

## Formation and Excretion of Pyrrole-2-Carboxylate in Man

Anne M. Heacock, Elijah Adams

*J Clin Invest.* 1974;54(4):810-818. <https://doi.org/10.1172/JCI107821>.

**Research Article**

A detailed investigation of the purification of pyrrole-2-carboxylate (PCA) from human and rat urine indicates that previously reported mean values overestimate the correct quantity of free PCA by a factor of approximately three for rat urine and approximately five for human urine. Although several criteria of purity were satisfied by a previous method, pyrrole-reactive impurities were still present in the final fractions. These impurities are separated from PCA by chromatography through an amino acid analyzer ion-exchange resin. With the corrected method, normal human values for endogenous urinary PCA in 16 individuals averaged 0.51  $\mu\text{mol/day}$ , with a range of 0.20-1.3  $\mu\text{mol}$  and a SD of 0.31  $\mu\text{mol}$ . The probable source of human PCA is free hydroxy-L-proline, as inferred from the high value for PCA in the urine of a subject with hereditary hydroxyprolinemia, and from the three- to eightfold elevation in PCA excretion by two normal subjects after a large oral load of hydroxy-L-proline. Subcutaneous administration of  $[2-^{14}\text{C}]\text{PCA}$  to a single human subject indicated almost complete conversion of the exogenous compound to derivatives, which are largely excreted in the urine. Data are discussed suggesting that much or all of the PCA in human urine may be formed in urine from a labile precursor, presumably  $\Delta^1$ -pyrroline-4-hydroxy-2-carboxylate.

**Find the latest version:**

<https://jci.me/107821/pdf>



# Formation and Excretion of Pyrrole-2-Carboxylate in Man

ANNE M. HEACOCK and ELIJAH ADAMS

*From the Department of Biological Chemistry, University of Maryland School of Medicine, Baltimore, Maryland 21201*

**ABSTRACT** A detailed investigation of the purification of pyrrole-2-carboxylate (PCA) from human and rat urine indicates that previously reported mean values overestimate the correct quantity of free PCA by a factor of approximately three for rat urine and approximately five for human urine. Although several criteria of purity were satisfied by a previous method, pyrrole-reactive impurities were still present in the final fractions. These impurities are separated from PCA by chromatography through an amino acid analyzer ion-exchange resin. With the corrected method, normal human values for endogenous urinary PCA in 16 individuals averaged 0.51  $\mu\text{mol/day}$ , with a range of 0.20–1.3  $\mu\text{mol}$  and a SD of 0.31  $\mu\text{mol}$ . The probable source of human PCA is free hydroxy-L-proline, as inferred from the high value for PCA in the urine of a subject with hereditary hydroxyprolinemia, and from the three- to eightfold elevation in PCA excretion by two normal subjects after a large oral load of hydroxy-L-proline. Subcutaneous administration of [2- $^{14}\text{C}$ ]PCA to a single human subject indicated almost complete conversion of the exogenous compound to derivatives, which are largely excreted in the urine. Data are discussed suggesting that much or all of the PCA in human urine may be formed in urine from a labile precursor, presumably  $\Delta^1$ -pyrroline-4-hydroxy-2-carboxylate.

## INTRODUCTION

Pyrrole-2-carboxylic acid was synthesized over a century ago (2), but its history as a compound of biological origin is rather recent. It was first identified as a degradation product of sialic acids (3), then as a

These studies comprise portions of a doctoral thesis (1) submitted to the University of Maryland by Anne M. Heacock. The thesis should be consulted for many details not presented in this paper.

Dr. Heacock's present address is Mental Health Research Institute, University of Michigan, Ann Arbor, Mich. 48104.

Received for publication 15 March 1974 and in revised form 3 June 1974.

derivative of the oxidation of the D-hydroxyproline isomers by mammalian D-amino acid oxidase (4). The latter relationship results from the lability of the direct oxidation product,  $\Delta^1$ -pyrroline-4-hydroxy-2-carboxylic acid, which loses water spontaneously to form the pyrrole (Fig. 1). A similar reaction is catalyzed by the more specific allohydroxy-D-proline oxidase of *Pseudomonas* (5).

In whole animal observations, pyrrole-2-carboxylate (PCA)<sup>1</sup> was identified in rat or human urine after administration of the D-isomers of hydroxyproline (6), a finding ascribable to the action of D-amino acid oxidase. The finding that radioactive hydroxy-L-proline, fed to rats, also labeled urinary PCA (7) was difficult to interpret because of the presence in rat colon of bacteria capable of catalyzing the conversion of hydroxy-L-proline to allohydroxy-D-proline (8). However, the conversion in rats of parenterally administered L-proline to PCA, reported by Gerber, Gerber, and Altman (9), was unexplained until our recent finding (10) that the L-amino acid oxidase of rat kidney is able to oxidize hydroxy-L-proline to  $\Delta^1$ -pyrroline-4-hydroxy-2-carboxylate at a rate sufficient to account for the endogenous production of PCA. Similar findings and the same conclusion were subsequently reported from another laboratory (11).

The excretion of endogenous PCA by man has been reported from three laboratories: by Gerber, Traelnes, Wood, and Altman (12), by Yaminishi, Iguchi, Ohyama, and Matsumura (13), and by ourselves (10). The methods for isolating urinary PCA varied, being based in two studies (12, 13) on conversion to pyrrole and its distillation, and in another study (10) by extraction of PCA into ether and removal of impurities by ion-exchange chromatography. Nevertheless, values from these laboratories were all in close agreement, averaging between 2 and 3  $\mu\text{mol}/24\text{ h}$  for healthy adults.

In the work described here, we have investigated

<sup>1</sup>Abbreviation used in this paper: PCA: pyrrole-2-carboxylate.

earlier methods for isolating and measuring urinary PCA, with the conclusion that all previously reported values considerably overestimate the quantity of free PCA excreted. We also report studies on the effect of elevated body levels of free hydroxy-L-proline on PCA excretion, on the excretion pattern after administering radioactive PCA to man, and on the source and site of PCA formation.

