Effects of spermine on mitochondrial Ca\(^{2+}\) transport and the ranges of extramitochondrial Ca\(^{2+}\) to which the matrix Ca\(^{2+}\)-sensitive dehydrogenases respond

James G. McCormack
Department of Biochemistry, University of Leeds, Leeds LS2 9JT, U.K.

INTRODUCTION

The Ca\(^{2+}\)-transport system of the inner membrane of mammalian mitochondria consists of an electrophoretic Ca\(^{2+}\) uniporter for uptake into the matrix driven by the membrane potential (about 180 mV, negative inside) set up through proton extrusion by the respiratory chain, and probably two egress mechanisms, the predominant of which is an electroneutral Na\(^{+}\)/Ca\(^{2+}\) exchanger that is ultimately also driven by the protonomotive gradient through subsequent Na\(^{+}\)/H\(^{+}\) exchange (for reviews see, e.g., Nicholls & Akerman, 1982; Crompton, 1985; Carafoli, 1987); there is also a less well-characterized Na\(^{+}\)-independent egress mechanism which may involve direct Ca\(^{2+}\)/2H\(^{+}\) exchange. The Ca\(^{2+}\) uniporter can be inhibited physiologically by Mg\(^{2+}\) and also artificially by Ruthenium Red, whereas the Na\(^{+}\)/Ca\(^{2+}\) egress mechanism can be inhibited physiologically by increases in extramitochondrial Ca\(^{2+}\), and also artificially by drugs such as diltiazem, which are more normally used as blockers of the plasma-membrane Ca\(^{2+}\) channel (see the above references).

There are two major functions, which are likely to be mutually exclusive, which have been proposed for this Ca\(^{2+}\)-transport system. The earlier, though still advocated (e.g., Chiesi et al., 1988), of these is that it plays a key role in buffering or setting the extramitochondrial (i.e. cytoplasmic) concentration of Ca\(^{2+}\) (see, e.g., Fiskum & Lehninger, 1982; Akerman & Nicholls, 1983). For this property to be exhibited, the egress pathways for Ca\(^{2+}\) have to be saturated, and this requires in excess of approx. 10–15 nmol of total Ca content per mg of mitochondrial protein (see the Discussion section). The alternative proposal is that its primary function is rather to regulate matrix Ca\(^{2+}\) concentration and relay changes in cytoplasmic Ca\(^{2+}\) into this compartment (see, e.g., Denton & McCormack, 1980, 1985; Hansford, 1985) and hence regulate oxidative metabolism, as there are three key Ca\(^{2+}\)-sensitive intramitochondrial dehydrogenases, namely the pyruvate (PDH), NAD\(^{+}\)-isocitrate (NAD-ICDH) and 2-oxoglutarate (OGDH) dehydrogenases. Thus it is envisaged that hormones and other agents which promote energy-requiring events such as contraction and secretion by raising cytosolic Ca\(^{2+}\) would also, as a result, promote energy production (see the above references). In the presence of physiological Na\(^{+}\) and Mg\(^{2+}\), activation of these enzymes can be demonstrated within intact coupled mitochondria from a variety of different mammalian sources as extramitochondrial Ca\(^{2+}\) is raised within the expected physiological ranges.
range (i.e. approx. 0.05-2 μM; see Denton & McCormack, 1985) and as total Ca content rises in the approximate range 0-4 nmol/mg of protein. It was therefore proposed (e.g. Denton & McCormack, 1985; Hansford, 1985) that the ability of mitochondria to buffer extramitochondrial [Ca^{2+}] may be reserved for more pathophysiological circumstances where there is an abnormal influx of Ca^{2+} across the plasma membrane, resulting in the capacity of the normal cellular Ca^{2+}-buffering systems being exceeded.

Nicchitta & Williamson (1984) reported that the polyamine spermine is a potent activator of mitochondrial Ca^{2+} uptake; this has since been confirmed by Lenzen et al. (1986) and Kroner (1988), though earlier Akerman (1977) had reported that it inhibited this process. In the studies by Nicchitta & Williamson (1984) and Lenzen et al. (1986), it was clearly demonstrated that spermine could thus lower the set-point at which the mitochondria buffer the extramitochondrial Ca^{2+} environment to the sub-micromolar range. Given these findings and the above controversies, it seemed important to establish the effects of spermine on the ranges of extramitochondrial Ca^{2+} to which the matrix Ca^{2+}-sensitive dehydrogenases would exhibit Ca^{2+} regulation under different conditions. A small part of the present work has been previously reported briefly as a meeting abstract (McCormack, 1987).

