Biochem. J. (1970) **119**, 575–585
Printed in Great Britain

Studies *in vivo* on the Biosynthesis of Collagen and Elastin in Ascorbic Acid-Deficient Guinea Pigs

EVIDENCE FOR THE FORMATION AND DEGRADATION OF A PARTIALLY HYDROXYLATED COLLAGEN

BY M. J. BARNES, B. J. CONSTABLE, L. F. MORTON AND E. KODICEK
Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council, Cambridge CB4 1XJ, U.K.

(*Received* 9 *April* 1970)

1. After the administration of L-[G-^{3}H]proline to guinea pigs deprived of ascorbic acid for increasing periods of time, the specific radioactivities of proline and hydroxyproline in skin collagen and aortic elastin were determined at various time-intervals after administration of the labelled compound with a view to studying the formation and degradation of collagen and elastin both deficient in hydroxyproline. 2. As judged from the incorporation of radioactivity into elastin proline, elastin synthesis was not decreased in the ascorbic acid-deficient animals. There was however, a rapid decline in the specific radioactivity of elastin hydroxyproline. The proline/hydroxyproline specific-radioactivity ratio was approx. 1.5:1 after 6 days and 20:1 after 12 days of ascorbic acid deprivation, in contrast with the ratio of 1:1 in controls. The results suggested that the effect of ascorbic acid deficiency on elastin biosynthesis could be regarded as simply an elimination of hydroxylation of elastin proline with the formation and retention of a polymer increasingly deficient in hydroxyproline. 3. Collagen proline and hydroxyproline specific radioactivities were derived from material that was soluble in hot trichloroacetic acid, non-diffusible and collagenase-degradable. In contrast with elastin, there was a rapid decline in the specific radioactivity of proline as well as hydroxyproline in collagen from the ascorbic acid-deficient animals. However, the proline/hydroxyproline specific-radioactivity ratio in all samples from scorbutic animals was consistently slightly above 1:1. The results suggest the appearance in place of collagen, but in rapidly diminishing amounts, of a partially hydroxylated collagen in which the degree of hydroxylation may be decreased only by approx. 10%. 4. Incorporation of radioactivity into the diffusible hydroxyproline in skin remained relatively high despite the rapid decline in the incorporation of radioactivity into collagen. This observation is interpreted as indicative of an increasing degree of degradation of partially hydroxylated collagen to diffusible peptides. An alternative explanation might be that partially hydroxylated peptides are released to an increasing extent from ribosomes before they attain a length at least sufficient to render them non-diffusible. In either case it implies the accumulation in scurvy of low-molecular-weight peptides enriched in proline and deficient in hydroxyproline and could explain the failure to accumulate a high-molecular-weight collagen deficient in hydroxyproline. 5. It is thought, however, that, in addition, an inhibition of ribosomal amino acid incorporation leading to decreased synthesis of partially hydroxylated collagen may also occur, perhaps secondarily to impaired hydroxylation.

The requirement for ascorbic acid in collagen biosynthesis is thought to be due to its involvement in the hydroxylation of proline and lysine to collagen hydroxyproline and hydroxylysine respectively.

Hydroxylation occurs after the incorporation of proline and lysine into peptide linkage. Hydroxylation can be prevented when, for example, isolated connective tissues are incubated in the absence of oxygen or presence of the chelating agent, $\alpha\alpha'$-bipyridyl. Under these conditions a high-molecular-weight polypeptide essentially free of hydroxyproline (and hydroxylysine), but, like collagen,

soluble in hot trichloroacetic acid and susceptible to collagenase (see Udenfriend, 1966; Prockop & Kivirikko, 1967, 1968; Chvapil & Hurych, 1968; Gould, 1968*a*,*b*; Juva, 1968), is formed. This polypeptide, which may perhaps be regarded as an unhydroxylated precursor of collagen, has been named protocollagen (Juva & Prockop, 1966). The cell-free enzymic hydroxylation of protocollagen has been shown to require atmospheric oxygen, ferrous iron, α-oxoglutarate and ascorbate (see Hurych & Chvapil, 1965; Peterkofsky & Udenfriend, 1965; Juva & Prockop, 1966; Hutton, Tappel & Udenfriend, 1967; Hausmann, 1967; Hurych & Nordwig, 1967; Kivirikko & Prockop, 1967*a*,*b*).

Although it has been possible by the use of labelled proline to obtain evidence for the formation of protocollagen in isolated granuloma tissue taken from scorbutic guinea pigs (Stone & Meister, 1962; Gottlieb, Kaplan & Udenfriend, 1966), attempts to detect by chemical analysis of appropriate tissue fractions the presence of a proline-rich hydroxyproline-deficient collagen *in vivo* in scorbutic guinea pigs have been unsuccessful (Robertson, Hewitt & Herman, 1959; Gross, 1959; Gould, Manner, Goldman & Stolman, 1960).

Further, Barnes, Constable & Kodicek (1969*a*) found that hydroxyproline excretion in scorbutic guinea pigs continued within the normal range for some considerable period after collagen synthesis is first affected by the avitaminosis. A subsequent decrease in hydroxyproline excretion may be due to inanition rather than ascorbic acid deficiency as such, since a comparable decrease was observed in pair-fed control animals. These findings are not readily compatible with the thesis that in ascorbic acid deficiency hydroxylation is prevented and hydroxyproline-free collagen (protocollagen) is synthesized. Studies by other workers also appear to point in the same direction (Mitoma & Smith, 1960; see also Efron, Bixby, Hockaday, Smith & Meshorer, 1968).

