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HORMONE ACTION AT THE MEMBRANE LEVEL

IV. EPINEPHRINE BINDING TO RAT LIVER PLASMA MEMBRANES AND RAT EPIDIDYMAL FAT CELLS

L. LESKO and G. V. MARINETTI

Department of Biochemistry, University of Rochester School of Medicine & Dentistry, Rochester, New York 14642 (U.S.A.)

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SUMMARY

[^{3}H]Epinephrine binding to isolated purified rat liver plasma membranes is a reversible process. An initial peak in binding occurs at about 15 min and a plateau occurs by 50 min. Optimal binding occurred at a membrane protein concentration of 125μg. Rat liver plasma membranes stored at −70 °C up to 4 weeks showed no difference in epinephrine binding capacity as compared to control fresh membranes.

Epinephrine binding to liver plasma membranes was decreased by 79 % by phospholipase A_2 (phosphatide acylhydrolase EC 3. 1. 1. 4), 81 % by phospholipase C (phosphatidylcholine choline phosphohydrolase EC 3.1.4.3) and 59 % by phospholipase D (phosphatidylcholine phosphatidohydrolase EC 3.1.4.4). Trypsin and pronase digestion of the membrane decreased epinephrine binding by 97 and 47 % respectively.

In the presence of 10^{-3}M Mg^{2+} ions, increasing concentrations of GTP decreased epinephrine binding to liver plasma membranes. A maximal effect was demonstrated with 10^{-5}M GTP, representing an inhibition of 52 % of the control. In a Mg^{2+}-free system, epinephrine binding was unaffected by GTP. However, in a Mg^{2+}-free system, increasing concentrations of ATP cause increasing inhibition of hormone binding. ATP at 10^{-3} M reduced epinephrine binding to 28 % of the control. GTP (10^{-5}M) was shown to inhibit epinephrine uptake rather than epinephrine release from the membrane.

[^{3}H]Epinephrine binding to isolated rat epididymal fat cells shows an initial peak within 5 min followed by a gradual rise which plateaus after 60 min. Epinephrine binding increased nearly linearly with increasing fat cell protein concentration (40–200 μg protein).

GTP (10^{-5}M) and ATP (10^{-4}M) decreased epinephrine binding to rat epididymal fat cells by 41 %. Nearly complete inhibition of binding was demonstrated with 10^{-2}–10^{-3}M ATP. Epinephrine analogs that contain two hydroxyl groups in the 3 and 4 position on the benzene ring act as inhibitors of [^{3}H]epinephrine binding

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[a] The Krebs-Ringer bicarbonate buffer contained the following: 118 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 4.3 mM glucose, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$ and 1.2 mM KH_2PO_4.

to rat adipocytes. Alteration of the epinephrine side chain has relatively little influence on binding. Analogs in which one of the ring hydroxyl groups is missing or methylated are poor inhibitors of [^{3}H]epinephrine binding.

Alpha-(phentolamine and phenoxybenzamine) and beta-(propranolol and dichorisoproterenol) adrenergic blocking agents were tested with respect to their ability to influence [^{3}H]epinephrine binding and their influence on epinephrine-stimulated lipolysis. Only dichloroisoproterenol significantly inhibited epinephrine binding (by 25 %). The two beta-adrenergic blocking agents caused an inhibition of epinephrine-stimulated glycerol release, with propranolol being most effective. Phentolamine and phenoxybenzamine had no significant effect on the epinephrine stimulation of glycerol release by fat cells.

INTRODUCTION

Epinephrine binds to proteins in the rat liver plasma membrane and this hormone-membrane interaction precedes the activation of adenylate cyclase [1, 2]. Rat adipocyte ghosts possess an adenylate cyclase system [3] which is activated by lipolytic hormones such as epinephrine, glucagon and adrenocorticotropin [4].

Previous studies [5–8] have shown that analogs of epinephrine containing two hydroxy groups in the three and four positions of the benzene ring act as competitive inhibitors of epinephrine binding whereas analogs in which one of the ring hydroxyl groups is missing or methylated exhibit a low affinity for the epinephrine binding sites. Added insulin and glucagon have no effect on epinephrine. At present it is believed that separate and distinct receptors exist for each hormone [9–12].

The interaction of catecholamines with isolated rat adipocyte membranes [13], turkey erythrocyte membranes [7, 8] sarcolemma plasma membranes prepared from rabbit skeletal muscle [14] and cardiac membranes [15–17] has been well documented.

Recently Cuatrecasas et al. [18] have reported the binding of catecholamines to microsomal membranes and suggest that in their systems a major part of the binding may be associated with the enzyme catechol-*O*-methyl transferase. However, Lefkowitz [19] has recently reported that in his systems the binding of norepinephrine is unrelated to catechol-*O*-methyl transferase.

This paper examines the properties of epinephrine binding to rat liver plasma membranes and to isolated fat cells and the effects of certain agents on this binding.

EXPERIMENTAL SECTION

Materials

l-Epinephrine, l-bitartrate (7-^{3}H(N)) (spec. act: 9.45–10.3 Ci/mol) was obtained from New England Nuclear Corporation. The following reagents were used: l-epinephrine-bitartrate (CalBiochem), ATP (disodium salt), GTP (sodium salt, (P-L Biochemicals, Inc.), and bovine serum albumin (fraction V, Sigma Chemical Company). Trypsin was purchased from Worthington Biochemical Corporation, pronase from Calbiochem. Phospholipase A_2 (*Naja naja*) was obtained from Miami Serpentarium Laboratories, phospholipase C (*Clostridium welchii*) and phospholipase D from Sigma Chemical Company. Collagenase was obtained from Worthington

Biochemical Corp. The following analogs were tested: l-phenylalanine, l-tyrosine, (+),l-3-methoxy-4-hydroxymandelic acid, (+),l-3,4-dihydroxymandelic acid, 1-3,4-dihydroxyphenylalanine, 1-3,4-dihydroxyphenylglycol, metanephrine, and l-isoproterenol-(+)-bitartrate (all from CalBiochem), Vasoxyl (methoxamine · HCL) (Burrough Wellcome Company), 6-hydroxydopamine-HBr (Regis Chemical Company) and 4-nitrocatechol (Aldrich Chemical Company). Alpha-adrenergic blocking agents phentolamine and phenoxybenzamine · HCL were purchased from Ciba Chemicals and Smith, Kline and French, respectively. Beta-adrenergic blocking agents propranolol and dichloroisoproterenol were obtained from Ayerst Labs and Eli Lilly, respectively. l-Cysteine · HCL, NAD^+(from Sigma Chemical Company), hydrazine (K and K Laboratories), EDTA (Fisher Chemicals), glycerol (Fisher Chemicals), glycerol-kinase and glycerolphosphate dehydrogenase (from Sigma Chemical Company) were used in the determination of epinephrine-stimulated glycerol release. Type EGWP Millipore filters, 25 mm diameter, 0.2 μ pore size from Millipore Corporation were employed in the binding assay.

