Biochem. J. (1973) **134**, 647–650
Printed in Great Britain

Short Communications

The Uptake of [^{45}Ca]Calcium Ions by Matrix Vesicles Isolated from Calcifying Cartilage

By S. Y. Ali and Lois Evans
Institute of Orthopaedics (University of London), Royal National Orthopaedic Hospital, Stanmore, Middx. HA7 4LP, U.K.

(*Received* 2 *March* 1973)

Extracellular membranous matrix vesicles, which contain various phosphatases and appear to initiate hydroxyapatite formation in growth cartilage, were isolated and incubated with $^{45}Ca^{2+}$ and shown to form mineral in the presence of ATP. There is enhanced calcification in the presence of serum and under alkaline conditions.

The sequence of biochemical events responsible for the mineralization of cartilage and its subsequent transformation into cancellous bone has not yet been fully elucidated (McLean & Urist, 1968). Earlier work indicated the importance in the matrix of alkaline phosphatase (Robison, 1932), collagen fibres (Glimcher & Krane, 1968), phospholipids (Irving & Wuthier, 1968) and protein–polysaccharides (Bowness, 1968), but there is as yet no unified concept that relates all these diverse participants in the mineralization process.

A significant contribution was made by the electron-microscopic observations by Bonucci (1967, 1970) and Anderson (1967, 1969), who noted that the longitudinal septa of epiphysial cartilage contain extracellular membranous matrix vesicles (100nm in diameter), and the very first crystals of hydroxyapatite are associated with these vesicles. Similar vesicles have been seen in immature bone, dentine (Bernard, 1969, 1972) and calcifying aorta (Kim & Huang, 1971). Electron-microscopic histochemistry indicated that these matrix vesicles were not lysosomes, as they do not react for acid phosphatase, but in fact contain alkaline phosphatase and adenosine triphosphatase (Anderson *et al.*, 1970; Matsuzawa & Anderson, 1971).

Selective enzyme digestion of cartilage and centrifugal fractionation permit the isolation of intact extracellular matrix vesicles, devoid of significant cellular or matrix-component contamination (Ali *et al.*, 1970). Biochemical characterization showed that the vesicles contained most of the alkaline phosphatase, adenosine triphosphatase and pyrophosphatase activity of the epiphysial cartilage. It was postulated that matrix vesicles, by virtue of their enzyme content, membranous nature and location in cartilage, might initiate calcification (*a*) by increasing the P_i concentration locally by hydrolysis of appropriate substrates, (*b*) by generating energy by the hydrolysis of ATP to provide an active transport of ions inside the lumen of the vesicle for the formation of hydroxyapatite and (*c*) by acting on PP_i, which is considered to be an inhibitor of calcification (Ali *et al.*, 1970, 1971).

To test these hypotheses we decided to study the uptake of $^{45}Ca^{2+}$ into isolated matrix vesicles from rabbit epiphysial cartilage and to determine the various conditions involved in the mineralization process and endochondral calcification.

Matrix vesicles were isolated from young (about 1 month old, 1 kg body wt.) New Zealand Red or White rabbit epiphysial cartilage as described in detail elsewhere (Ali *et al.*, 1970) except that 4h digestion of cartilage by collagenase at 37°C was found to be sufficient. Pooled centrifugal fractions 4, 5 and 6, sedimentating between 40000*g* for 20min and 150000*g* for 130min at 4°C (MSE Super 50 centrifuge, angle rotor, $r_{av.}$ 5.84cm) were used as a source of matrix vesicles. These combined fractions contained 40–60% of the total cartilage alkaline phosphatase activity with a specific activity about 10–20 times that of the original digest. Chemical and enzyme assay methods were as described previously (Ali & Evans, 1969; Ali *et al.*, 1970).

For $^{45}Ca^{2+}$-uptake studies isolated matrix vesicles (about 0.3mg on a protein basis) were incubated in 0.95ml of a defined medium TC199 (Wellcome Research Laboratories, Beckenham, Kent, U.K.) to which was added 0.04ml of $^{45}CaCl_2$ (3.5μCi; 1.6μg of Ca^{2+}; The Radiochemical Centre, Amersham, Bucks., U.K.) and 0.26ml of 0.25M-Tes [*N*-tris-(hydroxymethyl)methyl - 2 - aminoethanesulphonic acid] buffer, pH7.4, at 37°C (final concns. Ca^{2+} 1.6mM, P_i 0.8mM). Unless mentioned otherwise, 5mM-ATP and $MgCl_2$ were included. Where mentioned, 0.25ml of fresh serum, obtained from the same young rabbits as the cartilage matrix vesicles, was substituted in place of 0.25ml of medium TC199. This mixture was then incubated at 37°C for 2h. After incubation, 1.25ml of medium TC199 was

added, mixed, and a sample (1.5ml) was withdrawn with a syringe and immediately filtered through a 100nm-pore-size Millipore filter (25mm diameter).

