Biochimica et Biophysica Acta 806 (1985) 1–8
Elsevier

BBA 41665

Functional studies on in situ-like mitochondria isolated in the presence of polyvinyl pyrrolidone

Klaus Wrogemann [a], Edward G. Nylen [a], Ian Adamson [b] and Shri V. Pande [c]

Departments of [a] Biochemistry and [b] Pathology, University of Manitoba, Winnipeg, R3E 0W3 and [c] Clinical Research Institute of Montreal, 110 avenue des Pins, ouest, Montréal, H2W 1R7 (Canada)

(Received July 6th, 1984)

Key words: Poly(vinyl pyrrolidone); Mitochondrial structure; Electron microscopy

Mitochondria isolated and maintained in sucrose mannitol medium show a large intermembrane space and a condensed matrix unlike the appearance of in situ mitochondria. Mitochondria resembling in situ organelles are obtained when the isolation medium is supplemented with certain macromolecules such as polyvinyl pyrrolidone. We found that the in situ appearance was acquired also by the conventionally isolated mitochondria when they were exposed to 2% polyvinyl pyrrolidone supplemented medium. Paradoxically, however, these in situ looking mitochondria proved functionally inferior in that their brief incubation without substrates led to a marked loss of their ability to respire with subsequently added substrates such as pyruvate, acylcarnitines or glutamate. The oxidation of succinate was, however, not so affected. This phenomenon was shared by heart and skeletal muscle mitochondria of different animal species but not by rat liver mitochondria. The inhibition of respiration could not be related to the failure to oxidize NADH, to the tieing up of mitochondrial free CoASH, or to the increased matrix space of mitochondria that was observed in the presence of polyvinyl pyrrolidone. The polyvinyl pyrrolidone-exposed mitochondria regained their respiratory ability on being freed from polyvinyl pyrrolidone. The same phenomenon was seen also when the medium contained 2% albumin or 20% Ficoll.

Introduction

There is a growing tendency to study subcellular organelles in vitro under conditions where there behaviour is likely to reflect their in situ characteristics. Mitochondria isolated in conventional media exhibit a large intermembraneous space and appear swollen when compared to mitochondria in situ in tissue sections [1]. It has been known for some time that the in situ like appearance can be observed in isolated mitochondrial suspensions provided these are isolated in the presence of a high molecular-weight substance, such as polyvinyl pyrrolidone [1,2], and it was suggested that such mitochondria may have different functional characteristics compared to the conventionally isolated mitochondira [2]. We have found that, although in the presence of polyvinyl pyrrolidone and other macromolecules the mitochondrial structure resembled their appearance in situ, paradoxically this accompanied a deterioration in their respiratory ability. Observations relating to the understanding of the possible causes of this functional deterioration are also described below.

Materials and Methods

Normal Syrian hamsters (Rb animals) were obtained from the Trenton Experimental Laboratory Animal Company, Bar Harbor, Maine. Additional animals were bred locally by random mating.

Heart mitochondria were isolated as described previously in sucrose-mannitol-EDTA medium using the proteinase Nagarse [3,4]. In some experi-

ments, the isolation medium was supplemented with 2% polyvinyl pyrrolidone (Sigma Chemicals, average molecular weight, 40000). Skeletal muscle mitochondria were isolated similarly [5]. Respiration rates and oxidative phosphorylation parameters were determined polarographically at 28°C using a Clark-type electrode [6]. The test medium generally contained 0.23 M mannitol/0.07 M sucrose/20 mM Tris-Cl/20 μM EDTA/5 mM phosphate at pH 7.2. Where indicated this medium was supplemented with either 2% polyvinyl pyrrolidone, 2% bovine serum albumin (Fraction V, Armour Pharmaceutical Co.), or 20% Ficoll (Pharmacia). In most cases 5 mM pyruvate plus 1 mM malate were used as substrates. Other substrate combinations used were 4.5 mM succinate in the presence of 2.7 μM rotenone, 20 μM palmityl-L-carnitine plus 1 mM malate, 1.2 mM acetyl-L-carnitine plus 1 mM malate, 5 mM glutamate plus 1 mM malate, 2.1 mM α-ketoglutate with or without 8 mM malonate, or 5 mM pyruvate plus 1 mM malonate and 10 mM L-carnitine.