Methods

Epinephrine Binding to Plasma Membranes. Plasma membranes were isolated from the livers of 120–160 g male Sprague-Dawley rats (Blue Spruce Farms, Altamont, New York) fed ad libitum on Purina Chow. The animals were sacrificed by decapitation. Membranes were prepared by the method of Neville [20] as modified by Ray [21]. In the experiments studying the effects of ATP and GTP on epinephrine binding, the membrane preparations after isolation were washed once with 1 mM EDTA to remove membrane-bound calcium and magnesium ions.

The washed membrane pellets obtained by centrifugation at $2400 \times g$ were resuspended in Krebs Ringer Bicarbonate [a] buffer, pH 7.3. After incubation the samples were placed in a Millipore suction apparatus containing a 0.2 μ pore size type EGWP Millipore filter (25 mm diameter) which had previously been rinsed with 2 successive 5 ml aliquots of the buffer, pH 7.3. The mixture was completely filtered by vacuum within 8 s and then washed with 4 successive 5-ml portions of chilled buffer, pH 7.3. The filters were removed, allowed to dry and placed in scintillation vials containing 10 ml of Beckman's toluene/BBS fluor scintillation cocktail. Blanks containing all reagents except the membranes were run simultaneously in each experiment to correct for the amount of [^{3}H]epinephrine bound to the filters. Samples were counted in a Packard Tri-Carb Scintillation Spectrometer Model 314. [^{3}H] Epinephrine binding is expressed as pmol bound/mg membrane protein.

Epinephrine Binding to Rat Epididymal Fat Cells. Fat cells were isolated from epididymal fat pads of 120–160g male Sprague-Dawley rats by the method of Rodbell [22]. Hormone binding was determined by direct Millipore filtration as described in the previous section.

Protein concentration was determined by the method of Lowry et al. [23] with bovine serum albumin as the standard.

Determination of fat cell number.

Epididymal fat cell number was determined by counting a known volume of isolated cells in a 1.0 mm^2 area of a hemacytometer grid (Spencer Bright Line, American Optical Company) using an Ernst Leitz Wetzlar microscope ($35 \times$ magnification).

Determination of Epinephrine-Stimulated Glycerol Release From Rat Epididymal Fat Cells. Glycerol release, which reflects the lipolytic activity of the fat cell, was determined by the enzymatic fluorometric method described by Laurell and Tibbling [24]. Fat cells (50–90 μg fat cell protein) were incubated in 2.0 ml of Krebs Ringer bicarbonate/glucose buffer, pH 7.3, containing $1 \cdot 10^{-5}$M l-epinephrine-bitartrate and blocking agent at a final concentration of $1 \cdot 10^{-5}$M for 20 min at 37 °C. Controls in the absence of blocking agent and/or epinephrine were incubated simultaneously. The incubation was terminated by the addition of 1.0 ml of 3 % perchloric acid. After 15 min, the suspensions were centrifuged for 10 min in an IEC clinical centrifuge equipped with No. 221 rotor, at 3000 rev./min. One-ml aliquots of the deproteinized supernatants were neutralized to pH 7.0 with 1 ml of freshly prepared saturated $KHCO_3$ solution. The suspensions were centrifuged at 3000 rev./min for 7 min. Aliquots were removed for glycerol analysis [24].

The fluorescence was measured with a Perkin-Elmer Fluorescence Spectrophotometer Model MPF-3 at 350 nm exciting wavelength and 458 nm emitting wavelengths (slit arrangement No. 4 nm, sensitivity No. 30). Aliquots of the glycerol standards at concentrations of 0.0125–1 mM, were used for construction of a calibration curve.

Results

Exton et al. [25] found that the half maximal cyclic-AMP response in perfused rat liver required an epinephrine concentration of $1 \cdot 10^{-7}$M. This concentration was

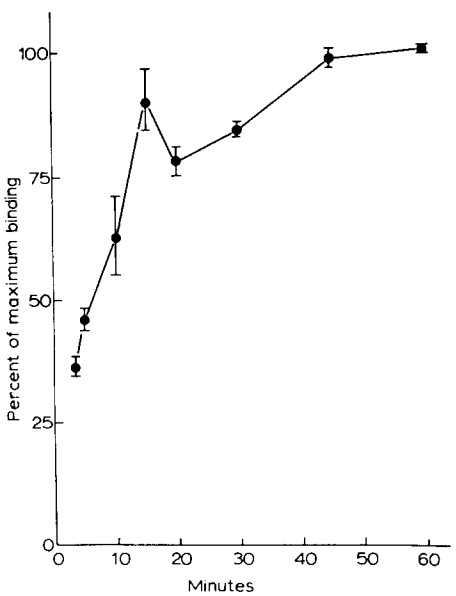

Fig. 1. Epinephrine binding to rat liver plasma membranes as a function of the length of incubation time. The plasma membranes (400–600 μg membrane protein/ml) suspended in 8.0 ml of Krebs Ringer bicarbonate buffer, pH 7.3, containing $4 \cdot 10^{-7}$ M [^{3}H]epinephrine (15 μCi) were incubated at 37 °C for 3, 5, 10, 20, 30, 45 and 60 min. Aliquots of these membranes containing 80–120 μg of membrane protein were applied to the filtering system and binding was determined by direct Millipore filtration as explained in the text. Each point represents the mean ±S.E. of triplicate determinations of three separate experiments.

taken as a reasonable physiological concentration for the binding studies of this work.

A binding time of 10 min was used in this study even though the binding of the hormone to liver membranes required about 1 h to reach a plateau (Fig. 1). The 10 min time was chosen in order to minimize catecholamine oxidation.

An initial sharp peak in epinephrine binding to liver membranes occurs at about 15 min (Fig. 1). After 50 min, the amount of [^{3}H]epinephrine bound had reached equilibrium. Each liver membrane preparation revealed an initial peak in binding at about 15 min. The nature of this initial peak has not been elucidated.

Epinephrine binding to adipocytes (Fig. 2) also revealed an initial peak within 5 min followed by a slower uptake which plateaued after 60 min. The peak in binding was also observed when binding was represented as pmol hormone bound/10^6 cells.

Hormone binding to liver membranes increased nearly linearly over the concentration range of 50–125 μg membrane protein (Fig. 3). When the membrane protein exceeds 125 μg, the amount of epinephrine bound decreases slightly. Optimal binding occurs at about 125 μg membrane protein.

