Effect of Fluorophenylalanine on Bacteriophage MS2 Replication

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A concentration of 10 μ g of fluorophenylalanine per ml added to a chemically defined medium reduced by 100-fold the number of bacteriophage MS2 produced on *Escherichia coli* C3000 and increased the latent period. Fluorophenylalanine was most effective when added concurrent with infection. Addition of a 10-fold greater concentration of phenylalanine reversed the inhibition caused by fluorophenylalanine. Radioactive fluorophenylalanine was incorporated into the coat protein. The four phenylalanine-containing chymotryptic peptides are not equally accessible to fluorophenylalanine. Only two of the peptides are highly labeled by fluorophenylalanine. Incorporation of fluorophenylalanine decreased the specific infectivity and the rate of adsorption but did not increase the sensitivity of the whole virus to ribonuclease. MS2 ribonucleic acid (RNA) functioned as messenger RNA for the incorporation of both phenylalanine and fluorophenylalanine in a cell-free incorporating system from *E. coli*.

Dunn and Leach (9) observed possible differences in the codewords for phenylalanine and fluorophenylalanine in *Escherichia coli* and suggested that only the UUU codon is accessible to fluorophenylalanine, whereas both the UUU and UUC codons function with phenylalanine. The ribonucleic acid (RNA) bacteriophages were selected as a system for further comparison of fluorophenylalanine and phenylalanine, since the consequences of the functioning of viral RNA both as messenger RNA in a cell-free system and as a template for viral replication may be determined (29). Amino acid analogues may be used to analyze the mechanism of protein synthesis (18) by observing the drift in protein functionality produced by their utilization.

The incorporation of fluorophenylalanine into an enzyme results in a spectrum of events depending upon the specific protein or parameter being examined. Westhead and Boyer (26) demonstrated that the incorporation of fluorophenylalanine into rabbit aldolase and glyceraldehyde-3-phosphate dehydrogenase with replacement of 25% of the phenylalanyl residues resulted in no change in enzymatic activity. In a chemically defined medium containing exponentially growing

cultures of either *E. coli* or yeast, the addition of fluorophenylalanine reduced the rate of growth and the inhibition was reversed by the addition of phenylalanine (5, 6). Halvorson and Spiegelman (11) found that fluorophenylalanine inhibited adaptation to maltose in yeast. The replacement of 75% of the phenylalanyl residues in exopenicillinase from *Bacillus cereus* resulted in a 50% decrease in enzyme specific activity (17). Yoshida (27) found that replacement of 10% of the phenylalanyl residues reduced the specific activity of *B. subtilis* α -amylase by 30%.

Although fluorophenylalanine has been extensively used in the analysis of animal virus replication, this paper constitutes the first extended examination of the effects of fluorophenylalanine on the replication of an RNA bacteriophage.

MATERIALS AND METHODS

The sodium salt of guanosine triphosphate (GTP) was obtained from Schwarz BioResearch, Inc. The sodium salt of adenosine triphosphate (ATP) was obtained from P-L Biochemicals, Inc. The silver barium salt of phosphoenolpyruvate, pyruvate kinase (EC 2.7.1.40), DL-*p*-fluorophenylalanine, and DL-*p*-fluorophenylalanine-1-¹⁴C were obtained from Calbiochem. ¹⁴C-L-phenylalanine (367 mc/mole) was obtained from New England Nuclear Corp. Ribonuclease (EC 2.7.7.16) was obtained from Mann Research Laboratories. DL-*p*-Fluorophenylalanine was labeled with tritium by the solvent exchange method at Volk Radiochemicals and was purified by column and

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paper chromatography. Bacteriophage MS2 and its host *E. coli* Hfr C3000 were kindly supplied by A. J. Clark, University of California, Berkeley.

Techniques used in handling the bacteriophage were those described by Adams (2). The complete medium (MS) and synthetic medium (TPG) were as described by Strauss and Sinsheimer (21), unless otherwise indicated for the particular experiment. The RNA was prepared by the sodium perchlorate procedure of Freifelder (10). The method of Katz and Comb (13) was used to determine the base composition of the RNA. Preparation of the *E. coli* 30,000 $\times$ *g* supernatant solution was as described by Nirenberg (15). The radioactivity of the sample was determined in a liquid-scintillation counter by using Bray's solution (14). Chymotryptic digestion of the coat protein was performed by the procedure of Notani et al. (16). The electrophoresis and chromatography of the resulting peptides were by the procedure of Dintzis (8).

