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PARTIAL PURIFICATION OF A CALCIUM IONOPHORE FROM HUMAN UREMIC PLASMA AND NORMAL URINE

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A fraction is isolated from human uremic plasma and normal urine using a three step chromatographic separation :gel permeation on Sephadex G15, then chromatography on hydroxyapatite and finally desalting operation on Sephadex G15. The fraction thus separated is ninhydrine positive and uncouples mitochondria principally releasing the resting respiration.Calcium potentiates the uncoupling,while red ruthenium and magnesium inhibit it. These results are in good agreement with a ionophorous activity of the isolated fraction. An hypothetic physiological role of this compound is there being discussed.

The patients suffering from chronic renal insufficiency treated by hemodialysis present important troubles of the calcium metabolism whose origin remains obscure. Under these conditions the aim of this work has been to isolate from these patients plasma one or several factors which might act on this metabolism. As the presence of such a factor in the plasma of patients suffering from chronic renal insufficiency lets us assume its presence in normal urine, we have also used this biological fluid. The mechanism of cellular calcium homeostasis remains obscure, yet there is no doubt that mitochondria are implicated in this regulation considering their capacity to accumulate calcium.

Besides, in the patients suffering from chronic renal insufficiency a significant decrease in mitochondrial calcium phosphate granulations in the renal proximal tubule can be noticed (1). Therefore we have studied the action on mitochondria of the compound (s) we isolated.

Our results show the existence in these biological fluids of one or several substances affecting mitochondrial functions by releasing intramitochondrial calcium.

These results are consistent with the ionophorous nature of the isolated substance.

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MATERIALS AND METHODS

a) Biological fluids

We used normal urine or plasmatic ultrafiltrates from uremic patients treated by hemodialysis. Ultrafiltrates were obtained at the beginning of dialysis by applying negative pressure in the dialyser compartment of a Dow Cordis capillary dialyser, fitted with a cellulose acetate membrane, before the dialysis fluid ran through the dialyser.Ultrafiltrates thus obtained were then concentred by partial lyophilisation. Urine from normal subjects was used after simple filtration. Experiments were performed with uremic plasmas from three patients and with a pool of normal urines.

b) Chromatography

The first step consisted in a gel permeation chromatography. 5 to 20 ml of biological fluid were applied at the top of a 2.6X100 cm column (Pharmacia Uppsala Sweden) packed with Sephadex G 15. Bidistilled water was used as an eluant. The eluate flow-rate of 100 ml/h was obtained with a peristaltic pump (Minipuls Gilson). The eluate was monitored at 254 nm with a LKB detector, using a fraction collector (Microcol TDC 80 Gilson) to collect 8 ml of fluid.

The second step was carried out on a 16X30 cm column (Pharmacia) packed with hydroxyapatite (HA Ultrogel IBF) equilibrated with sodium phosphate 1mM pH6. After applying the sample, the column was washed with 60 ml of the same buffer. Then it was eluted with sodium phosphate 100mM pH6. The eluate flow rate was 30ml/h and it was monitored at 254 nm and collected in 3 ml fractions.

The third step was a fraction desalting, carried out on 1.5X100cm glass column packed with Sephadex G15 bidistillated water as an eluant. The eluate flow rate was 50 ml/h. The eluate was monitored at 254 nm and collected in 3 ml aliquots. The efficiency of desalting was checked by measurements of the conductivity with a Tacussel CD 7N conductimeter. The calcium concentration of the fraction obtained was performed using a IL 751 atomic absorption spectrophotometer (Instrumentation Laboratory). Then the fractions were lyophilised and stored at - 20°C before use.

c) Rat liver mitochondria

Mitochondria were obtained as described by Johnson and Lardy (2). Measurements of oxygen consumption and pH were carried out at 25°C using a Clark microelectrode and a pH electrode fitted to a Gilson oxygraph. Mitochondria (2mg of protein) were incubated 3mn in 3.4 ml of a respiratory medium (saccharose 0.25M, KCl 10mM,$K_2H_2PO_4$ 8mM pH 7.2, rotenone 1 µM) supplemented with various concentrations of the ionophorous fraction. The substrate used was sodium succinate (8mM).Oxidative phosphorylation was started by ADP addition (0.15mM).

