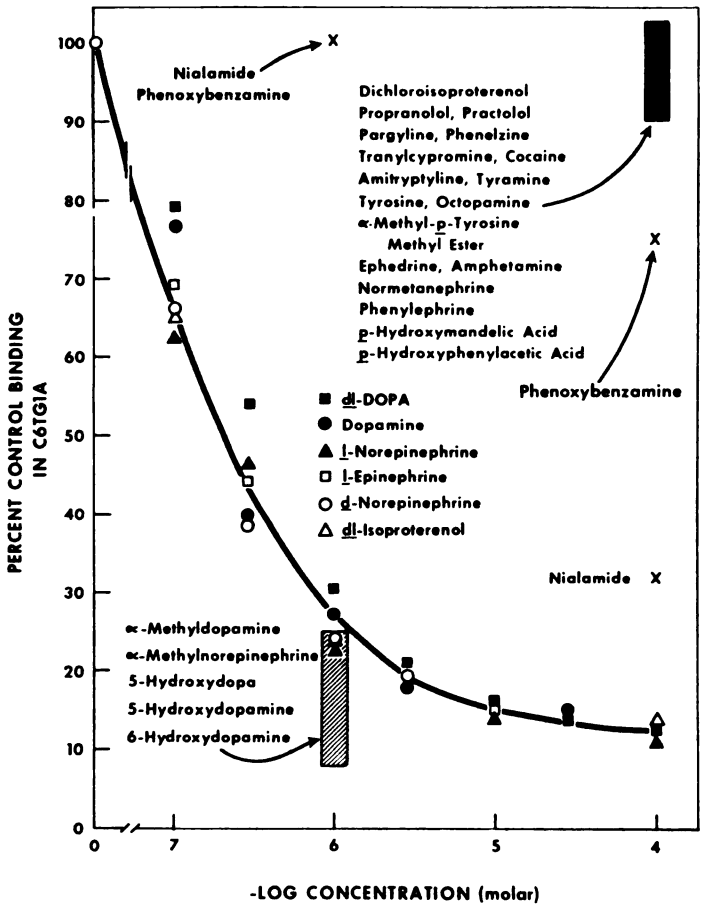


FIG. 4. *Effects of various drugs on reaction of [^{3}H]norepinephrine with C6TG1A particulate fraction (17 μg/200 μl)*

Results obtained with the canine ventricular preparation were essentially identical. At concentrations above 3 μM not all points are shown, for clarity.

tion does not occur with the *meta* isomer (e.g., resorcinol), since a stable resonance structure does not exist for an *m*-quinone.

These considerations suggested a necessity for oxidation at some point in the reaction sequence of [^{3}H]norepinephrine with the particulate fractions. Accordingly, the effects of other reducing agents were examined (Fig. 8). Ascorbic acid and sodium metabisulfite caused marked inhibition of the [^{3}H]norepinephrine reaction at concentrations of 0.1–1.0 μM. The [^{3}H]norepinephrine reaction with washed cardiac microsomes was more sensitive to inhibition by ascorbate than was the unwashed particulate fraction, perhaps because of removal of iron (see below) contained in myoglobin that was lost during the washing step.

Inhibition of the [^{3}H]norepinephrine reaction by reducing agents was pronounced at all time intervals (Fig. 9) and at both low (5 nM) and high (50 nM) [^{3}H]norepinephrine concentrations (Table 2). Notably, [^{3}H]norepinephrine-particulate complexes were essentially undetectable at relatively short incubation times in the presence of 100 μM ascorbate, metabisulfite, or hydroquinone. Furthermore, inhibition of the [^{3}H]norepinephrine reaction by other effective compounds was quantitatively and qualitatively similar at both 5 nM and 50 nM [^{3}H]norepinephrine concentrations (Table 2).

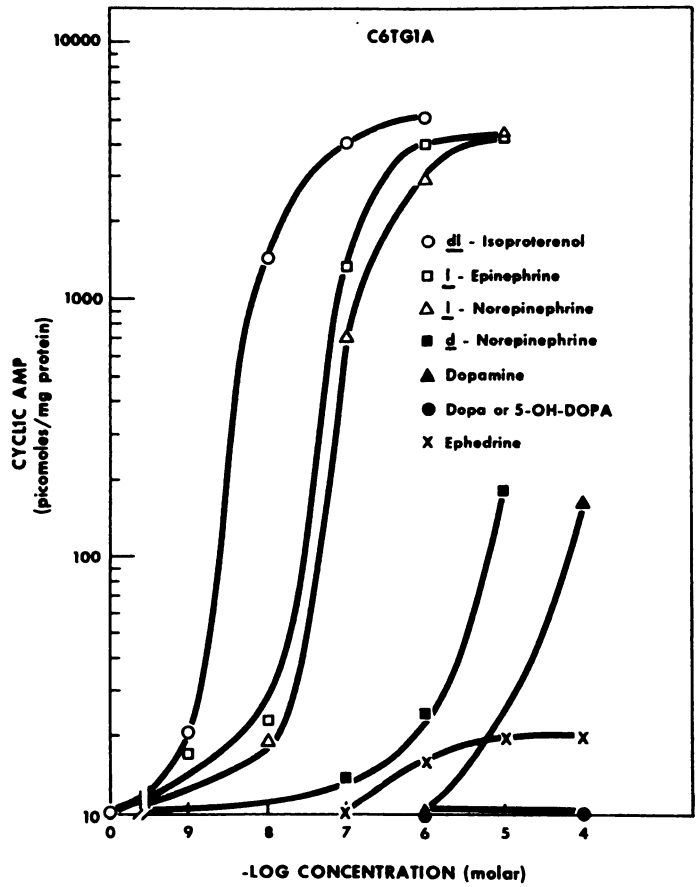


FIG. 5. *Effects of catecholamines on cyclic AMP accumulation by intact C6TG1A cells*
Cells were exposed to the indicated concentrations for 10 min.

