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SENSITIVITIES OF (Na$^+$-K$^+$)-ATPase AND Na$^+$ EXTRUSION MECHANISMS TO OUABAIN AND ETHACRYNIC ACID IN THE CORTEX OF THE GUINEA-PIG KIDNEY*

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SUMMARY

1. The effects of ouabain and ethacrynic acid on the transport of ions by cortex slices from guinea-pig kidney have been studied and compared with their effect on a microsomal (Na$^+$-K$^+$)-ATPase preparation from the same tissue.

2. The extrusion of Na$^+$ accompanied by Cl$^-$ is almost insensitive to ouabain, whereas the exchange of Na$^+$ for K$^+$ is 50 % inhibited at an ouabain concentration that is only slightly higher than that needed to inhibit the (Na$^+$-K$^+$)-ATPase by 50 %. The total inhibition of the Na$^+$–K$^+$ exchange is accomplished at the same drug concentration as that required to inhibit the (Na$^+$-K$^+$)-ATPase completely.

3. On the other hand, $2 \cdot 10^{-3}$ M ethacrynic acid has only a slight effect on the Na$^+$–K$^+$ exchange, whereas it completely inhibits the extrusion of Na$^+$ accompanied by Cl$^-$. The level of ethacrynic acid required to inhibit the (Na$^+$-K$^+$)-ATPase half maximally is 100 times greater than that of ouabain.

4. The residual ATPase activity in the absence of Na$^+$ and K$^+$ but in the presence of Mg^{2+} is completely insensitive to ouabain, but is nevertheless inhibited by high doses of ethacrynic acid.

5. The results suggest that two pumps are involved in Na$^+$ extrusion from the kidney cortex cell. One involves exchange for external K$^+$ and derives its energy from the (Na$^+$-K$^+$)-ATPase. The other, which should be most effective in cell volume regulation, expels Na$^+$ accompanied by Cl$^-$ without the involvement of (Na$^+$-K$^+$)-ATPase.

INTRODUCTION

The renal tubular cells are known to transport important quantities of Na$^+$ from the tubular lumen to the interstitial space. Numerous studies of the electrochemical potential profile have contributed to our understanding of the mechanisms

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responsible for the transtubular movement of Na^+ (refs. 1–6). From these studies, a model has emerged according to which Na^+ enters the tubular cell passively across the luminal membrane following its electrochemical gradient, and is then extruded by an energy-requiring mechanism situated in or very close to the peritubular membrane[1,4,6–11].

In a large variety of tissues, it has been demonstrated that the extrusion of Na^+ from the cell is coupled to the uptake of K^+ from the extracellular space[12,13]. Cl^- movement has previously been considered to be secondary to the fluxes provoked by such a pump. However, WHITTEMBURY AND PROVERBIO[14] recently demonstrated that 1 mM ouabain strongly inhibited the Na^+–K^+ exchange in cortex slices from guinea-pig kidney, but had little effect on the extrusion of Na^+ accompanied by Cl^- and water; on the other hand, 2 mM ethacrynic acid principally inhibited the Na^+ extruded with Cl^- and water, but only marginally affected the Na^+–K^+ exchange. These results led WHITTEMBURY AND PROVERBIO to postulate the existence of two separate Na^+ 'pumps' to remove excess Na^+ from the intracellular space, namely one pump that exchanged intracellular Na^+ for extracellular K^+, and the second that extruded Na^+ accompanied by Cl^- and water, thus being an important mechanism for volume regulation. This hypothesis was confirmed by results obtained by perfusing toad kidneys *in vivo* and observing the effect of the two drugs[15]. The existence of two different Na^+ pumps within the same cell has also been postulated in other cell types, namely the erythrocyte[16], the toad urinary bladder[17], and the intestinal mucosa[18]

Since the pioneering work of SKOU[19], much evidence has accumulated to implicate a Na^+-and-K^+-activated ATPase ((Na^+-K^+)-ATPase) in the transport of ions across biological membranes [12,13,20]. The activity of this enzyme is inhibited by ouabain[21,22] and furthermore a good correlation between the ouabain sensitivities of the ion transport system and of the (Na^+-K^+)-ATPase has been demonstrated in various cells[23,24]. In addition, cells that are unable to maintain a differential ionic gradient tend to lack this enzyme[25]. DUGGAN AND NOLL[26] have demonstrated an inhibitory effect of ethacrynic acid on the same enzyme, but others[27] have cast doubt on the physiological significance of this finding.

