Extracellular Polysaccharide Biosynthesis by *Pseudomonas* NCIB 11264. Studies on Precursor-forming Enzymes and Factors Affecting Exopolysaccharide Production by Washed Suspensions

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(Received 5 February 1979; revised 9 May 1979)

An assay was developed to measure the rate of exopolysaccharide formation by washed non-proliferating suspensions of *Pseudomonas* NCIB 11264 grown under a range of controlled environmental conditions. The specific activities of certain of the enzymes involved in the formation of the sugar nucleotide precursors of polysaccharide biosynthesis were also measured in steady-state populations. The level of enzyme activity did not reflect either the amount of extracellular polysaccharide produced or the rate at which glucose was incorporated into exopolysaccharide, which was dependent on medium composition, environmental factors, and the rate and stage of growth of the organism. The specific activities were not affected by either cultural conditions or the separate addition of actinomycin D and chloramphenicol, indicating a constitutive biosynthetic system.

INTRODUCTION

Norval (1969), using washed suspensions of *Klebsiella aerogenes* grown in several different media, was able to demonstrate similar rates of polysaccharide synthesis by preparations of both exponential and stationary phase cultures. Apart from these findings, there is relatively little comparative information available on the rate of exopolysaccharide formation by bacteria grown under a range of controlled environmental conditions. Studies on exopolysaccharide synthesis in washed suspensions of bacteria have generally been undertaken to examine the effects of nutrients on polysaccharide production (Wilkinson & Stark, 1956; Wilkinson, 1958) or, alternatively, to follow the resynthesis of capsular material following decapsulation (Bernheimer, 1953; Juni & Heym, 1961).

Pseudomonas NCIB 11264, initially isolated from a carbohydrate-rich industrial effluent, synthesizes an extracellular acylated heteropolysaccharide (Williams *et al.*, 1979). The influence of various growth parameters on exopolysaccharide production has been examined using both batch and continuous culture techniques (Williams & Wimpenny, 1977, 1978). The effects of environmental factors and medium composition on the rate of exopolysaccharide formation by washed, non-proliferating suspensions of *Pseudomonas* NCIB 11264 are considered in this paper. The results of preliminary induction studies are also discussed.

A preliminary report of certain aspects of this work has been presented (Williams & Wimpenny, 1975).

METHODS

Growth and maintenance of culture. *Pseudomonas* NCIB 11264 was routinely cultured in 200 ml chemically defined medium in 1 l baffled Erlenmeyer flasks with gyrotary incubation (120 rev. min⁻¹) at 30 °C for 48 h

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or in a 10 l capacity fermentation vessel as described previously (Williams & Wimpenny, 1977). The techniques and equipment for the continuous (chemostat) culture experiments have also been described previously (Williams & Wimpenny, 1978).

Determination of the rate of exopolysaccharide production in non-proliferating suspensions. The rate of exopolysaccharide production by washed suspensions was determined as follows. Bacteria were harvested by centrifugation (35000 g, 30 min), washed twice in 0.9% (w/v) NaCl buffered to pH 7.5 with 0.01 M-Tris/HCl (designated NaCl/Tris) and resuspended in 0.01 M-KH₂PO₄ (pH 7.0) to give a suspension containing approximately 1 to 2 mg protein ml⁻¹. All solutions and suspensions were kept at 0 to 4 °C throughout.

The assay was based on the incorporation of [U-¹⁴C]glucose into exopolysaccharide material under non-growing conditions. Protein synthesis inhibitors were not included because of possible non-specific interference in polysaccharide biosynthesis. The *A*₅₂₀ remained constant during the 30 min incubation period. The reaction mixture, containing nitrogen-free defined medium (pH 7.0), [U-¹⁴C]glucose (5 µCi; The Radiochemical Centre, Amersham) and glucose (28 nmol), was equilibrated at 30 °C before adding cell suspension to a total volume of 5 ml. The mixture was gently shaken at 30 °C and samples (0.5 ml) were removed at intervals and mixed with an equal volume of chilled formalin (2%, v/v) to stop the reaction. Bacteria were removed by centrifugation (5000 g for 15 min) and samples (50 µl) of the supernatant were applied to Whatman no. 1 paper which was irrigated for 15 h in ethanol/1.0 M-ammonium acetate (7:3, v/v; pH 7.5) to remove low molecular weight materials. The areas at the origin containing ¹⁴C-labelled polymeric material (Williams & Wimpenny, 1975) were cut out, suspended in toluene (10 ml) containing 0.5% (w/v) 2,5-diphenyloxazole, and their radioactivities were determined using a Beckman LS-100 ambient temperature scintillation spectrometer. The efficiency of ¹⁴C counting on paper was approximately 30%.

