The Quantitative Relationship of Urinary Peptide Hydroxyproline Excretion to Collagen Degradation

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ABSTRACT To determine the quantitative relationship of urinary hydroxyproline peptide excretion to collagen breakdown, known quantities of radioactive hydroxyproline peptides were administered to unlabeled animals and excertion of radioactivity in respiratory carbon dioxide, urine, and feces was measured. The major routes of excretion of collagen peptide metabolites were respiratory carbon dioxide (75%) and urine, as hydroxyproline-containing peptides (25%).

Since the predominant urine hydroxyproline peptide linkage is prolyl-hydroxyproline, L-prolyl-L-hydroxyproline-^{3}H was administered to unlabeled animals. Greater than 80% of the administered dipeptide was excreted in urine, suggesting that this peptide linkage is not hydrolyzed to a significant extent in vivo.

These data suggest that urinary hydroxyproline excretion is a "fairly" sensitive indicator of collagen breakdown and can be used at the clinical level to quantitate changes in collagen breakdown.

INTRODUCTION

Collagen represents about one-third of the body protein and is unique in its high content of the imino acid hydroxyproline. Approximately 14% of the amino acids in collagen are hydroxyproline, whereas elastin, the other major hydroxyproline-containing protein, has less than 2% hydroxyproline. Hydroxyproline is normally excreted in urine, about 97% in the form of peptides and 3% as the free imino acid (3). The dietary intake of gelatin has resulted in a marked increase of urinary excretion of bound hydroxyproline (3, 4). The amino acid composition of urinary hydroxyproline peptides before (5, 6) and after (6) gelatin feeding in normal subjects suggests that urinary hydroxyproline peptides are end-products of collagen degradation. The predominant urinary hydroxyproline peptides, prolyl-hydroxyproline and glycyl-prolyl-hydroxyproline, contain amino acid sequences known to occur frequently in the collagen molecule (7). To further implicate collagen breakdown as the source of urinary hydroxyproline peptides are the observations that excretion increases or decreases in a number of clinical conditions where an increase or decrease in collagen breakdown would be expected (8–11).

Urinary hydroxyproline peptide excretion would be most useful in the analysis of diseases of connective tissues at the clinical level if it represents a quantitatively significant and fairly constant fraction of the hydroxyproline released by collagen degradation. Since urinary hydroxyproline excretion is almost entirely in the form of peptides, its quantitative importance would appear to depend on what proportion of the peptides released by the degradation of collagen is excreted in urine, what proportion is further degraded to carbon dioxide, and whether this is a fairly constant relationship.

The present investigation approaches the question of the quantitative significance of urinary hydroxyproline peptide excretion by techniques similar to those used in the study of plasma proteins (12, 13). Known quantities of radioactive hydroxyproline peptides were administered to unlabeled animals and the distribution of excretion in urine, respiratory carbon dioxide, and feces was measured. This approach permits an estimation of the quantitative significance of each of these pathways from the distribution of excretion of radioactivity.

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These studies were initiated to determine the following: (*a*) the major routes of excretion of collagen metabolites; (*b*) the quantitative significance of urine excretion of collagen metabolites; and (*c*) the significance of the prolyl-hydroxyproline peptide linkage.

METHODS

Preparation of ^{14}C-labeled gelatin. Two male weanling Fischer rats were given a total of 200 μc of proline-L-^{14}C (Schwartz Bioresearch) intraperitoneally in four doses 1 wk apart. The animals were sacrificed by an intraperitoneal injection of sodium pentobarbital 4 wk after the last injection and all subsequent operations were carried out at 2°–4°C. The skin was removed, cleaned of hair and subcutaneous tissue by scraping with a scalpel, and the corium cut into small pieces with scissors. The corium was then homogenized with 0.07 M NaCl (20:1, v/w) in a Virtis "45" homogenizer at 40,000 rpm for 45 min in a circulating ice bath. The temperature of the homogenate did not exceed 8°C. The resultant milky suspension was stirred for 24 hr, centrifuged at 35,000 *g* for 1 hr in a Spinco model L centrifuge and the clear supernate removed and discarded. The residue was extracted and centrifuged two additional times in the same manner. The residue was then subjected to five extractions with cold 2.0 M NaCl and five extractions with cold 0.2 M sodium citrate buffer, pH 3.5, in the same manner to remove soluble collagen. These extracts were discarded. The residue of insoluble collagen was washed with cold distilled water and centrifuged. The supernate was discarded and the residue homogenized in 500 ml of distilled water for 15 min at 25,000 rpm in the Virtis "45" homogenizer. The resultant suspension was gelatinized by autoclaving for 1 hr at 15 lb. pressure (120°C). The gelatin solution was centrifuged for 45 min at 35,000 *g*. The residue was discarded, and the clear supernate filtered through glass wool and lyophilized. The lyophilized gelatin was dissolved in distilled water, neutralized with dilute NaOH, and made up to a concentration of 30 mg/ml.

