Biochimica et Biophysica Acta, 502 (1978) 289—297

BBA 47481

THE EFFECT OF CALCIUM ON THE RESPIRATORY RESPONSES OF CORN MITOCHONDRIA

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(Received September 12th, 1977)

Summary

Tightly coupled respiring corn mitochondria (*Zea mays* L.) respond to calcium addition with a transitory respiratory increase, proton extrusion, and Ca^{2+} binding. The extent of response is dependent upon the level of endogenous phosphate, and a large sustained respiratory increase can be obtained with addition of phosphate. However, calcium does not act as a permeant cation in that it will not penetrate with acetate. It appears that the transitory respiratory increase must be linked to the uptake of a calcium phosphate complex, but there is no evidence that transport of the complex serves to produce an electrophoretic calcium uniport. It is believed that calcium phosphate transport in corn is a constitutive property, and not produced by membrane damage.

Introduction

For vertebrate mitochondria there is a growing consensus that Ca^{2+} is transported electrophoretically down a gradient of electrical potential created by respiration or ATP hydrolysis [1—3]. Moyle and Mitchell [4] suggest that Ca^{2+} is transported as a positively charged phosphate complex, $[(Ca_2)HPO_4]^{2+}$. However, Ca^{2+} uptake can also be driven by the passive permeation of readily penetrating anions, such as thiocyanate, or by permeant acids, such as acetic (in the presence of uncoupler) [3,5]. At steady state, the Ca^{2+} uptake driven by succinate oxidation shows a Nernstian distribution between mitochondria and medium (ref. 6, cf. ref. 7). The transport of Ca^{2+} across the inner mitochondrial membrane is usually ascribed to a Ca^{2+}-binding porter or carrier, and the extraction of lipophilic divalent ionophores has been reported from beef heart mitochondria [8] and rat liver mitochondria [9]. The inhibitory effects of La^{3+}

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Abbreviation: TES, *N*-tris(hydroxymethyl)methyl-2-aminoethane sulphonic acid.

[10] and ruthenium red [11] are suggestive of inhibited carrier transport. Thus, Ca^{2+} in combination with the carrier becomes a "permeant cation" in vertebrate mitochondria, electophoretically fluxing with transported anions, and increasing respiration and osmotic swelling.

There is scant evidence that plant mitochondria transport Ca^{2+} by a similar uniport. All studies agree that Ca^{2+} uptake (when it occurs) is linked to phosphate uptake, and that other transported anions such as acetate or arsenate will not substitute [12—14]. There is an opinion that Ca^{2+} uptake may be the artifactual result of preparatory damage to the inner membrane [15], but it is difficult on the basis of reported data to see where the mung bean preparations of Wilson and Minton [16], which accumulate Ca^{2+}, are functionally inferior to those of Moore and Bonner [15], which do not. Conflicting results such as these may have their origin in experimental procedures, but as yet one cannot distinguish between loss of a calcium phosphate carrier and induction of Ca^{2+} permeability.

There are reported responses of plant mitochondria to Ca^{2+} which partially resemble those of vertebrate mitochondria. Chen and Lehninger [13] found sweet potato mitochondria to give Scatchard plots of Ca^{2+} binding resembling those of animal mitochondria, and both high and low affinity binding was largely inhibited by 15 μM La^{3+}. Ruthenium red inhibited respiration-driven Ca^{2+} uptake. Sweet potato mitochondria [13] and corn mitochondria [17,18] show a low level of respiratory stimulation in response to 300—500 μM Ca^{2+}, which declines after a minute or two.

Respiring corn mitochondria can bind about 100 nmol Ca^{2+}/mg protein in the absence of exogenous phosphate [19,20]. However, the binding proves to depend on the level of endogenous phosphate [21], which is also true for Sr^{2+} binding by bean mitochondria [22]. Associated with Ca^{2+} binding is the movement of endogenous phosphate from a readily leached phase to a leaching-resistant phase through a mersalyl sensitive site [21]. The Ca^{2+} and endogenous phosphate are rapidly released when respiration ceases [19,23]. Just after respiration ceases the addition of phosphate to the medium leads to Ca^{2+} retention. Loss of endogenous phosphate is also retarded, although there is exchange for labeled exogenous phosphate which is insensitive to mersalyl and uncouplers [23].

