Inhibition by glycosaminoglycans of CaCO_3 (calcite) crystallization

David GRANT, William F. LONG and Frank B. WILLIAMSON

Department of Biochemistry, University of Aberdeen, Marischal College, Aberdeen AB9 1AS, Scotland, U.K.

Of a range of glycosaminoglycans, heparin and heparan sulphate were the most effective inhibitors *in vitro* of CaCO_3 (calcite) crystallization as assayed by conductimetric measurements. The possible role of such glycosaminoglycans in modulating calcium-salt crystallizations *in vivo* is discussed.

INTRODUCTION

Calcifications, particularly those involving the formation of CaCO_3 crystals, are among the most common normal and pathological biomineralizations (Lowenstam & Weiner, 1983; Addadi *et al.*, 1987). It has been suggested that all or most tissues are potentially calcifiable; that, in many, endogenous modulatory factors normally preclude the process; that removal or degradation of these might be a prerequisite for biological calcifications; and that glycosaminoglycans may be among these modulatory factors (Kobayashi, 1971, and references cited therein). In addition, amorphous biological deposits of calcium salts, consisting of continuous random networks of small structural units related to the crystalline state or of microcrystalline/cluster networks have recently been described (Taylor *et al.*, 1986).

The association of various glycosaminoglycans with calcifications occurring in the lesions of progressive massive fibrosis and pleural plaques (Wusteman *et al.*, 1972), in the aortic ground substance during arteriosclerosis (Wexler & Thomas, 1967), from asbestos-dust nuclei (Davis, 1970), in cartilage (Hunter *et al.*, 1985) and of glycosaminoglycan-like sulphated carboxylated polysaccharides with calcification in marine algal coccoliths (Borman *et al.*, 1982) has been discussed. Changes in cardiac valve glycosaminoglycan metabolism precede degenerative valve calcification (Hallgrímsson *et al.*, 1970), and deficiencies in urinary glycosaminoglycans may lead to calcifications resulting in renal-stone formation (Gjaldbaek, 1982; Martelli *et al.*, 1985). Exogenous heparin restores to injured bladder urothelium its functional crystal anti-adhesion properties (Parsons *et al.*, 1980; Gill *et al.*, 1982). Calcium-containing crystals are mitogenic (Cheung *et al.*, 1986), and calcifications commonly occur in breast carcinomas (Winters *et al.*, 1986). Calcification in this situation may result from disarrangement of the crystal inhibitory function of epithelial-stroma-junction glycosaminoglycans (Barth *et al.*, 1977).

The present paper reports the effects of a range of glycosaminoglycans on CaCO_3 (calcite) crystallization in defined conditions *in vitro*.

EXPERIMENTAL

Materials

Heparin from porcine intestinal mucosa (preparation A) was supplied by Glaxo Operations U.K. Ltd.,

Runcorn, Cheshire, U.K., and was prepared as previously described (Woodhead *et al.*, 1986); *N*-desulphation of this preparation was carried out by the method of Nagasawa & Inoue (1980). Porcine intestinal mucosal heparin (preparation B, lot no. 46C0035) and whale cartilage chondroitin 4-sulphate (lot no. 77C0187) were from Sigma Chemical Co. Ltd., Poole, Dorset, U.K. Chondroitin 4-sulphate, chondroitin 6-sulphate, dermatan sulphate, keratan sulphate, heparan sulphate (preparation A) and hyaluronate, from river-sturgeon notochord, river-sturgeon cranial cartilage, porcine intestinal mucosa, bovine cornea, bovine lung and human umbilical cord were respectively produced under contract from the U.S.A. National Institutes of Health, and were gifts from Professor M. B. Mathews and Professor J. A. Cifonelli, Department of Pediatrics, University of Chicago, Chicago, IL, U.S.A., from whom analytical information is available in the form of a data sheet. Heparan sulphate from porcine intestinal mucosa (preparation B) was from Opocrin Research Laboratories, Corlo, Italy (lot no. HS 435). Aquarite DP 906 was from Albright and Wilson Ltd., Oldbury, Warley, West Midlands, U.K. Tripolyphosphate was from Monsanto Chemical Co., St. Louis, MO, U.S.A. Ethane-1-hydroxy-1,1-diphosphonic acid was prepared by the method of Grant (1979). CaCl_2 and NaHCO_3 were Analar grade.