Preparation of ^{14}C-labeled dialyzable collagen peptides. A male weanling Fischer rat was given a total of 750 μc of proline-L-^{14}C (New England Nuclear Corp.) by intraperitoneal injection two times a wk for 4 wk. Two months after the last injection the animal was sacrificed by ether inhalation and the four tail tendons were dissected free from surrounding tissues. The tendons were cut into small pieces with scissors and homogenized with 50 ml of distilled water in a Virtis "45" homogenizer for 30 min at 25,000 rpm at room temperature. The resultant fine suspension was gelatinized by autoclaving for 3 hr at 15 lb. pressure (120°C). The gelatin solution was then centrifuged for 15 min at 15,000 *g*, the residue discarded, and the supernate treated with 5.5% TCA (final concentration) at 2–4°C for 12 hr. The gelatin was then centrifuged as before, the fine TCA precipitate discarded, and the supernate dialyzed (Visking No. 20/32 tubing), first against cold running tap water (8–10°C) overnight, and then twice against distilled water for 24 hr at 2–4°C. After filtering through glass wool, the clear nondialyzable solution was lyophilized, dissolved in distilled water, the pH adjusted to 7.2 with dilute NaOH, and made to a concentration of 12 mg/ml.

60 mg (5 ml) of this gelatin was incubated at 37°C for 48 hr with 10 mg of crystalline trypsin (Worthington Biochemical Corp., Freehold, N. J.) and 3 mg of clostridial collagenase in 0.1 M Tris buffer, 0.01 M $CaCl_2$, and 1:10,000 merthiolate (final concentrations). After incubation this material was dialyzed (Visking No. 20/32 tubing) against repeated changes of distilled water. The peptide efflux was lyophilized, dissolved in a small volume of distilled water, neutralized with dilute NaOH, and made up to a concentration of 11 mg/ml.

Preparation of ^{3}H-labeled dipeptide L-*prolyl*-L-*hydroxyproline.* 25 mg of purified dipeptide L-prolyl-L-hydroxyproline (Cyclo Chemical Corp.) was exposed as dry powder to 3 c of tritium gas in an electric discharge for 30 min at room temperature (New England Nuclear Corp.) Labile tritium was removed by dissolving it in 5-ml of water and vacuum distillation at room temperature, and then by lyophilizing three times after dissolving in 20 ml of 0.01 N HCl.

Care of animals. All animals received Purina rat chow and water *ad lib.* until the time of administration of labeled material except those animals receiving ^{14}C-labeled gelatin orally. Food was withheld from these animals for 24 hr before beginning the experiment. After administration of labeled material to animals, water was given ad lib.

Bilateral nephrectomy. Under light ether anesthesia, the renal artery, renal vein, and ureter on each side were ligated and the kidneys removed.

Administration of labeled preparations. ^{14}C-labeled gelatin was administered to intact and bilaterally nephrectomized male Wistar rats (250 ±20 g). Intact animals received the gelatin intraperitoneally, subcutaneously, and orally (via gastric tube). Nephrectomized animals received the gelatin intraperitoneally.

^{14}C-labeled dialyzable collagen peptides were administered to intact male Wistar rats (180 ±10 g) subcutaneously.

^{3}H-labeled L-prolyl-L-hydroxyproline was administered to intact male Wistar rats (230 ±20 g) subcutaneously.

Sample collection and preparation. The animals were kept in glass metabolic cages. Respiratory $^{14}CO_2$ was collected by bubbling expired air through a fine sintered glass bubbler immersed in 10 ml of 1 M Hyamine (Packard Instrument Co.) for 30 min periods. In preliminary experiments, using two bubblers in series, it was found that the first bubbler trapped all of the $^{14}CO_2$ expired during a 30 min period. Therefore the second bubbler was not used in succeeding experiments. Collection of $^{14}CO_2$ for two successive 15 min periods gave the same result as a single 30 min collection. Timed urine collections were made using dilute HCl as a preservative. Feces was hydrolyzed in 6 N HCl, evaporated to dryness on a steam table, and dissolved in sodium citrate buffer.

Chromatographic analysis. Free and total proline and hydroxyproline of collagen peptides, dipeptide, and urines were separated by column chromatography and analyzed as previously described (14). Paper chromatography of L-prolyl-L-hydroxyproline, and its diketopiperazine, and rat urines were carried out on No. 1 Whatman paper in ascending fashion for 18 hr at room temperature. Unheated air was used for drying the applied samples. Solvent systems were the following: (*a*) phenol: water, 5:1; (*b*) *n*-butanol: glacial acetic acid: water, 18:2:5 and 4:1:5 (15); and (*c*) isobutanol: 3.3% ammonium hydroxide, 5:2. Chromatograms were stained for dipeptide with 0.2% isatin in *n*-butanol and for the diketopiperazine of prolyl-hydroxyproline with the starch-iodide spray of Pan and Dutcher (16).

The purity of dipeptide-^{3}H was checked by chromatography in three solvent systems listed above using L-prolyl-L-hydroxyproline and its diketopiperazine as reference compounds.

The rat urines were chromatographed with nonradioactive L-prolyl-L-hydroxyproline and its diketopiperazine in the following manner: the reference compounds L-prolyl-L-hydroxyproline and its diketopiperazine were applied (10 μg/10 μl) to the same segment of Whatman paper, and were then overlaid with 20 μl of urine from rats that had received dipeptide-^{3}H, to insure that the sample and the reference compounds were exposed to the same concentration of salts and urea during the development of the chromatogram. After development, the paper chromatogram was cut into 1-cm strips which were eluted with 1 ml of 0.1 M sodium citrate buffer (pH 2.90), and counted in a liquid scintillation spectrometer.

Radioactive counting procedures. Radioactivity was determined in a Packard liquid scintillation spectrometer as previously described (14). Observed counts per minute were converted to disintegrations per minute. Counting efficiencies for ^{14}C samples ranged from 60 to 75%, and for ^{3}H samples, from 20 to 24%. ^{14}C background was 20 cpm and ^{3}H background was 6 cpm. Observed cpm in samples from the gelatin-^{14}C experiment ranged from 2 to 10 times background; from the peptide-^{14}C experiments 5 to 10 times background; and from the dipeptide-^{3}H experiment, greater than 10 times background.