Massive accumulation of Ca^{2+} [24] or Sr^{2+} [22] by plant mitochondria requires an energy source, exogenous phosphate and 0.5—2 mM Ca^{2+}. The Ca : P_i accumulation ratio is 1.7 [25], compared to 2.1 for Ca^{2+} bound with the fluxing endogenous phosphate [21]. Proton release and Ca^{2+} uptake are a function of phosphate concentration, with H^+/Ca^{2+} ratios of 0.9 for endogenous phosphate and 0.8 with added phosphate, which is attributed to the formation of calcium phosphate precipitate [21]. Electron microscopy shows heavy precipitates with both Ca^{2+} [26] and Sr^{2+} [27] uptake.

Other than phosphate, the Class B (proton-conducting) anions [3] are ineffective in producing respiration-driven Ca^{2+} uptake and swelling [12—14]. However, corn mitochondria suspended in 100 mM potassium acetate show an initial, 30 s burst of Ca^{2+} uptake which is rapidly lost due to acetate coupling [21]. Energized acetate uptake under these conditions is very rapid and extensive in corn mitochondria, with very little selectivity as to accompanying cation [28].

In short, the transport system for Ca^{2+} and Sr^{2+} in those plant mitochondria where it occurs appears to be linked to co-transport with phosphate, endogenous or exogenous. We report here some additional observations on Ca^{2+} transport made in an effort to charaterize more closely the initial respiratory burst. It is probable that the burst is associated with electrophoretic movement of a positively charged calcium · phosphate complex, but interaction with the coupling mechanism is also possible.

Materials and Methods

Preparation of mitochondria. Mitochondria were isolated from 4-day-old, etiolated corn shoots as described [29]. It has been shown [30] that the quality of these mitochondria is at least the equal of that of gradient purified mitochondria. Typical respiratory control ratios (with malate and pyruvate as substrates) were 5—10, and high ADP/O ratios were observed (2.6—2.9). Outer membrane integrity was ensured using exogenous cytochrome reduction, as described [30].

Reaction media. The standard reaction medium consisted of 0.2 M sucrose, 10 mM TES buffer, 0.1% bovine serum albumin and 1 mM $MgSO_4$, adjusted to pH 7.2 with KOH. Phosphate (5 mM KH_2PO_4) was added for oxidative phosphorylation measurements. When pH changes were measured 0.5 mM TES buffer was used.

Oxygen consumption and swelling. Oxygen uptake was measured, in 4.5 ml of standard reaction medium, using a Clarke oxygen electrode (Yellow Springs Instrument Co., Cleveland Ohio) and a closed glass vessel at 25° C. Low concentrations of succinate plus pyruvate were used as substrates to avoid, as much as possible, complications due to substrate uptake. Swelling was monitored simultaneously by following percent transmission at 520 nm as described previously [31].

pH measurements. Changes in medium pH were followed simultaneously with percent transmission in a glass vessel at 25° C, using a Beckman Expandomatic pH meter and combination pH electrode. H^+ ejection was determined quantitatively by back titration with standard HCl and NaOH.

Protein determination. Protein was estimated by the method of Lowry et al. [32] using bovine serum albumin, fraction V, as the standard.

Calcium binding. Calcium binding was measured directly using the murexide technique of Mela and Chance [33]. An Aminco DW-2, dual wavelength spectrophotometer was employed and absorption at 540—507 nm was recorded at room temperature. An increase in absorbance occurs as Ca^{2+} is sequestered by the mitochondria, thus decreasing the concentration of the murexide · Ca complex in the medium [33].

The scatchard plot was constructed from $^{45}Ca^{2+}$ uptake data as described by Chen and Lehninger [13].

P_i determinations. The inorganic phosphate content of the mitochondria was estimated from perchloric acid extracts by the method of Marsh [34]. Mitochondria (0.1 ml) were added to 2 ml of 5.5% cold $HClO_4$ and allowed to stand for 1 min. Precipitated protein was removed by centrifugation and the supernatant analyzed for P_i.

Chemicals. Reagents were purchased from Sigma Chemical Co. (St. Louis, Mo.) and Calbiochem (Cleveland, Ohio).