RESULTS

Effect of fluorophenylalanine on MS2 replication: time of addition of fluorophenylalanine. Viral replication is under temporal control with different proteins synthesized for early and late functions in the replication cycle. Variation in the time of addition of fluorophenylalanine can provide information concerning the sensitivities of the various proteins to incorporation of the analogue. If differential sensitivities exist, they may be exploited to analyze the steps in replication. Figure 1 shows a study of the outcome of fluorophenylalanine addition at various times relative to infection. When the fluorophenylalanine was added at either 60 min before or 10 min after infection, replication was markedly inhibited. A greater inhibition was observed when fluorophenylalanine was added concomitant with infection. The reason for a lesser inhibition when the inhibitor was added 60 min before infection is unknown, but it is a consistent observation and does not depend upon the medium used. When radioactive fluorophenylalanine was incubated with *E. coli* cells during a 60-min period before infection, 90% of the radioactivity remained in the medium; thus, removal of the fluorophenylalanine from the solution cannot be the explanation. The addition of fluorophenylalanine at later times (20, 30, or 60 min) after infection resulted in decreasing inhibition. A low multiplicity of infection (MOI) was used. The concentration of fluorophenylalanine was selected as the one giving 2 logs of inhibition.

Reversal by phenylalanine. Figure 2 shows the effect of reversing the fluorophenylalanine inhibition by adding an excess of phenylalanine. There was only a slight inhibition when phenylalanine was added approximately 1 min after fluorophenylalanine. When reversal occurred after 10

FIG. 1. Effect of time of addition of fluorophenylalanine. Replicate flasks containing *E. coli* growing in TPG medium were infected at 0 time with 1.2×10^6 plaque-forming units (PFU) of MS2 virus per ml (MOI = 0.01). Fluorophenylalanine (10 μ g/ml) was added at the times indicated by the arrows, and samples were taken and treated with chloroform at the indicated times. No fluorophenylalanine was present in one sample (Δ), whereas fluorophenylalanine was added at -60 ($\bullet$), 0 ($\circ$), 10 ($\ominus$), 20 ($\ominus$), 30 ($\ominus$), and 60 ($\circ$) min relative to time of infection.

min, there was a 20-min increase in the latent period, but the yield of virus was not decreased. Addition of phenylalanine 30 min after infection and addition of fluorophenylalanine resulted in no change in the production of virus during 60 min of incubation when compared to an uninhibited culture. Rather than halting virus production as noted in the inhibited culture, there was a continued synthesis of virus until approximately the same final titer was reached as was obtained in an uninhibited infection. The reversal by phenylalanine shows the specificity of the inhibition and establishes that parental viral RNA was not degraded during the period when protein synthesis was inhibited by fluorophenylalanine.

Properties of fluorophenylalanine-containing phage: proof of incorporation by using density gradient centrifugation. The evidence presented above does not yield information regarding the possible incorporation of fluorophenylalanine into viral coat protein in place of phenylalanine. Therefore, experiments were done with radioactive fluorophenylalanine to establish such an incorporation. Radioactive ^{14}C -phenylalanine and ^3H -fluorophenylalanine were added to cultures of *E. coli* concomitant with infection. A

FIG. 2. Reversal of fluorophenylalanine inhibition by addition of phenylalanine. Replicate cultures of *E. coli* were infected with 2×10^7 PFU of MS2 per ml at 0 time (MOI = 0.5). Fluorophenylalanine (10 μ g/ml) was added to all cultures except the control (○) at 0 time. Phenylalanine (100 μ g/ml) was added to the cultures at the times indicated: ●, 0 time; ◐, 10 min; □, 30 min; ●, not added. Samples were taken at the indicated times, and the total phage concentration was determined after chloroform lysis.

