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The Effect of Lead on the Calcium-Handling Capacity of Rat Heart Mitochondria

By D. R. PARR and E. J. HARRIS
Biophysics Department, University College London, Gower Street, London WC1E 6BT, U.K.

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1. Very low concentrations of Pb^{2+} decrease the capacity of rat heart mitochondria, oxidizing pyruvate plus malate, to remove Ca^{2+} from the medium. 2. The primary effect is on the rate of Ca^{2+} sequestration; this is reflected in the overall extent of Ca^{2+} removal. 3. Pb^{2+} has at least two separate actions. Below about 0.5 nmol/mg of protein, it acts solely by competing with Ca^{2+} ($K_i = 0.4\,\mu M$); above this concentration it also inhibits the production or use of respiratory energy, so that at 1 nmol of Pb^{2+}/mg of protein, Ca^{2+} removal is almost completely abolished. 4. Pb^{2+} inhibits coupled and uncoupled respiratory O_2 use by mitochondria oxidizing pyruvate plus malate, but at higher concentrations than those that affect Ca^{2+} removal; similar concentrations of Pb^{2+} inhibit pyruvate uptake, but not malate uptake, by the mitochondria. 5. Mg^{2+} only decreases Ca^{2+} removal by competition, and is a far-less effective competitor than Pb^{2+} ($K_i = 0.15$ mM). It is possible that the primary cause of the second effect of Pb^{2+} is displacement of membrane Mg^{2+}. 6. The consequences of these results are discussed in terms of the possible involvement of heart mitochondria in excitation–contraction coupling, and the Pb^{2+} levels that might occur in heart tissue *in vivo*.

Mitochondria are increasingly being implicated in the regulation of cytosolic Ca^{2+} concentrations, and through this in the control of a variety of cellular processes. One such process is excitation–contraction coupling of cardiac muscle (Lehninger, 1974; Carafoli, 1975), a tissue in which the mitochondria have unique Ca^{2+}-handling characteristics (Jacobus *et al.*, 1975) and sarcoplasmic reticulum is scarce.

A flux of as little as 0.3 nmol of Ca^{2+}/mg of protein could be sufficient to cause contraction and relaxation (Carafoli, 1975), and although the ability of heart mitochondria to remove Ca^{2+} sufficiently fast has been questioned (e.g. Scarpa & Graziotti, 1973) it may be sequestered by binding to anionic sites on the membrane, rather than by true uptake through the membrane. Such binding occurs irrespective of the presence of a permeant anion, and is very rapid (Lehninger, 1974).

The involvement of mitochondria in excitation–contraction coupling probably varies between species, but the widespread Ca^{2+}-accumulating capacity of mitochondria strongly suggests that at very least they perform a scavenging role, ensuring that cytosolic Ca^{2+} does not exceed concentrations at which the activities of enzymes are altered, and other Ca^{2+}-handling mechanisms are unable to cope. Factors that decrease the rate or extent of Ca^{2+} removal are thus likely to disrupt heart function; in the present paper we show that Pb^{2+} is one such factor.

We have shown previously that about 10 nmol of Pb^{2+}/mg of protein greatly decreases the phosphorylation performance of heart mitochondria *in vitro*, in the presence of ATP (Parr & Harris, 1975*a*) or other chelating and complexing agents (Parr & Harris, 1975*b*). Now we report that one-tenth of that Pb^{2+} concentration significantly decreases the rate, and hence the extent, of Ca^{2+} removal by energized heart mitochondria in the absence of P_i. The only previous report is that of Scott *et al.* (1971) who comment briefly that Pb^{2+} competes with Ca^{2+} for uptake into de-energized mitochondria.

This work forms part of a programme investigating the effects of heavy metals on membrane function.

Experimental

Mitochondrial preparation and incubation

Rat heart mitochondria were prepared from female Sprague–Dawley rats, as described by Parr *et al.* (1975). The final wash was in 300 mM-sucrose, freed from Ca^{2+} by passage down a Dowex ion-exchange column and buffered at pH 7.4 with 10 mM-Tris/Hepes;* this was done to remove any residual EGTA carried over from the earlier preparative procedure.

* Abbreviations: Hepes, 2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid; EGTA, ethanedioxybis-(ethylamine)tetra-acetic acid.

