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EFFECT OF 2-AMINOETHANESULPHONIC ACID (TAURINE) AND 2-HYDROXYETHANE SULPHONIC ACID (ISETHIONIC ACID) ON CALCIUM TRANSPORT BY RAT LIVER MITOCHONDRIA

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Abstract—Taurine and isethionic acid at concentrations ranging from 3×10^{-5} to 6×10^{-4} M did not change oxygen uptake of liver mitochondria in the presence of various respiratory substrates. Taurine and isethionic acid on the contrary inhibited mitochondrial respiration in the presence of 4 mM $CaCl_2$. Maxive uptake of calcium and inorganic phosphate production measured after respiration experiments were not affected by taurine and isethionic acid. ATP-dependent calcium uptake in conditions of high external calcium (10 mM) was increased significantly by taurine and isethionic acid; they were also able to increase calcium binding in mediums without substrates or ATP, having external calcium concentrations of 0·5 and 0·75 mM. We can therefore suggest that taurine and isethionic acid react with calcium on the mitochondrial outer membrane and/or intermembrane spaces, increasing mitochondrial calcium-binding capacity and thus affecting mitochondrial oxygen uptake.

EVIDENCE suggesting the presence of taurine (2-aminoethane-sulphonic acid), in the tissues of some invertebrate and vertebrate species has been accumulated in recent years.[1] Taurine and taurinc biosynthesis and metabolism enzymes are ubiquitously present in the biological systems, but a definite physiological function of taurine beyond bile-acid formation has not yet been clearly established. The presence of a strong acidic group in taurine (pK_a 1·5) and in the product of its metabolic conversion, isethionic acid (2-hydroxyethane sulphonic acid) (pK_a 2–2·3),[1] suggests that the affinity between sulphonic acid and cell cations may play an important role in the regulation of ionic exchange in tissues. It has been shown that taurine can decrease potassium efflux from the heart, induced by toxic doses of epinephrine or digoxin,[2,3] and can counteract the depressive effect on cardiac contractility of low calcium media.[4]

It therefore seemed of interest to study the action of taurine and isethionic acid on calcium fluxes and calcium activated respiration at the level of rat liver mitochondria. These were chosen for investigation as an elementary model for calcium kinetics.

It is widely known that calcium stimulation of mitochondrial respiration is accompanied by H^+ ejection from intramitochondrial compartments.[5]

The stoichiometry between calcium and H^+ movements can be regulated, together with a number of factors, by the system of permeant anions, i.e. acetate and phosphate.

Although we have shown that taurine is not a permeant substrate at the level of mitochondrial intermembranes,* it is possible it may interfere with calcium kinetics due to its ionic properties.

* F. Buffoni, unpublished results.

METHODS

Male Sprague–Dawley rats, av. wt 250 g, were used throughout the experiments. Animals were killed by a blow on the head, their livers were removed and homogenized in a chilled 0·25 M sucrose solution using a Potter-Elvehjem Teflon glass homogenizer (clearance 0·07 in. max., 0·05 in. min.), at 800 rev/min. The homogenate was centrifuged at 450 *g* for 20 min at 4°. Mitochondrial pellets were obtained from the supernatant by differential centrifugation in 0·25 M sucrose at 8000 *g* for 20 min with an MSE centrifuge. Pellets were washed twice with 0·25 M sucrose.

Mitochondrial respiration was determined by the standard manometric technique in Warburg flasks, at 30°, with oxygen as the gas phase, in a total suspension volume of 1·5 ml, comprising of the following: 10 mM phosphate buffer, pH 7·5; 5 mM, KF; 20 mM, Tris–HCl, pH 7·5; 2 mM, ATP; 66 mM, glucose; 10 I.U., hexokinase; and 15 mM, substrate. A second groups of experiments were carried out in a different medium comprising of: 3 mM, ATP; 4 mM, $CaCl_2$; 10 mM, Tris–HCl, pH 7·4; 0·12 mM, phosphate buffer, pH 7·4; and 15 mM, substrate. Substrates were tipped from side arms after 5 min preincubation. Protein concentration was approx. 10 mg per flask. In some experiments ^{45}Ca was used as a calcium marker in the second medium; after 30 min incubation the Warburg flasks were chilled with ice and the mitochondrial suspension was immediately centrifuged at 15,000 *g* for 10 min at 0°.

