Influence of Ascorbic Acid on Ribosomal Patterns and Collagen Biosynthesis in Healing Wounds of Scorbutic Guinea Pigs

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Scorbutic guinea pigs were wounded and the influence of administering ascorbic acid 6 days later was studied with respect to cellular morphology, ribosomal distribution and protein synthesis. Electron-microscopic studies revealed that the dilated endoplasmic reticulum observed in the fibroblasts of scorbutic wound tissue had reverted to a normal configuration 24 h after intraperitoneal injection of 100 mg of ascorbate. Quantitative determination of the distribution of free and membrane-bound ribosomes indicated a significant increase in membrane-bound ribosomes in wound tissue from ascorbate-supplemented (recovery) animals. Sucrose-density-gradient centrifugation indicated a significant increase in the proportion of large membrane-bound polyribosomes in the range 300–350S and a concomitant decrease in 80S monoribosomes in the ribosome sedimentation profile of recovery tissue. Determination of the synthesis of non-diffusible [^3H]hydroxyproline in scorbutic and recovery wounds showed a 3–4-fold stimulation in peptidyl-proline hydroxylation in recovery tissues. Studies carried out in which scorbutic and recovery tissues were incubated with [^{14}C]leucine indicated that general protein synthesis, as measured by ^{14}C incorporated into non-diffusible material/ μg of DNA, was unaltered by ascorbate supplementation. Similar studies of [^3H]proline incorporation suggested that in recovery tissues there was a small but significant increase in [^3H]proline incorporated/ μg of DNA, which probably represents an increase in procollagen synthesis. This observation correlates well with the increase seen in recovery tissues of large polyribosomes on which collagen precursor polypeptides are known to be synthesized. Preliminary characterization of the repair collagen synthesized by recovery animals showed it to be a typical Type I collagen having the chain composition $(\alpha_1)_2\alpha_2$. The extent of glycosylation of the hydroxylysine of the newly synthesized collagen was greater than that reported for either normal guinea-pig dermal collagen or dermal scar collagen.

As well as being a common and fundamental pathological process, wound healing has been recognized for some time as a suitable experimental model for biochemical studies of connective tissue. Detailed light- and electron-microscopic studies of the sequential changes in the components of guinea-pig skin wounds have revealed the cytological and morphological changes that occur during the repair process (Ross & Benditt, 1961, 1962*a,b*, 1964). The chemical events have been studied in less detail, but it is apparent that wound repair requires a substantial synthesis and accumulation of collagen and subsequent cross-linking of the fibres to give new tensile strength to the damaged tissue. Of great significance in this context is the critical role played by ascorbic acid in both the formation and maintenance of collagen in healing wounds of man and guinea pigs (for review see Gould, 1968). For centuries it has been observed that wounds do not heal normally in scurvy, and the role of ascorbate in

wound repair and more particularly in participating as a cofactor in the hydroxylation of specific peptidyl-proline and peptidyl-lysine residues during collagen biosynthesis has since been established (for review see Barnes & Kodicek, 1972).

Other cofactors of the hydroxylase enzymes include molecular O_2 and ferrous iron and if either the O_2 is replaced by N_2 or the iron is chelated with $\alpha\alpha'$ -bipyridyl, then hydroxylation is inhibited and procollagen accumulates intracellularly (for review see Grant & Prockop, 1972*a,b,c*). A small but significant amount of underhydroxylated collagen has been found in connective tissue from scorbutic guinea pigs, and the results are consistent with the suggestion that scurvy produces an intracellular accumulation of severely underhydroxylated collagen which may after a time inhibit further synthesis of procollagen (Barnes *et al.*, 1970). Such a build-up might give rise to the distortion of the endoplasmic reticulum and changes in ribosomal patterns seen in electron-microscopic studies of scorbutic guinea-pig wounds

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(Ross & Benditt, 1964). However, these disturbances of the cellular apparatus for protein synthesis are rapidly restored to the normal conformation when ascorbic acid is administered to the scorbutic animal and a concomitant appearance of extracellular collagen fibres is observed in the wound tissue (Ross & Benditt, 1964).

Thus the scorbutic wound recovering from ascorbate deficiency offers the possibility of studying some of the factors involved in the initiation of collagen biosynthesis, the ribosomal and post-ribosomal events of collagen synthesis and the wound-healing process as a whole. Here we report some of the biochemical changes occurring in skin wounds of scorbutic guinea pigs recovering from ascorbate deficiency and we also present a preliminary characterization of the collagen involved in wound repair. A preliminary report of this work has been presented elsewhere (Harwood *et al.*, 1973).

In the interests of clarity it is useful to define some of the terms to be employed herewith for, in spite of the fact that the pathways and mechanisms of collagen biosynthesis have become clearer in recent years, the terminology has become more confusing. The term procollagen has found general acceptance as the triple-helical precursor which is cleaved enzymically to the collagen fibril monomer, tropocollagen. Terminology for the non-hydroxylated equivalents of procollagen, tropocollagen, and their respective pro- α and α -chains is ill-defined and confused, for in the past all these entities may have been termed procollagen. In the context of this paper, when reference to procollagen is made we are equating procollagen with the amino acid sequence coded in the collagen mRNA, and procollagen synthesis is synonymous with collagen mRNA translation. Since collagen contains hydroxyproline, hydroxylysine and covalently linked carbohydrate, when referring to collagen biosynthesis we imply that not only are the collagen mRNA species being translated but that the post-translational events of hydroxylation and glycosylation are also being effected.

