Such an action of propionate is not supported by the relatively small inhibition of acetylation obtained with the soluble preparations. It is, however, not necessarily excluded by this result; two possibilities might be suggested. Inhibition may be caused, not by propionate, but by a metabolite of propionate such as propionyl-coenzyme A, which may occur in much higher concentration at the active enzyme site in the mitochondria than is attained in the soluble system. The relatively slow oxidation of propionate by rat-liver homogenates may result in the accumulation of an intermediate in greater concentration than would occur if the metabolism of propionate proceeded normally. A second possibility is suggested by the work of Aisenberg & Potter (1956), who found that activation of acetate by the enzyme in rat-kidney mitochondria was more sensitive to fluoride than was activation by the enzyme in the supernatant fraction of the cells. It is conceivable that a parallel situation occurs in rat liver, i.e. that the cell contains two acetic thiokinases and the one within the mitochondria is more susceptible to inhibition by propionate. The present results could then be explained if, of the two, only the mitochondrial enzyme were linked with the enzymes of the Krebs cycle. Both of these possibilities should be open to testing.

SUMMARY

1. The production of $^{14}\text{CO}_2$ from carboxyl-labelled acetate by rat-liver homogenates was almost completely abolished by propionate in only one-tenth of the concentration of the acetate.

Butyrate was a less powerful inhibitor of oxidation of acetate.

2. The oxidation of $[2-^{14}\text{C}]$ pyruvate was affected to a much smaller extent by propionate, although the incorporation of isotope into acetoacetate was largely suppressed.

3. Oxidation of acetate was strongly inhibited by propionate in sheep-liver homogenates; in pigeon-liver homogenates the inhibition was much less.

4. The oxidation of propionate by rat-liver homogenates was much slower than its oxidation by sheep- or pigeon-liver homogenates.

5. The acetylation of sulphanilamide by a rat-liver extract, together with an acetone fraction of pigeon liver containing the amine-acetylating enzyme, was inhibited to only a relatively small extent by propionate.

6. The very low rate of oxidation of acetate by rat-liver homogenates in the presence of propionate was increased by the addition of coenzyme A.

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Connective Tissue Growth Stimulated by Carrageenin

3. THE NATURE AND AMOUNT OF POLYSACCHARIDE PRODUCED IN NORMAL AND SCORBUTIC GUINEA PIGS AND THE METABOLISM OF A CHONDROITIN SULPHURIC ACID FRACTION*

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It is well known that new connective tissue formed during the repair of wounds in scorbutic animals is abnormal histologically and in other respects, e.g. the tensile strength of this repair tissue is much less than that formed when adequate amounts of ascorbic acid are available (Wolbach & Bessey, 1942). Evidence of impaired collagen formation in the scorbutic tissue has been presented, e.g.

Robertson & Schwartz (1953), Kodicek & Loewi (1955), Dunphy & Udupa (1955). Abnormalities in the mucopolysaccharides in the ascorbic acid-deficient repair tissue have also been the subject of a number of reports during the past few years, but the exact nature of these changes, qualitative and quantitative, is incomplete and still the subject of some dispute. Persson (1953) found more hexosamine in scorbutic tissues than in normal tissues. Dunphy & Udupa (1955) also refer to increased

* Part 2: Slack (1957).

mucopolysaccharide in scorbutic granulation tissue. On the other hand, Kodicek & Loewi (1955) report similar hexosamine contents in normal and scorbutic repair tissue and Ludwig (1954) also found no difference in hexosamine content in new retrobulbar tissue in both normal and scorbutic guinea pigs. Reddi & Norström (1954) reported a diminished uptake of ^{35}S in scorbutic cartilage which has been confirmed by Kodicek & Loewi (1955). On the basis of histochemical studies Bradfield & Kodicek (1951) have postulated the presence of an abnormal non-sulphated mucopolysaccharide in scorbutic skin wounds. Gersh & Catchpole (1951) considered that the observed changes indicated a depolymerization of glycoproteins.

Robertson & Hinds (1956) report on a study of the mucopolysaccharides formed in granulation tissue resulting from the injection of carrageenin into guinea pigs. They found that the granuloma tissue forming in scorbutic guinea pigs contained about five times as much mucopolysaccharide as did the tissue produced in animals adequately supplied with ascorbic acid. The predominant polysaccharide which accumulated in the scorbutic granuloma tissue gave highly viscous solutions, suggesting a high degree of polymerization. The authors state that this polysaccharide was isolated and identified as hyaluronic acid, but sufficient experimental details to support this statement are not given in the paper. In a series of investigations into the pathology and biochemistry of the granulation tissue formed under the stimulus of carrageenin injections into guinea pigs [cf. Jackson (1957), Slack (1957), Williams (1957)] the formation of collagen and mucopolysaccharides has now been studied in this newly formed connective tissue in scorbutic guinea pigs. The present paper reports the separation and analysis of some polysaccharide fractions from these carrageenin granulation tissues in normal and scorbutic animals, and extends the interesting observations of Robertson & Hinds (1956).

Some information has been obtained about the incorporation of ^{35}S -labelled sulphate into the sulphated polysaccharides of the granulomata in scorbutic animals as compared with that in normal animals. At the same time the incorporation of labelled sulphate into the chondroitin sulphate of rib cartilage in the two groups of animals has been determined for comparison with that from the granulomata and with material isolated by Reddi & Norström (1954).