control contained ^{14}C -phenylalanine but was not infected. After 4 hr, all cultures were treated with chloroform and the virus (or similar fraction) was purified by repetitive cycles of centrifugation at $30,900 \times g$ for 30 min and $105,536 \times g$ for 4 hr in a Spinco 40 rotor. CsCl (0.55 g/ml) was added to the purified samples, and they were centrifuged for 60 hr in the SW 39 rotor (Fig. 3). Radioactivity from both ^{14}C -phenylalanine- and ^3H -fluorophenylalanine-labeled infected cultures sedimented to the same position in the density gradient as MS2 particles as determined by absorbancy (A_{260}) measurements. There was no radioactivity sedimenting at that position when there was no viral infection. When 10 μ g of radioactive fluorophenylalanine per ml was added to a sample of virus and the mixture was centri-

FIG. 3. CsCl density gradient centrifugation of labeled virus. Three samples were analyzed by 60 hr of centrifugation at $99,972 \times g$ in the SW 39 rotor. Carrier phage was added to the radioactive phage and was detected by A_{260} measurements (○). The radioactive cultures were purified by three cycles of centrifugation at $30,900 \times g$ for 15 min and $105,536 \times g$ for 4 hr. ^3H -fluorophenylalanine was present during MS2 infection (●), whereas ^{14}C -phenylalanine was present during infection (◐). An uninfected control with ^{14}C -phenylalanine is shown (□). The original density of the CsCl solution was 1.4, and two-drop fractions were collected.

fuged, the radioactive fluorophenylalanine was found at the top of the gradient and there was no localization at the viral peak. Fluorophenylalanine was not bound to the phage under the conditions of these experiments.

Precipitation of radioactive virus with antisera.

Antiserum for MS2 virus was prepared as described by Adams (2). Table 1 shows the precipitation of radioactivity from solution containing either ^{14}C -phenylalanine- or ^3H -fluorophenylalanine-labeled virus with antiserum prepared against bacteriophage MS2. No radioactivity was precipitated by antiserum against *E. coli*. The precipitation of radioactivity from labeled virus preparations with antiserum against the whole phage implies the incorporation of fluorophenylalanine into the coat protein.

Absorption of radioactive virus to membrane

filters. Lodish and Zinder (14) found that under proper ionic conditions bacteriophage f2 is retained by membrane filters, thereby making possible another assay for the incorporation of radio-

FIG. 4. Fingerprints of phenylalanyl- and fluorophenylalanyl-containing coat proteins. Chymotryptic digestion of the labeled coat proteins was followed by electrophoresis and chromatography. Ninhydrin spray was used to detect the peptides, and the appropriate areas were cut out and counted in a liquid scintillation spectrometer. Where no numbers are shown, the radioactivity measured was only background level.

TABLE 1. Precipitation of fluorophenylalanine-containing MS2 with antiserum^a

Antiserum against	Net counts/min precipitated	
	¹⁴ C-phenylalanine MS2	¹⁴ C-Fluorophenylalanine MS2
MS2.....	11,807	1,025
<i>E. coli</i>	0	0

^aRadioactive MS2 was prepared by adding approximately 5 μ g of the amino acid to *E. coli* cultures at the time of infection. The specific activity of the ¹⁴C-fluorophenylalanine was 0.01 that of ¹⁴C-phenylalanine. After 3 hr of incubation, chloroform was added and the bacterial debris was removed by centrifugation at $26,360 \times g$ for 30 min. The MS2 was then sedimented by 6 hr of centrifugation at $105,400 \times g$. Samples of the preparations were added to buffered saline to which 0.1 ml of the appropriate antiserum was added. Then the mixtures were incubated for 2 hr at 45 C. The precipitate was removed by centrifugation, dissolved in hyamine, and counted in a liquid scintillation counter. The nonspecific precipitation of labeled virus was corrected for by subtracting the counts per minute precipitated when no antiserum was present.

active fluorophenylalanine into the bacteriophage. With MS2, a higher ionic strength was required for maximum retention than was the case with f2. Table 2 shows the retention of 70 to 80% of the total trichloroacetic acid-precipitable

radioactivity when the phage-containing solution was made 0.3 M with respect to NaCl. Only 8 to 18% of the total radioactivity was retained on filtration of water or broth solutions of the virus. Addition of radioactive fluorophenylalanine (10 μ g/ml) to a solution (0.3 M NaCl) of phage followed by membrane filtration (Millipore Corp., Bedford, Mass.) showed no retention of radioactivity on the filter; therefore, fluorophenylalanine is incorporated into phage and not just bound. Thus, the amino acid analogue was incorporated into the intact viral particle as was demonstrated by density gradient centrifugation, precipitation by specific antiserum, and by attachment to membrane filters.

