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SOLUBILIZATION AND CHARACTERIZATION OF HEPATIC BILIRUBIN UDP-GLUCURONYLTRANSFERASE*,**

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SUMMARY

The hepatic enzyme, bilirubin UDPglucuronyltransferase (UDPglucuronate glucuronyltransferase, EC 2.4.1.17) has been solubilized in stable form by treating rat liver microsomes with 10% (by weight) dissolved digitonin at pH 7.8. All enzyme activity was associated with the rough membranes of the endoplasmic reticulum as shown by ultracentrifugation of selected density gradients in combination with electron microscopy. Proof of solubilization was determined by analytic ultracentrifugation, gel filtration, and electron microscopy which indicated that the enzyme was an homogeneous protein with a calculated molecular weight in the range of 150 000. Studies to characterize enzyme kinetics and assay requirements were performed and differ from those reported for liver homogenates.

INTRODUCTION

UDPglucuronyltransferase (UDPglucuronate glucuronyltransferase, EC 2.4.1.17) is found in the livers of vertebrates where it catalyzes the glucuronidation of carboxylic, phenolic, alcoholic, amino, and possibly sulfhydryl compounds. This reaction provides a major metabolic pathway for detoxifying or reducing the biological activity of many substances including alkaloids, steroids, thyroxine, bilirubin, and numerous drugs. While it is widely believed that more than one enzyme is involved in mediating the conjugation of these various substrates, direct evidence for substantiating this hypothesis will require the availability of a soluble and stable enzyme preparation. Although a number of solubilizing and activating agents have been used to extract enzymes from hepatic microsomes[1–6], the resulting unstable preparations have either contained only a fraction of the total starting activity or have failed to satisfy basic criteria for solubilization of a membranous enzyme[2–9]. Recently,

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Mowat and Arias[6] tested a number of solubilization methods and noted that even in their best preparations they were unable to disassociate enzyme activity from microsomal elements. Since a soluble enzyme preparation should be free of cellular debris, exhibit only components of molecular size during analytical centrifugation, and behave as a solute during gel filtration it seems clear that the transferase enzyme has not yet been solubilized.

The present study introduces a method that has resulted in the complete solubilization of this membranous enzyme. Proof of solubilization is presented, assay details are described, and kinetic characteristics of soluble transferase have been defined.

MATERIALS AND METHODS

Buffers

Stock maleate. Malcate 375 mM (Sigma) and 50 mM $MgCl_2$ (Fisher) were adjusted to pH 6.5 with 5 M NaOH.

Homogenizing buffer. 100 ml of stock maleate was added to 37.5 mM maleate, 0.5 M sucrose, 5 mM $MgCl_2$, 171.15 g of sucrose (Schwarz/Mann, ultra pure, No. 04000-9530), 10 g of dextran (Sigma No. 4751, Type 60C), and diluted with water to 1000 ml.

Gradient buffer 1. 25 ml of stock maleate was added to 106.97 g of sucrose, 2.5 g of dextran, and diluted to 250 ml with water (1.25 M with respect to sucrose).

Gradient buffer 2. This buffer is identical in composition to gradient buffer 1 except that it is made with 136.92 g of sucrose (1.60 M with respect to sucrose).

Triethanolamine buffer. 75 mM triethanolamine was adjusted to pH 7.8 with concentrated HCl.

Reagents

1.67 g of digitonin (Sigma No. 05628) was dissolved in 100 ml of water by boiling to remove traces of ethanol. The water lost by boiling was replaced.

145.2 mg of uridine diphosphoglucuronic acid (Sigma) was dissolved in water and made up to 5 ml (45 mM) (stable 1 week at 5 °C). 2 M glycine was prepared and the pH adjusted to 2.8 with concentrated HCl.

30% human albumin (Schwarz/Mann No. 7306) was dialyzed overnight against 1 l of 1 mM disodium EDTA, pH 7.20, and diluted with 125 ml of water to make a 2.4% concentration (stable for 6 months at 4 °C).

10.4 g of bilirubin (Nutritional Biochemicals No. 9324) was added to 2 ml of 0.05 M NaOH and diluted to 10 ml with 2.4% human albumin (1.8 mM with respect to bilirubin) (prepared daily).

