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2,5,2′,5′-TETRACHLOROBIPHENYL IMPAIRS THE BIOENERGETIC FUNCTIONS OF ISOLATED RAT LIVER MITOCHONDRIA

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Abstract—The effects of 2,5,2′,5′-tetrachlorobiphenyl (25TCB) on parameters related to the bioenergetic functions of isolated rat liver mitochondria were investigated. State 3 respiration was inhibited by 25TCB with both succinate and glutamate/malate as the respiratory substrates. The extent of inhibition with succinate was larger than that observed with glutamate/malate. The concentration of 25TCB required to cause 50% inhibition for succinate was 51 μM, but with glutamate/malate, only 53% inhibition was observed at 200 μM. 25TCB stimulated state 4 respiration after 1–2 min lag period; state 4 respiration in the presence of glutamate/malate was more intensely stimulated by 25TCB than in the presence of succinate. 25TCB dissipated the membrane potential across the mitochondrial membranes. Isolated rat liver mitochondria accumulate large amounts of Ca^{2+} at the expense of respiration-linked energy (substrate oxidation) or of that provided by the hydrolysis of ATP by the mitochondrial ATPase. The Ca^{2+} accumulation by mitochondria was severely depressed by 25TCB when the energy was supplied by respiration. Furthermore, the inhibition of Ca^{2+} accumulation by 25TCB with succinate was greater than that produced with glutamate/malate. On the other hand, with ATP as the source of energy, 25TCB inhibited Ca^{2+} accumulation at high concentrations. 25TCB also released Ca^{2+} from mitochondria that had already accumulated Ca^{2+}, indicating that mitochondrial membrane integrity was damaged by the intercalation of 25TCB. These results show that 25TCB impairs mitochondrial energy production, and inhibits Ca^{2+} sequestration by mitochondria.

Polychlorinated biphenyls (PCBs) consist of innumerable isomers whose position and degree of chlorination are different. They are readily absorbed into biological systems [1]. 2,5,2′,5′-Tetrachlorobiphenyl (25TCB) is one of the TCBs whose toxic events are well characterized [2–5]. Over 95% of the total ATP required in eukaryotic cells is produced by mitochondrial oxidative phosphorylation. Energy thus produced is utilized for various cellular activities such as active transport of ions [6]. Therefore, damage of mitochondrial energy-transducing functions by toxic chemicals causes deleterious effects on cellular activities [7, 8].

The administration of an acute lethal dose of 25TCB (1.7 g/kg body weight) to rats causes a marked inhibition of (Ca^{2+} + Mg^{2+})-ATPase located in plasma membranes of hepatocytes [5]. The decreased activity of this enzyme leads to an accumulation of Ca^{2+} in the cytosol. An increase in cytosolic free Ca^{2+} concentration is a potential mechanism of cell injury following contact with toxic agents [9–11]. In normal conditions, the major fraction of accumulated Ca^{2+} in cytosol is sequestered in mitochondria by an energy-dependent pathway [12–15]. However, it is known that impairment of Ca^{2+} sequestration by mitochondria takes place by the treatment with several chemically unrelated compounds such as menadione [16], phalloidin (hepatotoxic mushroom poison) [17], which leads to an increase in cytosolic free Ca^{2+} concentration. This closely relates to the toxic events by these agents.

The present study, therefore, was performed in order to determine whether mitochondrial energy transducing functions and the Ca^{2+} movements across the mitochondrial membranes are affected by the treatment with 25TCB, using isolated rat liver mitochondria. Evidence presented in this paper shows that 25TCB interferes with energy-transducing functions of mitochondria, and both inhibits the accumulation of Ca^{2+} by mitochondria and releases Ca^{2+} from them.

MATERIALS AND METHODS

Chemicals. 2,5,2′,5′-Tetrachlorobiphenyl (25TCB) was synthesized by the Ullman condensation of the 2,5-dichloroiodobenzene [18]. The purity of 25TCB was found to be more than 99% by gas liquid chromatography. 25TCB solution was prepared in ethanol, ATP, antimycin A, ruthenium red, oligomycin, and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO). Antipyrylazo III and tetraphenylphosphonium chloride (TPP^+) were purchased from Nakarai Chemicals (Kyoto, Japan) and Wako Pure Chemical Industries Ltd. (Osaka, Japan), respectively. Other chemicals were commercial products of the highest purity.

