L-Ascorbic acid

BOEHRINGER MANNHEIM / R-BIOPHARM
Enzymatic BioAnalysis / Food Analysis

Colorimetric method

for the determination of L-ascorbic acid in foodstuffs and other materials

Determination of iso-ascorbic acid: see pt. 3 and 13

For *in vitro* use only

Store at 2-8°C

Cat. No.10 409 677 035

Test-Combination for 21 determinations

For recommendations for methods see references (2)

Principle (Ref. 1)

L-Ascorbic acid (L-ascorbate) and some more reducing substances (x-H_2) reduce the tetrazolium salt MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] in the presence of the electron carrier PMS (5-methylphenazinium methosulfate) at pH 3.5 to a formazan. In the assay with the sample the sum of the reducing substances is measured (1).

(1) L-Ascorbate (x-H_2) + MTT $\xrightarrow{PMS}$ dehydroascorbat (x) + MTT-formazan + H^+

For the specific determination of L-ascorbate, in a sample blank assay only the L-ascorbate fraction as part of all reducing substances present in the sample is oxidatively removed by ascorbate oxidase (AAO) in the presence of oxygen from the air (2). The dehydroascorbate formed does not react with MTT/PMS.

(2) L-Ascorbate +½ O_2 $\xrightarrow{AAO}$ dehydroascorbate + H_2O

The absorbance difference between the sample and the absorbance difference of the sample blank is equivalent to the quantity of L-ascorbate in the sample. The MTT-formazan is the measuring parameter and is determined by means of its light absorbance in the visible range at 578 nm.

The Test-Combination contains

1. Bottle 1 with approx. 43 ml solution, consisting of: sodium phosphate/citrate buffer, pH approx. 3.5; MTT
2. Tube 2 containing approx. 20 ascorbate oxidase spatulas, approx. 17 U AAO each
3. Bottle 3 containing approx. 4 ml PMS solution

Preparation of solutions

1. Use the contents of bottle 1 undiluted.
2. Use the spatula from tube 2 unchanged.
3. Use the contents of bottle 3 undiluted.

Stability of reagents

Solution 1 is stable at 2-8°C stored in the dark (see pack label).
Bring solution 1 to 37°C before use.
The contents of tube 2 are stable at 20-25°C (see pack label).
Solution 3 is stable at 2-8°C stored in the dark (see pack label; a slightly red color of the solution does not influence accuracy of the result).

Procedure

Wavelength: (Hg) 578 nm
Glass cuvette[1]: 1.00 cm light path
Temperature: 37°C
Final volume: 2.700 ml
Read against air (without a cuvette in the light path), against water or against sample blank[2]
Sample solution: 0.5-20 µg L-ascorbic acid/assay[3] (in 0.100-1.600 ml sample volume, see also instructions)

For each sample a sample blank is to be carried out.

Pipette into cuvettes	Sample blank	Sample
solution 1 (warmed up to 37°C)	1.000 ml	1.000 ml
redist. water	1.500 ml	1.500 ml
sample solution*	0.100 ml	0.100 ml
tube 2 (AAO spatula)**	1 spatula	-
Mix**** and incubate for 6 min at 37°C. While incubating mix the contents of the sample blank assay every 2 min for about 5 s with the spatula (see pt. 2.4.). After removing the ascorbate oxidase spatula**, read the absorbances of the sample blank and sample (A_1). Start the reaction by the addition of:		
solution 3***	0.100 ml	0.100 ml
Mix**** and allow the solutions to stand for 15 min at 37°C. Read the absorbances of sample blank and sample immediately one after another (A_2); see pt. 2.5 and pt. 2.6.		

* Rinse the enzyme pipette or the pipette tip of the piston pipette with sample solution before dispensing the sample solution.
** Use the spatula only once.
*** The reaction system is sensitive to light after addition of solution 3 (MTT). The cuvettes should therefore not stand in the light.
**** For example, with a plastic spatula or by gentle swirling after closing the cuvette with Parafilm (trademark of the American Can Company, Greenwich, Ct., USA)

Determine the absorbance differences (A_2-A_1) for both, sample and sample blank. Subtract the absorbance difference of the sample blank from the absorbance difference of the sample.

$$\Delta A = (A_2-A_1)_{sample} - (A_2-A_1)_{sample\ blank}$$

The measured absorbance differences should, as a rule, be at least 0.100 absorbance units to achieve sufficiently precise results (see "Instructions for performance of assay" and "Sensitivity and detection limit", pt.4).

