Stimulation of Precipitation of Calcium Phosphate by Matrix Vesicles

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The ability of matrix vesicles isolated from the epiphyseal growth plate of 6-week-old chicks to facilitate the precipitation of calcium phosphate was studied *in vitro*. The vesicles lowered the minimum concentration product $[Ca^{2+}] \times [P_i]$ needed to induce crystal formation, thereby showing that the vesicles are nucleators of crystallization. After freezing and thawing the vesicles at pH 6.0, part but not all of this ability to nucleate disappeared. Freezing and thawing markedly decreased the Ca and P_i content of the vesicles, suggesting that part of the nucleating activity may have been due to mineral already present. After removal of the mineral the residual nucleating activity could be destroyed by extracting the vesicles with lipid solvents or by treatment with enzymes such as phospholipase C, neuraminidase or proteinase. Matrix vesicles obtained from chicks treated with 1-hydroxyethane-1,1-diphosphonate, a compound that inhibits calcification *in vivo*, showed impaired nucleating activity, both before and after treatment at pH 6.0. The vesicle preparation bound some diphosphonate *in vitro*, probably to the mineral present in the preparation, since no binding could be detected in vesicles preincubated at pH 6.0. No difference was found in the nucleating activity of vesicles isolated from rachitic chicks which had or had not received cholecalciferol 48 h before death. These results suggest that matrix vesicles possess intrinsic nucleating activity that may be important in biological calcification.

Matrix vesicles or 'calcifying globules' were first observed by Bonucci (1967) and Anderson (1969) in calcifying epiphyseal cartilage. Since deposits of crystals seemed to appear close to these organelles, these authors proposed that they were involved in the calcification process. A large amount of alkaline phosphatase, pyrophosphatase and adenosine triphosphatase activity is associated with the matrix vesicles (Ali *et al.*, 1970; Matsuzawa & Anderson, 1971). These activities, which may be part of a single enzyme complex (Majeska & Wuthier, 1975), are thought to play some role in the initial stages of precipitation of calcium phosphate. They might increase the concentration of P_i locally, by hydrolysis of phosphate substrates, or they might participate in the translocation and accumulation of Ca^{2+} (Ali & Evans, 1973; Ali, 1976) and/or P_i within the matrix vesicles (Anderson & Reynolds, 1973; Anderson *et al.*, 1975). Alternatively they might destroy inhibitors of mineralization (Fleisch & Neuman, 1961), such as PP_i (Felix & Fleisch, 1976b). Bonucci (1975) has proposed that the organic structure (the 'crystal ghost') probably functions as a template for crystal formation. A glycoprotein with a high affinity for Ca^{2+} and with alkaline phosphatase

activity has been isolated from calf scapula cartilage (Vittur & de Bernard, 1973). The matrix vesicles are rich in phosphatidylserine (Peress *et al.*, 1974; Wuthier, 1976), which has a high affinity for bivalent-metal ions such as Ca^{2+} (Papahadjopoulos, 1968), and lipids, especially acidic phospholipids, have been suggested to be involved in the initiation of calcification (Irving & Wuthier, 1968). At least part of the mechanism by which matrix vesicles initiate calcification could involve a nucleating action, whereby organic structures induce the precipitation of calcium phosphate from a solution supersaturated with respect to calcium and phosphate. Indeed nucleating activity has been demonstrated in interstitial fluid containing matrix vesicles and aspirated from calcifying rat cartilage (Howell *et al.*, 1975). In a previous preliminary report we showed that isolated vesicles possessed nucleating activity (Felix & Fleisch, 1976a).

In the present paper the nature of this nucleation property of vesicles isolated from epiphyseal growth plates has been studied under various conditions. These include studies *in vivo* and *in vitro* with disodium 1-hydroxyethane-1,1-diphosphonate, an analogue of PP_i that inhibits the precipitation of calcium phosphate *in vitro* (Fleisch *et al.*, 1970) and the calcification of the epiphyseal growth plate *in vivo* (Schenk *et al.*, 1973). For comparison we used a different diphosphonate, disodium dichloromethane-

Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; Tes, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulphonic acid.

diphosphonate, a compound that does not inhibit the calcification of the growth plate except to a minor degree at high concentrations (Schenk *et al.*, 1973), although it does inhibit the precipitation of calcium phosphate *in vitro* and ectopic calcification *in vivo* (Fleisch *et al.*, 1970).

Experimental

Materials

Hepes and Tes were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Bovine serum albumin was from Fluka, Buchs, Switzerland, butyl-PBD [5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole] from Ciba-Geigy, Basle, Switzerland, poly(vinyl alcohol) from Grogg, Berne, Switzerland, and neomycin sulphate from Médical de Toledo, Geneva, Switzerland. Disodium 1-hydroxyethane-1,1-diphosphonate, disodium dichloromethanediphosphonate, disodium 1-hydroxy[^{14}C]-ethane-1,1-diphosphonate (sp. radioactivity $1.9 \mu\text{Ci}/\mu\text{mol}$) and disodium dichloro[^{14}C]methanediphosphonate (sp. radioactivity $0.53 \mu\text{Ci}/\mu\text{mol}$) were from Procter and Gamble Co., Cincinnati, OH, U.S.A., and $^{45}\text{CaCl}_2$ (sp. radioactivity $1.1 \text{mCi}/\mu\text{mol}$) was from The Radiochemical Centre, Amersham, Bucks., U.K. All other reagents were analytical grade and were obtained from Merck, Darmstadt, West Germany.

Proteinase and neuraminidase were obtained from Sigma. Stated activities were as follows: 1 mg of proteinase (purified, type VI) liberated $3.4 \mu\text{mol}$ of tyrosine/min from casein at pH 7.5 at 37°C (3.4 units), 1 mg of neuraminidase (from *Clostridium perfringens* type V, purified) released $0.65 \mu\text{mol}$ of *N*-acetylneuraminic acid/min at pH 5.0 at 37°C (0.65 unit). The contamination of the latter by proteinase activity as defined above was 0.001 unit/mg of protein. Bacterial collagenase (CLS II) (125 units/mg) and phospholipase C (from *Clostridium perfringens*) were obtained from Worthington, Freehold, NJ, U.S.A.; hyaluronidase (from bovine testes, 300 N.F. units/mg) was from ICN, Nutritional Biochemicals, Cleveland, OH 44128, U.S.A. Phospholipase C liberated $2.1 \mu\text{mol}$ of phosphocholine from phosphatidylcholine at 25°C (2.1 units)/mg of protein. A unit of collagenase activity is defined as $1 \mu\text{mol}$ of amino acid produced in 5 h. The N.F. unit is defined according to a standard preparation described by Dorfman (1955).