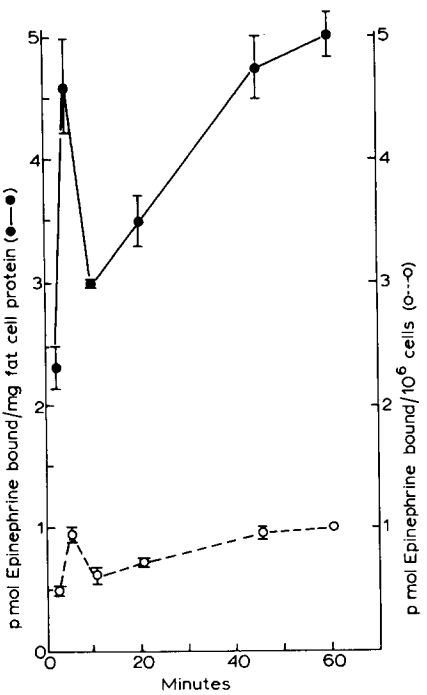

Fig. 2. Epinephrine binding to rat epididymal fat cells as a function of the length of incubation time. Epididymal fat cells (250–500 μg fat cells protein/ml) suspended in 7.0 ml of Krebs Ringer bicarbonate/albumin/glucose buffer, pH 7.3, containing $4 \cdot 10^{-7}$ M [^{3}H]epinephrine (15 μCi) were incubated at 37 °C. After timed intervals of 3, 5, 10, 20, 30, 45 and 60 min, aliquots of cells containing 80–120 μg fat cell protein were used for analysis of hormone binding. Each point represents the mean $\pm$S.E. of duplicate samples of one experiment. The same pattern was observed in three separate experiments. Hormone binding is expressed as pmol hormone bound/mg fat cell protein and pmol hormone bound/10^6 cells.

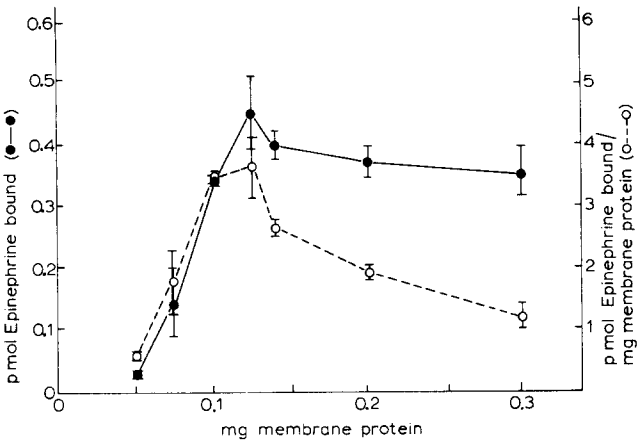

Fig. 3. Epinephrine binding to rat liver plasma membranes as a function of membrane protein concentration. The incubation system contained in a final volume of 2.0 ml Krebs Ringer bicarbonate buffer, pH 7.3, $1 \cdot 10^{-7}$ M [^{3}H]epinephrine (1.0–1.5 μCi) and varying amounts of membrane protein in aliquots of 50, 75, 100, 125, 200, and 300 μg. Incubation was carried out for 10 min at 37 °C. Binding was determined by the direct Millipore filtration as explained in the text. Each point represents the mean ± S.E. of triplicate determinations of four separate experiments.

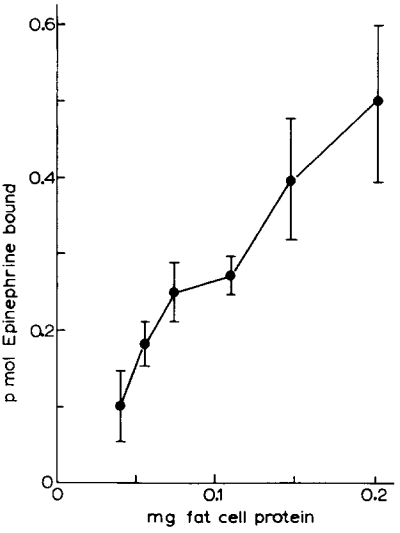

Fig. 4. Epinephrine binding to rat epididymal fat cells as a function of fat cell protein concentration. The incubation system contained in a final volume of 2.0 ml, Krebs Ringer bicarbonate/albumin/glucose buffer, pH 7.3, $1 \cdot 10^{-7}$ M [^{3}H]epinephrine (1.5 μCi) and varying amounts of fat cell protein (37, 55, 74, 111, 148 and 203 μg protein). After a 10 min incubation at 37 °C, binding was determined by Millipore filtration as described in the text. Each point represents the mean ± S.E. of triplicate determinations of three separate experiments.

The binding of [^{3}H]epinephrine as a function of fat cell protein concentration was studied (Fig. 4). The binding increased nearly linearly over the concentration range of 40–200 μg fat cell protein.

Isolated rat liver plasma membranes can be stored up to 4 weeks at -70 °C with no significant change in [^{3}H]epinephrine binding (data not shown). Freychet et al. [26] found that frozen liver plasma membanes are stable up to three months with respect to insulin binding.

Modification of the rat liver plasma membrane. The structure of the rat liver plasma membrane was modified by chemical, physical and enzymatic treatment. By altering the structure of the membrane lipids and proteins or by modifying protein-lipid interactions and measuring the changes in hormone binding, it may be possible to gain some insight into the nature of the hormone receptors in the membrane.

Binding of epinephrine at different temperatures showed a plateau up to 60 °C and an increase from 60 °C to 80 °C (Fig. 5). The increase in binding seen at the elevated temperatures is believed to represent in part non-specific covalent binding of oxidized catecholamine to membrane proteins, and in part represents entrapment of the hormone in denatured coagulated membrane particles.

The possible role of phospholipids in epinephrine binding to rat liver plasma membranes was investigated by the use of phospholipases A_2, C and D. Phospholipases A_2 and C decreased epinephrine binding by 79 to 81 %. Phospholipase D treatment resulted in a 59 % decrease in hormone binding (Table I). These results suggest that the presence of intact phospholipids are required for optimal epinephrine binding.

Structural modification of the membrane was also achieved by peptide bond hydrolysis using trypsin and pronase. The data in Table I show that proteolytic hydrolysis of the membrane proteins markedly decreases epinephrine binding. At a trypsin and pronase concentration of 50 μg/100 μg membrane protein, binding of epinephrine is decreased by 97 and 47 % respectively.