120 mM disodium EDTA (Fisher 5-311) was adjusted to pH 7.2 by adding NaOH. 1 M triethylamine was adjusted to pH 7.2 with concentrated HCl.

Enzyme preparation

The whole homogenate was prepared by exsanguinating the liver with buffer and homogenizing it in an iced vessel using 2.5 ml of buffer for each g of liver (10 min at speed 6 with a Sorval Omnimixer).

Homogenate pellet

This whole homogenate was weighed and centrifuged (IEC No. 2067 polycarbonate tubes, IEC head No. 494) at 120 000 × *g* for 1 h at 5 °C. The resulting homogenate pellet was weighed and the supernatant discarded. The pellet was resuspended in homogenizing buffer by adding 5 ml of the buffer for each g of pellet and grinding the preparation in a Teflon pestle tissue grinder (A. H. Thomas No.3431). 8 ml or less of this pellet suspension was placed on a discontinuous gradient formed in 30 ml polycarbonate tubes (IEC No. 2055) by superimposing 10 ml of gradient buffer 1 on 10 ml of gradient buffer 2 and centrifuging at 100 000 × *g* for 1 h at 5 °C in a swinging bucket head (IEC No. 485). All activity was collected in a viscid pink-brown band at the interface of the two gradient buffers. This band was aspirated and suspended in approximately 5 vol. of homogenizing buffer by grinding and centrifuged in weighed tubes at 120 000 × *g* for 30 min. The supernatant was discarded and the weight of the pelleted band determined. The pelleted band was resuspended by grinding with 5 ml of 75 mM triethanolamine buffer for each g of band. This constituted the active band. Equal volumes of digitonin solution and band suspension were mixed. (The suspension contained 0.167 g of band in each ml, so that the admixture of each supplied 10% of the band weight in digitonin.) This mixture was homogenized without delay and centrifuged immediately at 120 000 × *g* for 30 min. The supernatant from this mixture contained all of the enzyme and the pellet was discarded. Overnight storage of this soluble enzyme at 5 °C caused no loss of activity. When inactive detritis, mostly digitonin, flocculated during storage it was readily removed by centrifuging at 20 000 × *g* for 15 min.

The soluble enzyme was centrifuged in weighed tubes at 320 000 × *g* for 9 h at 5 °C. All activity was concentrated in the clear gelatinous brown pellet. The volume of the supernatant was estimated by weighing the tube before and after decantation and the supernatant discarded. The concentrated soluble enzyme kept for several months if the pellet was stored at 5 °C. Prior to assay, the pellet was reconstituted by grinding it with an amount of 75 mM triethanolamine buffer equal to the volume of supernatant that was discarded after the 320 000 × *g* centrifugation. Any turbidity in the solution was easily removed without loss of activity by centrifuging at 20 000 × *g* for 15 min.

Assay

3.5 ml of the homogenate was diluted with 2.5 ml of 75 mM triethanolamine buffer and homogenized with 6 ml of digitonin solution. The homogenate pellet suspension and the band suspension were prepared by grinding them with equal volumes of digitonin solution. The solubilized enzyme and the reconstituted 32 000 × *g* concentrate were assayed without further treatment.

Incubation mixture

200 μl of UDP glucuronic acid, 200 μl of bilirubin, and 50 μl of $MgCl_2$ were added to 50 μl of 1 M triethanolamine. A blank was prepared using the same reagents but substituting water for UDP glucuronic acid and disodium EDTA for $MgCl_2$. The reaction was started by adding 100 μl of the sample to each of the tubes followed by incubation in a water bath at 37 °C for 30 min. Addition of 1 ml of glycine–HCl stopped the reaction.

Assay procedure

The procedure for diazotization, extraction, and colorimetry has been detailed by Black *et al.*[10] and the molar extinction coefficient used for conjugated bilirubin was $44.36 \cdot 10^2\ M^{-1} \cdot cm^{-1}$ at 530 mM as determined by Van Roy and Heirwegh[11]. Nitrogen was determined by the method of Kjeldahl[12].

