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Binding of Iodinated *Beta* Adrenergic Antagonists to Proteins Derived from Rat Heart

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SUMMARY

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Hydroxybenzylpindolol [Aurbach, G. D., Fedak, S. A., Woodard, C. J., Palmer, J. S., Hauser, D. & Troxler, F. (1974) *Science*, 186, 1223-1224] and hydroxyphenyl derivatives of alprenolol and KL-255 have been prepared. These compounds were iodinated and studied as potential ligands for the investigation of *beta* adrenergic receptors *in vitro*. The iodinated derivatives were potent inhibitors of isoproterenol-stimulated adenylate cyclase (EC 4.6.1.1.) activity. Iodohydroxybenzylpindolol (IHYP) proved to be the most useful ligand, and the properties of the binding of IHYP to proteins derived from rat ventricular muscle have been characterized. The *l* stereoisomers of propranolol, isoproterenol, epinephrine and norepinephrine were more potent by an average of two orders of magnitude than the corresponding *d* isomers in the inhibition of IHYP binding. The K_d values of 10 antagonists were determined by direct binding assay and by inhibition of isoproterenol-stimulated adenylate cyclase. The K_d values of eight of these compounds, as determined by the two methods, were in good agreement, while in two cases they differed by approximately one order of magnitude. The EC_{50} values of isoproterenol, epinephrine, and norepinephrine for adenylate cyclase stimulation were determined and compared with their K_d values as determined by competition with IHYP for binding sites on rat ventricular muscle protein. The order of potency was the same in both cases, but the absolute potency of all three agonists was substantially lower in studies of adenylate cyclase than in binding studies. High concentrations of a variety of biologically active amines which would not be expected to interact with *beta* receptors did not affect the binding of IHYP. Specific binding was defined as the binding of IHYP in the presence of 1 μM *d*-isoproterenol minus its binding in the presence of 1 μM *l*-isoproterenol. When IHYP (0.02 nM) was incubated with 200-400 μg of protein, equilibrium was achieved in approximately 40-60 min. Dissociation of specific binding took place with a half-time of about 15 min. The K_d of IHYP was approximately 1.4 nM as determined both by Scatchard analysis and by direct measurements of rates of association and dissociation. The binding of IHYP was saturable, and there was approximately 0.16 pmole of IHYP binding sites per milligram of membrane protein. The binding sites have many of the properties expected of *beta* adrenergic receptors *in vitro*, and it is likely that these sites represent the physiologically significant receptors.

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INTRODUCTION

The use of radiolabeled ligands has made possible the study of a number of membrane receptors for hormones and neurotransmitters. The greatest successes have been achieved with the cholinergic receptor (2) and with receptors for the peptide hormones glucagon (3, 4) and insulin (5, 6). Similar approaches have resulted in the characterization of opiate-binding material in mammalian brain (7).

Attempts to isolate adrenergic receptors have been complicated by the lack of an irreversible ligand of the requisite specificity and by the sensitivity of catecholamines to oxidative destruction (8–11). This lability probably accounts for many of the results obtained in experiments in which catecholamines were incubated with membranes derived from several organs (12–18). The evidence which implicates oxidation as an obligatory intermediate step in the binding of catecholamines includes the fact that binding was inhibited by antioxidants such as ascorbate and metabisulfite (10, 11). Furthermore, the time course of binding was similar to the time course of oxidation of catecholamines as measured using alumina chromatography (11). The final proof that the binding was nonspecific and was not associated with either *beta* receptors or any other specifiable site came from experiments in which the binding of [^{3}H]epinephrine to bovine serum albumin was studied. The binding to albumin was the same as seen with similar amounts of membrane protein derived from rat heart. In both cases binding was slow, was not blocked by *beta* adrenergic blocking agents, and did not show stereospecificity. It was inhibited, however, by antioxidants, including ascorbate and catechol (11).

An approach to an assay of *beta* receptors which avoids the problems due to the susceptibility of the catecholamines to oxidative destruction involves the use of antagonists which do not contain the catechol substituent. Initial attempts to use tritiated antagonists were not successful (19, 20). The combination of a relatively low specific activity and the high lipid solubility of many of these agents made the experiments difficult. Levitzki *et al.* (21, 22) and Lefkowitz *et al.* (23, 24) recently reported experiments in which stereospecifically displaceable binding of tritiated antagonists was observed. This binding appears to be directed toward *beta* receptors.

A different approach was taken by Aurbach and his collaborators (1). This group used iodinated hydroxybenzylpindolol to study the *beta* receptors of turkey erythrocytes. Compounds of this type have several advantages over tritiated ligands. The specific activity of iodinated compounds is almost 200 times greater than that of tritiated compounds. Furthermore, it is relatively easy to separate the iodinated compound from its non-iodinated precursor. This makes it possible to perform binding studies with a pure and identifiable compound and to show that the compound used in the binding studies is in fact a *beta* antagonist. This capability is of only secondary importance if a compound is labeled by tritium exchange (21), but it may be of major importance if a compound is modified by iodination (1) or by catalytic reduction of a double bond (23, 24).

At least three iodinated derivatives of *beta* antagonists are now available (Fig. 1). In this report we present the results of experiments designed to test the usefulness of iodinated hydroxyphenyl derivatives of alprenolol and KL-255[4] and of an iodinated hydroxybenzyl derivative of pindolol (1). The last of these compounds proved to be the most useful ligand, and it has been used for partial characterization of the *beta* receptor of rat heart.

MATERIALS AND METHODS

Synthesis of hydroxyphenyl derivatives of alprenolol and KL-255. The general approach was that described by Schulz (25). For the synthesis of HPK[5] a phenolic precursor, 5-methyl-2-chlorophenol (1 Eq), was dissolved in 20% sodium hydroxide.

[4] B. B. Wolfe, T. K. Harden, J. Andrews, and P. B. Molinoff, manuscript in preparation.

[5] The abbreviations used are: HPK, hydroxyphenyl-KL-255; HPA, hydroxyphenylalprenolol; HYP, hydroxybenzylpindolol; the prefix I denotes iodination with ^{127}I or ^{125}I; cAMP, adenosine cyclic 3′,5′-monophosphate.

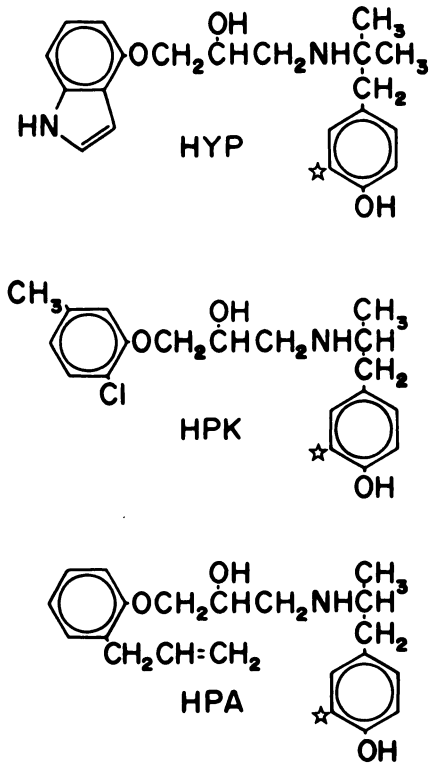

FIG. 1. *Structures of hydroxybenzylpindolol (HYP), hydroxyphenyl-KL-255 (HPK), and hydroxyphenylalprenolol (HPA).*
The sites of iodination are indicated by stars.