Abbreviations. In tables and calculations, the following abbreviations are used: pro, proline; hypro, hydroxyproline; dpm, disintegrations per minute; ip, intraperitoneal; and sc, subcutaneous.

Calculations

1. Total $^{14}CO_2$ excretion. Total $^{14}CO_2$ excretion was determined from the area under the curve of $^{14}CO_2$ excretion.

2. Total ^{14}C excretion = dpm respiratory $^{14}CO_2$ + dpm total urine ^{14}C.

3. Molar ratio of proline to hydroxyproline = (μg pro/μg hypro) × (1 μmole pro/115 μg) × (131 μg/1 μmole hypro).

4. Molar specific activity ratio of proline to hydroxyproline = (dpm/μg pro ÷ dpm/μg hypro) × (115 μg/1 μmole pro) × (1 μmole hypro/131 μg).

5. Molar ratio of urine proline to urine hydroxyproline derived from labeled material. Although labeled urine proline and hydroxyproline derived from both preparations are mixed with nonradioactive proline and hydroxyproline from endogenous sources, the amounts in urine derived from labeled gelatin and labeled dialyzable peptides can be calculated from the total radioactivity in urinary proline and hydroxyproline, and the specific activity of each in the administered preparation.

Molar ratio of urine proline to urine hydroxyproline = (total dpm ^{14}C in urine pro ÷ dpm ^{14}C/mole pro in labeled material) ÷ (total dpm ^{14}C in urine hypro ÷ dpm ^{14}C/mole hypro in labeled material).

6. Hydroxyproline-^{14}C dose (in dpm) = (μg hypro in dose of labeled material) × (dpm/μg hypro in dose of labeled material).

RESULTS

Purity of labeled preparations. 94% of the total radioactivity in the labeled skin gelatin was in proline and hydroxyproline (Table I). The molar ratio of proline to hydroxyproline was 1.13, and the molar specific activity ratio of proline to hydroxyproline was 1.05. All of the radioactivity in the dialyzable peptides was in proline and hydroxyproline. The molar ratio of proline to hydroxyproline was 1.54 and the molar specific activity ratio of proline to hydroxyproline was 0.87. The same ratios were observed in the tendon gelatin from which the dialyzable peptides were prepared.

TABLE I

Distribution of Radioactivity in ^{14}C-Labeled Collagen Peptides

	Amount	Specific activity	Total ^{14}C	Percentage
	μg	dpm/μg	dpm	%
^{14}C-labeled gelatin				
Total ^{14}C			187,500*	100.
pro-^{14}C	4000	24.0	96,000	51.2
hypro-^{14}C	4000	20.0	80,000	42.7
Unaccounted for			11,500	6.1
^{14}C-labeled dialyzable collagen peptides				
Total ^{14}C			190,000‡	100.
pro-^{14}C	2010	58.6	117,600	62.0
hypro-^{14}C	1470	60.4	88,800	46.8
Unaccounted for			(16,400)	(+8.8)

* 30 mg of ^{14}C-labeled gelatin.
‡ 11 mg of ^{14}C-labeled dialyzable collagen peptides.

The L-prolyl-L-hydroxyproline-^{3}H preparation contained no free proline or hydroxyproline radioactivity as determined by column chromatography. Paper chromatography revealed that 69% of the total radioactivity was in the area of isatin coloration of the reference standard L-prolyl-L-hydroxyproline (Fig. 1). After hydrolysis in 6 N HCl at 120°C for 3 hr and column chromatography, 12% of the total radioactivity was in proline plus hydroxyproline. This analytical loss was constant.

Respiratory and urinary excretion of radioactivity. Preliminary experiments demonstrated that less than 0.9% of the total radioactivity remained in the peritoneal cavity 72 hr after intraperitoneal administration of labeled gelatin, indicating that absorption was complete.

After oral or parenteral administration of 30 mg of labeled gelatin to intact animals, excretion of respiratory $^{14}CO_2$ occurred rapidly. A sharp peak in the $^{14}CO_2$ excretion was observed at 2–3 hr and excretion fell off rapidly thereafter (Fig. 2). Intraperitoneal administration (animal 1) led to the highest level of $^{14}CO_2$ excretion and the most rapid rate of decline, while subcutaneous administration (animal 3) led to a somewhat lower peak excretion with a slower rate of decline. After oral administration (animal 5), $^{14}CO_2$ excretion was qualitatively similar to that after subcutaneous administration (Fig. 2).

Excretion of radioactivity in urine after administration of 30 mg of ^{14}C-labeled gelatin was maximal during the 1st 8 hr, and decreased markedly during the

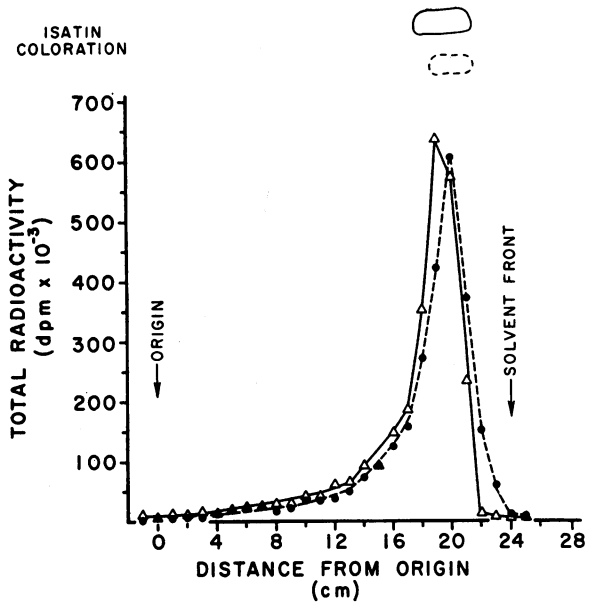


FIGURE 1 Paper chromatograph of injected L-prolyl-L-hydroxyproline-^{3}H and rat urine. Solid line represents injected dipeptide-^{3}H; dashed line represents urine excreted by rat (No. 14) after administration of dipeptide-^{3}H.

next 16 hr (Fig. 3). Total urine radioactivity during the period of 24–48 hr after administration was small (Fig. 3).

