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EFFECT OF CLORGYLINE ON TRINITROBENZENESULFONIC ACID INCORPORATION INTO MITOCHONDRIAL MEMBRANES

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Abstract—The stimulation of trinitrobenzenesulfonic acid (TNBS) into mitochondrial membranes by the MAO inhibitor clorgyline has been investigated. The incorporation of TNBS increased with increasing pH, whereas the stimulation of TNBS incorporation by clorgyline decreased with increasing pH. The stimulation effect could not be blocked by treatment of the membranes with phospholipase C or the chelating agent EDTA, but could be blocked by thiol reagents. However, no interaction between clorgyline and thiol groups could be detected. This stimulation of TNBS into mitochondrial membranes could not be attributed to a direct interaction of TNBS with clorgyline, a change in ionic strength, or a complex formation between clorgyline and the TNBS molecule. The compounds 1-anilinonaphthalene-8-sulfonic acid and 4-hydroxyquinone both blocked the stimulation of TNBS by clorgyline, suggesting that clorgyline interacts with protein and lipid hydrophobic regions. It is suggested that clorgyline interacts with mitochondrial membrane components other than MAO at concentrations higher than that required for enzyme inhibition.

It has been suggested that the acetylenic compounds, pargyline and clorgyline, are specific irreversible inhibitors for partially purified monoamine oxidase [monoamine: O_2 oxidoreductase (deaminating) EC 1.4.3.4; MAO][1,2]. Pargyline has also been suggested to be a specific inhibitor of MAO *in vivo*[3]. However, some of these acetylenic compounds have recently been shown to inhibit enzymes other than MAO, including plasma amine oxidase[4,5] and aldehyde dehydrogenase[6,7]. Furthermore, it has been found that deprenil, another acetylenic MAO inhibitor, blocks the uptake of norepinephrine into mouse brain slices and the depletion of norepinephrine from microsomes of the rat heart[8]. These observations suggest possible binding sites for these compounds other than MAO but may be classified as nonspecific as they mainly occur at concentrations higher than that required for MAO inhibition. Since MAO is strongly bound to the outer mitochondrial membrane[9,10], these acetylenic inhibitors may interact nonspecifically with components of the membrane other than the enzyme.

The rate of reaction of trinitrobenzenesulfonic acid (TNBS) with amino groups has been used to study lipid–protein interactions, and perturbation of the erythrocyte membrane by ions and drugs[11,12]. The present investigation uses the rate of TNBS incorporation into mitochondrial membranes in an attempt to characterize the interaction of membrane constituents with the MAO inhibitor, clorgyline.

MATERIALS AND METHODS

Determination of MAO activity. Assay tubes in ice contained 50 μl of enzyme preparation (0.1 to 2.5 mg protein/ml), 100 μl of 0.1 M sodium phosphate buffer at pH 7.5 containing 2 mM EDTA and 100 μl tyramine. The final tyramine concentration was 1 mM, containing 25 nCi [^{14}C] tyramine. Incubations were performed at 37° in a shaking water bath for 20 min. At the end of the incubation, 0.3 ml of 2 N HCl was added and the reaction products were extracted and counted, as described by Wurtman and Axelrod[13].

Preparation of mitochondria. Rat liver mitochondria were isolated by the method of Hunter *et al.*[14], and washed (resuspended and centrifuged at 10,000 *g* for 15 min) twice with 0.25 M sucrose and twice with 0.05 M Tris buffer at pH 7.5.

TNBS incorporation. A slightly modified procedure described by Godin and Ng[11] was used by following the increase in absorbance of the reaction mixture at 335 nm as a function of time. Experiments were carried out at 37° in a total volume of 3 ml containing 1.8 ml of 0.05 M Tris buffer at pH 7.5, unless otherwise indicated, 0.1 ml of 6 mM TNBS which had been adjusted to pH 7.0 before use, and the compound to be tested in aqueous solution; the volume was made up to 2.9 ml with distilled water. The reaction was initiated by the addition of 0.1 ml of 10 mg/ml of mitochondrial protein. Incubations were for 10 min unless otherwise indicated, at which time 2.0 ml of 5% (w/v) sodium dodecyl sulfate in 0.5 N HCl was added and the absorbance read at 335 nm against the appropriate blank without membranes. Experimental values were also corrected with blanks which contained membranes in the absence of TNBS.

