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ISOLATION AND IDENTIFICATION OF A RENIN INHIBITOR FROM OX BILE

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Abstract—A renin inhibitor in ox bile was purified by ammonium sulfate fractionation, dialysis and chromatography on Bio-Gel P-2 and Amberlite XAD-2 resin. The inhibitor was shown to be taurodeoxycholic acid by means of nuclear magnetic resonance spectroscopy, gas chromatography, amino acid and elemental analyses. Further confirmation of the assignment was established by the direct comparison of its infrared spectrum with that of an authentic sample. Taurodeoxycholic acid inhibited the reaction of rabbit renin with angiotensinogen *in vitro*. Glycocholic acid, also present in ox bile, however, had no inhibitory effect on the renin angiotensinogen reaction.

RENIN is an endopeptidase which splits a leucyl–leucine bond in an alpha-2-globulin substrate, angiotensinogen, to yield a decapeptide, angiotensin I. Angiotensin I is subsequently converted into an octapeptide, angiotensin II, by a converting enzyme contained in various organs and plasma. Angiotensin II has a pressor activity.

Three kinds of renin inhibitors have been reported. These are the naturally occurring renin preinhibitor, lysophospholipid, reported by Sen *et al.*[1,2] and Osmond *et al.*,[3] methyl or ethyl esters of synthetic tetrapeptides, Leu-Leu-Val-Tyr and Leu-Leu-Val-Phe, from our laboratory,[4] and heparin.[5] A recent report from our laboratory[6] indicated that another active inhibitor of renin occurred in rabbit bile.

In the present paper we report the purification, identification and some biochemical properties of an active inhibitor of renin occurring in ox bile.

MATERIALS AND METHODS

Materials. Amberlite XAD-2 resin, a styrene-divinylbenzene copolymer, was purchased from Rohm & Haas, Bio-Gel P-2 from Bio-Rad Laboratories, and Dowex 50-X8 from the Dow Chemical Company. Synthetic Val^5-angiotensin II amide was supplied by the Ciba Pharmaceutical Institute. Glycine, taurine, cholic acid and deoxycholic acid were purchased from Wako Pure Chemical Ind.

Preparation of rabbit renin and renin substrate. Renin was prepared from rabbit renal cortex using the method of Haas *et al.*,[7] followed by ammonium sulfate fractionation between 30 and 60 per cent saturation, and dialysis against physiological saline containing 2×10^{-3} M EDTA. The activity of renin was equivalent of 17·3 μg Val^5-angiotensin II amide/ml of the preparation (protein concentration 10·8 mg/ml),

according to a direct method[1] based on the pressor response by the intravenous injection into a rat as described below. The renin preparation was diluted 20-fold with physiological saline prior to use.

Renin substrate was prepared from the heparinized plasma of rabbits nephrectomized bilaterally according to the method of Sen *et al.*[1] 24 hr before use. This preparation contained renin substrate equivalent to 18·0 μg angiotensin/ml (protein concentration, 9·3 mg/ml), according to the indirect method of Pickens *et al.*[8] by incubation of renin substrate with an excess of rabbit renin in the presence of EDTA and DFP. The substrate used for experiments was diluted 5-fold with physiological saline.

Assay of angiotensin formed. The reaction mixture consisted of 0·2 ml of renin preparation, 0·5 ml of renin substrate, 1·0 ml of distilled water containing a test material, and 1·3 ml of 1/15 M phosphate buffer, pH 6·4. The mixture was incubated at 37° for 10 min and the reaction was stopped by heating in a boiling water bath for 5 min. The control assay system produced usually 0·15–0·16 μg/ml of angiotensin. The renin–renin substrate reaction in the assay condition was a first-order reaction as shown in Fig. 1. The angiotensin formed was assayed by means of its pressor response in rats weighing 170–180 g, anesthetized by intraperitoneal injection of pentobarbitone sodium (50 mg/kg), vagotomized, and treated subcutaneously with pentolinium tartrate (10 mg/kg). All assays were performed in comparison with Val^5-angiotensin II amide.

