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Iodohydroxybenzylpindolol: Preparation, Purification, Localization of Its Iodine to the Indole Ring, and Characterization as a Partial Agonist

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SUMMARY

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Hydroxybenzylpindolol (HYP) has been subjected to iodination using a chloramine-T method. We have studied the products of iodination and determined both the chemical and pharmacological properties of one of the iodinated species of HYP. Routine iodination consistently resulted in two labeled products separable by QAE-Sephadex, paper and high-pressure liquid chromatography. The pattern of elution on high-pressure liquid chromatograms varied with the source and age of the ^{125}I used. Optimized conditions led to a major peak of iodohydroxybenzylpindolol (I-HYP). This peak was relatively stable to incubation at 37°C (66% remaining after 2 h). It chromatographed on paper with an R_f similar to that reported for I-HYP by M. E. Maguire, R. A. Wiklund, H. J. Anderson and A. G. Gilman (*J. Biol. Chem.* **251:** 1221–1231, 1976). Ultraviolet titration curves and NMR spectra indicated that iodination occurs neither on the hydroxybenzyl moiety of HYP nor on the benzylic portion of the indole moiety of HYP. Spectra are consistent with iodination occurring at position 3 of the indole moiety of HYP. Pharmacological properties were studied both on kitten ventricular membranes and on isolated kitten atria and papillary muscles. I-HYP competitively blocked isoproterenol stimulation of kitten ventricular membrane adenylyl cyclase with pK_B's ($-\log K_B$) that ranged from 9.2 to 9.4, which were indistinguishable from pK_B's for HYP determined simultaneously. I-HYP acted as a partial agonist in the isolated organ assays. Calculations performed as described by M. Marano, and A. J. Kaumann (*J. Pharmacol. Exp. Ther.* **198:** 518–525, 1976) yielded pK_p's for I-HYP for competition against isoproterenol's interaction with chronotropic and inotropic β-adrenergic receptors in right atria, left atria, and papillary muscles of 9.2, 9.4, and 8.7, respectively. EC_{50}'s (effective concentrations giving 50% of maximum response for partial stimulation of chronotropic and inotropic receptors) in the same tissues, on the other hand, were ($-\log$ molar) 7.3, 7.3, and 7.1, respectively. Thus, I-HYP, as HYP (Kaumann, A. J., L. Birnbaumer and R. Wittman, in *Receptors and Hormone Action*, L. Birnbaumer and B. W. O'Malley, Eds., Vol. 3, 134–177, Academic Press, New York, 1978) behaves in kitten cardiac tissues as a partial agonist that initiates stimulation of response only at concentrations at which it causes better than 97.5% occupancy of β-receptors.

INTRODUCTION

Hydroxybenzylpindolol labeled with ^{125}I has been used extensively as a probe for characterizing the binding properties of β-adrenergic receptors (1–11). The iodinated derivative has been shown to retain its high affinity (0.03–4.9 nM) for the receptor in intact S49 lymphoma cells (1), rat glioma cells (2), rat ventricular proteins (3), and turkey erythrocyte membranes (4).

The development of a labeled derivative of HYP[1] is

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[1] Abbreviations used: HYP, hydroxybenzylpindolol; I-HYP, iodohy-

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particularly interesting due to HYP's property of being a partial agonist in kitten ventricular systems. Thus, although HYP functions as a pure β blocker in several systems (1, 2, 4), it has been shown that in kitten atria and papillary muscles HYP partially stimulates chronotropic and inotropic receptor-dependent responses (5). Partial activation of adenylyl cyclase by HYP in kitten heart membranes has also been found (5). It is not known whether the partial agonistic effect is mediated by β-adrenergic receptors, since the stimulating effect of HYP in the systems where it acts as a partial agonist proceeds with an EC_{50} (effective concentration giving 50% of maximal response) that is significantly higher than expected from its affinity for the β-adrenergic receptor assessed in studies of its blocking action (5). HYP was reported to be a partial agonist also in rat fat cells; its affinity for fat cell receptors was not reported, however (12). Receptor occupancy by HYP, therefore, does not parallel effect in kitten heart tissues. The reasons for this nonlinearity in kitten tissues must be sought in the coupling function, i.e., the mechanism by which receptor occupancy modifies adenylyl cyclase activity. Studies on the binding and kinetic aspects of the interaction of HYP with receptors and adenylyl cyclase would be likely to elucidate these aspects of the coupling phenomenon. In order to develop I-HYP as a probe for these studies, its characterization in terms of stability and classical pharmacologic activity must be accomplished. In this article we report a useful method for preparation of a stable iodinated species of I-HYP. This product is shown to have an affinity for adenylyl cyclase-coupled β-receptors of kitten ventricle membranes indistinguishable from that of HYP and to behave, like HYP, as a partial agonist. As prepared, the compound is stable and therefore useful for studies on receptor–ligand interactions. Its iodine was localized to position 3 of the indole ring.

MATERIALS

HYP, better than 99% pure as determined by high-pressure liquid chromatography (see below), and pindolol were obtained as gifts from Sandoz Pharmaceuticals, Hanover, New Jersey. Carrier-free $Na^{125}I$ was purchased from Amersham, Arlington Heights, Illinois, and from New England Nuclear, Boston, Massachusetts. [^{3}H]-cAMP was from Schwarz/Mann, Orangeburg, New York. GTP, ATP, cAMP, L-tyrosine, L-3-monoiodotyrosine, and myokinase were obtained from Sigma Chemical Company, St. Louis, Missouri; creatine phosphate and creatine phosphokinase were from CalBiochem, La Jolla, California; acetonitrile and ethyl acetate were from Burdick and Jackson, Muskegon, Michigan; QAE-Sephadex A-25 was from Pharmacia Fine Chemicals, Piscataway, New Jersey; and dimethylsulfoxide-d_6 (DMSO-d_6) was from Aldrich, Milwaukee, Wisconsin. All other reagents were of the highest grade of purity commercially available and were purchased from standard sources.

droxybenzylpindolol; DMSO-d_6, deuterated dimethylsulfoxide; EC_{50}, effective concentration giving 50% of maximum stimulation; pK_p, −log molar of the equilibrium dissociation constant of a partial agonist from its receptor; pK_B, −log molar of the equilibrium dissociation constant of a blocker from its receptor; hplc, high-pressure liquid chromatography; NMR, nuclear magnetic resonance; GMP-P(NH)P, guanyl-5′yl-imidodiphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether) *N,N′*-tetraacetic acid.

METHODS

High-pressure liquid chromatography (hplc). A C_{18}-μBondapak column from Waters Associates connected to a high-pressure pump (Waters Associates Model 6000A) was equilibrated with a 50:50 mixture of acetonitrile and 0.1 M ammonium bicarbonate as described by Brown *et al.* (4). The ethyl acetate extract, after iodination, was dried under a stream of nitrogen, resuspended in methanol, and injected onto the head of the column. The column was eluted at a flow rate of 1.0 ml/min and 0.5-ml fractions were collected. A uv detector (Waters Associates Model 440) continuously monitored the eluant at 254 nm.

QAE-Sephadex chromatography. The ethyl acetate extract was dried under N_2, redissolved in 100 μl methanol, and added to 500 μl running buffer (0.1 M ammonium formate) at pH 9.3. The sample was loaded onto a 5-cm × 0.25-cm^2 QAE-Sephadex column previously equilibrated with running buffer and 1-ml fractions were collected. Each fraction was monitored for uv absorbance at 269 nm in a Beckman spectrophotometer and for gamma emission in a Searle analytic gamma spectrometer. HYP eluted first (fractions 5 through 7), followed by both a minor and a major peak of radioactivity. A third peak of radioactivity could be eluted by changing the buffer pH from 9.3 to 8.0. Unless indicated otherwise, running buffers contained no phenol. Additions of phenol (1 mM) did not affect the elution patterns.