## METHODS

**Purification of PCA from human urine: method 1.** Urine from healthy volunteers was collected in bottles containing 2 ml of toluene and 20 ml of 1 M Tris, pH 9.0. No attempt was made to control diet or activity in these subjects. The 24-h collection began after the first morning urine was discarded and included the first morning urine of the following day. Samples were stored at  $-15^{\circ}\text{C}$  or analyzed within a day after collection. The volume was measured and a 50-ml sample was removed; to it was added [ $^3\text{H}$ ]-PCA ( $4\text{--}6 \times 10^4$  dpm). The urine sample was adjusted to pH 3–4 with 2 N HCl and extracted with 125 ml of ether in a continuous liquid-liquid extractor for at least 4 h (sometime overnight) at room temperature. The ether extract was brought almost to dryness in a flash evaporator at  $35^{\circ}\text{C}$ , taken up in 4 ml of water, adjusted to pH 2–4 if necessary, and applied to a  $1 \times 10\text{-cm}$  column of Dowex-50  $\text{H}^+$  (X8, 100–200 mesh, Dow Chemical USA, Membrane Systems Div., Midland, Mich.). The column was washed with distilled water and the first 50 ml of effluent were collected, evaporated to dryness, brought to 2 ml with water, and analyzed for PCA and radioactivity. By this method, PCA was stable in urine kept for 18 h at room temperature under collection conditions, as determined either by adding authentic PCA to a sample of urine or comparing measurements of endogenous PCA made immediately and after storage.

**Further purification of PCA.** The ether extract of urine was concentrated to 10–15 ml in a flash evaporator and extracted with 10 ml of 0.1 N NaOH by vigorous shaking in a screw-cap tube. The water phase was brought to 0.5 N in HCl, placed in a distilling flask, and heated over a flame almost to dryness; the distillate was collected and pyrrole-reactive material was measured with reference to a pyrrole standard, freshly distilled and appropriately diluted.

**Chromatography and electrophoresis.** The concentrated Dowex-50 effluent was chromatographed on  $20 \times 20\text{-cm}$  silica gel sheets (No. 6060, Eastman Kodak Co., Rochester, N. Y.) in any of several solvents: *n*-propyl alcohol: water (64:36); ethyl alcohol: water (75:25); *tert*-butyl alcohol: formic acid: water (70:15:15); *sec*-butyl alcohol: 3% aqueous  $\text{NH}_3$  (100:44); ethyl acetate: isopropyl alcohol: 3% aqueous  $\text{NH}_3$  (9:6:4); *n*-propyl alcohol: 1 N  $\text{NH}_3$  (20:30); and *tert*-butyl alcohol: 1 N  $\text{NH}_3$  (100:44). Corresponding  $R_f$  values for PCA were 0.51, 0.77, 0.96, 0.87, 0.35, 0.73, and 0.81, for the respective solvents given. Flat-bed electrophoresis (Savant apparatus, Savant Instruments, Inc., Hicksville, N. Y.) was carried out in 0.05 M sodium acetate, pH 5.6, on Whatman 3 MM paper ( $65 \times 15\text{ cm}$ ) at 2,500 V for 2 h, with cooling. Under these conditions, PCA moved 25 cm toward the anode. Radioactivity on paper or thin-layer sheets was located by cutting out  $1 \times 2.2\text{-cm}$  sections and counting these directly in scintillation vials.

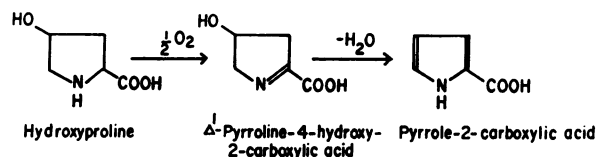


FIGURE 1 Formation of PCA from hydroxyproline. Formation of the pyrroline oxidation product results from enzymatic oxidation by L- or D-amino acid oxidase of the appropriate isomers of hydroxyproline. This product is unstable and readily loses a mole of water to yield PCA.

PCA was detected on paper or thin-layer sheets by dipping these in a *p*-dimethylaminobenzaldehyde solution (1 g of reagent in 90 ml of acetone and 10 ml of concentrated HCl) and heating the paper in an oven at  $120^{\circ}\text{C}$  for several minutes until a pink-purple color developed.

**Purification of PCA from human urine: method 2.** Method 1 was followed, except that a larger sample (360 ml) of urine was extracted with ether, to accommodate the extra purification step. To it was added approximately  $10^5$  dpm of [ $^{14}\text{C}$ ]PCA and the pH was adjusted to 3–4 with 2 N HCl. The sample was divided into six 60-ml portions and each was continuously extracted with ether as in method 1. The combined ether extract was evaporated almost to dryness and brought to 8 ml with water, and half was applied to each of two Dowex-50 columns, as above. The water effluent was collected as under method 1. The Dowex-50 effluents were combined and evaporated after adjusting the pH to 4–5. The residue was brought to 0.5 ml with water and applied to an amino acid analyzer column ( $0.6 \times 63\text{ cm}$ , Durrum DC-1 resin, Durrum Instrument Corp., Palo Alto, Calif.). The sample was washed in with Durrum Pico Buffer A, adjusted to pH 2.95. The column was operated at  $52^{\circ}\text{C}$  and eluted with the same buffer, pumped at a rate of 0.6 ml/min with a Milton-Roy minipump (Milton-Roy Co., St. Petersburg, Fla.), collecting fractions of 1.5 ml. Authentic PCA was eluted at 90–100 min; PCA in urine samples purified by method 1 or PCA added to such samples sometimes emerged from the column earlier, usually at 80–85 min. It should be emphasized that the elution position of PCA was somewhat variable, depending on the pH and complexity of the sample chromatographed (whether urine or a simpler solution) and on the history of the column. The binding forces that retard PCA on a cation-exchange column are not obvious, and it seems likely that they may be sensitive to small changes in conditions. It is therefore important to identify the PCA peak in such samples by cochromatography with radioactive PCA as an internal marker.

**Purification of PCA from rat urine.** Male Wistar rats (80–150 g) were kept in plastic metabolism cages permitting the gross separation of urine from feces. Up to five rats were kept in a cage and were offered water and food ad lib. Urine was collected in a vessel containing 1 ml of toluene and 2 ml of 1 M Tris, pH 9.0. Urine from several rats was pooled, filtered, and adjusted to pH 3–4 with 2 N HCl. For the determination of unlabeled endogenous PCA,  $4\text{--}6 \times 10^4$  dpm of radioactive PCA was added to the urine before extraction. For the purification of PCA by method 1 or 2, the rat urine was treated essentially as described above for human urine.

