The Effect of Scurvy on Hexosamine-Containing Substances in Healing Wounds in Guinea Pigs

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(Received 3 March 1969)

1. Granulation tissue from healing tendonectomy wounds in guinea pigs was analysed and the effects of inanition and ascorbic acid deficiency on this tissue were investigated. 2. Inanition produced no significant effect on either the glucosamine or the galactosamine content of the tissue. Ascorbic acid deficiency decreased the galactosamine content without affecting the glucosamine content. 3. Fractionation of papain-digested granulation tissue gave three major fractions, which behaved respectively as glycopeptide, hyaluronic acid and a sulphated glycosaminoglycan mixture. At least half of the sulphated glycosaminoglycan mixture behaved as dermatan sulphate. 4. Inanition produced no consistent effect on the fractions examined. In ascorbic acid deficiency, a decrease in the sulphated glycosaminoglycan fraction was observed, which accounted for the decreased galactosamine content of the tissue. This was accompanied by a decrease in hyaluronic acid and a slight increase in the glycopeptide fraction.

The involvement of ascorbic acid in the formation of collagen in healing wounds is well established (see reviews by Robertson, 1961; Kodicek, 1964). In addition to collagen, normal intercellular matrix may also contain glycosaminoglycans and glycoproteins. The structural role of these substances in the healing wound is poorly understood (see reviews by Chen & Postlethwait, 1964; Gardner, 1967), and studies of the effect of ascorbic acid on their synthesis have given apparently contradictory results (Kofoed & Robertson, 1966).

Granulation tissue formed after tendonectomy in hind legs of guinea pigs was chosen for study in our laboratory, because the new tissue can fairly readily be distinguished from pre-existing ones. Histological examination (Bradfield & Kodicek, 1951; Kodicek, 1961) has revealed abnormalities in scorbutic granulation tissue that indicate an abnormal polysaccharide content. Later studies (Hughes & Kodicek, 1960; Kodicek, 1964; Antonowicz & Kodicek, 1968) have shown that, although the glucosamine content of scorbutic tissue is unchanged, the galactosamine content is diminished.

The purpose of the present study was, first, to determine whether the decreased galactosamine content is a direct result of ascorbic acid deficiency, or a secondary effect of inanition in the deficient animals as reported by Friberg (1958) and Kofoed & Robertson (1966), and, secondly, to fractionate

and analyse the tissue in order to define the lesion more precisely.

MATERIALS AND METHODS

D-Glucosamine, D-galactose and D-glucuronolactone were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks. D-Galactosamine and N-acetylneuraminic acid were obtained from Sigma (London) Chemical Co., London S.W.6. Papain (twice crystallized) was obtained from Sigma (London) Chemical Co. and testicular hyaluronidase type HSEP was obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Hyaluronic acid, chondroitin sulphate A and dermatan sulphate were generously given by Dr J. A. Cifonelli. D-[1-¹⁴C]Glucosamine and D-[1-¹⁴C]-galactosamine (each 4 mc/m-mole) were obtained from The Radiochemical Centre, Amersham, Bucks. Cellulose acetate strips (31 cm. × 2.5 cm.) were obtained from Millipore Filter Corp., Bedford, Mass., U.S.A.

Male albino guinea pigs weighing 250–330 g. received a modified form of the ascorbic acid-free diet described by Reid & Briggs (1953), either with no added ascorbic acid (deficient group) or with 0.2% (w/w) ascorbic acid (controls fed *ad lib.* and pair-fed groups respectively). The food intake of the controls fed *ad lib.* and scorbutic animals was unrestricted; that of each pair-fed animal was restricted to the quantity eaten by the corresponding scorbutic animal on the previous day.

Ten days after the beginning of the experiment tendonectomy was performed under diethyl ether anaesthesia. All tendons were removed from both hind legs of each animal; sulphonamide was inserted in the wound to lessen the risk of infection, and the skin edges of the wound were

brought together with cotton sutures (Kodicek & Loewi, 1955). Ten days later the animals were killed, and the newly formed granulation tissue, freed from skin and fascia, was excised, weighed and defatted by mincing in acetone-diethyl ether (1:1, v/v), with three changes of solvent, each of 10 ml. The material was dried under reduced pressure over P_2O_5 and reweighed.

Total nitrogen was determined by the method of Johnson (1941), and hydroxyproline was measured by the method of Bergman & Loxley (1963).

Determination of hexosamines in dry defatted tissue. The granulation tissue from each animal was analysed separately.

