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Enhancement of Ribosomal Ribonucleic Acid Synthesis by Deoxyribonucleic Acid Gyrase Activity in *Escherichia coli*

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The effect of the deoxyribonucleic acid (DNA) gyrase inhibitors coumermycin A_1, novobiocin, and oxolinic acid on ribonucleic acid (RNA) synthesis in *Escherichia coli* was studied in vivo and in vitro. Preferential inhibition of ribosomal RNA (rRNA) synthesis was observed. No effect of oxolinic acid and coumermycin on rRNA synthesis was seen in mutants having a DNA gyrase which is resistant to these inhibitors. In a temperature-sensitive DNA gyrase mutant rRNA synthesis was decreased at nonpermissive temperatures. Thus, a functional DNA gyrase is required for rRNA synthesis. Purified DNA gyrase had no effect on rRNA synthesis in a purified system. However, DNA gyrase does show preferential stimulation of rRNA synthesis in a system supplemented with other proteins. Apparently, DNA gyrase stimulation of rRNA synthesis requires another protein.

In *Escherichia coli* the enzyme DNA gyrase (DNA topoisomerase II) introduces negative superhelical turns into circular double-stranded DNA molecules ([9]; reviewed in references 4 and 33). The enzyme is needed for replicative DNA synthesis (3). DNA gyrase is composed of two kinds of subunits, A and B, which are encoded on nonadjacent genes, the *gyrA* (formerly named *nalA*) gene and the *gyrB* (formerly named *cou*) gene, respectively. The enzyme is inhibited by the antibiotics oxolinic acid and nalidixic acid (8, 29), which act on the A subunit. The B subunit is the target for another set of DNA gyrase inhibitors, coumermycin and novobiocin. All of these inhibitors block the DNA supercoiling activity of DNA gyrase in vivo as well as in vitro (6, 8, 10).

Evidence has accumulated that supercoiling facilitates the transcription of a DNA template in vitro (2, 22, 32) and selectively activates some promoters (1, 2). DNA gyrase inhibitors strongly reduce the transcription of rRNA operons (18, 34), the lactose operon, and the colicin E1 gene, whereas other genes are not affected (34). Also in vivo, DNA gyrase inhibitors block the synthesis of a subset of mRNA species (5, 15, 17, 23, 28). Previously, we have demonstrated that the B-subunit inhibitor novobiocin strongly inhibits rRNA synthesis, whereas mRNA synthesis is only moderately affected (18). Results conflicting with ours have been reported by Wahle and Mueller (31), who state that DNA gyrase inhibitors reduce rRNA synthesis to a smaller extent than total RNA synthesis.

Antibiotic-resistant mutants (8, 34) and temperature-sensitive mutants (14) of DNA gyrase have become available. These mutants allowed us to investigate whether the activity of both DNA gyrase subunits is necessary for the occurrence of rRNA synthesis. The effect of purified DNA gyrase on rRNA transcription in vitro was also studied.

MATERIALS AND METHODS

Chemicals. Oxolinic acid, produced by Warner Lambert Co., was a gift of Substantia, Amsterdam, The Netherlands. Coumermycin A_1 was purchased from Godfrey Science & Design, Syracuse, N.Y. Novobiocin was from Sigma Chemical Co., St Louis, Mo. Oxolinic acid was dissolved in dilute NaOH (25 mg/ml); coumermycin was dissolved in dimethyl sulfoxide (25 mg/ml). The solutions were stored at −20°C. Casamino Acids were from Difco Laboratories, Detroit, Mich.

[4,5-^{3}H]leucine (52 Ci/mmol), [5-^{3}H]uracil (25 Ci/mmol), [5,6-^{3}H]UTP (54 Ci/mmol), and $^{32}P_i$ (40 Ci/ml) were from The Radiochemical Centre, Amersham, U.K. DNA gyrase was a generous gift of T. F. Meyer, Max-Planck-Institut für Medizinische Forschung, Heidelberg, Germany. One unit of DNA gyrase is defined as the amount of enzyme which converts 1 μg of relaxed closed circular DNA into the supercoiled form within 30 min. Wheat germ $tRNA^{gly}$ was a gift of G. Robillard, Department of Physical Chemistry, University of Groningen, Groningen, The Netherlands.

