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Reductive Carboxylation of Propionate to Butyrate in Methanogenic Ecosystems

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During the batch degradation of sodium propionate by the anaerobic sludge from an industrial digestor, we observed a significant amount of butyrate formation. Varying the initial propionate concentrations did not alter the ratio of maximal butyrate accumulation to initial propionate concentration within a large range. By measuring the decrease in the radioactivity of [1-^{14}C]butyrate during propionate degradation, we estimated that about 20% of the propionate was converted to butyrate. Labeled butyrate was formed from [1-^{14}C]propionate with the same specific radioactivity, suggesting a possible direct pathway from propionate to butyrate. We confirmed this hypothesis by nuclear magnetic resonance studies with [^{13}C]propionate. The results showed that [1-^{13}C]-, [2-^{13}C]-, and [3-^{13}C]propionate were converted to [2-^{13}C]-, [3-^{13}C]-, and [4-^{13}C]butyrate, respectively, demonstrating the direct carboxylation on the carboxyl group of propionate without randomization of the other two carbons. In addition, we observed an exchange reaction between C-2 and C-3 of the propionate, indicating that acetogensis may proceed through a randomizing pathway. The physiological significance and importance of various metabolic pathways involved in propionate degradation are discussed, and an unusual pathway of butyrate synthesis is proposed.

The conversion of complex organic matter to methane and carbon dioxide proceeds mainly through volatile fatty acids (12, 13); among these acids, propionate is often an important metabolic intermediate (6, 10). Thermodynamic considerations (13) imply that anaerobic acetogenesis from propionate is only feasible at a very low partial pressure of hydrogen. Difficulties in achieving propionate degradation in unadapted anaerobic systems have been often reported with either pilot (1) or full-scale (20) operations.

In anaerobic digestors propionate is converted to acetate by syntrophic association of obligate proton reducers such as *Syntrophobacter wolinii* (3) and hydrogenophilic methanogens and, to a much lesser extent, by sulfate reducers belonging to the genus *Desulfobulbus* (15, 21). Using labeled compounds, Koch et al. (11) demonstrated that propionate degradation to acetate by a highly enriched methanogenic culture proceeds via a randomizing pathway.

During batch degradation of propionate by sludge from an anaerobic digestor, we observed a transient accumulation of butyrate (14, 16). In this paper, we propose a new pathway of butyrate synthesis by reductive carboxylation of propionate.

MATERIALS AND METHODS

Chemicals. All chemicals were reagent grade and were purchased from Prolabo or Merck. ^{14}C-labeled compounds and ^{13}C-labeled sodium propionate were purchased from Amersham Corp. and Merck Sharp & Dohme, respectively. The labeling specificity was at least 95% for all of the ^{13}C-labeled sodium propionates.

Culture conditions. The sludges were sampled anaerobically from a 2,500-m^3 mesophilic anaerobic contact digestor treating vegetable cannery wastewaters (20) with an organic load of 4.2 kg of chemical oxygen demand per m^3 per day; its content of volatile suspended solids was about 11 g/liter.

Batch degradations of volatile fatty acids were performed in sealed 120-ml serum vials containing 20 ml of sludge under an N_2-CO_2 (80%:20%) atmosphere at 35°C. Before anaerobic addition of sodium propionate, the vials were incubated for 24 h to allow complete degradation of the few residual organic compounds present in the sludge; under such conditions, propionate consumption started without any lag. Samples (0.1 ml) were centrifuged for 5 min at 10,000 × *g*, and the volatile fatty acid concentrations in the supernatant were determined.

Analytical methods. Volatile fatty acids were assayed by gas chromatography as previously described (19).

Tracer studies with ^{14}C-labeled compounds were done in duplicate, and samples of 0.5 ml were taken to follow the radioactivity of individual volatile fatty acids, which were separated by high-performance liquid chromatography on two serial 10μ C18 Radial Pak columns (Waters Associates, Inc.). The sample volume was 50 μl, and elution was performed at 1.2 ml/min with 75 mM ammonium phosphate buffer adjusted to pH 3; the effluent was monitored at 210 nm and then continuously mixed with pseudocumene scintillation liquid Lumaflow II in a 1:3 ratio to determine the radioactivity of the separated volatile fatty acids (Flo-One HP counter; Radiomatic Instruments & Chemicals Co., Inc.). Standardization was done by periodic injection of solutions of 1-^{14}C-labeled volatile fatty acids of known specific radioactivity.