The O_2 concentration in air-equilibrated test medium was determined as proposed by Estabrook [7] using sonicated mitochondria and enzymatically standardized NADH. Respiratory control and ADP/O ratios were calculated according to Chance and Williams [8]. Protein was determined by the method of Lowry et al. [9]. Changes in mitochondrial pyridine nucleotide reduction were monitored fluorometrically [10] and CoA was estimated as described by Rabier et al. [11].

For electron-microscopy mitochondria in State-3 respiration were fixed in 2% glutaraldehyde with stirring for 20 s and then pelleted in an Eppendorf microcentrifuge. After 30 min the medium with glutaraldehyde was removed, the pellet rinsed with test medium, and post-fixed in 1% OsO_4. The pellet was loosened from the tube with a spatula, dehydrated through graded alcohols to 100%, then embedded in Spurr plastic. Three random blocks from each pellet were cut and ultrathin sections (stained by lead citrate) were examined by electron microscopy.

Results and Discussion

Heart mitochondria isolated in isotonic sucrose mannitol EDTA medium appear swollen with a rounded configuration and large intermembraneous spaces (Fig. 1A). When the isolation medium is supplemented with 2% polyvinyl pyrrolidone (polyvinyl pyrrolidone, M_r 40000) the mitochondria appear irregular in shape, with a much larger matrix space, and the intermembraneous space is greatly reduced (Fig. 1B). Mitochondria isolated in the presence of polyvinyl pyrrolidone thus looked more like the in situ mitochondria as has been previously described [1]. Mitochondria isolated in polyvinyl pyrrolidone medium behaved rather similar to but no better than the conventionally isolated mitochondria with regard to their respiratory ability, ADP/O ratios, and respiratory control ratios provided while testing for these parameters, mitochondria were added to medium that already contained the respiratory substrates. When these mitochondria were allowed a brief prior incubation in medium devoid of substrate (such as pyruvate-malate) to deplete their endogenous substrates and pyruvate-malate were then added (Fig. 2), the resulting respiratory rates were about 1/5 or less than of those seen in the absence of the substrate-free prior incubation step (Table I) and the respiratory control and the ADP/O ratios were also accordingly decreased. However, when polyvinyl pyrrolidone was absent, a similar prior incubation had little effect on these same parameters of mitochondrial respiration (Table I).

Further testing showed that the conventionally isolated (that is without polyvinyl pyrrolidone) mitochondria acquired the appearance of in situ organelles on simply being placed in 2% polyvinyl pyrollidone containing medium (Fig. 1C and D) and these mitochondria then showed the same susceptibility to the loss of respiratory ability on substrate free prior incubation (Table II) as that described above for the mitochondria isolated and tested in the presence of polyvinyl pyrrolidone. Table II shows that the substrate-free prior incubation in polyvinyl pyrrolidone containing medium lowered the ability of mitochondria to rspire with a number of substrates besides pyruvate-malate but that the oxidation of succinate was not affected. The effect of time of substrate-free prior incubation in polyvinyl pyrrolidone medium showed (Fig. 3) that the lowering of oxidation of pyruvate-malate was more susceptible than that of glutamate-malate, and that succinate

