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HORMONE ACTION AT THE MEMBRANE LEVEL

III. EPINEPHRINE INTERACTION WITH THE RAT LIVER PLASMA MEMBRANE*

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SUMMARY

Epinephrine binds to the isolated plasma membrane of rat liver. At least two types of binding sites have been demonstrated by Scatchard analysis having affinity constants of $1.85 \cdot 10^7$ M^{-1} and $1.36 \cdot 10^5$ M^{-1}. The binding of DL-epinephrine, D- and L-norepinephrine, 3,4-dihydroxyphenylethylamine and 3-methoxy-4-hydroxy-L-phenylethylamine demonstrate that the benzene ring and the two ring hydroxyl groups are important for binding and that the side chain does not play a major role in binding. Both D- and L-norepinephrine are bound equally well but 3-methoxy-4-hydroxyphenylethylamine is bound very slightly compared to 3,4-dihydroxyphenylethylamine.

Delipidized membranes bind epinephrine to the same extent as do intact membranes. Thus the hormone is bound primarily to protein. Alteration of the membrane by chemical agents which interact with SH groups inhibit the binding of epinephrine. Agents which iodinate tyrosine residues also inhibit binding. However, agents which acylate amino and hydroxyl groups do not inhibit hormone binding.

Several α- and β-adrenergic blocking agents were tested (phentolamine, phenoxybenzamine, dihydroergotamine, propranolol and dichloroisoproterenol) for their effect on epinephrine binding. Only phentolamine gave a moderate inhibition of epinephrine binding.

Attempts were made to purify the epinephrine receptor protein. A receptor fraction was isolated by treating membranes containing bound epinephrine with sodium dodecyl sulfate and chromatographing the membrane fragments on Sephadex G-200 and DEAE-cellulose. The latter column gave the best separation of the detergent treated membranes and yielded a fraction which contained the major part of the bound epinephrine and which had the highest binding capacity for the hormone.

INTRODUCTION

Epinephrine has been shown to bind to the rat liver plasma membrane, and to cause an increase in the activity of the membrane bound enzyme, adenyl cyclase[1].

Abbreviations: ACTH, adrenocorticotropic hormone; PCMB, *p*-chloromercuribenzoate; DTNB, 5',5'-dithio-bis-2-nitrobenzoic acid.

It has further been demonstrated that the binding of epinephrine to liver plasma membrane is inhibited by epinephrine analogues which contain two free hydroxyl groups on the aromatic ring[2]. The binding of epinephrine is inhibited to a much lesser extent by analogues in which one of these ring hydroxyls has been methylated. Other studies[2–5] have shown that epinephrine action on cells is inhibited by the beta blocking agent propranolol, but propranolol, does not inhibit the action of glucagon or adrenocorticotropic hormone (ACTH). It appears that epinephrine binds to a specific receptor protein in the plasma membrane and this reseptor differs from the receptors for other hormones. The aims of this research are to investigate the nature of the binding of epinephrine to rat liver plasma membrane and to purify and characterize the receptor protein.

MATERIALS AND METHODS

DL-[7-^{3}H]Epinephrine, 3,4-dihydroxy[2-^{3}H]phenylethylamine, and 3-methoxy-4-hydroxy-β-[5-^{3}H]phenylethylamine were obtained from New England Nuclear Corp. L-Norepinephrine [^{14}C]methylene D-bitartrate and D-norepinephrine [^{14}C]methylene D-bitartrate were obtained from Amersham–Searle. The following reagents were used: iodoacetamide (Sigma), phenylmethyl sulfonyl fluoride (Sigma), azobenzene-2-sulfenyl bromide (Nutritional Biochem), succinic anhydride and *N*-acetylimidazole (Eastman Kodak), mercaptoethanol (Pierce Chemical Co.), L-epinephrine (Cal Biochem), beef zinc insulin, crystallized (Eli Lilly Co.), phenoxybenzamine hydrochloride (Smith, Kline, French), dihydroergotamine methanesulfonate (Sandoz Pharmaceut.), dichloroisoproterenol hydrochloride (Eli Lilly Co.) propanolol hydrochloride (Ayerst Labs), L-norepinephrine (Sigma), phentolamine (Ciba).

