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HYDROXYPROLINEMIA

III. THE ORIGIN OF FREE HYDROXYPROLINE

IN HYDROXYPROLINEMIA. COLLAGEN TURNOVER. EVIDENCE FOR A BIOSYNTHETIC PATHWAY IN MAN

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SUMMARY

1. A patient with hydroxyproline oxidase deficiency offered a unique opportunity to investigate the origin of plasma and urine hydroxyproline, since most of the free hydroxyproline produced in this patient is accumulated in blood and excreted unchanged in the urine. Dietary origin had been ruled out previously.

2. The hydroxyproline-containing peptides in the urine were not different from normal. There was no evidence of abnormal collagen breakdown. Results indicate that the free hydroxyproline derives from a normal collagen turnover of about 2 g/day.

3. Trial of a scorbutic diet unexpectedly resulted in a striking increase in excretion of free and peptide-bound hydroxyproline in the urine, and a further increase in plasma hydroxyproline concentration. This is in contrast to the decreased urinary hydroxyproline observed in scorbutic guinea pigs. Administration of ascorbic acid after the period of depletion resulted in further increase in excretion of free hydroxyproline. This suggests that ascorbic acid depletion in man increases turnover of mature collagen or of partially hydroxylated collagen precursors.

4. When labeled glyoxylate was administered, a significant though small amount of urinary free hydroxyproline became labeled, indicating a slight degree of biosynthesis from glyoxylate in man, as has been described in rat livers.

INTRODUCTION

Hydroxyprolinemia is a metabolic disorder characterized by the accumulation of large amounts of free L-hydroxyproline in the plasma with overflow into the urine. The disorder has been described in only one patient^{1,2}, a mentally retarded girl with no obvious clinical, X-ray or histological evidence of disorders of bone or collagen.

The accumulation of free hydroxyproline in hydroxyprolinemia has been shown to result from deficient activity of the enzyme hydroxyproline oxidase, which nor-

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mally oxidizes hydroxyproline to Δ' -pyrroline-3-hydroxy-5-carboxylate², the first step in the degradative pathway of hydroxyproline to glyoxylate and pyruvate. The assumption is made in these studies that the sources of free plasma hydroxyproline in this patient are not different from those in normal human subjects, *i.e.* that the excess accumulation of hydroxyproline is due only to the deficiency of hydroxyproline oxidase².

Normal human subjects have no detectable excretion of free hydroxyproline in urine and a very low plasma hydroxyproline concentration (less than 0.015 $\mu\text{mole/ml}$ (ref. 3)). Hence studies of the origin of hydroxyproline in normal human subjects are very difficult. The hydroxyprolinemic patient, on the other hand, because of the accumulation of large amounts of free L-hydroxyproline in blood (0.21–0.41 $\mu\text{mole/ml}$) and urine (about 250 mg/day) offered a unique opportunity to investigate the origin of free hydroxyproline normally present in human blood.

Free hydroxyproline in plasma and urine could, a priori, be derived from (1) dietary hydroxyproline, (2) collagen breakdown or (3) biosynthesis of hydroxyproline by a pathway other than collagen.

The hydroxyprolinemic patient had no decrease in the plasma free or peptide-bound hydroxyproline concentration after 3 weeks on a hydroxyproline-free diet^{1,2}. This suggests that an ordinary diet is not normally a major source of the plasma and urinary hydroxyproline in man, confirming the findings of PROCKOP AND SJOERDSMA⁴.

Two approaches were used in the investigation of collagen as the probable source of the hydroxyproline. (1) Quantitative studies were made of the urinary excretion of free hydroxyproline and hydroxyproline-containing peptides by the hydroxyprolinemic patient. The total bound hydroxyproline and the pattern of excretion of hydroxyproline-containing peptides were found to be not different from normal, indicating that the free hydroxyproline is derived from normal collagen turnover (calculated to be about 2 g/day). (2) A scorbutic diet was tried with the hope of decreasing production of hydroxyproline by diminishing collagen turnover. This expectation was based on the report that in chick embryos collagen proline hydroxylase requires ascorbic acid as a co-factor⁵. In scorbutic guinea pigs there is reported a decrease in the excretion of hydroxyproline⁶. However, the hydroxyproline in the blood and urine of this patient was increased rather than diminished under the scorbutic regimen. These seemingly contradictory observations could be reconciled if in the scorbutic guinea pig there may be not a decrease but an increase in free hydroxyproline, which is masked by an accompanying increase in the degradation of the imino acid.