Calculation

The calculation is carried out with the aid of the extinction coefficient of MTT-formazan.

According to the general equation for calculating the concentration:

$$c = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times \Delta A\ [g/l]$$

V = final volume [ml]
v = sample volume [ml]
MW = molecular weight of the substance to be assayed [g/mol]
d = light path [cm]
ε = extinction coefficient for MTT-formazan at 578 nm
= 16.9 [l x $mmol^{-1} \times cm^{-1}$]

It follows for L-ascorbic acid:

$$c = \frac{2{,}700 \times 176{,}13}{16{,}9 \times 1{,}00 \times 0{,}100 \times 1000} \times \Delta A = 0.2814 \times \Delta A$$

[g L-ascorbic acid/l sample solution]

If the sample has been diluted during preparation, the result must be multiplied by the dilution factor F.

When analyzing solid and semi-solid samples which are weighed out for sample preparation, the result is to be calculated from the amount weighed:

$$Content_{L\text{-ascorbic acid}} = \frac{c_{L\text{-ascorbic acid}}\ [g/l\ sample\ solution]}{weight_{sample}\ in\ g/l\ sample\ solution} \times 100\ [g/100\ g]$$

1 If desired, disposable cuvettes may be used instead of glass cuvettes.
2 For example, when using a double-beam photometer
3 See instructions for performance of assay
4 If water is used the determination has to be performed immediately. For stabilization dilute sample, if necessary, with meta-phosphoric acid (e.g. from E. Merck, Darmstadt; Cat. No. 546), 1.5% (w/v), adjusted to pH 3.5-4.0 with KOH (10 M).

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1. Instructions for performance of assay

The amount of L-ascorbic acid present in the assay has to be between 0.5 µg and 20 µg. In order to get a sufficient absorbance difference, the sample solution is diluted to yield a concentration of L-ascorbic acid between 0.03 and 0.20 g/l.

Dilution table

Estimated amount of L-ascorbic acid per liter	Dilution with water[4]	Dilution factor F
< 0,20 g	-	1
0,20-2,0 g	1 + 9	10
2,0-20 g	1 + 99	100
> 20 g	1 + 999	1000

If the measured absorbance difference (ΔA) is too low (e.g. < 0.100), the sample solution should be prepared again (weigh out more sample or dilute less strongly) *or* the sample volume to be pipetted into the cuvette can be increased up to 1.600 ml (adjust sample solution to pH 3.5-4.0!). The volume of water added must then be reduced to obtain the same final volume in the assays for sample and sample blank. The new sample volume v must be taken into account in the calculation.

2. Technical information

2.1 In order to guarantee the stability of solution 1 (buffer/MTT), it is recommended that only the amount immediately needed be taken from bottle 1 and warmed up to 37°C.

2.2 To economize on pipetting during serial measurements, a mix of reagents can be prepared from solution 1 and water in a ratio of 2 parts solution 1 and 3 parts water.

2.3 Incubation with AAO at 37°C can take place:
a) in the temperable cuvette holder of the photometer
b) in a water-bath - using a cuvette holder or aluminium block
c) if necessary, also in a thermal cupboard.
During incubation the spatula remains inside the cuvette.

2.4 In the sample blank assay, one must "sufficiently" stir with the ascorbate oxidase spatula. Air must be mixed in and the ascorbate oxidase washed out of the spatula. If it is not sufficiently stirred, the results obtained will be false, i.e. too low.

2.5 After addition of PMS (solution 3), the reaction system is sensitive to light (daylight and artificial light). The following incubation (15 min at 37°C) **must** take place in the dark.
a) If incubating in the photometer, close cuvette space of the photometer and block out light.
b) Cover the cuvettes in the water-bath (Alu-block).
c) Store in a darkened thermal cupboard.
Light causes an uncontrolled "creep reaction". When the sample cuvette and the sample blank cuvette are exposed to light for the same amount of time, the sample and blank reactions run parallel, so that by successive measurement of the absorbances A_2 a creep extrapolation is unnecessary.