Mitochondrial calcium uptake was determined by the decrease of calcium concentration in the supernatants. The kinetics of calcium binding were determined according to Reynafarje and Lehninger.[6]

About 5 mg of mitochondrial protein, 2 mM, Tris–HCl, pH 7·4, $CaCl_2$ varying from 0·05 to 0·75 mM, were incubated in 0·25 M sucrose with air as the gas phase as described, and the supernatant radioactivity determined. Other calcium uptake experiments were carried out according to Rossi and Lehninger,[7] with 25 mM ATP and $CaCl_2$ concentrations varying from 1 to 10 mM, or according to Haugaard *et al.*,[8] with the following medium: 25 mM, Tris–HCl, pH 7·4; 4·1 mM, ATP; 0·54 mM, $CaCl_2$; 8·2 mM, succinate and 0·118 mM KCl. Lactic dehydrogenase activity, alcohol dehydrogenase, β-hydroxybutyrate dehydrogenase, oxalacetate transaminase, alkaline phosphatase, benzylamineoxidase and carbonic anhydrase were all determined according to methods described previously.[9–16]

Proteins were determined according to Waddel[17] and Lowry *et al.*[18] and phosphate according to Martin and Doty.[19] Radioactivity was determined with a Packard Tri-Carb spectrometer in Bray's liquid, with the usual corrections for quenching.

Reagents. Chemicals were obtained either from British Drug Houses or Merck and were of analytical grade; deionized, bidistilled water, Biochemia Boeringer enzymes and reagents were used throughout the experiments. Pig plasma benzylamine oxidase was prepared in this laboratory by Buffoni in crystalline form. A crude preparation of rat erithrocytes was used for carbonic anhydrase determinations.

EXPERIMENTAL RESULTS

Effect of taurine and isethionic acid on enzymatic systems in vitro. Taurine and isethionic acid have no direct effect on many enzymatic proteins of different structures and prosthetic groups. The enzymatic activities we have tested were a group of dehydrogenases (alcohol dehydrogenase, lactic dehydrogenase, glutamic dehydrogenase,

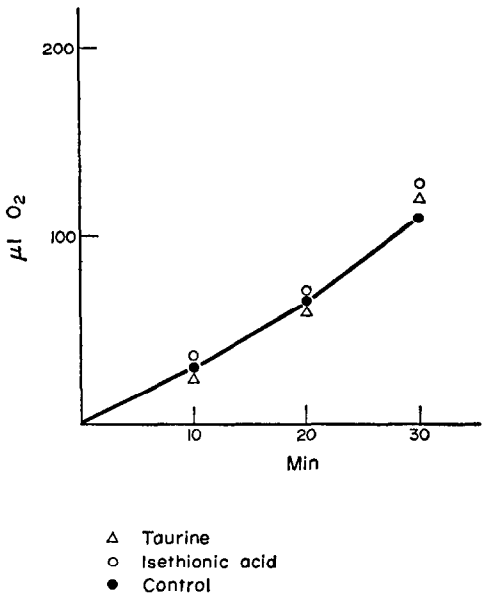

FIG. 1. Effect of taurine and isethionic acid on mitochondrial respiration. Warburg flasks, 30°, oxygen. Incubation mixture: 10 mM phosphate buffer pH 7·5, 5 mM $MgCl_2$, 5 mM KF, 20 mM Tris–HCl pH 7·5, 2 mM ATP, 66·6 mM sucrose, 30 mM glucose, 10 I.U. hexokinase, 15 mM α-keto-glutarate, 1 mM taurine or isethionic acid. Total volume 1·5 ml. Mitochondrial proteins about 10 mg. On abscissa, time (min); on ordinate, oxygen consumption (µl).