Effect on ribonuclease resistance. Incorporation of fluorophenylalanine into poliovirus makes the particles more susceptible to inactivation by ribonuclease than normal poliovirus (12). During 2 hr of incubation of virus with 10 μ g of ribonuclease per ml, there was no significant decrease in viral titer over control incubations in which ribonuclease was omitted for either phenylalanine- or fluorophenylalanine-containing MS2. Under these conditions, substitution of fluorophenylalanine for phenylalanine in the coat protein failed to increase the permeability of the virus particle to ribonuclease.

Specific infectivity. The incorporation of protein subunits containing fluorophenylalanine into the viral coats might produce noninfectious or less infectious particles which could probably be purified by the same procedure as normal phage. Thus, by comparing infectivity with some measure of the number of particles, it could be determined whether noninfectious or less infectious particles were produced in the presence of fluorophenylalanine. Measurement of the absorbance and viable virus titer of purified solutions should give evidence on this point. Table 3 shows that fluorophenylalanine-containing MS2 had a lower specific infectivity and thus more noninfectious particles were made in the presence of fluorophenylalanine.

Adsorption to the host. The incorporation of fluorophenylalanine into the coat protein might modify the adsorption reaction between phage and host bacteria. As was suggested by Lodish and Zinder (14), a modification of the membrane filtration technique enables rapid measurement of adsorption. The experiment was done in broth solution, and the host bacteria along with any adsorbed phage were retained by the membrane filter. Table 4 shows that a much smaller percentage of either ³H- or ¹⁴C-fluorophenylalanine-containing phage was adsorbed to the host bacteria. The total figure obtained with phenylalanine-con-

TABLE 2. Retention of labeled virus on membrane filters^a

Solution of virus	Counts/min		
	¹⁴ C-phenylalanine MS2	³ H-fluorophenylalanine MS2	¹⁴ C-fluorophenylalanine MS2
0.3 M NaCl.....	141,529 (81%)	806 (67%)	7,223 (81%)
Water.....	13,813 (8%)	176 (12%)	1,636 (18%)
5% Trichloroacetic acid.....	175,117 (100%)	1,209 (100%)	8,893 (100%)

^aSamples of solution containing radioactive virus were filtered through membrane filters (HA; Millipore Corp.) and washed with 100 ml of the corresponding solution. The filters were dried and counted in the liquid scintillation counter. Significantly greater retention of MS2 virus occurred in 0.3 M NaCl rather than in the 0.15 M NaCl found to result in maximum retention of f2 by Lodish and Zinder (14). The per cent radioactivity retained is shown in parentheses.

TABLE 3. Specific infectivity^a

Virus type	A_{257}/ml	Titer (PFU/ml) ^b	PFU/ A_{257}
Normal.....	15.7	5.1×10^{10}	3.2×10^9
Fluorophenylalanine-grown..	0.231	2.2×10^8	9.5×10^8

^aSamples of MS2 virus grown in TPG medium and TPG medium plus 10 μg of fluorophenylalanine per ml were purified and then diluted, and the absorbance was determined at 257 nm.

^bPlaque-forming units per milliliter.

TABLE 4. Effect of fluorophenylalanine on the adsorption of MS2 to *E. coli*^a

Type of phage	Percentage of added radioactivity adsorbed to bacteria
¹⁴ C-phenylalanine MS2.....	15.1
³ H-fluorophenylalanine MS2.....	2.4
¹⁴ C-fluorophenylalanine MS2.....	3.7

^aLabeled MS2 phage were added to growing cultures of *E. coli*, and after 10 min the solution was diluted 10-fold with TPG and then filtered through membrane filters (HA; Millipore Corp.). The bacteria-phage complexes retained on the filter were counted by using a liquid scintillation counter.

taining virus agrees well with the number of f2 phage in a stock capable of forming plaques (7).

The adsorption constant, K , was determined as described by Adams (2) for MS2 grown in the presence of phenylalanine (2.97×10^{-9} ml/min) or fluorophenylalanine (2.12×10^{-9} ml/min), and these results demonstrated that fluorophenylalanine-containing virus is defective in adsorption.