Preparation of mitochondria. Liver mitochondria were isolated from male Wistar rats (200–300 g) by the standard method in a medium containing 0.25 M sucrose, 5 mM Tris–HCl (pH 7.4), and 0.1 mM

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EDTA. EDTA was omitted in a final wash and resuspension [19]. Protein concentration was determined by the biuret method using bovine serum albumin as a standard [20].

Measurements of respiration rates. Respiration rates of mitochondria were measured polarographically using a Clark-type oxygen electrode in a 2 ml glass reaction vessel maintained at 25° in a water bath. The respiration buffer consisted of 0.2 M sucrose, 20 mM KCl, 3 mM $MgCl_2$, and 5 mM potassium phosphate (pH 7.4). The respiration substrate was either 5 mM succinate or 5 mM glutamate/5 mM malate, and the amount of mitochondrial protein added was 1 mg/ml. The effects of 25TCB on state 3 and state 4 respiration were evaluated as described previously [21].

Measurements of membrane potential. Membrane potential across the mitochondrial membranes was monitored at 25° with a TPP^+ electrode constructed according to Kamo *et al.* [22]. The reaction mixture was the same medium as used for the measurement of respiration rates plus 5 μM TPP^+ in a final volume of 2.5 ml. The calibration of the electrode was performed by multiple additions of known amount of TPP^+ before each experiment.

Measurements of Ca^{2+} movements across the mitochondrial membranes and mitochondrial swelling. Ca^{2+} movements were measured with the metallochromic indicator, antipyrylazo III [23] with a Shimadzu UV-300 dual-wavelength spectrophotometer, equipped with magnetic stirring and thermostatic control (final volume 2.5 ml, 25°). Wavelength pair was 720–790 nm. The net Ca^{2+}-uptake by mitochondria is accompanied by a decrease in absorbance, and the net release is reflected by an increase in absorbance. Calibration was performed by adding a known amount of $CaCl_2$ in the presence of uncoupler (25 μM 2,4-dinitrophenol) at the end of each experiment. Swelling of mitochondria was monitored with the same instrument as apparent absorbance changes at 540 nm (double beam mode) [24]. For these measurements, antipyrylazo III was omitted from the incubation medium. The incubation medium and further details are given in the figure legends.

In all experiments, the control contained the same volume of solvent (ethanol), and the final concentration of solvent was less than 1% (v/v); the concentration of solvent did not affect the activities assayed.

RESULTS

Effects on respiration rates

Figure 1 shows the effects of 25TCB on state 3 respiration (active respiration in the presence of ADP) of rat liver mitochondria. 25TCB caused strong inhibition of the respiration with succinate as the respiratory substrate, 51 μM indicating 50% inhibition (ID_{50}). By contrast, state 3 respiration was less affected by 25TCB when glutamate/malate was used as the substrate, with only 53% inhibition even at 200 μM. Since 25TCB does not inhibit the mitochondrial ATPase [25], the observed inhibition is due to the interference with the electron transport chain. Therefore, the electron transport chain is more intensely inhibited by 25TCB in the presence of succinate than with glutamate/malate.

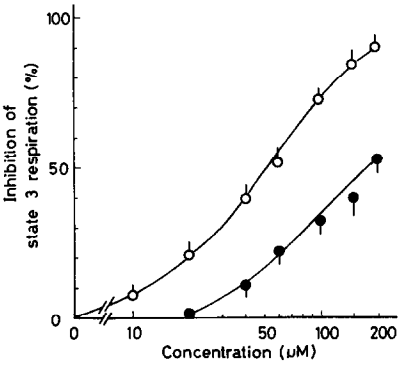

Fig. 1. Effects of 25TCB on state 3 respiration of rat liver mitochondria. The incubation medium consisted of 0.2 M sucrose, 20 mM KCl, 3 mM $MgCl_2$, 5 mM potassium phosphate (pH 7.4), and either 5 mM succinate (○) or 5 mM glutamate/5 mM malate (●). Mitochondria (1 mg/ml) were interacted with 25TCB for 3 min, then the state 3 respiration was initiated by the addition of 150 μM ADP. Each point is a mean ± SD of 3 separate experiments. Control rates were 132.5 ± 4.8 and 68.2 ± 2.9 natoms O/min/mg protein for succinate and glutamate/malate, respectively. Temp. 25°, vol. 2 ml.