However, if the (Na^+-K^+)-ATPase was the only system capable of providing the energy necessary for ion transport, it would be expected that inhibition of this enzyme complex would inhibit all Na^+ transport, *i.e.* Na^+–K^+ exchange and $Na^+ + Cl^-$ movement. Since this is not so, the possibility must be raised that other energy supplies are involved in bulk Na^+ transport in the kidney, as KESSLER *et al.*[28] have suggested. In an attempt to clarify this matter, we have determined the dose–response curves for the effect of ouabain and ethacrynic acid on the two modes of Na^+ extrusion from kidney cortex slices and on a microsomal preparation of (Na^+-K^+)-ATPase from the same tissue; the results indeed suggest that there may be two sources of energy involved in Na^+ extrusion from this cell.

MATERIALS AND METHODS

Source of inhibitors

Ouabain (Strophanthin-G) was purchased from the Sigma Chemical Company, St. Louis, U.S.A., and ethacrynic acid was generously provided by Merck, Sharp and Dohme, Rahway, N. J., U.S.A.

Preparation of kidney slices

The methods used to prepare the tissue have been described in detail elsewhere[14]. The experimental protocols and the physiological solutions employed were the same as in the previous work[14].

Immediately after preparation, the slices were preincubated with shaking (120 rev./min) for 2 h in a K$^+$-free medium at 0.5°. During this treatment, the cells take up Na$^+$ and Cl$^-$ and lose a considerable quantity of K$^+$ until equilibrium is reached. This treatment was followed by a second incubation of 30 min under similar conditions but with the addition of the appropriate inhibitor at the desired concentration. Finally, the tissues were incubated for 45 min (sufficient to attain equilibrium) at 25° in the same solution (in certain experiments with the addition of K$^+$); at this temperature, the control samples, incubated without external K$^+$, extrude Na$^+$ accompanied by Cl$^-$, and in the presence of external K$^+$, they are able to exchange intracellular Na$^+$ for this ion in addition to that extruded with Cl$^-$ (refs. 7 and 14). Throughout the incubations, the solutions were gassed with O_2–CO_2 (95:5, v/v).

For analysis, the slices were dried on filter paper, weighed, desiccated for 24 h at 100°, and re-weighed. They were then shaken for 24 h in 2 ml 1 M HNO_3, and the Na$^+$, K$^+$, and Cl$^-$ concentrations of the resulting extracts were determined. The cation concentrations were measured in a Baird atomic flame photometer (Model XY-2) with an internal lithium standard[29]. Cl$^-$ was determined by potentiometric titration[4,14].

Preparation of a (Na$^+$-K$^+$)-ATPase

The techniques used followed those employed by ROBINSON[18] for the intestinal mucosa. The outermost slices obtained as described[14] were homogenized at 0° in a solution of 0.25 M sucrose containing 20 mM Tris–HCl buffer (pH 7.2) and 1 mM EDTA (10 ml/g tissue). The homogenate was centrifuged for 20 min at 3000 × *g*, the sediment resuspended in the same volume of sucrose–Tris–EDTA and recentrifuged at the same speed. The two supernatants were then combined and recentrifuged for 1 h at 100000 × *g*. The sediment from this last centrifugation was suspended in 5 ml of the sucrose–Tris–EDTA solution, and used for the study of the ATPase activity. Electron micrographs confirmed that this preparation consisted almost exclusively of microsomes.

Assay of ATPase activity

Aliquots of the enzyme suspension were incubated for 10 min in the presence of 100 mM imidazole buffer (pH 7.2), 5 mM $MgCl_2$ and 2 mM Na$^+$-free Tris-ATP (Sigma Co.). When appropriate, 100 mM NaCl and 10 mM KCl were added. The inhibitors, ouabain and ethacrynic acid, were also added to certain samples at concentrations dictated by the experiment in question. The enzyme was only exposed to ouabain during the 10-min incubation period, but with ethacrynic acid, it was necessary to preincubate the enzyme with the inhibitor for 10 min before adding the ATP, as has been previously pointed out by DUGGAN AND NOLL[26]. Preliminary experiments demonstrated that under such conditions, a linear relationship existed between the liberation of phosphate and the time of incubation. The experiments were carried out at 37 and at 25°, but no difference was found between the results obtained at the different temperatures.