EXPERIMENTAL METHODS

Treatment of animals

Albino guinea pigs of both sexes, weighing between 350 and 450 g., were used. Considerable difficulties were en-

countered in arranging satisfactory experiments in which groups of guinea pigs on a diet with adequate ascorbic acid intake could be matched with guinea pigs which had become demonstrably deficient in vitamin C and which had been injected with carrageenin at the same time as the non-scorbutic group: a series of experiments was attempted first with diet no. 18 (Bruce & Parkes, 1946). For normal animals this was supplemented by hay and greens, and ascorbic acid; the 'scorbutic' animals received only the diet. These experiments proved quite unsatisfactory since we found great variations in the ascorbic acid contents of the commercial supplies of diet no. 18, which made it impossible to obtain groups of guinea pigs with a reasonably uniform state of scorbutus at predictable time intervals. Some animals were kept for many months on diet no. 18 only, and on this diet grew normally and remained in apparent good health. A satisfactory routine was eventually established with a diet described by Kodicek & Loewi (1955). This consisted of Sussex ground oats 525 g., fish meal 215 g., bran 130 g., dried yeast 80 g., salt mixture 20 g., given *ad libitum*. In normal animals the diet was supplemented by regular greens and ascorbic acid (50 mg. every other day from the time of injection of carrageenin). Not all groups of guinea pigs will eat this diet freely and it was found preferable to present the mixture to the animals in pellet form in order to ensure an adequate intake of protein. With guinea pigs weighing 350–450 g. satisfactory growth curves are obtained with ascorbic acid supplement (or greens). Without greens or ascorbic acid a state of vitamin C deficiency was reached in 7 days, as judged by a slowing down of the rate of increase in body weight, depletion of the adrenal ascorbic acid level and general appearance of the animals. In these experiments the groups of scorbutic guinea pigs were left 7 or 10 days without greens or ascorbic acid before injection of carrageenin. After the injection of 5 ml. of a 1% solution of carrageenin in 0.9% NaCl subcutaneously into the lower abdominal region under ether anaesthesia, the scorbutic guinea pigs generally survived a further 14 days. Longer term experiments became difficult because of the rapidly mounting death rate from ascorbic acid deficiency after 21 days. Older animals developed demonstrable ascorbic acid deficiency more slowly and at much more variable individual rates.

The animals were killed by ether inhalation. The abdominal skin was then peeled back, the mass of new connective tissue was dissected free from the abdominal and chest wall, macerated with scissors, crushed after freezing in liquid nitrogen, and weighed.

Isolation of mucopolysaccharide fractions

All extractions were carried out in the presence of octanol as preservative.

The first step in the extraction of the mucopolysaccharides consisted in digestion of the crushed granuloma tissue with papain for 24 hr. at 45°, as described previously (Slack, 1957). The granulomata from several animals at each interval of time were pooled as shown in Table 1. The cooled digest was then filtered through Filtercel (Johns-Manville Co.) which removed insoluble debris and free fat, giving a clear, straw-coloured filtrate with negligible loss of polysaccharides (chondroitin sulphate added to crude protein hydrolysates could be recovered quantitatively after filtration). After overnight dialysis against running

water the filtrate was reduced in volume at 50° under vacuum to 20–25 ml. Solid KCl was added to give a final concentration of 1.2M-KCl and a 1% (w/v) solution in water of cetylpyridinium chloride (CPC) slowly added dropwise, with shaking, until a persistent foam formed on the liquid surface, indicating excess of CPC. Under these conditions there is selective precipitation of carrageenin without loss of CPC complexes of less highly sulphated polysaccharides (Scott, 1955). After standing at 30° for at least 2 hr. or overnight, the CPC-carrageenin complex was centrifuged down at 500 *g* for 15 min. and discarded. The supernatant was then dialysed thoroughly, first against tap water overnight and then against several changes of 4 l. of distilled water for 48 hr. The volume of the diffusate was again reduced to 20 ml. under vacuum and adjusted to 0.005M concentration of sodium borate. Cetylpyridinium chloride (1%, w/v in water) was then slowly added to excess and the mixture allowed to stand for 2 hr. or more at 30°. Almost complete precipitation of all tissue polysaccharides was obtained under these conditions as CPC-borate complexes, and these were centrifuged at 1500 *g*. A small proportion of hyaluronic acid remained in the supernatant after centrifuging and this was recovered by thorough dialysis against distilled water containing 0.01% CPC; the dialysis required several days, with twice-daily changes of 4 l. of glass-distilled water containing CPC. The total CPC-borate-polysaccharide complexes first precipitated were now dissociated by shaking with 10 ml. or so of

methanol-saline at 30–37°. The methanol-saline consisted of equal parts (by vol.) of methanol, water and saturated NaCl solution and was used throughout this extraction procedure for releasing polysaccharides from their CPC complexes. After solution of the polysaccharide complexes in methanol-saline the free CPC was removed by shaking with fuller's earth. The amount of fuller's earth required was judged by the disappearance of persistent frothing on shaking the mixture, which indicated adsorption of all free CPC. The fuller's earth was then centrifuged, and the polysaccharides precipitated from the supernatant by the addition of 3 vol. of ethanol and standing overnight. The precipitated polysaccharides were dissolved in 10–15 ml. of water, dialysed against three changes, each of 4 l., of distilled water and reprecipitated by 3 vol. of ethanol. The polysaccharides were centrifuged down after standing overnight, washed with ethanol-ether (50:50, v/v) and dried *in vacuo* for 24 hr. over P₂O₅. The weight of this material, with the addition of a small amount of hyaluronic acid recovered above on dialysis, represents the total polysaccharide extracted by this procedure (Fig. 1, *T*). Resolution of the polysaccharides into hyaluronic acid and chondroitin sulphate was then effected according to the scheme given in Fig. 1, cetylpyridinium chloride fractionation from solutions of known ion concentration being used. In 0.2M-Na₂SO₄ there was complete inhibition of hyaluronic acid precipitation as the CPC complex. Addition of the CPC, as a 0.1% solution in 0.2M-Na₂SO₄, was best carried out slowly with

