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12β-Dehydrogenation of Bile Acids by *Clostridium paraputrificum*, *C. tertium*, and *C. difficile* and Epimerization at Carbon-12 of Deoxycholic Acid by Cocultivation with 12α-Dehydrogenating *Eubacterium lentum*

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12β-Hydroxysteroid dehydrogenating activities were detected in 13 strains of *Clostridium paraputrificum*, 1 strain of *C. tertium*, and 1 strain of *C. difficile*, together with a 3α- and 3β-hydroxysteroid dehydrogenase system in many strains. Redox reactions at C-12 of disubstituted and trisubstituted bile acids were performed unspecifically by representative strains of *C. paraputrificum*. 3α,12β-, 3β,12β-Dihydroxy-, 3α,7α,12β-trihydroxy-, and 3-keto,12β-hydroxy-5β-cholanoic acids, so far not known as bacterial bile acid metabolites, were identified. Epimerization of the 12α-hydroxyl group of deoxycholate via the 12-keto intermediate was achieved by cocultivation of *C. paraputrificum* and *Eubacterium lentum*, elaborating a 12α-hydroxysteroid dehydrogenase only. In addition, epimerization at C-12 was demonstrated with mixed human fecal cultures.

Human intestinal flora (approximately 10^{11} organisms per g of feces) intensively metabolize biliary bile acids, which have escaped enterohepatic circulation. Under the anaerobic conditions of the gut, deconjugation of conjugated bile acids and reductive dehydroxylation of the 7α-hydroxyl group prevail, but dehydroxylation at C-3, oxidation, and epimerization of the hydroxyl groups at C-3 and C-7, oxidation of the 12α-hydroxyl group, and corresponding reductions of keto functions have also been demonstrated. These transformations and others, including synthetic and degradative reactions by gut bacteria, have recently been reviewed (13).

Epimerization of bile acid hydroxyl groups at C-3 and C-7 was demonstrated with mixed fecal and pure cultures. *Eubacterium lentum* (3, 16) and *Clostridium perfringens* (11, 15) were shown to elaborate a 3α- and 3β-hydroxysteroid dehydrogenase (HSDH) system capable of epimerizing the 3α-hydroxyl group via the keto intermediate. Analogous results were reported for 7α-hydroxyl group epimerization by lecithinase-lipase-negative clostridia (2) and for *C. absonum* (14). Cooperation of bacteria e.g., strains of *E. aerofaciens* (17) and *Peptostreptococcus productus*-like organisms, with a stereospecific 7α-HSDH or 7β-HSDH (10), was shown to represent a further mechanism of epimerization at C-7. Intestinal bacteria able to epimerize the 12α-hydroxyl group of bile acids were hitherto unknown, although fecal bile acids with 12β-hydroxyl groups had been detected (1, 5). In this communication we report on the existence and properties of 12β-hydroxysteroid dehydrogenating activities in strains of *C. difficile*, *C. tertium*, and especially *C. paraputrificum* and present evidence for epimerization at C-12 of deoxycholate by binary cultures of *C. paraputrificum* and 12α-HSDH containing *E. lentum* strains.

(Part of this paper is a section of the thesis of A. Schneider, to be submitted to the Faculty of Medicine, Johannes Gutenberg Universität, Mainz, Federal Republic of Germany, for the degree of Doktor der Medizin.)

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MATERIALS AND METHODS

The following abbreviations are used: TFA, trifluoroacetyl; HFIP, hexafluoroisopropyl; GLC, gas-liquid chromatography; RRT, relative retention time. Bile acids are abbreviated by the location and configuration of the hydroxyl groups as well as by the location of the keto groups.

Bacterial strains. Strains of *C. difficile*, *C. paraputrificum*, and *C. tertium* were obtained from Elsa Haralambie, Institut für Medizinische Mikrobiologie des Universitätsklinikums der Gesamthochschule, D-4300 Essen, Federal Republic of Germany. With the exception of one strain, all strains of *C. paraputrificum* and *C. tertium* were isolated from the feces of patients with colon carcinoma or other gastrointestinal disorders (9). Strains of *C. difficile* were obtained from the culture collection of the institution. *E. lentum* K 6/2 and K19/23.2 were isolated from the feces of healthy subjects in our laboratory (3).