Growth and labeling. The bacterial strains used are listed in Table 1. Bacteria were grown in M9 medium (16) supplemented with glucose (0.2%), Casamino Acids (0.2%), and, where necessary, thymine (10 μg/ml). At an optical density at 450 nm of 0.4, the cultures were labeled with [^{3}H]uracil or [^{3}H]leucine as indicated in the legends of the figures. At the same

TABLE 1. *E. coli* strains

Strain	Genotype	Source
NF955	*thr leu thi ileC* λ *cI857 S7* d5*ilv*[a] λ *cI b2*	Jørgensen and Fiil (13)
N4156	*polA end thy gyrA*[b]	Gellert et al. (8)
N4182	Δ*lac-514 trp rpsL gyrB*[c]	Yang et al. (34)
KNK402	F[+] *polA1 endA2 thyA rpsL tsx-79 phx gyrA43*(Ts)	Kreuzer and Cozzarelli (14)

[a] Containing the *rrnX* operon.
[b] Phenotype: nalidixic acid resistant.
[c] Phenotype: coumermycin resistant.

time, coumermycin or oxolinic acid was added to a final concentration of 25 or 50 μg/ml, respectively. Samples of 100 μl were taken, and incorporation of label into acid-precipitable material was determined as described previously (18).

RNA synthesis in vitro. Transcription was performed in a standard reaction mixture (0.1 ml) with the following composition: 40 mM Tris-hydrochloride (pH 7.9); 10 mM $MgCl_2$; 100 mM KCl; 0.4 mM potassium phosphate; 10% glycerol; 1 mM ATP; 0.3 mM each of CTP and GTP; 0.1 mM [^{3}H]UTP (1 Ci/mmol); 0.1 mM EDTA; 0.1 mM ethyleneglycol-bis-β-(aminoethylether)-N,N'-tetraacetic acid; 0.1 mM dithiothreitol; wheat germ $tRNA^{gly}$ (5 μg/ml); and λ d5*ilv* DNA (10 μg/ml). Also present, where indicated, were novobiocin at a concentration of 10 μg/ml, oxolinic acid at 20 μg/ml, and DNA gyrase at 30 U/ml.

The mixture was preincubated for 10 min at 37°C unless otherwise specified. RNA polymerase was added (25 μg/ml), and, where indicated, S100 extract (10 μl) was added (for its preparation we refer to a previous paper [19]). After 30 min the reaction was stopped by addition of 0.1 ml of 4× SSC (SSC: 0.15 M NaCl–0.015 M sodium citrate, pH 7.0). Deproteinized samples were assayed for total RNA by acid precipitation and for rRNA by liquid hybridization competition as described earlier (21). The data were corrected for the base composition in rRNA and mRNA according to Giorno et al. (11).

Pulse-labeling and rRNA hybridization. To exponentially growing cultures of strain N4182 (absorbancy at 450 nm of 0.45) at 37°C, coumermycin or oxolinic acid was added to a final concentration of 35 μg/ml. After 8 min, 46 μCi of [^{3}H]uracil (specific activity, 25 Ci/mmol) was added, and RNA was labeled for 30 s. The cultures were transferred to a tube containing 0.25 ml of sodium dodecyl sulfate-lysis mixture (0.03 M Tris-hydrochloride, pH 7.5; 0.03 M EDTA; 0.2 M NaCl; 1.5% sodium dodecyl sulfate), which was placed in boiling water. After 2 min at 100°C the tubes were chilled on ice. The lysates were twice extracted with 1 volume of phenol saturated with 2× SSC. RNA was ethanol precipitated and dissolved in 0.27 ml of hybridization mixture: 6× SSC containing 0.025% sodium dodecyl sulfate and 0.01% aurintricarboxylic acid.

Total acid-precipitable RNA was determined as described earlier (21). The estimation of rRNA was carried out by hybridization competition using λ d5*ilv* DNA immobilized on nitrocellulose filters (pore size, 0.15 μm; Sartorius, Gottingen, Germany). Filters contained 30 μg of DNA. In a total volume of 1 ml of hybridization mixture, 60 μl of labeled RNA solution was hybridized with or without excess unlabeled rRNA for competition. ^{32}P-labeled rRNA was included as an internal standard. After incubation for 45 h, the filters were washed twice with 5 ml of 6× SSC, treated with an RNase mixture (21) for 1 h at 25°C, and washed twice with 5 ml of 6× SSC and 5 ml of 1% trichloroacetic acid. Determination of radioactivity was as described before (21). The hybridization efficiency was about 40%. The data were corrected as described above.