Fig 1. Fractionation of mucopolysaccharides. CPC, Cetylpyridinium chloride.

gentle shaking at 30° and in two or more stages, the mixture being set aside for 2 hr. or more on each occasion and centrifuged at each stage. This more readily ensured complete precipitation of the chondroitin sulphate complex since it was more certain that an adequate excess of CPC was present. Subsequent complete precipitation of the hyaluronic acid complex required very thorough dialysis to prevent inhibition by salt of the precipitation. The scheme given in Fig. 1, elaborated in collaboration with Dr J. E. Scott, was extended in the experiments in an attempt to separate keratosulphate if this were present. No polysaccharide soluble in 1% CPC, as is keratosulphate (Borelius, Gardell & Scott, 1958), was found.

The chondroitin sulphate was further fractionated by increasing ethanol concentrations in the presence of calcium acetate 5% (w/v) and 0.5*N*-acetic acid (Meyer, Linker, Davidson & Weissman, 1953).

Radioactivity experiments

Injection of sodium [³⁵S]sulphate. In all instances the labelled sulphate was administered as Na₂³⁵SO₄ (Radiochemical Centre, Amersham) dissolved in 0.9% NaCl at pH 6.8. The dose was approx. 1 μC/g. body wt., given in a single injection subcutaneously into the right thigh.

The observed counts have been corrected for differences in administered dose ³⁵S/g. body wt.

Measurement of radioactivities. Sulphate was liberated by hydrolysis from polysaccharide samples with aq. 25% (v/v) formic acid (98–100%) in sealed tubes at 100° for 18–24 hr. The free sulphate was precipitated as 4-chloro-4'-aminodiphenyl sulphate. The precipitate was dissolved in 1 ml. (more if required) of absolute methanol and portions (0.025 ml.) were pipetted on to four disks of thin lens paper cut to fit the wells of 1 cm.² polythene counting planchettes. After drying at 45° the planchettes were counted as negligible-thickness samples, as in the method of Henriques, Henriques & Neuberger (1955), standard scaling equipment and a thin end-window tube being used. By this method 0.005 μC of ³⁵S coprecipitated with amounts of 5–75 mg. of carrier sulphate gave, on the average, 870 counts/min. (maximum observed, 890/min.; minimum, 840/min.). With amounts of carrier sulphate between 75 and 200 μg. the observed counts rose linearly from 870/min. to 970/min. Increasing amounts of ³⁵S coprecipitated with a standard amount (25 μg.) of carrier sulphate showed a linear increase in recorded counts/min. from 40/min. for 0.00025 μC of ³⁵S to 1600/min. for 0.01 μC of ³⁵S. Amounts of ³⁵S of the order of 10⁻⁴ μC are therefore detectable as giving about twice the background counts of 8–9/min.

Chemical analyses

Estimation of total hexosamines. These were done according to the method of Boas (1953).

Separation and measurement of glucosamine and galactosamine. In some of the analyses these were separated on Dowex 50 ion-exchange columns, as described by Gardell (1953), and determined.

Estimation of hexuronic acid. The carbazole method of Dische (1947) was used, readings being taken at 535 mμ against glucuronic acid standards and known amounts of a highly purified sample of cartilage chondroitin sulphate.

Optical rotations. These were kindly determined by Dr Barker, Department of Chemistry, University of Manchester.

Measurement of sulphate. This was determined in samples of polysaccharides hydrolysed for 24 hr. in aq. 25% (v/v) formic acid (98–100%). The method has been developed from the use of 4-chloro-4'-aminodiphenyl, described by Belcher & Wilson (1956), to enable microgram amounts of sulphate to be determined, and details will be published elsewhere by Dr J. E. Scott. 4-Chloro-4'-aminodiphenyl is superior to benzidine as a sulphate precipitant: it is much less subject to interference from other constituents in hydrolysates and the crystalline 4-chloro-4'-aminodiphenyl sulphate precipitate aggregates and comes down from the solution very much more readily than does benzidine sulphate. In brief, the method consists in diluting portions of the hydrolysate approximately 1:10 with water and adding equal volumes of a 0.24% solution of 4-chloro-4'-aminodiphenyl hydrochloride in 0.05*N*-HCl. The precipitation is carried out in conical centrifuge tubes at between pH 1 and 2. After mixing and standing for a few hours, or overnight, the precipitate can be readily centrifuged down. The supernatant is decanted off carefully and the precipitate washed with water saturated with 4-chloro-4'-aminodiphenyl sulphate. After standing for ½ hr. or so the washed precipitate is recentrifuged, the supernatant decanted and the tubes are allowed to drain. The precipitate is then dissolved in a convenient volume of absolute methanol, e.g. 10 ml., and the absorption read at 254 mμ. Blanks are set up with similar amounts of 25% formic acid, diluted 1:10, and the 4-chloro-4'-aminodiphenyl hydrochloride solution, and adjusted to between pH 1 and 2. Suitable standard sulphate solutions are prepared so that an absorption curve in the range 0–50 μg. of sulphate can be plotted. The absorption at 254 mμ bears a linear relation to microgram amounts of 4-chloro-4'-aminodiphenyl sulphate and shows an intense, sharp peak at this wavelength. This method of measuring sulphate is particularly useful in experiments with ³⁵SO₄, since the radioactivity of the 4-chloro-4'-aminodiphenyl solution in methanol can be determined at the same time, with a known volume pipetted onto a lens-paper disk and counted as a negligible-thickness disk. I have been informed by Dr S. L. Rose of Imperial Chemical Industries Ltd. that 4-chloro-4'-aminodiphenyl is a most potent carcinogenic agent. Special care is therefore called for in using this reagent.