Samples (about 4 mg.) were suspended in 4 ml. of 4 M-HCl and hydrolysed for 4 hr. at 100° in Teflon-lined screw-capped tubes. A small amount of undissolved material was removed by centrifugation and the supernatant solutions were evaporated in a Buchler Rotary Evapo-mix at a temperature not exceeding 40°. The samples were re-dissolved in water and two portions (each 15% of the whole) were removed for analysis of total hexosamine by a modification of the method of Neuhaus & Letzring (1957). Two further portions (each 30% of the whole) were mixed with tracer amounts (0.0025 μ C) of D-[1- 14 C]glucosamine and D-[1- 14 C]galactosamine as markers and used for the analysis of glucosamine and galactosamine. Separation was achieved

by paper chromatography on sheets of Whatman no. 7 paper, 50 cm. in length, pretreated with 0.1 M-BaCl₂ solution (Heyworth, Perkins & Walker, 1961) to improve separation. The solvent was butan-1-ol-pyridine-water (6:4:3, by vol.), and development took place in the descending direction for 24 hr. The paper was dried and cut into strips, and the radioactive areas were located by a Nuclear-Chicago Actigraph II radiochromatogram scanner. The two hexosamine spots on each strip were cut out and analysed directly by a modified Neuhaus & Letzring (1957) assay. Glucosamine and galactosamine standards were treated with the same procedure. The quantity of amino sugar present in the tracer mixture was too small to be detected chemically. The advantages of this analytical procedure are that both amino sugars are assayed independently and a large number of samples can be processed together.

Fractionation of dry defatted tissue. The fractionation scheme is summarized in Scheme 1.

Granulation tissue from five or more animals in each group was pooled, to avoid ambiguities caused by individual variation and to obtain sufficient material for chemical analysis.

Stage 1. Samples (about 60 mg.) were mixed with 7.0 ml. of a solution, pH 5.5, containing sodium acetate (0.2 M), cysteine (5 mM), EDTA (5 mM) and crystalline papain

Scheme 1. Fractionation of granulation tissue.

Fig. 1. Distribution of total hexosamine and glycosaminoglycans in Sephadex fractions after digestion with papain of dry fat-free granulation tissue: (a) controls fed *ad lib.*; (b) pair-fed controls; (c) scorbatic animals. Hexosamine is expressed in extinction units obtained in the Neuhaus & Letzring (1957) assay. Glycosaminoglycans (shaded areas) are expressed in extinction units obtained in the Di Ferrante (1956) turbidimetric assay. Sephadex G-50 columns were used, with 0.05 M-pyridine-formate buffer, pH 6.0, as eluant.

(8.8 mg.). They were incubated for 17 hr. at 60°, after which a further 6.5 mg. of cysteine hydrochloride and 5.5 mg. of papain were added, and the samples were incubated for a further 7 hr. at 60°. In one experiment the tissue was heated at 100° for 5 min. in buffer alone before the other components were added: this appeared to improve subsequent digestion and to increase the yield of glycosaminoglycans. After the mixtures were cooled on ice, trichloroacetic acid was added (final concn. 10% [w/v]) and after 30 min. on ice the sediment, designated the 'papain-resistant fraction', was removed by centrifugation and washed with 2 × 1.0 ml. of 10% trichloroacetic acid. The trichloroacetic acid in the soluble fraction was removed by repeated extraction with diethyl ether, and the aqueous solution was evaporated to a small volume under reduced pressure and made up to 0.5 ml. with water. It was applied to a column (0.8 cm. × 25 cm.) of Sephadex G-50 previously equilibrated with 0.50 M-pyridine-formate buffer, pH 6.0, and was eluted with the same buffer. The first 3.0 ml. (void volume) was discarded, and the subsequent 16 ml. was collected in 1.0 ml. fractions. A 20 µl. sample was withdrawn from each fraction for the turbidimetric determination of glycosaminoglycans by the method of Di Ferrante (1956), scaled down to 0.6 ml. final

volume. A 50 µl. sample was withdrawn from each fraction for the determination of hexosamine: after hydrolysis with 4 M-HCl for 4 hr. at 100° and evaporation, the modified Neuhaus & Letzring (1957) assay was used.

