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Connective Tissue Growth Stimulated by Carrageenin

2. THE METABOLISM OF SULPHATED POLYSACCHARIDES*

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Robertson & Schwartz (1953) have shown that the subcutaneous injection of carrageenin in guinea pigs causes the formation of large amounts of easily separated connective tissue. In the course of preliminary experiments, which have been briefly reported by Jackson (1956), this method has been found to provide a very convenient experimental model for biochemical studies on connective-tissue formation and removal. The new tissue so formed is readily separated from surrounding skin, fascia and muscle and amounts of the order of 10–20 g. of granuloma tissue/guinea pig are regularly obtained in the early phase of the response to a single injection of 50 mg. of carrageenin. In the earlier paper of this series some observations on the formation and removal of collagen in the carrageenin-induced granuloma are reported (Jackson, 1957). The present paper is concerned with the metabolic behaviour of sulphated polysaccharides from the same granulation tissue, and from skin and rib cartilage of normal animals.

It has been shown by Layton (1950) that there is an increased uptake of sulphate *in vitro* by granulation tissue formed after muscle injury. Kodicek & Loewi (1955) studied the uptake of [³⁵S]sulphate by mucopolysaccharides of forming connective tissue after tendotomy in guinea pigs. They found that the uptake of ³⁵S reached a maximum from the 7th to 15th days and returned to low values on the 23rd day after operation. The radioactivities measured by them relate to total organic [³⁵S]sulphate in the granulation tissue after preliminary extraction with Ringer solution, and therefore loss of unknown amounts of soluble organic [³⁵S]sulphate in this

fraction. Some 67% of the [³⁵S]sulphate in the Ringer-washed granulation tissue was shown to be present in an alkali-extractable polysaccharide having a mobility on paper ionophoresis similar to chondroitin sulphuric acid and which was meta-chromatic to toluidine blue. There remained some 25% of [³⁵S]sulphate activity unaccounted for, of which apparently only a small part is contained in residue material. But paucity of extracted material made further characterization impossible. In the present experiments the uptake of [³⁵S]sulphate into three fractions of the granuloma and other tissues of guinea pigs has been studied. The first fraction is that extracted along with neutral salt-soluble collagen, i.e. by neutral 0.2M sodium chloride. The second fraction is brought into water or dilute salt solution by digestion with activated papain, which destroys the protein linkages. Finally, there is an organic sulphate fraction in residual material which resists dissolution even by papain digestion and which will be referred to as residue sulphate.

METHODS

Treatment of animals

Guinea pigs were treated as described in the preceding paper, Jackson (1957). In the main series of carrageenin experiments thirty-six guinea pigs were used, each was injected with 50 mg. of carrageenin subcutaneously, and killed in batches of three at each interval of time. A further twenty-one guinea pigs were used for the determination of specific activities in normal rib cartilage and skin, again three guinea pigs for each interval of time. The 625 μ C of ³⁵S injected was chosen to give a dosage of 1 μ C/g. body wt. Any corrections necessary in individual groups of animals to bring the dosage to this level were small, but have been made throughout where required.

* Part 1: Jackson (1957).

Isolation of polysaccharide fractions

Neutral salt-soluble polysaccharides. One-third of the total pooled granuloma tissue, macerated, was suspended in 10 times its weight of 0.2 M-NaCl (pH 7.4) at 2° and stirred occasionally during 24 hr. 2-Octanol was used as preservative. The supernatant was decanted off and extraction for a further 24 hr. was carried out. The combined supernatants were centrifuged at 1500 g for 15 min. and then dialysed first against running water for 24 hr. and finally against several changes of distilled water for 24 hr. The total dialysate was then brought to 1.5 M-KCl by addition of solid KCl and incubated overnight at 35° with 50 mg. of cetylpyridinium chloride in the presence of 2-octanol. By using the method of fractional precipitation of cetylpyridinium complexes (Scott, 1956) it was found that, at 1.5 M-KCl, all carrageenin present in the extract could be precipitated, and centrifuged off, with negligible loss of total ^{35}S radioactivity. Excess of cetylpyridinium chloride was removed from the supernatant by shaking for 1 hr. with 2 g. of fuller's earth. After centrifuging, the supernatant was reduced in volume to about 50 ml. by vacuum distillation on a water bath not exceeding 65°, and the polysaccharides were then precipitated on standing overnight at 2° with 3 vol. of ethanol. The polysaccharide-containing precipitate was centrifuged off, redissolved in 20 ml. of water and dialysed for 24 hr. against frequent changes of distilled water. After finally centrifuging at 1500 g for 15 min. the supernatant contains the neutral salt-soluble polysaccharides separated from carrageenin.

