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PROPERTIES OF SQUALENE-2(3),22(23)-DIEPOXIDE-α-ONOCERIN CYCLASE FROM *ONONIS SPINOSA* ROOT

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Key Word Index—*Ononis spinosa*; Leguminosae; α-onocerin; biosynthesis; cell-free system; squalene diepoxide cyclase.

Abstract—Investigation of the enzyme system converting squalene diepoxide to α-onocerin has shown that it occurs in the soluble fraction of the cells of *Ononis spinosa* and is stable to acetone powdering. A pH of 7·25 and a K^+ concentration of 0·15 M are required for optimal activity. The enzyme is particularly susceptible to detergents, being inhibited by all those examined. It is also affected by a number of other inhibitors, possibly due to a non-specific detergent effect. In many respects the properties of this cyclase differ from those of other known squalene epoxide cyclases. The use of substrate analogues and other evidence suggest that this enzyme requires both substrate epoxide groups for recognition and cyclization of the substrate. Some evidence has been obtained for the existence of an intermediate between squalene diepoxide and α-onocerin.

INTRODUCTION

IT HAS been well established that squalene-2(3)-epoxide is the immediate acyclic precursor of sterols and triterpenoids in many animals,[1,2] plants[3,4] and micro-organisms.[5–7] The enzymes involved in cyclizing squalene-2(3)-epoxide and related substrates, the cyclases, have recently been reviewed.[8] However, the properties of the enzymes involved have been reported in detail only in the cases of mammalian liver and yeast.[9–11] In a previous communication[12] we have shown that squalene-2(3),22(23)-diepoxide is converted to the triterpenoid α-onocerin by a cell free system from *Ononis spinosa*. We observed an additional product from squalene diepoxide which we incorrectly suspected to be cycloartenol, and which we now think is probably a partly cyclized intermediate. In the present paper we report some of the properties of this enzyme system and further characteristics of the additional product formed on incubating squalene-2(3),22(23)-diepoxide with extracts of *Ononis spinosa*.

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RESULTS AND DISCUSSION

Optimal Conditions for Enzyme Activity

The activities of cell free systems prepared with a number of buffers used for homogenization are shown in Table 1. It can be seen that both a reducing agent (such as metabisulphite or ascorbate) and a phenol binding agent (such as bovine serum albumin or soluble polyvinylpyrrolidone, PVP) are required for cyclase activity. Bovine serum albumin proved to be superior to soluble PVP in preserving enzyme activity but was not suitable for experiments in which an accurate determination of plant protein was required. On the other hand, insoluble PVP could not replace soluble PVP. It was observed that there was much variation (0·013 ± 0·012 nmmol/hr) between the activities of 15 different enzyme preparations although the experimental error involved in the assay of activity of a single preparation was much less [0·03 ± 0·0016 (4 determinations) nmmol/hr]. The activity of each preparation is dependent upon such variables as the concentration and type of phenols present, the activity of endogenous proteases and the physiological state of the plant. Clearly it is not possible to compare the results obtained from different preparations.

TABLE 1. HOMOGENIZATION BUFFERS

0·1 M Potassium phosphate buffer (pH 7·2) with the following additives	Activity observed (nmmol/hr × 10^{-2})
2% (w/v) soluble PVP and 0·05% (w/v) cysteine	2·5
2% (w/v) soluble PVP, 0·2 M sucrose, 0·05 M ascorbate and 0·05% (w/v) cysteine	2·1, 0·7
0·2 M sucrose, 0·05 M ascorbate and 0·05% (w/v) cysteine	0·0
Insoluble PVP (1 g dry wt. of polyclar AT per g fr. wt tissue), 0·2 M sucrose, 0·05 M ascorbate and 0·05% (w/v) cysteine	0·0
0·2 M sucrose and 0·1 M metabisulphite	0·4
1% (w/v) bovine serum albumin, 0·2 M sucrose, 0·05 M ascorbate and 0·05% (w/v) cysteine	1·2

The pH optimum of the enzyme was determined using buffer C (see Experimental). Prior to injection of the substrate the pH of each assay was adjusted to the required value by means of 1 M potassium phosphate to give a final phosphate concentration of 0·1 M. The pH optimum of this cyclase preparation was found to be 7·25 (with a small shoulder on the acid side). The same buffer was used to determine the optimum KCl concentration for cyclase activity. Figure 1 shows the effects of varying the concentration of KCl on cyclase activity. The optimum concentration was 0·15 M.