RESULTS

RNA and protein synthesis in DNA gyrase mutants in vivo. In previous experiments (18) we have shown that novobiocin, an inhibitor of the DNA gyrase subunit B, strongly inhibits rRNA accumulation. Now we have extended these studies, using antibiotic-resistant DNA gyrase mutants (see Table 1). In *E. coli* N4182, a strain with a mutant B subunit, the incorporation of [^{3}H]uracil into RNA is virtually unaffected by coumermycin, whereas, after a short period, it is completely inhibited by oxolinic acid (Fig. 1A). In strain N4156, an A-subunit mutant, the situation is reversed: coumermycin inhibits whereas oxolinic acid does not (Fig. 1B). Thus, the activity of both subunits is a necessary condition for RNA accumulation to occur.

Since the continuing incorporation of labeled precursors mainly measures the accumulation of stable RNA, it is expected that DNA gyrase at least sustains synthesis of rRNA and other stable RNAs. However, from such an experiment no conclusions can be drawn on mRNA synthesis. To investigate this more directly, we studied the effect of inhibitors on pulse-labeling and distinguished between rRNA and non-rRNA synthesis by hybridization with a rRNA-specific probe. In the *gyrB* mutant, oxolinic acid inhibited rRNA synthesis much more strongly than non-rRNA (presumably mRNA) synthesis (Table 2), which is in good agreement with the only partial inhibition of protein synthesis (Fig. 1C). As expected, coumermycin had only a very small effect on rRNA and protein synthesis.

In another approach to study the effect of DNA gyrase, we used the temperature-sensitive mutant *gyrA*(Ts). The effect of a temperature shift on RNA accumulation and protein synthesis is shown in Fig. 2. In contrast to the RNA accumulation in the parent strain, which was increased by 20% at the higher temperature (data not shown), the RNA accumulation in the mutant was clearly reduced, confirming similar findings by Kreuzer and Cozzarelli (14). However, contrary to what was observed with DNA gyrase inhibitors (Fig. 1), some rRNA synthesis

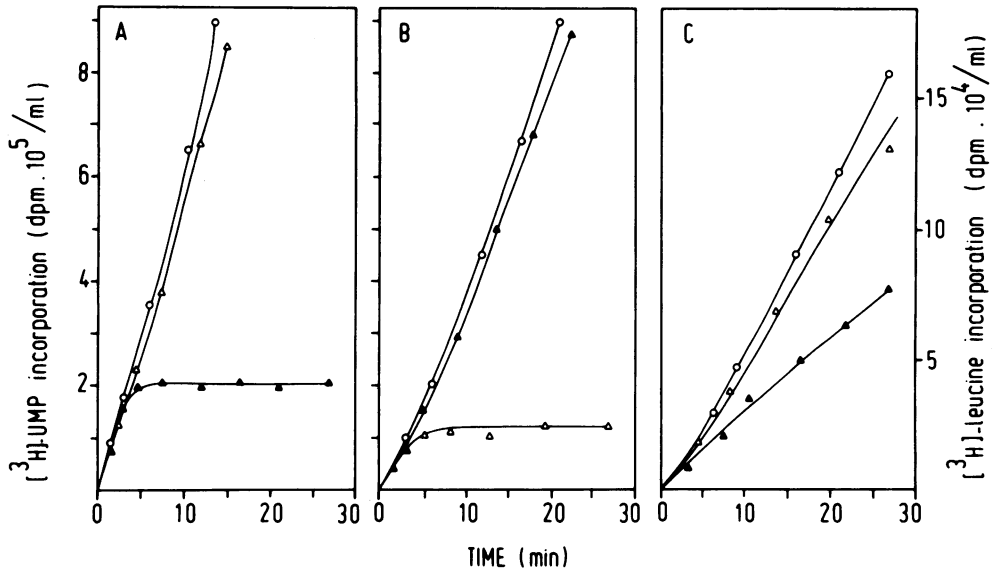

FIG. 1. *Influence of DNA gyrase inhibitors on RNA accumulation and protein synthesis. (A and B) To an exponentially growing culture of N4182 (A) or N4156 (B), [^{3}H]uracil (5 μCi/ml) was added to 0 min. (C) To an exponentially growing culture of N4182, [^{3}H]leucine (5 μCi/ml) was added at 0 min. DNA gyrase inhibitors were added at 0 min. At the indicated times 100-μl samples were removed. Symbols: ○, control; △, coumermycin; ▲, oxolinic acid.*

