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

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1. After the administration of labelled proline to guinea pigs deprived of ascorbic acid for 15 days, the dorsal skin was examined 5 days later in an attempt to detect the presence of hydroxyproline-deficient collagen (protocollagen). The extent of incorporation of proline into skin collagens indicated a severe impairment of collagen synthesis. 2. A comparison of proline and hydroxyproline specific radioactivities in diffusible peptides obtained by treatment with collagenase of either purified skin collagens or direct hot-trichloroacetic acid extracts of skin failed to indicate the presence of protocollagen. Possible reasons for this are discussed. 3. The incorporation results did not indicate an inability of normal collagen, i.e. collagen hydroxylated to the normal degree, to cross-link in scurvy. 4. Incorporation of labelled proline into aortic elastin isolated from the same animals did not indicate a decrease in elastin biosynthesis in ascorbic acid deficiency, beyond that attributable to the inanition accompanying the vitamin deficiency. The proline/hydroxyproline specific-radioactivity ratio in elastin from scorbutic guinea pigs was about 6:1 in contrast with the 1:1 ratio in control groups. It is concluded that the formation of elastin hydroxyproline was ascorbate-dependent and that a hydroxyproline-deficient elastin is formed and retained in scurvy. The formation of desmosines was unimpaired in scorbutic animals. 5. Studies with chick embryos confirmed the formation of elastin hydroxyproline from free proline. Incorporation of free hydroxyproline into elastin hydroxyproline was negligible. 6. Digestion of solubilized samples with collagenase indicated that the hydroxyproline in guinea-pig aortic elastin preparations was not derived from contamination by collagen. It is suggested that most if not all of the hydroxyproline in the guinea pig elastin preparations investigated can be considered an integral part of the elastin molecule.

The hydroxylation of specific prolyl and lysyl residues during collagen biosynthesis is believed to occur after the incorporation of proline and lysine into peptide linkage (see Udenfriend, 1966; Gould, 1968a). Under certain conditions, including ascorbic acid deficiency (see Udenfriend, 1966; Prockop & Kivirikko, 1967, 1968; Chvapil & Hurych, 1968; Gould, 1968a,b; Juva, 1968), the hydroxylation is inhibited, and it has been possible to demonstrate by isotope techniques under such conditions the formation *in vitro* of a hydroxyproline- and hydroxylysine-deficient collagen, termed protocollagen (Juva & Prockop, 1966). One of the purposes of the present study was to attempt to detect, in scorbutic guinea pigs, the formation of protocollagen *in vivo* and, if detected, to study its ability to undergo the same specific process of aggregation and cross-linking that is characteristic of tropocollagen. To this end, labelled proline was administered to scorbutic and control guinea pigs and a comparison of the specific radioactivities of

proline and hydroxyproline in collagen was made. When hydroxylation is unimpaired, the proline/hydroxyproline specific-radioactivity ratio in collagen isolated after the administration of labelled proline is 1:1 (Hausmann & Neuman, 1961). In the event of impaired hydroxylation and concomitant formation of a hydroxyproline-deficient collagen, the specific-radioactivity ratio in collagen samples containing labelled protocollagen would be greater than 1:1.

Elastin is known to contain a small percentage of hydroxyproline and it was decided therefore to examine the possibility that the formation of elastin hydroxyproline is also ascorbate-dependent.

The formation of desmosine and isodesmosine (Partridge, Elsdon & Thomas, 1963; Thomas, Elsdon & Partridge, 1963) from lysine previously incorporated into peptide linkage during elastin biosynthesis is known to be inhibited in copper deficiency (Weissmann, Shields & Carnes, 1963; Starcher, Hill & Matrone, 1964; Miller, Martin,

Mecca & Piez, 1965; O'Dell *et al.* 1966). It is believed that copper is required for the oxidation of the ϵ -amino group of specific lysyl residues during desmosine biosynthesis (Partridge, Thomas & Elsdon, 1965). It was decided to test whether the copper-dependent biosynthesis of the desmosines showed a requirement for ascorbic acid. A study of the incorporation of labelled lysine into the desmosines of elastin under conditions of ascorbic acid deficiency was therefore undertaken.

A preliminary account of some of this work was given by Barnes, Constable & Kodicek (1968).

MATERIALS AND METHODS

Chemicals. Amino acids used as standards were obtained as follows: L-proline and L-hydroxyproline (puriss grade) from Koch-Light Laboratories Ltd., Colnbrook, Bucks.; DL-hydroxylysine monohydrochloride from K & K Laboratories, Plainview, N.Y., U.S.A.; L-lysine monohydrochloride from British Drug Houses Ltd., Poole, Dorset; desmosine and isodesmosine as a generous gift from Dr S. M. Partridge, Agricultural Research Council Meat Research Institute, Langford, Somerset. Naphthalene and dioxan for scintillation-counting purposes were obtained from Nuclear Enterprises (G.B.) Ltd., Edinburgh. Pyridine (AnalaR, for chromatographic purposes) was obtained from British Drug Houses Ltd.; before use it was refluxed with ninhydrin (1g./l.) for 2 hr., and then collected by distillation.

Radioactive amino acids. L-[^3H]Proline (batch 8: 292 mc/m-mole; batch 9: 720 mc/m-mole; both at 1 mc/ml. of water), DL-[^3H]lysine monohydrochloride (476 mc/m-mole; 1 mc/ml. of water), L-[$^4,5\text{-}^3\text{H}_2$]lysine monohydrochloride (302 mc/m-mole; 1 mc/ml. of water), L-[U- ^{14}C]proline [120 mc/m-mole; 4 μC /ml. of 2% (v/v) ethanol] and DL-hydroxy[$^2\text{-}^{14}\text{C}$]proline [4.76 mc/m-mole; 8 μC /ml. of 2% (v/v) ethanol] were obtained from The Radiochemical Centre, Amersham, Bucks.