Epichlorohydrin (1.1 Eq) was added, and the solution was stirred at room temperature for 24 hr. The resulting phenoxyepoxide was extracted into ethyl ether and vacuum-distilled (93–103°, 0.1 mm). The yield of 1-(2-chloro-5-methylphenoxy)-2,3-epoxypropane was 31%. One equivalent of this product was refluxed in 1-propanol with 0.84 Eq of 4-hydroxyamphetamine for 3 hr. The amine (HPK) was extracted into 0.1 M hydrochloric acid, which then was washed with dichloromethane. Adjustment of the pH to 9.5 led to the formation of the free base of HPK, which separated from the solution as a white oil. HPK was extracted into ethyl ether, the ether was evaporated, and the oil was chromatographed on a silica gel (100–200 mesh) column, using 7.5% or 15% methanol in ethyl acetate as eluant. HPK was eluted from the column after the phenoxyepoxide but well before the 4-hydroxyamphetamine. The yield of the second reaction was 34%. The hydrochloride salt of HPK was obtained by dissolving the free base (an oil) in 1 Eq of hydrochloric acid and then adding concentrated hydrochloric acid. The melting point of the salt was 171–188°.

Identical methods were used to prepare HPA. The phenolic precursor of HPA was 2-allylphenol. The yield of the phenoxyepoxide, 1-(2-allylphenoxy)-2,3-epoxypropane, was 52%. It was collected as a clear, colorless oil which boiled at 87–90° (0.075 mm). The phenoxyepoxide was condensed with 4-hydroxyamphetamine as described above to form HPA (17% yield). The melting point of the hydrochloride salt of HPA was 125–127.5°.

Mass spectra and NMR spectra of the phenoxyepoxides and the hydroxyphenyl compounds were consistent with their postulated structures. Percentages of carbon, hydrogen, and nitrogen as determined by elemental analyses of HPK and HPA were identical with their theoretical values. A detailed description of the synthesis, purification, and iodination of HPK and HPA will be presented elsewhere.[4]

Iodination of hydroxyphenyl derivatives. HPA, HPK, and HYP were iodinated by the method of Hunter and Greenwood (26). Fifty equivalents of amine were used per equivalent of NaI. The amine and NaI were dissolved in 1.0 mM HCl to give a final concentration of 0.3 mM NaI. An equal volume of potassium phosphate (0.3 M), pH 7.6, was added. Chloramine-T (7 Eq) was used to oxidize the iodide to the reactive species. The reaction was allowed to proceed for 2 min at room temperature and was terminated by the addition of 800 Eq of sodium metabisulfite (1 mg/ml) in 1.0 M acetic acid. The iodinated derivatives were extracted three times with ethyl acetate containing 0.01% phenol, and the combined extract was washed three times with sodium metabisulfite in acetic acid.

Chromatography. Following iodination, the ethyl acetate extract was spotted on Whatman No. 3MM paper. Descending chromatography was performed at room temperature with 0.1 M ammonium formate, pH 8.5, containing 0.01% phenol. After development for 6–8 hr the paper

was cut, while still wet, into strips 1 cm wide. Each strip was placed in a vial, and the radioactive compounds were eluted with 5 ml of ethyl acetate containing 0.01% phenol. The fractions were stored at −70°. Prior to each experiment the ethyl acetate was removed from aliquots of the ligands under a stream of nitrogen so that the membranes were not exposed to solvent. Binding assays with the iodinated hydroxyphenyl derivatives were performed within 2 weeks of iodination.

Thin-layer chromatography was performed on silica gel plates at room temperature. The solvent systems used were toluene–diethylamine (6:5) and ethyl acetate–methanol (1:2, 1:1, and 2:1).

Instrumentation. Mass spectra were obtained on a Finnigan gas chromatograph–quadrupole mass spectrometer–computer system, model 3100D, with a model 600 data system. This instrument was used in the electron impact mode. NMR spectra were obtained on a Joelco NMR spectrometer at 60 MHz. Purifications using high-performance liquid chromatography were performed on a 36-inch C18 column eluted with methanol–water (2:1), utilizing a Waters Associates chromatography pump (model M6000) at 0.8 ml/min (400–600 psi).

Heart membrane preparation. Male Sprague-Dawley rats (120–175 g) were killed by cervical fracture. The ventricles were dissected free of atria and large vessels and were homogenized in 7 volumes of 8% (w/v) sucrose in 10 mM potassium phosphate buffer, pH 7.5, using a Polytron PT-10-ST tissue disrupter at setting 6 for 12–15 sec. The homogenate was centrifuged at 10,000 × *g* for 10 min in a refrigerated centrifuge, and the pellet was washed by resuspension in 7 volumes of sucrose–potassium phosphate. After a second centrifugation the pellet was suspended at a Polytron setting of 4.5 for 5–10 sec in 1.72 M sucrose–10 mM potassium phosphate, pH 7.5 (20 ml of sucrose per gram of heart). The suspension was transferred into cellulose nitrate tubes (8 ml/tube). A 5-ml layer of 0.2 M sucrose in 10 mM potassium phosphate buffer, pH 7.5, was placed above the layer of 1.72 M sucrose. The tubes were centrifuged at 100,000 × *g* for 70 min in a Beckman SW 40-Ti swinging bucket rotor. Membranes which collected at the interface of the two sucrose layers were removed with a Pasteur pipette and diluted with 50 mM Tris, pH 7.5, for adenylate cyclase assays, or with 50 mM potassium phosphate, pH 7.5, containing 4 mM $MgSO_4$, for binding studies. Approximately 15 mg of membrane protein were obtained from 1 g of heart. Recovery of adenylate cyclase activity ranged from 20% to 40%. The specific activity of the enzyme was increased approximately 3-fold by this fractionation procedure.

Adenylate cyclase assay. A modification of the method of Krishna *et al.* (27) was used for the determination of adenylate cyclase activity. A volume of 200 μl of heart membranes (about 600 μg of protein) was added to a reaction mixture containing the following final concentrations of reagents: Tris-HCl, pH 7.5, 40 mM; [8-^{3}H]ATP, 1.56 μCi, 0.4 mM; $MgSO_4$, 5 mM; cAMP, 7 mM; theophylline, 10 mM; phosphoenolpyruvate, 10 mM; GTP, 0.13 mM; and pyruvate kinase, 100 μg/assay. The final reaction mixture of 500 μl was incubated for 10 min at 37° in a shaking water bath. The reaction was terminated by placing the tubes in boiling water for 5 min. An additional 500 μl of water were added to each sample prior to the removal of heat-denatured proteins by centrifugation. [^{3}H]cAMP was purified by Dowex column chromatography, followed by $BaSO_4$ precipitation as previously described (27). The recovery of cAMP was greater than 85% as measured by absorbance at 260 nm. Adenylate cyclase activity was linear for up to 20 min of incubation.