Paper chromatography. Descending paper chromatography was developed in 0.1 M ammonium formate, pH 8.5, and 0.1 mM phenol as described by Maguire *et al.* (8). The paper was air-dried overnight, cut into 0.5-cm strips, and tested for gamma emission. R_f of HYP was 0.58.

Iodination of HYP. Method A: The procedure of Brown *et al.* (4) was followed. Briefly, 10 μl of 1.0 mM HYP in methanol was placed in a glass test tube and evaporated with N_2. To this tube the following were added in order: 5 μl of 1.0 M potassium phosphate, pH 7.5, and 10 μl of 1.0 mM KI in 0.1 N NaOH with trace ^{125}I (specific activity varied between 0.02 and 6.5 mCi/mmol). At time zero, 5 μl of freshly prepared 4 mM chloramine-T was added. The reaction was allowed to proceed at 37°C for 40 min. The remaining chloramine-T was reduced by the addition of 50 μl of 10 mM sodium metabisulfite and 1 ml of water was added to the reaction. HYP and its iodinated derivatives were extracted twice from the reaction mixture with 500 μl of ethyl acetate. This procedure separated free from bound iodide. The aqueous and organic phases were counted in a Searle analytic gamma spectrometer (efficiency 49%) to determine the percentage incorporation. Iodination products were stored in ethyl acetate at 4°C in the dark. For preparation of "large" quantities of I-HYP this method was scaled up 100-fold by increasing both concentrations and volumes 10-fold (see legend to Fig. 5).

Method B: This method, a modification of that used by Maguire *et al.* (8), was developed for synthesis of highly labeled ^{125}I-HYP useful in future binding studies.

The procedure was the same as Method A with the following exception: 0.1 mM KI (with trace ^{125}I) to give a specific activity of 6.5 mCi/mmol) or 0.1 mM $Na^{125}I$ (carrier free) in 10 μl of 0.1 N NaOH was substituted for 1.0 mM KI, such that the final ratio of HYP:KI:chloramine-T was changed from 1:1:2 to 10:1:20. The iodination was allowed to proceed at room temperature for 1 min and then stopped as described for Method A.

Stability studies. An aliquot of I-HYP in ethyl acetate was dried under N_2 and resuspended in 10 mM Tris-HCl, pH 7.5, 1.0 mM EGTA. Aliquots of 200 μl each were placed in separate test tubes, and total counts in each tube were determined. After incubation at 37°C for the specified times, tubes were removed from the water bath, counted to determine radioactivity remaining at time *t*, and extracted twice with ethyl acetate. The aqueous and organic phases were tested for radioactivity to estimate the amount of iodide bound to HYP. The ethyl acetate extracts were evaporated under N_2 and the samples were dissolved in 25 μl methanol. A 10-μl aliquot was injected onto the hplc column. An equal volume was counted and used to determine the percentage counts in each fraction eluted from the column. Plots were made of the percentage counts remaining at time *t*, the percentage of ethyl acetate-extractable counts, and the counts per minute of each fraction eluted from the hplc column.

Ultraviolet titration. Five compounds—tyrosine, 3-monoiodotyrosine, tryptophan, HYP, and I-HYP—were each dissolved in buffer (0.1 M KCl, 0.04 M lysine, 0.014 M histidine, pH 5.0) at a final concentration of approximately 0.1 mM. Each sample was titrated in spectrophotometer cuvettes by addition of 2 M KOH and the pH was measured directly. Spectra from 340 to 220 nm were obtained at each pH on a Heath spectrophotometer. Data were plotted as percentage of the maximum absorbance at 245 nm as a function of pH. Addition of ethyl acetate to the titrated samples of I-HYP resulted in extraction of 95–100% of the added radioactivity, consistent with the assumption that the I-HYP had remained intact.

NMR spectra. NMR spectra were obtained with a Varian XL-100-15A spectrometer using 10 mg tyrosine, 10 mg 3-monoiodotyrosine, 5 mg pindolol, 10 mg HYP or approximately 1 mg I-HYP purified by double hplc followed by an additional QAE-Sephadex chromatography at pH 9.3. I-HYP, HYP, and pindolol were dissolved in DMSO-d_6 which was used as the internal reference, with the center line of the multiplet of DMSO-d_6 being taken as 2.49 ppm from tetramethysilane. L-Tyrosine and L-3-monoiodotyrosine were dissolved in D_2O/NaOD. This solvent was employed because of the insolubility of L-3-monoiodotyrosine in DMSA-d_6. Spectral analyses of the aromatic regions of pindolol, HYP, and I-HYUP were performed using a version of the LAOCN3 algorithm adapted to a minicomputer (13).

Adenylyl cyclase assay. Adenylyl cyclase activity was assayed as previously described (13). The assay contained 25 mM Tris–HCl, pH 7.5; 3.5 mM $MgCl_2$; 0.1 mM [α-^{32}P]ATP (10^6 cpm); 10 μM GTP; 1.0 mM EGTA; 1.0 mM [^{3}H]cAMP (10^4 cpm); 1.0 mM ascorbic acid; an ATP-regenerating system composed of 20 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase, and 0.02 mg/ml myokinase; and various test compounds as indicated. Assays were initiated by adding 50 μg of kitten ventricle membrane protein prepared as previously described (14) to give a final assay volume of 0.05 ml. Assays were incubated at 32.5°C for 10 min and terminated by adding 100 μl of 40 mM ATP, 10 mM cAMP, and 1% sodium dodecyl sulfate, followed by boiling for 3 min. The [^{32}P]cAMP formed was purified and quantitated by the method of Salomon *et al.* (15) as modified by Bockaert *et al.* (16).

Isolated organ studies. Isolated atria and papillary muscles from reserpinized (5 mg/kg, sc, 24 h) kittens (0.8–0.9 kg) were used. These animals were anesthetized with chloroform, and their hearts were removed and rapidly washed free of blood with physiologic salt solution containing (millimolar concentrations): Na^+, 140; K^+, 5; Ca^{2+}, 2.25; Mg^{2+}, 1; Cl^-, 98.5; SO_4^{2-}, 1; HCO^{2-}, 29; HPO_4, 1; fumarate, 10; pyruvate, 5; 1-glutamate, 5; glucose, 10; and EDTA disodium salt, 0.04. The solution was equilibrated with 95% O_2 and 5% CO_2. The hearts were dissected in freshly oxygenated solution at room temperature. Only thin papillary muscles (less than 0.8 mm) were used. The muscles were mounted in a setup with a 50-ml bath as described by Blinks (17). The tissues were tied with Flexafil threads 6/9 (J. Pfrimmer and Co., Erlanger, West Germany) to stainless-steel wires attached to Statham G76-350 and Grass FT 0.03 strain gauge transducers. Inotropic effects were studied on left atria and papillary muscles driven with square-wave pulses of 5-ms duration and just threshold intensity was applied through a small platinum electrode in contact with the nontendinous end of the papillary muscles (12/min) or the thickest portion of the left atria (30/min). Papillary muscles were stretched to their optimal length (L_{max}) at which maximal contractile tension was developed. At L_{max} the resting tension of left atrial strips was very high and tended to creep downward with time. For this reason the strips were set to a length at which resting tension was approximately half that of L_{max}. Chronotropic effects were on spontaneously beating right atria suspended at a resting tension just sufficient for measurable development of tension. Tensions and frequency of contractions were recorded on a four-channel polygraph. The tissues were exposed to 5 μM phenoxybenzamine for 2 h, washed, and left phenoxybenzamine free thereafter. Phenoxybenzamine was used to prevent extraneuronal uptake of isoproterenol (5) and to block α-adrenergic receptors; administration of phenoxybenzamine causes irreversible potentiation of myocardial effects of isoproterenol (18, 19). To evaluate the interaction(s) and effect(s) of I-HYP, three cumulative concentration–effect curves were determined on each tissue in such a manner that after a first curve for isoproterenol alone, the agonist was washed out, a second curve for I-HYP alone was obtained, the highest concentration of which was left in the bath, and a third and last curve for isoproterenol in the presence of the high concentration of I-HYP was obtained. EC_{50} values for I-HYP were calculated from the cumulative concentration–effect curves obtained in response to I-HYP alone. Affinity values (pK_p's) for I-HYP were calculated from the shift in the concentration–effect curve for isoproterenol alone.