Recent *in vitro* studies have shown that rat liver can synthesize hydroxyproline from glyoxylate and pyruvate^{7–10}. Our patient afforded an excellent opportunity to determine whether that biosynthetic pathway exists in man. Evidence is presented here which indicates that the same biosynthetic pathway is present to a limited extent in the hydroxyprolinemic patient (and therefore, presumably also in normal man).

EXPERIMENTAL

Measurement of amino acids

Plasma and urine amino acids were measured on a Technicon automatic amino acid analyzer. Hydroxyproline was measured either on the analyzer using a modified

technique¹¹ or by the chemical method of PROCKOP AND UDENFRIEND¹². Good agreement was found between these two methods.

Separation and analysis of hydroxyproline-containing peptides

A 24-h urine specimen from the hydroxyprolinemic patient was collected while the patient was on a mixed diet. An aliquot of this urine specimen was analyzed in this laboratory by a modification of the method of MEILMAN, URIVETZKY AND RAPOPORT¹³ for hydroxyproline-containing peptides. Another aliquot was sent to Dr. MORTON URIVETZKY who kindly performed the same analysis. The following modification was used in this laboratory.

50 μ l of each effluent fraction from the column was spotted on filter paper and the specific stain of JEPSON AND SMITH¹⁴ was used to locate those fractions containing free hydroxyproline. Following this, one tenth of the effluent in alternate tubes was hydrolyzed at 124° for 3 h with an equal volume of conc. hydrochloric acid. 100 μ l of this hydrolysate was spotted on filter paper and tested by the specific stain for hydroxyproline. The fractions containing "bound hydroxyproline" then showed the specific reaction for free hydroxyproline. All of the fractions containing hydroxyproline were then analyzed for hydroxyproline by the chemical method. The analysis performed in this laboratory agreed well with that performed by Dr. M. M. URIVETZKY on the same urine sample by his method¹³.

Studies on an ascorbic acid-deficient diet

For 5 months our dieticians maintained the patient on an ascorbic acid-deficient 1800–2000-cal diet supplemented by vitamin B complex. At the end of this period there was the following evidence of ascorbic acid depletion: (1) The plasma ascorbic acid concentration fell from a control value of 0.45 mg/100 ml (low limit of normal) to 0.14 mg/100 ml. The concentration of ascorbic acid in white blood cells fell to 0.15 mg/100 ml. (2) At the end of the period of ascorbic acid depletion, the patient was given a standard ascorbic acid load test. After ingesting 500 mg of ascorbic acid, the patient excreted only 0.52 mg/h ascorbic acid in the first 2 h and 0.40 mg/h in the next 2 h. Normal controls without prior depletion of ascorbic acid excrete 29–49 mg of ascorbic acid per h after a similar load of ascorbic acid¹⁵. (3) The patient excreted *p*-hydroxyphenyllactic and *p*-hydroxyphenylacetic acids. This was demonstrated by paper chromatography of the urine in butanol–acetic acid–water (12:3:5, v/v/v) using diazotized sulfanilic acid reagent¹⁶. The urine also gave a positive ferric chloride test which was assumed to be due to the excretion of *p*-hydroxyphenylpyruvic acid.

After 6 weeks on the diet it was observed that the patient's gums bled easily on pressure. At this time she also developed furuncles which responded to penicillin therapy. 3 months after the diet was begun the patient ingested an orange. The plasma ascorbic acid rose to 0.28 mg/100 ml and the white cell ascorbic acid to 0.75 mg/100 ml. The diet was continued for 2 more months. The plasma and leucocyte ascorbic acid concentration promptly fell to very low levels. The condition of the patient remained good with no clinical signs of scurvy.

Plasma and urine hydroxyproline were measured at intervals before, during, and after the period of ascorbic acid depletion. The results are presented in Table I.