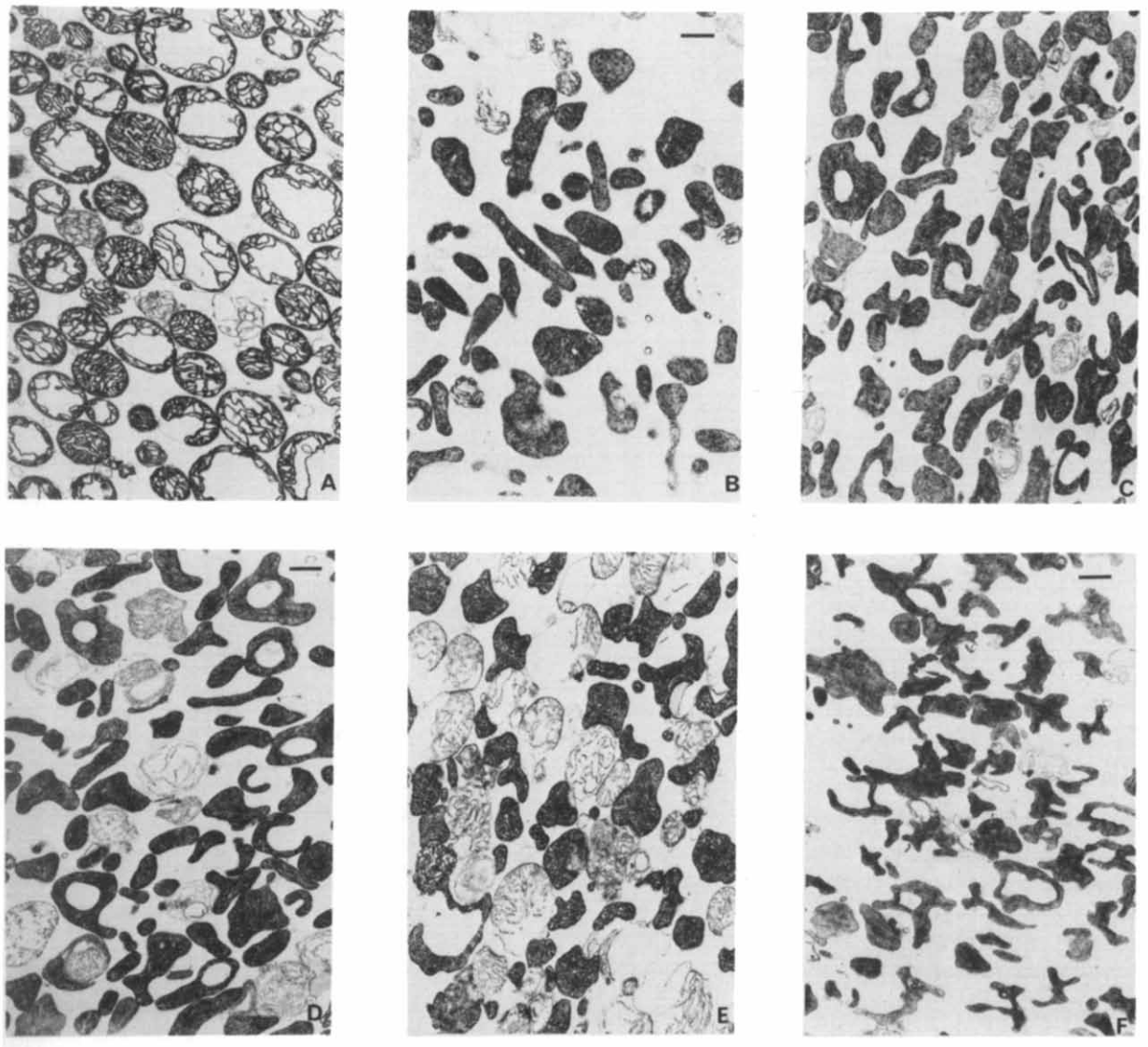

Fig. 1. Ultrastructure of hamster heart mitochondria under State-3 conditions with pyruvate-malate as substrates as affected by the presence of polyvinyl pyrrolidone and a substrate-free prior incubation step. In (A), polyvinyl pyrrolidone was absent from both the isolation and the incubation media and a 90 s substrate-free prior incubation step preceded pyruvate-malate addition. In (B), conditions were identical to A except that 2% polyvinyl pyrrolidone was present throughout. In (C) and (D), mitochondria isolated without polyvinyl pyrrolidone were incubated in polyvinyl pyrrolidone containing medium with (C), or without (D), a substrate-free prior incubation step. In (E) and (F), conditions were the same as in (D) except that the polyvinyl pyrrolidone was replaced by 2% albumin and 20% Ficoll, respectively. Electron micrographs, ×8000 (bar equals 1 μm).