Excretion patterns after administration of 90 mg of ^{14}C-labeled gelatin were qualitatively the same as those seen with a 30 mg dose.

After subcutaneous administration of labeled dialyzable collagen peptides, $^{14}CO_2$ excretion occurred rapidly, with excretion patterns almost identical to those seen after administration of 30 mg of ^{14}C-labeled gelatin (the total ^{14}C dose of both preparations was approximately the same; see Table I). Urine-^{14}C excretion was also similarly rapid, qualitatively following the same general pattern seen with subcutaneous administration of ^{14}C-labeled gelatin.

Measurement of respiratory $^{14}CO_2$ and urine-^{14}C excretion between 48 and 120 hr after parenteral administration of labeled gelatin and labeled dialyzable peptides showed that almost all excretion (greater than 95%) had occurred by 48 hr; therefore, all calculations of total excretion were based on 48 hr excretion.

After parenteral administration of either preparation, total ^{14}C excretion (respiratory $^{14}CO_2$ + urine-^{14}C) was a fairly constant percentage of the total ^{14}C dose (Table II), averaging 55% for gelatin and 59% for dialyzable peptides. After administration of labeled dialyzable peptides, total radioactivity in feces collected from 0 to 72 hr after injection was less than 0.2% of the total ^{14}C dose. Total $^{14}CO_2$ excretion ranged from 36 to 42% of the total ^{14}C dose. Urine-^{14}C excretion (Table II) was more variable after gelatin administration than after peptide administration, ranging from 11 to 17% after gelatin and from 21 to 22% after peptide administration.

Total ^{14}C excretion after oral administration of labeled gelatin was 44 and 46% of dose (Table II). $^{14}CO_2$ excretion was similar to that observed after parenteral

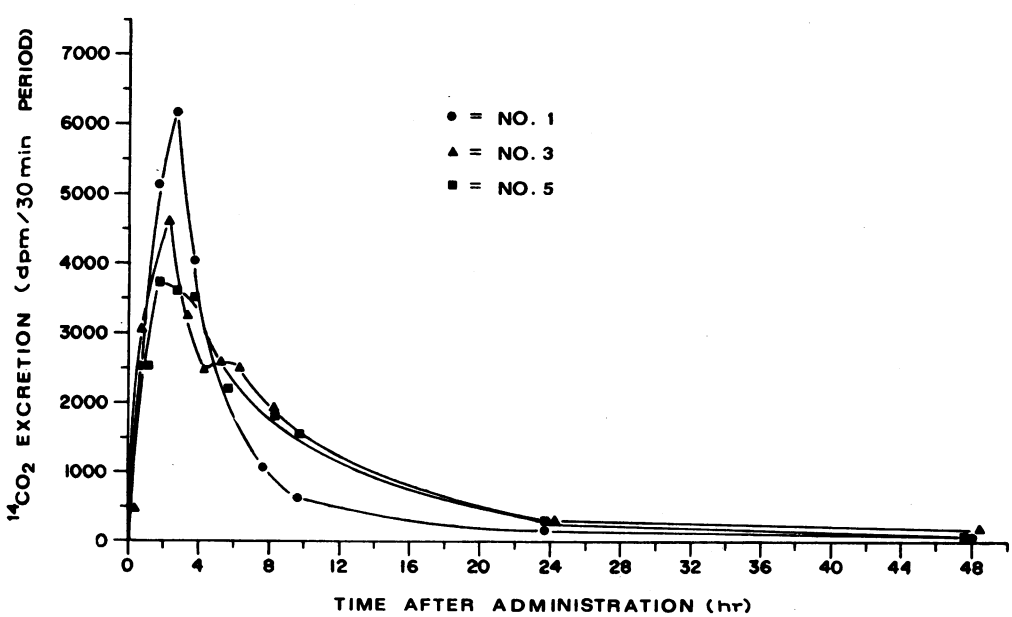


FIGURE 2 $^{14}CO_2$ excretion after administration of 30 mg of ^{14}C-labeled gelatin to intact rats. No. 1, intraperitoneal; No. 3, subcutaneous; No. 5, oral administration.