Binding assay. Membrane incubations were carried out at 37° in 50 mM potassium phosphate, pH 7.5, containing 4 mM $MgSO_4$ and 20 pM (30,000 cpm) IHYP. Following incubation, membrane samples were pipetted onto Whatman glass fiber filters (GF/C) supported in a 12-port filtering manifold (Millipore). The filters were washed at 37° under vacuum with 25 ml of 20 mM potassium phosphate, pH 7.5, containing 1 mM $MgSO_4$. The time required to filter and wash a sample was less than 20 sec. Over 90% of the protein was retained by the filters under these conditions. Radioactivity retained by the filters was

determined by liquid scintillation spectrometry in a scintillation fluid consisting of 4 g of 2,5-diphenyloxazole and 0.5 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene in 1 liter of toluene mixed with Triton X-100 (2:1).

Ascorbic acid (1 mM) was used as an antioxidant in experiments in which catechols were included in the incubation medium. This concentration of ascorbic acid had no effect on adenylate cyclase activity or on the binding of IHYP. When IHYP was incubated with membranes in the presence of the complete adenylate cyclase medium, the binding observed was identical with that seen in the medium described above.

The binding of IHYP in the presence of 1 μM *d*-isoproterenol minus its binding in the presence of 1 μM *l*-isoproterenol was defined as specific binding. This binding was usually greater than 75% of total binding. Specific binding of IHYP at a concentration of 0.02 nM was linear with protein concentrations up to 2 mg/ml. All the experiments described in this study were performed at a protein concentration at which less than 10% of the total radioactivity was bound. A small amount of IHYP was retained by washed filters in the absence of tissue. A blank containing no tissue was included in each experiment. These blanks ranged from 5% to 10% of the specific binding.

Protein determination. Protein concentration was determined by the method of Lowry *et al.* (28). Bovine serum albumin was used as a standard.

Materials. [8-^{3}H]ATP (tetralithium salt, Schwarz/Mann; specific activity, 21 Ci/mmole) was purified on the day of each adenylate cyclase assay on Dowex 50W-X8 (200–400 mesh, H^+ form, Bio-Rad). ATP, phosphoenolpyruvate tricyclohexylammonium salt, pyruvate kinase (type I, 115 units/mg), GTP sodium salt (type III), *l*-epinephrine, *l*-norepinephrine, and *l*-isoproterenol were purchased from Sigma Chemical Company. Na^{127}I, ammonium formate, diethylamine, L-ascorbic acid, Tris-HCl, and crystalline bovine serum albumin were obtained from J. T. Baker Chemical Company. Theophylline was purchased from Nutritional Biochemicals, and cAMP, from Schwarz/Mann. Na^{125}I was obtained from Amersham/Searle. Aldrich Chemical Company was the source of 2-chloro-5-methylphenol, 2-allylphenol, and epichlorohydrin. The *d* stereoisomers of isoproterenol, epinephrine, and norepinephrine were gifts from Dr. F. C. Nachod of Sterling-Winthrop Research Institute. Dr. Henry L. LeMien, of Ayerst Laboratories, provided the *d* and *l* isomers of propranolol. 4-Hydroxyamphetamine was a gift of Smith Klein & French Laboratories. *dl*-KL-255 was provided by Dr. Cordes of Sanol-Arzneimittel (Germany), and *dl*-alprenolol, by Hassle Pharmaceuticals. HYP was a generous gift of Drs. A. G. Gilman and G. D. Aurbach.

RESULTS

Mass spectrum of HYP. Trimethylsilyl derivatives of HYP were prepared. Two major peaks were seen by gas chromatographic analysis. The mass spectrum of the peak with the longer retention time showed cleavage products consistent with its identification as bis(trimethylsilyl)-HYP (Fig. 2). The mass spectrum of the other gas chromatographic peak showed shifts of +72 mass units for all fragments containing the indole moiety, indicating the addition of a trimethylsilyl group to the indole nitrogen. The molecular weight of bis(trimethylsilyl)-HYP is 498. The molecular ion which corresponds to the loss of 15 mass units ($-CH_3$) is 483 (Fig. 2).

Chromatography of iodinated compounds. HYP and HPA were iodinated with Na^{125}I, and in each case the monoiodinated product was separated from its precursor by paper chromatography (0.1 M ammonium formate, pH 8.5). Thus the purified iodinated derivatives had a specific activity equal to that of the ^{125}I (2.2 Ci/μmole). The R_F values of IHPA, IHYP, HYP, and HPA were 0.15, 0.2, 0.6, and 0.75, respectively. Binding studies were carried out with the purified IHYP and IHPA (Fig. 3, insets). Similar amounts of each ligand were bound to the heart membranes. The binding of IHYP (fraction 5) was inhibited by propranolol in a stereospecific manner, with the *l* isomer being approximately 2 orders of magnitude more potent than the *d* isomer. Material in frac-

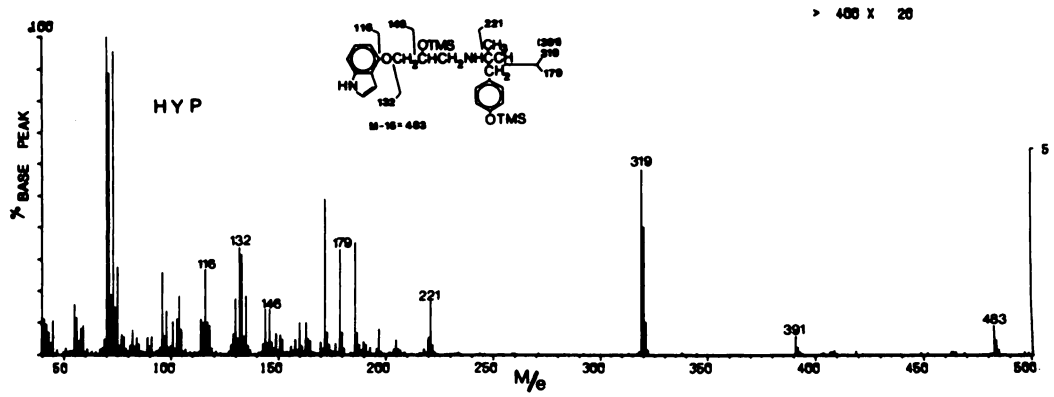

FIG. 2. *Mass spectrum of bistrimethylsilyl derivative of HYP*

HYP (20 μg) was incubated with 25 μl of bistrimethylsilyltrifluoroacetamide at 60° for 30 min. An aliquot of this mixture (5 μl) was injected into a Finnigan gas chromatograph–mass spectrometer equipped with a 36-inch column packed with 3% OV 17. Two major peaks were seen by gas chromatography. These peaks correspond to the bis- and tristrimethylsilyl (TMS) derivatives of HYP. The mass spectrum of the peak with the longer retention time is shown. The likely cleavage sites are indicated in the inset.

tions 2 and 3 (Fig. 3A) bound to heart membranes, but the binding was not inhibited even by high concentrations of *dl*-propranolol. The binding of IHPA was not affected by *l*-propranolol or several other *beta* receptor ligands at concentrations as high as 0.1 mM. IHPK (Fig. 1) was prepared and purified using high-performance liquid chromatography. The binding properties of IHPK were identical with those of IHPA, in that binding was not inhibited by high concentrations of either *beta* receptor agonists or antagonists.