Results

Ca^{2+} and respiration

In the earlier reports of transitory Ca^{2+}-stimulated respiration in the absence of phosphate [17,18], the mitochondria were isolated in phosphate buffer which increases the endogenous phosphate content. Fig. 1 compares the respiratory stimulation in mitochondria isolated in phosphate and TES buffer, and possessing high and low endogenous phosphate (115 and 45 nmol/mg protein, respectively). The initial increases in respiration due to Ca^{2+} addition were 82 and 32%. The transmission trace shows a larger contraction or shrinkage associated with the larger respiratory increase (see also Table II). Previous work [21] has shown Ca^{2+} uptake of 140 and 70 nmol/mg protein with comparable high and low endogenous phosphate content. Maintenance of the Ca^{2+} load is dependent upon continued respiration [19,23]. Hence, if this transitory respiratory burst is due to electrophoretic influx of Ca^{2+}, the Ca^{2+} must be transported as a phosphate complex as suggested by Wilson and colleagues [16,22] for plant mitochondria, and by Moyle and Mitchell [4] for animal mitochondria.

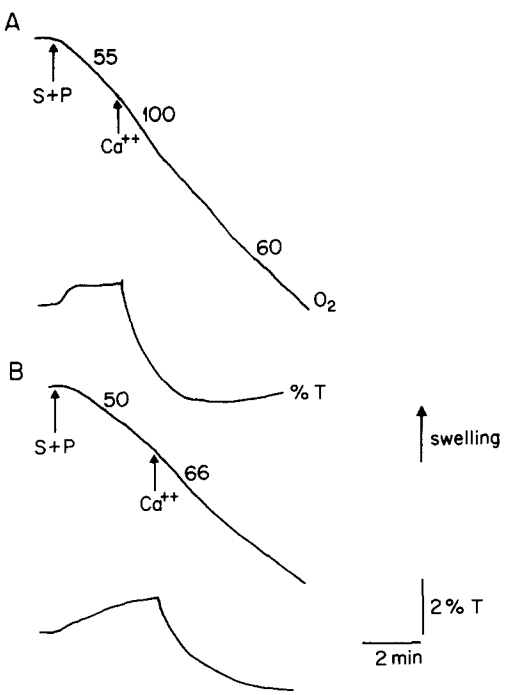

Fig. 1. Effect of endogenous P_i on the calcium response of respiring corn mitochondria. The incubation mixture consisted of 4.5 ml of standard reaction medium to which had been added 0.2 ml of mitochondrial suspension (1.8 mg protein in A, and 1.9 mg in B), 50 μM CoA, 50 μM thiamine pyrophosphate, 1 μmol ATP and 1 μg/ml oligomycin. Other additions, as indicated, were 2 mM succinate + pyruvate (S + P) and 1 mM $CaCl_2$. (A) Mitochondria isolated in KH_2PO_4 buffer; endogenous P_i = 115 nmol/mg protein. (B) Mitochondria isolated in TES buffer; endogenous P_i = 45 nmol/mg protein. Rates of oxygen consumption are given as nmol/min per mg protein.