Fingerprints of phenylalanine- and fluorophenylalanine-containing coat proteins. Figure 4 shows

fingerprints of chymotryptic peptides from MS2 coat proteins grown in the presence of radioactive phenylalanine and fluorophenylalanine, respectively. The solid line shows the outline of the ninhydrin-positive areas in the chromatogram. The figures show the radioactivity in total counts per minute obtained from the various areas of the chromatogram when cut out and counted in a liquid scintillation counter. In radioautographs, only four areas of the fingerprint from phenylalanine-labeled protein were radioactive. These four highly radioactive peptides gave counts greater than 2,800, but only two of these peptides were radioactive when labeled fluorophenylalanine was used for growth of the phage. The lower specific activity of the coat protein precluded radioautography. This observation suggests that two of the phenylalanine-containing peptides are not accessible to fluorophenylalanine.

RNA from MS2. Viral RNA can serve as messenger RNA for the in vitro synthesis of protein; therefore, it would be important to establish whether MS2 RNA can serve as a template for the incorporation of fluorophenylalanine into protein. Below are summarized the properties of MS2 isolated by the perchlorate method and experiments in which this RNA was used as messenger.

Properties of MS2 RNA. RNA was isolated from MS2 by the perchlorate method of Freifelder (10). The ultraviolet absorbance spectrum showed a maximum at 257 nm and a minimum at 232 nm, values which are similar to those published by Strauss and Sinsheimer (21) for RNA prepared by the phenol procedure. A melting curve of MS2 RNA in 0.132 M NaCl and 0.01 M tris (hydroxymethyl) aminomethane (Tris) (pH 7.0) showed a T_m of 62 C. The lower ionic strength accounts for the lower melting temperature than that obtained by Strauss and Sinsheimer (21); however, both melting curves showed the same total hyperchromic effect, suggesting that

both samples of RNA had similar degrees of ordered structure. The base composition was guanine, 0.263; uracil, 0.260; cytosine, 0.250; and adenine, 0.226. This composition was similar to that found by Strauss and Sinsheimer (21), and all measured parameters agree with their findings.

Function of messenger RNA. Table 5 shows the activity of MS2 RNA as messenger RNA in the cell-free system from *E. coli*. The amino acid substrates used were labeled phenylalanine and fluorophenylalanine, and the messenger activity was compared to polyuridylylate. There was more than a 70-fold stimulation of phenylalanine incorporation by 50 μ g of polyuridylylate and almost a 40-fold stimulation of fluorophenylalanine in-

corporation. As expected, this incorporation was energy-dependent and inhibited by puromycin. MS2 RNA-stimulated incorporation showed the same characteristics, but the extent of stimulation was much less since most amino acids must be incorporated when MS2 RNA is the messenger. There was an average 2.5-fold stimulation of phenylalanine incorporation and a 1.6-fold stimulation of fluorophenylalanine incorporation by the MS2 RNA over those obtained in the absence of added messenger RNA. Thus, fluorophenylalanine can be incorporated into protein as directed by MS2 RNA; however, under the present experimental conditions, this incorporation is too low to be used to label MS2 proteins in vitro with fluorophenylalanine and then to isolate and study the position of fluorophenylalanine incorporation.

TABLE 5. Utilization of MS2 RNA as messenger RNA in cell-free system^a

Messenger		Additions	Counts/min incorporated	
Type	Amt (μ g)		Phenylalanine	Fluorophenylalanine
	0	Complete	789	301
Poly U	10	Complete	35,936	6,086
Poly U	50	Complete	63,209	11,447
Poly U	50	- Energy	216	119
Poly U	50	+ Puromycin	680	373
MS2	10	Complete	1,499	426
MS2	50	Complete	2,190	508
MS2	50	- Energy	190	125
MS2	50	+ Puromycin	321	166

