REFERENCES

- Anderson, T. F. (1956). In *Physical Techniques in Biological Research*, vol. 3, p. 178. Ed. by Oster, G. & Pollister, A. W. New York: Academic Press Inc.
- Bensusan, H. B. & Hoyt, B. L. (1958). *J. Amer. chem. Soc.* **80**, 719.
- Collins, F. C. & Leinweber, J. P. (1956). *J. phys. Chem.* **60**, 389.
- Duke, F. R. & Brown, L. M. (1954). *J. Amer. chem. Soc.* **76**, 1443.
- Frisch, H. L. & Collins, F. C. (1953). *J. chem. Phys.* **21**, 2158.
- Gross, J. (1956). *J. biophys. biochem. Cytol.* **2**, 261.
- Gross, J. (1958). *J. exp. Med.* **107**, 265.
- Ham, F. S. (1958). *J. Phys. Chem. Solids*, **6**, 335.
- Jackson, D. S. (1957). *Counc. int. Org. Med. Sci. Symp.: Connective Tissue*, p. 69. Ed. by Tunbridge, R. E. Oxford: Blackwell Scientific Publications Ltd.
- Johnson, R. A. & O'Rourke, J. D. (1954). *J. Amer. chem. Soc.* **74**, 2124.
- Nielsen, A. E. (1955). *J. Colloid Sci.* **10**, 576.
- Nielsen, A. E. (1958). *Acta chem. scand.* **12**, 951.
- Nielsen, A. E. (1959). *Acta chem. scand.* **13**, 784.
- Schmitt, F. O. (1959). *Rev. mod. Phys.* **31**, 349.
- Schmitt, F. O., Gross, J. & Highberger, J. H. (1955a). *Symp. Soc. exp. Biol.* **9**, 326.
- Schmitt, F. O., Gross, J. & Highberger, J. H. (1955b). *Exp. Cell Res. Suppl.* **3**, 326.
- Waugh, D. F. (1957). *J. cell. comp. Physiol.* **49**, Suppl. 1, 145.
- Wood, G. C. & Keech, M. K. (1960). *Biochem. J.* **75**, 588.

Biochem. J. (1960) **75**, 605

The Formation of Fibrils from Collagen Solutions

3. EFFECT OF CHONDROITIN SULPHATE AND SOME OTHER NATURALLY OCCURRING POLYANIONS ON THE RATE OF FORMATION

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The fact that mucopolysaccharides are abundant in developing connective tissue and the observation that these substances precipitate collagen fibrils from collagen solutions have led to suggestions that they may be important in collagen fibrillogenesis (Meyer, 1946, 1955-56; Gross, 1956).

Except for brief reports by Gross (1956, 1959) and Gross & Kirk (1958) little attention has been paid to the effect of mucopolysaccharides on the kinetics of fibril formation. These workers found that, with two doubtful exceptions, a large number of preparations of chondroitin sulphate, hyaluronic acid, keratosulphate and heparitin sulphate had little or no effect on the rate of fibril formation under the experimental conditions they used. Previous work has shown that the process of collagen-fibril formation may be divided into two phases (Bensusan & Hoyt, 1958; Wood & Keech, 1960), which have been termed nucleation and growth (Wood, 1960). In this paper this work is extended to a study of the effect of the presence of low concentrations of certain mucopolysaccharides in the reaction mixture on the kinetics of fibril formation. Particular attention is paid to the effect of chondroitin sulphate on the overall precipitation process and on the growth step alone. It has been suggested (Wood & Keech, 1960) that

different methods of preparing collagen solutions and different methods of pretreating the same collagen solution may result in starting materials, for precipitation experiments, of different degrees of molecular aggregation and therefore having different precipitation properties. A further aim of the present work was therefore to investigate the possibility that the effect of chondroitin sulphate on rate of precipitation depends on the pretreatment of the collagen solution.

The ability to precipitate collagen fibrils is also shown by the nucleic acids (Gross, 1956; Randall, Booth, Burge, Jackson & Kelly, 1955), which, like the mucopolysaccharides, are polyanionic in nature, and the effect of these substances on the rate of fibril formation has therefore also been investigated.

Part of this work has been reported briefly elsewhere (Wood, 1958).