In the experiments with ^{13}C-labeled sodium propionate, small vials (25 ml) with 10 ml of sludge were used; two 3-ml samples were withdrawn after 8 and 32 h, and the supernatants were analyzed by nuclear magnetic resonance (NMR) spectroscopy. The ^{13}C-NMR spectra of sludge supernatants were recorded with a Brucker AM-300 NMR spectrometer operating in the Fourier transform mode at a frequency of

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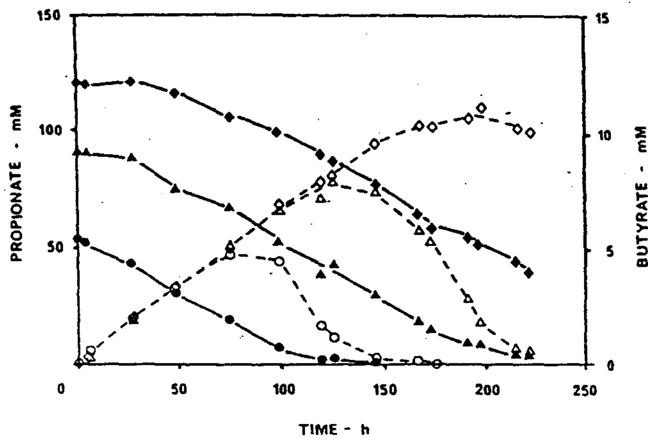

FIG. 1. Effect of various initial propionate concentrations (closed symbols) on butyrate accumulation (open symbols).

75.45 MHz. Spectra were usually recorded with a spectral width of 200 ppm, corresponding to a digital resolution of 1 Hz per point, a relaxation delay of 1 s, and a pulse width of 90° under broad-band proton decoupling. Deuterium (20%, vol/vol) was added for the field frequency lock, and 400 to 20,000 scans were needed for an optimal signal-to-noise ratio. An exponential filter corresponding to 2-Hz broadening was applied before Fourier transformation. For quantitative recordings, gated irradiation was used, with a 10-s relaxation delay; the spin-lattice relaxation times of protonated carbons were about 2 s under our conditions. A trace of dioxane was added as a chemical shift reference. Spectra were identified by comparison with spectra of authentic compounds recorded under similar conditions (pH ≈ 7).

RESULTS

Kinetics of propionate degradation. During batch degradation of sodium propionate, transient accumulations of butyrate and acetate were observed (Fig. 1). The acetate concentration was always less than 1 mM, and the maximal accumulated butyrate concentration increased proportionally with increasing initial propionate concentration but was only 10% of the amount of propionate added. The propionate degradation rate and the apparent butyrate formation and degradation rates were independent of the initial propionate concentration and were, respectively, 44, 6.1, and 10.4 μmol/h per g of volatile suspended solids.

Estimation of the true rates of butyrate formation and degradation. A trace amount (10 μCi) of sodium [1-^{14}C]butyrate was added during the batch degradation of 26 mmol of unlabeled sodium propionate per liter. For small increments of time (Δt), the decrease in the radioactivity of butyrate [$\Delta(\mathrm{dpm}_{\mathrm{but}})$] and an average specific radioactivity of butyrate (Sp $\mathrm{Act}_{\mathrm{but}}$) were determined. The actual degradation rate of butyrate ($\Delta[\mathrm{butyrate}]/\Delta t$) was then calculated from the following equation: $\Delta(\mathrm{dpm}_{\mathrm{but}})/\Delta t = (\Delta[\mathrm{butyrate}]/\Delta t) \times \mathrm{Sp\ Act}_{\mathrm{but}}$. The butyrate consumed during each time interval was calculated, and, by comparison with the variation in butyrate concentration measured in the medium, the butyrate produced during the same period was estimated. The kinetics of formation and degradation of butyrate (Fig. 2) were obtained by integration of these values and showed that the transient accumulation of butyrate resulted from an almost constant production rate and from an increasing degradation rate at low propionate concentrations. The calculated total production and consumption of butyrate were 5.1 and 5.5 mmol/liter, respectively. The difference was due partly to the butyrate already present in the medium when the tracer was added and partly to routine experimental error. The actual ratio between the formed butyrate and the consumed propionate (26 mmol/liter) was about 20%, and we can assume by the difference that the main pathway of propionate catabolism remained acetogenesis.