Calculations of pK_p values and resulting occupancy curves were performed according to Marano and Kaumann (20). For further details on the statistics and evaluation of effects of partial agonists, see Marano and Kaumann (20) and Kaumann *et al.* (5).

RESULTS

Iodination and purification of I-HYP. Iodination of HYP by Method A followed by subsequent purification over QAE-Sephadex A-25 consistently resulted in two major iodinated peaks. The first peak eluted with pH 9.3 buffer after a minor peak, and the second eluted with pH 8.0 buffer (Fig. 1). The ethyl acetate extract of the iodination mixture could be separated into two peaks of radioactivity by paper chromatography (Fig. 2). Reverse-phase chromatography over a Waters Associates C_{18}-μBondapak column routinely yielded four peaks of radioactivity from ethyl acetate extracts derived from HYP iodination by Method A. The first (minor) peak eluted with the solvent front and was no longer extractable into ethyl acetate. It was considered to be free iodine. The second peak varied somewhat in relative abundance and eluted close to standard uniodinated HYP. It was not studied. The next two peaks were termed I and II relative to their elution from the hplc column after standard HYP (lower left panel of Fig. 3 and left panel of Fig. 5). Separated peaks I and II rechromatographed over the hplc column in the same position, indicating that the two derivatives were not spontaneously interconvertible. Peak I from hplc chromatographed on QAE-Sephadex as the major pH 9.3 peak. Although Method A always appeared to yield Peaks I and II, initial experiments showed variability in the stability and the elution pattern of the iodinated products from hplc. This variability was eventually ascribed to the batch of ^{125}I used to trace label I-HYP (see below). Satisfactory results were obtained consistently with ^{125}I from Amersham, provided it was used within a week of receipt or it was diluted upon receipt with ^{127}I to specific activities less than *100* mCi/mmol (Fig. 3 and Fig. 5 lower right panel). Iodination by

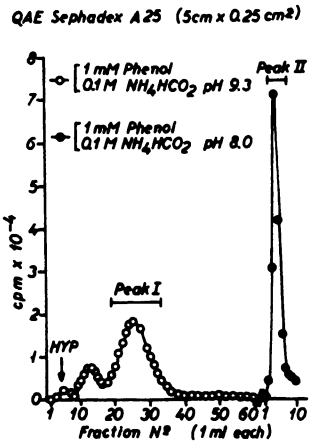


FIG. 1. *QAE-Sephadex A-25 elution pattern of ethyl acetate extracts obtained from iodination by Method A of 10 nmol HYP with iodide from Amersham as described under Methods*

Peak I was eluted with running buffer at pH 9.3. Peak II was eluted with running buffer at 8.3. Fractions of 1 ml were collected.

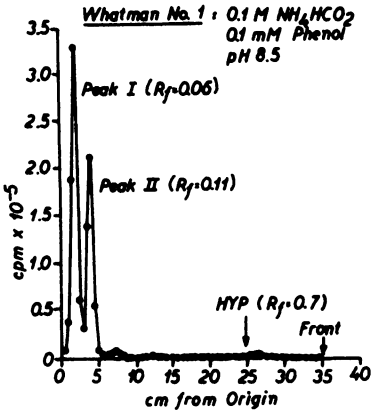


FIG. 2. *Paper chromatography of ethyl acetate extracts obtained from iodination by Method A of 10 nmol of HYP with iodide from Amersham as described under Methods*

Method B resulted in a single major peak of radioactivity (Fig. 4) which cochromatographed upon hplc with purified Peak I prepared by Method A (Fig. 5). Peak I is referred to as I-HYP for the remainder of this article.

Stability of I-HYP. The stability of I-HYP at 37°C was explored to determine the suitability of the analog for both binding and cyclase assays. It was found that both the elution pattern and the stability of iodinated products obtained by Method A vary with the age and source of ^{125}I used. Figure 3 presents a comparative study carried out in November 1977 using fresh batches of ^{125}I from New England Nuclear and Amersham to iodinate HYP by Method A. Thus, using ^{125}I from New England Nuclear we found that although 73% of the initially incorporated (and ethyl acetate extractable) iodine was recovered in what appeared as I-HYP, only 23% of this material (17% of the initially incorporated iodide) eluted as I-HYP after a 2-h incubation at 37°C (Fig. 3, inset and lower right panel). In contrast, only 24% of the initially incorporated and ethyl acetate-extractable iodide was found to elute as Peak I upon hplc when fresh Amersham ^{125}I and Method A was used, but 67% of this material (16% of the initial) was identified as Peak I on hplc after the 2-h incubation (Fig. 3, inset and lower left panel). Thus, although the radioisotope preparation from New England Nuclear gave higher yields of Peak I-like material, the product was unstable under conditions similar to those that would be used for studying binding and adenylyl cyclase. On the other hand, the less abundant product obtained with Amersham iodide was stable enough to be used in such studies.

Preparation of I-HYP for analysis of chemical structure and pharmacological properties. Approximately 1-mg batches of Peak I I-HYP were prepared and purified by repeating the 100-fold scaled-up procedure (Method A) three times, chromatographing the products from each iodination separately over C_{18}-μBondapak, pooling the separated I-HYP peaks, chromatographing the combined materials a second time over C_{18}-μBondapak with a slightly different solvent, and pooling I-HYP followed by extraction into ethyl acetate and evaporation to dryness. For details see Fig. 5 and its legend.