Experimental

Materials

Duncan-Hartley guinea pigs were obtained from D. Hall, Darley Oaks Farm, Newchurch, Burton-on-Trent, Staffs., U.K. The vitamin C-deficient diet was purchased from Scientific Products Farm Ltd., Canterbury, Kent, U.K. Tego antiseptic aerosol was obtained from Hough, Hoseason and Co. Ltd., Manchester, U.K., and Polybactrin spray was from Calmic Ltd., Crewe, Cheshire, U.K. [^3H]Proline (10 Ci/mmol), [^{14}C]leucine (150 mCi/mmol) and [$^4,^5\text{-}^3\text{H}$]lysine (5.3 Ci/mmol) were obtained from

The Radiochemical Centre, Amersham, Bucks., U.K. $\alpha\alpha'$ -Bipyridyl was purchased from Calbiochem, Los Angeles, Calif., U.S.A., and all other chemicals were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. Purified bacterial collagenase was obtained from Sigma (London) Chemical Co., London S.W.6, U.K., and rat tail-tendon tropocollagen was a gift from Dr. F. S. Steven of this Department.

Preparation of wound tissue

Male guinea pigs weighing 400–500 g were fed *ad libitum* on a vitamin C-deficient diet (Kodicek & Loewi, 1955) for 7 days before being wounded. At this time the animals were anaesthetized with ether, the skin of the flanks was shaved with electric clippers and the shaved area sprayed with Tego antiseptic aerosol, and then circular (3 cm diam.) full-thickness skin wounds penetrating to the fascia of the underlying muscle were made in both flanks under aseptic conditions. The wounds were sprayed with Polybactrin and left uncovered. The animals were maintained on the scorbutigenic diet for a further 6 days, at which time the 'recovery' animals were given an intraperitoneal injection of 4 ml of ascorbic acid (25 mg/ml unless otherwise indicated) in water adjusted to pH 7.4 with 0.1 M-NaOH. Then 24 h later both the scorbutic and 'recovery' animals were killed by decapitation and the repair tissue within the wound was carefully dissected out and placed in ice-cold modified Krebs medium (Dehm & Prockop, 1971).

Infected wounds were discarded but, as reported by Forrest & Jackson (1971), the above techniques did not result in a high incidence of infection. Animals fed on the scorbutigenic diet showed little if any weight increase, and after wounding weight losses of the order of 7–10% were observed. The scorbutic wounds were particularly fragile and prone to bleeding when the animals were handled, but the survival rate was approx. 85%.

For electron microscopy, samples of wound tissue were fixed in buffered glutaraldehyde solution and post-fixed in buffered 1% (v/v) OsO_4 . The samples were then dehydrated in a graded ethanol series and propylene oxide and embedded in Araldite. Ultrathin sections were cut by using an LKB Ultratome III, stained with uranyl acetate (0.2%) and lead citrate (0.02%) and examined in a Phillips EM 201 electron microscope.

Analysis of the distribution of free and membrane-bound cytoplasmic ribosomes

Analysis of the relative proportions of free and membrane-bound ribosomes in scorbutic wounds and wounds from 'recovery' animals (recovery

wounds) was carried out by one of the following two methods.

Method I. This procedure was essentially that devised by Blobel & Potter (1967). Wound tissues from 18 scorbutic or 'recovery' guinea pigs were excised and homogenized (20 strokes at speed setting 7) in 0.25M-sucrose in 0.05M-Tris-HCl buffer (pH 7.5) containing 0.025M-KCl and 0.5mM-MgCl₂ by using a motor-driven Teflon-glass homogenizer (Tri-R Instruments Inc., New York, N.Y., U.S.A.). Nuclei and mitochondria were sedimented at 4°C by centrifugation at 10000g (r_{av} , 6.5cm) for 10min in the 10×10ml titanium angle rotor of an MSE Superspeed 65 ultracentrifuge. The pellet was re-homogenized, centrifuged at 10000g for 10min and the two supernatants were combined. Free and total cytoplasmic ribosomes were obtained by centrifugation on a discontinuous sucrose gradient as in the method of Blobel & Potter (1967).

Method II. The above method I is subject to the possible criticism that some bound ribosomes may sediment with the nuclear/mitochondrial pellet and hence the relative proportions obtained may not genuinely reflect the distribution prevailing in the cell. Therefore an alternative procedure which overcomes this difficulty was also used in assessing the relative proportions of free and bound ribosomes. In this case wound tissues from ten scorbutic or 'recovery' guinea pigs were homogenized in 0.25M-sucrose in the above Tris-KCl-MgCl₂ buffer and pellets of free and membrane-bound ribosomes obtained as described by Venkatesan & Steele (1972).

RNA determinations were carried out on the pellets obtained by methods I and II. The ribosomal pellets were hydrolysed with 0.3M-KOH for 1h at 37°C and the RNA present was determined by using a modified Schmidt-Tannhauser technique. The samples were neutralized with cold 1.2M-HClO₄, acidified with an equal volume of 0.6M-HClO₄ and centrifuged at 17000g for 20min at 4°C. RNA in the supernatant was assayed by measuring the extinction at 260nm and made quantitative by using the relationship 32µg/ml = 1.00 E_{260} unit (Fleck & Munro, 1962; Munro & Fleck, 1966).