β-hydroxybutirate dehydrogenase), carbonic anhydrase as a model of a zinc-containing enzyme, benzylamineoxidase as a model of a cupric copper-enzyme, and glutamic-oxalacetate transaminase as a model of a pyridoxal phosphate enzyme. No direct effect was demonstrated with concentrations up to 10^{-2} M of taurine and isethionic acid.

Effect of taurine and isethionic acid on mitochondrial respiration. Taurine and isethionic acid did not alter the oxygen uptake of liver mitochondria in the presence of alpha-ketoglutarate (Fig. 1) or other respiratory substrates, i.e. under conditions in which oxidative phosphorilation is operative in presence of the hexokinase–glucose system. However, mitochondrial oxygen uptake was inhibited by isethionic acid and taurine where respiration was calcium-activated using glutamate as the substrate (Fig. 2).

The degree of inhibition between each substance was significantly different; maximum inhibition was higher with isethionic acid (85 per cent, 6×10^{-4} M isethionic acid), whereas taurine showed a marked effect at low concentrations (45 per cent $1{\cdot}5 \times 10^{-4}$ M), but no further effect could be detected at higher concentrations. Total phosphate production, which normally was maximal, remained unchanged in these conditions. Similar respiratory inhibition, in the presence of 3×10^{-4} M isethionic acid could also be observed with other respiratory substrates (Table 1), showing no variation in the total phosphate production. Sodium sulphate, similarly to taurine did not penetrate the mitochondrial matrix, but was able to inhibit mitochondrial respiration at concentration of 3×10^{-3} M (Fig. 3).

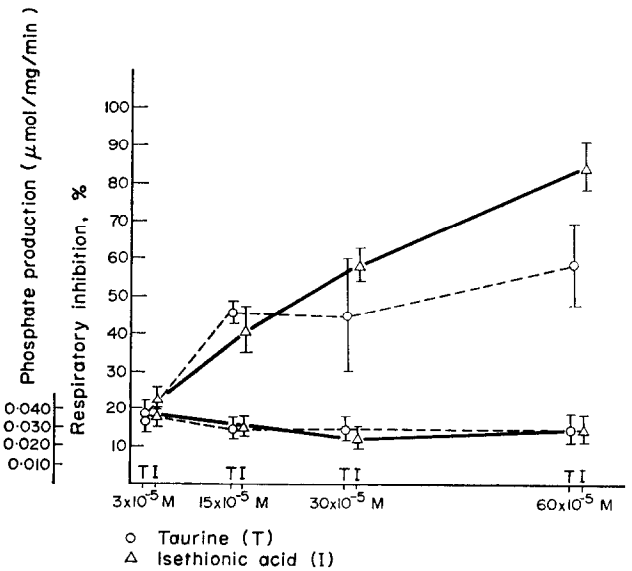

FIG. 2. Effect of taurine and isethionic acid on mitochondrial calcium activated respiration and phosphate production. Warburg flask, 30°, oxygen. Incubation medium: 3 mM ATP, 4 mM $CaCl_2$, 10 mM $MgCl_2$, 80 mM NaCl, 10 mM Tris–HCl pH 7·4, 0·12 mM phosphate buffer pH 7·4, 15 mM glutamate. Total volume 1·5 ml. After 30 min incubation the reaction was stopped with 0·5 ml of 14% perchloric acid and phsophate production was measured. On abscissa, taurine and isethionic acid concentrations; on ordinate, percentage of oxygen uptake inhibition compared to control values and inorganic phosphate production (μmoles/mg/min). Data are expressed as mean ±S.E. ($n = 8$ for isethionic acid, $n = 4$ for taurine).

