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Oxidation of Carbon Monoxide by *Pseudomonas carboxydohydrogena*

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*Pseudomonas carboxydohydrogena*에 의한 일산화탄소의 산화

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ABSTRACT

The stoichiometry between the consumption of CO and O_2 and the production of CO_2 ($2CO + O_2 \rightarrow 2CO_2$) showed that *Pseudomonas carboxydohydrogena* grows as a typical aerobic CO oxidizer with CO. The optimal concentration of CO for growth was found to be 30% in gas mixture with air. The initial buffer concentration of the culture medium did not affect the growth of this bacterium. *P. carboxydohydrogena* is an obligate aerobe and does not use nitrate as a terminal electron acceptor. The CO dehydrogenase is an inducible and soluble enzyme. The reaction rate and stability were maximal at pH 7.5, and the Arrhenius plot revealed an activation energy of 37.7 kJ/mol (9.0 kcal/mol). The crude enzyme used methylene blue, thionin, and toluylene blue as electron acceptors for the oxidation of CO to CO_2 under anaerobic conditions. It was found that water must be the source of the second oxygen atom for CO oxidation.

INTRODUCTION

Pseudomonas (*Seliberia*) *carboxydohydrogena* is a carboxydobacterium which is able to grow aerobically with carbon monoxide(CO) as the sole carbon and energy source (Hegeman, 1981; Zavarzin and Nozhevnikova, 1977). It was first isolated by Sanzhieva and Zavarzin (1971). The organism was polymorphic and its small, rod-shaped cells were often seen combined in stellate aggregations, on the basis of which and of its ability to oxidize not only hydrogen but also CO, they named it *Seliberia carboxydohydrgoena*. However, Meyer *et al.* (1980) proposed that *S. carboxydohydrogena* be transferred from the genus *Seliberia* to the genus *Pseudomonas* as *Pseudomonas carboxydohydrogena* on the basis of morphological study, its strictly aerobic metabolism, and some other traits tested.

CO was oxidized by water to CO_2 before assimilation (Kim and Hegeman, 1981a; Romanova *et al.*, 1977) in this bacterium and the key reaction of the Calvin cycle was detected in extracts of cells from this organism, namely, the carboxylation of ribulose-1, 5-diphosphate (Nozhevnikova and Saval'eva, 1972; Zavarzin

and Nozhevnikova, 1977). The carbon monoxide dehydrogenase (CO-DH) which is responsible for the oxidation of CO in this bacterium consists of at least three nonidentical subunits and contains noncovalently bound flavin adenine dinucleotide (Kim and Hegeman, 1981a). Cytochromes of the a, b, and c type and functional terminal oxidase(s) were found in cells grown on CO (Kim and Hegeman, 1981b) and increased production of respiratory chain components and induction of CO-insensitive terminal oxidases may be responsible for detoxification of CO in this organism (Cypionka and Meyer, 1982).

The investigations reported herein for *P. carboxydohydrogena* were intended to test general properties of cell growth on CO, inducibility and localization of the CO-DH, and factors affecting the CO-DH activity. Furthermore, we tried in this study to give a direct evidence that water is the source of the second oxygen atom in the oxidation of CO to CO_2.

MATERIALS AND METHODS

Organism and standard culture conditions

P. carboxydohydrogena (DSM 1083, Z-1062) which was obtained from J. Schmidt (University of Arizona, Tempe) was grown CO-autotrophically in mineral medium under the conditions described by Kim and Hegeman (1981a). Cells were harvested in midexponential growth phase, washed once in 0.05 M Tris-hydrochloride buffer (pH 7.5, standard buffer), and stored at −20°C (Kim and Hegeman, 1981a).

CO and O_2 consumption

To measure CO and O_2 uptake and CO_2 production by autotrophically growing *P. carboxydohydrogena*, 5 standard mineral medium (S MM) (Doudoroff, 1940) agar plates were inoculated heavily and placed into a serum stoppered desiccator. The gas mixture of 40% CO (C.P., 99.5% min., Matheson) and 60% air in the desiccator was established by using a manometer, and krypton (research purity, 99.995% min., Matheson) gas was introduced to a final concentration of 5% in the gas mixture as an inert reference gas. Cells were grown at 30°C. Samples of gas (0.2 m*l* each) were taken using a gas tight syringe (Pressure-lock, Precision Sampling Corp.) and injected into a Hewlett-Packard Gas Chromatograph 5710A (temperature: oven, 45°C for 8 min, then increased to 200°C by temperature programming; injection port, 100°C; thermal conductivity detector, 100°C; filament current, 168 mA; 1/8 in. × 6 ft. stainless steel column filled with Spherocarb [80/100 mesh, Analabs]). The carrier gas was helium, with flow-rate of 30 m*l* per min.

