BINDING OF 1-NOREPINEPHRINE TO ISOLATED RAT FAT CELL PLASMA MEMBRANES. EVIDENCE AGAINST COVALENT BINDING AND BINDING TO CATECHOL-O-METHYL TRANSFERASE.

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SUMMARY: Binding of 1-norepinephrine to isolated fat cell plasma membranes is rapid, saturable and reversible. Albumin gives an apparent decrease in norepinephrine binding and prevents saturation of the binding sites. The binding of norepinephrine is 10,000 fold less sensitive than is catechol-O-methyl transferase activity to inhibition by syringic acid and syringaldehyde, demonstrating that catechol-O-methyl transferase is not a significant binding protein in this system. Approximately 65% of the norepinephine is dissociable by incubation in Krebs-Ringer bicarbonate buffer pH 7.4 for 30 min at 37°, and all the remaining bound hormone is dissociated by 1N HCl. The dissociated material was shown to be norepinephrine by chromatography in two different solvent systems. Norepinephrine binding was inhibited by ferricyanide and by ascorbic acid, metabisulfite, and butylated hydroxytoluene.

INTRODUCTION: The possible role of NE[2] oxidation and covalent binding and the possibility of binding to COMT[2] has been investigated. These studies were done in light of the tendency of catecholamines to oxidize in aqueous solution (1), and the report that COMT may be present to some extent in the plasma membranes of fat cells (2). Cuatrecasas et al. (3) have recently invoked covalent binding to COMT to explain irreversible binding of catecholamines in their membrane systems. We now report that NE binding to the isolated rat fat cell plasma membrane is rapid, saturable and reversible, is decreased by albumin, is decreased by oxidizing and reducing agents, and does not involve COMT.

MATERIALS AND METHODS: Isolated fat cells were prepared from the epididymal fat pads of 160 gm Sprague-Dawley rats by the method of Rodbell (4). Fat cell ghosts were prepared by the method of Rodbell (5) as modified by R. Gorman (personal communication). The modification of the procedure involves vigorous,

1 - Taken in part from the Ph.D. thesis of S. Koretz.

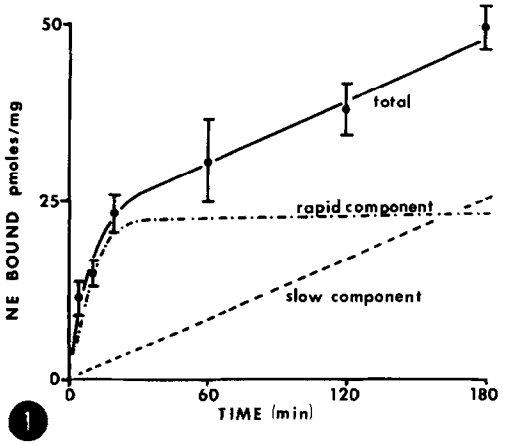
2 - NE, norepinephrine; COMT, catechol-O-methyl transferase

rather than gentle aggitation of the fat cells in the hypotonic lysing medium. The resulting fragmented ghosts were isolated by the two-phase aqueous polymer system of Lesko, et al. (6). The plasma membranes were washed three times in Krebs-Ringer-bicarbonate (KRB) buffer*, pH 7.4, with centrifugation at 9000 xg for 10 minutes at 4°C. l-Norepinephrine-7-H^3 (sp. act. 5 Ci/mmole) was obtained from New England Nuclear Corp.

Hormone binding was determined as follows: after incubation of membranes (50 µg protein) with labeled NE, the membranes were immediately chilled in ice and spun at 900 xg for 15 min at 4°C. The membrane pellets were resuspended in 2.0 ml of cold KRB buffer and immediately filtered through 0.2 µ EGWP Millipore filters (Millipore Corp.). The filters were washed rapidly three times with 5 ml of cold KRB buffer and counted in 10 ml of Beckman Biosolv/TLA toluene cocktail. Controls containing no membrane were also run and the counts in these controls were subtracted from the binding with membranes present. Counting was measured with a Packard Tri-Carb Liquid Scintillation Spectrometer.

The membrane bound NE which was dissociated by incubation in 2.0 ml of KRB buffer or 1N HCl was examined by chromatographic analysis in two separate solvent systems. System A consisted of chromatography on cellulose phosphate paper (Whatman P81) using n-butanol/ethanol/water, 1:1:1 v/v as described by Levin (7). System B consisted of thin layer chromatography on silica gel plates. Cat. No. 5763. (EM Laboratories, Inc.) using chloroform/methanol/acetic acid/water, 50:15:4:2 v/v. Five µg each of norepinephrine, normetanephrine and noradrenochrome (all from Sigma Chem. Co.) were run as standards and were detected by spraying the chromatograms with 10% $FeCl_3$/0.5% $K_3Fe(CN)_6$ (7). The chromatograms were divided into sections to include the visible components as well as those areas containing no visible components. The areas were removed and counted in 10 ml of Beckman Biosolv/TLA toluene cocktail in a Packard Tri-Carb liquid scintillation counter. The counts were corrected for quenching.