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 the authors with bovine plasma albumin as substrate.

Ascorbic acid-deficient guinea pigs. Male albino guinea pigs, 2-3 weeks old and weighing 120-160 g., were made scorbutic by the use of a scorbutogenic diet, which was basically that described by Reid & Briggs (1953) and full details of which were given by Constable (1960). All animals were first kept for 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 divided into three groups. One group continued to be fed *ad libitum* with the ascorbic acid-supplemented diet (normal controls), a second was fed *ad libitum* with the basal diet free of ascorbic acid (ascorbic acid-deficient group) and a third (pair-fed controls) received the ascorbic acid-supplemented diet but was pair-fed individually with animals of the ascorbic acid-deficient group. On day 15 of the experiment animals were injected intraperitoneally with the appropriate labelled amino acids. Animals were killed on day 20, and the aorta was removed

(as far as the bifurcation) and a sample of dorsal skin of approximately equal surface area was collected from each animal (the skin was shaved before the animals were killed).

Chick embryos. Fertilized eggs (from Thornber's 505 White Leghorn stock, and supplied by the Department of Animal Pathology, School of Veterinary Medicine, University of Cambridge) were incubated for 19 days in a Western incubator at 39°. They then received L-[U- ^{14}C]proline or DL-[$^2\text{-}^{14}\text{C}$]hydroxyproline by injection on to the chorio-allantoic membrane. Incubation was continued for a further 6 hr. Embryos were then killed and the aorta was removed from each.

Determination of ascorbic acid in adrenal glands. The wet weight of the adrenal glands was measured immediately after their removal and the glands were then ground in a suitable volume of 20% (w/v) metaphosphoric acid. After dilution to 6% (w/v) metaphosphoric acid and filtration, ascorbic acid in a portion of the extract was measured by a colorimetric procedure essentially as described by Hughes (1956).

Determination of hydroxyproline. The colorimetric method of Bergman & Loxley (1963) was employed. Samples were first hydrolysed in 6M-HCl in sealed tubes at 100° for 16 hr. The HCl was removed from hydrolysates by rotary evaporation *in vacuo*.

Preparation of guinea-pig dorsal-skin collagens. All operations were performed at 4° unless otherwise indicated. Skin samples, immediately after removal, were scraped free of epidermis and subcutaneous tissue, weighed, cut into small pieces and disintegrated in 0.45M-NaCl in sodium phosphate buffer, pH 7.2 and *I* 0.02, for four periods of 15 sec. in a Silverson micro laboratory emulsifier-mixer. Disintegrated samples were then extracted twice for 24 hr. on a mechanical shaker with the phosphate buffer. Neutral-salt-soluble collagen was precipitated from the combined extracts by addition of an equal volume of 5M-NaCl and then purified by repeated precipitation and redissolution, as described by Orloff & Gross (1963). The residue was extracted twice for 24 hr. with 0.5M-acetic acid, and acid-soluble collagen was precipitated from the combined extracts by addition of NaCl to 5% (w/v) and purified by repeated precipitation and redissolution as described by Piez, Eigner & Lewis (1963). After extraction of acid-soluble collagen, insoluble collagen was dissolved from the residue as gelatin by two extractions with 0.3M-trichloroacetic acid at 60° for 1 hr. Gelatin was purified from the combined extracts by ethanol precipitation as described by Popenoe & Van Slyke (1962).

Hot-trichloroacetic acid extraction of guinea-pig dorsal skin. Samples of dorsal skin were disintegrated in 0.17M-NaCl in sodium phosphate buffer, pH 7.2 and *I* 0.02, as described above. An equal volume of 10% (w/v) trichloroacetic acid was added to the skin homogenate, extraction performed at 60° for 1 hr., the extract collected after centrifugation and the residue re-extracted with 5% (w/v) trichloroacetic acid at 60° for 1 hr. The combined extracts were dialysed exhaustively at 4° at first against water containing excess of free proline and hydroxyproline and finally against water alone, and the dialysis residue was freeze-dried.

Preparation of elastin. Aortae were freed of any adhering extraneous tissue, cut into small pieces and extracted several times at 4° with a large volume of 0.17M-NaCl, and the tissue was then washed with water and defatted by successive extractions with ethanol, ethanol-diethyl ether

(1:1, v/v) and diethyl ether. Elastin was prepared from the dried defatted tissue by extraction with 0.1 M-NaOH at 98° for 45 min. (Lansing, Rosenthal, Alex & Dempsey, 1952), and the residue was extracted several times with hot water and dehydrated by extraction with ethanol and diethyl ether as described above. Elastin preparations were finally dried *in vacuo* over conc. H₂SO₄ and NaOH pellets.

Soluble elastin. Elastin before collagenase digestion was brought into solution by partial hydrolysis with dilute oxalic acid as described by Partridge, Davis & Adair (1955). Oxalic acid was removed by dialysis and the dialysis residue freeze-dried.

Collagenase digestion. Samples of soluble elastin and gelatins were dissolved in 0.33 M-calcium acetate in 0.05 M-tris-HCl buffer, pH 7.2. Acid-soluble collagen was suspended in the buffer. A sample of collagenase dissolved in the same buffer was then added (substrate/enzyme ratio approx. 100:1, w/w) and the mixture incubated at 37°, in the presence of 1 drop of CHCl₃. Incubation times are given below. After incubation, the mixture was dialysed thoroughly against water and the combined diffusates were evaporated to dryness on a rotary evaporator *in vacuo* before hydrolysis.