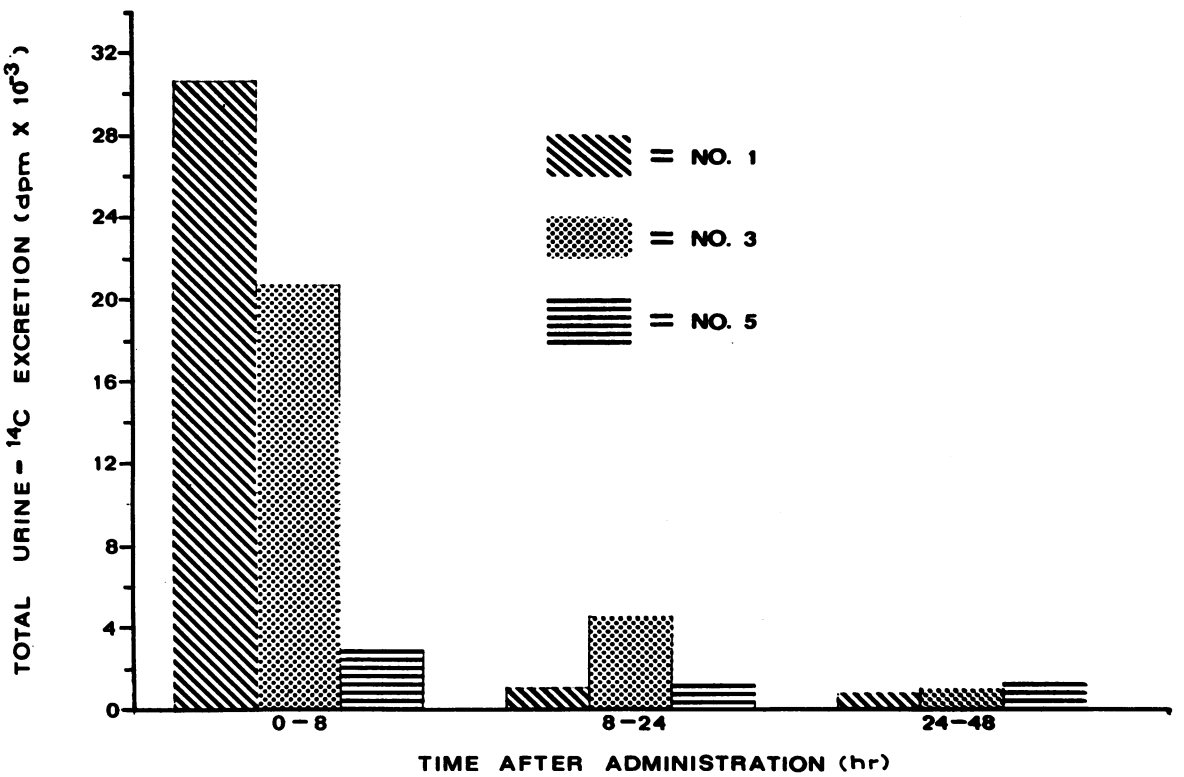


FIGURE 3 Total urine-^{14}C excretion after administration of 30 mg of ^{14}C-labeled gelatin to intact rats. No. 1, intraperitoneal; No. 3, subcutaneous; No. 5, oral administration.

administration (41 and 43% of dose), but urine-^{14}C excretion was markedly decreased (3% of dose).

After parenteral administration of labeled gelatin, 67 to 78% of the total excretion was as respiratory $^{14}CO_2$, and 22 to 33% as urine-^{14}C. After oral administration of gelatin, 94% of the total ^{14}C excretion was as respiratory $^{14}CO_2$, and 6% as urine-^{14}C. After administration of dialyzable peptides, $^{14}CO_2$ excretion accounted for 62 to

TABLE II

Summary of ^{14}C Excretion after Administration of ^{14}C-Labeled Collagen Peptides to Intact Rats

Rat No.	Route	^{14}C dose	Respiratory $^{14}CO_2$ excretion		Urine-^{14}C excretion		Total ^{14}C excretion*	
			Radioactivity	Percentage of dose	Radioactivity	Percentage of dose	Radioactivity	Percentage of dose
		dpm	*dpm*	%	*dpm*	%	*dpm*	%
^{14}C-labeled gelatin								
1	ip	187,500	67,800	36.2	32,500	17.3	100,300	53.5
2	ip	562,500	226,600	40.3	63,000	11.2	289,600	51.5
3	sc	187,500	79,100	42.2	26,200	14.0	105,300	56.2
4	sc	562,500	227,000	40.4	94,300	16.8	321,300	57.1
5	oral	187,500	80,800	43.1	5,180	2.8	85,980	45.9
6	oral	562,500	233,000	41.4	14,600	2.6	247,600	44.0
^{14}C-labeled dialyzable collagen peptides								
10	sc	175,400	64,300	36.7	35,900	20.5	100,200	57.2
11	sc	190,000	77,400	40.7	39,600	20.8	117,000	61.5
12	sc	190,000	71,800	37.8	40,500	21.3	112,300	59.1
13	sc	190,000	69,100	36.4	42,100	22.2	111,200	58.6

* Total ^{14}C excretion = respiratory $^{14}CO_2$ + urine-^{14}C.

