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PHARMACOLOGICAL AND BIOCHEMICAL ACTIVITIES OF SOME MONOMETHYLXANTHINE AND METHYLURIC ACID DERIVATIVES OF THEOPHYLLINE AND CAFFEINE*

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Abstract—Some monomethylxanthine and methyluric acid derivatives of theophylline and caffeine have been studied to explore whether they possess pharmacological and biochemical activities similar to those of their parent compounds. Both 3-methylxanthine and 1-methylxanthine, but not 1,3-dimethyluric acid or 3-methyluric acid, produced the same maximal relaxation of guinea pig tracheal muscle as did theophylline. The EC_{50} values for theophylline and 3-methylxanthine were not significantly different, whereas those for 1-methylxanthine, 1,3-dimethyluric acid and 3-methyluric acid were significantly higher than that of theophylline. In the Langendorff guinea pig heart, theophylline and 3-methylxanthine caused essentially identical increases in cardiac contractile force. Although less effective than theophylline, 1-methylxanthine and caffeine produced equivalent increases in cardiac contractility. At concentrations higher than those effective for the methylxanthines, 1,3-dimethyluric acid markedly increased contractile force. 3-Methylxanthine inhibited cyclic AMP phosphodiesterase to a lesser extent than did theophylline at both 1.4 and 400 μM cyclic nucleotide concentrations. However, at the higher substrate concentration, cyclic GMP phosphodiesterase activity was inhibited by 3-methylxanthine more than by theophylline. Thus, it appears that the monomethylxanthine and methyluric acid derivatives of theophylline and caffeine possess a spectrum of pharmacological activity similar to that of their parent compounds, a finding which raises important questions about various aspects of the current therapeutic use of methylxanthines.

The methylxanthines, caffeine, theophylline and theobromine, comprise an important group of pharmacologically active agents consumed by the general populace in beverages, and used for various therapeutic purposes. The effects of these agents on myocardial tissue, smooth muscle, the central nervous system (CNS), and on inhibition of cyclic 3′:5′-nucleotide phosphodiesterase (EC 3.1.4.17) have been extensively investigated [1, 2]. Numerous studies have also examined the activity of a wide variety of substituted xanthine congeners [3–6]. However, the only investigations to explore whether the variety of mono- and dimethylxanthine and methyluric acid derivatives which are formed during biotransformation reactions [7–10], do possess pharmacologic activity have been limited to studies of their mitogenic and anti-adenosine activities in cell culture [11, 12]. For this reason, the present study compares the activity of some of the known methylxanthine metabolites to that of theophylline with respect to relaxation of tracheal smooth muscle, stimulation of cardiac muscle, and inhibition of cyclic nucleotide phosphodiesterase.

MATERIALS AND METHODS

Materials. Theophylline, acetyl-β-methylcholine Cl (methacholine), histamine, cyclic 3′,5′-adenosine monophosphate (cyclic AMP) and cyclic 3′,5′-guanosine monophosphate (cyclic GMP) were purchased from Sigma Chemical Co. (St. Louis, MO.). Caffeine was obtained from Matheson, Coleman & Bell (East Rutherford, NJ.) and 3-methylxanthine from Aldrich Chemical Co. (Milwaukee, WI.). Initial samples of 1-methyl- and 1,3-dimethyluric acid were a gift from Dr. H. T. Nagasawa, V.A. Hospital, Minneapolis, MN. Additionally, the two methyluric acids as well as 1-methylxanthine and 3-methyluric acid were obtained from Adams Chemical Co. (Round Lake, IL.), cAMP[^{3}H-g] (38.4 Ci/m-mole) and cGMP[8-^{3}H] (10.2 Ci/m-mole) were purchased from New England Nuclear (Boston, MA.) and ICN Pharmaceuticals, Inc. (Irvine, Ca.) respectively.