Removal of ionic sulphate by the dialysis procedure was considered to be complete since (a) a known amount of radioactivity added as $\text{Na}_2^{35}\text{SO}_4$ to the macerated tissues could be demonstrated to have been completely removed from the dialysis sac at the end of the periods stated above, and (b) the final dialysate water gave no trace of cloudiness on addition of barium chloride.

Polysaccharides obtained by papain digestion. After the above extraction the tissue was suspended in 100 ml. of citrate buffer, pH 6.0, with 10 mg. of dry papain powder (Griffin and Tatlock)/g. wet wt. of tissue. The papain was activated by addition of cystine and ethylenediaminetetraacetic acid to give a concentration of 0.005 M of each and incubated under 2-octanol for 24 hr. at 45°. After cooling, the insoluble debris was centrifuged off at 1500 g for 15 min. The residue was washed thoroughly twice with 25 ml. of water and the washing combined with the supernatant. The combined extracts were then dialysed for 24 hr. and again centrifuged. The supernatant was brought to 1.5 M-KCl with solid KCl, and the precipitation of remaining intracellular carrageenin repeated by using the cetylpyridinium chloride method described above. Polysaccharides were separated, after removal of excess of cetylpyridinium with fuller's earth, by precipitation with 3 vol. of ethanol, redissolving in 20 ml. of water, further dialysis against distilled water and final centrifuging. About the same yields of $^{35}\text{SO}_4$ were obtained from equal amounts of the same granuloma tissue when 0.6 mg./g. of wet tissue of twice-recrystallized papain (Kimmel & Smith, 1954) was substituted for the commercial papain powder.

Residual sulphate fraction. Water suspensions of the washed residual material were dialysed against distilled water for 24 hr., and as such constitute the residue sulphate fraction. Examination of this residual material under the

light microscope shows it to be largely amorphous, having no particular staining characteristics. Some rough-branching fibrous material could occasionally be distinguished but it is impossible to say whether this material is elastin or not. Further digestion with fresh papain in buffer, pH 6, did not result in further dissolution of this residue material or reduce appreciably the small amount of ^{35}S activity contained in it.

Measurement of radioactivities

Samples of the three sulphate fractions above were hydrolysed for 24 hr. in 6 N- HNO_3 under reflux. After cooling, the hydrolysates were neutralized with KOH, adjusted to pH 5-6 and heated to 90° in centrifuge tubes in a water bath. To these tubes 5 ml. of 0.05 N- Na_2SO_4 and, after stirring, 3 ml. of N-BaCl₂ were then added and the precipitate of BaSO₄ was allowed to form for 1-2 hr. at 90°; the tubes were quickly centrifuged whilst hot. The BaSO₄ precipitate was washed first with water and finally with ethanol-ether (50:50, v/v) and dried at 110°. The radioactivity of solid disks of the BaSO₄ of 1 cm.² surface area and 'infinite' thickness was estimated by counting with a standard thin mica end-window Geiger-Müller tube and scaling equipment.

Correction for decay time of $^{35}\text{SO}_4$ has been made throughout; no correction for self-absorption is required.

Chemical analysis

Measurement of sulphate. Sulphate determinations were done on samples of the three fractions after 24 hr. hydrolysis with 6 N- HNO_3 . The hydrolysates were dried *in vacuo* in a desiccator over solid KOH. Sulphate analyses were carried out by the method of Dodgson & Spencer (1953) with the following modifications: (1) precipitation of the benzidine sulphate was on to no. 3 sintered-glass filters sealed into straight tubes (Townson and Mercer Ltd.) holding 10 ml. and fitting into centrifuge tubes. This greatly facilitates the collection of the small amount of benzidine sulphate. (2) Preliminary double washing of the precipitate is made with acetone saturated with benzidine sulphate and the final washing is with acetone alone. Reproducible results were more easily attained with these modifications, and by the use of HNO_3 rather than HCl for hydrolysis, provided that the greatest care is exercised in cleaning glassware, purifying reagents where required, and if a strictly standardized technique in the method itself is used.

