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# Calcification of isolated matrix vesicles and reconstituted vesicles from fetal bovine cartilage

(mineralization/bone/epiphyseal plate/phosphatase)

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ABSTRACT **Ca deposition by isolated matrix vesicles from fetal calf growth plate cartilage and by a deoxycholate extract from matrix vesicles that included their phosphatase was studied under defined *in vitro* conditions. Electron microscopy showed that after removal of deoxycholate and lyophilization of the vesicle extract, new vesicles were reconstituted, often with multiple membrane layers. Both intact calf vesicles and reconstituted vesicles initiated Ca deposition maximally when supplied with ATP, GTP, CTP, or UTP. Only nucleoside triphosphates supported Ca deposition well; mono- and diphosphoesters, although hydrolyzed, were ineffective as substrates. Nucleoside triphosphates supported Ca deposition even if the final [Ca] × [P] reached in the reaction mixture was below a metastable level (3.5 $mM^2$), suggesting that matrix vesicles or reconstituted vesicles promote calcification by localizing Ca or $PO_4$ or both. ATP or GTP supported Ca deposition readily at concentrations ranging from 0.25 to 1.0 mM but, at 2.5 and 5.0 mM, Ca deposition was inhibited. The ATPase of intact matrix vesicles and reconstituted vesicles was stimulated by addition of $Ca^{2+}$ and $Mg^{2+}$. Ca deposition did not require additional $Mg^{2+}$. These results lend support to the hypothesis that matrix vesicles and their phosphatases play an important role in mineralization.**

Investigations in various laboratories indicate that extracellular, submicroscopic matrix vesicles play a role in mineralization of cartilage (1–4), bone (5, 6), and teeth (7–10). Evidence supporting this conclusion is as follows: (*i*) matrix vesicles are preferentially located in the zone of incipient calcification in all of these tissues (1–10); (*ii*) the first recognizable deposits of crystalline hydroxyapatite have been identified in and on matrix vesicles (2, 4); (*iii*) matrix vesicles contain abundant cation-binding phosphatidylserine (11, 12) which can serve as a calcium trap; and (*iv*) matrix vesicles contain ATPase, pyrophosphatase, and other phosphatases (13–15) which can promote mineralization by increasing local phosphate concentration (16, 17) and by hydrolyzing calcification inhibitors such as ATP or $PP_i$ (16–22).

*In vitro* mineralization by isolated rabbit matrix vesicles was reported by Ali and Evans (23) and subsequently confirmed by Felix and Fleisch (24) and by ourselves (22). Our studies have also confirmed the observation by Ali (25) that rabbit vesicles hydrolyze various phosphate esters including ATP, ADP, AMP, and glucose 1-phosphate to support calcium deposition.

Realizing that species differences could exist, we began to study vesicles from another species, fetal calf, to confirm the presence of $Ca^{2+}$-stimulated ATPase (22, 26) and to determine the ability of calf vesicles to deposit calcium *in vitro*. Unexpectedly, we found a difference between rabbit and fetal calf vesicles with respect to substrate specificity for calcification. Also, we found that a deoxycholate extract with phosphatase activity from either rabbit or calf vesicles was able to assume a vesicle-like structure and to stimulate calcium deposition. This paper reports details of these findings.

## MATERIALS AND METHODS

**Isolation of Matrix Vesicles.** The isolation of matrix vesicles was modified from a previous method of Ali *et al.* (13). Epiphyseal growth plates were removed aseptically, minced into 3- to 5-mm pieces, and then digested in a solution (10 ml/g tissue) containing 1000 units of crude collagenase per ml, 0.12 M NaCl, 0.01 M KCl, 1000 units of penicillin per ml, 1 mg of streptomycin per ml, and 0.02 M 2-{[tris(hydroxymethyl)-methyl]amino}ethanesulfonate (TES) buffer at pH 7.45. The digestion was carried out at 37° for 3 hr. In order to remove cells, nuclei, and mitochondria in one step, the digest was centrifuged in a Spinco SW 50.1 rotor at 30,000 × *g* for 10 min, and the resulting precipitate was discarded. The vesicle-containing supernate was then spun at 300,000 × *g* for 20 min to sediment vesicles and this precipitate was washed once with 10 mM Tris-buffered saline at pH 7.6 (TBS), and reprecipitated at 300,000 × *g* for 20 min. The washed vesicle precipitate was resuspended in 0.6 ml of saline per g of original tissue to give a final concentration of about 0.8 mg of matrix vesicle protein per ml. Protein concentration was determined by the method of Lowry *et al.* (27). Vesicle suspensions were stored frozen at −20° until used.