In a previous study (Barnes, Constable & Kodicek, 1969*b*) we attempted to detect by isotopic techniques the formation of protocollagen *in vivo* in the skin of scorbutic guinea pigs. The collagen proline/hydroxyproline specific-radioactivity ratio after the administration of labelled proline is normally 1:1 (Hausmann & Neuman, 1961), but in the event of impaired hydroxylation and the concomitant formation of a hydroxyproline-deficient collagen would be greater than 1:1. Labelled proline was administered on day 15 of ascorbic acid deprivation and the skin examined 5 days later. It was concluded that, despite a severe impairment of collagen synthesis, little if any protocollagen was present.

This work has now been extended to include the period from day 6 to day 14 of ascorbic acid deprivation to assess the situation before day 15. Collagen proline and hydroxyproline specific radioactivities were measured at various intervals after the administration of labelled proline and, in addition, incorporation of radioactivity into the diffusible proline and hydroxyproline in the skin was measured, to obtain an indication of the extent of degradation of newly synthesized collagen or protocollagen in ascorbic acid deficiency.

In contrast with the impaired formation of collagen together with the virtual absence of protocollagen, Barnes *et al.* (1969*b*) found that a hydroxyproline-deficient elastin was synthesized and retained by the scorbutic guinea pig at a concentration comparable with that found for elastin in control animals. In the present study the extent of inhibition of elastin hydroxyproline formation as the severity of the ascorbic acid deficiency increased was investigated.

MATERIALS AND METHODS

Radioactive amino acids. L-[G-^{3}H]Proline, 266mCi/mmol, was obtained, as an aqueous solution (1mCi/ml), from The Radiochemical Centre, Amersham, Bucks., U.K.

Collagenase. This was highly purified collagenase (CLSPA) from Worthington Biochemical Corp., Freehold, N.J., U.S.A., reported by the suppliers to be free of non-specific proteolytic activity. No reaction, as judged by ninhydrin assay of the incubation mixture, could be detected by us with bovine plasma albumin as substrate.

Ascorbic acid-deficient guinea pigs. Young male albino guinea pigs, weighing 120–140g, were obtained from A. Tuck and Son, Animal Breeding Station, Rayleigh, Essex, U.K. Animals were made scorbutic by use of the scorbutogenic diet employed in previous studies (Barnes *et al.* 1969*a*,*b*). All animals were first kept for approx. 1 week on the basal diet supplemented with ascorbic acid (0.2%, w/w). At the commencement of the experiment, animals of comparable growth rate and body weight were selected and divided into two groups, one of which, the ascorbic acid-deficient group, was fed *ad libitum* on the basal diet free of ascorbic acid and the other (the control group) continued to receive the basal diet supplemented with ascorbic acid. The control group was fed *ad libitum* until day 8 of the experiment. Thereafter this group was pair-fed individually with animals in the ascorbic acid-deficient group to allow for the decrease in food intake in the latter group, generally commencing at about days 10–13 of vitamin deficiency.

Animals were injected intraperitoneally, on the appropriate day of the experiment, with a solution of L-[G-^{3}H]-proline, and killed at various intervals after injection. The dorsal skin was shaved before the animals were killed. A portion of dorsal skin and the aorta (as far as the bifurcation) were removed from each animal after it had been killed.

Determination of hydroxyproline. The colorimetric procedure of Bergman & Loxley (1963) was employed. Samples were first hydrolysed in 6M-HCl in sealed tubes at 100°C for 18h. The HCl was removed from hydrolysates by rotary evaporation *in vacuo*.

Hot-trichloroacetic acid extraction of guinea-pig dorsal skin: examination of non-diffusible and diffusible fractions. Dorsal skin samples of equal weight (approx. 0.15g/animal) were combined within each group for each experimental period, cut into small pieces (over ice) and disintegrated at 4°C in 0.17M-NaCl in sodium phosphate buffer, pH 7.2 and I 0.02, for four periods of 15s in a Silverson laboratory micro emulsifier–mixer. An equal volume of 10% (w/v) trichloroacetic acid containing approx. 10mg of L-proline was added to the skin homogenate and extraction was then performed at 60°C for 30min. The extract was collected after centrifugation and the residue re-extracted with 5% (w/v) trichloroacetic acid containing approx. 1mg of L-proline/ml at 60°C for 30min. The combined extracts (volume approx. 80ml) were dialysed three times against 2vol. of water. The three diffusates were combined (to form the diffusible fraction) and retained for further treatment as described below. The dialysis residue was further dialysed, initially against water containing excess of free proline and hydroxyproline and finally against water alone, the diffusates were discarded, and the dialysis residue (the non-diffusible fraction) freeze-dried. A sample of the freeze-dried product (approx. 15mg) was digested with collagenase for 2h at 37°C as previously described (Barnes *et al.* 1969*b*) except that 0.01M-calcium acetate in 0.05M-tris–HCl buffer, pH 7.2, was used. After digestion, the incubation mixture was dialysed against water (4×10vol.), and the combined diffusates were evaporated to dryness on a rotary evaporator *in vacuo* and then hydrolysed by refluxing under N_2 with constant-boiling HCl for 18h. Proline and hydroxyproline specific radioactivities were then determined after separation of the amino acids by ion-exchange chromatography as described below.