The purified Peak I I-HYP prepared by the scaled-up

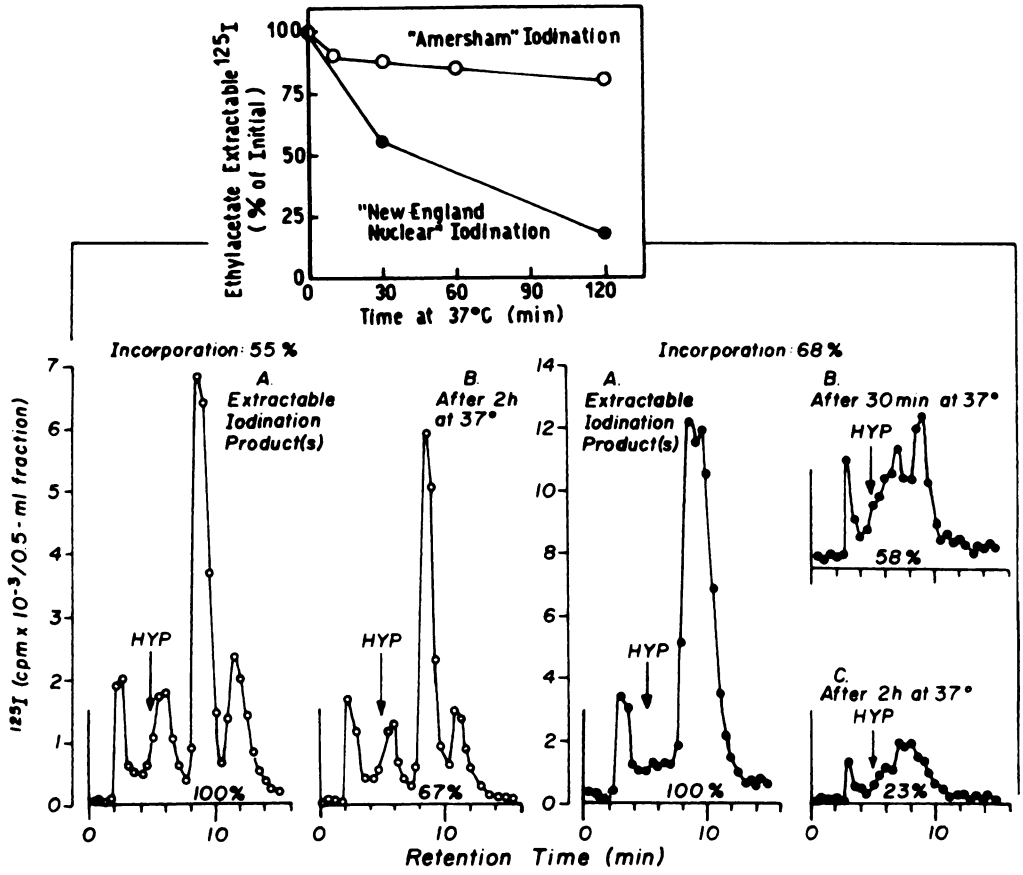


FIG. 3. *High-pressure liquid chromatogram of ethyl acetate extracts obtained from iodination of HYP with iodides from Amersham and New England Nuclear*

HYP (10 nmol) was iodinated by Method A using 10 nmol of KI to which was added K[125]I from either Amersham (open circles, Amersham iodination) or New England Nuclear (closed circles, New England Nuclear iodination) to give a final specific activity of 0.73 or 0.85 mCi/mmol, respectively. Extractable iodination products were evaporated, resuspended, incubated at 37°C for the indicated times, reextracted into ethyl acetate, evaporated, resuspended into methanol, and chromatographed over C_{18}-μBondapak column as described under Methods. *Upper inset*: Stability of extractable iodination products to incubation at 37°C. *Lower panel* (*left and right*): Elution profiles of extractable iodination products before and after incubation at 37°C for the indicated times.

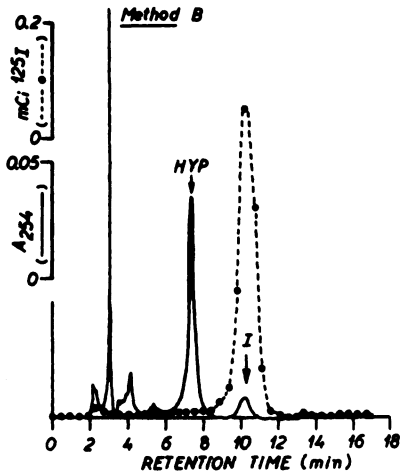


FIG. 4. *High-pressure liquid chromatography of ethyl acetate-extractable iodinated products obtained by subjecting HYP (10 nmol) to iodination by Method B using carrier-free [125]I (Amersham, 2.45 mCi)*

The iodination reaction was stopped after 1 min at room temperature by addition of 50 μl of 10 mM sodium metabisulfide. After addition of 500 μl of H_2O, iodinated products were extracted twice into ethyl acetate (500 μl each time). The extracted [125]I-labeled compounds (83% of the initially added [125]I) were evaporated to dryness under a stream of N_2 and resuspended into 100 μl of methanol. The methanol solution as well as three 100-μl washes of the tube were injected onto a C_{18}-μBonadpak (30 × 0.4 cm, Waters) column. The column was developed as described under Methods with a 1:1 mixture of acetonitrile:0.1 M $(NH_4)HCO_3$, pH 8.0, at a flow rate of 1.0 ml/min. Elution profiles of absorbance at 254 nm and of [125]I (0.5-ml fractions) are shown. The major iodine-containing peak was extracted into ethyl acetate, evaporated to dryness, resuspended into 1.0 ml of methanol, and stored without further additions at −20°C until used. Final yield: 1.7 mCi, i.e., 68%.

version of Method A chromatographed upon hplc with the same retention time as the major iodination product obtained by Method B (compare upper and lower sections of right panel of Fig. 5). I-HYP prepared in this manner (middle panel of Fig. 5) was used without further purification in most of the spectral titration analysis and the adenylyl cyclase, inotropic and chronotropic assays. Further QAE-chromatography at pH 9.3 was necessary (see Methods) to separate a minor contaminant which was released by the C_{18}-μBondapak column and which interfered in NMR determinations. The additional puri-

