The Effect of Scurvy on Glycosaminoglycans of Granulation Tissue and Costal Cartilage

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1. The effect of ascorbic acid deficiency on glycosaminoglycans of granulation tissue and cartilage of guinea pigs was investigated by determination of the changes in the glucosamine and galactosamine contents 12 days after tendonectomy. 2. In normal granulation tissue, the glucosamine and galactosamine contents rose to a peak at 5 and 10 days respectively, whereas the hydroxyproline and proline contents continued to rise throughout the 20 days after tendonectomy. 3. The galactosamine in scorbutic granulation tissue, but not in that of pair-fed controls, decreased significantly in absolute amount and relatively to glucosamine, which remained practically unchanged; the cartilage galactosamine did not decrease during the 22 days of deficiency owing to the presence of excess of preformed galactosaminoglycans, which masked the small amount of newly formed glycosaminoglycans. 4. The chemical results were confirmed by radioactivity studies *in vivo* of incorporation of [^{14}C]glucose into galactosamine and glucosamine of scorbutic granulation tissue and cartilage. The incorporation of ^{14}C into galactosamine decreased significantly in scurvy in both tissues. 5. The results indicated in both tissues a decreased formation of galactosamine during scurvy, although an increased degradation of polymerized glycosaminoglycans could not be entirely ruled out. It is concluded that, if lack of ascorbic acid causes an impaired galactosamine formation, the most likely position for the block may be in the UDP-*N*-acetylglucosamine 4-epimerase reaction.

The role of ascorbic acid in the formation of the two main components of connective tissue, namely collagen and glycosaminoglycans (mucopolysaccharides), has been studied by many investigators. Both *in vivo* and *in vitro*, the vitamin appears to have a direct effect on the formation of the collagen molecule (Gould, 1958; Robertson & Hewitt, 1961; Hurych & Chvapil, 1962), but its effect on the metabolism of the mucopolysaccharides of the connective tissue remains obscure. Histochemically, granulation tissue of scorbutic animals contains decreased amounts of acid mucopolysaccharides (Penney & Balfour, 1949; Bradfield & Kodicek, 1951; Stein & Wolman, 1958; Fuks, Vinogradov, Shishkin & Maksimovskii, 1965). A diminished uptake of [^{35}S]sulphate into scorbutic granulation tissue (Kodicek & Loewi, 1955; Upton & Odell, 1956) and cartilage (Friberg & Ringertz, 1954; Reddi & Norström, 1954; Kodicek & Loewi, 1955) was observed. The impaired sulphate uptake could be caused by several factors. The absence of an effect of ascorbic acid on the sulphating mechanism of liver and the failure of ascorbic acid to improve the diminished uptake *in vitro* (Kodicek & Loewi, 1955) suggested that the impairment in scorbutic

connective tissue might be due to a decreased amount of substrate, chondroitin, rather than a block in the sulphating mechanism (Kodicek, 1958). Therefore a study was undertaken to examine the effect of ascorbic acid deficiency on glycosaminoglycans by the determination of changes in the glucosamine and galactosamine content of granulation tissue and cartilage, since particularly galactosamine is a characteristic constituent of the repeating units of acid mucopolysaccharides. The work was organized into three parts, to determine (a) the glucosamine and galactosamine content in normally developing granulation tissue in guinea pigs at various periods after tendonectomy, with simultaneous estimation of proline and hydroxyproline, (b) the effect of dietary ascorbic acid on the content of glucosamine and galactosamine in granulation tissue and cartilage, and (c) the incorporation of [^{14}C]glucose *in vivo* into these two hexosamines in tissues of normal and scorbutic guinea pigs.

Short accounts of these results have been presented previously (Antonowicz & Kodicek, 1964; Kodicek, 1965).