While the patient was scorbutic and again after ascorbic acid had been adminis-

TABLE I

EFFECT OF SCORBUTOGENIC DIET ON BLOOD AND URINE HYDROXYPROLINE

Date	Clinical condition of patient	Hydroxyproline			Ascorbic acid		
		Plasma, free (μ moles/ml)	Urine, total (mg/day)	Urine, free (mg/day)	Urine, peptide bound (mg/day)	Plasma (mg/100 ml)	Leucocytes (mg/100 ml)
7- 4-62 to 12-10-63 (6 dates)	Good, except for mental deficiency	0.27-0.34	301-325	243-325	39-50	—	—
5- 8-64	Good (control for low ascorbic acid diet)	0.34	—	—	—	0.45	—
5-17-64	9th day on diet	0.33	—	—	—	—	—
5-22-64	Good	0.33	—	—	—	0.25	—
6- 8-64	Good	0.45	—	—	—	0.25	—
6-29-64	Gums bled easily when touched; developed furuncles; penicillin therapy begun	0.43	—	—	—	0.14	0.14
7- 8-64	Good; off penicillin	0.38	839	671	158	0.14	—
7-13-64	Abscess over sacrum; penicillin therapy 5 days	0.80	—	—	—	—	—
7-22-64	Good	0.61	—	—	—	0.20	0.40
7-25-64	Furuncles recurred; cleared without antibiotics	—	831	675	156	—	—
7-30-64	Patient believed to have eaten an orange	0.37	—	—	—	—	—
8- 6-64	Good	0.33	—	—	—	0.28	0.75
9-17-64	Good	0.44	—	—	—	0.07	0.10
9-26-64	Good	—	838	724	114	—	—
9-28-64	Good	0.54	—	—	—	0.13	0.15
10- 1-64	Gums bled easily when touched; skin normal	—	—	—	—	—	—
10- 6-64	Ascorbic acid administered	—	—	—	—	0.14	0.15
10- 9-64	Good; on ascorbic acid therapy 2 days	—	1312	1156	166	—	—
11- 5-64	Good; on ascorbic acid	0.30	—	—	—	—	—
5-10-66	Good	0.41	—	—	—	—	—
5-23-66	Good	0.33	—	386	—	—	—
6- 1-66	Good	—	—	513	—	—	—
7-10-66	Good	0.39	—	—	—	—	—
10-14-66	Good	0.45	—	—	—	—	—
10-16-66	Good	—	—	442	—	—	—

tered for 5 months, skin biopsies from comparable areas on both thighs were performed. The specimens were studied by histological techniques and electron microscopy. The appearance of the skin collagen from the hydroxyprolinemic patient was not different from normal and there was no demonstrable change when she was considered to be scorbutic.

Studies with labeled glyoxylate

10 μ C of [14 C]glyoxylate, labeled in the first carbon atom¹⁷ was administered intravenously to the hydroxyprolinemic patient. An attempt was made to collect urine in 1-h timed periods for 4 h. However, this was not possible with the mentally retarded patient and the collection periods were not exactly 1 h in duration (Table II). One third of the urine from each collection period was treated with 2,4-dinitrophenylhydrazine, in order to form the stable dinitrophenylhydrazone derivative of α -keto- γ -hydroxyglutaric acid. These samples were then frozen at -15° until analysis. The remaining two thirds of the urine samples were frozen until analyzed for radioactivity in hydroxyproline and γ -hydroxyglutamic acid.

For analysis of the radioactivity in α -keto- γ -hydroxyglutaric acid 0.2 ml of a standard solution of α -keto- γ -hydroxyglutaric acid (1.52 mg/50 ml) was added to each urine sample as a carrier. The keto acid dinitrophenylhydrazone derivatives were extracted according to the method of ABDEL-TAWAB, BRODA AND KELLNER¹⁸. The α -keto- γ -hydroxyglutaric acid was isolated by paper chromatography, using three solvents successively; the first, toluene-acetic acid-water (4:3:1, v/v/v); the second, *n*-butanol-3% aq. ammonia (1:1, v/v); and the third, *n*-butanol-acetic acid-water (12:3:5, v/v/v). This third solvent system gives excellent separation of α -keto- γ -hydroxyglutaric acid from glyoxylate and all other keto acids. The method gave a recovery of 23% (range 20-26%) in three separate determinations of non-radioactive standard α -keto- γ -hydroxyglutaric acid. After chromatographic purification, the dinitrophenylhydrazone derivative of α -keto- γ -hydroxyglutaric acid was eluted and the radioactivity counted in a Tri-carb scintillation counter.

For analysis of hydroxyproline, γ -hydroxyglutamic acid and the other amino acids, one fiftieth of each timed urine collection was combined, desalted on Dowex

TABLE II

RECOVERY OF GLYOXYLATE AS α -KETO- γ -HYDROXYGLUTARIC ACID

Collection period	Time after injection (min)	Time of collection period (min)	Urine vol. (ml)	Counts/min recovered as α -keto- γ -hydroxyglutaric acid
1	0-50	50	91	0
2	50-62	12	93	0
3	62-88	26	94	814
4	88-135	47	262	1134
5	135-163	28	127	0
6	163-226	63	97	0
			Uncorrected total	1948
			Corrected total	8396*

* Corrected for 23% yield of α -keto- γ -hydroxyglutaric acid by the method.