The radioactivity of the diluted incubation mixture (pre-filtrate) and of the filtrate were analysed by taking duplicate 0.05ml samples, diluting with 0.2ml of water and adding 10ml of the dioxan-based scintillant NE250 (Nuclear Enterprises, Edinburgh, U.K.). The radioactivities of the vials were counted in an NE8312 automatic scintillation spectrometer. The $^{45}Ca^{2+}$ uptake was calculated on the basis of the difference between the radioactivities in the pre-filtrate and filtrate and expressed as a percentage of the radioactivity in the pre-filtrate. Two other methods were also used to confirm this experimental approach, by direct assay of radioactivity on the filter and by centrifugal sedimentation after two washings.

Martonosi & Feretos (1964), in their experiments on the uptake of $^{45}Ca^{2+}$ by isolated sarcoplasmic-reticulum fragments, used Millipore filters with an average pore size of 450 to 300nm. An experiment was therefore done, under the conditions described above, to determine the filter of the right pore size to retain the matrix vesicles. Alkaline phosphatase was used as a marker for matrix vesicles, and the percentages of total enzyme activity retained on filters of different pore sizes were as follows: 100nm, 98%; 220nm, 49%; 300nm, 40%; 450nm, 16%; 650nm, 13%; 800nm, 3%; 8000nm, 4%. It was therefore decided to use 100nm-pore-size filters for uptake studies. The total retention of matrix vesicles on 100nm-pore-size filters was a clear confirmation of the diameter of matrix vesicles, which has been indicated by electron microscopy to be about 100nm (Anderson, 1969; Ali *et al.*, 1970; Bonucci, 1970).

In preliminary experiments it was noticed that the uptake of $^{45}Ca^{2+}$ by matrix vesicles was variable, and this was ascribed to poor buffering by the carbonate buffer system in medium TC199, because the hydrolysis of ATP tended to lower the pH. With improved buffering of the medium (Tes) it was found that the uptake of $^{45}Ca^{2+}$ by matrix vesicles, in the presence of serum and ATP, is highly dependent on the pH. There is little or no uptake at or below pH7.2 (at 37°C) and the uptake rises rapidly above this pH (Fig. 1). Howell *et al.* (1968) have demonstrated by micro-puncture studies that the extracellular fluid of epiphysial cartilage has pH7.58, and thus matrix vesicles seem to have the right environment in the longitudinal septa for rapid hydroxyapatite formation. Our studies therefore further stress the importance of alkaline conditions for the mineralization process, as emphasized by others (Howell *et al.*, 1968; Brookes *et al.*, 1970; Barzel, 1970; Bernstein *et al.*, 1970; Bassett, 1971).

The uptake of $^{45}Ca^{2+}$ by the matrix vesicles could be prevented by preheating the matrix vesicles at 80°C for 15min (Table 1 and Fig. 1). When matrix vesicles were preheated at a range of temperatures (4–80°C) there was a rapid loss of $^{45}Ca^{2+}$ uptake above 50°C, and this paralleled closely the inactivation of alkaline phosphatase activity of the vesicles. When the uptake was measured at various temperatures there was a maximum between 37° and 40°C. There was no uptake at 4°C, thus ruling out mere binding of $^{45}Ca^{2+}$ to phospholipids etc. The uptake of $^{45}Ca^{2+}$ was proportional to the matrix-vesicle concentration and to the time of incubation at 37°C. Preliminary electron-microscopic studies of the Millipore membrane, after filtration and $^{45}Ca^{2+}$-uptake experiment, indicate an increase in the hydroxyapatite-like microcrystals associated with the matrix vesicles, which form a layer on the filter. There was no uptake of $^{45}Ca^{2+}$ by the vesicles after ultrasonic disruption for 4min, and this implied that the integrity of the membranous structure was important for apatite formation (Table 1). There was complete inhibition of $^{45}Ca^{2+}$ uptake in the presence of 10mM-cysteine, which has been shown to be an inhibitor of alkaline phosphatase (Agus *et al.*, 1966), although cysteine may also be acting as a chelator of essential ions. The enhancement of $^{45}Ca^{2+}$ uptake by the matrix vesicles in the presence of a small amount of serum (Table 1) may imply a role for humoral factors in the mineralization process. Serum may thus provide additional