The paper chromatogram of the products obtained by iodination of HYP showed a shoulder with a low R_F followed by a single peak (Fig. 3). Thin-layer chromatograms (Fig. 4) were developed with toluene–diethylamine (6:5) for each of the paper chromatographic fractions which had significant levels of radioactivity (Fig. 3A, fractions 2–6).

A single peak of radioactivity was seen with fractions 5 and 6 (Fig. 4D and E). The thin-layer chromatogram of fractions 2–4 showed that these fractions contained a mixture of several radioactive compounds. Fraction 5 migrated as a single peak on thin-layer chromatography in three ratios of ethyl acetate and methanol (1:2, 1:1, and 2:1, Fig. 4F). There was no change in the chromatographic pattern of the ligand used for binding studies (fractions 5 and 6) for at least 3 weeks.

Characterization of filtration assay. Heart membranes were incubated at 37° for 60 min in the presence of 1 μM *d*- or *l*-isoproterenol and IHYP (30,000 cpm). Approximately 40% of the total radioactivity was removed by a 5-ml wash (Fig. 5). A further small reduction in the binding observed in the presence of both stereoisomers of isoproterenol was obtained by washing with 25 ml of buffer. Stereospecific binding was approximately 0.45 fmole in the unwashed samples, and it was unchanged by washing with up to 75 ml of buffer. A small amount of IHYP was retained by washed filters in the absence of tissue.

Time course of stereospecific binding. The time course of binding of IHYP to rat heart membranes was determined (Fig. 6). Binding in the presence of 1 μM *l*-isoproterenol (nonspecific binding) was complete within 30 sec at 37° and was unchanged during a 90-min incubation. Binding of IHYP in the presence of 1 μM *d*-isoproterenol required 40–60 min to reach equilibrium. The rate of stereospecific binding was temperature-dependent. No stereospecific binding occurred at 0°, even after 120 min of incubation, and binding was slower at 23° than at 37°.

Reversal of stereospecific binding. In order to determine the rate of dissociation of stereospecifically bound IHYP, binding was allowed to take place for 60 min. Dis-

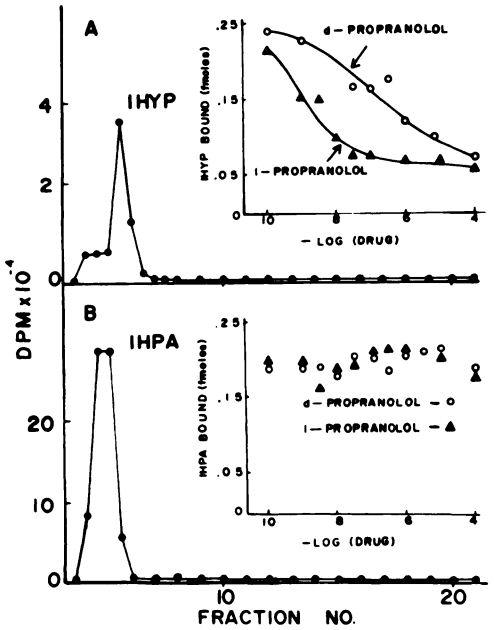

FIG. 3. *Paper chromatographic purification of IHYP and IHPA*

The ethyl acetate extracts from simultaneous iodinations of HYP (A) and HPA (B) were spotted on Whatman No. 3MM paper. Each chromatogram was developed for 6 hr at room temperature with 0.1 M ammonium formate, pH 8.5, containing 0.01% phenol.

Inset: Heart membranes (1.15 mg/ml) were incubated for 30 min at 37° with fraction 5 from the IHYP chromatogram (A) and fraction 4 from the IHPA chromatogram (B). Samples (500 μl) containing either *d*- or *l*-propranolol at the indicated concentrations were filtered and washed as described in MATERIALS AND METHODS. Each point is the mean of duplicate determinations.

sociation was then initiated by the addition of 1 μM *l*-isoproterenol. The decrease in the amount of IHYP bound was followed over the next 100 min (Fig. 7). Dissociation followed first-order kinetics (Fig. 7, inset), with a half-time of 14.6 min. Over 90% of the specific binding had dissociated within 100 min. Similar results were obtained when dissociation was initiated by a 100-fold dilution of the reaction mixture.

Identification of bound [^{125}I]*ligand.* In order to determine whether the radioactivity bound to membranes was unchanged IHYP, samples were incubated with [^{125}I]ligand for 60 min in the presence of 1 μM *d*-isoproterenol. The bound ligand was quantitatively removed (94 ± 2%) from the

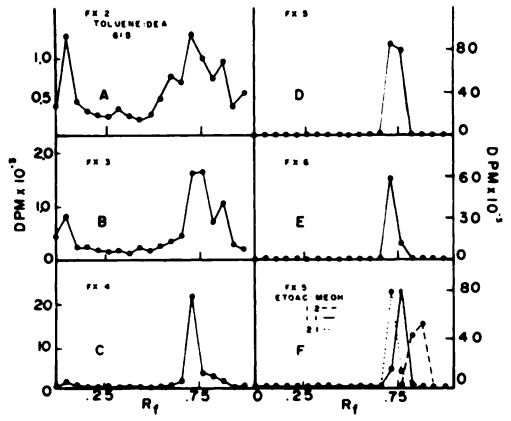

FIG. 4. *Thin-layer chromatograms of IHYP*

A–E. Aliquots (5 μl out of 5 ml) of fractions 2–6 from a paper chromatographic purification of IHYP (Fig. 3) were spotted on Eastman silica gel plates and developed in toluene–diethylamine (6:5). The plates were cut into equal sections (1.0 × 2 cm), and the radioactivity in each section was determined.

F. Aliquots (5 μl) of fraction 5 (Fig. 3) were spotted on silica gel plates, which were developed with ethyl acetate–methanol (1:2, 1:1, and 2:1).

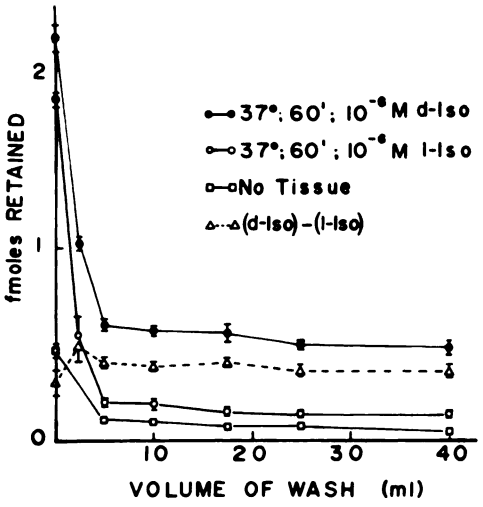

FIG. 5. *Effect of volume of wash on binding of IHYP*

Heart membranes (1.0 mg/ml) were incubated for 60 min at 37° under standard conditions with either 1 μM *l*-isoproterenol (l-Iso, O——O) or 1 μM *d*-isoproterenol (d-Iso, ●——●). Samples were washed with the indicated volumes of wash buffer. An identical series of samples were incubated in the absence of tissue (□——□). The difference in binding in the presence of 1 μM *d*- and *l*-isoproterenol is also shown (△- - -△). Each point is the mean ± standard error of triplicate determinations.