oxidation remained relatively unaffected even after 5 min prior incubation. Mitochondria from heart of rat and pigeon, and skeletal muscles of hamster and rat, showed a similar susceptibility to pyruvate-malate oxidation following a substrate-free prior incubation in polyvinyl pyrrolidone medium as that of hamster heart mitochondria. The mitochondria of rat liver, however, behaved differently as their ability to respire with pyruvate-malate, palmitoylcarnitine-malate, or glutamate-

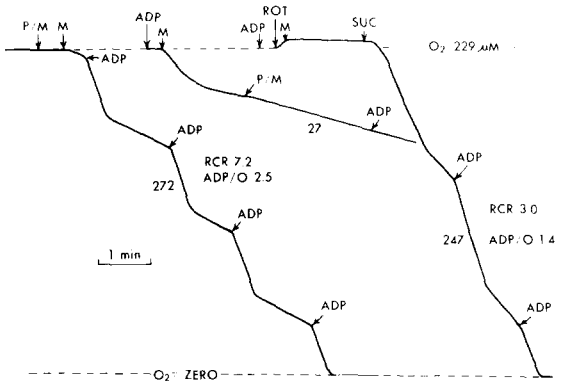

Fig. 2. Inhibition of pyruvate but not that of succinate oxidation by a substrate-free prior incubation in the polyvinyl pyrrolidone medium. Conventionally isolated hamster heart mitochondria were incubated at 28°C in 1.5 ml of air-saturated medium that contained 230 mM mannitol/70 mM sucrose/20 mM Tris-HCl/20 μM EDTA/5 mM P_i at a final pH of 7.2. Additions were: 5 mM pyruvate plus 1 mM L-malate (P/M), 758 μg (protein) mitochondria (M), 224 μM ADP, 2.7 μM rotenone (ROT) and 4.5 mM succinate (suc). The numbers along the curve indicate oxygen uptake in μmoles/min per g protein. RCR, respiratory control ratio [8].

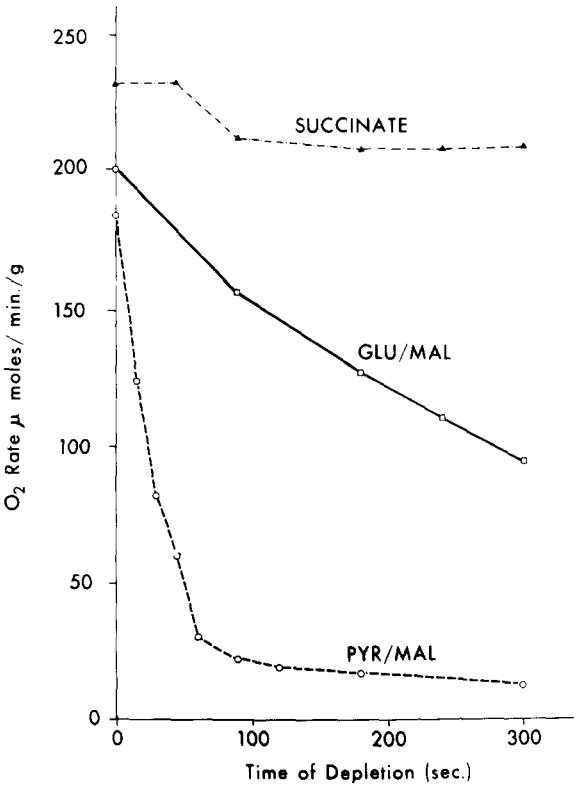

Fig. 3. Effect of time of substrate-free prior incubation step in polyvinyl pyrrolidone medium on the subsequent rate of State-3 respiration. Other conditions were as in the second experiment of Fig. 2. Pyruvate-malate (PYR/MAL); glutamate-malate (GLU/MAL).