Diffusible fraction. The initial three diffusates (see above), after being combined, were freeze-dried and the product was redissolved in a small volume of water. A portion was then hydrolysed as above. After removal of HCl from hydrolysates by rotary evaporation *in vacuo*, carrier L-hydroxyproline (approx. 500μg) was added before the determination of proline and hydroxyproline radioactivity as described below.

Elastin. Aortic elastin was isolated and hydrolysed as described previously (Barnes *et al.* 1969*b*). Proline and hydroxyproline specific radioactivities were determined as described below.

Determination of the radioactivity in proline and hydroxyproline. Samples were first hydrolysed as indicated above. Hydrolysates were freed of HCl by rotary evaporation *in vacuo*. Proline and hydroxyproline were separated by ion-exchange chromatography at 50°C on the Technicon Amino Acid AutoAnalyzer, with a pyridine–formate buffer system, measured and the radioactivities counted as described previously (Barnes *et al.* 1969*b*). All results are expressed at the same efficiency of counting (40%).

RESULTS

Elastin

Guinea pigs deprived of ascorbic acid for increasing periods of time were killed at 4.5 and 24h after administration of labelled proline and the specific radioactivities of proline and hydroxyproline in isolated aortic elastin were determined (Table 1). Values at 24h in both ascorbic acid-deficient animals and controls were generally appreciably higher than the corresponding values at 4.5h.

Proline specific radioactivities in the ascorbic acid-deficient group were consistently higher than those in the corresponding control animals, which did not suggest any decrease in elastin biosynthesis in scurvy. The decrease on day 12 in specific radioactivities of proline in the ascorbic acid-deficient group, also observed in the controls, is thought to be due to inanition.

The proline/hydroxyproline specific-radioactivity ratio in control animals was close to unity. The observed variation (from 0.8:1 to 1.4:1) is most likely due to a greater degree of experimental error in assessing the ratio in elastin samples in view of the low content of hydroxyproline and the relatively low specific radioactivities. Nevertheless the approximation to unity indicated a relatively constant degree of hydroxylation of elastin proline during elastin biosynthesis. In contrast, in the ascorbic acid-deficient animals, the ratio showed a progressive increase from 1.5–1.6:1 to the value of 18–20.3:1 on day 12, suggesting progressive impairment of hydroxylation with increasing severity of ascorbic acid deficiency. The decreased hydroxylation of elastin proline would also appear to be reflected in the chemical composition of the protein since there appeared to be an increase in the proline/hydroxyproline percentage composition ratio as the period of ascorbic acid deficiency was increased (Table 1). The mean value for this ratio on days 10–14 (including both 4.5h and 24h values) in the controls was 8.24 (S.E.M. ±0.24):1 and in the ascorbic acid-deficient group 10.05 (S.E.M. ±0.45):1 ($P<0.01$).

Collagen

Collagen was extracted from guinea-pig dorsal skin with hot trichloroacetic acid under conditions that were found to yield 90–95% of the total hydroxyproline in the skin. Any protocollagen in the skin should also be soluble in hot trichloroacetic acid (Gottlieb *et al.* 1966). Extracts were separated by dialysis into a high-molecular-weight non-diffusible fraction (dialysis residue) and a low-molecular-weight diffusible fraction (diffusate). The results from two experiments are described.

The dialysis tubing used in this study (Visking 18/32 tubing) did not allow the passage of cytochrome *c* (molecular weight approx. 12000) and is reported to retain completely insulin of molecular weight 6000 (Craig, King & Stracher, 1957).

Expt. 1: *non-diffusible hot-trichloroacetic acid-soluble fraction (dialysis residue).* Proline and

Table 1. *Elastin: specific radioactivities of proline and hydroxyproline in elastin from control and scorbutic guinea pigs*

Each animal received on the appropriate day of the experiment 0.1 mCi of L-[G-^{3}H]proline in a single dose. Animals were killed 4.5 or 24 h later and the aortae were removed and combined within each set of animals before the isolation of elastin. Elastin proline and hydroxyproline were determined and the specific radioactivity of each was measured as described in the text.

		4.5 h*				24 h*			
	Day of expt.	Proline/ hydroxyproline % composition ratio	Specific radioactivity (c.p.m./μmol) Proline	Specific radioactivity (c.p.m./μmol) Hydroxyproline	Proline/ hydroxyproline specific-radioactivity ratio	Proline/ hydroxyproline % composition ratio	Specific radioactivity (c.p.m./μmol) Proline	Specific radioactivity (c.p.m./μmol) Hydroxyproline	Proline/ hydroxyproline specific-radioactivity ratio
Ascorbic acid-deficient group	6	8.7 (6)	102	65	1.6	8.9 (6)	222	149	1.5
	8	8.6 (6)	92	54	1.7	7.3 (6)	132	62	2.1
	10	8.8 (5)	69	6	11.5	9.7 (5)	142	15	9.5
	12	9.7 (6)	36	2	18.0	12.1 (6)	61	3	20.3
	14	9.9 (6)	—	—	—	10.1 (6)	—	—	—
Control group†	6	8.3 (6)	66	56	1.2	8.8 (6)	129	125	1.0
	8	8.8 (6)	46	40	1.2	8.6 (6)	111	131	0.9
	10	8.7 (5)	27	30	0.9	7.9 (4)	80	57	1.4
	12	9.5 (5)	17	21	0.8	8.1 (6)	35	31	1.1
	14	8.1 (6)	—	—	—	8.4 (6)	—	—	—

* Time after administration of labelled proline.