2.6 Should creep reactions appear despite light exclusion, then these are caused by the sample. As the sample and sample blank cuvettes contain the same sample volume, the creep reactions are identical. In this case an extrapolation may be neglected. Care must be taken, however, that the absorbances A_2 are read successively one after another.

Important note
Normally, in the analysis of a L-ascorbate assay control solution an absorbance difference $(A_2-A_1)_{\text{sample blank}} < 0.020$ absorbance units is an indication for:
a) good storage conditions for solution 3 (PMS) in the dark. The solution has not been exposed to direct sun or artificial light when taken out of the bottle.
b) sufficient mixing with the AAO spatula (see pt. 2.4).
c) Exclusion of light from the cuvettes after the addition of solution 3 (PMS)(see pt. 2.5).

2.7 An aqueous L-ascorbic acid solution, e.g. for assay control, is extremely unstable. Therefore, it is recommended to dissolve the L-ascorbic acid with meta-phosphoric acid (1.5%, w/v; pH adjusted to 3.5-4.0 with KOH, approx. 10 M). Do not use an oxalate solution for stabilization; the ascorbate oxidase is inhibited by oxalate.

2.8 Water can be used to dilute the sample solution only, however, when the measurement takes place immediately after the dilution. The dilution with meta-phosphoric acid (see pt. 2.7.) is less problematic. In this case, a stabilization of the L-ascorbic acid is attained; furthermore a pH adjustment of the sample to 3.5-4.0 may not be necessary.

2.9 In carrying out the calculation, a clear indication should be given as to whether the results are to be given as L-ascorbic acid (molar mass 176.13 g/mol) or as L-ascorbate (molar mass 175.12 g/mol). (In enzymatic determinations, the L-ascorbate ion is measured.)

2.10 L-Ascorbyl palmitate cannot be measured with the enzymatic method because free L-ascorbic acid is sponaneously destroyed under the alkaline reaction conditions which are necessary for hydrolysis.

3. Specificity (Ref. 1)

Under the stated assay conditions this method is specific for L-ascorbic acid. Only D-arabo-ascorbic acid (iso-ascorbic acid) is determined simultaneously (see pt. 13) with reduced speed.

Results of 94 to 100% will be achieved in the analysis of freshly produced L-ascorbic acid when air and moisture are excluded during storage. (The "quality" of the results depends on the age of the solid L-ascorbic acid, the solvent used for the production of solutions (meta-phosphoric acid, 1.5% (w/v); pH 3.5-4), the storage conditions for L-ascorbic acid and the solutions, as well as the working conditions in the analytical determination. (One has to pay attention especially to the sufficient mixing with the AAO spatula (see pt. 2.4) and the exclusion of light after the addition of PMS (see pt. 2.5); the light absorbance difference for the sample blank should be small (e.g. < 0.020)).

When interpreting data from recovery experiments, the low stability of L-ascorbic acid solutions, especially in the presence of heavy metal ions e.g. of iron and copper and of oxygen, has to be considered. (Results of less than 100% may not indicate an inclomplete conversion of L-ascorbic acid in the determination; they may result from the loss of L-ascorbic acid in sample and sample solution.)

Ascorbyl palmitate does not react. The determination of ascorbate after saponification makes no sense because ascorbate is destroyed under the conditions of alkaline hydrolysis.

4. Sensitivity and detection limit (Ref. 1.3)

The smallest differentiating absorbance for the procedure is 0.005 absorbance units. This corresponds to a maximum sample volume v = 1.600 ml of an L-ascorbic acid concentration of approx. 0.1 mg/l sample solution (if v = 0.100 ml, this corresponds to 1.4 mg/l sample solution).

The detection limit of approx. 0.3 mg/l is derived from the absorbance difference of 0.015 and a maximum sample volume v = 1.600 ml).

5. Linearity

Linearity of the determination exists from 0.5 µg L-ascorbic acid/assay (0.3 mg L-ascorbic acid/l sample solution; sample volume v =1.600 ml) to 20 µg L-ascorbic acid/assay (0.2 g L-ascorbic acid/l sample solution; sample volume v = 0.100 ml).