Treatment with phospholipase C. Suspension of mitochondria (10 mg protein/ml) in 0.05 M Tris buffer, at pH 7.8, were made 1 mM in $CaCl_2$, and 0.5 mg phospholipase C/10 mg of protein was added. The mixtures were incubated at 37° for 20 min and then centrifuged at 20,000 *g* for 20 min. The pellet was then resuspended in Tris buffer at pH

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7.5 for determination of TNBS incorporation.

Estimation of phospholipid content. Phospholipids were determined by a modification of the method developed by Raheja *et al.*[15]. The chromogenic solution was prepared as described by Vaskovsky and Kostetsky[16] and the phospholipids were extracted from the protein samples by the method of Folch *et al.*[17]. The chloroform–methanol extract was evaporated to dryness; subsequently, 0.4 ml chloroform and 0.1 ml of the chromogenic reagent were added to each tube. A blank was prepared with chloroform and chromogenic solution only. The tubes were placed in a boiling water bath for 1 to 1.5 min and then cooled to room temperature. After standing for 5 min, 1 ml chloroform and 2 ml 1,2-dichloroethane were added and the tubes were shaken for 1 min followed by centrifugation at 500 g for 3 min. The organic layer was read at 710 nm against the blank. A calibration curve was prepared using phosphatidylcholine as a standard, and was linear through 300 μg phospholipid.

Other methods. Protein was determined by the method of Lowry *et al.*[18] using bovine serum albumin as a standard. Protein thiol groups were determined by the method described by Ellman[19] and the results were expressed as μg cysteine/mg of protein.

Materials. [Side chain-2-^{14}C] tyramine hydrochloride (50 μCi; 55 mCi/m-mole) was obtained from the Radiochemical Centre, Amersham, United Kindgdom. The following were obtained from Sigma Chemical Co., Ltd., Surrey, U.K.: tyramine HCl, 2,4,6-trinitrobenzenesulfonic acid, bovine serum albumin, DL-α-phosphatidylcholine dipalmitoyl, phospholipase C from *Clostridium welchii* and 5,5'-dithiobis-(2-nitrobenzoate). Monopropargylamine was purchased from Aldrich Chemical Co., Inc., Milwaukee, WI, U.S.A. Clorgyline, *N*-methyl-*N*-propargyl-3-(2,4 dichlorophenoxy) propylamine, was a gift from Dr. D. R. Maxwell, May & Baker Ltd., Dagenham, U.K. 1-Anilinonaphthalene-8-sulfonic acid magnesium salt and all other reagents were obtained from BDH Chemicals, Poole, U.K.

RESULTS

Clorgyline potentiation of TNBS incorporation. The reaction of 0.2 mM TNBS with mitochondrial protein and lipid amino groups was linear over the range of 0.5 to 10 mg protein/3.0 ml of reaction mixture. The time course of TNBS incorporation in the presence and absence of 0.5 mM clorgyline is shown in Fig. 1. Clorgyline increased the amount of TNBS incorporated and 10 min was chosen as the standard time of incubation. The stimulation of TNBS incorporation into mitochondrial membranes by varying concentrations of clorgyline (0.1 to 1.5 mM) is shown in Fig. 2. The stimulation increased up to 1.0 mM clorgyline, after which a plateau was reached.

The incorporation of TNBS alone increases linearly over the pH range of 7.0 to 8.0, as shown in Fig. 3. However, over this pH range of 7.0 to 8.0, there is a difference in the stimulation of TNBS incorporation into the mitochondrial membrane by clorgyline compared to that shown in Fig. 3. This effect is shown in Fig. 4, and it can be observed that, as the pH is raised from 7.0 to 8.0, the per cent stimulation of TNBS incorporation by 0.5 mM clorgyline is decreased from 55 to 5 per cent.