† Controls were individually pair-fed with animals in the ascorbic acid-deficient group after day 8. Numbers in parentheses denote the number of animals available at the time of killing from an original total of six.

Table 2. *Collagen: Expt.* 1: *proline and hydroxyproline specific radioactivities in the non-diffusible, hot-trichloroacetic acid-soluble fraction (dialysis residue) of dorsal skin from control and scorbutic guinea pigs*

The results are for the same animals as are listed in Table 1. Approximately equal samples of dorsal skin were combined within each set of animals before hot-trichloroacetic acid extraction as described in the text; extracts, after dialysis and freeze-drying, were digested with collagenase and the specific radioactivities of proline and hydroxyproline in the resulting diffusible material determined.

		4.5 h*			24 h*		
		Specific radioactivity (c.p.m./μmol)		Proline/hydroxyproline specific-radioactivity ratio	Specific radioactivity (c.p.m./μmol)		Proline/hydroxyproline specific-radioactivity ratio
	Day of expt.	Proline	Hydroxyproline		Proline	Hydroxyproline	
Ascorbic acid-deficient group	6	547	492	1.11	739	667	1.11
	8	430	352	1.22	357	289	1.24
	10	119	105	1.13	74	69	1.07
	12	29	24	1.21	21	17	1.24
	14	36	31	1.16	13	12	1.08
Control group†	6	614	669	0.92	614	583	1.05
	8	357	361	0.99	785	828	0.95
	10	302	292	1.03	553	563	0.98
	12	293	292	1.0	224	217	1.03
	14	254	248	1.02	415	417	1.0

* Time after administration of labelled proline.

† See Table 1.

hydroxyproline specific radioactivities were determined in the diffusible peptides obtained after incubation of the non-diffusible, hot-trichloroacetic acid-soluble fraction with highly purified collagenase. Under our conditions approx. 70% of the hydroxyproline was rendered diffusible. The use of collagenase was introduced to obtain a complete separation of the proline radioactivity in collagen or protocollagen from any proline radioactivity from other sources.

Proline and hydroxyproline specific radioactivities at 4.5 and 24h after administration of labelled proline after various periods of ascorbic acid deprivation are given in Table 2. There was a sharp decrease in 4.5 and 24h values of both proline and hydroxyproline, commencing at about days 8–10 of vitamin deficiency. The relatively slight decrease in values in the control animals at about days 12–14 of the experiment is thought to be due to inanition. The specific-radioactivity values generally increased between 4.5 and 24h in control samples, whereas in the ascorbic acid-deficient animals, after day 6 of deficiency, the 24h values were consistently lower than those obtained 4.5h after injection of labelled proline, suggesting the possibility of a more rapid turnover rate.

The proline/hydroxyproline specific-radioactivity ratio was very close to unity in all control samples but consistently slightly above unity in all samples from scorbutic animals. The mean of all values shown in Table 2 for the control group was 1.00 (S.E.M. $\pm$0.013) and for the ascorbic acid-deficient group 1.16 (S.E.M. $\pm$0.021) ($P<0.001$).

Expt. 1: *diffusible hot-trichloroacetic acid-soluble fraction* (*diffusate*). The incorporation of radioactivity into the diffusible proline and hydroxyproline derived from hot-trichloroacetic acid extracts of dorsal skin at 4.5 and 24h after the administration of L-[G-^{3}H]proline at various periods of ascorbic acid deficiency is shown in Table 3.

For comparison, Table 3 also shows the incorporation of L-[G-^{3}H]proline into the non-diffusible hydroxyproline in the hot-trichloroacetic acid extracts. This was calculated from a knowledge of the specific radioactivities given in Table 2 and from colorimetric determination of the total non-diffusible hydroxyproline in the extract. Although these specific radioactivities relate only to the hydroxyproline rendered diffusible by collagenase digestion, it was found that the hydroxyproline remaining non-diffusible after collagenase action was of a similar specific radioactivity.

As already inferred in the specific-radioactivity data (Table 2), the incorporation of L-[G-^{3}H]proline into the non-diffusible hydroxyproline showed a rapid decrease in the ascorbic acid-deficient animals. Surprisingly, however, this decrease was not reflected in the incorporation of radioactivity into the hydroxyproline in the diffusible fraction, which remained relatively constant and comparable with that in control animals. Further, in agreement with the continued incorporation of radioactivity within the normal range, there was no decrease in the actual amount, as determined colorimetrically, of diffusible hydroxyproline in the skin of the scorbutic animals (Table 3).

Diffusible proline and hydroxyproline both showed a large decrease in radioactivity from 4.5 to 24h indicative of a rapid turnover rate. It should be noted that at 24h, diffusible proline radioactivity in the ascorbic acid-deficient group, particularly at day 10 and also to a lesser extent at days 12 and 14, was appreciably higher than the corresponding control values.

Expt. 2: *non-diffusible hot-trichloroacetic acid-soluble fraction* (*dialysis residue*). In a second experiment proline and hydroxyproline specific radioactivities were determined, after collagenase digestion, in dialysed hot-trichloroacetic acid extracts of dorsal skin at a number of time-periods after administration of L-[G-^{3}H]proline to guinea pigs on day 9 of ascorbic acid deprivation. This experiment was undertaken primarily to assess the decrease, if any, of specific-radioactivity values with time after injection in the ascorbic acid-deficient group and thus to obtain an indication of the turn-over rate.

