ISOLATION OF A DIVALENT CATION IONOPHORE FROM BEEF HEART MITOCHONDRIA

George A. Blondin[1]

Institute for Enzyme Research
University of Wisconsin
Madison, Wisconsin 53706

Received November 7, 1973

SUMMARY: A divalent cation ionophore has been isolated from a partial tryptic digest of mercurial treated-lipid depleted mitochondria by extraction with butanol:acetic acid:water (4:1:5). The partially purified ionophore interacts with both calcium and magnesium ions and facilitates their transfer across the mitochondrial inner membrane or across a carbon tetrachloride phase separating two discontinuous aqueous compartments. The mitochondrial divalent cation ionophore shares many of the properties of previously reported divalent cation ionophores of microbiological origin, e.g., X537A and A23187.

The idea of natural ionophores in animal membrane systems was first given experimental support in 1971 with the reported (1) isolation of a Na^+/K^+ ionophore from beef heart mitochondria by extraction with organic solvents. Since that time, progress in this area has been painfully slow due, in part, to the vanishingly small yields of ionophoretically active fractions and to the lack of reproducibility which characterized such studies. However, we have recently found and reported (2,3) the totally unexpected observation that the mitochondrial Na^+/K^+ ionophore, unlike ionophores of microbiological origin, is largely unavailable to simple organic solvent extraction, but is readily extractable with organic solvent systems after membrane perturbation with trypsin, organic mercurials, or ionic detergents. The emerging notion that natural ionophores represented a departure from the classically monodispersed nature of analogous microbiologically derived ionophores has correspondingly altered our experimental approach to this area of inquiry in the direction of empirically determining the most effective methods of releasing natural ionophores. The present communication is an account of our efforts along these lines which have led to an efficient and highly reproducible

[1]Established Investigator of the American Heart Association, Inc.

procedure for the release of a mitochondrial divalent cation ionophore (DCI)[2].

MATERIALS AND METHODS

Freshly prepared HBHM (10 gms) are suspended in 200 ml of 20 mM Tris-Cl (pH 7.4) and incubated for 15 minutes at 25° in the presence of 0.48 mM potassium FMA. After removal of lipid with acetone:ammonia according to the procedure of Fleischer *et al.* (4), the dried (acetone powder) protein residue is added with vigorous stirring to 195 ml of a solution at 38° containing 4.0 mM dithiothreitol, 25 mM Tris-Cl (pH 8.3), and butylated hydroxy toluene (25 μgm/ml) Trypsin (100 mg/gm protein) is then added and digestion is allowed to proceed for one hour at 38° while the system is backtitrated to pH 7.8 with 1N KOH at 15 and 60 minutes. Calcium chloride (200 μmoles) and additional trypsin (50 mg/gm protein) are added after one hour and the digestion continued for 1.0 additional hour. The reaction mixture is then rapidly cooled to 20° and the total volume is estimated. Glacial acetic acid (0.2 volumes) and secondary butanol (0.8 volumes) are then added while stirring vigorously. Extraction with butanol:acetic acid:water is carried out for one hour at room temperature and the resulting suspension is centrifuged for 30 minutes at 2400 rpm in the 284 head of an International centrifuge. The supernatant (S_1) is decanted, followed by the addition to it of 0.625 volumes S_1 of chloroform and stirring for 10 minutes. An additional 0.625 volumes S_1, each of chloroform and distilled water, are then added and the resulting biphasic mixture is allowed to mix well for 10 minutes after which it is immediately poured into a separatory funnel. After 10-20 minutes, the phase separation is complete and the lower chloroform-butanol phase is withdrawn and evaporated to dryness at 40° under reduced pressure (water vaccuum) in the presence of 50 ml to toluene. The residue is then washed according to the procedure of Bligh and Dyer (5) as follows: after solution in 200 ml methanol, 100 ml chloroform, and 80 ml

[2]Abbreviations: DCI, divalent cation ionophore; HBHM, heavy beef heart mitochondria; FMA, fluorescein mercuric acetate.

distilled water, chloroform (100 ml) is then added with constant stirring followed by 100 ml 5% K_2CO_3. After mixing well for 10 minutes, the mixture is poured immediately into a separatory funnel and the resulting lower chloroform phase (LP-2) is withdrawn, cleared with absolute ethanol (circa 20-30 ml), and evaporated to dryness. The LP-2 residue is either fractionated directly (see Figure 1) or stored frozen in 10-15 ml of 30% ethanol in benzene.