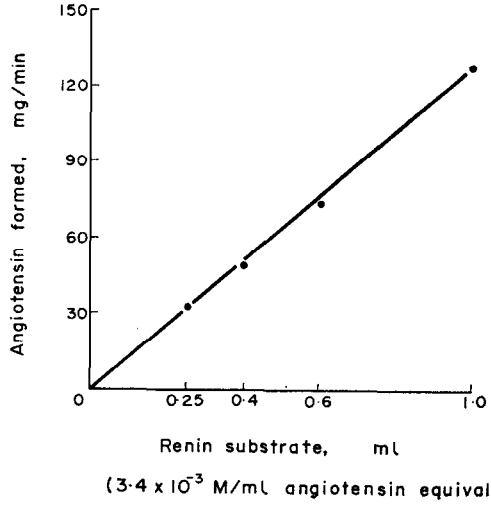

FIG. 1. Angiotensin formation of renin in the presence of various amounts of renin substrate.

Analytical methods. Amino acid analyses were performed by the method of Spackman *et al.*[9] in a Hitachi type KLA-3B analyzer. A sample (1 mg) was hydrolyzed with 6 N HCl (2 ml) for 24 hr in a sealed tube at 105°. The hydrolyzed sample was dried over NaOH pellets under reduced pressure and was taken up in 0·2 N citrate buffer (pH 2·2) for introduction into the analyzer.

Gas–liquid chromatography was performed on a Yanagimoto type GCG-550 gas chromatograph equipped with a hydrogen-flame ionization detector. Samples (1–20

mg) were hydrolyzed with 5 N NaOH at 120° for 7 hr. After acidification with hydrochloric acid, the hydrolyzed samples were extracted with chloroform, methylated with diazomethane, treated with trifluoroacetic anhydride, and injected into the column. The column was a 1·2 m × 2·3 mm (i.d.) glass tube packed with 0·6% QF-1 on Gas-Chrom Q (100–200 mesh). The flow rate of helium carrier gas was kept constant at 13 ml/min. The flash heater was maintained at 210 ± 10° and the detector at 230°. The column temperature was maintained at 200°.

Nuclear magnetic resonance spectra were determined with a Varian model HA-100 spectrometer using tetramethylsilane as an internal standard. Infrared spectra were obtained using a Hitachi type EPI-2 spectrometer with samples prepared in KBr pellets.

Taurodeoxycholic acid was prepared essentially according to the procedure of Norman.[10]

RESULTS

Isolation of a renin inhibitor. The isolation procedure is outlined in Fig. 2.

Ammonium sulfate fractionation. Ox bile was obtained immediately after slaughter. To about 2 l. of fresh bile, ammonium sulfate was added slowly with stirring to give 45% saturation. The resulting dark green viscous precipitate was then separated by centrifugation at 6500 *g* for 20 min.

Dialysis. The precipitate was dissolved in water and dialyzed against water for 20 hr and then against 7 l. of acetone–water (2:1, v/v) for 15 hr. The outer solution

