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ACTION OF ORGANOPHOSPHORUS COMPOUNDS ON CELL ORGANELLES—I

EFFECT OF TETRAETHYL DITHIO PYROPHOSPHATE ON LYSOSOMAL HYDROLASES

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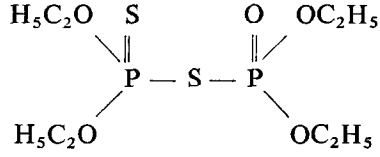
Abstract—The effect of asymmetric tetraethyl dithio pyrophosphate, a potent anticholinesterase compound, on rat brain and liver acid hydrolases from lysosomes was studied *in vivo* and *in vitro*. In both situations activation of "latent" acid hydrolases and labilization of lysosomal membrane have been observed. Three other derivatives, tetraethyl pyrophosphate, tetraethyl monothiono pyrophosphate and tetraethyl dithiono pyrophosphate, were tested for anticholinesterase action as well as for the action on cell organelles. The correlation between the data obtained *in vivo* and *in vitro*, as well as the relationship between this effect and anticholinesterase power is discussed.

IN A previous paper the anticholinesterase activity and the toxicity of asymmetric tetraethyl dithio pyrophosphate* was studied.[1] Synthesized by Almasi *et al.*[2] TETPP is a liposoluble compound, a potent inhibitor of AcChE *in vitro* ($pI_{50} = 7{\cdot}3$ on rat brain homogenate) and *in vivo*. Its DL_{50} is 5·5 mg/kg (mice, i.m. injection). It has a strong myotic activity.[1]



Asymmetric tetraethyl dithio pyrophosphate

As a derivative of TEPP, the interest for its investigation is particularly great, keeping in mind that important changes in the antiChE activity occur by replacing the oxygen atom with sulphur in the molecule of TEPP.[3] On the other hand a change in the structure of an OPC brings about not only changes in the inhibitory potency but it may be followed by the appearance of other interaction capacities as the binding to other proteins[4,5] or esterases.[6–8]

* *Abbreviations*: TETPP, asymmetric tetraethyl dithio pyrophosphate TEPP, tetraethyl pyrophosphate; TEMPP, tetraethyl monothiono pyrophosphate; TEDTPP, tetraethyl dithiono pyrophosphate; OPC, organophosphorus compound; AcChE, acetylcholinesterase; BuChE, butyrylcholinesterase; DTNB, 5,5′-dithiobis(2 nitrobenzoic acid).

We have proposed to investigate the effects of TETPP on the cell organelles. The present paper is a study of the action of OPC on the brain and liver lysosomal hydrolases. Experiments *in vivo* were carried out with sublethal doses of anti-ChE compound which induces a 50–80 per cent inhibition of brain AcChE. Three other known OPC were also used in order to investigate the correlation between the anti-ChE activity and the effect on cell organelles.

MATERIALS AND METHODS

Chemicals. The following substances were synthesized by published procedures: TEPP,[9] TEDTPP,[10] TETPP[2] and TEMPP.[3] A number of materials were purchased from the commercial suppliers as indicated: phenolphtalein-β-monoglucuronide, DTNB (Sigma Chemical Co., St. Louis, U.S.A.), acetylcholine chloride, acetylthiocholine iodide and butyrylcholine iodide (BDH Chemicals Ltd., Poole, England), horse heart cytochrome *c* (Serva, Feinbiochemica, Heidelberg, Germany).

Animals. Male and female albino rats weighing between 150 and 180 g were used.

Administration of TETPP and the control of the intoxication. The animals were injected intramusculary with a single dose of TETPP suspended in vegetable oil, in a dose of 3 mg/kg. After 1 hr the rats were killed by decapitation, their brain and liver (previously perfused) chilled and rinsed in cold sucrose. The degree of intoxication was estimated for each experiment measuring the inhibition of brain AcChE. We considered suitable for biochemical investigation the animals having between 50 and 80 per cent inhibition of AcChE.

