Phosphatidylcholine metabolism in neonatal mouse calvaria

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Phosphatidylcholine metabolism was examined in neonatal mouse calvaria in vitro. Incorporation of choline into phosphatidylcholine was slow in this tissue. At 2 h after a pulse of [methyl-3H]choline only 30% of the tissue radioactivity was in the organic phase. Chromatography of the aqueous phase of the tissue extract revealed that more than half of the radioactivity was present as choline at this time. There was no accumulation of phosphocholine, which would have been expected if the cytidylyltransferase were the rate-limiting step in the CDP-choline pathway in the tissue. Choline kinase activity in calvarial cytosol was lower than choline kinase activity in liver cytosol of the same animals. No evidence for significant phosphatidylcholine synthesis through the methylation pathway was found in the calvarial tissue. Although rates of choline–phosphatidylcholine base exchange were higher in bone microsomes than in microsomes from liver, the rate of phosphatidylcholine production through this pathway appeared to be too slow to account for the phosphatidylcholine produced by the calvaria. Phosphatidylcholine synthesis in the calvaria was unaffected by 2 h of treatment with 10 nm-parathyroid hormone, 0.1 nm–0.1 μM-1α,25-dihydroxycholecalciferol, 5 μM-prostaglandin E$_1$ or 2.5 nm-salmon calcitonin, or by 17 h of treatment with 10 nm-parathyroid hormone or 0.1 nm-1α,25-dihydroxycholecalciferol.

INTRODUCTION

Calcemic hormones have been shown to alter phospholipid metabolism in target tissues. Phosphatidylcholine metabolism is affected by parathyroid hormone (PTH) and calcitonin in bone (Rappaport & Stern, 1986) and by PTH in kidney (Lo et al., 1976; Farese et al., 1980; Bidot-Lopez et al., 1981; Meltzer et al., 1982). Bone resorption by PTH was accompanied by phosphatidylcholine breakdown (Crues et al., 1979). Treatment of vitamin D-deficient chicks with 1α,25-dihydroxycholecalciferol [1,25-(OH)$_2$D$_3$] altered the pattern of phosphatidylcholine metabolism in intestine (Matsumoto et al., 1981). Changes in the fatty acid composition of phosphatidylcholine in intestinal brush border membranes fractions resulted from treatment of vitamin D-deficient chicks with 1α-hydroxycholecalciferol (Max et al., 1978) or 1,25-(OH)$_2$D$_3$ (O'Dougherty, 1979). In studies in vitro, vitamin D metabolites stimulated phosphatidylcholine transfer from liposomes into isolated renal tubular brush border membranes (Eglavish et al., 1983). Vitamin D-deficient, phosphate-deficient rats had lower amounts of phosphatidylserine in the metaphyses of their long bones than did treated rats (Boskey & Timchak, 1983), and treatment of the osteogenic sarcoma cell line UMR 106 with 1,25-(OH)$_2$D$_3$ increased serine incorporation into phosphatidylserine while decreasing choline incorporation into phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine (Matsumoto et al., 1985).

To determine whether the synthesis of phosphatidylcholine in bone is affected by calcemic hormones, we examined the incorporation of choline into phosphatidylcholine as well as into intermediates in the GDP-choline pathway of phosphatidylcholine synthesis.

EXPERIMENTAL

Animals

Neonatal (4–6 day) mice were obtained from Charles River, Montreal. The animals were killed by cervical dislocation, and the calvaria (frontal and parietal bones) dissected.

Incorporation studies

For incorporation studies, calvariae were rinsed in Dulbecco's modified Eagle's medium (DMEM; Grand Island Biological Co.) containing 10% heat-inactivated horse serum (Flow Laboratories) and 10 mM-Hepes (Sigma). The tissues were then preincubated in the same medium for 1–3 h at 37 °C in CO$_2$/air (1:19) in individual wells of 24-multiwell culture dishes (Corning 25820). For 17 h cultures, tissues were incubated in culture tubes containing 2 ml of medium and the indicated additions. Tubes were gassed for 1 min with O$_2$/air (1:1), stoppered and placed in a roller drum at 37 °C. For some studies the medium was supplemented with 280 μM-choline to ensure that the tissue choline concentration was not rate-limiting. At the end of the preincubation, bones were transferred from the culture tubes or culture dish wells into other wells containing 1 ml of medium to which 10 μCi of [Me-3H]choline, [Me-3H]ethanolamine or [Me-3H]methionine (Amer-

Abbreviations used: 1,25-(OH)$_2$D$_3$, 1α,25-dihydroxycholecalciferol (1α,25-dihydroxyvitamin D$_3$); PTH, parathyroid hormone; DMEM, Dulbecco's modified Eagle's medium.