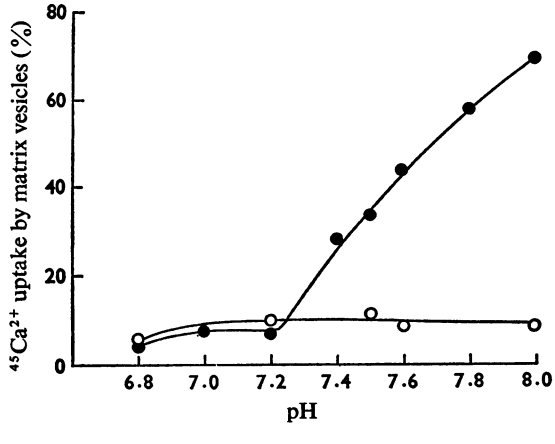

Fig. 1. *Effect of pH on $^{45}Ca^{2+}$ uptake by matrix vesicles*

Isolated matrix vesicles were incubated in a defined medium containing $^{45}Ca^{2+}$, ATP and serum, at various pH values, for 2h at 37°C. Uptake of $^{45}Ca^{2+}$ was measured by the filtration method as described in the text. The results are expressed as the percentage of total radioactivity retained on the filter calculated from the difference between the filtrate and pre-filtrate. ●, Fresh matrix vesicles; ○, heated (80°C for 15min) matrix vesicles.

Table 1. *Effect of ATP and serum on the uptake of $^{45}Ca^{2+}$ by matrix vesicles*

Isolated matrix vesicles were incubated in a defined medium containing $^{45}Ca^{2+}$ at 37°C for 2h. Uptake of $^{45}Ca^{2+}$ was then measured by either filtration or centrifugation as described in the text. The results are expressed as the percentage of total radioactivity (*a*) retained on the filter or (*b*) found in the sediment after centrifugation. The averages of two experiments are given.

	Without matrix vesicles	With heated (80°C for 15min) matrix vesicles	With fresh matrix vesicles
(*a*) Filtration method:			
Incubation medium+5mM-ATP	6.7	2.3	50.0
Medium+serum+5mM-ATP	6.9	5.3	57.1
Medium+serum (no ATP)	5.4	5.4	13.5
Matrix vesicles disrupted by ultrasonic treatment+serum+5mM-ATP	—	4.6	8.0
(*b*) Centrifugation method:			
Incubation medium+5mM-ATP	0.1	1.5	8.9
Medium+serum+5mM-ATP	0.1	0.7	14.1
Medium+serum (no ATP)	0.1	0.2	0.4

substrates for the enzymes and perhaps essential vitamins and hormones.

The uptake of $^{45}Ca^{2+}$ by matrix vesicles was proportional to the concentration of ATP between 0.1 and 5mM. This substantiated our postulate that the formation of P_i by the hydrolysis of ATP by the adenosine triphosphatase present in the matrix vesicles increases the ion product $[Ca^{2+}]\cdot[HPO_4^{2-}]$ and facilitates calcification (Anderson *et al.*, 1970; Ali *et al.*, 1970). However, the lack of apatite formation in the presence of ATP after the vesicles were disrupted by ultrasonic treatment, without loss of alkaline phosphatase activity, suggests that the uptake of $^{45}Ca^{2+}$ by matrix vesicles may be an active process, dependent on the energy provided by ATP hydrolysis, as has been shown to be the case for sarcoplasmic reticulum (Hasselbach & Makinose, 1960; Martonosi & Feretos, 1964), isolated peripheral-nerve vesicles (Lieberman *et al.*, 1967) or the Ca^{2+}-concentrating potential of mitochondria (Lehninger, 1970; Matthews *et al.*, 1968). Cartier (1952) and Cartier & Picard (1955) first showed the importance of ATP and adenosine triphosphatase in calcification of cartilage. Alkaline phosphatase is now known to hydrolyse ATP, PP_i and other simple phosphate esters (Fernley & Walker, 1967; Jibril, 1967; Russell *et al.*, 1972), and interest in the role of phosphatases in calcification has been reawakened. Alkaline phosphatase, adenosine triphosphatase and pyrophosphatase are localized in membranes of matrix vesicles, and membrane-bound particles have been shown to have the potential for concentrating, selectively, specific cations. Given the fact that alkaline phosphatase gets phosphorylated during the hydrolysis of its substrate (Fernley & Bisaz, 1968) and that it is membrane-bound, it is possible that the structure of the matrix-vesicle double-membrane may be important in promoting apatite formation.