Animals

Leghorn chickens (7 weeks old) weighing 1.5 and 2.0 kg were obtained from local farms. They were killed by decapitation, the legs were removed, and the cartilage of the epiphyseal growth plate was dissected as described below.

When the chickens were treated with diphosphonates, a dose of $161 \mu\text{mol}/\text{day}$ per kg was injected

subcutaneously daily for 7 days from day 41 to day 47 as a solution containing the compound at a concentration of $161 \mu\text{mol}/\text{ml}$. Control birds were injected with 0.15M -NaCl, and all birds were killed 24 h after the last injection.

To obtain rachitic chicks, 1-day-old chicks, obtained from a local breeder, were kept in a laminar-flow rack in the dark for 20 days and fed with a diet deficient in vitamin D_3 (0.5 g% calcium, 0.58 g% phosphorus, obtained from Station de Recherches Avicoles INRA, B.P. 1, 37 Nouzilly, France), and 48 h before they were killed they were given orally 0.2 ml of arachis oil (Sais, Horn, TG, Switzerland) with or without $12.5 \mu\text{g}$ (500 units) of cholecalciferol.

Methods

Nucleation measurements. The tests were performed by a modification of the method of Fleisch & Neuman (1961). Three stock solutions of the following composition were prepared and filtered through Millipore filters, pore size $0.45 \mu\text{m}$: (a) 10.7mM - KH_2PO_4 , 0.117M -KCl, 10mM -Hepes; (b) 5.6mM - CaCl_2 , 0.138M -KCl, 10mM -Hepes; (c) 0.155M -KCl, 10mM -Hepes. Solution (a) was always freshly prepared, whereas solutions (b) and (c) were kept up to 1 month if stored at 4°C . All solutions were adjusted to pH 7.60 at 37°C , a pH found by Howell *et al.* (1968) in fluid aspirated from calcifying rat cartilage. The composition of these three solutions is such that mixed in any proportion the ionic strength and the pH of the mixtures remain constant.

The three stock solutions were pipetted into dust-free conical 1.5 ml plastic tubes so that the final mixture contained Ca^{2+} at 1.70mM . The total P_i concentration was varied so that the $[\text{Ca}^{2+}] \times [\text{P}_i]$ product increased by increments of 0.8mM^2 , covering the range of 0.8 – 6.4mM^2 . Furthermore $10 \mu\text{l}$ of $^{45}\text{CaCl}_2$ ($0.01 \mu\text{Ci}$)/neomycin ($10 \text{mg}/\text{litre}$) to inhibit bacterial growth, and in one series of experiments diphosphonates, and matrix vesicles suspended in solution (c) were added. The final total volume was 0.59ml . The tubes were then covered with caps and shaken for 19 h at 37°C (amplitude 0.15mm , 1470 oscillations/min). After the incubation the tubes were kept at 25°C for 15 min and centrifuged for 6 min at $7000g_{av.}$. Samples ($100 \mu\text{l}$) of the supernatant were taken, and added to 10ml of scintillation fluid (0.6litre of toluene, 0.4litre of 2-methoxyethanol, 7g of butyl-PBD and 80g of naphthalene) and counted for radioactivity in a Packard liquid-scintillation counter. When no precipitation occurred the radioactivity remaining in $100 \mu\text{l}$ of supernatant was 4000 – 5000c.p.m. A decrease in radioactivity (at least down to 2000c.p.m.) was used to indicate that precipitation of calcium phosphate had occurred (Felix & Fleisch, 1976a). The results were expressed as the minimum product of $[\text{Ca}^{2+}] \times [\text{P}_i]$ necessary for calcium to be precipitated. In most cases the transition

from no precipitation to full precipitation occurred over an increment of 0.8mm^2 , for example no precipitation (4000–5000 c.p.m.) might be found at 2.4mm^2 , and a complete precipitation (<2000 c.p.m.) at 3.2mm^2 . In this case a value of 3.2mm^2 would be given for the formation product. Sometimes precipitation did not occur so sharply, e.g. no precipitation (4000–5000 c.p.m.) might be found at 2.4mm^2 , some (2000–3000 c.p.m.) at 3.2mm^2 and complete precipitation (<2000 c.p.m.) at 4.0mm^2 . In this case a value of 3.6mm^2 would be given. More exact numbers could not be obtained with this system, but the precision was somewhat improved if the mean of several individual values was calculated.

Isolation of matrix vesicles. This was performed essentially by the method of Majeska & Wuthier (1975) with some modifications described in detail elsewhere (Felix & Fleisch, 1976b). In short, epiphyseal growth plates from the femur, tibia and metatarsus were dissected out and cut into slices $0.1\text{--}0.2\text{mm}$ thick, washed with cold Gey's balanced-salt solution, dried between filter papers and weighed. Gey's balanced-salt solution for slide cultures (Gey & Gey, 1936) was used for the entire isolation procedure. Phosphate was replaced by 2mM-Tes, pH 7.4, to prevent any possible precipitation of calcium phosphate. After digestion with hyaluronidase and collagenase (full details in Felix & Fleisch, 1976b), cells were spun down at $1500g_{av.}$ for 10 min (fraction I) and cell fragments at $20000g_{av.}$ for 10 min (fraction II). The supernatant was divided into batches according to the experimental design planned and centrifuged at $100000g_{av.}$ for 30 min. The pellet was resuspended in Gey's balanced-salt solution and centrifuged once more at $100000g_{av.}$ for 60 min. The pellet (matrix vesicles; fraction III) was then suspended in 1 ml of solution (c)/g of cartilage and subsequently used for the nucleation test. When the test could not be done on the day of isolation, the vesicles pellet obtained by the centrifugation at

$100000g_{av.}$ for 30 min was suspended in Gey's balanced-salt solution and kept frozen overnight. The next day it was thawed, the vesicles were spun down at $100000g_{av.}$ for 60 min and the pellet was suspended in solution (c) as described above. The nucleating activity was the same whether the test was done on the day of the isolation or 1 day later.