Measurement of CaCO_3 crystal formation

Seed crystals of CaCO_3 were prepared from CaCl_2 and NaHCO_3 by the method of Reddy & Nancollas (1971). I.r. spectroscopy confirmed that the crystals formed were of calcite. Glycosaminoglycan or commercial crystallization inhibitor in water (30 mm³) was added to a suspension of seed crystals in water (550 mm³) for experiments at a saturation index of approx. 4; 183 mm³ for experiments at a saturation index of approx. 31). The saturation index is the product of the initial calcium and carbonate ionic activities divided by the value of this product under equilibrium conditions. The mixture was then either directly added to 60 cm³ of a supersaturated solution of CaCO_3 in a polypropylene vessel containing CaCl_2 and NaHCO_3 prepared as described by Reddy & Nancollas (1971), or added after preincubation of the seed crystal mixture for a period specified in the Results section. Preincubation of seed crystal mixtures, and incubation of CaCO_3 solutions at temperatures ($\pm 0.005^\circ\text{C}$) specified in the Results section, were in a Techne water bath (model TU-16D). N_2 was bubbled through the CaCO_3 solutions at 22 cm³·min⁻¹.

Crystal formation was monitored by measuring the decrease in conductivity of solutions after seed-crystal addition by a method derived from that of Little & Nancollas (1970), and using a Philips conductivity meter (model PW 9527) fitted with a Chandos dip-type conductivity cell (model X100). Control experiments established that the rates of CaCO_3 crystallization measured were directly proportional to the quantity of seed crystals added and to the concentrations of CaCl_2 and NaHCO_3 present. A similar proportionality was observed when crystallization occurred in the presence of modulators, except that, in solutions of low CaCO_3 saturation index (i.e. containing in the region of 1×10^{-4} mol of CaCl_2 and 2×10^{-2} mol of $\text{NaHCO}_3/\text{dm}^3$) containing heparin, heparan or tripolyphosphate, a more complex relationship existed between CaCl_2 concentration and crystallization rate, probably because of complex-formation between modulator and Ca^{2+} .

RESULTS

Preliminary experiments involved addition of CaCO_3 seed crystals to CaCO_3 solutions with a saturation index of approx. 4 at 25°C . At this relatively low saturation index, the possibility of crystallization due to non-specific spontaneous nucleation is reduced; the temperature selected is that most frequently used in previous crystallization studies (e.g. Reddy & Nancollas, 1971). A commercial CaCO_3 -crystallization-inhibitor preparation, Aquarite, prevented crystallization in this experimental system (Fig. 1). The gradual increase in conductivity after addition of the inhibitor suggests that some dis-

solution of the seed crystals occurred. The crystallization rate was decreased in the presence of heparin; this effect was dependent upon heparin concentration, and was more marked after preincubation of the seed crystals with heparin (Fig. 1).

The effects of other glycosaminoglycans and of components of commercial CaCO_3 -crystallization inhibitors were examined at an operating temperature of 37°C (Table 1). Of the glycosaminoglycans, heparin and heparan sulphate were the most effective in decreasing both the initial crystallization rate and the total amount of CaCO_3 crystallized. The effectiveness of heparin was decreased by *N*-desulphation.

Crystallization in experiments in which a low CaCO_3 saturation index was used showed complex kinetics, probably because of the generation of secondary crystallization centres (cf. Nancollas & Reddy, 1971). In addition, in experiments at a low CaCO_3 saturation index, complex-formation between Ca^{2+} and glycosaminoglycan may contribute to the observed effects. Because of this, and because the use of a higher saturation index allowed larger conductivity changes to be measured after seed-crystal addition, a series of experiments was carried out at a CaCO_3 saturation index of approx. 31. Fig. 2(a) shows the inhibitory effect on crystallization of representative concentrations of heparin under these conditions. Second-order rate plots generated straight lines (Fig. 2b). Rate constants calculated from these plots, and from similar plots generated from experiments carried out under the same conditions but involving other glycosaminoglycans, are shown in Table 2. The kinetics of crystallization occurring in the presence of chondroitin 6-sulphate, dermatan sulphate and keratan

Fig. 1. Effect of heparin on crystallization from a CaCO_3 solution (saturation index approx. 4)