malate was not so affected by a similar prior incubation step (data not shown). The observed phenomenon was not specific to polyvinyl pyrrolidone as similar appearances and respiratory characteristics were observed when 2% albumin or 20% Ficoll replaced polyvinyl pyrrolidone (Fig. 1E and F). The respiratory deterioration with pyruvate-malate was found to manifest following a substrate-free prior incubation regardless of the presence of P_i and/or ADP at the prior incubation step. Presence of malate alone during the prior incubation step was unable to offer any protection but that of pyruvate alone was.

Once the State-3 inhibition had occurred fol-

TABLE I

EFFECT OF 2% PVP IN THE ISOLATION AND THE INCUBATION-MEDIUM ON OXIDATIVE PHOSPHORYLATION OF HEART MITOCHONDRIA WITH PYRUVATE MALATE AS SUBSTRATE

Each value shows mean ± S.E. with *n* in parenthesis. PVP, polyvinyl pyrrolidone.

Media	Substrate-free prior incubation step	State-3 rates (μmol O_2/min per g protein)	Respiratory control ratio	ADP/O
Lacking PVP	not allowed	209 ± 20 (4)	8.7 ± 0.6 (4)	2.6 ± 0.06 (4)
	allowed	202 ± 11(16)	8.7 ± 0.4(16)	2.5 ± 0.04(16)
Having PVP	not allowed	150 ± 11(10)	5.7 ± 0.5(10)	2.5 ± 0.02(10)
	allowed	29 ± 6(10) [a]	1.3 ± 0.1(10) [a]	0.6 ± 0.31(10) [a]

[a] $P \ll 0.05$ compared to 'not allowed'.

TABLE II

EFFECT OF PVP IN THE INCUBATION MEDIUM AND OF SUBSTRATE-FREE PRIOR INCUBATION STEP ON THE ABILITY OF HEART MITOCHONDRIA TO RESPIRE WITH DIFFERENT SUBSTRATES

Each value shows mean ± S.E. with n in parenthesis.

Respiratory substrate	2% PVP in medium	Respiratory rates with substrate-free prior incubation (μmol O_2/min per g protein)	
		Not allowed	Allowed
Pyruvate/malate	−	209 ± 20 (4)	202 ± 11(16)
	+	211 ± 8(31)	25 ± 2(31) [a]
Pyruvate-malonate/carnitine	−	108 ± 8 (4)	104 ± 6 (4)
	+	102 ± 9 (4)	19 ± 3 (4) [a]
Palmityl-carnitine/malate	−	–	230 ± 13 (4)
	+	177 ± 26 (4)	31 ± 8 (4) [a]
Acetyl-L-carnitine/malate	−		120 ± 8 (4)
	+	87 ± 9 (4)	13 ± 4 (4) [a]
Glutamate/malate	−	213 ± (1)	236 ± 20 (5)
	+	226 ± 11 (6)	180 ± 16 (6) [a]
Glutamate	−	268 ± 12 (4)	251 ± 14 (4)
	+	254 ± 8 (4)	62 ± 8 (4) [a]
α-Ketoglutarate/	−	213 ± 9 (3)	216 ± 2 (3)
	+	207 ± 2 (3)	114 ± 13 (3) [a]
α-Ketoglutarate/malonate	−	108 ± (1)	129 (1)
	+	95 ± 8 (4)	75 ± 6 (4)
Succinate	−	–	260 ± 7 (5)
	+	244 ± 9 (6)	243 ± 8 (6)

[a] $P < 0.001$ or $P \ll 0.001$ compared to the PVP lacking set.