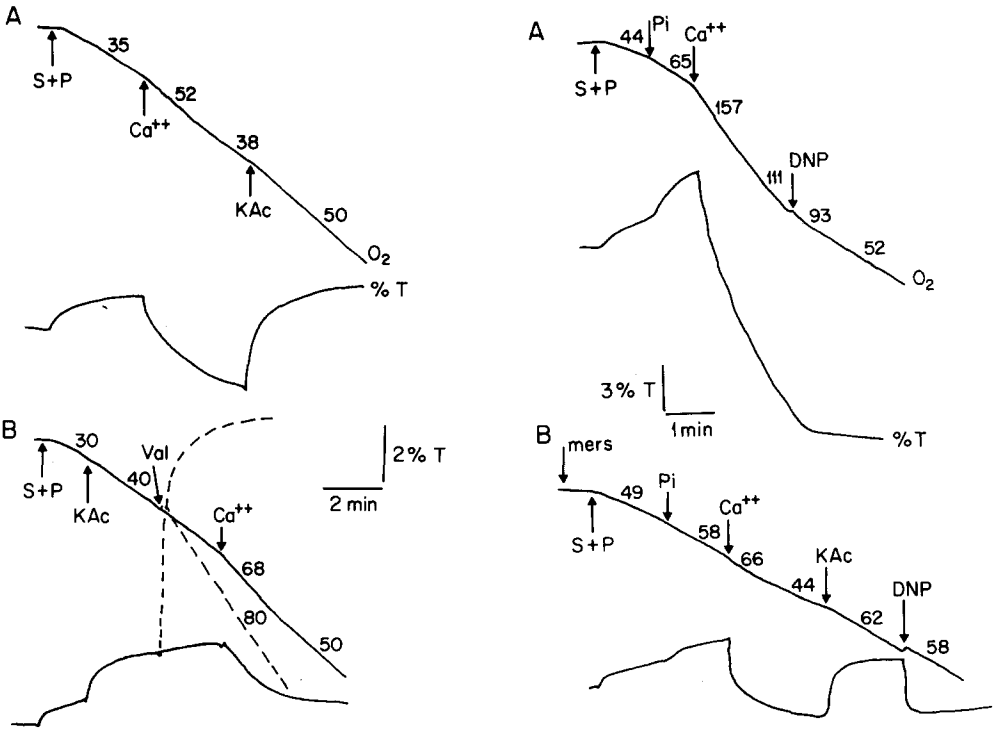

Fig. 2. Effect of acetate on the calcium response of corn mitochondria. Assay conditions ase described in Fig. 1. Additions, as shown, were: 2 mM succinate + pyruvate, 1 mM $CaCl_2$, 10 mM potassium acetate (K Ac) and 0.3 μg valinomycin (val). Rates are expressed as nmol O_2 /min per mg protein.

Fig. 3. Effect of calcium on respiring corn mitochondria in the presence of added P_i. Assay conditions were the same as those in Fig. 1 except that 2 mM KH_2PO_4, 20 μM mersalyl (mers) and 20 μM dinitrophenol were added as shown.

The addition of acetate does not produce the extensive swelling one would expect if Ca^{2+} were a permeant cation (Fig. 2). However, the presence of acetate does increase Ca^{2+}-stimulated respiration slightly but consistently (Figs. 2A and 2B). Addition of valinomycin, which increases the permeability coefficient of K^+, demonstrates the increased acetate uptake and swelling which occurs in the presence of a permeating cation (Fig. 2B).

A number of cations were tested for the ability to produce a transitory rise in respiration rate (Table I). Only Ca^{2+}, Sr^{2+} and Ba^{2+} were effective. It was previously found that only these cations were effective in increasing respiration of corn mitochondria in the presence of exogenous phosphate [35], and they are the divalent cations taken up by mung bean mitochondria in the presence of phosphate [16].

If phosphate is added to the medium there is a large respiratory burst on addition of Ca^{2+} (Fig. 3A), which is suppressed by the presence of mersalyl (Fig. 3B). Mersalyl does not suppress acetate uptake, and the Ca^{2+} would be free to enter and produce swelling if it were simply a permeant cation. The sweeping decline in light transmission in Fig. 3A has previously been shown to

TABLE I

RESPIRATORY RESPONSES OF CORN MITOCHONDRIA TO ADDITION OF VARIOUS CATIONS

Experiments performed as in Fig. 2 with the addition of 1 mM chloride salt of the cations, except for 1 mM $ZnSO_4$ and $La(NO_3)_3$. Respiration rates after cation addition are the initial rate (burst) followed by the sustained rate at 1 min.

Cation	Respiration (nmol O_2/min per mg protein)	
	+ acetate	+ cation
Ca^{2+}	81	149, 93
Sr^{2+}	74	105, 81
Ba^{2+}	93	118, 81
Mn^{2+}	93	81, 81
Zn^{2+}	70	13, 13
Cu^{2+}	70	6, 6
Co^{2+}	80	80, 74
Al^{3+}	75	88, 88
La^{3+}	75	57, 57

be associated with massive calcium phosphate uptake, and is probably due to light scattering and absorption by calcium phosphate deposits [20].