Sucrose gradient centrifugation of free and total cytoplasmic ribosomes

Sucrose-gradient-centrifugation profiles of the populations of free and total ribosomes from the wound tissues were obtained. Ribosomal pellets obtained by method I were resuspended in 0.02M-Tris-HCl buffer (pH 7.4) containing 0.24M-KCl and 0.0075M-MgCl₂, and 0.2ml portions were layered on 4.5ml linear 15–40% (w/v) sucrose density gradients prepared in the same buffer. These gradients were then centrifuged at 112000g (r_{av} , 7.4cm) for 1h at 4°C in the 3×5ml aluminium swing-out

rotor of an MSE Superspeed 65 ultracentrifuge. After centrifugation the gradients were analysed at 254nm by piercing the centrifuge tube and displacing the gradient upwards through the flow cell of an LKB Uvicord I using 60% sucrose. The sucrose gradients were calibrated by using the relationship of Martin & Ames (1961) with a preparation of 80S monoribosomes from mouse Ehrlich ascites cells as the standard.

Synthesis of collagen

The wound tissue from two or three animals was pooled and preincubated at 37°C in 2.5ml of modified Krebs medium for 30min before the addition of [5-³H]proline. Incubation was continued with shaking at 37°C for 3h, at which time the flasks were cooled to 0°C and 0.2ml of medium containing 270µg of cycloheximide and 2.7µmol of $\alpha\alpha'$ -bipyridyl was added to stop any further incorporation of [³H]proline and hydroxylation of [³H]procollagen (Rosenbloom *et al.*, 1967). The tissues were homogenized in the incubation media and the homogenates dialysed against running tap water for 48h before hydrolysis and further analysis. All samples were hydrolysed in sealed tubes in 6M-HCl at 105°C for 24h. The hydrolysates were evaporated to dryness by rotary evaporation, redissolved in water, and separate samples were taken for assay of total [³H]proline and for [³H]hydroxyproline (Juva & Prockop, 1966).

Collagen characterization

Wound tissue from 19 'recovery' animals was incubated in 15ml of modified Krebs medium containing 50µg of ascorbic acid/ml and after a preincubation of 30min 150µCi of [5-³H]proline was added and the incubation continued for 3h at 37°C. β -Aminopropionitrile (64µg/ml) was also included in the incubation medium to inhibit the formation of both intra- and inter-molecular cross-links between the collagen polypeptides (Siegel & Martin, 1970). The synthesis of labelled collagen was stopped by addition of [¹²C]proline to a final concentration of 100mg/ml and incubation was continued for 60min to facilitate conversion of [³H]procollagen into [³H]tropocollagen (Lapière *et al.*, 1971; Bornstein *et al.*, 1972). The tissue was homogenized in the modified Krebs medium, then made 0.2M in acetic acid and extracted for 18h at 4°C before being dialysed for 48h against two changes of 8litres of 0.2M-acetic acid. The non-diffusible material was centrifuged at 21000g for 30min at 4°C and the supernatant taken for CM-cellulose chromatography.

Approx. 100mg of rat tail-tendon tropocollagen dissolved in 0.2M-acetic acid was pooled with the

[^3H]collagen extract and dialysed against 0.06M-sodium acetate buffer, pH 4.8, before chromatography on a CM-cellulose column (2.5 cm $\times$ 10 cm) by using a linear gradient from 0–0.12M-NaCl under the conditions described by Piez *et al.* (1963). The extinction of the carrier tropocollagen was monitored at 230 nm and the total ^3H radioactivity measured by taking 0.2 ml samples out of the 10 ml fractions for assay in an Intertechnique SL 40 scintillation counter (Juva & Prockop, 1966).

Determination of the extent of glycosylation of repair collagen

The wounds from 20 'recovery' animals were pooled and preincubated at 37°C for 30 min in 15 ml of modified Krebs medium containing ascorbic acid (50 μg /ml), and then 50 μCi of [^3H]lysine was added and the incubation continued for 4 h. The tissue was homogenized in the modified Krebs medium, made 0.2M in acetic acid and extracted for 24 h at 4°C before being dialysed for 48 h against two changes of 8 litres of 0.2M-acetic acid. The non-diffusible material was centrifuged at 21 000g for 30 min at 4°C, the supernatant removed and made 5% (w/v) in NaCl and left at 4°C for 18 h to precipitate the collagen. The precipitate was collected by centrifugation at 21 000g for 30 min at 4°C, resuspended in 5 ml of 20 mM-Tris-HCl (pH 7.6) containing 0.17M-NaCl and 5 mM-CaCl₂ and incubated at 37°C with bacterial collagenase (200 Mandl units/ml) for 48 h. The incubated material was then dialysed for a total of 48 h against two changes of 50 ml of water at 4°C and the diffusate was rotary-evaporated to dryness. Alkaline hydrolysis and subsequent desalting were carried out as described by Grant *et al.* (1972). [^3H]Lysine, [^3H]hydroxylysine and [^3H]hydroxylysine glycosides were separated by a modification of the method of Askenasi & Kefalides (1972). The hydrolysate was dissolved in 0.1M-HCl and chromatographed at pH 5.28 on the long column of a Jeol JLC 6AH amino acid analyser. Fractions (2.5 ml) were collected and samples (1 ml) were counted in 10 ml of scintillant [2 vol. of toluene containing 5 g of 2,5-diphenyloxazole/l and 0.3 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene/l and 1 vol. of Triton X-100] to which was added 0.2 ml of 2M-HCl to avoid precipitation of the sample.