No bovine serum albumin was added to the final mitochondrial preparation.

Incubations were at 22.5°C in small plastic pots, with mitochondria at a final concentration of 0.75mg/ml in about 1ml of medium containing 150mM-KCl, 10 mM-Tris/Hepes, 3mM-Tris pyruvate and 3mM-Tris malate, pH7.4. Incubations were stirred to ensure rapid mixing, and had a large surface area/volume ratio to achieve aeration.

Treatment with Pb^{2+} and Mg^{2+}

Pb^{2+} was added as 0.5mM- or 10μM-$Pb(NO_3)_2$ and Mg^{2+} as 300mM-$MgCl_2$. In both cases the cation was added at a fixed time, usually 5min before either Ca^{2+} or substrate addition, or sampling for ATP assay.

Ca^{2+}-removal experiments

To measure Ca^{2+}-removal rates, about 55 or 155nmol of $^{45}Ca^{2+}$/mg of protein was added to mitochondrial suspensions. After exactly 10s, 50μl of 100mM-EGTA was added to halt Ca^{2+} movement, as described by Reed & Bygrave (1974*a*). The suspension (1ml) was then centrifuged for 1min in a conical plastic tube in a Coleman Microfuge. The supernatant was discarded, and the pellet washed by filling the tube with medium, which was then also discarded. The pellet was extracted by grinding in 100μl of 1.5M-$HClO_4$, and the $^{45}Ca^{2+}$ radioactivity in samples counted after dilution and centrifugation. Appropriate corrections were made for carry-down by separate experiments with [^{14}C]dextran. Reed & Bygrave (1974*a*,*b*), using rat liver mitochondria, sometimes included Ruthenium Red in their quench medium. With the procedure described here, omission of Ruthenium Red made no significant difference to the measured Ca^{2+} removal rates. Ca^{2+} associated with mitochondrial pellets after EGTA quench was stable for at least 7min, although in practice centrifugation and washing of the pellet was completed within 2min.

In experiments to measure removal rates, calcium buffers, as recommended by Reed & Bygrave (1975) were not necessary, because the Ca^{2+} concentrations used were above the saturating level of the uptake, so that small amounts of endogenous chelating agents had no significant effect on removal rates.

Total Ca^{2+} removal was usually measured with incubations as described above. The suspension was left for 5min after the Ca^{2+} addition, by which time removal was completed. After centrifugation, samples of the supernatant were assayed for $^{45}Ca^{2+}$.

ATP assays

Samples (1ml) of suspension were extracted by adding 0.5ml of cold $HClO_4$. The precipitated protein was removed, samples neutralized, and decanted from the precipitated perchlorate before assaying the ATP fluorimetrically by following the conversion of $NADP^+$ into NADPH by a coupled hexokinase–glucose 6-phosphate dehydrogenase system.

Substrate-uptake measurements

Mitochondria were suspended in medium lacking substrates; Tris [^{14}C] pyruvate or Tris [^{14}C] malate were added to give a final concentration of 0.8mM. Their uptake was terminated after 5s by the addition of α-cyano-3-hydroxycinnamic acid or butylmalate respectively, to final concentrations of 10 and 30mM respectively. The radioactivity in the mitochondrial pellets was determined as described for ^{45}Ca, with corrections for carry-down.

Results

In the absence of added permeant anion, rat heart mitochondria remove between 70 and 130nmol of Ca^{2+}/mg of protein from their medium; this amount is not exceeded, however high the Ca^{2+} concentration of the medium. We refer in the present paper solely

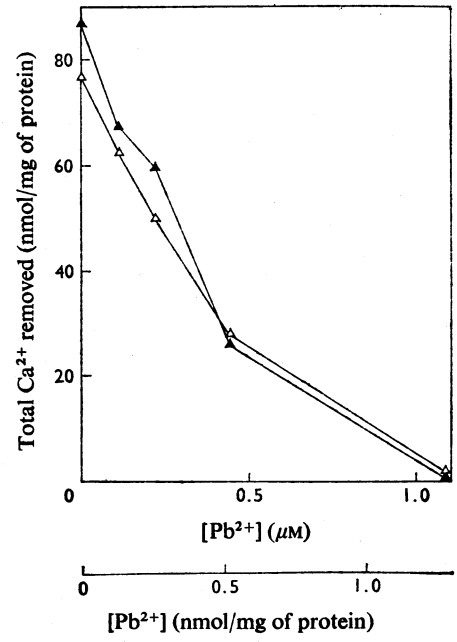

Fig. 1. *Effect of Pb^{2+} concentration on the total extent of Ca^{2+} removal by rat heart mitochondria*