The results are presented in Table 4. Again, as in Expt. 1, the specific radioactivities of both proline and hydroxyproline in the ascorbic acid-deficient animals were appreciably decreased in comparison with control values. Further, the proline/hydroxyproline specific-radioactivity ratio in all samples from scorbutic animals was again slightly greater than unity. However, a continuous decline in specific radioactivities with time after injection, as might have been anticipated from the results of Expt. 1, was not observed in the scorbutic group.

Expt. 2: *diffusible hot-trichloroacetic acid-soluble fraction* (*diffusate*). The incorporation of radioactivity into the diffusible proline and hydroxyproline derived from the hot-trichloroacetic acid extracts is shown in Table 5. There was a rapid decrease in radioactivity with time, indicative of the rapid turnover of the diffusible proline and hydroxyproline. As in Expt. 1, despite the decreased incorporation of radioactivity into the non-diffusible hydroxyproline in the ascorbic acid-deficient animals, incorporation into the diffusible hydroxyproline was not decreased and was of the same order as that in controls. Proline radioactivity in the ascorbic acid-deficient group was again in certain instances appreciably higher than in the corresponding controls.

Table 3. *Collagen: Expt.* 1: *comparison of the incorporation of* L-[G-^{3}H]*proline into diffusible and non-diffusible hydroxyproline in hot trichloroacetic acid extracts of dorsal skin from control and scorbutic guinea pigs*

The results are for the same animals as those listed in Table 1. Hot-trichloroacetic acid extracts of skin were separated by dialysis into diffusible and non-diffusible fractions (diffusate and dialysis residue). Proline and hydroxyproline radioactivity in the diffusate was determined as described in the text. Total hydroxyproline in the diffusate was determined colorimetrically; after hydrolysis, HCl was removed by rotary evaporation *in vacuo* and any residual acidity due to trichloroacetic acid neutralized by means of NaOH. Incorporation of radioactivity into the hydroxyproline in the dialysis residue was calculated from the specific radioactivities given in Table 2 and the concentration of hydroxyproline in the dialysis residue as determined colorimetrically.

		4.5 h*				24 h*		
		Incorporation of radioactivity (c.p.m./g wet wt. of skin)				Incorporation of radioactivity (c.p.m./g wet wt. of skin)		
		Diffusate		Dialysis residue	Total hydroxyproline in diffusate	Diffusate		Dialysis residue
	Day of expt.	Proline	Hydroxyproline	Hydroxyproline	(μg/g wet wt. of skin)	Proline	Hydroxyproline	Hydroxyproline
Ascorbic acid-deficient group	6	20211	1806	42637	57	4577	478	55935
	8	17967	2192	28593	56	3500	478	16715
	10	14016	1860	7914	77	10355	266	5510
	12	10163	1041	2022	86	5201	253	1821
	14	14368	1555	2310	61	5761	151	1259
Control group†	6	27402	1663	51018	58	4194	406	44139
	8	18404	1477	35486	47	3567	250	45797
	10	16863	1597	27524	72	2357	324	52528
	12	15171	1474	31083	65	3591	203	23312
	14	18799	1673	21901	62	2681	310	43118

* Time after administration of labelled proline.

† See Table 1.

Table 4. *Collagen: Expt. 2: proline and hydroxyproline specific radioactivities in the non-diffusible hot-trichloroacetic acid-soluble fraction (dialysis residue) of dorsal skin from control and scorbutic guinea pigs*

Each animal received, on day 9, 0.1 mCi of L-[G-^{3}H]proline in a single injection. Animals were killed at various intervals after injection. Six animals were used at each time-interval for each group. Animals to be killed at 12, 24 and 48 h received intraperitoneally 0.5 ml of an aq. soln. of 3% (w/v) L-proline at 5, 6.5 and 8 h after administration of labelled proline. Approximately equal samples of dorsal skin were combined within each set of animals before hot-trichloroacetic acid extraction as described in the text; extracts, after dialysis and freeze-drying, were digested with collagenase and the specific radioactivities of proline and hydroxyproline in the resulting diffusible material were determined.

	Time after injection (h)	Specific radioactivity (c.p.m./μmol)		Proline/hydroxyproline specific-radioactivity ratio
		Proline	Hydroxyproline	
Ascorbic acid-deficient group	2	45	35	1.29
	4.5	96	77	1.25
	12	79	70	1.13
	24	26	22	1.18
	48	75	62	1.21
Control group†	2	188	189	0.99
	4.5	145	154	0.94
	12	210	199	1.06
	24	209	205	1.02
	48*	139	140	0.99

* One animal died before completion of the experiment.
† See Table 1.

Table 5. *Collagen: Expt. 2: incorporation of* L-[G-^{3}H]*proline into the diffusible and non-diffusible hydroxyproline of dorsal skin from control and scorbutic guinea pigs*

The results are for the same animals as those listed in Table 4. Hot-trichloroacetic acid extracts of skin were separated by dialysis into diffusible and non-diffusible fractions (diffusate and dialysis residue). Proline and hydroxyproline radioactivity in the diffusate was determined as described in the text. Incorporation of radioactivity into the hydroxyproline in the dialysis residue was calculated from the specific radioactivities given in Table 4 and the concentration of hydroxyproline in the dialysis residue as determined colorimetrically.