Properties: All mitochondrial swelling studies were carried out essentially as described in the legend of Figure 1 under assay. Measurement of DCI induced calcium and magnesium efflux from mitochondria was performed as described by Reed and Lardy (6) on pellets obtained after centrifugation of mitochondrial suspensions for 1 minute at 20,000 rpm in a Misco Table centrifuge. A Perkin Elmer Model 303 Atomic Absorption spectrophotometer was used to determine the residual concentration of calcium and magnesium after resuspension of the harvested mitochondrial pellets in 0.2 M HNO_3. Measurement of the bulk transport of calcium and magnesium was performed as described by Pressman (7).

RESULTS:

Sec-butanol:acetic acid:water extraction of FMA treated lipid-free mitochondria which have been submitted to partial tryptic digestion consistently yields 53 ± 2 mg/gm protein of chloroform soluble residue. When this residue is submitted to chromatographic fractionation on silicic acid/alumina, the elution profile shown in Figure 1 is characteristically obtained. The two principle features are (1) the emergence of a very sharp band (fraction 18 and 19) of UV absorbing material (280 nm) on the tailing end of a much broader band (fractions 14-18) between 125 and 175 ml into the 19:1 -$CHCl_3$:MeOH elution schedule, and (2) the emergence of magnesium ionophoretic activity coincident with the fraction 18-19 band as shown in Figure 1 by the dashed line. The mass yield of this band usually falls between 2.0 and 4.0 mg per gm mitochondrial protein depending on the extent of its overlap with the fraction 14-18 band. Recycling of pooled ionophoretically active material yields approximately 0.5-0.6 mg of UV absorbing material (280 nm) per gm mitochondrial

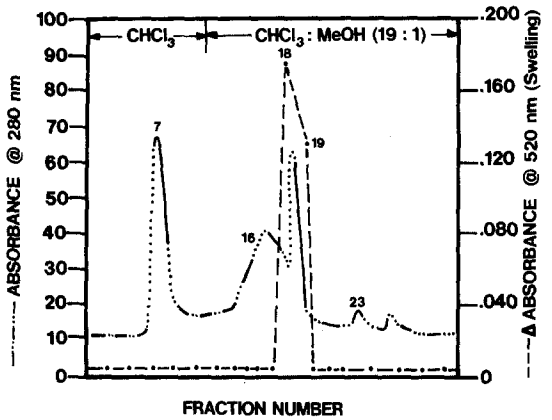

Figure 1. Chromatography of butanol:acetic acid:H_2O isolate on silicic acid: alumina for the preparation of partially purified DCI. The residue from LP-2 (not more than 500 mg) is charged onto a column (3.0 x 29 cm) containing 40 grams dried (120° for 18 hours) silicic acid in chloroform layered over 40 gms neutral alumina II with a 2 mm layer of sea sand interposed. The residue is charged in 10 ml chloroform. The elution sequence is (a) 150 ml of $CHCl_3$, and (b) 300 ml of $CHCl_3$:MeOH - 19:1. Fractions of approximately 15 ml each are collected at a flow rate of approximately 1 ml per minute and the effluent is continuously monitored at 280 nm by means of an LKB-Uvicord II. Pooled active fractions from several experiments were recycled through the same type column in order to obtain sufficient material for the study of properties as outlined in the text.

Assay: Chromatographic fractions were evaporated to dryness at 40° under a stream of nitrogen, each of the residues dissolved in 1.0 ml absolute ethanol, and 20 µl aliquots of the resulting solutions were assayed under the following conditions: freshly prepared HBHM (0.1 ml of a suspension in 0.25 M sucrose, 10 mM Tris-Cl (pH 7.4) containing 6 mg of mitochondrial protein per ml) were added to 2.9 ml of incubation medium which was 0.1 M in $Mg(NO_3)_2$ and 10 mM in Tris-NO_3 (pH 7.4). Antimycin and rotenone were present at a concentration of 0.33 µgms/ml. After the mixture was incubated for 1 minute, the test fractions were added and the decrease in absorbance (swelling) at 520 nm was determined at 25° in a Beckman DU spectrophotometer. Readings were taken at time 0 and 5 minutes after the addition of fraction aliquots and the decrease in absorbance over this time is plotted for each fraction.

protein for an enrichment factor of approximately 2000. The latter, which we refer to as partially purified DCI is that which has been used for the study of properties as outlined below.