The matrix vesicles from 1 g of cartilage were always resuspended in a final volume of 1 ml and the results of the nucleation test therefore expressed in relation to the wet weight of cartilage from which the vesicles had been isolated. In some Tables the alkaline phosphatase activity is also given. The pooled results of many experiments showed that digestion of the cartilage yielded [given as mean $\pm$ S.E.M. (n)] $1.49 \times 10^8 \pm 0.61 \times 10^8$ cells/g of cartilage (27). The vesicle fraction obtained from 1 g of cartilage contained 0.28 ± 0.023 (4) mg of protein and 13.2 ± 0.42 (27) units ($\mu\text{mol/min}$) of alkaline phosphatase activity.

Chickens treated with 1-hydroxyethane-1,1-diphosphonate had a much wider zone of hypertrophic cells than did normal chickens (Bisaz *et al.*, 1975). Therefore, when the cartilage from chickens treated with 1-hydroxyethane-1,1-diphosphonate was cut into slices, only a small part of this hypertrophic cartilage was taken, to yield about the same amount of hypertrophic tissue as from normal chickens, so that the total amount of tissue taken from normal birds and from treated birds was also similar in both cases. In chickens treated with dichloromethanediphosphonate the size of unmineralized cartilage layers was not different from that in normal birds. The epiphyseal growth plates from vitamin D-deficient chickens looked macroscopically the same whether or not they had received cholecalciferol 48 h before death. Table 1 shows the number of cells obtained after digestion and the distribution of the alkaline phosphatase found when the matrix vesicles

Table 1. Cell number and distribution of alkaline phosphatase in normal, diphosphonate-treated and rachitic chickens. The numbers of cells obtained from 1 g of cartilage and the distribution (% of initial activity) of the alkaline phosphatase among the different fractions are given as the means $\pm$ S.E.M. (n) or as individual values. Matrix vesicles were isolated as described under 'Methods' from vitamin D-replete chickens that had been treated with NaCl or diphosphonates, or from vitamin D-deficient chickens that had or had not received cholecalciferol. Fraction I contains cells ($1500g_{av.}$ for 10 min), fraction II cell debris ($20000g_{av.}$ for 10 min) and fraction III vesicles ($100000g_{av.}$ for 30 min).

Treatment of chickens	Vitamin D-replete			Vitamin D-deficient	
	+NaCl	+1-Hydroxyethane-1,1-diphosphonate	+Dichloromethane-diphosphonate	–Cholecalciferol	+Cholecalciferol
Cell number	$(1.12 \pm 0.12) \times 10^8$ (5)	$(0.95 \pm 0.39) \times 10^8$ (4)	$(1.15 \pm 0.04) \times 10^8$ (3)	1.2×10^8	1.37×10^8
Alkaline phosphatase					
Fraction I	39.5 ± 0.85 (5)	30.6 ± 1.3 (4)	37.1 ± 2.3 (3)	34.2, 35.8	36.2, 41.2
Fraction II	15.1 ± 0.81 (5)	14.4 ± 2.3 (4)	16.0 ± 0.5 (3)	18.3, 18.4	18.0, 16.1
Fraction III	22.2 ± 1.34 (5)	26.1 ± 1.4 (4)	23.5 ± 0.8 (3)	24.1, 21.8	23.1, 20.6
Supernatant	20.2 ± 0.78 (5)	25.7 ± 1.0 (4)	20.9 ± 1.9 (3)	20.8, 20.8	20.2, 19.5

were isolated from chickens treated with 0.15% NaCl or diphosphonate, or rachitic ($\pm$ cholecalciferol) chickens. The values for the controls and treated birds did not differ and were similar to those reported by Felix & Fleisch (1976b).

Treatment of matrix vesicles at pH 6.0. Since any nucleation effect might have been induced by apatite crystals already present in the isolated matrix vesicles, preincubation in an acid solution was used to dissolve any crystals present. The vesicles that were collected after centrifugation at $100000g_{av}$ for 30 min were resuspended in Gey's balanced-salt solution with 5 mM-maleic acid, but without NaHCO_3 and P_i . The pH of this solution was adjusted to 6.0 with NaOH. The suspension was then frozen and thawed five times, after which it was left on ice for 30–45 min. Thereafter the vesicles were spun down at $100000g_{av}$ for 60 min, and the pellet was resuspended in solution (c) as described above and used for the nucleation test. When the test was not done on the day of isolation, the vesicle suspension at pH 6.0 was kept frozen overnight and the procedure was completed the next day as described above.

Lipid extraction. The lipids were extracted as described by Fleischer *et al.* (1967). After the matrix vesicles had been either frozen in Gey's balanced-salt solution or frozen and thawed five times at pH 6.0, they were centrifuged at $100000g_{av}$ for 60 min. The pellets were resuspended in one of the following ice-cold solvents: ethanol; butan-1-ol; 10% (v/v) acetone in water; 10% (v/v) water in acetone; the latter to which $10\mu\text{l}$ of 25% (w/v) ammonia per 74.4 ml had been added. The suspensions were left in an ice bath for 15 min and were then centrifuged for 60 min at $220000g_{av}$. The pellets were then resuspended in solution (c) and used for the nucleation test.

Treatment of matrix vesicles with enzymes. (a) Phospholipase C. After the matrix-vesicle suspension had been frozen and thawed at pH 6.0, it was centrifuged at $100000g_{av}$ for 60 min. The pellet was suspended in a solution containing 10 mM-Hepes, 1.0 mM- CaCl_2 and 0.163 M-NaCl, pH 7.3, and incubated for 45 min at 37°C with 0, 0.021, 0.21 or 2.1 units of phospholipase C/ml. The final volume was 2.0 ml and contained the matrix vesicles isolated from 1.3 g of cartilage. After the 45 min incubation, 7 ml of cold Gey's balanced-salt solution was added and the mixture was centrifuged at $100000g_{av}$ for 60 min. The pellets were resuspended in 1.3 ml of solution (c) and used for the nucleation test.