Since the reducing agents and the oxidizable polyhydric phenols effectively prevented reaction of [^{3}H]norepinephrine with membranes, their ability to affect catecholamine-stimulated accumulation of cyclic AMP was evaluated (Fig. 10). In the presence of 100 μM ascorbate, metabisulfite, catechol, hydroquinone, or pyrogallol, full responsiveness to 1 nM–1 μM isoproterenol was observed. In fact, although none of these reducing agents was an agonist (at 0.1 mM), they all appeared to potentiate responses to submaximal concentrations of isoproterenol slightly, perhaps via protecion of the agonist from oxidation.

The mechanism of inhibition of the [^{3}H]-norepinephrine reaction was investigated further by incubation of the C6TG1A particulate fraction with reducing agents, followed by centrifugation to remove the soluble compounds (Table 3). Membranes so treated were fully reactive with [^{3}H]norepinephrine, indicating the absence of any irreversible effect on this particulate fraction. All compounds inhibiting the reaction still did so after the initial treatments indicated (Table 3).

Additional experiments were performed to alter the concentrations of potential oxidants present in the standard reaction mixture. Incubation under an atmosphere of N_2 significantly inhibited the [^{3}H]norepinephrine reaction (Fig. 11). Furthermore, certain heavy metals are well known to promote oxidation of catecholamines. Previous studies have indicated severe inhibition of the [^{3}H]norepinephrine reaction with particulate fractions by EDTA, and full restoration

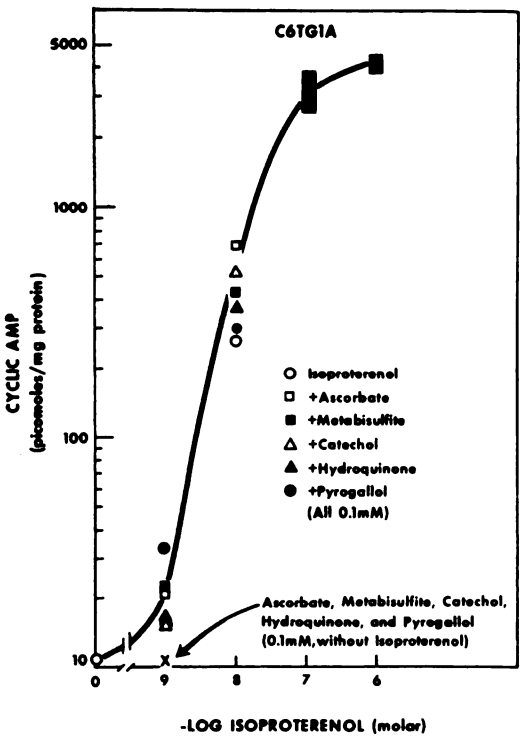


FIG. 6. *Response of C6TG1A cells to isoproterenol in the presence of catecholamines*

At concentrations of 0.1 and 1 μM isoproterenol, the solid bars indicate the range of absolute responses, which were essentially identical.

was not obtained with 1 mM Mg^{++} or Ca^{++} (3). With either C6TG1A or ventricular particulate preparations, striking inhibition of the [³H]norepinephrine reaction was seen when the concentration of EDTA exceeded 0.1 μM, suggesting an important role of trace concentrations of metals (Fig. 12). In the presence of 10 μM EDTA neither 15 μM Mg^{++}, Ca^{++}, nor Ba^{++} could reverse the inhibition. However, Cu^{++}, Fe^{++}, Zn^{++}, Co^{++}, and Mn^{++} were able to restore full reactivity or to stimulate the reaction in excess of the original (minus EDTA) level. The metals that were effective either are oxidants or have extraordinarily high affinity constants for EDTA and could thus displace an effective metallic oxidant. Inactive metals are poor oxidants and/or have affinities for EDTA that are orders of magnitude less than are those for Zn^{++}, Cu^{++}, Fe^{++}, Mn^{++}, and Co^{++} (18). The inhibitory effects of EDTA and a lack of oxygen were additive, resulting in nearly complete inhibition of the [³H]norepinephrine reaction with the particulate fraction (data not shown).

Characterization of reaction product. Features of the data presented to this point suggest a reaction scheme more complex than simple equilibrium between macromolecule and ligand. Most disturbing are the time required to reach presumed equilibrium, the specificity of inhibition by polyhydric phenols, the inhibitory effects of reducing agents, and the stimulation by low concentrations of certain heavy metals. Since quinones formed by oxidation of *o*- and *p*-dihydric phenols are highly susceptible to ring addition (e.g., the cyclization of oxidized catecholamines to the corresponding adrenochrome derivatives), we considered that covalent bonding between oxidation products of norepinephrine and reactive groups of the particulate fractions was possible. This seems even more likely in view of the description by Saner and Thoenen (19, 20) of covalent reaction between the *p*-quinone of 6-hydroxydopamine and bovine serum albumin. Significant reaction was also observed by these workers between albumin and norepinephrine or dopamine.

