Inactivation of Metronidazole by Anaerobic and Aerobic Bacteria

E. D. RALPH AND DENISE A. CLARKE

Department of Medicine, University Hospital, The University of Western Ontario, London, Ontario N6A 5A5 Canada

The rate of inactivation of metronidazole in vitro was determined during the course of time-kill curves against anaerobic and aerobic bacteria in the stationary phase of growth. Metronidazole at a concentration of 10 µg/ml, as measured by bioassay, was rapidly inactivated in broth culture by susceptible anaerobic bacteria (minimum bactericidal concentration ≤ 3 µg/ml), and this correlated closely with its bactericidal activity. In contrast, the drug was neither inactivated nor had any bactericidal activity against a resistant strain of *Propionibacterium acnes* (minimum bactericidal concentration > 1,500 µg/ml). Three of four aerobic bacteria also inactivated metronidazole, although at generally slower rates than the anaerobes, but this was not associated with a bactericidal effect against these organisms. The presence of aerobic bacteria in mixed cultures with *Bacteroides fragilis* did not, moreover, inhibit the bactericidal activity of metronidazole against the latter organism. However, the possibility still remains that, in vivo, aerobic bacteria capable of inactivating metronidazole could inhibit the action of the drug against anaerobes in mixed infections.

It has been well established that metronidazole is consistently bactericidal in vitro against many anaerobic bacteria (2, 13) and appears to be effective clinically in the treatment of anaerobic infections (1, 14). Against aerobic organisms, however, metronidazole is relatively inactive (2), and, moreover, earlier studies have shown that it is inactivated by certain aerobic bacteria (6, 8).

A study was devised to examine the rate of inactivation of metronidazole by both anaerobic and aerobic bacteria and the potential inhibitory effect of aerobic organisms on its activity against anaerobes.

MATERIALS AND METHODS

Bacterial organisms. Six clinical anaerobic isolates (*Bacteroides fragilis, Fusobacterium nucleatum, Eubacterium lentum, Peptostreptococcus anaerobius, Clostridium perfringens,* and *Propionibacterium acnes*) and four aerobic strains (*Staphylococcus aureus, Escherichia coli, Proteus morganii,* and *Streptococcus faecalis*) were used as the test organisms. All were maintained on blood agar in an anaerobic glove box (Coy Manufacturing, Ann Arbor, Mich.). The gas mixture within the anaerobic glove box consisted of 85% nitrogen, 10% hydrogen, and 5% carbon dioxide. The bacteria were subcultured in brucella broth (Difco) containing 5 µg per ml of hemin(BB+H) 18 to 48 h before each experiment.

The minimum inhibitory concentrations (MICs) of metronidazole for these organisms were determined by the tube dilution method in BB+H in the anaerobic glove box at an inoculum size of approximately 10^5 colony-forming units per ml (CFU/ml). The minimum bactericidal concentrations (MBCs) were determined after subculture of tubes showing no growth above to brucella agar (Difco) containing hemin (BA+H) and defined as the lowest concentration at which no growth occurred.

Time-kill curves. All procedures were performed within the anaerobic glove box (AGB) except the pouring of plates for colony counting. The anaerobic and aerobic bacteria were in the stationary phase of growth in broth culture (BB+H) before the time-kill curve experiment. Metronidazole (supplied as laboratory powder by Poulenc Ltd., Montreal, Quebec, Canada) was added to the culture at a concentration of 10 µg/ml, and sampling of viable colony counts was performed by Eppendorf pipet (Eppendorf Geratebau Nethler + Hinz GmbH, Hamburg, Germany) every 2 h for 8 h and at 24 h with portions added to phosphate-buffered saline, pH 7.2. Colony counts were made by the pour plate method in BA+H and incubated either anaerobically for the anaerobic organisms or aerobically for the aerobes. For the *P. anaerobius* and *P. acnes* strains, surface colony counting procedures were performed within the AGB because they were too fastidious to tolerate pour plate procedures under aerobic conditions. The concentration of metronidazole in the plates for colony counting was reduced below the MICs of the organisms by the combined effect of dilution in phosphate-buffered saline and in the agar. Control tubes without metronidazole were also run simultaneously for each of the organisms.

