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CONVERSION OF D-ARABINOSE TO D-ERYTHROASCORBIC ACID AND OXALIC ACID IN *SCLEROTINIA SCLEROTIORUM*

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SUMMARY: D-*glycero*-Pent-2-enono-1,4-lactone (trivial name: D-erythroascorbic acid) occurs in the phytopathogen, *Sclerotinia sclerotiorum* (Lib.) de Bary, where it has a potential role as precursor of oxalic acid. On Glc/yeast/malt medium, *S. sclerotiorum* produces only nominal amounts of D-erythroascorbic acid but even partial replacement of Glc by D-Ara increases production of erythroascorbic acid and oxalic acid. Use of D-[1-^{14}C]-, -[3-^{14}C]-, or -[6-^{14}C]Glc and D-[5-^{3}H]-, -[2-^{14}C,5-^{3}H]-, or -[UL-^{14}C]Ara provide additional information on erythroascorbic acid biosynthesis and cleavage. The latter process resembles that obtained by peroxygenation of erythroascorbic acid in alkaline solution. An unknown erythroascorbic acid-like compound also occurs in both Glc- and Ara-based cultures.

In certain phytopathogenic species of fungi, OxA accelerates plant tissue maceration through synergism with polygalacturonase (1-5). The possibility that OxA biosynthesis in these organisms is analogous to that of L-ascorbic acid-derived OxA formation in plants (6-7) arose from the discovery that AA is rare or absent in yeasts and fungi but an analog, EAA (D-*glycero*-pent-2-enono-1,4-lactone) is present (8-14). Treatment in alkaline hydrogen peroxide cleaves AA to OxA and L-threonic acid (15-17). AA and EAA have identical ring structures. Murakawa et al. (8) found EAA in *Candida* species and proposed a pathway: D-Ara → D-arabinono-1,5,-lactone → D-arabinono-1,4-lactone → EAA. The final enzymic step in this process has been characterized (13). The possibility that D-Ara and its oxidation product, EAA, might be involved in OxA biosynthesis prompted this study in *Sclerotinia sclerotiorum*.

MATERIALS AND METHODS

Reagent grade chemicals were used throughout. AA was recrystallized from water (1g ml^{-1}) with a 10-fold excess of glacial acetic acid. EAA was provided by Professor P.A. Seib (18). Stock solutions of AA and EAA (2 mg ml^{-1}) in 5 mM DTT remained stable at –20°C. Working standards (200 ng ml^{-1}) were prepared daily. A stock solution of OxA (20 mg ml^{-1}) was

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The abbreviations used are: AA, L-ascorbic acid; Ara, D-arabinose; DTT, dithiothreitol; EAA, D-erythroascorbic acid; OxA, oxalic acid; Ukn, unknown erythroascorbic acid-like compound.

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prepared from freshly sublimed crystals and stored at –20°C. Working standards (20-200 μg ml^{-1}) were prepared daily.

Source of organism and cultural conditions. *Sclerotinia sclerotiorum* (Lib.) de Bary (ATCC #18687) *(=Whetzelinia sclerotiorum)* (19) was maintained on YM agar (Difco #0712-01-8) at 4°C and transferred to YM broth (Difco #0711-01) for experiments. Mycelial mats were grown on agar for 2 weeks. Aerobic cultures were grown at 23°C from 7 mm agar disks in floating rafts of microporous polypropylene (Sigma Chem Co, St Louis, MO) on 100 ml of media. In experiments involving radiolabeled substrates, respired $^{14}CO_2$ was recovered on filter paper (2 cm x 8 cm) saturated with 0.6 ml of 1 M KOH. Mycelium covered the raft surface in 4 days and produced sclerotia within 5 days when grown on 1% Glc. Replacement of Glc with 1% Ara slowed growth slightly. Droplets of exudate accumulated during sclerotial development (20).