* The KRB buffer contained 118 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 1.2 mM KH_2PO_4.



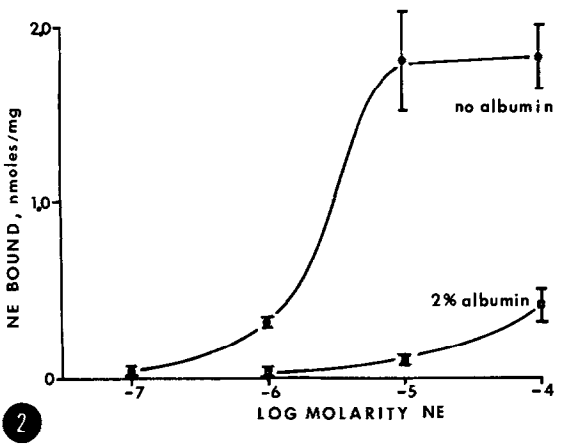


Figure 1. Time Course of Binding of NE to Fat Cell Plasma Membranes.

50 µg of membrane protein were incubated with 1 x 10^{-7}M l-NE (0.5 µ Ci) at 37° for various times in 2.0 ml KRB buffer, pH 7.3. The tubes were then chilled in ice, and hormone binding was carried out as given in the text. The values represent the mean ± SD of triplicate determinations.

Figure 2. Dose Response Curves for the Binding of NE to Fat Cell Plasma Membranes in the Presence and Absence of Albumin.

50 µg of membrane protein were incubated at 37° for 10 minutes with varying concentrations of l-NE in 2.0 ml KRB buffer pH 7.4 with or without 2% albumin (bovine serum albumin, Sigma Fraction V). The tubes were immediately chilled in ice, and hormone binding carried out as given in the text. The values represent the mean ± SD of triplicate determinations.

RESULTS AND DISCUSSION: The time course of NE binding is shown in Figure 1 There is a rapid saturable component having a T 1/2 of 10 min and a slower non-saturable component. At 10 min, the time used in all other experiments, the rapid component comprises 90% of the total binding. The dose response curves for the binding of NE to membranes in the presence and absence of albumin are shown in Figure 2. Albumin causes a shift of the curve to the right and prevents saturation of binding. This effect is most likely due to NE binding albumin, which decreases the effective concentration of free NE in the medium. Epinephrine binding to albumin has been reported by Powis (8). This effect of albumin may explain why high levels (about 10^{-5}M) of catecholamines are required to achieve maximal lipolysis in isolated fat cell systems (9-11).

The relative abilities of several NE analogues to inhibit NE binding are compared with their abilities to inhibit COMT (12) (Table I). The binding of NE is 10,000 fold less sensitive than COMT activity to inhibition by syringic acid and syringaldehyde. This finding is inconsistent with the hypothesis that COMT is the binding protein.

In contrast to the results of Cuatrecasas et al. (3), but in agreement with those of Lefkowitz et al. (13) and Bilezikian and Aurbach (14), we find that the bound NE is totally dissociable by 1N HCl within 5 minutes at 37°. We also find that 65% of the bound NE is dissociated by NE-free KRB buffer within 30 minutes at 37°, as shown in Figure 3. In addition, 75-79% of the labeled ma-

Table I

Ability of COMT Inhibitors to Inhibit NE Binding to Fat Cell Plasma Membranes

	BINDING DATA[a]		COMT DATA[b]	
COMT Inhibitor	(cpd)/(NE)	% inhib. of BINDING	(cpd)/(NE)	% inhib. of COMT
Syringaldehyde	1000/1	45±6 (3)	1/10	49
Syringic Acid	1000/1	38±11 (3)	1/10	38
Vanilmandelic Acid	1000/1	28±1 (6)	1/10	12
			1/1	39
Normetanephrine	1000/1	34±1 (6)	1/10	0
			1/1	12
Pyrogallol	1000/1	52±5 (3)		

[a] Fat cell plasma membranes were incubated in 2.0 ml of KRB buffer pH 7.4 with 10^{-7} M l-NE (0.5μ Ci) with and without 10^{-4}M COMT inhibitors listed in the table. Hormone binding was carried out as given in the text. The values represent the mean ± SD of 3 or 6 determinations (given in parenthesis). Syringic acid (3,5-dimethoxy-4-hydroxybenzoic acid) and syringaldehyde were obtained from K and K Fine Chemicals. The other agents were obtained from Sigma Chem. Co. The (cpd)/NE designates the molar ratios of these agents. (cpd represents the COMT inhibitor)

[b] Taken from Nikodejevic et al. (12).