Time-kill curves were also performed on mixed cul-

tures of *B. fragilis* with each of the four aerobic bacteria. On the day before each experiment, both the *B. fragilis* and the aerobic strain (either *S. aureus, E. coli, P. morganii,* or *S. faecalis*) were subcultured in the same tube of BB+H. On the next day, 10 μg of metronidazole per ml was added, and a time-kill curve was performed on the stationary phase mixed culture as well as on a control containing only *B. fragilis*. The latter control kill curve was always performed simultaneously with each of the mixed cultures because there was some variation in the total log count decrement in experiments done at different times. Colony counts on the aerobe and *B. fragilis* were carried out as previously described. Pour plates of the aerobic organisms were incubated aerobically and counted because *B. fragilis* did not grow on these plates. To count the *B. fragilis* colonies on the anaerobic plates without interference from the aerobic bacteria, the following antibiotics were incorporated into the BA+H to inhibit the growth of the latter organisms: polymyxin B, 15 μg/ml for *E. coli*; gentian violet, 2 μg/ml for *S. aureus*; vancomycin, 5 μg/ml for *S. faecalis*; and nalidixic acid, 30 μg/ml for *P. morganii*. These antimicrobials were shown not to interfere with the growth of the *B. fragilis* strain.

Inactivation of metronidazole. At the various sampling times of the above time-kill curves, portions of the cultures were filtered through 0.20 μm filters (Nalge-Synbron Corp., Rochester, N.Y.), and assayed for metronidazole by a bioassay technique previously described (11). The accuracy of the assay was ±10%.

The effect of aerobic (rather than anaerobic) incubation on the inactivation of metronidazole by the *E. coli* and *P. morganii* strains was also studied. The methodology was similar to that described above except the organisms were incubated aerobically. In addition, the effect of cell-free filtrates of stationary phase cultures of the four aerobic bacteria on metronidazole was determined. The organism was initially grown anaerobically to the stationary phase, the cultures were filtered through 0.20-μm filters to remove the bacteria, and the filtrates were then incubated anaerobically with 10 μg of metronidazole per ml. The concentration of metronidazole was determined at various sampling times over 24 h.

RESULTS

MICs and MBCs. MICs and MBCs are shown in Table 1. For all anaerobic bacteria except *P. acnes*, the MICs and MBCs were 1 μg/ml or less and 3 μg/ml or less, respectively, and were considered in the susceptible range. For *P. acnes* the MIC and MBC were greater than 1,500 μg/ml. The most susceptible organism was *F. nucleatum*, which had an MIC and MBC of less than 0.01 μg/ml.

For the aerobic organisms, the MICs and MBCs were all equal to or greater than 500 μg/ml. The most resistant organism was *S. faecalis*, with an MIC and MBC greater than 2,000 μg/ml, and the most susceptible was *P. morganii*, which had an MIC of 500 μg/ml and an MBC of 750 μg/ml.

TABLE 1. *MICs and MBCs of metronidazole for six anaerobic and four aerobic organisms[a]*

Organism	MIC (μg/ml)	MBC (μg/ml)
Anaerobic bacteria		
F. nucleatum	<0.01	<0.01
P. anaerobius	0.07	0.07
B. fragilis	1	2
E. lentum	1	1
C. perfringens	1	3
P. acnes	>1,500	>1,500
Aerobic bacteria		
P. morganii	500	750
E. coli	1,000	1,000
S. aureus	1,000	>2,000
S. faecalis	>2,000	>2,000

[a] Inoculum size was approximately 10^5 CFU/ml.

Time-kill curves and inactivation of metronidazole. (i) Anaerobes. The time-kill curves for the six anaerobic bacteria are shown in Fig. 1. The inoculum sizes of the stationary phase organisms ranged from 5×10^7 CFU/ml for *P. anaerobius* to 5×10^{10} CFU/ml for *F. nucleatum*. The addition of 10 μg of metronidazole per ml reduced the viable cell counts of the five susceptible organisms from 2 to 5 logs within 4 h, and by 8 h the bactericidal action was generally finished. In repeat time-kill curves on these organisms, there was some variability in the total log count reduction, although the pattern of rapid killing within 2 to 4 h and cessation of bactericidal effect by 6 to 8 h was consistent.

In contrast to the above kill curves, no reduction in counts occurred with *P. acnes*, as noted by the flat curve at the top of Fig. 1.