**Deoxycholate Extraction of Matrix Vesicles.** Matrix vesicles were extracted with 10 mM deoxycholate (0.8 mg of vesicle protein per ml) in TBS at 37° for 30 min (28). The dispersed solution was then centrifuged in BEEM capsules (BEEM Inc., Bronx, NY) with a Beckman SW 50.1 rotor with adaptors at 250,000 × *g* for 10 min. The precipitate (20% of total protein), containing negligible ATPase, was discarded. The supernate was then dialyzed against two changes of 10 mM Tris buffer, pH 7.6, for 36 hr at 4°. The dialyzed fraction was then lyophilized for storage. Later, to test residual phosphatase activity and calcium depositing ability, the solid was taken up in water to a final protein concentration of about 1.6 mg/ml. The resulting solutions recovered about 50% of the ATPase activity of the original extract.

**Calcification of Matrix Vesicles and Reconstituted Deoxycholate Vesicle Extract.** Calcium deposition was achieved by exposing 25 μl samples of whole matrix vesicles or 10-μl samples of deoxycholate extract (17 μg of protein) to a calcifying solution for 2 hr at 37°. Unless otherwise specified, the standard calcifying medium consisted of matrix vesicles or extract, 2.2 mM $CaCl_2$ (containing 50,000 cpm of $^{45}Ca$), 1.6 mM

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Abbreviations: TES, 2-{[tris(hydroxymethyl)methyl]amino}ethanesulfonic acid; TBS, 10 mM Tris-buffered saline, pH 7.6.