50 (H^+) and the amino acid fraction eluted with ammonia. This procedure separated the amino acids from glyoxylate and glycolate. The amino acid fraction was evaporated to dryness, placed on the 150-cm column of a Beckman-Spinco automatic amino acid analyzer, and the amino acids separated according to the method of SPACKMAN, STEIN AND MOORE¹⁹. The effluent was collected in 2-ml fractions and the identity of each amino acid peak confirmed by paper chromatography.

The effluent corresponding to each single amino acid peak was pooled, desalted on Dowex 50 (H^+) to remove citrate buffer, and concentrated in a rotary evaporator. The radioactivity was then counted using a Tri-carb scintillation counter.

In order to ascertain whether the counts in the hydroxyproline fraction were actually associated with hydroxyproline, and not with some other contaminant, one half of the urine from collection period 3 (see Table II) was chromatographed on the 150-cm column of the amino acid analyzer. The fractions which contained hydroxyproline were located by the specific stain of JEPSON AND SMITH¹⁴. Only those tubes of effluent which gave the specific stain for hydroxyproline were pooled.

The pooled fractions containing hydroxyproline were desalted on Dowex 50 (H^+) to remove citrate buffer and then subjected to combined high-voltage paper electrophoresis at a pH of 1.6 in 8% formic acid, and chromatography in lutidine-water (2:1, v/v). The paper was stained with ninhydrin to locate the yellow hydroxyproline spot. Several other unknown spots were present on the chromatogram, all staining purple with ninhydrin. The paper was then exposed to an X-ray film for 1 month before development of the film. Only the yellow hydroxyproline spot gave a positive radioautographic spot (Fig. 1).

In order to be certain that the radioactivity in the hydroxyproline was not due to Δ' -pyrroline-3-hydroxy-5-carboxylate, which might have been produced by the biosynthetic pathway, the following procedure was used. One fiftieth of the urine from each collection period was combined, concentrated to 10 ml *in vacuo* without heat, acidified to pH 1-2 with hydrochloric acid, and treated with 10 ml of a 1% solution of *o*-aminobenzaldehyde in 95% alcohol. The mixture was allowed to stand for 1 h at room temperature to allow complete conversion of any Δ' -pyrroline-3-hydroxy-5-carboxylate present to its *o*-aminobenzaldehyde derivative. The urine was then desalted and the hydroxyproline fraction eluted from the column of the amino acid analyzer. An electrophoretogram was made as described above. The *o*-aminobenzaldehyde derivative of Δ' -pyrroline-3-hydroxy-5-carboxylate separates very well from hydroxyproline by high-voltage electrophoresis and by ion-exchange and paper

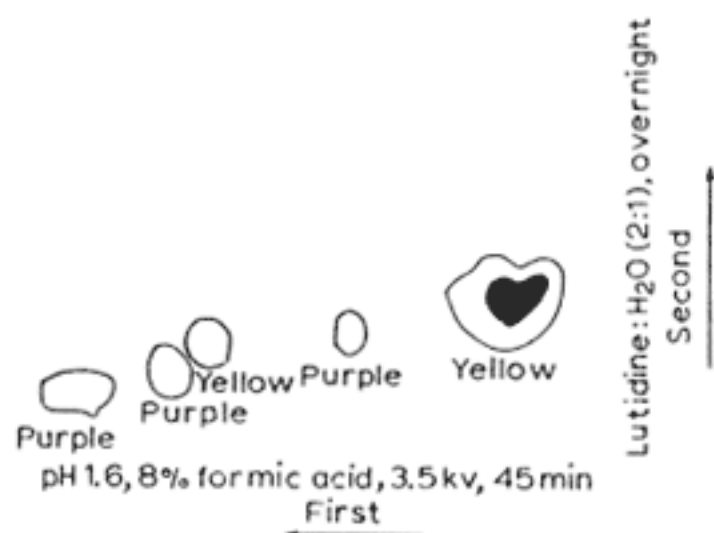


Fig. 1. Radioautogram showing positive area in position of hydroxyproline isolated from urine 62-88 min after intravenous administration of labeled glyoxylate.

chromatography. A radioautogram was made as before. Only the yellow hydroxyproline spot gave a positive radioautogram. It was concluded that the radioactivity was in fact associated with the hydroxyproline component of the effluent from the column and not with contaminating Δ' -pyrroline-3-hydroxy-5-carboxylate.