Figure 2 shows the effects of 25TCB on state 4 respiration (resting respiration after the expenditure of ADP) of rat liver mitochondria. A 1–2 min lag period was observed before stimulation of the respiration became obvious. This was in contrast to 2,4-dinitrophenol (DNP), a protonophoric uncoupler, which showed an instantaneous stimulation. Values presented in Fig. 2, therefore, are maximum rates attained after a lag phase. As the concentration of 25TCB was increased, oxygen consumption was stimulated, reaching a peak where maximum rate was observed (more than 4-fold increase with glutamate/malate, 2-fold increase with succinate at

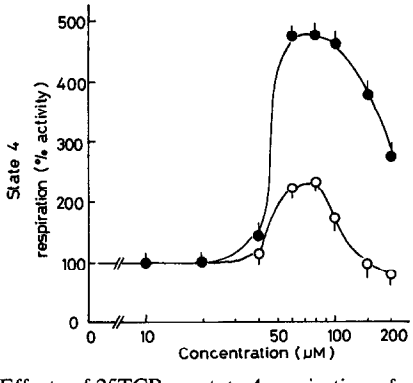

Fig. 2. Effects of 25TCB on state 4 respiration of rat liver mitochondria in the presence of either succinate (○) or glutamate/malate (●). The incubation medium was as described in Fig. 1. Shown are the maximum rates of respiration (% activity) 3–4 min after the addition of 25TCB during state 4 respiration. Each point is a mean ± SD of 3 separate experiments. Control rates were 25.6 ± 1.8 and 9.8 ± 1.6 natoms O/min/mg protein for succinate and glutamate/malate, respectively.

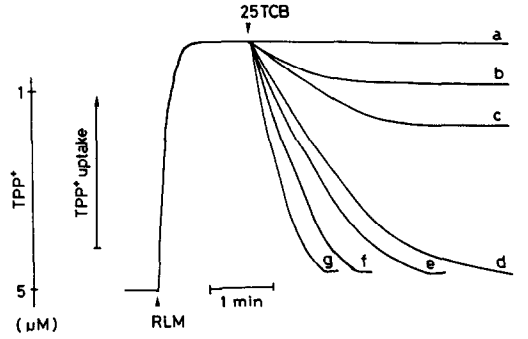

Fig. 3. Effects of 25TCB on the movement of TPP^+ across the mitochondrial membranes. Rat liver mitochondria (RLM, 1 mg/ml) were incubated with various concentrations of 25TCB in the respiration buffer containing 5 mM succinate and 5 μM TPP^+. a, none; b, 20; c, 40; d, 60; e, 80; f, 100; g, 200 μM. Temp. 25°, vol. 2.5 ml.

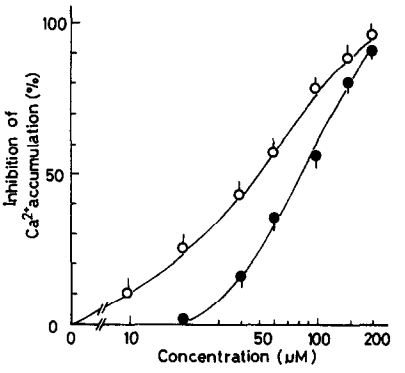

Fig. 4. Effects of 25TCB on the respiration-driven Ca^{2+} accumulation by mitochondria under the condition of blockade of Ca^{2+} accumulation driven by ATP hydrolysis. The incubation medium contained 0.2 M sucrose, 20 mM KCl, 3 mM $MgCl_2$, 5 mM potassium phosphate (pH 7.4), 2.5 μg oligomycin, 150 μM antipyrylazo III, and 5 mM succinate (○) or 5 mM glutamate/5 mM malate (●) as the respiratory substrate. Mitochondria (1 mg/ml) were interacted with 25TCB for 3 min, then the Ca^{2+} accumulation was initiated by adding 150 μM $CaCl_2$. The results are expressed as percentage inhibition of initial rate of Ca^{2+} uptake (mean ± SD of 3 experiments). The control rates for succinate- and glutamate/malate-driven Ca^{2+} uptake were 16.8 ± 1.3 and 8.7 ± 0.9 nmoles Ca^{2+}/sec/mg protein, respectively.