Using this assay procedure, the rate of glucose incorporation into polysaccharide [$\mu\text{g (mg cell protein)}^{-1} \text{ h}^{-1}$] by washed non-proliferating bacterial suspensions was proportional to the protein content (0.1 to 1.0 mg ml⁻¹) and was determined for a range of controlled steady-state growth conditions, which, except when under investigation, were maintained as follows: pH 7.0, temperature 30 °C, aeration rate 500 ml min⁻¹, impeller speed 900 rev. min⁻¹, glucose input 20 mg ml⁻¹, NH₄Cl input 0.5 mg ml⁻¹, KH₂PO₄ input 5.44 mg ml⁻¹.

Experiments with chloramphenicol and actinomycin D. (i) *Effect of protein synthesis inhibitors on exopolysaccharide formation.* The organism, previously shown to be sensitive to chloramphenicol and actinomycin D at 100 to 250 µg ml⁻¹ and 15 to 25 µg ml⁻¹, respectively, was grown in continuous culture under conditions of carbon or nitrogen limitation. Bacteria were harvested aseptically, washed twice with NaCl/Tris (pH 7.5), resuspended in 0.01 M-KH₂PO₄ (pH 7.0) and used to inoculate batch cultures in defined media (with and without added chloramphenicol at 500 µg ml⁻¹). Identical results were obtained when the bacteria were washed with the KH₂PO₄ solution prior to resuspension in the same buffer. Polysaccharide concentrations were determined gravimetrically after propan-2-ol precipitation, or viscometrically at concentrations below 500 µg ml⁻¹ (Williams & Wimpenny, 1977).

Polysaccharide production was also assayed by the [U-¹⁴C]glucose incorporation procedure. The nitrogen-free reaction mixture was supplemented with chloramphenicol (100 µg ml⁻¹) and the total volume, including the inoculum, was 5 ml.

(ii) *Effect of inhibitors on the rate of exopolysaccharide formation.* Shaken flask cultures (500 ml) grown for 48 h under carbon-limiting or carbon-excess conditions (1 and 20 mg ml⁻¹ glucose, respectively) were harvested at 4 °C by centrifugation (35000 g, 30 min). Bacteria were washed twice with chilled NaCl/Tris (pH 7.5) and resuspended in 15 ml 0.01 M-KH₂PO₄ (pH 7.0). Aliquots (5 ml) were transferred to each of three flasks containing 20 ml defined medium supplemented with glucose (40 mg ml⁻¹) and Casamino acids (0.2%, w/v). Protein synthesis inhibitors were included as follows: (1) control, no inhibitor present; (2) actinomycin D (15 µg ml⁻¹); (3) chloramphenicol (100 µg ml⁻¹). The flasks were shaken in a water bath at 30 °C. At intervals between 0 and 5 h, samples (5 ml) were removed and bacteria were recovered by centrifugation. After washing twice in NaCl/Tris (pH 7.5) they were resuspended in 5 ml 0.01 M-KH₂PO₄ (pH 7.0). The rates of polysaccharide production by the two types of bacteria (carbon-limited and carbon-excess) under the three different incubation conditions were measured by the [¹⁴C]glucose incorporation technique.

Preparation of cell-free extracts and enzyme assays. Steady-state chemostat-grown bacteria from 500 ml culture were harvested by centrifugation (35000 g, 30 min), washed twice with NaCl/Tris (pH 7.5) and resuspended in 10 ml 0.01 M-KH₂PO₄ (pH 7.0). A portion (1 ml) of the suspension was used to determine the rate of [¹⁴C]glucose incorporation into exopolysaccharide. The remainder of the washed suspension was passed twice through a French press and treated with DNAase; unbroken organisms were removed by centrifugation (6000 g, 15 min). The crude cell-free extract thus obtained was centrifuged (115000 g, 60 min) and the supernatant fraction was carefully decanted and stored at 4 °C. Assays were always performed on freshly prepared samples, not on fractions that had been stored or deep frozen.