**Assay for PCA.** PCA was measured by a modification of the Neuman-Logan assay for hydroxyproline (14), with

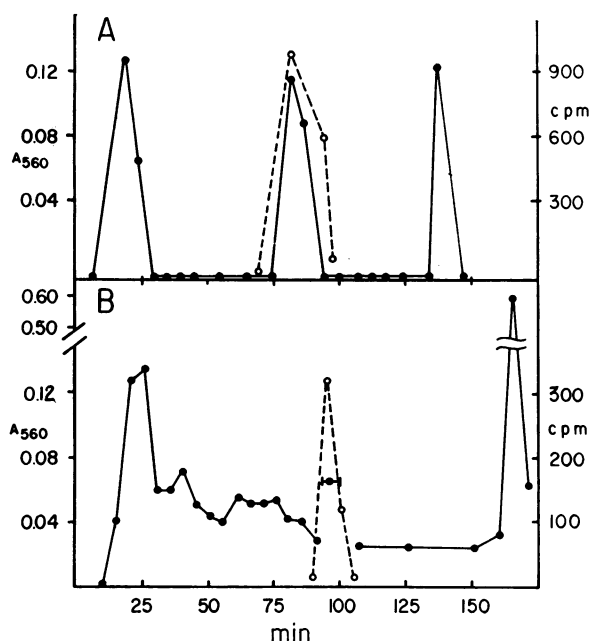


FIGURE 2 Final purification of PCA by ion-exchange chromatography. Each panel shows the elution of PCA (purified by method 1) from an amino acid analyzer column. The left ordinate is absorbance (*p*-dimethylaminobenzaldehyde assay) of 0.5-ml samples (●). The right ordinate is radioactivity in counts per minute (○) of 0.1-ml samples. A represents a 24-h urine collection (from eight rats), to which  $7 \times 10^4$  dpm of  $[2\text{-}^{14}\text{C}]\text{PCA}$  was added before further processing. B represents 300 ml of human urine to which  $7 \times 10^4$  dpm of  $[2\text{-}^{14}\text{C}]\text{PCA}$  was added before further processing. In B, the contents of several tubes representing the radioactive peak were pooled for a single PCA analysis. See the text for other details.

omission of the oxidation step that converts hydroxyproline to  $\Delta^1$ -pyrroline-4-hydroxy-2-carboxylate (4). The method was slightly modified from that described earlier (5). The sample was diluted to 0.5 ml and treated with 0.2 ml of *p*-dimethylaminobenzaldehyde solution (5% in *n*-propyl alcohol) and 0.3 ml of 3.6 N  $\text{H}_2\text{SO}_4$ . The mixture was heated for 5 min at  $75^\circ\text{C}$  and the absorbance at 560 nm was read in a Zeiss PMQ II spectrophotometer (Carl Zeiss, Inc., New York). The molar absorptivity is  $4.2 \times 10^4$  at 560 nm and the assay was linear with PCA to an absorbance of 0.6. Calculations of endogenous PCA in human or rat urine were corrected for losses during purification by the recovery of radioactive PCA that had been added initially.

**Radioactivity measurements.** These were carried out in a Packard Tri-Carb instrument (Packard Instrument Co., Inc., Downers Grove, Ill.) with plastic mini-vials with 6 ml of Aquasol (New England Nuclear, Boston, Mass.) as scintillation fluid.

**Preparation of radioactive PCA.**  $[\text{G}\text{-}^3\text{H}]\text{PCA}$  was prepared by incubation of  $[\text{G}\text{-}^3\text{H}]\text{allohydroxy-D-proline}$  with hog-kidney *D*-amino acid oxidase.  $[\text{G}\text{-}^3\text{H}]\text{Allohydroxy-D-proline}$  was obtained in turn by the enzymatic epimerization of the correspondingly labeled hydroxy-L-proline, a commercial product. The latter was incubated with hy-

droxyproline-2-epimerase (100  $\mu\text{Ci}$  of substrate, representing about 20 nmol, 200 U of enzyme, fraction VI or VII [Table II, ref. 15]). Incubation was carried out in 0.05 M Tris, pH 8.1, in a final volume of 1 ml for 4 h at room temperature. The reaction was stopped by adding 0.1 ml of 2 N HCl and the suspension was centrifuged at 100,000 *g* for 15 min. The supernatant solution was applied to the Durrum DC-1 column described above and eluted with the pH 2.95 buffer. An initial peak, presumably  $^3\text{H}_2\text{O}$  (15), was not retarded on the column; the second and third peaks eluted at 70–75 min and 80–85 min, respectively, corresponded to hydroxy-L-proline and allohydroxy-D-proline. The latter fractions were pooled and desalted by elution from a  $1 \times 10$ -cm column of Dowex-50  $\text{H}^+$  (8X, 100–200 mesh) with 1 N  $\text{NH}_4\text{OH}$ . The identity and purity of  $[\text{G}\text{-}^3\text{H}]\text{allohydroxy-D-proline}$  were confirmed by paper electrophoresis at pH 2 (16), or by thin-layer chromatography in *n*-propyl alcohol:water:methanol (7:3:2) (hydroxy-L-proline,  $R_f$  0.39; allohydroxy-D-proline,  $R_f$  0.19).

$[\text{G}\text{-}^3\text{H}]\text{Allohydroxy-D-proline}$ , prepared and purified as above, was incubated with hog kidney *D*-amino acid oxidase as follows: 50  $\mu\text{Ci}$  substrate ( $6.5 \times 10^9$  dpm/ $\mu\text{mol}$ ), 70 U of *D*-amino acid oxidase (Sigma Chemical Co., Inc., St. Louis, Mo., crystalline, 15 U/mg, which had been dialyzed against one liter of 0.01 M sodium pyrophosphate, pH 8.3, for 4 h at  $4^\circ\text{C}$ ), 20,000 U of catalase (beef liver, Worthington Biochemical Corp., Freehold, N. J.), 30  $\mu\text{g}$  of FAD, and 70  $\mu\text{mol}$  of sodium pyrophosphate, pH 8.3, in a total volume of 5.2 ml. The mixture was incubated at  $37^\circ\text{C}$  in air, with shaking. At 4 and 8 h, 70 U of *D*-amino acid oxidase, 20,000 U of catalase, and 30  $\mu\text{g}$  of FAD were added. After 22 h, the incubation was stopped by adding 1 ml of 2 N HCl, the volume was brought to 50 ml with water, the pH was adjusted to 3.5–4.0, and the solution was extracted with 125 ml of ether in a liquid-liquid extractor for 4 h. The ether layer was evaporated to dryness on a flash evaporator, brought to 4 ml with water, and applied to a  $1.1 \times 25$ -cm column of Dowex-50  $\text{H}^+$ . The column was eluted with water, collecting 1.5-ml fractions. Usually two peaks of radioactivity emerged from the column; the second, larger peak was PCA. After concentration in a flash evaporator, its identity and purity was checked by paper electrophoresis at pH 5.6 (see above); labeled material traveled with authentic PCA, detected by staining the paper with *p*-dimethylaminobenzaldehyde as described above. The tritiated PCA represented 10–15% of the initial  $[\text{G}\text{-}^3\text{H}]\text{allohydroxy-D-proline}$  and had a specific radioactivity 50% that of the initial hydroxy-L-proline. In the enzymatic conversion of hydroxy-L-proline to allohydroxy-D-proline, the specific activity diminished by 35%, a measure of the tritium in the  $\alpha$ -position (15). A further drop of 15% occurred on oxidation and/or dehydration to PCA.