6. Precision

In a double determination using one sample solution, a difference of 0.005 to 0.010 absorbance units may occur. With a sample volume of v = 0.100 ml this corresponds to an L-ascorbic acid concentration of approx. 1.5-3 mg/l. (If the sample is diluted during sample preparation, the result has to be multiplied by the dilution factor F. If the sample is weighed in for sample preparation, e.g. using 1 g sample/100 ml = 10 g/l, a difference of 0.015-0.03 g/100 g can be expected.)

The following data have been published in the literature:

x = 0,059 g/l	CV = 2,4 %	n = 15	in series
x = 0,192 g/l	CV = 1,8 %	n = 15	in series
x = 0,380 g/l	CV = 1,2 %	n = 15	in series
x = 0,059 g/l	CV = 3,8 %	n = 15	from day to day
x = 0,192 g/l	CV = 2,2 %	n = 15	from day to day
x = 0,380 g/l	CV = 2,0 %	n = 15	from day to day (Ref. 1.1)

7. Interference/sources of error

Sugars usually found in foodstuffs do not interfere with the assay up to a quantity of 30 mg each per assay.

Of the sugar alcohols, only D-sorbitol interferes, at a quantity above 20 mg/assay by inhibition of the ascorbate oxidase.

Similarly, alcohols at high concentrations inhibit the ascorbate oxidase (e.g. > 100 mg ethanol/assay). By prolonging the reaction time of the incubation with AAO to 10 min, most interferences can be excluded.

High amounts of sulfur dioxide (> 50 µg SO_2/assay) react with the MTT and PMS and may be the reason for a creep reaction. If the presence of SO_2 is suspected, it is recommended to remove it as stated under pt. 11 "Determination of L-ascorbic acid in wine".

Metal ions are adequately complexed in the assay system. Higher concentrations (>100 µg/assay) may, however, inhibit the ascorbate oxidase.

Whilst nitrite ions do not interfere with the assay, their presence leads to spontaneous decomposition of the L-ascorbic acid.

Oxalate ions already inhibit the ascorbate oxidase markedly at a concentration of 30 µg/assay. Higher oxalate concentrations should be removed from the sample with a slight excess of Ca-ions in a weakly acid medium (pH 5-6).

8. Recognizing interference during the assay procedure

8.1 If the conversion of L-ascorbic acid has been completed according to the time given under "Procedure", it can be concluded in general that no interference has occurred.

8.2 On completion of the reaction, the determination can be restarted by adding L-ascorbic acid (qualitative or quantitative): if the absorbance is altered subsequent to the addition of the standard material, this is also an indication that no interference has occurred.

8.3 Operator error or interference of the determination through the presence of substances contained in the sample can be recognized by carrying out a double determination using two different sample volumes (e.g. 0.100 ml and 0.200 ml): the measured differences in absorbance should be proportional to the sample volumes used.

When analyzing solid samples, it is recommended that different quantities (e.g. 1 g and 2 g) be weighed into 100 ml volumetric flasks. The absorbance differences measured and the weights of sample used should be proportional for identical sample volumes.

8.4 Possible interference caused by substances contained in the sample can be recognized by using an internal standard as a control: in addition to the sample, blank and standard determinations, a further determination should be carried out with sample **and** assay control solution in the same assay. The recovery can then be calculated from the absorbance differences measured.

8.5 Possible losses during the determination can be recognized by carrying out recovery tests: the sample should be prepared and analyzed with and without added standard material. The additive should be recovered quantitatively within the error range of the method.

9. Reagent hazard

The reagents used in the determination of L-ascorbic acid are not hazardous materials in the sense of the Hazardous Substances Regulations, the Chemicals Law or EC Regulation 67/548/EEC and subsequent alteration, supplementation and adaptation guidelines. However, the general safety measures that apply to all chemical substances should be adhered to.

After use, the reagents can be disposed of with laboraty waste, but local regulations must always be observed. Packaging material can be disposed of in waste destined for recycling.