The effect of cations and nucleotides on [^{3}H]epinephrine binding. Rodbell et al. [27–29] have shown that nucleotides can modify hormone binding to cell membranes and the hormone stimulation of adenylate cyclase. Moreover, the cell plasma membrane contains several enzymes which act on nucleotides. The isolated rat liver plasma membrane has been shown to contain Mg^{2+}-stimulated ATPase, Na^+,K^+-activated ATPase, ATP pyrophosphorylase, 5′-mononucleotidase and adenylate cyclase [2, 21]. Since the plasma membrane is active in nucleotide metabolism and also binds epinephrine, the effect of nucleotides on this binding was examined.

In the presence of Mg^{2+} ions (10^{-3}M), increasing concentrations of GTP decreased [^{3}H]epinephrine binding to rat liver plasma membranes (Table II). A maximal effect was demonstrated with 10^{-5}M GTP, representing an inhibition of 52%. In a Mg^{2+}-free system, epinephrine binding was unaffected by this nucleotide. These results raised the question whether GTP preferentially inhibited the uptake of epinephrine or enhanced the dissociation of membrane-bound hormone. It was demonstrated that 10^{-5} M GTP did not have any significant effect on the rate of dissociation of membrane-bound epinephrine over a period of 60 min (Fig. 6). However, this concentration of GTP did inhibit the uptake of epinephrine to the plasma membrane (Fig. 7). This inhibition was maintained throughout the hour-long incubation, and the main effect of GTP was to eliminate the early peak in epinephrine binding.

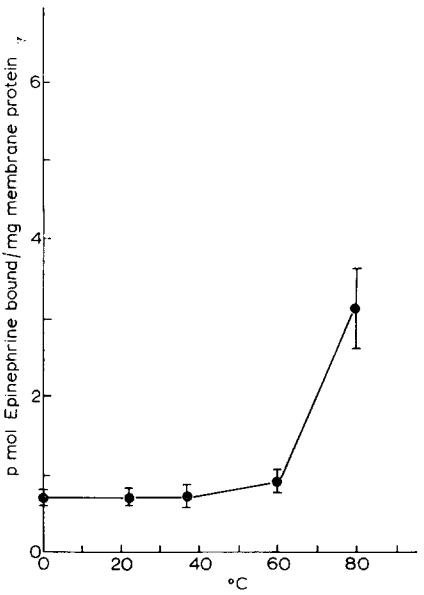

Fig. 5. Epinephrine binding to rat liver plasma membranes as a function of temperature of incubation. The incubation system contained 90–120 μg of membrane protein and $1 \cdot 10^{-7}$ M [^{3}H]epinephrine (1.0–1.5 μCi) in Krebs Ringer bicarbonate buffer, pH 7.3 in a final volume of 2.0 ml. This mixture was incubated for 10 min at 0, 37, 60 and 80 °C. The amount of epinephrine bound to the membrane was determined by direct Millipore filtration as described in the text. Each point represents the mean $\pm$ S.E. of triplicate determinations of three separate experiments.

[^{3}H]Epinephrine binding to plasma membranes (sarcolemma) prepared from rabbit skeletal muscle has been reported by Vallieres et al. [14] to be inhibited by GTP at concentrations above $8 \cdot 10^{-8}$M. GTP and GDP at concentrations of $5 \cdot 10^{-8}$M stimulate the rate and degree of dissociation of bound [^{123}I]-labeled glucagon from rat liver plasma membranes and decrease the affinity of the binding sites for glucagon to the membranes [27–29]. It was demonstrated that the rate of dissociation of [^{125}I]-labeled glucagon is more affected by GTP than the rate of glucagon association. In contrast to this effect of GTP on glucagon we find that epinephrine uptake is primarily affected by GTP rather than its dissociation.

Data presented in Table II show that in the presence of 10^{-3}M Mg^{2+} ions, only 10^{-2}M ATP caused a marked decrease in [^{3}H]epinephrine binding to liver plasma membranes. Concentrations of 10^{-3}M and 10^{-4}M ATP appeared to decrease slightly the epinephrine binding. In a Mg^{2+}-free system, increasing concentrations of ATP produced the maximal (87 %) decrease in binding. This is in contrast to GTP where no significant inhibition of hormone binding occurred in the absence of added Mg^{2+} ions.

Tomasi et al. [1] reported inhibition of [^{3}H]epinephrine binding to rat liver plasma membranes with 1–2 mM ATP. ATP at a concentration of 1–5 mM has been shown by Lefkowitz et al. [17] to decrease [^{3}H]norepinephrine binding to a subcellular fraction from canine cardiac muscle by 74 %.

TABLE I

THE EFFECT OF PHOSPHOLIPASES AND PROTEASES ON EPINEPHRINE BINDING TO RAT LIVER MEMBRANES

Phospholipase A_2 (*Naja naja*) (5 μg/100 μg membrane protein), phospholipase C (*Cl. welchii*) (5 μg/100 μg membrane protein) or phospholipase D (5 μg/100 μg membrane protein) was added to 4.0 ml of plasma membrane suspension (450–600 μg membrane protein) in Krebs Ringer bicarbonate buffer, pH 7.3. The membranes were incubated at 37 °C for 45 min and then centrifuged and washed 3 times with 4.0 ml of Krebs Ringer bicarbonate buffer, pH 7.3. Aliquots of the phospholipase-treated membrane (90–120 μg membrane protein) were incubated in 2.0 ml of Krebs Ringer bicarbonate buffer, pH 7.3, containing $1 \cdot 10^{-7}$ M [^{3}H]epinephrine (1.0–1.5 μCi) for 10 min at 37 °C. Trypsin (50 μg of trypsin/100 μg membrane protein) or pronase (50 μg of pronase /100 μg membrane protein) were added to 4.0 ml of membrane suspension (500–600 μg membrane protein/ml) in Krebs Ringer bicarbonate buffer, pH 7.3. The membrane suspension was incubated for 1 h at 37 °C and then centrifuged and washed three times with 4.0 ml of Krebs Ringer bicarbonate buffer, pH 7.3. Aliquots of the treated membranes (90–130 μg of membrane protein) were then incubated in 2.0 ml of Krebs Ringer bicarbonate buffer, pH 7.3 containing $1 \cdot 10^{-7}$ M [^{3}H]epinephrine (1.0–1.5 μCi) for 10 min at 37 °C. Binding was determined by direct Millipore filtration. Each value represents the mean $\pm$ S.E. of triplicate determinations of three separate experiments.