$[\text{G}\text{-}^3\text{H}]\text{PCA}$  was prepared similarly from  $[\text{G}\text{-}^3\text{H}]\text{hydroxy-L-proline}$ . The latter was converted to  $[\text{G}\text{-}^3\text{H}]\text{allohydroxy-D-proline}$  essentially as described above. Oxidation of the *D*-epimer ( $4\text{--}8 \times 10^7$  dpm), however, was carried out with the addition of 500  $\mu\text{mol}$  of unlabeled allohydroxy-D-proline and was catalyzed by crude bacterial allohydroxy-D-proline oxidase (5). The PCA formed was isolated as described above and represented 28% of the initial allohydroxy-D-proline. Unexpectedly, specific activity of the PCA was equal to that of the allohydroxy-D-proline and hydroxy-L-proline, indicating no loss of tritium from the 5-position of hydroxyproline. Tentatively, we attribute this to an isotope effect resisting the loss of tritium on tautomerization of the pyrrole ring.

[2-<sup>14</sup>C]PCA was prepared from 50  $\mu$ Ci of the mixed isomers of [2-<sup>14</sup>C]hydroxy-DL-proline. In this case, the epimerization step was omitted and the oxidation step was carried out as described for the preparation of [G-<sup>3</sup>H]PCA.

Radioactive PCA was stored in 0.001 M NaOH at -15°C. Some decomposition occurred over a period of several months, as inferred from the appearance of increasingly prominent peaks, which were not clearly retarded on the Durrum DC-1 column, but emerged at about 12 and 30 min.

**Amino acid analysis.** For the experiments described here, urinary  $\gamma$ -hydroxyglutamate, free hydroxyproline, and other relevant amino acids were determined with the Technicon amino acid analyzer (Technicon Instruments Corp., Tarrytown, N. Y.), with a column (0.6  $\times$  63 cm) of Durrum DC-1 resin. The elution system began with Durrum Pico Buffer A adjusted to pH 2.95, and followed after 45 min with the same buffer at pH 3.30. The column operation otherwise was as noted under method 2 for PCA. Approximately half the column output was pumped into the auto-analyzer/recorder portion of the analyzer, in the usual way, while the remainder was collected in a fraction collector.

**Mass spectra.** Mass spectra of authentic PCA and of PCA purified from human urine by method 2 were obtained with a Dupont 490 mass spectrometer (E. I. Dupont de Nemours & Co., Wilmington, Del.), operating at a source temperature of 180°C; samples in aqueous solution were introduced via the batch inlet. The urine sample, corresponding to the middle peak of Fig. 2B, was passed through a small Dowex 50H<sup>+</sup> column as in method 1; the slight retardation of PCA on Dowex 50H<sup>+</sup> permitted essentially complete separation from the citrate buffer in the sample applied.

**Alkalinization and NaBH<sub>4</sub> treatment of urine.** To maintain an alkaline urine during the collection of human urine, 4.2 g of NaHCO<sub>3</sub> was ingested at the beginning of each day's urine collection; 1.2 g of NaHCO<sub>3</sub> was taken every 2-3 h throughout the day. All urine samples, collected at 1-2 h intervals, were at pH 7-8. At each collection, urine samples were divided into equal portions and each was brought to 0.1 N in NaOH. To one sample, NaBH<sub>4</sub> (4 mg/ml) was added; the other was not treated. Both samples were then acidified to pH 3.5 with 2 N HCl, and each was extracted with ether and analyzed for PCA by method 2.

**Sources and purity of compounds.** Allohydroxy-D-proline and hydroxy-L-proline were purchased from Sigma Chemical Co. Hydroxy-L-proline was recrystallized twice from water-ethanol. It was judged essentially free of PCA by adding radioactive PCA to an acidified solution of 30 mmol of hydroxyproline and extracting this solution with ether. The recovery of labeled PCA in the ether extract (40-50%) was associated with no detectable PCA by colorimetric assay; the sensitivity of this assay indicated that contamination of hydroxy-L-proline with PCA could not have exceeded 0.00006%. Hydroxy-L-proline was also found to contain less than 0.0005% of a D-isomer of hydroxyproline by tests with hog kidney D-amino acid oxidase, as described in detail elsewhere (1, 17). *Threo*- $\gamma$ -hydroxy-L-glutamate was purchased from Calbiochem (San Diego, Calif.). *Erythro*- $\gamma$ -hydroxy-D-glutamate was a sample originally donated by Dr. M. Winitz (18). PCA was purchased from Aldrich Chemical Co., Inc. (Milwaukee, Wis.) Its UV spectrum and molar absorptivity at 255 nm (12,700) agreed with that reported earlier (4). [G-<sup>3</sup>H]Hydroxy-L-

proline (10<sup>6</sup> dpm/ $\mu$ mol) was purchased from New England Nuclear and purified through a 0.6  $\times$  63-cm column of Durrum DC-1 resin under the conditions noted above. The pH 2.95 buffer eluted two radioactive contaminants at 25 min and 95 min, in addition to the hydroxyproline peak at 70-80 min. The latter material, representing 99% of the radioactivity, was desalted by elution from a Dowex-50 column as noted above. [5-<sup>3</sup>H]Hydroxy-L-proline (3-5  $\times$  10<sup>8</sup> dpm/ $\mu$ mol) was purchased from New England Nuclear and was purified and desalted as for [G-<sup>3</sup>H]hydroxy-L-proline. [2-<sup>14</sup>C]Hydroxy-DL-proline, mixed isomers (4.07  $\times$  10<sup>7</sup> dpm/ $\mu$ mol), was purchased from Calatomic (Div. of Calbiochem, San Diego, Calif.) and was not further purified before use. *p*-Dimethylaminobenzaldehyde was the American Chemical Society certified product of Fisher Scientific Co. (Pittsburgh, Pa.). Reagents for the Technicon amino acid analyzer were purchased from the Pierce Chemical Co. (Rockford, Ill.) DC-1 cation-exchange resin and the Durrum Pico buffers were purchased from the Durrum Instrument Corp.