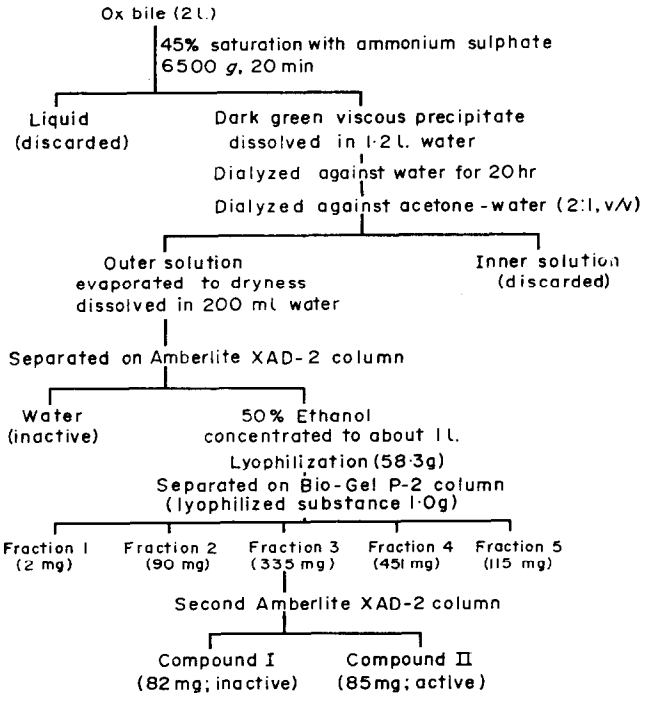

FIG. 2. Outline of fractionation used to obtain an ox bile renin inhibitor.

was evaporated under reduced pressure to dryness. The resulting residue was dissolved in 200 ml water and purified further.

Amberlite XAD-2 chromatography. The solution was applied to a column (10 × 70 cm) of Amberlite XAD-2 (100–200 mesh). The column was developed with 15 l. water and then 15 l. of 50% ethanol. The eluate obtained with 50% ethanol contained the renin inhibitor. The eluate was evaporated to about 1 l. under reduced pressure, followed by lyophilization (yield, 58·3 g).

Bio-Gel P-2 chromatography. One of the lyophilized residue was dissolved in a small amount of water and applied to a Bio-Gel P-2 column (200–400 mesh, 2·6 × 100 cm). Elution was carried out with water and detected by a Waters model R-4 Refractive Index Monitor (Fig. 3). Fraction 3 containing the renin inhibitor was pooled and evaporated to dryness.

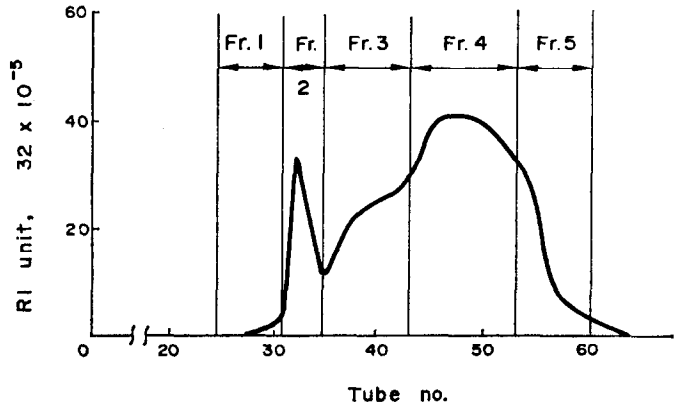

FIG. 3. Chromatogram obtained from gel filtration on Bio-Gel P-2 column. Eluant, water; each 10-ml fraction was collected; detection, refractive index.

Second Amberlite XAD-2 column chromatography. The residue was dissolved in 20% ethanol and was rechromatographed on an Amberlite XAD-2 column (2·6 × 100 cm) pre-equilibrated with 20% ethanol. Gradient elution (20–90% ethanol) was carried out, whereupon two fractions were obtained (Fig. 4). The second fraction (II; tubes 38–47) possessed the high activity. No activity was detected in the first fraction (I; tubes 22–31). Fraction I was dissolved in a small amount of water by slight heating. Acetone was added slowly to the solution to a final concentration of 90%. The solution was allowed to stand in a refrigerator overnight. Fine colorless needles, m.p. 143–145°, were obtained. Crystallization of II was achieved in a similar way (the final concentration of acetone was 95%); m.p. 149–151°.