TABLE 2. *Effect of DNA gyrase inhibitors on RNA synthesis in a coumermycin-resistant strain in vivo*[a]

Inhibitor	[^{3}H]uracil incorporated (10^3 dpm)			rRNA (%)
	Total RNA	Non-rRNA	rRNA	
None	82.0	49.6	32.4	44.7
Coumermycin	74.8	47.2	27.7	42.1
Oxolinic acid	28.9	26.9	2.0	8.4

[a] RNA was pulse-labeled in strain N4182 (*gyrB*). Hybridization competition was as described in the text.

still occurred after the temperature shift. Although protein synthesis appeared only slightly reduced (Fig. 2B), the real effect must be larger since protein synthesis in the parent strain was increased about 20% at the higher temperature (data not shown). Again, the effect of the temperature-sensitive mutation on protein synthesis was less than the reduction by DNA gyrase inhibitors, as shown in Fig. 1. Even so, we clearly saw a selective effect on transcription of rRNA.

Effect of DNA gyrase on rRNA synthesis in vitro. The influence of novobiocin and oxolinic acid on rRNA synthesis in a system containing purified λ d5*ilv* DNA isolated from strain NF955 (see Table 1) and RNA polymerase as the only macromolecular components is shown in Table 3. These inhibitors of DNA gyrase have no effect on rRNA synthesis. In vitro, rRNA constitutes a much lower percentage of the total RNA synthesized than in vivo at high growth rates (20, 30). However, as was described earlier, the relative rate of rRNA synthesis in vitro is stimulated to physiological values by addition of a crude protein fraction derived from the S100 supernatant (19). Analogous protein fractions of *E. coli* N4182 and N4156 stimulated as well (Table 3). The enhancement of rRNA synthesis by N4182 extract was completely abolished by oxolinic acid and unaffected by novobiocin; with an extract of strain N4156 the effects were reversed. The effect of DNA gyrase inhibitors on the stimulated rRNA synthesis strongly suggests that in the extract DNA gyrase is one of the active components stimulating rRNA synthesis in vitro.

To demonstrate the involvement of DNA gyrase more directly, purified enzyme was added to our transcription system. No influence of purified DNA gyrase on rRNA synthesis is found in a system with RNA polymerase and DNA as the only macromolecular components (Table 4). This result, at first sight puzzling, may be due to the absence of other factor(s) necessary for the DNA gyrase effect. Indeed, DNA gyrase stimulated when suboptimal amounts of extract were present (Fig. 3). Apparently, DNA gyrase and at least one other (protein) component are needed.

In another approach to distinguish between DNA gyrase and the putative factor, differential heat inactivation was applied. The stimulation