MATERIALS AND METHODS

All biochemicals and chemicals used in this study were of the highest grade commercially available and were purchased from either Sigma Chemical Co. (Poole, Dorset, U.K.) (including spermine (diphosphate salt)), BDH Chemicals (Poole, Dorset, U.K.), or Boehringer Corp. (Lewes, East Sussex, U.K.), except for p-(p-aminophenyl)-azobenzenesulphonic acid (used in the PDH assay; see below) which was from Pfaltz and Bauer, Stanford, CT 06902, U.S.A., diltiazem (cis(-)-3-acetyloxy-5-[2-(dimethylamino)ethyl]-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepin-4(5H)-one), which was kindly gifted by Dr. G. Satzinger of Goedecke A. G., Freiburg, Germany, and fura-2 and its acetoxymethyl ester, which were from Calbiochem (Cambridge, U.K.). The radioisotopes used were from Amersham International (Ameringham, Bucks., U.K.).

Mitochondria were prepared from rat heart (Denton et al., 1980), liver (McCormack, 1985a) and kidney (McCormack et al., 1988) after Polytron homogenizations of the tissues as described in the appropriate reference; the liver and kidney preparations included a purification step through a Percoll gradient to remove endoplasmic-reticulum contamination, in particular (Assimacopoulos-Jeannet et al., 1986). Mitochondria were incubated (at approx. 0.5-2 mg of protein/ml) at 30 °C in a basic medium consisting of 120 mM-KCl, 20 mM-Tris and 5 mM-KH_{2}PO_{4} (pH 7.3) together with appropriate amounts of EGTA-Ca buffers to generate the required extramitochondrial Ca^{2+} concentrations (see McCormack, 1985b), and other additions as indicated in legends. Potassium salts were used throughout unless indicated. The addition of extra phosphate alone (as spermine diphosphate was used) had no effects on the results obtained. Where appropriate, rat heart mitochondria were loaded with fura-2 as fully described in McCormack et al. (1989), which also details how the Ca^{2+}-dependent changes in fluorescence were monitored and calibrated.

The Ca^{2+}-sensitive properties of the PDH system and of OGDH and NAD-ICDH were assayed for within the intact heart (Denton et al., 1980), liver (McCormack, 1985a,b) and kidney (McCormack et al., 1988) mitochondria as described previously. The extraction of mitochondria and the assay of the Ca^{2+}-sensitive enzymes were also as described previously: PDH phosphate phosphatase (Marshall et al., 1984), NAD-ICDH (Denton et al., 1978) and OGDH (McCormack & Denton, 1979). A unit of enzyme activity is the amount that catalyses the conversion of 1 μmol of substrate/min at 30 °C.

![Image](https://example.com/image.png)

Fig. 1. Effects of spermine on the Ca^{2+}-dependent activation of PDH in coupled rat heart mitochondria incubated with or without Na^{+} and Mg^{2+}

Rat heart mitochondria (approx. 0.5 mg of protein/ml) were incubated for 4 min at 30 °C in KCl-based medium (see the Materials and methods section) containing 10 mM-2-oxoglutarate, 0.1 mM-L-malate and EGTA-Ca buffers (at 5 mM-EGTA) to give the concentrations of free extramitochondrial Ca^{2+} shown, and with other additions as follows: none ( ), 0.5 mM-spermine ( ), 10 mM-NaCl plus 2 mM-MgCl_{2} ( ), 10 mM-NaCl plus 0.5 mM-spermine ( ). PDH_{4} and total PDH activities were measured as described in the Materials and methods section (see Denton et al. (1980) for full details; total activity was unaffected by spermine, and was 101 ± 6 munits/mg protein (mean ± s.e.m. for 24 observations)). Similar results were obtained after incubations for 8 min, and essentially similar results could be obtained with liver or kidney mitochondria (see Table 1), except that with the former some pyruvate (1 mM) also had to be added (see McCormack, 1985b). ATP content was unaffected by spermine or by any of the other conditions over the ranges of Ca^{2+} shown, and averaged 8.2 ± 0.3 (52) nmol/mg of protein. However, it should be noted that, if these Ca^{2+} ranges were exceeded, then there were apparent time-dependent diminutions of mitochondrial ATP content, as has been noted previously, and which are presumably the result of saturation of Ca^{2+} egress and resultant net Ca^{2+} accumulation (to a great extent from the EGTA-Ca buffer at 5 mM-EGTA), leading to generalized perturbation of mitochondria (see McCormack, 1985b). Each point represents the mean of observations made on 3-5 different preparations.