After this work was completed, Kofoed &

Robertson (1966) reported that the depression of sulphated glycosaminoglycan synthesis in scurvy is secondary to inanition, but later Schafer, Sullivan, Svejcar, Kofoed & Robertson (1966, 1968) demonstrated, using cell-culture techniques, that fibroblasts taken from patients with Hurler's syndrome showed a dependence on ascorbic acid for the formation of sulphated glycosaminoglycans, particularly that of dermatan sulphate. Schafer, Silverman, Sullivan & Robertson (1967) showed that normal human fibroblasts displayed a similar behaviour. Workers in several other Laboratories studied the effect of ascorbic acid deficiency on the metabolism of glycosaminoglycans with varying success (Gore, Tanaka, Fujinami & Goodman, 1965; Kirchheiner, 1965*a,b*, 1966*a,b,c*; Fuks *et al.* 1965; Reynolds, 1966).

MATERIAL AND METHODS

Experimental animals. Young male albino guinea pigs (The War Department, Allington Farm, Wilts.) weighing 250–300 g. were used throughout the experiments. The animals were kept in separate cages at 21°. All animals received a modified scorbutogenic diet of Reid & Briggs (1953), which was supplemented for the control animals with ascorbic acid (0.2% of the diet) (Constable, 1960). In all experiments there was free access to drinking water. The animals were weighed twice weekly.

Four experiments were performed. Expt. 1 was conducted on 12 guinea pigs. All animals were given the ascorbic acid-supplemented diet *ad lib.* After they had reached a weight of 320–350 g. they were tendonectomized in both hind legs by the procedure of Kodicek & Loewi (1955) and killed in groups of four on the fifth, tenth and twentieth days after operation.

Expt. 2 was conducted on 126 guinea pigs: 82 deficient and 44 control animals. All animals were given diets *ad lib.* Tendonectomy was performed on the tenth day after the dietary regimen started, and the animals were killed 12 days after the operation.

To investigate the effect of partial inanition (Expt. 3), eight guinea pigs were used: four deficient, three pair-fed control and one control animal fed *ad lib.* The pair-fed controls were given the ascorbic acid-supplemented diet, but in an amount corresponding to that consumed by the deficient group the previous day. All other guinea pigs were given diets *ad lib.* The operation and management of the guinea pigs was the same as in Expt. 2. After the animals were killed, the granulation tissue from hind legs was analysed in each animal separately.

Expt. 4 was carried out as two sets of trials, on five normal and five deficient guinea pigs in all. Carrier-free [^{14}C]glucose (specific radioactivity 79.7 mc/m-mole) was obtained from The Radiochemical Centre (Amersham, Bucks.). The [^{14}C]glucose was administered in a single intraperitoneal injection to each animal, 24 hr. before death, after 22 days on control or deficient diet (i.e. 12 days after tendonectomy). The labelled glucose was given in a dose of 50 μC /animal to three normal and three scorbutic guinea pigs in the first trial and in a dose of 150 μC /animal to two normal and two scorbutic animals in the second trial.

Preparation of granulation tissue. The granulation tissue from both hind legs, freed from surrounding skin and fascia, was excised immediately after death. The granulation tissue from the animals of the same group was pooled, dried *in vacuo* over P_2O_5 at 21° to constant weight, pulverized, extracted with acetone exhaustively to remove fat, and finally dried *in vacuo* to constant weight.

Preparation of costal cartilage. The ribs were removed from the animals. The surrounding muscle and fascia were trimmed from the ribs, and the costal cartilage was cut into small pieces, weighed and defatted by the method used for the granulation tissue.

Chemical methods. To separate and determine glucosamine and galactosamine, the column-chromatographic (Gardell, 1953) and paper-chromatographic (Fischer & Dörfel, 1954; Fischer & Nebel, 1955) techniques were adapted to allow measurement on small amounts of granulation tissue. Each sample was analysed by both methods. Dry fat-free tissue (50 mg.) was hydrolysed with 5 ml. of 6*N*-HCl at 100° for 16 hr., evaporated *in vacuo* until free of HCl and dissolved in 0.5 ml. of water, of which 0.3 ml. was used for paper chromatography and the remaining 0.2 ml. for column chromatography.

Column chromatography. The column (7 mm. diam.) was filled to a height of 40 cm. with the cation-exchange resin Dowex AG 50 W (X8; 200–400 mesh) (Bio-Rad Laboratories, Berkeley, Calif., U.S.A.). The hexosamines were eluted with 0.3*N*-HCl at a flow rate of 7–8 ml./hr., and 50 fractions (1 ml.) were collected, after the first 45 ml. of the eluate had been discarded. The glucosamine was eluted between 52 and 59 ml. followed by the galactosamine peak between 62 and 70 ml. The 1 ml. fractions were analysed individually for the separated hexosamines by the method of Elson & Morgan (1933).