Heparin (preparation A) (Δ , $\blacktriangle$, ∇ , $\blacktriangledown$), Aquarite ($\circ$) or water ($\bullet$) was added to seed crystals, and the mixture preincubated for 30 min (∇) or 16 h ($\blacktriangle$), or not preincubated ($\circ$, $\bullet$, Δ , $\blacktriangledown$), before incubation with a solution containing NaHCO_3 (2×10^{-2} mol $\cdot$ dm $^{-3}$) and CaCl_2 (1×10^{-4} mol $\cdot$ dm $^{-3}$). Preincubation and incubation were at 25°C . Concentrations of modulators in the final incubation mix were 0.006% ($\circ$, Δ , ∇ , $\blacktriangle$) or 0.6% ($\blacktriangledown$) (w/v). Seed crystals were present in the final reaction mix at 0.38 mg $\cdot$ cm $^{-3}$. Symbols on ordinate scale refer to the difference between conductivity measured at time after addition of seed crystals (S), and initial conductivity (S_0); K is the conductivity cell constant (1.45).

Table 1. Effect of glycosaminoglycans on crystallization from a CaCO₃ solution (saturation index approx. 4)

Modulators were added to seed crystals, and the mixture added either directly, or after a 16 h preincubation, to a mixture of NaHCO₃ and CaCl₂. The concentration of modulators in the final incubation mix was 60 µg/ml. Other conditions were as described in Fig. 1. Initial crystallization rates and amounts of CaCO₃ crystallized are expressed as percentages of the rate (5.6 µS·min⁻¹) and amount (13.2 µS) respectively observed after seed crystal addition in the absence of modulator. Abbreviations: ND, not determined; EHDP, ethane-1-hydroxy-1,1-diphosphonic acid.

Glycosaminoglycan	Initial crystallization rate (%)		Amount of CaCO ₃ crystallized (%)	
	No preincubation	Preincubation	No preincubation	Preincubation
Chondroitin 6-sulphate	75	43	ND	24
Hyaluronate*	68	53	ND	64
Dermatan sulphate	58	36	ND	13
Chondroitin 4-sulphate (river sturgeon)	48	23	116	35
Heparin				
Preparation A	25	12	81	6
Preparation B	ND	ND	ND	5
N-Desulphated heparin	112	57	99	52
Heparan sulphate (preparation A)	25	10	54	5
Tripolyphosphate	21	ND†	70	ND†
EHDP	29	ND†	110	ND†

* Inhibitory effects dependent upon viscosity of hyaluronate solution.

† Dissolution of seed crystals.

Table 2. Effect of glycosaminoglycans on crystallization from a CaCO₃ solution (saturation index approx. 31)

Experimental conditions were as described in Fig. 2. Rate-constant units are mol⁻¹·min⁻¹·dm³·(mg of seed)⁻¹·100 cm³ (Nancollas & Reddy, 1971).

Glycosaminoglycan	Final concentration (µg/ml)	Second-order rate constant
None		4.11
Chondroitin 4-sulphate (river sturgeon)	60	4.19
Hyaluronate	60	4.03
Chondroitin 6-sulphate	60	3.86
Keratan sulphate	60	3.60
Chondroitin 4-sulphate (whale)	60	3.29
	180	2.70
	720	1.22
Dermatan sulphate	60	3.30
Heparin		
Preparation A	60	1.52
	180	0.84
Preparation B	20	2.70
	40	1.93
	60	1.44
	180	0.84
	240	0.63
	480	0.33
	540	0.32
	600	0.26
N-Desulphated heparin	60	4.40
Heparan sulphate		
Preparation A	60	1.52
Preparation B	60	1.39
	300	0.42

sulphate were complex (results not shown); for chondroitin 6-sulphate, an initial short slow decrease in conductivity was followed by a major decrease; for dermatan sulphate, an initial short fast decrease was followed by a slower, but prolonged, decrease; for keratan sulphate, two processes, with differing kinetics, appeared to be superimposed. For the first two of these glycosaminoglycans, the rate constant in Table 2 is that of the major secondary process; for keratan sulphate, a pseudo-second-order rate constant was calculated by assuming that the two processes were superimposed zero- and second-order reactions.

An additional property of heparin was perceived in experiments involving very high CaCO₃ saturation index (approx. 400; results not shown). Under these conditions, spontaneous crystallization in the absence of exogenous seed crystals occurred. When heparin was present during this process, a slow increase in conductivity was observed after the initial crystallization event. This suggests that crystals formed in these circumstances may have a surface area/volume ratio that allows small co-precipitated ions to leach from the crystals. This did not occur in the absence of heparin.

