Flavonoids are a group of phenolic compounds that are diverse in chemical structure and characteristics. They occur naturally in fruit, vegetables, nuts, seeds, flowers, and bark and are an integral part of the human diet. Over 4,000 different flavonoids have been described, and they are categorized into flavonols, flavones, catechins, flavanones, anthocyanidins, and isoflavonoids. All these compounds are characterized by a phenyl benzoylpyrone-derived structure and they differ by 1) modifications of the nucleus, especially the pyronic cycle saturation, 2) the number and position of phenol functions, and 3) the degree of methylation and glycosylation, which affects various properties of these flavonoids, particularly their hydrophobicity (21).

Flavonoids are ingested daily in human diet; the consumption of 4-oxo-flavonoids has been estimated at ~26 mg/day in The Netherlands (13). Flavonoids have long been considered inert and nonessential for human health; however, in the last few years it has been shown that these compounds affect a wide variety of biological systems in mammals, exhibiting antioxidant, anti-inflammatory, antiviral, antiproliferative, and anticarcinogenic effects (23, 25). Recently, much attention has been paid to their antioxidant properties and to their inhibitory role in various stages of tumor development in animal studies. Even if epidemiological studies failed to put forward an association between flavonoid consumption and cancer mortality, the intake of flavonoids and flavones was inversely associated with coronary heart diseases (14); this could possibly be explained by the antioxidant effects of flavonoids.

Among flavonoids, flavonols seem particularly interesting because they are abundant in plant foods (12) and have most of the biological properties of flavonoids. In foods flavonols naturally occur as O-glycosides, with a sugar usually bound at the C-3 position (36). Only free flavonoids with no sugar molecule, the so-called aglycones, were thought to be able to pass through the gut wall. No enzyme that can split the predominantly β-glycosidic bonds is secreted in the gut or is present in the intestinal wall of mammals (10), so hydrolysis only occurs in the large intestine by microflora (1). Less is known about the absorption and metabolism of flavonoids in humans at the usual levels of dietary intake. Despite extensive bacterial metabolism, the absorption of flavonols in rats adapted to a diet containing 0.2% quercetin brings up to ~100 µmol/l accumulation in blood plasma (20). In humans, the ingestion of fried onions containing quercetin glucosides equivalent to 64 mg of quercetin aglycone led to a mean peak plasma level of quercetin of 650 nmol/l after 3 h (15).

In the liver, flavonoids and their metabolites may undergo modifications such as methylation or hydroxylation (11). The liver also synthesizes conjugated derivatives by coupling with a sulfate or a glucuronic acid molecule. Consequently there is an accumulation of various conjugated derivatives in the plasma of rats fed a diet containing quercetin or rutin (19, 20). The conjugated flavonoid derivatives are excreted in urine and bile, and the major route depends on the species. Even if the liver seems to be the main tissue involved in flavonoid metabolism, other tissues that contain conjugative enzymes, such as intestinal mucosa or kidneys, could also be implicated (11, 38).

Quercetin is absorbed in humans and is only slowly eliminated through the day (15). Thus quercetin could significantly contribute to the antioxidant defenses present in blood plasma. The aim of the present work was to 1) characterize in rats the circulating metabolites of quercetin and 2) test in vitro and in vivo the antioxidant capacities of these conjugated derivatives.

MATERIALS AND METHODS

Animals and Diets

Male Wistar rats used for the experiments weighed ~170 g. They were housed two per cage in temperature-controlled
rooms (22°C), with a dark period from 0900 to 2100 and access to food from 0900 to 1700. Twenty-four rats were divided into two groups and adapted during 3 wk to semipurified diets: 1) a control diet and 2) a 0.2% quercetin diet; the detailed compositions of these diets are given in Table 1. Animals were maintained and handled according to the recommendations of the Institut National de la Recherche Agronomique Institutional Ethics Committee, in accordance with decree no. 87–848.