Pathway of butyrate formation from propionate. When 10 μCi of sodium [1-^{14}C]propionate was added to sludge incubated in presence of the same unlabeled acid (32 mM), radioactivity was detected first in acetate and then in butyrate after 2 and 7 h, respectively (Fig. 3). The specific radioactivity of propionate remained constant at 6×10^4 dpm/μmol during the experiment; the specific radioactivity of butyrate reached this value within 10 h, while that of acetate was 3×10^4 dpm/μmol. Radioactivity was also found in CO_2 and CH_4 but was not quantified. These results suggested that butyrate was formed from propionate by the addition of one unlabeled carbon atom.

The mechanism of this conversion was studied with ^{13}C-labeled propionate. When the reaction was started with

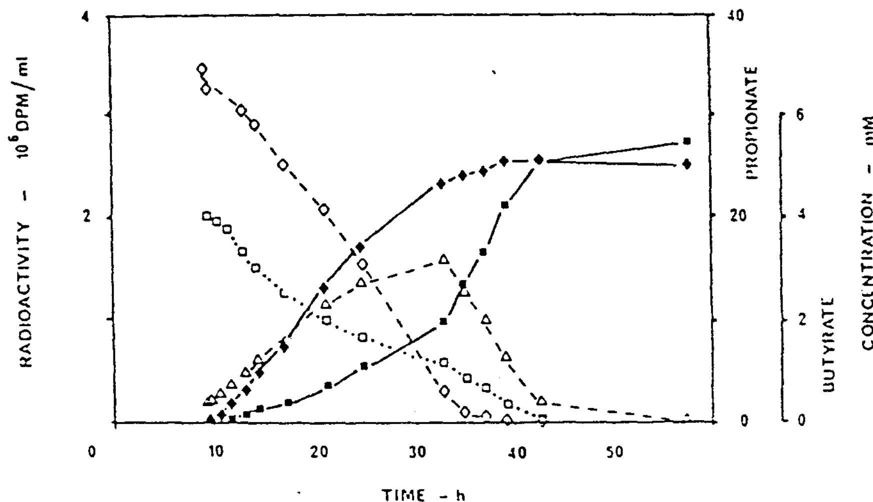

FIG. 2. Butyrate metabolic rates during propionate degradation with 10 μCi of [1-^{14}C]butyrate (△). The calculated butyrate production (◆) and consumption (■) and the observed propionate (◇) and butyrate (△) concentrations in the medium are shown.

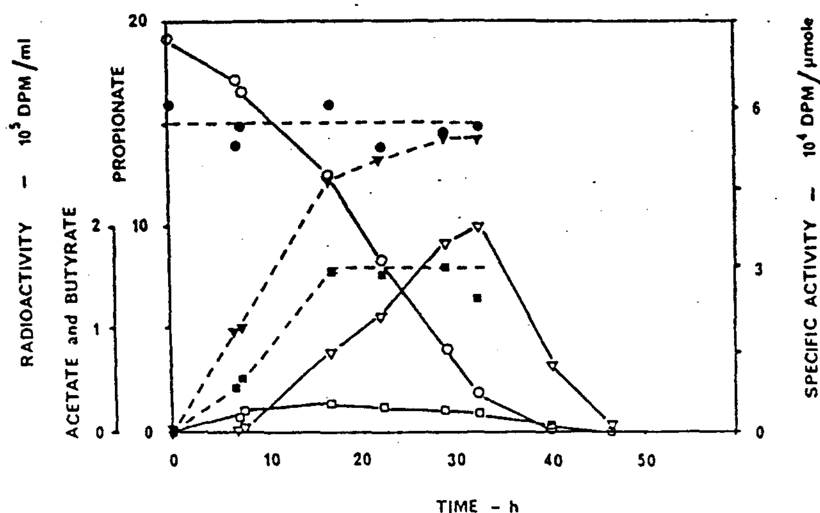

FIG. 3. Butyrate (▽) and acetate (□) labeling during degradation of propionate in the presence of [1-^{14}C]propionate (○). The specific radioactivities of propionate (●), butyrate (▼), and acetate (■) are shown.