Hydrolysis. Before ion-exchange chromatography, collagen samples were hydrolysed by refluxing under N₂ with constant-boiling HCl for 24 hr. Elastin samples were hydrolysed under identical conditions for 40 hr. Diffusible material derived from collagenase digests was hydrolysed for 18 hr. The HCl was removed from hydrolysates by rotary evaporation *in vacuo*.

Amino acid analysis. This was performed with a Technicon Amino Acid AutoAnalyzer.

Determination of the specific radioactivities of proline and hydroxyproline in collagen and elastin hydrolysates. Proline and hydroxyproline were separated by ion-exchange chromatography at 50° on the Technicon Amino Acid AutoAnalyzer, with a pyridine-formate buffer system (see Padieu, Malekina & Schapira, 1965). Samples were applied to a column (140 cm. × 0.6 cm.) containing Technicon type A Chromo-Beads that had previously been regenerated with 2 M-pyridine and then equilibrated with 0.1 M-pyridine-formate buffer, pH 2.9, containing 20% (v/v) of methanol. The column was eluted with the same buffer at approx. 30 ml./hr. Hydroxyproline and proline were eluted at approx. 2½ hr. and 4 hr. respectively. Fractions (2-3 ml.) were collected on a fraction collector and a portion of each was diluted and placed in the Sampler II module of the Technicon AutoAnalyzer to be assayed with ninhydrin. Standard solutions (in the appropriate dilution of the buffer) of L-proline and L-hydroxyproline were similarly assayed to allow determination of proline and hydroxyproline in the eluate. Peak fractions were combined and dried *in vacuo* over conc. H₂SO₄ and NaOH pellets. The residue was dissolved in 0.2 ml. of water and counted in 10 ml. of Bray's (1960) solution in a Packard Tri-Carb liquid-scintillation spectrometer (model 3375).

With elastin hydrolysates it was found more convenient, in view of the much lower concentration of hydroxyproline, to assay those fractions containing hydroxyproline by the procedure of Bergman & Loxley (1963).

Determination of the specific radioactivities of lysine, desmosine and isodesmosine in elastin hydrolysates. Lysine and the desmosines were separated by ion-exchange chromatography on the Technicon AutoAnalyzer. In Expt.

1 a modified citrate buffer gradient was employed. It was necessary to modify the standard Technicon buffer gradient to separate lysine from unidentified components occurring in guinea-pig elastin hydrolysates. The composition of the nine-chamber Autograd was as follows: chambers 1 and 2: 75 ml. of pH 2.88 buffer, containing 5% (v/v) of methanol in each chamber; chamber 3: 37.5 ml. of pH 2.88 buffer, containing 5% (v/v) of methanol, and 37.5 ml. of pH 5.0 buffer; chambers 4-9, 75 ml. of pH 5.0 buffer in each chamber. Chromatography was at 60° and elution was at approx. 30 ml./hr. Isodesmosine and desmosine were eluted at approx. 7½ and 7½ hr. respectively (just before ammonia) and lysine (after ammonia) at approx. 9 hr. Fractions containing lysine or the desmosines were assayed with ninhydrin on the Technicon AutoAnalyzer against appropriate standards as described above. Main fractions were combined within each peak and a 1.4 ml. sample was counted directly in 20 ml. of Bray's (1960) solution containing 5% of thixotropic gel powder.

In Expt. 2 lysine and the two desmosines were separated by means of the pyridine-formate buffer system. After the elution of proline and hydroxyproline by 0.1 M-pyridine-formate buffer, pH 2.9, containing 20% (v/v) of methanol, a gradient was applied as follows: chambers 1 and 2: 250 ml. of 0.1 M-pyridine-formate buffer, pH 2.9, containing 20% (v/v) of methanol in each chamber; chambers 3 and 4: 250 ml. of 1 M-pyridine-formate buffer, pH 5.0, in each chamber. Chromatography was at 50° and elution was at approx. 30 ml./hr. Isodesmosine, desmosine and lysine were eluted after ammonia at approx. 17½ hr., 18 hr. and 19½ hr. respectively. Fractions of the eluate containing isodesmosine, desmosine and lysine were assayed with ninhydrin against a standard solution of the appropriate amino acid. Peak fractions were combined and dried, and the residue was dissolved in 0.2 ml. of water and counted in 10 ml. of Bray's (1960) solution.

RESULTS

Effect of ascorbic acid deficiency. In a parallel experiment, animals survived for approx. 21 days on the scorbutogenic diet, during which time a rapid depletion of the ascorbic acid of the adrenal glands occurred, the concentration per 100 g. wet wt. of gland falling from approx. 120 mg. to 46 mg. after 5 days, 11 mg. after 10 days, 4 mg. after 15 days and 2 mg. after 21 days on the diet. Normal control animals given the ascorbic acid-supplemented diet *ad libitum* showed, over the same period, a slight increase in concentration to 140 mg./100 g. wet wt. of adrenals.

Controls fed *ad libitum* showed a regular increase in weight over the entire experimental period, the total average increase amounting to about 100 g. Animals fed on the ascorbic acid-free diet gained weight at a comparable rate until days 10-13, at which time a fall in food intake and body weight first appeared and continued thereafter to an increasing degree for the remaining period of vitamin deficiency. On day 20, when the animals were killed, the average body weight had fallen to about

Table 1. Total collagen extracted and radioactivity incorporated into collagen hydroxyproline from the skin of control and scorbutic guinea pigs

Each animal received, on day 15 of the experiment, a total of 1 mc of L-[G-³H]proline in five 0.2 mc doses at 4 hr. intervals. Animals were killed 5 days later. Collagen extracted from the dorsal skin was calculated from the hydroxyproline content of the extracts before purification, assuming collagen to contain 13.4% of hydroxyproline. Purified collagens were isolated from the dorsal-skin extracts and the radioactivities of collagen proline and hydroxyproline determined as described in the text.