Subcellular fractionation of rat brain and liver. The tissue was homogenized in 10 vol. 0·25 M sucrose, buffered with 10 mM Tris–HCl, pH 7·4, in a glass homogenizer fitted with a tight teflon pestle. An aliquot of the homogenate was frozen for later assay of enzyme activity. The homogenate was centrifuged at 600 *g* for 10 min. The sediment (fraction N) was washed twice with 0·25 M sucrose and suspended to a final protein concentration of about 10 mg/ml. The supernatant was centrifuged at 8000 *g* for 15 min and the sediment designated as ML_1 fraction (mitochondria and lysosomes) was washed twice in the same solution and suspended to about 20–30 mg of protein/ml. The supernatant obtained from 8000 *g* centrifugation was submitted at 22,000 *g* for 30 min, the sediment retained without further washings (ML_2 fraction) and the resulted supernatant was called MS fraction (cellular supernatant containing microsomes).

Enzyme activity determinations. ChE activity of brain and liver fractions was measured by means of the Warburg method at 37° in a Krebs–Ringer bicarbonate medium (pH 7·4) equilibrated with a mixture of 95% N_2 and 5% CO_2. The reaction mixture at a final vol. of 2 ml contained about 2 mg of protein. The substrate (acetylcholine and butyrylcholine when brain and respectively liver fractions were used) at a final concentration of 10^{-2} M was put in the side arm of Warburg flasks. The reaction was started by tipping the substrate into the main compartment after thermoequilibration for 10 min. The readings were performed every 5 min for 30–40 min. The spectrophotometric assay of brain AcChE was carried out using the procedure of Ellmann *et al.*[11] The reaction mixture contained at 1 ml final volume and 1 cm light path length, 0·1 M phosphate buffer, pH 7·4, 2×10^{-4} M DTNB and about 0·1 mg of protein. The reaction was started with acetylthiocholine (10^{-3} M final concentration) and the absorption increase at 412 nm was recorded with a C. Zeiss, Jena, VSU-1 model

spectrophotometer. In these conditions to an extinction increase of 0·1 correspond 7.35 nmoles of hydrolysed substrate. Cytochrome oxidase was followed spectrophotometrically at 550 nm.[12] The spectrophotometric cell contained 1 ml of 5×10^{-5} M ferrocytochrome *c* in 0·075 M phosphate buffer, pH 7·2. The reaction was started by addition of subcellular fractions (0·01–0·04 mg of protein) previously solubilized with Na deoxycholate (1 mg/mg of protein). The enzymatic activity is expressed in terms of the first order kinetic constant ($min^{-1} \times$ mg $prot^{-1} \times$ ml).

β-Glucuronidase was assayed by colorimetric determination of phenolphtalein ($\epsilon_{mM}^{550} = 26{\cdot}6$) from phenolphtalein-β-monoglucronide.[13] The incubation medium contained at a final volume of 1 ml: 0·1 M acetate–acetic acid buffer, pH 5·0, 10^{-3} M substrate and 0·4 mg of protein. The reaction (20 min at 37°) was stopped by the addition of 1 ml 5% trichloroacetic acid solution and the colour was developed with alcaline glycine solution. When "total" or "free" activity was measured, to this basal medium (A), 1 mg of nonionic detergent Lubrol XW (medium B) or 0·2 M sucrose (medium C) were respectively added. Acid phosphatase was determined from the inorganic phosphorus released from Na β-glycerophosphate at 37° and 20 min. The incubation mixture (0·5 ml) contained 0·1 M acetate–acetic acid buffer, pH 5·0, 1–2 mg of protein and 0·05 M substrate. When "free" activity was measured, the medium was supplemented with 0·2 M sucrose. Inorganic phosphorus was determined by the procedure of Lowry and Lopez.[14]

Protein determinations. Proteins were assayed by the procedure of Gornall *et al.*[15] Since samples turbidity due to lipids (especially for the brain) is an important source of errors, the proteins were previously precipitated with cold ethanol (final concentration 80%). After centrifugation for 20 min at 3000 *g* and 0°, the supernatant was discarded and proteins were solubilized in 0·45 ml of 1 N NaOH and 0·05 ml 5% Na deoxycholate. By this procedure a good reproducibility was obtained. Control experiments with bovine serum albumin or with rat liver mitochondria, showed that ethanol precipitation is not complete, recorded values being about 3–5 per cent lower. We preferred this way because other methods as lipid extraction with diethylether did not give satisfactory reproducibility.