Adrenal ascorbic acid. This was determined in adrenal tissue shortly after death, by the method described by Glick (1954).

Nitrogen. The method of Chibnall, Rees & Williams (1943) was used.

RESULTS

Before considering the results obtained in these experiments some further comment on the methods used for extracting the mucopolysaccharides may be considered relevant. The present procedure for isolating chondroitin sulphate and hyaluronic acid, with the quaternary ammonium derivative cetylpyridinium chloride, is based on a considerable preliminary experience in collaboration with Dr J. E. Scott. Samples of isolated chondroitin sulphate from rib cartilage and umbilical cord

Table 1. *Details of animal experiments*

The number of days of scorbutus includes the period after injection of carrageenin. The guinea pigs were killed at the times given below and granulomata were pooled from the stated number of animals for extraction of polysaccharides. Figures for adrenal-gland ascorbic acid are averages of pooled adrenal glands. In 50 individual normal animals the lowest ascorbic acid level found was 0.52 mg./g. of adrenal tissue; the average was 1.26 mg./g. Total polysaccharide is the fraction indicated by *T* in the fractionation scheme, Fig. 1. Either mean figures ($\pm$ s.d.), or the values obtained for tissue pooled from several animals, are given.

No. of expt.	Condition of animals	No. of animals	Duration of granuloma (days)	Average wet wt. of granuloma/animal (g.)	Average water content (g./100 g. of tissue)	Total polysaccharide* (mg./g.)		Adrenal gland ascorbic acid (mg./g.)
						Wet tissue	Dry tissue	
A 1	Scorbutic (16 days)	9	6	14.4 $\pm$ 4.2	89 $\pm$ 0.8	1.01	9.2	0.24
2	Normal	5	6	16.8 $\pm$ 3.0	86 $\pm$ 0.6	0.39	2.8	1.28
3	Scorbutic (19 days)	9	9	14.0 $\pm$ 2.9	89 $\pm$ 0.7	1.25	11.4	0.11
4	Normal	5	9	18.4 $\pm$ 5.1	85 $\pm$ 0.4	0.30	2.0	1.21
5	Scorbutic (22 days)	9	12	7.7 $\pm$ 1.6	87 $\pm$ 1.4	0.69	5.3	0.04
6	Normal	5	12	14.0 $\pm$ 2.7	83 $\pm$ 0.3	0.28	1.7	1.03
B 1	Scorbutic (13 days)	8	6	11.1 $\pm$ 4.4	88 $\pm$ 1.1	0.67	5.6	0.29
2	Normal	6	6	14.0 $\pm$ 3.8	85 $\pm$ 0.6	0.33	2.2	1.32
3	Scorbutic (16 days)	8	9	6.8 $\pm$ 3.1	88 $\pm$ 0.9	0.97	8.1	0.18
4	Normal	6	9	9.7 $\pm$ 2.3	84 $\pm$ 0.5	0.29	1.8	1.40
C 1	Scorbutic (14 days)	17	7	14.2	87	0.25	9.6	0.36
2	Normal	12	7	16.7	84	0.26	1.6	1.37

* Dried by ethanol-ether.

hyaluronic acid, together incubated overnight with muscle tissue and papain, can both be recovered separately in good yield by this method. The recoveries of chondroitin sulphate were of the order of 85 %. Recovery of hyaluronic acid appeared to be somewhat lower and more variable, 70–80 %, but the sulphate content of the recovered hyaluronic acid was always less than the original umbilical cord sample, e.g. 0.19 as against 1.2 % of sulphate in the original. At least some of the loss in recovery of hyaluronic acid may therefore be attributed to a 'purification' during the recovery procedure. In previous experiments in this series on the carrageenin-induced granuloma (Slack, 1957) the fractionation procedure was designed to separate fractions of sulphated polysaccharides with different metabolic behaviour. That is, polysaccharides extractable by neutral salt solution (0.2M-NaCl, pH 7.4) are presumably more immediately available in the tissues for metabolic interactions, and therefore may have a more active metabolism than those requiring papain digestion to release them. In the present experiments the chondroitin sulphate fraction obtained is derived from the total chondroitin sulphate originally present in the tissue and is probably a mixture of components with different metabolic behaviours. Further experiments are required to apply this method of fractionating mucopolysaccharides to tissue material previously (Slack, 1957) separated into neutral salt-soluble fraction, papain-digest fraction and residue material.