Intracellular Distribution of Cyclase Activity

Table 2 shows that almost all of the *Ononis spinosa* cyclase activity occurs in the 100 000 *g* fraction of the cell. Reconstituted acetone powders were compared with the original homogenate. A portion of the acetone powder suspension was centrifuged at 100 000 *g* for 2 hr and the squalene diepoxide-α-onocerin cyclase activity of both supernatant and pellet examined. The results are shown in Table 3. Acetone powders showed increased (approx. two-fold) specific activities over the original homogenate. Although centrifugation at 100 000 *g* did not increase specific activity, it did not precipitate activity either. It is

interesting to note that we have been unable to demonstrate the presence of any squalene-2 (3)-epoxide-cycloartenol cyclase activity in these cell-free extracts. (*O. spinosa* contains appreciable quantities of phytosterols). This suggests that the properties and location of the α-onocerin and cycloartenol forming cyclases are markedly different. These properties may well be significant in the control of α-onocerin and phytosterol biosynthesis.

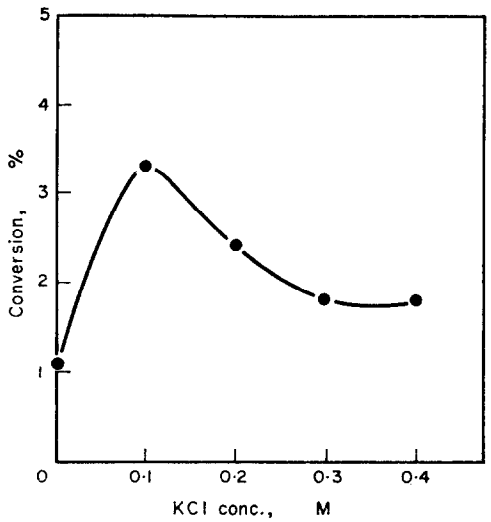


FIG. 1. THE EFFECT OF POTASSIUM CHLORIDE OF THE α-ONOCERIN CYCLASE FROM *Ononis spinosa*.

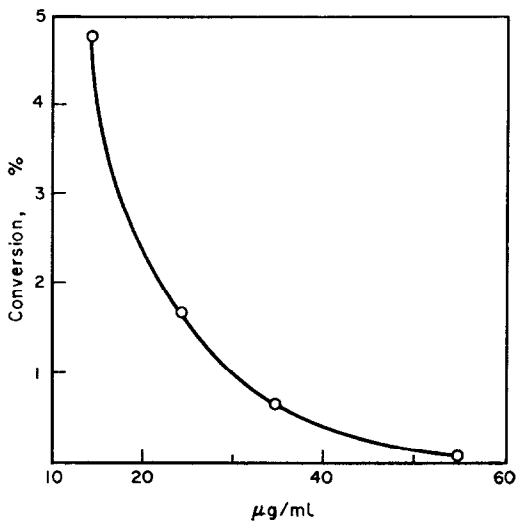


FIG. 2. THE EFFECT OF TWEEN 80 ON ENZYMIC ACTIVITY.

The Effect of Detergents upon Cyclase Activity

A 5:1 (w/w) ratio of Tween 80 to substrate was normally used for emulsification of the substrate. The effect of greater concentrations of Tween 80 is shown in Fig. 2. If $\log_{10}$ percentage conversion is plotted against the concentration of Tween 80, a straight line is