MATERIALS AND METHODS

Collagen solutions. For most of the experiments solutions of collagen (0.1%) in 0.1M-NaCl containing 0.05M-sodium acetate buffer, pH 4.2, were prepared from one sample of purified calf dermis (dermis X), as described previously (Wood & Keech, 1960). For a small number of experiments solutions were prepared in a similar way from another

sample of purified dermis (dermis Y₂). The latter solutions behaved in essentially the same manner as the former but the two differed in one respect. Under the experimental conditions used, collagen from dermis X precipitated completely but only 50–60% of the collagen in solutions from dermis Y₂ precipitated.

Mucopolysaccharides. Chondroitin sulphates prepared from different tissues may be divided into three types. According to Meyer, Linker, Davidson & Hoffman (1956), all are polymers whose repeating units contain *N*-acetyl-D-galactosamine, ester sulphate and hexuronic acid. In chondroitin sulphate A and chondroitin sulphate C the last-named is D-glucuronic acid and in chondroitin sulphate B it is L-iduronic acid. The three substances differ in certain physical properties and in susceptibility to testicular hyaluronidase. Two commercial samples of chondroitin sulphate were used (chondroitin sulphate-1 from Evans Medical Supplies, chondroitin sulphate-2 from L. Light and Co. Ltd.). Both had been prepared from cattle trachea and therefore consisted of chondroitin sulphate A plus a little chondroitin sulphate C (Meyer *et al.* 1956). Samples of chondroitin sulphate A, chondroitin sulphate B and chondroitin sulphate C were kindly supplied by Professor K. Meyer. Heparin and hyaluronic acid were commercial preparations (from British Drug Houses Ltd. and L. Light and Co. Ltd. respectively). Two further, impure, polysaccharide preparations from bovine intervertebral disk were kindly supplied by Dr P. F. Lloyd. Both resulted from deproteinization of aqueous extracts of the tissue. The first (F1) contained predominantly keratosulphate and was similar to fraction A extracted from human nuclei pulposi (Hall, Lloyd, Haphey, Horton & Naylor, 1957). The second (F2) contained two components: one had the ionophoretic properties of hyaluronic acid whereas the other was probably a mucoprotein.

Nucleic acids. Two commercial samples of calf-thymus deoxyribonucleic acid (DNA) (DNA-1 from British Drug Houses Ltd., DNA-2 from California Foundation for Biochemical Research) and high-molecular-weight preparations from calf thymus and rat liver (DNA-3 and DNA-4 respectively) were used. Two commercial samples of ribonucleic acid (RNA) (RNA-1 from British Drug Houses Ltd., RNA-2 from Nutritional Biochemicals Corp.) and high-molecular-weight preparations from rat liver and ox liver (RNA-3 and RNA-4 respectively) were employed.

Analytical methods. In addition to the methods described previously (Wood & Keech, 1960) the procedure of di Ferrante (1956) was used to estimate low concentrations of chondroitin sulphate and heparin.

Procedure for studying the effect of the polyanions on the rate of precipitation of collagen. The polyanion was incorporated into the phosphate buffer added to the collagen solution at zero time and the course of precipitation followed turbidimetrically. The composition of the various solutions and the experimental procedure were as described previously (Wood & Keech, 1960).

EXPERIMENTS AND RESULTS

Effect of different polyanions on the overall precipitation

Fig. 1 (a) shows the effect of chondroitin sulphate A and heparin on the rate of precipitation at

pH 7.1, *I* 0.23 and 25°. Thus chondroitin sulphate A, at a concentration one-tenth of that of the collagen, accelerated precipitation whereas heparin, at a concentration one-hundredth of that of the collagen, retarded it. The general shape of the precipitation curve was not altered by the presence of either mucopolysaccharide but the extinction attained different final values (E_{∞}) in each case. Since in all three cases the collagen was completely precipitated the extent of precipitation is given by $p = E/E_{\infty}$.