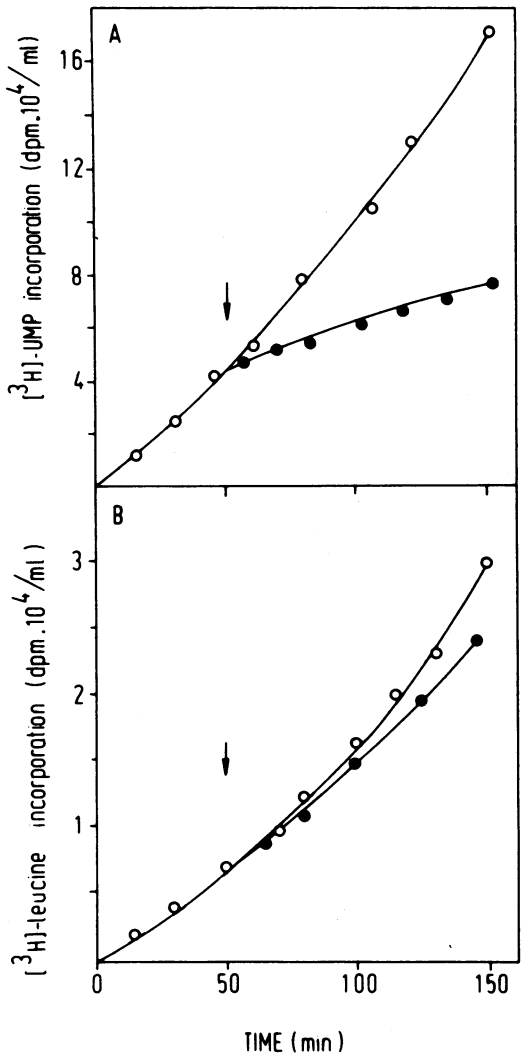

FIG. 2. *RNA and protein synthesis in a temperature-sensitive DNA gyrase mutant. Strain KNK402 was labeled with (A) [³H]uracil (5 μCi/ml, 0.25 Ci/mmol) or (B) [³H]leucine (5 μCi/ml, 0.5 Ci/mmol) at 0 min. At 50 min, part of the cultures were shifted to 39°C. Symbols:* ○, *30°C;* ●, *39°C.*

of rRNA synthesis was abolished when the extract was heated for 3 min at 50°C, but was restored by the addition of DNA gyrase (Table 4). Extract which was kept at 60°C for 3 min could not be reactivated by adding DNA gyrase. Clearly, the extract contains another protein factor which is needed besides DNA gyrase.

The *gyrA*(Ts) mutant offered another possibility to differentiate between DNA gyrase and the protein factor. As mentioned above (Fig. 2), the incorporation of [³H]uracil into stable RNA was depressed after a temperature shift in vivo. In vitro, at permissive temperatures, the addition of mutant extract had a normal positive effect on the transcription of rRNA genes (Table 5). At 39°C, however, the stimulation of the rRNA synthesis almost disappeared. Thus, inactivation of DNA gyrase in the mutant extract reduces the rRNA synthesis. Addition of wild-type DNA gyrase to the mutant extract activated the synthesis of rRNA at 39°C. Purified DNA gyrase without extract had no effect.

DISCUSSION

The experiments reported here show that DNA gyrase participates in RNA synthesis in vivo. mRNA synthesis is only partly inhibited by DNA gyrase inhibitors. These findings are in agreement with other reports that protein syn-

TABLE 3. *Effect of DNA gyrase inhibitors on RNA synthesis in vitro*[a]

Extract	Inhibitor		[³H]UMP incorporated (10^3 dpm)		rRNA (%)
	Novo[b]	Oxo[c]	Non-rRNA	rRNA	
None	−	−	87.7	21.9	23.6
	+	−	90.6	23.6	24.4
	−	+	96.5	21.1	21.3
N4182	−	−	66.9	82.8	60.5
	+	−	60.6	68.6	58.3
	−	+	69.0	25.2	31.1
N4156	−	−	68.8	69.0	55.4
	+	−	75.9	29.1	31.4
	−	+	70.8	60.3	51.3

[a] RNA was synthesized at 37°C in a standard reaction mixture with or without the indicated extract and with or without DNA gyrase inhibitors.
[b] Novo, Novobiocin.
[c] Oxo, Oxolinic acid.

TABLE 4. *Effect of DNA gyrase and heated extract on rRNA synthesis in vitro*[a]

Extract	Temp (°C)	DNA gyrase	[³H]UMP incorporated (10^3 dpm)		rRNA (%)
			non-rRNA	rRNA	
−		−	69.6	14.3	20.3
−		+	72.1	15.5	21.0
+		−	58.2	58.1	55.3
+	50	−	60.1	16.1	24.9
+	50	+	62.1	43.1	46.2
+	60	−	56.8	13.0	22.2
+	60	+	54.1	15.7	26.4

[a] RNA was synthesized with or without extract added and with or without DNA gyrase. Before addition to a standard reaction mixture the extract of strain N4182 was heated, where indicated, for 3 min at 50 or 60°C.