TABLE 2. INTRACELLULAR DISTRIBUTION OF SQUALENE DIEPOXIDE-α-ONOCERIN CYCLASE IN *Onocerin spinosa*

Fraction	Volume (ml)	Protein conc. (mg/ml)	Conversion (nmmol/hr per assay × 10^{-2})	Specific activity (nmol/mg/hr × 10^{-2})	Total activity (nmol/hr per fraction)
20 000 *g* Pellet	5	1·760	0·04	0·03	0·0025
20 000 *g* Supernatant	31	0·876	1·2	1·71	0·465
Microsomal	2	0·668	0·1	0·19	0·0025
Soluble	31	0·816	1·1	1·68	0·425

obtained which intercepts the Tween 80 = 0 axis at a conversion equivalent of 21%. At similar concentrations Triton X100 produces much less inhibition although at higher concentrations it inhibits strongly (Fig. 3). However, it was found that Triton X100:substrate ratio of 2:1 gave a good emulsion and thus this detergent is certainly superior to Tween 80 for

the solubilization of the substrate. The effects of a number of different detergents used to solubilize the substrate for the cyclase are shown in Table 4; Triton X100 stands out not only for its ability to produce efficient emulsification at relatively low ratios to substrate, but also for the fact that it causes significantly less inhibition of the enzyme.

TABLE 3. EFFECT OF ACETONE TREATMENT UPON ENZYMIC ACTIVITY

Fraction	Conversion (%)	*Specific activity (nmmol/mg/hr × 10^{-3})
Crude homogenate	11·6	4·5
Total acetone powder	6·9	8·3
Acetone powder 100 000 *g* pellet	1·1	0·0
Acetone powder 100 000 *g* supernatant	6·1	8·0

* The specific activities are low due to the 1% BSA present in the homogenisation buffer.

TABLE 4. EFFECT OF DIFFERENT DETERGENTS AS SOLUBILIZING AGENTS FOR SQUALENE-2(3),22(23)-DIEPOXIDE-α-ONOCERIN CYCLASE

Solubilizing agent	Ratio of detergent to substrate	Conversion (%)
Water		3·0
Tween 80	1:1*	6·4
	5:1	8·4
Brig 35	5:1	6·3
Triton X100	1:1	9·9
Serum Fraction DF1V-1	1 mg/ml	7·9

* Emulsion not clear.

Variation of the concentration of the serum protein fraction DF1V-1 as a solubilizing agent had no effect on the percentage conversion of squalene diepoxide over quite a wide range—at concentrations lower than 1 mg/ml it did affect the efficiency of solubilization.

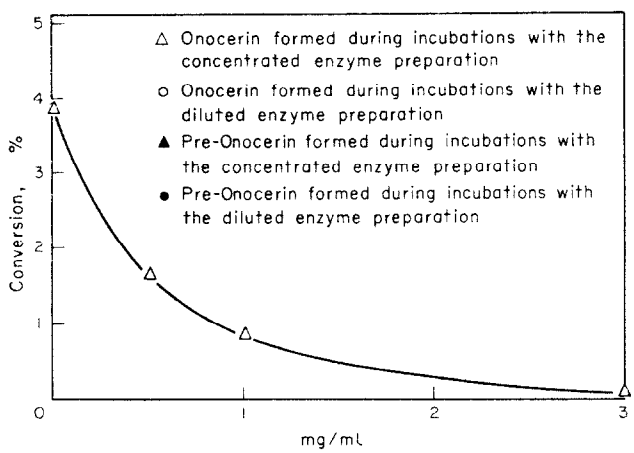


FIG. 3. THE EFFECT OF TRITON X100 ON ENZYMIC ACTIVITY.

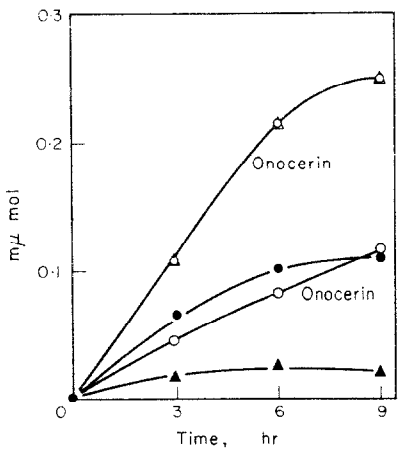


FIG. 4. THE EFFECT OF DILUTION OF THE ENZYME PREPARATION ON THE PRODUCTS OF THE ENZYMIC REACTION.

0·15% (w/v) deoxycholate (which produces a threefold activation of mammalian liver cyclase) also inhibits *O. spinosa* cyclase preparations by 83%. Table 5 shows the effect of a number of other inhibitors upon the activity of the enzyme. The general inhibition, even by those compounds such as mepyrapone, AMO 1618 and SKF 525A which would not be

expected to inhibit on the basis of their known activities, may be due to the detergent-like structures of these inhibitors.