HPLC Analysis

Sample treatment. Plasma or reaction mixture (for glucuronidation and sulfation studies) was spiked with 25 µM of dioxemetin, used as internal standard and acidified to pH 4.9 with 0.1 vol of 0.58 M acetic acid solution. Samples were incubated for 30 min at 37°C in the absence (unconjugated) or presence (total) of 5 × 10⁶ U/l β-glucuronidase and 2.5 × 10⁶ U/l sulfatase (final volume, 200 µl). The reactions were stopped by adding 7.5 vol of acetone, and the resulting mixtures were centrifuged. Supernatants were evaporated to a volume equivalent to two times the initial volume of the samples (recovery >85%), then 20 µl were injected and analyzed by HPLC. The amounts of conjugated derivatives were calculated by subtracting the unconjugated values from the total ones.

Chromatographic conditions. The HPLC system used consisted of an autosampler, 2 ultraviolet detectors and a software system that controlled all the equipment and carried out data processing. The system was fitted with a 5-µm C18 Hypersil based desactivated silicat analytic column (150 × 4.6 mm ID) (Life Sciences International, Cergy, France). The ultraviolet detector was set at 370 nm, and the mobile phase consisted of 73% solvent A and 27% solvent B, where solvent A = H₂O/H₃PO₄ (99.5:0.5) and solvent B = acetonitrile. For deconjugated samples, elution was isocratic (flow rate 1.5 ml/min). To visualize and separate the conjugated metabolites of flavonoids, the chromatographic conditions of elution were as follows (flow rate 1 ml/min): 0–2 min, 85% solvent A/15% solvent B; 2–22 min, 85% solvent A/15% solvent B → 60% solvent A/40% solvent B; 22–24 min, isocratic for 3 min, then returned to initial conditions and equilibration for 8 min.

In Vitro Assay of Flavonoid Glucuronidation

Intestinal and liver microsomes from rats adapted to the quercetin diet were prepared by differential ultracentrifugation at 105,000 g at 4°C for 1 h. To prepare intestinal microsomes, mucosal scrapings were homogenized in ice-cold buffer of a composition similar to that used for liver homogenization (50 mM Tris-HCl, pH 7.2, 100 mM sucrose, 10 mM EDTA, 2 mM diethytheritol, and 1 µM leupeptin), except that trypsin inhibitor (25 mg/100 ml) was added to the buffer to prevent UDP-glucuronosyltransferase inactivation by pancreatinzymes. The final microsomal pellet was resuspended in a buffer containing 100 mM HEPES, pH 7.2, and 100 mM sucrose (supplemented with trypsin inhibitor for intestinal fractions) and kept in a frozen state at −20°C until use. The final preparation was adjusted to have a final protein concentration of ~5 mg/ml, measured according to the Pierce bicinchoninic acid (BCA) protein reagent kit (Interchim, Montluçon, France).

Incubations were carried out as follows. In a final volume of 750 µl, 540 µl of buffer (75 mM HEPES and 10 mM MgCl₂, pH 7.3), 50 µl of UDP-glucuronic acid (4.5 mM final) and 100 µl of microsomal suspension (50 µg protein) were activated in situ by 60 µl of a 0.2% solution of Triton X-100. The reaction was started by the addition of 2 µl of an aglycone solution (18.75 mM in DMSO). Incubations were performed at 37°C for 3 h, then aliquots of the reaction mixture were taken and treated (with or without β-glucuronidase/sulfatase) for HPLC analysis, exactly as described above.

In Vitro Assay for Flavonoid Sulfation

Fresh liver was homogenized in 3 vol of ice-cold 0.1 M Tris-HCl, pH 7.2, and 0.25 M sucrose. The homogenate was centrifuged for 20 min at 12,500 g, and the supernatant was further centrifuged at 105,000 g for 1 h. The final protein concentration in the cytosolic extract was ~25 mg/ml.

The in vitro sulfitation of quercetin andisorhamnetin (3’-O-methyl quercetin) by cytosolic sulfotransferases of rat liver was followed using 3’-phosphadenosine 5’-phosphosulfate (PAPS) as the sulfate donor. The standard assay mixture consisted of 20 µM of the flavonoid substrate dissolved in DMSO (final concentration in the assay 0.4%), 25 µM PAPS, 5 mM MgCl₂, and the enzyme protein (0.5 mg) in 25 mM Tris-HCl buffer, pH 7.2, in a total volume of 500 µl. The reaction was initiated by addition of the cytosolic extract and incubated for 60 min at 37°C, and then aliquots of the reaction mixture were taken and processed as described for HPLC analysis.