It has been shown by Wood (1960) that the sigmoid sections of precipitation curves obtained in the absence of added polyanion are closely described by equations of the form:

$$\frac{dp}{dt} = K_2 C_0^{\frac{1}{2}} p^{\frac{1}{2}} (1-p), \quad (1)$$

where C_0 is the initial collagen concentration, p is the extent of precipitation and K_2 is a constant. The smooth curves shown in Fig. 1 (b) represent the best fit of equation (1) to the data, indicating that it also provides a satisfactory description of the results when these polyanions are added. Similar

Fig. 1. Effect of chondroitin sulphate A and heparin on the rate of precipitation of collagen at pH 7.1, *I* 0.23, 25° and with collagen 0.05%. Reaction mixtures contained: ○, 0.005% of chondroitin sulphate A; +, 0.0005% of heparin; ●, no added polysaccharide (control). (a) Time course of change of E . (b) Time course of extent of precipitation ($p = E/E_{\infty}$, where E_{∞} is final value of E); the curves represent the best fit of equation (1) (see text) to the experimental data.

Table 1. *Effect of chondroitin sulphate and heparin on the rate of precipitation of collagen*

Experimental conditions: pH 7.1; I 0.23; temp. 25°; collagen 0.05%; polysaccharide 0.005%, except in one experiment with heparin where the concentration was as indicated (0.0005%). For definition of t_c , K_2 and E_∞ see text. Each set of data was obtained with a different preparation of collagen solution. Since the protein in the collagen solution was about 93% of collagen (Wood & Keech, 1960) the data in column 4 indicate complete precipitation of the collagen.

Polysaccharide	t_c (min.)	K_2	Extent of precipitation of protein (%)	E_∞
—*	10.0	0.93	—	0.90
Chondroitin sulphate A*	1.5	1.28	—	0.62
Heparin (0.0005%)*	16.0	0.59	—	1.00
Heparin†	> 60	—	89	—
—	7.7	1.17	92	0.61
Chondroitin sulphate A	0.2	2.34	92	0.53
—	0.8	1.68	—	0.54
Chondroitin sulphate B	10.3	1.02	94	0.49
—	12.5	0.88	—	0.56
Chondroitin sulphate C	1.2	2.05	92	0.50
—	1.8	2.17	—	0.49
—	7.5	1.17	92	0.63
Chondroitin sulphate-1	0.5	2.27	93	0.50
Chondroitin sulphate-2	0.9	2.20	94	0.59

* Data for curves shown in Fig. 1.

† In this experiment the precipitate was flocculent and rose to the top of the cell so that the precipitation curve could not be determined turbidimetrically.

Table 2. *Effect of mucopolysaccharides on the rate of precipitation of collagen*

Experimental conditions: pH 7.1; I 0.23; temp. 25°; collagen 0.05%; polysaccharide 0.005%. For definitions of t_c , K_2 and E_∞ see text. As in Table 1, column 4 indicates complete precipitation of collagen.

Polysaccharide	t_c (min.)	K_2	Extent of precipitation of protein (%)	E_∞
—	7.3	1.17	92	0.63
Hyaluronic acid	5.1	1.25	93	0.71
—	8.2	1.03	—	0.70
F1	1.4	1.32	94	0.62
—	3.3	1.32	—	0.50
F2	3.6	0.81	92	0.70

results were obtained with other polyanions, whose effect may therefore be conveniently stated in terms of K_2 , E_∞ and the lag period t_c . The parameter K_2 is, from equation (1), proportional to the slope of the graph of p against t for a given value of p .

As shown in Table 1, the accelerating effect of chondroitin sulphate A is indicated by decrease of t_c and increase of K_2 , whereas the retarding effect of heparin is shown by increase of t_c and decrease of K_2 . Chondroitin sulphate-1, chondroitin sulphate-2 and chondroitin sulphate C also accelerated precipitation but chondroitin sulphate B did not have a significant effect on K_2 , although it increased t_c slightly. Table 2 shows that F1 (keratosulphate) accelerated precipitation in a similar way to chondroitin sulphate A and chondroitin sulphate C [in Wood (1958) keratosulphate was erroneously

Table 3. *Effect of nucleic acids on the rate of precipitation of collagen*

Experimental conditions: pH 7.1; I 0.23; temp. 25°; collagen 0.05%; nucleic acid 0.005%. Each set of results was obtained with a different preparation of collagen solution from dermis X. For definitions of t_c and K_2 see text.

Nucleic acid	t_c (min.)	K_2
—	6.0	2.05
DNA-1	12.0	1.32
DNA-2*	> 60	—
DNA-3	11.2	0.66
DNA-4	10.5	0.66
—	5.0	1.61
RNA-1	7.2	1.76
RNA-2	6.1	1.61
RNA-3	9.8	0.88
RNA-4	8.8	1.83

* DNA-2 gave a flocculent precipitate on which turbidity measurements could not be made (cf. heparin, Table 1).

stated to retard precipitation] whereas hyaluronic acid and F2 did not have a marked effect.