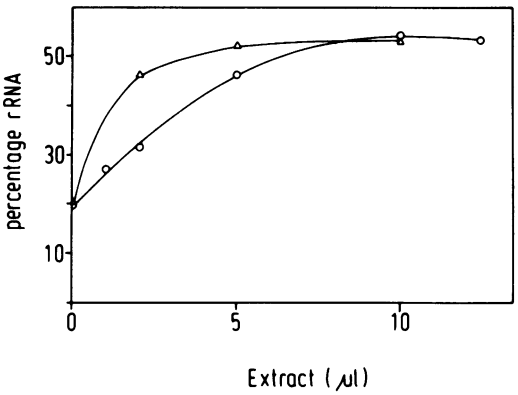

FIG. 3. *Effect of DNA gyrase and extract on rRNA synthesis in vitro. RNA was synthesized in a standard reaction mixture with various amounts of extract of strain N4182. Symbols: O, without DNA gyrase; Δ, with DNA gyrase.*

TABLE 5. *Effect of extract of strain KNK402 [gyrA(Ts)] on rRNA synthesis at different temperatures in vitro*[a]

Extract	Temp (°C)	DNA gyrase	[^{3}H]UMP incorporated (10^3 dpm)		rRNA (%)
			Non-rRNA	rRNA	
−	30	−	73.4	17.7	23.0
+	30	−	65.1	59.1	52.9
−	39	−	98.2	27.7	25.9
−	39	+	95.2	25.3	24.8
+	39	−	75.1	33.7	35.4
+	39	+	72.2	47.1	44.6

[a] RNA was synthesized at the indicated temperatures with or without extract of KNK402 and purified wild-type DNA gyrase.

thesis is only moderately affected (17) and only a subset of mRNA species is inhibited (5, 7, 15, 23, 25, 28), whereas some mRNA's are stimulated (17, 23). Conflicting results were reported by Wahle and Mueller, who found that mRNA synthesis was more inhibited by DNA gyrase inhibitors than rRNA synthesis (31). From our experiments it is obvious that the transcription of the rRNA operons is dependent on DNA gyrase and that the permanent activity of the enzyme is required. Specific effects of DNA gyrase on rRNA synthesis are also suggested by the experiments of Yang et al. (34) and of Kreuzer and Cozzarelli (14). Our experiments with DNA gyrase mutants clearly demonstrate that both subunits of DNA gyrase are needed for rRNA synthesis to occur. Moreover, DNA gyrase mutants have allowed us to show that DNA gyrase is involved in rRNA synthesis in vitro.

It is far from clear how DNA gyrase acts selectively on transcription. It is likely that gene expression is influenced by the conformation of the DNA template. The transcription of phage and plasmid DNA in vitro has been shown to be affected by the degree of superhelicity (2, 33). DNA gyrase could activate promoters by influencing the physical state of the intracellular DNA. RNA polymerase is believed to display preferential affinity for promoters which are negatively supercoiled, because then unwinding of the DNA is favored (24). Differences in ease of opening of various promoters could explain operon-specific effects of DNA gyrase inhibitors.

Data presented by Sinden and Pettijohn (27) show that DNA in the *E. coli* chromosome is organized into independent, supercoiled domains. The number of domains per genome equivalent appears to depend on the growth conditions. Possibly, the metabolic state of the cells modulates the extent of supercoiling of the template, thereby influencing the transcription activity of genes in a different manner. Coumermycin causes relaxation of DNA in vivo (6, 26). Thus, lowering of DNA supercoiling is coupled with a reduction of rRNA synthesis. Both DNA gyrase and an additional factor are required to sustain high levels of rRNA synthesis in vitro. We tested whether ligase could substitute for the additional factor in the extract. To this end, we added T_4 ligase to our purified transcription system, either solely or in combination with purified DNA gyrase. No effect was observed on the amount of RNA synthesized or on the fraction consisting of rRNA (data not shown).

The effect of DNA gyrase on transcription of linear DNA in vitro is puzzling. The enhancement by DNA gyrase of transcription on linear DNA (5, 34) and of replication of linear DNA (5, 12) has been reported. It is conceivable that an equivalent of a superhelical strand can be built up by hindering the axial rotation of the DNA molecules. On the other hand, our data do not exclude circularization of DNA in the presence of the extract.

In conclusion, it appears that the degree of supercoiling of the DNA differently affects the affinity of the various promoters for RNA polymerase and other regulatory elements and therefore plays an important role in the selectivity of transcription.

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