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Mitochondria were loaded with 45Ca (but with total Ca loads within the Ca2+-regulatory ranges of the enzymes) as described by McCormack & Denton (1984) (heart) and McCormack (1985c) (liver) for subsequent studies on Ca2+ egress, which were carried out as described in the above references. Mitochondrial total Ca content was measured as described by Assimacopoulos-Jeannet et al. (1986) and mitochondrial protein was measured by the method of Gornall et al. (1949). Calculations were carried out as detailed in McCormack (1985b); statistical significance was assessed by Student's t test.

RESULTS

The major advantages of using the intramitochondrial Ca2+-sensitive enzymes as probes for matrix Ca2+ are that this allows the monitoring of changes in Ca2+ fluxes and steady-state Ca2+ distributions across the inner membrane to be undertaken while the mitochondria are incubated with buffered and low concentrations of Ca2+ within the physiological range and at physiological Ca loads (see Denton & McCormack, 1985), i.e. to match most closely physiological circumstances. These advantages are also afforded by fura-2-loaded rat heart mitochondria (see McCormack et al., 1989). This is in contrast with other techniques which have been used to monitor the effects of spermine on these parameters (Nicchitta & Williamson, 1984; Lenzen et al., 1986), which principally used external Ca2+ electrodes or indicators to monitor changes in the extramitochondrial concentration of Ca2+ set by the mitochondria (i.e. when the egress pathways are saturated).

A concentration of 0.5 mM-spermine was chosen for most of the experiments. This was partly due to spermine solubility in stock solutions, but largely because the reported $K_{0.5}$ values for its effects on Ca2+ uptake were 170 μM (Nicchitta & Williamson, 1984) and 50 μM (Lenzen et al., 1986), with both reporting saturation at about 0.4 mM. However, Kroner (1988) reported a $K_{0.5}$ value of around 0.2 mM, which is closer to that found here (see below). Nevertheless, 0.5 mM is certainly close to saturating (Fig. 5, below). Also in common with these previous reports, spermine was found to be much less effective (5–10 times) than spermine, and putrescine was ineffective (results not shown).

Fig. 1 and Table 1 show that 0.5 mM-spermine produced marked decreases in the $K_{0.5}$ values for extramitochondrial Ca2+ in the Ca2+-dependent activations of PDH in fully coupled mitochondria prepared from rat heart, kidney or liver. Fig. 2 and Table 1 show that very similar effects of 0.5 mM-spermine were evident on the Ca2+-dependent activations of OGDH under similar circumstances. No techniques are yet available to assess the Ca2+-sensitivity of NAD-ICDH in intact rat heart mitochondria, for reasons discussed previously (Denton et al., 1980), and there are also data-interpretation problems with assessing the Ca2+-sensitivity of this enzyme in liver (McCormack, 1985b) and kidney (McCormack et al., 1988) mitochondria, largely because there is as yet no satisfactory way of eliminating subsequent flux through OGDH. There are also reports that this enzyme may respond to a higher Ca2+-concentration range, of up to an order of magnitude, than PDH and OGDH (Rutter & Denton, 1988). However, bearing these provisos in mind, the data of Fig. 3 and Table 1 suggest that spermine has very similar effects on the Ca2+-sensitivity of this enzyme to changes in extramitochondrial Ca2+ as it has on PDH and OGDH, at least in kidney and liver mitochondria. Rutter & Denton (1988) also showed that the Ca2+-sensitivity of NAD-ICDH and OGDH may be altered by changes in the ATP/ADP ratio; however, spermine (0.5 mM) was found not to bring about any changes in mitochondrial ATP content [assayed as in Denton et al., 1980] and McCormack (1985b) in the present study (see Fig. 1 legend). It should also be noted that the O2 electrode can be used to monitor Ca2+-dependent effects on OGDH in heart (Denton et al., 1980) and kidney (McCormack et al., 1988) mitochondria, and on NAD-ICDH in kidney

Table 1. Effects of spermine (0.5 mM) on the $K_{0.5}$ values for extramitochondrial Ca2+ in the Ca2+-dependent activations of the matrix Ca2+-sensitive dehydrogenases within intact mitochondria from rat heart, liver and kidney incubated under different conditions