Tracheal muscle preparations. Tracheal smooth muscles were obtained from Brevital (Eli Lilly & Co., Indianapolis, IN.) anesthetized female guinea pigs of the Hartley strain (300–500 g) and mongrel dogs, and after removal of fatty deposits were sliced into rings. Guinea pig tracheal chain [13] and dog trachealis muscle preparations [14] were suspended in 10-ml organ bath containing Krebs bicarbonate buffer (118.4 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 24.9 mM $NaHCO_3$, 2.5 mM $CaCl_2$, and 11.1 mM glucose), pH 7.4, oxygenated with 95% O_2 and 5% CO_2, and maintained at 37°. The tissues were mounted with an initial tension of either 0.5 g (guinea pig) or 1.0 g (dog) and after a 30-min equilibration period were made to

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Table 1. Geometric mean EC_{50} values for methylxanthines and methyluric acids on guinea pig and dog tracheal preparations

Compound	Geometric mean EC_{50} (95% confidence interval) Molar concn	N*	Dog trachealis muscle	N*
Theophylline	6.40×10^{-5} (5.03–8.13)	19	1.18×10^{-3} (0.48–2.92)	6
3-Methylxanthine	1.25×10^{-4} (0.88–1.77)	18	3.81×10^{-4} (1.81–8.09)	6
1-Methylxanthine	4.09×10^{-4} (3.23–5.16)†	8		
1,3-Dimethyluric acid	6.03×10^{-4} (3.28–11.10)†	6		
3-Methyluric acid	6.16×10^{-4} (5.20–7.31)†	5		

* Number of tracheal preparations employed to determine geometric mean EC_{50} values according to the procedure of Fleming *et al.* [16].

† Significantly different from theophylline values ($P < 0.05$).

contract with 10^{-6} M histamine and 0.5×10^{-6} M methacholine respectively. Cumulative concentration–response curves [15] for the relaxing effect of theophylline and the various methylxanthine and methyluric acid congeners were recorded by a Grass force-displacement transducer and polygraph. When addition of test substance elicited no further relaxation, 10^{-3} M theophylline was added to indicate maximal muscle relaxation. All compounds were dissolved in 25 mM $LiCO_3$. Addition of equivalent volumes of 25 mM $LiCO_3$ to the organ bath had no effect on the muscle relaxation. Results are expressed as the geometric mean EC_{50} values and their 95 per cent confidence intervals [16].

Isolated guinea pig hearts. Female guinea pigs of the same strain and weight as above were injected with sodium heparin (2000 I.U./kg, i.p.) 60 min prior to sacrifice. The animals were stunned by a blow to the head and the heart was rapidly removed. After the atria were excised, the hearts were perfused by the Langendorff technique with 3 mM HEPES buffer, pH 7.4, containing: NaCl, 140 mM; KCl, 4.0 mM; $MgCl_2$, 1.0 mM; $CaCl_2$, 1.8 mM; and glucose, 5.6 mM. The temperature of the perfusion solution was maintained at 30°; perfusion pressure was held constant at 60 mm H_2O and the perfusion rate was adjusted by means of a Harvard peristaltic pump. Cardiac contractility was monitored by a nylon suture at the apex of the heart attached to a Grass force-displacement transducer and recorded on a polygraph. Diastolic tension was adjusted to 5.0 g. The heart was stimulated at twice threshold voltage (180 stimuli/min, 3-msec duration) by two platinum electrodes placed across the ventricles.

After an equilibration period of 45 min, the heart was perfused stepwise for 7 min with a buffer solution of increasing drug concentrations. The order of drug additions from one heart preparation to the next was varied according to a Latin square design. Solubility of the various methylxanthine and methyluric acids in the perfusion buffer was increased by the addition of 1.3×10^{-4} M ethylenediamine, a concentration that had practically no effects on the pattern or force of contractility, nor did ethylenediamine alter the effect of theophylline on the same parameters.

Phosphodiesterase assay. Tracheal smooth muscle was obtained from 43 female dogs anesthetized with 35 mg/kg of sodium pentobarbital. The tracheal tissue was homogenized (Broeck tissue grinders) in 50 mM Tris–HCl, pH 7.5, containing 0.25 M sucrose, and the homogenate was centrifuged at 5000 *g* for 30 min; the resulting supernatant was centrifuged at 105,000 *g* for 1 hr at 4°. The 105,000 *g* supernatant fluid from twelve dogs was then pooled. The phosphodiesterase assay was a modification of the procedure of Thompson and Appleman [17], as previously reported [18]. The conversion at 37° of [^{3}H]cyclic AMP or [^{3}H]cyclic GMP to their labeled products was determined in 100 μl of reaction mixture containing the tissue extract, excess alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1, Sigma type III from *Escherichia coli*), 50 mM Tris–HCl (pH 7.5) and 5 mM $MgCl_2$, with or without theophylline or 3-methylxanthine. Cyclic nucleotide concentrations of 1.4 and 400 μM were used since the phosphodiesterase activity in the 105,000 *g* supernatant fractions exhibit multiple K_m values for cyclic AMP and cyclic GMP [19]. The labeled products formed during the incubation were separated from the substrates by the addition of 1 ml of BioRad 1 × 8 resin (1:3 suspension in 1 mM HCl). All determinations were performed in triplicate.

