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EFFECTS OF TRIETHYLCHOLINE ON THE OUTPUT OF ACETYLCHOLINE FROM THE ISOLATED DIAPHRAGM OF THE RAT

BY

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Triethylcholine [triethyl(2-hydroxyethyl)ammonium] produces a failure of neuromuscular transmission; the rate of onset and final magnitude increase with increasing frequency of stimulation. At the time of maximal depression of the twitches, contractions of the muscle produced by close-arterially injected acetylcholine are not depressed, suggesting that the site of the transmission failure is prejunctional (Bowman & Rand, 1961b; Bowman, Hemsworth & Rand, 1962a). Triethylcholine inhibits the synthesis of acetylcholine by the choline acetyltransferase in organized brain tissue (Bull & Hemsworth, 1963) but has very little effect on acetylcholine synthesis when the particles containing the enzyme are disrupted (Hemsworth & Morris, 1964). The effects of triethylcholine on neuromuscular transmission and on acetylcholine synthesis are reversed by an excess of choline. These results suggest that triethylcholine acts like hemicholinium which is believed to inhibit acetylcholine synthesis by preventing the transport of choline to the sites of its acetylation by choline acetyltransferase (MacIntosh, Birks & Sastry, 1956; Schueler, 1960; Gardiner, 1961).

The neuromuscular transmission failure produced by triethylcholine is often preceded by a slight increase in the amplitude of maximal indirectly elicited twitches, and it has been suggested that the drug produces an initial increase in the release of acetylcholine (Roberts, 1962; Bowman *et al.*, 1962a) which is followed by a decreased release when synthesis is impaired (Bowman *et al.*, 1962a). In the experiments described in this paper, the effects of triethylcholine on contractions of, and on acetylcholine output from the rat phrenic nerve–hemidiaphragm preparation have been studied simultaneously.

Hayes & Riker (1963) have recently cast some doubt on the acetylcholine theory of neuromuscular transmission. According to them, the directly stimulated chronically denervated hemidiaphragm releases the same amount of acetylcholine as the indirectly stimulated contralateral muscle, suggesting that skeletal muscle, rather than motor nerve, may be the source of acetylcholine. It was therefore of interest, if these observations could be repeated, to compare the effects of triethylcholine on innervated and denervated muscles.

METHODS

The experiments were carried out on forty-two albino rats (Wistar strain) weighing 150 to 200 g.

Innervated rat diaphragm. The rat was killed by a blow on the head, and a hemidiaphragm together with its phrenic nerve was removed and placed in Tyrode solution at room temperature while superficial connective tissue and all but a small portion of rib were removed. The Tyrode solution had the following composition in g/litre: NaCl 8.0, KCl 0.2, $CaCl_2$ 0.2, $MgCl_2$ 0.1, NaH_2PO_4 0.1, $NaHCO_3$ 1.0 and glucose 1.0. The hemidiaphragm was attached to a glass muscle holder by threads tied round the rib. When acetylcholine was to be collected, the muscle and its holder were fitted into a small Perspex organ-bath (total volume 3 ml.) which was suspended on the surface of a heated water-bath at 38° C. The organ-bath contained 2 ml. of Tyrode solution which was continuously bubbled with oxygen and maintained at a constant temperature of about 37° C. The central tendon of the diaphragm was attached to a spring-loaded lever and contractions were recorded on a kymograph. Twitches and tetani of the diaphragm were elicited by stimulation of the phrenic nerve with rectangular pulses of 100 μsec duration and of two- to three-times the strength required to evoke a maximal twitch. Stimulating electrodes of the type described by Burn & Rand (1960) were used.