Optimal concentration of CO for cell growth

Small-size, liquid culture system was used to determine the optimal CO centration for cell growth at 30°C. Three 250 m*l* flasks which contained 100 m*l* of SMM and a magnetic bar were inoculated with *P. carboxydohydrogena* to initial turbidities of around 10 Klett units. These flasks were placed into three different desiccators, and atmospheres of 20%, 30%, and 40% CO were introduced into respective desiccators. Each flask was stirred by a magnetic stirrer at 30°C.

Effect of buffer concentration on growth

The same growth conditions were used as in the small-size, liquid culture method except that the CO concentration in the gas phase was 30%. The initial concentration of Na_2HPO_4-KH_2PO_4 (pH 6.8) added to the medium was 0.005 M and 0.1 M, respectively.

Test for the presence of anaerobic metabolism of CO

Cells were grown with CO in SMM liquid medium containing 0.2% KNO_3. A butyl rubber-stoppered side-arm flask (125 m*l*) containing the medium was inoculated with cells and was

flushed aseptically with CO at least for 20 min. Cells were incubated at 30°C by shaking with a Gyrotory shaker (New Brunswick Scientific Co.) at 200 rpm.

The presence or absence of nitrate reductase in this organism was then tested. Cells were grown with 0.2% sodium succinate as substrate under the same conditions as those of the preceeding observation, except that the medium was flushed with argon gas (prepurified, 99.998% min., Metheson) instead of CO for 20 min.

Inducibility of CO dehydrogenase

Autotrophically grown cells with CO for 2 weeks, heterotrophically grown cells with 0.2% sodium succinate for 2 weeks, and cells first grown with 0.2% sodium succinate for 5 days followed by growth under CO atmosphere for 2 weeks were tested for their oxygen consumption with CO as substrate. The rate of oxygen uptake by whole cells were measured at 30°C using a YSI model 53 biological oxygen monitor.

Preparation of cell extracts

The thawed cells grown on CO were resuspended in cold standard buffer and disrupted by sonic oscillation as described by Kim and Hegeman (1981a).

The suspension was centrifuged for 30 min at 10,000 × g. The supernatant was referred to as crude extract. The crude extract was then sedimented for 90 min at 100,000 × g. The resulting supernatant fluid and precitate were referred to as soluble and particulate fraction, respectively (Kim and Hegeman, 1981a).

Standard enzyme assay

CO dehydrogenase was assayed by measuring the CO-dependent decrease of absorbance of thionin dye at 30°C using anaerobic silica cuvettes and a Beckman DU spectrophotometer (Kim and Hegeman, 1981a).

Reduction of artificial electron acceptors

The standard enzyme assay method was used to determine the range of electron acceptors and to select the best acceptor for CO oxidation with crude cell extracts.

Localization of CO dehydrogenase

Crude extracts, soluble fractions, and particulate fractions (washed once with the standard buffer) from CO-autotrophically grown cells were tested for the intracellular localization of CO-DH by the standard assay method.

Protein determination

Protein was determined by modified biuret reaction (Gornall *et al.*, 1949) from a standard curve prepared previously using bovine serum albumin (Sigma) standards. The protein contents of suspensions of whole cells and crude extracts were measured by the same method after boiling the samples in 20% NaOH for 10 min (Meyer and Schlegel, 1978).

CO_2 evolution under anaerobic conditions

Anaerobic reaction conditions were established by use of Hungate tubes. The reaction mixture contained 5 m*l* of standard buffer containing 20 μM thionin and 0.5 m*l* of soluble fraction (1.0 mg protein per m*l*). After flushing with CO for at least 6 min, 10 μl of 25 mM sodium hydrosulfite solution was injected into the reaction mixture. The tube was shaken at 100 rpm for 1 hour at 30°C. 1.0 m*l* of 8.5% H_3PO_4 was then added to the mixture and 0.5 m*l* of gas sample was drawn from each tube for gas chromatography. The gas chromatograph running conditions were the same as described before except for using a column temperature of 100°C and filament current of 215 mA.