## RESULTS

**PCA excretion by two methods.** Initial data for the urinary excretion of PCA by man were obtained by method 1, which appeared valid by a number of criteria. First, the method had been tested with rat urine in the following ways: (a) Endogenous PCA in rat urine, to which radioactive PCA was added, was purified by method 1; a further purification step, paper electrophoresis, then failed to alter the specific radioactivity. (b) Urinary PCA, labeled from radioactive precursors (proline or hydroxyproline) administered to the rats, was purified by method 1; the labeled material failed to separate from authentic unlabeled PCA by paper electrophoresis or by thin-layer chromatography in three solvents. (c) [<sup>14</sup>C]PCA in rat urine, labeled from administered L-[U-<sup>14</sup>C]proline, was purified by method 1, diluted with unlabeled PCA, then reduced catalytically (10% Pd catalyst on charcoal, hydrogenation at 55 lb/in<sup>2</sup>, room temperature, 44 h). Proline, formed in 20% yield, was separated from the residual PCA by retention on a Dowex-50 H<sup>+</sup> column and elution with 2 N HCl. It was then purified on the Durrum DC-1 column by elution with 0.066 M sodium citrate at pH 3.30 (amino acid analyzer conditions otherwise as under Methods). After a further purification step by paper electrophoresis (as noted for hydroxyproline, under Methods), the proline was eluted and found to have the same specific radioactivity as the PCA (purified by method 1) from which it had been derived.

In observations with endogenous PCA purified by method 1 from human urine, paper electrophoresis after addition of radioactive PCA similarity failed to alter the specific radioactivity. Like the PCA so purified from rat urine, the *p*-dimethylaminobenzaldehyde-reactive material purified by method 1 from human urine appeared to move with authentic PCA in several sol-

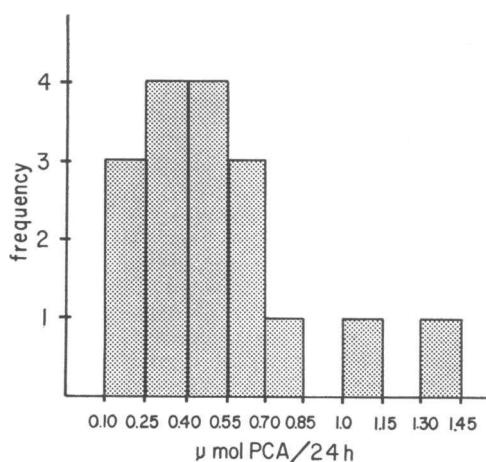


FIGURE 3 Frequency distribution of PCA value in 17 determinations from 16 individuals. Data from Table II, method 2.

vents on thin-layer chromatography. Finally, the determination of this material purified by method 1 was compared with the pyrrole obtained by distillation of the ether extract; values by both methods were in good agreement. In addition, as has been noted, our values for the 24-h excretion of PCA in human urine, de-

TABLE I  
PCA by Two Methods

Subject	Age	Sex	PCA	
			Method 1	Method 2
			<i>μmol/24 h</i>	
1	27	F	1.7	0.27
			2.3	0.49
2	55	M	2.7	0.40
3	26	F	3.3	0.64
4	26	F	1.3	0.35
5	35	F	2.2	0.29
6	38	M	2.2	0.55
7	15	F	1.1	0.20
8	26	M	2.5	0.63
9	25	F	1.2	0.44
10	29	M	1.4	0.18
11	49	F	3.8	1.3
12	52	F	1.5	0.17
13	30	F	3.4	0.75
14	38	F	4.8	0.54
15	58	F	3.0	1.1
16	40	F	2.7	0.40
Mean			2.4 ± 0.91	0.51 ± 0.31
±SD				

For each individual, values by each method were determined from portions of the same 24-h urine. Separate analyses (subject 1) were from separate 24-h urines.

termined by method 1, were in agreement with those based on two variations of a distillation method (12, 13).

Despite these numerous indications of purity of the PCA obtained by method 1, several later findings led us to question the validity of this method. On subjecting PCA so purified (and labeled by the addition of radioactive PCA) to further chromatography on Biogel P-2 columns (Bio-Rad Laboratories, Richmond, Calif.), the peak of chromogenic material (*p*-dimethylaminobenzaldehyde assay) was eluted slightly ahead of the peak of radioactivity; in contrast, chromatography with authentic PCA always showed coincidence of chromogenic material and radioactivity. Similar findings were made with cellulose chromatography (Whatman cellulose powder, eluted with *tert*-butyl alcohol: 1 N NH<sub>4</sub>OH, 4:1) or with silicic acid chromatography (eluted with toluene:ethanol, 7:3), in that there was some separation of purified "PCA" and radioactive PCA. A possible isotope effect, attributable to the use of [<sup>3</sup>H]PCA as the radioactive marker, was not borne out when [2-<sup>14</sup>C]PCA behaved in the same manner. Possible polymerization or other alteration of urinary PCA during the purification procedure was excluded as an explanation, by trials in which authentic PCA, added to the initial urine samples and carried through all the procedures of method 1, failed to separate from added radioactive PCA during the various forms of chromatography noted above.

Because of slight retardation of PCA on Dowex-50 H<sup>+</sup>, the greater resolution of an amino acid analyzer cation exchange column was tested. This procedure succeeded in separating the "PCA" fraction of method 1 into three chromogenic peaks (Fig. 2). Only one of these coincided with authentic PCA, either labeled or unlabeled, and whether added immediately before this column procedure or carried through all previous steps. The peak coinciding with authentic PCA was also consistent with PCA in that the spectrum of its chromogen with *p*-dimethylaminobenzaldehyde was identical with that from PCA. An additional identification of material in this peak was based on a comparison of its mass spectrum with that of authentic PCA. The latter compound, whose mass spectrum we were unable to find reported, gave peaks at the following *m/e* ratios (relative intensities in parentheses): 28(100), 31(30), 32(20), 29(19), 44(19), 76(16), 69(14), and 111(0). Material from urine gave a spectrum consistent with that of the standard.