Group	Guinea pig no.	Collagen extracted (mg./g. wet wt. of skin)			10 ⁻⁵ × Radioactivity incorporated into hydroxyproline (c.p.m./g. wet wt. of skin)		
		Neutral-salt-soluble collagen	Acid-soluble collagen	Insoluble collagen	Neutral-salt-soluble collagen	Acid-soluble collagen	Insoluble collagen
Controls fed <i>ad lib.</i>	1	14.0	43.9	85.9	1.22	2.49	—
	2	16.9	30.4	127.6	1.59	2.81	4.96
	3†	3.4	26.0	123.8	0.52	0.51	0.91
Pair-fed* controls	4	7.9	20.9	135.0	1.44	1.21	—
	5	3.6	21.3	152.2	0.68	0.67	1.31
	6	3.1	28.8	156.7	0.58	2.42	5.16
Scorbutic	7	1.2	30.5	165.6	0.05	0.02	0.07
	8	1.9	25.6	240.2	0.08	0.02	0.08
	9	1.5	35.1	235.7	0.06	0.02	0.11

* Guinea-pigs nos. 4, 5 and 6 were pair-fed with nos. 7, 8 and 9 respectively.

† See the text.

150g., having attained previously a maximum weight of approx. 200g. Pair-fed controls showed a growth pattern similar to that of scorbutic animals.

Studies on collagen

Incorporation of L-[G-³H]proline into skin collagens of control and scorbutic guinea pigs. There was a marked decrease in the amount of neutral-salt-soluble collagen in the skin in ascorbic acid deficiency (Table 1), indicative of decreased collagen synthesis. Although partial inanition itself caused some decrease, as judged by comparison of pair-fed controls with controls fed *ad libitum*, the decrease in the scorbutic animals was greater. This finding is in accord with the observations made by Gross (1959). The only anomaly was the control animal no. 3, which may have been adversely affected by the injections since there was only a poor growth rate thereafter. This may account for the relatively low values for this animal in comparison with the other controls fed *ad libitum*.

The wet weight per unit area of skin samples from pair-fed and scorbutic animals was only approx. 80% and 60% respectively of that of normal controls fed *ad libitum*. It seems probable that the increased concentration of insoluble collagen in the skin of pair-fed and scorbutic animals can be attributed to this decrease in skin wet weight. Further, the depletion of neutral-salt-soluble skin collagen in pair-fed and scorbutic animals would be correspondingly greater than indicated in Table 1.

A further indication of the marked inhibition of

collagen synthesis in ascorbic acid deficiency was the decreased incorporation of [G-³H]proline (Table 1) into skin collagens of scorbutic animals. Incorporation values for the scorbutic group were in all instances much lower than those of either control group. Similarly, a decrease in the specific radioactivities of proline and hydroxyproline was observed in the scorbutic group (Table 2).

The ratio of specific radioactivities of proline and hydroxyproline in all collagen fractions of the two control groups was 1:1, indicative of normal hydroxylation. In the scorbutic guinea pigs, however, the acid-soluble and insoluble collagen fractions showed specific-radioactivity ratios greater than 1:1. The possible presence of non-collagenous protein was suspected, and in Table 3 the results are shown of treatment with collagenase for 21 hr. of acid-soluble and insoluble collagen fractions. Over 90% of the hydroxyproline became diffusible after incubation of the insoluble (gelatinized) collagen samples with collagenase. Digestion of the acid-soluble (native) collagen sample with collagenase released approx. 70% of the hydroxyproline in a diffusible form. The determination of the specific radioactivity of proline and hydroxyproline in the collagen-derived diffusible peptides revealed that the specific-radioactivity ratio became 1:1 after treatment.

Collagenase digestion of hot-trichloroacetic acid extracts of skin. The possibility existed that proto-collagen might have been left in solution and therefore lost when collagens were precipitated during purification. Skin samples, after disintegration,

Table 2. *Specific radioactivities of proline and hydroxyproline in skin collagens from control and scorbutic guinea pigs*

The results are for the same animals as are listed in Table 1.

Group	Guinea pig no.	Neutral-salt-soluble collagen			Acid-soluble collagen			Insoluble collagen					
		Specific radioactivity (c.p.m./μmole)		Proline/hydroxyproline specific-radio-activity ratio	Specific radioactivity (c.p.m./μmole)		Proline/hydroxyproline specific-radio-activity ratio	Specific radioactivity (c.p.m./μmole)		Proline/hydroxyproline specific-radio-activity ratio			
		Proline	Hydroxyproline		Proline	Hydroxyproline		Proline	Hydroxyproline				
Controls fed <i>ad lib.</i>	1	8207	8518	0.96	5370	5629	0.95	—	—	—			
	2	8936	9178	0.97	8860	9029	0.98	3644	3796	0.96			
	3*	15531	15014	1.03	1850	1934	0.96	732	716	1.02			
Pair-fed controls	4	17574	17796	0.99	5401	5661	0.95	—	—	—			
	5	17792	18610	0.96	2821	3061	0.92	793	838	0.95			
	6	15560	18400	0.85	7934	8214	0.97	3322	3221	1.03			
Scurbutic	7	4260†	4125†	1.03†	136	69	1.97	105	43	2.44			
	8				191	63	3.03	65	31	2.10			
	9				107	51	2.10	90	44	2.05			

* See the text.

† Neutral-salt-soluble collagen extracts from the ascorbic acid-deficient animals were combined before purification.

were therefore extracted with hot trichloroacetic acid, and the extracts, after dialysis and freeze-drying, were treated directly for 2 hr. with collagenase. Over 90% of the hydroxyproline became diffusible. The specific radioactivities of proline and hydroxyproline in the diffusible products obtained by this treatment are given in Table 4. The results again reflect the much greater decrease of collagen biosynthesis in the ascorbic acid-deficient animals than in the pair-fed controls. The proline/hydroxyproline specific-radioactivity ratios were in all cases close to 1:1, indicating that little if any procollagen could be detected in the skin of scorbutic animals.