Then identical experiments were carried out with a medium supplemented either with calcium alone (50 nmoles), or with calcium and various concentrations of the isolated fraction.

Finally the activity of the ionophorous fraction was assayed in the presence of 2mM $MgCl_2$ or 3.2 nmoles of ruthenium red as an inhibitor of the calcium entry (3)

d) Expression of results

Oxidative rates, respiratory control and $P/_O$ were determined according to the classic technique (4). Oxidation rates are given as nmoles of oxygen consumed per minute per milligram of mitochondrial protein. The respiratory control(RC)is the oxidation rate ratio between states 3 and 4. The P/O ratio gives the quantity of ADP molecules phosphorylated per oxygen atom consumed.

RESULTS

a) Chromatography

Gel permeation chromatography of biological fluid provided 8 fractions. The peak indicated in figure 1A was collected. Then a 400 ml sample of this peak (about 100 mg) from four of the above steps on Sephadex G15 was applied to the hydroxyapatite column. The ultraviolet absorbing frac-

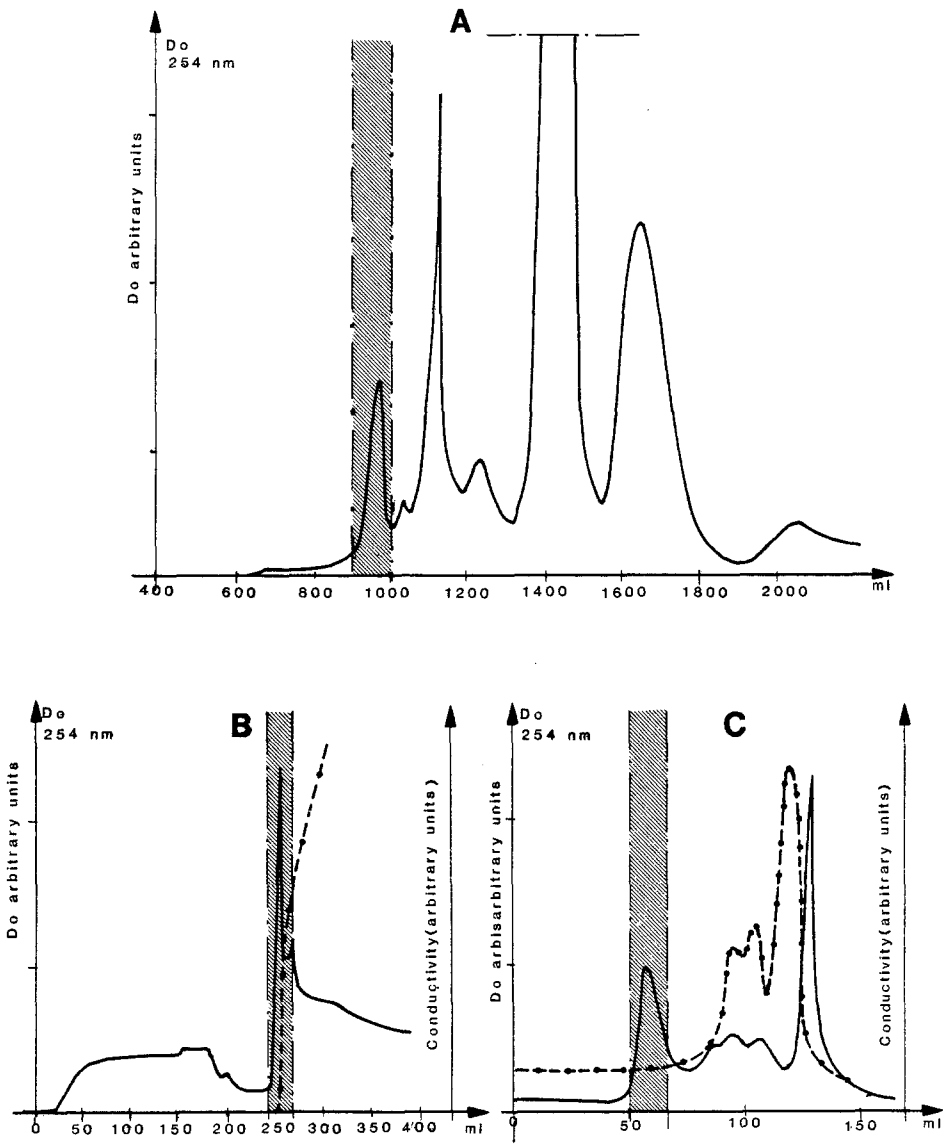

Figure 1 : Typical chromatograms
A. Gel permeation of biological fluid on Sephadex G15
B. Chromatography on hydroxylapatite
C. Desalting on Sephadex G 15.

——————— Optical density at 254 nm
–●–●–●–●– Conductivity

Table 1: Isolated fraction activity on mitochondria. Experimental conditions are described in Materials and Methods.

Isolated fraction µg per mg protein	RC *	P/O	Oxydation rate state 3	Oxydation rate state 4
0	4.2	1.7	105	25
25	3.1	1.5	100	32
50	1.3	0.7	122	97
75	1.0	0	159	159

tion eluted with phosphate 100 mM was collected (figure 1B) lyophilised and submitted to a desalting operation (figure 1C).The first peak, free from salt was collected and represents about 5 mg after lyophilisation.Its calcium concentration varies from 0.6 to 2% according to the samples. If this peak undergoes another chromatography on Sephadex G15, it divides again into several 254 nm absorbing peaks similar to those in figure 1C. All these peaks act upon mitochondria as peak 1; the experiments described below were performed with peak 1 because of its easier separation. Finally this fraction is ninhydrin positive.