From the results of this added step, which constituted method 2, it appeared that method 1 had overestimated the daily excretion of PCA in the rat by a factor of 2-3, and the daily excretion of PCA in man by a factor of 5-6. Table I present values for the 24-h excretion

of PCA in a number of individuals, as estimated by both methods, carried out on portions of the same urine sample for each individual. Fig. 3 presents data by method 2 in the form of a histogram.

*Effect of an oral load of hydroxy-L-proline.* The effect of an oral load of hydroxy-L-proline on PCA excretion by two subjects is summarized in Table II. Urine was collected for 24 h; hydroxyproline ingestion began shortly after the start of the urine collection and was divided into equal doses of 100 mg/kg of body weight, taken every 2 h for 12–15-h period. It is notable that both subjects experienced nausea and dizziness beginning approximately 12 h after the first dose of hydroxyproline and lasting for several hours. Scriver and Goldman (19) reported muscle aching, mild pyrexia, and nausea beginning about 12 h after infusing hydroxyproline in human subjects. As shown in Table II, free PCA in the urine excreted by both individuals during the period of hydroxyproline loading greatly exceeded their own control values and fell at the limit of or beyond the range of 16 control values (Table I).

*Level of PCA excretion in a hydroxyprolinemic subject.* It was of interest to examine PCA excretion in an individual with impaired catabolism of hydroxy-L-proline via the major pathway, and consequent marked plasma elevation of free hydroxyproline. A 24-h urine was obtained<sup>2</sup> from a patient who has been the subject of several extensive studies (20–22). Our determination of free hydroxy-L-proline in this sample (301 mg/24 h) agreed with previous measurements (22). Two assays of the PCA content of this patient's urine by method 1 averaged 2.9  $\mu\text{mol}$ , a value not significantly different from the mean of 22 values reported earlier by this method (10) or by the values reported in the present paper (Table I). However, the more accurate value obtained by method 2 was 1.4  $\mu\text{mol}$ , a value approximately three times higher than the normal mean by this method.

*Excretion of radioactivity after injection of [2-<sup>14</sup>C]-PCA.* [2-<sup>14</sup>C]PCA was dissolved in sterile 0.9% saline and filtered through a sterile 0.45- $\mu\text{m}$  Millipore filter (Millipore Corp., Bedford, Mass.). A sample of this solution, chromatographed on the Durrum DC-1 column as outlined for method 2, indicated that essentially all the radioactivity was eluted in the same position as authentic PCA; less than 1% was eluted in the 15–30-min fractions. The [2-<sup>14</sup>C]PCA (7.8  $\mu\text{Ci}$ , 0.42  $\mu\text{mol}$ ) was administered subcutaneously in two sites to a single subject (subject 2, Table I). Urine was col-

lected for two consecutive 24-h periods, the first period beginning at the time of PCA administration. The first 24-h urine contained 91% of the radioactivity injected; the second 24-h urine contained no detectable radioactivity. A 60-ml portion of the first 24-h urine, to which 1  $\mu\text{mol}$  of unlabeled PCA had been added, was extracted with ether in the usual way; about 20% of the urine radioactivity was ether-extractable. The ether extract was dried, taken up in water, and passed through a Dowex-50 H<sup>+</sup> column (1  $\times$  10 cm). The radioactive effluent was chromatographed through the Durrum DC-1 column as under method 2. Most of the radioactivity was eluted in a peak at 25 min. The free carrier PCA, which eluted at 85 min, contained only a trace of radioactivity. After these tubes were pooled and losses of radioactivity in PCA were corrected by the recovery of carrier PCA, only 0.33% of the total radioactivity in urine was present as free PCA.

*Endogenous PCA in alkaline urine.* Data presented elsewhere (1) suggested that much or all of the PCA found in the urine of rats given hydroxy-L-proline or alhydroxy-D-proline may be formed in the urine itself rather than in the tissues. The basis for this inference was that after administration to rats of radioactive PCA, urinary radioactivity was found largely in water-soluble form. In contrast, PCA in the urine after precursor administration was present largely as ether-extractable free PCA. These findings could be most easily explained if the presumptive intermediate between hydroxyproline and PCA,  $\Delta^1$ -pyrroline-4-hydroxy-2-carboxylate (Fig. 1), were readily excreted in the urine but were then rapidly converted to PCA at the usual acid pH of bladder or voided urine. The acid catalysis of the pyrroline compound to PCA was first reported by Radhakrishnan and Meister (4).

TABLE II  
Excretion of PCA,  $\gamma$ -Hydroxyglutamate, and Hydroxy-L-Proline after an Oral Load of Hydroxy-L-Proline

	Subject	
	1	2
Total hydroxyproline ingested, g	25	42
Total hydroxyproline, g/per kg	0.48	0.58
Urinary hydroxy-L-proline		
Total, g	1.2	3.9
Fraction of dose, %	4.7	9.2
Urinary PCA		
Total, $\mu\text{mol}/24\text{ h}$	1.5*	3.3*
Fold-increase over endogenous	3–5	8
Urinary $\gamma$ -hydroxyglutamate, mg	76	18

Subjects 1 and 2 are the same individuals as in Table I.

\* Measured by method 1, respective values for urinary PCA were 3.4  $\mu\text{mol}$  (subject 1) and 4.5  $\mu\text{mol}$  (subject 2).

<sup>2</sup> We acknowledge with thanks the kindness of Dr. Vivian E. Shih, Massachusetts General Hospital, in sending us this sample.

To examine this question for endogenous PCA in man, urine was collected from a single subject (subject 1, Table I) during a period of ingestion of  $\text{NaHCO}_3$  sufficient to maintain an alkaline urine, as noted under Methods. Half of each urine sample was treated with  $\text{NaBH}_4$  to reduce any  $\Delta^1$ -pyrroline-4-hydroxy-2-carboxylate to hydroxyproline (23). The value for PCA in a control sample of urine was  $0.46 \mu\text{mol}/24 \text{ h}$ ; the value for an  $\text{NaBH}_4$ -treated sample was  $0.34 \mu\text{mol}/24 \text{ h}$ , or about 25% less.