When the nucleic acids were studied the outstanding feature of the results was the marked retarding effect of all the samples of DNA, the magnitude of which varied from one preparation of DNA to another (Table 3). All the preparations of RNA increased t_c slightly. Three of them had little effect on K_2 but RNA-3 decreases it. The effect of DNA-3 and RNA-3 on the rate of precipitation in collagen solutions prepared from dermis Y_2 is shown in Table 4. DNA-3 retarded precipitation even when its concentration was as low as 0.005%. RNA-3 increased t_c slightly but had no significant effect on K_2 .

The variation of rate of precipitation from one preparation of collagen solution to another, noted previously (Wood & Keech, 1960), is also apparent in the data for the control experiments in Tables 1-4. The magnitude of the effect of the different polyanions also varied appreciably from one experiment to another but there is no doubt that under these conditions chondroitin sulphate A, chondroitin sulphate C and F1 (keratosulphate)

accelerated precipitation and that DNA and heparin retarded it.

Tables 1 and 2 show that in spite of the accelerating or retarding effect of any of the mucopolysaccharides the collagen was completely precipitated. The variation of E_∞ must therefore be due to the effect of these substances on the structure of the precipitates. Those mucopolysaccharides which accelerated precipitation (chondroitin sulphate A, chondroitin sulphate C and F1) caused E_∞ to be lower than in control experiments, whereas heparin, which retarded precipitation, increased it. Chondroitin sulphate B also lowered E_∞ and hyaluronic acid and F2 increased it, although these three compounds had little effect on rate of precipitation.

Effect of chondroitin sulphate on the overall precipitation at different values of pH and ionic strength

The effect of chondroitin sulphate-1 at four values of pH (I 0.23) and three values of I (pH 7.1) is shown in Table 5. The effect of these variables in the absence of chondroitin sulphate-1 (controls) agrees with that described previously (Wood & Keech, 1960). The shortening of the lag period by chondroitin sulphate-1 was not affected appreciably by changing pH but became more pronounced on increasing I . The increase of K_2 caused by chondroitin sulphate was enhanced by lowering the pH from 8 to 6 and by increasing I . In those experiments where the accelerating effect of chondroitin sulphate-1 was most marked E_∞ was lower than in the controls, but at pH 8, I 0.23 and at pH 7, I 0.13, E_∞ was higher when chondroitin sulphate-1 was present.

Effect of chondroitin sulphate-1 on the growth step of precipitation

Fig. 2 shows the results of three experiments designed to test the effect of chondroitin sulphate-1 on the growth step. In each experiment four

Table 4. *Effect of different concentrations of RNA-3 and DNA-3 on the rate of precipitation of collagen*

Experimental conditions: pH 7.1; I 0.23; temp. 25°; collagen 0.05%. Each set of results was obtained with a different preparation of collagen solution from dermis Y_2 . For definitions of t_c and K_2 , see text.

Nucleic acid	Concn. (%)	t_c (min.)	K_2
DNA 3	—	6.5	1.04
	0.0005	9.7	0.81
	0.0025	11.2	0.66
	0.0050	41.0	0.37
RNA 3	—	7.0	0.96
	0.0005	11.6	0.88
	0.0025	11.6	0.88
	0.0050	8.8	0.96

Table 5. *Effect of chondroitin sulphate-1 on the rate of precipitation of collagen at different values of pH and ionic strength*

Collagen 0.05%. Chondroitin sulphate-1 0.005%. Temp. 25°. Each set of results was obtained with a different preparation of collagen solution. For definitions of t_c , K_2 and E_∞ see text.