lowing the prior incubation it was not relieved by subsequent hypotonic swelling, by valinomycin plus K^+, by uncouplers like dinitrophenol or MCCP, by reducing agents like dithiothreitol, by sonication, by low concentrations of nonionic detergents, or by the addition of cofactors ATP, magnesium, coenzyme A, or NAD^+. This polyvinyl pyrrolidone effect was, however, reversible; mitochondria that showed loss of their State-3 respiratory ability with pyruvate-malate following a substrate free prior incubation, regained much of their respiratory ability when they were then freed of polyvinyl pyrrolidone by pelleting and washing before being retested with pyruvate-malate. This observation eliminated the possibility that the substrate-free prior incubation led to a major loss of the mitochondrial cofactor(s) to the medium. Upon the removal of polyvinyl pyrrolidone, the mitochondrial ultrastructure was found to have returned to the configuration of conventionally isolated mitochondria that show a swollen appearance (not shown but similar to Fig. 1A).

Although the substrate-free prior incubation lowered the respiratory rates with glutamate alone to a greater extent than with glutamate-malate (Table II), a loss of citric acid cycle intermediates was not the major cause of respiratory inhibitions. This was shown by the observation that the inhibition of the oxidation of pyruvate in the presence of carnitine and malonate, which does not depend on citric acid cycle operation [12], was just as marked as that of pyruvate-malate oxidation (Table II).

A similar pattern of the inhibition of the oxidation of several substrates has been reported in maleate intoxication, where the inhibition was traced to the enzyme CoA transferase (originally succinyl-CoA: 3-ketoacid Coenzyme A transferase, E.C. 2.8.3.5) mediated tieing up of the mitochondrial CoASH [13]. And significantly this mitochondrial enzyme is found in heart and skeletal muscles but not in liver [14]. This prompted us to examine whether a sequestering of

mitochondrial CoASH was occurring under the conditions of inhibited respiration. In mitochondria respiring under State-3 conditions with pyruvate-malate as substrate in polyvinyl pyrrolidone medium, the free CoASH values obtained as nmol per mg protein, respectively, for mitochondria that were given a substrate-free prior incubation and those that were not ($n = 5$ in both), were, 1.5 ± 0.11 and 1.6 ± 0.08. The corresponding total CoA values were 2.2 ± 0.14 and 2.1 ± 0.17. Although the levels of free CoA were not altered after substrate free prior incubation in polyvinyl pyrrolidone medium the possibility that a subtle change in mitochondrial configuration in polyvinyl pyrrolidone medium interfered with the ready access of CoA to its respective enzymes cannot be ruled out. Since the respiratory inhibition was observed with substrates oxidation of which involved the mitochondrial NAD-NADH system, a possibility of interferences with these steps was considered. No interference of NADH oxidation per se was observed. When mitochondria respiring under the inhibitory state were sonicated and then offered NADH as a substrate, they respired briskly (data not shown). Monitoring of NADH fluorescence in intact mitochondria showed (Fig. 4) that under conditions of respiratory inhibition when polyvinyl pyrrolidone was present, the steady-state NADH production was indeed markedly decreased, compared to that in test medium without polyvinyl pyrrolidone. This suggests that the observed respiratory inhibitions of oxygen consumption somehow resulted from the failure of substrate dehydrogenases to feed reducing equivalents to the respiratory chain.