(b) Neuraminidase. The experiments were carried out by the procedure described above for phospholipase C, except that the treatment with neuraminidase was at two pH values, pH 7.0 and pH 6.0, the latter being closer to the pH optimum for the enzyme. The incubation media were either 10 mM-Tris/maleate/HCl at pH 6.0 and 0.150 M-NaCl, or 10 mM-Hepes at pH 7.0 and 0.161 M-NaCl. The

matrix vesicles isolated from 1.3 g of cartilage were incubated for 60 min at 37°C in 1 ml final volume with 0, 0.01, 0.1 or 1.0 unit of neuraminidase. At the end of the incubation 8 ml of cold Gey's balanced-salt solution was added and the mixture was centrifuged at $100000g_{av}$ for 60 min.

(c) Proteinase. The experiments were performed as described above for phospholipase C. The matrix vesicles of 1.3 g of cartilage were incubated in a final volume of 1 ml containing 10 mM-Hepes at pH 7.5 and 0.160 M-NaCl for 45 min at 37°C with 0, 0.34, 3.4 or 34 units of proteinase. Thereafter 8 ml of Gey's balanced-salt solution was added and the mixture was centrifuged at $100000g_{av}$ for 60 min.

All these enzyme solutions were centrifuged at $180000g_{av}$ for 60 min before the matrix vesicles were added.

About $30\mu\text{l}$ of incubation medium diluted with Gey's balanced-salt solution was left in the tube, after the supernatant was poured off after centrifugation, so that some enzyme was left with the vesicles. For this reason, the effect of enzyme alone on the nucleation test was studied. The concentrations used were 0.7, 1.4 and 2.8 times the amount of enzyme expected to be present after the vesicles had been treated with the highest amount of enzyme.

Uptake of diphosphonate by vesicles in vitro. The vesicles obtained from 0.9–1.2 g of cartilage, either treated or untreated at pH 6.0, were resuspended in 2.0 ml of Gey's balanced-salt solution containing phosphate and 2 mM-Tes, pH 7.6, to which either 0.1 mM-1-hydroxy ^{14}C ethane-1,1-diphosphonate, 0.1 mM-dichloro ^{14}C methanediphosphonate or no diphosphonate had been added. The mixture was incubated for 1 h at 37°C . Then 7 ml of cold Gey's balanced-salt solution (containing Tes, but no phosphate) was added and the mixture was centrifuged at $180000g_{av}$ for 35 min. The pellet was washed with $2\times 9\text{ ml}$ of Gey's balanced-salt solution. The final pellet was resuspended in solution (c) and used for the nucleation test, and $100\mu\text{l}$ samples of all the supernatants and of the vesicle suspension were counted for radioactivity in 10 ml of scintillation fluid as described above for ^{45}Ca .

Ca^{2+} determination. Ca^{2+} was measured fluorimetrically by a modification of the method of Bandrowski & Benson (1972). After centrifugation of the matrix vesicles, the pellet obtained from 1.2–1.9 g of cartilage was suspended in 0.2–0.4 ml of 5% (w/v) trichloroacetic acid, left for 1 h and centrifuged at $7000g_{av}$ for 5 min. Then $10\mu\text{l}$ of supernatant or standard in 5% (w/v) trichloroacetic acid was added to 1.25 ml of 0.8 M-KOH containing $7.5\mu\text{g}$ of calcein {2,7-bis[bis(carboxymethyl)aminomethyl]-fluorescein}. Fluorescence was measured on an Aminco 4-7440 solid-state Blanksubtract Fluorocolorimeter with excitation at 460 nm (Wratten filters 2A, 96 and 48) and emission at 510 nm and higher

(Wratten filter 2A+12). The concentrations of standards and samples were between 0.125 mM- and 0.875 mM- Ca^{2+} .

Phosphate determination. P_i was measured with Malachite Green by the method of Altmann *et al.* (1971), with 40-times smaller volumes. The 5% (w/v) trichloroacetic acid extract of vesicles, prepared for the calcium determination, was used: 10 μl of sample was added to 1 ml of 0.3375 M- H_2SO_4 and 0.25 ml of a mixture of 5 g of poly(vinyl alcohol) and 185 mg of Malachite Green dissolved in 1 litre of water; 10 μl samples of standard solutions containing 5% (w/v) trichloroacetic acid were used. All solutions had a concentration between 0.1 mM- and 0.5 mM- P_i .

Alkaline phosphatase determination. This enzyme was measured as described by Morgan *et al.* (1973): 10 μl of solution with enzyme activity was incubated in 0.5 ml of incubation medium for 8 min. A unit represents 1 μmol of *p*-nitrophenol produced/min.

Protein determination. This was performed as described by Lowry *et al.* (1951), with bovine serum albumin as a standard.

Electron micrographs. After centrifugation at 100000 $g_{av.}$ for 60 min the pellet was fixed for 2 h in the tube with 1.8% (v/v) glutaraldehyde in 0.1 M-cacodylate buffer, pH 7.4, at an osmolality of 380 mosmol/l. After this it was washed three times with 0.1 M-cacodylate buffer, pH 7.4, containing sufficient sucrose to bring the solution to 340 mosmol/l, and post-fixed with 1% OsO_4 in the same buffer. After dehydration in graded ethanol and propylene-oxide the pellets were embedded in Epon 812. Sections were cut on a Reichert OMU 3 microtome, and then stained with uranyl acetate and lead citrate before examination in a Philips 300 electron microscope.

Measurement of width of epiphyseal plate. Sections of the upper part of the tibia were stained by Von Kossa's technique (Romeis, 1968). The width of the epiphyseal plate was measured with a ruler.

Results

Nucleating activity of normal vesicles

As shown in Table 2 and as described earlier (Felix & Fleisch, 1976a), isolated matrix vesicles have a strong nucleating activity. They induce calcium phosphate precipitation at a $[\text{Ca}^{2+}] \times [\text{P}_i]$ product as low as 1.6 mm^2 , compared with control values of 5.08 mm^2 in the absence of vesicles.