Control curves (not shown) for each of the strains showed less than a 1-log change over the 24-h periods, and the organisms were therefore considered to be in the stationary phase of growth and did not undergo a significant loss of viability over the sampling period.

The rate of inactivation of 10 μg of metronidazole per ml for the six anaerobic bacteria during their time-kill curves is shown in Fig. 2. For all organisms except *P. acnes*, a rapid decrease in the amount of metronidazole in the broth was noted. For *C. perfringens, F. nucleatum,* and *P. anaerobius*, no detectable (<0.5 μg/ml) metronidazole remained after 4 h. Less than 1 μg of metronidazole per ml was present for *B. fragilis* and *E. lentum* by 8 h, and none was detectable by 24 h. The inactivation of metronidazole was therefore followed closely by its bactericidal effect as shown previously in Fig. 1. No significant inactivation occurred in the presence of *P. acnes*, and this correlated with the lack of bactericidal effect of metronidazole against this organism (Fig. 1).

(ii) Aerobes. The time-kill curves of the four aerobic strains (not shown) exposed to 10 µg of metronidazole per ml showed no significant loss of viability over 24 h and were similar to the control tubes. Inoculum sizes averaged approximately 10^9 to 10^{10} CFU/ml.

As shown in Fig. 3, however, three of the four aerobic organisms inactivated metronidazole to a variable extent over the 24-h time interval. The most rapid inactivation occurred with *S. faecalis*, followed by *P. morganii* and *E. coli.*

No inactivation was noted with the *S. aureus* strain. By 24 h, both *S. faecalis* and *P. morganii* had reduced the concentration of metronidazole to less than 1 µg/ml, and *E. coli* reduced the concentration to less than 2 µg/ml.

The effect of aerobic conditions on the inactivation of metronidazole by *P. morganii*, compared with anaerobic incubation, is shown in Fig. 4. The rate of inactivation was more rapid initially under anaerobic conditions (especially within 2 to 4 h), but after 8 to 24 h no significant
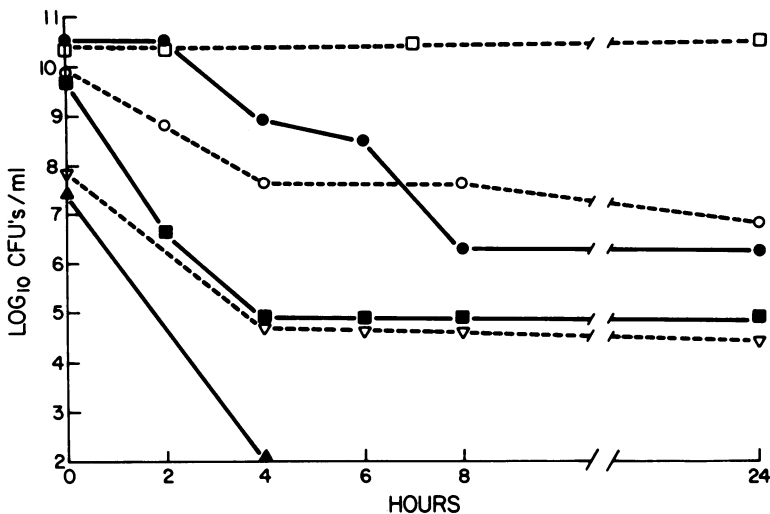

FIG. 1. *Time-kill curves for the six anaerobic organisms in the stationary phase of growth. Metronidazole (10 µg/ml) was added at zero time. Symbols: □, P. acnes; ●, F. nucleatum; ○, C. perfringens; ■, B. fragilis; ▽, E. lentum; ▲, P. anerobius.*
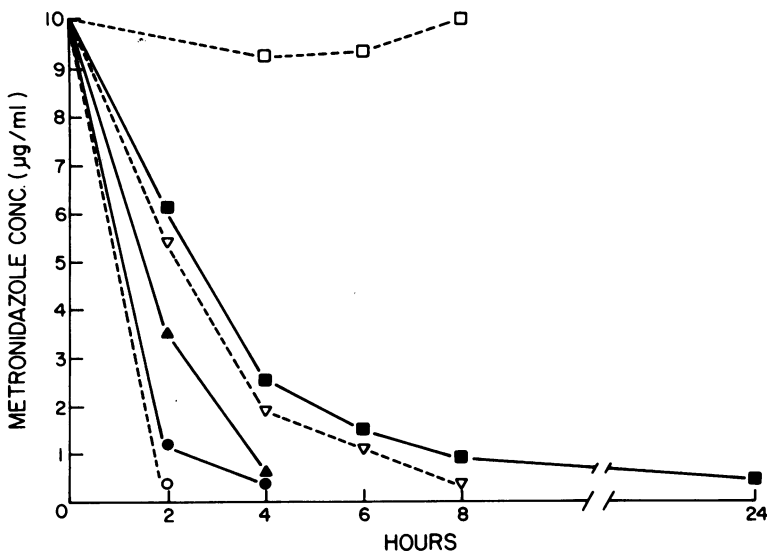