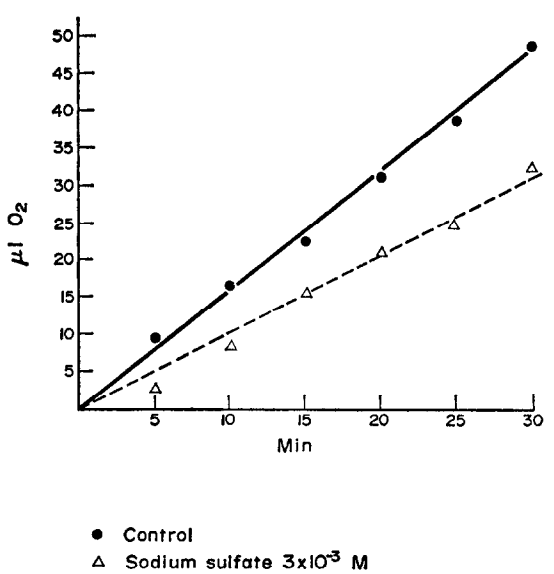

FIG. 3. Effect of sulphate on calcium activated respiration. Experimental conditions as in Fig. 2. On abscissa, time (min); on ordinate, oxygen consumption (μl).

TABLE 1. EFFECT OF ISETHIONIC ACID ON MITOCHONDRIAL RESPIRATION AND INORGANIC PHOSPHATE PRODUCTION*

Substrates	Oxygen uptake (μl/mg/30 min)		Phosphate production (μmoles Pi/mg/30 min)		No. of experiment
	Control	Isethionic acid 3×10^{-4} M	Control	Isethionic acid 3×10^{-4} M	
Glutamate	2·67 ± 0·3	1·70 ± 0·3	1·07 ± 0·12	1·22 ± 0·12	7
α-Ketoglutarate	1·93 ± 0·3	1·11 ± 0·35	1·24 ± 0·17	1·25 ± 0·13	7
Succinate	8·29 ± 1·10	6·33 ± 0·52	1·33 ± 0·14	1·41 ± 0·14	7

* Experimental conditions as in Fig. 2. Data expressed as mean ± S.E.

Effect of taurine and isethionic acid on calcium uptake by liver mitochondria: energy-linked "maxive uptake of calcium". Total calcium uptake was measured in conditions of mitochondrial calcium-dependent respiration, exposing isolated mitochondria to massive concentrations of calcium. At the end of respiration experiments total calcium uptake was measured and found not to be significantly affected by taurine and isethionic acid (Table 2). Taurine, isethionic acid and sulphate when measured under the same conditions caused no variation in mitochondrial swelling.

TABLE 2. EFFECT OF ISETHIONIC ACID AND TAURINE ON MITOCHONDRIAL ENERGY-LINKED MASSIVE UPTAKE OF CALCIUM

	Calcium uptake (nmoles/mg/30 min)	No. of experiment
Control	289 ± 50	8
Isethionic acid 4×10^{-3} M	287 ± 50	8
Taurine 4×10^{-3} M	254 ± 73	8

Experimental conditions as in Fig. 2. Incubation stopped after 30 min. Data expressed as mean ± S.E.

Effect of taurine and isethionic acid on calcium uptake by liver mitochondria: energy-linked "limited uptake of calcium". Limited uptake of calcium (energy-linked) was studied as described by Haugaard[8] with an external calcium concentration of 0·54 mM, in the presence of ATP, succinate, $MgCl_2$ and KCl. In these conditions the maximum uptake of calcium after 3-min incubation was not affected by taurine, isethionic acid or sulphate (Table 3).

Effect of taurine, isethionic acid and sodium sulphate on ATP-dependent calcium uptake. When energy for calcium uptake was supplied by ATP hydrolysis only, with various external calcium concentrations, taurine, isethionic acid and sodium sulphate increased the calcium uptake significantly at the highest concentrations (10 mM) (Table 4).