pH	I	t_c (min.)		K_2		E_∞		Percentage change due to chondroitin sulphate		
		Control	Chondroitin sulphate	Control	Chondroitin sulphate	Control	Chondroitin sulphate	t_c	K_2	E_∞
6.0	0.23	2.0	0.5	4.42	10.08	0.94	0.50	-75	+129	-47
7.1	0.23	7.0	1.0	1.03	2.05	0.83	0.73	-86	+99	-12
7.5	0.23	7.5	1.5	0.96	1.85	0.70	0.72	-80	+93	+3
8.0	0.23	6.8	1.5	1.33	1.68	0.46	0.73	-78	+26	+59
7.1	0.13	1.0	1.0	7.44	7.44	0.46	0.61	0	0	+33
7.1	0.23	2.5	1.7	1.18	1.39	1.08	1.00	-32	+18	-8
7.1	0.31	25.3	1.0	0.45	1.07	1.10	0.91	-96	+138	-17

samples of collagen solution (1.5 ml.) were equilibrated at 25°. On two of these, precipitation runs were done by the usual procedure, one with chondroitin sulphate present (curve 2) and one without (curve 1). To each of the remaining two samples 1.55 ml. of buffer was added at zero time and the mixtures were allowed to stand at 25° for the duration of the lag period, determined from curve 1. To one of them 0.15 ml. of chondroitin sulphate-1 (1% in 0.1M-NaCl) was added with stirring and to the other 0.15 ml. of 0.1M-NaCl. The course of subsequent precipitation is given by curves 3 and 4 respectively. All the reaction mixtures had identical volume and salt composition after the lag period.

Comparison of curves 1 and 2 in each set indicates the accelerating effect of chondroitin sulphate-1 on the over-all process (nucleation + growth) described above. Comparison of curves (3) and (4) indicates that when chondroitin sulphate-1 was added at the end of the lag period at pH 6 and 7 (I 0.23), it had no appreciable effect

either on the rate of growth or on E_{∞} , and that at pH 7, I 0.31 it retarded growth slightly. Comparison of curves (1) and (4) shows that the slight difference in the composition of the reaction mixtures during the lag period between (1) and (2) on the one hand and (3) and (4) on the other, together with the stirring of (3) and (4) at the end of the lag period, had little effect on subsequent precipitation. It is concluded that, under these conditions, the acceleration of the over-all precipitation by chondroitin sulphate-1 and its effect on E_{∞} is due mainly, if not wholly, to its action during the lag period.

Effect of chondroitin sulphate-1 on the rate of precipitation from collagen solutions pretreated with phosphate buffer, pH 7

The following experiments (Fig. 3) were done to investigate the possibility that the effect of chondroitin sulphate on the kinetics of fibril formation depends on the pretreatment of the collagen solution. In each experiment 1.55 ml. of buffer, pH 7.1, cooled to 0°, was added to each of

Fig. 2. Effect of chondroitin sulphate-1 on the growth step of precipitation. Collagen concn. 0.05%; chondroitin sulphate concn. 0.005%; temp. 25°. (a) I 0.23, pH 7.1; (b) I 0.23, pH 6.0; (c) I 0.31, pH 7.1. In (a), (b) and (c): (1) chondroitin sulphate absent; (2) chondroitin sulphate present throughout precipitation; (3) chondroitin sulphate absent during lag period, the end of which is indicated by the arrow; (4) chondroitin sulphate absent, mixture stirred at end of lag period as in (3). In (a) curve (4) coincides with curve (1).

Fig. 3. Effect of chondroitin sulphate-1 on the rate of precipitation of collagen from collagen solutions previously kept at pH 7.0 for 16 hr. at 0°. Collagen concn. 0.05%; chondroitin sulphate concn. 0.005%; pH 7.0. (a) I 0.23, 25°; (b) I 0.31, 25°; (c) I 0.31, 37°. In (a), (b) and (c): (1) chondroitin sulphate absent; (2) chondroitin sulphate present. For comparison curves (3) and (4) show the effect of chondroitin sulphate on rate of precipitation from collagen solutions not previously adjusted to pH 7.0: (3) chondroitin sulphate absent; (4) chondroitin sulphate present. The collagen concentration and salt composition of the medium were the same in all four experiments.

two samples of collagen solution (1.15 ml.) at 0° and the mixtures were allowed to stand at 0° for 16 hr. To one, 0.15 ml. of 0.1M-NaCl was added with stirring, and to the other, 0.15 ml. of 1% chondroitin sulphate-1 in 0.1M-NaCl was added. The temperature was rapidly raised to 25° or 37° and precipitation followed (curves 1 and 2 respectively). Thus when, as described above, the pH of the collagen solution was first brought to neutrality and precipitation was then initiated by raising the temperature, chondroitin sulphate-1 retarded precipitation at 25° and had no significant effect at 37°. On the other hand, when, as in preceding experiments, the temperature was raised first and precipitation initiated by adjusting pH (curves 3 and 4), chondroitin sulphate-1 accelerated precipitation at both 25° and 37°.