RESULTS AND DISCUSSION

The distribution of enzymatic activities in fractions obtained by differential centrifugation. Table 1 shows the distribution of proteins, cytochrome oxidase, acid hydrolases and ChE in the fractions obtained by differential centrifugation of brain and liver homogenates. Our results are in agreement with classical works[16,17] most of the rat liver and brain cytochrome oxidase, acid phosphatase and β-glucuronidase being associated with ML_1 fraction. The highest specific activity of AcChE and BuChE was found in ML_2 fraction of brain and liver homogenates, in agreement with previous works of Koeppen *et al.*[18] on brain and Goutier *et al.*[19] on liver tissue. Koeppen *et al.*[18] have shown that the separation of an AcChE enriched cell fraction from brain requires some special treatments. They think that during the separation, the cell fractions could be contaminated with fragments of myelin sheats which bind the enzyme.

Since the inhibition of AcChE after TETPP injection had been observed in all the brain fractions, the degree of enzyme inhibition by OPC injection was determined

TABLE 1. DISTRIBUTION OF ENZYMATIC ACTIVITIES AND PROTEIN IN LIVER AND BRAIN SUBCELLULAR FRACTIONS

			Cytochrome oxidase (6)		Cholinesterase (6)		Acid phosphatase (6)		β-Glucuronidase (8)	
Tissue	Fraction	Protein (4)	Distrib. (%)	Sp. act.	Distrib. (%)	Sp. act.	Distrib. (%)	Sp. act.	Distrib. (%)	Sp. act.
Liver	Whole homogenate			11·9		11·5		16·7		18·2
	N	17·6 ± 0·8*	10·8 ± 0·5	7·3	10·4 ± 0·9	6·8	8·3 ± 0·5	7·9	5·5 ± 0·4	5·7
	ML_1	20·5 ± 1·4	80·3 ± 2·9	46·6	10·3 ± 0·6	5·8	56·1 ± 3·2	37·5	65·1 ± 1·7	58·0
	ML_2†	10·4 ± 0·7	9·1 ± 1·8	10·4	21·2 ± 1·7	23·4	13·8 ± 1·6	22·1	16·5 ± 1·7	28·9
	MS	48·1 ± 1·8	0	0	57·6 ± 1·9	13·8	24·5 ± 1·5	8·5	16·6 ± 1·6	6·3
	Recovery	96·6	100·2		99·5		102·7		103·6	
Brain	Whole homogenate			18·7		75·2		8·7		
	N	30·1 ± 1·9	8·6 ± 0·8	5·3	10·5 ± 1·1	26·3	11·8 ± 0·9	3·2		
	ML_1	30·1 ± 1·0	64·0 ± 2·8	39·0	31·2 ± 2·4	76·5	46·2 ± 4·0	14·0		
	ML_2†	7·4 ± 0·3	10·6 ± 1·3	26·7	17·7 ± 1·5	180·2	13·2 ± 1·6	15·5		
	MS	30·3 ± 1·2	0	0	21·4 ± 1·7	53·1	26·1 ± 1·8	7·5		
	Recovery	98·5	83·2		80·8		97·3			

Specific activity is expressed as nmoles substrate/min/mg of protein. Number of experiments are indicated in parentheses.
* Standard error of the mean.
† Four experiments.

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TABLE 2. DISTRIBUTION OF ACID HYDROLASES IN LIVER AND BRAIN SUBCELLULAR FRACTIONS AFTER TETPP ADMINISTRATION (3 mg/kg)

Fraction	Liver β-glucuronidase (6)		Liver acid phosphatase (6)		Brain acid phosphatase (6)	
	Distrib. (%)	Sp. act.	Distrib. (%)	Sp. act.	Distrib. (%)	Sp. act.
Whole homogenate		17·8		14·6		7·6
N	6·2 ± 0·5*	5·9	8·8 ± 0·9	6·9	13·8 ± 1·1	3·3
ML_1	42·4 ± 1·9 ($P < 0·01$)	41·0	32·7 ± 2·2 ($P < 0·01$)	26·0	21·7 ± 1·8 ($P < 0·01$)	5·6
ML_2†	11·8 ± 2·1 ($P > 0·05$)	23·4	9·4 ± 0·7 ($P < 0·01$)	15·2	12·5 ± 0·9 ($P > 0·1$)	15·9
MS	51·4 ± 2·3 ($P < 0·01$)	17·1	46·9 ± 2·3 ($P < 0·01$)	13·1	45·5 ± 3·5 ($P < 0·01$)	11·5
Recovery	111·8		97·8		93·5	

Specific activity is expressed as nmoles substrate/min/mg of protein. Number of experiments are indicated in parentheses. The incubation medium contained acetate-acetic acid buffer, enzyme and substrate (medium A).