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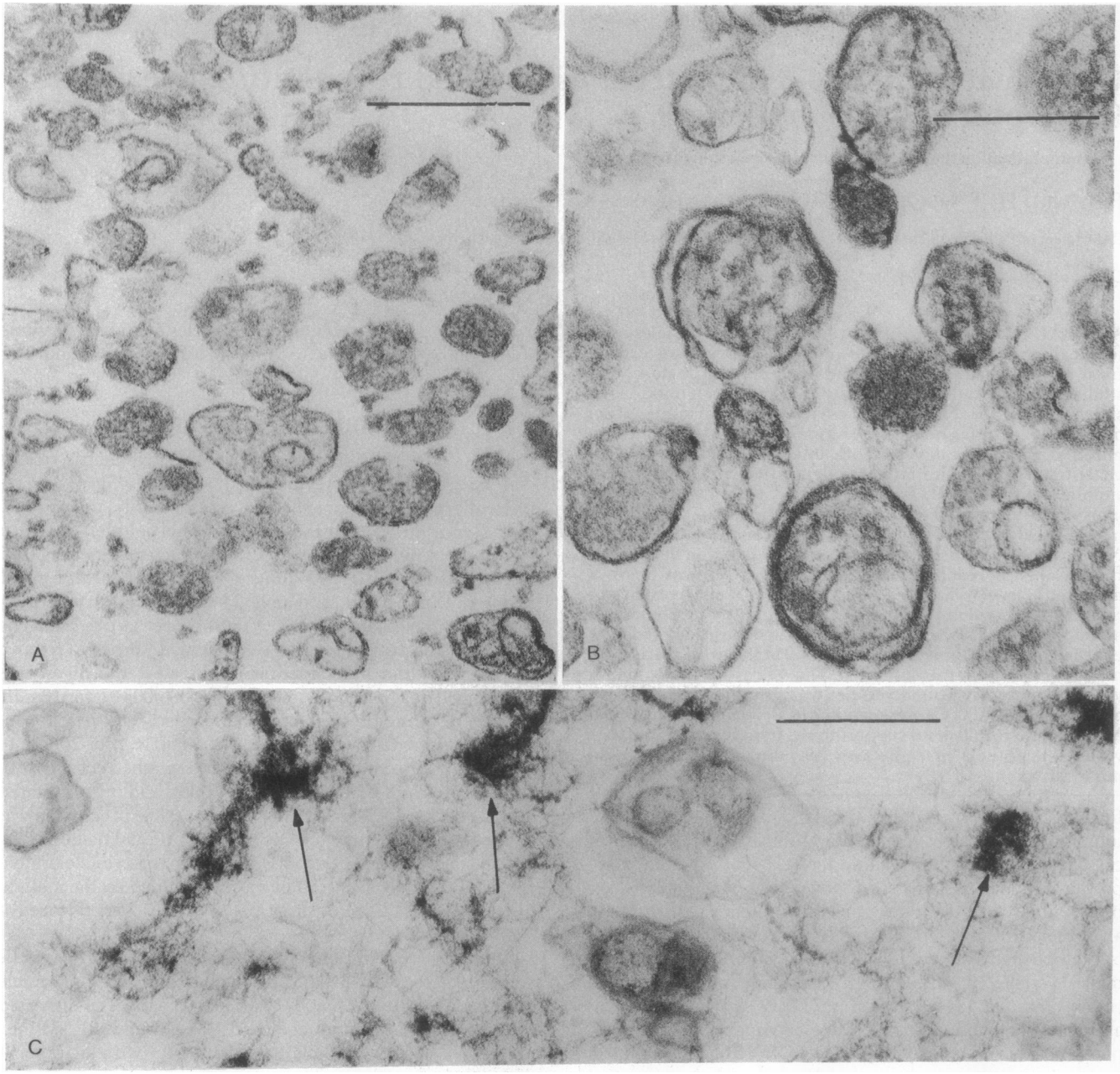


FIG. 1. Electron micrographs. Bars represent 0.3 μm. (Uranyl acetate and lead citrate in *A* and *B*; ×87,900.) (*A*) Freshly isolated matrix vesicles from fetal calf. (*B*) Vesicles reconstituted from a deoxycholate extract of calf matrix vesicles. These vesicles were 2 to 3 times larger in diameter than intact matrix vesicles and often showed multilayered investing membranes. (*C*) Reconstituted vesicles from a deoxycholate extract after exposure to the standard calcifying solution for 120 min. This section was intentionally left unstained in order to demonstrate the intrinsic electron density of precipitates of presumed calcium phosphate, indicated by arrows. During exposure to the calcifying solution there was breakdown of vesicles and concomitant development of a filamentous network between calcium deposits. These deposits were located mostly outside of vesicles and appeared to be noncrystalline. A similar picture of vesicle breakdown and calcium deposition was seen after exposure of intact calf vesicles to the calcifying solution.

$KH_2PO_4$, 1 mM nucleotide triphosphate, 1 mM $MgCl_2$, 85 mM NaCl, 15 mM KCl, 10 mM $NaHCO_3$, and 50 mM TES at pH 7.6, in a total volume of 0.58 ml (22). At the end of incubation, the reaction was centrifuged in BEEM capsules at 250,000 × *g* for 6 min. Aliquots (10 μl) of the supernate were taken for $P_i$ determination (29). The resulting precipitate was rinsed twice with 0.5 ml of TBS. The precipitate was then resuspended in 100 μl of distilled water, and radioactivity was assayed in Bray's scintillation fluid (30).