Fig. 1 presents a typical result: in each case there was a high-molecular-weight hexosamine-containing peak containing material giving turbidity in the Di Ferrante assay, and a lower-molecular-weight hexosamine-containing peak, partially separated from it, which does not react in the Di Ferrante assay. Tubes 3–5, the high-molecular-weight peak, were pooled, and are referred to below as the glycosaminoglycan fraction, and tubes 6–10, the low-molecular-weight peak, were also pooled and are referred to below as the glycopeptide fraction. Inorganic salts and other low-molecular-weight material practically devoid of hexosamine were eluted in fractions 10–16; this material was discarded.

Stage 2. The glycosaminoglycan fractions were further fractionated by precipitation of the cetylpyridinium salts on micro-columns containing Whatman Chromedia CF 11 (Antonopoulos, Gardell, Szirmai & de Tyssonsk, 1964; Švejar & Robertson, 1967). They were eluted with salt solutions containing cetylpyridinium chloride, three fractions being obtained: (1) material not precipitable by cetylpyridinium ion; (2) material extractable with 0.3 M-NaCl, designated the hyaluronic acid fraction; (3) material insoluble in 0.3 M-NaCl but extractable with 1.25 M-MgCl₂, designated the sulphated glycosaminoglycan fraction. Cetylpyridinium ion was removed by precipitation with potassium thiocyanate, followed by filtration through a bed of Celite (Korn, 1959). The filtrates were evaporated to a small volume under reduced pressure, and salt was removed by fractionation on columns of Sephadex G-50 as described above for the original fractionation. The high-molecular-weight glycosaminoglycan material (located by the Di Ferrante assay) was evaporated under reduced pressure and redissolved in 1.0 ml. of a buffer, pH 4.6, containing 0.1 M-sodium acetate and 0.15 M-NaCl. Part was used for the determination of total hexosamine and uronic acid; the remainder (of the hyaluronic acid and sulphated glycosaminoglycan fractions) was treated with testicular hyaluronidase as described below.

Stage 3. Each hyaluronic acid and sulphated glycosaminoglycan fraction (0.7 ml.) was mixed with 50 µg. of testicular hyaluronidase and incubated at 37°. The course of hydrolysis was followed by the Di Ferrante assay. After 1 hr. the turbidity of the hyaluronic acid fractions had fallen to less than 5% of the initial value and that of the sulphated glycosaminoglycan fractions had fallen to less than 30%. After 18 hr. the incubation was terminated and the material was refractionated on Sephadex G-50 as described above. The first 3.0 ml. was discarded and the following 12 ml. was collected in 1.0 ml. fractions. A 0.5 ml. sample from each fraction was evaporated under reduced pressure and hydrolysed, and portions were withdrawn for the analysis of glucosamine and galactosamine. The remaining 0.5 ml. portions of unhydrolysed material were combined into two peaks, fractions 3–5 (hyaluronidase-resistant material) and fractions 6–10 (hyaluronidase-sensitive material), evaporated, redissolved in water and portions were used for the assay of uronic acid and of dermatan sulphate.

Chemical assays. Apart from the studies on unfractionated tissue and on fractions from the first Sephadex column (see above), total hexosamine and glucosamine/galactosamine ratios were measured by the method of Ludowieg &

Benmaman (1967). The hydrolysis conditions were 4M-HCl for 4 hr. at 100° in all cases, and the hydrolysates were evaporated under reduced pressure at a temperature not exceeding 40°. Those known to contain a high proportion of material likely to interfere in the assay, i.e. the glycopeptide and papain-insoluble fractions, were first partly purified by the method of Boas (1953). The calculations are based on the assumption that glucosamine and galactosamine are the only hexosamines present in the material examined.

Hexuronic acid was determined by the method of Galambos (1967) with D-glucuronolactone as standard.

Dermatan sulphate, as a percentage of the total uronic acid-containing material present, was determined from the ratio of the extinctions obtained in the Pelzer & Staib (1957) and Galambos (1967) assays (Schafer, Silverman, Sullivan & Robertson, 1967; Švejcar & Robertson, 1967).

Sialic acid was determined by the method of Warren (1959) with *N*-acetylneuraminic acid as standard, and with the appropriate correction for deoxyribose.

Neutral sugar was determined by the anthrone method, with D-galactose as standard (Roe, 1955).

RESULTS

Body weights. Fig. 2 shows the changes in body weight in a representative experiment involving 15 animals divided into three groups. In the group fed *ad lib.* there was a general upward trend both before and after tendonectomy, whereas in the pair-fed and scorbutic groups there was an upward trend before and a downward trend after 10 days.

Whole-tissue analyses. The weight changes of all

the animals examined and the results of analyses performed on unfractionated granulation tissue are given in Table 1.