From Table 1 it will be seen that the total amount of new connective tissue formed is less in the scorbutic than in the normal animal. Also this granuloma tissue in the scorbutic guinea pigs has a higher water content than has the tissue found in normal animals. The differences in amounts of granuloma found in normal and scorbutic animals given in these experiments are not very large. If, however, the duration of ascorbic acid deficiency is extended to 21 days or more before injection of carrageenin then the amount of new connective tissue formed 6 days after carrageenin was greatly reduced in comparison with the normal animals. Experimental results for these longer periods of ascorbic acid deficiency are not included because of (a) the high mortality rate and (b) loss of appetite in the animals and therefore the difficulty in assessing effects due to ascorbic acid deficiency and those due to protein and other deficiencies. From the figures of abnormal ascorbic acid contents given in Table 1 it would seem probable that a state of real ascorbic acid deficiency had been established by the dietary regime in the vitamin C-deficient animals. The highest average figure in the scorbutic animals, 0.36 mg./g. of adrenal, was considerably below the lowest value found in a normal animal (0.52 mg./g. of adrenal) and only about one-quarter of that in the average normal animal (1.26 mg./g. of adrenal).

There is a striking difference in the amounts of total polysaccharide material extracted from the

normal and scorbutic granuloma tissue. Robertson & Hinds (1956) found a fivefold increase in production of polysaccharides in granulomata from scorbutic animals in comparison with that from normal animals. The present experiments confirm their finding. From Tables 1 and 2 it will be seen that there is a variable increase in total polysaccharide material. In terms of unit wet weight of tissue the increase varies from about two to five times the amount produced in normal animals. Expressed as g. of total polysaccharide/g. of dry tissue, the increase in polysaccharide amounts to 2½–6 times that found in the normal granuloma. It will also be seen from Table 2 that the amount of sulphate found/unit weight of total polysaccharide in the scorbutic tissue is only about 25 % of that in the normal animals. This suggests that the greatly increased production of polysaccharides in the scorbutic granuloma is largely, if not entirely, accounted for by increased production of some non-sulphated polysaccharide.

In Tables 2 and 3 are given analytical figures for chondroitin sulphate and hyaluronic acid isolated from the total polysaccharide material of the granuloma tissue. Considering the chondroitin sulphate first, it will be seen that the amount isolated/g. of dry tissue is generally smaller from the scorbutic animals, but the differences from the amounts in normal tissue are not very large and show considerable variation. The contents of hexosamine and sulphate are similar in the samples of chondroitin sulphate from both the normal and scorbutic animals. The figures for uronic acid content and nitrogen content also showed no

marked differences between the chondroitin sulphate from normal and from scorbutic tissue. Further analysis of the hexosamine showed this to consist very largely of galactosamine in the chondroitin sulphate from both sources. Some detectable glucosamine was found in all the chondroitin sulphate samples, but in chondroitin sulphate from scorbutic tissues the amount of glucosamine was sufficiently large to permit quantitative estimation. Some attempts to obtain further information about the normal and scorbutic chondroitin sulphate fractions are given in Table 4. The amount of polysaccharide available for these experiments was small and the results must therefore be regarded as tentative. Fractional precipitation by ethanol, according to the technique of Meyer *et al.* (1953), resulted in at least half of the chondroitin sulphate from both normal and scorbutic tissue being precipitated by 20 % ethanol. This fraction would appear to be a chondroitin sulphate (A) fraction since it underwent some hydrolysis with testicular hyaluronidase. A further 10–20 % of chondroitin sulphate both from scorbutic and normal tissue was precipitated by 30 % ethanol and the remainder, 25–30 % of the total chondroitin sulphate, was precipitated by 50 % ethanol. This latter fraction from both types of tissue was hydrolysed by testicular hyaluronidase more readily than was fraction A, and would therefore seem to correspond to chondroitin sulphate (C). The amounts of chondroitin sulphate fractions available were insufficient for further analyses. Although this experimental evidence is admittedly tentative the chondroitin sulphate produced in

Table 2. *Analyses of total and separated polysaccharides from granuloma tissue*

Hexosamine was estimated by the method of Boas (1953) and sulphate by the 4-chloro-4'-aminodiphenyl method. The combined recovery of polysaccharide as separated chondroitin sulphate and hyaluronic acid was, on the average, rather more than 50 % of the original total polysaccharide.

No. of expt.	Condition of animals	Total polysaccharide			Chondroitin sulphate			Hyaluronic acid		
		Amount (mg./g. of dry tissue)	Hexos- amine Sul- (g./100 g. of polysaccharide)		Amount (mg./g. of dry tissue)	Hexos- amine Sul- (g./100 g. of polysaccharide)		Amount (mg./g. of dry tissue)	Hexos- amine Sul- (g./100 g. of polysaccharide)	
A 1	Scorbutic (16 days)	9.2	24.6	2.1	1.0	25.2	11.8	4.4	33.6	1.6
2	Normal	2.8	26.1	8.9	1.4	27.4	13.3	0.54	30.3	0.9
3	Scorbutic (19 days)	11.4	28.2	1.9	1.06	23.5	9.6	5.2	29.2	1.9
4	Normal	2.0	31.4	8.2	1.1	33.4	12.7	0.26	35.8	0.14
5	Scorbutic (22 days)	5.3	29.8	2.2	0.6	31.0	12.9	3.5	36.2	1.9
6	Normal	1.7	33.4	8.0	1.05	30.5	11.6	0.2	29.8	0.27
B 1	Scorbutic (13 days)	5.6	27.7	2.4	0.8	26.2	11.3	2.6	32.8	—
2	Normal	2.2	30.6	8.6	1.06	25.9	10.9	0.53	28.4	—
3	Scorbutic (16 days)	8.1	29.1	2.35	1.5	32.0	10.6	4.0	27.9	—
4	Normal	1.8	32.0	9.4	0.9	34.2	11.2	0.37	37.4	—
C 1	Scorbutic (14 days)	9.6	34.3	1.8	0.84	30.7	12.2	4.4	34.3	1.2
2	Normal	1.6	28.1	8.3	1.15	24.6	13.1	0.25	28.9	0.8