10. General information on sample preparation

In carrying out the assay:
Use **clear, colorless or colored liquid samples** directly, or after dilution according to the dilution table, and of a volume up to 1.600 ml;
Filter **turbid solutions**;
Degas **samples containing carbon dioxide** (e.g. by filtration);
Treat **"strongly colored" samples** that are used undiluted or with a higher sample volume with polyvinylpolypyrrolidone (PVPP) - (e.g. 1 g/100 ml);
Crush or homogenize **solid or semi-solid samples,** extract with meta-phosphoric acid[4] or dissolve in meta-phosphoric acid[4] and filter if necessary;
Deproteinize **samples containing protein** with meta-phosphoric acid (15% w/v), adjust the to 3.5-4 with potassium hydroxide (2 M), and dilute with water to a meta-phosphoric acid concentration of 1.5% (w/v);
Extract **samples containing fat** with an aqueous solution of meta-phosphoric acid[4] (extraction temperature should be above the melting point of the fat involved). Cool to allow the fat to separate, make up to the mark, place the volumetric flask in a ice bath for 15 min and filter.

Important note
The Carrez-clarification cannot be used in the sample preparation for the L-ascorbic acid determination due to a too low recovery rate.

11. Application examples

Determination of L-ascorbic acid in fruit juices, vegetable juices and beverages
Use clear, light-colored juices for the assay after adjustment to pH 3.5-4.0 and after dilution with water or with meta-phosphoric acid[4] according to the L-ascorbic acid content (see dilution table) (see pt. 2.8.).

Decolorize dark juices with 1% polyvinylpolypyrrolidone (PVPP) (e.g. 10 ml juice, pH 3.5-4.0, + approx. 100 mg PVPP), stir for 1 min and filter. Use the generally clear solution for the assay. Decolorization can often be omitted on dilution.

Filter turbid juices.

Determination of L-ascorbic acid in alcoholic beverages

Wine
Adjust wine to pH 3.5-4.0 and dilute with water or meta-phosphoric acid[4] to a suitable L-ascorbic acid concentration (see dilution table). Red wine does not have to be decolorized.

Wine which contains sulfur dioxide (SO_2) has to be treated with formaldehyde as follows:
Mix 10 ml wine with 1 drop of formaldehyde solution (approx. 5%, w/v), mix and allow to stand for 5 min at 20-25°C. Adjust to pH 3.5-4.0 and dilute, if necessary (see dilution table), and use the solution for the assay.

If a higher sample volume is used (0.200-0.500 ml) the spatula (AAO) should be left in the sample blank assay mixture for 10 min.

Beer
Stir approx. 10 ml beer with a glass rod or filter to remove the carbon dioxide. Adjust the sample to pH 3.5-4.0.

Determination of L-ascorbic acid in milk
Adjust 100 ml milk to pH 3.5-4.0 by addition of approx. 1 g citric acid (monohydrate). Filter and discard the first part of the filtrate. Use the slightly opalescent solution (up to v = 0.500 ml) immediately for the assay.

Determination of L-ascorbic acid in meat products
Homogenize approx. 4 g sample which has previously been minced in a mixer (3 times for 20 s, followed by waiting time of 40 s) with 10ml meta-phosphoric acid (15%, w/v) in a homogenizer for 1 min. (The metal parts of the apparatus should be cleaned before use with acid as iron ions can interfere with the assay). Adjust the pH of the mixture with potassium hydroxide solution (2 M) to pH 3.5-4.0 and transfer quantitatively to a 100 ml volumetric flask. Rinse the apparatus with water and add to the flask. Fill up to the mark and mix. To remove the fat, keep the volumetric flask for 15 min at 2-8°C, then filter or centrifuge (about 3000-5000 rpm). Use the supernatant solution, diluted if necessary, for the assay.

Determination of L-ascorbic acid in potatoes
Mince approx. 50 g potatoes with 50 ml meta-phosphoric acid (15% w/v) and 0.1 ml n-octanol with a household mixer (approx. 1 min).

Adjust to pH 3.5-4.0 with potassium hydroxide solution (2 M). Transfer the mixture quantitatively to a 500 ml volumetric flask with water, fill up to the mark and mix. Filter and use 0.500 ml of the sample solution for the assay.

Incubate the sample blank assay for 15 min with the AAO spatula. Take the altered sample volume into account in the calculation.

Determination of L-ascorbic acid in flour
Accurately weigh approx. 20 g flour into a 100 ml Erlenmeyer flask and add 100 ml meta-phosphoric acid[4]. Shake until a homogeneous dispersion is obtained. Filter and use the clear solution (up to v = 1.500ml) immediately for the assay.