Several explanations for the stimulation of TNBS incorporation by clorgyline are possible. No direct interaction between the TNBS and clorgyline molecules could be detected and, as shown in Fig. 5, the apparent K_{diss} of clorgyline for stimulation of TNBS into mitochondrial membranes is unchanged over a 6-fold range of TNBS concen-

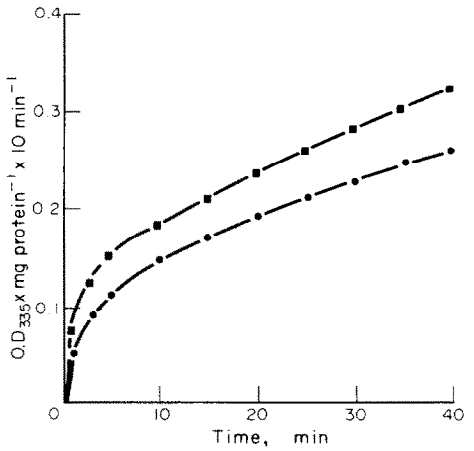

Fig. 1. Time course of TNBS incorporation into mitochondrial membranes in the absence and presence of clorgyline. Assays of both control TNBS incorporation (●) and incorporation in the presence of 0.5 mM clorgyline (■) were performed in 0.05 M Tris buffer at pH 7.5 with a final TNBS concentration of 0.2 mM. At the times indicated, reactions were terminated with 5% sodium dodecyl sulfate/0.5 M HCl and the absorbance was determined at 335 nm. Each point on the graph is the mean of four determinations which varied by less than 4 per cent.

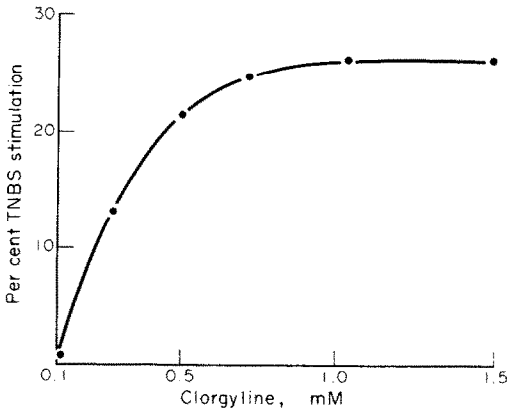

Fig. 2. Concentration dependence of clorgyline on the rate of TNBS incorporation into mitochondrial membranes. Incubations were for 10 min at 37° in 0.05 M Tris buffer at pH 7.5 with a final TNBS concentration of 0.2 mM, as described in Materials and Methods. Control values for TNBS incorporation in the absence of clorgyline were $0.162 \pm 0.003 \times$ mg protein$^{-1} \times 10$ min^{-1}. Each point on the graph is the mean of four determinations which varied by less than 4 per cent.

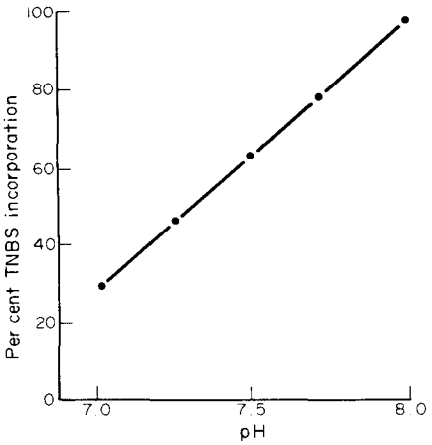

Fig. 3. Effect of pH on TNBS incorporation into mitochondrial membranes. All assays were performed with a final TNBS concentration of 0.2 mM in 0.05 M Tris buffer at the pH indicated. Values are expressed as a per cent of the control value ($0.238 \pm 0.007 \times$ mg protein$^{-1} \times$ 10 min^{-1}) set at pH 8.0. Each point on the graph is the mean of four determinations which varied by less than 4 per cent.

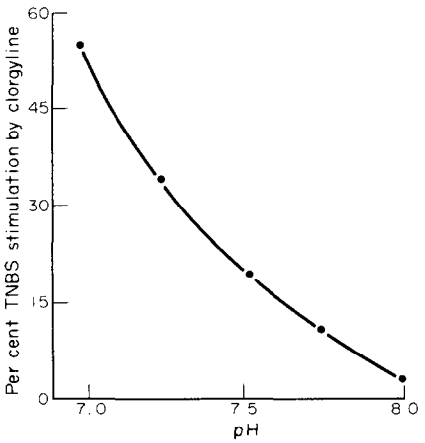

Fig. 4. Effect of pH on the clorgyline stimulation of TNBS incorporation into mitochondrial membranes. All assays were performed with a final TNBS concentration of 0.2 mM in 0.05 M Tris buffer at the pH indicated. Results are expressed as per cent stimulation of TNBS incorporation by 0.5 mM clorgyline compared to controls in the absence of clorgyline. Each point on the graph is the mean of four determinations which varied by less than 4 per cent.

