THE APPLICABILITY OF THE DONNAN RELATION TO THE DISTRIBUTION OF CERTAIN ANIONS BETWEEN MITOCHONDRIA AND MEDIUM

E.J.HARRIS and Celia BERENT
Department of Biophysics, University College, London, WC 1E 6BT, England

Received 27 July 1970

1. Introduction

When two compartments are separated by a membrane, the steady state distribution of permeating ions not undergoing energised transport reflects the electrical potential difference between them. The ratio between the activities on the two sides (subscripts 1 and 2) depends on the charge the ion bears and the Gibbs-Donnan relations subsist

$$(A_1^-/A_2^-) = (B_1^{2-}/B_2^{2-})^{1/2} = (C_1^{3-}/C_2^{3-})^{1/3}$$

between anions A^-, B^{2-} and C^{3-}. The potential difference can arise from the dissociation of a molecule having one non-permeating ion as in the Donnan system, but the relation applies as well to potentials generated by the expenditure of metabolic energy. Although specific examples of the applicability of Donnan-like relations to certain ions in their distribution across cell membranes have long been known, the extension of the principle to small membrane-bounded systems has not attracted much attention. It was pointed out briefly that the distributions of some permeating anions across the mitochondrial membrane when they are present together, accord with the Donnan equations [1]. The present communication provides evidence that a number of anions become distributed in a way approximating to Donnan equations over a wide range of concentrations.

The importance of showing that the Donnan equations apply to certain anions is that one can then predict mitochondrial/cytoplasmic ratios of other penetrant anions from knowledge of the distribution of one of them.

The generality of the anion distribution relation does not depend on any particular theory about the origin of the electrical asymmetry. It has been argued [2] that even if anion accumulation occurs by reason of anion-hydroxyl exchange, the minimum potential energy is still met when the various anions follow the Donnan-like equations.

2. Methods

Most results were obtained using rat liver mitochondria prepared by Schneider's method [3]. The 250 mM sucrose used for homogenisation and washing was supplemented with 0.5 mM EGTA and 0.05% BSA. Incubations were made at about 4 mg mitochondrial protein/ml in a medium having 120 mM KCl, 20 mM $NaHCO_3$, mannitol 60 mM. A stream of 95% O_2 with 5% CO_2 was directed onto the top of the suspension during incubation at 20°. Samples (0.2 ml) were taken at intervals. The mitochondria were separated by centrifugation through silicone as described in detail before [4]. Aliquots of the suspension medium recovered from above the silicone, and of the acid extract from beneath the silicone were used for enzymatic assay for selected compounds by fluorimetric methods [5].

Supplementary experiments were made to find the simultaneous distributions of a number of different anions using, in turn, one of them with a ^{14}C label along with other unlabelled anions. This method is more convenient for succinate and acetate.

Routinely, a trace of tritiated water was used to

North-Holland Publishing Company – Amsterdam

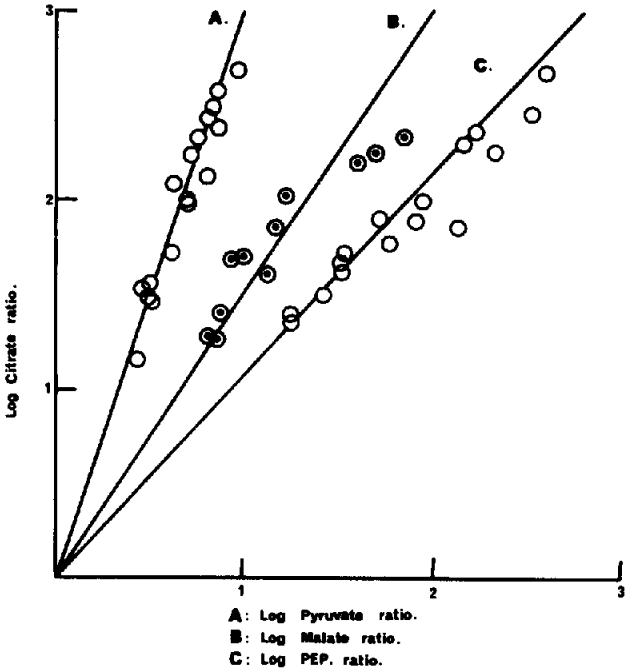

Fig. 1(A) The relation between the logarithms of the respective ratios of the mitochondrial contents/concentrations in the medium of citrate and pyruvate. Specimen values of the individual figures will be found in table 1A. (B) The same for citrate against malate, refer to table 1B. (C) The same for citrate against phosphoenolpyruvate, refer to table 1C. The lines drawn have slopes of 3/1, 3/2, 3/2.8.