	Time after injection (h)	Incorporation of radioactivity (c.p.m./g wet wt. of skin)		
		Diffusate		Dialysis residue
		Proline	Hydroxyproline	Hydroxyproline
Ascorbic acid-deficient group	2	25060	1120	3008
	4.5	15822	1294	5971
	12	5560	588	5566
	24	3756	172	1947
	48	2370	109	5163
Control group*	2	26761	1278	14636
	4.5	11077	901	9647
	12	3880	458	14841
	24	2407	159	18366
	48	1680	120	11743

* See Table 1.

DISCUSSION

Elastin. As observed previously (Barnes *et al.* 1969*b*) ascorbic acid deficiency does not appear to cause a decrease in elastin biosynthesis. In the present study the proline specific radioactivities in the ascorbic acid-deficient group were always higher than the corresponding values in the control group. The reason for this is not clear. It could represent an increased rate of elastin synthesis in

ascorbic acid deficiency but no increased yield of elastin relative to controls was observed nor did we observe previously (Barnes *et al.* 1969*b*) an increase in proline specific radioactivity when elastin was isolated 5 days after the administration of labelled proline to guinea pigs on day 15 of ascorbic acid deficiency. It is possible that the increase reflects a different rate of formation of cross-links in the two groups (see Barnes *et al.* 1969*b*). The increase in specific-radioactivity values between 4.5 and 24h is presumably attributable to continued incorporation of labelled proline into elastin.

Although elastin synthesis is not decreased in scurvy, it is clear from the proline/hydroxyproline specific-radioactivity ratio that the formation of elastin hydroxyproline is greatly decreased and that this effect becomes progressively more marked as the period of vitamin deficiency is extended. Thus formation of elastin hydroxyproline is already affected after 6 days of ascorbic acid deficiency and at 12 days the degree of hydroxylation is almost negligible.

There seems little doubt now that hydroxyproline in elastin preparations is an integral part of the elastin molecule (see Barnes *et al.* 1969*b*; Bentley & Hanson, 1969; Sandberg, Weissman & Smith, 1969). It also appears very probable that, as suggested previously (Barnes *et al.* 1969*b*), the hydroxyproline is formed by the hydroxylation of specific prolyl residues, previously incorporated into peptide linkage during elastin biosynthesis, by the enzyme protocollagen hydroxylase. Thus it has been shown that the content of hydroxyproline in elastin can be increased (by approx. 5 residues/1000) by incubation with this enzyme and appropriate cofactors, including ascorbic acid (Rhoads & Udenfriend, 1969). Nevertheless, the impaired formation of elastin hydroxyproline in ascorbic acid deficiency appears to have no deleterious effect on the biosynthesis of elastin.

Collagen. Collagen proline and hydroxyproline specific radioactivities refer, in this study, to the specific radioactivities of proline and hydroxyproline in hot-trichloroacetic acid-soluble non-diffusible collagenase-degradable material.

At no period of ascorbic acid deficiency, irrespective of the time-interval between administration of labelled proline and isolation of the tissue, did we observe high incorporation of radioactivity into collagen proline accompanied by low incorporation into hydroxyproline. The specific radioactivities of both proline and hydroxyproline decreased sharply after 8–10 days of ascorbic acid deprivation. This does not suggest that the effect of ascorbic acid deficiency on collagen biosynthesis can be explained simply in terms of the formation and retention of a polymer increasingly deficient in hydroxyproline due to a gradual elimination of hydroxylation.

However, the proline/hydroxyproline specific-radioactivity ratio in collagen from scorbutic animals did reflect impaired hydroxylation, since even at day 6 it was consistently slightly above unity, in contrast with the 1:1 ratio in all controls. It is not considered that this was due to the presence of radioactive proline from a source other than collagen or protocollagen, since the specific radioactivities refer to proline and hydroxyproline in diffusible peptides obtained by the action of highly purified collagenase. Further, the increased ratio was observed even in those samples in which the concentration of proline and hydroxyproline radioactivity was of the same order of magnitude as that of controls; if due to contaminating proline radioactivity, it would have been expected that control ratios would also reflect the presence of such a contaminant. It might also have been expected that as incorporation of radioactivity into collagen proline and hydroxyproline decreased, any contaminating proline radioactivity would become increasingly obvious, i.e. the proline/hydroxyproline specific-radioactivity ratio should increase substantially. However, the ratio remained relatively constant despite the sharp decrease in specific radioactivities. It is therefore considered that the statistically significant increase in the ratio can be regarded as indicative of impaired hydroxylation and it is suggested that it represents the synthesis of a partially hydroxylated collagen in which the overall degree of hydroxylation is decreased from the normal amount by perhaps about 10%. Noteworthy in this context are studies (Bornstein, 1967; Butler, 1968; Miller, Lane & Piez, 1969) that show that some proline and lysine residues are normally only partially hydroxylated.

It must be concluded, however, in view of the rapid decline of the specific radioactivities of both proline and hydroxyproline, that the amount, not only of collagen, but also of the under-hydroxylated collagen appearing in a non-diffusible form extractable with hot trichloroacetic acid, rapidly diminishes to almost negligible concentrations as the period of ascorbic acid deficiency is extended.

The failure of the partially hydroxylated collagen to accumulate in ascorbic acid deficiency could be due to a decreasing synthesis, an increasing rate of degradation or both, possibly as a consequence of an increasing impairment of hydroxylation.