Hexokinase activity was assayed by the method reported by Norval & Sutherland (1973) and UDP-galactose 4-epimerase by the procedure of Maxwell *et al.* (1962). The formation of dTDP-L-rhamnose from

dTDP-D-glucose was assayed by the method of Glaser & Kornfeld (1966), and GDPmannose pyrophosphorylase activity was estimated by the method of Munch-Petersen (1962). Sugar nucleotides were purchased from Sigma.

Analytical methods. The protein content of the cell-free fractions and of the washed suspensions following lysis (1 M-NaOH:cell preparation, 1:1; lysed at 100 °C for 5 min) was determined by the method of Lowry, with a bovine serum albumin standard.

RESULTS

Rate of polysaccharide production during the growth cycle

Samples were collected at various stages throughout the growth cycle of the organism in a chemically defined medium, and the rate of [U-¹⁴C]glucose incorporation into exopolysaccharide by washed suspensions was determined. The incorporation rate remained low [20 to 55 µg glucose incorporated (mg protein)⁻¹ h⁻¹] and did not increase until the late-exponential growth phase when activity had trebled. The incorporation rate in the stationary phase [196 µg glucose incorporated (mg protein)⁻¹ h⁻¹] was approximately nine times higher than during the early-exponential phase. This incorporation pattern parallels the appearance of exopolysaccharide (Williams & Wimpenny, 1977).

Effect of growth rate and growth conditions on the rate of exopolysaccharide formation

The organism was grown in a chemically defined medium under strictly controlled conditions in a single-stage continuous culture system and the rate of glucose incorporation by washed suspensions was determined for the wide range of steady-state growth conditions reported by Williams & Wimpenny (1978).

(i) *Growth rate.* The ability of the organism to form exopolysaccharide was related to its growth rate (Table 1) and showed an apparent optimum value at $D = 0.04$ to 0.06 h⁻¹. This may have arisen as a result of limitation due to the high culture viscosity which developed at these lower dilution rates. As the dilution rate in the chemostat approached the maximum specific growth rate in batch culture the rates of glucose incorporation became very similar [30 and 22 to 27 µg glucose incorporated (mg protein)⁻¹ h⁻¹, respectively].

(ii) *Medium composition and growth conditions.* The maximum rates of glucose incorporation into polysaccharide were measured in bacteria grown under conditions of nitrogen limitation with excess phosphate and glucose at pH 7.0 and 30 °C (Table 1).

Effect of protein synthesis inhibitors on exopolysaccharide formation

Washed bacteria grown in a chemostat under conditions of carbon or nitrogen limitation were re-inoculated into defined medium with or without chloramphenicol (500 µg ml⁻¹) and grown as a batch culture. In the absence of chloramphenicol the final polysaccharide concentrations after 12 h were similar for both carbon- and nitrogen-limited organisms (5.6 and 5.9 mg ml⁻¹, respectively) (Fig. 1*a*). In the presence of chloramphenicol, polysaccharide synthesis by both types was considerably impaired and could only be detected in culture supernatants by viscometry (Fig. 1*b*). The chemical composition of the material formed during the incubation was not determined. Although the cultures of nitrogen-limited organisms continued to produce exopolysaccharide the amount was very low (approx. 30 µg ml⁻¹ after 12 h incubation) and with cultures of carbon-limited organisms none was detected. Growth of both types of culture was suppressed (A_{520} changes over the 12 h period were 1.25 to 1.75 and 3.0 to 3.6, respectively) but the pH of the cultures was not inhibitory to polysaccharide formation.

The rate of glucose incorporation by cultures of nitrogen-limited organisms [78 µg (mg protein)⁻¹ h⁻¹] was reduced in the presence of chloramphenicol (100 µg ml⁻¹) to 42 µg (mg protein)⁻¹ h⁻¹, but was considerably higher than the rate measured in cultures of carbon-limited organisms [6.8 µg (mg protein)⁻¹ h⁻¹] (Fig. 2). The rate of incorporation by the latter cultures was unaltered in the presence of the inhibitor.