trations. This indicates that the stimulatory effects of clorgyline do not depend on complex formation with TNBS. Propargylamine, over the concentration range 0.1 to 10 mM, did not affect the incorporation of TNBS, indicating that a change in ionic strength was not responsible for the stimulation effect of clorgyline. It seems, therefore, that clorgyline may be interacting with some membrane components in a way that increases the reaction of TNBS with membrane amino groups.

Effect of phospholipase C treatment on the stimulation of TNBS incorporation. The possibility of a clorgyline–phospholipid phosphoryl group interaction was tested by treating mitochondrial

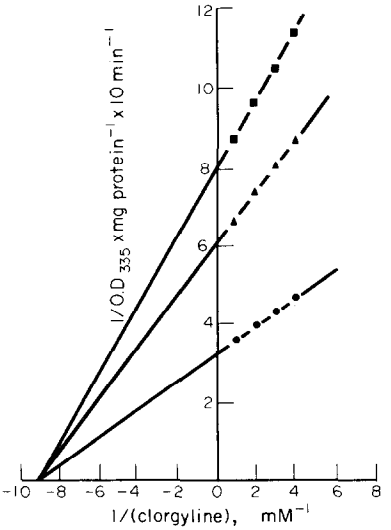

Fig. 5. Effect of increasing TNBS concentrations on the apparent K_{diss} of clorgyline for the stimulation of TNBS incorporation into mitochondrial membranes. Assays were performed as described in Materials and Methods except that the final TNBS concentrations were 0.2 mM (■), 0.33 mM (▲) and 1.2 mM (●). Each point on the graph is the mean of four determinations which varied by less than 4 per cent.

membranes with phospholipase C. This treatment reduced the lipid phosphoryl groups by 66 per cent (144 ± 8 μg phospholipid/mg of protein to 49 ± 11 μg phospholipid/mg of protein) and reduced the rate of TNBS incorporation but had no effect on the magnitude of the clorgyline stimulation of TNBS (Table 1). In contrast, chlorpromazine at a concentration of 0.1 mM stimulated TNBS incorporation by 71 per cent, but, after treatment with phospholipase C, this effect was abolished.

Effect of thiol reagents and other compounds. When arsenite (50 mM), a compound that interacts with vicinal thiol groups[20], was present in the incubations, the clorgyline-induced stimulation of TNBS incorporation was reduced by half (Table 2). In addition, mercuric chloride (5 mM), which itself causes an increase in TNBS incorporation, does not allow clorgyline to produce any further stimulation. However, treatment of the membranes with clorgyline over the concentration range 0.1 to 1 mM did not result in any decrease in the thiol content (8 ± 0.3 and 8 ± 0.5 μg cysteine/mg of protein for control and 1 mM clorgyline-treated membranes respectively) as measured by their reaction with 5,5′-dithiobis-(2-nitrobenzoate).

Acetylene compounds that inhibit MAO have been suggested to react with metal ions[21]. However, as shown in Table 2, EDTA (1 mM) had no effect on the clorgyline stimulation of TNBS. 1-Anilinonaphthalene-8-sulfonic acid and 4-hydroxyquinone (1 mM) both reduced the TNBS incorporation and also reduced the clorgyline stimulation of TNBS incorporation to control values (Table 2).

Clorgyline stimulation of TNBS after MAO inhibition. In order to investigate the possibility of the clorgyline–MAO interaction as part of the

Table 1. Effect of phospholipase C on the stimulation of TNBS incorporation by chlorpromazine and clorgyline*

Compounds present	Control membranes	Phospholipase C-treated membranes
	$O.D._{335} \times mg\ protein^{-1} \times 10\ min^{-1}$	
Control	0.147 ± 0.004 (100)	0.129 ± 0.003 (100)
Chlorpromazine (0.1 mM)	0.251 ± 0.006† (171)	0.131 ± 0.005‡ (102)
Clorgyline (0.5 mM)	0.181 ± 0.004† (129)	0.162 ± 0.002† (126)

* Assays were performed in 0.05 M Tris buffer at pH 7.5 with a final TNBS concentration of 0.2 mM as described in Materials and Methods. Each value is the mean of four determinations ± standard deviation. Numbers in parentheses are percentages.