Media and microbial methods. Stock cultures of clostridia were maintained in a cooked-meat medium (CM 81; Oxoid Ltd., London, England) supplemented with Schaedler broth (CM 497; Oxoid) and 1 ml of 0.1% resazurine solution (pH 7.6) per liter. Stock cultures of *E. lentum* were maintained in this medium, further supplemented with 0.1% arginine hydrochloride and 100 μM KH_2PO_4 (pH 7.0). These media, containing 250 μM bile acids, were used for transformation experiments.

Unless otherwise indicated, all media used were prereduced and anaerobically sterilized. Bacteria were transferred under protective gas (CO_2) as described previously (2), by using a multiple gas supply head with glass Pasteur pipettes in a sterile hood. Feces were likewise diluted. The relatively aerotolerant clostridia could also be cultivated by applying a conventionally prepared medium, freshly boiled before inoculation, and overlaying liquified agar (Table 1). Qualitatively identical bile acid patterns were obtained by both methods.

Bile acids. Cholic acid (3α,7α,12α) and deoxycholic acid (3α,12α) were purchased from E. Merck AG, Darmstadt, Federal Republic of Germany; chenodeoxycholic acid (3α,7α) and 3α,7α-dihydroxy-12-keto-5β-cholanoic acid were

TABLE 1. Reduction of 3,12-diketo-5β-cholanoate by selected strains of *C. paraputrificum* and by atypical strains of *C. tertium* and *C. difficile*[a]

Strain	% of total bile acids present in stationary phase:					
	3β,12β	3α,12β	3-keto,12β	3β,12-keto	3α,12-keto	3,12-diketo
C. paraputrificum[b] (group I)						
D 2459-16	0.9[c,d]	30.4[c,d]	48.7[c]	0.5	8.0	11.5
D 2660-02		9.4	43.8	0.2	3.9	42.7
D 717-07		17.6	8.0	—	6.4	68.0
D 203-05	1.2	14.4	19.5	0.1	10.9	51.5
C. paraputrificum[e] (group II)						
D 2923-09	4.0–4.4[c,f]	2.9–2.5[c,f]	66.8[c]	0.8	0.3	5.2
D 762-06		<1.0	44.1	1.7	2.1	51.8
C. tertium D 464-06	—	7.4	36.4	1.0	5.2	50.0
C. difficile D 1193-04	38.1	1.7	7.4	19.8	6.7	26.3

[a] The cholanoic acids were abbreviated by the location and configuration of the hydroxyl groups as well as by the location of the keto groups.
[b] Three other strains of *C. paraputrificum*, labeled as D 34-6, D 1917-3, and D 2665-1, produced transformation patterns similar to that of strain D 2550-2; one other strain, D 136-12, reacted as strain D 2459-16.
[c] Sum of the corresponding transformation product and of its artifactual degradation products, generated during GLC.
[d] With this strain the peak areas of peaks b through d (Fig. 1) were added exclusively to 3α,12β, since in another chromatogram without these artifacts, the peak area corresponding to 3β,12β had not increased.
[e] Four other strains of *C. paraputrificum*, labeled as D 1451-6, D 1310-5, D 762-6, and D 1133-4, generated qualitatively identical transformation patterns.
[f] Since retention times of thermal artifacts of 3β,12β and 3α,12β were identical, the respective peaks were added either to 3α,12β (first value) or to 3β,12β (second value).

purchased from Calbiochem-Behring Frankfurt, Federal Republic of Germany; 3α-hydroxy-7-keto-, 3α-hydroxy-12-keto-, and 3,12-diketo-5β-cholanoic acids were obtained from Steraloids, Wilton, N.H.; and 3β,12β-dihydroxy-, 3α,12β-dihydroxy-, 3α,7α,12β-trihydroxy-, and 3β-hydroxy-12-keto-5β-cholanoic acids were generous gifts of F. C. Chang, University of South Alabama, Mobile. We are also obliged to P. Klein, Argonne National Laboratory, Argonne, Ill., for a sample of 3-keto-12α-hydroxy-5β-cholanoic acid. Apocholic acid (3α,12α-dihydroxy-5β-chol-8,14-enoic acid) was available in the laboratory from previous work.