The pair-fed group differed from the group fed *ad lib.* in change in body weight, but its granulation tissue did not differ chemically in the parameters examined. The scorbutic group also differed from the group fed *ad lib.* in changes in body weight, and in addition its granulation tissue differed in total hexosamine, galactosamine and hydroxyproline content.

Fractionation of granulation tissue. Table 2 shows the distribution of hexosamine after fractionation of granulation tissue, and Tables 3 and 4 show the results of tests designed to provide further information on the identity of the fractions. Since more than 95% of the hexosamine in the tissue was soluble in 10% trichloroacetic acid after digestion with papain, most of the hexosamines were amenable to fractionation. In the group fed *ad lib.* and the pair-fed group the material soluble in trichloroacetic acid after digestion with papain was distributed about equally between the high-molecular-weight glycosaminoglycan fraction and the lower-molecular-weight glycopeptide fraction.

The glycopeptide fraction was slightly increased in amount in the scorbutic tissue. It contained significant amounts of sialic acid and neutral sugars, and most of the hexosamine appeared to be glucosamine. Uronic acid was difficult to measure in the

Fig. 2. Changes in body weight: (a) controls fed *ad lib.*; (b) pair-fed controls; (c) scorbutic animals. Results are given for five animals, separately, in each group.

Table 1. Total changes in body weight, and glucosamine, galactosamine, total hexosamine, hydroxyproline and total nitrogen contents of granulation tissue after tendonectomy in control and scorbutic guinea pigs

The results are average values $\pm$ s.e.m. calculated for the numbers of samples given in parentheses, except the values for hydroxyproline and total nitrogen content, which are averages of two determinations on pooled tissue of five animals. Determination of total hexosamine was performed separately from the glucosamine and galactosamine assays. The total changes in body weight are those from the beginning of the experiment until the time of death.							
	Total changes in body wt. (g.)	Granulation tissue					Total nitrogen
		Dry fat-free wt. (mg./100 mg. of wet tissue)	Content (mg./100 mg. of dry fat-free tissue)			Hydroxy- proline	
			Glucosamine	Galactosamine	Total hexosamine		
Controls fed <i>ad lib.</i>	+ 89.2 $\pm$ 11.4 (6)	13.0 $\pm$ 0.34 (6)	0.59 $\pm$ 0.040 (6)	0.38 $\pm$ 0.020 (6)	0.95 $\pm$ 0.040 (6)	3.3	13.3
Pair-fed controls	- 17.8 $\pm$ 9.2 (12)	13.1 $\pm$ 0.50 (10)	0.51 $\pm$ 0.020 (9)	0.34 $\pm$ 0.032 (9)	0.99 $\pm$ 0.034 (9)	3.7	13.9
Scorbutic animals	- 49.1 $\pm$ 7.4 (14)	14.3 $\pm$ 0.52 (16)	0.55 $\pm$ 0.031 (13)	0.15 $\pm$ 0.009 (13)	0.72 $\pm$ 0.031 (13)	1.1	12.3
<i>P</i> values							
Between <i>ad lib.</i> -fed and pair-fed	< 0.001	—	> 0.5	0.4-0.5	0.4-0.5	—	—
Between pair-fed and scorbutic	< 0.025	—	> 0.5	< 0.001	< 0.001	—	—

The results are average values ± s.e.m. calculated for the numbers of samples given in parentheses, except the values for hydroxyproline and total nitrogen content, which are averages of two determinations on pooled tissue of five animals. Determination of total hexosamine was performed separately from the glucosamine and galactosamine assays. The total changes in body weight are those from the beginning of the experiment until the time of death.

crude material because of interference by other compounds; its concentration was low, with a uronic acid/hexosamine ratio less than 0.2:1.0.

The glycosaminoglycan fraction, on the other hand, was markedly decreased in amount in the scorbutic tissue. In all three groups about 70-80% of this fraction was recovered in the cetylpyridinium chloride-soluble hyaluronic acid and sulphated glycosaminoglycan subfractions; the remainder was probably lost during fractionation. In other analyses, where a final extraction with 3M-sodium chloride was performed, less than 1% of the hexosamine was eluted at this high salt concentration.

The cetylpyridinium chloride-soluble subfraction was small and variable in amount. Since its uronic acid/hexosamine ratio was close to 1.0:1.0, it may consist of glycosaminoglycans whose molecular weight is too low for precipitation by cetylpyridinium ion. No consistent differences between the various groups were observed in this subfraction.