Studies on elastin

Incorporation of free [¹⁴C]proline and hydroxy-[¹⁴C]proline into chick-embryo aortic elastin (Table 5). Free proline was a much more efficient precursor of elastin hydroxyproline than free hydroxyproline. Further, with free proline as precursor, the elastin proline/hydroxyproline specific-radioactivity ratio was 1:1.

Embryos received either labelled proline or hydroxyproline in identical amounts in terms of the L-isomer. The pool size of free proline in the 10-day-old chick embryo is approximately double that of free hydroxyproline (Decker, Levene & Gross, 1959), and therefore in the present study it was probable that there would be a greater dilution of the labelled proline administered. Consequently the difference in the extent of incorporation between the two compounds is likely to be even greater than indicated in Table 5.

Amino acid composition of guinea-pig aortic elastin (Table 6). The amino acid pattern of guinea-pig aortic elastin preparations resembled closely that reported for elastin preparations from other species (see Seifter & Gallop, 1966). The hydroxyproline content of a number of samples examined was approx. 1.5–1.6% (w/w).

Incorporation of L-[G-³H]proline into proline and hydroxyproline of aortic elastin in control and scorbutic guinea pigs (Table 7). The specific radioactivity of proline in elastin samples isolated from scorbutic guinea pigs was of the same order of magnitude as that from control animals and did not reveal the marked decrease found for the proline of skin collagen from the same scorbutic animals. In contrast, the specific radioactivity of hydroxyproline in elastin from the scorbutic animals was considerably decreased, so that the proline/hydroxyproline specific-radioactivity ratio was about 6:1. The ratio in the control animals was 1:1, indicative of normal (i.e. unimpaired) hydroxylation.

Collagenase digestion of soluble elastin. Soluble elastin prepared from aortic elastin isolated from

Table 3. *Specific radioactivities of proline and hydroxyproline of collagen fractions before and after treatment with collagenase*

After incubation with collagenase for 21 hr., the specific radioactivities of proline and hydroxyproline in the diffusible material were determined as described in the text.

Guinea pig no. (see Table 2)	Collagen fraction	Before collagenase treatment			After collagenase treatment		
		Specific radioactivity (c.p.m./ μ mole)		Proline/ hydroxyproline specific-radio- activity ratio	Specific radioactivity (c.p.m./ μ mole)		Proline/ hydroxyproline specific-radio- activity ratio
		Proline	Hydroxyproline		Proline	Hydroxyproline	
3	Insoluble	732	716	1.02	657	720	0.91
9	Acid-soluble	107	51	2.1	51	51	1.0
9	Insoluble	90	44	2.05	36	39	0.92

Table 4. *Specific radioactivities of proline and hydroxyproline of collagen in hot-trichloroacetic acid extracts of guinea-pig skin after treatment with collagenase*

Each animal received on day 15 of the experiment 0.5 mc of L-[G-³H]proline in five 0.1 mc doses at 4 hr. intervals. Animals were killed 5 days later. A sample of dorsal skin was extracted with hot trichloroacetic acid as described in the text; the extracts, after dialysis and freeze-drying, were digested with collagenase for 2 hr. and the specific radioactivities of proline and hydroxyproline in the diffusible material determined.

Group	Guinea pig no.	Specific radioactivity (c.p.m./ μ mole)		Proline/ hydroxyproline specific-radio- activity ratio
		Proline	Hydroxyproline	
Controls fed <i>ad lib.</i>	10	3105	3136	0.99
	11	2861	2930	0.98
	12	1467	1570	0.93
Pair-fed controls*	13	1269	1325	0.96
	14	847	909	0.93
	15	—	—	—
Scorbutic	16	52	50	1.04
	17	49	45	1.09
	18	53	48	1.10

* Guinea pigs nos. 13, 14 and 15 were pair-fed with nos. 16, 17 and 18 respectively.

Table 5. *Incorporation of free [¹⁴C]proline and hydroxy[¹⁴C]proline into chick-embryo aortic elastin*

A group of thirteen 19-day-old embryos injected with 0.4 μ C of DL-hydroxy[2-¹⁴C]proline/embryo and a group of seven 19-day-old embryos injected with 0.2 μ C of L-[U-¹⁴C]proline/embryo were killed 6 hr. after injection, and the aortae were collected and combined within each group before the isolation of elastin. The specific radioactivities of elastin proline and hydroxyproline were determined as described in the text.

Radioactive amino acid injected	Specific radioactivity (c.p.m./ μ mole)		Proline/ hydroxyproline specific-radio- activity ratio
	Proline	Hydroxyproline	
DL-Hydroxy[2- ¹⁴ C]proline	2	7	0.3
L-[U- ¹⁴ C]Proline	75	74	1.0

normal control guinea pigs was incubated with collagenase for 21 hr. under conditions described in the Materials and Methods section. After digestion, the mixture was dialysed and the diffusate analysed

for hydroxyproline. It was found that only 10–15% of the hydroxyproline of soluble elastin became diffusible after collagenase digestion. Digestion of collagen samples with collagenase under the same

conditions rendered over 90% of the hydroxyproline diffusible. The diffusible material derived from soluble elastin was examined on the Technicon Amino Acid AutoAnalyzer and found to be of a composition similar to the original sample except for the absence of desmosines; it did not resemble collagen.