Rat Lipoprotein Preparation

Blood from eight rats (weighing ~150 g) fed a standard nonpurified diet (AO3 pellets; Usine d’Alimentation Rationnelle, Villemoisson/Orge, France) was collected into tubes containing EDTA (1 g/l). Equal volumes of plasma samples were pooled for lipoprotein separation. Because the LDL fraction is poorly represented in rat plasma, a very low density lipoprotein (VLDL) fraction was isolated. Samples (protected by EDTA, 1 g/l) were overlaid with 0.15 M NaCl (density = 1.066 kg/l), and chylomicrons were discarded after two 30 min-long centrifugations at 12,000 g and 15°C. To isolate the VLDL + LDL fraction, the remaining plasma was adjusted to a density of 1.050 kg/l with solid KBr. Centrifugation was performed at 100,000 g and 15°C for 20 h. The VLDL + LDL fraction was then washed by a further period of ultracentrifugation at the same density.

Measurement of the Oxidative Susceptibility of Lipoproteins

Before oxidation experiments, the purified lipoprotein fraction was dialyzed against deoxygenated PBS (10 mmol/l, pH 7.4) for 24 h. The final protein concentration (BCA protein

Table 1. Composition of diets

<table>
<thead>
<tr>
<th>Component</th>
<th>Control diet</th>
<th>Quercetin diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat starch</td>
<td>680</td>
<td>678</td>
</tr>
<tr>
<td>Casein</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Peanut oil</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Mineral mixture</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

Values are g/kg diet. Wheat starch and casein were from Louis François (Paris, France). Peanut oil was from Comptoir Industriel des Glénieux (Genay, France). Mineral mixture (per kg of diet) was composed of the following: 15 g CaHPO₄, 2.5 g K₂HPO₄, 5 g KCl, 5 g NaCl, 2.5 g MgCl₂, 2.5 mg Fe₂O₃, 125 mg MnSO₄, 0.2 mg CuSO₄·7H₂O, 100 mg ZnSO₄·7H₂O, and 0.4 mg KI, purchased from Usine d’Alimentation Rationnelle (UAR) (Villemoisson, Epinay sur Orge, France). Vitamin mixture (per kg of diet) was composed of the following: 19,800 IU retinol, 2,500 IU cholecalciferol, 20 mg thiamine, 15 mg riboflavin, 70 mg niacin, 10 mg pyridoxine, 0.05 mg cobalamin, 800 mg ascorbic acid, 170 mg tocopherol, 40 mg menadione, 100 mg nicotinic acid, 1.36 g choline, 5 mg folic acid, 50 mg p-aminobenzoic acid, and 10 mg biotin, purchased from UAR.
The reagent kit (Pierce) in lipoprotein fraction was adjusted to 20 mg/l. Lipoprotein concentration was calculated from the mass of protein plus individual lipids. The lipoprotein fraction was supplemented with ethanolic solutions of aglycones or with the aqueous solutions of the conjugated derivatives of these molecules. Oxidation was initiated by the addition of freshly prepared CuSO4 solution (10 µmol final concentration) at 37°C. The kinetics of lipoprotein oxidation were determined by continuously monitoring at 37°C the changes in absorbance at 234 nm, corresponding to dienes formation, on an Uvikon 930 spectrophotometer.

Determination of Total Antioxidant Status

Total antioxidant status was assayed on heparinized plasma samples using the total antioxidant status kit (Randox Laboratories, Roissy, France), according to specified recommendations. The principle of this assay is based on the incubation of 2,2'-azino-bis(3-ethylbenzthiazoline sulfonate) (ABTS) with peroxidase and H2O2 to produce the radical cation ABTS*+, leading to the appearance of a blue-green color, which is measured at 600 nm. The antioxidant capacity (expressed as trolox equivalent) of the sample was inversely proportional to the coloration intensity.

Statistics

Values are means ± SE, and significance of the differences between mean values was determined by one-way ANOVA coupled with the Student-Newman-Keuls multiple-comparison test. Values of P < 0.05 were considered significant.