In order to be certain that the counts detected in the fraction which contained γ -hydroxyglutamic acid were in fact in this compound and not in threonine which overlaps it on this column chromatographic system, the combined γ -hydroxyglutamic acid and threonine peak was desalted and subjected to paper electrophoresis at pH 5.4. In this electrophoretic system threonine remains with the neutral band while γ -hydroxyglutamic acid migrates toward the anode and is separated from all other amino acids. The electrophoretogram was exposed to an X-ray plate for 1 month before development. The radioautogram was negative in the area of threonine, but a distinct band was visible in the exact position occupied by a standard marker of γ -hydroxyglutamic acid. It was concluded that the counts in this fraction were actually in γ -hydroxyglutamic acid and not in threonine or some other contaminating amino acid. Apparently some biosynthesized γ -hydroxyglutamic acid is excreted by the patient after loading with glyoxylate, even though there was not enough of it to give a ninhydrin-positive band on the paper in the position of the radioactivity. Without glyoxylate load the patient excretes no detectable γ -hydroxyglutamic acid.

RESULTS

The urinary pattern of hydroxyproline-containing peptides

Fig. 2 shows the pattern of excretion of hydroxyproline-containing peptides and of free hydroxyproline in the urine of the patient on a normal diet. Comparison with the normal pattern as reported by MEILMAN, URIVETZKY AND RAPOPORT¹³ shows that the four peptide peaks A, C, D, and E were not different from normal. There also was an additional peak, A', which was ninhydrin negative but which gave a positive test for hydroxyproline after hydrolysis, and contained no proline or other amino acids.

It is probable that this peak represents the diketopiperazine of free hydroxyproline, which might be expected to be formed as an artefact on the column. MEILMAN,

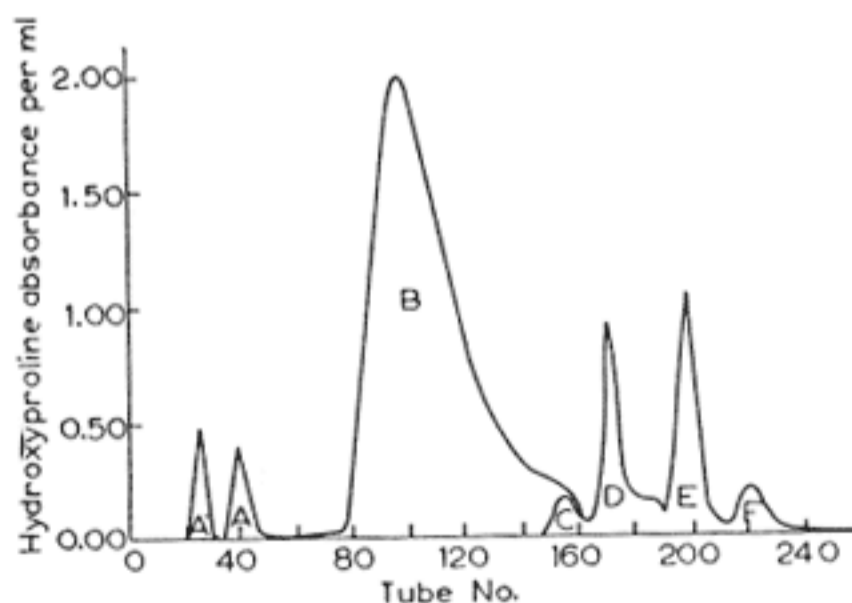


Fig. 2. Urinary-free hydroxyproline and hydroxyproline-containing peptide excretion by the hydroxyprolinemic patient. Comparison with the normal pattern reported by MEILMAN, URIVETZKY AND RAPOPORT¹³ indicates that the only significant differences are the large peak of free hydroxyproline (Peak B) and the presence of Peak A', probably the diketopiperazine of hydroxyproline.

URIVETZKY AND RAPOPORT¹³ stated that the A peak in normals probably represented a diketopiperazine of proline-hydroxyproline (D peak).

It is concluded that the total bound hydroxyproline and the pattern of excretion of hydroxyproline-containing peptides in the patient is not different from normal. In contrast, unbound free hydroxyproline is markedly increased in the urine (B peak).