At the start of each experiment, the Tyrode solution was removed by suction and replaced by 2 ml. of Tyrode solution containing 5 μg/ml. of physostigmine or neostigmine. The nerve was then stimulated at a frequency of 1 shock/sec for 1 hr. At the end of this period stimulation was stopped and the tissue was washed with 2 ml. of Tyrode solution containing anticholinesterase. The bath was then refilled with 2 ml. of the same solution and the nerve was stimulated for 15 min at a frequency of 1, 5, 10 or 20 shock/sec. Stimulation was then stopped for 2 min to allow the acetylcholine to diffuse out of the tissue (Krnjević & Mitchell, 1961). This period of 15 min stimulation followed by 2 min rest was constant throughout all experiments and is subsequently referred to as the collection period. The bath fluid was removed by suction and one drop of B.D.H. Universal Indicator was added to it. Sufficient N-hydrochloric acid was added to render the solution pink/red at *p*H 4 and it was set aside for assay. The bath was refilled with Tyrode solution containing anticholinesterase and, in control experiments, the whole procedure was repeated at least eight times at hourly intervals. During the 43 min between each collection period, in all experiments, the preparation was stimulated at 1 shock/sec and the bath fluid was replaced with fresh Tyrode solution containing anticholinesterase (and other drugs where appropriate) every 20 min to avoid the accumulation of muscle metabolites in the small organ-bath.

When the effects of triethylcholine and choline were studied, frequencies of stimulation of 1, 5 and 20 shocks/sec were used, but throughout any one experiment the frequency during each collection period was the same. The procedure incorporated the points mentioned above and the experiments were carried out according to the following design. At least two control samples were collected at hourly intervals in the absence of triethylcholine. Triethylcholine was added 2 min after the start of the next collection period to observe any initial effect on acetylcholine release. Subsequent replacements of the bath fluid contained the same concentration of triethylcholine. When the frequency for the collection period was 1 or 5 shocks/sec a second sample was taken 1 hr later. When 20 shocks/sec was used, a third sample was taken after a further hour had elapsed. At 35 min before the next collection period, choline was added to the bath without removing the triethylcholine. Following this collection period, the tissue was washed twice and the bath fluid was replaced with Tyrode solution containing anticholinesterase only. A final sample was collected after the usual time interval. Triethylcholine and choline were dissolved in Tyrode solution containing anticholinesterase such that the required dose was contained in 0.1 ml. Allowance was made for this small increase in the volume of the bathing fluid in calculating the release of acetylcholine in the presence of these drugs.

Chronically denervated rat diaphragm. The left phrenic nerve of fifteen rats was sectioned aseptically during ether anaesthesia about 1 cm from the diaphragm, through a small incision made in the intercostal muscles. The rats were killed 10 to 12 days later and the chest was opened. Stimulation of the right nerve *in situ* evoked a clear twitch of the corresponding half of the diaphragm but stimulation of the left nerve was without effect on this muscle which was clearly fibrillating. Both phrenic nerves and the whole diaphragm were removed from the animal and then separated into halves.

When acetylcholine collections were not made, both hemidiaphragms were set up in separate 50-ml. organ-baths and the contractions were recorded on a kymograph. When acetylcholine collections were to

be made, both hemidiaphragms were studied simultaneously, each mounted in a separate small organ-bath. The chronically denervated muscles were stimulated directly by means of two fine silver wires; one was coiled round the tissue holder and made contact with the intercostal margin, the other was inserted through the muscle at its tendinous junction and held in position by the thread attached to the recording lever. Contractions were elicited by rectangular pulses of 100 μsec duration and supramaximal strength. Both hemidiaphragms were treated simultaneously in exactly the same way. The design of the experiments for acetylcholine collection was the same as that already described. In several experiments, samples were collected in the absence of any anticholinesterase.

Assay methods. The following assay preparations were used: (1) The guinea-pig ileum suspended in 2 ml. of Tyrode solution at 32° C containing physostigmine (25 μg/l.) and morphine sulphate (10 mg/l.) after the method of Paton (1957). (2) The frog rectus abdominis muscle suspended in a 1-ml. bath of frog-Ringer solution containing physostigmine (10 μg/ml.) as described by Hayes & Riker (1963). In our hands, the limit of sensitivity of this preparation was 10 ng of acetylcholine. The less-sensitive preparations were improved by the addition of 0.5 to 1 mg/ml. of adenosine triphosphate to the Ringer solution (Torda & Wolff, 1946) but on the whole this preparation was found insufficiently sensitive and was abandoned in favour of the other two. (3) The dorsal muscle of the leech suspended in 1 ml. of leech solution (g/l.: NaCl 7.0, KCl 0.33, $CaCl_2$ 0.18 and $NaHCO_3$ 0.12) containing physostigmine (10 μg/ml.) as described by MacIntosh & Perry (1950). This preparation was used most often but when choline was present in the assay solution the guinea-pig ileum was preferred because choline itself caused a small contraction of the leech muscle.