Rat liver plasma membranes were prepared according to the method of RAY[6]. The binding of epinephrine to the membrane was measured by three methods. In the first method a mixture of L-epinephrine and DL-[7-^{3}H]epinephrine was incubated with a membrane suspension (50–100 μg) for 15 min at 37°, the membrane was precipitated with 5 % trichloroacetic acid, and washed 4 times with 5 % trichloroacetic acid as described previously[1]. Equilibrium dialysis was the second method. Varying quantities of membrane or membrane fractions (50–100 μg) were dialyzed with an initial concentration of [^{3}H]epinephrine ranging from 10^{-8} to 10^{-5} M. 1 ml of the membrane-epinephrine suspension to the dialyzed was placed in a 1-inch length of Union Carbide dialysis tubing and equilibrated in 140 ml of 0.05 M Tris–HCl buffer (pH 7.5) for 24 h at 5°. The equilibration vessel consisted of a 125-ml erlenmeyer flask stirred with a 5/16 inch × 2 inch teflon magnetic stirring bar which allowed for complete equilibration in the 24-h dialysis period. At the end of the dialysis period 0.1-ml aliquots (in duplicate) were obtained from both the dialysate and the dialysis bag. [^{3}H]Epinephrine was counted in 10 ml of BRAY's[7] scintillation cocktail in a Packard Tri Carb liquid scintillation counter.

The millipore technique was the third method used for the analysis of hormone binding. 450-nm millipore 25-mm filter discs (Millipore Corp.) were washed with 250 mM KCl and water. The membrane suspension (50–100 μg) was added to the filter apparatus and the membrane was collected on the filter disc by vacuum filtration. The membrane was then washed three times with 10-ml aliqouts of 0.05 M Tris buffer (pH 7.5). Controls were also run with hormone alone.

The rat liver plasma membrane was fractionated by column chromatography using Sephadex and DEAE-cellulose. In both cases approximately 4–7 mg of membrane were solubilized in 3 ml of 0.1 % sodium dodecyl sulfate, 0.05 M Tris–HCl buffer (pH 7.5), and sonicated at 0° with three 15-sec pulses. The solubilized membrane was applied to a Sephadex G-200 column equilibrated with 0.1 % sodium dodecyl sulfate, 0.05 M Tris–HCl buffer (pH 7.5). The second procedure for fractionating the membrane was to apply the sonicated membrane preparation in 0.1 % sodium dodecyl sulfate to a DEAE-cellulose column. The column was prepared from Whatman DE 11 powder, (W. R. Balston Ltd), washed successively with 0.05 M NaOH, water, 0.5 M HCl, water, and then equilibrated with 0.2 M Tris–HCl buffer. Column separations were done at 5°. 2-ml fractions were collected. The Sephadex column measured 24 cm × 2 cm and the DEAE-cellulose column measured 12 cm × 1.5 cm. Protein was analyzed by the method of LOWRY *et al.*[8].

Electrophoresis in polyacrylamide gels was done by the methods of MAIZEL[9] and SHAPIRO *et al.*[10]. Membrane or membrane fractions were first dialyzed against 0.1 % sodium dodecyl sulfate in 0.01 M phosphate buffer (pH 7.1) for 16 h at 5°. Bromphenol blue was used as a marker. The gels were fixed in 20 % sulfosalicylic acid and stained with 0.25 % Coomasie blue. The relative migrations of the membrane components were compared with the migration rates of proteins with known molecular weights. The following proteins were used as standards: trypsin (Worthington Biochem.), γ-globulin, pepsin and chymotrypsin (Nutritional Biochem.), and malate dehydrogenase (Schwarz Biochem.). The molecular weights for these proteins are given elsewhere[11,12].

Samples for amino acid analysis were hydrolyzed under vacuum in 6 M HCl for 24 h and then analyzed on a Beckman Model 120 C amino acid analyzer. Whole membrane samples were extracted with ethanol–ether (1:1, v/v) or chloroform–methanol (2:1, v/v) prior to hydrolysis in order to remove lipid.

Membranes were modified by treating with various reagents, and the epinephrine-binding capacity of these altered membranes was compared to the epinephrine-binding capacity of the standard membrane preparation. These studies were designed to determine whether specific functional groups on the membrane are associated with epinephrine binding. The conditions and mode of action of these reagents with proteins as studied by other investigators are as follows: mercaptoethanol to reduce disulfide bonds[13]; urea as a denaturing agent; phenylmethyl sulfonyl fluoride to react with the hydroxyl group of serine[14,15]; azobenzene-2-sulfenyl bromide as a selective agent for cysteinyl residues[16]; succinic anhydride to succinylate amino groups (and hydroxy groups) of proteins[17]; iodoacetamide to carboxymethylate imidazole and SH groups[18]; KI–I_2 treatment to iodinate tyrosine groups[19], and *N*-acetylimidazole to acetylate tyrosine hydroxyl groups[20]. In interpreting the results of these studies it is recognized that some of these reagents are not absolutely specific for the functional groups indicated.

RESULTS

The amino acid composition of the membrane is given in Table I. The membrane has an appreciable content of hydrophobic amino acids but a relatively small amount of cysteine or cystine. The percent of acidic amino acids (glutamic acid and aspartic acid) is 22.9 as compared with 11.5 % basic amino acids (histidine, lysine, arginine).