Measure of normal collagen turnover

Because of the metabolic block in the hydroxyproline degradative pathway, the hydroxyprolinemic patient accumulates and excretes large amounts of free hydroxyproline (about 250 mg/day), besides a normal amount (39–62 mg/day) of "peptide-bound" hydroxyproline. Assuming that all of this hydroxyproline is derived from collagen and that the hydroxyproline oxidase deficiency is complete, since hydroxyproline constitutes about 14% of collagen²⁰, it can be calculated that the total collagen turnover in the patient (and presumably in a normal child of the same age) is about 2 g/day, with about one sixth (50:300) degraded to peptides and five sixths (250:300) degraded to free amino acids.

Effect of ascorbic acid depletion

The alteration in the plasma and urine hydroxyproline concentration when the patient was placed on a diet deficient in ascorbic acid for a period of 5 months was in direct contrast to that in scorbutic guinea pigs, who have a decreased hydroxyproline excretion. The hydroxyprolinemic patient had an increase in the fasting plasma hydroxyproline concentration to values higher than any we had observed previously. There was also an increased excretion of free and bound hydroxyproline into the urine (Table I). The excretion of free hydroxyproline was increased much more than the peptide-bound hydroxyproline. There was no comparable increase in the excretion of other amino acids, hence the increased free hydroxyproline excretion could not be attributed to a renal general aminoaciduria. There was further increase in hydroxyproline excretion when ascorbic acid was administered at the end of the period of depletion.

¹⁴C-Labeled hydroxyproline and γ -hydroxyglutamic acid from [¹⁴C]glyoxylate

Table III shows the counts and the per cent conversion of [¹⁴C]glyoxylate to ¹⁴C-labeled amino acids. There was an insignificant number of counts in the amino acid fractions other than those listed in Table III as evidenced by the chromatograms.

The recovery of the four amino acids which were labeled is virtually complete when the amino acid analyzer is used, hence these counts can be compared directly. On the other hand, the yield of α -keto- γ -hydroxyglutaric acid is incomplete, so that comparison of recovery of counts in the keto acid may not be directly comparable to that in the amino acids but can be corrected.

It is apparent that the glycine, alanine, hydroxyproline and γ -hydroxyglutamic acid became labeled when [¹⁴C]glyoxylate was administered. As a part of another study¹⁷, the specific activity of labeled glycine was measured in one normal patient after administration of labeled glyoxylate. The recovery of ¹⁴C as glycine was 0.03%, a figure identical to that in the hydroxyprolinemic patient. It is concluded that the conversion of glyoxylate to glycine is not different in the hydroxyprolinemic patient from the normal.

TABLE III

CONVERSION OF GLYOXYLATE TO AMINO ACIDS

Total counts/min in administered glyoxylate: $22.00 \cdot 10^6$. Total counts/min excreted in urine in 4 h: $4.81 \cdot 10^6$. Counts/min: recovered in amino acids separated by column chromatography.

	Counts/min	Per cent of administered counts/min
Hydroxyproline	17 372	0.079
γ -Hydroxyglutamic acid	14 797	0.067
Glycine	6 313	0.029
Alanine	2 374	0.011
α -Keto- γ -hydroxyglutaric acid	8 396*	0.038*
Other amino acids	Insignificant	Insignificant

* Corrected for 23% yield by the procedure.

Production of [^{14}C] α -keto- γ -hydroxyglutaric acid from [^{14}C]glyoxylate

Table II shows the counts/min recovered from the urine in each collection period as α -keto- γ -hydroxyglutaric acid. The total is corrected for the poor yield (20–26%) of this compound by the procedure. It is apparent that α -keto- γ -hydroxyglutaric acid excretion was increased and was labeled after administration of [^{14}C]glyoxylate and that the maximum excretion was in urine collected in the 88–135-min period after the [^{14}C]glyoxylate was administered. Ordinarily the hydroxyprolinemic patient has no detectable excretion of the α -keto- γ -hydroxyglutaric acid in the urine and does not excrete detectable amounts even after oral ingestion of hydroxyproline, whereas T. D. R. H. has observed that for normal persons α -keto- γ -hydroxyglutaric acid is very easily detectable in the urine after oral ingestion of hydroxyproline²¹.

DISCUSSION

The total excretion of hydroxyproline in peptide form and the pattern of excretion of hydroxyproline peptides are found to be no different in the hydroxyprolinemic patient than in control patients. There is no evidence that collagen degradation is altered in hydroxyprolinemia. Histological and electron microscopic examination of skin collagen showed no abnormality. It is concluded that this patient has normal collagen metabolism, and therefore, that the estimate of her collagen turnover, about 2 g/day, is of the correct order of magnitude as an estimate of the normal collagen turnover in a child of the same age (12 years).