Effect of treatment at pH 6.0

When the isolated matrix vesicles were frozen and thawed at pH 6.0, the nucleating activity decreased, but a significant amount of activity remained (Table 2). This procedure resulted in dissolution of calcium phosphate already present, as shown in Table 3. The same loss of nucleating activity was also

Table 2. Nucleating activity of isolated matrix vesicles

Influence of various amounts of vesicles on the minimum product of $[\text{Ca}^{2+}] \times [\text{P}_i]$ necessary for inducing precipitation of calcium phosphate <i>in vitro</i> . Incubation was for 19 h at 37°C in a final volume of 0.59 ml. Results are means $\pm$ s.e.m. for the numbers of observations in parentheses.		$[\text{Ca}^{2+}] \times [\text{P}_i]$ (mm^2)			
		0 μl	25 μl	50 μl	200 μl
Treatment of vesicles	Alkaline phosphatase activity (units/ml)	13.1 $\pm$ 0.44 (19)	13.1 $\pm$ 0.44 (19)	13.1 $\pm$ 0.44 (19)	13.1 $\pm$ 0.44 (19)
	Suspension of matrix vesicles added	...	...	...	...
Kept at pH 7.4		5.16 $\pm$ 0.08 (33)	2.34 $\pm$ 0.08 (27)	2.18 $\pm$ 0.08 (33)	1.6 (1)
Frozen and thawed five times at pH 6.0		5.00 $\pm$ 0.08 (17)	—	4.00 $\pm$ 0.24 (9)	3.14 $\pm$ 0.08 (6)

Table 3. *Calcium and phosphate content of isolated matrix vesicles*

The values are given as μmol of Ca^{2+} or P_i in the vesicles obtained from 1 g of cartilage. Results are means $\pm$ S.E.M. for the numbers of determinations given in parentheses.

Treatment of vesicles	Content ($\mu\text{mol/g}$ of cartilage)		Ratio $\text{Ca}^{2+}/\text{P}_i$
	Ca^{2+}	P_i	
Kept at pH 7.4	0.084 ± 0.006 (4)	0.054 ± 0.009 (4)	1.63 ± 0.18 (4)
Frozen and thawed five times at pH 6.0	0.008 ± 0.005 (3)	0.009 ± 0.004 (3)	—

Table 4. *Effect on Ca^{2+} and P_i content of fixation and dehydration for electron microscopy*

The values for individual pellets are given and represent the mineral content of matrix vesicles obtained from 1.2 g of cartilage.

Procedure	Ca^{2+} (μmol)	P_i (μmol)	$\text{Ca}^{2+}/\text{P}_i$
Directly isolated	0.118, 0.126	0.094, 0.084	1.26, 1.50
After glutaraldehyde and OsO_4	0.085, 0.091	0.006, 0.006	14.0, 15.1
After glutaraldehyde, OsO_4 and dehydration	0.094, 0.088	0.0055, 0.006	17.05, 14.7

observed when matrix vesicles were left in a suspension of pH 6.0 at 0°C for 1 h without freezing. Freezing and thawing at pH 7.4, however, had no effect (Felix & Fleisch, 1976a).

Nucleation at physiological concentrations of Ca^{2+} and P_i

In a further study, instead of determining the $[\text{Ca}^{2+}] \times [\text{P}_i]$ product necessary for precipitation, the time required for induction of calcium phosphate precipitation at a physiological $[\text{Ca}^{2+}] \times [\text{P}_i]$ product was measured. The concentration of $[\text{Ca}^{2+}] \times [\text{P}_i]$ chosen was that reported by Howell *et al.* (1968) in the fluid aspirated from rat epiphyseal cartilage. Vesicles obtained from 1.8 g of cartilage and frozen and thawed at pH 6.0 were incubated at 37°C in a final volume of 2.0 ml, which contained 1.2 mM- CaCl_2 , 2.0 mM- NaH_2PO_4 , 5.0 mM- KCl , 0.125 M- NaCl , 0.25 mM- MgCl_2 , 0.25 mM- MgSO_4 , 5.5 mM-D-glucose, 10 mg of neomycin/litre and 25 mM-Hepes adjusted to pH 7.6 at 37°C with NaOH . In the presence of vesicles, calcium phosphate precipitation occurred after 38–48 h, whereas no precipitation was observed in the control.

Morphological appearance of the vesicles

Morphologically (Plate 1) the matrix vesicles did not change after being frozen and thawed at pH 7.4. Exposure to pH 6.0 also had no observable effect. Crystals were sometimes observed in vesicles treated at pH 7.4, but never at pH 6.0. The procedure used

Table 5. *Nucleating activity after lipid extraction*

The isolated matrix vesicles were either directly extracted with lipid solvents (a) or were first frozen and thawed five times at pH 6.0 and then extracted with lipid solvents (b). For details see under Methods.

(a) Vesicle preparation added	$[\text{Ca}^{2+}] \times [\text{P}_i]$ (mm ²)		
Suspension of matrix vesicles added	...	0	25 μl 50 μl
Not extracted			2.4 2.4
Extracted with:	}		
90% acetone			3.2 2.8
90% acetone+NH ₃		5.2	3.2 3.2
Ethanol			2.8 2.8
Butan-1-ol			3.2 3.2
$[\text{Ca}^{2+}] \times [\text{P}_i]$ (mm ²)			
(100 μl of matrix vesicle suspension added)			
(b) Vesicle preparation	Expt. 1		Expt. 2
No vesicles	5.0		4.0
Vesicles not extracted	4.0		2.4
Vesicles extracted with:			
10% acetone	4.8		4.0
90% acetone	> 5.6		4.0
90% acetone+NH ₃	> 5.6		3.6

for fixation and staining probably dissolved much of the mineral, as suggested by the data in Table 4, which shows that Ca^{2+} and especially P_i markedly decreased after fixation with glutaraldehyde and OsO_4 .

Effect of lipid solvents

Extraction with lipid solvents of normal matrix vesicles not pretreated at pH 6.0 either had no effect or only slightly decreased the nucleating activity (Table 5a). In contrast, when matrix vesicles were first frozen and thawed at pH 6.0 and then extracted with lipid solvents, the nucleating activity disappeared completely (Table 5b).

Effect of enzymes

Matrix-vesicle preparations frozen and thawed at pH 6.0 were incubated with phospholipase C,

EXPLANATION OF PLATE I

Electron micrograph of isolated matrix vesicles

(a) Vesicles that had not been further treated. (b) Vesicles that had been frozen and thawed five times at pH7.4. (c) Vesicles that had been left at pH6.0 for 1 h. (d) Vesicles that had been frozen and thawed five times at pH6.0 and then left in ice for 45 min.

Table 6. *Nucleating activity after treatment at pH 6.0 and with enzyme*
After the isolated matrix vesicles had been frozen and thawed five times at pH 6.0, they were incubated with phospholipase C, proteinase or neuraminidase, and 100 μ l of vesicle suspension was then tested for nucleating activity. As controls the enzymes alone were tested for a possible effect on nucleation. Each value represents the mean of the duplicates of an individual experiment. For details, including the choice of enzyme concentration used, see under 'Methods'.