find the total water carried down by the mitochondrial protein, and in addition frequent measurements of the volume of sucrose-accessible water were made. The latter were used to correct the total quantities found in the protein extract for the solute carried down with the particles.

Inorganic phosphate distributions were measured using a trace of ^{32}P-labelled phosphate along with added carrier. Aliquots of the suspension medium and of the acid extracts of the mitochondria were thoroughly mixed with 2 ml of 1% ammonium molybdate–3% sulphuric acid solution and 2 ml of isopropyl acetate. An aliquot of the isopropyl acetate was used for counting.

3. Results

3.1. *Metabolite assays*

The object of making assays of samples in various conditions of metabolism was to obtain values for the inside/outside ratios for pairs of anions. Results from 4 assays, 2 of medium and 2 of the mitochondrial extract, furnish two ratios which are plotted against each other on the final graphs. In order to detail typical conditions, specimen results of assays have been collected in table 1A, B and C for 3 pairs of anions. The inter-anion relations are best tested by plotting the logarithms of the inside:outside ratios of one compound against another. The slope of the lines so obtained measures the ratio of the respective net charges n_1 and n_2 on the ions.

Values of ratios between pyruvate, malate and phosphoenolpyruvate are plotted in fig. 1, in each case related to the citrate ratio. Most, but not all, of the relevant separate contents and concentrations are given in table 1, parts A to C. With pyruvate it was not found possible to obtain reliable assays at low concentrations, so values in the latter case were obtained using ^{14}C-labelled pyruvate. Whether there is a certain amount of bound or occluded pyruvate is not certain but the assays indicated high ratios between endogenous pyruvate and its external concentration.

3.2. *Results of radioactivity distribution method*

When a number of anionic compounds are applied together the relative ratios to which each one is accumulated could be estimated by a multiple incubation with one in turn of the mixture labelled with radioactivity. A separate but chemically identical incubation included ^{14}C-sucrose to measure the sucrose accessible water carried down with the protein.

Sets of the ratios of the quantity of substrate (or other anion) accumulated, duly corrected for the amount carried down in the sucrose space are shown in table 2. Rotenone was present to stop oxidation of NAD-coupled substrates, but succinate is oxidised as far as malate. Since experimentally it was found that the ratios holding for these two substances were closely similar, it follows that the radioactivity distribution whether measuring ^{14}C-succinate, or a mixture of this and the ^{14}C-malate formed from it, will remain the same. To verify the point, however, one ex-

Table 1
Mitochondrial anion contents and the respective anion concentrations in the medium under various conditions.