RESULTS AND DISCUSSION

CO and O_2 consumption by growing cells

As shown in Fig. 1 the stoichiometry between CO consumption and CO_2 production is exactly 1 : 1. However, the stoichiometry between CO and O_2 uptake shows that CO uptake

is more than double that of O_2; i.e., the overall stoichiometry does not follow the proposed overall equation for carboxydobacterial CO oxidation: $2CO + O_2 \rightarrow 2CO_2$ (Kistner, 1953; Sanzhieva and Zavarzin, 1971).

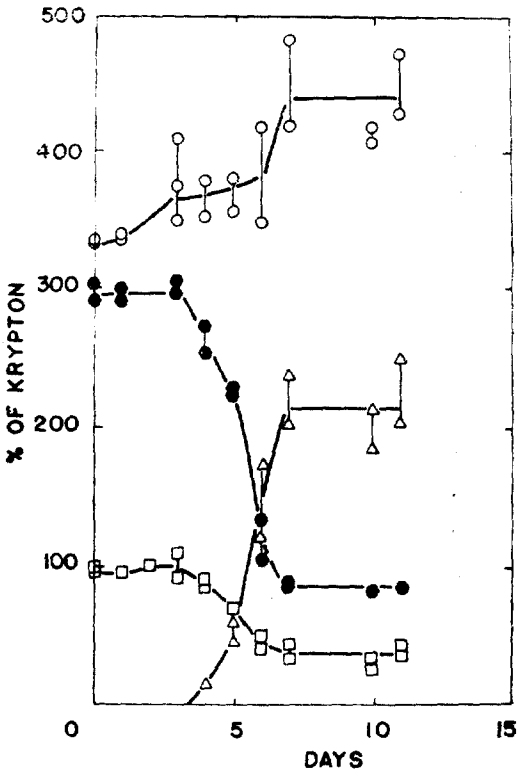

Fig. 1. CO and O_2 consumption by autotrophically growing cultures of *P. carboxydohydrogena*. Symbols: nitrogen (—○—), oxygen(—□—), carbon monoxide(—●—), and carbon dioxide (—△—).

But if we note that the increase in the relative volume of N_2 in the gas phase, this anomaly can be easily resolved. The desiccator used for this test was wrapped with Parafilm to prevent leakage, but an inward leakage occurred nevertheless during cell growth probably because the consumption of CO and O_2 resulted in a lower pressure in the desiccator.

If one corrects the amount of O_2 consumed by the factor of air input to the desiccator during growth, it can be seen that the overall stoichiometry of gas uptake and production in growing cultures of *P. carboxydohydrogena* at the expense of CO follows the proposed equation, indicating that this bacterium grows as a typical aerobic CO oxidizer.

Optimal concentration of CO for growth

As shown in Fig. 2 the best doubling time and final cell yield in liquid media were obtained with 30% CO in gas mixture with air among those concentrations tested. This concentration is higher than that (20%) of Zavarzin and Nozhevnikova (1977), which may be due to different culture conditions. When cells were grown under a higher concentration of CO, the growth rate was slower than that with 30% of CO. This indicates that CO is still inhibitory for cell growth even in carboxydobacteria as previously noted (Zavarzin and Nozhevnikova, 1977).

As *P. carboxydohydrogena* grew optimally with 30% CO in the air mixture, subsequent cultivation of this bacterium was carried out with this concentration.