60–80 μM), after which further increases in concentration repressed the respiration. The extent of stimulation due to 25TCB with succinate was less than that observed with glutamate/malate as the substrate. This is attributed to the increased inhibition of the electron transport chain with succinate when compared to glutamate/malate. The result indicates that 25TCB acts as a good uncoupler in the presence of NAD^+-linked substrate rather than FAD^+-linked substrate.

Effects on membrane potential

The permeant cation TPP^+ was taken up from the reaction medium into the inner side of mitochondria when they are energized with succinate, indicating the formation of membrane potential (Fig. 3). An addition of 25TCB induced a concentration-dependent release of incorporated TPP^+ into the incubation medium (dissipation of membrane potential). Below 40 μM of 25TCB that had no stimulatory effect on state 4 respiration (Fig. 2), the dissipation of membrane potential was also small (traces a–c). Above this concentration, 25TCB completely collapsed the membrane potential (traces d–g), which led to the stimulation of state 4 respiration (Fig. 2).

Effects on Ca^{2+} accumulation

Ca^{2+} movements across the mitochondrial membranes were measured by monitoring the changing level of Ca^{2+} in the incubation medium with a metallochromic indicator, antipyrylazo III. Figure 4 shows the effects of various 25TCB concentrations on the Ca^{2+} accumulation, when the energy needed for this process is provided by the substrate oxidation under the condition of blockade of Ca^{2+} accumulation driven by the hydrolysis of ATP (i.e. in the presence of oligomycin, a specific inhibitor of mitochondrial ATPase [26]). 25TCB inhibited Ca^{2+} accumulation in a concentration-dependent manner with both succinate and glutamate/malate as the substrate. The inhibition of Ca^{2+} accumulation with succinate (ID_{50}, 50 μM) is greater than that obtained with glutamate/malate (ID_{50}, 82 μM). The Ca^{2+} accumulation by mitochondria also takes place by energy from ATP hydrolysis. Figure 5 shows the effects of 25TCB on the Ca^{2+} accumulation driven by the ATP hydrolysis in the presence of respiratory inhibitors, antimycin A and NaN_3. At concentrations below 60 μM, 25TCB did not affect the Ca^{2+} accumulation, but above this concentration, Ca^2 accumulation was inhibited sharply in a concentration-dependent manner, 75% inhibition was observed at 200 μM, with ID_{50}, 145 μM.

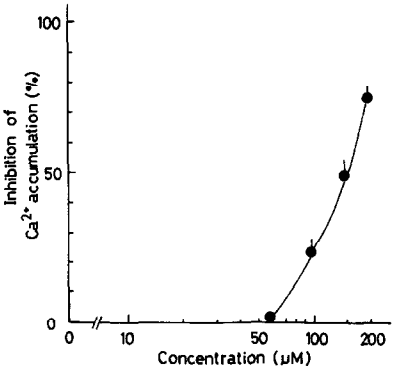

Fig. 5. Effects of 25TCB on the ATP-driven Ca^{2+} accumulation by mitochondria under the condition of blockade of respiration-driven Ca^{2+} accumulation. The incubation medium contained 0.2 M sucrose, 20 mM KCl, 3 mM $MgCl_2$, 5 mM potassium phosphate (pH 7.4), 5 μg antimycin A, 2 mM NaN_3, 150 μM antipyrylazo III, and 3 mM ATP. Mitochondria (1 mg/ml) were interacted with 25TCB for 3 min, then the Ca^{2+} accumulation was initiated by the addition of 150 μM $CaCl_2$. The results are expressed as percentage inhibition of the initial rate of Ca^{2+} uptake (mean ± SD of 3 experiments). The control rate for Ca^{2+} uptake was 8.4 ± 0.7 nmoles Ca^{2+}/sec/mg protein.