Agent	Epinephrine Bound % of Control
Phospholipase A_2	20.8 $\pm$ 4.6
Phospholipase C	18.8 $\pm$ 3.0
Phospholipase D	40.7 $\pm$ 8.3
Trypsin	3.3 $\pm$ 0.0
Promase	53.3 $\pm$ 16.3

TABLE II

EFFECT OF GTP AND ATP ON EPINEPHRINE BINDING TO RAT LIVER PLASMA MEMBRANES

Rat liver plasma membranes were washed with 1 mM EDTA and resuspended in Krebs Ringer bicarbonate buffer, pH 7.3 containing either no Mg^{2+} ions or $1 \cdot 10^{-3}$ M Mg^{2+} ions. Aliquots of the membrane suspension (90–130 μg of membrane protein) were added to an incubation volume of 2.0 ml containing Krebs Ringer bicarbonate buffer, pH 7.3 (Mg^{2+}-free or $1 \cdot 10^{-3}$ M Mg^{2+} ions), $1 \cdot 10^{-7}$ M [^{3}H]epinephrine (1.5 μCi) and varying concentrations of GTP or ATP. The incubation was carried out for 10 min at 37 °C. Binding was determined by direct Millipore filtration. Each value represents the mean $\pm$ S.E. of triplicate determinations of three separate experiments. *P* values are relative to the control.

	Epinephrine bound (pmol/mg membrane protein)	
	10^{-3} M Mg^{2+}	Mg^{2+}-free
Control	4.3 $\pm$ 0.7	4.6 $\pm$ 1.2
10^{-6} M GTP	3.6 $\pm$ 0.7 $P < 0.50$	3.1 $\pm$ 0.8 $P < 0.25$
10^{-5} M GTP	2.7 $\pm$ 0.2 $P < 0.10$	3.3 $\pm$ 1.3 $P < 0.50$
10^{-4} M ATP	3.3 $\pm$ 1.2 $P < 0.50$	2.3 $\pm$ 0.7 $P < 0.10$
10^{-3} M ATP	2.1 $\pm$ 0.7 $P < 0.10$	1.3 $\pm$ 0.1 $P < 0.005$
10^{-2} M ATP	0.8 $\pm$ 0.3 $P < 0.005$	0.6 $\pm$ 0.1 $P < 0.005$

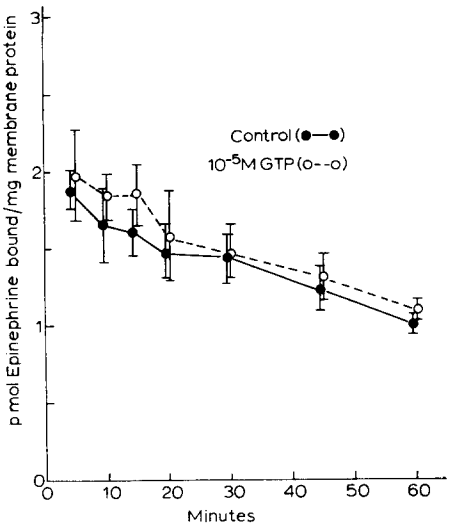

Fig. 6. The effect of GTP on the dissociation of membrane-bound epinephrine. Fresh plasma membranes were prepared as described in the text. Two 5 ml volumes of membrane (240 μg membrane protein/ml) suspended in Krebs Ringer bicarbonate buffer, pH 7.3, ($1 \cdot 10^{-3}$ M Mg^{2+} ions) were incubated with $4 \cdot 10^{-7}$ M [^{3}H]epinephrine (15 μCi) for 15 min at 37 °C. The membrane-hormone complex was washed three times with 4 ml Krebs Ringer bicarbonate buffer, pH 7.3. $1 \cdot 10^{-5}$ M GTP was added to one of the two tubes and the mixture was incubated. At timed intervals of 5, 10, 15, 20, 30, 45 and 60 min, 0.5 ml aliquots (120 μg membrane protein) were removed and applied to the filtering apparatus. Controls were incubated in a GTP-free assay system. Epinephrine dissociation from the membrane was measured by direct Millipore filtration. Each value represents the mean $\pm$ S.E. of duplicate determinations of one experiment which was representative of three such experiments.

The addition of 10^{-6}M GTP did not affect [^{3}H] epinephrine binding to epididymal fat cells (Table III). However, inhibition of binding was demonstrated with 10^{-5}M GTP. Epinephrine binding per mg fat cell protein was decreased by 41 % ($p < 0.10$) and binding/10^6 cells was decreased by 72 % ($p < 0.05$). Inhibition of epinephrine binding was also shown with 10^{-4}M ATP; epinephrine binding per mg fat cell protein was lowered by 38 % ($p < 0.05$). When the concentration of ATP was increased to 10^{-2}M, epinephrine binding was completely inhibited. Cryer et al. [30] demonstrated an inhibitory effect of GTP, CTP, UTP as well as GDP and GMP on the adenylate cyclase system of fat cell membranes.

Tomasi et al. [1] and Dunnick and Marinetti [5] demonstrated that certain functional groups of the epinephrine molecule were involved in its binding to rat liver plasma membranes. It was found that analogs containing two hydroxyl groups in positions 3 and 4 of the benzene ring act as inhibitors of epinephrine binding. Similar observations have been made by Schramm et al. [7] and Bilezikian et al. [8] in turkey erythrocytes.

To extend these studies, several epinephrine analogs were tested as potential inhibitors of epinephrine binding to isolated rat epididymal fat cells (Table IV).

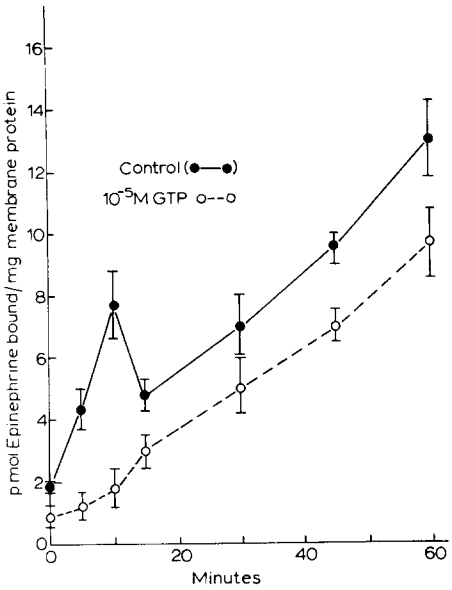

Fig. 7. The effect of GTP on the rate of epinephrine binding to rat liver plasma membranes. A 10 ml aliquot of fresh rat liver plasma membranes (240 μg membrane protein/ml) suspended in Krebs Ringer bicarbonate buffer, pH 7.3 containing 10^{-3} M Mg^{2+} was divided into two portions. One half was incubated for 15 min at 37 °C with $1 \cdot 10^{-5}$ M GTP and the other one half served as the control. After 15 min of incubation, $4 \cdot 10^{-7}$ M [^{3}H]epinephrine (15 μCi) was added to both membrane suspensions. At timed intervals of 5, 10, 15, 20, 30, 45 and 60 min, 0.5 ml aliquots (120 μg of membrane protein) were removed and applied to the filtering system. Uptake of hormone to the GTP-treated and control membrane was measured by direct Millipore filtration. Each value represents the mean $\pm$ S.E. of duplicate determinations of three separate experiments.