Estimation of hexosamine. The method was that of Blix (1948), the reaction, however, being carried out at 90° for 1 hr. Three estimations were made on each of the three fractions from the pooled granulomata at the various time intervals after injection of carrageenin.

The absorption maximum of the colour produced from representative samples of the tissue fractions, isolated as described above, varied between 526 and 538 m μ . The readings were all made at 530 m μ .

RESULTS

After the subcutaneous injection of carrageenin there is a very rapid, and massive, growth of new connective tissue. Even by the third day, upwards of 10 g. of fresh tissue is regularly obtained, and by the fifth and sixth days amounts of 30 g. and more

are not uncommon. As will be seen from Table 1 there follows from the seventh day onwards an equally rapid resorption of the granuloma, which is eventually largely replaced by loose fatty tissue. The amounts of sulphate found in the three polysaccharide-containing fractions isolated from the granuloma are also shown in Table 1. It will be seen that the total sulphate is already at a maximum 3 days after injection of carrageenin, and falls steadily after the sixth day, reaching the levels found in the normal guinea-pig skin by the ninth to the 14th day. The amount of sulphate in the salt (0.2M-NaCl) fraction is also highest at the third day, then falls with time, more slowly from about the seventh day. The sulphate in fraction 2, that extracted by papain digestion, is high during the whole period from the third to the seventh day and shows no definite peak. From the seventh to the

14th day there is a fall in the sulphate content of this papain fraction. On the other hand, the residue sulphate shows only small alterations in amount, being slightly higher from about the ninth day onwards.

Hexosamine determinations in the three fractions extracted from the granuloma are shown in Table 2. Total hexosamine is already high by the third day after carrageenin. It remains high until the sixth day and then falls to the amount found in normal guinea-pig skin by about the 14th day. Hexosamine in the three fractions of granuloma shows alterations with time similar to those found in sulphate from the three fractions. Hexosamine in the salt fraction 1 is already high at the third day. It then falls with time after the fifth day, at first sharply but more slowly from the seventh day onwards. In the papain fraction 2 the hexosamine content does

Table 1. *Amounts of sulphate/100 g. wet wt. of tissue in three fractions of granuloma tissue, and total wet wt. of tissue obtained, after subcutaneous injection of 50 mg. of carrageenin*

Figures are means of at least three determinations on pooled tissue from three guinea pigs at each time interval.

Time after injection of 50 mg. of carrageenin (days)	Wet wt. of tissue extracted (g.)	Salt fraction 1 0.2M-NaCl (pH 7.4)	Papain fraction 2	Residue sulphate 3	Totals 1, 2 and 3
			10 mg. of papain/g. of tissue	by hydrolysis remainder	
			(mg./100 g. wet wt. of tissue)		
3	13	23	11	5	39
5	18.5	15	12	5	32
5.5	33	15	13	6	34
6	38	12	13	6	31
7	33	9	11	5	25
9	8.5	7	6	7	20
14	8.5	4	5	8	17
21	3.5	2	3	7	12
28	2.0	1	4	8	13
Normal guinea-pig skin	—	7	9	3	19

Table 2. *Amounts of hexosamine/100 g. of wet tissue in three fractions of the granuloma*

Figures are means of three or more determinations on pooled tissue from three guinea pigs at each time interval.

Time after injection of 50 mg. of carrageenin (days)	Salt fraction 1 0.2M-NaCl (pH 7.4)	Papain fraction 2 10 mg. of papain/g. of tissue	Residue sulphate 3 by hydrolysis remainder	Totals 1, 2 and 3
		(mg./100 g. wet wt. of tissue)		
3	116	71	10	197
5	104	74	10	188
5.5	120	79	9	208
6	85	90	12	187
7	61	80	13	154
9	32	80	10	122
14	25	70	9	104
21	15	50	9	74
28	14	54	10	78
Normal guinea-pig skin	37	65	8	110

not show such large changes with time. As with the sulphate content, hexosamine in the residue fraction 3 varies little in amount throughout the time interval studied.