The non-energized DCI induced mass transfer of $Mg(NO_3)_2$ across the inner membrane and into the matrix space of isolated mitochondria was determined by measurement of associated mitochondrial swelling as shown in Figure 2A. The

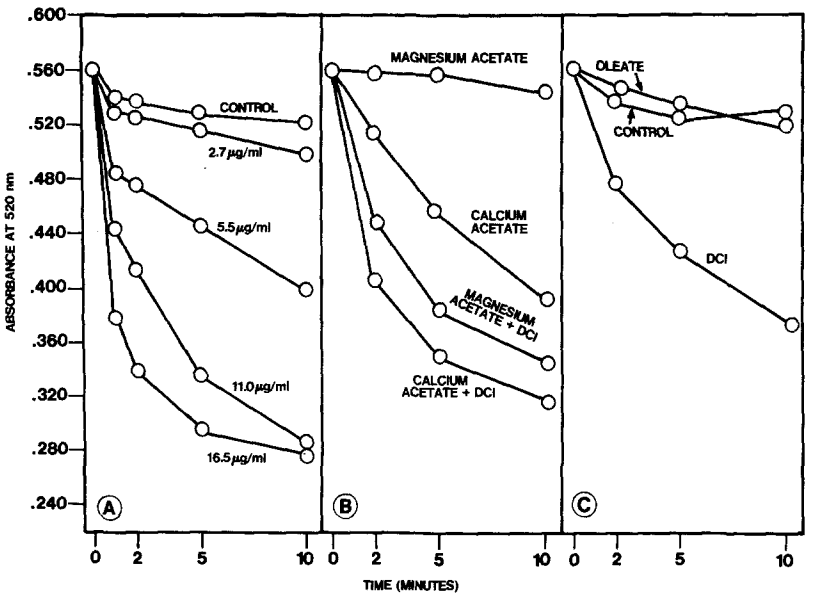

Figure 2. The effects of DCI on swelling of isolated mitochondria suspended in decimolar divalent cationic solutions. All incubations were carried out essentially as described under assay in the legend to Figure 1. The media were (2A) 0.1 M $Mg(NO_3)_2$, 10 mM Tris-NO_3 (pH 7.4), (2B) 0.1 m Mg^{++} or Ca^{++} acetate, 10 mM Tris acetate (pH 7.4), and DCI at a level of 8.5 µg/ml, and (2C) 0.1 M Mg $(NO_3)_2$, 10 mM Tris-NO_3 (pH 7.4), 0.3% albumin, and DCI or oleate both at a level of 8.5 µg/ml. All incubations contained in addition antimycin and rotenone at a concentration of 0.33 µgm/ml.

rate of $Mg(NO_3)_2$ transport is seen to be proportional to the concentration of DCI between 2.7 and 16.5 µg DCI per ml incubation medium. The data of Figure 2B represent an attempt to demonstrate the DCI induced transport of divalent cation in both calcium and magnesium media. Although the background rate is considerably faster in the presence of calcium acetate over that of the magnesium acetate, nevertheless, it is clear that DCI is capable of inducing an increase in the rate of transport of both calcium and magnesium acetate. The data of Figure 2C are included in order to allay suspicion of the involvement of free fatty acids as the active component of DCI. All three of the experiments shown have been performed in the presence of albumin. Whereas oleate, at a concentration of 8.5 µgms/ml is without effect on the transport of $Mg(NO_3)_2$

in the presence of albumin, the enhanced rate in the presence of the same concentration of DCI appears to be unaffected by the presence of albumin.

Since DCI appeared to function in both calcium and magnesium media, its effect on endogenous mitochondrial ions was examined. Figure 3A shows that

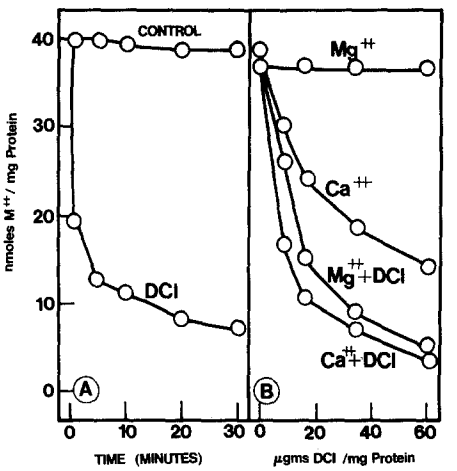

Figure 3. DCI induced release of calcium and magnesium ions from isolated mitochondria. 3A describes the time dependent release of Mg^{++} from mitochondria suspended in 0.25 M sucrose, 15 mM Tris Cl pH 7.4, 0.5 mM Tris EDTA, HBHM at 1.44 mg protein per ml, and antimycin and rotenone at 2.0 µg per mg protein. 3B describes the DCI concentration dependence of both calcium and magnesium efflux in identical media to 3A. Measurements were made at 20 min.

mitochondria retain all their Mg^{++} ions during a control incubation in sucrose-EDTA whereas addition of DCI to a level of 25 µgms/mg protein produced a time dependent loss of Mg^{++} ions which was complete at this level in 20 minutes. Figure 3B shows the concentration dependence of DCI relative to its ability to induce or enhance the loss of both endogenous Ca^{++} ions and Mg^{++} ions from mitochondria suspended in sucrose-EDTA.