Accordingly, the stability of the reaction product was investigated. Addition of large quantities of unlabeled norepinephrine at various time intervals after initiation of the reaction with [³H]norepinephrine resulted in only a small loss of radioactivity adhering to the filter (Fig. 13). The amount of apparent reversal decreased steadily with increasing incubation time. With both C6TG1A and cardiac particulate preparations there was no significant reversal of reaction even after hours of incubation with unlabeled norepinephrine (not shown).

The effects of acids (HCl, 1 N; TCA,[1] 5%) were more complex (Fig. 13 and Table 4). HCl and TCA treatment at 37° for extended periods resulted in loss of 30–50% of the tritium adhering to the filter, although relatively large quantities of reaction product were still detectable by filtration even after prolonged exposure to 1 N HCl or 5% TCA

[1] The abbreviation used is: TCA, trichloroacetic acid.

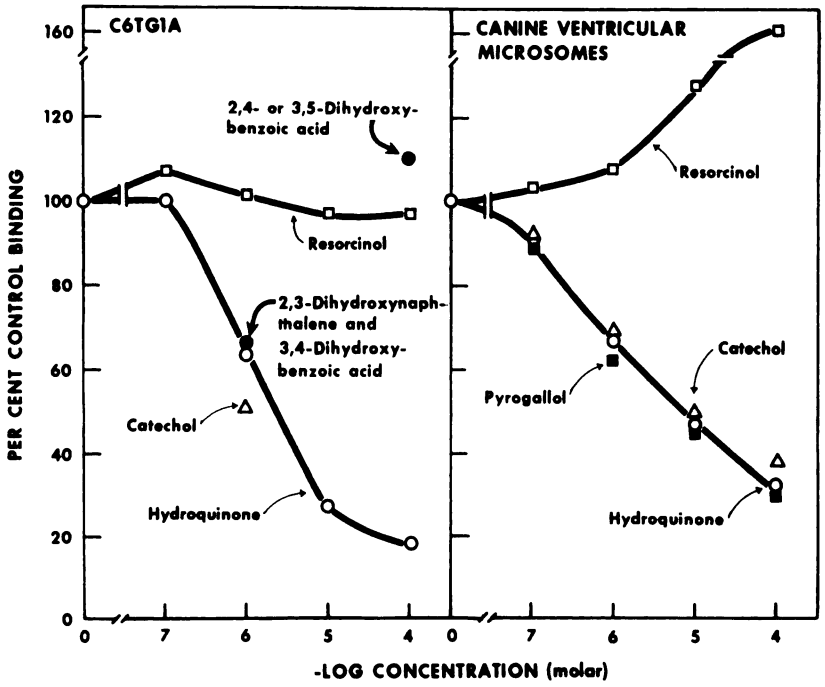


FIG. 7. *Inhibition of [³H]norepinephrine reaction with C6TG1A (17 μg/200 μl) and ventricular (30 μg/200 μl) particulate fractions by polyhydric phenols*

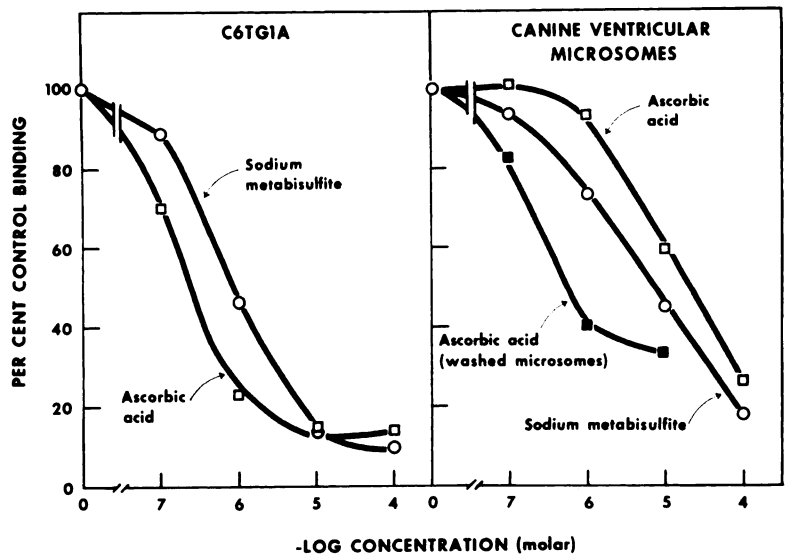


FIG. 8. *Inhibition of [³H]norepinephrine reaction with C6TG1A (17 μg/200 μl) and ventricular (30 μg/200 μl) particulate fractions by ascorbate and metabisulfite*

The effect of ascorbate was tested on both the original and washed ventricular fractions.

at 100° (Table 4). When, however, carrier albumin was added and radioactive reaction products were recovered by addition of TCA and centrifugation, product was obtained in a quantity similar to that seen by filtration prior to the addition of HCl (Fig. 13). Presumably, reaction products or fragments thereof are more soluble and/or are not ad-