Characterization of fractions I and II. The nuclear magnetic resonance (n.m.r.) spectra of I and II, determined in d_6-dimethyl sulfoxide solution, are shown in Fig. 5. The outstanding feature of the spectra was the sharp resonances attributed to angular methyl groups. This feature is common with steroids and it was deduced that I and II might be steroidal compounds. The angular methyl signals, however, were undetectable when the n.m.r. spectra were taken in deuterium oxide. Bile acids in water at physiological pH are known to associate into micelles.[11] Under the circumstance of n.m.r. measurements, non-equivalence of three protons of the angular methyl

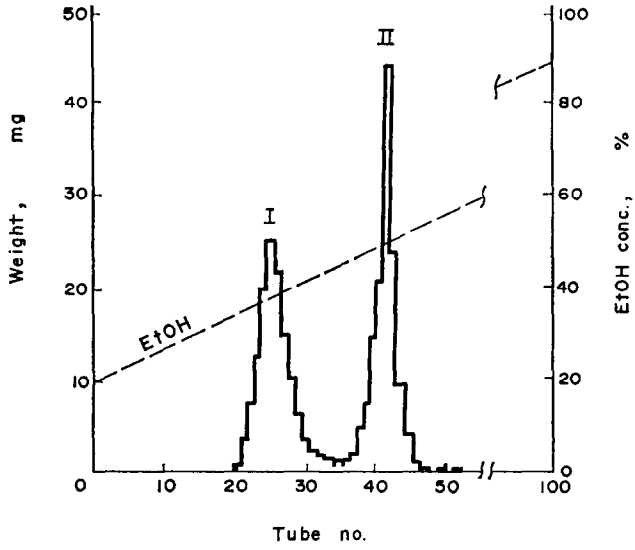

FIG. 4. Chromatogram obtained from Amberlite XAD-2 column. Eluant, gradient elution of ethanol concentration from 20 to 90 per cent; each 18-ml fraction was collected.

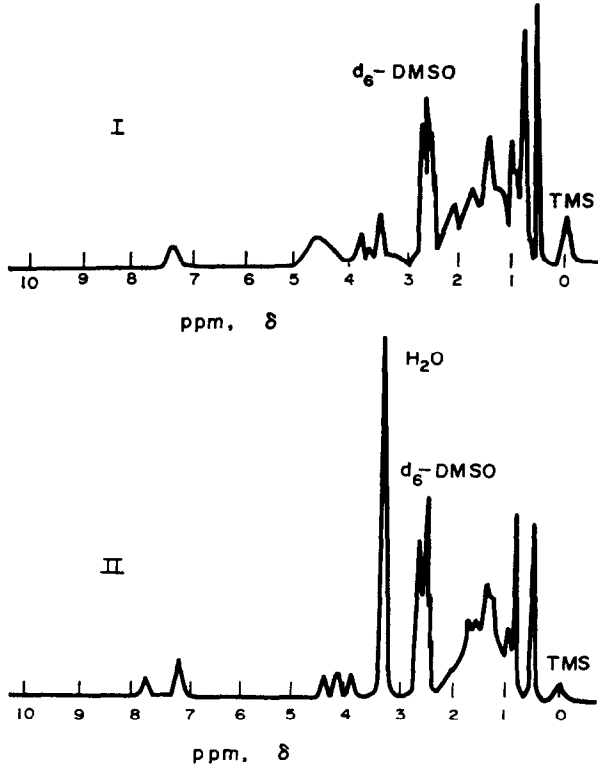

FIG. 5. Nuclear magnetic resonance spectra of compounds I and II.

group may arise, resulting in the disappearance of the sharp signal for the angular methyl group.

Gas chromatographic analyses, conducted with alkaline hydrolysates of I and II, showed the presence of cholic acid in I and of deoxycholic acid in II. On amino acid analysis, glycine and taurine were detected in the acid hydrolysates of I and II respectively. These facts strongly suggest that I is glycocholic acid and II is taurodeoxycholic acid.

Confirmation of the assignment was obtained from the elemental analyses and infrared (i.r.) spectra. A sample (6 mg) was dissolved in water and the solution was passed through a column of Dowex 50–X8, free acid form (0·5 × 10 cm), to remove ammonia. The eluate was evaporated to dryness and subjected to elemental and i.r. spectroscopic analyses. As shown in Table 1, the analytical data of I and II were identical with those of glycocholic acid and taurodeoxycholic acid respectively. The i.r.