Previous adjustment of the pH to neutrality had itself a marked retarding effect on precipitation (compare curves 1 and 3). The magnitude of this effect is too great to be accounted for merely by the time required to raise the temperature from 0° to 25° or 37°.

*Incorporation of mucopolysaccharides
into the collagen precipitates*

Precipitates were prepared in the presence of different concentrations of chondroitin sulphate-1 or heparin, at pH 7.1, I 0.23, by allowing the reaction mixtures to stand at 25° for 16 hr. The precipitates were coagulated by stirring, centrifuged and the concentration of mucopolysaccharide in the supernatant was estimated. Little, if any, chondroitin sulphate-1 was incorporated into the precipitate (Table 6). An appreciable fraction of the added heparin was retained in the precipitate but the ratio of heparin to collagen in the precipitate was, nevertheless, low.

DISCUSSION

The results show that low concentrations of some mucopolysaccharides and nucleic acids have a marked effect, under certain experimental conditions, on the rate of precipitation of collagen and on the final extinction (E_{∞}) of the precipitate, but

do not affect the amount of collagen ultimately precipitated.

In a parallel electron-microscope study M. K. Keech (in preparation) has shown that precipitates prepared at pH 7, I 0.23, in the presence of the mucopolysaccharides and nucleic acids used in the present work, were in the form of fibrils bearing the 640Å striations characteristic of native collagen. When those polyanions which lower E_{∞} relative to the controls at pH 7, I 0.23 (chondroitin sulphate-1, chondroitin sulphate-2, chondroitin sulphate A, chondroitin sulphate B and keratosulphate) were present during precipitation the fibrils were thinner than in controls and when those were present which increase E_{∞} (heparin, hyaluronic acid and DNA) the fibrils were thicker than in controls. Keech's results thus indicate that there is a qualitative correlation between E_{∞} and fibril width similar to that observed previously when precipitation of collagen in the absence of added polyanion was studied (Wood & Keech, 1960).

The polyanions which accelerate precipitation at pH 7, I 0.23 (chondroitin sulphate-1, chondroitin sulphate-2, chondroitin sulphate A, chondroitin sulphate C and keratosulphate) decrease E_{∞} and fibril width, whereas those which retard precipitation (heparin, DNA) increase E_{∞} and fibril width. Chondroitin sulphate B and hyaluronic acid, however, which have no appreciable effect on rate of precipitation under these conditions, decrease and increase respectively E_{∞} and fibril width. In addition, chondroitin sulphate-1 acting at higher pH still accelerates precipitation but increases E_{∞} and therefore, presumably, fibril width. Thus the lack of simple correlation between rate of precipitation and fibril width observed at different ionic strength and pH (Wood & Keech, 1960) is also found when rate of fibril formation is altered by the polyanions.

It was concluded from earlier work that precipitation is the result of two effectively consecutive processes, one occurring during the lag period (nucleation) and the other during the growth phase (growth of nuclei into fibrils) (Wood, 1960). Since, in spite of the presence of the polyanions in the reaction mixtures, all the precipitation curves

Table 6. *Incorporation of chondroitin sulphate and heparin into collagen precipitates*

Experimental conditions: pH 7.1; I 0.23; temp. 25°; collagen 0.05%.

Polysaccharide	Concn. of polysaccharide in reaction mixture (%)	Polysaccharide remaining in precipitate (%)	Ratio (w/w) polysaccharide collagen in precipitate
Chondroitin sulphate-1	0.0055 0.00275	4 2	0.004 0.002
Heparin	0.0047 0.00235 0.00094	16 24 45	0.015 0.011 0.008

are described by equation (1), it is concluded that these substances do not alter the way in which the soluble collagen particles aggregate during growth. This conclusion is supported by the observation that all the precipitates consisted of fibrils with 640 Å striations.