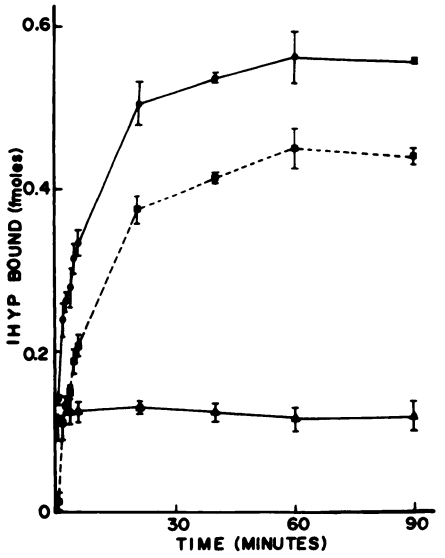

FIG. 6. *Time course of binding of IHYP to heart membranes*

Heart membranes (0.287 mg/ml) were incubated under standard conditions with either 1 μM *d*-(●——●) or *l*-isoproterenol (▲——▲) for up to 90 min at 37°. Aliquots (1 ml) were filtered and washed at the indicated times. The amount of IHYP bound in the presence of *d*-isoproterenol minus the amount bound in the presence of *l*-isoproterenol is also shown (■---■). Each point is the mean ± standard error of quadruplicate determinations.

membranes with 1 mM HCl in ethanol. The samples were concentrated under nitrogen and subjected to thin-layer chromatography. Ninety-two per cent of the radioactivity of the incubated samples migrated with the same R_F as that of a fresh IHYP standard. In another experiment IHYP was incubated with membranes and the bound ligand was extracted with ethanolic HCl. The binding characteristics of the ligand recovered from the membranes were identical with those of fresh ligand.

Determination of number of binding sites. Stereospecific binding assays were carried out with increasing concentrations of IHYP. A 250-fold range of IHYP concentrations was produced by the addition of low specific activity IHYP (10 Ci/mole) to a constant amount (90,000 cpm) of high specific activity ligand. The data, plotted by the method of Scatchard (29) (Fig. 8), gave a K_d of 1.4 nM. The number of IHYP binding sites was calculated to be 0.16 pmole/mg of membrane protein. A double-reciprocal plot (30) of the same data resulted in a calculated K_d of 2.3 nM and 0.24 pmole of IHYP binding sites per milligram of protein (Fig. 8, inset).

Inhibition of IHYP binding by beta receptor antagonists and agonists. Figure 9A shows the inhibition of IHYP binding by the *d* and *l* isomers of propranolol. The *l* isomer was approximately 300 times more potent than the *d* isomer. The concentration-dependent inhibition of IHYP binding by the iodinated derivatives of HYP, HPA, and HPK was determined (Fig. 9B). IHYP appeared to be slightly more potent than

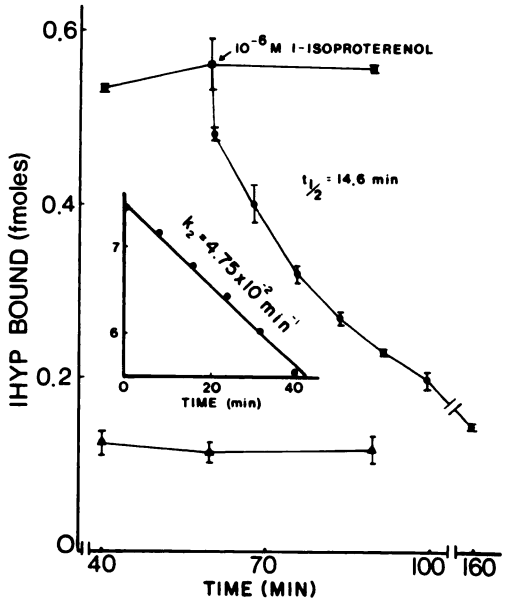

FIG. 7. *Dissociation of stereospecific binding of IHYP*

Heart membranes (0.29 mg/ml) were incubated at 37° under standard conditions with either 1 μM *d*-(■——■) or *l*-isoproterenol (▲——▲). Following a 60-min incubation *l*-isoproterenol (final concentration, 1 μM) was added to an incubation tube containing *d*-isoproterenol. Aliquots (1 ml) were filtered and washed with 25 ml of wash buffer at the indicated times (●——●). Each point is the mean ± standard error of quadruplicate samples.

Inset: The same data were plotted on a first-order rate plot. The ordinate is the natural logarithm of the amount bound at time *t* after the addition of *l*-isoproterenol minus the amount bound at 160 min. The abscissa is time in minutes after the addition of *l*-isoproterenol. The slope is equal to the rate constant for dissociation.

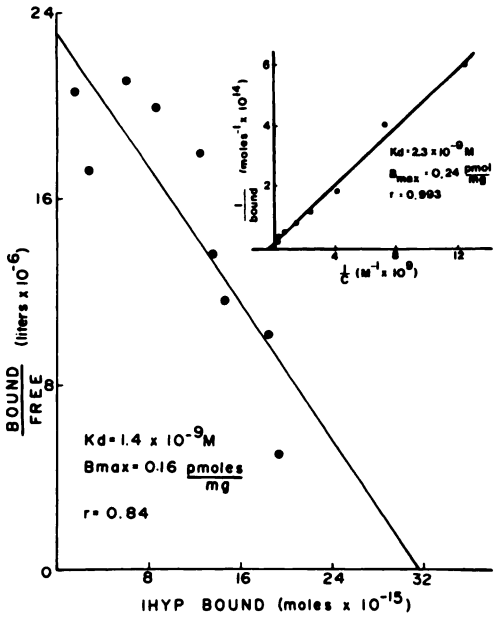

FIG. 8. *Determination of number of binding sites and dissociation constant for IHYP*

Stereospecific binding of IHYP was determined over a wide range of ligand concentrations by adding a variable amount of low specific activity IHYP (10 Ci/mole) to a constant amount of [^{125}I]HYP (2.2 Ci/μmole, 90,000 cpm/sample). Heart membranes (0.196 mg/ml) were incubated with IHYP (0.08–3.8 nM) under standard conditions with either 1 μM *d*- or *l*-isoproterenol for 60 min at 37°. Each point is the mean of triplicate determinations. The data were plotted according to the method of Scatchard, yielding a slope of -0.73 ± 0.06.

Inset: The same data were plotted as the reciprocal of bound IHYP vs. the reciprocal of the free concentration (C) of IHYP. This plot gave a line with a slope of 0.481 ± 0.007. The K_d value, number of binding sites for IHYP (B_{max}), and correlation coefficient of the least-squares line (r) are shown for each part of the figure.