Paper chromatography. The hydrolysate was first purified in an ion-exchange column by the method of Boas (1953). After adsorption hexosamines were eluted with 8 ml. of 1*N*-HCl from a column (10 cm. long $\times$ 7 mm. diam.) of Dowex 50 (X8; 200–400 mesh) (Bio-Rad Laboratories) and 6 ml. was evaporated *in vacuo* at 40° to remove HCl. Descending chromatograms on Whatman no. 541 paper, cut into nine strips (2 cm. wide $\times$ 36 cm. long), were developed with ethyl acetate–water–pyridine (2:2:1, by vol.). After 10 hr. the well-separated spots of glucosamine and galactosamine were made visible by the method of Fischer & Dörfel (1954) and eluted in the dark with methanol–acetic acid (10:1, v/v), and E_{482} was read after 1 hr. on a Unicam SP. 600 spectrophotometer.

Other determinations. Hydroxyproline was determined by the method of Bergman & Loxley (1963), proline by that of Troll & Lindsley (1955) and ascorbic acid by that of Bessey (1938).

Measurement of ^{14}C radioactivity incorporated into glucosamine and galactosamine. All the tissues were prepared and treated as in the experiments with non-radioactive material. The paper-chromatographic method was used both for the chemical determination of hexosamines and for the radioactivity measurements. The samples were applied to paper strips as follows: 1, glucosamine standard, 10 μg .; 2, galactosamine standard, 10 μg .; 3–7, samples equivalent to 2–4 mg. of dry fat-free tissue; 8, combined glucosamine and galactosamine standards, 10 μg . each; 9, the blank.

After the chromatograms had been developed, strips 4, 5 and 6 were used for counting and, with the aid of strips 3

and 7, which were stained with triphenyltetrazolium for the chemical determination, the regions of glucosamine and galactosamine spots were marked in the dark room over blue light. The glucosamine and galactosamine spots from strips 4, 5 and 6 were excised and the rest was cut into 2 cm. pieces. The three corresponding pieces from the three strips were transferred into counting vials containing 5 ml. of toluene with 0.4% 2,5-diphenyloxazole (scintillation grade; Packard Instrument Co. Inc., La Grange, Ill., U.S.A.) and counted three times for 30 min. each time in a Tri-Carb liquid-scintillation counter (Packard Instrument Co. Inc.). A correction for the blank count and quenching was made. Three 2 cm. pieces of strip 9 served as a blank. Strips 3 and 7 were used for the chemical determination of glucosamine and galactosamine.

RESULTS

Good agreement, for glucosamine and galactosamine determinations, was obtained between the paper - chromatographic and column - chromatographic techniques on 30 samples with a standard error of 4.9% and 5.1% of the mean respectively. All the results are means of values obtained with both methods.

Normal developing granulation tissue 5, 10 and 20 days after tendonectomy. Table 1 summarizes the changes in the constituents of newly developing granulation tissue between the fifth and the twentieth days after tendonectomy. The glucosamine content decreased, after a peak on the fifth day, to about one-third by the twentieth day of healing. On the other hand, galactosamine maintained its peak value until the tenth day and decreased only by the twentieth day after tendonectomy. The glucosamine/galactosamine concentration ratio remained constant at 1.1-1.2 from the tenth day after the operation. The amount of hydroxyproline increased gradually during the whole period of healing.

Effect of ascorbic acid deficiency. The initial mean weights of both control and deficient groups were about 280g. During the 10 days before tendonectomy, the animals of both groups gained weight at about the same rate. Thereafter the scorbutic

group rapidly lost weight, with a mean of 238g. at death 12 days after tendonectomy, whereas the control group continued to gain in weight and reached a mean value of 378g. The average weights for Expt. 2 are given in Fig. 1.