Table 1. *Effect of growth rate and growth conditions on the steady-state specific activities of enzymes involved in sugar nucleotide formation*

Activities are expressed as μmol substrate transformed $(\text{mg protein})^{-1} \text{ h}^{-1}$ except for those of dTDP-L-rhamnose synthetase where the units are $\text{nmol} (\text{mg protein})^{-1} \text{ h}^{-1}$. Rates of $[\text{U-}^{14}\text{C}]\text{glucose}$ incorporation into the exopolymeric material by washed cell suspensions are also included and are expressed as μg glucose incorporated $(\text{mg protein})^{-1} \text{ h}^{-1}$.

Experimental variable	Steady state growth conditions						Hexokinase	UDPg galactose 4-epimerase	GDPmannose pyrophosphorylase	dTDP-L-rhamnose synthetase	[U- ¹⁴ C]Glucose incorporation rate
	<i>D</i> (h ⁻¹)	pH	Temp. (°C)	Glucose concn* (mg ml ⁻¹)	NH ₄ Cl concn* (μg ml ⁻¹)	KH ₂ PO ₄ concn* (mg l ⁻¹)					
Dilution rate <i>D</i>	0.023	7.0	30.0	13.8	1.10	ND	0.84	0.21	0.56	24.0	230
	0.038			14.9	2.68		0.83	0.14	0.42	31.0	290
	0.057			16.4	1.87		1.18	0.11	0.49	ND	350
	0.115			20.4	2.14		1.28	ND	0.53	70.0	114
	0.176			19.8	15.0		1.31	0.31	0.50	32.2	62
	0.235			19.3	29.0		1.16	0.62	0.31	21.7	30
Temperature	0.08	7.0	15.0	18.0	101	ND	1.96	0.48	0.31	10.8	18
			20.0	20.8	8.03		1.88	0.77	0.54	44.8	141
			25.0	18.4	8.56		1.42	0.45	0.51	31.8	271
			35.0	15.3	1.07		1.22	0.77	0.26	32.2	81
			37.5	15.5	0.21		1.47	0.23	0.46	46.6	61
			40.0	18.0	375		1.39	0.043	0.36	6.6	3
pH	0.08	4.6	30.0	15.3	490	ND	ND	ND	0.058	ND	< 1
		5.5		15.3	490		2.22	0.10	0.095	17.7	6
		6.4		13.9	0.11		1.89	0.62	0.59	73.9	135
		7.0		17.0	0.32		1.86	0.48	0.30	107	201
		8.0		17.0	0.27		1.49	0.68	0.19	64	155
		8.7		16.7	0.16		0.96	0.39	0.34	45.7	87
Phosphate input concn	0.14	7.0	30.0	15.5	802	0.54 (0.06)	3.83	0.18	0.73	33.4	36
				11.8	1.50	106 (0.27)	3.10	0.42	0.81	36.2	109
				12.9	2.60	490 (0.65)	3.22	0.21	0.69	42.6	105
				11.8	1.91	1340 (1.51)	2.27	0.23	0.66	38.6	91
				11.2	0.64	4030 (4.22)	2.34	0.30	0.67	23.5	95

Table 1 (*cont.*)

NH ₄ Cl input concn	0.03	7.0	±0.0	16.7	0.43 (58)	ND	1.15	0.75	0.50	46.6	188
				15.4	1.72 (480)		0.92	0.61	0.60	58.0	322
				13.4	0.27 (910)		1.08	0.58	0.41	60.0	360
				10.5	22.5 (1250)		0.96	0.43	0.58	47.0	396
				8.9	796 (2080)		0.92	0.89	0.56	101	224
				10.7	1450 (2460)		0.73	0.59	0.59	85.0	214
				0.0 (1.5)	510 (810)		ND	0.18	0.23	38.6	11.8
				0.0 (2.0)	3000 (3430)		ND	0.17	0.35	47.0	14.4
				0.0 (2.9)	3900 (4380)		ND	0.18	0.25	43.0	12.5
				0.0 (0.92)	204		2.40	0.22	0.27	42.6	27
Glucose input concn	0.10	7.0	30.0	0.13 (2.7)	6.4	ND	3.60	0.36	0.52	66.8	185
				0.78 (3.8)	1.3		1.69	0.45	0.62	70.5	230
				16.1 (19.0)	0.44		1.88	0.34	0.63	66.4	224
				27.5 (30.5)	0.48		2.40	0.24	0.62	74.5	212
				0.0 (1.3)	860 (1600)		3.19	0.37	0.47	61.9	32
				0.0 (2.9)	613 (1600)		1.28	0.47	0.23	34.7	30
				0.11 (6.3)	406 (1600)		0.93	0.22	0.28	25.6	51

ND, Not determined.