* Standard error of the mean.

† Four experiments.

in each experiment in brain homogenate or MS fraction, immediately after sacrification of the rats. The control of the inhibition was required by following conditions: (a) the absorbtion rate of the toxic is not the same in all experiments; (b) the correlation between AcChE inhibition and possible effects on cell organelles must be determined.

Effects of TETPP in vivo *on the acid hydrolases.* The doses of OPC which inhibit between 50 and 80 per cent the AcChE activity had been shown to increase significantly the activity of liver and brain acid phosphatase and β-glucuronidase in MS fraction (Table 2). This was accompanied by a decrease of activity in particulate fraction (ML_1). Such changes could be taken as indicative about a lysosomal damage. The same significance has the increasing of "free" β-glucuronidase activity in ML_1 fraction of liver (Table 3).

TABLE 3. CHANGES OF "FREE" β-GLUCURONIDASE ACTIVITY OF ML_1 FRACTION OF RAT LIVER AFTER TETPP ADMINISTRATION (3 mg/kg)

Animals	Exp.	Medium			"Free activity"
		A	B	C	
Untreated	1	50·0	51·2	3·6	7·0
	2	55·3	57·6	8·7	15·1
	3	52·6	59·3	7·8	13·2
Injected	1	38·8	40·5	15·6	38·5
	2	51·4	49·2	15·8	32·3
	3	40·3	40·9	12·6	31·2

The incubation medium contained the reagents described in the experimental section. To the basal medium (A) lubrol XW (B) or sucrose (C) were added. "Free" β-glucuronidase activity is expressed as per cent of activity in medium C compared to the activity in medium B considered as 100 per cent.

The question arises, how the OPC can bring about these changes on lysosomal membrane. The following possibilities will be taken in consideration: (a) the hypoxia that often follows the intoxication by OPC; (b) the effect of acetylcholine accumulation; (c) the direct action of TETPP on lysosomes.

(a) An earlier work of Sellinger *et al.*[20] had shown that severe hypoxia (as the 2 hr pinching of hepatic artery) resulted in a release of lysosomal hydrolases from particulate fraction. This was accompanied by a marked depressing of cytochrome oxidase activity up to 10 per cent of control value. But, in our experiments we did not observe any symptoms of respiratory failure and no significant changes of mitochondrial cytochrome oxidase.

(b) In order to control whether accumulated acetylcholine can bring about lysosomal damage, in three experiments the determination of liver β-glucuronidase was made after simultaneous administration of TETPP with atropine sulphate (3 mg/kg). In all cases we observed a similar increase of β-glucuronidase activity in MS fraction.

(c) The direct action of TETPP on cell organelles was controlled *in vitro*, by measuring the acid hydrolases after previous incubation of organelles with different concentrations of OPC.

TABLE 4. EFFECT OF TETPP ON PROTEIN CONTENT AND ENZYMATIC ACTIVITIES OF ML_1 FRACTION

	Liver				Brain		
TETPP (moles/l)	Protein (mg/ml	(%))	β-Glucuronidase	Acid phosphatase	Protein (mg/ml	(%))	Acid phosphatase
	0·65	3·8	50·3	34·4	0·50	4·9	13·2
10^{-6}	0·64	3·7	50·5	32·8			
10^{-5}	0·69	4·0	48·4	32·5	0·45	4·4	14·0
10^{-4}	0·66	3·8	46·9	30·2	0·48	4·7	13·6
10^{-3}	0·73	4·2	44·6	28·9	0·50	4·9	13·1

Two ml of ML_1 fraction from rat liver and brain (17·2 and 10·2 mg of protein/ml) were incubated during 30 min with TETPP at final concentration between 10^{-6} and 10^{-3} M. Thereafter the mixture was centrifuged at 22,000 *g* for 10 min. The supernatant was used for protein and enzymatic assays as described in the experimental section (medium A and B). The enzymatic activities recovered in the supernatant were expressed as nmoles/min/milligram of protein.