TABLE I

AMINO ACID COMPOSITION OF THE PLASMA MEMBRANE AND MEMBRANE FRACTIONS OBTAINED BY COLUMN CHROMATOGRAPHY

Amino acid	*Mole %*		
	Membrane *	*Sephadex* ** *receptor fraction*	*DEAE-cellulose* ** *receptor fraction*
Lysine	6.14	6.35	6.91
Histidine	1.94	2.24	2.32
Arginine	3.32	3.47	3.23
Aspartic acid	9.91	10.49	10.22
Threonine	6.23	6.87	4.78
Serine	7.56	8.64	14.63
Glutamic acid	13.27	12.65	11.30
Proline	5.23	5.57	3.32
Glycine	7.92	7.01	16.03
Alanine	8.11	9.01	6.72
Half-cystine	1.22	Trace	Trace
Valine	6.70	6.88	6.18
Methionine	0.51	Trace	Trace
Isoleucine	5.61	4.33	3.00
Leucine	10.25	11.26	6.32
Tyrosine	2.38	1.70	1.67
Phenylalanine	4.14	4.20	3.36

* Average of 3 determinations.
** Analysis of one sample.

Our amino acid analysis is similar to that reported by EVANS[22] for the rat liver plasma membrane. BENSON[23] has summarized data on the amino acid composition of other membranes and has also found that these membranes contain a greater number of acidic amino acids.

Analysis of the sodium dodecyl sulfate-treated membrane by gel electrophoresis

TABLE II

BINDING OF [^{3}H]EPINEPHRINE TO THE PLASMA MEMBRANE AND MEMBRANE FRACTIONS

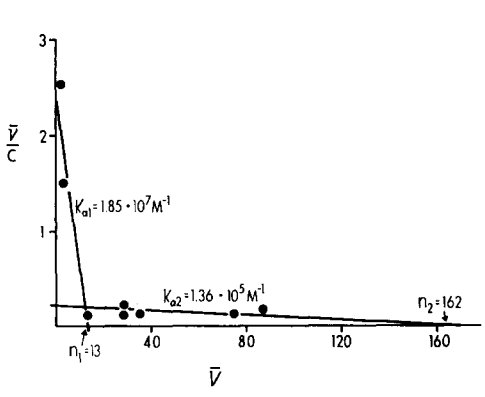
	pmoles/mg protein *
A. *Membrane*	
Membrane (precipitation method)	84 (82–86)
Membrane (equilibrium dialysis)	82 (76–87)
B. *Membrane fractions* **	
Column Fraction I	2 (2–2)
Column Fraction II	21 (18–23)
Column Fraction III	8 (2–13)

* Average of duplicate determinations (range of values shown in parentheses).
** Column fractions from DEAE-cellulose. The column fractions require 2 days to obtain. The membranes were tested in 0.1% sodium dodecyl sulfate since this concentration of sodium dodecyl sulfate is used to solubilize the membranes prior to column fractionation. The membranes used in B were from a different preparation from that studied in A. Equilbrium dialysis was used in B for testing epinephrine binding. The epinephrine concentration in A was 7.10^{-7} M (0.625 μC) and in B was 1.10^{-6} M (0.625 μC).

showed that it was composed of many components with molecular weights ranging from approximately 40000 to well over 200000. The molecular weights were estimated according to the method of SHAPIRO *et al.*[10].

The amount of epinephrine bound to the plasma membrane is given in Table II. The precipitation method gave an average value of 84 pmoles/mg membrane protein and the equilibrium dialysis method gave an average value of 82 pmoles/mg membrane protein. These values were obtained at a concentration of $7 \cdot 10^{-7}$ M epinephrine. In order to determine the number of binding sites for epinephrine and the binding constants, the amount of epinephrine bound to the membrane was studied as a function of the concentration of free epinephrine added to the system and the data was analyzed by a SCATCHARD[21] plot. The data in Fig. 1 shows that there are at least two types of binding sites for epinephrine. Assuming there is no interaction between these sites, the association constants are $1.36 \cdot 10^5$ M^{-1} and $1.85 \cdot 10^7 M^{-1}$. The extrapolated maximum number of pmoles of epinephrine bound per mg membrane protein are 162 and 13, respectively.