† Values significantly different from their respective controls evaluated by Student's t-test ($P < 0.01$).

‡ Not significant.

Table 2. Effect of some compounds on the stimulation of TNBS incorporation by clorgyline*

Compounds present in control and clorgyline-treated membranes	Control membranes	Clorgyline-treated membranes
	$O.D._{335} \times mg\ protein^{-1} \times 10\ min^{-1}$	
None	0.153 ± 0.003 (100)	0.200 ± 0.005† (131)
Arsenite (50 mM)	0.156 ± 0.005	0.184 ± 0.003† (116)
Mercuric chloride (5 mM)	0.200 ± 0.007	0.203 ± 0.005‡
EDTA (1 mM)	0.160 ± 0.008	0.205 ± 0.006† (128)
4-Hydroxyquinone (1 mM)	0.127 ± 0.003	0.132 ± 0.007‡
1-Anilinonaphthalene-8-sulfonic acid (1 mM)	0.137 ± 0.004	0.145 ± 0.009‡

* Assays were performed in 0.05 M Tris buffer at pH 7.5 with a final TNBS concentration of 0.2 mM as described in Materials and Methods. Each value is the mean of four determinations ± standard deviation. Numbers in parentheses are percentages.

† Values significantly different from their respective controls evaluated by Student's t-test ($P < 0.01$).

‡ Not significant.

Table 3. Clorgyline stimulation of TNBS incorporation after irreversible inhibition of MAO by clorgyline and removal of free inhibitor*

Concentration of clorgyline used to inhibit MAO (μM)	% MAO inhibition after dialysis to remove free clorgyline	TNBS incorporation in the presence of 0.5 mM clorgyline after dialysis ($O.D._{335} \times mg\ protein^{-1} \times 10\ min^{-1}$)
None	0	0.185 ± 0.003
0.1	60	0.180 ± 0.006
1.0	95	0.187 ± 0.004
10	100	0.190 ± 0.005
100	100	0.184 ± 0.006
500	100	0.181 ± 0.005

* Mitochondrial membranes were incubated for 10 min at 37° with the indicated concentration of clorgyline and then dialyzed to remove free inhibitor. MAO activity using tyramine as substrate and TNBS incorporation were determined as described in Materials and Methods. Control values for TNBS incorporation in the absence of 0.5 mM clorgyline were 0.151 ± 0.008 × mg protein^{-1} × 10 min^{-1}. MAO determinations were performed in triplicate and varied by less than 3 per cent. Each TNBS incorporation value is the mean of four determinations ± standard deviation.

stimulation of TNBS incorporation, mitochondrial membranes were incubated for 10 min with from 0.1 to 500 μM clorgyline and then dialyzed 36 hr to remove any free inhibitor. These membrane preparations were then used to test for the stimulation of TNBS incorporation by clorgyline. As shown in Table 3, MAO is progressively inhibited after dialysis by clorgyline concentrations up to 10 μM, after which complete inhibition was observed. However, after dialysis, the same stimulation of TNBS incorporation was observed by 0.5 mM clorgyline as with membranes that had not been treated with the compound prior to dialysis. Furthermore, after complete inhibition of MAO and removal of free inhibitor, the same time course of TNBS incorporation with and without 0.5 mM clorgyline was observed as that shown in Fig. 1.

DISCUSSION

Trinitrobenzenesulfonic acid has been used for the study of amines and lipids, and as a probe for the erythrocyte membrane[11,22,23]. In this study the rate of trinitrophenylation of mitochondrial membrane protein and lipid amino groups was used to monitor alterations in membrane structure induced by the MAO inhibitor clorgyline.