RESULTS

Circulating Metabolites of Quercetin

The chromatographic conditions were adapted to separate the circulating conjugated metabolites (see MATERIALS AND METHODS); with this gradient elution procedure, quercetin, isorhamnetin (3'-O-methyl quercetin), and tamarixetin (4'-O-methyl quercetin) were eluted at 15.7, 19.2, and 19.8 min, respectively. Figure 1, A and B, shows representative chromatograms of the plasma from control rats and from rats adapted to a 0.2% quercetin diet. The HPLC profile from rats adapted to the quercetin diet is characterized by: the absence of quercetin and the presence of three unidentified peaks, noted 1, 2, and 3 (retention times: 6.2, 7.1, and 12.9 min), corresponding to the conjugated metabolites of quercetin. These metabolites had spectra (from 250 to 450 nm) that fitted with flavonoid structure (data not shown). On the other hand, the HPLC profile for control rats did not show any trace of such metabolites.

The β-glucuronidase treatment of plasma from rats adapted to the quercetin diet (Fig. 1C) revealed that some circulating metabolites corresponded to glucuronated forms of quercetin (5.4 ± 0.8 µM), isorhamnetin (1.2 ± 0.4 µM), and tamarixetin (3.0 ± 1.1 µM). However, two major and unidentified peaks (4 and 5) were also recovered after the action of β-glucuronidase (retention time = 12.23 and 12.74 min; Fig. 1C). Moreover, when the plasma was treated in the presence of β-glucuronidase plus sulfatase (Fig. 1D), these latter peaks (4 and 5) practically disappeared, showing that they corresponded to sulfated derivatives, whereas those of quercetin (20.1 ± 2.5 µM) and isorhamnetin (93.3 ± 2.1 µM) appeared.

These data showed that in rats fed a quercetin diet the glucurono-sulf derivatives of isorhamnetin (89.1 ± 2.1 µM) and of quercetin (14.7 ± 1.7 µM) are the major circulating forms (91.5%), whereas glucuronides of quercetin and of its methoxylated forms are the other minor metabolites (8.5%).

In Vitro Studies on Glucuronidation of Quercetin and Isorhamnetin

The capacity of rat liver and cecal microsomal fractions to transfer in vitro glucuronic acid from UDP-glucuronic acid to quercetin and isorhamnetin has been tested using microsomal fractions obtained from rats adapted to the 0.2% quercetin diet (n = 6).

After the hepatic microsomal glucuronoid conjugation of 50 µM quercetin, the reaction products were analyzed by HPLC using the gradient elution procedure. Four metabolites of quercetin, labeled a, b, c, and d according to their increasing hydrophobicity, were present in the sample (Fig. 2A). Microsomes isolated from the cecal wall also exhibited an UDP-glucuronyl transferase activity and the chromatographic profile (Fig. 2B) presented the same four peaks (a, b, c, and d) as those obtained with liver microsomal fractions; however, the relative amounts of each peak were different. In both conditions, the reaction of glucuronitdation was not complete and the capacity of the cecal wall to metabolize quercetin to glucuron conjugates (83 ± 3%) appeared significantly greater than that of liver (69 ± 2%).

As for quercetin, the hepatic and cecal microsomal glucuronidation of isorhamnetin led to quite similar chromatographic profiles (Fig. 3, A and B), except for the relative amount of each peak. Because of their higher hydrophobicity, the four glucuronon conjugates of isorhamnetin (a’, b’, c’, and d’) presented greater retention times than those of quercetin. Moreover, as for quercetin, the conversion of isorhamnetin to glucuron derivatives was slightly more efficient with microsomes from cecal wall (87 ± 4%) than with microsomes from liver (78 ± 2%). This result suggests that in vivo the cecal wall could play a noticeable role in flavonol glucuronidation.

In Vitro Assay for Sulfation of Quercetin and Isorhamnetin

Liver cytosolic extracts have been prepared to test their capacity to produce sulfoderivatives from quercetin and isorhamnetin. As it was previously found (31), our experiments showed that the hepatic phenolsulfotransferase involved in the sulfation of quercetin was sensitive to substrate inhibition (for concentration >35 µM, data not shown). Consequently, present experiments have been performed using 25 µM of quercetin or isorhamnetin. The in vitro sulfation of these compounds led, for each of them, to the formation of three sulfoconjugates (Fig. 4, A and B); the corresponding peaks were noted e, f, and g for quercetin and e’, f’, and...
Among these peaks, the last one (namely \( g^8 \) and \( g^8 \)) was markedly higher than the others.