Additions	Incubation with phospholipase C				Incubation with neuraminidase				Incubation with proteinase			
	at pH 6.0				at pH 7.0							
	Phospho- lipase C (units/ml)	[Ca ²⁺] $\times$ [P _i] (mm ²)	Alkaline phosphatase (units/ml)	Neur- aminidase (units/ml)	[Ca ²⁺] $\times$ [P _i] (mm ²)	Alkaline phospha- tase (units/ml)	[Ca ²⁺] $\times$ [P _i] (mm ²)	Alkaline phospha- tase (units/ml)	Proteinase (units/ml)	[Ca ²⁺] $\times$ [P _i] (mm ²)	Alkaline phospha- tase (units/ml)	Proteinase (units/ml)
No vesicles	—	4.8, 4.8, 5.2	—	—	4.8, 4.8	—	4.8	—	—	4.8, 4.8	—	—
Vesicles not incubated	—	3.2, 3.2, 3.8	10.5, 12.8, 9.4	—	3.2, 3.2	14.1, 11.7	3.2	11.7	—	4.0, 3.2	10.4, 9.4	—
Vesicles incubated with or without enzyme	0	3.2, 3.2, 3.6	11.4, 10.6, 8.0	0	4.0, 3.6	15.9, 11.8	3.2	10.9	0	3.6, 3.2	8.3, 8.6	—
	0.021	3.2, 3.2	10.0, 11.3	0.01	3.6	14.1	—	—	0.34	4.0, 3.2	9.6, 8.3	—
	0.21	4.0, 3.2	10.7, 10.6	0.1	4.0, 4.0	15.4, 9.4	—	—	3.4	4.8, 4.6	9.7, 8.1	—
	2.1	4.8, 5.6	10.2, 7.4	1.0	4.8, 5.6	16.2, 8.3	4.8	7.9	34.0	4.8, 4.8	7.6, 8.7	—
Enzyme alone	1.5	4.8, 5.4, 5.2	—	0.7	5.6	—	4.8	—	24.0	4.8, 5.6	—	—
	3.0	5.6	—	1.4	5.6	—	4.8	—	48.0	4.8, 5.4	—	—
	6.0	5.6, 4.8	—	2.8	6.0	—	5.2	—	96.0	4.8, 4.8	—	—

Table 7. *Nucleating activity of matrix vesicles obtained from chickens that had been treated with diphosphonates*
Matrix vesicles were obtained from 7-week-old chickens that had been injected subcutaneously for 7 days with disodium 1-hydroxyethane-1,1-diphosphonate or disodium dichloromethanediphosphonate (161 $\mu\text{mol/day}$ per kg) or with 0.15M-NaCl. The means $\pm$ S.E.M. (*n*) are given.

Amount of matrix vesicle suspension added	[Ca ²⁺] $\times$ [P _i] (mm ²)		
	NaCl	1-Hydroxyethane-1,1-diphosphonate	Dichloromethane-diphosphonate
0	4.68 $\pm$ 0.16 (5)	4.60 $\pm$ 0.20 (4)	4.8 $\pm$ 0 (3)
25 μl } pH 7.4-treated	2.34 $\pm$ 0.27 (5)	4.98 $\pm$ 0.14 (3)†	2.56 $\pm$ 0.35 (3)
50 μl }	2.10 $\pm$ 0.23 (5)	4.70 $\pm$ 0.14 (3)†	1.88 $\pm$ 0.27 (3)
100 μl }	1.85 $\pm$ 0.10 (5)	3.77 $\pm$ 0.27 (3)†	1.75 $\pm$ 0.14 (3)
50 μl } pH 6.0-treated	4.40 $\pm$ 0.19 (5)	4.80 $\pm$ 0 (4)	4.70 $\pm$ 0.14 (3)
100 μl }	3.87 $\pm$ 0.16 (5)	4.60 $\pm$ 0.20 (4)*	3.77 $\pm$ 0.27 (3)
200 μl }	3.20 $\pm$ 0 (5)	4.00 $\pm$ 0 (4)†	3.60 $\pm$ 0.23 (3)
Alkaline phosphatase in matrix-vesicle preparation obtained from 1 g of cartilage (μmol of <i>p</i> -nitrophenyl phosphate/min)	13.0 $\pm$ 1.1 (5)	34.7 $\pm$ 3.3 (4)†	21.4 $\pm$ 2.4 (3)*
Alkaline phosphatase activity in 1 g of cartilage (μmol of <i>p</i> -nitrophenyl phosphate/min)	64.5 $\pm$ 2.1 (5)	153.0 $\pm$ 8.3 (4)†	111.3 $\pm$ 14.9 (3)†

* Significance of difference from control (NaCl), $P < 0.02$.

† Significance of difference from control (NaCl), $P < 0.01$.

‡ Significance of difference from control (NaCl), $P < 0.001$.

Table 8. *Effect of diphosphonates on the nucleation activity when added in vitro*

The diphosphonates were added to the nucleation test system together with or without matrix vesicles isolated from normal chicken.

Treatment	[Ca ²⁺] $\times$ [P _i] (mm ²)							
	No vesicles		With vesicles					
	No diphosphonates	No diphosphonates	1-Hydroxyethane-1,1-diphosphonate			Dichloromethane-diphosphonate		
			1 μM	0.1 μM	0.01 μM	10 μM	1 μM	0.1 μM
Expt. 1								
50 μl , treated at pH 7.4	4.8, 4.8	2.4, 2.4	6.4, 7.2	—	—	8.8, 8.8	—	—
100 μl , frozen and thawed five times at pH 6.0	4.8, 4.8	4.0, 4.0	7.2, 7.2	—	—	7.6, 8.0	—	—
Expt. II:								
50 μl , treated at pH 7.4	4.8, 4.8	2.8, 3.2	—	3.6	3.2	—	3.2	3.2
100 μl , frozen and thawed five times at pH 6.0	4.8, 4.8	4.0, 4.0	—	4.8	4.0	—	4.8	4.0
No vesicles	4.0, 4.0, 4.0		5.6, 6.4, 5.6	4.0, 4.0	4.0, 4.0	7.2, 7.2, 7.2	4.8, 4.0	4.0, 4.0

neuraminidase or proteinase. Whereas low concentrations of enzymes did not influence the nucleating activity, high concentrations of enzymes resulted in complete loss (Table 6). Additions of enzymes alone in some cases increased the [Ca²⁺] $\times$ [P_i] product necessary for precipitation from the control value of 4.8 mm² to a mean of 5.2 mm².