Further evidence for the two-step nature of fibril formation is given by the observation that chondroitin sulphate-1 accelerates precipitation and affects fibril width only if it is present during the lag period. This is another demonstration that the ultimate fibril width is determined in the very early stages of precipitation, and, if the two-step mechanism is correct, must mean that chondroitin sulphate-1 accelerates precipitation by affecting nucleation. In view of this the fact that chondroitin sulphate-1 did not accelerate precipitation (at pH 7, I 0.23 and 25°), when the collagen solution had previously been adjusted to pH 7 for 16 hr. at 0°, suggests that in this experiment nucleation had occurred before chondroitin sulphate-1 was added, i.e. during the period at pH 7 and 0°. The occurrence of nucleation before addition of chondroitin sulphate may well account for Gross's observation (Gross, 1956, 1959; Gross & Kirk, 1958), confirmed in the present work, that a number of preparations of chondroitin sulphate did not accelerate precipitation at 37°, for his experimental procedure involved prior dialysis of the collagen solutions against cold neutral phosphate buffer.

Some indication of the possible mode of action of chondroitin sulphate and the other polyanions may be gained by further consideration of the two-step mechanism of fibril formation. According to this, nucleation may be affected in two ways. First, chondroitin sulphate might accelerate nucleation, increase the number of nuclei formed and hence accelerate subsequent precipitation and decrease the size of the ultimate fibrils. Secondly, it might alter the shape of the nuclei formed. Since the nuclei are assumed to grow at a rate proportional to their surface area, increase in the axial ratio of the nuclei must cause an increase in their rate of growth as well as a decrease in the width of the fibrils produced. Since chondroitin sulphate-1 is able both to accelerate precipitation by acting during the period of nucleation and to retard it when present only during the growth step (see Fig. 2c), the first possibility, acceleration of nucleation, is therefore likely only if nucleation and growth are fundamentally different processes. In order to achieve the increase of axial ratio of the nuclei, required by the alternative mechanism, chondroitin sulphate-1 would have to promote end-to-end aggregation of the soluble collagen particles. It would appear, from the marked dependence of the effect of chondroitin sulphate-1 on the pH and

ionic strength of the medium, that electrostatic intermolecular forces play an important part. It has been suggested (Kühn, Grassmann & Hofmann, 1957; Kühn, Hofmann & Grassman, 1959; Hodge & Schmitt, 1958) that amino acids with basic side chains (arginine, lysine) are concentrated in the end regions of the soluble collagen macromolecules, and it is suggested that a chondroitin sulphate molecule might promote end-to-end aggregation by the interaction of its negatively-charged sulphate groups with the positively-charged groups of two collagen macromolecules. Only a very small fraction of the added chondroitin sulphate-1 is incorporated into the collagen precipitate, but, since the nuclei constitute only a very small fraction of the fibrils into which they grow, it is possible that chondroitin sulphate constitutes an appreciable fraction of each nucleus.

The retardation of fibril growth by chondroitin sulphate-1, and the even more pronounced retardation by DNA and heparin, might be due to the formation of collagen-polyanion complexes which, because of the high negative charge conferred by the polyanion, aggregate less readily than free collagen particles. This complex formation would have to be reversible since only little polyanion is incorporated into the precipitate although the collagen is completely precipitated.

The mechanisms suggested above, particularly that with reference to the shape of the nuclei, would be expected to have rather specific requirements of the chemical structure, size, shape and charge distribution of the polyanion molecules and this is consistent with the diversity of the effects of the various mucopolysaccharides and nucleic acids. Even such closely related compounds as chondroitin sulphate A and chondroitin sulphate C on the one hand and chondroitin sulphate B on the other have different effects on rate of precipitation.

It is well known that the presence of acid mucopolysaccharides in connective tissues, as indicated by metachromasia and hexosamine determinations, is particularly noticeable in the early stages of collagen-fibril formation (Dunphy & Udupa, 1955; Jackson, 1955). The present work suggests that these mucopolysaccharides might be concerned with regulating nucleation and growth and hence help to determine the rate of formation of fibrils and their ultimate size. Differences in mucopolysaccharide content of different tissues might be connected in this way with variation of fibril width from tissue to tissue; pronounced metachromasia is associated with small fibril diameter in certain tissues (Van den Hooff, 1957).

The interest in DNA arose in the present work from its polyanionic nature. It does not usually occur extracellularly and the observations of its

effect on fibril formation *in vitro* does not at the moment appear to have any physiological implications.