Table 3. *Specific activities ($^{35}\text{SO}_4$) in counts/min./0.1 mg. of sulphate found in three fractions from carrageenin-induced granuloma in guinea pigs*

$\text{Na}_2^{35}\text{SO}_4$, $1\mu\text{C/g.}$ body wt. was injected intraperitoneally 5 days after 50 mg. of carrageenin had been injected subcutaneously. Radioactivities were counted with 29 ± 0.3 mg. of carrier BaSO_4 as 'infinitely' thick disks 1 cm.^2 surface area.

Time after injection of $\text{Na}_2^{35}\text{SO}_4$	Salt fraction 1 0.2M-NaCl (pH 7.4)	Papain fraction 2 10 mg. of papain/g. of tissue	Residue sulphate 3 by hydrolysis remainder
2 hr.	12	14	26
6 hr.	354	23	22
8 hr.	462	44	20
12 hr.	672	128	22
24 hr.	586	327	19
2 days	438	363	13
4 days	53	245	8
9 days	22	199	12
12 days	28	143	7
16 days	10	115	6

Fig. 1. Specific activity-time curves of three fractions of organically bound sulphate from carrageenin-induced granuloma in guinea pigs, and of papain-extracted polysaccharide from normal guinea-pig skin. Zero is the point of injection of $1\mu\text{C}$ of $\text{Na}_2^{35}\text{SO}_4/\text{g.}$ body wt. and is 5 days after injection of carrageenin. $\circ$, Neutral-salt-fraction polysaccharide; $\bullet$, papain-fraction polysaccharide; $\triangle$, residue sulphate; $\times$, papain-fraction polysaccharide from normal guinea-pig skin.

Specific activities of $^{35}\text{SO}_4$, in counts/min./0.1 mg. of sulphate found, in the three fractions of granuloma tissue are shown in Table 3. The highest activity was found in the salt fraction at 12 hr., but if the figures are plotted as in Fig. 1 it will be seen that the steeply rising part of the curve is bisected, at about 10 hr., when the falling part of the curve is extrapolated backwards from the 12 hr. point. Maximal activity of the salt fraction 1 sulphate is therefore probably at about 10 hr. The specific activity of the salt fraction then falls very rapidly so that it has already reached a low level by the fourth day after injection of $\text{Na}_2^{35}\text{SO}_4$. From the fourth to the 16th days there is a slow fall of the remaining activity in this fraction.

Maximal activity in the papain-extracted fraction was found at the 2-day point. But again the ascending part of the specific activity-time curve (see Fig. 1) was steeper than the descending portion, and if the latter is extrapolated backwards, it will be seen to bisect the ascending curve at about 36 hr. This is about the time required for maximal activity to be reached in the chondroitin sulphate of rib cartilage, see Boström (1952), Slack (1955). It will be seen from Table 3 and Fig. 1 that the maximal activity of the papain fraction is less than one-half that of the salt fraction. Furthermore, the fall in specific activity with time occurs at a slower rate than in the salt fraction.

By contrast the specific activities of the residue sulphate are low at all the time intervals studied and show relatively small fluctuations with time. The specific activity-time curve shows no definite peak and the half-life cannot be calculated on the available data.

Polysaccharides were extracted from rib cartilage and skin of normal guinea pigs after injection intraperitoneally of $1\mu\text{C}$ of $\text{Na}_2^{35}\text{SO}_4/\text{g.}$ body wt. After injection the animals were killed at the time intervals shown in Table 4 and portions of rib cartilage and skin, cleaned free of adjacent muscle,

Table 4. *Specific activities (counts/min./0.1 mg. of sulphate) found in normal guinea-pig rib cartilage and skin extracted with papain*

Radioactivities were counted with 29 ± 0.3 mg. of carrier BaSO_4 as 'infinitely' thick disks. Three guinea pigs were taken for each time interval.