RESULTS

Theophylline, in the range of 10^{-7} to 10^{-3} M, had a concentration-dependent relaxing effect on both the histamine-contracted guinea pig tracheal chain and methacholine-contracted dog trachealis smooth muscle preparations. Cumulative additions of the various methylxanthine metabolites also elicited a concentration-dependent relaxation of the smooth muscle. Representative log concentration–response curves obtained with guinea pig tracheal muscle are shown in Fig. 1; 3-methyl- and 1-methylxanthine, but not the methyluric acids, relaxed the tissue to the same extent as did theophylline. In most experiments, theophylline and 3-methylxanthine were almost equally effective. The geometric mean EC_{50} values and their 95 per cent confidence intervals determined on the basis of the per cent of maximal response elicited by the individual compounds in the guinea pig and dog smooth muscle preparations are given in Table 1. The ratio of the molar concentrations of theophylline to 3-methylxanthine required to produce a 50 per cent relaxation (EC_{50}) ranged from

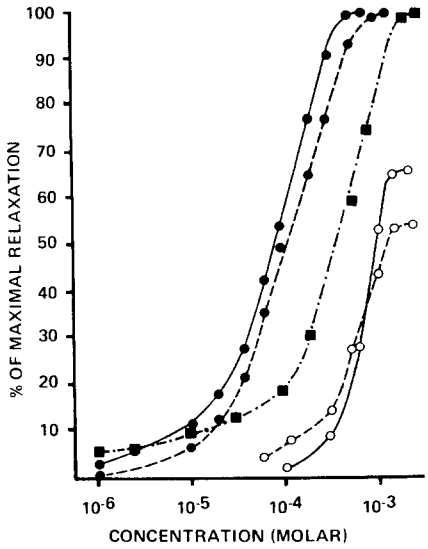

Fig. 1. Log concentration–response curves representing guinea pig tracheal chain relaxations produced by theophylline (●——●), 3-methylxanthine (●– – –●), 1-methylxanthine (■– – – –■), 1,3-dimethyluric acid (○——○) and 3-methyluric acid (○– – –○). Tracheal chains were equilibrated in gassed (95% O_2, 5% CO_2) Krebs bicarbonate solution (37°) for 30 min, then placed "in tone" by the addition of 10^{-6} M histamine. Test drugs were added in serially increasing concentrations to generate concentration–response curves which are plotted as the per cent of maximal relaxation obtained by 10^{-3} M theophylline. The representative response curves shown were obtained for each of the agonists compared with theophylline in different guinea pig tracheal preparations. The mean ± S. E. for all the experiments reported (n = 37) for the increase in tone produced by histamine and for the maximal relaxation elicited by theophylline was 330.0 ± 20.3 mg and 726.3 ± 26.2 mg respectively.

0.5 to 1.0. The addition of theophylline and 3-methyl- or 1-methylxanthine to the tissue bath resulted in additive effects on tracheal muscle relaxation (data not shown).

In the experiments on isolated guinea pig heart, the activities of various methylxanthines and methyluric acids were compared to theophylline's ability to increase cardiac contractile force (Fig. 2). With the exception of 1-methyluric acid, each agent studied caused a concentration-dependent increase in the force of contraction. Theophylline and 3-methylxanthine elicited approximately equivalent increases in the contractile force up to 1.0 and 1.5 mM concentrations respectively. At higher concentrations, both compounds caused a decrease in developed tension (not shown). Caffeine and 1-methylxanthine produced approximately equivalent effects, but both were less active than theophylline and 3-methylxanthine. Relative to its relaxing effect on the tracheal preparations, somewhat unexpected was the marked positive inotropic activity observed with high concentrations of 1,3-dimethyluric acid. Limited solubility of this methyluric acid in the perfusion medium prevented further studies. The geometric mean EC_{125} values and their 95 per cent confidence intervals for the inotropic effects of the compounds in the perfused guinea pig heart are presented in Table 2.