Harvest and extraction. Mycelial mats were drained on filter paper, weighed and lyophillized. Spent media from the raft and the outer vessel were recovered separately and stored at –20°C. Droplets associated with sclerotia were collected by micropipet, pooled, and stored at –70°C. Lyophillized mycelium was pulverized in liquid N_2 and taken up in 2 mM DTT (3 ml g^{-1}). Following extraction, samples were centrifuged at 10,000g for 10 min. Supernatant (1 ml) was applied to a column of 2 ml of Dowex 50 H^+ exchange resin and eluted with sufficient distilled water to recover 5 ml. Eluate was filter-centrifuged (0.45 μm) and analyzed for EAA and OxA by HPLC. Aliquots were loaded on tandem columns of Dowex-50 H^+/Dowex-1-formate exchange resin (10 x 0.9 cm), flushed through with water, and then the anionic column was eluted with a linear gradient of 250 ml water/500 ml 0.1 M formic acid which partially resolved an unknown EAA-like compound and glyceric acid, and fully resolved EAA. Each was characterized by HPLC. Radiolabeled EAA was recovered by dilution with authentic unlabeled EAA and recrystallized from hot acetonitrile (21).

HPLC of erythroascorbic acid and oxalic acid. EAA was separated on a HPX-87 (250 x 4.6 mm) organic acid column, (Bio-Rad Labs, Richmond CA) with 2 mM H_2SO_4 at 0.7 ml min^{-1}, 23°C. An alternative procedure utilized a PLRP-S (250 x 4.6 mm) polymeric, reverse phase, C_{18} column, (Polymer Labs, Amherst MA) with 0.3% metaphosphoric acid and 1.8% tetrahydrofuran in water at 0.5 ml min^{-1}, 23°C (22). Detection was either amperometric at 0.7 V (BioAnalytical Systems Inc, West Lafayette, IN) or spectrophotometric at 254 nm. OxA was separated on an IC-Pak anion column (50 x 4.6 mm, Waters, Milford, MA) 1.2 ml min^{-1}, 23°C with eluant B as described by Jones et al. (23).

Strategies used to compare carbon sources for EAA and OxA production. Three strategies were undertaken to compare Glc and Ara as carbon sources for production of EAA, the Ukn, and OxA. ***First strategy***: Duplicate rafts containing 4-day-old, glucose-grown, mycelial mats were drained of media and then resupplied with YM broth containing 1% Glc, 0.5% Glc + 0.5% Ara, or 1% Ara. During the initial 4 day growth and, again, during the subsequent 6 day growth on substituted media, the pH of the media decreased from 6 to 4. After 6 days on the new broth, the mycelial mat from one raft was drained of excess media and stored at –70°C. The other mat was lyophillized and stored at –20°C. Both were analyzed for EAA , the Ukn, and oxalate. Although both conditions of storage gave comparable results, the lyophilized mat was chosen for illustration since it permitted separation of sclerotia from mycelial tissue. ***Second strategy***: Three sets of raft-grown *S. sclerotiorum* were initiated from Glc-, Glc + Ara-, or Ara-based agar cultures and then grown 4 days on the same carbon source at 23°C, a period sufficient to allow the fungal mat to fill the raft but insufficient for sclerotial development. Mats were harvested, lyophillized, and analyzed as described above. ***Third strategy***: Two sets of raft cultures were grown from Glc-based and Ara-based YM agar. Within each set, comparisons were made among carbon sources composed of 1% Glc, 0.5% Glc + 0.5% Ara, 0.5% Glc + 0.5% D-arabinono-1,4-lactone, 1% Ara, 0.5% Ara + 0.5% D-arabinono-1,4-lactone, or 1% D-arabinono-1,4-lactone. Cultures were held four days at 23°C and then separated into mycelia and spent media. The mats were lyophilized and stored at –20°C prior to extraction and analysis.

Radioactive substrates and analysis. D-[1-^{14}C]-, -[3-^{14}C]-, -[6-^{14}C]-, and -[UL-^{14}C]Glc (New England Nuclear Corp, Boston MA) were repurified by thin layer chromatography prior to use. D-[5-^{3}H]Ara was purchased from ICN Biochem., Irving, CA. D-[2-^{14}C]-, and -[UL-^{14}C]-Ara were prepared from D-[3-^{14}C]- and -[UL-^{14}C]Glc, respectively, by oxidation of the latter sugar with Glc oxidase plus catalase (Sigma), hydrolysis of the resultant D-gluconolactone to D-gluconic acid, chromatography on anionic exchange resin and further oxidation with cerium(IV) sulfate (24).