Spontaneous crystallization occurred, even at a low saturation index, when the same crystallization vessel and electrode were repeatedly used. In this case, second-order rate constants were dependent on N₂-bubbling rates, and the effect was attributed to the formation and release of CaCO₃ crystals on the surfaces of vessel and electrode. This effect might account for reported variations in crystallization second-order rate constant with the amount of seed crystals added (Nancollas & Reddy, 1971). Glycosaminoglycans, particularly heparin and heparan sulphate, inhibited crystallization under these conditions.

Fig 2. Effect of heparin on crystallization from a CaCO_3 solution (saturation index approx. 31)

(a) Heparin (preparation B) was added to seed crystals and the mixture was incubated with a solution containing NaHCO_3 ($2 \times 10^{-2} \text{ mol}\cdot\text{dm}^{-3}$) and CaCl_2 ($8 \times 10^{-4} \text{ mol}\cdot\text{dm}^{-3}$) at 37°C . Heparin concentrations in the final reaction mix were 0.054 (○), 0.018 (●), 0.006 (△), 0.002 (▽) and 0 (▲) % (w/v). Seed crystals were present in the final reaction mix at $0.13 \text{ mg}\cdot\text{cm}^{-3}$. (b) Second-order rate plots were constructed by the method of Nancollas & Reddy (1974). On the ordinate scale, S_0 is the final conductivity after addition of seed crystals; other symbols are defined in Fig. 1. $\mu\text{S}^{-1} (\times 10^{-2})$ is equivalent to $\text{mol}\cdot\text{dm}^{-3} (\times 10^{-4})$.

DISCUSSION

Interest in the crystallization from aqueous solutions of sparingly soluble salts such as CaCO_3 is stimulated by the involvement of such crystallizations in biomineralization, during desalination processes and in oil-field scale formation (Nancollas, 1979). Crystallization consists of ill-understood nucleation events, followed by growth of nuclei to macroscopic dimensions through mechanisms for which several models have been proposed. Models of CaCO_3 crystallization have been discussed by House (1981).

The mechanism by which glycosaminoglycans inhibit CaCO_3 crystallization is not clear. It has been suggested that chondroitin sulphate inhibits skeletal calcification by complexing calcium and sulphate ions (Dunstone, 1962; Bowness *et al.*, 1967). Glycosaminoglycans, particularly heparins and heparan sulphate, together with the commercial crystallization inhibitors used in the

present study, form solution-phase complexes with Ca^{2+} , and, for heparin, this property depends, as does the crystallization-inhibitory property demonstrated here, upon the retention of the *N*-sulphonate group (Ayotte & Perlin, 1986). This solution-phase property is unlikely to be related directly to the anti-crystallization properties, however, since we find that reagents such as EDTA are not CaCO_3 -crystallization inhibitors. The variation, with concentration of an organic-phosphonate CaCO_3 -crystallization inhibitor, of the second-order rate constant of CaCO_3 crystallization, has been interpreted in terms of a decrease in effective crystal surface area in the presence of the inhibitor (Nancollas, 1979). Similarly, the kinetics of crystallization in the presence of heparins and heparan sulphate (Fig. 3) generate simple Freundlich (1926) adsorption isotherms.

Mast-cell heparins are of uncertain physiological function; chemically related heparan sulphates are

Fig. 3. Effect of heparin and heparan sulphate on crystallization from a CaCO₃ solution (saturation index approx. 31): adsorption isotherms (Freundlich, 1926)

○, Heparin preparation A; ●, heparin preparation B; □, heparan sulphate preparation A; ■, heparan sulphate preparation B. Experimental conditions were as described in Fig. 2. $k_{c,0}$ and $k_{c,i}$ are the second-order rate constants in the absence or presence of inhibitor respectively, derived from the gradients shown in Fig. 2(b).

components of mammalian pericellular matrices. The amounts and fine structures of heparan sulphate alter during changes in the growth patterns of cells and tissues, including neoplasia (Long & Williamson, 1979, 1983, 1984; Eldor *et al.*, 1987; Grant *et al.*, 1987; Nakajima *et al.*, 1988). We suggest that the possibility that such alterations may be causally related to the crystallogenic potential of tissues deserves consideration.

This work was supported by a grant from the Cancer Research Campaign.