SUMMARY

1. Low concentrations of heparin and deoxyribonucleic acid have been found to retard fibril formation in solutions of acid-soluble collagen at pH 7, *I* 0.23 and 25°. Two types of chondroitin sulphate (A and C) and a preparation rich in kerato-sulphate accelerated fibril formation. Another type of chondroitin sulphate (B) and hyaluronic acid had no effect. Those polyanions which accelerated precipitation reduced fibril width and those which retarded precipitation increased it. The accelerating effect of chondroitin sulphate A increased with decreasing pH in the range pH 6–8 and with increasing *I* in the range 0.13–0.31.

2. Chondroitin sulphate A accelerated precipitation only when present during the lag period. This observation supports the conclusion from earlier work that collagen fibrils form as the result of two consecutive processes, nucleation and growth, and it is concluded that chondroitin sulphate A accelerates precipitation by affecting nucleation. Only a very small proportion of the added chondroitin sulphate was incorporated into the collagen precipitate.

3. When collagen solutions were pretreated with phosphate buffer, pH 7, at 0° addition of chondroitin sulphate A retarded subsequent precipitation at 25°, indicating that nucleation had taken place during pretreatment and that the observed retardation was due to the effect of chondroitin sulphate A on growth.

4. The mode of action of the polyanions is considered in the light of the two-step mechanism of fibril formation and the findings are discussed in relation to the possible role of mucopolysaccharides in the formation of collagen fibrils *in vivo*.

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REFERENCES

- Bensusan, H. B. & Hoyt, B. L. (1958). *J. Amer. chem. Soc.* **80**, 719.
 di Ferrante, N. (1956). *J. biol. Chem.* **220**, 303.
 Dunphy, J. E. & Udupa, K. N. (1955). *New Engl. J. Med.* **253**, 847.
 Gross, J. (1956). *J. biophys. biochem. Cytol.* **2**, 261.
 Gross, J. (1959). *Connective Tissue, Thrombosis and Atherosclerosis*, p. 77. Ed. by Page, I. H. New York: Academic Press Inc.
 Gross, J. & Kirk, D. (1958). *J. biol. Chem.* **233**, 355.
 Hall, D. A., Lloyd, P. F., Happey, F., Horton, W. G. & Naylor, A. (1957). *Nature, Lond.*, **179**, 1078.
 Hodge, A. J. & Schmitt, F. O. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 418.
 Jackson, S. F. (1955). *Proc. Roy. Soc. B*, **144**, 556.
 Kühn, K., Grassmann, W. & Hofmann, U. (1957). *Naturwissenschaften*, **44**, 538.
 Kühn, K., Hofmann, U. & Grassmann, W. (1959). *Naturwissenschaften*, **46**, 512.
 Meyer, K. (1946). *Amer. J. Med.* **1**, 675.
 Meyer, K. (1955–56). *Harvey Lect.* **51**, 97.
 Meyer, K., Linker, A., Davidson, E. A. & Hoffman, P. (1956). *Biochim. biophys. Acta*, **21**, 506.
 Randall, J. T., Booth, F., Burge, R. E., Jackson, S. F. & Kelly, F. C. (1955). *Symp. Soc. exp. Biol.* **9**, 127.
 Van den Hooff, A. (1957). *Counc. int. Org. Med. Sci. Symp.: Connective Tissues*. Ed. by Tunbridge, R. E. Oxford: Blackwell Scientific Publications Ltd.
 Wood, G. C. (1958). *Abstr. Comm. 4th int. Congr. Biochem., Vienna*, p. 26, 2–92.
 Wood, G. C. (1960). *Biochem. J.* **75**, 598.
 Wood, G. C. & Keech, M. K. (1960). *Biochem. J.* **75**, 588.

Biochem. J. (1960) **75**, 612

Steroid Sulphatase, Arylsulphatase and β -Glucuronidase in the Mollusca

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In recent years considerable interest has been shown in the use of enzymes from molluscan tissues for the hydrolysis of urinary steroid conjugates (Henry & Thevenet, 1952; Stitch & Halkerton, 1953*a, b*, 1956; Jayle & Baulieu, 1954).

Although these tissues are known to be rich sources of β -glucuronidase and arylsulphatase (Dodgson, Lewis & Spencer, 1953) and to possess steroid sulphatases, the specificity of the latter has been investigated in only two species. An enzyme