The separation of sodium dodecyl sulfate-treated membranes on Sephadex G-200 is illustrated in Fig. 2. The membrane fraction that bind epinephrine emerges in the high molecular weight fractions 5–10 which is immediately after the column void volume. When this fraction is isolated from membranes not pretreated with epinephrine and tested for epinephrine binding, it binds 21–23 pmoles L-epinephrine per mg membrane protein. This fraction will be designated Sephadex receptor



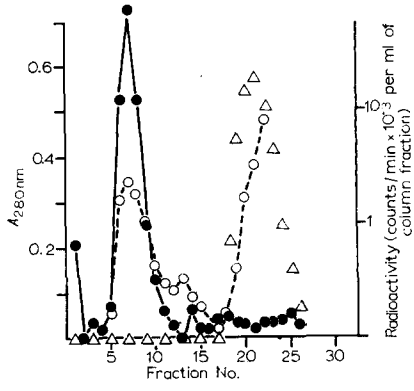


Fig. 1. Scatchard plot for the binding of epinephrine to the plasma membrane. The amount of [^{3}H]epinephrine bound to the membrane was determined at different concentrations of added epinephrine (10^{-5}–10^{-8} M) by equilibrium dialysis. The membrane concentration was 0.4 mg protein. $\bar{v}$ = amount of bound epinephrine (pmoles/mg membrane protein); c = molar concentration of free epinephrine; K = affinity constant; n = number of binding sites (pmoles/mg membrane protein). The equation for two noninteracting binding sites is $\bar{v}/c = (n_1K_1 - K_1\bar{v}) + (n_2K_2 - K_2\bar{v})$.

Fig. 2. Sephadex G-200 column fractionation of membranes pre-treated with [^{3}H]epinephrine. Membranes (4 mg protein) were incubated with 0.62 μC [^{3}H]epinephrine ($2 \cdot 10^{-7}$ M) for 15 min at 23°. The membranes were centrifuged, solubilized in 3 ml 0.05 M Tris buffer (pH 7.5) containing 0.1% sodium dodecyl sulfate and sonicated at 0° with three 15-sec pulses. The membranes were then fractionated on a 2 cm × 24 cm column of Sephadex G-200 at 0°. 2-ml fractions were collected and analyzed for absorption at 280 nm (●—●) and for radioactivity (○—○). A [^{3}H]epinephrine control (0.5 mg unlabeled epinephrine *plus* 0.62 μC of labeled epinephrine) was run under identical conditions. Its elution profile is indicated by the triangles △.

fraction. A control curve of the elution pattern of free epinephrine (Fig. 2) shows that free epinephrine does not appear until Fraction 18 and has a peak at Fraction 22. Thus the epinephrine in Sephadex receptor fraction is not free and can be presumed to be bound to the membrane.

Analysis of the Sephadex receptor fraction by gel electrophoresis showed that this fraction has many components. The amino acid composition of the Sephadex receptor fraction is similar to that of the total membrane (Table I). This finding is not unexpected since Sephadex receptor fraction accounts for the major portion of membrane protein, though it has a much lower lipid content than the total membrane. Most of the lipid has been displaced by sodium dodecyl sulfate[2].

Attempts to further purify the membrane receptor for epinephrine were accomplished by separating the detergent-treated membrane on DEAE-cellulose. A typical elution pattern is seen in Fig. 3. The three areas in which the major peaks appear are designated I, II, and III. The number of smaller peaks, particularly in the fractions between Peaks I and II, varied somewhat in different membrane preparations. Fractions II and III had a higher specific activity for epinephrine binding than did Fraction I.

Since some radioactivity accompanies each of the major protein peaks, the binding properties of the proteins in these fractions were analyzed by equilibrium dialysis. Using an initial epinephrine concentration of $1 \cdot 10^{-6}$ M, the membrane protein fractions I, II, and III (isolated from membranes not exposed to epinephrine)

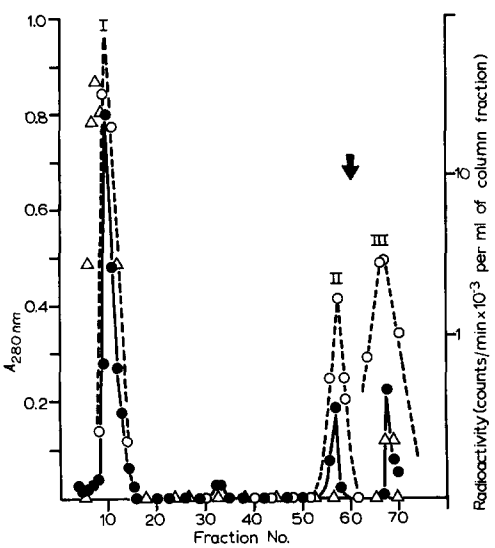


Fig. 3. DEAE-cellulose column fractionation of membranes pre-treated with [^{3}H]epinephrine Membranes (6.7 mg protein) were incubated with 1.2 μC of [^{3}H]epinephrine ($6 \cdot 10^{-7}$ M) for 15 min at 23°. The membranes were centrifuged, solubilized in 3 ml of 0.05 M Tris buffer (pH 7.5) containing 0.1% sodium dodecyl sulfate and sonicated at 0° with three 15-sec pulses. The membranes were fractionated on a 1.5 cm × 12 cm column of DEAE-cellulose. Elution was carried out with a linear gradient of Tris buffer (pH 7.5) starting with 0.2 M buffer and ending with 2.0 M buffer (the latter indicated by the arrow). 2-ml fractions were collected and analyzed for absorption at 280 nm (●—●) and radioactivity (○—○). An [^{3}H]epinephrine control (0.85 mg unlabeled epinephrine *plus* 1.2 μC of labeled epinephrine) was run under identical conditions. Its elution profile is indicated by the triangles △.