The ascorbic acid concentration of adrenals was determined in seven control and 12 scorbutic guinea pigs. The average value for deficient animals was 4.4 ± 0.71 mg./100g. wet wt. compared with 135.9 ± 10.1 mg./100g. wet wt. of adrenals in the controls.

Granulation tissue. The average dry fat-free weight of granulation tissue derived from 17 control animals was about 2.5 times (52.7 ± 4.3 mg.) that of 18 deficient animals (20.4 ± 1.7 mg.).

There was no significant difference (Table 2) in

Fig. 1. Average weight curves of ascorbic acid-deficient (●) and control (○) guinea pigs of Expt. 2.

Table 1. Glucosamine, galactosamine, proline and hydroxyproline in normal granulation tissue after tendonectomy

The results were obtained from pooled samples of four animals in each group. The hexosamine values are the averages of results obtained with both paper-chromatographic and column-chromatographic techniques.

Group no.	Days after tendonectomy	Hexosamine content (mg./100g. of dry fat-free tissue)		Glucosamine Galactosamine concentration ratio	Imino acid content (mg./100g. of dry fat-free tissue)	
		Glucosamine	Galactosamine		Proline	Hydroxyproline
1	5	0.61	0.21	2.9	7.3	4.7
2	10	0.34	0.29	1.2	8.0	6.0
3	20	0.19	0.17	1.1	8.6	7.5

Table 2. *Glucosamine, galactosamine and total hexosamine of granulation tissue and costal cartilage in control and scorbutic animals*

The glucosamine and galactosamine results are mean values obtained from paper-chromatographic and column-chromatographic estimations. Total hexosamine was determined by the Boas (1953) method with glucosamine standard for granulation tissue and galactosamine standard for cartilage to correspond to the major sugar component. The S.E.M. values are calculated for numbers of samples stated.

	No. of samples	Hexosamine content (mg./100g. of dry fat-free tissue)		Glucosamine Galactosamine concentration ratio	No. of samples	Total hexosamine content (mg./100g. of dry fat-free tissue)
		Glucosamine	Galactosamine			
Granulation tissue						
Control	20	0.37 ± 0.038	0.27 ± 0.033	1.4	13	1.02 ± 0.063
Scorbutic	16	0.41 ± 0.054	0.10 ± 0.026	4.1	9	0.76 ± 0.041
<i>P</i> value		> 0.5	< 0.001			< 0.01
Costal cartilage						
Control	15	0.34 ± 0.033	2.50 ± 0.259	0.14	13	3.23 ± 0.180
Scorbutic	17	0.40 ± 0.064	2.58 ± 0.248	0.15	9	3.08 ± 0.102
<i>P</i> value		> 0.5	> 0.5			> 0.1

Table 3. *Effect of scurvy or partial inanition on hexosamines in granulation tissue*

Results are average values ± S.E.M. calculated from the numbers of samples given in parentheses.

	Hexosamine content (mg./100 mg. of dry fat-free tissue)		Glucosamine Galactosamine concentration ratio
	Glucosamine	Galactosamine	
Expt. 3			
Control fed <i>ad lib.</i>	0.43	0.25 (2)	1.7
Pair-fed control	0.41 ± 0.047 (6)	0.21 ± 0.026 (6)	2.0
Scorbutic	0.32 ± 0.016 (8)	0.06 ± 0.014 (8)	5.3
<i>P</i> value	> 0.025 < 0.05	< 0.005	
Hughes & Kodicek (1960)			
Control fed <i>ad lib.</i>	0.61	0.27	2.3
Pair-fed control	0.50	0.27	1.9
Scorbutic	0.36	< 0.06	—

glucosamine concentration between control and scorbutic animals (0.37 and 0.41 mg./100g.), but the two groups differed significantly in galactosamine content (0.27 and 0.10 mg./100g.). The glucosamine/galactosamine concentration ratio increased about threefold in deficiency (from 1.4 to 4.1).

The results of Expt. 3, designed to compare the effect of partial inanition (pair-fed controls) with that of scurvy, are shown in Table 3. Significant differences were obtained between the galactosamine values of granulation tissue from deficient and pair-fed animals, thus confirming the results of Hughes & Kodicek (1960).