Over a 40-min incubation period the amount of TNBS incorporated into mitochondrial membranes was increased by clorgyline (Fig. 1) and was dependent on the concentration of clorgyline used (Fig. 2). This effect could not be attributed to a direct interaction of clorgyline with the TNBS molecule or to changes in ionic strength. Furthermore, the K_{diss} of clorgyline (Fig. 5) was unchanged over a 6-fold range of TNBS concentrations, indicating that a complex formation between clorgyline and the TNBS molecule does not enhance the reactivity of TNBS or increase its accessibility to any binding sites. Therefore, the stimulation of TNBS incorporation by clorgyline is most likely a reflection of the way clorgyline interacts with the mitochondrial membrane, producing a change (perturbation) which results in TNBS reacting faster and with more membrane amino groups.

The rate of TNBS incorporation into mitochondrial membranes increased with increasing pH (Fig. 3), which, as suggested by Gordesky *et al.*[24], may be due to a greater chemical reactivity of the nonprotonated amino groups at higher pH values. However, it is possible that the conformation of the membrane may be altered in such a way, at higher pH values, as to expose more amino groups for reaction. The stimulation of TNBS incorporation by clorgyline decreases with increasing pH (Fig. 4); this may reflect an alteration in the mitochondrial membrane structure with increase in pH or alternatively may reflect the possibility that the concentration of soluble clorgyline is higher at lower pH values.

It has been shown that chlorpromazine greatly increased the TNBS incorporation into erythrocyte membranes and that this effect could be reduced by pretreatment of the membranes with phospholipase C[11]; this indicated that chlorpromazine interacted with the phospholipid phosphoryl groups. A similar effect was observed with chlorpromazine on mitochondrial membranes; however, phospholipase C treatment had no effect on the clorgyline stimulation of TNBS incorporation, indicating that a clorgyline–phosphoryl group interaction was not responsible for the TNBS stimulation (Table 1).

Belleau and Morgan[25] suggested that the acetylenic groups of MAO inhibitor drugs may react with thiol groups. The possibility of clorgyline interacting with the membrane by a reaction with protein thiol groups and thereby increasing the accessibility of TNBS to its reaction sites has been suggested in the present work by the observation that arsenite reduced by 50 per cent the stimulation of TNBS incorporation by clorgyline. Furthermore, mercuric chloride, which increased the rate of TNBS incorporation, abolished the clorgyline effect (Table 2). However, after membrane treatment with clorgyline, the number of thiol groups did not decrease, indicating that clorgyline does not potentiate TNBS incorporation by an interaction with thiol groups. Acetylenic MAO inhibitors have been shown by others not to interact with thiol groups[26]. Acetylene groups are known to react with metal ions but this type of interaction cannot explain the clorgyline effect because treatment of the membranes with EDTA does not decrease the clorgyline stimulation of TNBS incorporation.

1-Anilinonaphthalene-8-sulfonic acid, a compound which interacts with hydrophobic regions[27] and 4-hydroxyquinone, which due to its ring structure is also probably hydrophobic in nature, abolished the clorgyline effect (Table 2). This suggests that clorgyline perturbs the mitochondrial membrane through either protein and/or lipid hydrophobic regions with the aromatic moiety of the molecule. This is further supported by the observation that propargylamine, which has no aromatic ring, does not affect the rate of TNBS incorporation in concentrations up to 10 mM.

Prior irreversible inhibition of MAO by clorgyline (0.1 to 500 μM) and removal of any free inhibitor by dialysis have no effect on the clorgyline stimulation of TNBS incorporation. This observation indicates that: (1) the alteration of the mitochondrial membrane by clorgyline is reversible, whereas MAO inhibition is irreversible, and (2) the interaction of MAO with clorgyline is not part of the increase observed in TNBS incorporation. However, MAO is totally inhibited at clorgyline concentrations of 10 μM whereas the stimulation of TNBS incorporation is measurable only at clorgyline concentrations greater than 0.1 mM. Therefore, if the clorgyline–MAO interaction results in a greater reactivity of the amino groups of the MAO enzyme, the TNBS assay is not sufficiently sensitive to measure this effect.

In conclusion, the present results indicate that, at low concentrations, clorgyline inhibits MAO and at higher concentrations interacts hydrophobically with other membrane components. Similar results have recently been obtained by McCauley[28], who found that [^{14}C]pargyline, an acetylene compound structurally similar to clorgyline, binds to membrane components other than

MAO at concentrations above the limit where MAO is completely inhibitied.

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