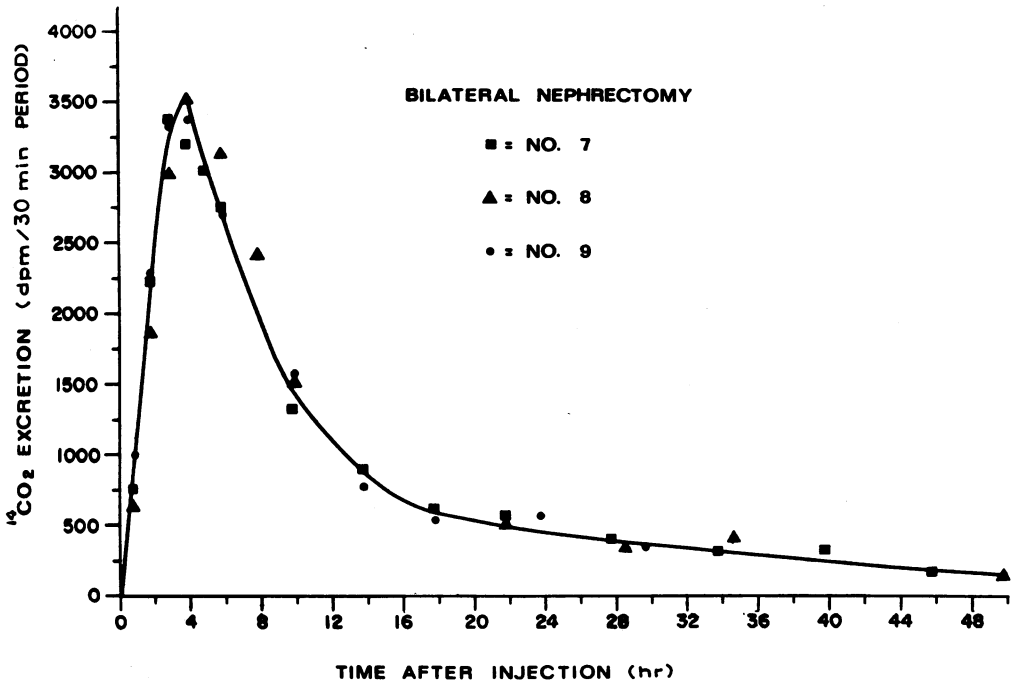


FIGURE 4 $^{14}CO_2$ excretion after intraperitoneal administration of 30 mg of ^{14}C-labeled gelatin to bilaterally nephrectomized rats.

66% of the total excretion, and urine-^{14}C for 34 to 38% of the total excretion.

Although the ^{14}C-labeled gelatin and ^{14}C-labeled dialyzable collagen peptides were prepared from different collagens (skin and tendon, respectively) and differed in molecular size, the data indicate that they were excreted in similar fashion. The data for both experiments indicate that after parenteral administration about

TABLE III

Characterization of Urinary ^{14}C Excretion after Parenteral Administration of ^{14}C-Labeled Collagen Peptides to Intact Rats

Rat No.	Urine hypro-^{14}C excretion	Urine pro-^{14}C excretion	Ratio*	Urine imino acid-^{14}C excretion‡	Total urine ^{14}C excretion	Imino acid-^{14}C excretion as a percentage of total urine-^{14}C excretion
	dpm	*dpm*		*dpm*	*dpm*	%
^{14}C-labeled gelatin						
1	10,600	11,600	1.03	22,200	30,600	72.5
2	22,900	18,700	0.77	41,600	57,400	72.5
3	9,120	7,620	0.79	16,740	20,700	80.9
4	12,600§	11,300	0.85	23,900	27,700	86.3
4	19,300§	20,200	0.99	39,500	47,900	82.5
^{14}C-labeled dialyzable collagen peptides						
10	12,600	8,780	0.81	21,380	29,300	73.0
11	9,280	8,760	1.10	18,040	30,800	58.6
12	16,700	11,200	0.78	27,900	37,100	75.2
13	14,900	11,750	0.92	26,650	38,500	69.3

* Molar ratio of pro-^{14}C to hypro-^{14}C = $\frac{\text{total dpm } ^{14}C \text{ in urinary pro}}{\text{dpm } ^{14}C/\text{mole pro in gelatin}} \div \frac{\text{total dpm } ^{14}C \text{ in urinary hypro}}{\text{dpm } ^{14}C/\text{mole hypro in gelatin}}$.

‡ Urine imino acid-^{14}C excretion = urine pro-^{14}C + urine hypro-^{14}C.

§ Urines from the same animal, successive timed collections.

70% of the total excretion was as respiratory $^{14}CO_2$ and 30% as urine-^{14}C. Fecal radioactivity was insignificant (less than 0.2% of dose). These observations indicate that the major routes of excretion of collagen peptide metabolites are respiratory carbon dioxide and urine.

Excretion of radioactivity in nephrectomized animals. A 30 mg dose of ^{14}C-labeled gelatin was administered to bilaterally nephrectomized rats in order to determine whether rapid excretion in urine prevented complete catabolism of some of the administered gelatin. Since preliminary experiments had shown that there was a depression of $^{14}CO_2$ excretion soon after nephrectomy under ether anesthesia (within 2 hr), the ^{14}C-labeled gelatin was administered to the animals 2-1/2 to 8 hr after anesthesia, when this effect is not seen.

After intraperitoneal administration of 30 mg of ^{14}C-labeled gelatin to nephrectomized animals, $^{14}CO_2$ excretion reached a peak slightly later (Fig. 4), but was qualitatively similar to the excretion patterns observed with parenteral and oral administration in intact animals (Fig. 2). Quantitatively, $^{14}CO_2$ excretion after bilateral nephrectomy was the same (42 to 44% of dose) as that in intact animals.

Characterization of urinary radioactivity. The amounts of urine proline and hydroxyproline derived from the labeled gelatin or labeled peptides can be compared by dividing the total radioactivity in urine proline and the total radioactivity in urine hydroxyproline by the specific radioactivity of each in the administered labeled gelatin or labeled peptides, since the only source of radioactivity in the animals was the administered labeled gelatin or peptides. This ratio is expressed on a molar basis in Table III. The average molar ratios of proline to hydroxyproline of 0.89 after gelatin and 0.90 after dialyzable peptide administration indicate that proline and hydroxyproline from both preparations were excreted in urine in approximately equimolar amounts.