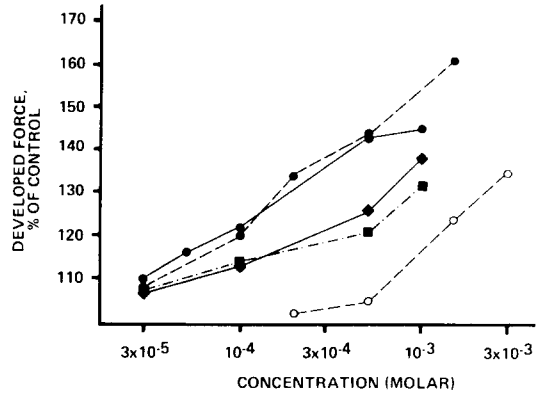

Fig. 2. Effect of theophylline (●——●), 3-methylxanthine (●— —●), caffeine (◆——◆), 1-methylxanthine (■— —■), and 1,3-dimethyluric acid (○— —○) perfusion on contractile force of the isolated guinea pig heart. Values are means of six to eleven hearts at each drug concentration (standard errors have been omitted for clarity) expressed as percentages of control contractile force. The mean ± S. E. for the control contractile force was 7.51 ± 0.44 (n = 28).

In view of the similar potencies of theophylline and 3-methylxanthine in the smooth and cardiac muscle preparations, it was of interest to compare the ability of these two compounds to inhibit cyclic 3′:5′-nucleotide phosphodiesterase. Varying concentrations of the xanthines were used, up to 0.5 mM, which was the limit of solubility of 3-methylxanthine in the assay mixture.

Theophylline and 3-methylxanthine produced a concentration-dependent inhibition of both cyclic AMP and cyclic GMP phosphodiesterase activities. At a cyclic nucleotide concentration of 1.4 μM, theophylline was a slightly more potent inhibitor of the hydrolysis of both cyclic AMP and cyclic GMP than was 3-methylxanthine (Fig. 3). For example, 0.5 mM concentrations of theophylline and 3-methylxanthine inhibited cyclic AMP hydrolysis 48 and 32 per cent and inhibited cyclic GMP hydrolysis 42 and 30 per cent respectively. Neither of the methylxanthines showed any preferential inhibition

Table 2. Geometric mean EC_{125} values for methylxanthines and 1,3-dimethyluric acid in the isolated perfused guinea pig heart preparation

Compound	Geometric mean EC_{125} (95% confidence interval) Molar concn	N*
Theophylline	0.92×10^{-4} (0.55–1.54)	7
3-Methylxanthine	1.32×10^{-4} (0.71–2.24)	7
Caffeine	3.70×10^{-4} (1.83–6.92)†	5
1-Methylxanthine	5.51×10^{-4} (2.28–13.4)†	6
1,3-Dimethyluric acid	10.71×10^{-4} (8.69–13.26)†	3

* Number of isolated heart preparations used to determine geometric mean EC_{125} values according to the procedure of Fleming *et al.* [16].

† Significantly different from theophylline values ($P < 0.05$).

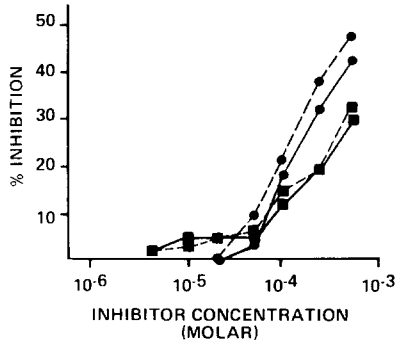

Fig. 3. Inhibitory effects of increasing concentrations of theophylline (circles) and 3-methylxanthine (squares) on cyclic AMP phosphodiesterase activity (dashed line) and cyclic GMP phosphodiesterase activity (solid line). The adopted standard concentration of cyclic nucleotides used as substrate was 1.4 μM. Values are means of triplicate determinations using 105,000 g supernatant fractions of dog trachealis smooth muscle homogenates as the enzyme source. Control rates of cyclic AMP and cyclic GMP phosphodiesterase hydrolysis were 640.1 and 1039 pmoles/min/mg of protein in the 105,000 g supernatant fractions respectively.