It is recommended to perform sample preparation and assay as fast as possible to avoid precipitation of starch in the assay system.

12. Further applications

The method may also be used in the examination of pharmaceuticals (e.g. vitamin tablets and solutions, analgetica, antipyretica, Ref. 3.2-3.4), and in research when analyzing biological materials.

Determination of L-ascorbic acid in vitamin tablets
Dissolve vitamin C containing tablets with meta-phosphoric acid[4], dilute solution according to the dilution table and use it for the assay.

Important note
If the sample to be analyzed for L-ascorbic acid contains also heavy metals (e.g. copper and iron in the case of pharmaceuticals or additives to animal feed), it is absolutely necessary to prepare the sample solution immediately before pipetting into the cuvettes (because of the high instability of L-ascorbate in solutions containing metal ions), otherwise the results would be too low.

13. Determination of iso-ascorbic acid in meat products

Iso-ascorbic acid reacts with reduced speed under the given assay conditions (see pt. 3).

The **sample preparation** for the analysis of meat products is performed as described under pt. 11. 0.500 to 1.000 ml of filtrate are used for the assay, dependent on the concentration of iso-ascorbic acid in the sample solution.

In **Procedure**, the incubation time is approx. 20 min at 37°C (instead of 6 min for the determination of L-ascorbic acid). While incubating, the contents of the sample blank assay should be mixed for 5 s every 3 min.

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L-Ascorbic acid assay control solution

The assay control solution serves as a control for the enzymatic determination of L-ascorbic acid in foodstuffs and other materials.

Reagents

L-Ascorbic acid (Vitamin C), A.R.
meta-Phosphoric acid (e.g. Merck Darmstadt, Cat.No. 546), 1.5% (w/v); adjusted to pH 3.5-4.0 with KOH (10 M).

Preparation of the assay control solution

Accurately weigh approx. 200 mg L-ascorbic acid to the nearest 0.1 mg into a 100 ml volumetric flask, fill up to the mark with meta-phosphoric acid, and mix thoroughly. Dilute solution 1:10 (1 + 9) with meta-phosphoric acid in a volumetric flask and mix thoroughly.

The solution is stable for 1 day at 20°C, for 3 days at 2-8°C, and for 1 week at -15 to -25°C.

Application:

1. Addition of L-ascorbic acid assay control solution to the assay mixture:
Instead of sample solution the assay control solution is used for the assay. (The measurement of the assay control solution is not necessary for calculating the results.) (For recovery see pt. 3)

2. Restart of reaction, quantitatively:
After completion of the reaction with sample solution and measuring A_2, add 0.050 ml assay control solution to the assay mixture. Read absorbance A_3 after the end of the reaction (approx. 15 min). Calculate the concentration from the difference of (A_3-A_2) according to the general equation for calculating the concentration. The altered total volume must be taken into account. Because of the dilution of the assay mixture by addition of the assay control solution, the result differs insignificantly from the result got according to pt. 1.

3. Internal standard:
The assay control solution can be used as an internal standard in order to check the determination for correct performance (gross errors) and to see whether the sample solution is free from interfering substances:

Pipette into cuvettes:	Sample blank Sample	Sample	Sample blank Standard	Standard	Sample blank Sample + Standard	Sample + Standard
solution 1 (37°C)	1,000 ml	1,000 ml	1,000 ml	1,000 ml	1,000 ml	1,000 ml
redist. water	1,500 ml	1,500 ml	1,500 ml	1,500 ml	1,500 ml	1,500 ml
sample solution	0,100 ml	0,100 ml	-	-	0,050 ml	0,050 ml
assay control sln.	-	-	0,100 ml	0,100 ml	0,050 ml	0,050 ml
tube 2 (AAO-spatula)	1 spatula	-	1 spatula	-	1 spatula	-
Mix and incubate for 6 min at 37°C. Continue as described in the pipetting scheme under "Procedure". Follow the instructions given under "Instructions for performance of assay" and the footnotes.						

The recovery of the standard is calculated according to the following formula:

$$\text{recovery} = \frac{2 \times \Delta A_{\text{sample + standard}} - \Delta A_{\text{sample}}}{\Delta A_{\text{standard}}} \times 100\ [\%]$$

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