It was thought that increased degradation might possibly occur as the result of the absence, due to impaired hydroxylation of lysine (Hausmann, 1967; Hurych & Nordwig, 1967; Kivirikko & Prockop, 1967*a*,*b*), of hydroxylysine residues essential for intermolecular cross-linking (Bailey & Peach, 1968; Bailey, Fowler & Peach, 1969). Measurement of proline and hydroxyproline specific radioactivities at various time-intervals after the

administration of labelled proline did suggest some increase in degradation, relative to controls, in Expt. 1, but a similar conclusion could not be reached from the results of Expt. 2. The fact is that the marked fall in radioactivity incorporation occurring at about days 8–10 of vitamin deprivation was already apparent in the specific-radioactivity values at the earliest time-period after administration of labelled proline examined, and it is not considered that this situation can be explained solely on the basis of increased degradation due to impaired formation of cross-links. Even in the absence of cross-linking, it would still be expected that, at least at the earlier time-periods investigated, an appreciable incorporation of radioactivity into a high-molecular-weight non-diffusible moiety would be observed, since in conditions where cross-linking is known to be impaired, e.g. lathyrism, copper deficiency and after administration of penicillamine, there is continued synthesis and an accumulation of high-molecular-weight material as neutral-salt-soluble collagen (see Levene & Gross, 1959; Weissman, Shields & Carnes, 1963; Chou, Savage & O'Dell, 1969; Rucker, Parker & Rogler, 1969; Deshmukh & Nimni, 1969).

Since it is considered that the occurrence in tissues of free hydroxyproline and low-molecular-weight peptides containing hydroxyproline arises from the degradation of collagen (see Prockop & Kivirikko, 1968), an examination of the incorporation of radioactivity into diffusible hydroxyproline was undertaken as another approach to assessing the extent of degradation in ascorbic acid deficiency. It was found that, despite the marked decrease in radioactivity incorporated into collagen in the scorbutic animals, incorporation into the diffusible hydroxyproline remained relatively high. There was no decrease in the actual amount of diffusible hydroxyproline in the skin. This is in accord with the excretion of hydroxyproline within the normal range by the scorbutic animals over the entire period of the experiment (M. J. Barnes, B. J. Constable, L. F. Morton & E. Kodicek, unpublished work). The same situation, it is thought, can be inferred from the results of Robertson *et al.* (1959), who studied carrageenan granuloma formation *in vivo* in scorbutic guinea pigs.

The above findings can be interpreted as indicative of the degradation of an increasing proportion of newly synthesized hydroxyproline-containing material to a diffusible form in the scorbutic animals. In view of the evidence for the involvement of ascorbic acid in collagen proline and lysine hydroxylation, and for the formation of partially hydroxylated collagen described above, it is suggested that the incorporation of radioactivity into the diffusible hydroxyproline in the scorbutic animals may be indicative of the degradation of recently formed partially hydroxylated polypeptides (see Efron *et al.* 1968) rather than the degradation of newly synthesized collagen. It is also suggested that this may be occurring at an increasing rate with an increasing impairment of hydroxylation since it could account for the low incorporation of radioactivity into collagen accompanied by undiminished incorporation into diffusible hydroxyproline. Since the sharp decline in the radioactivity incorporation into collagen is reflected in the specific-radioactivity values even as shortly as 4.5h after administration of labelled proline, degradation to a diffusible state must presumably be very rapid. Alternatively the diffusible hydroxyproline in the scorbutic animals may conceivably arise, in part and to an increasing extent, by the release from ribosomes of partially hydroxylated peptides, progressively deficient in hydroxyproline, before such peptides attain a size sufficient to render them non-diffusible. Possibly the increase in diffusible proline radioactivity noted in certain instances in the scorbutic group can also be regarded as indicative of the presence of diffusible proline-rich hydroxyproline-deficient peptides.

The formation of such peptides by either of the mechanisms discussed above might account for the lack of accumulation in ascorbic acid deficiency of a collagen becoming progressively deficient in hydroxyproline. However, an inhibition of ribosomal amino acid incorporation, resulting in decreased synthesis, might also be a contributing factor. It has been suggested (Hagopian, Bosmann & Eylar, 1968) that glycosylated hydroxylysine residues, occurring in collagen, are essential for the extrusion of the collagen molecule from the cell. If, due to impaired hydroxylation, these residues are absent, partially hydroxylated collagen may accumulate to some extent within the cell (see Cooper & Prockop, 1968) and thereby in some way interfere with ribosomal function, either preventing amino acid incorporation or increase in chain length. Electron-microscopic studies have indicated changes in the state of aggregation of ribosomes on the surface of membranes of the rough endoplasmic reticulum in scorbutic fibroblasts (see Ross, 1968).

Richmond & Stokstad (1969) have studied the incorporation *in vivo* of labelled proline into proline and hydroxyproline of skin collagens of control and scorbutic guinea pigs. Their results differ from ours in a number of respects. In particular they found that neutral-salt-soluble collagen isolated from control animals 24h or 3 days after the administration of labelled proline was incompletely hydroxylated and concluded that hydroxylation continued during the process of collagen maturation into an insoluble form. In contrast, they observed in scorbutic guinea pigs an impairment of proline hydroxylation, the degree of which was greatest in insoluble colla-

gen and least in neutral-salt-soluble collagen, which would seem to imply a preferential cross-linking of the under-hydroxylated collagen. We believe their results must be interpreted with caution since they were derived without the use of collagenase to ensure separation of collagen proline radioactivity from possible contaminating proline radioactivity from proteins other than collagen or protocollagen (see Barnes *et al.* 1969*b*).