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methanol/water (5:4, v/v) and 3.0 were rinsed quickly in motorized pestle. Tubes transferred were homogenized with an additional 0.5-4 ml of medium at -4 °C for 1 h, by vol. At the end of the incubation, bones were homogenized in phosphate-buffered saline and transferred to tubes containing 1 ml of methanol/water (5:4, v/v). Either immediately or after storage at -20 °C, tissues were homogenized in Dounce homogenizers with a motorized pestle. For some experiments, tissues that had been stored at -20 °C in methanol/water were decalcified for 15 min at -4 °C in multiwell dishes containing 0.5 ml of 0.5 mM-EDTA. They were then rinsed in water and homogenized in methanol/water (5:4, v/v). These variations in tissue preparation did not affect the response observed. After homogenization, 1.7 ml of methanol/water (5:4, v/v) and 3.0 ml of chloroform were added to give final proportions of methanol/water/chloroform of 15:12:25, by vol. The mixture was vortex-mixed, centrifuged and then washed three times with a theoretical upper phase mixture. Radioactivity in the medium and upper and lower phases was determined by previously published techniques (Pritchard & Vance, 1981).

**Chromatography**

The upper phase and medium were chromatographed on Silica G60 with methanol/0.6% NaCl/aq. NH₃ (10: 10: 1, by vol.). The lower phase was chromatographed on Silica G60 with chloroform/methanol/acetic acid/water (50:30:8.3, by vol.).

**Subcellular fractions**

Homogenates were prepared from calvaria and livers of the same neonatal mice. Tissues were homogenized in 0.145 mM-NaCl/10 mM-Tris/HCl (pH 7.4)/10 mM-NaF/1 mM-EDTA. Livers were prepared as 20-30% (w/v) homogenates, whereas bones were prepared as 50% (w/v) homogenates. Homogenates were centrifuged at 12000g for 15 min. The resulting supernatants were centrifuged at 100000g for 1 h to obtain cytosol and microsomal pellets. Microsomal pellets were resuspended in homogenizing medium. Average yields from the isolation were: bone, 8.5 mg of cytosolic protein/g of tissue (0.44 mg of cytosolic protein/calvarium) and 1.77 mg of microsomal protein/g of tissue (0.09 mg of microsomal protein/calvarium); liver, 33.2 mg of cytosolic protein/g of tissue and 13.6 mg of microsomal protein/g of tissue.

**Enzyme assays**

Choline kinase (EC 2.7.1.32) was measured in bone and liver cytosol by a method similar to that described previously (Weinhold & Rethy, 1974). The reaction mixture contained 0.1 mM-Tris/HCl (pH 8.5), 10 mM-MgCl₂, 10 mM-ATP, 0.8 μM-1 mM-[Me-³H]choline chloride (0.2 μCi) and 0.002-0.2 mg of cytosolic protein in 0.1 ml. Choline-phosphotidylcholine base exchange was measured in bone and liver microsomes by the method of Saito et al. (1975). The reaction mixture contained 50 mM-Hepes (pH 8.5), 1 mM-CaCl₂, 0.8 μM-1 mM-[Me-³H]choline chloride (0.5 μCi) and 0.25-5 mg of microsomal protein in 0.2 ml.

**Statistics**

Data were analysed by analysis of variance (Snedecor & Cochran, 1967).

**RESULTS**

**Incorporation studies**

Continuous incubation of calvaria over a 1 h period in medium containing [Me-³H]choline resulted in a progressive increase in radioactivity in both the upper and lower phases (Fig. 1). Bones that had been killed prior to incubation by heating showed little radioactivity in the upper phase and none in the lower phase. Fig. 2 illustrates the distribution of radioactivity in upper phase, lower phase and medium when the bones were transferred to fresh medium at the end of a 30 min pulse with [Me-³H]choline. Radioactivity in the upper phase fell rapidly during the initial 30 min time period, declined more slowly over the next 90 min, and then was stable for the next 60 min. Radioactivity in the lower phase increased progressively. At the end of 120 min,
Fig. 2. Distribution of radioactivity in culture medium (▲) and aqueous (upper, ●) and organic (lower, ×) phases of neonatal mouse calvaria during the chase period following a 30 min pulse with [Me-3H]choline

Bones were incubated in 1 ml of DMEM + 15% heat-inactivated horse serum for both the pulse and chase periods. Tissues were rinsed in phosphate-buffered saline, homogenized in methanol/water (5:4, v/v) and partitioned into upper and lower phases at a final solvent ratio of methanol/water/chloroform of 15:12:25, by vol. Values are means ± S.D. of responses from three calvaria per group. Bones were preincubated with 280 μM-choline for 2.5 h prior to the pulse period and were then rinsed with incubation medium containing 28 μM-choline.