**Electron Microscopy.** Pellets of matrix vesicles and deoxycholate vesicle extract were fixed and embedded *in situ* in BEEM capsules by addition of 2.5% glutaraldehyde followed by postfixation in osmium tetroxide (13) and embedding in Epon. Thin sections (approximately 500 Å thick) were examined and photographed in a Philips EM-300 electron microscope.

## RESULTS AND DISCUSSION

**Electron Microscopy of Calf Matrix Vesicles and Reconstituted Deoxycholate Vesicle Extract.** Pellets of isolated calf matrix vesicles were similar to those already described (13) (Fig. 1*A*). Needle-like hydroxyapatite crystals were rare within the

Table 1. Substrate specificity for calcium deposition by calf matrix vesicles and reconstituted vesicles

| Substrates* | Final $P_i$ in supernates, mM | | Final [Ca] × [P] in supernates, mM | | Ca deposited, nmol/0.58 ml | |
|---|---|---|---|---|---|---|
| | | | Matrix vesicles | | | |
| ATP, 1 mM | 2.6 ± 0.3 | (17) | 5.0 ± 0.7 | (16) | 161 ± 24 | (23) |
| GTP, 1 mM | 3.2 ± 0.2 | (3) | 5.8 ± 0.4 | (3) | 202 ± 18 | (5) |
| CTP, 1 mM | 2.1 ± 0.4 | (4) | 4.1 ± 0.8 | (4) | 136 ± 11 | (4) |
| UTP, 1 mM | 1.8 ± 0.1 | (4) | 3.2 ± 0.2 | (4) | 270 ± 31 | (4) |
| ADP, 1.5 mM | 2.9 ± 0.1 | (4) | 6.4 ± 0.2 | (4) | 4.5 ± 0.6 | (4) |
| GDP, 1.5 mM | 2.2 ± 0.2 | (4) | 2.7 ± 0.5 | (4) | 4.9 ± 0.6 | (4) |
| AMP, 3 mM | 4.3 ± 0.4 | (4) | 9.2 ± 0.7 | (4) | 20 ± 2.5 | (4) |
| CMP, 3 mM | 4.1; 3.9 | (2) | 9.1; 8.6 | (2) | 27; 13 | (2) |
| UMP, 3 mM | 4.3; 4.4 | (2) | 9.2; 8.6 | (2) | 28; 14 | (2) |
| *CP*, 3 mM | 4.2 ± 0.3 | (4) | 9.1 ± 0.6 | (4) | 18 ± 2 | (4) |
| *P*EA, 3 mM | 2.7; 2.7 | (2) | 5.9; 5.9 | (2) | 4; 4 | (2) |
| Glc-1-*P*, 3 mM | 3.0; 2.2 | (2) | 6.6; 4.9 | (2) | 3; 3 | (2) |
| *β*Gro*P*, 3 mM | 2.8; 2.8 | (2) | 6.2; 6.3 | (2) | 4.0 ± 1.4 | (4) |
| None | 1.7 ± 0.2 | (15) | 3.6 ± 0.7 | (13) | 3.6 ± 0.7 | (25) |
| | | | Reconstituted vesicles | | | |
| ATP, 1 mM | 2.1 ± 0.1 | (7) | 4.3 ± 0.3 | (7) | 130 ± 29 | (18) |
| GTP, 1 mM | 2.4; 2.5 | (2) | 4.4; 4.6 | (2) | 204; 205 | (2) |
| CTP, 1 mM | 1.9 ± 0.2 | (6) | 3.8 ± 0.3 | (6) | 138 ± 13 | (6) |
| CMP, 3 mM | 4.2; 4.2 | (2) | 9.0; 9.1 | (2) | 35; 14 | (2) |
| UMP, 3 mM | 4.0; 3.9 | (2) | 8.7; 8.5 | (2) | 24; 11 | (2) |
| GDP, 1.5 mM | 2.2; 2.1 | (2) | 4.7; 4.7 | (2) | 5; 4 | (2) |
| *CP*, 3 mM | 4.7; 3.7 | (2) | 10.1; 8.0 | (2) | 23; 16 | (2) |
| None | 1.7 ± 0.1 | (9) | 3.7 ± 0.04 | (9) | 3.4 ± 0.6 | (13) |

Values are the mean ± SD for the number of determinations in parentheses; individual data are given for two determinations.