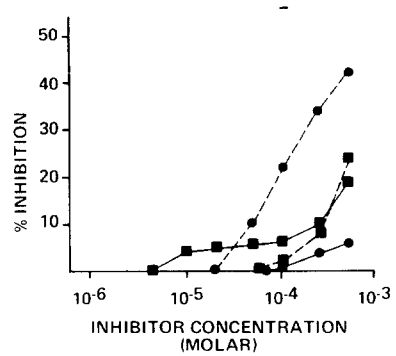

Fig. 4. Inhibitory effects of increasing concentrations of theophyline (circles) and 3-methylxanthine (squares) on cyclic AMP phosphodiesterase activity (dashed line) and cyclic GMP phosphodiesterase activity (solid line). The adopted standard concentration of cyclic nucleotides used as substrates was 400 μM. Values are means of triplicate determinations using 105,000 g supernatant fractions of dog trachealis smooth muscle homogenates as the enzyme source. Control rates of cyclic AMP and cyclic GMP phosphodiesterase hydrolysis were 9.35 and 4.88 nmoles/min/mg of protein in the 105,000 g supernatant fractions respectively.

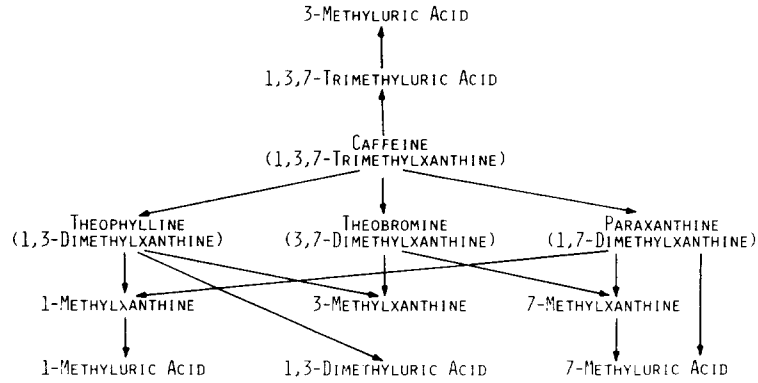

Fig. 5. Composite of proposed degradative pathways for methylxanthines. Significance of a particular metabolite varies with the animal species [7–10, 20].

of either cyclic AMP or cyclic GMP phospodiesterase at this low cyclic nucleotide concentration. In contrast, at the 400 μM cyclic nucleotide concentration, theophylline, but not 3-methylxanthine, inhibited cyclic AMP more than cyclic GMP metabolism (Fig. 4). Thus, comparing the effects of 0.5 mM theophylline and 3-methylxanthine, theophylline and 3-methylxanthine inhibited cyclic AMP hydrolysis 48 and 25 per cent and cyclic GMP hydrolysis 8 and 20 per cent respectively. It appears, therefore, that at a 400 μM substrate concentration, theophylline is a better inhibitor of cyclic AMP hydrolysis than is 3-methylxanthine, but the latter compound is a better inhibitor of cyclic GMP hydrolysis than is theophylline.

DISCUSSION

A composite of proposed degradative pathways for the methylxanthines is shown in Fig. 5. Depending upon the particular xanthine ingested, most of the various metabolites except 3-methyluric acid have been recovered from man. The appearance of trace quantities of 1,3,7-trimethyluric and 3-methyluric acid in rat urine after caffeine administration has been reported by Khanna *et al.* [20]. Macht and Ting [21] recognized the desirability of testing the antispasmodic effect of the monomethylxanthines on the bronchi, but such compounds were unavailable at that time.