Effect of treatment with diphosphonates given in vivo

Table 7 shows the nucleating activity of matrix vesicles isolated from chickens treated with diphosphonates. Matrix vesicles from chickens treated with 1-hydroxyethane-1,1-diphosphonate had lost both the nucleating activity induced by the membrane as well as that induced by the calcium phosphate.

Table 9. *Uptake of diphosphonates by isolated matrix vesicles*

Matrix vesicles from 0.9 g of cartilage were treated at pH 7.4 and vesicles obtained from 1.2 g of cartilage were treated at pH 6.0. Both preparations were incubated for 1 h at 37°C in 2.0 ml of Gey's balanced-salt solution containing 2 mM-Tes, pH 7.6, and 0.1 mM-1-hydroxy[¹⁴C]ethane-1,1-diphosphonate or 0.1 mM-dichloro[¹⁴C]methanediphosphonate. Then 7.0 ml of ice-cold Gey's balanced-salt solution without phosphate was added followed by centrifugation at 180000g_{av} for 35 min. The supernatant was decanted, and the pellet washed twice with 9 ml of Gey's balanced-salt solution (for details see under 'Methods'). The amounts of diphosphonates found in the supernatant, in the washings and in the washed matrix vesicles are given in nmol. The values of two individual experiments are given.

	1-Hydroxyethane-1,1-diphosphonate (nmol)				Dichloromethane-diphosphonate (nmol)			
	pH 7.4		pH 6.0		pH 7.4		pH 6.0	
Supernatant	188.1,	190.0	196.1,	195.2	195.7,	195.0	196.3,	197.0
First washing	5.12,	5.6	2.76,	4.14	2.14,	3.56	1.64,	2.52
Second washing	1.44,	1.16	0.12,	0.26	0.66,	0.46	0.72,	0.04
Vesicles	5.24,	3.18	0.46,	0.40	1.52,	0.96	1.28,	0.40

Table 10. *Effect of vitamin D*

Chickens had been fed on a diet deficient in vitamin D, and 2 days before they were killed they received orally either 12.5 µg (500 units) of cholecalciferol (+D) or no cholecalciferol (-D). The values of two experiments are given, the plasma Ca²⁺ was determined only in the second experiment. In the absence of matrix vesicles the [Ca²⁺] × [P_i] product necessary for precipitation was 4.8 (mm²) in both experiments.

(a) Nucleating activity

Vesicle suspension added	[Ca ²⁺] × [P _i] (mm ²)	
	-D	+D
pH 7.4-treated		
50 µl	4.0, 4.0	3.2, 3.2
100 µl	3.2, 3.2	3.2, 3.2
200 µl	2.8, 2.4	2.4, 2.4
pH 6.0-treated		
100 µl	4.0, 4.0	4.0, 4.0
200 µl	3.2, 3.2	3.2, 3.2
Alkaline phosphatase in matrix vesicles obtained from 1 g of cartilage (µmol of p-nitrophenyl phosphate/min)	13.5, 24.3	17.9, 18.4

(b) Plasma Ca²⁺ and epiphyseal width

The means ± S.E.M. (numbers of animals) are given.

	-D	+D
Plasma Ca ²⁺ (mm)	1.22 ± 0.04 (4)*	1.97 ± 0.17 (4)*
Expt. 2		
Epiphyseal width (mm)		
First experiment	2.66 ± 0.19 (5)	2.31 ± 0.19 (5)
Second experiment	5.00 ± 0.96 (4)	3.00 ± 0.46 (4)

* The values are significantly different (*P* < 0.01).

was higher in the diphosphonate-treated birds, especially with 1-hydroxyethane-1,1-diphosphonate.

Effect of diphosphonates added in vitro

Diphosphonates were also added together with the matrix vesicles directly to the nucleation test system in concentrations ranging from 10 nM to 10 µM. As shown in Table 8, 1-hydroxyethane-1,1-diphosphonate inhibited the precipitation of calcium phosphate at 1 µM and dichloromethanediphosphonate at 10 µM. At lower concentrations both diphosphonates had little or no effect.

Uptake of diphosphonates by vesicles in vitro

Table 9 shows the uptake of 1-hydroxy[¹⁴C]-ethane-1,1-diphosphonate and of dichloro[¹⁴C]-methanediphosphonate added to isolated matrix vesicles *in vitro*. About 2% of the 1-hydroxyethane-1,1-diphosphonate added remained in the vesicles kept at pH 7.4. However, about 10 times less remained in matrix vesicles that had been frozen and thawed at pH 6.0. Less dichloromethanediphosphonate than 1-hydroxyethane-1,1-phosphonate was retained in the vesicle, and the difference between vesicles treated at pH 7.4 and pH 6.0 was less. It seemed that the diphosphonates could be completely eluted from the vesicles treated at pH 6.0, but not from those treated at pH 7.4; thus the logarithm of the radioactivity in each wash decreased linearly when expressed as a function of the number of washes at pH 6.0, but had the tendency to flatten off at pH 7.4.

The matrix vesicles that had been kept at pH 7.4 and that had been incubated with 1-hydroxyethane-1,1-diphosphonate or dichloromethanediphosphonate induced calcium phosphate precipitation at 4.0 mm², instead of at 3.2 mm² as occurred in the control incubated without diphosphonates. There was no difference in nucleating activity between diphosphonate-treated and untreated matrix vesicles

Treatment of the chickens with dichloromethanediphosphonate caused no change in the vesicles.

Alkaline phosphatase activity expressed as the activity in matrix vesicles derived from 1 g of cartilage

if they had been frozen and thawed at pH 6.0 before incubation with diphosphonates.

Effect of vitamin D

The nucleating activities of matrix vesicles isolated from chickens that had been kept on a diet deficient in vitamin D and that had or had not received cholecalciferol 48 h before they were killed were not different, although the vitamin treatment did significantly increase the plasma Ca^{2+} (Table 10).