Growth rate

When *P. carboxydohydrogena* was grown under the standard culture conditions, the doubling time was found to be 25 hours with a cell yield of 5 g wet weight per liter after 2 weeks

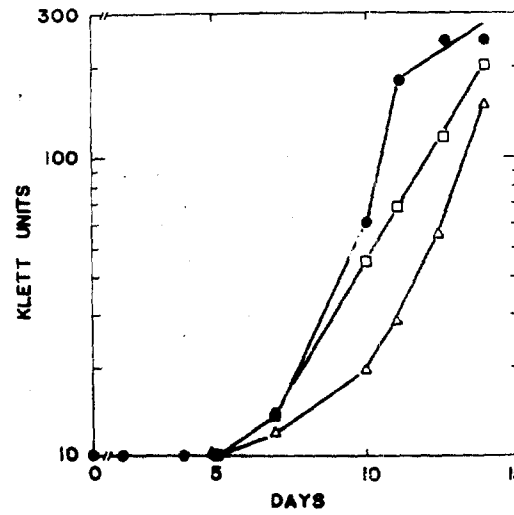

Fig. 2. Effect of CO concentration in air on the cell growth. Symbols: 20% (—□—), 30% (—●—), and 40%(—△—) of CO in gas mixture with air.

of cultivation from a small inoculum (data not shown).

Effect of buffer concentration on cell growth

When *P. carboxydohydrogena* was grown with two different initial concentrations of phosphate buffer, cells grown under poorly-buffered conditions (0.005 M) did not show any difference in growth rate and cell yield compared with cells grown in well-buffered medium until 10 days, but the pH of the culture medium dropped from 6.75 to 3.3, although there was almost no change in pH in well-buffered medium (data not shown).

The drop in pH in poorly-buffered culture medium was of interest. As CO-DH could reduce thionin with CO as substrate under anaerobic conditions (Table 2), water was thought to be the donor of the second oxygen atom for CO oxidation. Even though free formate was not considered to be an intermediate during CO oxidation (Kim and Hegeman. 1981a) it was thought that the complex CO-DH must cause the CO to become a formate-equivalent (e.g., a formyl group) on the surface of the enzyme and then be released by hydrolysis as formic acid (or similar acid) and be secreted into the medium under poorly-buffered conditions. Several attempts to separate and to identify the possible acid in the culture medium were unsuccessful. It was therefore concluded that the change in pH was brought about solely by the assimilation into cellular material of ammonia from ammonium sulfate added to the medium as a nitrogen source, leaving behind sulfuric acid (data not shown).

Absence of anaerobic metabolism of CO

There was no increase in turbidity when *P. carboxydohydrogena* was cultured at the expense of CO under anaerobic conditions with nitrate as terminal electron acceptor (data not shown). Furthermore, it was found that this bacterium could not grow with sodium succinate under the same culture conditions, indicating that *P. carboxydohydrogena* does not use nitrate as a terminal electron acceptor under anaerobic conditions with CO or succinate as substrate and that this bacterium is an obligate aerobe as previously reported (Meyer *et al.*, 1980).

Table 1. Localization of the CO-DH in cells of *P. carboxydohydrogena* grown with CO[a]

Fractions	Specific activity[b]
Crude extracts	30.3
Soluble fractions	33.8
Particulate fractions	<0.00303

[a]Enzyme activity in each fraction was measured using standard assay method.
[b]μmol thionin reduced per mg protein·min

CO dehydrogenase is inducible

Cells grown heterotrophically with sodium succinate did not take up any measurable amount of O_2 with CO as substrate (data not shown). When those heterotrophically grown cells were transferred to CO gas phase and grown for 2 weeks, they showed CO-stimulated O_2 uptake. Cells grown autotrophically with CO exhibited greater O_2 consumption than the hetero-autotrophically grown cells. These results are in accord with other report (Meyer *et al.*, 1980) that the CO oxidizing activity was measurable only in cells grown with CO; i.e., the CO-DH is an inducible enzyme.

Localization of enzyme activity

The CO dehydrogenase was found to be completely soluble and none of the CO-DH activity was detectable in particulate fractions (Table 1). This supports those previous reports that CO-DH in cells of carboxydobacteria is soluble enzyme (Cypionka *et al.*, 1980; Meyer *et al.*, 1980; Meyer and Schlegel, 1979)

Electron acceptors

When various electron acceptors were tested to determine the range of possible electron acceptors and to select the best reacting acceptor for CO oxidation with crude extracts from

Table 2. CO-dependent reduction of electron acceptors by crude extracts of *P. carboxydohydrogena*

Electron acceptors	Conc.	E'₀(mV)	nm measured	CO oxidation rate (% of rate with thionin)[a]
Methyl viologen	2.5 mM	−440	605	<0.001
NAD (NADP)	5.0 mM	−320 (−324)	340	<0.005
FAD (FMN)	20 μM	−219	450	<0.005
Methylene blue	20 μM	+ 11	610	73
Thionin	20 μM	+ 64	595	100
Toluylene bule	30 μM	+110	650	15
DCPIP[b]	40 μM	+217	610	<0.001
Ferricyanide	40 μM	+360	410	<0.005

[a]Reduction rate (CO oxidation rate) of each acceptor was measured photometrically; activity with thionin dye was set as 100%.
[b]2,6-dichlorophenol-indophenol.