An addition of 149nmol of $^{45}Ca^{2+}$/mg of mitochondrial protein was made to the medium (final concn. 127μM) 5min after treatment with Pb^{2+}. When removal was complete (after a further 5min), EGTA (to 5mM) (△) or water (▲) was added to the medium, before separation of mitochondria from the medium; the supernatant was counted for radioactivity.

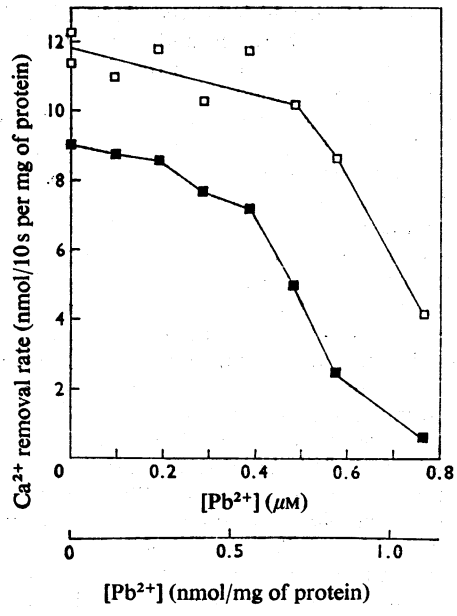

Fig. 2. *Effect of Pb^{2+} concentration on the rate of Ca^{2+} removal by rat heart mitochondria*

After the addition of Pb^{2+}, mitochondrial suspensions were left for 5 min before adding 153 (□) or 59 (■) nmol of Ca^{2+}/mg of mitochondrial protein (final concn. 109 or 42 μM-Ca^{2+} respectively). The rate of Ca^{2+} removal was determined as described in the Experimental section.

to removal or sequestration of Ca^{2+} by mitochondria from their medium in order to avoid using the terms 'uptake' and 'binding' (which imply matrix and membrane loading respectively), because of the difficulty in distinguishing between the two, if indeed a rigid distinction can be drawn, and because the relative importance may depend on the origin of the mitochondria, and perhaps other factors.

When about 150 nmol of Ca^{2+}/mg of protein was given, the extent of its removal was greatly decreased by less than 1 nmol of Pb^{2+}/mg of protein (Fig. 1). The addition of EGTA after completion of removal further decreased the apparent extent by about 10 nmol/mg of protein at low Pb^{2+} concentrations, but at concentrations above 0.5 nmol of Pb^{2+}/mg of protein had no effect. The difference at low Pb^{2+} concentrations is presumably due to surface-bound EGTA-removable Ca^{2+}. In a KCl medium the rate of removal of either 55 or 155 nmol of Ca^{2+}/mg of protein was also greatly decreased by less than 1 nmol of Pb^{2+}/mg of protein (Fig. 2), and there was a similar effect in a sucrose medium. The decrease in extent was not due to incomplete measurement of a slower removal, as would be expected since, in the absence of P_i, the overall extent of removal is a reflection of the equilibrium between removal and release. For this reason, we believe the decrease in the rate of removal is the primary effect, resulting in the establishment of a new equilibrium position favouring the medium.

Similar large decreases in Ca^{2+}-removal rate were observed with respiratory inhibitors (rotenone plus antimycin), an uncoupler tetrachlorotrifluorobenzimadazole and at low temperature (4°C).

Fig. 3 shows that the effect of Pb^{2+} on Ca^{2+}-removal rate was not instantaneous, but reached its full extent within a few minutes. The maximum effect was reached rather more rapidly as the amount of Pb^{2+} increased, although only a narrow range of Pb concentration–time situations exist in which the time of exposure was of critical importance.

The endogenous ATP content of a mitochondrial suspension fell rapidly from its usual value of about 3 nmol/mg of protein following the addition of Pb^{2+}, reaching 1 nmol/mg of protein within 1 min. We have found that the total ATP concentration rarely drops below this value, whatever the treatment.