Labeling procedure. Five rafts each bearing a 7 mm disk of *S. sclerotiorum* from Glc/YM agar were floated on 25 mL of sterile YM broth containing 1% Glc and grown for 4 days at 23°C. The initial pH of 6.2 decreased to 4 during this period. Spent media in each vessel (16-17 ml) was removed (leaving original media that had been drawn into the raft undisturbed) and 15 ml of fresh, sterile 1% Glc/YM broth containing a selected, sterile-filtered radiolabeled sugar (Table I) was added to each vessel. Aliquots were removed and analyzed for radioactivity. The filter paper $^{14}CO_2$ trap was charged with KOH at this time. Cultures were grown four more days, each day removing an aliquot of media to follow uptake of radioactivity. At the end of the growth period, exudate droplets were collected from each mat and stored at –70°C. Mycelial mats were removed, weighed, frozen, lyophilized, and stored at –20°C. Spent media within the raft was removed and stored separately from that collected from the vessel, both at –20°C. After extraction of mats with 2 mM DTT, insoluble residues were analyzed for ^{14}C and ^{3}H by combustion in a sample oxidizer (Model 206, Packard Instr. Co.) using ^{14}C- and ^{3}H-methyl methacrylate standards for comparison. To obtain precise values of ^{14}C content in OxA, aliquots from each sample were diluted with 50 mg of OxA as carrier and precipitated as calcium Ox by treatment with calcium acetate. OxA was regenerated, converted into its dicyclohexylamine salt (25) and recrystallized from water / methanol / ethyl acetate (1:7:7, v/v/v) to constant specific radioactivity.

RESULTS AND DISCUSSION

Peroxygenation of EAA. In 0.1 M CAPS buffer at pH 10, 30 °C, 37 mM H_2O_2 cleaved 0.75 mM EAA to yield an equivalent amount of OxA within 2 h. Increasing EAA to 6 mM delayed stoichiometric release of OxA to 12 h and higher concentrations of EAA were incompletely oxidized due to increasing acidity of the reaction mixture. OxA production from EAA was similar to alkaline peroxygenation of AA in which the products were OxA and L-threonic acid (16).

Raft culture of *S. sclerotiorum*. A mycelial mat covered the raft surface in 4 days at 23°C and drops of clear exudate appeared. One to two days later, dense, black sclerotia (3-7 mm dia) formed in exudatic regions. Exudate, sclerotia, mat, and spent medium were harvested for analysis of EAA, OxA and the Ukn.

In Glc/YM broth, *S. sclerotiorum* secreted over 80 mg of OxA g^{-1} dry wt of cells. EAA (approximately 10 μg g^{-1} dry wt) and the Ukn (100-200 μg g^{-1} dry weight) were resolved on the organic acid HPLC column. The Ukn had a R_{AA} = 0.87 as compared to R_{AA} = 1.10 for EAA (where R_{AA} = 1).

Ara was a better carbon source than Glc for EAA biosynthesis and OxA formation. In Ara/YM broth *S. sclerotiorum* cultures grew slightly slower and reached sclerotial development in 6-7 days. To compare Glc and Ara as carbon sources for OxA production, three strategies were employed: In the first, Glc/YM broth was replaced after 4 days with broth containing Glc, Glc + Ara, or Ara. Mats of Glc-grown *S. sclerotiorum* (dry weight 0.8±0.1 g) were approximately 1.5 times greater than Ara-grown tissue; the Glc + Ara-grown mats were intermediate. Most of the