* Input concentrations are given in parentheses.

Fig. 1. Exopolysaccharide production by washed suspensions of carbon- or nitrogen-limited chemostat-grown *Pseudomonas* NCIB 11264 on transfer to glucose defined medium with or without chloramphenicol (500 $\mu\text{g ml}^{-1}$). (a) Polysaccharide production by cultures of nitrogen-limited ($\circ$) and carbon-limited ($\bullet$) organisms in the absence of chloramphenicol were estimated by precipitation with propan-2-ol. (b) Exopolymer production by cultures of nitrogen-limited ($\square$) and carbon-limited ($\blacksquare$) organisms in the presence of chloramphenicol was followed viscometrically at $25 \pm 0.2^\circ\text{C}$ using a Zimm-Crothers viscometer.

Fig. 2. Exopolysaccharide production, determined using the [$\text{U-}^{14}\text{C}$]glucose incorporation assay, by washed suspensions of nitrogen-limited chemostat-grown *Pseudomonas* NCIB 11264 in the standard incubation mixture ($\bullet$) and in the presence of chloramphenicol (100 $\mu\text{g ml}^{-1}$) ($\circ$). Incorporation by suspensions of carbon-limited organisms in the standard incubation mixture is also shown ($\blacksquare$).

Effect of protein synthesis inhibitors on the rate of exopolysaccharide formation

The rate of polysaccharide formation by washed suspensions of carbon- or nitrogen-limited *Pseudomonas* NCIB 11264 was determined over a 5 h period following transfer to defined media containing protein synthesis inhibitors (Table 2). The rate of glucose incorporation by organisms grown under carbon-excess conditions increased fivefold throughout the incubation period. The original low incorporation rate may be attributed to the low environmental pH (5.8) that had developed during the 48 h initial batch culture. After 5 h the measured rate was reduced by 10% in the presence of chloramphenicol, whereas no reduction in the rate occurred in the presence of actinomycin D.

Initially the activity under carbon limitation was reduced by about tenfold as compared with the rate measured in polysaccharide-producing cultures. However, at the end of the

Table 2. *Effect of the protein synthesis inhibitors chloramphenicol and actinomycin D on the rates of glucose incorporation into exopolysaccharide by suspensions of nitrogen-limited and carbon-limited Pseudomonas NCIB 11264 following transfer to environmental conditions favouring exopolysaccharide formation*

Rates of polysaccharide production are expressed as μg glucose incorporated $(\text{mg protein})^{-1} \text{ h}^{-1}$.

Time (h)	Carbon-excess			Carbon-limited		
	Control	Actinomycin D (15 $\mu\text{g ml}^{-1}$)	Chloram- phenicol (100 $\mu\text{g ml}^{-1}$)	Control	Actinomycin D (15 $\mu\text{g ml}^{-1}$)	Chloram- phenicol (100 $\mu\text{g ml}^{-1}$)
0	59.5	74.8	63.1	3.4	17.9	5.8
1.5	160	212	195	162	178	149
3.0	215	219	237	222	221	179
5.0	292	305	264	264	230	210

5 h period it had risen to within 10% of the latter value. The inclusion of chloramphenicol led to a 20% reduction in activity.

At the end of the incubation period polysaccharide could be recovered by propan-2-ol precipitation from the cell-free supernatants of each of the six incubation flasks. Slight growth was observed only in flasks containing organisms originally grown under carbon-excess conditions; this growth was partially inhibited by chloramphenicol or actinomycin D.

Apparently, the inclusion of a protein synthesis inhibitor prevented neither the formation of the exopolysaccharide nor the increase in activity of the synthesizing system, indicating that the system was constitutive rather than inducible.