Bile acid analysis. Bacterial suspensions were acidified to pH 1 to 2 and extracted three times with 1.5 volumes of ether. The pooled ether phases were then washed twice with 0.5 volumes of water, the combined water washings were reextracted once with ether, and the ether extract was evaporated to dryness. For GLC analysis, bile acids were converted to TFA-HFIP derivatives by incubation in a 2:1 mixture of trifluoroacetic anhydride and hexafluoroisopropanol for 60 min at 37°C. These compounds were chromatographed on columns of 3% QF-1 (Macherey-Nagel, Düsen, Federal Republic of Germany) at 230°C (2, 4). Substrate bile acids and deoxycholate were used as internal standards. Bile acids were identified by comparing RRTs with those of reference bile acids, and the structures were verified by GLC-mass spectrometry. Selected mass spectra were compared with those of authentic reference compounds, and fragmentation pathways were investigated as described previously (4). The RRTs of reference 12β-hydroxy bile acids, first available for the experimentsdiscussed here, were determined by six estimations with deoxycholate as the internal standard: 3β,12β-dihydroxy-5β-cholanic acid (3β,12β), 1.07; 3α,12β-dihydroxy-5β-cholanic acid (3α,12β), 1.16; 3α,7α,12β-dihydroxy-5β-cholanic acid (3α,7α,12β), 2.20; 3β,12α, 0.86; 3α,12α, 1.00; 3α,7α,12α, 2.22.

RESULTS

Reduction of 3,12-diketo-5β-cholanoate. We screened strains of various clostridial species for their ability to transform cholate, chenodeoxycholate, 3α-hydroxy-7-keto-, 3-keto,7α,12α-dihydroxy-, and 3,12-diketo-5β-cholanoate. This set of commercially available bile acids should allow the detection of all conceivable α and β HSDHs. We found that 13 strains of *C. paraputrificum* (all strains tested) reduced the 12-keto function of 3,12-diketo-5β-cholanoate stereospecifically to a 12β-hydroxyl function, whereas the 3-keto group was reduced to a 3α- and a 3β-hydroxyl group; 3β,12β, 3α,12β, 3-keto,12β, 3β,12-keto, and 3α,12-keto were generated as reduction products (Fig. 1; Table 1). Table 1 shows that most strains formed all conceivable reduction products but in widely differing amounts. The fraction of the sum of the three 12β-hydroxy bile acids produced by the 13 *C. paraputrificum* strains varied between 13.2 and 87.3%, with an average value of 47.8%, whereas the respective data for the two 3β-hydroxy bile acids were 0.2 to 4.8%, with an average value of 1.3%. Within the eight strains of group I, an average value of 25.7% was obtained for the two 3α-hydroxy bile acid transformation products, with a variation between 11.9 and 63.3%.

On account of the 3-keto group reduction, *C. paraputrificum* strains were subdivided into two groups: group I (eight strains), which intensively reduced the 3-keto function preferentially to a 3α-hydroxyl group; and group II (five strains), which produced 3α- and 3β-hydroxy bile acids in similar but minor amounts. The presence of a 3α- and 3β-HSDH system in most strains was confirmed with 3-keto,7α and by trace epimerization of 3α-hydroxyl groups. Screening experiments with bile acids cited above showed that all strains of *C. paraputrificum* were devoid of any measurable 7α- or 7β-HSDH.

In many transformation experiments, peaks b through d and f through h of Fig. 1, the chromatogram with the largest number of individual peaks, were either not detectable or were detected in trace amounts only. Subsequent repetitions of GLC analyses with identical samples obtained by various strains resulted in the observation that peaks corresponding to peaks b through d (group I) and f through h (group II) of Fig. 1 were irregularly visible in chromatograms of the same samples. Variations in peak areas frequently appeared. Two observations were especially distinct: first, the complete