Table 3. Analysis of chondroitin sulphate and hyaluronic acid separated from granulomatous tissue

Glucosamine and galactosamine were isolated and recovered by the method of Gardell (1953). Uronic acid was estimated according to Dische (1947), N by the method of Chibnall, Rees & Williams (1943) and sulphate by the 4-chloro-4'-aminodiphenyl method. In experiments A 1 and 3 nine animals were used, in A 2 and 4 five animals, in C 1 seventeen animals and in C 2 twelve animals. CS, Chondroitin sulphate; HA, hyaluronic acid; Glu., glucosamine; Gal., galactosamine; UA, uronic acid; Sul., sulphate.

No. of expt.	Condition of animals	Chondroitin sulphate						Hyaluronic acid									
		Amount recovered			Glu.	Gal.	UA	Sul.	N	Amount recovered			Glu.	Gal.	UA	Sul.	N
		Total (mg.)	Dry tissue (mg./g.)	(g./100 g. of polysaccharide)						Total (mg.)	Dry tissue (mg./g.)	(g./100 g. of polysaccharide)					
A 1	Scorbutic (16 days)	14.2	1.0	1.5	26	38	11.8	—	62.5	4.4	32	4.9	38	1.6	3.02	$[\alpha]_D^{23}$ (1% w/v)	
2	Normal	18.8	1.4	Trace	28	27	13.3	3.0	7.25	0.54	34	1.6	37	0.90	—	CS	
3	Scorbutic (19 days)	14.6	1.06	1.6	23	35	9.6	2.92	72.0	5.2	38	8.7	42	1.9	3.6	HA	
4	Normal	15.2	1.1	0.5	29	31	12.7	—	3.6	0.26	—	—	—	0.14	—	~	
C 1	Scorbutic (14 days)	26.2	0.84	1.8	32	29	12.2	2.75	137.6	4.4	40	4.4	39	1.2	3.13	~	
2	Normal	36.8	1.15	Trace	30	30	13.1	2.81	8.0	0.25	30	1.2	32	0.80	—	~	

Table 4. Some further observations on the isolated chondroitin sulphate and hyaluronic acid from granulomatous tissue

Ethanol precipitation, in the presence of 5% (w/v) calcium acetate and 0.5N acetic acid, was done according to the method of Meyer *et al.* (1953). Chromatography was carried out in 2-methylpropan-2-ol-water (40:60, v/v), with lithium borate, pH 9. Toluidine blue staining was carried out in the presence of 0.1% of 5-amino-acridine hydrochloride to prevent diffusion of polysaccharide. Hyaluronidase activity was measured after 6 hr. at 37°: —, not tested; + +, reducing sugars found; +, trace of reducing sugars only.

No. of expt.	Condition of animals	Precipitation by increasing ethanol concn.		Type of precipitate in ethanol	Action of hyaluronidases		Viscosity	Chromatography	Toluidine blue staining reaction
		Proportion insoluble (%)	Ethanol (% v/v)		Testicular	Bacterial			
Chondroitin sulphate C 1	Scorbutic	65	20	} Finely granular	+	Nil	.	Fast Similar Similar	Strong metachromasia
		10	20-30		-	.	.		
		25	50		+	Nil	.		
C 2	Normal*	50	20	} Finely granular	+	Nil	.	Fast Similar	
		20	20-30		-	.	.		
		30	50		+	Nil	.		
Hyaluronic acid A 3	Scorbutic	95	65	Rough fibrous	+	+	Very viscous	Origin mainly trace of some mobile component	No metachromasia (stains Alcian blue)
		95	65	Rough fibrous	+	+	Very viscous	Origin	Very slight metachro-

* Portions of these specimens were examined by the chromatographic technique of Stoffyn & Jeanloz (1954). The chondroitin sulphate (C 2) yielded D-lyxose only, the hyaluronic acid (C 1) gave D-arabinose but also some D-lyxose.

both normal and scorbutic granulomata appears to be qualitatively similar. The amounts produced in the two types of tissue do not differ greatly, although in general the scorbutic granuloma seems to produce rather less chondroitin sulphate than does the normal.

Considering now the hyaluronic acid fraction, it will be seen from Tables 2 and 3 that much larger amounts of hyaluronic acid are produced in the scorbutic granuloma than in granuloma of the normal animal. The amounts actually recovered/unit dry weight of tissue from the granulomata of scorbutic animals varied between 5 and 20 times the amounts of hyaluronic acid recovered from granulomata in normal animals. No marked differences in glucosamine or uronic acid contents are found in this polysaccharide fraction from either normal or scorbutic tissue. On the other hand, the sulphate content was in all cases higher in the scorbutic hyaluronic acid fraction and was not appreciably reduced by a further fractionation (Table 3). A further fractionation of the hyaluronic acid from the normal granuloma, Table 3 (C2), reduced the content of sulphate from 0.80 to 0.28 g./100 g. of polysaccharide. A little galactosamine was found in the two samples of hyaluronic acid fraction from normal granulomata which were analysed. One of these, C2, was refractionated, resulting in the galactosamine being reduced to a trace only. On the other hand, the samples of hyaluronic acid from scorbutic tissues contained from 4.4 to 8.7 g./100 g. of galactosamine and these amounts were not substantially reduced by a further refractionation.