Proline-^{14}C and hydroxyproline-^{14}C accounted for 73–86% of the urine radioactivity after parenteral administration of gelatin to intact rats. After parenteral administration of labeled dialyzable peptides, proline-^{14}C and hydroxyproline-^{14}C accounted for 69 to 75% of the total urine-^{14}C in three animals, and 59% in the fourth (Table III).

After administration of labeled peptides, urine free hydroxyproline-^{14}C was 5 to 13% of the total urine hydroxyproline-^{14}C. Urine free hydroxyproline-^{14}C was not determined after administration of labeled gelatin because of insufficient samples.

Excretion of ^{3}H-labeled dipeptide L-*prolyl*-L-*hydroxyproline.* In order to examine the possibility that the prolyl-hydroxyproline peptide linkage might be excreted quantitatively in urine, tritiated L-prolyl-L-hydroxyproline was administered to animals and urinary excretion of radioactivity measured. The injected dose and urinary excretion of dipeptide were determined on the basis of (*1*) total imino acid radioactivity (proline plus hydroxyproline) using column chromatography, and (*2*) dipeptide radioactivity using paper chromatography. Excretion of tritiated L-prolyl-L-hydroxyproline (Table IV) averaged 81% of dose, determined on either basis. Paper chromatograms of urine demonstrated that the peak of radioactivity was identical with that of the injected dipeptide radioactivity, as well as with the isatin coloration of the unlabeled reference dipeptide (Fig. 1).

TABLE IV

Excretion of Radioactivity in Urine after Administration of L-*Prolyl*-L-*Hydroxyproline-^{3}H to Intact Rats**

	Urine pro-^{3}H + hypro-^{3}H‡	Urine L-pro-L-hypro^{3}H§
	% of dose	*% of dose*
14	82	82
15	76	77
16	85	89
17	78	80
Average	80	82

* Administration was by subcutaneous injection; dose = 300 × 10^4 dpm. 8.3 μg of dipeptide.

‡ Imino acids determined by column chromatography after hydrolysis.

§ Dipeptide determined by paper chromatography.

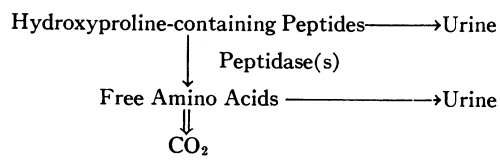
DISCUSSION

The present approach is similar to that used in the study of plasma protein breakdown, where a known amount of ^{14}C-labeled plasma protein was administered to unlabeled animals and excretion of radioactivity in urine, respiratory carbon dioxide, and feces was measured (12, 13). Using this approach, it is not necessary to independently determine the amount of protein broken down in order to estimate the quantitative significance of pathways of metabolite excretion, as would be the case if only one excretory route were measured.

It has been recognized (17) that the significance of urinary hydroxyproline excretion depends on the relationship of catabolism of hydroxyproline peptides to carbon dioxide and excretion in urine. If urine excretion represents a fairly significant and constant excretory route of hydroxyproline peptides, changes in collagen breakdown can be calculated from changes in urinary hydroxyproline excretion.

Available data indicate that peptides are not directly catabolized by enzymes (18, 19) but first must be hydrolyzed to their constituent free amino acids before further degradation to carbon dioxide. If a peptide is

not hydrolyzed to its constituent amino acids, the alternatives would appear to be reutilization or excretion in urine. At present there is no evidence for the reutilization of peptides (20, 21). The distribution of excretion of collagen peptides is schematized below:



Different forms of hydroxyproline containing peptides were used to study the distribution of excretion of collagen metabolites: ^{14}C-labeled gelatin (thermally denatured collagen), ^{14}C-labeled dialyzable collagen peptides, and L-prolyl-L-hydroxyproline-^{3}H.

Distribution of excretion. The present data agree with the data for plasma proteins (12, 13) that feces appears to be an insignificant route of excretion of protein metabolites, and that respiratory carbon dioxide and urine are the major routes.

A comparison of oral and parenteral administration of labeled gelatin demonstrated a difference in the distribution of excretion of radioactivity between respiratory carbon dioxide and urine. After parenteral administration of gelatin, about 30% of the total excretion was in urine and 70% as respiratory $^{14}CO_2$. Similar results were obtained for labeled peptides. Labeled gelatin was administered orally to demonstrate that more complete cleavage of peptide linkages to amino acids could be affected with catabolism to carbon dioxide. The distribution of excretion after *oral* administration suggested that more complete breakdown was effected, since over 90% of the excretion was $^{14}CO_2$. The oral route is considered to be unphysiologic, since there is little evidence that endogenous collagen catabolism occurs via the gastrointestinal tract (10).

In order to be certain that the parenteral excretion pattern was not affected by escape from proteolysis because of rapid urine excretion, the gelatin was administered to bilaterally nephrectomized rats so that maximal exposure to parenteral proteolytic conditions could be effected. With urine excretion prevented, $^{14}CO_2$ excretion both in time and quantity was almost identical to that observed in intact animals. If parenteral proteolysis were bypassed in the intact animal by rapid urine excretion, excretion of respiratory $^{14}CO_2$ in the nephrectomized animals should have increased. The observation that no increase occurred suggests that only a certain, relatively constant fraction of the excretion of collagen peptide metabolites occurs as respiratory carbon dioxide. Since urine excretion of radioactivity after parenteral administration of gelatin or dialyzable peptides was relatively constant, these data support the suggestion that there is a fairly constant distribution of excretion of collagen peptide metabolites between respiratory carbon dioxide and urine, with about 70% excreted by the lung and 30% by the kidney.