In a further experiment, labelled aortic elastin was isolated from normal control guinea pigs 5 days after the simultaneous administration of L-[G-³H]-proline and L-[4,5-³H₂]-lysine. Soluble elastin was prepared and a sample then digested with collagenase for 21 hr. After dialysis, the dialysis residue was freeze-dried and the specific radioactivities of proline and hydroxyproline were determined. The specific radioactivities (Table 8)

were identical with those of the soluble elastin before digestion. In contrast, there was a change in the specific radioactivities of lysine and the desmosines after incubation at 37°. The increased specific radioactivity of desmosine and isodesmosine in the non-diffusible material obtained after collagenase digestion (Table 8) is thought to indicate some formation of cross-links during incubation *in vitro* at 37°.

Table 6. Amino acid composition of guinea-pig aortic elastin

Results are expressed as residues/1000 total residues.

Amino acid	Content
Hyp	12.8
Asp	4.3
Thr	8.7
Ser	7.1
Glu	13.4
Pro	114.3
Gly	346.7
Ala	237.9
Val	125.6
Ile	23.9
Leu	51.9
Tyr	24.6
Phe	14.1
Isodesmosine	1.1
Desmosine	1.2
Lys	6.9
His	0.3
Arg	5.4

Table 8. Specific radioactivities of proline, hydroxyproline, lysine, desmosine and isodesmosine of soluble elastin before and after treatment with collagenase

Six guinea pigs each received a total of 0.75 mc of L-[G-³H]-proline and 0.5 mc of L-[4,5-³H₂]-lysine monohydrochloride, given together in five equal doses at 4 hr. intervals. Animals were killed 5 days later. Aortae were combined before the isolation of elastin. Specific radioactivities in soluble elastin and in the non-diffusible material obtained after incubation of soluble elastin with collagenase for 21 hr. at 37° were determined as described in the text.

	Before incubation	After incubation at 37° with collagenase and dialysis
Specific radioactivity (c.p.m./μmole)		
Proline	635	648
Hydroxyproline	536	537
Specific-radioactivity ratio		
Proline/hydroxyproline	1.2	1.2
Specific radioactivity (c.p.m./μmole)		
Lysine	1840	1158
Desmosine	875	1726
Isodesmosine	918	1724
Specific-radioactivity ratio		
Desmosine/lysine	0.5	1.5
Isodesmosine/lysine	0.5	1.5

Table 7. Incorporation of L-[G-³H]-proline into proline and hydroxyproline of aortic elastin in control and scorbutic guinea pigs

In Expt. 1 the three groups of animals listed in Tables 1 and 2, and in Expt. 2 those listed in Table 4, were used. Aortae were combined within each group before the isolation of elastin. The specific radioactivities of proline and hydroxyproline of elastin were determined as described in the text.

Expt. no.	Group	Specific radioactivity (c.p.m./μmole)		Proline/hydroxyproline specific-radioactivity ratio
		Proline	Hydroxyproline	
1	Controls fed <i>ad lib</i> .	646	521	1.2
	Pair-fed controls	642	653	1.0
	Scorbutic	501	86	5.8
2	Controls fed <i>ad lib</i>	490	490	1.0
	Pair-fed controls	214	173	1.2
	Scorbutic	274	42	6.5

Table 9. Incorporation of [G-³H]- and [4,5-³H₂]-lysine into the lysine and desmosines of aortic elastin in control and scorbutic guinea pigs

In Expt. 1 each animal received, on day 15, 1 mc of DL-[G-³H]lysine monohydrochloride in five 0.2 mc doses at 4 hr. intervals. Animals were killed 5 days later, and the aortae were removed and combined within each group before the isolation of elastin. Lysine and desmosines were separated by ion-exchange chromatography with a citrate buffer system, and the specific radioactivities were determined as described in the text. In Expt. 2 the same animals as those described in Table 4 were used. In addition to [G-³H]proline each animal received at the same time 1 mc of L-[4,5-³H₂]lysine monohydrochloride given as in Expt. 1. Aortae were combined within each group before the isolation of elastin. Lysine and desmosines were separated by ion-exchange chromatography with a pyridine-formate buffer system, and the specific radioactivities were determined as described in the text.

Expt. no.	Group	No. of animals	Specific radioactivity (c.p.m./μmole)			Specific-radioactivity ratio	
			Lysine	Desmosine	Isodesmosine	Desmosine	Isodesmosine
						Lysine	Lysine
1	Controls fed <i>ad lib.</i>	3	500	682	633	1.4	1.3
	Pair-fed controls	3	537	418	446	0.8	0.8
	Scorbutic	3	444	486	519	1.1	1.2
2	Controls fed <i>ad lib.</i>	3	3050	3309	3523	1.1	1.2
	Pair-fed controls	3	1762	1358	1171	0.8	0.7
	Scorbutic	3	1346	1859	1860	1.4	1.4

Incorporation of DL-[G-³H]- and L-[4,5-³H₂]-lysine into the lysine and desmosines of aortic elastin in scorbutic guinea pigs. The results obtained in two experiments are given in Table 9. In agreement with the studies of proline incorporation (Table 7), the incorporation of lysine into elastin did not indicate a marked decrease in biosynthesis of elastin associated with ascorbic acid deficiency, like that observed in collagen biosynthesis. The desmosines/lysine specific-radioactivity ratios in the scorbutic animals were not lowered below control values, indicating that the formation of desmosine and isodesmosine was not impaired in scurvy.

DISCUSSION

Collagen. It was one of the aims of the present study to determine whether ascorbic acid deficiency *in vivo* gave rise to impaired hydroxylation of collagen proline with the concomitant formation of a proline-rich collagen. To this end, the specific-radioactivity ratio of proline to hydroxyproline in collagen was measured, after the administration of labelled proline to scorbutic and control guinea pigs.