were shown to bind 2.2, 20.8, and 7.7 pmoles L-epinephrine per mg protein, respectively (Table I). This shows that the protein in Peak II has the greatest affinity for epinephrine and will be designated DEAE receptor fraction. An elution profile of free epinephrine is also shown in Fig. 3. Free epinephrine elutes as a major peak very close to membrane Peak I and a minor peak close to Peak III. No free epinephrine emerges near Peak II.

Analysis of DEAE receptor fraction by gel electrophoresis in the presence of sodium dodecyl sulfate showed that this fraction has several components. The molecular weight range of the components was from 33000–300000. Electrophoresis of the DEAE receptor fraction without sodium dodecyl sulfate gave a slow moving diffuse band. This data suggests that the DEAE receptor fraction may consist of several subunits which have a tendency to aggregate and that sodium dodecyl sulfate dissociates the aggregates.

Amino acid analysis of DEAE receptor fraction is given in Table I, The amino acid composition differs markedly from the total membrane. It contains a larger amount of serine and glycine but a lower content of leucine and isoleucine. DEAE receptor fraction represents approximately 6% of the total membrane protein. The total epinephrine binding capacity of this fraction (or of Sephadex receptor fraction) is not greater than the binding capacity of the total membrane. The possible reasons for this will be given in DISCUSSION.

Another method which was used to determine the epinephrine binding of the membrane and membrane fractions, involved gel electrophoresis of samples of membrane previously incubated with [^{3}H]epinephrine. The gels were cut in 0.5-cm slices, solubilized with NCS solution (Nuclear Chicago) at 37°, and counted in Bray's cocktail. This study showed that with intact membranes the labeled epinephrine was bound only to the top band of the gel, indicating that epinephrine binds to a very high molecular weight structure which does not penetrate the gel. Epinephrine binding to DEAE receptor fraction was also demonstrated by this method. However, DEAE receptor fraction entered the gel. Free epinephrine run as a control was found to rapidly migrate through the gel and enter the buffer reservoir.

In a previous paper it was observed that chloroform–methanol extraction of membranes containing bound epinephrine released about 20–25% of the bound epinephrine into the organic phase. These experiments indicated two different types

TABLE III

BINDING OF [^{3}H]EPINEPHRINE TO DELIPIDIZED MEMBRANES

% *of control* *	
Chloroform–methanol **	*Ethanol–ether* **
102 ± 7 (3)	109 ± 10 (3)

* ± S.E.; the number of experiments indicated in parentheses.

** Membrane delipidized at 0° for 10 min by treating 1 ml of membrane suspension with either 4 ml of ethanol–ether (1:1, v/v) or 4 ml of chloroform–methanol (2:1, v/v). The delipidized protein was obtained by centrifugation, suspended in 0.5 M Tris–HCl buffer (pH 7.5) and tested for binding of [^{3}H]epinephrine. A control membrane was tested for binding at the same time. The precipitation method was used. The epinephrine concentration was 7.10^{-7} M (0.625 μC).

of binding sites for epinephrine but did not give definitive information whether the binding was all on proteins or in part on lipids of the membrane. Consequently the membranes were delipidized in the cold with organic solvents (ethanol–ether and chloroform–methanol) and the delipidized protein residue tested for epinephrine binding. The data in Table III shows that there is no loss in epinephrine binding to the membranes after lipid extraction. Hence the binding of the hormone is entirely with the proteins. This is supported by the findings that trypsin treatment of the membrane markedly decreases the binding of epinephrine[2].

It was of interest to see how stereospecific was the binding of epinephrine since epinephrine exists in D- and L-forms. Inasmuch as labeled D- and L-epinephrine are not commercially available we tested D- and L-[^{14}C]norepinephrine. We had previously shown that norepinephrine blocks the binding of epinephrine to the membrane[2]. The data in Table IV shows that both the D- and L-isomers of norepinephrine are bound to the membrane essentially to the same extent. Hence the stereochemistry of the side chain is not critical to the binding. These results support our earlier observations that epinephrine analogues containing a modified side chain were not effective inhibitors of epinephrine binding but that the binding of epinephrine was due mainly to the benzene ring and the two ring hydroxyl groups[2].