<table>
<thead>
<tr>
<th>Condition</th>
<th>Heart</th>
<th>Liver</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PDH</td>
<td>OGDH</td>
<td>PDH</td>
</tr>
<tr>
<td>Control</td>
<td>35 ± 2</td>
<td>29 ± 3</td>
<td>114 ± 9</td>
</tr>
<tr>
<td>0.5 mM-Spermine</td>
<td>15 ± 1</td>
<td>16 ± 1</td>
<td>31 ± 2</td>
</tr>
<tr>
<td>10 mM-NaCl</td>
<td>133 ± 16</td>
<td>92 ± 6</td>
<td>n.d.</td>
</tr>
<tr>
<td>Na+ spermine</td>
<td>72 ± 9</td>
<td>41 ± 4</td>
<td>n.d.</td>
</tr>
<tr>
<td>2 mM-MgCl2</td>
<td>192 ± 20</td>
<td>149 ± 14</td>
<td>402 ± 16</td>
</tr>
<tr>
<td>Mg2+ spermine</td>
<td>74 ± 6</td>
<td>66 ± 6</td>
<td>106 ± 8</td>
</tr>
<tr>
<td>Na+2, Mg2+</td>
<td>551 ± 36</td>
<td>504 ± 32</td>
<td>586 ± 27</td>
</tr>
<tr>
<td>Na+, Mg2+, spermine</td>
<td>295 ± 12</td>
<td>266 ± 25</td>
<td>333 ± 20</td>
</tr>
<tr>
<td>1 μM-FCCP</td>
<td>1110 ± 56</td>
<td>966 ± 29</td>
<td>1249 ± 40</td>
</tr>
<tr>
<td>FCCP, spermine</td>
<td>975 ± 32</td>
<td>930 ± 17</td>
<td>1066 ± 51</td>
</tr>
</tbody>
</table>

Data from various experiments of the types shown in Figs. 1–3 have been combined to give the $K_{0.5}$ values ± s.d. shown; values were derived from 20–50 observations over suitable [Ca2+] ranges, which were made on at least three different preparations of mitochondria in each case: n.d., not determined. For the experiments with uncoupler for PDH measurements, 2 mM-MgATP was present, together with oligomycin (5 μg/ml) and rotenone (0.2 μg/ml).
mitochondria, in the presence of ADP or uncoupler; similar results to those shown in Figs. 1–3 and Table 1 were obtained with this technique (not shown).

Control experiments established that spermine had no effects at all on any of the kinetic parameters (including Ca<sup>2+</sup>-sensitivities) of OGDH and NAD-ICDH in extracts of mitochondria. Spermine was also found not to affect the sensitivity of the free-acid form of fura-2 to Ca<sup>2+</sup> (see below). The observations by Damuni et al. (1984) and Thomas et al. (1986) that spermine activated extracted PDH phosphate phosphatase by decreasing its K<sub>e</sub> for Mg<sup>2+</sup> in both the presence and the absence of Ca<sup>2+</sup> were confirmed in the present study (results not shown).

Damuni & Reed (1987) also reported a spermine-activated bivalent-cation-independent phosphatase from bovine kidney mitochondria which showed some activity towards PDH phosphate. However, spermine had no effects on the amounts of PDH<sub>a</sub> [or the activities of OGDH and NAD-ICDH (Figs. 2 and 3)] in intact mitochondria incubated with EGTA alone, and in either the absence (Fig. 1) or the presence (results not shown) of pyruvate, which activates PDH through its inhibition of PDH<sub>a</sub> kinase. Spermine also had no effects on the Ca<sup>2+</sup>-sensitivity of PDH, OGDH or NAD-ICDH in uncoupled mitochondria (Table 1). Also, spermine uptake by mitochondria is very slow (Toninello et al., 1985). Thus, overall it would appear to be entirely valid to use these enzymes' activities (and fura-2) in intact mitochondria to assess the effects of spermine on this Ca<sup>2+</sup>-transport system.