b) Mitochondrial activity

Each isolated fraction was assayed for mitochondrial activity as indicated in Materials and Methods and gave similar results.In each table we reported the experimental results from only one experimental series. In each experimental series the mitochondria used came from the same preparation.

The isolated fraction inhibits phosphorylation and coupled respiration (table 1).It increases the succinate oxidation rate principally at state 4 as mitochondria can still phosphorylate ADP. When mitochondria are totally uncoupled the respiratory rate is higher than that wich accompanies the phosphorylation of ADP (table 1). These effects are dose-dependant.

The results reported in table 2 indicate the experiments performed in the presence of calcium (50 nanomoles). Because of the slightly uncoupling activity of calcium, the mitochondria incubated in its presence alone exhibit a respiratory control lower than the previous one. The activity of the isolated

Table 2: Isolated fraction activity on mitochondria in the presence of 50 nmoles Ca^{++} in the medium. Other experimental conditions are described in Materials and Methods.

Isolated fraction µg per protein	RC *	P/O	Oxydation rate state 3	Oxydation rate state 4
0	2.7	1.3	115	42
0.75	1.4	0.8	112	81
2.5	1.3	0.7	110	88
5	1.4	0.7	118	80
12.5	1.3	0.8	127	100
25	1.0	0	140	140

Table 3: Effect of Ruthenium Red and Mg^{++} upon isolated fraction activity on mitochondria. Other experimental conditions are described in Materials and Methods.

Isolated fraction µg per mg protein	R.Ruthe n Mole	Mg^{++} n Mole	RC*	P/O	Oxydation rate State 3	State 4
0	0	0	4.1	1.5	97	23
25	0	0	1	0	138	138
25	3.2	0	4	1.5	94	23
25	0	2	4.2	1.5	96	23

* RC = Respiratory control

fraction is largely potentiated by addition of calcium as 0.75 µg are sufficient to induce strong uncoupling.