FIG. 2. *Inactivation of metronidazole (10 µg/ml) by the six anaerobic organisms. Symbols: □, P. acnes; ●, F. nucleatum; ○, C. perfringens; ■, B. fragilis; ▽, E. lentum; ▲, P. anaerobius.*
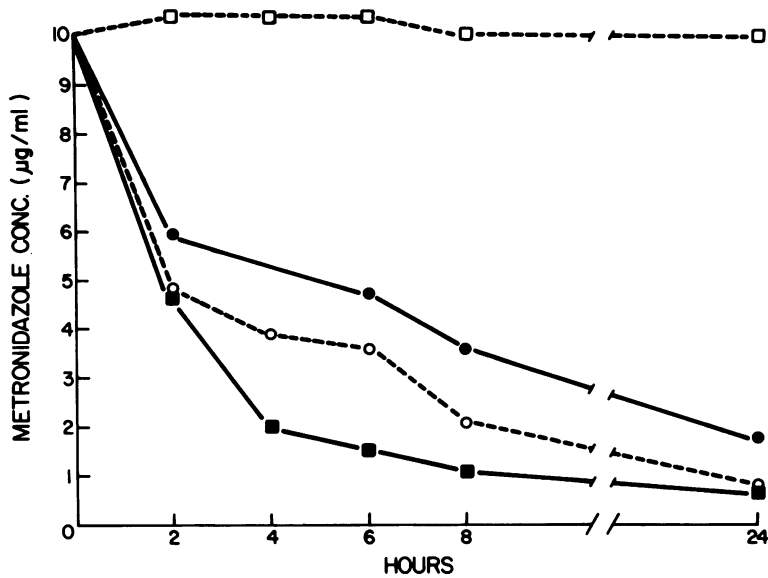
FIG. 3. *Inactivation of metronidazole (10 µg/ml) by the four aerobic organisms. Symbols: □, S. aureus; ●, E. coli; ○, P. morganii; ■, S. faecalis.*
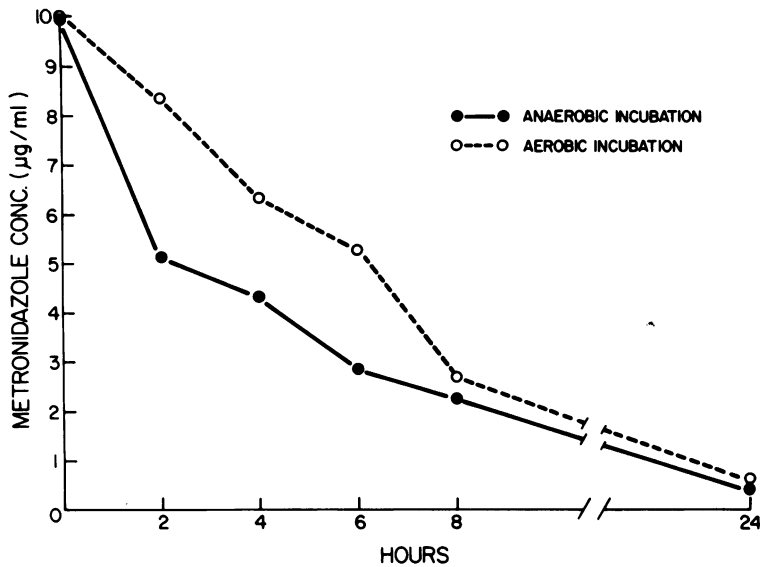

FIG. 4. *Inactivation of metronidazole (10 µg/ml) by P. morganii under anaerobic and aerobic conditions.*

difference was noted in the amount of metronidazole remaining under anaerobic or aerobic conditions. A similar pattern was noted for *E. coli* (not shown), but the initial difference in the rate of inactivation with anaerobic and aerobic incubation was smaller.