In both control groups of animals the ratio was always close to 1:1, whereas in acid-soluble and insoluble collagens prepared from scorbutic guinea pigs values greater than 1:1 were observed. When, however, such collagens were analysed after collagenase digestion, the proline/hydroxyproline specific-radioactivity ratio in diffusible peptides derived from such samples was 1:1. Since protocollagen as well as collagen is known to be susceptible to collagenase (Gottlieb, Peterkofsky & Udenfriend,

1965; Gottlieb, Kaplan & Udenfriend, 1966; Prockop & Juva, 1965), and therefore, if present in the incubation sample, would be expected to be rendered diffusible, it can be assumed that protocollagen was absent from the purified collagen samples isolated from the scorbutic animals. It seems probable that the ratios greater than 1:1 observed in samples without collagenase treatment were due to the presence of non-collagenous protein containing radioactive proline, which was not detected in collagen samples from control animals owing to the much greater extent of incorporation of radioactivity into their collagen proline.

It might be noted that at least for normal collagen, i.e. collagen hydroxylated to the normal extent (a proline/hydroxyproline specific-radioactivity ratio 1:1), the relative distribution of incorporated radioactivity into the three collagen fractions of scorbutic animals (Table 1) did not indicate an inability to aggregate or cross-link or both in scurvy. Bentley & Jackson (1965) reached a similar conclusion from a study of the distribution of incorporated radioactivity between 0.14M- and 1.0M-sodium chloride-extractable collagen.

To examine the possibility that protocollagen was lost during the purification of collagen extracts, skin samples were extracted with hot trichloroacetic acid, since protocollagen has been shown to be soluble under these conditions (Gottlieb *et al.* 1966), and the extracts, after dialysis and freeze-drying, digested directly with collagenase. It was found that the proline/hydroxyproline specific-radioactivity ratio in the diffusible peptides was close to 1:1 in all samples. However, all samples from scorbutic animals showed values slightly above 1:1,

but the significance of this, particularly in view of their much lower specific radioactivities, is not clear and it was concluded that little if any protocollagen was present in the scorbutic skin.

Recent isotope-incorporation studies from a number of laboratories (reviewed by Udenfriend, 1966; Prockop & Kivirikko, 1967, 1968; Chvapil & Hurych, 1968; Gould, 1968a,b; Juva, 1968) have clearly demonstrated the formation *in vitro* of a proline-rich collagen precursor (protocollagen) when hydroxylation was prevented by various means, including, apparently, a lack of ascorbic acid (Stone & Meister, 1962; Gottlieb *et al.* 1966). Further evidence for the participation of ascorbic acid in the hydroxylation of peptide-bound proline during collagen biosynthesis has been obtained from studies that demonstrated the cell-free enzymic hydroxylation of protocollagen, whereby peptidyl-[¹⁴C]proline is converted into peptidyl-hydroxy[¹⁴C]proline (Peterkofsky & Udenfriend, 1965; Juva & Prockop, 1966; Hutton, Tappel & Udenfriend, 1967).

However, protocollagen has not been shown to accumulate *in vivo* in collagen-forming tissues under conditions of ascorbic acid deficiency. Fractionation of extracts of such tissues isolated from scorbutic animals has failed to produce unequivocal evidence for the presence of a proline-rich hydroxyproline-deficient collagen precursor (Robertson, Hiwett & Herman, 1959; Gross, 1959; Gould, Mannert, Goldman & Stolman, 1960).

Mitoma & Smith (1960) concluded from their studies *in vivo* of incorporation of labelled proline into granuloma tissue that proline hydroxylation was not impaired in scurvy. The proline/hydroxyproline specific-radioactivity ratio in collagen, isolated by them from scorbutic granuloma tissue 24 hr. after the administration of labelled proline, was in fact slightly greater than 1:1 (1.14 and 1.36 in two separate experiments), being rather less than 1:1 in control samples. Similarly, Robertson *et al.* (1959) observed, 12 hr. after administration of labelled proline, a collagen proline/hydroxyproline specific-radioactivity ratio greater than 1:1 in the scorbutic granuloma. They considered that their results might be due to the presence of non-collagenous proteins. In the absence of collagenase treatment it is difficult to exclude this possibility.

The apparent absence of protocollagen under the conditions of the present study (administration of labelled proline on day 15 and examination of the skin on day 20 of vitamin deficiency) does not necessarily imply that none is formed *in vivo* in scurvy. It may be that its formation is only short-lived and that in the continued absence of ascorbic acid its formation, like that of collagen, ceases *in vivo*. However, Bhatnagar, Kivirikko & Prockop (1968) observed in tissue cultures of embryonic

cartilage the continuous formation of protocollagen for at least 72 hr., when $\alpha\alpha'$ -bipyridyl was used to prevent hydroxylation.

Alternatively labelled protocollagen might have been formed after the administration of labelled proline (on day 15), but was completely removed during the next 5 days before the skin was examined. The nature and fate of protocollagen are still uncertain. It is believed that protocollagen is of a size at least comparable with that of the α -chains of tropocollagen (Lukens, 1966; Kivirikko & Prockop, 1967). Prockop and co-workers (Juva, Prockop, Cooper & Lash, 1966; Bhatnagar *et al.* 1968) state that protocollagen remains essentially intracellular. But Hurych, Chvapil & Brokes (1968) detected protocollagen formed in granuloma tissue by incubation in the presence of $\alpha\alpha'$ -bipyridyl, in the neutral-salt-soluble, acid-soluble and insoluble collagen fractions, and suggested that it is extruded from the cell and is able to aggregate or cross-link or both in a similar manner to tropocollagen. If protocollagen remains essentially in a soluble form, it seems possible that it could be readily removed by proteolytic digestion (Hurych, Chvapil, Tichy & Beniac, 1967). Relevant to this point is the observation by Efron, Bixby, Hockaday, Smith & Meshorer (1968) of an increased urinary excretion of hydroxyproline in a human patient fed on a scorbutogenic diet. The authors concluded that this might arise as a result of degradation of partially hydroxylated collagen precursors formed as a consequence of the vitamin deficiency and rapidly eliminated as a consequence of their abnormality.