Standard solutions of acetylcholine chloride were prepared in Tyrode solution containing one drop of B.D.H. Universal Indicator per 2 ml. of solution. Where appropriate the standard solutions also contained concentrations of anticholinesterase, triethylcholine and choline the same as those present in the test samples. Immediately before assay, the test solutions were neutralized to the same *p*H as the standards. The solutions were assayed directly on the guinea-pig ileum preparations. For assays on the leech muscle, 0.2 to 0.5 ml. of test and standards were each made up to 1 ml. with leech solution before assay. The whole of these 1-ml. volumes were added to the leech muscle. Apart from the slight effect of choline on the leech muscle already described, standard solutions prepared with the acetylcholine omitted did not cause contractions of the assay preparations. Triethylcholine exerted a weak atropine-like action on the guinea-pig ileum preparation, but this affected both standard and test solutions equally. The assays were carried out by matching responses of standard and test solutions.

In most experiments tests were made to check that the substance present was acetylcholine. The tests used were: (1) the omission of anticholinesterase from the diaphragm chamber; (2) abolition of both test and standard responses after 1 drop of 0.5 N-sodium hydroxide solution had been added to each ml. of the solutions, which were then allowed to stand for 30 min before being neutralized with N-hydrochloric acid; (3) block of standard and test responses by atropine (20 ng/ml.) in the guinea-pig ileum and by tubocurarine (4 to 6 μg/ml.) in the leech muscle.

The drugs used were triethylcholine chloride (Ward Blenkinsop), choline chloride (B.D.H.), acetylcholine chloride (Roche), neostigmine methylsulphate (Roche), physostigmine salicylate (B.D.H.), tubocurarine chloride (Burroughs Wellcome) and atropine sulphate (B.D.H.). With the exception of acetylcholine, the concentrations of these drugs are given in terms of the salts.

RESULTS

In the absence of triethylcholine, the amount of acetylcholine released from the indirectly stimulated diaphragms remained constant, for a given frequency of stimulation, during at least eight collections, that is over a period of at least 8 hr. Table 1 gives the mean amounts of acetylcholine released during stimulation at different frequencies. The results agree fairly closely with those of Straughan (1960) and Krnjević & Mitchell (1961).

Table 1 combines the results of assays on the guinea-pig ileum and on the leech muscle. There was general agreement between the results obtained with these two assay preparations, although the leech muscle gave figures for acetylcholine about 10% lower than those for the

guinea-pig ileum. There was no difference between the results obtained using neostigmine or physostigmine in the diaphragm bath, but physostigmine was the anticholinesterase used in most experiments because it was less likely to cause irreversible spasm of the ileum.

TABLE 1

RELEASE OF ACETYLCHOLINE (AS CATION) BY DIFFERENT FREQUENCIES OF STIMULATION

Figures in parentheses show the number of hemidiaphragms from which each result is calculated

Stimulus frequency (shocks/sec)	Release of acetylcholine: Total in 15 min (ng)	Release of acetylcholine: Mean per impulse (pg)
1	12 (16)	13·3
5	20 (6)	4·4
10	25 (4)	2·8
20	32 (18)	1·8

Anticholinesterase drugs antagonize the effects of triethylcholine (Bowman, Hemsworth & Rand, 1962b). Since physostigmine or neostigmine was present in the bath fluid it was necessary to use larger concentrations of triethylcholine (0.5 mg/ml.) than those normally found effective (Bowman *et al.*, 1962a). The concentration of choline chloride used (125 μg/ml.) was correspondingly increased. The histograms in Fig. 1 express the mean