Calcium binding

A Scatchard plot of Ca^{2+} bound to non-respiring mitochondria in the absence of exogenous phosphate is shown in Fig. 4. As in the report of Chen

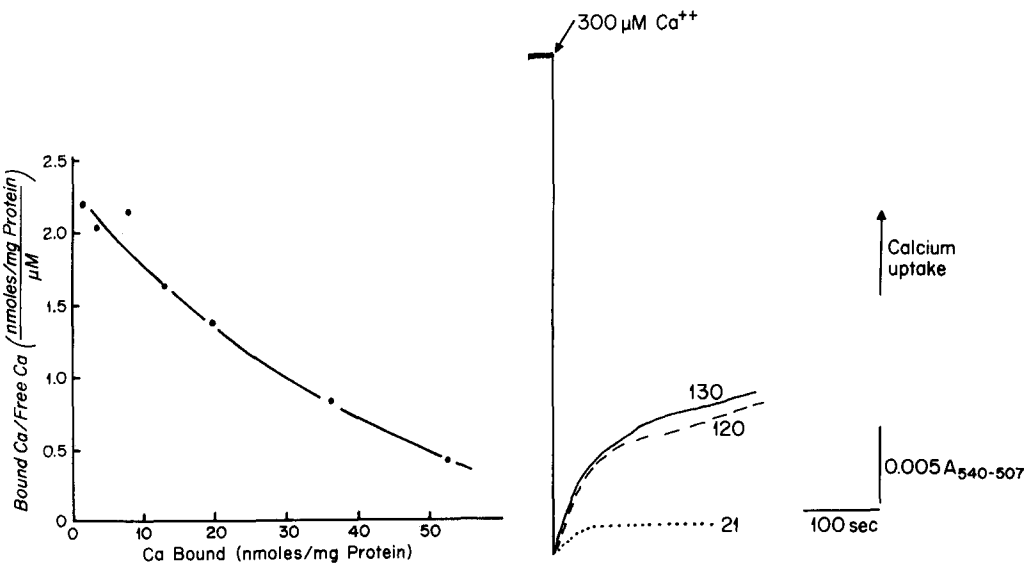

Fig. 4. Scatchard plot of Ca^{2+} bound to corn mitochondria. Experimental details are given in Materials and Methods.

Fig. 5. Spectrophotometric measurement of Ca^{2+} uptake by corn mitochondria. The reaction mixture consisted of 2.8 ml standard reaction medium, 0.2 ml mitochondrial suspension (2.2 mg protein), 50 μM CoA, 50 μM thiamine pyrophosphate, 2 mM succinate + pyruvate and 40 μM murexide. $CaCl_2$ (0.3 mM) was added as shown; the numbers on the traces indicate nmol Ca^{2+} taken up/mg protein. ———, control; - - - - - -, +10 mM potassium acetate or KCl; · · · · · ·, +5 μM antimycin A.

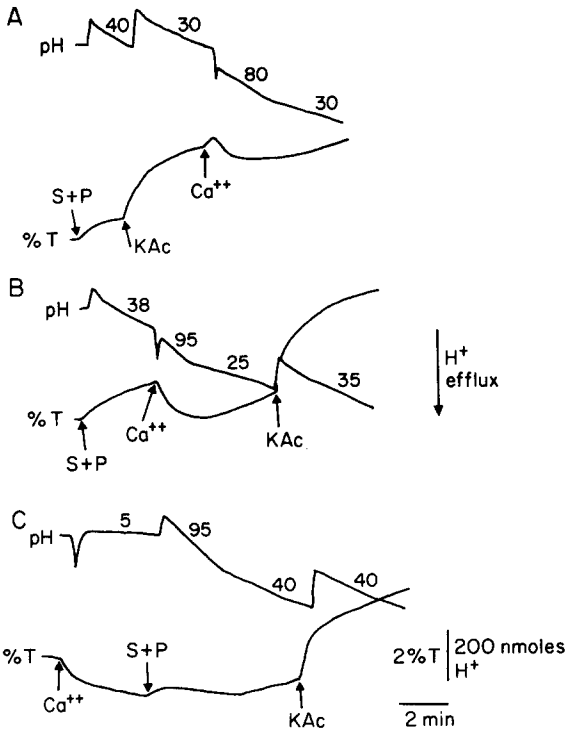

Fig. 6. Effect of Ca^{2+} on H^+ efflux. Assay conditions were as in Fig. 1 except that the standard reaction medium contained only 0.5 mM TES buffer (pH 7.2). Additions, as shown, were: 1 mM succinate + pyruvate, 10 mM potassium acetate, 1 mM $CaCl_2$. Numbers of traces indicate the rate of H^+ efflux in nmol/min per mg protein. H^+ ejection was calibrated by back titration with standard HCl and NaOH.

and Lehninger [13] there is little evidence for high affinity binding sites.