* *CP*, creatine phosphate; *P*EA, phosphoethanolamine; *β*Gro*P*, *β*-glycerophosphate; Glc-1-*P*, glucose 1-phosphate.

isolated vesicles, suggesting that the heavier, mineral-containing, matrix vesicles may have been sedimented with the larger particulates (cells, nuclei, mitochondria, etc.). The deoxycholate extract of vesicles was able to resume vesicular structure after removal of the deoxycholate, lyophilization, and resuspension (Fig. 1*B*). These reconstituted vesicles were about twice the size of intact matrix vesicles and often had double- or triple-layered investing membranes. After exposure of the reconstituted vesicles to a calcifying solution for 120 min, many of the vesicles seemed broken or empty, and those remaining were associated with an apparently noncrystalline electron-dense precipitate, presumably calcium phosphate, that was mostly outside vesicles (Fig. 1*C*).

**Substrate Requirements for Calcium Deposition.** Although rabbit matrix vesicles can utilize various mono-, di-, and triphosphate esters equally well to support calcium deposition (22, 25), intact calf vesicles and reconstituted vesicles from deoxycholate extract could use only nucleoside triphosphates (ATP, CTP, GTP, and UTP) as substrates to achieve maximal calcium deposition (Table 1). The highest final [Ca] × [P] values in the supernate often were achieved with AMP, CMP, and UMP, but these substrates were associated with only minor calcium de-

Table 2. Effect of [Ca] × [P] on calcium deposition by whole matrix vesicles or reconstituted vesicles

| Substrate at 1 mM | Initial $P_i$, mM | Final $P_i$ in supernate, mM | Final [Ca] × [P] in supernate, $mM^2$ | Ca deposition, nmol/reaction mixture per 2 hr | |
|---|---|---|---|---|---|
| | | | Whole matrix vesicles | | |
| ATP | 1.6 | 2.3 ± 0.1 | 4.9 ± 0.5 | 137 ± 20 | (4) |
| ATP | 0.8 | 1.5 ± 0.04 | 3.2 ± 0.2 | 91 ± 18 | (4) |
| ATP | 0.0 | 0.7 ± 0.04 | 1.6 ± 0.2 | 32 ± 8 | (4) |
| None | 1.6 | 1.7; 1.7 | 3.6; 3.7 | 2; 3 | (2) |
| GTP | 1.6 | 2.7 ± 0.5 | 4.6 ± 1.1 | 268 ± 32 | (4) |
| GTP | 0.8 | 2.1 ± 0.4 | 3.8 ± 0.8 | 248 ± 18 | (3) |
| GTP | 0.0 | 1.4 ± 0.3 | 2.6 ± 0.6 | 202 ± 20 | (6) |
| None | 1.6 | 1.6 ± 0.2 | 3.5 ± 0.4 | 3.6 ± 1.1 | (5) |
| | | | Reconstituted vesicles | | |
| ATP | 1.6 | 2.1 ± 0.2 | 4.0 ± 0.3 | 163 ± 12 | (3) |
| ATP | 0.8 | 1.4 ± 0.3 | 2.7 ± 0.5 | 132 ± 5 | (3) |
| ATP | 0.0 | 0.8 ± 0.3 | 1.6 ± 0.5 | 109 ± 33 | (4) |
| None | 1.6 | 1.7 ± 0.1 | 3.7 ± 0.1 | 3.7 ± 0.6 | (3) |

Values are the mean ± SD for the number of determinations indicated in parentheses.