Costal cartilage. In contrast with findings on granulation tissue there was no significant difference in the glucosamine and galactosamine content between control and deficient guinea pigs (Table 2)

and the ratio of glucosamine to galactosamine was apparently unchanged.

The average total hexosamine concentration of granulation tissue and costal cartilage for both control and scorbutic guinea pigs was determined by the method of Boas (1953). There was no significant change in hexosamine of the scorbutic costal cartilage, but there was a significant decrease in the scorbutic granulation tissue (Table 2).

Incorporation of [U-¹⁴C]glucose in vivo into hexosamine of normal and scorbutic tissue. (a) Granulation tissue. Table 4 shows the ¹⁴C radioactivity, expressed as counts/min./100 mg. of dry fat-free tissue, incorporated into each hexosamine of normal and scorbutic granulation tissue, 24 hr. after intraperitoneal injection of D-[U-¹⁴C]glucose (50 μC and 150 μC in trial 1 and trial 2 respectively). The degree of incorporation into each animal

Table 4. *Radioactivity incorporated into glucosamine and galactosamine of normal and scorbutic granulation tissue 24 hr. after intraperitoneal injection of [U-¹⁴C]glucose*

Because of the small amounts of granulation tissue and low radioactivities pooled samples from two or three animals were used in two instances.

		Radioactivity (counts/min./100mg. of dry fat-free tissue)		<u>Glucosamine</u>
		Glucosamine	Galactosamine	<u>Galactosamine</u> radioactivity ratio
Trial 1 (dose injected: 50 μ C/animal)				
Control	1	263	284	0.9
	2	209	209	1.0
	Mean	236	247	1.0
Scorbutic	3	985	281	3.5
Trial 2 (dose injected: 150 μ C/animal)				
Control	1	2106	1928	1.1
	1	1947	1134	1.7
	Mean	2027	1532	1.4
Scorbutic	1	638	125	5.1
	1	675	225	3.0
	Mean	657	175	4.1

Table 5. *Specific radioactivities of hexosamines of normal and scorbutic granulation tissue 24 hr. after intraperitoneal injection of [U-¹⁴C]glucose*

Because of the small amounts of granulation tissue and low radioactivities pooled samples from two or three animals were used in two instances.

	No. of pooled samples	Specific radioactivity (counts/min./mg. of hexosamine)		<div>Glucosamine Galactosamine specific radioactivity ratio</div>
		Glucosamine	Galactosamine	
Trial 1 (dose injected: 50 μ C/animal)				
Control	1	412	519	0.79
	2	481	823	0.58
	Mean	447	671	0.69
Scorbutic	3	1582	1778	0.88
Trial 2 (dose injected: 150 μ C/animal)				
Control	1	5198	6743	0.77
	1	2908	3909	0.74
	Mean	4053	5326	0.76
Scorbutic	1	2113	—*	—
	1	2053	—*	—
	Mean	2083		

* The chemical values for galactosamine were too low and therefore not accurate for calculation of specific radioactivity (see the text).

depends on a number of factors, such as body weight, precursor pool size etc., and therefore only the glucosamine/galactosamine radioactivity ratios for individual animals or pooled sample were considered, although the values within individual treatments were remarkably consistent. The ratios showed an approx. threefold increase in scorbutic tissue in both trials, rising from 1.0 to 3.5 and 1.4 to 4.1 respectively.

The specific radioactivities in normal and scorbutic tissues (Table 5), expressed as counts/min./mg. of glucosamine or galactosamine respectively, were again fairly consistent within individual treatments. Since the galactosamine content in the scorbutic tissue of trial 2 decreased to barely detectable values (1 and 2.3 μ g./100mg. of dry fat-free tissue), the specific radioactivities were not calculated in view of the possible large errors.

Table 6. *Radioactivity incorporated into hexosamines of normal and scorbutic costal cartilage 24 hr. after intraperitoneal injection of [U-¹⁴C]glucose*The dose injected was 150 μ C/animal.