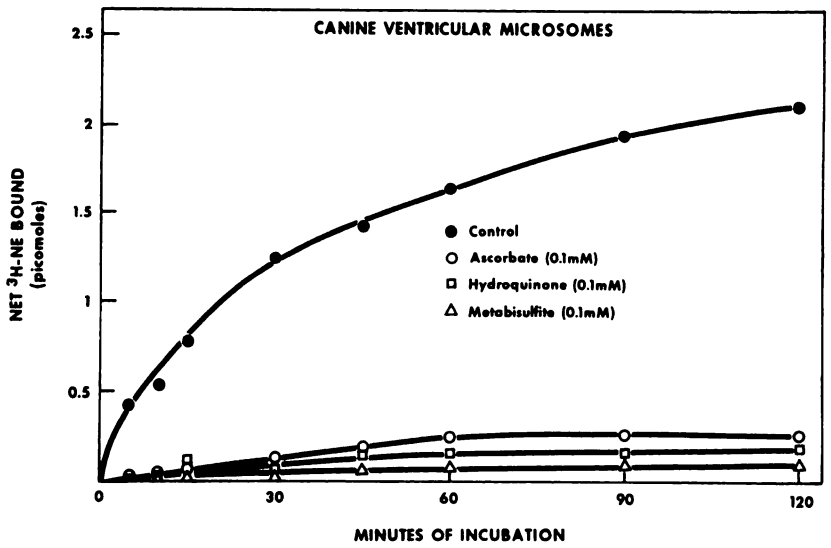


FIG. 9. *Time course of [^{3}H]norepinephrine (^{3}H-NE) reaction with canine ventricular fraction (30 μg/200 μl) in the presence and absence of indicated agents*

TABLE 2

Effect of [^{3}H]norepinephrine concentration on [^{3}H]norepinephrine-particulate reaction

Samples of canine heart microsomes (30 μg/200 μl) were incubated with either 5 nM or 50 nM [^{3}H]-norepinephrine in 20 mM potassium phosphate, pH 7.4, for 120 min at 37°. Blank values of 317 cpm (50 nM) or 52 cpm (5 nM) were subtracted. Control reaction values were 4154 cpm (50 nM) and 687 cpm (5 nM).

Sample	Concentration	5 nM [^{3}H]-norepinephrine	50 nM [^{3}H]-norepinephrine
	μM	*% control reaction*	
Control		100	100
Ascorbate	1	38	35
	100	9	8
Sodium metabisulfite	1	63	97
	100	23	28
Catechol	1	60	54
	100	18	8
Hydroquinone	1	57	45
	100	12	7
l-Norepinephrine	1	44	44
	100	11	4

sorbed to filters after addition of HCl (see below for discussion of product solubility).

In contrast to the inhibitory effects of micromolar concentrations of ascorbate and metabisulfite when present during the reac-

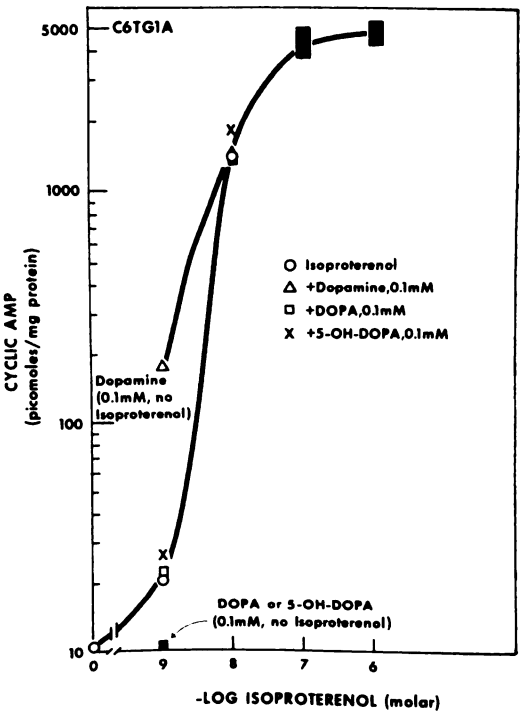


FIG. 10. *Effect of 10-min exposure to isoproterenol on cyclic AMP levels in intact C6TG1A in the presence and absence of reducing agents and polyhydric phenols*

At concentrations of 0.1 and 1 μM isoproterenol, solid bars indicate the range of absolute responses, which were essentially identical.

TABLE 3

Effect of prior treatment with various compounds on [^{3}H]norepinephrine reaction with C6TG1A particulate preparation

Samples containing 20 mM potassium phosphate, pH 7.4, and C6TG1A particulate fractions (10 μg/200 μl) were incubated for 2 hr at 37° with and without the indicated compounds (prior treatment) before centrifugation at 78,000 × *g* for 60 min. Pellets were resuspended in the original volume and incubated in the presence of 50 nM [^{3}H]norepinephrine for 2 hr at 37° with and without inhibitory compounds (secondary treatment) before filtration. Protein incubated in both treatments without agents is the control, set at 100% ("none" in the table). All other values are relative to the control. No corrections were made for differential loss of protein in the samples. Control binding of 100% corresponds to 1900 cpm bound per resuspended pellet derived from 200 μl of the initial incubation medium, or 0.4 pmole/10 μg of C6TG1A protein (before centrifugation). The same amount of protein without prior incubation or centrifugation bound approximately 0.8 pmole/10 μg of protein.

Prior treatment	Secondary treatment					
	None	Ascorbate, 100 μM	Metabisulfite, 10 μM	Hydroquinone, 100 μM	Dopamine, 100 μM	5-Hydroxydopa, 100 μM
	%	%	%	%	%	%
None	100[a]	2	15	18	9	−1
Ascorbate, 100 μM	93	2	13	22	12	0
Metabisulfite, 10 μM	85	12	14			
Hydroquinone, 100 μM	55	2		28	15	0
Dopamine, 100 μM	76	10		43	11	0
5-Hydroxydopa, 100 μM	75	7		41	13	−1

[a] Control.

tion, these reducing agents at 1 mM concentrations were ineffective in reversing the reaction (Table 4). Additional experiments showed similar failure of reversal after a 22-hr incubation with reducing agents.