A Dixon plot (Dixon, 1953) of the reciprocal of Ca^{2+}-removal rate against Pb^{2+} concentration (Fig. 4), shows that up to about 0.5 nmol of Pb^{2+}/mg of protein the decrease in rate has the characteristics of competitive inhibition (the first effect), with $K_i = 0.4\,\mu$M. Above this concentration, however, there is a sudden profound change that indicates a second, completely distinct effect of Pb^{2+}. A similar experiment done in the presence of added ATP results

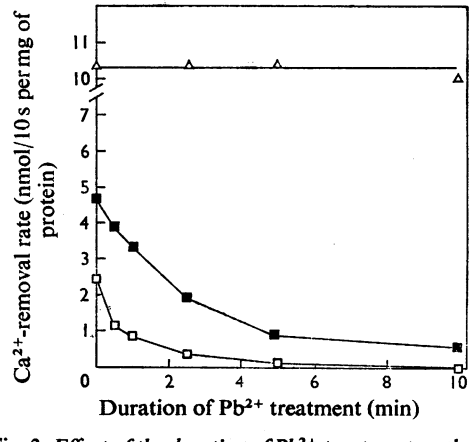

Fig. 3. *Effect of the duration of Pb^{2+} treatment on the Ca^{2+}-removal rate of rat heart mitochondria*

In the absence of Pb (△) and after the addition of 1.1 (■) or 5.6 (□) nmol of Pb^{2+}/mg of protein, various times elapsed before the Ca^{2+} removal rate was determined after the addition of 52 nmol of Ca^{2+}/mg of protein (final concn. 38 μM).

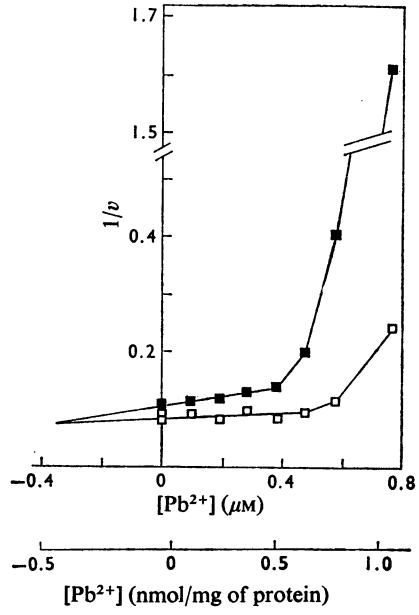

Fig. 4. *Dixon plot of the effect of Pb^{2+} on the reciprocal of Ca^{2+}-removal rate (1/v) of rat heart mitochondria*

The data and experimental details are as in Fig. 2. The units of Ca^{2+}-removal rate are nmol of Ca^{2+}/10s per mg of protein.

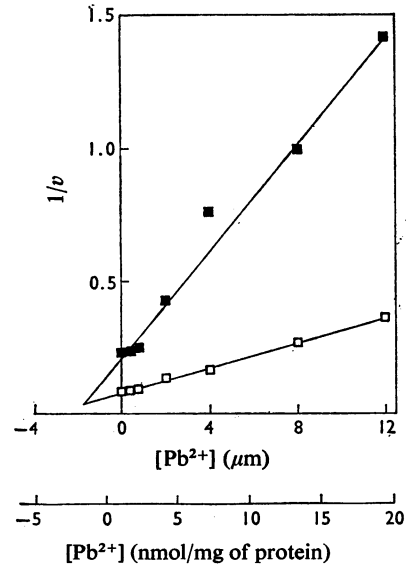

Fig. 5. *Dixon plot of the effect of Pb^{2+} in the presence of added ATP on the reciprocal of Ca^{2+}-removal rate (1/v) of rat heart mitochondria*

Conditions were as in Fig. 4, except that 1.2μmol of ATP/mg of protein was added to the mitochondrial suspension 30s before the Pb^{2+}. The removal rates were determined after the addition of 159 (□) or 53 (■) nmol of Ca^{2+}/mg of protein (final concn. 102 or 34μM-Ca^{2+} respectively).

in a Dixon plot from which the second phase is lacking (Fig. 5), with $K_i = 1.7\mu M$. The addition of ATP 5min after the Pb^{2+} does not restore the Ca^{2+}-removing capacity of the mitochondria.