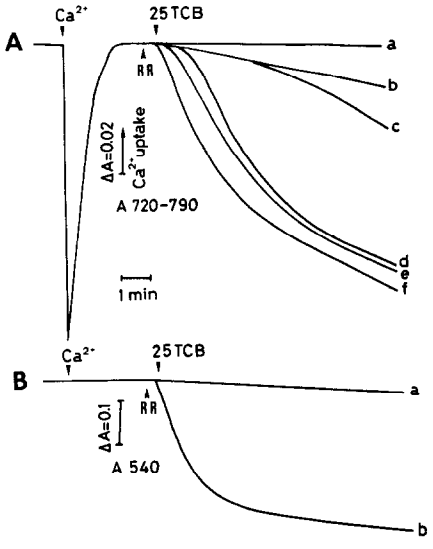

Fig. 6. The release of Ca^{2+} from mitochondria and mitochondrial swelling induced by 25TCB. The incubation medium consisted of 0.2 M sucrose, 20 mM KCl, 3 mM $MgCl_2$, 10 mM potassium acetate, 5 mM Tris–HCl (pH 7.4), and 150 μM antipyrylazo III. Mitochondria (1 mg/ml) were loaded with 150 μM Ca^{2+} with 3 mM ATP as the source of energy. After Ca^{2+} load was complete, 1 μM ruthenium red (RR) was added. (A) Concentration-dependence of the effects of 25TCB on Ca^{2+} release: trace a, −RR, −25TCB; trace b, +RR, −25TCB; trace c, +RR, 30 μM 25TCB; trace d, +RR, 40 μM 25TCB; trace e, +RR, 60 μM 25TCB; trace f, +RR, 100 μM 25TCB. (B) 25TCB-induced swelling of mitochondria: trace a, +RR, −25TCB; trace b, +RR, 60 μM 25TCB.

Effects on Ca^{2+} *release*

Figure 6A shows 25TCB-induced release of Ca^{2+} from mitochondria that have accumulated Ca^{2+}. Firstly, mitochondria were allowed to accumulate 150 nmoles Ca^{2+}/mg protein from the medium with ATP as the source of energy. Once accumulation was complete, ruthenium red (a potent inhibitor of Ca^{2+} uniporter [27]) was added to block the re-uptake of Ca^{2+}. After ruthenium red addition, various concentrations of 25TCB were added, and Ca^{2+} movements were monitored. Trace b shows an experiment illustrating the control release of Ca^{2+} which became apparent by blocking re-uptake ("basal Ca^{2+} release"). The release of Ca^{2+} is in contrast to the retention observed in the absence of ruthenium red (trace a). At concentrations below 30 μM 25TCB, the efflux rate of Ca^{2+} was very similar to that of basal Ca^{2+} release (trace c). At 40 μM 25TCB, a large increase in the efflux was observed after a 2 min lag period (trace d). When 25TCB concentrations were further raised, rate of Ca^{2+} efflux was increased, and lag period before Ca^{2+} efflux became obvious decreased (traces e and f). Figure 6B shows the absorbance at 540 nm of an identical suspension of mitochondria. A downward deflection indicates mitochondrial swelling. Mitochondria underwent large swelling when 25TCB was added to the incubation medium. Thus, 25TCB-induced Ca^{2+} release is accompanied by the swelling of mitochondria.

DISCUSSION

2,5,2′,5′-Tetrachlorobiphenyl (25TCB) is classified as a phenobarbital (PB)-type inducer of microsomal drug metabolizing enzymes [28]. The toxicity of 25TCB is less than that of 3,4,3′,4′-TCB (34TCB) which categorized into a methylcholanthrene (MC)-type inducer [29]. Although 25TCB is less toxic than 34TCB, a single oral dose of 25TCB in excess of 1.5 g/kg body weight to rats produces heavy mortality within 2–3 days [4]. Regarding the hepatotoxicity caused by 25TCB, Lin *et al.* reported an acute lethal dose (1.7 g/kg body weight) of 25TCB to rats produced swollen hepatocytes that had conformationally altered mitochondria, a large degree of intracellular vacuolization, membrane fragmentation, and an increase in cytosolic matrical materials [5]. Despite the mechanism of toxicity for PCBs remaining unknown, Poland *et al.* suggested that toxic responses of TCBs classified as MC-type inducer are mediated by a binding protein, refered to as the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) receptor [30]. 34TCB binds strongly to this receptor, and its toxicity may be due to biochemical events mediated by this receptor [31]. However, 25TCB does not bind to the TCDD receptor, its toxicity may be exerted by another mechanism.