If protocollagen is incorporated into an insoluble form, its rapid removal may be less likely. In this context Bhatnagar *et al.* (1968) reported that some protocollagen that was retained for up to 72 hr. in embryonic cartilage cultures became progressively less soluble, possibly owing to the formation of complexes with glycosaminoglycans (Kivirikko & Prockop, 1967).

Hutton *et al.* (1967) showed that ascorbic acid, as a cofactor for protocollagen hydroxylase, can be replaced in some measure by reduced pteridines. If such compounds were to function *in vivo* in this way in ascorbic acid deficiency, it might be necessary to consider that the impairment of collagen synthesis in scurvy implies that ascorbic acid has some role other than that of a direct cofactor in hydroxylation. However, it has been reported (Gabuzda, Phillips, Schilling & Davidson, 1952) that there is a decreased conversion of pteroylglutamic acid into *N*⁵-formyl-5,6,7,8-tetrahydropteroylglutamic acid in scurvy.

Elastin. In contrast with collagen, biosynthesis of elastin, as judged by the incorporation of labelled proline or lysine, did not appear to be markedly affected by ascorbic acid deficiency. Formation of

desmosines was also unimpaired. The desmosines/lysine specific-radioactivity ratios in the pair-fed controls suggested some inhibition of cross-linking under conditions of inanition. In the scorbutic animals, however, despite the occurrence of inanition, the desmosines/lysine specific-radioactivity ratios were not decreased, and this may be indicative of some stimulation of desmosine formation in ascorbic acid deficiency, offsetting the inhibition to be expected from the accompanying inanition.

The theoretical desmosines/lysine specific-radioactivity ratios should be 4:1 (Partridge *et al.* 1966). The lower ratios obtained in this study are attributable in part to loss of ^3H during the formation of desmosines from lysine, and also probably to the fact that cross-linking was not completed during the 5 days between administration of labelled lysine and isolation of elastin (Partridge *et al.* 1966).

Although biosynthesis of elastin does not appear to be depressed in scurvy, the incorporation of labelled proline into hydroxyproline of elastin preparations was clearly decreased, suggesting that the formation of this hydroxyproline, like that of collagen hydroxyproline, requires ascorbic acid as a cofactor. In contrast with the findings with collagen, the results suggested the synthesis and retention of a hydroxyproline-deficient elastin in ascorbic acid deficiency. As already noted, there was no evidence that cross-linking (necessary for the retention of elastin) was impaired in ascorbic acid deficiency.

The studies with chick embryos confirmed that elastin hydroxyproline was derived primarily from free proline. Incorporation of free hydroxyproline into elastin hydroxyproline was very slight. It seems probable by comparison with collagen biosynthesis that elastin hydroxyproline is formed by the hydroxylation of proline residues after incorporation of the latter into peptide linkage. The proline/hydroxyproline specific-radioactivity ratio of 1:1 in elastin samples isolated from both guinea pigs and chick embryos is compatible with this possibility. It is possible that a small number of proline residues may be incorporated during elastin synthesis into a sequence of amino acids that fulfils the requirements of protocollagen hydroxylase.

In the foregoing discussion the assumption has been made that the hydroxyproline in elastin preparations is present as an integral part of the elastin molecule rather than arising by contamination of the elastin preparations with collagen. If the hydroxyproline in elastin preparations were derived entirely from collagen, then, since the proline/hydroxyproline specific-radioactivity ratio in elastin preparations is 1:1 it would be necessary to postulate the unlikely possibility that the specific-radioactivity of contaminating collagen

proline in the elastin preparations was identical with that of the elastin proline.

The studies with collagenase were undertaken to investigate whether the hydroxyproline was derived from collagen. Elastin was first solubilized, since it was considered that this would facilitate the removal of collagen by collagenase. The results indicated that most of the hydroxyproline in elastin preparations was not released as diffusible peptides by collagenase. Further, examination of the diffusible material after collagenase digestion indicated that its amino acid composition resembled that of elastin and not that of collagen. The significance of the release of this diffusible material during incubation with collagenase is not at present clear. Samples incubated in the absence of collagenase also revealed this phenomenon in some measure. The finding that the specific radioactivity of the hydroxyproline remaining in the dialysis residue after dialysis following collagenase treatment was identical with that in the elastin before collagenase digestion seems to rule out the possibility that a small proportion of the hydroxyproline was derived from collagen and that this portion contained the incorporated radioactivity. In this event the decrease in the specific radioactivity of hydroxyproline in ascorbic acid deficiency would only reflect a diminished collagen biosynthesis. Thus, unless the collagen is present in a form that is resistant to collagenase, it seems probable that the hydroxyproline in elastin preparations is an integral part of the elastin molecule.

Further evidence for this is the constant finding of a small percentage of hydroxyproline in elastin samples, even after most rigorous purification techniques (LaBella, Vivian & Thornhill, 1966). Solubilization of elastin and repeated precipitation of the soluble α -elastin by coacervation still leaves hydroxyproline present (Partridge & Davis, 1955). Thus, if the hydroxyproline is derived from collagen, it seems that the collagen must be very tightly bound to elastin.

Amino acid analyses of samples of elastin isolated, as described in the Materials and Methods section, from both bovine ligamentum nuchae and guinea pig aorta have failed to reveal the equivalent amount of hydroxylysine to be expected if the hydroxyproline in elastin originated primarily from collagen (M. J. Barnes, B. J. Constable & E. Kodicek, unpublished work). Samples were chromatographed in amounts such that, if all the hydroxyproline of the sample was derived from collagen, a well-defined hydrolysine peak should have been observed.

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