Mg^{2+} at concentrations a thousand times those used for Pb^{2+}, had relatively little effect on the rates of removal of 55 or 155nmol of Ca^{2+}/mg of protein, and a Dixon plot (Fig. 6) indicates simple competition between the two cations, with $K_i = 0.15$mM. Pb^{2+} (10nmol/mg of protein) displaced only 5nmol or less endogenous mitochondrial Mg^{2+}/mg of protein.

When mitochondrial oxidation of pyruvate and malate was uncoupled, the rate of O_2 use was inhibited by Pb^{2+}, but only at concentrations greater than those that affected the Ca^{2+}-removal rate (Table 1), and complete inhibition was not achieved. Coupled resting respiration was also partially inhibited, and we never observed any Pb^{2+}-induced respiratory stimulation. We considered the possibility that Pb^{2+} was exerting its effect by inhibiting substrate entry into the mitochondria and found that 15nmol of Pb^{2+}/mg of protein decreased the rate of pyruvate entry from 18 to about 9nmol of pyruvate/mg of protein per 5s. Malate entry was unaffected.

Pb^{2+} treatment resulted in the release of NAD^+ from the mitochondrial membranes. We have observed a similar release following Ca^{2+} damage and

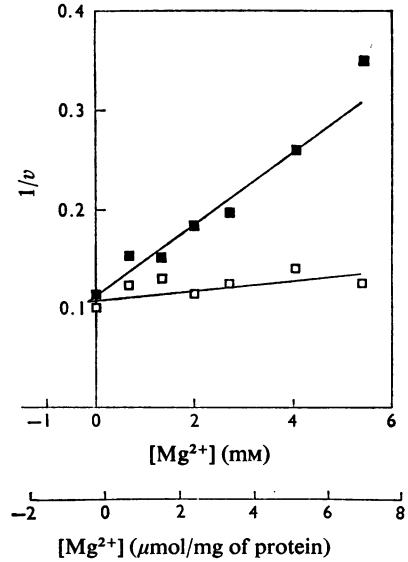

Fig. 6. *Dixon plot of the effect of Mg^{2+} on the reciprocal of Ca^{2+}-removal rate (1/v) of rat heart mitochondria*

Conditions were as in Fig. 4, with the replacement of Pb^{2+} with Mg^{2+}. Removal rates were determined after the addition of 166 (□) or 64 (■) nmol of Ca^{2+}/mg of protein (final concn. 124 or 48μM-Ca^{2+} respectively).

Table 1. *Effect of Pb^{2+} concentration on the rate of O_2 use by uncoupled rat heart mitochondria oxidizing pyruvate plus malate*

Tetrachlorotrifluorobenzimidazole (0.18 μM) was used to uncouple respiration, the rate of which was then 54.6 natoms of O_2/mg of protein per min.

Pb^{2+} concn.		Inhibition of uncoupled respiratory rate
(nmol/mg of protein)	(μM)	(%)
0.0	0.0	0
2.3	0.8	11
4.6	1.5	41
6.8	2.3	35
9.1	3.1	35

feel that this is probably a general consequence of mitochondrial swelling, rather than a direct effect of these cations on the membrane.

Discussion

The rate, and hence the extent of Ca^{2+} removal by heart mitochondria is decreased by Pb^{2+}. Dixon plots of the effect have two phases; the first, below 0.5 nmol of Pb^{2+}/mg of protein, has the characteristics of competitive inhibition ($K_i = 0.4\,\mu$M). Above this level a more potent effect is apparent that results in almost complete inhibition of Ca^{2+} sequestration at about 1 nmol of Pb^{2+}/mg of protein. In the presence of added ATP, however, only one phase is present, which also has the kinetics of competitive inhibition, but with a different K_i (1.7 μM). Although it is possible that added ATP abolishes the first effect and alters the second, making it a competitive phenomenon, we believe it more probable that ATP overcomes the second effect, and that the action of Pb^{2+} over an extended range in the presence of added ATP is essentially the same as the first effect seen in its absence. The difference in K_i may reflect some ATP-induced alteration in the affinity of the membrane for Pb^{2+} or Ca^{2+}, or be due to differential chelation of Ca^{2+} and Pb^{2+} by ATP. Abolition of the second effect by added ATP suggests an action of Pb^{2+} on energy supply or use in Ca^{2+} removal. This is supported by the rapid drop in mitochondrial ATP concentration that follows Pb^{2+} treatment, and by the similar effects of respiratory inhibitors, low temperature and uncoupler.