TABLE 1. ANALYTICAL DATA OF COMPOUNDS I AND II

	I (m.p. 143·5–145·0)		II (m.p. 149·0–151·0)	
Analysis	Found	Calculated for glycocholic acid-3/2 H_2O	Found	Calculated for taurodeoxycholic acid-H_2O
C	62·96	63·38	60·16	60·32
H	9·40	9·41	9·15	9·15
N	2·81	2·84	2·69	2·70
S			6·83	6·19
Amino acid analysis	Glycine		Taurine	
Gas chromatography	Cholic acid		Deoxycholic acid	
Inhibitory activity against renin	−		+	

spectra indicated that absorption of I occurred at 1740 (carboxylic acid), 1660 (amide I band) and 1550 cm^{-1} (amide II band), while that of II occurred at 1660 (amide I band) and 1550 cm^{-1} (amide II band; Fig. 6). Further confirmation of the structure of I was established by the direct comparison of its i.r. spectrum with that of an authentic sample.

Biological results of compounds I and II. The inhibitory effects of compounds I and II on angiotensin formation by renin *in vitro* were examined. The control assay system produced 0·158 μg/ml of angiotensin. The addition of compound II (3·0 mg) to the incubation system resulted in a significant inhibition of angiotensin formation by renin (0·012 μg/ml), that is, 92 per cent inhibition, but no inhibition was observed when compound I (3·0 mg) was added. Addition of sodium taurodeoxycholate (3·0 mg) to the system showed a similar inhibitory effect on angiotensin formation (0·013 μg/ml), that is, 92 per cent inhibition. Figure 7 shows the inhibitory effects in the presence of various concentrations of compound II and synthetic sodium taurodeoxycholate (final concentration: 5×10^{-5} M, 10^{-4} M, 2×10^{-4} M, 5×10^{-4} M, 10^{-3} M and 2×10^{-3} M) on the angiotensin formation of renin. The inhibitory effect was expressed as a percentage reduction in the angiotensin formation of the control assay

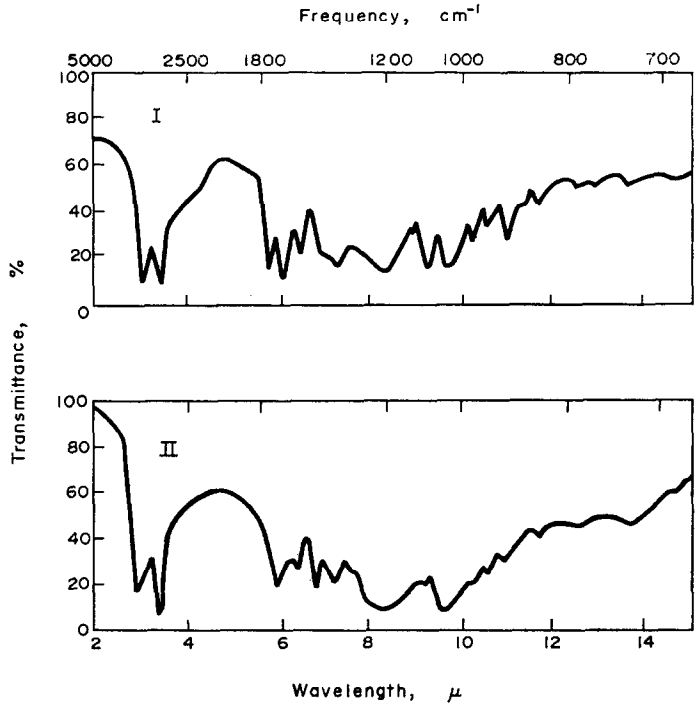

FIG. 6. Infrared spectra of compounds I and II.