1-Tyrosine, 1-phenylalanine, (+), 1-3-methoxy-4-hydroxy-mandelic acid, metanephrine and methoxyamine did not inhibit epinephrine binding. On the other hand, epinephrine, norepinephrine, isoproterenol, (+),1-3,4-dihydroxymandelic acid, dihydroxyphenylalanine, 1-3,4-dihydroxyphenylglycol and 6-hydroxydopamine were strong inhibitors. These data demonstrate that analogs having the 3,4-dihydroxybenzene (catechol) structure possess a high affinity for the epinephrine binding site, whereas analogs in which one of the ring hydroxyls is missing or is methylated are poor inhibitors of epinephrine binding. Alteration of the epinephrine side chain had little effect on binding. The side chain is believed to play a role in coupling the hormone binding to its physiological action.

The catecholamine-stimulated lipolysis in adipose tissue of most species of animals is believed to involve adrenergic beta receptors [31]. It was of interest to test whether alpha- and beta- blocking agents inhibit the binding of epinephrine to rat epididymal fat cells and thus act at this primary site on the membrane or whether they uncouple the binding. At 10^{-5}M, only dichloroisoproterenol significantly inhibited binding (Table V). However, both of the beta-adrenergic blocking agents tested caus-

TABLE III

THE EFFECT OF GTP AND ATP ON EPINEPHRINE BINDING TO RAT EPIDIDYMAL FAT CELLS

Aliquots of a fat cell suspension (50–90 μg fat cell protein) were added to 2.0 ml of Krebs Ringer bicarbonate/albumin/glucose buffer, pH 7.3, containing $1 \cdot 10^{-7}$ M [^{3}H]epinephrine (1.0–1.5 μCi) and varying concentrations of GTP: $1 \cdot 10^{-5}$ M and $1 \cdot 10^{-6}$ M, or varying concentrations of ATP: $1 \cdot 10^{-4}$ M, $1 \cdot 10^{-3}$ M and $1 \cdot 10^{-2}$ M. All incubations were performed for 10 min at 37 °C. Fat cells were incubated simultaneously in a nucleotide-free system. Binding was determined by the direct Millipore filtration method. Each value represents the mean±S.E. of triplicate analyses from three separate experiments. *P* values are relative to the controls. The system contained 1.2 mM Mg^{2+}.

	Amount of epinephrine bound	
	(pmol/mg fat cell protein)	(pmol/10^6 cells)
Control	15.6±1.6	3.6±0.8
$1 \cdot 10^{-6}$ M GTP	15.1±3.5 $P < 0.50$	2.9±0.7 $P < 0.50$
$1 \cdot 10^{-5}$ M GTP	9.2±2.3 $P < 0.10$	1.0±0.3 $P < 0.05$
$1 \cdot 10^{-4}$ M ATP	9.7±1.7 $P < 0.05$	2.3±0.6 $P < 0.25$
$1 \cdot 10^{-3}$ M ATP	no binding	no binding
$1 \cdot 10^{-2}$ M ATP	no binding	no binding

TABLE IV

THE EFFECT OF EPINEPHRINE ANALOGS ON THE BINDING OF [^{3}H]EPINEPHRINE TO RAT EPIDIDYMAL FAT CELLS

Fat cells were added in aliquots of 50–90 μg fat cell protein to 2.0 ml of Krebs Ringer bicarbonate/albumin/glucose buffer pH 7.3 containing $1 \cdot 10^{-4}$ M concentration of analog. Following a 10 min incubation at 37 °C, [^{3}H]epinephrine was added to a final concentration of $1 \cdot 10^{-7}$ M (1.0 μCi). The incubation proceeded for an additional 10 min; the suspension was applied to the Millipore filtration apparatus. Each value represents the mean percent±S.E. of triplicate analysis of three separate experiments.

Hormone analog added	Epinephrine bound % of the control
unlabeled l-epinephrine	7.4±1.6
metanephrine	24.1±6.8
isoproterenol	3.2±2.4
l-norepinephrine	15.1±2.4
l-3,4-dihydroxyphenylalanine	7.8±3.3
l-3,4-dihydroxyphenylglycol	8.5±4.0
6-hydroxydopamine	6.3±1.8
(+), l-3,4-dihydroxymandelic acid	1.3±1.3
(+), l-3-methoxy-4-hydroxymandelic acid	68.4±5.8
methoxamine (vasoxyl)	107.0±6.5
l-phenylalanine	97.9±3.3
l-tyrosine	85.8±5.2
4-nitrocatechol	0–5

TABLE V

THE EFFECT OF ALPHA AND BETA ADRENERGIC BLOCKING AGENTS ON EPINEPHRINE BINDING TO RAT EPIDIDYMAL FAT CELLS AND GLYCEROL RELEASE

Fat cells (50–90 μg fat cell protein) isolated from rat epididymal fat pads as described in the text were incubated in 2.0 ml of Krebs Ringer bicarbonate/albumin/glucose buffer, pH 7.3, containing $1 \cdot 10^{-7}$ M [^{3}H]epinephrine (1.5 μCi) and alpha and beta adrenergic blocking agents at a concentration of $1 \cdot 10^{-5}$ M for 10 min at 37 °C. Control samples without blocker were incubated simultaneously. The amount of epinephrine bound was determined by direct Millipore filtration. Each value for $1 \cdot 10^{-5}$ blocker represents the mean ±S.E. of triplicate determinations of eight separate experiments. Glycerol was determined as given in the text.

	Epinephrine bound % of control	Epinephrine-stimulated glycerol release % of control
Alpha Blockers:		
Phentolamine	103.0±8.4	89.0±2.6
Phenoxybenzamine	95.6±8.1	94.2±2.2
Beta Blockers:		
Propranolol	98.9±8.9	44.4±5.6
Dichlorisoproterenol	74.6±6.1	77.5±2.4

ed an inhibition of epinephrine-stimulated glycerol release, with propranolol being the most effective. Propranolol and dichloroisoproterenol, at 10^{-5}M concentration, decreased epinephrine-stimulated glycerol release by 56 and 22 % respectively. Dichloroisoproterenol which gave the greatest decrease in [^{3}H]epinephrine binding was not as effective as propranolol in inhibiting epinephrine-stimulated glycerol release. The extent of inhibition of epinephrine binding by dichloroisoproterenol was similar to the extent of inhibition of epinephrine-stimulated glycerol release. By contrast, propranolol did not inhibit epinephrine binding but did inhibit the epinephrine-stimulated glycerol release. In contrast to these results, Gorman et al. [13] demonstrated a 51 % decrease in epinephrine binding to rat epididymal fat cell membranes with 10^{-4}M propranolol. Phentolamine did not affect epinephrine binding.