Both the hyaluronic acid fractions from normal and scorbutic tissues gave viscous solutions in water. The viscosities were not actually determined but this fraction from the scorbutic animals in all

cases gave markedly viscous solutions in water and formed rough fibrous precipitates from ethanol. The optical rotations of the two samples of scorbutic hyaluronic acid which were determined, -50° and -60° , are also lower than the accepted figures for hyaluronic acid. On chromatography in borate buffer, pH 9, the hyaluronic acid fractions remained largely at the origin, as does umbilical cord hyaluronic acid, but there was some streaming from the origin in one of the hyaluronic acid samples (A3), indicating the presence of a more mobile component.

The specific radioactivities of the chondroitin sulphate fractions from both normal and scorbutic granulomata and from rib cartilage of the normal and scorbutic animals are given in Table 5. All isotope injections were given 5 days after injection of carrageenin, at which time the scorbutic animals had been maintained on an ascorbic acid-deficient diet for 10 days. Although the experimental data are too restricted to permit accurate estimates of the metabolic rates it is sufficient to enable some general estimates of probabilities. Considering first the chondroitin sulphate fraction in the normal guinea pigs, there is a close similarity in the initial specific activity at 24 hr. in chondroitin sulphate from the granuloma and that from rib cartilage. During the following 6 days of observation the level of activity has fallen to lower levels in granuloma chondroitin sulphate than in the rib cartilage chondroitin sulphate, indicating a more active rate of metabolism. In the scorbutic animals the initial level of activity at 24 hr. is somewhat lower in the granuloma chondroitin sulphate than in the normal granuloma chondroitin sulphate. Furthermore, the loss of activity from the scorbutic granuloma chondroitin sulphate during the ensuing 6 days is less than in this fraction from

Table 5. *Specific radioactivities of chondroitin sulphate separated from granulomata and rib cartilage of normal guinea pigs and guinea pigs maintained for 10 days on ascorbic acid-deficient diet before carrageenin injection*

Measurement of radioactivity was done on 1 cm.² negligible-thickness disks as 4-chloro-4'-aminodiphenyl sulphate. Under these counting conditions the activity of 0.005 μ C of ³⁵S is of the order of 870 counts/min. above background. All counts are corrected for dose of ³⁵S, body wt., for background and for the decay time of ³⁵S. The figures are the average for at least three separate determinations in each case. The granuloma tissue was pooled from nine or more scorbutic animals and from five normal animals at each time interval. The highest count found in any of the purified hyaluronic acid fractions was approximately twice the background counts, i.e. 18/min./0.1 mg. of hyaluronic acid.

Time from injection of		Specific activities of chondroitin sulphate separated from granulomata		Specific activities of chondroitin sulphate separated from rib cartilage	
Carrageenin	³⁵ S	Normal animals	Scorbutic animals	Normal animals	Scorbutic animals
(days)		(counts/min./0.1 mg. of sulphate)		(counts/min./0.1 mg. of sulphate)	
6	1	920	805	905	205
7	2	400	530	650	170
9	4	205	415	520	155
12	7	95	310	230	125

normal animals. It seems probable that there is a slower rate of metabolism in the chondroitin sulphate fraction in the scorbutic animals than in the normal animals. This reduced metabolic activity is shown more clearly by the chondroitin sulphate fraction from rib cartilage in the scorbutic animals. The 24 hr. level of activity is only one-quarter of that in normal rib-cartilage chondroitin sulphate, and at the seventh day the loss of activity is much smaller than that found in normal cartilage chondroitin sulphate. This reduced metabolic activity of sulphated polysaccharides in cartilage is in agreement with results of previous work, Reddi & Norström (1954), Kodicek & Loewi (1955).

DISCUSSION

The experimental evidence presented here lends support to the finding by Robertson & Hinds (1956) of a massive increase in polysaccharide production in carrageenin-induced granuloma tissue in guinea pigs under conditions of ascorbic acid deficiency. These authors suggested that most of the increase in polysaccharide production was in fact due to an increased output of hyaluronic acid. The evidence of the present experiments would seem to confirm this suggestion. The hyaluronic acid fraction from these scorbutic-granuloma tissues certainly shows some similarities to umbilical cord hyaluronic acid. It formed very viscous solutions in water, indicating a high degree of polymerization, precipitation by ethanol produced an immediate fibrous coagulum, it was not depolymerized by papain and the nitrogen, uronic acid and glucosamine contents were similar to those found in umbilical cord hyaluronic acid. Nevertheless, the present experiments show some evidence of dissimilarity between the hyaluronic acid fractions from (a) scorbutic granuloma, (b) umbilical cord and (c) normal granulomata. The first contained some 1–2 % of sulphate and about 4–9 % of galactosamine, both higher than the amounts found in the hyaluronic acid fraction from normal granuloma. Also both the sulphate and galactosamine contents fell markedly when the normal granuloma hyaluronate fraction was repurified, whereas repurification had little effect on the amount of these constituents in the scorbutic hyaluronic acid fractions. The optical rotation is also lower than are the accepted figures for umbilical cord hyaluronic acid.