TABLE 3. EFFECT OF ISETHIONIC ACID, TAURINE AND SULPHATE ON ENERGY-LINKED LIMITED UPTAKE OF CALCIUM

	Calcium uptake (nmoles/mg/min)	No. of experiment
Control	95·4 ± 7·6	16
Isethionic acid		
0·15 mM	88·6 ± 11·9	8
0·30 mM	99·5 ± 6·5	10
0·60 mM	109·5 ± 8·9	8
Taurine		
0·15 mM	122 ± 11·9	6
0·30 mM	94·8 ± 15	6
0·60 mM	103 ± 8·7	6
Sulphate		
0·30 mM	115 ± 6·9	6
0·60 mM	125 ± 10·6	6

Medium composition according to Haugaard *et al.*,[10] 4·1 mM $MgCl_2$, 0·54 mM $CaCl_2$, 4·1 mM ATP, 8·2 mM succinate, 0·118 mM KCl, 25 mM Tris–HCl pH 7·4, mitochondrial proteins about 5 mg. Total volume 3 ml. Incubation time 3 min. Data expressed as mean ± S.E.

TABLE 4. EFFECT OF ISETHIONIC ACID, TAURINE AND SULPHATE ON ATP DEPENDENT CALCIUM UPTAKE*

	Mitochondrial uptake (nmoles calcium/mg/min)				
External $CaCl_2$	1 mM	2 mM	5 mM	10 mM	No. of experiment
Control	68·6 ± 16·15	99·84 ± 18·7	303·86 ± 85·5	535·4 ± 159·6	8
Isethionic acid					
3×10^{-4} M	93·7 ± 14	103·6 ± 24	257·99 ± 61·9	648·1 ± 107·1†	8
Taurine					
3×10^{-4} M		93·81 ± 34·7		792·8 ± 90·8†	4
Sulphate					
3×10^{-4} M		92·42 ± 40·8		620·85 ± 92·9†	4

* Incubation medium according to Rossi and Lehninger,[9] 25 mM ATP, 2 mM Tris–HCl pH 7·4, 0·25 M sucrose, $CaCl_2$ varying from 1 to 10 mM, about 5 mg mitochondrial protein. Incubation time 1 min at 25°. Data expressed as mean ± S.E.

† Values different from control $P < 0·05$. Variance analysis according to Croxton.[20]

Effect of taurine and isethionic acid on mitochondrial calcium accumulation in the absence of substrate and ATP. Calcium accumulation was measured according to Reynafarje and Lehninger[6] in media lacking substrate and ATP, at external calcium concentrations ranging from 0·05 to 0·75 mM; these were lower than those used in previous experiments (Fig. 4). In these conditions the kinetics of calcium accumulation was modified by isethionic acid (Table 5) and taurine (Fig. 5) with an increase of maximum binding capacity. The apparent affinity constant of the process decreased, as can be seen from the apparent K_m-value for calcium accumulation in the presence of 3×10^{-4} M isethionic acid, which increased from 2·72 ± 0·29 of the controls to

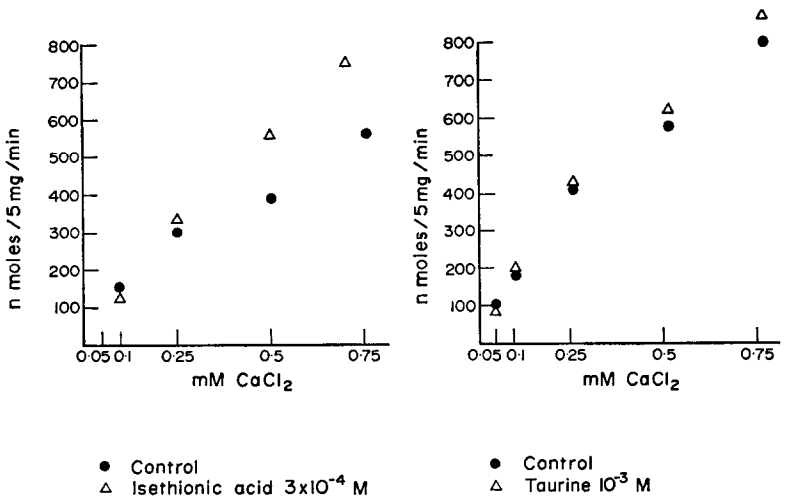

FIG. 4. Effect of taurine and isethionic acid on mitochondrial calcium accumulation in the absence of substrate and ATP. Mitochondrial protein about 5 mg. Incubation in 0·25 M sucrose for 1 min, with air as gas phase, 25°. Total volume 2 ml. On abscissa, external calcium concentrations; on ordinate, total calcium accumulation (nmoles/5 mg/min).