sodium [1-^{13}C]propionate (32 mM), [2-^{13}C]butyrate and [2-^{13}C]acetate were formed (Fig. 4a). This demonstrated a carboxylation on the carboxyl group of propionate. The NMR recordings showed also an important peak of [^{13}C]carbonate in the medium, which resulted from the decarboxylation of propionate to acetate. After 8 h of incubation in the presence of [2-^{13}C]propionate (32 mM), [3-^{13}C]butyrate and a small amount of [3-^{13}C]propionate (10% of the propionate labeling) were detected (Fig. 4b). This exchange of propionate labeling increased to 30% after 32 h (Table 1), and a peak of [4-^{13}C]butyrate was observed. Both samples also contained [^{13}C]carbonate, which resulted from acetogenesis of [2-^{13}C]propionate and further splitting of the formed [1-^{13}C]acetate by methanogens. [2-^{13}C]acetate resulting from the β oxidation of the [4-^{13}C]butyrate was also observed. The low acetate concentration in the medium and the low sensitivity of the NMR method, even with 99% enriched initial products and long recording times, explain why the labeling of the carboxyl group of acetate remained unresolved from the background signal. After 32 h of incubation in the presence of [3-^{13}C]propionate, [4-^{13}C]butyrate was detected and an exchange of labeling in propionate was observed (Table 1). This exchange was much lower than that obtained with [2-^{13}C]propionate and represented only 10% of the labeling of this acid.

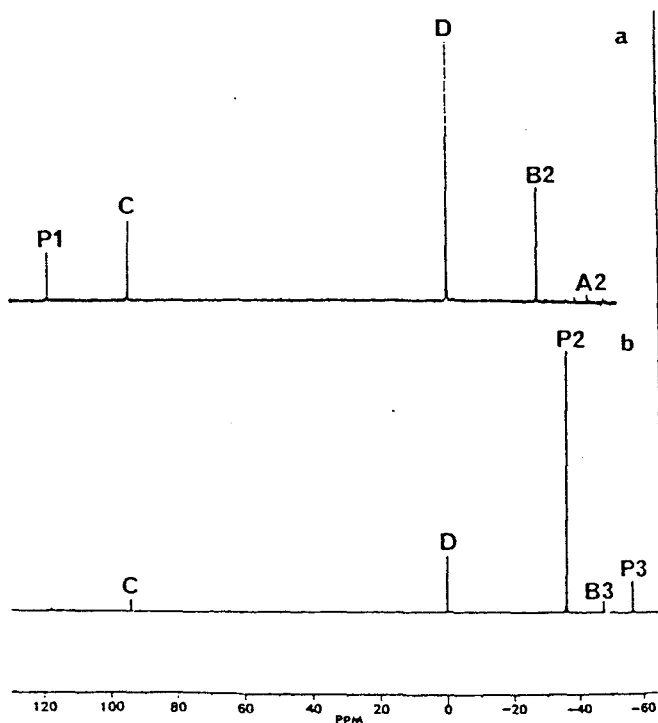

FIG. 4. NMR recordings of anaerobic sludge supernatants during the degradation of [1-^{13}C]propionate after 32 h of incubation (a) and of [2-^{13}C]propionate after 8 h of incubation (b). Peaks: P1, [1-^{13}C]propionate; C, carbonate; D, dioxane; B2, [2-^{13}C]butyrate; P2, [2-^{13}C]propionate; A2, [2-^{13}C]acetate; B3, [3-^{13}C]butyrate; and P3, [3-^{13}C]propionate.

DISCUSSION

The results presented here clearly demonstrate the existence of a butyric fermentation of propionate: the accumulation of butyrate was proportional to the initial concentration of propionate, and the degradation of [1-^{14}C]propionate gave [^{14}C]butyrate with the same specific radioactivity. Since NMR studies with [1-^{13}C]propionate showed no exchange between the carboxyl group and the other carbon atoms of propionate, all the radioactivity found in butyrate must have arisen from the carboxyl group of [1-^{14}C]propionate. These results strongly suggest that butyrate is synthesized by an elongation of the carbon chain of propionate.