	No. of samples	Radioactivity (counts/min./100 mg. of dry fat-free tissue)		Glucosamine Galactosamine radioactivity ratio
		Glucosamine	Galactosamine	
Control	1	232	601	0.39
	1	206	695	0.30
	Mean	219	648	0.35
Scorbutic	1	258	266	0.97
	1	264	561	0.47
	Mean	261	414	0.72

Table 7. *Specific radioactivities of hexosamines of normal and scorbutic costal cartilage 24 hr. after intraperitoneal injection of [U-¹⁴C]glucose*The dose injected was 150 μ C/animal.

	No. of samples	Specific radioactivity (counts/min./mg. of hexosamine)		Glucosamine Galactosamine specific radioactivity ratio
		Glucosamine	Galactosamine	
Control	1	964	546	1.8
	1	503	286	1.8
	Mean	734	416	1.8
Scorbutic	1	588	160	3.7
	1	557	204	2.7
	Mean	573	182	3.2

Because of variation in pool size it is considered that the glucosamine/galactosamine specific radioactivity ratios reflect their relative metabolism more accurately than the individual specific radioactivities. No difference was found between the ratios in normal and deficient animals.

(b) Costal cartilage. Table 6 shows the radioactivity (counts/min./100 mg. of dry fat-free tissue) incorporated into each hexosamine of normal and scorbutic costal cartilage. An increase in the glucosamine/galactosamine radioactivity ratio from 0.35 in normal to 0.72 in the scorbutic cartilage was observed.

Table 7 shows the specific radioactivities of both hexosamines of normal and scorbutic costal cartilage. Here, in contrast with granulation tissue, the specific radioactivity of galactosamine was lower than that of glucosamine, and this lowering was reflected in the higher glucosamine/galactosamine specific radioactivity ratio (1.8 for normal, 3.2 for scorbutic cartilage).

DISCUSSION

In this investigation it was found that the glucosamine content in normally developing

granulation tissue in guinea pigs is highest in the first 5 days after the tendonectomy. This is in good agreement with the results reported by Dunphy, Udupa & Edwards (1956) and Taylor & Sanders (1957). Radioisotope studies and chemical determinations (Boström & Aquist, 1952; Kodicek & Loewi, 1955; Curran & Kennedy, 1955; Dunphy *et al.* 1956; Slack, 1957; Jackson, 1958) have confirmed the rapid synthesis of mucopolysaccharides in granulation tissue and indicate that the rate of synthesis declines as collagen fibres are deposited. There is so far no evidence that the hexosamines in granulation tissue are present in any other form but polymers (Kodicek & Loewi, 1955).

In scorbutic guinea pigs, the decreased content of galactosamine in granulation tissue reported in this study indicates an impairment in mucopolysaccharide metabolism. This is in keeping with the decreased uptake of radioactive sulphate by scorbutic granulation tissue *in vivo* and *in vitro* demonstrated by Kodicek & Loewi (1955) and Upton & Odell (1956). The significantly lower value for total hexosamines obtained by the method of Boas (1953) was due mainly to the decrease in galactosamine, since glucosamine did not change

significantly. Consequently there was a concomitant threefold increase in the glucosamine/galactosamine concentration ratio over that of normal granulation tissue (Tables 2 and 3).

The low content of galactosamine in granulation tissue of scorbutic guinea pigs, as well as the low uptake of [^{35}S]sulphate (Kodicek & Loewi, 1955), indicates the possibility of a block in the formation of galactosamine-containing mucopolysaccharides (galactosaminoglycans).

It has been suggested (Kodicek, 1958, 1965) that, since the formation of glucosamine does not appear to be impaired, according to the biosynthetic pathway of hexosamines the lowered formation of galactosamine might be due to an impairment of the UDP-*N*-acetyl-D-glucosamine 4-epimerase reaction (Kalckar & Maxwell, 1956, 1958). This step catalyses the conversion of UDP-*N*-acetylglucosamine into UDP-*N*-acetylgalactosamine by an oxidoreduction mechanism that is not yet clearly understood and that has been shown to exist in liver (Maley & Maley, 1959; Maley, Tarentino, McGarrah & Del Giacco, 1968). The formation of UDP-*N*-acetylgalactosamine from galactose 1-phosphate via galactosamine 1-phosphate, also by an epimerization, is an alternative pathway suggested by Dorfman (1965); however, at present there is no evidence that this is of major physiological significance.