P. carboxydohydrogena, acceptors with a negative potential (methyl viologen, NAD, NADP, FAD, and FMN) were not reduced by the CO-DH. However, acceptors with a positive potential (methylene blue, thionin, and toluylene blue) between + 10 mV and + 100 mV were reduced by the enzyme when CO was used as substrate (Table 2). This was also true in experiments with the purified enzyme, supporting the notion that ubiquinone is aphysiological electron acceptor during CO oxidation (Cypionka *et al.*, 1980; Kim and Hegeman, 1981a and 1981b).

As the reduction rates were highest with thionin and the reduction of this dye was completely dependent both on added CO and CO-DH, this dye was used for routine enzyme activity determination in the photometric assay.

pH dependence

Between the two buffer systems tested, the CO-DH is the most active at pH 7.5 in 0.05 M Tris-HCl (Fig. 3). The pH activity curve shows no inflection point and is quite different from that reported for CO oxidation catalyzed by extracts of *Clostridium pasteurianum* (Thauer *et al.*, 1974) or *Methanobacterium thermoautotrophicum* (Daniels *et al.*, 1977). The curve for *P. carboxydohydrogena*, however, is similar to that of another carboxydobacterium, *P. carboxydovorans*, which shows no inflection point and gives maximal methylene blue reduction rates with CO in the range of pH 6.5 to 7.2 in several different buffer systems (Meyer and Schlegel, 1979).

The CO-DH of *P. carboxydohydrogena* was also found to be most stable at pH 7.5 in 0.05 M Tris-HCl at 4°C (data not shown). This coincidence in pH values for maximum activity and for stability simplified selection of

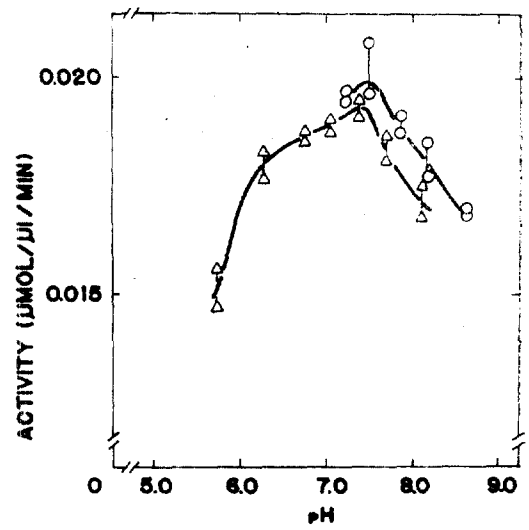

Fig. 3. Effect of pH on the CO dehydrogenase activity. The optimal pH for the enzyme activity was determined by the standard assay method using various pH buffers of 0.05 M Tris-HCl (—○—) and 0.05 M phosphate (—△—).

the standard buffer for enzyme assay and for cell extracts preparation.

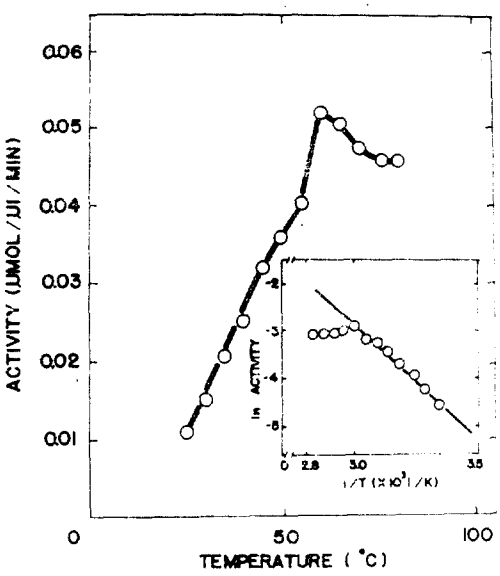

Fig. 4. Effect of temperature on the CO dehydrogenase activity. Temperature dependence of the enzyme activity was tested at various temperatures employing the standard enzyme assay. Activation energy = 37.7kJ/mol (9.0 kcal/mol).