			Citrate-pyruvate relation (fig. 1, curve A) (citrate produced metabolically)			
A	Additions to medium (fluorocitrate present at 17 μM)		Citrate		Pyruvate	
			Content (μmole/g)	Concn. (μM)	Content (mole/g)	Concn. (μM)
	Pyruvate, ATP (0.8 mM)		4.85	50	13.3	2100
	Same, after 12 nmoles each Ca and Mn per mg protein		7.30	55	12.6	1960
	Pyruvate, malate (200 μM)		5.8	19	14.4	2100
	Same, after total of 25 nmoles Ca and Mn per mg protein		15.4	73	11.2	1980
	Pyruvate, Mg (1.2 mM)		2.1	9	17.8	2400
	Pyruvate		5.6	33	9.3	1770
	Same with ADP		3.1	87	5.1	1600
	Pyruvate, ATP (300 μM)		6.2	64	3.7	740
	Pyruvate, phosphate (1 mM), Mg (0.67 mM), Mn (66 μM)		5.0	42	7.0	1000
	Pyruvate, ATP (50 μM)		9.3	25	5.7	780
	Same, after 11 nmoles each Ca and Mn per mg protein		16.0	35	7.0	750
			Using ^{14}C citrate with ordinary pyruvate, or ^{14}C pyruvate with ordinary citrate			
	Pyruvate, arsenite (0.5 mM)		4.8	168	0.21	65
	Citrate, rotenone (0.25 μg/mg) protein		2.45	173	0.40	145
	Pyruvate, citrate, succinate (1 mM)		10.8	174	0.54	130
	Rotenone (0.25 μg/mg protein)		15.2	161	1.60	305
			Citrate-malate relation (fig. 1, curve B)			
B			Citrate		Malate	
	Pyruvate (2 mM), Mg (1.3 mM)	at 1 min	1.45	8.5	0.43	8.5
		at 20 min	3.5	90	2.2	155
	Same	at 1 min	3.1	15	0.9	12.5
		at 15 min	4.7	45	1.46	88
	Phosphate (0.8 mM), Mg (0.5 mM)	at 1 min	4.3	62	19.4	1320
		at 15 min	9.0	346	7.5	1000
	Malate, oxoglutarate (2.2 mM) phosphate (0.8 mM), Mg (0.5 mM)		3.94	210	8.35	1270
	Malate, valinomycin (2 μg/g protein), phosphate (0.8 mM), Mg (0.5 mM)		3.45	70	10.3	1200
	Malate, $MnCl_2$ (120 μM) phosphate (0.8 mM) $MgCl_2$ (0.5 mM)		4.0	83	12.6	1270

Table 1 (continued)

C	Additions to medium	Citrate-phosphoenolpyruvate relation (fig. 1, curve C)			
		Citrate		Phosphoenolpyruvate	
		Content (nmole/mg)	Concn. (μM)	Content (nmole/mg)	Concn. (μM)
	Oxoglutarate (0.87 mM), fluorocitrate (17 μM)	1.92	84	0.26	15
	plus 30 nmole/mg Ca and Mn	3.65	76	0.68	16
	Oxoglutarate (0.91 mM), oleate (125 μM), oligomycin (1 μg/mg)	3.48	66	2.18	62
	Oxoglutarate (0.91 mM), ATP (1 mM)	2.64	45	0.50	8
	plus Ca (15 nmole/mg)	6.2	34	0.90	7
	Oxoglutarate (1.45 mM), malonate (5 mM)	0.8	10	1.0	12
	plus ADP	0.6	8	2.2	15
	Malate (3.5 mM), Mg (0.5 mM), phosphate (3.mM), fluorocitrate (25 μM)	2.2	11	1.2	8
	plus ADP, hexokinase, glucose	1.8	18	1.2	13
	Malate (2 mM), phosphate (2 mM), Mg (0.5 mM), Mn (66 μM)plus ADP, hexokinase, glucose	4.2	23	2.4	11
		4.7	16	2.15	6
	Malate (1.3 mM), phosphate (2 mM), Mg (0.6 mM)	4.3	56	1.3	19

These and other unlisted ratios of the contents to the concentrations have been used in plotting figs. 1 and 2. The contents noted have been corrected for the quantities of anion carried down in the sucrose accessible space.

periment was carried out in presence of antimycin to stop succinate oxidation.

The first two experiments in table 2 show concordant ratios, that is, equal $1/n$th roots for citrate, malate and pyruvate when these anions are present together. Experiments 2 and 3 include results showing the near equality of succinate and malate ratios. Experiment 5 shows concordance between succinate, malonate and pyruvate ratios; in this set of results, as in experiment 7, the citrate ratio is appreciably higher than the $1/n$th root requires. Experiment 6 shows concordant citrate and succinate ratios. Turning now to other anions included in these experiments it can be seen that acetate (experiments 4 and 6) is accumulated to a higher ratio than expected by com-