The hyaluronic acid subfraction had a uronic acid/hexosamine ratio close to 1.0:1.0, the Pelzer & Staib (1957) assay on it indicated a complete absence of dermatan sulphate, its hexosamine was almost entirely glucosamine and testicular hyaluronidase caused complete degradation of it to low-molecular-weight fragments (Tables 3 and 4). On electrophoresis on cellulose acetate strips (Stefanovich & Gore, 1967) a single Alcian Blue-staining band was obtained, with the mobility of hyaluronic acid. The amount obtained from scorbutic granulation tissue was consistently about half that derived from pair-fed animals. In two experiments the amount obtained from animals fed *ad lib.* in the sample examined was similar to that obtained from pair-fed controls, but in a third experiment it was close to that of the scorbutic group.

The sulphated subfraction behaved as a mixture of sulphated glycosaminoglycans when subjected to electrophoresis on cellulose acetate strips. The uronic acid/hexosamine ratio was 1.0:1.0, and in all groups about half of the material was susceptible to hyaluronidase (Tables 3 and 4). The distribution of hexosamine among Sephadex fractions after digestion with hyaluronidase is shown in Fig. 3. Most of the hyaluronidase-resistant material behaved as dermatan sulphate in the Pelzer & Staib (1957) assay, and about half of the hyaluronidase-susceptible material behaved as dermatan sulphate. Both fractions contained galactosamine as the major amino sugar. The sulphated glycosaminoglycan subfraction obtained from tissue of pair-fed animals was similar in quantity to that obtained from animals fed *ad lib.*, but the quantity obtained from scorbutic animals was much smaller. Nevertheless, the composition of this material appeared to be identical in all three groups.

Table 2. *Distribution of hexosamine between principal fractions obtained from granulation tissue*

The results, which are expressed as μ moles of hexosamine/100 mg. of dry fat-free tissue, are the average of two experiments with different batches of pooled tissue from a total of 41 animals.

		Papain-resistant	Soluble in trichloroacetic acid after digestion with papain			
			Glycopeptide	Glycosaminoglycan subfractions		
				Total	Cetylpyridinium chloride-soluble	Hyaluronic acid Sulphated glycosaminoglycan
Controls fed <i>ad lib.</i>	0.15	2.9	3.1	0.22	0.85	0.96
Pair-fed controls	0.21	2.8	3.5	0.36	1.35	1.03
Scorbutic animals	0.19	3.3	1.8	0.27	0.65	0.30

Table 3. *Further analysis of principal fractions and subfractions obtained from granulation tissue*

The results are expressed as molar ratios of the substance assayed to total hexosamine present in each fraction. They were obtained either in a single experiment (1) or as an average of two experiments (2).

Fraction	Assay	Controls fed <i>ad lib.</i>	Pair-fed controls	Scorbutic animals
Papain-resistant	Galactosamine (1)	0.8	0.7	0.7
Glycopeptide	Galactosamine (1)	0.07	0.09	0.02
	Sialic acid (2)	0.30	0.28	0.32
	Neutral sugar (1)	0.38	0.40	0.30
Glycosaminoglycans				
Hyaluronic acid subfraction	Uronic acid (2)	1.0	1.1	1.2
	Galactosamine (1)	0.07	0.06	0.02
Sulphated glycosaminoglycan subfraction	Uronic acid (2)	1.0	1.0	1.0
	Galactosamine (1)	0.8	0.8	0.6

Table 4. *Digestibility with testicular hyaluronidase, and dermatan sulphate content, of hyaluronic acid and sulphated glycosaminoglycan subfractions*

Fraction	Assay	Controls fed <i>ad lib.</i>	Pair-fed controls	Scorbutic animals
Glycosaminoglycans				
Hyaluronic acid subfraction	% digestible	98	97	99
Sulphated glycosaminoglycan subfraction	% digestible	60	55	52
	Digestible			
	Dermatan sulphate content (%)	40	50	60
Resistant fraction	Dermatan sulphate content (%)	> 90	> 90	> 90

DISCUSSION

Changes in body weight during the experimental period indicate that the effects of inanition in the ascorbic acid-deficient animals can be simulated by pair-feeding, although the weight loss in the ascorbic acid-deficient group was significantly greater ($P < 0.025$) than in the pair-fed group.

Analyses performed on unfractionated granulation tissue clearly indicate that the decrease in the galactosamine content of the scorbutic tissue is not an effect of inanition, and that it is quantitatively

comparable with the decrease in the hydroxyproline content.