IHPA or IHPK. The capacity of a group of antagonists to inhibit isoproterenol-stimulated adenylate cyclase activity as determined by Schild analysis (31) was similar to the potency of these compounds in inhibiting IHYP binding to rat heart membranes (Table 1A).

The stimulation of adenylate cyclase by the *d* and *l* stereoisomers of isoproterenol, epinephrine, and norepinephrine was determined (Fig. 10A). The order of potency of these compounds in terms of their ability to stimulate adenylate cyclase was similar to their order of potency as inhibitors of IHYP binding to heart membranes (Fig. 10 and Table 1B). In terms of absolute values, the EC_{50} values of agonists for adenylate cyclase were substantially higher than the corresponding K_d values determined in binding studies.

Effects of other agents on binding of IHYP. Alpha adrenergic receptor blockers, cholinomimetics, inhibitors of catecholamine uptake, and a number of biologically active amines, including several psychoactive drugs, were tested for their ability to inhibit the binding of IHYP (Table 2). At a concentration of 10 μM only histamine and desmethylimipramine had any significant effect. At 100 μM significant

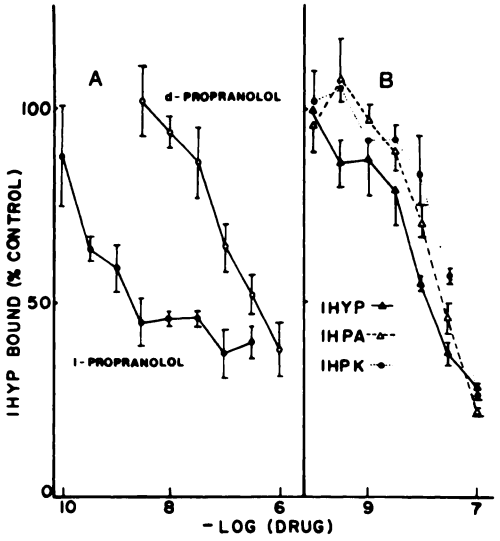

FIG. 9. *Inhibition of IHYP binding by beta receptor antagonists*

A. Heart membranes (1.20 mg/ml) were incubated under standard conditions with either *d*- or *l*-propranolol at the indicated concentrations. After 60 min at 37°, 500-μl samples were filtered and washed with 25 ml of wash buffer. The results are expressed as the percentage of IHYP bound in the absence of any competing antagonist. Control binding (100%) was 0.37 fmole of IHYP. Each point is the mean ± standard error of triplicate determinations.

B. IHPA, IHPK, and IHYP at the indicated concentrations were incubated under the conditions described in part A. Control binding of IHYP ranged from 0.36 to 0.53 fmole. The results are expressed as the mean ± standard error of triplicate determinations. Protein concentration ranged from 1.11 to 1.30 mg/ml.

TABLE 1

Effects of beta antagonists and beta agonists on adenylate cyclase activity and on IHYP binding

A. *Antagonists*. The values of K_d from binding studies were determined from experiments similar to those depicted in Fig. 9. The concentration of antagonist required to inhibit specific binding by 50% provides a measure of the K_d. The values of K_d from the inhibition of isoproterenol-stimulated adenylate cyclase were calculated from the intercept on the abscissa of a Schild plot (31), where $\log (a/a' - 1) = 0$.

B. *Agonists*. The values of K_d from binding studies were determined from experiments similar to those depicted in Fig. 10. The EC_{50} values listed are the concentrations of agonists which caused half-maximal stimulation of adenylate cyclase activity.

A. Antagonists	K_d (binding)	K_d (adenylate cyclase)
	M	*M*
l-Propranolol	7.6×10^{-10}	1.3×10^{-9}
d-Propranolol	4.2×10^{-6}	6.8×10^{-7}
dl-IHYP	7.1×10^{-9}	4.9×10^{-9}
dl-IHPA	1.5×10^{-8}	2.9×10^{-8}
dl-IHPK	2.5×10^{-8}	2.3×10^{-9}
dl-HYP	7.9×10^{-9}	2.0×10^{-8}
dl-HPA	1.8×10^{-8}	1.9×10^{-8}
dl-HPK	4.0×10^{-8}	2.8×10^{-8}
dl-Alprenolol	5.6×10^{-9}	5.5×10^{-8}
dl-KL-255	3.8×10^{-9}	1.3×10^{-8}

B. Agonists	K_d (binding)	EC_{50} (adenylate cyclase)
	M	*M*
l-Norepinephrine	2.5×10^{-6}	2.5×10^{-5}
d-Norepinephrine	1.6×10^{-3}	1.0×10^{-3}
l-Epinephrine	5.0×10^{-8}	1.3×10^{-5}
d-Epinephrine	1.6×10^{-5}	2.0×10^{-4}
l-Isoproterenol	2.5×10^{-9}	6.3×10^{-7}
d-Isoproterenol	4.0×10^{-5}	1.6×10^{-4}

inhibition was also seen with dopamine, *d*-amphetamine, and chlorpromazine.

The binding of IHYP was not affected by NaCl (10–300 mM), KCl (1.0–10 mM), $CaCl_2$ (0.5–2.5 mM), or $MgSO_4$ (0.5–6.0 mM). Changes in the pH of the incubation medium from 6.8 to 8.1 caused no significant alterations in the specific or nonspecific binding of IHYP.

DISCUSSION

Receptors are usually defined operationally in terms of the physiological response which is elicited when they are activated by an appropriate hormone or neurotransmitter. In the case of the *beta* adrenergic receptor the physiological response is an activation of the enzyme adenylate cyclase.

This enzyme serves as a useful marker for *beta* receptor-enriched membrane fragments, but a direct binding assay is necessary for detailed studies of these receptors *in vitro*. *Beta* receptor assays utilizing agonists as radioactive ligands (8, 12–18, 32) have not been successful. Several groups of investigations have therefore turned to the use of radiolabeled antagonists. Levitzki and co-workers (21, 22) described the stereospecific binding of [^{3}H]propranolol to turkey erythrocyte membranes, while Lefkowitz *et al.* (23) studied the binding of [^{3}H]alprenolol to frog erythrocytes. More recently a study of the stereospecific binding of [^{3}H]alprenolol to canine heart membranes was carried out (24). A high ratio of nonspecific to specific binding was encountered in the [^{3}H]propranolol studies (19–22) and in the investigation of dog heart *beta* receptors (24). The high concentrations of ligand made necessary by the use of tritiated compounds may account for the large amounts of nonspecific binding. The results of Aurbach *et al.* (1) and those reported here suggest that the use of lower concentrations of an iodinated ligand may be important in reducing the degree of nonspecific binding in a *beta* receptor assay. On the other hand, tritiated ligands are more convenient to use. The short half-life of ^{125}I makes it necessary to prepare, purify, and standardize the radioligand at frequent intervals. Furthermore, iodinated compounds are more susceptible to autodestruction than are tritiated compounds.