Since it was previously shown[2] that 3,4-dihydroxyphenylethylamine was a strong inhibitor of epinephrine binding but 3-methoxy-4-hydroxyphenylethylamine was not, it seemed reasonable to expect that 3,4-dihydroxyphenylethylamine was

TABLE IV

THE BINDING OF L-[^{14}C]NOREPINEPHRINE AND D-[^{14}C]NOREPINEPHRINE TO THE PLASMA MEMBRANE

	L-*Norepinephrine* * (*pmoles/mg protein*)	D-*Norepinephrine* * (*pmoles/mg protein*)
Precipitation ** method	89 ± 9.8 (3)	80 ± 7.5 (3)
Millipore *** method	162 (138–187)	145 (120–169)

* L-Norepinephrine $7.0 \cdot 10^{-5}$ M (0.20 μC); D-norepinephrine, $6.3 \cdot 10^{-5}$ M (0.065 μC).
** ± S.E.; the number of experiments is indicated in parentheses.
*** Average of two experiments. The values are given in parentheses.

TABLE V

THE BINDING OF 3,4-DIHYDROXY[2-^{3}H]PHENYLETHYLAMINE, 3-METHOXY-4-HYDROXY-β-[5-^{3}H]-PHENYLETHYLAMINE TO THE PLASMA MEMBRANE

The concentrations were as follows: 3,4-dihydroxy[2-^{3}H]phenylethylamine, $6 \cdot 10^{-7}$ M (2.5 μC); 3-methoxy-4-hydroxy-β-[5-^{3}H]phenylethylamine, $4 \cdot 10^{-7}$ M (2.5 μC). The precipitation method was used to test binding.

pmoles/mg protein *	
3,4-Dihydroxy-[2-^{3}H]phenylethylamine	*3-Methoxy-4-hydroxy-β-[5-^{3}H]phenylethylamine*
77 ± 11 (3)	2.7 ± 0.5 (3)

* ± S.E.; the number of experiments indicated in parentheses.

TABLE VI

EFFECT OF VARIOUS AGENTS ON THE BINDING OF [^{3}H]EPINEPHRINE TO THE PLASMA MEMBRANE

Agent *	% *of control* **
Azobenzene sulfenyl bromide, $2 \cdot 10^{-3}$ M (SH)	50 ± 9 (5)
Iodoacetamide, 10^{-3} M (SH, histidine)	80 ± 10 (4)
Mercaptoethanol, 10^{-3} M (SH) (S–S)	54 ± 4 (4)
Urea, 1 M + mercaptoethanol, 10^{-3} M	54 ± 5 (4)
Urea, 1 M (hydrogen bonds)	153 ± 6 (3)
Phenylmethyl sulfonyl fluoride, 10^{-3} M (OH)	104 ± 11 (4)
Succinic anhydride, 10^{-2} M (NH_2, OH)	100 ± 7 (4)
KI–I_2, 10^{-2}–$2 \cdot 10^{-4}$ M (tyrosine ring)	28 ± (4)
N-Acetylimidazole, 10^{-2} M (NH_2, OH)	100 ± (3)
Acetic acid buffer (pH 5.0), 0.2 M	101 ± 7 (5)

* The functional groups affected by these agents are indicated in parentheses.

** ± S.E. and the number of experiments in parentheses. The precipitation technique was used. The epinephrine concentration was $7 \cdot 10^{-7}$ M (0.625 μC).

TABLE VII

EFFECT OF α- AND β-ADRENERGIC BLOCKING AGENTS ON THE BINDING OF [^{3}H]EPINEPHRINE TO THE PLASMA MEMBRANE

	% *of control* ** *Millipore technique*	*Precipitation technique*	
	10^{-4} *M*	10^{-5} *M*	10^{-4} *M*
α-Blockers *			
Phentolamine	84 ± 8.5 (4)	86 (80–91)	80 (76–84)
Phenoxybenzamine	—	98 ± 4 (3)	
Dihydroergotamine	94 ± 8.3 (3)	93 ± 17 (3)	
β-Blockers *			
Propranolol	102 ± 2.7 (4)	98 (84–111)	95 (89–101)
Dichloroisoproterenol	101 ± 3.2 (4)	108 (93–124)	

* The epinephrine concentration was $7 \cdot 10^{-7}$ M (0.625 μC).

** ± S.E.; values in parentheses refer to number of experiments or range of duplicate experiments.

binding to the membrane more firmly than was 3-methoxy-4-hydroxyphenylethylamine and at the same site where epinephrine binds. We therefore studied the binding of labeled 3,4-dihydroxyphenylethylamine and 3-methoxy-4-hydroxyphenylethylamine to the plasma membrane. The data in Table V show that 3,4-dihydroxyphenylethylamine binds more extensively than does 3-methoxy-4-hydroxyphenylethylamine and confirms our earlier conclusions and stresses the importance of the ring hydroxyl groups in the binding.