In certain experiments we attempted to assess product formation by centrifugation, in addition to filtration. Under these circumstances we noted that large amounts of product (detectable by filtration) failed to sediment at 78,000 × *g* for 60 min (Table 5). This centrifugation was the same as that used originally for the isolation of the canine ventricular preparation and was almost 3 times the force used to prepare the C6TG1A particulate fraction. Such spontaneous (or trypsin-stimulated) apparent solubilization of binding activity was also noted by Bilezikian and Aurbach (8). That these observations do indeed represent solubilization of reactive protein is indicated by the following considerations.

1. Following reaction with [^{3}H]norepinephrine, most of the filterable radioactivity could be sedimented at 250,000 × *g* for 20 hr but not at 78,000 × *g* for 60 min, the original isolation condition.

2. Both the extent of the [^{3}H]norepinephrine reaction and the amount of radioactivity not sedimentable at 78,000 × *g* for 60 min were increased by an elevated potassium phosphate concentration (Table 5).

3. If particulate fractions were incubated for 2 hr in 20 mM potassium phosphate without [^{3}H]norepinephrine and then centrifuged at 78,000 × *g* for 60 min, large amounts of reactive material were present in the supernatant fraction as determined by subsequent incubation with [^{3}H]norepinephrine. Furthermore, the reaction of [^{3}H]norepinephrine with the pellet so obtained was somewhat reduced (see legend to Table 3).

4. Subsequent to the reaction of particulate fractions with [^{3}H]norepinephrine, bound ^{3}H which did not sediment at 78,000 × *g* for 60 min was incubated with trypsin or Pronase. After centrifugation in 2–10% continuous sucrose gradients, the mobility of the

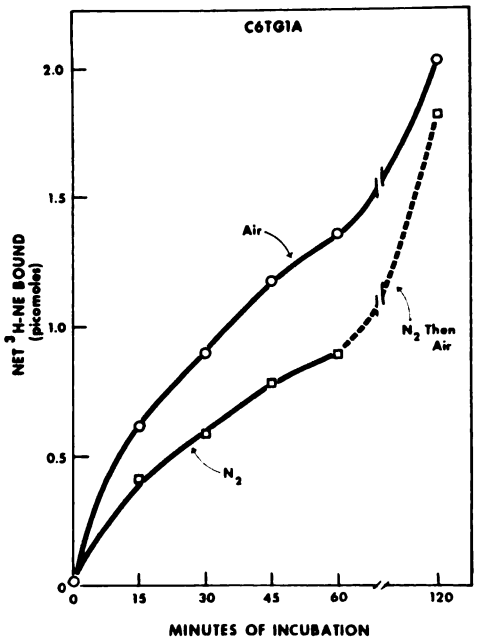


FIG. 11. *Inhibition of [³H]norepinephrine (³H-NE) reaction with C6TG1A particulate fraction (17 μg/200 μl) by N_2*

The oxygen concentration in the buffer was minimized by bubbling with N_2 before addition of protein. Test tubes were briefly gassed and filled with N_2 before sealing with a rubber stopper and incubation for the indicated time. After 60 min a sample treated with N_2 was exposed to air for 60 min before filtration as indicated by the dashed line.

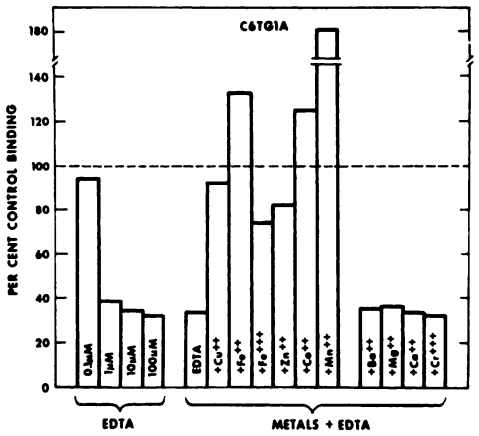


FIG. 12. *Inhibition of [³H]norepinephrine reaction by EDTA and stimulation by cations*

C6TG1A particulate fraction (17 μg/200 μl) was incubated in the presence of the indicated concentrations of EDTA. For incubation with cations, 10 μM EDTA containing 15 μM metallic chlorides or sulfates was added. Canine ventricular microsomes gave similar results.

protease-treated material was markedly impaired when compared to radioactive product not treated with protease.

5. Radioactive product that was not sedimented at 78,000 × *g* was not dialyzable against 20 mM potassium phosphate, pH 7.4, or 1 N HCl for 24 hr at 0°. However, if this material was dialyzed against water, the radioactive product adhered almost entirely to the dialysis membrane. Much of the radioactive product was dialyzable after treatment with trypsin or Pronase.

It seems possible that many of the anomalous results in the literature are due to the alterations in the rate of spontaneous solubilization of macromolecules, which are then more capable of reaction. There are other experimental pitfalls. For example, if reaction mixtures are treated with 1 N HCl, most of the protein will sediment at low centrifugal force (1000 × *g*). However, most of the radioactive macromolecular product sediments only at forces greater than those required to pellet the bulk of the protein present.