REFERENCES

Bailey, A. J., Fowler, L. J. & Peach, C. M. (1969). *Biochem. biophys. Res. Commun.* **35**, 663.

Bailey, A. J. & Peach, C. M. (1968). *Biochem. biophys. Res. Commun.* **33**, 812.

Barnes, M. J., Constable, B. J. & Kodicek, E. (1969*a*). *Biochim. biophys. Acta*, **184**, 358.

Barnes, M. J., Constable, B. J. & Kodicek, E. (1969*b*). *Biochem. J.* **113**, 387.

Bentley, J. P. & Hanson, A. N. (1969). *Biochim. biophys. Acta*, **175**, 339.

Bergman, I. & Loxley, R. (1963). *Analyt. Chem.* **35**, 1961.

Bornstein, P. (1967). *Biochemistry, Easton*, **6**, 3082.

Butler, W. T. (1968). *Science, N.Y.*, **161**, 796.

Chou, W. S., Savage, J. E. & O'Dell, B. L. (1969). *J. biol. Chem.* **244**, 5785.

Chvapil, M. & Hurych, J. (1968). *Int. Rev. connect. Tissue Res.* **4**, 67.

Cooper, G. W. & Prockop, D. J. (1968). *J. Cell Biol.* **38**, 523.

Craig, L. C., King, T. P. & Stracher, A. (1957). *J. Am. chem. Soc.* **79**, 3729.

Deshmukh, K. & Nimni, M. E. (1969). *J. biol. Chem.* **244**, 1787.

Efron, M. L., Bixby, E. M., Hockaday, T. D. R., Smith, L. H. & Meshorer, E. (1968). *Biochim. biophys. Acta*, **165**, 238.

Gottlieb, A. A., Kaplan, A. & Udenfriend, S. (1966). *J. biol. Chem.* **241**, 1551.

Gould, B. S. (1968*a*). In *Treatise on Collagen*, vol. 2A, p. 139. Ed. by Gould, B. S. London and New York: Academic Press.

Gould, B. S. (1968*b*). In *Treatise on Collagen*, vol. 2A, p. 323. Ed. by Gould, B. S. London and New York: Academic Press.

Gould, B. S., Manner, G., Goldman, H. M. & Stolman, J. (1960). *Ann. N.Y. Acad. Sci.* **85**, 385.

Gross, J. (1959). *J. exp. Med.* **109**, 557.

Hagopian, A., Bosmann, H. B. & Eylar, E. H. (1968). *Archs Biochem. Biophys.* **128**, 387.

Hausmann, E. (1967). *Biochim. biophys. Acta*, **133**, 591.

Hausmann, E. & Neuman, W. F. (1961). *J. biol. Chem.* **236**, 149.

Hurych, J. & Chvapil, M. (1965). *Biochim. biophys. Acta*, **97**, 361.

Hurych, J. & Nordwig, A. (1967). *Biochim. biophys. Acta*, **140**, 168.

Hutton, J. J., Tappel, A. L. & Udenfriend, S. (1967). *Archs Biochem. Biophys.* **118**, 231.

Juva, J. (1968). *Acta physiol scand*, Suppl. no. 308.

Juva, K. & Prockop, D. J. (1966). In *Biochemie et Physiologie du Tissu Conjonctif*, p. 417. Ed. by Comte, P. Lyons: Société Ormeco et Imprimerie du Sud-Est.

Kivirikko, K. I. & Prockop, D. J. (1967*a*). *Archs Biochem. Biophys.* **118**, 611.

Kivirikko, K. I. & Prockop, D. J. (1967*b*). *Proc. natn. Acad. Sci. U.S.A.* **57**, 782.

Levene, C. I. & Gross, J. (1959). *J. exp. Med.* **110**, 771.

Miller, E. J., Lane, J. M. & Piez, K. A. (1969). *Biochemistry, Easton*, **8**, 30.

Mitoma, C. & Smith, T. E. (1960). *J. biol. Chem.* **235**, 426.

Peterkofsky, B. & Udenfriend, S. (1965). *Proc. natn. Acad. Sci. U.S.A.* **53**, 335.

Prockop, D. J. & Kivirikko, K. I. (1967). *Ann. intern. Med.* **66**, 1243.

Prockop, D. J. & Kivirikko, K. I. (1968). In *Treatise on Collagen*, vol. 2A, p. 215. Ed. by Gould, B. S. London and New York: Academic Press.

Rhoads, R. E. & Udenfriend, S. (1969). *Archs Biochem. Biophys.* **133**, 108.

Richmond, V. & Stokstad, E. L. R. (1969). *J. dent. Res.* **48**, 83.

Robertson, W. van B., Hewitt, J. & Herman, C. (1959). *J. biol. Chem.* **234**, 105.

Ross, R. (1968). *Biol. Rev.* **43**, 51.

Rucker, R. B., Parker, H. E. & Rogler, J. C. (1969). *J. Nutr.* **98**, 57.

Sandberg, L. B., Weissman, N. & Smith, D. W. (1969). *Biochemistry, Easton*, **8**, 2940.

Stone, N. & Meister, A. (1962). *Nature, Lond.*, **194**, 555.

Udenfriend, S. (1966). *Science, N.Y.*, **152**, 1335.

Weissman, N., Shields, G. S. & Carnes, W. H. (1963). *J. biol. Chem.* **238**, 3115.