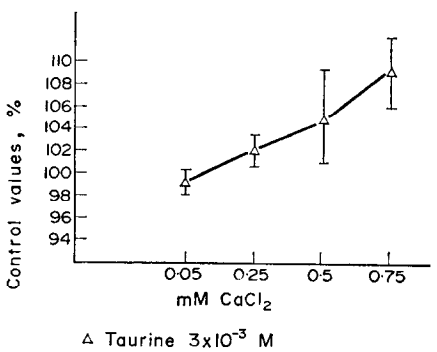

FIG. 5. Effect of taurine on mitochondrial calcium accumulation. Experimental conditions as in Fig. 4. On abscissa, external calcium concentration; on ordinate percentage of mitochondrial binding in the presence of taurine compared to controls values. Data expressed as mean ± S.E. ($n = 8$).

TABLE 5. EFFECT OF ISETHIONIC ACID ON MITOCHONDRIAL CALCIUM ACCUMULATION IN THE ABSENCE OF SUBSTRATE AND ATP*

	Mitochondrial calcium binding					
External $CaCl_2$	0·05 mM	0·1 mM	0·25 mM	0·5 mM	0·75 mM	No. of experiment
Control	13·6 ± 2·8	23·4 ± 4·7	55 ± 8·2	57·1 ± 6·5	75·6 ± 12	10
Isethionic acid 3×10^{-4} M	14·3 ± 3·3	24·3 ± 4·3	53·3 ± 7·1	78·3 ± 8·8‡	91·2 ± 18†	10

* Medium composition according to Reynafarje and Lehninger,[8] 0·25 M sucrose, 2 mM Tris–HCl pH 7·4, calcium concentrations varying from 0·05 to 0·75 mM, mitochondrial proteins about 5 mg. Incubation time 1 min at 25°. Data expressed as mean± S.E.

† Values different from control $P < 0·05$.

‡ Values different from control $P < 0·01$. Variance analysis according to Croxton.[20]

3·94 ± 0·02 (mean ± S.E., $n = 10$), when determined according to Lineweaver and Burk.[21]