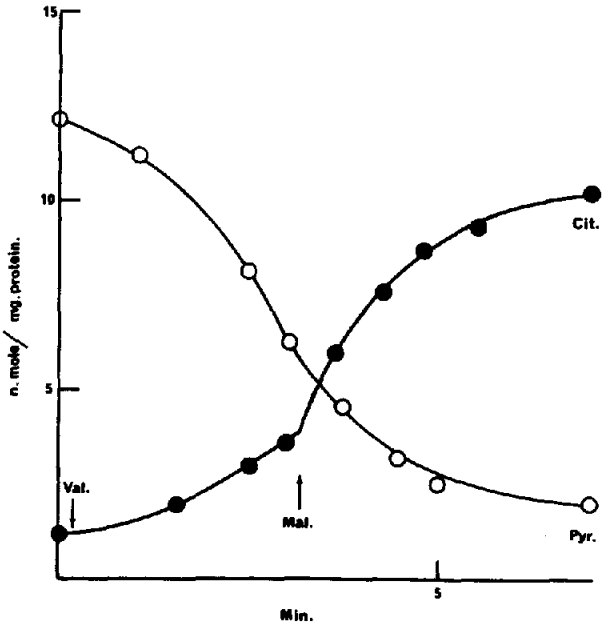

Fig. 2. Illustration the displacement of internal pyruvate by citrate generated in a valinomycin-stimulated system. At 3 min an addition of malate (to 2 mM) was made, which enhanced citrate accumulation and pyruvate expulsion. The medium contained initially tris pyruvate 2 mM, KCl 120 mM, tris chloride pH 7.4 20 mM, protein 7.3 mg/ml and valinomycin 50 ng/mg protein.

Table 2
Distribution ratios for anions between mitochondria and medium determined when several are present together.

	Anions measured and concentrations used (μM)									
	Citrate	Succinate	Malate	Malonate	*Oxoglutarate*	*Acetate*	Glutamate	Pyruvate	dl-Hydroxybutyrate	Phosphate
	$n = 3$	$n = 2$	$n = 2$	$n = 2$	$n = 2$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	pH 7.4
No. 1	40		200					500		
Ratio	263		27					7.8		
1/nth root	6.4		5.2					7.8		
No. 2	200	150	150					500		
Ratio	19.7	5.7	5.7					2.45		
1/nth root	2.7	2.4	2.4					2.45		
No. 3	60	120	180				760			
Ratio	27.6	6.9	5.0				2.7			
1/nth root	3.0	2.6	2.2				2.7			
No. 4	100	120			150	6000				
Ratio	26.5	10.3			3.5	5.5				
1/nth root	3.0	3.2			1.9	5.5				
No. 5	70	130		90	90			260		
Ratio	106	8.25		8.2	3.55			3.1		
1/nth root	4.7	2.8		2.8	1.9			3.1		
No. 6	40	130			200	375				
Ratio	134	27.8			7.6	11.4				
1/nth root	5.1	5.2			2.7	11.4				
No. 7	40	100			320		140	280		60
Ratio	414	35.8			15.1		5.8	9.1		8.9
1/nth root	7.4	6.0			3.9		5.8	9.1		
No. 8	14	30							1000	400
Ratio	20.3	5.35							3.35	5.7
1/nth root	2.7	2.3							3.35	

To test the Donnan-like behaviour, the 1/nth roots of the ratios are compared, where n is the presumed net charge. The ratios are of the mitochondrial content (μmole/g protein) corrected for the amount of solute carried down in the sucrose accessible space divided by the external concentration in mM. In experiments 1–7, rotenone was present at 0.15 μg/mg. and in No. 8, antimycin was present at 0.26 μg/mg protein. Anions which do *not* appear to fit in the scheme are shown in *italics*.

parison with the anions previously mentioned, while oxoglutarate (experiments 4–7) attains a ratio nearer to that holding for singly charged acids than for a doubly charged acid. Glutamate (experiments 3 and 7) behaves, as might be expected, as a singly charged anion. It is likely that part of the oxoglutarate accumulated is converted to glutamate.

3.3. *Displacement of one anion by another*

Sharing by the mitochondrial anions of a limited capacity, set by the mitochondrial cation content, implies that increasing the content of one anion will tend to cause a displacement of another. Citrate, by reason of its treble charge, is particularly effective, because it is more accumulated than, say, pyruvate. When citrate is being produced metabolically from pyruvate the latter is displaced. Fig. 2 illustrates this point. Metabolism is stimulated with valinomycin and carboxylation of pyruvate to oxalacetate proceeds so that citrate is formed up to the third minute while pyruvate is expelled. At the third minute, malate is added, which provides a greater concentra-

tion of oxalacetate, more citrate is formed and pyruvate expulsion occurs to an extent that evidently limits further reaction.