The major criteria which are desirable for a receptor ligand are a high affinity for the receptor and a high specific activity after labeling with either tritium or iodine. These are not, however, sufficient characteristics to ensure success. For example, HYP, HPA, and HPK have been labeled with ^{125}I to a specific activity of 2.2 Ci/μmole. The iodinated compounds are *beta* receptor antagonists with K_i values of 5, 29, and 2 nM, respectively (Table 1), and they bound to the cardiac mem-

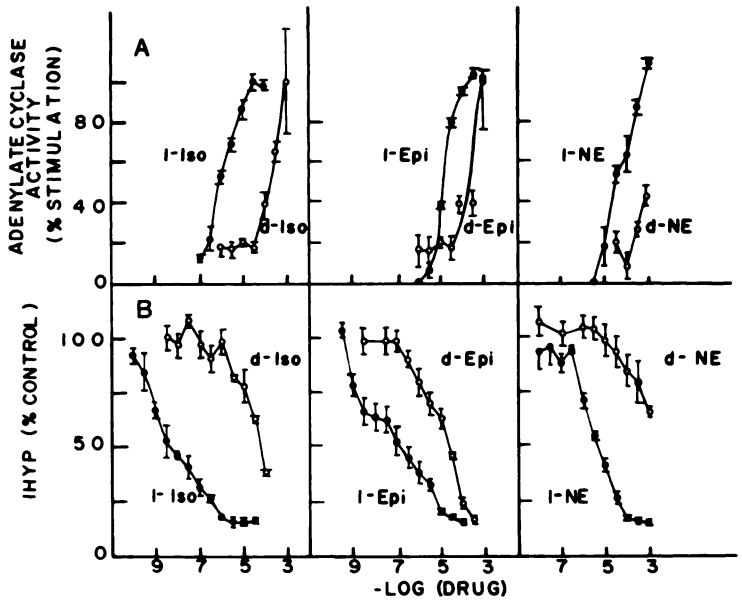

FIG. 10. *Effect of beta adrenergic agonists on adenylate cyclase activity and IHYP binding*

A. Adenylate cyclase activity was determined in the presence of the indicated concentrations of the *d* (O——O) and *l* (●——●) stereoisomers of isoproterenol (Iso), epinephrine (Epi), and norepinephrine (NE). The results are expressed as the percentage of stimulation above basal ± standard errors for triplicate samples. Basal activity was 6.5 pmoles/mg/min.

B. Heart membranes were incubated at 37° for 60 min in the presence of the indicated concentrations of the *d* (O——O) and *l* (●——●) stereoisomers of isoproterenol, epinephrine, and norepinephrine. After 60 min at 37°, 500-μl samples were filtered and washed with 25 ml of wash buffer. The results are expressed as the percentage of IHYP bound in the absence of any competing agonist. Each point is the mean ± standard error of triplicate determinations. The range of protein concentration in the cyclase and binding studies was 1.2–1.5 mg/ml. Control binding of IHYP ranged from 0.41 to 0.58 fmole.

branes used in these studies. The properties of the binding of IHYP were similar to those which would be predicted of *beta* receptors *in vitro*. However, the binding of IHPK and IHPA was not inhibited by either propranolol or isoproterenol. These results were difficult to explain, since both compounds inhibited the binding of IHYP to membranes derived from rat heart (Fig. 9) as well as to membranes derived from glial cells.[6] The most reasonable explanation for the inability of iodinated derivatives of alprenolol and KL 255 to serve as ligands in our binding assay is based on the fact that these compounds are considerably more lipophilic (33) than is IHYP, which appears to be a very useful ligand for investigations of *beta* adrenergic receptors. IHPA and IHPK may still prove useful in studies of *beta* adrenergic receptors which have been made lipid-free with the use of detergents. An alternative explanation for the finding that IHPK and IHPA did not show stereospecifically displaceable binding is that these compounds have such a slow rate of association that no binding is seen in 1 hr, or such a fast rate of dissociation that no specific binding could be observed with the binding assay used.

The best results in terms of purifying IHYP were obtained with descending paper chromatography using 0.1 N ammonium formate at pH 8.5. This system made it possible to separate IHYP quantitatively from its noniodinated precursor. Since the iodination was carried out in the presence of a 50-fold molar excess of amine over iodine, this separation was essential in order to know the specific activity of the compound used in the binding assays. The conditions of the iodination were such that nearly all the iodinated ligand was in the monoiodo form. The same approaches used

[6] A. G. Gilman, personal communication.

TABLE 2

Effects of non-beta adrenergic drugs on IHYP binding

Drugs at the indicated concentrations were incubated in standard medium containing heart membranes (0.86 mg/ml). After a 60-min incubation at 37°, 500-μl samples were filtered and washed with 25 ml of wash buffer. The results are presented as a percentage of the IHYP bound in the absence of drug (0.46 ± 0.08 fmole). Each value is the mean ± standard error of triplicate determinations.

Drug	Concentration	Binding
	μM	*% control*
Phenoxybenzamine	100	90 ± 11
Phentolamine	100	107 ± 9
Physostigmine	100	111 ± 9
Carbachol	100	107 ± 4
Cocaine	100	110 ± 4
Morphine	100	100 ± 13
Serotonin	100	105 ± 14
Catechol	100	94 ± 13
Sodium ascorbate	1000	102 ± 7
Dopamine	100	75 ± 4
Acetylcholine	100	97 ± 3
Desmethylimipramine	1	95 ± 9
	10	84 ± 6
	100	66 ± 5
d-Amphetamine	1	85 ± 12
	10	93 ± 9
	100	61 ± 7
Chlorpromazine	1	103 ± 15
	10	107 ± 7
	100	74 ± 8
Histamine	1	111 ± 10
	10	81 ± 5
	100	71 ± 5
d-Isoproterenol	1	107 ± 8
l-Isoproterenol	1	24 ± 2

to obtain high specific activity ligand for use in binding assays were used to obtain IHYP of a known low specific activity. The low specific activity ligand was used in experiments designed to determine the number of binding sites and also in experiments in which the effect of IHYP on isoproterenol-stimulated adenylate cyclase was determined.

^{125}I is a γ emitter which decays with a half-life of about 60 days to the stable isotope ^{125}Te. The product of ^{125}I decay is a divalent element in the same family in the periodic table as oxygen. Once formed, the ^{125}Te could accept a proton from water or could, conceivably, form a dimer with a second molecule of IHYP. In order to minimize complicating effects from the ^{125}Te derivative of IHYP, the radioligand was always used within 2 weeks of its iodination, so that at most only 15% of the iodine would have decayed. The concentration of any product formed as a consequence of decay was always less than 3 pM; thus it is unlikely that decay products had substantial effects.

In any receptor assay some definition of specific as opposed to nonspecific binding is required. Specific binding has been defined in several studies as propranolol-displaceable binding (21, 23). The definition of specific binding used in the present study was based on the difference between the amount of binding seen in the presence of 1 μM *d*-isoproterenol and the binding observed at the same concentration of the *l* stereoisomer. The difference in potency of the two isomers (Fig. 10) was sufficiently great that at 1 μM the *l* stereoisomer inhibited at least 95% of the displaceable binding while at the same concentration the *d* isomer was without any significant effect. The advantage of this approach is that it does not assume that all binding which is displaceable by a *beta*-active ligand is specific. This is not simply a theoretical problem, since epinephrine-displaceable binding of [^{3}H]epinephrine to Millipore filters has been described (11).