## DISCUSSION

The data presented correct previously reported (10, 12, 13) estimates of the 24-h urinary excretion of PCA in man. Similar studies in the rat (1), to be presented elsewhere, also revise earlier data for the level of endogenous PCA in rat urine. The revised values for man indicate a range of only 0.2–1.3  $\mu\text{mol}/\text{day}$  for the group studied, with a mean value of 0.5  $\mu\text{mol}$ . Earlier values with a mean five times that of the present corrected values were in agreement with data obtained by different methods in two other laboratories. It is worth emphasizing the difficulties in reliably measuring these low levels of PCA in urine, especially in the presence of other compounds similar in extraction behavior and in the capacity to react with *p*-dimethylaminobenzaldehyde. Presumably these interfering compounds may also be pyrrole derivatives. The evidence presented suggests that earlier methods were measuring compounds other than free PCA, but similar enough to migrate with it on paper electrophoresis and on several chromatographic solvents, and to be degraded similarly to volatile compounds which could be distilled with the pyrrole derived from PCA. The interfering components, however, were separable from PCA by refined ion-exchange chromatography.

A natural consideration is that the interfering compounds separable only by method 2 are actually degradation or polymerization products of PCA, formed either metabolically or under the separation conditions. Against this possibility, however, was the finding that on chromatographing free PCA from rat urine, which had been labeled by the administration of labeled allohydroxy-D-proline, only the true PCA peak, and not the early or late peaks (Fig. 2), was labeled. In addition, as can be seen by comparing endogenous PCA values by methods 1 and 2 (subjects 1 and 2, Table I) with corresponding values after a large hydroxy-L-proline load (Table II), there was no significant increase in these other pyrrole-reactive compounds, which are estimated by the *difference* between PCA by method 1 and PCA by method 2. A similar but more striking conclusion comes from experiments with rats (1): large hydroxy-L-proline loads, which elevated true PCA over

30 times the control levels, failed to increase peaks 1 and 3 (Fig. 2A).

Radioactive PCA, added to urine and processed through the final column separation, also yielded only a single radioactive peak in the "true" PCA position. These findings would seem to exclude either the metabolic or chemical derivation from PCA of the interfering components separated by the final step of ion-exchange chromatography.

The metabolic derivation of PCA in rat urine from hydroxy-L-proline is based on its marked increase by a hydroxy-L-proline load, its labeling from labeled hydroxy-L-proline, and the finding of an enzymatic basis for the oxidation of hydroxy-L-proline to  $\Delta^1$ -pyrroline-4-hydroxy-2-carboxylate by rat kidney L-amino acid oxidase (1, 10, 11). A similar inference can be drawn from the human data presented here. While the daily excretion of PCA on a body-weight basis is some 15-fold less in man (approximately  $0.008 \mu\text{mol}/\text{kg}$ ) than in young rats ( $0.12 \mu\text{mol}/\text{kg}$  (1)), the daily release from collagen of free hydroxyproline may also show a similar relationship, as calculated from the data of Weiss and Klein for the rat (24) and from Efron, Bixby, and Pryles for man (21). Urinary PCA measurements from a hydroxyprolinemic subject gave a value three times the average value for our control series. Ingestion of a hydroxyproline load by two normal subjects should have elevated plasma levels of hydroxyproline to the range of the hydroxyprolinemic subject (0.2–0.5 mM) for much of the period of urine collection, by analogy with the findings of Efron et al. (21) for the effect of a single oral hydroxyproline load. In agreement with this inference and the possible relationship to PCA formation, the two subjects showed respective elevations of excreted PCA four and eight times their own control values.

Present evidence indicates that in the rat exogenously administered PCA is largely converted to water-soluble conjugates. In the first such report (25), after intraperitoneal injection in rats of 10 mg of  $[1\text{-}^{14}\text{C}]\text{PCA}$ , 23–37% of the radioactivity was excreted as free PCA, the remaining urinary radioactivity being largely present in water-soluble conjugates. In comparable experiments of ours (1) with significantly smaller doses of labeled or unlabeled PCA, only about 5% of the administered compound was recovered as free PCA. In the single human experiment reported here, less than 1% of administered radioactive PCA was recovered as free PCA. In aggregate, data for both rat and man indicate that exogenous PCA is essentially all excreted in the urine, most of it in conjugated form. From our single human case, it is not clear if the extremely efficient conjugation observed is a species characteristic or is characteristic of the small dose given.



A final question concerns the pathway of formation of PCA in man. Preliminary studies of human kidney homogenates, described in more detail elsewhere (1), failed to demonstrate definitive enzyme activity for the conversion of hydroxy-L-proline to the pyrroline product characteristic of the action of L-amino acid oxidase (10) or D-amino acid oxidase (17). In contrast, the capacity of rat kidney homogenate to oxidize hydroxy-L-proline to a PCA precursor was clearly adequate to account for the observed excretion of PCA by the rat (1). Yet the demonstration of increased PCA excretion with an increased hydroxyproline load in man implies such a pathway, which may be present in man in nonrenal tissues, or whose in vivo capacity may for unknown reasons be masked in the homogenate studies.

If there is such an enzymatic origin, however, it is likely that the immediate oxidation product,  $\Delta^1$ -pyrroline-4-hydroxy-2-carboxylate, is not entirely converted to PCA in human tissues. This is suggested by the finding that 25% of the PCA measured in acid urine is present in alkaline urine as a borohydride-reducible compound. It seems plausible that more than 25%, or even all, of the PCA measured in urine is excreted initially as the pyrroline precursor, whose conversion to PCA is catalyzed by the complex components of urine even at a neutral or slightly alkaline pH. This inference is based in part on the finding that free PCA administered to man is almost entirely converted to other compounds or conjugates. The finding of appreciable although small endogenous quantities of unchanged free PCA in human urine, therefore, seems more compatible with its generation in urine than in the tissues. Earlier analytic methods for urinary PCA did not give correct values, but included pyrrole-reactive components that were probably unrelated excretion products. For this reason, earlier attempts to correlate PCA excretion with metabolic disorders such as hyperthyroidism (13) are difficult to interpret. It will be of interest, with an apparently more reliable method for PCA, to determine if PCA excretion may be an additional indicator of collagen turnover, as is hydroxyproline excretion (26). Our findings suggest that PCA will not be a sensitive indicator, since extremely high levels of body free hydroxyproline were required to increase the excretion of PCA significantly. Further data will be required to determine if the measurement of PCA excretion can provide any qualitatively different metabolic insights than the measurement of free hydroxyproline itself.