27% of the radioactivity was in the lower phase. Radioactivity in the medium accounted for approx. two-thirds of the radioactivity from the first incubation period through the termination of the experiment. To determine the extent to which the radioactivity in the bone was in a loosely associated (trapped) state, a separate experiment was carried out in which the tissues were transfered into fresh medium after the first 30 min of the chase period. Only 13% additional radioactivity was released into the medium during the next 90 min, suggesting that little of the radioactivity in the bone was freely exchangeable (results not shown).

Fig. 3 illustrates the distribution of radioactivity in the intermediates of the CDP-choline pathway in the bone tissue during the course of the 2 h chase. The major proportion of the radioactivity in the upper phase was in the form of choline. Small amounts of phosphocholine and CDP-choline were produced and were present in roughly equal amounts. The combined amounts of these two intermediates never accounted for more than 50% of the radioactivity in the upper phase by the end of 2 h. The radioactivity in the medium during the chase was relatively stable and in experiments in which the medium was chromatographed at the end of a 2 h incubation, approx. 90% was found to be unmetabolized (results not shown).

The radioactivity in the lower phase at the end of a 2 h incubation was predominantly phosphatidylcholine (80%), with smaller amounts of sphingomyelin (14%) and lysophosphatidylcholine (5%). Similar proportions of phosphatidylcholine and sphingomyelin were found when total phospholipids were assayed (results not shown).
Table 1. Lack of effects of calcaemic hormones on [Me-3H]-choline incorporation into lipid in neonatal mouse calvaria

For the 2 h incorporation studies, bones were incubated in stationary cultures in 1 ml of medium (DMEM + 15% heat-inactivated horse serum). For the 17 h studies, tissues were incubated in 2 ml of medium, gassed with O2/air (1:1), stoppered and incubated in a roller drum at 37 °C. Tissues were rinsed in phosphate-buffered saline, homogenized in methanol/waters (5:4, v/v) and partitioned into upper and lower phases at a final solvent ratio of methanol/water/chloroform of 15:2:25, by vol. Values are the means ± s.d. of the responses from three bones. Medium calcium values (means ± s.d.) from the 17 h incubations were as follows: control, 1.97 ± 0.17 mm; 1,25-(OH)2D3, 2.40 ± 0.05 mm; PTH, 2.32 ± 0.05 mm. For the first two experiments shown, the 2 h incubation with hormone followed a 30 min pulse in the absence of hormone. The 17 h incubation with hormone was followed by a 2 h pulse with [3H]choline in the absence of hormone and a 90 min chase, also in the absence of hormone.

<table>
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<th>Time (h)</th>
<th>Treatment</th>
<th>[3H]Choline incorporated (nmol/bone)</th>
</tr>
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<tbody>
<tr>
<td>2</td>
<td>Control</td>
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</tr>
<tr>
<td></td>
<td>1,25-(OH)2D3 (2 nM)</td>
<td>0.71 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>0.42 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>PTH (10 nM)</td>
<td>0.43 ± 0.02</td>
</tr>
<tr>
<td>17</td>
<td>Control</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>1,25-(OH)2D3 (0.1 nM)</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>PTH (10 nM)</td>
<td>0.14 ± 0.02</td>
</tr>
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</table>

When [Me-3H]ethanolamine was added instead of [Me-3H]choline, radioactivity in the lower phase was predominantly phosphatidylethanolamine with small (less than 5%) amounts chromatographing with phosphatidylcholine (results not shown). No incorporation of radioactivity into lipid was detected when [Me-3H]methionine was used as the precursor (results not shown).

Several experiments assessed the direct effects of calcaemic hormones on phosphatidylcholine synthesis and the pattern of metabolites in the CDP-choline pathway in the cultured calvaria (Table 1). The treatments tested included the following: PTH, 1.0 and 10 nM; 1,25-(OH)2D3, 0.1 nM–0.1 μM; prostaglandin E2, 5 μM; salmon calcitonin, 2.5 nM. Most of the studies involved 2 h incubation with the hormones. In one study, tissues were preincubated with either 10 nM-PTH or 0.1 nM–1,25-(OH)2D3 for 17 h under conditions which produced resorption, as shown by increased medium calcium concentration. Results from two 2 h experiments and from the 17 h experiment are shown in Table 1. None of the treatments altered incorporation of [Me-3H]choline into neonatal mouse calvaria. Other factors which were tested and found to have no effect on [3H]choline incorporation in the calvaria during a 2 h chase were the source of the serum (fetal calf versus horse), the age of the animals (1 week versus 5 days), and the presence of 1 mM-Mn2+ during the chase period (results not shown). In one experiment carried out with ROS (rat osteosarcoma) 8/10 cells (provided by Dr. C. Owen Parkes) no effect on choline incorporation was elicited by 2 h incubation with 100 nM-PTH (results not shown).