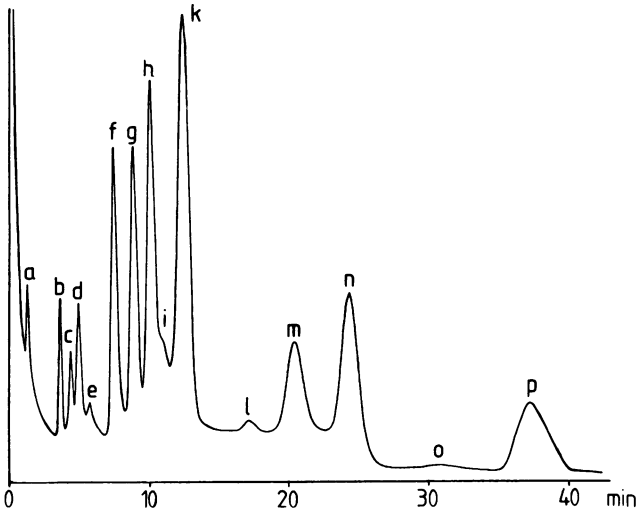

FIG. 1. Reduction of 3,12-diketo-5β-cholanoate by *C. paraputrificum* D 2459-16. Bile acids were analyzed by GLC as described in the text. Peaks: a, solvent impurity; b, c, d, thermal artifacts of 3α,12β or 3β,12β; e, unknown; f, g, h, thermal artifacts of 3-keto,12β; i, 3β,12β; k, 3α,12β; l, 3β,12-keto; m, 3α,12,keto; n, 3-keto,12β; o, unknown substrate impurity; p, 3,12-diketo. Structures were verified by GLC-mass spectrometry except peaks a, e, and o. Mass spectra of peaks b through d and k were identical, which was true also for peaks f through h and n. A detailed discussion is presented in the text.

absence of peak group I in another chromatogram of the sample of *C. paraputrificum* D 2459-16, shown in Fig. 1; and second, the appearance of peak group II in another chromatogram of the same sample of *C. difficile* D 1193-04, shown in Table 1. In this chromatogram the relative fraction of the peaks of group II was 6.9%, clearly generated at the expense of the peak corresponding to 3-keto,12β, which was reduced to 0.7%.

The obvious interpretation that these peaks should be thermal artifacts of 12β-hydroxy bile acids was further substantiated by using reference compounds. With the TFA-HFIP derivatives of 3β,12β and 3α,12β, frequent formation of peaks of group I was observed, reaching 25 and 10.9% of the total peak area, respectively, whereas with 3α,7α,12β, artifacts reached 4.4%. These results agreed with and extended earlier observations that the TFA-methyl ester of 3β,12β may decompose during high-temperature GLC (6).

Mass spectra of the TFA-HFIP derivatives of 3α,12β and 3β,12β differed only in the intensities of fragment ions (common for spectra recorded during GLC separation) from each other and from that of deoxycholic acid published earlier (4). These findings agree with the established fact that in general, epimeric bile acids cannot be distinguished by their mass spectra (20). The mass spectra of the three by-products (group I) eventually present were all identical with those of the parent compounds, the ions of highest molecular weight always corresponding to the loss of one TFA group. If this loss occurs during high-temperature GLC, at least one cholenoic acid with an RRT between 0.20 (cholanoic acid) and 0.60 (lithocholic acid) should be produced. Epimerization of the remaining TFA groups (12) and different positions of the double bond may explain the formation of the three actually observed peaks. Analogous results were obtained for peaks b through d and k of Fig. 1 as well as with samples of other strains.

Metabolite n (Fig. 1) was tentatively identified as 3-keto,12β on the basis of chromatographic and mass spectral properties. The mass spectrum of this compound had diagnostically important ions at *m/z* 636 (I; 9.2% relative abundance in terms of base peak), 533 (II; 9.2%), 271(III; 100%), and 253 (IV; 43.3%). These ions correspond to the molecular ion of the TFA-HFIP derivative of a monoketo-monohydroxycholanoic acid (I), loss of one TFA group (II), loss of a TFA group and the side chain (III), and loss of a TFA group, one molecule of H_2O, and the side chain (IV) (4). Additionally, the fragmentation pathways observed indicate that the keto function cannot be a 12-keto function because of the absence of certain ions characteristic of the presence of such a group (20). The spectrum was, however, identical with that of the TFA-HFIP derivative of authentic 3-keto,12α.

Mass spectra of peaks f through h (Fig. 1) and corresponding peaks in other tests were all identical with one another and with that of compound n. The obvious conclusion that peaks of group II are thermal artifacts of 3-keto,12β, analogous to the results obtained with 3α,12β and 3β,12β, could not be definitely demonstrated because this bile acid was not available as reference compound.