When the filtrates of stationary phase cultures of the four aerobes were incubated with 10 µg of metronidazole per ml for 24 h anaerobically, no

inactivation of metronidazole occurred in the cell-free medium; therefore, the presence of bacterial organisms was required for inactivation to occur.

(iii) Mixed anaerobic-aerobic cultures. The time-kill curves for *B. fragilis* alone and in a mixed culture with *S. faecalis* are shown in Fig. 5. After 10 µg of metronidazole per ml was added to the cultures, the bactericidal effect on

B. fragilis alone in culture (1.5-log decrease) was slightly less than that when in a mixed culture with *S. faecalis* (2-log decrease). Metronidazole had no bactericidal effect on *S. faecalis* in the mixed culture. For the other three aerobic organisms (not shown), there was also no inhibition of the bactericidal effect of metronidazole against *B. fragilis* in mixed culture as noted in the rate or total log count decrement, nor did

metronidazole have any significant activity against the aerobes.

The rate of inactivation of metronidazole by the mixed culture of *B. fragilis* and *S. faecalis* compared with *B. fragilis* alone is shown in Fig. 6. The inactivation was somewhat faster by the mixed culture initially, but was similar by 6 h onwards. The more rapid initial inactivation correlated with a more rapid bactericidal effect on
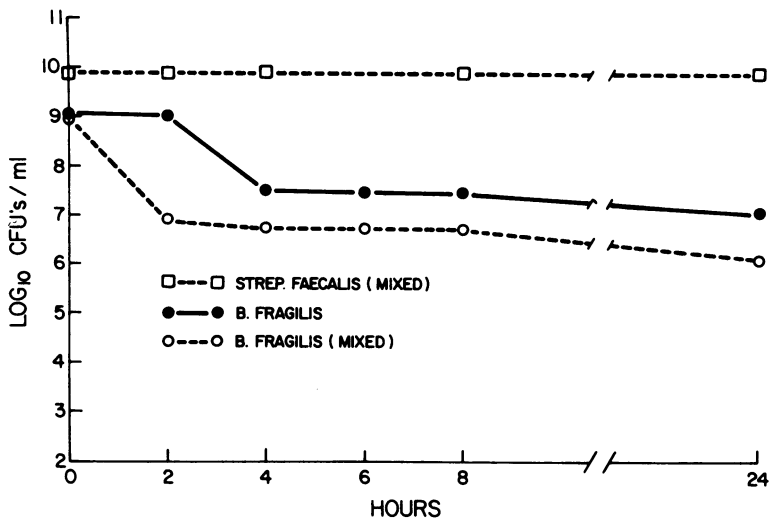

FIG. 5. *Time-kill curves for B. fragilis alone and in the mixed culture with S. faecalis. Time-kill curve for S. faecalis in the mixed culture is also shown.*
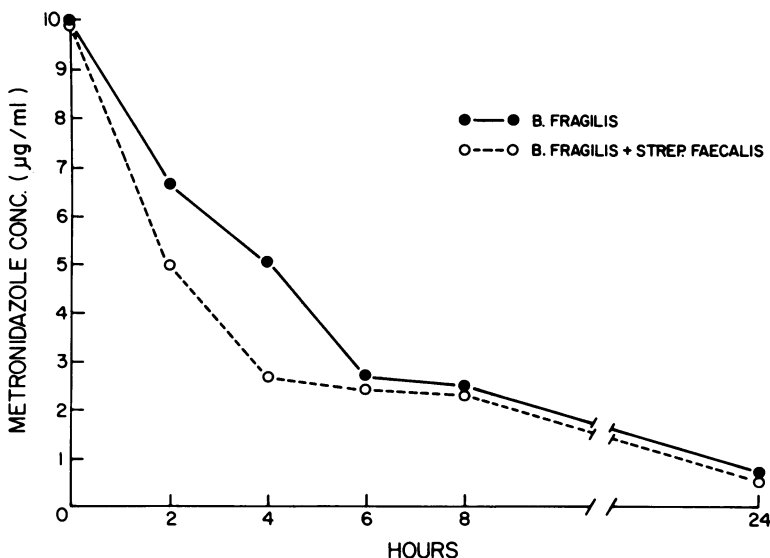

FIG. 6. *Inactivation of metronidazole (10 μg/ml) by B. fragilis alone and by B. fragilis and S. faecalis in mixed culture.*

B. fragilis in the mixed culture as noted in Fig. 5 during the first 2 to 4 h of incubation. For the other mixed cultures (data not shown), the inactivation of metronidazole was similar to that of *B. fragilis* alone, and this correlated with the similar time-kill curves alluded to above.