The findings reported here show that 3-methylxanthine and theophylline have approximately equal effects in relaxing guinea pig trachea and in stimulating cardiac muscle. In addition, the present observation that 3-methylxanthine inhibits cyclic nucleotide phosphodiesterase establishes yet another pharmacological activity shared by this metabolite and theophylline. 3-Methylxanthine was only slightly less active than theophylline as an inhibitor of both cyclic AMP and cyclic GMP phosphodiesterase when a 1.4 μM substrate concentration was used. However, at a 400 μM cyclic nucleotide

concentration, theophylline was more active than 3-methylxanthine as an inhibitor of cyclic AMP phosphodiesterase whereas 3-methylxanthine was more active than theophylline as an inhibitor of cyclic GMP phosphodiesterase. The potency ratio for theophylline and 3-methylxanthine as inhibitors of cyclic AMP and cyclic GMP hydrolysis using a 1.4 μM substrate concentration was approximately the same as the ratio for their relaxing activities in tracheal smooth muscle. However, for both compounds approximately five times higher concentrations were required to inhibit phosphodiesterase activity than to relax the smooth muscle tissue, suggesting the possible contribution of amplification mechanisms in the sequence of reactions between cyclic AMP and its ultimate effect on contractile elements [18]. Of the other monomethylxanthine and methyluric acids tested, the effects of 1-methylxanthine are of particular interest. Like theophylline and 3-methylxanthine, 1-methylxanthine was also capable of completely relaxing tracheal smooth muscle and, in addition, was as active as caffeine in causing an increase in cardiac contractile force. Although 1,3-dimethyluric acid and 3-methyluric acid both possess the ability to at least partially relax tracheal smooth muscle and 1,3-dimethyluric acid also stimulates cardiac muscle, the effective concentration of these compounds was considerably higher than that of 1-methylxanthine. In addition, no particular activity of 1-methyluric acid on cardiac muscle could be demonstrated.

Of the major methylxanthines, theophylline is of particular therapeutic importance in the management of chronic obstructive airway disease. Significant variations in the serum half-life of theophylline exist in patients, and it has been suggested that the half-life differences are related to the rate and/or pathway of theophylline degradation [22, 23]. In man, the major urinary excretion products of theophylline are 1,3-dimethyluric acid, 1-methyluric acid and 3-methylxanthine [9]. Thus, the metabolite found in the present study to be almost equipotent with theophylline occurs in man. Therefore, attempts to adjust the plasma concentration for optimum theophylline dosage may require quantitation of 3-methylxanthine as well.

In addition, other situations exist where the knowledge of the presence of an active metabolite may be of critical importance. The elimination of 3-methylxanthine and other metabolites is dependent on adequate renal function, whereas elimination of theophylline is primarily dependent on hepatic metabolism. In patients with impaired renal function but normal hepatic function, 3-methylxanthine may accumulate and contribute to the effects of administered theophylline. Potentialy toxic reactions due to additive effects of the two xanthines may occur even though a patient's serum theophylline levels were maintained within the recommended range of 8–20 μg/ml [22]. The observation of Lohmann and Miech [24] on the effect of allopurinol on theophylline metabolism in the rat represents yet another consideration. These investigators showed that, whereas 1-methyluric acid was a product of theophylline metabolism in the control rat, 1-methylxanthine was produced in the presence of allopurinol. They concluded that theophylline was *N*-demethylated to 1-methylxanthine by the hepatic microsomal drug-metabolizing enzyme system and then 1-methylxanthine was converted to 1-methyluric acid by xanthine oxidase. This latter step is blocked by allopurinol. Such a drug interaction could result in plasma levels of an active rather than an inactive metabolite. Furthermore, variations in the plasma concentrations of active metabolites may also be expected if the rate of theophylline disposition is altered. Indeed, the serum half-life of theophylline has been shown to be affected by agents known to induce the hepatic microsomal enzyme system [24–27].

The knowledge that metabolites of the methylxanthines possess pharmacological activity may also help to interpret some of the current discrepancies between the observed pharmacological and biochemical activities of these agents. For example, higher concentrations of theophylline *in vitro* are required to inhibit phosphodiesterase than are the documented plasma levels of theophylline which appear to be associated with symptomatic relief in obstructive lung disease [22, 23, 28]. Since 3-methylxanthine is now also shown to inhibit phosphodiesterase, an additive effect *in vivo* with that of theophylline might be one possible explanation for the differences between enzymatically and therapeutically effective concentrations of theophylline.

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