To confirm the presence of hitherto unknown 12β-HSDH in *C. paraputrificum*, detected with 3,12-diketo, we cultivated representative strains, D 2660-02 and D 762-06, with deoxycholate or 3α,12-keto, with cholate or 3α,7α,12-keto, respectively, as substrates. Transformation of 3α,12β and 3α,7α,12β was only tested with strain D 2660-02 because insufficient amounts of these bile acids were available.

In all experiments, a 12α-hydroxyl group was not attacked, whereas a 12β-hydroxyl function was oxidized to a 12-keto group, resulting in the formation of 10.1% 3α,12-keto and 9.9% 3α,7α,12-keto, respectively (3β,12β [0.5%] and 3-keto,12β [1.4%] were also detected as by-products). A 12-keto function was effectively reduced to a 12β-hydroxyl group, resulting in the formation of 78.6% 3α,12β and 68.9% 3α,7α,12β (strain D 2660-02) and 79.3% 3α,12β and 64.0% 3α,7α,12β (strain D 763-06). It is concluded that the reduction product of 3α,7α,12-keto is identical with 3α,7α,12β because of the stereospecific β-reduction of 3α,12-keto to 3α,12β, the nonoxidation of cholate and deoxycholate at C-12 by these strains, and the 12β-hydroxyl group oxidation by one of these strains.

We were also able to demonstrate the presence of an NADP-dependent 12β-HSDH in cell extracts of *C. paraputrificum* D 762-06 with 3α,12β as substrate.

Epimerization at C-12 by binary cultures of *C. paraputrificum* and 12α-HSDH containing *E. lentum*. To confirm the epimerization of the 12α-hydroxyl function to a 12β-hydroxyl function by cooperation of organisms with a stereospecific α-HSDH and a stereospecific β-HSDH, we cocultured *C. paraputrificum* D 2660-02 or D 762-06 in the presence of deoxycholate or 3α,12-keto with *E. lentum* K 6/2 or K 19/23.2. The single bile acid-transforming activities of the latter strains were powerful 12α-HSDHs.

With these binary cultures, we were able to demonstrate 12α-hydroxyl group epimerization and 12-keto group reduction to a 12α- and a 12β-hydroxyl function. Figure 2 shows the time course of deoxycholate epimerization to 3α,12β by the combination *C. paraputrificum* D 2660-02, and *E. lentum* K 19/23.2. Attention should be paid to the fact that the concentration of 3α,12-keto passed a maximum during the log phase, whereas the concentration of 3α,12β increased continously towards a plateau. In the late stationary phase, however, some reconversion of 3α,12β to deoxycholate occurred. This observation is notable, since transformation experiments with 3α,12β could not be performed on account

of the lack of this bile acid. The same bacterial combination reduced 3α,12-keto, producing plateau concentrations of 58.1% for 3α,12β, 8.8% for deoxycholate, and 33.0% for 3α,12-keto after 24 h. Again it is noteworthy that deoxycholate was generated already in the early log phase, while appreciable amounts of 3α,12β were not formed before the late log and early stationary phases. These kinetic experiments also revealed the intermediary formation of 3β,12-keto—a maximum of about 12% was reached—during the early log phase.

Epimerization at C-12 of bile acids by human stool suspensions. 12α-Hydroxyl group epimerization by mixed human fecal cultures was demonstrated. In 2 of 10 transformation experiments with cholate as starting material, inoculated with 1 ml of fresh fecal suspensions (dilution degree 1:10) and incubated for 7 days, we detected 3α,12β in amounts of 4.8 and 15.4% (person K 26, a healthy matched control) beside deoxycholate in amounts of 72.4 and 69.5%. The total of 7-dehydroxylation products of cholate was about 91% both times. Corresponding experiments with deoxycholate and apocholate confirmed epimerization at C-12 but with cultures from person K 26 only. Apocholate conversion, hydrogenation of the C-8=C-14 double bond, as well as epimerization of the 12α-hydroxyl function, was unusually extensive, as indicated by the presence of 37.4% deoxycholate and 62.2% 3α,12β. Although chance had played its part here, the possibility of bile acid epimerization at C-12 by human mixed fecal cultures was clearly demonstrated.