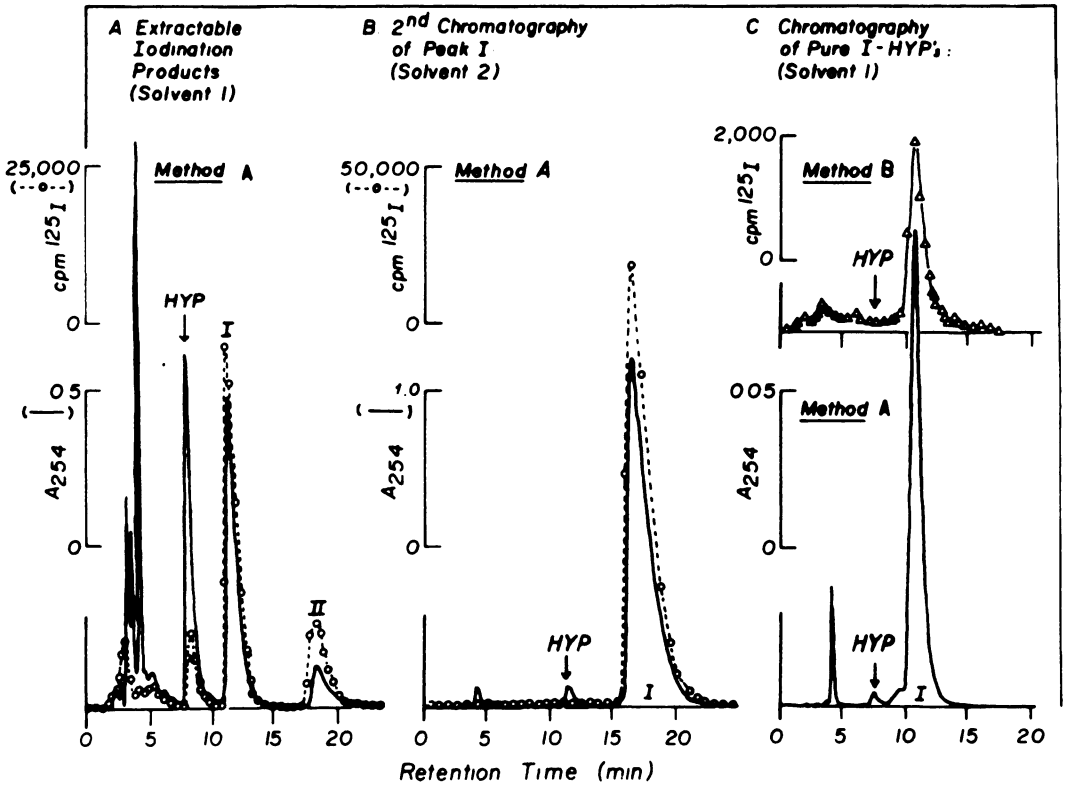


FIG. 5. *"Large-scale" preparation of iodo-HYP*

Method A was as follows: 100 μl of HYP solution (1000 nmol) were mixed with 200 μl 1 M P_i(K) pH 7.5, and 100 μl of 10 mM IK (620,000 cpm of ^{125}I from Amersham/Searle) in 0.1 N NaOH. Iodination was started by the addition of 50 μl of fresh 20 mM chloramine-T (1000 nmol). The reaction mixture was incubated at 37°C for 40 min. At 10, 20, and 30 min, three more additions of 50 μl of the chloramine-T solution were made. The reaction was quenched at t = 40 min by addition of 500 μl of 20 mM sodium metabisulfite. Iodination products, unreacted HYP, and other extractable products were extracted into ethyl acetate, evaporated to dryness, dissolved into 150 μl of methanol, and injected onto a C_{18}-Bondapak (Waters) column of 0.4 × 30 cm. The column was developed at 1.0 ml/min (850 psi) with solvent 1 (50:50 mixture of acetonitrile:0.1 M NH_4HCO_3, pH 8.0). Elution profiles of absorbance at 254 nm (flow cell measurement) and ^{125}I radioactivity (0.5-ml fractions) are shown in the left panel. Peak I was extracted into ethyl acetate, combined with two similar peaks from additional iodinations, evaporated to dryness, dissolved in 150 μl of methanol, and rechromatographed using solvent 2 (40:60 mixture of acetonitrile:0.1 M NH_4HCO_3, pH 8.0). Elution profiles of absorbance at 254 nm and ^{125}I radioactivity (0.5-ml fractions) are shown in the middle panel of the figure. Peak I was extracted into ethyl acetate, combined with three other such peaks, and dissolved in 100 μl methanol. Ten microliters of this solution was analyzed by hplc over the C_{18}-μBondapak column using solvent 1. The lower right panel shows the absorbance pattern at 254 nm of this product. The upper right panel shows the elution profile of a stable ^{125}I-labeled HYP peak obtained in carrier-free form by the Method B illustrated in Fig. 4. This radioactive peak had been stored for 3 weeks in methanol at −20°C. Overall yield of I-HYP by Method A, illustrated above, was 67%.

fication step did not alter either the uv spectrum or the pharmacological characteristics of the Peak I I-HYP preparation.

Ultraviolet titration. The effect of pH change on the spectra of five different compounds was measured, and four of these are shown in Fig. 6. Spectral titrations of tyrosine, 3′-monoiodotyrosine, HYP, and I-HYP were obtained at 245 nm, i.e., that part of the I-HYP and HYP spectra that is due solely to their hydroxybenzyl moieties. As shown in Figs. 6 and 7, 3-monoiodotyrosine has a lower pK than tyrosine. However, both HYP and I-HYP titrations coincided with that for tyrosine at 245 nm.

NMR spectra. NMR spectra of HYP, I-HYP, pindolol, tyrosine, and 3-monoiodotyrosine were compared. The spectra for HYP and I-HYP differ in the aromatic region which spans about 150 Hz of the scan at 100 MHz (Fig. 8). The NMR peaks could be identified by comparison to NMR spectra of indole in CS_2 (21), of pindolol in DMSO-d_6 (Fig. 8), and of tyrosine and 3-monoiodotyrosine in D_2O (Fig. 9). Theoretical spectra were calculated by iterative comparison with observed spectra and provided the refined assignments shown in Table 1.

Inhibition of adenylyl cyclase stimulation by HYP and I-HYP. A comparison was made of the affinities of HYP and I-HYP for β-adrenergic receptors. In four separate experiments, each determining the pK_B of a separate batch of I-HYP in comparison to HYP, pK_B values for I-HYP varied between 9.2 and 9.4 and did not differ from those for HYP by more than 0.1 unit. Figure 10 shows one such experiment. Under the assay conditions used here (0.1 mM ATP, 3.5 mM $MgCl_2$, 10 μM GTP, pH 7.5), the apparent K_A for (−)-isoproterenol was 1.6×10^{-8} M. A Schild plot of the data shown in Fig. 10 gives a slope of 1.11, an intercept of 9.3, and affinity constants (K_B's) of 0.40 and 0.44 mM for HYP and I-HYP, respectively (22).

Partial agonism of I-HYP. Isolated organ studies on kitten heart tissues showed that I-HYP is a partial ago-

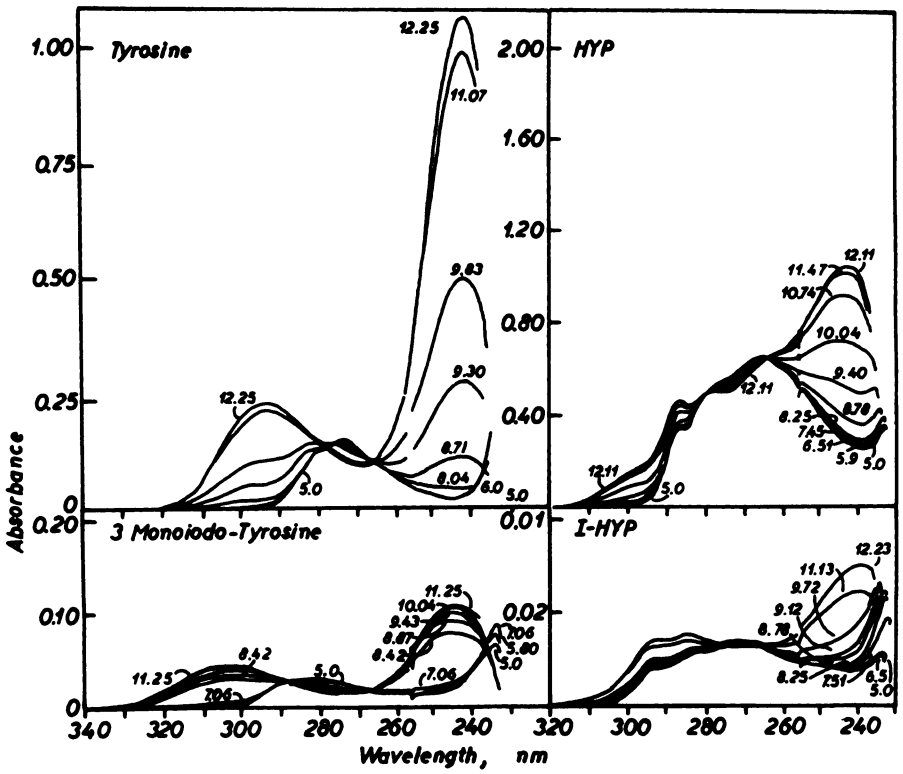


FIG. 6. *Ultraviolet spectra at varying pH values of* L-*tyrosine, 3-monoiodotyrosine, HYP, and I-HYP*
Numbers indicate pH's at which spectra were obtained.

nist in stimulating chronotropic receptors in right atria and inotropic receptors in both left atria and papillary muscles (Fig. 11).

Other. Commercially available ^{125}I-HYP was tested for its chromatographic properties and stability. It was found to cochromatograph upon hplc with I-HYP; 91% of its label was extractable in ethyl acetate after a 2-h incubation at 37°C. Of this, 82% migrated as Peak I upon hplc, yielding an overall recovery of 75% of the incubated label as material cochromatographing with I-HYP.