Figs. 1–3 and Table 1 show that the effects of spermine on the distribution of Ca<sup>2+</sup> across the inner membrane are evident in both the absence and the presence of either or both Na<sup>+</sup> and Mg<sup>2+</sup>, and that the effects appear to be of similar magnitude in all of these different conditions (around 2–3-fold).
Spermine effects on mitochondrial Ca\(^{2+}\) transport

Fig. 4. Effects of increasing spermine concentration on the activation of OGDH within rat liver mitochondria

Rat liver mitochondria were incubated in a fluorimeter exactly as was described in Fig. 3 for kidney mitochondria, except that 0.25 mM-malonate replaced the hydroxymalonate. The following additions were then made as indicated: at arrow x, 100 \(\mu\)M-2-oxoglutarate; at arrow y, 1 mM-EGTA plus 1 mM-CaCl\(_2\) (resultant free extramitochondrial [Ca\(^{2+}\)] of 26 nm); at arrow z, concentrations of spermine as follows: 1, none; 2, 0.05 mM; 3, 0.1 mM; 4, 0.2 mM; 5, 0.5 mM; 6, 2 mM. A typical experiment is shown; see Fig. 5 and Table 2.

Fig. 5. Concentration-dependency of the effects of spermine on the Ca\(^{2+}\)-dependent activations of the Ca\(^{2+}\)-sensitive intramitochondrial dehydrogenases within intact rat heart, liver and kidney mitochondria

Data were combined from experiments of the types shown in Figs. 1–3 (but at different [spermine]) and Fig. 4, which were carried out on rat heart (○), liver (■) and kidney (▲) mitochondria. Data on the sensitivity to changes in extramitochondrial Ca\(^{2+}\) of PDH, OGDH and NAD-ICDH have been combined for each type of mitochondria for clarity, as no significant differences between enzymes, or indeed type of mitochondria, were evident (see Table 2). The presence or absence of Na\(^{+}\) and/or Mg\(^{2+}\) did not substantially affect the results.

Table 2. \(K_{0.5}\) values for spermine in its effects on the distribution of Ca\(^{2+}\) across the inner membrane of mitochondria from rat heart, liver and kidney

Data from various experiments of the types shown in Figs. 1–3 and 6 (below) (but at different concentrations of spermine) and in Figs. 4 and 5 have been combined to give the \(K_{0.5}\) values (±S.D.) shown; each value was derived over suitable [spermine] ranges and is based on 15–30 observations made on at least two different preparations of mitochondria in each case. The presence or absence of Na\(^{+}\) and/or Mg\(^{2+}\) did not appreciably affect the data given below (results not shown): n.d., not determined.

<table>
<thead>
<tr>
<th>Experimental approach (see other Figs.)</th>
<th>Heart (K_{0.5}) (µM)</th>
<th>Liver (K_{0.5}) (µM)</th>
<th>Kidney (K_{0.5}) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDH, Ca(^{2+})-sensitivity (Fig. 1)</td>
<td>246 ± 12</td>
<td>194 ± 20</td>
<td>302 ± 15</td>
</tr>
<tr>
<td>OGDH, Ca(^{2+})-sensitivity (Figs. 2 and 4)</td>
<td>295 ± 20</td>
<td>324 ± 18</td>
<td>224 ± 21</td>
</tr>
<tr>
<td>NAD-ICDH, Ca(^{2+})-sensitivity (Fig. 3)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>315 ± 30</td>
</tr>
<tr>
<td>Fura-2-loaded (Fig. 6)</td>
<td>251 ± 16</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

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Ruthenium Red (2 μM) was found to block all the effects of increasing extramitochondrial Ca\(^{2+}\) within the ranges used, in either the absence or the presence of spermine (0.5 mM), and likewise the uptake of \(^{45}\)Ca (see below) (results not shown). Also, Nicchitta & Williamson (1984) reported rather enhanced egress of Ca\(^{2+}\) by spermine in the presence of Ruthenium Red; however, again no evidence for this was found in the present study (see below).

The effects of spermine on mitochondrial Ca\(^{2+}\) egress were thus explored in the present work by pre-loading mitochondria with \(^{45}\)Ca, but with overall loads of Ca\(^{2+}\) still within the enzymes’ activatory ranges (around 2–4 nmol/mg of protein), as has been described previously (McCormack, 1985c). This means that the Ca loads used in the present study were always lower than those used by Nicchitta & Williamson (1984) (4–60 nmol/mg of protein). No effects at all of spermine were evident on Ca\(^{2+}\) egress from rat heart, liver or kidney mitochondria in either the absence or the presence of Na\(^{+}\) and/or diltiazem, and with or without Ruthenium Red (results not shown), even though losses over the whole range from 20 to 90% were achieved under the conditions used. This suggests that spermine has no direct effects on mitochondrial Ca\(^{2+}\) egress. It should also be noted that spermine caused clear effects on mitochondrial \(^{45}\)Ca uptake in the present investigations when mitochondria were incubated with low or lightly buffered concentrations of Ca\(^{2+}\) (results not shown).