In order to study the nature of the binding of epinephrine to the membrane, *i.e.* what functional groups on the membrane are involved in the binding, a variety of agents were tested. The data is shown in Table VI. Agents which interact with or compete for SH groups* (iodoacetamide, azobenzene sulfenyl bromide, mercaptoethanol) are the most effective inhibitors whereas agents which alkylate NH_2 and OH

* We had previously shown that *p*-chloromercuribenzoate(PCMB) and 5′,5′-dithio-bis-2-nitro-benzoic acid (DTNB) also inhibit epinephrine binding[2].

groups do not inhibit the epinephrine binding. Urea gave an unexpected stimulation of epinephrine binding and the stimulation was completely neutralized by mercaptoethanol. It appears that urea and sodium dodecyl sulfate alter the membrane mainly by dissociation to make more binding sites available and that SH groups are involved in the binding. KI–I_2 which iodinates tyrosine rings was a strong inhibitor of epinephrine binding but *N*-acetylimidazole which acetylates tyrosine hydroxyl groups gave no inhibition.

It was of interest to determine if the receptor for epinephrine was an alpha or beta receptor. To answer this question we tested several α and β-adrenergic blocking agents. The data in Table VII shows that except for phentolamine which gave a small inhibition, none of these agents blocked the binding of epinephrine to the membranes.

It was shown earlier[24] that epinephrine and glucagon stimulated adenyl cyclase activity in isolated liver plasma membranes but that insulin inhibited the enzyme and counteracted the glucagon stimulation. In order to elucidate the antagonistic effects of insulin on glucagon and epinephrine action in liver we tested the effect of insulin on epinephrine binding. The results in TableVIII indicate that insulin has no significant effect on epinephrine binding. Under the same conditions norepinephrine gives a marked inhibition of epinephrine binding.

Circular dichroism studies on the DEAE receptor fraction was carried out by Dr. G. Hammes and D. Tallman at Cornell University. The analysis of the CD spectrum by the method of GREENFIELD AND FASMAN[25] gave the following values (approximate): 10 % α-helix, 30 % β-structure and 60 % disordered coil.

DISCUSSION

Epinephrine binds to the isolated plasma membrane to at least two different sites having binding constants of $1.85 \cdot 10^7$ M^{-1} and $1.36 \cdot 10^5$ M^{-1}. One type of binding site occurs in smaller numbers but has a 100-fold greater binding affinity than the other site. How epinephrine binding is related to its function is not known at present but it seems reasonable to assume that epinephrine binding has more than a circumstantial connection with its ability to stimulate the membrane bound adenyl cyclase.

The present studies show that the two ring hydroxyl groups are important for epinephrine binding since analogues which contain this structure inhibit epinephrine binding, whereas analogues in which one of the ring hydroxyls is methylated are poor inhibitors. Moreover, the analogue 3,4-dihydroxyphenylethylamine, which has the same ring structure with two hydroxyl groups, binds to the membrane as well as does epinephrine, whereas 3-methoxy-4-hydroxyphenylethylamine, in which one hydroxyl group is methylated, shows poor binding to the membrane. The stereochemistry of the side chain of epinephrine is not critical for binding since both the D- and L-isomers of norepinephrine bind to the membrane to the same extent. It was also shown in a previous study[2] that analogues in which the side chain is modified but still have 2 hydroxyls in the ring are good inhibitors of epinephrine binding.

The binding of epinephrine is inhibited by a variety of agents which interact with SH groups (PCMB, DTNB, azobenzene sulfenyl bromide, mercaptoethanol) but not by agents which acylate amino or hydroxyl groups. Thus SH groups appear to be important in the binding but this presents an enigma since the intact membrane

has a relatively low content of cysteine and moreover, the partially purified DEAE-cellulose receptor fraction has only a trace amount of cysteine. We provisionally conclude from these results that maximal binding of epinephrine to the receptor protein requires a unique conformation and that SH groups play a role in maintaining this conformation of the membrane near or at the site of epinephrine binding.

The KI–I_2 inhibition of epinephrine binding indicates an interaction of the benzene ring of epinephrine with the phenyl ring of tyrosine. The failure of *N*-acetyl-imidazole to inhibit the binding of epinephrine however indicates that the tyrosine hydroxyl groups are not important in the binding. These studies point to a hydrophobic interaction between these two ring systems.