The cause of the decreased galactosamine content in scurvy might have other explanations. There could be in scurvy an increased turnover of galactosamine after it had been formed, or increased degradation of polymerized galactosaminoglycans.

If [^{14}C]glucose is injected into control and scorbutic animals, one would expect that a similar impairment would appear in the radioactivity associated with the hexosamines. That glucose is the precursor of glucosamine has been shown by Roseman, Moses, Ludowieg & Dorfman (1953), Roseman, Ludowieg, Moses & Dorfman (1954) and Dorfman, Roseman, Moses, Ludowieg & Mayeda (1955).

In both tissues a lower rate of synthesis of galactosaminoglycans occurred in scorbutic animals. In cartilage, the total concentration of galactosamine was unaffected by scurvy (Table 2), in view of the presence of large amounts of preformed galactosaminoglycans, and therefore a lower specific radioactivity was observed (Table 7). On the other hand, in newly formed granulation tissue in scurvy, the galactosamine was formed during the healing period, resulting in a lower concentration (Table 2), and the final specific radioactivity was therefore the same as in the control group (Table 5).

Robertson & Hinds (1956) and Slack (1958) found an increased amount of glucosamine in scorbutic granulomas induced by carrageenin. Granulation

tissue originating from a healing process in surgical wounds is a physiological repair mechanism and therefore differs in many respects from the irritation granuloma produced by implantation of foreign substances (Gilman, 1957). This is likely to be reflected in differences in the nature and time of onset of connective-tissue synthesis and of response to scurvy (Kirchheiner, 1968), so that results obtained from the two techniques are not strictly comparable. Even the positioning of the surgical wound in the form of a surface skin wound in contrast with a deep-seated tendon wound will affect certain biochemical and histochemical parameters (Dunphy *et al.* 1956).

Kirchheiner (1968) studied changes in mucopolysaccharides in carrageenin granulomas after a 14-day period of ascorbic acid deficiency, and reported an increase in hyaluronic acid, glycoproteins and [^{35}S]sulphate incorporation. From the growth curves published it appears that the animals were barely deficient. Unpublished results from our Laboratory (I. Antonowicz & E. Kodicek) indicate that changes in mucopolysaccharide content, as described in this study, occur only when the adrenal ascorbic acid decreases significantly and the weight starts to decline.

The apparent effects of inanition on acid mucopolysaccharides (Friberg, 1958; Kofoed & Robertson, 1966) have obscured the interpretation of studies on mucopolysaccharide metabolism in scurvy just as until recently they interfered with the interpretation of collagen changes (Gross, 1959). The specific involvement of ascorbic acid in collagen biosynthesis is indicated by the more detailed knowledge of the hydroxylating mechanism of proline (Hutton, Kaplan & Udenfriend, 1967; Hutton, Tappel & Udenfriend, 1967; Kivirikko & Prockop, 1967). In mucopolysaccharides, Gore *et al.* (1965) did not find any absolute changes in sulphated glycosaminoglycans except in dermatan sulphate in guinea-pig aortas. This result is not surprising in view of their slow turnover in preformed tissue and agrees with our chemical finding on cartilage. The results of Friberg (1958) and Kofoed & Robertson (1966) on the role of inanition are in direct contrast with our results on pair-fed controls, which showed no decrease in galactosaminoglycan content and were similar to normal controls fed *ad lib*. These findings confirm observations of Hughes & Kodicek (1960) that the wounds of pair-fed animals had a normal histochemical and histological appearance. They are also in keeping with results of more extensive chemical studies reported by Bates, Levene & Kodicek (1968).

The demonstration that normal human fibroblasts and those obtained from patients with Hurler's syndrome (Schafer *et al.* 1966, 1967, 1968) display a requirement for ascorbic acid for the

formation of some acid galactosaminoglycans indicates that ascorbic acid may play a role in their intermediary metabolism. In contrast, Reynolds (1966) did not observe any differences in sulphated mucopolysaccharides between chick-bone rudiments grown *in vitro* in a medium with added ascorbic acid or without such a supplement.

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