Temperature dependence

The maximum CO-DH activity occurred at 60°C and the temperature dependence of the reaction rate followed the Arrhenius equation with an activation energy of 37.7 kJ/mol (9.0 kcal/mol) (Fig. 4). These optimal temperature and activation energy values are comparable to those of *P. carboxydovorans* (Meyer and Schlegel, 1979), but the activation energy is smaller than that of *M. thermoautotrophicum* (Daniels *et al.*, 1977).

Even though the enzyme was most active at 60°C, this temperature has not been used for routine enzyme assay since almost 50% of the initial activity was lost in 50 min at this temperature (data not shown). The enzyme was quite stable at 30°C and this temperature was used for the standard assay.

Mechanism of CO oxidation

During an experiment that measured evolution of CO_2 under anaerobic reaction conditions, the volume of CO_2 evolved in the reaction mixture was double that of the control under the given experimental conditions (Table 3). This result, together with the fact that we used a completely anaerobic system to measure the CO-DH activity, implying that the second oxygen for the oxidation of CO does not come from molecular oxygen but must come from water as in the case of other carboxydobacteria (Cypionka *et al.*, 1980; Meyer and Schlegel, 1979 and 1980) and the clostridia (Drake *et al.*, 1980).

Table 3. CO_2 evolution under anaerobic conditions[a]

Reaction system	Thionin[b]	CO[c]	Enzyme[d]	CO_2 peak area[e]	Relative CO_2(%)[f]
Control 1	−	+	−	17.0	100
Control 2	+	−	+	17.0	100
Experimental	+	+	+	34.4	202

[a] CO_2 formed under anaerobic conditions was measured using gas chromatograph.
[b] + : 20 μM thionin in standard buffer flushed with N_2. − : no thionin in standard buffer flushed with N_2.
[c] + : flushed with CO. − : flushed with N_2.
[d] + : 0.5ml of soluble fraction(1.0mg protein/ml) flushed with N_2 was added. − : no enzyme added.
[e] CO_2 peak area = peak height × retention time.
[f] % of CO_2 peak area of control; control value was set as 100%.

To confirm this result, the stable isotope experiment with ^{12}CO and $H_2{}^{18}O$ was carried out under the same anaerobic experimental conditions, but there was no significant increase in m/e ratio of 46/44 compared with control and the observed ratio was far below the anticipated value (data not shown), as was true for Kirkconnell and Hegeman (1978). Certain problems in the experimental system including the GC/MS analysis may be the reason or the isotope effect in this system is much greater than we think.

ACKNOWLEDGMENT

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적 요

소비된 일산화탄소(CO)및 산소(O_2)와 발생된 이산화탄소(CO_2) 사이의 양적인 상관관계 ($2CO + O_2 \longrightarrow 2CO_2$)는 *Pseudomonas carboxydohydrogena*가 CO를 이용하여 전형적인 호기성 CO 산화세균으로 성장함을 나타냈다. 이 세균은 30%의 CO 와 70%의 공기의 혼합기체 속에서 가장 잘 자랐으며, 배지속의 완충액의 최초 농도는 이 세균의 성장에 영향을 미치지 않았다. *P. carboxydohydrogena*는 절대호기성균이며 nitrate를 전자전달계의 말단전자수용체의 하나로 사용하지 않는다. 가용성이며 유도효소인 CO dehydrogenase는 pH 7.5에서 반응속도와 안정성이 가장 크고, Arrhenius plot에 의하면 효소의 활성에너지는 37.7 kJ/mol (9.0 kcal/mol)로 나타났다. 순화되지않은 효소는 methylene blue, thionin, 그리고 toluylene blue 등을 무기성 조건 하에서 CO를 CO_2로 산화시키기 위한 전자수용체로 사용하였으며, CO의 산화에 필요한 두번째 산소의 공급원은 물로 밝혀졌다.

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