Calcium binding in the absence of phosphate was also followed by the murexide technique (Fig. 5). No difference in Ca^{2+} uptake could be detected in the presence of acetate. The same result was obtained with use of ^{45}Ca; after 2 min of active uptake there were 111 and 109 nmol of Ca^{2+} bound per mg protein in the presence and absence of 10 mM potassium acetate, respectively (data not shown).

H^+ efflux

Fig. 6 shows the change in proton efflux upon addition of Ca^{2+} in the

TABLE II

EFFECT OF ENDOGENOUS P_i CONTENT ON LIGHT SCATTERING AND H^+ EJECTION RESPONSES TO CALCIUM

Experiments were performed as described in Fig. 6B.

P_i content (nmol/mg protein)	$\Delta\%\ T$ (per mg protein)	Increase in H^+ ejected (nmol/min per mg protein)
38	1.6	28
96	2.4	50

absence of added phosphate. Again, acetate was added to see if the presence of a transportable anion would alter the Ca^{2+} response, but again there was none. The transitory respiratory burst (Figs. 1 and 2) is thus associated with a burst of proton efflux. As shown in Table II, the initial increase in proton efflux upon adding Ca^{2+} was greater with high endogenous phosphate, in concurrence with increased light scattering and respiratory release (Table II and Fig. 1).

Discussion

These experiments affirm previous conclusions that some species of plant mitochondria do accumulate Ca^{2+}. The uptake is energy dependent and phosphate dependent. In the sense in which the term is used for vertebrate mitochondria there is no evidence that Ca^{2+} is a permeant cation, it does not produce massive swelling with acetate. The limited Ca^{2+} uptake in the absence of exogenous phosphate is limited by the level of endogenous phosphate. There is a transitory increase in respiration and H^+ efflux upon addition of Ca^{2+}, but this must be due to binding or transport of a Ca^{2+} · phosphate complex. There is an undefined energy demand associated with the binding or transport of the complex which gives the respiratory burst, and sustained respiration is needed to maintain the complex [19,23].

The early opinion of our laboratory was that an intermediate of oxidative phosphorylation was the complex (i.e. Ca : X ~ P). A major reason for this was that the Ca : Mg ratio of the medium governed whether phosphate was transported or used to form ATP [17,19], suggesting that Mg : X ~ P was the stable intermediate for phosphorylation [18,19]. Calcium was believed to form an unstable intermediate which dissociated to give transport [18,19]. The fact that only Ca^{2+}, Sr^{2+} and Ba^{2+} were active in promoting phosphate transport was attributed to the high rates of water substitution in complex formation shown by these cations [19,35]; that is, they formed water-unstable metal: phosphate intermediates, allowing rapid uptake and release to aqueous phases (i.e. transport). This hypothesis required a site for oligomycin action inhibitory to phosphoryl transfer in ATP formation [18].

The ascendency of the chemiosmotic hypothesis would appear to negate the above hypothesis, but since there is no evidence for electrophoretic Ca^{2+} uniport it is difficult to know what to substitute. The most attractive hypothesis is that of Moyle and Mitchell [4] for electrophoretic symport of a positively charged carrier · Ca · phosphate complex. The Ca : P_i and Ca : H^+ ratios observed with corn mitochondria (see Introduction) support this idea. But why in this case is there not release of the Ca^{2+} as a companion ion to acetate, producing swelling, and recycling of the phosphate for continued transport? By deduction, this is what must happen in liver mitochondria (see, however, ref. 36).

These questions await more sophisticated inquiries. The point of importance is that some tightly coupled plant mitochondria do have a well-documented transport system for Ca^{2+} which is not a simple electrophoretic Ca^{2+} uniport resulting from membrane permeability changes (cf. ref. 15). If the techniques of mitochondrial isolation have introduced membrane changes leading to Ca^{2+} transport, they are changes which expose an energy-linked calcium phosphate transport system.

Acknowledgements

This research was supported by the U.S. Energy Research and Development Administration (E-11-1-790).

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