A renal aminoaciduria has been demonstrated in infants with scurvy²² and it is possible that some of the increased excretion of free hydroxyproline was due to an increased renal clearance of this amino acid. This could not, however, explain the increased concentration of free hydroxyproline in the plasma or the increased excretion of hydroxyproline-containing peptides. The patient had no significant increase in the excretion of any free amino acid other than hydroxyproline, which indicates that increased collagen turnover, rather than aminoaciduria, was the major cause of the hydroxyprolinemia.

Almost all of the hydroxyproline in the body is in bound form in collagen²⁰.

Collagen is known to be degraded, at least in part, to hydroxyproline-containing peptides, which are excreted in the urine. This peptide-bound hydroxyproline excretion is commonly used as an index of collagen turnover; it is increased by growth²³, bone disease²⁴, hyperthyroidism²⁵, and in the *post-partum* period when the uterus is undergoing involution²⁶. It is not easy to estimate how much of the collagen hydroxyproline is degraded to free rather than to peptide-bound hydroxyproline in normal subjects, since any free hydroxyproline released from the metabolically-active soluble collagen or from breakdown of mature collagen would normally be degraded by the enzyme system present in human liver and kidney^{2,7}.

It is conceivable that the patient might have an abnormal metabolism of the soluble pool of collagen such that more of the hydroxyproline derived from collagen is degraded to free hydroxyproline and less to peptides than in normal subjects, or that the individual peptides excreted might be different from normal. This possibility was considered unlikely since there is no reason to suppose that abnormal degradation of free hydroxyproline would in any way be related to abnormal synthesis of collagen or to the breakdown of mature collagen or of its unused precursors. The total bound hydroxyproline and the pattern of excretion of hydroxyproline peptides were found to be not different from normal.

It has been postulated that the primary biochemical defect in scurvy is a failure of hydroxylation of proline^{27,28}. Collagen hydroxyproline is known to be derived from endogenous proline; little or none is derived from endogenous hydroxyproline²⁹. Until recently it was not known at what stage of collagen synthesis the hydroxylation of proline occurred. Some investigators concluded that the proline destined to become collagen hydroxyproline was hydroxylated when the proline was in the form of an "active" intermediate such as prolyl-transfer-ribonucleic acid (prolyl-sRNA)^{28,30-32}. This was then believed to be incorporated into the pre-collagen molecule. Recent studies have clearly shown, however, that the hydroxylation of proline occurs only after the proline is bound in the form of "procollagen", which is identical to collagen except for an absence of hydroxyproline and hydroxylysine and a corresponding increase in proline and lysine³³⁻³⁷.

The exact role of ascorbic acid in proline hydroxylation has also been investigated recently. It has been shown that an enzyme, collagen proline hydroxylase, is responsible for the hydroxylation of collagen proline in chick embryos⁵, and there is indication that this enzyme requires ascorbic acid as a co-factor. It seems reasonable to consider, therefore, the possibility that deficient activity of proline hydroxylase, secondary to ascorbic acid deficiency, may be the primary defect in scurvy. In scorbutic guinea pigs there is a decrease in the excretion of hydroxyproline⁶. This is compatible with failure of hydroxylation of procollagen. The pattern of excretion of free and bound hydroxyproline in human scurvy is not known.

It was postulated that, if ascorbic acid deficiency does result in significant failure of hydroxylation of procollagen in human subjects, it might be possible to bring about a decrease in the concentration of free and bound hydroxyproline in blood and urine of the hydroxyprolinemic child by maintaining her on an ascorbic acid-poor diet.

It is not clear whether hydroxyprolinemia is causally related to mental retardation, since no other case of this disorder has been reported, and since this patient was detected by screening a retarded population^{1,2}. If hydroxyprolinemia does cause

brain damage and if maintenance on an ascorbic acid-poor diet would decrease the plasma hydroxyproline concentration, it was thought that a trial of a scorbutic diet might be helpful. Accordingly, concentrations of free and bound hydroxyproline in the blood and urine of the hydroxyprolinemic patient were studied after biochemical evidence of scurvy had been produced by means of a diet deficient in ascorbic acid.

However, the increase in plasma and urinary hydroxyproline on a scorbutogenic diet was contrary to expectation; it suggests that the hydroxylation of procollagen in man is not totally blocked by ascorbic acid depletion. The findings are comparable with those found by I. A. SCHAFER (personal communication, 1967) for an infant with Hurler's syndrome, who, on a scorbutogenic diet and with no detectable ascorbic acid levels, continued to grow and showed no decrease or increase in hydroxyproline excretion in the urine, free or bound.