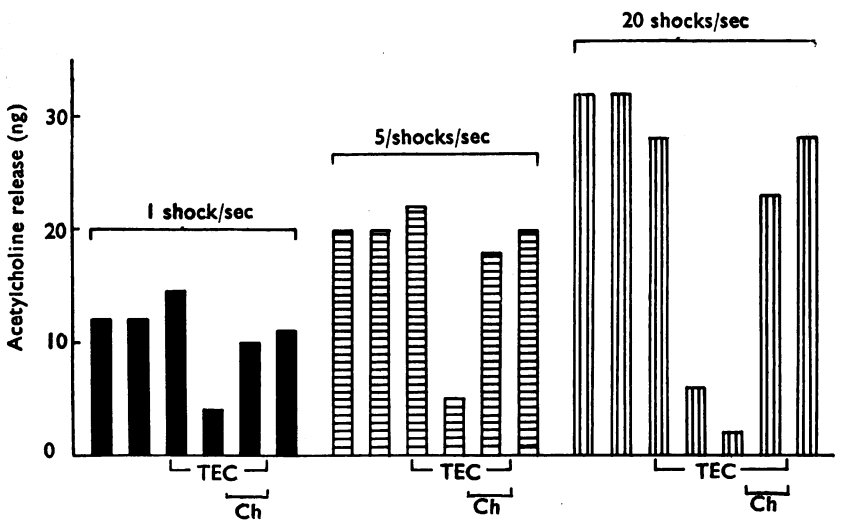

Fig. 1. Each column represents the acetylcholine (as cation) released during a 15-min period of nerve stimulation at the frequencies shown (ordinates in nanograms). Collections made in the presence of triethylcholine and choline are marked TEC and Ch respectively. The last column for each frequency represents the release after washing out both triethylcholine and choline.

results of the assays from all experiments. The slight initial potentiation of the twitches produced by triethylcholine was accompanied by a small increase in the amount of acetylcholine released which was detectable when frequencies of 1 or 5 shocks/sec were used during the collection periods, but not when 20 shocks/sec was used. After 1 hr, when the

twitches were completely blocked, acetylcholine output was depressed by from 66 to 81% depending on the stimulus frequency used during the collection period. After a further hour in the presence of triethylcholine the acetylcholine release was depressed by over 90% when a stimulus frequency of 20 shocks/sec was used. The addition of choline restored the acetylcholine release to 72 to 90% of the control value at the start of the experiment; when both triethylcholine and choline were washed out of the tissue, release was restored to 82 to 100% of the original control value.

Large amounts of choline may block the motor end plates by a depolarizing action (Hutter, 1952), but this effect is not normally evident with the amounts of choline necessary to restore twitches of the rat diaphragm depressed by triethylcholine (Bowman *et al.*, 1962a). However, the amounts of choline used in the present experiments were larger than usual and, although choline restored the release of acetylcholine, the twitches were rarely increased in size probably because, under the conditions of the experiments, the postjunctional blocking action of choline masked its effect at the nerve endings.

Chronically denervated muscles. Direct stimulation of skeletal muscle has been shown to produce normal contractions when applied at the height of the block of indirectly elicited contractions produced by triethylcholine (Bowman & Rand, 1961b). However, the effect of triethylcholine on contractions elicited by continuous direct stimulation has not been previously studied.

Triethylcholine chloride, in a concentration of 250 to 300 μg/ml., caused an initial slight potentiation followed by a slowly developing block of the twitches elicited by indirect stimulation but, apart from a small and short-lasting contracture immediately after its

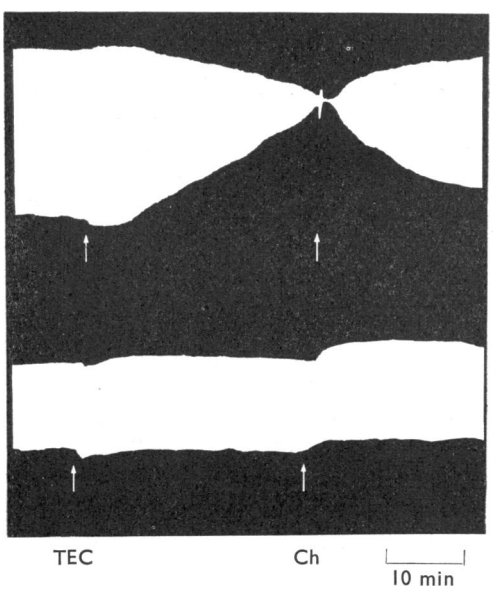

Fig. 2. Maximal twitches of both isolated hemidiaphragms from the same rat elicited at a frequency of 1 shock/sec. Upper trace, innervated right hemidiaphragm stimulated through its phrenic nerve. Lower trace, chronically denervated left hemidiaphragm stimulated directly. At TEC, 250 μg/ml. of triethylcholine chloride and at Ch, 75 μg/ml. of choline chloride were added to each bath.

addition, was usually completely without effect on the twitches of the contralateral denervated hemidiaphragm. In a few experiments the addition of triethylcholine was followed by a small and gradually developing increase in the amplitude of the twitches of the denervated muscle but, even when the concentration was doubled and the drug left in contact with the tissue for several hours, there was no depression of the denervated muscle contractions. Choline chloride in a concentration of 75 μg/ml. restored the contractions of the indirectly stimulated muscle and produced a small contracture of the denervated muscle, but was without any other effect. Fig. 2 illustrates the effects of triethylcholine and choline on innervated and denervated hemidiaphragms.