Our experiments with isolated matrix vesicles from epiphysial cartilage demonstrate that these extracellular membranous particles appear to form hydroxyapatite under physiological conditions in the presence of ATP. There is enhanced calcification in the presence of dilute serum, and the mineral formation increases rapidly under alkaline conditions.

This work is supported by a generous grant from the Nuffield Foundation.

Agus, S. G., Cox, R. P. & Griffin, M. J. (1966) *Biochim. Biophys. Acta* **118**, 363–370
Ali, S. Y. & Evans, L. (1969) *Biochem. J.* **112**, 427–433
Ali, S. Y., Sajdera, S. W. & Anderson, H. C. (1970) *Proc. Nat. Acad. Sci. U.S.* **67**, 1513–1520
Ali, S. Y., Anderson, H. C. & Sajdera, S. W. (1971) *Biochem. J.* **122**, 56P
Anderson, H. C. (1967) *J. Cell Biol.* **35**, 81–101
Anderson, H. C. (1969) *J. Cell Biol.* **41**, 59–72
Anderson, H. C., Matsuzawa, T., Sajdera, S. W. & Ali, S. Y. (1970) *Trans. N.Y. Acad. Sci.* **32**, 619–630
Barzel, U. S. (1970) in *Osteoporosis* (Barzel, U. S., ed.), pp. 119–206, Grune and Stratton, London
Bassett, C. A. L. (1971) in *The Biochemistry and Physiology of Bone* (Bourne, G. H., ed.), 2nd edn., vol. 3, pp. 1–76, Academic Press, London
Bernard, G. W. (1969) *J. Dent. Res.* **48**, 781–788
Bernard, G. W. (1972) *J. Ultrastruct. Res.* **41**, 1–17
Bernstein, D. S., Wachman, A. & Hattner, R. S. (1970) in *Osteoporosis* (Barzel, U. S., ed.), pp. 207–216, Grune and Stratton, London
Bonucci, E. (1967) *J. Ultrastruct. Res.* **20**, 33–50
Bonucci, E. (1970) *Z. Zellforsch.* **103**, 192–217

Bowness, J. M. (1968) *Clin. Orthop.* **59**, 233–247
Brookes, M., Richards, D. J. & Singh, M. (1970) *Angiology* **21**, 355–367
Cartier, P. (1952) *Exposés Annu. Biochim. Med.* **14**, 73–86
Cartier, P. & Picard, J. (1955) *Bull. Soc. Chim. Biol.* **37**, 661–675
Fernley, H. N. & Bisaz, S. (1968) *Biochem. J.* **107**, 279–283
Fernley, H. N. & Walker, P. G. (1967) *Biochem. J.* **104**, 1011–1018
Glimcher, M. J. & Krane, S. M. (1968) in *Treatise on Collagen* (Gould, B. S. & Ramachandran, G. N., eds.), vol. 2b, p. 67, Academic Press, London
Hasselbach, W. & Makinose, M. (1960) *Biochem. Z.* **333**, 518–528
Howell, D. S., Pita, J. C., Marquez, J. F. & Madruga, J. E. (1968) *J. Clin. Invest.* **47**, 1121–1131
Irving, J. T. & Wuthier, R. E. (1968) *Clin. Orthop.* **56**, 237–260
Jibril, A. O. (1967) *Biochim. Biophys. Acta* **141**, 605–613
Kim, K. K. & Huang, S. (1971) *Lab. Invest.* **25**, 357–366
Lehninger, A. L. (1970) *Biochem. J.* **119**, 129–138
Lieberman, E. M., Palmer, R. F. & Collins, G. H. (1967) *Exp. Cell Res.* **46**, 412–418
Martonosi, A. & Feretos, R. (1964) *J. Biol. Chem.* **239**, 648–658
Matsuzawa, T. & Anderson, H. C. (1971) *J. Histochem. Cytochem.* **19**, 801–808
Matthews, J. L., Martin, J. H., Lynn, J. A. & Collins, E. J. (1968) *Calcif. Tiss. Res.* **1**, 330–336
McLean, F. C. & Urist, M. R. (1968) *Bone*, 3rd edn., University of Chicago Press, Chicago
Robison, R. (1932) *The Significance of Phosphoric Esters in Metabolism*, pp. 40–95, New York University Press, New York
Russell, R. G. G., Monod, A., Bonjour, J. P. & Fleisch, H. (1972) *Nature (London)* **240**, 126–127