DISCUSSION

I-HYP has been used by several investigators as a high-affinity probe for agonist, antagonist, receptor, and adenylyl cyclase interactions with β-adrenergic receptors. I-HYP has the advantage of being labeled to a high specific activity while retaining its high affinity for β-adrenergic receptors (1–11). In our own efforts to develop this probe, we have defined some important characteristics of the labeled ligand.

Two methods for the iodination of HYP were used. Method A was applied predominantly to the production of relatively large amounts of I-HYP trace-labeled with ^{125}I so as to be able to study its pharmacological properties. Method B serves for the iodination of ^{125}I-HYP of high specific activity in small quantities for use in future binding studies. Using iodide from Amersham, Method A routinely gave 40–50% incorporation of ^{125}I into ethyl acetate-extractable products. Subsequent purification produced two main radioactively labeled products discernible on hplc, QAE-Sephadex chromatography and paper chromatography. The most abundant peak eluting from hplc, termed Peak I, contained about 60% of the counts extractable by ethyl acetate (24–30% of the original ^{125}I) and the second major peak (Peak II) contained between 20 and 30% of the extractable counts (i.e., 8–15% of the original ^{125}I). The hplc elution profile was similar to that obtained by Brown *et al.* (4). Their results showed a major peak of radioactivity eluting immediately after unlabeled HYP and followed by a smaller second peak of labeled product. The quantity of material in the smaller second peak was directly proportional to the length of the iodination reaction. On the other hand, Method B

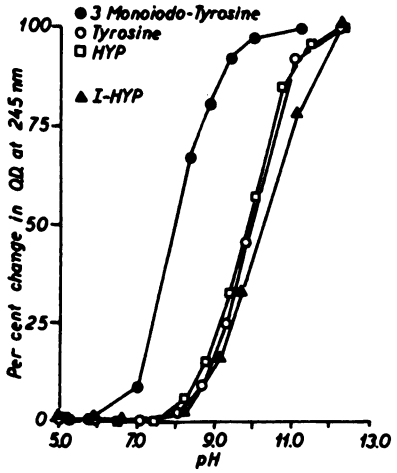


FIG. 7. *Effect of varying the pH on absorbance of I-HYP, HYP, tyrosine, and 3-monoiodotyrosine at 245 nm*

yielded only one radioactive product which cochromatographed with Peak I on hplc. Thus, Peak I became the focus of our study.

Because binding studies often require long incubations at 32.5°C, the reproducibility of the iodination and the stability of the iodinated analog were determined. It was found that certain iodides, while giving higher percentages of incorporation into ethyl acetate-extractable materials by Method A, yielded product(s) that after a 2-h incubation at 37°C resulted in a 77% loss of ethyl acetate-extractable label (Fig. 5). Thus, the usefulness of this type of material in biological studies is questionable. On the other hand, fresh carrier-free $Na^{125}I$ such as obtained during these studies from Amersham gave reproducible incorporations of 40–50% into ethyl acetate extracts and always resulted (by Method A) in at least two main radioactive peaks on hplc. Peak I is fairly stable at 37°C; a 2-h incubation resulted in a 33% loss of label from this peak. These findings make it imperative to check the stability of Peak I whenever a new source of iodide is used. The net effect of loss of label was to decrease the apparent specific activity of [^{125}I]I-HYP with time. Thus, studies in which net cpm are measured as indicative of [^{125}I]I-HYP binding should be corrected for degradation of [^{125}I]I-HYP with time. The ^{125}I-labeled material obtained by iodinating HYP with Method B was found to cochromatograph both on hplc and on QAE-Sephadex with I-HYP obtained with Method A (Fig. 4) and to be stable to incubation at 37°C for 2 h (not shown). In all probability the same material is synthesized by both methods.

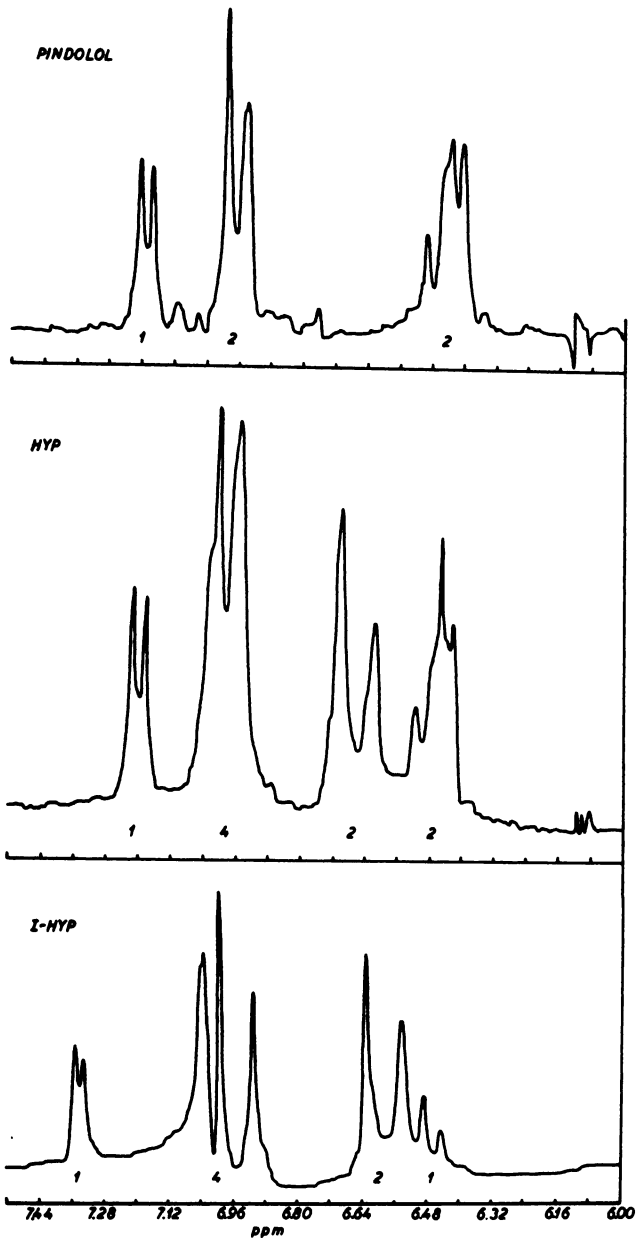


FIG. 8. *Chemical shifts (in ppm from tetramethylsilane) of resonances of protons from the aromatic rings of HYP and I-HYP*

Spectra were determined at 100 MHz in DMSO-d_6. Numbers under doublets and multiplets indicate the corresponding integrals. For further details see Methods and text.