The technique for Sephadex chromatography has been previously described[13]. The column used was formed with Sephadex G-200 (Pharmacia Fine Chemicals) by equilibrating it with 0.1 M Tris (Fisher T-370), pH 8.0. Column length was 13.4 cm and the diameter 1.5 cm ($V_t = 23.7$ ml). Void volume was 4.16 ml as determined by eluting blue dextran (Sigma D5751). All samples were eluted with Tris buffer. Flow rate through the column was 0.46 ml/h. A 1% solution of bovine γ-globulin (Sigma BG-11), mol. wt 160 000 in 0.1 M Tris, pH 8.0, was used as a marker protein.

RESULTS

The wet weight of the active band was 42.3 ± 4.4 (S.D.)% of the wet liver weight. Table I shows that it retained 68.2% of the total activity and 26.1% of the liver nitrogen. The gain in specific activity was 2.6-fold. Electron microscopy of the band showed that it was composed predominantly of rough endoplasmic reticulum in association with bile canaliculi.

TABLE I

NITROGEN CONTENT AND UDPGLUCURONYLTRANSFERASE ACTIVITY IN VARIOUS RAT LIVER PREPARATIONS

Results are shown as the mean and standard deviation ( ) of preparations from 6 rats. The nitrogen content of wet liver was 18.3 ± 1.6 mg of N/g of wet liver.

Sample	*Whole homogenate*	*Homogenate pellet*	*Active band*	*Soluble enzyme*	*Concentrate*
Fraction of liver nitrogen	1	0.506 (0.088)	0.261 (0.040)	0.162 (0.036)	0.066 (0.010)
Fraction of total UDPglucuronyltransferase activity	1	1.018 (0.136)	0.682 (0.010)	0.628 (0.133)	0.493 (0.076)
μmole(s) of bilirubin conjugated/mg of N/h	0.132 (0.014)	0.272 (0.063)	0.344 (0.022)	0.437 (0.050)	1.047 (0.094)

Solubilization of bilirubin UDPglucuronyltransferase

The active band suspension contained 0.167 g of band per ml and was treated with equal volumes of digitonin solutions yielding enzyme preparations ranging between 0.000835 and 0.0668 g/ml which contained digitonin in amounts ranging from 0.5 to 40% of the band weight. Assays were performed on the digitonized band prior to centrifugation and following centrifugation on the resulting supernatant and pellet. These results are shown in Fig. 1. Although maximum activation of the band occurred with 1% digitonin, very little solubilization was evident with most of the activity remaining in the pellet. Maximum solubilization was achieved when the weight of digitonin equaled 10% of the band weight. With digitonin concentrations of 10% and higher the activities in the whole preparation and in the supernatant were essentially identical. At concentrations greater than 10% there was progressive loss

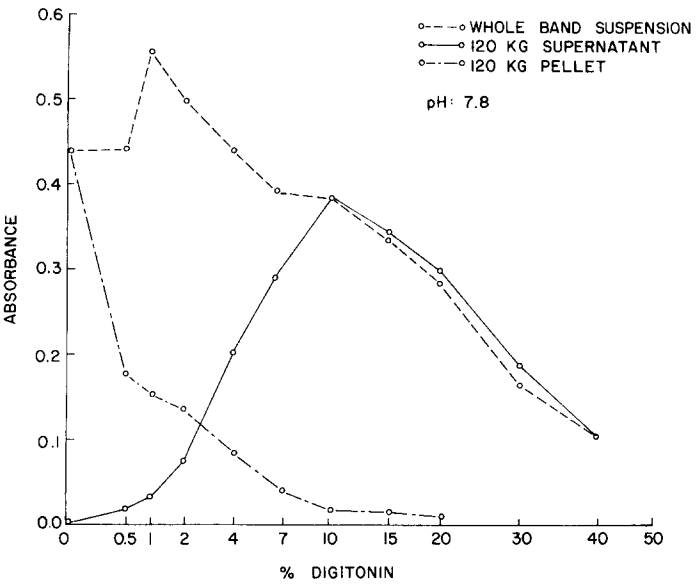

Fig. 1. The concentration of digitonin is expressed as the per cent of digitonin relative to active band weight and plotted on the abscissa using a square root scale. Absorbance at 530 nm measures the concentration of azo pigments produced by the glucuronyltransferase enzyme. All assays were performed at optimal pH, 7.8. Maximal transferase activity appeared in the supernatant at 10% digitonin concentration at which point all activity had disappeared from the pellet. At 1% digitonin the whole band suspension showed maximal activity; however, little enzyme was released into the supernatant at this concentration. At digitonin concentrations exceeding 10%, transferase activity was inhibited.

of activity. When the digitonin concentration was fixed at 10% of the band weight and the pH varied over a wide range, the optimal solubilizing pH was established at 7.8. 10% digitonin not only yielded maximum solubilization of UDPglucuronyltransferase, it also resulted in maximum specific activity.