Because the mitochondrial ultrastructure changed greatly in the presence of polyvinyl pyrrolidone, the possibility was considered that a further change might become discernible under the inhibited respiratory state compared to mitochondria that were not subjected to a substrate-free prior incubation and were actively respiring. However, no noticeable difference was observed between the mitochondria fixed under the two respective conditions (compare Fig. 1C and D). Measurements of mannitol space, which is a better indicator of extramatrix space of mitochondria than is sucrose space [15], and matrix space showed (Table III) that although the presence of polyvinyl

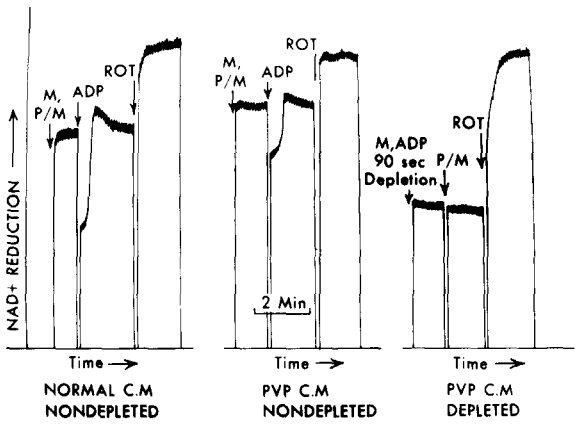

Fig. 4. Changes in pyridine nucleotide reduction recorded fluorimetrically. Conventionally isolated hamster heart mitochondria were tested as in Fig. 2 in medium without polyvinyl pyrrolidone and not allowed a substrate-free prior incubation (normal C.M nondepleted), under the same conditions but in 2% polyvinyl pyrrolidone (PVP) supplemented medium (PVP C.M nondepleted), and in polyvinyl pyrrolidone supplemented medium after a 90 s substrate-free prior incubation step (PVP C.M depleted).

pyrrolidone caused about 50% increase in the matrix space in line with the results of electron micrographs, this was not further altered by the intervention of a substrate-free prior incubation step.

Although the results of present investigation allowed exclusion of certain possibilities, the exact nature of the alteration(s) responsible for the inhibition of respiration following a substrate-free prior incubation of mitochondria in the presence of polyvinyl pyrrolidone could not be localized. We wish to point out that an inhibition of respiration was observed by Harris et al. [2] also when 3% albumin was present but in their study the oxidation of succinate was also impaired. A condensation of mitochondrial matrix was assumed to be the cause of these inhibitions because the effects were overcome by introducing mitochondrial swelling, for example with valinomycin [2]. In the present experiments, the inhibition of respiration was not overcome by exposing mitochondria to known swelling agents or by the inclusion of 1 mM EDTA or EGTA. Moreover, our direct measurements revealed that polyvinyl pyrrolidone caused an expansion (cf. Ref. 1) and not a condensation of the matrix. These considerations sug-

TABLE III

EFFECT OF INCUBATION CONDITIONS ON THE MANNITOL AND MATRIX WATER SPACES OF RAT HEART MITOCHONDRIA

Constantly stirring incubation system in 1170 μl at 30°C contained 100 mM mannitol, 50 mM KCl, 25 mM Tris-HCl (pH 7.4), 5 mM ADP, 5 mM Pi, 1 μCi [^{14}C]mannitol or 3 μCi [^{3}H]H_2O, and where shown, 2% polyvinyl pyrrolidone (PVP), 5 mM pyruvate plus 1 mM malate. Incubations were started by the addition of mitochondria (20 μl, 1.0 mg protein). 120 s later, pyruvate and malate (10 μl) were added to tubes that did not have these compounds already. Final volume was 1200 μl at this stage. 1 ml of the reaction mixtures was carefully transferred to 1.5 ml Eppendorf tubes containing from bottom to top, 50 μl of formic acid: water (1 : 1.11, v/v) mixture and about 150 μl of a silicone oil (sp. gravity 1.048). Exactly 160 s later, mitochondria were brought into the formic acid layer by starting rapid centrifugation and then processed as in Ref. 16 except that the 90°C heating step was omitted, and that each tube received 40 μl of H_2O and 1.1 ml of Beckman's EP-scintillant. For the liquid scintillation counting, 25 ml glass counting vials were used as holders of the Eppendorf tubes. The values (mean ± S.E. of $n = 5$) are expressed on the basis of the protein recovered (approx. 64%, monitored separately) below the oil layer.