In using a reversible ligand to study receptor binding it is useful to wash the filter (or pellet) to remove unbound or occluded ligand. On the other hand, it is possible that the specifically bound ligand might also be removed from the receptor. In order to examine the feasibility of a wash, the amount of radioactivity in a series of identical assays was determined as a function of the volume of wash applied to the filters (Fig. 5). The observation that the amount of stereospecifically displaceable binding was the same without a wash as with a 40-ml wash implied that the stereospecific binding was only slowly dissociable and that washing the filters did not remove specifically bound ligand. This conclusion is supported by the calculated half-life of dissociation of specific binding

(Fig. 7). The binding observed in the presence of 1 μM *l*-isoproterenol was also seen with higher concentrations of isoproterenol and propranolol. This nonspecific binding usually accounted for 20% of the total binding observed (range, 10–40%). In the absence of a wash the amount of radioactivity retained on the filters was considerably lower without tissue than with tissue incubated at 0° or in the presence of 1 μM *l*-isoproterenol (Fig. 5). Our initial interpretation of these results was that a substantial amount of ligand was occluded on the filters or within the membranes. However, experiments carried out with [^{14}C]inulin showed that little ligand was occluded. This suggested that a substantial amount of the ligand was associated with some constituent of the membranes. This association apparently was weak, since a wash with as little as 5 ml of buffer, which consumed only a few seconds, was able to remove nearly all this associated ligand. Since this association was not affected by *beta* receptor ligands, it has not been further characterized.

The binding of IHYP to proteins derived from rat heart was complete within 40–60 min. The slowness of the binding reaction was probably a result of the very low concentration of ligand used in these studies. The half-time of dissociation of IHYP was 14.6 min (Fig. 7). In addition to providing a direct measure of the rate constant for dissociation, this experiment demonstrated the essentially complete reversibility of the specific binding. Only 7% of the specific binding did not dissociate during the 100 min during which dissociation was allowed to occur. A slow rate of dissociation of IHYP was also observed by Maguire *et al.*,[7] who showed that the reversal of the inhibition of adenylate cyclase by IHYP is also very slow.

The affinity of IHYP for the specific binding sites was determined by adding increasing amounts of IHYP of low but known specific activity to a constant amount of [^{125}I]HYP (Fig. 8). Graphical analysis of the data (29, 30) provided estimates of both the K_d (1.4 and 2.3 nM) and the density of binding sites (0.16 and 0.24 pmole/mg of protein). The value for the density of binding sites is in good agreement with the value reported for canine heart membranes (24). A second method of determining the K_d was based on the equation $K_d = k_2/k_1$. The dissociation rate constant k_2 was determined from the slope of the first-order rate plot (Fig. 7, inset). The data presented in Fig. 6 were employed for the determination of k_1, using the equation $k_1 = k_2 (DR_e)/(R - DR_e)(D - DR_e)$ which is derived from $D + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} DR$ at equilibrium. D is the initial concentration of IHYP, and R is equal to the initial concentration of receptors. DR_e is the concentration of IHYP bound at equilibrium (60 min). The calculated values were $k_2 = 4.75 \times 10^{-2}$ min^{-1}, $k_1 = 3.0 \times 10^7$ M^{-1} min^{-1}, and $K_d = 1.6$ nM. The values of K_d determined by the two methods are in obvious agreement. This suggests that the data obtained are internally consistent and that the values of k_1 and k_2 accurately describe the kinetics of the interaction of IHYP with its binding sites.

The concentrations of IHYP and of IHYP binding sites were much lower than the K_d of the ligand for its receptor. This means that the concentration of a competing ligand which displaces half the specifically bound IHYP is equal to the K_d of the competing ligand. This relationship can be derived by methods similar to those of enzyme kinetics, and it is intuitively reasonable that in the absence of a competing ligand and at an IHYP concentration of 20 pM only a small percentage of the specific sites were filled by radioactive ligand. A competing ligand, at a concentration equal to its K_d, will occupy half the total sites, and, since IHYP was not occupying a significant percentage of the total number of sites, half the specifically bound IHYP was displaced. This approach requires that the ligands act reversibly, that they bind at the same site, and that the concentrations of both receptor and radioligand are much lower than the K_d of the radioligand for its binding site.

The ability of a series of agonists and antagonists to inhibit binding was deter-

[7] M. E. Maguire, R. A. Wiklund, H. J. Anderson and A. G. Gilman, unpublished observations.

mined, and the K_d values of the competing ligands were compared with the EC_{50} values for activation of adenylate cyclase (for agonists) or with the K_d values for inhibiting isoproterenol stimulation of adenylate cyclase (for antagonists, see ref. 31 and Table 1). There was good agreement with regard to most of the antagonists. The worst agreement was with IHPK and *dl*-alprenolol, whose K_d values differed by approximately one order of magnitude. There was no systematic difference in the K_d values determined by inhibition of IHYP binding as opposed to inhibition of adenylate cyclase. This is consistent with the conclusion that the differences are due primarily to the difficulty in accurately determining the inflection point of a curve. *l*-Propranolol was the most potent ligand studied, with K_d values of 0.8 and 1.3 nM as determined by binding and inhibition of adenylate cyclase, respectively.

The high degree of stereospecificity exhibited by propranolol was also seen with the three pairs of stereoisomers of agonists investigated. Similar potency ratios for the *d* and *l* stereoisomers of isoproterenol, epinephrine, and norepinephrine were seen in studies of binding and in studies of their ability to activate adenylate cyclase. However, even though *l*-isoproterenol was the most potent agonist tested in either system, the agreement for agonists was poor in absolute terms. The potencies of the three *l* stereoisomers were greater by 1–2.5 order of magnitude in terms of inhibiting binding of IHYP than in activation of adenylate cyclase. The effect of catecholamines on adenylate cyclase is frequently different when the formation of cAMP is measured in an intact cell than after disruption of cellular integrity. In some cases the major change was in terms of the power of the agonists (34, 35), while in others the potency of the catecholamines decreased on homogenizing the tissue (36–38). We are unable to explain this discrepancy. A decrease in the maximum effect of a catecholamine on adenylate cyclase can be explained by a loss of receptors or by a dissociation of receptors from the enzyme. Neither change however, can explain a decrease in potency. The existence of a substantial number of spare receptors would lead to a situation in which the ED_{50} for cyclase was smaller than the K_d as determined in studies of binding. This is obviously the opposite result to that reported here. Despite our lack of an explanation for these results, it is reasonable to suggest that it is the data obtained with cyclase which are misleading, and not the data obtained in the binding studies. Studies with intact preparations in which cAMP-dependent processes such as cardiac contractility are studied (37, 38) have shown that the responses occur at concentrations of catecholamines which are very similar to those which affect the binding of IHYP. These concentrations are very much lower than those required to activate adenylate cyclase in the disrupted preparation used in these studies.

The properties of the binding of IHYP to proteins derived from rat heart are similar to those expected of *beta* adrenergic receptors. It is likely that this compound will provide a useful tool for the study of cardiac *beta* receptors *in vitro*.

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