Results

Assessment of scorbutic and recovery status by electron microscopy and ascorbic acid determinations

Electron-microscopy studies carried out on wound tissue from scorbutic and 'recovery' animals revealed marked differences in cellular morphology particularly with regard to the endoplasmic reticulum. In

all the sections of scorbutic tissue examined the fibroblasts showed a significant swelling and rounding-up of the endoplasmic reticulum, whereas in recovery tissue the endoplasmic reticulum had reverted to the configuration observed in normal fibroblasts. There was little evidence of extracellular collagen fibrils in scorbutic tissue, but in several instances an apparently homogeneous extracellular material similar to that previously described (Ross & Benditt, 1962b; Peach, 1962) was observed around the scorbutic fibroblasts. These observations on the dilated endoplasmic reticulum in scorbutic tissue are in agreement with earlier electron-microscopic studies on scurvy and wound healing in the guinea pig (Peach, 1962; Ross & Benditt, 1964). Further demonstration of the scorbutic nature of the guinea pigs used and an assessment of their recovery from vitamin C deficiency 24 h after intraperitoneal administration of ascorbate was given by determining the concentrations of ascorbic acid in the adrenals of the animals. These concentrations were assayed by the method of Roe (1954) by using pooled tissue from eight animals. The adrenals from scorbutic animals contained 27.5 μg of ascorbate/g wet wt., and comparable values for 'recovery' animals which had received total doses of 100 mg and 250 mg of ascorbic acid were 68.2 and 74.1 μg of ascorbate/g wet wt. respectively. These values are in the range reported by Bentley & Jackson (1965) for the ascorbic acid content of adrenals of scorbutic and normal guinea pigs and clearly indicate that the scorbutic animals were genuinely vitamin C-deficient and that concentrations of ascorbate in the 'recovery' animals had returned to a normal value of approx. 70 μg /g wet wt. of adrenal tissue.

It should be noted that several other workers, e.g. Barnes *et al.* (1969) and Hughes & Jones (1971), have reported the ascorbic acid content of adrenals of normal guinea pigs to be in the range 400–1200 μg /g wet wt. of tissue. However, these values were obtained for animals which had not been subjected either to surgery as quoted here or to the injection of carrageenan (Bentley & Jackson, 1965). Since both wound healing and carrageenan injection give rise to a stimulation of collagen synthesis, these differences in the concentrations of ascorbate in adrenals from non-scorbutic animals can be readily explained by the demand for ascorbate (Robertson & Schwartz, 1953) arising from the induced trauma.

Distribution of ribosomes in wound tissues

Not only did the electron micrographs of the wound tissues reveal a dilation and distortion of the endoplasmic reticulum in scorbutic fibroblasts, but examination of numerous sections gave the overall impression that these cells had relatively more free ribosomes than did the fibroblasts in the recovery

Table 1. *Distribution of RNA in free and bound ribosomes in scorbutic and recovery tissue*

The wounds from six to nine animals were pooled and free and membrane-bound ribosomes were isolated either by the method of Blobel & Potter (1967) (method I) or of Venkatesan & Steele (1972) (method II). RNA determinations were carried out on the ribosomal pellets as described in the text. Values in parentheses after 'Recovery' denote mg of ascorbate administered 24h before the experiment.

Method	Experiment	Condition of wound	Total ribosomes (mg of RNA)	Free ribosomes (mg of RNA)	100×free ribosomes	100×bound ribosomes
					total ribosomes (%)	total ribosomes (%)
I	A	Scorbutic	3.61	2.38	65.8	34.2
		Recovery (100)	3.42	1.84	53.9	46.1
	B	Scorbutic	3.25	2.28	70.0	30.0
		Recovery (100)	3.75	1.86	49.7	50.3
II	A	Scorbutic	4.93	2.82	57.1	42.9
		Recovery (100)	5.06	1.96	38.7	61.3
		Recovery (250)	4.27	1.61	37.6	62.4
	B	Scorbutic	4.86	2.90	59.6	40.4
		Recovery (100)	4.98	1.76	35.4	64.6
		Recovery (250)	5.11	1.78	34.9	65.1

tissues. Thus it appeared that the administration of ascorbic acid resulted in a restoration of ribosomal morphology which approaches the configuration of a normal fibroblast. Similar qualitative observations of electron micrographs of scorbutic guinea-pig wounds were first reported by Ross & Benditt (1964), who also concluded that vitamin C deficiency led to a shift in ribosomal distribution. In order to determine more precisely the extent of these changes a quantitative analysis of free and membrane-bound ribosomes in scorbutic and recovery tissues was undertaken.

The data presented in Table 1 show that a high proportion of the ribosomes of scorbutic wound tissues are not membrane-bound. As measured by the method of Blobel & Potter (1967) 73% of cellular ribosomal RNA was present in free ribosomes, whereas analysis by the method of Venkatesan & Steele (1972) gave a value of 66%. However, when ascorbic acid had been supplied 24h before analysis then the recovery tissues had a markedly increased proportion of their ribosomes membrane-bound. By the method of Blobel & Potter (1967) 43% of the cellular ribosomal RNA was present as membrane-bound ribosomes. Analysis of the recovery tissue by the method of Venkatesan & Steele (1972) suggested that 57% of the ribosomes were bound, but an increased ascorbic acid dosage to 250mg/animal gave no significant further increase in the proportion of bound ribosomes (Table 1).