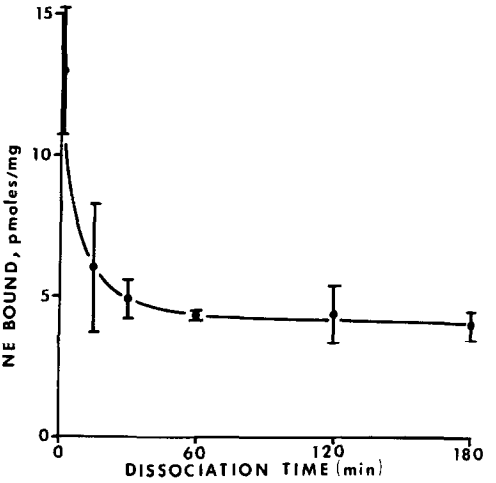


Figure 3. Dissociation of Membrane-Bound NE.

50 μg of membrane protein were incubated in 2.0 ml KRB buffer containing 1 x 10^{-7} M l-NE (0.5 μ Ci) at 37°C for 10 minutes. The membranes were immediately chilled and centrifuged at 900 xg for 15 min at 4°C and resuspended in 2.0 ml fresh KRB buffer. The membranes were incubated for various times at 37°C, centrifuged, resuspended in 2.0 ml of fresh buffer, filtered through 0.2 μ EGWP millipore filters, and counted. The values represent the mean ± SD of triplicate determinations.

terial which is released within 30 minutes by KRB buffer-co-chromatographed with NE. The labeled material that is not dissociated within 30 minutes by KRB buffer is completely dissociated by 1N HCl, and this material co-chromatographed with NE. The 75-79% recovery is a minimal value, since the NE undergoes oxidation to a small extent during the 10 min incubation with membranes and during the 30 min time required for dissociation. Most of the NE oxidation occurs during the spotting of the samples for chromatography. This is evidenced by streaking of the counts ahead of the major NE spot. The streaking pattern is similar to that observed with noradrenochrome. NE oxidation is evident during the spotting of the samples for chromatography, since the spots appeared yellow-orange.

Evidence that catecholamine oxidation to a cyclized product is not a prerequisite for binding is our observation that noradrenochrome fails to compete with NE for binding. Furthermore, oxidation of NE with a 2-fold excess of

$K_3Fe(CN)_6$ markedly inhibits binding (Table II). However, the reducing agents ascorbic acid and sodium metabisulfite and the anti-oxidant butylated hydroxytoluene also inhibit binding when present at 10^{-6}M to 10^{-4}M. The possibility that NE quinone, the intermediate oxidation product between NE and noradrenochrome, is in part the bound species has not yet been ruled out. The fact that all of the bound hormone is released by 1N HCl rules out covalent binding of NE via an oxidation mechanism proposed by Saner and Thoenen (1).

The biological significance of the binding of NE to cell membranes has yet to be determined. The requirement of a catechol group for high affinity binding is evident from previous work (15-20) and is in agreement with the observation of

Table II

Effect of Reducing Agents and Oxidizing Agents on NE Binding to Fat Cell Plasma Membranes[a]

Test Agent		Binding, % of Control
Butylated Hydroxytoluene,	10^{-6}M	83 ± 6
	10^{-4}M	75 ± 5
Ascorbic acid,	10^{-6}M	58 ± 2
	10^{-4}M	50 ± 4
Sodium Metabisulfite,	10^{-6}M	52 ± 11
	10^{-4}M	35 ± 2
$K_3Fe(CN)_6$	2×10^{-7}M	23 ± 10
	1×10^{-3}M	8 ± 4

[a] Membranes (50 μg protein) were incubated for 10 min at 37°C with 10^{-7}M NE (0.5 μ Ci) in 2.0 ml of KRB buffer pH 7.4 with and without the test agents given in the Table. Hormone binding was carried out as given in the text. The values represent the mean ± SD of 3 determinations.

Furchgott (21) that a catechol group is required for full agonistic β-receptor activity in heart muscle. However, Feller and Finger (9) conclude that a catechol ring is not obligatory for producing a maximal release of free fatty acids in adipose tissue. Their results indicated that a phenolic group in either the meta or para position was sufficient to produce a maximal lipolytic response when the β-carbon atom was also hydroxylated. Moreover, N-substitution with a bulky alkyl or aralkyl group greatly enhanced the lipolytic activity of the phenethylamine molecule. Trimetoquinol shows the dramatic effect of a large aralkyl group as discussed by Fain (22). Lincova et al. (11) also stress the importance of N-substitution of catecholamines and oxedrines for maximal lipolytic activity in adipose tissue.

After this paper was submitted for publication a report appeared by Lefkowitz (23) giving evidence that norepinephrine binding to microsomal membranes from dog skeletal muscle, cardiac muscle, spleen, kidney and liver was unrelated to COMT.

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