The incubation was terminated by the addition of 1 ml of 6% $HClO_4$, and the liberated phosphate was determined in the deproteinized solution by the method of KING[30]. The protein content of the original suspension was measured by means of the Folin reagent[31].

RESULTS

Inhibition of ATPase activity

Two components of ATPase activity were determined in the microsomal suspension of guinea-pig kidney cortex, namely that activated by Mg^{2+} alone (residual ATPase) and that activated by Na^+ and K^+ in addition to Mg^{2+} (total ATPase). The difference represents the (Na^+-K^+)-ATPase. The mean total ATPase activity was 165 ± 3 nmoles phosphate liberated per min·mg protein, whereas the mean residual ATPase activity was 84 ± 6 (S.E.M.; $n = 6$ in each group).

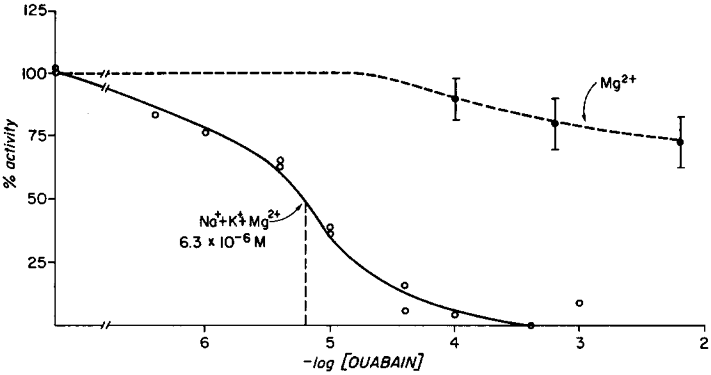

Fig. 1. Dose-response curve of the inhibition of residual ATPase (●) and (Na^+–K^+)-ATPase (○) by ouabain. The vertical lines represent ± 1 S.E.M.

In Fig. 1, the effect of different concentrations of ouabain on the activities of the ATPase components is presented. The residual ATPase remained practically unaffected by this drug. However, the (Na^+-K^+)-stimulated component was 50% inhibited by a concentration of $6.3 \cdot 10^{-6}$ M ouabain, and was completely inhibited by $1 \cdot 10^{-4}$ M.

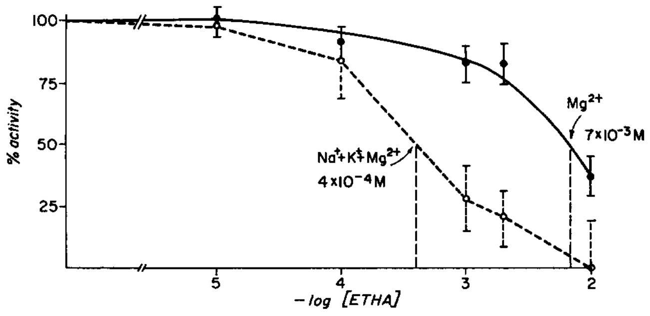

Fig. 2. Dose-response curve of the inhibition of residual ATPase (●) and the (Na^+-K^+)–ATPase (○) by ethacrynic acid (ETHA).

On the other hand, ethacrynic acid (Fig. 2) had a markedly different effect on the ATPases. The residual ATPase activity was half maximally inhibited by a concentration of $7 \cdot 10^{-3}$ M ethacrynic acid, whereas the (Na^+-K^+)-stimulated component was 50 % inhibited by $4 \cdot 10^{-4}$ M, and almost completely inhibited by $1 \cdot 10^{-2}$ M ethacrynic acid. These values agree with those reported by other authors[26].

Inhibition of tissue ion movements

It was previously demonstrated[7,10,14,32–34] that on being rewarmed in a K^+-free medium, kidney cortex slices extrude an important quantity of Na^+ and Cl^- and lose a small amount of K^+. It has also been found[7,14,34,35] that when K^+ is present in the incubation medium, the slices extrude practically the same quantity of Na^+ and Cl^-, but also exchange a considerable quantity of intracellular Na^+ for external K^+, experiencing a net gain of the latter ion.