Finally we can notice that magnesium and ruthenium red inhibit the fraction activity (Table 3).

DISCUSSION

a) Chromatography

As indicated previously, the isolated fraction has an unsteady behaviour during desalting on Sephadex G15, Indeed, it can be eluted in the void volume or retained by the column (fig 1C).

These results suggest that the same compound is present in all these fractions. This might be accounted for by a polymerisation of the molecule(s) studied, and the calcium concentration of the first fraction is consistent with this hypothesis. Indeed the synthetic ionophore A 23187 is known to be able to form a dimer by binding with an atom of calcium (5) and we think this might be the case too with our substance. Thus, according to its calcium dependent polymerisation, the isolated fraction might be eluted differently from Sephadex G15. Moreover, as active fraction may be eluted after phosphate salt from Sephadex G15 (figure 1C), the molecular weight of the compound(s) must have been low. Furthermore it may have a peptidic character according to the positive ninhydrin reaction.

b) Mitochondrial activity

Our results indicate that, when mitochondria are strongly uncoupled, both state 3 and 4 succinate oxidation rates increase and these respiratory stimulations are higher than that which accompanies the phosphorylation of ADP. Such respiratory stimulations were noticed by various authors (6) when mitochondria accumulate high Ca^{++} concentrations.Moreover the uncoupling induced by the isolated fraction is potentiated by addition of calcium and inhibited by magnesium and ruthenium red. According to numerous studies on mitochondrial activity

of ionophorous substances for divalent cation (7,5), these results are consistent with a ionophorous activity for calcium of the fraction studied. Thus the increase of the respiratory rate can be accounted for by the energy dependant reaccumulation of calcium discharged from mitochondria by the fraction. Apparently, the substance(s) acts in concert with the calcium carrier of mitochondria to establish an energy dissipating cyclic flux of calcium hence by passing the phosphorylated pathway.

The results of table 1 are in good agreement with such a recycling of Ca^{2+} across the membrane by the mitochondrial carrier: calcium will be provided in the respiratory medium by its presence in the isolated fraction as a contaminent (75µg of this fraction supply the medium with 40 nanomoles of calcium with a 2% contamination in this case).

Experiments in the presence of 50 nanomoles of Ca^{+} corroborate that the uncoupling activity of the isolated fraction is Ca^{2+} dependent. In this experimental series, mitochondria are strongly uncoupled with 0.75µg of added substance which provides the medium with only 0.4 nanomole of Ca^{2+}. So the activity of the fraction is no longer due to its Ca^{2+} contamination.

The calcium recycling is confirmed by the third experimental series. Indeed, ruthenium red, by inhibiting the energy-dependant calcium entry in the mitochondria prevents uncoupling.

Finally, since the affinity of magnesium for the isolated substance is probable, at the studied concentration this ion must act by binding to the substance(s) thus preventing the uncoupling. Similar results with the synthetic ionophore A 23187 have been observed(7). The concentration of this fraction in the serum of some patients suffering from renal insufficiency is about 50µg per ml. In normal urine the concentration is about 200µg per ml. Those quantities are relatively important and suggest that the ionophore is probably present at low concentrations in the plasma of normal subjects. So, we think this substance might play a physiological role by participating in the mechanisms of cellular calcium homeostasis. Particularly this natural factor might induce a physiological release of intramitochondrial calcium. This physiological release at present is accounted for with difficulty, however some workers such as Carafoli(8) searched for a calcium releasing factor. Moreover, the decrease in mitochondrial calcium granulation in renal tubules of uremic patients (1) might be correlated to the accumulation of the ionophorous substance.

It is obvious that this hypothetical activity must no be related only to mitochondria, but also to calcium dependent effect in cells. Studies with the synthetic ionophore A 23187 support that idea (5,9).

Obviously the fraction isolated is not yet pure (see results of table 1 and 3) but this work is a preliminary step and further investigations are

being conducted in order to determine the structure of this ionophorous compound(s) present in normal urine and uremic plasma.

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