Up to the time malate was added, the ratio of pyruvate is close to the cube root of the ratio for citrate. After malate addition, it appears that the generation of citrate is so fast that the pyruvate is driven down to a lower content than corresponds to the Donnan relation.

In a preliminary experiment with rat heart mitochondria it was found that succinate was accumulated to a factor of 11 times. The uptake of citrate (in 5 min) only corresponded to a ratio of 7 times; this is in accordance with the low permeability of heart mitochondria to citrate [6].

4. Discussion

These results provide a reason to consider that the mitochondrial interior behaves like a Donnan system in respect of the anions citrate, malate, succinate, malonate, pyruvate, glutamate and phosphoenolpyruvate. The last anion has an effective negative charge of about 2.8 at pH 7.4. If the interior were held positive by an electrogenic action of inward energised cation transport, the distributions of these anions, as well as of H^+, which is less concentrated in the interior [7, 8] would be accounted for. However the fact of the simple relations between these anions does not rest on the explanation of the phenomenon.

It is not clear why the relative accumulation of oxoglutarate is less than that of the other anions, nor why that of acetate should be greater. It has however been observed that acetate vastly exceeds the other substrate anions in its ability to accompany K^+ into the matrix under the influence of valinomycin, e.g., K^+ uptakes of 300 μmole/g protein with acetate occur, compared with only 60 with citrate (see table 3 in Harris [9]).

Two considerations stem from the Donnan-like distributions; the first is that the inside:outside ratios will depend on the quantity of the most highly charged anion (citrate) in the system, because it is most accumulated, and the second is that in seeking to determine the distributions of metabolites between mitochondria and cytosol it would appear that the respective ratios of a series of anions can be deduced if the ratio of one of the series is known. There is always a proviso that rapid metabolism must not be in progress because rapid consumption or generation of a metabolite will displace the existing ratio from the steady state value.

The important anion, phosphate, in the preliminary experiments appears to be distributed in both the singly and doubly charged forms in ratios lower than hold for the Donnan-like system. This result is given with the reservation that the calculation depends on assuming an internal pH higher by 0.6 units than outside to be consistent with other data [7, 8] which give the difference to be between 0.5 and 0.7 units.

Turning to the application of the relations we have established in determining the distributions between the cytosol and mitochondrion, they will entail a considerable shift from present calculated figures. For example, values given by Williamson [10] in table 2 which depended on assuming a uniform distribution of glutamate and aspartate led to a calculated malate ratio of 1.5 when the citrate was 92. We should expect the malate ratio to be about 20 when the citrate is 92 and the glutamate ratio instead of being unity, as he assumed, should be 4.5. It remains to be seen whether the Donnan-like system holds when the mitochondria are in the cell because it is possible that their permeabilities to the anions are modified during the preparative procedures. However a systematic basis for predicting certain anion adjustments occurring with isolated mitochondria has been provided.

Acknowledgements

This work was supported by grants from the Medical Research Council, the Wellcome Trust, and the Muscular Dystrophy Association of America.

References

[1] E.J. Harris, in: The Energy Level and Metabolic Control in Mitochondria, eds. S.Papa et al. (Adriatica Editrice, Bari, 1969) p. 135.
[2] E.J.Harris and B.C.Pressman, Biochim. Biophys. Acta 172 (1969) 66.
[3] W.S.Schneider, J. Biol. Chem. 176 (1948) 259.
[4] E.J.Harris and K.Van Dam, Biochem. J. 106 (1968) 759.
[5] J.R.Williamson and B.E.Corkey, in: Methods in Enzymology, Vol. 13, ed. J.M.Lowenstein (Academic Press, New York, 1969) p. 434.

[6] P.J.England and B.H.Robinson, Biochem. J. 112 (1969) 8P.
[7] S.Addanki, F.Cahill and J.F.Sotos, J. Biol. Chem. 243 (1968) 2337.
[8] E.J.Harris, FEBS Letters 5 (1969) 50.
[9] E.J.Harris, in page 31 of ref. [1].
[10] J.R.Williamson, page 385 of ref. [1].