TABLE 5. EFFECT OF INHIBITORS ON SQUALENE DIEPOXIDE-α-ONOCERIN CYCLASE

Inhibitor (0·5 mM)	Activity remaining (%)
None	100
S.K.F. 525A	83
S.K.F. 7997A$_3$	72
Phosphon S	59
S.K.F. 3301A	59
β-Hydroxyethyl hydrazine	55
Mepyrapone	37
AMO 1618	37
S.K.F. 7732A$_3$	34

TABLE 6. EFFECTS OF SQUALENE EPOXIDE AND IMINOSQUALENE ON SQUALENE DIEPOXIDE-α-ONOCERIN CYCLASE

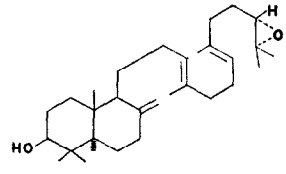
Inhibitor concn (μg/ml)	Inhibitor:substrate ratio	Activity remaining (%) Squalene epoxide	Iminosqualene
0		100	100
0·3	1/10:1	65	73
3	1:1	38	22
30	10:1	11	11

The Effect of Substrate Analogues upon Cyclase Activity

Squalene-2(3)-epoxide and 2(3)-iminosqualene both inhibit to about the same extent (Table 6) but 2(3),22(23)-diiminosqualene is a much more potent inhibitor (Corey, Dean and Rowan, to be published).

The Effect of Dilution upon Cyclase Activity

The effect of an eight-fold dilution upon cyclase activity is shown in Fig. 4. It can be seen that in addition to α-onocerin another radioactive product accumulates. When analysed by TLC on silica gel G in 2% methanol in chloroform, this product lies between squalene diepoxide and α-onocerin and behaves like a monohydroxy compound. A small quantity of the unknown was prepared using a large scale incubation and purified by repeated preparative TLC (see Experimental). Even after three TLC steps the unknown compound was still contaminated with 10% squalene diepoxide. This material was reincubated with the enzyme preparation and was converted to α-onocerin in 6% yield. The same enzyme preparation gave 5% conversion of authentic squalene diepoxide to α-onocerin under conditions of saturating substrate. Thus it is unlikely that the 6% conversion of the unknown product is due solely to its squalene diepoxide content.



(I)

We have tentatively named this unknown product 'pre-onocerin' and suggest the possible structure (I) on biogenetic grounds. There are a number of mechanisms by which it may arise. (i) The enzyme may have two cyclization sites on one unit. On dilution a conformational change may occur masking one of these sites. (ii) The enzyme may exist as a dimer with one

active site on each subunit. On dilution the dimer may dissociate, each monomer then catalysing the half cyclization of squalene diepoxide. (iii) Two enzymes may exist (squalene diepoxide–pre-onocerin cyclase and pre–onocerin–α-onocerin cyclase) as a multienzyme complex with pre-onocerin as an enzyme-bound intermediate. On dilution the complex may dissociate, pre-onocerin becoming a free intermediate and accumulating in the medium. (iv) Pre-onocerin may exist as a carrier protein-bound intermediate between two enzymes. Dilution may then interfere with its binding to the carrier.

It is clear that even though pre-onocerin can be converted to α-onocerin its precise role as an intermediate is far from certain. Indeed its rather poor conversion to α-onocerin suggests that it may not be significant *in vivo*. However, further study of its structure and synthesis *in vitro* may throw light on the mechanism of action of the cyclase and its relationship to other enzymes in this biosynthetic pathway. It proved impossible to synthesize sufficient pre-onocerin to study the squalene diepoxide–pre-onocerin and pre-onocerin-α-onocerin reactions separately. Thus all the work on the properties of the enzyme system was done on the squalene diepoxide–α-onocerin conversion.