We do not believe the second effect is due to inhibition of pyruvate uptake or oxidation since, although both were subject to inhibition by Pb^{2+}, neither was decreased by more than 50%, even with 10 nmol of Pb^{2+}/mg of protein. Scott *et al.* (1971) deduced from swelling studies that Pb^{2+} increases mitochondrial K^+ permeability. We are loth to ascribe the second effect to increased competition for available energy between Ca^{2+} and K^+ transport, since this would require the precedence of K^+ transport in the use of respiratory energy. The lack of respiratory stimulation by Pb^{2+} in KCl and the similar effects of Pb^{2+} on Ca^{2+} removal in KCl and sucrose media all suggest that the swelling observed by Scott *et al.* (1971) was another symptom of the inhibition of the production or use of respiratory energy by Pb^{2+}.

In various conditions Mg^{2+} has been reported to have no effect on Ca^{2+} uptake by heart mitochondria (e.g. Scarpa & Graziotti, 1973) or to compete with it (e.g. Sordahl, 1974; Carafoli *et al.*, 1975). In our conditions Mg^{2+} inhibits the Ca^{2+}-removal rate by a process that exhibits the kinetics of competition; it is considerably less efficient that Pb^{2+} ($K_i = 150\,\mu$M). Leblanc & Clauser (1974) found that some Mg^{2+} is required for Ca^{2+} uptake, and suggest that it is essential for a site involved in energy coupling between accumulation and O_2 use. The second effect of Pb^{2+} in our work may be due to the displacement of small amounts of endogenous membrane-bound Mg^{2+}, an idea supported by the fact that Mg^{2+} restores the function of mitochondria isolated from Pb^{2+}-fed rats (Krall *et al.*, 1971).

Estimates of the mitochondrial content of heart tissue (Carafoli & Azzi, 1972; Scarpa & Graziotti, 1973) permit the calculation that 0.5 nmol of Pb^{2+}/mg of protein is equivalent to between 3 and 9 p.p.m. of Pb^{2+} in the whole tissue. Normal human heart contains less than 0.3 p.p.m. (Barry & Mossman, 1970; Barry, 1975; Gross *et al.*, 1975), but Pb^{2+} accumulates in the aorta more than in any other soft tissue, reaching as much as 28 p.p.m. (Barry & Mossman, 1970). Occupational exposure may cause higher levels (e.g. Petkau *et al.*, 1974), and rats drinking water containing Pb^{2+} at the concentrations found in some Glasgow tap water accumulate up to 6 p.p.m. in their cardiac tissue (Moore *et al.*, 1975). Thus, if the Pb^{2+} sometimes found in heart tissue were mitochondrially located, it could significantly impair the Ca^{2+}-handling capacity of these organelles. It is established both *in vivo* and *in vitro* that mitochondria are one of the major subcellular loci of Pb^{2+} accumulation (e.g. Scott *et al.*, 1971; Murakami & Hirosawa, 1973; Ophus & Gulvåg, 1974; Asokan, 1974; Moore *et al.*, 1975). A nuclear protein that binds Pb^{2+} forming inclusion bodies may fulfill a detoxifying role in the kidney, preventing the action of Pb^{2+} on mitochondria (Goyer, 1971). However, no such protein has been found in cardiac cells.

The effect of Pb^{2+} in the absence of P_i may be somewhat artificial in view of the precipitating action of this anion on Pb^{2+}, but mitochondria do carry endogenous P_i, and various natural chelating and complexing agents, notably ATP, increase the effective solubility

and toxicity of lead to mitochondria in the presence of P_i (Parr & Harris, 1975*a*,*b*). Mitochondrially produced ATP thus has two conflicting effects; although it increases the effective solubility of Pb^{2+} in the presence of P_i, it also provides energy for continued Ca^{2+} removal, which Pb^{2+} would otherwise have stopped.

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