REFERENCES

- Addadi, L., Moradian, J., Shay, E., Maroudas, N. G. & Weiner, S. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 2732–2736
- Ayotte, L. & Perlin, A. S. (1986) *Carbohydrate Res.* **145**, 267–277
- Barth, V., Franz, E.-D. & Schöll, A. (1977) *Naturwissenschaften* **64**, 278–279
- Borman, A. H., de Jong, E. W., Huizinga, M., Kok, D. J., Westbroek, P. & Bosch, L. (1982) *Eur. J. Biochem.* **129**, 179–183
- Bowness, J. A. T. & Lee, K. M. (1967) *Biochem. J.* **103**, 382–390
- Cheung, H. S., Van Wyk, J. J., Russell, W. E. & McCarty, D. J. (1986) *J. Cell Physiol.* **128**, 143–148.
- Davis, J. M. G. (1970) *Exp. Mol. Pathol.* **12**, 133–147
- Dunstone, J. R. (1962) *Biochem. J.* **85**, 336–351
- Eldor, A., Bar-Ner, M., Yahalom, J., Fuks, Z. & Vlodavsky, I. (1987) *Semin. Thromb. Haemostasis* **13**, 475–488
- Freundlich, H. (1926) *Colloid and Capillary Chemistry*, p. 337, Methuen, London
- Gill, W. B., Jones, K. W. & Ruggiero, K. J. (1982) *J. Urol.* **127**, 152–154
- Gjaldbaek, J. C. (1982) *Clin. Chim. Acta* **120**, 363–365
- Grant, D. (1979) *Eur. Polym. J.* **15**, 1161–1165
- Grant, D., Long, W. F. & Williamson, F. B. (1987) *Med. Hypotheses* **23**, 67–71
- Hallgrimsson, J., Friberg, U. & Burke, J. F. (1970) *Exp. Mol. Pathol.* **12**, 70–83
- House, W. A. (1981) *J. Chem. Soc. Faraday Trans. 1* **77**, 341–359
- Hunter, G. K., Allen, B. L., Grynepas, M. D. & Cheng, P.-T. (1985) *Biochem. J.* **228**, 463–469
- Kobayashi, S. (1971) *Int. Rev. Cytol.* **30**, 257–371
- Little, D. M. S. & Nancollas, G. H. (1970) *Trans. Faraday Soc.* **66**, 3103–3112
- Long, W. F. & Williamson, F. B. (1979) *IRCS J. Med. Sci. Libr. Compend.* **7**, 429–434
- Long, W. F. & Williamson, F. B. (1983) *Med. Hypotheses* **11**, 285–308
- Long, W. F. & Williamson, F. B. (1984) *Med. Hypotheses* **13**, 385–394
- Lowenstam, H. A. & Weiner, S. (1983) in *Biomaterialization and Biological Metal Accumulation* (Westbrook, P. & de Jong, E. W., eds.), pp. 191–203, D. Reidel Publishing Co., Dordrecht
- Martelli, A., Marchesini, B., Buli, P., Lambertini, F. & Rusconi, R. (1985) in *Urolithiasis and Related Clinical Research* (Schwilli, P. O., Smith, L. H., Robertson, W. G. & Vahlensieck, W., eds.), pp. 355–358, Plenum Publishing Co., New York
- Nagasawa, K. & Inoue, Y. (1980) *Methods Carbohydr. Chem.* **8**, 291–294
- Nakajima, M., Irimura, T. & Nicholson, G. L. (1988) *J. Cell. Biochem.* **36**, 157–167
- Nancollas, G. H. (1979) *Adv. Colloid Interface Sci.* **10**, 215–252
- Nancollas, G. H. & Reddy, M. M. (1971) *J. Colloid Interface Sci.* **37**, 824–830
- Nancollas, G. H. & Reddy, M. M. (1974) *Soc. Pet. Eng. J.* **14**, 117–126
- Parsons, C. L., Stauffer, C. & Schmidt, J. D. (1980) *Science* **208**, 605–607
- Reddy, M. M. & Nancollas, G. H. (1971) *J. Colloid Interface Sci.* **36**, 166–172
- Taylor, M., Simkiss, K. & Greaves, G. N. (1986) *Biochem. Soc. Trans.* **14**, 549–552
- Wexler, B. C. & Thomas, L. L. (1967) *Nature (London)* **214**, 243–245
- Winters, C., Davies, R. Ll., Morgan, A. J. & Gravelle, I. H. (1986) *Micron Microsc. Acta* **17**, 11–23
- Woodhead, N. E., Long, W. F. & Williamson, F. B. (1986) *Biochem. J.* **237**, 281–284
- Wusteman, F. S., Gold, C. & Wagner, J. C. (1972) *Am. Rev. Respir. Dis.* **106**, 116–118