In order to establish whether these changes involved polyribosomes of a particular size, sucrose-density-gradient centrifugation studies were carried out on total and free polyribosomes from scorbutic and recovery wounds (Fig. 1). The results obtained are consistent with a reorganization of ribosomal structures after administration of ascorbic acid.

The analysis of total ribosomes (Fig. 1a) revealed a significant increase in the proportion of large polyribosomes in the range 300–350S and a concomitant decrease in 80S monoribosomes in recovery tissue. Analysis of the free ribosomes indicated that the only effect of ascorbate administration was to decrease the proportion of 80S monoribosomes (Fig. 1b).

Incorporation of [³H]proline and [¹⁴C]leucine by wound tissues incubated in vitro

Collagen synthesis was measured by incubating wound tissues with [³H]proline and then assaying the labelled non-diffusible peptide material for total ³H incorporated and [³H]hydroxyproline synthesized. Table 2 reports two typical experiments, and although the amount of tissue obtained per wound was subject to some variation, the results indicate a significant increase in the synthesis of [³H]hydroxyproline in recovery wounds when compared with scorbutic wounds. Some variation in the percentage of proline hydroxylated occurred between batches of animals (cf. Expts. I and II in Table 2), but in all experiments there was a 3–4-fold increase in the percentage of proline hydroxylated by recovery tissue over that seen in the scorbutic wounds. Since [³H]hydroxyproline is synthesized by the hydroxylation of [³H]proline in a polypeptide precursor of collagen, the increase in [³H]hydroxyproline synthesis is indicative of a stimulation of the hydroxylation of peptidyl-proline in the recovery wounds.

To test whether ascorbic acid was the only factor limiting peptidyl-proline hydroxylation in the 7-day scorbutic wounds, hydroxyproline synthesis was

Fig. 1. Polyribosomal profiles of wound tissue

Wound tissue from recovery and scorbutic animals was homogenized and post-mitochondrial supernatants were obtained by centrifugation as described in the text. (a) Total ribosomes were isolated and analysed as follows. Samples of the post-mitochondrial supernatants were treated with 0.1% (v/v) Triton X-100 and layered over a discontinuous sucrose gradient consisting of 3 ml of 0.5M-sucrose in 0.05M-Tris-HCl (pH 7.5) containing 0.025M-KCl and 0.5mM-MgCl₂ on 3 ml of 2.0M-sucrose in the same buffer. Total ribosomes were sedimented at 4°C by centrifugation at 105000g for 24h. The ribosomal pellets were resuspended in 0.02M-Tris-HCl (pH 7.4) containing 0.24M-KCl and 0.0075M-MgCl₂ and samples analysed by sucrose-density-gradient centrifugation as described in the text. (b) Free ribosomes were prepared by the above method except that detergent treatment was omitted, in which case membrane-bound ribosomes remained at the 0.5M-sucrose/2.0M-sucrose interface on centrifugation. The pellets of free ribosomes were again analysed by sucrose-density-gradient centrifugation. —, Recovery tissue; ----, scorbutic tissue.

examined in tissues from 40 scorbutic wounds which were incubated *in vitro* with and without ascorbic acid. Samples of four wounds were incubated in 2.5 ml of modified Krebs medium with 3 μ Ci of [³H]-proline for 3 h at 37°C with or without 100 μ g of ascorbic acid/ml and the [³H]hydroxyproline synthesized was assayed as described above. In this experiment the percentage hydroxylation of proline by scorbutic wounds was 2.89 ± 0.47 (5) and those wounds receiving 100 μ g of ascorbate/ml in the medium gave percentage hydroxylation of 6.71 ± 1.11

(5). Thus, supplementation of scorbutic wounds *in vitro* with ascorbic acid markedly increased the amount of [³H]hydroxyproline synthesized, and the extent of hydroxylation of incorporated [³H]-proline virtually reached the values seen in recovery wound tissues.

The question posed by these experiments is whether the stimulation of [³H]hydroxyproline synthesis in recovery wounds is also accompanied by a concomitant increase in procollagen synthesis, or whether the rate of procollagen synthesis remains unaltered and the increased [³H]hydroxyproline synthesis reflects merely an increased rate of hydroxylation after ascorbate administration. The results in Table 2 suggest that in recovery wounds there is probably a small increase in total ³H incorporated per wound. To assess whether this apparent increase arose from an increase in either procollagen synthesis or general protein synthesis, the incorporation of both [³H]proline and [¹⁴C]leucine per μ g of DNA was determined for scorbutic and recovery tissues (Table 3). No discernible difference was observed between the incorporation of [¹⁴C]leucine into non-diffusible peptide material by scorbutic or recovery tissues. In contrast there was a small but consistent increase (8–10%) in the incorporation of [³H]proline per μ g of DNA in the recovery wounds.