Choline kinase

The observations (a) that the greatest amount of radioactivity in the phospholipid precursors was present as choline throughout the incubation and (b) that there was no accumulation of phosphocholine (Fig. 3) suggested that choline kinase, rather than CTP: phosphocholine cytidylyltransferase, might be the rate-limiting step in the CDP-choline pathway in the bone tissues. Choline kinase activity was therefore assayed in cytosolic fractions from neonatal mouse bone and liver. Fig. 4 illustrates results from two experiments examining the dependence of the activity on choline concentration. Kinetic analysis of these two experiments indicated that the Km values for choline for the enzymes in neonatal bone cytosol and rat liver cytosol were 0.38 mM and 0.29 mM, respectively. The Vmax for the enzyme in bone, 44.9 nmol/h per mg of protein, was approx. 18% of that
Fig. 5. Effect of tissue concentration on choline kinase activity in cytosolic fractions from neonatal mouse calvaria (○, ○) and livers (▲, △) from the same animals.

Choline kinase activity was measured by a method similar to that described by Weinhold & Rethy (1974). Closed and open symbols are from two different tissue isolations. Values are single determinations. Activity was assayed at 20 min.

in liver (241.6 nmol/h per mg of protein). The effect of cytosolic protein concentration on activity at 1 mM-choline is shown in Fig. 5. Table 2 illustrates that removal of MgCl₂ at ATP from the system resulted in a decrease in phosphocholine production.

Choline–phosphatidylcholine base exchange

Base exchange activity was measured to determine the contribution of this pathway to the generation of phosphatidylcholine in bone. Choline–phosphatidylcholine base exchange in microsomes isolated from calvaria was approximately 2.7-fold lower than the activity in liver microsomes from the same animals (Table 3). EDTA, 2.5 mM, abolished base-exchange activity in both tissues (results not shown). No significant base-exchange activity was found in cytosol from any of the three tissues (results not shown).

DISCUSSION

The present studies indicate that [Me³H]-choline is slowly incorporated into phosphatidylcholine in neonatal mouse calvaria in vitro. The proportions of phosphatidylcholine, sphingomyelin and lysophosphatidylcholine in the neonatal bones was similar to that previously reported in bovine fetal bone (Wuthier, 1968). Measurement of the intermediates in the CDP-choline pathway revealed constant low radioactivity in phosphocholine and CDP-choline, and slow conversion of labelled choline into phosphocholine. There was no accumulation of phosphocholine, the expected finding if cytidylyltransferase were the rate-limiting step for phosphatidylcholine synthesis in bone as it is in a number of other tissues (Pelech & Vance, 1984). Rather, the data support the proposal that choline kinase is the rate-limiting enzyme. Assay of choline kinase in bone cytosol revealed that, at the choline concentration present in the medium, i.e. 0.028 mM, bone choline kinase activity was approx. 4 nmol/h per mg of protein. Since approx. 0.44 mg of cytosolic protein was obtained from each calvarium, the production of phosphatidylcholine by this pathway would be in the order of 1.8 nmol/h per calvarium. At the reported intracellular choline concentrations in other tissues, i.e. 0.1–0.2 mM (Pelech & Vance, 1984) the rate would be approximately twice as high. Either rate of synthesis would be more than adequate to account for the observed rates of choline metabolism through the CDP-choline pathway in the calvaria in the present studies.

Experiments comparing the rates of choline kinase in neonatal liver and bone showed that the rate of choline metabolism at this step was slower in bone than in liver. The Vₘₐₓ, in bone, 44.9 nmol/h per mg of protein, was 18% of that in liver. Conceivably, this could account for the low rates of phosphocholine production in the intact bone tissue.