Attempts to fractionate the granulation tissue were hampered by scarcity of material: each animal yields 20–70 mg. of dry defatted tissue, of which about 1% by weight is glycosaminoglycans. The fractionation procedure was designed to obtain the maximum information from the small amounts of material available.

In general, no qualitative or quantitative differences were detected between the granulation tissue from animals fed *ad lib.* and from pair-fed animals.

Fig. 3. Distribution of hexosamine in Sephadex fractions after hyaluronidase digestion of the sulphated glycosaminoglycan fractions: (a) controls fed *ad lib.*; (b) pair-fed controls; (c) scorbatic animals. Hexosamine is expressed in extinction units obtained in the Neuhaus & Letzring (1957) assay. Sephadex G-50 columns were used with 0.50M-pyridine-formate buffer, pH 6.0, as eluent.

The only exception to this was a difference, observed in one experiment only, between the hyaluronic acid subfractions of these two groups. The reason for this inconsistent result is not known.

Between the pair-fed and scorbutic groups, however, the hyaluronic acid and sulphated glycosaminoglycan subfractions both showed a consistent quantitative difference. Since the total glucosamine content of whole granulation tissue was identical in all three groups, the difference in amount of hyaluronic acid may reflect a change in its degree of polymerization or association with other molecules (cf. Thunell, 1967). However, since the sulphated glycosaminoglycan subfraction alone contained significant amounts of galactosamine, its decrease by 70% in the scorbutic granulation tissue cannot represent redistribution of material as a result of a change in physical properties. In this instance the decrease seems to imply a more fundamental effect of scurvy, and is consistent with the observed decrease in galactosamine content of the whole tissue, as

reported previously (Hughes & Kodicek, 1960; Kodicek, 1964; Antonowicz & Kodicek, 1968) and confirmed by the results in Table 1.

Of the sulphated subfraction, about 50% behaved in every respect as dermatan sulphate: it was resistant to testicular hyaluronidase and it gave the typical reaction of dermatan sulphate in the Pelzer & Staib (1957) assay. The remaining 50% was sensitive to testicular hyaluronidase, as measured by the behaviour of the products of digestion on a column of Sephadex G-50, but about half of this material still gave the typical Pelzer & Staib reaction of dermatan sulphate. This result may reflect the sensitivity to hyaluronidase of occasional glucuronic acid residues in dermatan sulphate (Fransson & Rodén, 1967), and suggests that the sulphated glycosaminoglycan fraction is probably about 75% dermatan sulphate. Smaller amounts of other glycosaminoglycans may also be present, particularly in the sulphated subfraction (Meyer, Davidson, Linker & Hoffman, 1956; Dörner, Antonopoulos & Gardell, 1968), but lack of material prevented us from performing a more detailed study of these minor components.

The observed decrease in amount of glycosaminoglycans in scorbutic granulation tissue may be attributable to a variety of causes. One is the lack of one or more essential building blocks, such as galactosamine (Kodicek, 1964; Antonowicz & Kodicek, 1968), or of energy sources, resulting from a disturbance in intermediary metabolism (Banerjee & Kawishwar, 1959; Ginter, Bobek, Kopec, Ovečka & Cerey, 1967; Schafer *et al.* 1967). Another possibility is the inhibition of glycosaminoglycan synthesis at the protein or polysaccharide level, possibly connected with the observed disturbances of the endoplasmic reticulum and other cell organelles (reviewed by Ross, 1968). Other possible causes include effects secondary to hormonal disturbances (Kitabchi, 1967; Sulimovici & Boyd, 1968), increased degradation of glycosaminoglycans, possibly associated with an abnormal release or activation or proteases or polysaccharidases (Messina, Girlando & Bruccheri, 1968; Reppert, Donegan & Hines, 1951), and decreased extracellular fixation of glycosaminoglycans, possibly resulting from a lack of nascent collagen fibres, which may be required to bind them into a permanent stable structure.

It is not yet possible to attribute the observed changes to any one particular mechanism on the basis of the available evidence. A survey of the literature on the effect of scurvy on glycosaminoglycans (Antonowicz & Kodicek, 1968; Bates & Levene, 1969) suggests that the changes observed are highly dependent on the type of system chosen, and that different glycosaminoglycans may behave in different ways. It is clearly of great importance

to select for study systems in which individual glycosaminoglycans can be identified and independently observed, and in which the secondary effects of scurvy, such as inanition, can be avoided or controlled.

We are indebted to Mr B. J. Pope and Miss J. Boughen for their valuable technical assistance.

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