Chromatography of reaction mixtures. Since we were not able to reverse the [³H]norepinephrine reaction and extract labeled material from the particulate fractions, we cannot confirm previous reports (2–4) that bound catecholamine can be extracted in unmodified form (at least after 2 hr of incubation at 37°). However, we were able to examine the chemical state of the unbound tritiated material remaining after 2 hr of incubation. The reaction mixture was immediately chromatographed sequentially on an alumina and a cation-exchange column (Table 6). The column procedures utilized are standard methods for purification of catecholamines prior to chemical analysis (21, 22). After incubation with the C6TG1A particulate fraction only 25% of the [³H]norepinephrine remained, while 65% of the [³H]norepinephrine was present if 0.1 mM ascorbate and metabisulfite were present. Similar results were obtained when the unbound tritiated material was subjected to

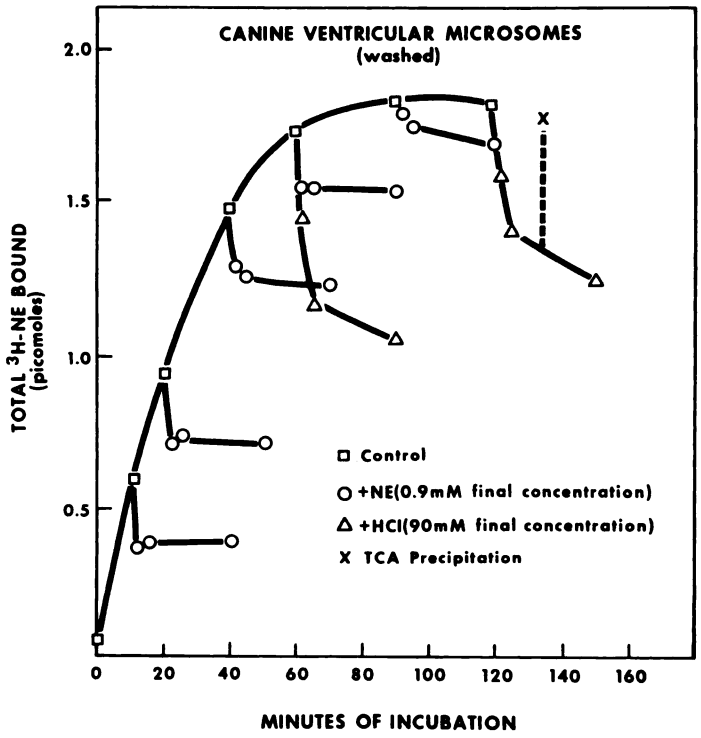


FIG. 13. *Attempted reversal of [³H]norepinephrine (³H-NE) reaction*

Washed canine ventricular microsomes (30 μg/200 μl) were incubated for the indicated times, after which an aliquot was filtered (□). Separate aliquots were added simultaneously to tubes containing 0.1 volume of either 10 mM *l*-norepinephrine (NE) (○) or 1 N HCl (△), and were filtered 2, 5, and 30 min later. The zero-time sample was filtered within 20 sec after addition of protein. After incubation for 120 min, certain samples were exposed to HCl (90 mM) for 15 min. Albumin (500 μg) and TCA (5%) were then added, and the protein was recovered by centrifugation. The pellet was dissolved in NaOH and counted (×). Blanks were not subtracted from these values, since they presumably are represented by the zero-time point. Similar data were obtained for unwashed canine ventricular microsomes and the C6TG1A particulate fraction (Table 4).

high-voltage electrophoresis (data not shown). The greater degradation of [³H]norepinephrine incubated at 37° without protein (Table 6) could be due to protein binding of metallic oxidants or perhaps to nonspecific binding of [³H]norepinephrine to protein, thus rendering the catecholamine less accessible to potential oxidants. It is interesting that while reducing agents eliminate the covalent reaction of [³H]norepinephrine (or its oxidized products) with the particulate fractions, they do not completely prevent degradation of the norepinephrine. Such degradation as occurs in the presence of protein and reducing agents may be in part enzymatic. Michaelson *et al.* (23) have observed that [³H]norepinephrine was deaminated to the aldehyde during incubation with a canine ventricular preparation identical with that utilized in this study.

DISCUSSION

These data demonstrate that the reaction observed with both C6TG1A and ventricular particulate preparations is not an equilibrium one, and strongly suggest the requirement for oxidation as an intermediate step. A plausible hypothesis involves a mechanism (Fig. 14) similar to that proposed by Saner and Thoenen (19, 20), wherein oxidation of catecholamine to the corresponding quinone is followed by nucleophilic attack on the aromatic ring. Mechanistically similar reactions of catecholamines with amino acids

TABLE 4

Stability of [³H]norepinephrine reaction product as measured by Millipore filtration

Particulate fractions were incubated with 50 nM [³H]norepinephrine in 20 mM potassium phosphate, pH 7.4, for 120 min before treatment as indicated and filtration. Control samples were filtered after 120 min without further treatment. "Heated" particulate preparations were heated at 100° for the indicated time before incubation with [³H]norepinephrine. Samples treated with TCA did not contain carrier BSA and were diluted 10-fold with buffer prior to filtration. Control binding (100%) was 13,200 cpm for C6TG1A membranes (21 μg/200 μl) and 9700 cpm for the canine ventricular preparation (30 μg/200 μl).