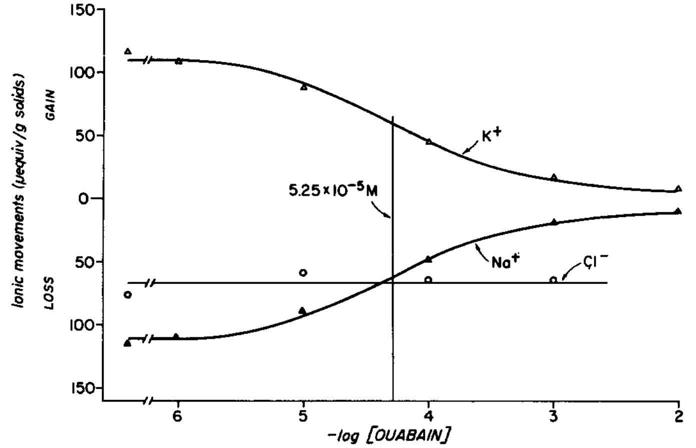

Fig. 3. Effect of different ouabain concentrations on the changes in cellular ionic content of cortex slices rewarmed in the presence of 16 mM K^+. Cellular uptake is represented by values above, and cellular loss by values below, the zero line. ○, movements of Na^+ accompanied by Cl^-; △, movements of K^+; ▲, movements of Na^+ in exchange for K^+. The total Na^+ movement observed can be obtained by adding the movement shown by ○ to that shown by ▲.

Fig. 3 shows the effect of different doses of ouabain on the ionic content of kidney slices rewarmed in a medium containing 16 mM K^+. Note that, in Fig. 3, to obtain the total Na^+ movement observed in the experiment, the movement shown by the empty circles must be added to that shown by the full triangles. In agreement with the results of WHITTAM AND WILLIS[36], the exchange of Na^+ for K^+ was 50 % inhibited by an ouabain concentration of $5.2 \cdot 10^{-5}$ M and almost completely inhibited by $1 \cdot 10^{-3}$ M. On the other hand, the extrusion of Na^+ accompanied by Cl^- was unaffected by the ouabain in the medium. When the rewarming medium contained only 8 mM K^+ (Fig.4), half maximal inhibition of the Na^+–K^+ exchange occurred at a concentration of $1.4 \cdot 10^{-5}$ M; this value is only twice the concentration required to inhibit the (Na^+-K^+)-ATPase by 50 %. The difference between the values obtained in Figs. 3 and 4 can be attributed to the well-known competition between ouabain and K^+ (refs. 12, 13 and 20). Since, in the absence of external

K^+, there is little or no exchange of Na^+ for K^+, no ouabain sensitivity curves could be determined.

The presence of ethacrynic acid in the incubation medium has a very different effect from that of ouabain. In Fig. 5, it can be appreciated that on rewarming in the absence of K^+, this drug did not influence the cellular K^+ content, but inhibited the extrusion of Na^+ accompanied by Cl^-, a half maximal inhibition being observed at a drug concentration of $3.8 \cdot 10^{-3}$ M. On rewarming in the presence of 16 mM

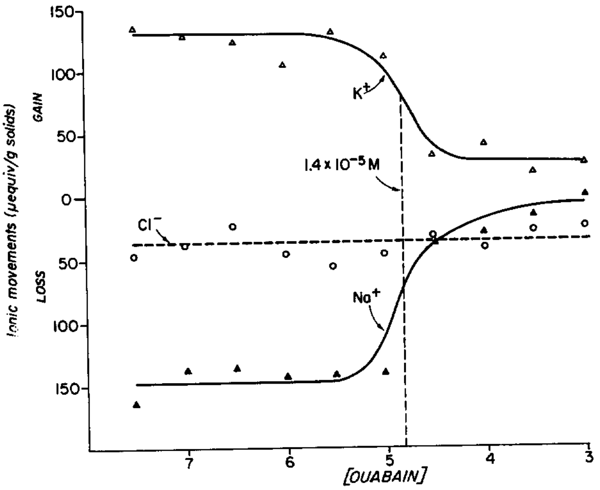

Fig. 4. Effect of different ouabain concentrations on the cellular ionic content of cortex slices rewarmed in the presence of 8 mM K^+. Details as in legend to Fig. 3.