Effect of growth conditions on steady-state enzyme levels

The specific activities of the enzymes assayed did not exhibit the pronounced variations shown by the glucose incorporation assay and were not indicative of an inducible system. In particular the activity of hexokinase was unaffected by environmental and nutritional changes (Table 1). The specific activities of the other three enzymes were slightly reduced under conditions of carbon limitation, and at extremes of both temperature and pH. On changing from carbon to nitrogen limitation, the rate of exopolysaccharide synthesis, as measured by glucose incorporation, was increased 8.5- to 9-fold. However, the assays showed little difference in the specific activities of these enzymes in preparations from bacteria grown under conditions which caused large changes in the rate of exopolysaccharide formation.

DISCUSSION

The rate of exopolysaccharide formation by *Pseudomonas NCIB 11264* was affected not only by environmental factors such as pH and temperature, but also by the concentrations of essential nutrients. Maximum incorporation rates were measured with bacteria grown under conditions of nitrogen limitation and with excess carbon substrate at pH 7 and at 30 °C, as indicated in the fermentation studies (Williams & Wimpenny, 1977, 1978). Incorporation rates were reduced under conditions of phosphate or glucose limitation. Similarly, the production of xanthan gum by *Xanthomonas campestris*, although not requiring active growth, was reduced at low bacterial concentrations (Moraine & Rogovin, 1966, 1973), and the specific product formation rate declined at higher viscosities.

Polysaccharide formation was also influenced by both the growth rate and stage of growth of the organism. Whereas the rate of polysaccharide production by *Klebsiella aerogenes* in liquid media was greatest during the exponential phase of growth (Duguid & Wilkinson, 1953), and washed suspensions of both exponential and stationary phase organisms of a *K. aerogenes* slime-forming mutant formed polysaccharide at very similar rates (Norval,

1969), *Pseudomonas* NCIB 11264 produced the exopolymer maximally during the late-exponential and stationary growth phases and thus resembles other strains of *Pseudomonas* (Haynes, 1951; Eagon, 1956) and *Zoogloea* (Parsons & Dugan, 1971; Unz & Farrah, 1976).

Although actinomycin D did not suppress either exopolysaccharide formation or the increase in activity of the synthesizing system in carbon-limited organisms on transfer to carbon-excess conditions, exopolymer formation by washed suspensions was adversely affected by chloramphenicol. Chloramphenicol is an effective inhibitor of bacterial protein synthesis and at higher concentrations will affect other cell processes (Hahn, 1967). The transfer of monosaccharides from nucleotide precursors to the lipid carrier has been shown to be inhibited by chloramphenicol (Stow *et al.*, 1971; Sutherland *et al.*, 1971), and exopolysaccharide production by *Pseudomonas* NCIB 11264 may be similarly affected by chloramphenicol.

The specific activities of the enzymes examined remained relatively constant and did not reflect either the amount of polysaccharide produced or the rate at which glucose was incorporated into exopolymeric material, although all of the enzymes assayed are involved in various metabolic processes, none being restricted solely to the synthesis of exopolysaccharide. Similarly, Norval & Sutherland (1973) were unable to observe any correlation between the loss of exopolysaccharide formation and the activities of the enzymes forming the nucleotide precursors in a number of non-mucoid mutant strains of *K. aerogenes*. Our findings and the results of the inhibitor studies are indicative of a constitutive biosynthetic system. Pullulan elaboration by *Pullularia pullulans* is inducible and can be inhibited by cycloheximide (Catley, 1972).

The rate at which *Pseudomonas* NCIB 11264 incorporated [^{14}C]glucose into exopolysaccharide increased markedly after the mid-exponential growth phase although activity was detectable throughout the complete growth cycle. The rate also decreased as the growth rate of the organism was increased in the continuous culture system. Many of the enzymes and precursors of exopolysaccharide biosynthesis are involved in the elaboration of other cellular polysaccharides and the decreased synthetic activity of the exopolymer system at higher growth rates may reflect competition for these enzymes and precursors in branched pathways leading to the synthesis of the more essential cellular polysaccharides. It is also possible that the organism may resemble the conditional (CR) mutants of *K. aerogenes* (Norval & Sutherland, 1969) which are believed to contain depleted levels or activity of the glycosyl carrier.

A.G.W. acknowledges the financial support of a C.A.P.S. studentship from the S.R.C. and Tate & Lyle Ltd. We thank Dr I. W. Sutherland for advice leading to the development of the [^{14}C]exopolysaccharide assay.

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