The data of Table I show the DCI catalyzed transfer of both calcium and magnesium from one aqueous phase, through a CCl_4 phase, and into a receiving aqeuous phase under conditions supporting cation-proton exchange. The calcium

TABLE I. Ionophore mediated transport of calcium and magnesium ions across a bulk phase of carbon tetrachloride.

Time (Hours)	nmoles Cation Transported	
	Calcium	Magnesium
0.0	0	0
1.0	389	90
2.0	898	741
4.0	1537	1161
6.0	2267	1938

The procedure employs a glass cylinder with a glass partition vertically sealed across the top 2/3 of the cylinder as diagramed in Reference 7. The bottom half of the vessel is filled with a heavier than water organic solvent (6.0 ml CCl_4) containing DCI (1 mg/ml), thereby creating two discontinuous compartments above the organic level, which are then filled, one (donor) with 2.0 ml aqueous buffer (25 mM Tricine-tetramethyl ammonium hydroxide, pH 8.5) containing the test cation (10 mM $CaCl_2$ or $MgCl_2$) and the other (receptor) with 2.0 ml buffer alone (50 mM citrate-tetramethyl ammonium hydroxide, pH 5.4) for the measurement of the appearance of the test cation. The result of this arrangement is that two separately exposed aqueous phases communicate with one another only via an organic phase. Aliquots of 50 µl were withdrawn from the aqueous receptor compartment at times indicated in the Table and assayed for the appearance of calcium or magnesium by atomic absorption spectrophotometry.

versus magnesium rates appear to be very similar and in both experimental systems, omission of DCI in the CCl_4 phase produced no detectable transfer.

DISCUSSION:

It is evident from the present results that DCI satisfies four major criteria by which classical ionophores may be clearly distinguished from all other natural products.

(1) DCI enhances the rate of transfer of divalent cations (calcium and magnesium) across a biological membrane as measured by its effect on both influx (Figure 2A and 2B) and efflux (Figure 3A and 3B) of divalent cations in isolated mitochondria. The similar pattern of induction of mitochondrial calcium and magnesium efflux by the classical microbiologically derived divalent cation

ionophore A23187 has already been fully documented by Reed and Lardy (6). While effects similar to those reported here of A23187 on calcium and magnesium influx into respiratory inhibited mitochondria have not yet appeared in the literature, the antibiotic has been shown to produce uptake of calcium in erythrocytes and spermatozoa (8).

(2) DCI is a lipophilic substance as evidenced by the entire range of its experimental manipulation. Both of the known classical divalent cation ionophores (A23187 and X537A) are characteristically lipophilic (6-8).

(3) The ability of DCI to form lipid soluble divalent cation (calcium and magnesium) complexes is implicit in the data of Table I. The formation of lipid soluble complexes of calcium and magnesium with both A23187 and X537A has been fully documented by equilibrium extraction methods (6-7).

(4) While the ability to form a lipid soluble cation complex is a necessary condition for a molecule to act as an ion carrier, both complexation and decomplexation must have favorable kinetic properties for a complexing agent to serve as an efficient ion carrier. The data of Table I support the notion that DCI possesses such properties versus both Ca^{++} and Mg^{++} ions as has been previously documented in a similar fashion for the ionophore X537A (7).

ACKNOWLEDGEMENTS:

I gratefully acknowledge the fine technical assistance of Mrs. Poonsin Olson, Ms. Carol Sakowski, and Mrs. Wanda Fechner. I am especially indebted to Dr. David E. Green for his advice and unstinting interest throughout the course of this work. This investigation was supported in part by Program Project Grant No. GM-12847 of The National Institute of General Medical Science and by a generous contribution from The Merck, Sharp and Dohme Research Laboratories.

REFERENCES

1. Blondin, G. A., A. F. DeCastro, and A. E. Senior, Biochem. Biophys. Res. Commun., 43, 28 (1971).
2. Blondin, G. A., Ann. N.Y. Acad. Sci., in press (1973).

3. Southard, J. H., G. A. Blondin, and D. E. Green, J. Biol. Chem., in press (1974).
4. Fleischer, S. B., B. Fleischer, and W. Stoeckenius, J. Cell Biol., 32, 193 (1967).
5. Bligh, E. G., and W. J. Dyer, Can. J. Biochem. Physiol., 37, 911 (1959).
6. Reed, P. W., and H. Lardy, J. Biol. Chem., 247, 6970 (1972).
7. Pressman, B. C., Fed. Proc., 32, 1698 (1973).
8. Reed, P. W., and H. Lardy, in The Role of Membranes in Metabolic Regulation, M. A. Mehlman and R. W. Hanson, editors, pp. 111-131, Academic Press, N.Y. (1972).