#### ACKNOWLEDGMENTS

We thank Dr. George Wright and Mr. James Tegeler, of the Department of Medicinal Chemistry, University of

Maryland School of Pharmacy, for their help in obtaining and interpreting mass spectra.

This work was supported by Research Grant G-M-11105 from the National Institutes of Health.

#### REFERENCES

1. Heacock, A. M. 1974. L-Hydroxyproline as a source of urinary pyrrole-2-carboxylate. Ph.D. thesis. University of Maryland.
2. Schwanert, H. 1860. Über einige Zersetzungsproducte der Schleimsäure. *Justus Liebigs Ann. Chem.* **116**: 257-287.
3. Gottschalk, A. 1955. 2-Carboxypyrrole: its preparation from and its precursor in mucoproteins. *Biochem. J.* **61**: 298-307.
4. Radhakrishnan, A. N., and A. Meister. 1957. Conversion of hydroxyproline to pyrrole-2-carboxylic acid. *J. Biol. Chem.* **226**: 559-571.
5. Yoneya, T., and E. Adams. 1961. Hydroxyproline metabolism V. Inducible aldehydehydroxy-D-proline oxidase of *Pseudomonas*. *J. Biol. Chem.* **236**: 3272-3279.
6. Letellier, G., and L. P. Bouthillier. 1956. The formation of 2-pyrrolinecarboxylic acid from hydroxy-D- and aldehydehydroxy-D-proline. *Can. J. Biochem. Physiol.* **34**: 1123-1129.
7. Wolf, G., and C. R. A. Berger. 1958. The metabolism of hydroxyproline in the intact rat. Incorporation of hydroxyproline into protein and urinary metabolites. *J. Biol. Chem.* **230**: 231-240.
8. Adams, E. 1970. Metabolism of proline and of hydroxyproline. *Int. Rev. Connect. Tissue Res.* **5**: 1-91.
9. Gerber, G. B., G. Gerber, and K. I. Altman. 1960. Some interrelated aspects of proline and hydroxyproline metabolism. *Nature (Lond.)*, **185**: 767-768.
10. Heacock, A. M., and E. Adams. 1973. Source of pyrrole-2-carboxylate in mammalian urine. *Biochem. Biophys. Res. Commun.* **50**: 392-397.
11. Iguchi, M., M. Kikuo, H. Ohyama, and Y. Matsumura. 1973. Collagen metabolism. II. Enzymic formation of pyrrole-2-carboxylic acid from L-hydroxyproline in the rat. *Biochim. Biophys. Acta.* **315**: 285-292.
12. Gerber, G. B., H. R. Traehnes, D. Wood, and K. I. Altman. 1954. The quantitative determination of pyrrole-2-carboxylic acid in urine. *Clin. Chim. Acta.* **9**: 185-187.
13. Yaminishi, Y., M. Iguchi, H. Ohyama, and Y. Matsumura. 1972. Collagen metabolism. I. Significance of urinary pyrrole-2-carboxylic acid. *J. Clin. Endocrinol. Metab.* **35**: 55-58.
14. Neuman, R. E., and M. A. Logan. 1950. The determination of hydroxyproline. *J. Biol. Chem.* **184**: 299-306.
15. Finlay, T. H., and E. Adams. 1970. Kinetic and structural studies of hydroxyproline-2-epimerase. *J. Biol. Chem.* **245**: 5248-5260.
16. Gryder, R. M., and E. Adams. 1969. Inducible degradation of hydroxyproline in *Pseudomonas putida*: pathway regulation and hydroxyproline uptake. *J. Bacteriol.* **97**: 292-306.
17. Heacock, A. M., and E. Adams. 1974. Hydroxy-L-proline as a substrate for hog kidney D-amino acid oxidase. *Biochem. Biophys. Res. Commun.* **57**: 279-285.
18. Benoiton, L., M. Winitz, S. M. Birnbaum, and J. P. Greenstein. 1957. Studies on diastereomeric  $\alpha$ -amino acids and corresponding  $\alpha$ -hydroxy acids. IX. Con-

- figuration of the isomeric  $\gamma$ -hydroxyglutamic acids. *J. Am. Chem. Soc.* **79**: 6192-6198.
19. Scriver, C. R., and H. Goldman. 1966. Renal tubular transport of proline, hydroxyproline and glycine. II: Hydroxy-L-proline as substrate and as inhibitor *in vivo*. *J. Clin. Invest.* **45**: 1357-1363.
  20. Efron, M. L., E. M. Bixby, L. G. Palattao, and C. V. Pryles. 1962. Hydroxyprolinemia associated with mental deficiency. *N. Engl. J. Med.* **267**: 1193-1194.
  21. Efron, M. L., E. M. Bixby, and C. V. Pryles. 1965. Hydroxyprolinemia. II. A rare metabolic disease due to a deficiency of the enzyme "hydroxyproline oxidase." *N. Engl. J. Med.* **272**: 1299-1309.
  22. Efron, M. L., E. M. Bixby, T. D. R. Hockaday, L. H. Smith, Jr., and E. Meshorer. 1968. Hydroxyprolinemia. III. The origin of free hydroxyproline in hydroxyprolinemia. Collagen turnover. Evidence for a biosynthetic pathway in man. *Biochim. Biophys. Acta.* **165**: 238-250.
  23. Singh, R. M. M., and E. Adams. 1965. Enzymatic deamination of  $\Delta^1$ -pyrroline-4-hydroxy-2-carboxylate to 2,5-dioxovalerate ( $\alpha$ -ketoglutaric semialdehyde). *J. Biol. Chem.* **240**: 4344-4351.
  24. Weiss, P. H., and L. Klein. 1969. The quantitative relationship of urinary peptide hydroxyproline excretion to collagen degradation. *J. Clin. Invest.* **48**: 1-10.
  25. Letellier, G., and L. P. Bouthillier. 1957. Synthesis of 2-pyrrolinecarboxylic ( $C^{14}O_2H$ ) acid and study of its metabolism in the intact rat. *Can. J. Biochem. Physiol.* **35**: 811-817.
  26. Kivirikko, K. I. 1970. Urinary excretion of hydroxyproline in health and disease. *Int. Rev. Connect. Tissue Res.* **5**: 93-163.