The nitrogen compositions and enzymatic activities at each stage in the preparation of soluble enzyme are compared in Table I. It can be seen that 49.3% of the initial activity remained in the concentrate and that the specific activity in the final preparation was enhanced by approximately 8-fold.

Proof of solubilization

UDPglucuronyltransferase activity eluted in a single peak from Sephadex G-200 columns coincident with the major protein fraction (Fig. 2). The K_{av} of this enzyme peak was calculated to be 0.325 and is compatible with a molecular weight of 130 000 (ref. 13). Under the same conditions and in the same column, bovine γ-globulin (mol.wt = 160 000) eluted with a K_{av} of 0.280. Two minor fractions with K_{av} values of 0.621 (mol. wt = 30 000) and 0.862 (mol. wt = 10 000) were also discernible; these were devoid of enzymatic activity. The specific activity of the eluate as determined in the most active fraction was 0.489 μM/mg N per h which was only slightly higher than that of the starting material which was 0.430 μM/mg N per h. Total activity of the eluted fraction was 44% of the activity in the sample

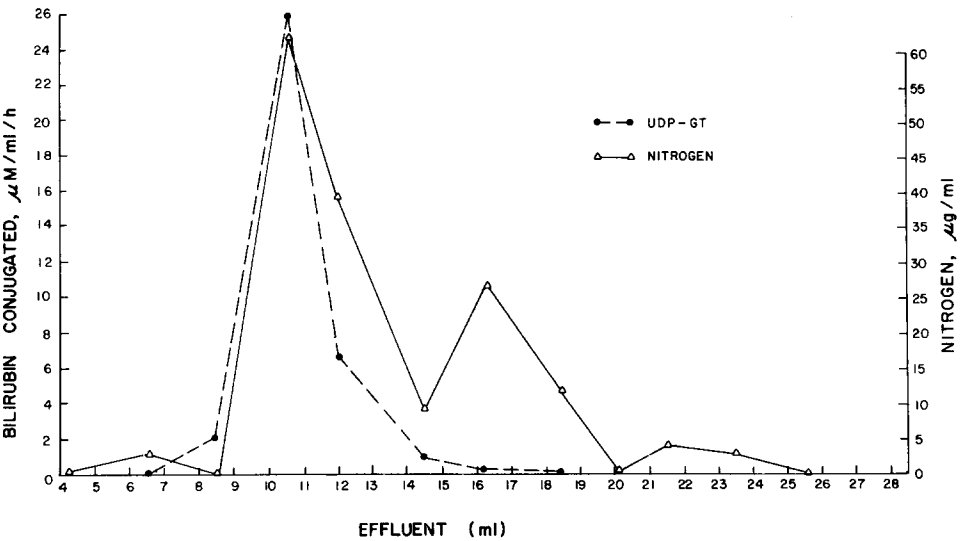

Fig. 2. Elution of bilirubin UDPglucuronyltransferase (UDP-GT) from Sephadex G-200.

applied to the column. This loss was attributed to enzyme deterioration during column transit.

Analytical ultracentrifugation of the concentrated enzyme revealed a major component with a sedimentation coefficient of 6.9 S which corresponds to a molecular weight in the region of 135 000 for a globular protein (Fig. 3). Minor components of higher molecular weight were also present.

Electron microscopic examination of the concentrated preparation revealed no formed elements; these should have been present had the preparation contained membranes or other microsomal fragments.

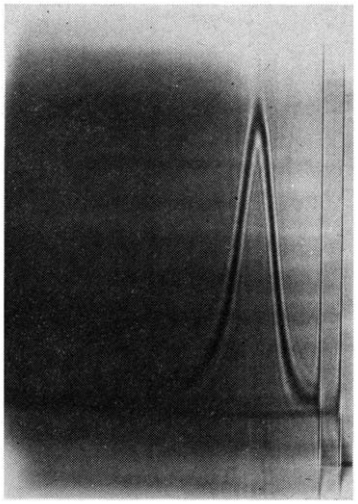

Fig. 3. Sedimentation pattern of soluble UDPglucuronyltransferase.