Previous studies of phospholipid synthesis in neonatal rat bone have demonstrated incorporation of [³¹C]acetate, [¹⁴C]glucose, [¹⁴C]glycerol, [⁳₂P]phosphate and [¹⁴C]choline into phosphatidylcholine in bone tissue (Dirksen, 1969, 1971, 1975; Dirksen et al., 1970a,b). These authors proposed that most of the incorporation was through the CDP-choline pathway, with no evidence for phospholipid methylation and low choline–phospha-

![Graph showing effect of tissue concentration on choline kinase activity in neonatal mouse calvaria and livers.](image-url)

**Table 2. Choline kinase activity in neonatal mouse calvaria**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Expt...</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<td>Choline (1 mM)/Tris/</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>MgCl₂/ATP</td>
<td></td>
<td></td>
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<tr>
<td>−MgCl₂</td>
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<tr>
<td>−ATP</td>
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<td></td>
</tr>
<tr>
<td>No added choline</td>
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<tr>
<td>(choline 0.66 μM)</td>
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<tr>
<td>−MgCl₂</td>
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<td>−ATP</td>
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<table>
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<tr>
<th>Conditions</th>
<th>Choline kinase activity (nmol/h per mg of cytosolic protein)</th>
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<tr>
<td>Choline (1 mM)/Tris/ MgCl₂/ATP</td>
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<td>−ATP</td>
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<td>No added choline (choline 0.66 μM)</td>
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<td>−ATP</td>
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tidylcholine base exchange in the tissue. Our results in mouse calvaria support a similar pattern of phosphatidylcholine synthesis. The rate of base exchange in bone microsomes at a choline concentration of 0.03 mM was 0.13 nmol/h per mg of microsomal protein, and at 0.1 mM was 0.45 nmol/h per mg of microsomal protein. Approx. 0.09 mg of microsomal protein was obtained from each calvarium. Thus, the rate of phosphatidylcholine synthesis through this pathway would be 0.012–0.04 nmol/h. This would only account for a fraction (≤10%) of the phosphatidylcholine synthesis that was observed in the calvaria. It is interesting that the rate of base exchange in bone microsomes was higher than that in liver microsomes from the same neonatal mice. An amplified base-exchange pathway may be important in handling of phospholipids other than phosphatidylcholine in bone. For example, phosphatidylserine, which is synthesized predominantly by base exchange in eukaryotes (Vance, 1985) may play a significant role in bone mineralization (Boskey, 1981).

Recent studies by Matsumoto et al. (1985) demonstrated a substantial effect of 1,25-(OH)2D3 on phosphatidylserine–phosphatidylinositol and phosphatidylserine–phosphatidyl ethanolamine base exchange in rat osteosarcoma cells. No significant effects were seen on choline incorporation. The results are consistent with our present observations, which showed a lack of effect of 1,25-(OH)2D3 on choline incorporation.

The current results suggest that phosphatidylcholine metabolism in bone is not altered by treatment of the tissues with PTH or 1,25-(OH)2D3. No effects of these hormones on incorporation of [Me-3H]choline into chloroform/methanol-soluble products or into intermediates in the CDP-choline pathway were observed. The results suggest that phosphatidylcholine synthesis in bone is stable and not readily altered by external stimuli. This contrasts with phosphatidylinositol metabolism, which we have found to be affected by parathyroid hormone and calcitonin at both early (2 h) and later (24 h) time points (Rappaport & Stern, 1986). Treatment of animals with vitamin D has been reported to alter intestinal phosphatidylcholine metabolism. Modifications in fatty acid content, as well as in the ratio of phosphatidylcholine to phosphatidylethanolamine, have been observed (Max et al., 1978; Matsumoto et al., 1981). Measurement of radioactivity in intermediates in the CDP-choline pathway has led to the hypothesis that vitamin D treatment alters the activity of CDP-choline:1,2-diacylglycerol choline-phosphotransferase. Our studies in vitro in bone suggest that this is not a universal effect in all vitamin D target tissues.

This work was supported by research grants from the Medical Research Council of Canada and the National Institutes of Health, U.S.A. We express our appreciation to Dr. Steven Pelech for useful suggestions at the outset of these studies.

**REFERENCES**


**Table 3. Effect of choline concentration on choline–phosphatidylcholine base exchange in 100000 g pellets from calvaria and livers of 5-day neonatal mice**

Choline–phosphatidylcholine base exchange was measured by the method of Saito et al. (1975). The reaction mixture contained 50 mM-Hepes, pH 8.5, 1 mM-CaCl2, 0.8 μM–1 mM-[Me-3H]choline chloride (0.5 μCi) and 0.25–5 mg of microsomal protein in 0.2 ml. Activity was assayed at 30 min.

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<td>67.2</td>
<td>34.6</td>
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Mean ± s.d. 2.7±0.97
Bone phosphatidylcholine metabolism

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