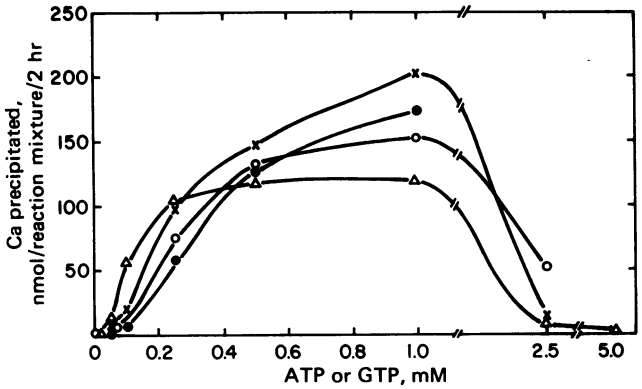


FIG. 2. Effect of concentration of ATP and GTP on calcium deposition by intact matrix vesicles (MV) or reconstituted vesicles (RV) from deoxycholate extract. O, MV plus ATP as substrate; △, RV plus ATP; ×, MV plus GTP; ●, MV plus GTP with no added $Mg^{2+}$.

position, about one-seventh the amount seen with ATP. Thus, calcium deposition by calf vesicles or reconstituted calf vesicles appears not to reflect simply the final [Ca] × [P] in the supernate but may require a concentration of these ions within the vicinity of vesicles during the local hydrolysis of nucleoside triphosphates.

To test the ability of matrix vesicles or reconstituted vesicles to concentrate Ca or $P_i$ or both, we decreased the final [Ca] × [P] in the incubation mixture by lowering the initial $P_i$. As shown in Table 2, with ATP or GTP as substrate, even though the final [Ca] × [P] was in some cases lower than the metastable level (3.5 $mM^2$) that we usually use as an initial ion product, there was still considerable calcium deposition. In these experiments the concentration of calcium was kept constant at 2.2 mM, a level used in our previous work with isolated matrix vesicles (22) and rachitic cartilage slices. This calcium level is higher than that observed in ultrafiltrates of cartilage fluid by Howell *et al.* (31) and Wuthier (32) who found a calcium concentration of approximately 1.4 mM. Although, in the present experiments, we were primarily interested in the final [Ca] × [P] ion product reached in vesicle suspensions and controlled this by lowering the $P_i$ concentration, we also have carried out experiments (data not shown) in which the initial calcium concentration was 1.4 mM and in these experiments we found a significant calcium deposition. Taken together with the data in Table 1 showing that high [Ca] × [P] values do not necessarily lead to calcium deposition, these findings suggest that, when the appropriate nucleoside triphosphate is present, fetal calf vesicles or reconstituted vesicles not only increase ambient [Ca] × [P] but also concentrate these ions locally to allow mineral deposition.

The standard concentration of nucleoside triphosphate, 1 mM, used in these experiments would appear to be somewhat high in comparison to data on growth plate cartilage fluid (31, 32) that show a total value for nucleotides, nucleosides, and their bases (measured at 260 nm) of 1 mM. Therefore, it is likely that the physiological concentration of nucleoside triphosphate is probably below 1 mM, and we thought it was important to test the effect of lower concentrations of nucleosides. Significant calcium deposition was supported with ATP and GTP concentrations as low as 0.2 mM which is probably near the physiological level (Fig. 2). Calcium deposition increased with increasing substrate concentration to 1 mM where maximal activity was observed. At 2.5 mM or above, both ATP and GTP inhibited calcium deposition, suggesting a dual effect with stimulation predominating at lower, near physiological, concentrations of ATP or GTP (32). The inhibition of calcium deposition by high concentrations of ATP may reflect the operation of several factors including ATP substrate inhibition of vesicle ATPase (22), a $Ca^{2+}$-chelating effect of ATP, and inhibited formation of hydroxyapatite (19). Fig. 2 also shows that added $Mg^{2+}$ was not required for Ca deposition.

From the present data, we conclude that the mechanism of calcification in calf matrix vesicles requires not only nucleoside triphosphate substrate but also the concerted effect of interactions of nucleoside triphosphatase, cation-binding phospholipids, and probably the integral structure of matrix vesicles.

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