Effects of TETPP on cellular organelles. The comparison of data obtained *in vivo* with the data concerning the direct action of the OPC on cellular organelles would show some light on the mechanism of lysosomal membrane labilization and on the relationship between this effect and the anticholinesterase power.

Liver ML_1 fraction was incubated at 0° with different concentrations of TETPP between 10^{-6} and 10^{-3} M. The "released" protein, "free" and "total" β-glucuronidase and acid phosphatase were determined (Table 4 and Fig. 1). In some control

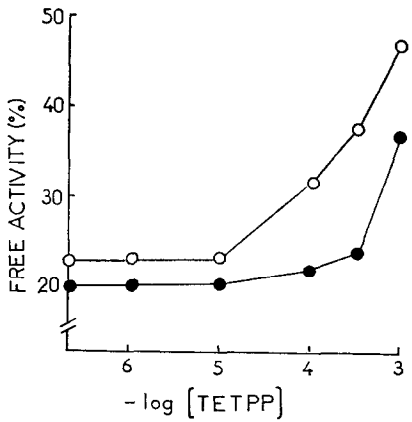

FIG. 1. Effect of TETPP *in vitro* on "free" activity of acid hydrolases from ML_1 fraction of rat liver. Two ml of ML_1 fraction (10·8 mg of protein/ml) were incubated during 30 min at 0° with 0·01 ml TETPP in ethanol, so that its final concentration varies between 10^{-6} and 10^{-3} M. The reference sample contained 0·01 ml ethanol. Thereafter the enzyme activity was measured, "free" activity being expressed as described in Table 3. (●) β-glucuronidase; (○) acid phosphatase.

experiments the effect of "aging "at 25° and of some "labilizing" agents as ascorbic acid or reduced glutathion (GSH) was determined. Although the amount of "released" protein did not exceed that from reference samples even at 10^{-4} to 10^{-3} M TETPP, the increase of "free" acid hydrolases activity could be however observed (Fig. 1). The incubation of organelles with 5×10^{-3} M ascorbic acid or GSH was accompanied by increase in "free" hydrolases activity and the release of enzymatic protein from particulate fraction.

We have also investigated the resistence of organelles to variation in sucrose concentration of the medium after a previous incubation for 30 min at 0° with 10^{-4} and 10^{-3} M TETPP. Figure 2 is a plot of variation of "free" activity of β-glucuronidase as a function of sucrose concentration. It is clear that the previous incubation with TETPP markedly decreases the osmotic resistence of lysosomal membrane.

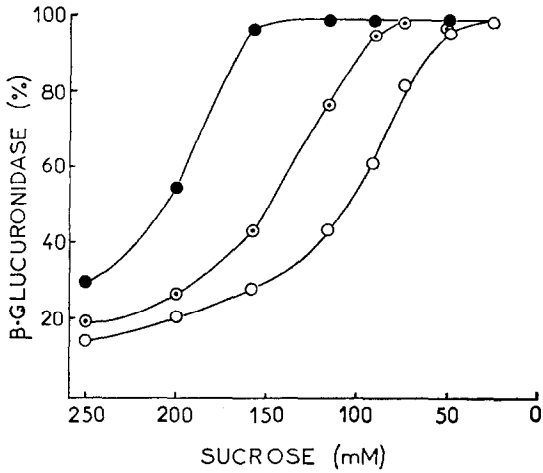

FIG. 2. Effect of TETPP on osmotic resistence of liver lysosomes. Two ml of ML_1 fraction from rat liver (13·4 mg of protein/ml) were incubated during 30 min at 0° with 0·01 ml TETPP in ethanol, its final concentration being 10^{-4} (⊙) or 10^{-3} M (●). The reference sample contained 0·01 ml ethanol (○). Thereafter from each sample 1/10 dilutions were made so that the final concentration of sucrose in the medium was between 0·025 and 0·25 M. After 10 min of incubation at 0°, β-glucuronidase activity was measured in a mixture containing acetate–acetic acid buffer, pH 5·0, substrate and 0·2 M sucrose. The results are expressed as per cent from the activity of organelles previously treated with lubrol.