The chromographic profiles obtained after the sulfation of quercetin (Fig. 4A) and isorhamnetin (Fig. 4B) have been compared with that of the plasma treated with \( \beta \)-glucuronidase (C) or with \( \beta \)-glucuronidase/sulfatase (D). D, diosmetin (internal standard); Q, quercetin; I, isorhamnetin; T, tamarixetin. 1–5, conjugated metabolites.

Antioxidant Properties of the Conjugated Forms of Quercetin

The antioxidant properties of quercetin and its conjugated derivatives have been studied by following the formation (at 234 nm) of conjugated dienes on rat VLDL + LDL fractions after induction of the oxidation with CuSO4. These experiments were performed using quercetin glucuronides, obtained via an in vitro procedure (the efficiency of glucuronidation was \( \sim 96\% \)). Because the in vitro sulfation of quercetin provided insufficient amounts of sulfocjugates, the assays on their antioxidant effects were realized using a sulfocjugate commercially available (quercetin-3-O-sulfate).

Figure 5 shows that quercetin increased, in a concentration-dependent manner, the lag phase of conjugated diene formation: +130 min at 0.25 µM and +255 min at

\[ g \] for isorhamnetin. Among these peaks, the last one (namely \( g \) and \( g' \)) was markedly higher than the others.

The chromatographic profiles obtained after the sulfation of quercetin (Fig. 4A) and isorhamnetin (Fig. 4B) have been compared with that of the plasma treated with \( \beta \)-glucuronidase (Fig. 1C). Taking into account the respective relative retention times compared with diosmetin, it appears that peaks 4 and 5, resulting from the hydrolysis of plasma quercetin metabolites by a \( \beta \)-glucuronidase, exhibit perfect coincidence with the sulfoconjugates of quercetin, \( f \) and \( g \), obtained in vitro. Moreover, peaks \( f' \) and \( g' \), corresponding to the sulfo-derivatives of isorhamnetin, were contained in the large peak (5) resulting from the hydrolysis of the plasma by a \( \beta \)-glucuronidase. These data show that the two large peaks (4 and 5) resulting from the hydrolysis of plasma by a \( \beta \)-glucuronidase corresponded to at least three different forms of sulfoconjugates.
0.5 µM. When present at 0.5 µM, quercetin glucuronides or quercetin-3-O-sulfate also significantly delayed copper-induced lipoprotein oxidation (1100 min or 1185 min, respectively); however, the magnitude of their effects was lower than that of the aglycone. The simultaneous presence of 0.25 µM quercetin glucuronides and 0.25 µM quercetin-3-O-sulfate induced an inhibition of lipoprotein oxidation of the same magnitude as that observed with 0.25 µM quercetin. It could be noted that, even if all these compounds significantly prolonged the lag phase, they did not affect the propagation rate of VLDL-LDL oxidation. Figure 6 shows that, when present at 0.5 µM, isorhamnetin delayed the onset of the propagation phase, 75 min, so it was less potent than quercetin (even when present at 0.25 µM). As in quercetin, the glucuronides of isorhamnetin at 0.5 µM or 1 µM significantly delayed copper-induced lipoprotein oxidation (35 or 65 min, respectively). These whole data show that, in vitro, the glucuronide or sulfate forms of quercetin and isorhamnetin exhibit a substantial protective effect on lipoprotein oxidation.

The antioxidant properties of quercetin and its conjugates were compared with those of trolox (a watersoluble form of vitamin E) using the present in vitro lipoprotein oxidation model. For each compound, IC50 relative to a control was determined according to the specifications detailed in Table 2. In agreement with the above results, the IC50 for the aglycone was generally lower than those of their glucuronides or sulfate forms (Table 2); however, these conjugates were four times more potent than trolox to inhibit lipoprotein oxidation.