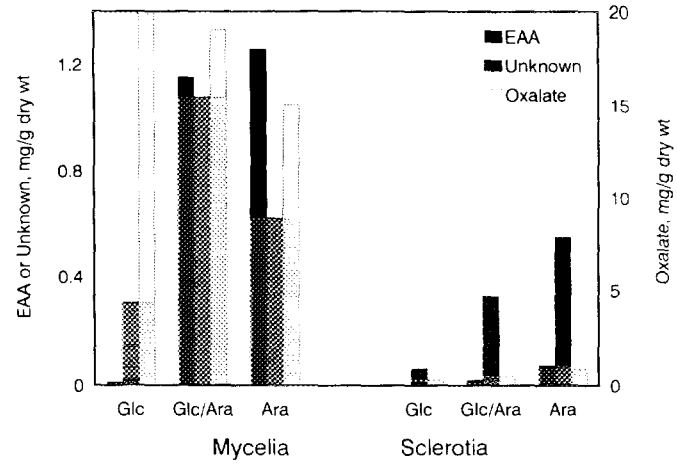

Fig. 1. Accumulation of EAA, the Ukn, and OxA in raft-cultured *S. sclerotiorum*. Fungus was first grown for 4 days on 1% Glc/YM media and then resupplied with 1% Glc, 0.5% Glc + 0.5% Ara, or 1% Ara for 6 days. The bar graph compares amounts which were recovered in soluble extracts of mycelia or sclerotia.

OxA (87±9 mg raft^{-1}) was recovered in spent broth (Fig. 1). EAA and the Ukn were present in Glc-grown mycelia; replacement of Glc by Ara, even partly, enhanced production of both. Sclerotia from Ara-grown cultures contained the Ukn but very little EAA. EAA (77 ng ml^{-1} of

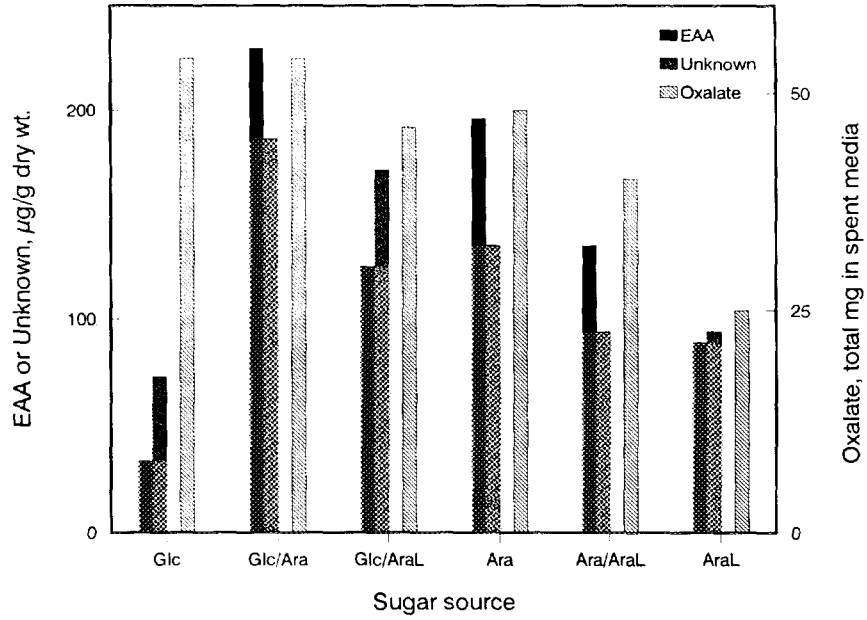

Fig. 2. Accumulation of EAA, the Ukn, and OxA (isolated striped bar) in raft-cultured *S. sclerotiorum*. Fungus was grown for 4 days on designated carbon sources in YM medium. OxA refers to total OxA in spent medium. Carbohydrate sources were 1% Glc, 0.5% Glc + 0.5% Ara, 0.5% Glc + 0.5% D-arabinonolactone, 1% Ara, 0.5% Ara + 0.5% D-arabinonolactone, or 1% D-arabinonolactone.