DISCUSSION

The bactericidal effect of metronidazole appears to be dependent upon its uptake by bacteria and conversion to a "reactive intermediate" which then interferes with cellular DNA, resulting in cell death (3–5). Other cellular events may also be inhibited, but these have not been well defined. In this study, the inactivation of metronidazole by five susceptible anaerobic bacteria correlated closely with its bactericidal action against these organisms. As the concentration of metronidazole in the medium fell below its MBC (usually by 4 h), little or no further killing occurred. In contrast, the *P. acnes* strain tested was resistant to metronidazole (MBC > 1,500 µg/ml) and did not inactivate the drug.

Metronidazole had no bactericidal effect on the four aerobic bacteria, but interestingly, was inactivated by three of them, although at generally slower rates than the anaerobes. This confirms by bioassay the previous studies of McFadzean et al. (6) who had shown that metronidazole was inactivated (as determined polarographically) to a variable extent after 24 h of incubation by certain gram-positive and -negative aerobic organisms, including *E. coli, Proteus* sp., *Klebsiella* sp., and *S. faecalis*. The degree of inactivation varied from 30 to 100% and appeared to be dependent upon the amount of metronidazole in the medium. They suggested that inactivation of metronidazole by vaginal bacteria was a possible mechanism for its rare failure in the treatment of trichomoniasis. Nicol et al. (8) also reported inactivation of metronidazole (as determined polarographically and biologically) by an organism of the genus *Mimae* that was isolated from the vaginal flora of a case of trichomoniasis that responded poorly to metronidazole.

The inactivation or disappearance of bioactive metronidazole in the medium presumably results from uptake of the drug by bacteria. This was demonstrated by Ings et al. (3), Müller and Lindmark (7), and Tally et al. (12) in which [¹⁴C]metronidazole was shown to be taken up by anaerobic organisms. The latter investigators also demonstrated that [¹⁴C]metronidazole was taken up more slowly by an *E. coli* strain compared with a *B. fragilis* strain. We also observed that the inactivation of metronidazole by aerobic bacteria is not dependent upon strict anaerobic conditions and also appears to be associated

with cellular processes because a cell-free filtrate of stationary phase organisms produced no inactivation of metronidazole.

The explanation as to why metronidazole had no bactericidal effect on the aerobic bacteria that inactivated it is not known. One possibility is that the biotransformation of metronidazole differs within anaerobic and aerobic bacteria and the compound formed in the latter is not active. Alternatively, the metabolism of the drug may be similar in both types of organisms, but only cellular processes in anaerobes are susceptible to its bactericidal action.

Metronidazole has a rapid bactericidal effect against *B. fragilis* and *C. perfringens* in the log phase of growth (10), and this study demonstrates its activity against stationary phase organisms. Bacteria in this phase of growth at large inoculum sizes were used to ensure maximum inactivation of metronidazole and, therefore, accentuate any differences among the different organisms. Also, bacteria in the stationary phase may simulate to some extent their microbiological state in abscesses.

Although metronidazole was inactivated by certain aerobic bacteria used in this study, their presence in mixed culture with *B. fragilis* did not inhibit the bactericidal action of metronidazole against this organism. This may have resulted from the more rapid uptake of metronidazole by *B. fragilis* before any significant inactivation by the aerobes, and/or the relatively large (10 µg/ml) concentration of metronidazole used compared with the MBC for the *B. fragilis* strain. However, although no inhibitory effect was demonstrated in vitro, the possibility remains that in vivo certain aerobic organisms in mixed infections could inhibit the effect of metronidazole against anaerobic bacteria. This would occur most likely under conditions in which the level of the drug was close to its bactericidal concentration.

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