In experiments on acetylcholine release both innervated and denervated hemidiaphragms from the same rat were studied simultaneously but mounted in separate baths. They were subjected to the same pattern of stimulation as that already described and Table 1 and Fig. 1 include the results obtained from the innervated halves.

A substance which caused contraction of the leech muscle was released from the stimulated denervated diaphragm. When assayed in terms of acetylcholine the quantity of this substance, released during stimulation at 20 shocks/sec for 15 min, was less than the equivalent of 3 ng. The substance did not appear to be acetylcholine since it was not destroyed by treatment with alkali and it did not require the presence of an anticholinesterase for its detection. Furthermore, the response of the leech muscle to this substance was more

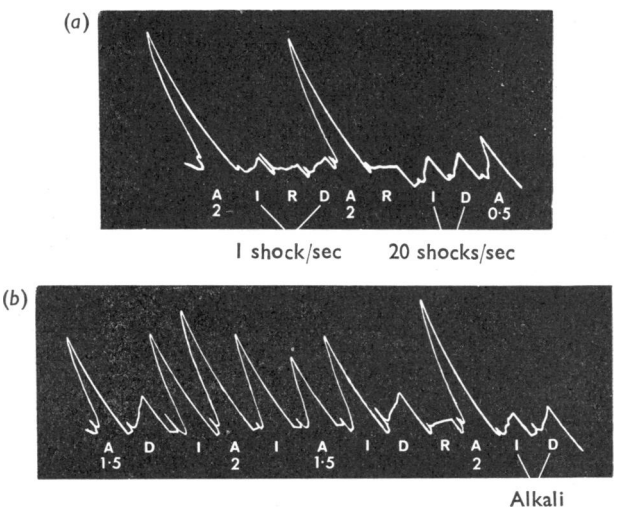

Fig. 3. Responses of leech muscle assay preparations to acetylcholine chloride at A, to 0.2 ml. volumes of fluid which had bathed stimulated innervated or denervated diaphragms at I and D respectively, and to 0.2 ml. volumes of fresh Tyrode solution at R. The numbers denote the amounts of acetylcholine chloride in nanograms. In (*a*) the Tyrode solution which had bathed the diaphragms, and the fresh Tyrode solution (R), did not contain anticholinesterase. After stimulation at 20 shocks/sec, 0.2 ml. of the fluids which had bathed both innervated and denervated muscles caused contractions of the leech muscle which were smaller than the response to 0.5 ng of acetylcholine chloride. (*b*) Shows part of an assay of solutions which had bathed innervated and denervated diaphragms stimulated at 1 shock/sec in the presence of physostigmine. The fresh Tyrode (R) also contained physostigmine. The last two responses are to solutions which had been treated with alkali. The acetylcholine in I was destroyed and the response to it now resembled that to D.

gradual than that to acetylcholine. The same substance was present in Tyrode solution which had bathed indirectly stimulated preparations; it could be detected after destroying the acetylcholine with alkali treatment or when acetylcholine was absent because of the omission of anticholinesterase from the bath fluid. The amount of the substance present increased slightly with increase in the frequency of stimulation. It was absent from solutions which had bathed an indirectly excited muscle which was completely paralysed by triethylcholine, and its presence, therefore, appeared to be the result of muscle contraction. Fig. 3, *a* illustrates responses of the leech muscle preparation to solutions which had bathed stimulated innervated and denervated hemidiaphragms not treated with anticholinesterase. Fig. 3, *b* shows part of the bioassay of solutions collected from innervated and denervated hemidiaphragms treated with physostigmine. After alkali treatment, the response of the leech muscle to the fluid from the innervated preparation resembled that to the fluid from the denervated muscle.