^aThe complete reaction system contained, in 0.25 ml, the following: Tris (pH 7.8), 25 μ moles; NH_4Cl , 12.4 μ moles; magnesium acetate, 2.5 μ moles; β -mercaptoethanol, 1.0 μ mole; ATP, 0.5 μ mole; GTP, 0.1 μ mole; phosphoenolpyruvate, 1.0 μ mole; 20 amino acid mix without phenylalanine, 0.0025 μ mole each; pyruvate kinase, 10 μ g; and *E. coli* 30,000 $\times$ g supernatant solution, 2.94 mg. Where indicated, 0.02 μ mole of puromycin was added, and GTP, ATP, phosphoenolpyruvate, and pyruvate kinase were omitted for the (-) energy samples. Radioactive substrates used were 0.2 μ Ci of phenylalanine-uniformly labeled- ^{14}C (1.55 pmoles) or 1.0 μ Ci of ^3H -fluorophenylalanine (11 pmoles). Messenger RNA was present as indicated. After 30 min of incubation at 37 C, 0.1-ml samples were placed on 2.3-cm discs of Whatman 3MM and dried in a stream of warm air. The discs were immersed in cold 5% trichloroacetic acid-0.25% sodium tungstate solution and allowed to stand for 10 min. Then the discs were treated for 7 min with 90 C, 5% trichloroacetic acid-0.25% sodium tungstate solution, washed twice with 95% ethyl alcohol, and finally dried with diethyl ether. Radioactivity was determined in Bray scintillating fluid.

DISCUSSION

The results obtained with fluorophenylalanine in MS2 infection will be compared with the results obtained with animal viruses.

The addition of fluorophenylalanine to a medium or suspending solution has no effect on the stability of MS2. In Fig. 1, the addition of fluorophenylalanine at 60 min after infection did not reduce the titer of phage from that produced in control cultures during this time when measured during a subsequent infection. Ackerman, Rabson, and Kurtz (1) demonstrated that the presence of fluorophenylalanine in the medium or suspending solution does not influence the stability of intact poliovirus.

When phenylalanine was added 30 min after infection and addition of fluorophenylalanine (Fig. 2), there was a reversal of the inhibition which indicates that the presence of fluorophenylalanine does not interfere with the adsorption of MS2. Measurement of the adsorption constant in the presence and absence of fluorophenylalanine (in the medium) also revealed that fluorophenylalanine does not influence the adsorption reaction. Tanami and Pollard (22) found that the initial stages of adsorption and penetration of psittacosis virus are not inhibited by fluorophenylalanine.

Since phenylalanine addition reverses the inhibition, the specificity of the inhibition is demonstrated. Zimmermann and Schafer (28) were able to differentiate formation of any viral material, production of S antigen, production of hemagglutinin, and finally production of new infectious particles of fowl plague virus by adding fluorophenylalanine at different times. Venwoerd and Hausen (24) found that there are two groups of early proteins in Me virus that may be differenti-

ated by time of addition of fluorophenylalanine. In several cases [Scharff et al. (20) and Baltimore and Franklin (3)], the continued synthesis of the viral-specific RNA polymerase is required for continuing viral production; therefore, addition of fluorophenylalanine late in the replication cycle is still effective in inhibiting production of infectious progeny.

Fluorophenylalanine is incorporated into the coat of bacteriophage MS2 as is demonstrated above. Since the specific infectivity and the adsorption constant are completely changed for fluorophenylalanine-containing viral proteins, the incorporation of fluorophenylalanine into coat protein results in a detectable alteration of the particle. Hummeler and Wecker (12) found structurally altered poliovirus particles produced by fluorophenylalanine incorporation; the particles retained the gross morphology, diameter, and shape of mature virus, but the capsid was permeable to phosphotungstate and ribonuclease. Such an increased sensitivity of the fluorophenylalanine-containing MS2 to ribonuclease was not observed in the present study.

Townsend (23) found that fluorophenylalanine is incorporated into tobacco mosaic virus at a slower rate than phenylalanine. From the amount of fluorophenylalanine required to produce a detectable effect on MS2 replication and from the comparative amounts of radioactive phenylalanine and fluorophenylalanine incorporated, a definite preference for phenylalanine was observed in the present study.

Weisblum et al. (25) found that a particular leucyl transfer RNA is capable of introducing leucine into only a single position in the α -chain of rabbit hemoglobin. Richmond found random replacement of the eight phenylalanine residues in *E. coli* alkaline phosphatase (19). The results obtained with fingerprints of fluorophenylalanine-containing peptides suggest a degeneracy of the phenylalanine code and that only one transfer RNA is accessible to fluorophenylalanine.

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