Time after injection of $\text{Na}_2^{35}\text{SO}_4$	Normal rib cartilage (papain fraction 2)	Normal skin (papain fraction 2)
12 hr.	84	128
1 day	138	163
2 days	150	149
4 days	124	114
9 days	109	89
12 days	94	62
16 days	79	48

fat and fascia, were extracted first with water in the cold for several hours and then digested for 24 hr. with papain, as described above under Methods. The specific activities found are shown in Table 4. The sulphate extracted by this method from both rib cartilage and skin in the normal guinea pig shows only one-half as much incorporation of $^{35}\text{SO}_4$ as the papain fraction of the carrageenin granuloma. The specific activity-time curve of the papain fraction from rib cartilage is very similar to that of normal cartilage in the rat. In the normal guinea-pig skin the peak of activity appears to be a little earlier than in the cartilage, at about 24 hr.

DISCUSSION

The granuloma produced by subcutaneous injection of small amounts of the polygalactose carrageenin has provided a most useful means of studying the active formation and breakdown of new connective tissue *in vivo*. In the preceding paper, Jackson (1957) has shown that in collagen formation there is early production of a neutral salt-soluble collagen which behaves metabolically as the precursor of formed collagen fibres.

During the early phase of the granuloma formation, in the first 5 days or so after carrageenin injection, there is a rapid build-up of polysaccharides. After the fifth or sixth day there follows a rapid fall in the polysaccharide contents, as determined by the methods described, so that from the 14th day onwards the total hexosamine and sulphate fall to about the levels found in normal guinea-pig skin. This is in general agreement with the recent experiments on wound healing in otherwise healthy rats by Dunphy & Udupa (1955). They showed that following upon wounds made in the abdominal cavity there is an early 'productive' or 'substrate' phase lasting from 1 to 5 days, during which there is a rapid formation of polysaccharides, followed by a 'collagen' phase lasting from the fifth or sixth day until healing is complete. During the 'collagen' phase they found a rapid fall in the polysaccharide content up to about the ninth day, after which the decrease occurred much more slowly. As regards total hexosamine and total sulphate in the carrageenin-induced granuloma the results are very similar. Unlike normal wound healing, however, the later stages of the carrageenin granuloma result in complete removal of the previously formed connective tissue. This is indicated by the fall in both total hexosamine and total sulphate below the values found in normal guinea-pig skin from the 21st day onwards.

When we come to consider the separate fractions of the granuloma tissue, two points stand out. First, both the hexosamine and sulphate of the residue fraction are low in amount and show little

fluctuation with time. This residue fraction would appear to play little part in the dynamics of the granuloma formation or breakdown and would appear to be related to already existing elements of the subcutaneous tissue. Microscopical examination of this fraction reveals a largely amorphous debris with no characteristic staining reactions. Occasionally, however, rough branching fibrous material can be discerned which may represent pre-existing elastin (elastin is very resistant to papain digestion). The hexosamine and sulphate content of this fraction may therefore, in part at least, be in association with elastin. Secondly, the very early production of polysaccharides with a subsequent very rapid fall in both hexosamine and sulphate is most clearly seen in the salt-soluble fraction 1 of the granuloma. The build-up and breakdown of the papain fraction appears to be a somewhat steadier, more delayed process.

These differences are clearly amplified by the dynamics of the process as indicated by the specific activities found in the three fractions. The salt-soluble fraction with its high level of specific activity follows a specific activity-time curve closely similar to that of the neutral salt-soluble collagen. It may, indeed, be the soluble sulphated-polysaccharide precursor of the papain fraction, which latter is closely associated with proteins which are both water- and neutral-salt insoluble (presumably largely insoluble and acid-soluble collagen). That this possible 'precursor', neutral salt-soluble polysaccharide, itself occurs largely in association with soluble protein and not in free solution, is evident from the fact that its presence is not readily shown by paper chromatography until the protein components are denatured by heating to 70° and by ethanol precipitation. The delayed fall in specific activity of the papain fraction and its close association in solubility properties with the insoluble collagen fraction suggest its association dynamically with this collagen fraction in its formation and subsequent breakdown.