Apparently there was in our patient an increase in the turnover of normal or abnormal collagen or collagen precursors which had been hydroxylated. This could result from (1) increased degradation of collagen which had been hydroxylated normally before the ascorbic acid depletion, or (2) increased breakdown of poorly hydroxylated abnormal collagen or collagen precursor, which might have an increased turnover because of its abnormality. The most marked increase in hydroxyproline excretion was during the early healing phase of the scurvy, following administration of ascorbic acid.

It appears that ascorbic acid depletion in guinea pigs may produce effects on collagen metabolism and turnover different from those observed in human hydroxyprolinemia. We have no data on the effect of ascorbic acid depletion on hydroxyproline excretion in normal man, but on the basis of the findings in human hydroxyprolinemia, one might expect to find an increase in the hydroxyproline excretion in normal human scorbutic subjects. However, the seeming discrepancy between observations of decreased free hydroxyproline for the scorbutic guinea pig and the increased free hydroxyproline for the hydroxyprolinemic patient on a scorbutic regimen might be explained if the lowered hydroxyproline for the guinea pig should be due not to its decreased production in scurvy, but to an increased degradation accompanying an increased production of the imino acid. The increase in hydroxyproline for the hydroxyprolinemic patient when on a scorbutogenic diet is evident, because she lacks the hydroxyproline oxidase for degrading it².

The fact that hydroxyproline, γ -hydroxyglutamic acid and α -keto- γ -hydroxyglutaric acid became labeled after administration of labeled glyoxylate indicates that the biosynthetic pathway described by GOLDSTONE AND ADAMS⁸ in rat liver exists also in man. The magnitude of this pathway appears to be small, as it is in rat liver⁸. Less than one thousandth of the administered [¹⁴C]glyoxylate was recovered in the urine as hydroxyproline. This was almost 3 times the recovery of the glyoxylate as urinary glycine (a well known metabolic product of glyoxylate produced by direct transamination). Glycine, however, would be expected to undergo rapid breakdown while the hydroxyproline, in the absence of hydroxyproline oxidase, is essentially the end product of metabolism and would be excreted unchanged in the urine.

It seems unlikely, in view of the very small order of magnitude of the biosynthetic pathway, that biosynthesis could account for a significant percentage of the very large amount of hydroxyproline excreted by the hydroxyprolinemic patient. Rather, the evidence supports the accepted view that most of the urinary hydroxy-

proline is derived from normal collagen breakdown, without normal subsequent degradation of the hydroxyproline. We have not, however, ruled out the possibility that the biosynthetic pathway for hydroxyproline may be smaller in the patient than in normal man because of inhibition of the enzymes in the biosynthetic pathway by the accumulated hydroxyproline.

It is difficult to understand why an independent pathway for biosynthesis of free hydroxyproline exists in man, since the hydroxyproline produced is apparently useless. Present evidence suggests that the condensation between pyruvate and glyoxylate to form α -keto- γ -hydroxyglutaric acid is mediated by a specific enzyme^{9,10}. The transamination between α -keto- γ -hydroxyglutaric acid and γ -hydroxyglutamic acid may be a reversible reaction with a non-specific enzyme involved^{9,10}. The conversion of γ -hydroxyglutamic acid to Δ' -pyrroline-3-hydroxy-5-carboxylate and the reduction of the latter to hydroxyproline are apparently non-reversible reactions⁸⁻¹⁰. It may be that the same enzymes which produce proline from glutamic acid are involved in this reaction, with a lesser affinity for hydroxyproline and Δ' -pyrroline-3-hydroxy-5-carboxylate than for proline and Δ' -pyrroline-5-carboxylate. In other words, the main role of the biosynthetic pathway may be in the synthesis of proline, with a little hydroxyproline also synthesized because of lack of complete specificity of the biosynthetic pathway. This would not, however, explain the physiological role of the enzyme which synthesized α -keto- γ -hydroxyglutaric acid.

KURATOMI AND FUKUNAGA⁹ and KURATOMI, FUKUNAGA AND KOBAYASHI¹⁰ have suggested that the condensation of pyruvate and glyoxylate may have a role in the regulation of the rate of oxidation of pyruvate in the tricarboxylic acid cycle, since α -keto- γ -hydroxyglutaric acid is a potent inhibitor of α -ketoglutarate oxidation. Further studies are necessary to define the actual biological role of this biosynthetic pathway.

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