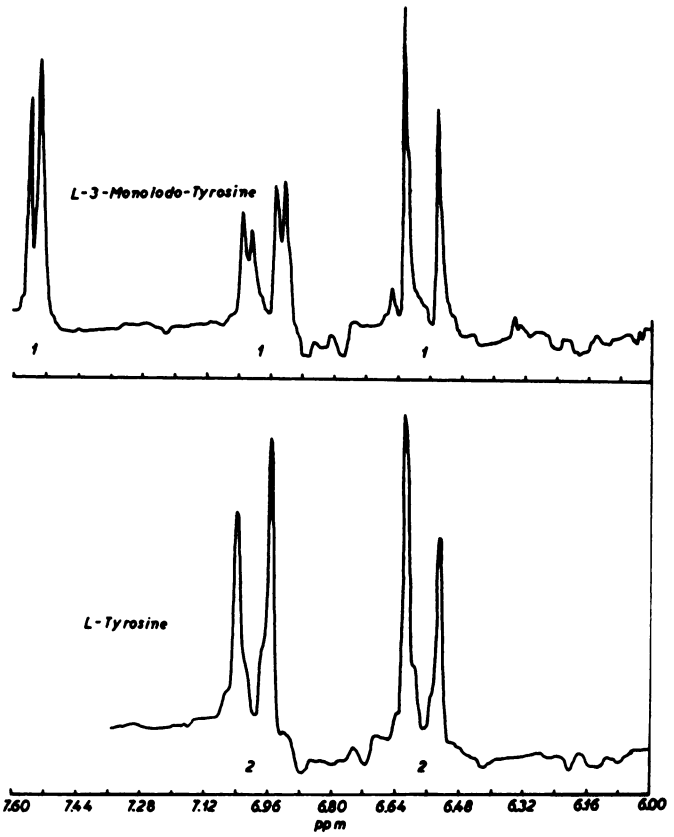


FIG. 9. *Chemical shifts [in ppm from 3-[trimethylsilyl]propanesulfonic acid (sodium salt)] of resonances of protons from the aromatic rings of tyrosine and 3-monoiodotyrosine*

Spectra were determined at 100 MHZ in D_2O/NaOD. Numbers under doublets and multiplets indicate the corresponding integrals. For further details, see Methods and text.

Two approaches were used to study the position of the iodine on I-HYP. The first approach was to measure the effect of iodination on the pK of the hydroxyl group of the *para*-hydroxylbenzyl moiety. It is known that iodination on carbon-3 of tyrosine lowers the pK of the phenolic hydroxyl group and that this effect can be measured by spectrophotometric titration (24). To test the feasibility of the technique with I-HYP, spectra of tyrosine and 3-monoiodotyrosine were determined in so-

TABLE 1

^{1}H-NMR assignments

^{1}H-NMR data from computer analysis of the aromatic proton spectra. The upper half of the spin Hamiltonian is presented. Chemical shifts in Hertz are on the diagonal and spin–spin coupling constants on the off diagonals.

a. Pindolol

	2I	3I	5I	6I	7I
2I[a]	718.92	3.12	0.13	0.37	−0.41
3I		641.88	−0.14	−0.34	0.34
5I			645.65	7.48	1.51
6I				695.91	8.36
					696.85

b. HYP

	3,5T	2,6T	2I	3I	5I	6I	7I
3,5T	667.00	8.56					
2,6T		698.30					
2I			720.14	3.12	−0.02	−0.36	−0.12
3I					−0.06	0.40	0.16
5I					645.83	5.88	0.95
6I						697.67	6.83
7I							698.98

c. I-HYP

	3,5T	2,6T	2I		5I	6I	7I
3,5T	657.19	8.41					
2,6T		692.34					
2I			731.49		647.14	4.51	4.01
5I						699.03	−0.01
6I							698.98
7I							

d. Tyrosine[b]

	3,5T	2,6T
3,5T	657.00	9.00
2,6T		699.00

e. I-Tyrosine[b]

	2T	5T	6T
2T	755.00	—	2.00
5T		695.00	8.00
7T			698.00

[a] The letter *I* represents protons on the indole moiety and the letter *T*, protons of the *para*-hydroxybenzyl.

[b] Computer simulation not done for tyrosine or I-tyrosine.

lutions adjusted to a range of pH values. At a wavelength of 245 nm, a p*K* for tyrosine of 9.9 and for 3-monoiodotyrosine of 8.0 were determined from plots of percentage variation in absorbance vs pH (Figs. 6 and 7). These values correlated well with those of 10.1 and 8.2, respectively, reported in the literature (23). In a preliminary experiment we determined that under our conditions of titration, the absorbance of tryptophan does not vary with pH at 245 nm. Thus, the uv titration spectra for HYP and I-HYP at this wavelength were solely dependent on the phenol moiety. As shown in Figs. 6 and 7, the uv titration for HYP and its iodinated derivative correlated with that for tyrosine, implying that the iodination of HYP does not occur on the phenol ring.

The second approach used to locate the iodination site on HYP utilized NMR spectroscopy. Peak assignments for pindolol were made by comparison to spectra of indole. The doublet farthest downfield at 7.17–7.21 ppm was identified as the proton on the 2 position of indole. The integral was set as one for this doublet. Based on this assignment, the two remaining multiplets integrated as two protons each. Because of shielding effects and values described in the literature (21, 24), the protons on the 3 and 5 positions were assigned to the farthest upfield multiplet at 6.41–6.50 ppm. Protons on the 6 and 7 positions were assigned to the peaks at 6.94–6.99 ppm. Assignments for HYP were made by comparison to the spectra for pindolol and tyrosine. The only differences in the HYP and pindolol spectra were the increase in the integral of the multiplet at 6.95–7.03 ppm from 2 to 4 and the appearance of a new doublet at 6.62–6.71 ppm with an integral of 2. These changes are consistent with the addition of a hydroxybenzyl moiety to the spectrum. Based on the tyrosine spectrum and the shielding effect of the oxygen in the hydroxyl group, the new doublet at 6.62–6.71 ppm was assigned to the 3 and 5 benzyl protons, and the increase in the multiplet at 6.95–7.03 ppm was attributed to the 2 and 6 benzyl protons.

Several changes occur in the NMR spectrum upon iodination of HYP: (i) the multiplet at 6.41–6.50 ppm with an integral of 2 reduces to a doublet with an integral of 1; (ii) the multiplet at 6.95–7.03 ppm resolves into a multiplet and a singlet peak at 6.91–7.05 ppm; (iii) the 1H doublet at 7.18–7.21 ppm shifts downfield to 7.33–7.35 ppm and its spin–spin coupling constant decreases from 3.1 to 2.1 Hz.

Two observations rule out the benzyl moiety as the site of iodination. First, the integral of 2 for the *ortho*-benzyl protons remains 2 after iodination. A value of 1 would be expected if the benzyl ring was iodinated, as is the case with tyrosine and monoiodotyrosine. Second, the complexity of the HYP spectrum simplifies on iodination. Iodination of tyrosine increases the complexity of the spectrum from two doublets each with an integral of 2 to two doublets and one multiplet each with an integral of 1.

The loss of a proton is evident in the multiplet at 6.43–6.52 ppm indicating iodination on either the 3 or the 5 position of the indole ring. Several observations are consistent with position 3 as the site of iodination. First, the downfield shift of the 2-indole proton is consistent with halogenation at position 3 and the changes in the coupling constant from 3.1 to 2.1 Hz of the 2-indole peak is not inconsistent with substitution on position 3. In HYP a $J_{2,3}$ of 3.1 Hz is comparable to published values in substituted indoles (25, 26). The spin–spin coupling constant of 2.1 Hz in I-HYP and the slant of the doublet downfield is consistent with the 2 position proton coupling to the nitrogen proton (26) which, although not observed in HYP, may be due to sample preparation. Finally, the remaining doublet at 6.45–6.50 ppm in the spectrum of I-HYP has a spin–spin coupling constant of 4.3 Hz, compatible with the splitting distances in the multiplet assigned to the 6- and 7-indole protons, i.e., 4.6 Hz. The refined assignments shown in Table 1 are in complete agreement with the above interpretation. Thus, on the basis that no changes occur in spectra relating to the *para*-hydroxybenzyl moiety and that NMR spectra show an alteration in the indole ring of I-HYP, we conclude that I-HYP is iodinated most probably on position 3 of its indole moiety with Structure I being that of Peak I I-HYP prepared here.