The demonstrations that fura-2 can be entrapped into the matrix of rat heart mitochondria (see Davis et al., 1987; Lukacs et al., 1988; McCormack et al., 1989) have allowed another independent means of monitoring spermine effects on Ca\(^{2+}\) distribution across the inner membrane of intact mitochondria under physiological incubation conditions. The results of a typical experiment are shown in Fig. 6, and derived \(K_{p.s.}\) values for extramitochondrial Ca\(^{2+}\) and for spermine from such experiments are given in Tables 1 and 2 respectively. Fig. 7 shows the effects of spermine on the distribution of Ca\(^{2+}\) across the inner membrane over the range of extramitochondrial [Ca\(^{2+}\)] expected in mammalian cells in mitochondria incubated with physiological concentrations of Na\(^{+}\) and Mg\(^{2+}\). While the present manuscript was in preparation, Moreno-Sanchez & Hansford (1988) published a paper on indo-1–loaded rat heart mitochondria in which some effects of spermine on Ca\(^{2+}\) uptake were demonstrated, which correlated with changes in PDH\(_{c}\).

Fig. 8 shows that spermine did not appear to affect the total Ca content of the mitochondria over the activatory ranges for the enzymes.

**DISCUSSION**

The study by Nicchitta & Williamson (1984) was on rat liver mitochondria, and Lenzen et al. (1986) reported spermine effects on rat liver, heart and brain mitochondria. However, Kroner (1988), although confirming effects on liver, reported no effects on heart. This is clearly at variance with the present work and that of Lenzen et al. (1986), which rather suggest that this is likely to be a widespread phenomenon for mitochondria.
Spermine effects on mitochondrial Ca\(^{2+}\) transport

Fig. 8. Effects of spermine on the total Ca content of rat heart and liver mitochondria over the Ca\(^{2+}\)-dependent activatory ranges of the intramitochondrial PDH and OGDH

Rat heart (●, ○) or liver (■, □) mitochondria were incubated (at 2–4 mg of protein/ml) at 30 °C in KCl-based medium (see the Materials and methods section) for experiments of the type shown in Figs. 1–3 and in either the absence (●, ■) or the presence (○, □) of 0.5 mM-spermine, and with various concentrations of CaCl\(_2\) (0–60 μM) or EGTA so that the full ranges of the Ca\(^{2+}\)-dependent effects on PDH or OGDH were observed, and then total Ca contents were measured as described previously (Assimacopoulos-Jeannet et al., 1986). The data from each enzyme were very similar, and have therefore been combined for clarity.

from mammalian tissues. The reasons underlying the negative report on heart by Kroner (1988) remain unknown, except perhaps that albumin was exclusively present in these heart incubations.

The data in the present report clearly indicate that spermine is a potent activator of mitochondrial Ca\(^{2+}\) uptake under conditions where the Ca\(^{2+}\)-sensitive properties of the matrix dehydrogenases are exhibited; i.e. this property is evident when mitochondria are incubated under conditions where they do not buffer, but rather respond to, extramitochondrial Ca\(^{2+}\), as well as when they are in their buffering mode. Therefore spermine leads to a decrease in the \(K_{m}\) values for extramitochondrial Ca\(^{2+}\) in the activation of the enzymes in coupled mitochondria under all conditions tested (Table 1). The effective extramitochondrial Ca\(^{2+}\) ranges, which run from approx. 5-fold lower to about 5-fold higher than the \(K_{m}\) values, are correspondingly decreased. In the most physiological circumstance, i.e. with both Na\(^{+}\) and Mg\(^{2+}\), the effective range for each type of mitochondria with spermine was from about 30–50 nM up to about 800–1200 nM, with \(K_{m}\) values of the order of 250–400 nM. This is still well within the expected cytosolic range for Ca\(^{2+}\) (e.g. Carafoli, 1987) and therefore, together with the other supporting evidence (see the Introduction), it is still concluded that the primary function of the Ca\(^{2+}\)-transport system is to relay hormonally induced changes in cytoplasmic Ca\(^{2+}\) into the matrix for regulation of the dehydrogenases and hence oxidative metabolism. The previous studies by Nicchitta & Williamson (1984) and Lenzen et al. (1986) did not test this circumstance (i.e. plus Na\(^{+}\) and Mg\(^{2+}\)), which is therefore most likely to be the explanation for the rather low values of buffering set-point which they report with spermine (0.2–0.3 μM). In the present study, there was a slight indication that spermine may be less effective in the presence of Na\(^{+}\) and Mg\(^{2+}\) (Table 1). As spermine did not appear to affect the total Ca content of the mitochondria over the activatory ranges of the enzymes (Fig. 8), this suggests that spermine does not alter the fact that the two functions of the Ca\(^{2+}\)-transport system are still most likely to be mutually exclusive (see the Introduction), and it is the matrix Ca\(^{2+}\)-regulatory role which is likely to be the more physiologically relevant. It is also worth noting that the values for total Ca content over the enzymes' activatory range, as opposed to the higher values required for buffering, are much closer to estimates made in situ by using X-ray probe microanalysis (e.g. Somlyo et al., 1985; Wendt-Gallitelli, 1986; LeFurgey et al., 1986). These data are thus in some ways contrary to the conclusion of Nicchitta & Williamson (1984) that the effects of spermine on Ca\(^{2+}\) egress would allow mitochondria to buffer extramitochondrial Ca\(^{2+}\) at lower Ca content; however, it is unclear how a process which depends on egress saturation would be affected by a change in \(K_{m}\).