Incubations	Mannitol space (μl/mg recovered protein)	Matrix water space (μl/mg recovered protein)
(A) PVP absent, pyruvate-malate added 2 min after mitochondria	5.61 ± 0.067	1.13 ± 0.096
(B) PVP present, pyruvate-malate added 2 min after mitochondria	5.20 ± 0.192	1.67 ± 0.154 [a]
(C) PVP present, pyruvate-malate present from the very beginning	5.42 ± 0.031	1.64 ± 0.079

[a] $P < 0.025$ compared to PVP absent.

gest that despite similar ultrastructural appearances imparted by polyvinyl pyrrolidone and albumin, these two macromolecules affect the mitochondrial functions differently. Although, intuitively one hopes that the in situ looking mitochondria would show better functional characteristics clearly this has not been the case. Under no conditions did mitochondria tested in the presence of polyvinyl pyrrolidone exhibit higher parameters of oxidative phosphorylation. In fact, in the presence of polyvinyl pyrrolidone these parameters were either the same or slightly poorer, even when tested under conditions of substrate present from the beginning. In addition, a decline of the State-3 respiration in successive cycles of State-3/State-4 transitions was seen during the oxidation of acylcarnitines plus malate even when these substrates were added to the incubation medium prior to that of mitochondria but this was never seen in the absence of polyvinyl pyrrolidone (data not shown). Whether these observed effects of macromolecules relate to their ability to exert colloid pressure [1,2], to change the medium viscosity, or to other factor(s) remains to be found as does the answer to the question of what provides the protection to mitochondria against a similar effect of macromolecules in their native environment in vivo. Until then the use of conventionally isolated mitochondria in studies in vitro would seem justified despite lack of their structural resemblance to the in situ organelles.

Acknowledgements

We thank Ms. S. Finlayson, Mr. R. Torres and Mr. Tak-Sing Lee for expert technical help. This work was supported by grants from the Medical Research Council of Canada, the Muscular Dystrophy Association of Canada, the Manitoba Heart Foundation and the Quebec Heart Foundation.

References

1 Bakeeva, L.E., Chentsov, Y.S., Jasaitis, A.A. and Skulachev, V.P. (1971) Biochim. Biophys. Acta 275, 319–331
2 Harris, E.J., Tate, C., Manger, J.R. and Bangham, J.A. (1971) Bioenergetics 2, 221–232
3 Wrogemann, K., Blanchaer, M.C. and Jacobson, B.E. (1972) Am. J. Physiol. 222, 1453–1457
4 Wrogemann, K. and Blanchaer, M.C. (1968) Can. J. Biochem. 46, 323–329
5 Jacobson, B.E., Blanchaer, M.C. and Wrogemann, K. (1970) Can. J. Biochem. 48, 1037–1042

6 Wrogemann, K., Blanchaer, M.C. and Jacobson, B.E. (1970) Life Sciences, Part II, 9, 1167–1173
7 Estabrook, R.W. (1967) Methods Enzymol. 10, 41–47
8 Chance, B. and Williams, G.R. (1956) Adv. Enzymol. 17, 65–134
9 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
10 Wrogemann, K., Jacobson, B.E. and Blanchaer, M.C. (1973) Arch. Biochem. Biophys. 159, 267–278
11 Rabier, D., Briand, P., Petit, F., Kamoun, P. and Cathelineau, L. (1983) Anal. Biochem. 134, 325–329
12 Pande, S.V. (1971) J. Biol. Chem. 246, 5384–5390
13 Pacanis, A., Strzelecki, T. and Rogulski, J. (1981) J. Biol. Chem. 256, 13035–13038
14 Pacanis, A. and Rogulski, J. (1977) 24, 3–12
15 Halestrap, A.P. and Quinlan, P.T. (1983) Biochem. J. 214, 387–393
16 Pande, S.V. and Parvin, R. (1978) J. Biol. Chem. 253, 1565–1573