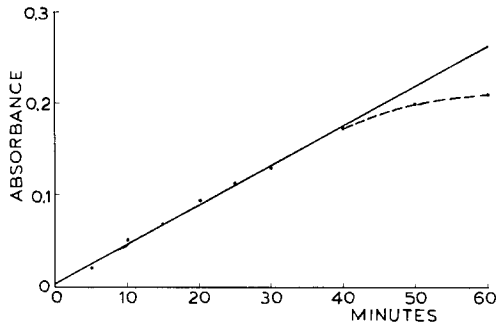

Fig. 4. Linearity of azo pigment production with time.

Assay characteristics of the soluble enzyme

The optimum pH for the inculation mixture was established by using 1M triethanolamine buffers ranging from pH 6.8–8.6. In the assay mixtures this range shifted from 6.9–8.2. The optimum pH was 7.8.

Mg^{2+} was essential for the reaction but its concentration did not require careful regulation. Any concentration of Mg^{2+} between 5 and 20 mM yielded satisfactory results. In the absence of Mg^{2+}, enzyme activity was negligible; with Mg^{2+} above 20 mM there was progressive inhibition.

Both bilirubin and uridine diphosphate glucuronic acid in excess caused inhibition of enzyme activity. For the amounts of enzyme used, the optimum concentration of bilirubin in the reaction mixture was $6 \cdot 10^{-4}$ M over a wide range of UDP glucuronic acid concentrations. The optimum concentration of UDP glucuronic acid was $15.0 \cdot 10^{-3}$ M over a wide range of bilirubin concentrations. Using these optimal substrate concentrations and a fixed amount of enzyme, the rate of glucuronidation was linear with respect to time for 30 min (Fig. 4). Progressively increasing amounts of enzyme resulted in a corresponding linear increase in glucuronidation (Fig. 5).

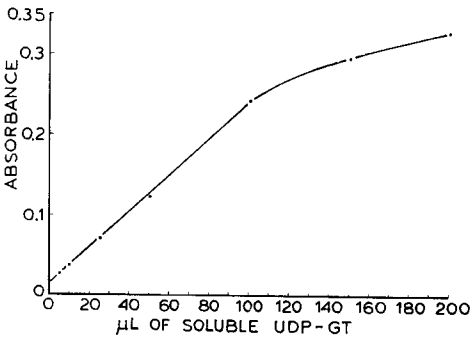

Fig. 5. Linearity of azo pigment production with varying enzyme concentrations. UDP-GT, UDPglucuronyltransferase.

The values of the Michaelis constants, K_m, for both substrates and of the maximal velocity, V, obtained by the graphical method of Lineweaver and Burk[14] are given in Table II.

TABLE II

MICHAELIS–MENTEN CONSTANTS AND MAXIMUM VELOCITIES

Solubilized enzyme was incubated for 30 min under the specified conditions of the assay. Bilirubin concentration was varied between $0.3 \cdot 10^{-3}$ and $1.6 \cdot 10^{-3}$ mM with the Lineweaver–Burk plot being linear up to $0.8 \cdot 10^{-3}$ mM. The range of UDPglucuronic acid concentrations was $0.3 \cdot 10^{-3}$ mM to $62.7 \cdot 10^{-3}$ mM. The Lineweaver–Burk plot was linear up to $5 \cdot 10^{-3}$ mM. Every concentration of each substrate was used as a fixed concentration and plotted against the range of concentrations for the variable substrate.

Substrate	$K_m \times 10^{-4}$	*V (μmoles/mg N/h)*
Bilirubin	0.95	0.47
UDPglucuronic acid	3.85	0.44

Optimal incubation temperature was 37 °C with loss of activity when the temperature exceeded 40 °C.

In the whole liver, homogenate, and the active band, storage at −15 °C resulted in enhanced activity when measured at 7 and 31 days. Data for frozen storage of the solubilized enzyme and of the concentrate are not available but storing either preparation for 7 days at 4 °C causes negligible loss of activity in the solubilized preparation and only a 12% loss in the concentrate (Table III).