These values will, of course, be influenced by the respective intracellular levels of free proline and leucine in the tissues. Because it was not possible to measure these levels directly it was considered that the measurement of free proline and leucine in the blood plasma of these animals might be an indirect indicator of the nutritional status of these vascular tissues. Blood samples from scorbutic and 'recovery' animals were collected in EDTA-treated vials and the plasma was obtained by centrifugation at 2500g for 10 min. Protein was precipitated by the addition of 50 mg of solid sulphosalicylic acid/ml of plasma. The protein precipitate was removed by centrifugation and a portion of the supernatant was analysed directly on a Jeol JLC 6AH amino acid analyser, with norleucine as an internal standard. Analysis of five samples of plasma from both scorbutic and 'recovery' animals gave mean values of free proline of 100 pmol/ml and 106 pmol/ml respectively and mean values of free leucine of 143 pmol/ml and 144 pmol/ml respectively.

Thus the evidence presented above suggests that the rate of general protein synthesis (measured as incorporation of [¹⁴C]leucine into non-diffusible material) is unaffected by ascorbate administration. Since procollagen contains a much higher proportion of proline than most other proteins, changes in procollagen synthesis are likely to give rise to changes in the incorporation of proline, and consequently the results shown in Table 3 appear to indicate that in recovery wounds there is an increase

Table 2. *Extent of hydroxylation of [³H]proline incorporated in vitro by scorbutic and recovery wound tissue*

Each sample, containing tissue from four wounds, was incubated with 2.5 μ Ci of [³H]proline for 3 h as described in the text. Non-diffusible [³H]hydroxyproline was assayed by the method of Juva & Prockop (1966).

Experiment	Condition of wound	$10^{-3} \times$ Total non-diffusible radioactivity (d.p.m.)	$10^{-3} \times$ Non-diffusible [³ H]hydroxyproline (d.p.m.)	$100 \times$ [³ H]hydroxyproline total ³ H (%)
I	Scorbutic	416.0	10.4	2.5
		474.4	15.2	3.2
		520.0	9.2	1.8
		560.0	14.4	2.6
		358.4	10.4	2.9
	Recovery	574.0	58.5	10.2
		773.2	70.4	9.1
		719.2	76.8	10.7
		858.4	89.2	10.4
		545.2	52.3	9.6
II	Scorbutic	482.0	8.7	1.8
		430.0	9.9	2.3
		660.8	12.4	1.9
		613.7	9.2	1.5
	Recovery	410.4	22.6	5.5
		522.0	36.0	6.9
		736.4	42.4	5.8
		470.0	29.6	6.3

Table 3. *Incorporation in vitro of [³H]proline and [¹⁴C]leucine by scorbutic and recovery wound tissue*

Each sample, containing the wound tissue from three or four animals, was incubated with 2.5 μ Ci of labelled amino acid for 3 h at 37°C as described in the Experimental section. Tissues were homogenized and a portion was taken for DNA determination by the method of Burton (1956).

Experiment	Condition of wound	$10^{-3} \times$ Non-diffusible [³ H]proline (d.p.m./ μ g of DNA)	$10^{-3} \times$ Non-diffusible [¹⁴ C]leucine (d.p.m./ μ g of DNA)
I	Scorbutic	47.2	69.3
		49.1	
		47.2	
	Recovery	54.5	69.7
		53.9	
II	Scorbutic	50.4	
		42.2	61.5
		42.8	62.5
	Recovery	46.7	63.0
		44.5	60.2
III	Scorbutic	—	65.1
			63.6
	Recovery	—	66.2
			65.0

extent of hydroxylation of procollagen. This conclusion is supported by preliminary data on the levels of activity of procollagen proline hydroxylase in the scorbutic and recovery wounds which indicate an approximate increase of 50% in the hydroxylase activity 24 h after ascorbate administration.

Partial characterization of collagen involved in wound repair

Co-chromatography on CM-cellulose of rat tail-tendon tropocollagen with the [³H]proline-labelled acetic acid extract of recovery wound tissue is shown in Fig. 2. Two peaks of radioactivity were eluted in positions corresponding to the α_1 and α_2 chains of the carrier tropocollagen. The elution position of the labelled guinea-pig and rat tail-tendon carrier α_2 chains are not coincident, an observation which may indicate slight differences in the composition of the α_2 chain from the different species. The ratio of d.p.m. in α_1 chain:d.p.m. in α_2 chain was almost exactly 2:1. Analysis of the peak fractions for [³H]hydroxyproline indicated that for α_1 and α_2 chains the values for $100 \times$ [³H]hydroxyproline d.p.m./total ³H d.p.m. were 44% and 42% respectively. These values approximate to the theoretical maximum for the percentage of imino acids hydroxylated in interstitial collagen (Eastoe, 1967).

Determination of the extent of glycosylation of the collagen synthesized by recovery tissue was carried out. The results presented in Table 4 confirm that the collagen synthesized during wound repair

in procollagen synthesis. This increase will provide additional substrate for hydroxylation, but the three- to four-fold stimulation in [³H]hydroxyproline synthesis must be attributed mainly to an increased

Fig. 2. Elution pattern from CM-cellulose of an acetic acid extract from recovery wound tissue

Wounds were incubated with [^3H]proline for 3 h, 'chased' with an excess of unlabelled L-proline, and then incubated for a further 60 min. Newly synthesized collagen solubilized by acetic acid extraction was co-chromatographed on CM-cellulose with rat tail-tendon tropocollagen under the conditions described in the text. The start of the linear gradient (0–0.12 M-NaCl) is indicated and the elution positions of the carrier α_1 and α_2 chains and the intramolecularly cross-linked β_{11} and β_{12} dimers are also arrowed. —, E_{230} pattern of carrier tropocollagen; ----, radioactivity.