TABLE 7. A COMPARISON OF THE CYCLASES FROM PIG LIVER, YEAST *Ochromonas malhamensis* AND *Ononis spinosa*

Source	Liver[11]	Yeast[11]	*Ochromonas malhamensis*[14]	*Ononis spinosa*
Product	Lanosterol	Lanosterol	Cycloartenol	α-Onocerin
Activity (nmmol/mg/hr)	4–47	1	12	0·2
Cell fraction	Microsomal	Soluble	Microsomal	Soluble
Effect of 0·15% (w/v) deoxycholate	3 Fold activation	None	2 Fold activation	80% Inhibition
Effect of 0·2% (w/v) Triton X100	None	6 Fold activation	Not determined	85% Inhibition
Optimum K^+ concentration (M)	0·4	*ca.* 0·01	0·35	0·15
Effect of acetone powdering	Enzyme activity lost but restored by deoxycholate	Enzyme retains activity but is precipitated	Not determined	Enzyme remains soluble and active

Comparison of the Properties of Some Other Cyclases with the Enzyme System from Ononis spinosa

The liver cyclase has been shown to be microsomal; the same may also be true of the yeast enzyme. Shechter *et al.*[11] found that although the latter enzyme occurred in the soluble fraction on differential centrifugation many of its properties were more characteristic of particulate enzymes: it was unstable to a number of purification procedures and was rendered insoluble by acetone powdering. They concluded that the enzyme may be microsomal in origin and that it may have been 'solubilized' on homogenization by some component on the yeast. Although *O. spinosa* is known to contain a high concentration of saponins[13] which might similarly release microsomal enzymes on homogenization, the behaviour of the cyclase on acetone powder treatment suggests that it may be the first truly soluble cyclase.

[13] A. S. GLADKIKH, I. A. GUBANOV and A. A. MESCHERYAKOV, *Izv. Akad. Nauk. Turkm. S.S.R. Ser. Biol. Nauk* **1**, 22 (1965).
[14] G. H. BEASTALL, H. H. REES and T. W. GOODWIN, *Febs Letters* **18**, 175 (1971).

This difference in solubility may be reflected in the other properties of the cyclase. Bloch *et al.*[9,10] have shown that the activation of the liver enzyme by deoxycholate is due to the release of the cyclase from microsomal membranes and that once in soluble form both deoxycholate and a high potassium ion concentration are required to prevent re-aggregation and inactivation of the enzyme. Thus the differences shown in Table 7 between these three cyclases in their behaviour towards detergents and potassium ions may reflect differences in their structural organization rather than fundamental differences in their modes of action.

Further purification of the cyclase from *O. spinosa* was carried out by attaching squalene diepoxide to a column of 'hexamethylene diaminecellulose' (see Experimental) and passing the enzyme solution through the column. Subsequent elution with increasing concentrations of KCl produced a five-fold increase in the specific activity (Table 8). However, a control column composed of the 'diamine-cellulose' effected a fifteen-fold purification and whilst it is possible that using these supports consecutively a further increase in purification might be hoped for, this attempt at affinity chromatography of cyclases failed because it was impossible to eliminate the purely ion exchange properties of the polymer used.

TABLE 8. AFFINITY CHROMATOGRAPHY OF α-ONOCERIN CYCLASE FROM *Ononis spinosa*

	Hexamethylene diamine column	Squalene oxide column
Total protein recovered	1·69 mg	1·83 mg
% Protein recovered	47%	51%
Total enzyme activity recovered	0·161 nm-mol/hr	0·173 nm-mol/hr
% Activity recovered	123%†	133%†
Fractions giving best purifications	0·4 M KCl	0·2, 0·3, 0·4, 0·5, 1 M KCl
Purification achieved	15-fold	4·5-fold
Activity recovered in these fractions*	77%	124%†

* As a percentage of activity applied.

† These high activities probably reflect the removal of phenolic inhibitors.

Amount of protein applied to both columns = 3·58 mg; enzymic activity applied to both columns = 0·1308 nmmol/hr.

EXPERIMENTAL

Preparation of cell free extracts. Cell-free extracts of 2-month-old *O. spinosa* seedling roots were prepared as described previously[12] using one of three homogenization buffers. (a) 0·1 M potassium phosphate (pH 7·2) containing 2% (w/v) soluble PVP, 0·05% (w/v) cysteine, 0·2 M sucrose and 0·05 M ascorbate. (b) 0·1 M potassium phosphate (pH 7·2) containing 1% (w/v) bovine serum albumin (BSA), 0·05% (w/v) cysteine, 0·2 M sucrose and 0·05 M ascorbate. (c) 0·01 M potassium phosphate (pH 7·2) containing 1% (w/v) BSA, 0·05% (w/v) cysteine, 0·2 M sucrose and 0·05 M ascorbate. Buffer (a) was used except where specifically stated otherwise. For some experiments a 'soluble fraction' was prepared by subjecting the 20 000 *g* supernatant fraction[12] to centrifugation at 100 000 *g* (av.) for 3 hr. An acetone powder was made by the standard method[15] using a 100 000 *g* supernatant fraction prepared with buffer (b).