DISCUSSION

The existence of intestinal microorganisms able to epimerize the 12α-hydroxyl group of bile acids was suggested, since 3α,12β and 3β,12β were detected as fecal bile acids in humans (1, 5). Although these acids may exist elsewhere in nature, they have not as yet been found, nor was it possible to demonstrate epimerization at C-12 of bile acids in vitro by mixed fecal or pure cultures until now. This communication is the first in which 12α-hydroxyl group epimerization of dihydroxy- bile acids of human mixed fecal cultures can be reported, together with the ability of strains of *C. paraputrificum*, *C. tertium*, and *C. difficile* to participate in 12α-hydroxyl group epimerization. With these strains, possessing a 12β-HSDH, hydroxyl group epimerization takes place only by cooperation with a bacterium possessing a 12α-HSDH via a 12-keto intermediate.

Although HSDHs of intestinal bacteria are usually α stereospecific, stereospecific β-HSDHs seem to be rare: one strain of *E. aerofaciens* (17) and three isolates of *P. productus*-like anaerobes (10) with a 7β-HSDH were isolated recently from human feces, and one strain of *E. lentum* with a 6β-HSDH (7) was isolated from rat feces. The epimerization at C-12 of bile acids by this type of reaction is in accordance with these reports. The detection of clostridia with stereospecific β-HSDHs is a novelty. All clostridia known so far, which were found to be able to epimerize the hydroxyl groups at C-3, C-7, or C-6 of bile acids, *C. perfringens* (11, 15), *C. absonum* (14), lecithinase-lipase-negative clostridia (2), and an unidentified *Clostridium* sp. (19), did so by themselves. Indeed, additional but rather weak abilities to epimerize bile acids at C-3 by the latter way were found to be present in 8 of 13 strains of *C. paraputrificum* and in the one strain of *C. tertium* and *C. difficile* mentioned above.

C. difficile and *C. tertium* have been detected in human feces (9) but belong to those numerous species and unspecified clostridia found in specific individuals only. *C. paraputrificum*, however, was more frequently isolated, at counts of 10^4 to 10^{11} per g of feces, and seems to be common in certain populations (8, 9). This species as the total fecal clostridial flora may become part of the predominant flora of humans in specific individuals, reaching counts of 10^9 to 10^{11} per g. In general, clostridia are only part of the accompanying or remaining flora, with counts of 10^3 to 10^6 per g of feces (18). Furthermore, since fecal counts of clostridia were found to rise under the influence of diets rich in fats and since 12β-hydroxy bile acids reported above were detected in appreciable amounts in the feces of men on such diets, we also expect an in vivo participation of *C. paraputrificum* (and eventually other bacteria) in 12α-hydroxyl group epimerization, at least in specific individuals.

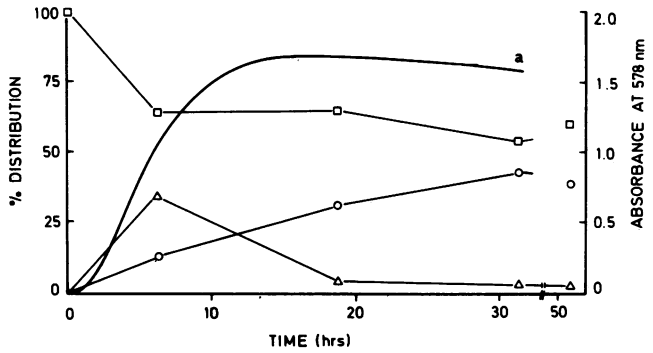

FIG. 2. Time course of deoxycholate epimerization to 3α,12β-dihydroxy-5β-cholanoate via 3α-hydroxy-12-keto-5β-cholanoate by a binary culture of *C. paraputrificum* D 2660-02, and *E. lentum* K19/23.2. A set of tubes containing 10 ml of medium were inoculated with standard volumes of overnight cultures of *C. paraputrificum* and *E. lentum* strains as described in the text. Growth was followed by measurement of culture turbidity at 578 nm with an Eppendorf photometer (a). One 10-ml tube each was taken at the times indicated by test points and analyzed for bile acids as described. Symbols: □, 3α,12α; ○, 3α,12β; △, 3α,12-keto.

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