The effect of sodium dodecyl sulfate, urea, and freeze thawing on the binding of epinephrine are interesting since they all increase the amount of epinephrine binding. These agents alter and/or disrupt the membrane in different ways but it appears that the common denominator is fragmentation of the membrane super macromolecular structure to smaller fragments which now have more exposed sites for binding. It is also possible that these agents decrease membrane association and thereby make more binding sites available. Although sodium dodecyl sulfate or sodium dodecyl sulfate *plus* urea solubilize the membrane and yield many fractions, the separation of these fractions on Sephadex G-200 shows that epinephrine is bound to a major fraction which comes out with or close to the void volume and is thus a very large molecular weight fragment. A similar result is obtained when membranes containing bound epinephrine are treated with sodium dodecyl sulfate and subjected to polyacrylamide gel electrophoresis. The bound epinephrine does not enter the gel whereas free epinephrine run under identical conditions readily migrates through the gel.

Attempts to purify the epinephrine receptor protein (or proteins) were carried out by treating the membranes containing bound epinephrine with 0.1 % sodium dodecyl sulfate and chromatographing these membranes on DEAE-cellulose. In both cases the fraction which contains the bound epinephrine or which shows the highest binding affinity emerges from the column rather late (Fractions 51–54) and accounts for about 6 % of the total membrane protein. This protein fraction (DEAE receptor fraction) is thus an acidic protein which is rather firmly bound to the column. Amino acid analysis of the fraction shows that in comparison to the total membrane protein it has a higher content of serine and glycine but a lower content of leucine, isoleucine and cysteine. Unfortunately the specific binding of this purified fraction is lower than that of the intact membrane. There are several explanations for this observation. First, maximal binding may require an intact membrane or a membrane fragment of a fairly large size indicating a super macromolecular structure with interaction between several protein components. Secondly, the long time required to isolate the receptor and the relatively high concentration of Tris buffer required for elution may alter the structure of the receptor and thus decrease its binding affinity. The receptor may be a very labile protein which is stabilized when it is associated with other membrane proteins. Thirdly, the membrane has some protease activity[26] and proteolysis may inactivate the receptor protein. We have now found that washing the membrane with 1 M NaCl removes the protease[26]. Membranes washed with 1 M NaCl still bind epinephrine and might be more suited for purification of the epinephrine receptor.

The membrane receptor for epinephrine apparently is neither of the α- or β-adrenergic type since α- and β-blockers do not appreciably inhibit the binding of epinephrine. Phentolamine, an α-blocker did inhibit to about 15–20%. Earlier we reported that propranolol, a β-blocker inhibited epinephrine binding by 50%. We were unable to confirm this finding in the present studies. Propranolol has given variable results and occasionally shows inhibition at 10^{-4} M concentration but the effect is not reproducible. Studies are continuing to resolve this problem. The effect of α- and β-blockers in liver by other workers has been frought with conflicting results[27]. It is of interest that phentolamine contains a benzene ring with a hydroxyl group indicating that this structure binds to the same site where epinephrine binds but to a much lesser extent.

Extraction of the plasma membrane with organic solvents has no effect on the binding of epinephrine. These results show that the proteins are involved in the binding and supports the finding that trypsin markedly inhibits epinephrine binding[2]. In a previous study[2] it was shown that chloroform–methanol extraction of membranes at 25° containing epinephrine led to a loss of 20–25% of the bound epinephrine and that this epinephrine occured in the organic solvent extract. In view of our present finding that delipidized membranes bind epinephrine to the same extent as do intact membranes, it is concluded that chloroform–methanol extraction of membranes containing bound epinephrine disrupts some of the protein bound epinephrine which then enters the organic phase since epinephrine is soluble in these solvents.

Insulin has an antagonistic effect on the action of epinephrine and glucagon in liver. How this antagonism occurs is not understood today. Glucagon and epinephrine stimulate adenyl cyclase in liver[24,28]. WICKS [29] and WICKS *et al.*[30] give data which strongly supports the hypothesis that the effects of these two hormones in inducing enzyme induction in liver are mediated by cyclic AMP. We previously showed[24] that insulin inhibits adenyl cyclase activity in isolated rat liver plasma membranes and also counteracts the glucagon stimulation of adenyl cyclase. Since both insulin and glucagon are polypeptide hormones, it is possible that part of the insulin antagonism to glucagon action in liver is at the membrane level. Insulin may inhibit glucagon binding to the membrane and thereby inhibit the glucagon stimulation of adenyl cyclase.

Insulin and epinephrine on the other hand have entirely different structures and hence one would not except them to bind to the same site on the membrane. The present studies confirm this concept since insulin had no significant effect on epinephrine binding. Hence it appears that the antagonistic action of insulin on epinephrine action in liver must be at the intracellular level. Insulin may induce the production of certain enzymes which counteract the epinephrine action.

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