The results presented in this paper show that 25TCB exerted its deleterious effect on the energy-transducing functions of mitochondria. Namely, 25TCB strongly inhibited state 3 respiration with succinate as the respiratory substrate. In addition, uncoupling of oxidative phosphorylation became evident when succinate was replaced by glutamate/malate. With both substrate, therefore, mitochondrial energy-production was impaired by 25TCB.

The uncoupling by 25TCB was directly related to the dissipation of inside-negative membrane potential (Figs. 2 and 3). With a protonophoric uncoupler such as DNP, the dissipation of membrane potential is performed by carrying protons across the membrane with an acid-dissociable group within the molecule. However, the dissipation by this agent is another mechanism, since 25TCB does not possess an acid-dissociable group. It is likely that 25TCB dissipates membrane potential by producing a non-specific increase in mitochondrial ion permeability as evidenced by the Ca^{2+}-release from mitochondria (Fig. 6). 25TCB possesses nonplanar structure because of the chlorine atoms attached to *ortho. ortho′* positions of the biphenyl ring [32]; intercalation of such molecules into the lipid phase of the membrane is expected to cause membrane damage, leading to impairment of the ion-compartmentation processes which are essential to form membrane potential.

25TCB severely depressed the accumulation of Ca^{2+} by mitochondria when the energy required for this process was provided by substrate oxidation. Furthermore, Ca^{2+} accumulation by mitochondria in the presence of succinate was more intensely inhibited by 25TCB than in the presence of NAD^+-linked substrate, glutamate/malate. On the other hand, with ATP as the source of energy, inhibition was only seen at high 25TCB concentrations (more than 60 μM); the chance, however, to use ATP as

the source of energy may be few *in vivo*, since ATP synthesis from ADP and inorganic phosphate is inhibited by 25TCB. 25TCB did not inhibit the mitochondrial ATPase which catalyses the ATP hydrolysis [25]. It therefore appears that the inhibition of Ca^{2+} accumulation by 25TCB with ATP as the source of energy is probably due to direct action of 25TCB on the Ca^{2+} uptake carrier, Ca^{2+} uniporter. Moreover, 25TCB inhibited electron transport which is responsible for producing inside-negative membrane potential (driving force for Ca^{2+} uptake by substrate oxidation); succinate oxidation was more sensitively inhibited by 25TCB than glutamate/malate oxidation [33], which was also parallel to the inhibition of Ca^{2+} accumulation by 25TCB seen with substrate as the source of energy (Fig. 4). Accordingly, when the energy is supplied by the substrate oxidation, the inhibition of Ca^{2+} accumulation due to 25TCB is also attributed to interference with the electron transport chain, in addition to the inhibition of Ca^{2+} uniporter seen at relatively high concentrations.

The experiments also show that 25TCB released Ca^{2+} that was already accumulated in mitochondria (Fig. 6). The release of Ca^{2+} due to 25TCB was accompanied by the swelling of mitochondria. This indicates that 25TCB caused rather nonspecific alterations in membrane permeability. Thus, 25TCB impairs Ca^{2+} sequestration by liver mitochondria by both the inhibition of Ca^{2+} accumulation and the releasing Ca^{2+} from mitochondrial stores. This and together with the inhibition of plasma membrane ($Ca^{2+} + Mg^{2+}$)-ATPase of hepatocytes [5] may increase cytosolic free Ca^{2+} concentration in hepatocytes (perturbation of intracellular Ca^{2+} homeostasis).

From the above facts, it is suggested that disruption of mitochondrial energy production and subsequent perturbation of intracellular Ca^{2+} homeostasis is a suitable candidate for the toxic mechanism of 25TCB. The latter is spotlighted in relation to toxic mechanism due to chemicals; a common pathway to cell death [34]. Recently, bromobenzene was shown to disrupt hepatocyte Ca^{2+} homeostasis [35]. As 25TCB is a structurally related compound of bromobenzene, it is plausible that 25TCB also perturbs hepatocyte Ca^{2+} homeostasis.

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