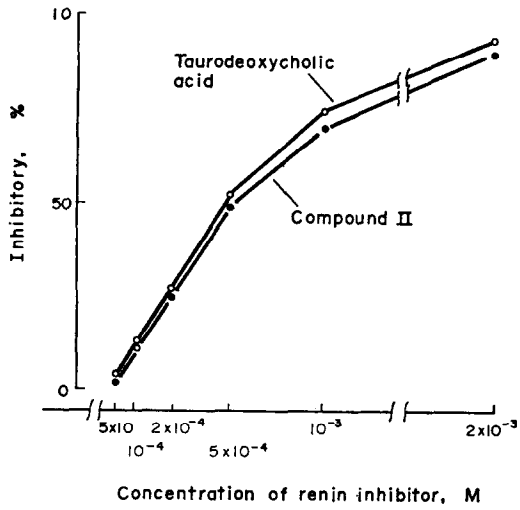

FIG. 7. Inhibitory effects in the presence of various concentrations of compound II and synthetic sodium taurodeoxycholic acid on the angiotensin formation of renin.

system, that is, inhibitory per cent. As shown in Fig. 7, the inhibitory per cent increased as the amount of the test samples added to the assay system increased. About 50 per cent inhibition in angiotensin formation was found at a concentration of 5×10^{-4} M of the materials in the assay system.

Val^5-angiotensin II amide (0·3 μg) was mixed with compound I (3·0 mg) or glycocholic acid (3·0 mg) and incubated at 37° for 30 min. The pressor response of these mixtures was the same as that of angiotensin II amide. The results indicated that compound I and glycocholic acid neither modified the physiological pressor activity of angiotensin nor showed a hypotensive effect when injected intravenously. One-half ml of renin substrate was incubated with 3·0 mg compound II or synthetic sodium taurodeoxycholate at pH 6·4, 37° for 2 hr. After the incubation, the concentration of renin substrate was measured by a modification of the method of Pickens *et al.*[8] Incubation of renin substrate with these compounds was sustained without any reduction of the amount of angiotensin formed as compared to control experiments. Compound II and taurodeoxycholic acid, therefore, do not inactivate renin substrate under the conditions of the experiment. One-fifth ml renin was incubated with 3·0 mg compound II or taurodeoxycholic acid at pH 6·4, 37° for 90 min. The preincubation of renin with these compounds showed no reduction of enzyme activity *in vivo* (pressor response) or *in vitro* (angiotensin formation). In these experiments, these compounds were removed from the incubation mixtures by dialysis against distilled water before estimation of the amount of renin substrate or renin activity. The results suggested that compound II and sodium taurodeoxycholate inhibited the reaction of renin with angiotensinogen.

DISCUSSION

Our previous experiments[6] proved that native rabbit bile had a potent inhibitory effect on the reaction of renin with angiotensinogen *in vitro*, and the inhibitor was obtained in the precipitate of 40 per cent saturation with ammonium sulfate. This inhibitory effect of rabbit bile on angiotensin formation by renin could be attributed to a renin inhibitor and to a substance that inactivated renin substrate.

The present study was designed to isolate and identify an inhibitor from ox bile. Two compounds, I and II, were obtained by ammonium sulfate fractionation and chromatography on Bio-Gel P-2 and Amberlite XAD-2 resin columns. The analytical results showed that compound I was glycocholic acid and compound II was taurodeoxycholic acid. The biological tests indicated that compound II produced a significant inhibition of renin activity, while compound I had no inhibitory effect. From the results, it was shown that the renin inhibitory effect of bile was due to taurodeoxycholic acid. Taurodeoxycholic acid may inhibit the reaction of renin with angiotensinogen, because the renin substrate and enzyme activity were not affected by the compound at all.

Recently, we have reported[12] that sodium deoxycholate inhibited renin activity in a competitive manner. These facts suggest that the 7-deoxy form of bile acids is essential to renin inhibition. A small amount of bile acids is present in normal blood, but the biological importance in blood pressure regulation is not clear. Another report concerning the renin inhibition by various bile acids will be published elsewhere.

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