DISCUSSION

The data reported here demonstrate that taurine, isethionic acid and sodium sulphate have no direct effect on different enzymatic proteins and do not interfere with several mechanisms of enzymatic reaction. The minimal effect on carbonic anhydrase and benzylamine oxidase, indicates that the sulphonic groups of both substances do not react with protein-bound metals. The minimal effect of taurine and isethionic acid on mitochondrial respiration in the presence of ATP, glucose–hexokinase and respiratory substrates is further evidence towards this hypothesis. However taurine, isethionic acid and sulphate markedly inhibit mitochondrial respiration when oxygen uptake is measured in the presence of calcium. This selective inhibition of mitochondrial calcium-activated respiration, suggests that taurine, isethionic acid and sulphate may interfere with some electrolyte fluxes at mitochondrial level. Ulrich and Kormendi[22] have previously observed that sulphate can increase the loss of endogenous potassium from mitochondria in the absence of ATP and substrates. The fact that sulphates as well as taurine and isethionic acid are able to inhibit mitochondrial oxygen uptake in the presence of calcium, indicates that the terminal sulphonic groups are responsible for the observed effect. The presence of an amino group in taurine could suggest why it induces a respiratory inhibition comparitively lower than isethionic acid. In fact, the anionic properties of the sulphonic groups of taurine are partially neutralized by the cationic properties of the terminal amino group. From the above we can postulate further on the possible interference of taurine and isethionic acid with calcium fluxes at mitochondrial level. This interference is not demonstrable on respiration-dependent mitochondrial maxive uptake of calcium or on limited uptake of calcium in the absence of inorganic phosphate. This may be due to the fact that taurine and isethionic acid do not affect the overall function of the electron driven mitochondrial calcium pump. On the contrary, taurine and isethionic acid increased mitochondrial calcium accumulation when it was measured either in the presence of ATP or at low external calcium concentrations in the absence of added ATP and respiratory substrates. The increased mitochondrial calcium accumulation which is evident in the forementioned conditions may be explained as a result of increased mitochondrial binding sites for calcium. The specific affinity of these additional binding sites must be very low, as the overall affinity constant is reduced. Possibly the sulphonic groups of taurine and isethionic acid act as "receptors" for calcium at the mitochondrial intermembrane space-or the outer membrane level. Taurine and isethionic acid cannot penetrate the mitochondrial matrix, but could attach to the outer membrane and penetrate into the intermembrane space.* These substances do not behave as permeant anions as they do not modify the light scattering changes which accompany mitochondrial calcium uptake. The effective concentration ranges of taurine and isethionic acid on calcium binding are in the range of 3×10^{-5} M and 6×10^{-4} M (5–100 nmoles/mg mitochondrial protein), i.e. in agreement with the number of maximum mitochondrial binding sites for calcium (40 nmoles/mg mitochondrial protein).[6] The presence of these additional binding sites for calcium may become evident only when calcium carriers are saturated by high

* F. Buffoni, unpublished results.

external calcium, or when binding capacity is measured in the absence of substrate and ATP, when active transport is very low.

We may draw the following general conclusion from the above: calcium is accumulated to a level of approx. 100 nmoles/mg mitochondrial protein by an energy-linked process in the absence of inorganic phosphate and other permeant anions. When inorganic phosphate is present, it is taken-up with calcium. Under these conditions, massive calcium loading can be achieved. In the absence of inorganic phosphate, the amount of calcium that can be taken-up by rat liver mitochondria is limited (Table 3). When approx. 100 nmoles of calcium/mg of mitochondrial protein are taken-up, the energy-linked uptake process stops spontaneously. Evidently, the binding sites in the membrane are saturated at this concentration of calcium. The extra calcium accumulated when phosphate is present, does not increase the saturation level, since calcium and phosphate precipitates in the matrix. If we assume that taurine, isethionic acid and sulphate increase the number of binding sites in the membrane, we may also assume that our hypothesis stating that inorganic phosphate might be preferentially able to transfer calcium inside mitochondrial matrix, removing it preferentially from these additional binding sites of lower affinity is correct. This could explain the fact that respiration is inhibited in this condition, the degree of inhibition being proportional to the concentration of these binding substances. The amount of calcium removed by phosphate at the level of high affinity binding sites is related to the electron flux and could, in fact, be reduced by taurine and isethionic acid.

One feature of the high affinity reaction is the inhibition produced by 2,4-dinitrophenol.[6] Calcium is assumed to be bound to a carrier not involved in the process of energy conservation but only in the energy-dependent calcium translocation. Inhibition of this binding by 2,4-dinitrophenol could be due to displacement of calcium from high affinity sites by proton excess induced to move across the membrane by 2,4-dinitrophenol.

Taurine, isethionic acid and sulphate may interfere with calcium phosphate mitochondrial transfer.

The physiological implication of our results are open to debate. Taurine, and in some cells, isethionic acid, are known to be present in the cytoplasm of cells in concentrations similar to those described in the experiments of this paper. It therefore seems justified to assume that these substances have intracellular functions. Their effect on the binding capacity of mitochondria to calcium, might offer a possible explanation of the protective effect on contraction observed with taurine in low calcium media and suggests a role for taurine and isethionic acid in modulating calcium fluxes.

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