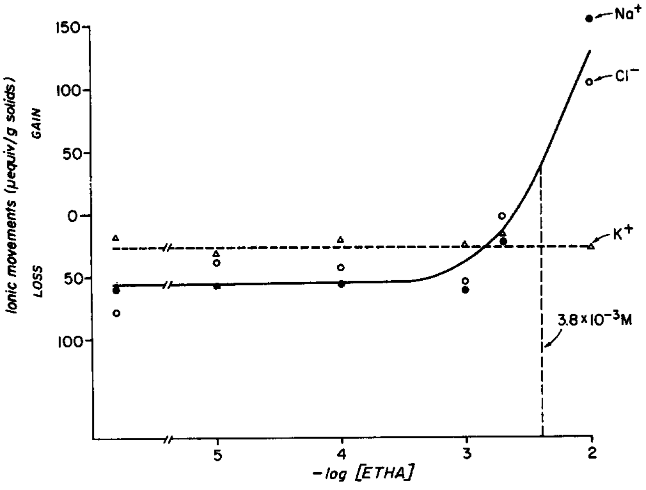

Fig. 5. Effect of different ethacrynic acid (ETHA) concentrations on the cellular ionic content of cortex slices rewarmed in the absence of K^+. △, movements of K^+; ● and ○, movements of Na^+ and Cl^-, respectively.

external K$^+$ (Fig. 6), the effect of ethacrynic acid on the expulsion of Na$^+$ with Cl$^-$ was practically identical with its effect in the absence of external K$^+$; this result emphasizes the lack of dependence on external K$^+$ of the system responsible for this ionic movement. On the other hand, ethacrynic acid did have a slight effect here on the Na$^+$–K$^+$ exchange, though this mechanism was not entirely inhibited even at a drug concentration of $1 \cdot 10^{-2}$ M.

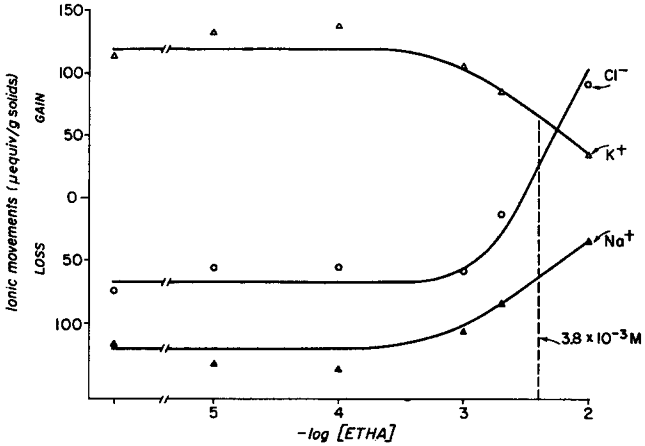

Fig. 6. Effect of different ethacrynic acid (ETHA) concentrations on the cellular ionic content of cortex slices rewarmed in the presence of 16 mM K$^+$. ○, movements of Cl$^-$ (with Na$^+$); △, movements of K$^+$; ▲, exchange of Na$^+$ for K$^+$.

DISCUSSION

Cardiac glycosides, such as ouabain, have been known for some time to be inhibitors of ion transport across cellular membranes[37]; more recently their specific effect on (Na$^+$-K$^+$)-ATPase has been demonstrated[21,22,38]. In the guinea-pig kidney cortex, the present results confirm and extend previous findings[14] that ouabain inhibits the exchange of Na$^+$ for K$^+$ across the cellular membrane, without affecting the extrusion of Na$^+$ with Cl$^-$. Ouabain has also been shown to inhibit a microsomal (Na$^+$-K$^+$)-ATPase preparation from the same tissue, a half maximal effect being observed at a concentration of $6.3 \cdot 10^{-6}$ M. This value correlates fairly closely with the half maximal inhibition of Na$^+$–K$^+$ exchange noted at a concentration of $1.4 \cdot 10^{-5}$ M (in the presence of 8 mM K$^+$), especially when the poorer accessibility of the drug to the active sites of the enzyme in experiments with whole tissue is considered. High doses of ethacrynic acid also partially inhibit the (Na$^+$-K$^+$)-ATPase and partially inhibit the Na$^+$–K$^+$ exchange, comparable concentrations having comparable effects. These results allow us to conclude without reasonable doubt that the (Na$^+$-K$^+$)-stimulated component of the ATPase activity is somehow involved in the exchange of Na$^+$ for K$^+$ across the peritubular membrane of the kidney cell.