DISCUSSION

The present studies show that epinephrine binding to isolated purified liver plasma membranes is a reversible process. An initial peak in binding occurs at about 15 min when the direct Millipore binding procedure is used. Why this peak occurs is not know but may be related to the instability of a particular receptor among several types of receptors or to the activation of an inhibitor substance during incubation. At the present time it is not known whether a single type of receptor or whether several different kinds of receptors occur in the membrane. Scatchard plots from previous studies [5, 6] have indicated at least two different types of binding sites.

Optimal hormone binding occurred at a given amount of membrane protein (125 μg) indicating that excess protein may lead to hormone degradation.

Phospholipases and proteases were found to inhibit epinephrine binding to liver plasma membranes. These observations are consistent with previous studies [1, 6]

and indicate a role for phospholipids in epinephrine binding to protein receptors.

Inasmuch as the adenylate cyclase of mammalian cells is localized in the plasma membrane and the plasma membrane contains a high content of lipid, in particular phospholipid, it is not surprising that phospholipids play a role in the action of this enzyme. Whether phospholipids play a specific role or whether they play a non-specific structural role in helping to maintain a particular conformation of the enzyme complex in the membrane is not easy to unravel. Birnbaumer [32] in a recent review article takes the view that phospholipids may play a specific role in coupling the hormone-receptor interaction to adenylate cyclase activation.

The effect of GTP and ATP on epinephrine binding suggest a possible regulatory function of these nucleotides in catecholamine action at the membrane level. The inhibition of binding would be expected to cause a decreased biological response of the hormone. It is possible that as the level of high energy nucleotides in the cell increases and thus the demands of the cell for mobilizing fat and carbohydrate are no longer required, the inhibition of these nucleotides on catecholamine binding represents a feedback control mechanism for inhibiting the action of these hormones.

Catecholamine binding to membranes is much higher than binding of certain polypeptide hormones such as insulin and glucagon. The in vivo concentration of different hormones varies widely. The concentration of catecholamines at synaptic junctions or nerve endings may be quite high by nature of the way in which they are released. Exton et al. [25] have shown that the concentration for half maximal response of cyclic AMP production in perfused liver is 10^{-8}M for glucagon and 10^{-7}M for epinephrine. The lowest concentration of glucagon giving a significant glucose mobilization was $2 \cdot 10^{-10}$M as contrasted to $1.4 \cdot 10^{-8}$M for epinephrine. Half-maximal glucose mobilization was observed at about $1 \cdot 10^{-9}$M glucagon as compared to $1 \cdot 10^{-7}$M epinephrine. It was of particular interest that although glucagon gave a much higher fold stimulation of cyclic AMP (60 fold) as compared to 2–4 fold for epinephrine, epinephrine was able to elicit glucose mobilization as great as or greater than glucagon. These observations point out the difficulty in attempting to correlate hormone binding with adenylate cyclase activity.

Epinephrine binding to isolated rat epididymal fat cells requires 1 hour to reach equilibrium at 37 °C at a hormone concentration of 10^{-7}M. However, an initial peak in binding is seen at about five minutes. A similar profile in epinephrine binding is seen with rat liver plasma membranes. The cause of this initial peak in binding is not understood. The slower gradual increase in binding from 10 minutes to 1 h may represent slow uptake by the cell, non-specific binding to lower affinity sites, or non-specific covalent binding of oxidized catecholamine to proteins on the cell membrane or inside the cell.

GTP and ATP both inhibited epinephrine binding to fat cells, the former at 10^{-5}M and the latter at 10^{-2}–10^{-4}M. At 10^{-2}–10^{-3}M, ATP completely abolished epinephrine binding. The inhibitory effects of ATP and GTP may represent an allosteric control mechanism whereby the energy balance of the cell is regulated.

Correlation of catecholamine binding to its biological response

The binding of [^{3}H]epinephrine to isolated rat liver plasma membranes with the concomitant stimulation of membrane bound adenylate cyclase was demonstrated by Marinetti et al. [2, 6] and Tomasi et al. [1]. The time course studies showed that

epinephrine binding precedes adenylate cyclase activation. The epinephrine stimulation of adenylate cyclase was small relative to the stimulation produced by glucagon, a finding confirmed in isolated perfused liver by Exton et al. [25] and in isolated liver cell membranes by Pohl et al. [33].

Tomasi et al. [1] studied the binding of [^{3}H] epinephrine and [^{125}I]-labeled glucagon to isolated liver plasma membranes. They found a relatively high localization of binding to the plasma membrane as compared to other cell fractions. Sulfhydryl agents *p*-chloromercuribenzoate and 5′ 5′-dithio-bis-2-nitrobenzoic acid at 10^{-4}M gave a marked inhibition of both epinephrine binding (by 90–98 %) and glucagon binding (by 40–49 %). A study of epinephrine analogs [1, 5] showed that analogs which possessed the catechol ring were the most potent inhibitors of binding and consequently this part of the epinephrine molecule appears to be essential for binding. The present study of epinephrine binding to fat cells confirmed the findings on rat liver membranes. The most potent inhibitors of epinephrine binding are those containing a catechol ring. The side chain does not appear to play an important role in binding but is believed to play a role in coupling the binding to the hormone response.

Gorman et al. [13] found that with fat cell ghosts, norepinephrine and isoproterenol were as effective as epinephrine in stimulating adenylate cyclase and gave nearly complete inhibition of epinephrine binding to fat cell ghosts. On the other hand DOPA gave no stimulation of adenylate cyclase but inhibited epinephrine binding by 85 %.

Schramm et al. [7] have studied catecholamine binding to turkey erythrocytes and its activation of adenylate cyclase. These workers reported that some analogs of epinephrine which were effective in displacing epinephrine binding caused only a small activation of adenylate cyclase but were able to inhibit the epinephrine stimulation of adenylate cyclase. The authors concluded that hormone binding was an essential but not sufficient condition to elicit a hormone reponse.

The correlation of catecholamine binding to its activation of adenylate cyclase in turkey erythrocytes was examined by Bilezikian and Aurbach [8]. These workers concluded that the catechol ring of the catecholamine was required for binding and that the side chain bearing the secondary hydroxyl group and an amine or substituted amine was required for adenylate cyclase activation.