Discussion

As described by Felix & Fleisch (1976a), isolated matrix vesicles strongly stimulate the precipitation of calcium phosphate. However, as shown in the present paper, a large part of this nucleating activity disappears when the matrix vesicles are frozen and thawed at pH 6.0, a procedure that dissolves the mineral present. There was no striking decrease in alkaline phosphatase activity, suggesting that loss of this enzyme was not responsible for the diminished nucleating activity. The effectiveness of this treatment with acid in dissolving calcium phosphate already present is shown by the fact that the content of these ions was greatly decreased after this treatment. Thus it appears that part of the nucleating activity of the vesicles is due to the presence of the mineral. Therefore the effect of other components of the vesicles on nucleation can be examined only after dissolution of the mineral. This is shown by the fact that extraction of lipid did not influence the nucleating activity when the matrix vesicles had not been pretreated at pH 6.0, but nucleation no longer occurred when they had been exposed to the low pH, and the mineral thereby dissolved. This fact makes it difficult to study the properties of matrix vesicles *in vitro*, because artifacts originating from crystals tend to occur. Previous work did show, however, that the nucleating activity could be destroyed by heating the matrix vesicles in a solution free of Ca^{2+} and P_i (Felix & Fleisch, 1976a). Heating in this way inactivates the alkaline phosphatase and disrupts the membrane, but is also likely to dissolve the deposits of calcium phosphate. Electron microscopy only partially helps to clarify the situation, since the procedure for fixation seems itself to dissolve the mineral, as has been noted by others (Termine, 1972). If one tries to avoid dissolution of mineral during fixation, one thus runs into the well-recognized risk of inducing additional precipitation of calcium phosphate during preparation of the material for electron microscopy (Schenk *et al.*, 1967).

After pretreatment at pH 6, acetone extraction resulted in complete loss of nucleating activity. This suggests that lipids are required for the nucleating activity caused by the organic component of the vesicles. However, it is possible that the acetone

treatment denatured proteins and extracted small peptides required for this activity. Phospholipase C, neuraminidase and proteinase also destroyed the nucleating activity remaining after pretreatment at pH 6.0. The amounts of these enzymes required were relatively high. Thus phospholipase C at 0.021 unit/ml, the lowest concentration used for incubation with matrix vesicles, lysed erythrocytes completely within 12 min. Since the three enzymes hydrolyse different molecules, and since they work only at high concentrations, it seems likely that more than one matrix component is responsible for nucleation, and that the integrity of the membrane itself is important for activity. It is noteworthy that alkaline phosphatase activity was unaltered by these treatments. Furthermore, it is notable that phospholipase C does not, at least in erythrocyte 'ghosts', attack phosphatidylserine (Coleman *et al.*, 1970), the lipid that has been suggested to be important for nucleation (Irving & Wuthier, 1968). The three enzymes themselves seemed to inhibit slightly the precipitation of calcium phosphate, but the marked decrease in nucleating activity (from 3.2 mm^2 to at least 4.8 mm^2) is too great to be explained in this way.

It is also noteworthy that the matrix vesicles isolated from chickens that had been treated with 1-hydroxyethane-1,1-diphosphonate had weaker nucleating activity, whether tested before or after pH 6.0 treatment, compared with the control. The matrix vesicles from chickens treated with dichloromethanediphosphonate were similar to those of the controls. This observation is consistent with results obtained *in vivo*, which show that 1-hydroxyethane-1,1-diphosphonate inhibits mineralization of bone and cartilage and induces abnormal calcification to produce an experimental condition reminiscent of rickets (King *et al.*, 1971; Jowsey *et al.*, 1971; Schenk *et al.*, 1973; Bisaz *et al.*, 1975), whereas dichloromethanediphosphonate has no such effect (Schenk *et al.*, 1973). When the diphosphonates were added with the matrix vesicles directly to the test system they had the same inhibitory activity as found earlier (Fleisch *et al.*, 1970) when tested *in vitro* in the absence of matrix vesicles, 1-hydroxyethane-1,1-diphosphonate being 10 times more potent than dichloromethanediphosphonate.

Since apatite binds diphosphonates, especially 1-hydroxyethane-1,1-diphosphonate (Jung *et al.*, 1973), it was expected that these compounds might be taken up by the vesicle preparation, that 1-hydroxyethane-1,1-diphosphonate might be bound to a greater extent than dichloromethanediphosphonate, and that dissolving the mineral by exposure to pH 6.0 might lessen this uptake. However, uptake of the diphosphonates *in vitro* had a smaller inhibitory effect on the precipitation of calcium phosphate than did administration *in vivo*.

The alkaline phosphatase in the matrix-vesicle

fraction and in the total cartilage itself was increased after diphosphonate treatment, particularly with 1-hydroxyethane-1,1-diphosphonate. However, the nucleation activity was diminished or unchanged, which suggests that the nucleation activity is independent of alkaline phosphatase.

Administration of cholecalciferol to rachitic chickens 48 h before they were killed had no effect on the nucleating activity. This might be because vitamin D does not act by changing nucleating activity, or that it takes more than 48 h for its effects to be observed. Although plasma calcium had increased at this time, there was no change in the epiphyseal width.

The nucleating activity remaining after pretreatment at pH 6.0 was small, but nonetheless it was sufficient to induce precipitation of calcium phosphate within 40 h at a $[Ca^{2+}] \times [P_i]$ product similar to that found in rat epiphyseal cartilage (Howell *et al.*, 1968). It is therefore possible that the matrix vesicles can function *in vivo* as nucleators of calcium phosphate precipitation. The fact that the matrix vesicles isolated from chickens treated with 1-hydroxyethane-1,1-diphosphonate had less nucleating activity supports this idea.

Apart from their nucleating activity, there are other mechanisms by which vesicles might induce precipitation. These include the accumulation of Ca^{2+} (Ali & Evans, 1973; Ali, 1976) and/or of P_i (Anderson & Reynolds, 1973; Anderson *et al.*, 1975), possibly by active processes or the destruction of substances such as PP_i that inhibit the precipitation of calcium phosphate (Fleisch *et al.*, 1966). PP_i could be removed by the pyrophosphatase present in large amounts in the matrix vesicles (Ali *et al.*, 1970; Majeska & Wuthier, 1975; Felix & Fleisch, 1976b), and this would allow the vesicles to express their full nucleating potential.

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