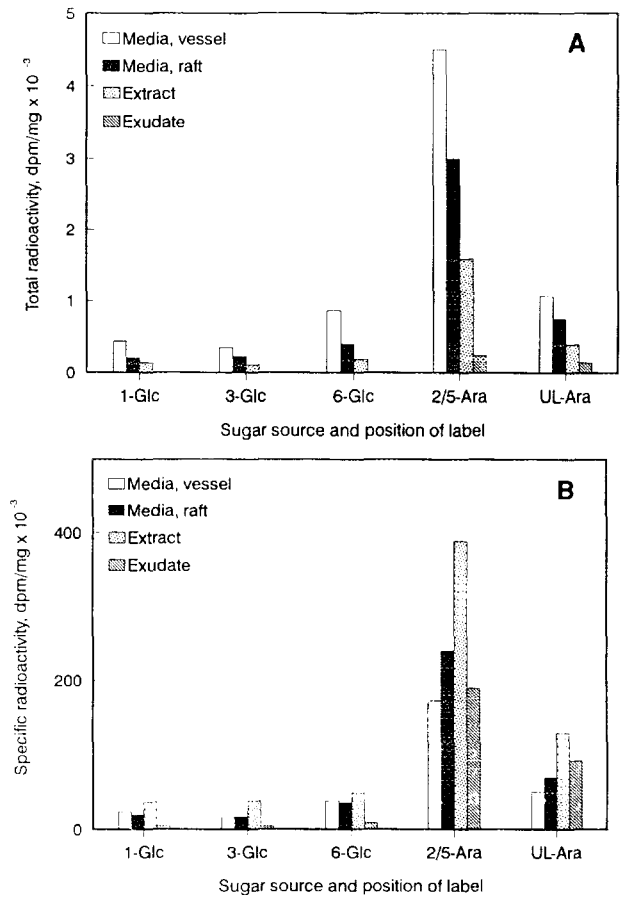

Fig. 3. Recovery of [^{14}C]OxA from raft-cultured *S. sclerotiorum* after 4 days of growth in media containing labeled Glc and/or Ara. Data are presented for [^{14}C]OxA in spent media (vessel or raft), soluble extract, and exudate which collectively accounted for <97% of the OxA produced. Panel A: Total radioactivity. Panel B: Specific radioactivity.

exudate) was found in exudate collected from sclerotial regions of Ara-grown mats but was undetectable in Glc-grown mats.

To avoid sclerotial development, a second stratagy involved 4-day-old mycelial mats which were grown from agar inoculum containing the same carbon sources used in the final broth culture. Over 97% of the OxA was secreted into the medium. EAA and the Ukn were present solely within mycelium. Ara-grown cultures produced 1.4±0.2 mg of EAA g^{-1} dry weight of mycelium whereas Glc-grown mycelium yielded less than 0.04 mg g^{-1}. Ara-grown cultures doubled OxA production over Glc-grown cultures on a per weight basis. The Ukn accumulated in Ara cultures to about twice that found with Glc.

In the third strategy, mycelia grown on Glc, Glc + Ara, or Glc + arabinono-1,4-lactone produced comparable masses of mycelia (237±28 mg raft^{-1}); those grown on Ara and/or arabinono-1,4-lactone produced 94±15 mg raft^{-1}. EAA production was very low (2.2 µg raft^{-1}) when Glc-based medium was used (Fig. 2). Only OxA from spent media is reported in Fig. 2 since OxA recovered from soluble extract and mycelia were only 1.8±0.8 and, 0.3±0.1 mg

Table 1. Forms and radioactivities of sugars used in radiolabeled experiments*

Radiolabeled sugar(s) used	Abbreviation used to designate labeled sugar used	Total radioactivity added (μCi)	Specific radioactivity after addition (μCi mmol^{-1})
D-[1-^{14}C]Glc	1-Glc	8.6	118
D-[3-^{14}C]Glc	3-Glc	8.3	119
D-[6-^{14}C]Glc	6-Glc	10.1	120
D-[2-^{14}C]Ara + D-[5-^{3}H]Ara	2/5-Ara	8.5	1,710
		20.0	7x10^{6}
D-[UL-^{14}C]Ara	UL-Ara	10.7	582
D-[5-^{3}H]Ara	5-Ara (Exper. A)	25	117x10^{3}
D-[5-^{3}H]Ara	5-Ara (Exper. B)	25	11.7x10^{3}

* Lyophilized fungal mats from each labeled experiment had similar dry weights (207±16 mg raft^{-1}). Spent media recovered in raft (7.4±0.4 ml raft^{-1}) and outer vessel (14.8±0.3 ml vessel^{-1}) were comparable among the five ^{14}C experiments.

raft^{-1}, respectively. Cultures grown in Ara and/or D-arabinono-1,4-lactone produced the most EAA.