The correlation between in vitro *and* in vivo *experiments.* A separate analysis of the data obtained *in vivo* and of those obtained *in vitro* allows the assumption that TETPP acts on lysosomal membrane activating and releasing acid hydrolases. We don't know whether these two groups of data reflect the same event, and whether the effect on organelles is correlated with anti-ChE action or with toxicity. In order to answer this question we followed two possibilities: (a) by comparing the concentration of the toxic at which alteration of the organelles are brought about *in vivo* and *in vitro*; (b) by comparing the action on the organelles of some OPC with similar structure but with different anti-ChE potency and toxicity.

(a) Assuming as first approximation that the injected TETPP (3 mg/kg) is distributed to various tissues as another OPC (^{32}P-Sarin),[5] a final concentration of about 10^{-5} M could be found in brain or liver, after 1 hr from the injection. But it is to be noted that only 50–80% of brain AcChE inhibition have been observed after 1 hr, despite the great anti-ChE potency of this compound *in vitro* ($pI_{50} = 7{\cdot}3$). We have no information about the rate of absorbtion, distribution and metabolization of this OPC, but we may suppose that in the tissue, the concentration of the toxic are more lower than those necessary to induce the similar effects on organelles *in vitro* (10^{-4} to 10^{-3} M). The above mentioned data show that the correlation between two kinds of experiments is only apparent.

Similar observations were made by Jovič *et al.*,[22] who studied the effects of some OPC on the oxygen uptake of rat brain slices, Na^+ and K^+-dependent ATPase, succinate dehydrogenase and aldolase activity. They found that there are necessary great concentrations of soman or DFP (3×10^{-3} M), in order to induce *in vitro* the depression of enzymatic activities observed with lethal doses of OPC. They explained the enzymatic changes as a consequence of respiratory or circulatory failure.

(b) Table 5 shows the effect on lysosomal enzymes of four OPC, derivatives of TEPP, in which the oxygen was replaced by one or two sulphur atoms. These structural changes result in great differences of anti-ChE potency and toxicity of the compounds, as it can be seen from the values of the second order rate constant ($s^{-1}M^{-1}$) of AcChE phosphorylation* or from DL_{50} values. It is noteworthy that the weakest anti-ChE compound (dithiono symmetric) has the most evident action on the organelles *in vitro*. It results that any correlation between cholinesterase inhibiting

TABLE 5. RELEASE OF LATENT ACTIVITY OF ACID HYDROLASES FROM ML_1 FRACTION OF RAT LIVER AS FUNCTION OF ANTICHOLINESTERASE POTENCY OF SOME OPC

Compound	K′ (s^{-1} M^{-1})	β-Glucuronidase a	b	c	Acid phosphatase a	b	c
TEPP	44,800				32		36
TEMPP	53,100	23	28	33	26	30	32
TETPP	6600	22	24	37	32	38	47
TEDTPP	531	23	31	35	29	39	64

(a) OPC concentration 10^{-4} M.
(b) OPC concentration 3×10^{-4} M.
(c) OPC concentration 10^{-3} M.
Experimental conditions are described in the legend of Fig. 1 and in the experimental section. Protein content of ML_1 fraction was between 12 and 15 mg/ml. "Free" activity of acid hydrolases (mean of four experiments) was expressed as per cent of activity in medium C compared to the activity in medium B considered as 100 per cent. "Free" activity of the reference sample was 19·0 for β-glucuronidase and 22·9 for acid phosphatase, respectively. Second order rate constant (s^{-1} M^{-1}) for AcChE phosphorylation was determined with purified enzyme from bovine erythrocyte.

* T. Bârzu and B. Cuparencu, in preparation.

potency and the effect on organelles cannot be found. Our data are not in agreement with a preliminary report of Ntiforo and Stein,[23] who had studied the labilizing effect of Malathion on the lysosomal membrane. Their results indicate that the labilizing effect is qualitatively related to anti-ChE action.

It may be concluded that high concentration of OPC exhibit an activation of lysosomal acid hydrolases. This action seems to be not correlated with anti-ChE effect, other factors such as the higher liposolubility of thio or thiono compounds, or the binding to organelles membrane, could be responsible for such an action. The last possibility is taken in our attention.

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