Determination of Total Antioxidant Status

Experiments were performed on plasma from rats fed a control diet or a diet supplemented with 0.2% quercetin. It appeared that the total antioxidant status of the plasma from rats fed the quercetin diet (0.963 ± 0.031 µM) was markedly higher than the total antioxidant status of control plasma (0.605 ± 0.025 µM) (Fig. 7). This interesting result shows that the circulating metabolites of quercetin possess antioxidant properties.

DISCUSSION

The present study shows that in rats adapted to a diet containing 0.2% quercetin, the circulating metabolites were not quercetin itself but glucurono and/or sulfo conjugates. The enzymatic hydrolysis of the conjugated metabolites yielded chiefly isorhamnetin (the 3’-O-methylated form of quercetin) (80%) and, in lesser part, quercetin (20%). This particularly high level of isorhamnetin is in accordance with the high capacity of the liver to methylate quercetin (38). The extensive O-methylation of quercetin, in addition to other conjugation reactions such as glucuronide and sulfate forma-
tion, may well be a major cause for the lack of toxicity of these compounds (6). Moreover, conjugation improves the water solubility of flavonoids and consequently favors their elimination.

A previous study performed on the hepatic microsomal glucuronidation of some flavonoids (3) reported that this important class of natural compounds might be conjugated by a specific isoform of UDP-glucuronosyl transferase. The liver does not constitute the unique site for flavonol glucuronidation because the present study shows a greater activity of glucuronidation in the cecal wall than in the liver. This result is in accordance with a recent study that reported that the in vivo glucuronidation of an isoflavone (genistein) occurred in the intestinal wall rather than in liver (29). The hepatic and cecal activity of quercetin glucuronidation led to the formation of the same glucuronides; however, the relative amount of each glucuronide depended on the tissue.

Sulfation constitutes another important pathway for phenolic compound conjugation. Although cytosolic sulfotransferases are present in numerous tissues, the highest level is found in the liver (26). The sulfation of quercetin by perfused rat liver gave two double conjugates, containing sulfate and glucuronic acid, and one sulfate derivative (31). This study is in agreement with our in vitro experiments, performed with liver cytosolic extracts, showing that the sulfation of quercetin led to the formation of three different compounds. Moreover, some of them could correspond to sulfated derivatives resulting from the hydrolysis of circulating metabolites by a β-glucuronidase.

Although the possibilities of glucuronidation and sulfation of flavonoids are numerous, it is quite striking to find only three peaks on HPLC analysis, corresponding to the plasma metabolites of quercetin. Two of them have retention times corresponding to multiconjugated metabolites (containing both sulfates and glucuronic acid(s)) of isorhamnetin and quercetin. However, our
and quercetin (22,362). The binding of phospholipid bilayer is not exclusive to the consumption of lipophilic aqueous oxygen radicals, and thereby they prevent the surface of phospholipid bilayers suitable for scavenging aglycones (quercetin or catechin) are localized near the 20, 34). According to Terao et al. (32), it is likely that noids can inhibit oxidative modification of LDLs (7, 9, systems (6), and there is evidence that several flavo-

cation to LDLs, and possibly other lipoproteins, is Increasing evidence suggests that the oxidative modifi-
occur in vivo.

ttributes. In this view, it has been shown that a flavonoid-rich extract, like pure flavonoids, they could prevent LDL oxidation by taking up the water-soluble free radicals generated by copper through the Fenton reaction, and therefore they could decrease the consumption of the LDL antioxidant contained in the lipid-water interface. Furthermore, it has been shown that a flavonoid-rich extract, like pure flavonoids, increases LDL resistance to oxidation by decreasing consumption of vitamin E (27, 33). The circulating metabolites of quercetin could also exert antioxidant properties, as shown by the higher total antioxidant status of the plasma from rats adapted to a diet supplemented by quercetin than those receiving a control diet. In the same way, it has been reported that the in vitro antioxidant activity of the rat serum was enhanced in the presence of quercetin in the diet (17).

Perspectives

This study shows that ubiquitous quercetin, found in fruits and vegetables, is conjugated in vivo and that its circulating derivatives, by inhibiting LDL oxidation, should contribute to the antioxidant pool in the blood.
and thus slow down atherosclerosis processes. Further studies performed to determine the mechanisms of the beneficial effects of flavonoids in foods on human health should take into account the complexity of their circulating metabolites.

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