Table 4. Extent of glycosylation of hydroxylysine synthesized *in vitro* by recovery wound tissue

Newly synthesized collagen was extracted from recovery wound tissue incubated with [^3H]lysine for 4 h and the extent of glycosylation of [^3H]hydroxylysine determined as described in the text. The values shown below for recovery tissue represent the relative distribution of radioactive label between [^3H]hydroxylysine and the [^3H]hydroxylysine glycosides; values for dermis and dermal scar are taken from Forrest *et al.* (1972a).

Tissue	Free hydroxylysine (%)	Glycosylated hydroxylysine (%)	Distribution of glycosylated units (%)	
			Hyl-Gal	Hyl-Gal-Glc
Dermis	73.4	26.6	39	61
Dermal scar	74.0	26.0	20	80
Recovery tissue*	58.0	42.0	20	80

* The radioactivities obtained for the recovery tissue were 14618 d.p.m., 2115 d.p.m. and 8468 d.p.m. for free [^3H]hydroxylysine, [^3H]Hyl-Gal and [^3H]Hyl-Gal-Glc respectively.

contains a higher proportion of the disaccharide unit (glucosylgalactose-hydroxylysine) than normal dermal collagen. However, the extent of glycosylation of hydroxylysine residues in the collagen synthesized by recovery tissue was found to be markedly higher than that reported for mature dermal-scar collagen.

Discussion

Work carried out in several laboratories in recent years suggests that hydroxylysine is an important precursor of the intermolecular cross-links which stabilize the collagen fibril *in vivo* (for review see Tanzer, 1973), and that hydroxyproline has a role

to play in the stabilization of the collagen triple helix (Sakakibara *et al.*, 1973; Berg & Prockop, 1973; Jimenez *et al.*, 1973b). The synthesis of these two hydroxy-amino acids is believed to involve ascorbic acid as a cofactor in the hydroxylation process, and hence the major effect of ascorbate deficiency is likely to be a derangement of collagen biosynthesis; indeed an 80–85% decrease in collagen synthesis has been observed in several studies using severely scorbutic guinea pigs (Gould, 1968). Such results, however, demonstrate that even in severe scurvy some synthesis of collagen takes place, a fact which may well be due to difficulties in achieving an absolutely scorbutic state and also to the

possibility that alternative compounds, e.g. tetrahydrofolate, may be used, if less efficiently, as cofactors in the hydroxylation process (Hutton *et al.*, 1967).

The guinea pigs used in the present study were demonstrably scorbutic but not severely so, as judged by ascorbate determinations carried out on the adrenals, and consequently wound tissue from these animals exhibited detectable collagen synthesis (Table 2). An increase in the degree of ascorbate deficiency would have led to a concomitant and unacceptable increase in the death rate of the wounded animals, and since the wound fibroblasts from the scorbutic animals exhibited the morphological features associated with ascorbate deficiency (Ross & Benditt, 1964) the degrees of deficiency were judged to be satisfactory for the present study.

Quantitative methods are available for assessing the relative proportions of free and membrane-bound ribosomes present in tissues. These proportions are of particular interest in the context of this study of collagen biosynthesis, since it is generally considered that free ribosomes are involved in the synthesis of intracellular proteins whereas proteins destined for export are synthesized on membrane-bound ribosomes (Palade, 1966; Campbell, 1970). Thus these techniques have been applied to the scorbutic and recovery wound tissues after our electron-microscopic studies, which suggested differences in ribosomal distribution in the ascorbate-deficient and ascorbate-supplemented wound fibroblasts. The results (Table 1) demonstrate that in recovery wound tissues there is an increase in the proportion of membrane-bound ribosomes, and sucrose gradient centrifugation of the total ribosomal population revealed that it was large polyribosomes of the size range 300–350S that were affected by ascorbate supplementation. Since it has been demonstrated (Lazarides & Lukens, 1971) that in embryonic chick limbs collagen is synthesized on heavy polyribosomes (approx. 330S) these results suggest that it is the collagen-synthesizing polyribosomes that are disorganized by vitamin C deficiency and reassembled after ascorbate administration.

From these observations it can be tentatively concluded that the polyribosomes involved in the synthesis of proteins other than collagen are unaffected by the concentration of ascorbate, a conclusion which is supported by the [^{14}C]leucine incorporation studies with scorbutic and recovery tissues which indicated identical amounts of general protein synthesis. Similar conclusions relating to the effects of ascorbate on the synthesis of non-collagenous proteins have been reported after studies conducted with some strains of fibroblasts in culture (Peterkovsky, 1972a,b; Bates *et al.*, 1972b).