Treatment	Interval	C6TG1A	Canine ventricular preparation
	min	*% control binding*	
HCl, 1 N, 37°	15	68	59
	60	65	57
	120	66	46
HCl, 1 N, 100°	5	70	49
	30	52	36
TCA, 5%, 37°	0.5	74	62
	5	67	55
	15	63	52
	30	65	52
TCA, 5%, 100°	5	68	39
	30	59	35
Ascorbate, 1 mM	15	96	91
	60	93	92
	120	84	93
Metabisulfite, 1 mM	15	87	97
	60	82	91
	120	74	83
Heated, 100°	2	132	68
	5	135	61
	15	140	39
	30	141	32

TABLE 5

Sedimentation of reacted [³H]norepinephrine

C6TG1A (105 μg/ml) and canine ventricular (300 μg/ml) particulate preparations were incubated for 120 min at 37° in 50 nM [³H]norepinephrine at the indicated concentrations of potassium phosphate (total volume, 20 ml). After filtration of aliquots for determination of total [³H]norepinephrine bound, the samples were centrifuged at 5×10^6 $g \times$ min, and aliquots of the supernatant fraction were filtered for determination of unsedimented, bound ³H. Pellets were dissolved in NaOH before determination of sedimented, bound ³H. Data are based on total ³H bound by each fraction obtained after centrifugation.

Particulate fraction added	Potassium phosphate, pH 7.4	[³H]Norepinephrine reacted	Sedimentation of reacted material in	
			Pellet	Supernatant
	mM	*pmoles/mg protein*	%	%
C6TG1A	2	76	35	65
	20	84	29	71
	200	178	18	82
Canine ventricle	2	94	45	55
	20	102	43	57
	200	104	36	64

and peptides have also been reported (24, 25). All features of the data detailed above seem consistent with such a mechanism. In order to test this hypothesis further, it would be desirable to oxidize the catecholamine to the quinone prior to addition of the particulate fraction. However, the oxidized catecholamine rapidly cyclizes internally to an adrenochrome, making such an experiment impossible.

It is not entirely clear what factors limit the extent and rate of reaction. The rate of the proposed oxidation step appears to be particularly important. The extent of reaction may also be markedly influenced by the number of macromolecular sites available for nucleophilic substitution. This may be dependent on the physical and chemical state of the particulate fractions and the rate of solubilization of protein from these fractions. Such factors may explain the remarkable stability of the binding activity of such particulate fractions to a variety of manipulations, including urea, heat, sonication, and degradative enzymes, since these procedures, by denaturing or solubilizing protein, would make more sites available for reaction.

The question remains as to the relation-

TABLE 6

Column fractionation of norepinephrine

[^{3}H]Norepinephrine in 20 mM potassium phosphate, pH 7.4, was incubated with and without (blank) the C6TG1A particulate fraction (100 μg/ml) for 120 min at the temperatures indicated before fractionation on alumina columns (see MATERIALS AND METHODS). The fraction eluted as norepinephrine was then chromatographed on Dowex 50-X8. The last column ("net norepinephrine") is the per cent eluted from the alumina column multiplied by the per cent eluted from the Dowex column. Ascorbate and metabisulfite were present at concentrations of 0.1 mM. [^{3}H]Norepinephrine bound to protein did not bind to the alumina column and could be recovered by Millipore filtration of the material eluting with water.

Sample	Norepinephrine on alumina	Norepinephrine on Dowex 50	Net norepinephrine
	%	% *alumina fraction*	%
Stock [^{3}H]norepinephrine	96	98	94
Blank, 37°	5	34	2
Blank, 0°	70	93	65
C6TG1A particulate, 37°			
+Ascorbate + metabisulfite	77	84	65
−Ascorbate − metabisulfite	46	54	25

ship between the final reaction product observed and any initial binding to a functionally important site. We can find no evidence to suggest that any such meaningful relationship exists.

This conclusion is supported by the kinetic experiments and those in which the reaction capacity of various cell lines was examined. A maximal rate of production of cyclic AMP begins essentially instantaneously, while the [^{3}H]norepinephrine reaction is at a fraction of final levels. Although troublesome, this argument can be countered if the number of spare receptors is large, i.e., if only a fraction of receptors need be occupied for maximal *beta* adrenergic response. The lack of correlation between adrenergic responsiveness of cells and their reactivity with [^{3}H]-norepinephrine could also be taken as evidence against the significance of the reaction. However, it must be kept in mind that the reaction sequence between the *beta* adrenergic receptor and the adenylate cyclase is unknown, and the unresponsive cells tested could lack essential hypothetical components of the system other than the receptor.

More significant are experiments in which those compounds capable of markedly inhibiting [^{3}H]norepinephrine reaction fail to stimulate cyclic AMP production or to inhibit catecholamine-stimulated cyclic AMP production in an experimental system exquisitely capable of detecting such activities. Thus brief exposure to isoproterenol (0.1 μM) results in accumulation of cyclic AMP to concentrations 400-fold above control. Dopa and 5-hydroxydopa, when tested as agonists, are clearly less potent by a factor of more than 10^5, since no stimulatory activity was observed at the high concentration tested (0.1 mM). When tested as antagonists, these compounds are inactive when present in excess of isoproterenol by a factor of 10^5. Yet isoproterenol, dopa, and 5-hydroxydopa are essentially equipotent inhibitors of the [^{3}H]norepinephrine reaction. These data strongly strain the credulity of hypotheses based on ring-specific and side chain-specific portions of the receptor (4, 8, 9). Similar arguments can be made for structurally unrelated reducing agents and for polyhydric phenols. Since these agents have no capacity to antagonize cyclic AMP accumulation at time intervals when the [^{3}H]norepinephrine reaction is markedly if not completely inhibited, the putative binding phenomenon observed must be questioned.