Two pathways of propionate synthesis, the acrylate and succinate pathways, have been described; anaerobic degradation of propionate is less well documented. It has been demonstrated that in the propionate-oxidizing sulfate reducer *Desulfobulbus propionicus*, the formation and probably the degradation of propionate proceed via the succinate pathway (17). The degradation of [2-^{14}C]- and [3-^{14}C]propionate by methanogenic propionate enrichment led to the formation of acetate with the carboxyl and the methyl groups equally labeled (11). This result suggested that propionate is degraded through a randomizing pathway, and recent NMR studies have confirmed this suggestion by demonstrating the

TABLE 1. Labeling positions of propionate and butyrate during batch degradation of ^{13}C-labeled sodium propionate[a]

Initial labeling of sodium propionate	Time of sampling (h)	Propionate degraded (%)	Fractional distribution of ^{13}C atoms in:			
			Propionate		Butyrate	
			2-^{13}C	3-^{13}C	3-^{13}C	4-^{13}C
2-^{13}C	8	43	0.90	0.10	1.00	0.00
	32	81	0.70	0.30	0.90	0.10
3-^{13}C	8	35	0.05	0.95	—[b]	—
	32	76	0.10	0.90	0.00	1.00

[a] No [1-^{13}C]butyrate, [1-^{13}C]propionate, or [2-^{13}C]butyrate was seen during these experiments.
[b] —, The butyrate concentration in the medium 8 h after the addition of [3-^{13}C]sodium propionate was too low to be observed.

formation of [2-^{13}C]propionate and succinate from [3-^{13}C]propionate in a dense suspension of a propionate-utilizing methanogenic coculture (9). The exchange between C-2 and C-3 of propionate observed in our NMR experiments indicates that propionate oxidation to acetate may proceed in sludge via the succinate pathway. However, methanogenic sludges contain non-propionate-degrading fermentative bacteria which are able to catalyze an exchange between the carboxyl group of propionate and CO_2 (2). This exchange reaction probably arises from the reversible formation of succinate from propionate and thus implies an exchange between C-2 and C-3 of propionate. Therefore, the exchange observed in our sludge samples does not necessarily mean that propionate is degraded through a randomizing pathway.

Until now, the only described pathway of butyrate formation in bacteria has been the condensation of two molecules of acetyl coenzyme A (acetyl-CoA) into acetoacetyl-CoA, with subsequent reduction to crotonyl-CoA and butyryl-CoA. If such a pathway is involved in propionate conversion, [1-^{13}C]- and [1-^{14}C]propionate will be fermented into unlabeled butyrate. Ferredoxin-dependent carboxylation of succinyl-CoA and propionyl-CoA, leading, respectively, to the formation of 2-oxoglutarate (5) and 2-oxobutyrate (4), have been described for photosynthetic microorganisms and for anaerobes (8, 18); therefore, two hypothetical pathways can be proposed. The first pathway would involve the reduction and activation of 2-oxoglutarate to glutaconyl-CoA, the decarboxylation of this compound to crotonyl-CoA as observed for *Pseudomonas fluorescens*, and the reduction of crotonyl-CoA to butyryl-CoA and butyrate (7). However, in this hypothesis, the [2-^{13}C]propionate would give [4-^{13}C]butyrate or a mixture of [4-^{13}C]- and [3-^{13}C]butyrate. Because the skeleton of the propionate molecule is conserved, 2-oxoglutarate and thus succinyl-CoA are not intermediates in the formation of butyrate. In the second hypothesis, the 2-oxobutyrate, formed by carboxylation of propionate after its activation to propionyl-CoA, is reduced to 2-hydroxybutyrate, with a further dehydration to crotonate. To our knowledge such reactions have never been observed in bacteria, but a 2-oxo-carboxylate reductase activity has been detected in proteolytic *Clostridia* spp. (8). By analogy with the acrylate pathway, the 2-hydroxybutyrate could be activated to its CoA ester and then lead by dehydration to the formation of crotonyl-CoA. This second hypothesis, which needs to be confirmed by enzymatic studies, appears more likely to be correct, since the propionate molecular skeleton is conserved as observed in NMR experiments. The presence of small amounts of [4-^{13}C]- and [3-^{13}C]butyrate formed from [2-^{13}C]- and [3-^{13}C]propionate, respectively, may be due to the exchange between C-2 and C-3 of propionate during acetogenesis.