Protein determination. BSA containing extracts could not be assayed due to the excess of BSA over plant protein. PVP containing extracts were precipitated with 6% (w/v) trichloracetic acid and the precipitate extracted with 3 × 1 ml of ice-cold $CHCl_3$ to remove PVP. The protein was then assayed using a slight modification of the method of Lowry *et al.*[16]

[15] R. K. MORTON, in *Methods in Enzymology*, Vol. 1, p. 25, Academic Press, New York (1955).

[16] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).

Substrates and inhibitors. Radioactive and non-radioactive substrates were prepared as described previously.[12] 2(3)-Iminosqualene was prepared by the method of Corey *et al.*,[17] 2(3), 22(23)-diiminosqualene was a gift from Prof. Corey. The serum protein fraction DF IV-1 was a gift from Prof. Boyd. Squalene diepoxide was prepared by reacting 2 mol of *N*-bromosuccinimide with 1 mol of radioactive squalene.[12] The mono and dibromohydrins were separated on TLC (silica gel) (R_f mono 0·27; R_f di 0·05 using benzene). The dibromohydrin was converted to the diepoxide using anh K_2CO_3 in MeOH (N_2). Incubation procedures used were as described by Dean[18] and the chromatographic separations of the products carried out as described by Rowan *et al.*[12] A small amount of the intermediate, pre-onocerin, was prepared by means of a large scale incubation of about 1 μCi of squalene diepoxide with 50 ml of the diluted enzyme preparation. The compound was purified by preparative TLC on silica gel G buffered at pH 7·5 (solvent, 2% MeOH in $CHCl_3$). After three such steps the pre-onocerin still contained about 10% of squalene diepoxide.

Preparation of squalene diepoxide attached to cellulose. Hexamethylenediamine was linked to cellulose (donated by Whatman Biochemicals, Maidstone, Kent) by the method of Axen *et al.*[19] Squalene diepoxide was attached to the amino groups by an adaptation of the method of Porath and Fornstedt.[20] Hexamethylenediamine-cellulose (*ca.* 2 g wet wt) was suspended in tetrahydrofuran (3·5 ml) and 0·035 M NaOH (1 ml) and squalene diepoxide (110 mg) in the same mixture (5 ml) added. The resulting mixture was stirred at 60° in N_2 for 2 hr and then at room temp. overnight. The cellulose was filtered off and washed, first with tetrahydrofuran and then with H_2O. The product contained about 10 mg of bound squalene diepoxide per g wt of cellulose.

Chromatography of a crude enzyme extract on squalene diepoxide-hexamethylenediamine-cellulose. A 100 000 *g* supernatant fraction was prepared using buffer (a). This was dialysed against two changes of 0·01 M potassium phosphate (pH 7·2) containing 0·05 M ascorbate, 0·2 M sucrose and 0·05 % (w/v) cysteine and then concentrated by ultrafiltration using an Amicon model 50 ultrafiltration cell. The concentrate (2 ml, 1·79 mg/ml) was applied to two separate columns; (a) a short column (3 cm) of squalene diepoxide-hexamethylenediamine-cellulose; and (b) a control column containing hexamethylenediamine-cellulose. Each column was equilibrated with the buffer described above and after application of the enzyme preparation, the columns were eluted with a stepwise gradient of KCl in this buffer. 2 ml each of the following solutions of KCl 0·1, 0·2, 0·3, 0·4, 0·5 and 1·0 M were added consecutively and the eluant fractions corresponding to these steps were collected. Each fraction was dialysed against 0·1 M potassium phosphate (pH 7·2) containing 0·05 M ascorbate, 0·2 M sucrose and 0·05% (w/v) cysteine and then assayed for protein and enzyme activity.

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