SUMMARY

1. New connective tissue, in a highly active metabolic state, was induced by subcutaneous injection of carrageenin in guinea pigs. The wet weight of the granulation tissue so induced increased rapidly to a maximum about the seventh day after carrageenin. There followed a rapid resorption of the new tissue and by the sixth week only a little fatty tissue remained at the injection site.

2. Sulphate and hexosamine determinations suggest an early, rapid synthesis of polysaccharide. This was already high on the third day, maximal about the fifth or sixth day, and then declined rapidly to reach normal levels about the 14th day.

3. $\text{Na}_2^{35}\text{SO}_4$ was injected intraperitoneally on the fifth day after carrageenin and animals were killed at several intervals of time afterwards. The highest specific activity found was in a neutral salt-soluble extract at 10 hr. after isotope administration. The activity of this fraction then fell rapidly with time. Sulphate extracted by papain digestion had a maximal specific activity about 30 hr. after isotope administration. Loss of activity in this fraction was less rapid than in the neutral salt-soluble extract. A residual sulphate fraction had low specific activities throughout and an indefinite half-life.

4. It is suggested that neutral salt (0.2M-NaCl) extract of the granuloma tissue may contain a precursor sulphated polysaccharide. The very high rate of metabolism of this neutral-salt extract parallels that of neutral salt-soluble collagen and points to a close metabolic interrelationship of these two connective-tissue components.

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Studies on Glucosaminidase

N-ACETYL- β -GLUCOSAMINIDASE IN RAT KIDNEY

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The occurrence of *N*-acetyl- β -glucosaminidase in mammalian tissues was described by Watanabe (1936*a*) and noted by Hahn (1945) and Roseman & Dorfman (1951). Watanabe (1936*b*) and East, Madinaveitia & Todd (1941) studied the properties of the enzyme in partially purified preparations from ox liver and testis.

Hahn (1945) and East *et al.* (1941) showed that *N*-acetyl- β -glucosaminidase and hyaluronidase, which are present together in crude testicular extracts, are different enzymes. Linker, Meyer & Weissmann (1955) have shown that the oligosaccharides formed from hyaluronic acid by hyaluronidase ('poly-*N*-acetyl- β -glucosaminidase') are further degraded stepwise by β -glucuronidase and *N*-acetyl- β -glucosaminidase. In view of the probable importance of *N*-acetyl- β -glucosaminidase in the biochemistry of mucopolysaccharides and in studies on the structure of derivatives of *N*-acetylglucosamine, further work on the preparation and properties of this enzyme seemed desirable. A preliminary survey of rat tissues showed that kidney was the most active source of the enzyme.

The kinetics of hydrolysis of phenyl *N*-acetyl- β -D-glucosaminide by a partially purified preparation of *N*-acetyl- β -glucosaminidase from rat kidney

have been investigated and a study has been made of some factors which influence the activity of the enzyme.

METHODS

Preparation of substrate. Phenyl *N*-acetyl- β -D-glucosaminide was prepared by a modification of the method of Helferich & Iloff (1933).

Preparation of the enzyme. Fresh rat kidney was ground for 1 min. with water in a stainless-steel homogenizer. The homogenate (2%, w/v) was brought to pH 4.8 with citrate buffer (final concn. 0.1M) and centrifuged for 15 min. at 1500 g. The supernatant was fractionated at 0–4° by addition of saturated ammonium sulphate. The fraction precipitated between 20 and 50% saturation was dissolved in water and dialysed overnight at 0–4°. After centrifuging to remove inactive, insoluble material, the preparation was diluted to the original volume of homogenate to give an enzyme concentration suitable for assay by the method described below. Such preparations contained about 70–80% of the activity of the homogenate. The activity fell about 10% on storage for 1 week at 0–4°.

Estimation of enzyme activity

This was carried out by a modification of the method of East *et al.* (1941). Tubes containing substrate (0.01M), buffer (0.05M citrate, pH 4.3) and enzyme (0.1 ml.) in a final volume of 1.0 ml. were incubated at 37° for 1 hr. Each