The reductive carboxylation of propionate to butyrate is a reaction that accepts six electrons, which is highly exergonic under the standard conditions (Table 2, reaction 2) and which can allow the growth of a microorganism with hydrogen as the electron donor if the partial pressure of hydrogen is not too low. However, in our experiment, the oxidation of propionate to acetate, which is only feasible at a very low hydrogen concentration (Table 2, reaction 1), is the only electron-donating reaction, and the hypothesis of an interspecies hydrogen transfer between an obligate proton-reducing acetogen and a hydrogen-utilizing, propionate-reducing bacterium has to be excluded for thermodynamic reasons.

The butyric fermentation of propionate must thus be regarded as a dismutation of propionate carried out by a single microorganism. If this dismutation was complete, an acetate/butyrate ratio of 1 would be obtained and the presence of obligate proton-reducing acetogenic bacteria such as *S. wolinii* in sludge would explain the higher ratio observed in our experiments. The free energy change of this reaction

TABLE 2. Influence of hydrogen partial pressure on the free energy change of hypothetical redox reactions for butyric fermentation of propionate

Reaction no. and reaction	$\Delta G^{\circ\prime}$ (kJ/reaction)[a] at P_{H_2} of:		
	1	3.55×10^{-5}	10^{-5}
1 Propionate + $3H_2O \rightarrow$ acetate + HCO_3^- + $3H_2$ + H^+	+76.1	0	−9.4
2 Propionate + HCO_3^- + H^+ + $3H_2 \rightarrow$ butyrate + $3H_2O$	−76.1	0	+9.4
3 Propionate $\rightarrow$ 0.5 acetate + 0.5 butyrate	0	0	0
4 Propionate + $1.8H_2O \rightarrow$ 0.8 acetate + 0.2 butyrate + 0.6 HCO_3^- + $1.8H_2$ + $0.6H^+$	+45.8	0	−5.5
5 Propionate + HCO_3^- + $H_2 \rightarrow$ 2 acetate + H_2O	−28.1	−3.1	−0.2
6 Propionate + 0.5 $HCO_3^- \rightarrow$ 1.75 acetate + $0.25H^+$	−2.9	−2.9	−2.9

[a] Calculated by pH 7 and at 25°C from the data of Thauer et al. (18) with each component at 1 M.

(Table 2, reaction 3) is zero under the standard conditions and is independent of the hydrogen concentration. Bacterial growth associated with this fermentation thus appears unlikely and would be thermodynamically possible only in the presence of a high concentration difference between propionate and its fermentation products.

The butyric fermentation of propionate coupled with acetate and hydrogen production (Table 2, reaction 4) is energetically possible only at a low hydrogen concentration. However, under these conditions, propionate degradation to acetate and hydrogen (Table 2, reaction 1) is more exergonic, and butyrate formation does not present a thermodynamic advantage which could explain the physiological significance of this hypothetical fermentation.

One may also assume that a single microorganism could grow by propionate conversion to acetate via a reductive carboxylation pathway. In this hypothesis, crotonyl-CoA, which is likely to be an intermediate in the synthesis of butyrate from propionate, could be hydrated to 3-hydroxybutyryl-CoA and, after subsequent oxidation to acetoacetyl-CoA, could lead to the formation of two acetyl-CoA molecules. One mole of propionate and one mole of CO_2 could thus be converted into two moles of acetate (Table 2, reaction 5). This electron-consuming reaction must be coupled with the oxidative decarboxylation of propionate (Table 2, reaction 6). In this scheme, the butyrate is not an obligate intermediate, and the reason for its excretion into the medium is unclear.

None of the last three hypotheses proposed here can be definitively excluded, and further studies are required to fully understand this new pathway of anaerobic propionate degradation. On the other hand, the sludges used in this study were obtained from a continuously fed anaerobic digestor in which the concentration of volatile fatty acids is maintained at a very low level (20). Since the conversion of propionate to butyrate was observed in batch cultures with a high initial substrate concentration, it is not possible to determine whether this new pathway of propionate degradation plays an important role in anaerobic digestors.

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