Since our data strongly indicate that the observed reaction of [^{3}H]norepinephrine with particulate fractions that undoubtably contain *beta* adrenergic receptors bears no relationship to such receptors, we feel obligated to examine the theoretical feasibility of detection of *beta* adrenergic receptors in such preparations by radioactive ligand binding. There are two questions of primary importance: (a) the specific activity of receptors in available sources of material and (b) the physicochemical nature of the catecholamine-receptor interaction.

If one is willing to make two assumptions—the turnover number of adenylate cyclase and the stoichiometry between the enzyme and related receptors—interesting

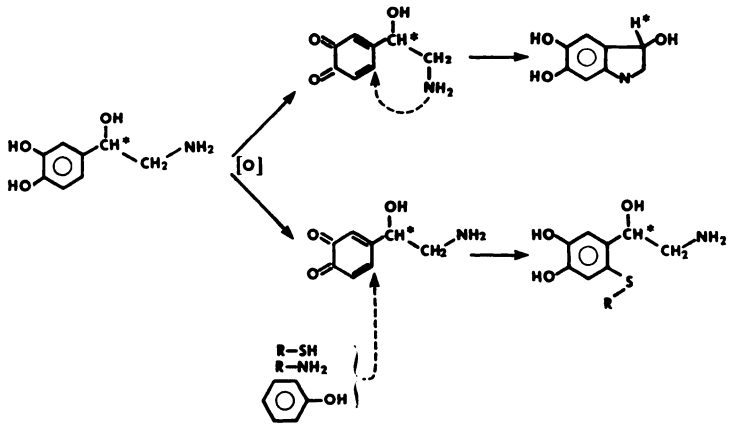


FIG. 14. *Possible reaction pathway for interaction of [^{3}H]norepinephrine with macromolecules*
The asterisks denote the positions of ^{3}H on the norepinephrine used in this study. This hypothetical pathway is based on the proposal of Saner and Thoenen (19, 20).

TABLE 7

Calculation of hypothetical adenylate cyclase-receptor relationships

The number of adenylate cyclase molecules per milligram of protein was calculated from the maximal rate of net cyclic AMP accumulation in intact C6TG1A cells in response to isoproterenol (500 pmoles/min/mg) (see ref. 11, also Fig. 2) and the assumed range of turnover numbers. A wide range of possible stoichiometry between the enzyme and the receptor is then additionally assumed. A counting efficiency of 100% is assumed.

Turnover No. for stimulated adenylate cyclase (assumed)	No. of adenylate cyclase molecules (calculated)	Molar ratio of receptor to adenylate cyclase (assumed)	No. of receptor molecules (calculated)	[^{3}H]Catecholamine bound at 10 Ci/mMole (calculated)
molecules/sec	*molecules/mg protein*		*molecules/mg protein*	*cpm/mg protein*
10^0	5×10^{12}	10^0	5×10^{12}	5×10^4
		10^2	5×10^{14}	5×10^6
		10^4	5×10^{16}	5×10^8
		10^6	5×10^{18}	5×10^{10}
10^2	5×10^{10}	10^0	5×10^{10}	5×10^2
		10^2	5×10^{12}	5×10^4
		10^4	5×10^{14}	5×10^6
		10^6	5×10^{16}	5×10^8
10^4	5×10^8	10^0	5×10^8	5×10^0
		10^2	5×10^{10}	5×10^2
		10^4	5×10^{12}	5×10^4
		10^6	5×10^{14}	5×10^6
10^6	5×10^6	10^0	5×10^6	5×10^{-2}
		10^2	5×10^8	5×10^0
		10^4	5×10^{10}	5×10^2
		10^6	5×10^{12}	5×10^4

generalizations can be made. Intact C6TG1A cells can generate at least 500 pmoles of cyclic AMP per minute per milligram of protein in response to isoproterenol, corresponding to 5×10^{12} molecules/sec/mg of protein (see Fig. 2). If we assume a range of turnover numbers for adenylate cyclase in these cells (maximally stimulated) and, in addition,

various molar ratios of receptor to cyclase, the calculations shown in Table 7 result. It is apparent that if the stimulated cyclase turnover number is relatively large and if there is a conservative stoichiometry (implying tight coupling) between the receptor and the cyclase, simplistic attempts to observe binding of [^{3}H]catecholamines to true receptors are doomed, even with highly active preparations such as C6TG1A and with saturating concentrations of catecholamine. The problem is obviously more acute either with particulate preparations of lesser activity or if minimal concentrations of [^{3}H]-catecholamine are needed to reduce nonspecific binding to acceptable levels. The initial approach seems clear: (a) to use the most active and homogeneous preparations available, (b) to use high concentrations of catecholamine (preferably isoproterenol) of high specific activity, (c) to search diligently for means to eliminate non-receptor binding, (d) to hope for low turnover numbers and a less conservative stoichiometry between receptor and cyclase, and (e) to attempt to obtain strict correlations between catecholamine binding data and adenylate cyclase activation. If this fails, novel ligands of higher specific activity may be required.

The final question concerns the reaction rates for association and dissociation of the catecholamine-receptor complex. It is clear that activation of adenylate cyclase is virtually instantaneous, and thus at least a fraction of the receptor population must be readily accessible. It is also clear that adenylate cyclase activity returns toward basal levels very quickly following removal of the catecholamine, implying rapid rates of dissociation (26).[2] These observations have obvious and important implications in choosing both incubation times and methods for separation of free and bound ligand.

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[2] M. E. Maguire and A. G. Gilman, unpublished observations.

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