The fact that it appears to be collagen-synthesizing

polyribosomes that are disorganized in the scorbutic tissues would argue that ascorbate is involved directly in the collagen synthetic process. It has been suggested (Fernandez-Madrid & Pita, 1970) that the enhancement of collagen synthesis by ascorbate may be a consequence of a direct influence of ascorbate on polyribosomal formation or stability. However, in view of the known role of ascorbate in the synthesis of hydroxyproline and hydroxylysine (Barnes & Kodicek, 1972), it seems more likely that changes in polyribosome conformation are secondary to changes in the hydroxylation process. Since some, if not all, of the hydroxylations of proline, and presumably lysine, normally occur on nascent collagen polypeptides (Miller & Udenfriend, 1970; Lazarides *et al.*, 1971) the hydroxylating enzymes must be located in the proximity of the membrane-bound collagen-synthesizing ribosomes, and since the enzymes themselves may be membrane-bound (Guzman & Cutroneo, 1973; Harwood *et al.*, 1974), any interference with hydroxylation may result in a disorganization of the translational machinery. In addition, because proline hydroxylase has an exceedingly high affinity for protocollagen (Juva & Prockop, 1969), the formation of stable enzyme-substrate complexes in the absence of hydroxylating cofactors could inhibit subsequent processing and may specifically influence the collagen-synthesizing polyribosomes by slowing down the translation of collagen mRNA. Such an effect could possibly alter the balance of the ribosome cycle and result in the decrease in the proportion of large collagen-synthesizing polyribosomes and the increase in single 80S polyribosomes observed in scorbutic tissue.

The formation and accumulation of enzyme-substrate complexes in the absence of ascorbate may explain the dilation of the endoplasmic reticulum in the scorbutic fibroblasts and also the diminished secretion of protocollagen reported by other workers. This failure of cells to secrete underhydroxylated collagen at a normal rate has been studied in fibroblasts in culture (Gribble *et al.*, 1969; Margolis & Lukens, 1971; Ramaley & Rosenbloom, 1971) and more recently in matrix-free embryonic chick tendon cells (Jimenez *et al.*, 1973a). In this latter study an intracellular accumulation of non-hydroxylated procollagen was observed when embryonic chick tendon cells were incubated for short-term periods in the presence of $\alpha\alpha'$ -bipyridyl, and only small peptides resulting from degradation of procollagen were found in the medium. However, the reports of Bates *et al.* (1972a) and Ramaley *et al.* (1973) suggest that underhydroxylated high-molecular-weight collagen polypeptides can be found extracellularly when fibroblasts are cultured under conditions where hydroxylation is inhibited. This discrepancy in the size of secreted peptides can probably be explained by the presence of extracellular

proteases in the media of cells freshly isolated by enzymic digestion (Grant *et al.*, 1973; Uitto & Prockop, 1973). These proteases would digest procollagen which would be present in a random coil conformation at 37°C because of the absence of hydroxylated proline residues which stabilize the collagen triple helix (Berg & Prockop, 1973; Jimenez *et al.*, 1973b). Since triple-helix formation normally occurs intracellularly (Rosenbloom *et al.*, 1973) and may be necessary for an optimal rate of secretion (Uitto & Prockop, 1974), the observed, reduction in the rate of secretion of underhydroxylated procollagen may also be related to its random coil conformation under physiological conditions.

The results described in the present study demonstrate the rapid recovery from the scorbutic state of the wound fibroblasts *in vivo*. Thus, 24h after ascorbate administration *in vivo*, the configuration of the endoplasmic reticulum, as observed in the electron microscope, has reverted to normal; there has been an increase in the relative proportion of bound ribosomes; and the degree of hydroxylation of non-diffusible proline has risen to a value 3–4 times that observed in the scorbutic state (Table 2). The rapid recovery of wound tissues from ascorbate deficiency was also observed *in vitro*. When scorbutic wounds were incubated with [³H]proline in the presence of ascorbic acid for 3h, there was a 2–4-fold stimulation of [³H]hydroxyproline synthesis above the values in unsupplemented tissues, and the degree of hydroxylation of peptidyl-proline was almost equal to that observed in recovery wounds. Because of the disorganization of collagen-synthesizing polyribosomes and the lowered hydroxylase activity in scorbutic wounds, this result suggests that the collagen-synthesizing machinery is readily re-assembled after ascorbate supplementation. This observation correlates well with the rapid recovery of proline hydroxylase activity reported in scorbutic fibroblasts (Stassen *et al.*, 1973), the rapid re-formation of collagen-synthesizing polyribosomes in scorbutic granulomas (Fernandez-Madrid & Pita, 1970) and the rapid recovery of cellular morphology in scorbutic wound fibroblasts (Ross & Benditt, 1964) within 1–4h of the administration of ascorbic acid to the tissues.

The nature of the collagen synthesized by recovery tissues was investigated, since previous studies carried out in this department have revealed marked differences in the physicochemical properties of the collagen from guinea-pig dermis and that from dermal scar tissue (Forrest & Jackson, 1971; Forrest *et al.*, 1972a,b). Because the repair collagen is synthesized not by fibroblasts of dermal origin but probably by fibroblasts derived from undifferentiated cells in the smooth muscle (Grillo, 1963), these differences could arise from different collagen molecules. The increased degree of hydroxylation

of lysine and glycosylation of hydroxylysine seen in scar tissue (Forrest *et al.*, 1972b) and the increased glycosylation seen in the recovery wound collagen (Table 4) could be attributable to more extensive post-translational modification of a Type I collagen gene product or, alternatively, to the synthesis of a Type III collagen which has tentatively been identified in embryonic skin (Miller *et al.*, 1971; Trelstad *et al.*, 1971). The results of CM-cellulose chromatography of the collagen synthesized during wound healing indicate that the collagen involved in repair is a typical Type I collagen having the normal chain composition of (α_1)₂ α_2 . Further study will be required to elucidate the significance of the increased extent of glycosylation observed in the collagen synthesized by the recovery wound tissue.

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