The concentrations of spermine used in the present and the previous (see above) studies are within the range expected for cellular contents of the polyamine (Pegg, 1986). However, there is no information as to the compartmentation or binding of spermine inside cells, so the actual concentration in the vicinity of the mitochondrial inner membrane is unknown. Therefore remains the intriguing possibility that changes in the cytosolic concentration of spermine may produce significant changes in the relationship between extra- and intra-mitochondrial Ca\(^{2+}\), and that this may contribute to the stimulation of oxidative metabolism.
observed in stimulated cells. Relevant to this are reports that the \( \beta \)-adrenergic stimulation of rat kidney (Koenig et al., 1983) and heart (Fan & Koenig, 1988) involves an increase in ornithine decarboxylase activity and a rise in cellular polyamine content.

The interpretation of the existing data on the effects of spermine on Ca\(^{2+} \) uptake by mitochondria would suggest that it has a direct effect on the Ca\(^{2+} \) uniporter. This suggests that it has an allosteric site on the uniporter (Kroner, 1988) rather than, for example, bringing about its effects through the shielding of other Ca\(^{2+} \)-binding sites on the surface of the inner membrane; the results reported here with uncoupler support this. Nor is it likely that the reported effects on Ca\(^{2+} \) uptake are due to the general protective effects of spermine on mitochondria, as spermidine appears to behave similarly in this (e.g. Tabor, 1960; Chaffee et al., 1977), and also because the effect of spermine is on the apparent \( K_m \) for Ca\(^{2+} \) (Nicchitta & Williamson, 1984).

Nicchitta & Williamson (1984) suggested that spermine effects on Ca\(^{2+} \) uptake were independent of the effects of Mg\(^{2+} \) as an inhibitor of uptake; however, Lenzen et al. (1986) reported evidence for interaction of Mg\(^{2+} \) and spermine. In the present work spermine did not affect the \( K_m \) for Mg\(^{2+} \) in its inhibition of Ca\(^{2+} \) uptake, nor did Mg\(^{2+} \) affect spermine \( K_{0.5} \) values (results not shown in full; Table 2). This is in better agreement with Nicchitta & Williamson (1984); the latter, it is suggested, were rather perhaps examining effects of Mg\(^{2+} \) on uptake itself, rather than any inhibition of spermine effects.

The differences between the present work and Nicchitta & Williamson (1984) with regard to Ca\(^{2+} \) egress is perhaps because in the latter only Na\(^{-} \)-independent egress was monitored, and also that there was some extramitochondrial Ca\(^{2+} \) present, which has been shown by Hayat & Crompton (1982) to be an inhibitor of Na\(^{-} \)-dependent Ca\(^{2+} \) egress. There thus remains the possibility that spermine may interact with this Ca\(^{2+} \)-regulated site on the Na\(^{+}/Ca\(^{2+} \) exchanger to bring about the effects noted by Nicchitta & Williamson (1984). Another obvious difference is the large differences in Ca loads of the mitochondria in the two studies (see above).

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