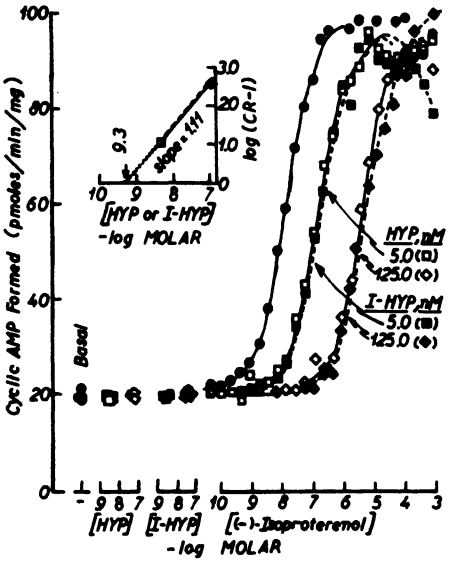


FIG. 10. *Effect of molar concentrations of HYP, I-HYP, and (−)-isoproterenol on amount of cyclic AMP formed*

Kitten ventricle heart membranes (35 μg) were incubated for 10 min at 32.5°C in 50 μl of medium containing 0.1 mM [α-^{32}P]ATP (sp act 435 cpm/pmol), 5.0 mM $MgCl_2$, 1.0 mM EDTA, 1.0 mM [^{3}H]cAMP (ca. 10,000 cpm), a nucleoside triphosphate-regenerating system consisting of 20 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase, 0.02 mg/ml myokinase, 0.1 mM ascorbic acid, 10 μM GTP, and the indicated concentrations of (−)-isoproterenol in the absence (●) and the presence of 5 nM HYP (□), 125 nM HYP (◇), 5 nM I-HYP (■), and 125 nM I-HYP (◆). The reactions were initiated by addition of the membranes and stopped by addition of 100 μl of 40 mM ATP, 10 mM cAMP, and 1% sodium dodecyl sulfate. Concentration ratios (CR values) of equieffective concentrations of isoproterenol in the absence and presence of theindicated concentrations of HYP and I-HYP were calculated and plotted according to Arunlakshana and Schild [(22), inset), yielding a Schild plot with a slope of 1.11 and an intercept value of $10^{-9.3}$ M for either HYP or I-HYP. Concentration of added HYP was determined by spectrophotometry using a molar absorption coefficient of 7000 at 269 nm. Concentration of added I-HYP was calculated on the basis of the specific activity of the [^{125}I]KI (550 cpm/nmol) used to synthesize the particular batch of I-HYP used in this experiment.

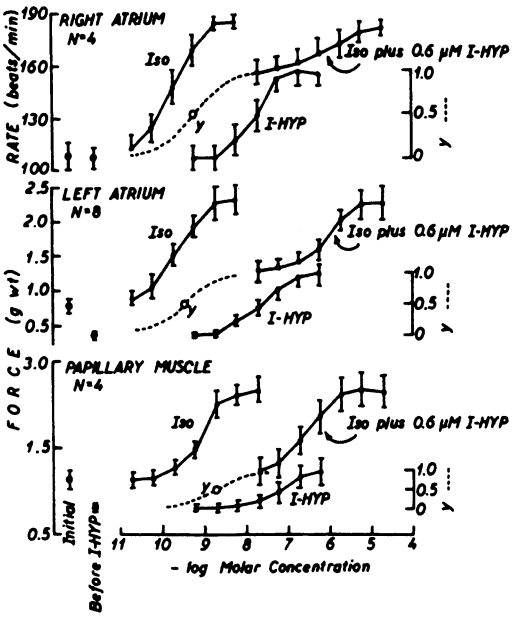
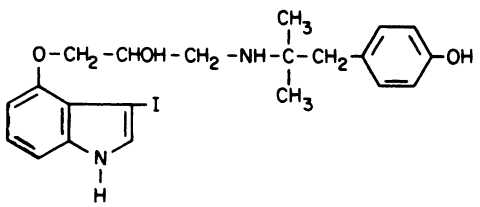


FIG. 11. *Concentration–effect curves for the action of (−)-isoproterenol (Iso), I-HYP, and (−)-isoproterenol plus 0.6 μM I-HYP on isolated spontaneously beating right atria and on isolated electrically driven left atria and papillary muscles*

For details see Methods. *N*, Number of tissues (animals); *y*, occupancy of receptors by I-HYP calculated according to $y = 1/(1 + K_p/x)$, where x = concentration of I-HYP and K_p = corrected equilibrium dissociation constant of the β-adrenergic receptor for I-HYP (○). Corrected K_p values were calculated in two steps: First, apparent pK_p values were determined from plots of equieffective concentrations of (−)-isoproterenol in the absence *versus* the same in the presence of 0.6 μM I-HYP as described by Marano and Kaumann (20). These plots (not shown) gave apparent pK_p values for right atria, left atria, and papillary muscles of 9.38 ± 0.06, 9.73 ± 0.23, and 8.88 ± 0.24, respectively. Then, these values were corrected for the slight desensitization that occurred between a first and a second concentration–effect curve for (−)-isoproterenol without I-HYP, yielding values of 9.21 ± 0.06, 9.45 ± 0.23, and 8.71 ± 0.24, respectively. This latter set of values was used to construct the occupancy curves as shown in the figure



STRUCTURE I

Peaks I and II could easily be separated and correlated upon chromatography over C_{18}-μBondapak, QAE-Sephadex, or paper. We did not locate, however, the minor peak eluting close to HYP from the reverse-phase column, upon chromatography on paper or QAE-Sephadex. Further studies on properties of this peak are not necessary if hplc is used to purify products of iodination of HYP. However, if QAE-Sephadex and/or paper chromatography are the sole purification methods used, it may be advisable to determine the chromatographic properties of this peak as well.

An iodinated product was described by Brown *et al.* (4) which had a pattern of elution from hplc similar to that of Peak I. These authors concluded that the phenolic, rather than the indole, moiety was the site of iodination. However, there were not sufficient data presented in their article to determine whether this material is the same as any of the materials prepared in our laboratory.

Pharmacological activity as measured by the blockade of isoproterenol stimulation of adenylyl cyclase in kitten ventricle membranes was identical for HYP and I-HYP. Our finding of a K_B of 0.4 nM for both HYP and I-HYP is close to the value of 0.13 nM reported in the literature for intact S49 cells (1), but is 10-fold lower than the value of 4.9 nM measured with rat ventricle proteins (3) and 10-fold greater than the value of 0.03 nM found with intact rat glioma cells (2) and 0.024 nM for turkey erythrocyte membranes (11). While these differences most probably reflect differences in the properties of the β-adrenergic receptors involved, they may also reflect difficulties in stability of the iodinated probe used. Experiments to be reported elsewhere with S49 cell membranes[2] showed that ^{125}I-HYP does indeed interact with *beta*-adrenergic receptors of these cells. Binding exhibited a K_D for I-HYP (prepared by Method B) of 0.02 nM, was displaced stereospecifically by isoproterenol and propranolol and displacement by isoproterenol was influenced by guanine nucleotides. Taken together these findings demonstrate the usefulness of the ^{125}I-HYP prepared by us as a probe for *beta*-adrenergic receptors.

Studies on the interaction of I-HYP with chronotropic and inotropic receptors of kitten heart (Figure 11), revealed that I-HYP, like HYP, is a partial agonist (5). We are currently investigating pharmacologic characteristics of this interesting type of compound, the list of which not only includes I-HYP and HYP, but also pindolol (the parent compound of HYP and I-HYP) and alprenolol (5). Among the questions that need to be asked is whether the agonistic effects of these compounds is indeed due to interaction with *beta*-adrenergic receptors and, if so, what kind of coupling features give rise to this phenomenology. The availability of a well characterized compound labeled to a high specific activity should aid in these investigations.

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