On the other hand the fact that, even in the presence of 1 mM ouabain, the cells are capable of extruding Na$^+$, not in exchange for K$^+$, but accompanied by Cl$^-$, under conditions such that the (Na$^+$-K$^+$)-ATPase is totally inhibited, permits the conclusion that Na$^+$ extrusion with Cl$^-$ is independent of the action of a (Na$^+$-K$^+$)-

ATPase. Although the (Na$^+$-K$^+$)-ATPase is to a certain extent sensitive to ethacrynic acid, as shown in Fig. 2 and demonstrated by others[26,39], it is about 100 times less sensitive to it than to ouabain. This low sensitivity of the (Na$^+$-K$^+$)-ATPase to an extremely potent diuretic has among other factors been invoked[27] to cast doubt on the possibility that the primary site of action of ethacrynic acid is indeed the (Na$^+$-K$^+$)-ATPase. This argument is weakened by the present findings that, nevertheless, relatively high concentrations of ethacrynic acid are required to inhibit the Na$^+$ + Cl$^-$ component of Na$^+$ transport. However, the extremely different action of ethacrynic acid on renal Na$^+$ transport, as compared with that of ouabain, forces the conclusion that the site of action of the two drugs is not the same, and that the source of energy involved may be very different. Studies on the effects of analogues of ethacrynic acid on Na$^+$ efflux and on (Na$^+$-K$^+$)-ATPase in erythrocytes[40] have served to demonstrate that this enzyme is not involved in the ethacrynic acid sensitive component of Na$^+$ transport in those cells, results that are in full accord with those reported in the present work.

It has previously been shown[14] that the inhibitory action of ouabain and ethacrynic acid on the net Na$^+$ efflux is due to a primary action on the active fluxes and not on the leaks. Thus both ouabain and ethacrynic acid inhibit the (active) Na$^+$ efflux (and K$^+$ influx), and do not increase the (passive) influx of Na$^+$ or Cl$^-$.

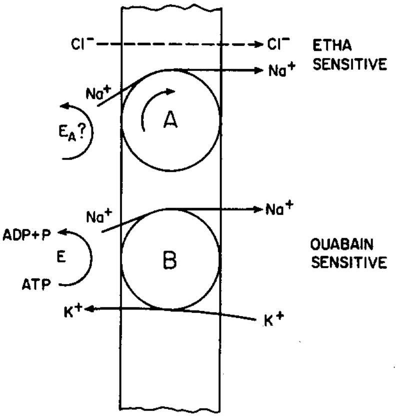

Fig. 7. Schematic representation of the properties and possible energy sources ot the two Na$^+$ pump mechanisms of the cortex cell from the guinea-pig kidney (ETHA, ethacrynic acid.)

The results obtained and conclusions drawn are represented in Fig. 7. Na$^+$ ions are expelled from kidney cells by two pumps, one (Pump B) being an exchange mechanism whereby intracellular Na$^+$ is interchanged with extracellular K$^+$, and the other (Pump A), a volume-regulating mechanism, being an electrogenic pump[41,42] which extrudes Na$^+$ accompanied by Cl$^-$. In view of the nature of the electrochemical potential gradients, Cl$^-$ should move passively out of the cell, driven by the active Na$^+$ movement, as has been discussed previously[43,44]. The energy responsible for the functioning of Pump B appears to come from the hydrolysis of ATP through the mediation of an enzyme complex known as the (Na$^+$-K$^+$)-ATPase, since both Pump B and the enzyme complex are inhibited by similar concentrations of ouabain.

Pump A is not affected on complete inhibition of the (Na+-K+)-ATPase by ouabain, though it is sensitive to ethacrynic acid; thus Pump A and its energy source appear to be independent of the operation of the (Na+-K+)-ATPase. Since both pumps are inhibited by 2,4-dinitrophenol and anoxia[7], there may nevertheless be a common link between the two systems.

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