To answer the question whether hormone binding is coupled or uncoupled to its physiological action, epinephrine binding to isolated fat cells was examined in the presence and absence of α-adrenergic and β-adrenergic blocking agents. The data in Table IV show that α-adrenergic blocking agents do not influence epinephrine binding nor do they influence significantly the epinephrine stimulation of lipolysis. However the β-blocking agent propranolol had no influence on epinephrine binding but did inhibit the epinephrine stimulated lipolysis. These observations are consistent with an uncoupling effect of propranolol. On the other hand, the β-blocking agent dichloroisoproterenol which has a structural resemblance to epinephrine, inhibited both the binding of epinephrine and the epinephrine stimulation of lipolysis to the same extent.

Current problems in epinephrine binding

Catecholamine oxidation. Saner and Thoenen [34] have postulated a covalent reaction of oxidized 6-hydroxydopamine with proteins. In view of the susceptibility of catecholamines to undergo oxidation, one must try to exclude oxidation of the

catecholamine during the binding studies. Some catecholamines such as 6-hydroxydopamine are much more susceptible to oxidation than others such as norepinephrine [34]. Indeed, it is also possible that the reduced catecholamines can undergo electrophilic substitution reactions with proteins and other membrane constituents. These types of covalent reactions with extraneous proteins would constitute non-specific binding which must be minimized or eliminated. One way to minimize this non-specific binding is to use a short incubation time. One presumes that the specific binding is high affinity binding and thus should be rapid. We therefore chose to use a 10 min incubation time which allows sufficient time for appreciable binding and which is relatively short so that oxidation of the epinephrine is very small. Saner and Thoenen [34] found by spectral analysis little oxidation of norepinephrine at pH 7.4 over 2 h. Under our conditions, over 95 % of the catecholamine binding is displaced by excess unlabeled hormone. Hormone binding which can be displaced is not covalent binding and hence cannot represent binding of the type reported by Saner and Thoenen.

Reversibility of catecholamine binding. We have found that about 50 % of the epinephrine bound to liver plasma membranes is dissociated within 1 h by incubation in the Krebs Ringer bicarbonate buffer. However, essentially all the bound hormone is dissociated within 1 min with 1M HCl (unpublished data). Lefkowitz [19] has also observed the complete dissociation of membrane bound norepinephrine by 1M HCl but partial dissociation with buffer. Bilezikian and Aurbach [8] found 80 % dissociation of isoproterenol bound to turkey erythrocytes. The dissociation was rapid and occurred within 2–5 min. Schramm et al. [7] also reported a 99 % elution by 0.1M acetic acid of epinephrine bound to turkey erythrocytes. Jarett et al. [35] found that dissociation of epinephrine bound to rat adipocyte subcellular membranes involved a rapid and slow component. The above work from four different laboratories is in conflict with the work of Cuatrecasas et al. [18] who observed no dissociation of bound norepinephrine in their microsomal membrane preparations, even with 0.4M HCl.

Relationship of catecholamine binding to catechol-O-methyl transferase. Cuatrecasas et al. [18] have concluded that in their microsomal membrane systems norepinephrine is bound covalently to the enzyme. Their evidence is indirect and no assays were done on catechol-*O*-methyl transferase activities. Recently Lefkowitz [19] has challenged this report and has found no correlation of binding of norepinephrine to catechol-*O*-methyl transferase. Furthermore, Lefkowitz could not repeat the findings of Cuatrecasas et al. [18] on the effects of *S*-adenosylmethionine and soterenol on catecholamine binding. We have recently studied the effect of two inhibitors of catechol-*O*-methyl transferase on norepinephrine binding [36]. These studies have shown that syringic acid and syringaldehyde give no inhibition of norepinephrine binding at a level where catechol-*O*-methyl transferase activity was completely inhibited. The inhibition of catechol-*O*-methyl transferase activity by these inhibitors was 10000 fold more sensitive than the inhibition of binding of norepinephrine.

Effect of biologically inert catechols on catecholamine binding and catecholamine stimulation of adenylate cyclase. Cuatrecasas [9, 18] has pointed out that (−) norepinephrine, dihydroxyphenylalanine and dihydroxymandelic acid possess no adrenergic pharmacologic activity (as agonists or antagonists) but they bind to tissue

preparations just as well as the fully active hormone (−) norepinephrine. This statement is incorrect, since Bilezikian and Aurbach [8] found that dihydroxyphenylalanine and dihydroxymandelic acid inhibited to about the same extent both the binding of isoproterenol to turkey red cell ghosts and the isoproterenol stimulation of adenylate cyclase in this membrane. Moreover, these catechol analogs do not bind to membranes as well as norepinephrine, epinephrine or isoproterenol. In addition, these analogs do possess some biological activity although less than the catecholamine hormones [37, 38]. Patel et al. [38] have found(+) isomers of norepinephrine and epinephrine to be biologically active in contraction of the vas deferens. The $-\log ED_{50}$ for (−) norepinephrine was 5.23 compared to 4.51 for (+) norepinephrine. The $-\log ED_{50}$ for (−) epinephrine was 5.78 compared to 4.51 for (+) epinephrine. Hence the (+) isomers have significant biological activity and it is erroneous to state that these (+) isomers are biologically inert.

NOTE ADDED IN PROOF (received January 21st, 1975)

Since this manuscript was submitted for publication, the stereospecific binding of alprenolol to frog erythrocyte membranes [39] and the stereospecific binding of propranolol to turkey erythrocyte ghosts [40] has been reported. The binding of hydroxybenzylpindolol to turkey erythrocyte ghosts has also been published [41]. Erythrocytes appear to have a more defined and simpler catecholamine receptor than do fat cells. The affinity constants of alprenolol, propranolol and hydroxybenzylpindolol in erythrocytes are considerably higher than the affinity constant of naturally occurring catecholamines such as epinephrine and norepinephrine. The above-mentioned work also showed that L(−)-catecholamines are more effective than D(+)-catecholamines in inhibiting the binding of these analogs. We have recently found that the binding constant of L-norepinephrine to chicken erythrocyte ghosts is approximately $7 \cdot 10^{-9}\ M^{-1}$. However, we find the binding constant of L-norepinephrine in fat cell membranes is approximately $4 \cdot 10^{6}\ M^{-1}$. This latter finding is in agreement with the concentration of L-norepinephrine required to stimulate lipolysis in fat cells. Erythrocytes thus appear to be far more sensitive to catecholamines than fat cells. This may be related in part to the difference in concentration of catecholamines released at nerve endings as compared to the concentration of catecholamines in plasma.

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