Proline and hydroxyproline derived from the labeled gelatin and labeled dialyzable peptides were excreted in urine in almost equimolar amounts (see Methods for calculation). Free urine proline-^{14}C and hydroxyproline-^{14}C averaged 8.3% of total urinary proline-^{14}C and hydroxyproline-^{14}C suggesting that the major urine excretory product of the labeled gelatin and labeled dialyzable peptides was a peptide (or peptides) containing approximately equal amounts of proline and hydroxyproline.

These observations are consistent with what is known about *endogenous* urinary hydroxyproline excretion in man, where most of the hydroxyproline excreted is in peptide form (3) and the predominant peptide linkage is prolyl-hydroxyproline (6), and indicate that urinary excretion of the *exogenous* gelatin and dialyzable peptides was qualitatively similar to urinary excretion of *endogenous* collagen metabolites.

The uniformity of the distribution of excretion in parenterally injected animals suggests that if these findings apply to the destruction of fibrous collagen, approximately 25% of the hydroxyproline released when collagen is destroyed should remain in peptide form, and 75% should be found in the free form. This suggestion is supported by data on tissue cultures of actively resorbing bone (22, 23). It was found under a variety of conditions that a maximum of 81 to 83% (range = 51 to 83%) of the hydroxyproline released in the medium was free amino acid, indicating that in the cellular environment of resorbing bone, 20–50% of the hydroxyproline peptide linkages were not hydrolyzed.

The clinical studies of Efron, Bixby, and Pryles (24) provide further evidence that the data from the present investigation apply to collagen breakdown in man. These authors studied a patient with a deficiency of hydroxyproline oxidase, an enzyme involved in the initial catabolism of free hydroxyproline. If catabolism of free hydroxyproline could be blocked experimentally, the ratio of *free* to *peptide*-bound hydroxyproline in urine would represent the distribution of excretion between respiratory carbon dioxide (75%) and urine (25%) in the normal animal. The patient of Efron et al. (24) excreted a markedly increased level of urinary free hydroxyproline, with normal levels of urinary peptide hydroxyproline. Urinary free hydroxyproline was consistently about 80% of the total hydroxyproline excretion, and urinary peptide hydroxyproline 20% of the total (25), supporting the present suggestion that the distribution of excretion of collagen metabolites in man

is approximately three-fourths by the lung and one-fourth by the kidney.

Significance of the prolyl-hydroxyproline peptide linkage. Peptide-bound proline and hydroxyproline accounted for most of the urine excretion of radioactivity and were excreted in approximately equimolar amounts. This becomes more significant in light of the observation that about 23% of the hydroxyproline in collagen is in a prolyl-hydroxyproline sequence (7). This suggests that when collagen is degraded, the prolyl-hydroxyproline sequence is excreted almost quantitatively in urine. The excretion in urine of about 81% of a parenteral dose of ^{3}H-labeled L-prolyl-L-hydroxyproline supports the conclusion that the prolyl-hydroxyproline sequence is excreted almost quantitatively in urine.

The resistance to metabolic attack of excreted urinary hydroxyproline peptides is supported by preliminary evidence (1) that ^{14}C-labeled urinary hydroxyproline peptides from proline-^{14}C labeled rats are almost quantitatively excreted in urine after injection into unlabeled animals. In addition, Ansorge and Hanson (26, 27) have demonstrated that 35 different peptides from human urine (18 of which are derived from collagen) are resistant to proteolysis by chymotrypsin, trypsin, carboxypeptidase A, and leucine amino-peptidase.

Since prolyl-hydroxyproline and glycyl-prolyl-hydroxyproline account for most of the urinary hydroxyproline excretion (6), the prolyl-hydroxyproline peptide linkage appears to be unique among peptide linkages involving hydroxyproline in its escape from proteolysis.

The predominance of the prolyl-hydroxyproline linkage in urinary hydroxyproline could be explained in the following ways: (*a*) the absence of the appropriate peptidase(s); (*b*) inaccessibility of the appropriate peptidase(s); or (*c*) the presence of peptidase(s) with a slow rate of hydrolysis. The second and third possibilities appear to be the most likely. The dipeptidase prolidase is the only known enzyme that will hydrolyze the prolyl-hydroxyproline linkage (28), and it is widely distributed in the body. It is found intracellularly in skeletal and uterine muscle, kidney, lung, pituitary, intestinal mucosa, and erythrocytes; and extracellularly in serum (28). Intestinal mucosa, kidney, and erythrocytes are apparently the richest sources of prolidase. Christensen and Rafn (29) have observed that erythrocytes are impermeable to peptides, indicating that peptides in the vascular system are out of contact with a major source of prolidase. However, *oral* administration of peptides would expose them to the intestinal prolidase, and thus account for the difference in oral and parenteral administration. It has been demonstrated (28) that prolidase hydrolyzes prolyl-hydroxyproline very slowly (one-tenth to one-twentieth of the rates observed with other dipeptides containing either proline or hydroxyproline).

It is concluded that the major routes of excretion of collagen metabolites are lung and kidney. About three-fourths of the peptides released by the degradation of collagen are hydrolyzed to their constituent amino acids and catabolized to carbon dioxide with excretion by the lung. About one-fourth of the peptides released are excreted in urine. The prolyl-hydroxyproline peptide linkage, the predominant urinary hydroxyproline peptide linkage, appears to be excreted almost quantitatively, presumably because of ineffective peptidase activity. It is suggested that this relationship is fairly constant, therefore changes in collagen breakdown can be quantitated by measurement of changes in urinary excretion of peptides containing the prolyl-hydroxyproline linkage.

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