Experiments with radiolabeled substrates. Table 1 summarizes forms and amounts of radioactivity used. For 1-, 3-, or 6-Glc, the OxA content of spent medium (vessel), spent medium (raft), soluble mycelial extract, and exudate was 21.3±2.1, 11.9±1.4, 3.5±0.5, and 0.7±0.16 mg raft^{-1}, respectively. For 2/5- and UL-Ara, the corresponding values were 23.7±3.4, 11.6±1.2, 3.6±0.6 and 1.4±0.1 mg raft^{-1}, respectively. Total and specific radioactivities in OxA from spent media (vessel + raft), mycelial extract, and exudate are summarized in Fig. 3. In Glc-labeled cultures, dilution by the medium reduced the amount of label appearing in OxA. When 2/5-Ara was supplied, 50% of the ^{14}C was recovered as OxA from media, mycelial extract, and exudate. Additional [^{14}C]OxA or its salts probably remained in the insoluble residue which contained 9% of the ^{14}C supplied. The specific radioactivity of OxA from 2/5-Ara revealed a pattern of OxA biosynthesis and transfer to be expected if OxA formed in the mycelium, then secreted into raft medium, and diffused into spent medium in the vessel. Exudate from sclerotial regions of mycelia contained OxA with a specific radioactivity approximating that in the soluble mycelial extract. The same observation applied to the UL-Ara experiment where total and specific radioactivities of OxA were lower since only two of five labeled carbons in UL-Ara appeared in OxA; moreover, the specific activity of UL-Ara supplied to the culture was lower.

Fractionation of soluble mycelial extract from 0.1% 5-Ara-grown *S. sclerotiorum* (Exper. A, Table 1) on Dowex 1 formate resin revealed that ^{3}H remained undisturbed through steps leading to EAA and its C_3 cleavage product, D-glyceric acid (Fig. 4). When pooled fractions from the three major peaks were analyzed with amperometric detection, the Ukn eluted at 200 ml, followed at 260 ml by glyceric acid and at 400 ml by EAA. The latter (88 μg) contained 74,000 dpm, about 8% of the ^{3}H in the extract. The identity of the labeled EAA was confirmed by dilution with authentic EAA, and recrystalization from acetonitrile to yield EAA accounting for 85% of the ^{3}H under the EAA peak. Comparable results were obtained in Exper. B (Table 1). In the 2/5-Ara experiment, the ^{3}H/^{14}C ratio was 2.5. This increased to 9 in the region of the elution profile corresponding to glyceric acid (refer to Fig. 4) as one might expect since glyceric acid retained

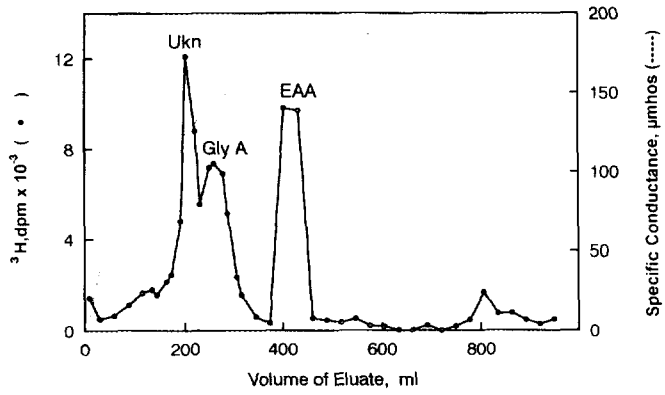

Fig. 4. HPLC elution profile of an extract from mycelia of raft-cultured *S. sclerotiorum* after 4 days of growth on 0.1% Ara/YM media containing D-[5-^{3}H]Ara. Upon completion of the water / 0.1 M formic acid gradient (250/500 ml), 1 M formic acid was added to the column. Three major peaks were detected, in order of elution, the Ukn partially merged with glyceric acid, and then EAA. The change in specific conductance of the eluate during the initial 600 ml of gradient is indicated by (----).

only ^{3}H. In the UL-Ara experiment, 0.14 mg of EAA (9.6 µCi mmol^{-1}) and 3.2 mg of OxA (5.3 µCi mmol^{-1}) were recovered from the soluble extract, quantities and specific radioactivities indicative of a path of conversion from Ara through EAA to OxA. Whether the Ukn is a byproduct or intermediate on the pathway to EAA is undetermined but its properties resemble EAA, possibly an open chain form of EAA or a 5-carbon analog of D-glucosone which functions as a precursor of AA in higher plants (7, 26).

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