TABLE III

STORAGE OF UDPGLUCURONYLTRANSFERASE PREPARATIONS

Numbers are mean (and standard deviation) of the fraction of initial activity in each preparation that remained after the stated time. The activity increased or remained constant except in the concentrate. Five rats were used to determine the mean activities shown.

Days	*Sample:*	*Whole liver (−15 °C)*	*Homogenate (−15 °C)*	*Active band (−15 °C)*	*Soluble enzyme (4 °C)*	*Concentrate (4 °C)*
7		1.02 (0.17)	1.05 (0.10)	1.16 (0.06)	1.00 (0.23)	0.88 (0.14)
31		1.51 (0.21)	1.13 (0.12)	1.75 (0.17)		

DISCUSSION

Evidence that bilirubin glucuronyl transferase has been solubilized is presented for the first time. Proof of solubilization has been established by gel filtration and ultracentrifugation experiments. While microsomal preparations have been used as a source of glucuronyl transferase[2–5,8,11], concentration of the enzyme into a microsomal subfraction has not been previously described. Electron microscopy of this microsomal subfraction revealed that it consisted primarily of rough endoplasmic reticulum, an observation which confirms earlier reports[15,16]. However, the presence of associated bile canaliculi has not been noted previously and their significance with respect to bilirubin transferase activity is not known at this time.

The activating effect of digitonin on microsomal transferase activity has been recognized by others[5,7,8,10]. Partial enzyme solubilization with this detergent has also been reported; however, each of these studies has failed to present clear evidence that the enzyme had been completely disassociated from cellular membranes. As

shown in the data presented *(vide supra)*, complete disruption of the enzyme–membrane complex was achieved with minimal loss of enzyme activity by selecting the optimum concentration of dissolved digitonin. This is clearly illustrated in Fig. 1 where three distinct effects of digitonin on microsomal transferase activity can be discerned: (1) Activation was most pronounced when added digitonin was 1% of microsomal weight and probably resulted from disruption of masking lipid membranes by the detergent properties of digitonin. This has also been shown with a number of other similar agents including deoxycholate, triton X, deoxytaurocholate, triton, Nonidet, sodium octyl sulfate, and sodium decyl sulfate[4–6]. (2) Increasing the concentration of digitonin to 10% resulted in complete solubilization of the enzyme. While the nature of solubilization is not completely understood, it seems likely that a mode of action in addition to unmasking active sites was involved. Formation of a soluble digitonin–enzyme complex could have resulted with an hydroxy group of the digitonin molecule serving as an acceptor site for the glucuronidation reaction. (3) The final digitonin effect observed was that of progressive transferase inactivation in proportion to increments of increasing digitonin concentrations above 10%. If a digitonin–enzyme complex were formed, this latter phenomenon could be the result of competitive inhibition.

The Sephadex experiments permitted preliminary estimates of molecular weight and provided convincing evidence that the enzyme had been disassociated from microsomal membranes. If microsomal release had not occurred, enzyme activity should have eluted at the solvent front and would not have been retained by Sephadex G-200. The homogeneous Schlieren pattern obtained by analytical ultracentrifugation was additional evidence of complete enzyme–membrane separation. From this pattern the sedimentation coefficient of the major component was estimated to be 6.9 S. Electron microscopy confirmed the absence of formed membranous fragments. Although the molecular weight of glucuronyl transferase could be calculated at approximately 150 000, a purer preparation will be needed before this estimate can be validated.

The assay characteristics of soluble transferase differs in several ways from that observed in homogenates. The pH optimum for soluble enzyme was 7.8 which agrees with Black *et al.*[10] and differs from that of 6.6 found by Lathe and Walker[17]. It has been generally recognized that Mg^{2+} is essential for enzyme activity[9]; however, its concentration could be varied from 5–20 mM without affecting results. Exposure to temperatures in excess of 45 °C for 3 min reduced enzyme activity by 80%. Solubilized preparations were stable for at least 7 days when stored at 4 °C.

This work makes available a stable and soluble preparation of hepatic glucuronyl transferase. It should now be possible to proceed with studies concerned with the important question of substrate specificity. However, as a prerequisite for investigating additional substrates, further enzyme purification must be achieved by the classical methods of protein separation.

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