Meyer *et al.* (1953) have reported finding, in various tissues, polysaccharide fractions similar to hyaluronic acid with a low, but persistent, sulphate content. The fact that the greater part of the hexosamine consists of glucosamine suggests that most of this scorbutic hyaluronic acid fraction is, in fact, hyaluronic acid closely associated with some sulphated fraction. If, however, this was a

simple admixture some measure of separation would be expected, as evidenced by decreasing sulphate content, as is found in repurifying the hyaluronic acid from normal tissue. In view of this difference in behaviour and without further experimental evidence it would seem best to regard this polysaccharide, produced in abundance by the scorbutic granuloma, as a type of hyaluronic acid not necessarily identical with hyaluronic acid from umbilical cord.

In contrast with the large amounts of low-sulphate or non-sulphated polysaccharide produced in the scorbutic granuloma, the amounts of chondroitin sulphate-like polysaccharide found were generally smaller than those in normal granuloma. The chondroitin sulphate polysaccharide appears to be identical or very similar in both normal and scorbutic granulation tissue, although further information is required to determine more closely whether they each consist of two or three types of chondroitin sulphate; and, also, whether definite quantitative differences recur in the proportions of types *A*, *B* and *C*.

The experimental data on the metabolism of the chondroitin sulphate fraction of polysaccharide isolated from normal and scorbutic granuloma tissue is admittedly scanty and any differences noted between the activities in chondroitin sulphate from normal tissues and from scorbutic tissues must be interpreted with due caution.

That at least some measure of reliance may be placed on these results would seem to follow from the much reduced incorporation of ^{35}S found in the scorbutic rib cartilage in comparison with that in normal rib cartilage, which is in agreement with previous work by Reddi & Norström (1954). By contrast the incorporation of ^{35}S into the chondroitin sulphate fraction from scorbutic granuloma appears to be only slightly reduced from the level of incorporation found in normal granuloma chondroitin sulphate. The subsequent loss of specific activity appears to take place more slowly, however, in the scorbutic than in the normal chondroitin sulphate, and this lower rate of loss of specific activity is also found in chondroitin sulphate from the scorbutic rib cartilage. Unfortunately only one point in time of injection of isotope in relation to duration of scorbutus is available, i.e. on the fifteenth day of ascorbic acid-deficient diet. At this stage the level of incorporation of ^{35}S into chondroitin sulphate from scorbutic tissue is only a little less than is that from normal tissue. This would seem to indicate that synthesis of chondroitin sulphate polysaccharide continues, at least during the first 14 days of ascorbic acid deficiency, at a rate not much below that in granuloma tissue of normal animals. There appears, from the present experiments, to be a

decrease in the rate of metabolism of chondroitin sulphate in the scorbutic animals during the subsequent 7 days of ascorbic acid deficiency. But at what level of ascorbic acid deficiency these alterations in polysaccharide metabolism first appear, how they are affected by increasing severity of ascorbic acid deficiency or whether they are, in fact, due wholly to ascorbic acid deficiency, are questions which cannot at present be adequately answered.

SUMMARY

1. Growth of new connective tissue was stimulated by the injection of 50 mg. of carrageenin beneath the abdominal skin of guinea pigs. Groups of guinea pigs were maintained on an ascorbic acid-deficient diet for 7 or 10 days before injection with carrageenin and killed at intervals from 6 to 12 days later. Corresponding groups of normal guinea pigs were given ascorbic acid supplements during the periods of granuloma production.

2. The granulomata were dissected out and pooled from groups of normal and scorbutic animals at each time interval of killing. After digestion of the granulation tissues with papain, polysaccharide materials were extracted by a fractionation scheme utilizing cetylpyridinium chloride. By this means three groups of polysaccharide material were obtained: (1) total polysaccharides; (2) a chondroitin sulphate fraction; (3) a hyaluronic acid-like fraction.

3. The granuloma tissue from the scorbutic animals showed a greatly increased production of total polysaccharides in comparison with that from normal animals. Evidence is presented that most of this increased polysaccharide production is due to greatly increased amounts of a polysaccharide fraction with properties similar to those of hyaluronic acid. Production of the chondroitin sulphate fraction was generally slightly reduced in the scorbutic granuloma in comparison with the normal animals.

4. Although most of the polysaccharide material obtained could be accounted for in terms of the chondroitin sulphate and hyaluronic acid fractions the presence of other mucopolysaccharides is not excluded.

5. Normal and scorbutic guinea pigs, 5 days after injection with carrageenin, were injected with $1 \mu\text{C/g. body wt. of Na}_2^{35}\text{SO}_4$ and killed at intervals of time from 1 to 7 days later. The specific activity of the chondroitin sulphate fraction from rib cartilage in the scorbutic animals was much less at 24 hr. than that in corresponding normal animals.

By contrast, incorporation of [^{35}S]sulphate into chondroitin sulphate from the granuloma of scorbutic guinea pigs at 24 hr. was only a little less than into chondroitin sulphate from granuloma of normal animals. The rate of loss of specific activity in the scorbutic granuloma, however, was less rapid than in the normal granuloma and suggests some reduction in the rate of metabolism of chondroitin sulphate in scorbutic granulation tissue.

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