Tubocurarine (5 μg/ml.) blocked the responses of the leech muscle to acetylcholine, to the fluid from the innervated preparation stimulated in the presence of physostigmine, and to the fluid from the innervated or denervated preparations stimulated in the absence of anticholinesterase. This large amount of tubocurarine was necessary to block the response of the leech muscle to acetylcholine and this drug could not, therefore, be used to distinguish acetylcholine from whatever substance was released from the contracting muscle. Since the amount of this substance was small, no corrections were made for it in the figures referring to acetylcholine release. The error involved could not have been greater than 10%.

DISCUSSION

The experiments support the previous suggestions, made on the basis of indirect evidence, that the effects of triethylcholine on neuromuscular transmission arise chiefly from changes in acetylcholine output from the nerve endings (Bowman & Rand, 1961a, b; Roberts, 1962; Bowman *et al.*, 1962a). The initial slight increase in twitch tension caused by repetitive firing of some muscle fibres (Bowman *et al.*, 1962a) may be explained by an increased output of acetylcholine. The rapid onset of this effect suggests that it is due to an action on the transmitter release mechanism rather than on synthesis. The closely related drug, tetraethylammonium, has been shown to produce a similar effect (Collier & Exley, 1963).

The secondary slowly developing depression of contractions, which is dependent on the frequency of stimulation, was accompanied by a reduced output of acetylcholine and may be largely explained on this basis. Bull & Hemsworth (1963) have shown that triethylcholine depresses the synthesis of acetylcholine by organized nervous tissue and this effect, occurring at the motor nerve terminals, probably accounts for the decreased output of transmitter. Choline restored the output of acetylcholine and, in muscles not previously treated with anticholinesterase, choline also reverses the depression of contractions. Furthermore, choline antagonizes the inhibitory action of triethylcholine on acetylcholine synthesis (Bull & Hemsworth, 1963). The results obtained with triethylcholine therefore indicate that it interrupts neuromuscular transmission in the same way as does hemicholinium (MacIntosh *et al.*, 1956; Schueler, 1960; Gardiner, 1961).

The maximal twitches of the diaphragm were completely abolished when acetylcholine output was still about 35% of normal. Two factors probably contributed to this apparent

discrepancy. Firstly, endplate potential is more directly related than is contraction to transmitter release, and contraction will fail while there is still sufficient release to produce sub-effective endplate potentials. Secondly, triethylcholine possesses a weak curare-like action (Bowman *et al.*, 1962a). While this action is too weak to be of importance when transmission is normal, it will become relatively more important as acetylcholine output diminishes and any safety margin in transmitter release disappears.

Experiments on chronically denervated muscle showed that triethylcholine did not depress contractions elicited by direct stimulation. This result indicates either that the small amount of acetylcholine released from chronically denervated muscles (Straughan, 1960; Mitchell & Silver, 1963) is unimportant in the contraction process, or that the synthesizing mechanism for this acetylcholine is not susceptible to inhibition by triethylcholine. We did not attempt to detect the small amounts of acetylcholine released from resting denervated muscles and any acetylcholine released from contracting denervated muscles was masked by the presence of some other substance which caused contraction of the assay preparations. This substance was shown not to be acetylcholine but no attempt at identification was made.

We were unable to confirm the observations of Hayes & Riker (1963) that directly stimulated denervated muscle releases as much acetylcholine as indirectly excited muscle. Krnjević & Straughan (1964) and B. Collier (personal communication) have recently obtained the same result as we have, and we therefore support their observations, as well as the original findings of Dale, Feldberg & Vogt (1936) on perfused cat muscles.

SUMMARY

1. The effects of triethylcholine on the output of acetylcholine from isolated phrenic nerve-hemidiaphragm preparations of rats have been studied.

2. Triethylcholine caused an initial small increase in acetylcholine output which was followed by a decrease of up to more than 90% depending upon the frequency of stimulation of the nerve.

3. The effects on acetylcholine output paralleled the effects on the twitches of the diaphragm. Choline, which restores contractions depressed by triethylcholine, also restored the output of acetylcholine.

4. Direct stimulation of chronically denervated muscles did not cause the release of detectable amounts of acetylcholine, and triethylcholine did not depress the contractions of these muscles.

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