PROTEINS DEALING WITH *N*-ETHYLMALEIMIDE INHIBITION OF PIG HEART MITOCHONDRIAL PHOSPHATE TRANSPORT SYSTEM

Yves BRIAND, Sylvie TOURAILLE, Roger DEBISE and Roger DURAND*
Laboratoire de Biochimie, Université de Clermont-Ferrand, B.P. 45, 63170 Aubiere, France

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1. Introduction

Phosphate transport system of pig heart mitochondria was found to be inhibited by mercurial, maleimide [1] or CPDS [2] like rat liver mitochondria [3–8]. A more intensive protective effect of phosphate against maleimide inhibition was observed in pig heart [1] than in rat liver mitochondria [9].

As described by Fonyo [10] mersalyl can protect against NEM reaction in rat liver mitochondria. A similar result has been observed by Coty and Pedersen [11] with *p*-MB in rat liver mitochondria allowing them to detect a major protein component (mol. wt. 32 000) of the NEM-sensitive phosphate transport system.

Consequently NEM-sensitive protein components can be obtained by first protecting either by phosphate or by mersalyl and then by irreversibly blocking non-protected SH groups by addition of radioactive NEM.

However one may ask a question: does the protective effect of phosphate against [^{3}H] NEM incorporation reflect the polypeptides linked to the P_i transport and especially does it act at the level of the same proteins as mersalyl.

It has been previously demonstrated [12,13] that protein fractions with the specific effect of phosphate on [^{3}H] NEM incorporation were obtained from whole pig heart mitochondria after hypotonic treatment and sonication. Moreover this fraction was found to be valinomycin NEM-sensitive and grisorixin (a new ionophore of nigericin's group).

We report here data regarding the comparative protective effects of phosphate, nigericin, and mersalyl against NEM inhibition of pig heart mitochondria phosphate transport system. Electrophoresis analysis of [^{3}H] NEM-labeled proteins is given in each case.

2. Material and methods

Pig heart mitochondria were prepared according to Crane et al. [14] but instead of 10 mM phosphate buffer, 10 mM Tris-HCl buffer was used. Proteins were determined by the biuret method [15].

Mitochondria swelling was followed by optical density change as described by Chappell and Crofts [16]; decreasing optical density was measured in the Eppendorf photometer at 546 nm.

Mitochondrial proteins were fractionated according to Briand et al. [13] (see fig.2 for details).

For [^{3}H] NEM determination, three samples of each protein fraction were taken off and proteins precipitated by 50% trichloracetic acid in 1.5 ml Eppendorf centrifuge tube. After centrifugation the pellets were dissolved in 0.4 ml formic acid for scintillation counting.

Polyacrylamide gel electrophoresis in SDS was carried out according to Weber and Osborn [17] and

* To whom reprint requests should be addressed.

Abbreviations: CPDS, 6,6'-dithionicotinic acid; NEM, *N*-ethylmeleimide; *p*-MB; parachloromercuribenzoate; Tris, Tris (hydroxymethyl) aminomethane; TCA, Trichloracetic acid; SDS, sodium dodecyl sulfate; NIG, nigericin; TEMED, *N,N,N',N'*,-tetramethylenediamine.

Packer [18] with some modifications: 6 X 120 nm gels with 6% acrylamide; 0.16% methylenebisacrylamide; 0.1% ammonium persulfate; 0.05% TEMED; 0.1% SDS; 0.1% mercaptoethanol in 0.1 M sodium phosphate buffer pH 7.2.

Samples containing 80 μg of proteins with 2% SDS, 0.1% mercaptoethanol were heated to 90°C for 3 min prior to application to the gels.

The gels were stained with Coomassie brilliant blue and destained according to Weber and Osborn [17].

The apparent mol. wt. of proteins was determined by used pyronin Y as dye and five calibration standard proteins (trypsin inhibitor: 21 500; bovine serum albumin: 68 000; RNA polymerase from *E. coli* α subunit 39 000; β subunit 155 000; β subunit 165 000).

3. Results and discussion

3.1. *Inhibition by mersalyl and NEM of phosphate transport*

As shown in fig.1 phosphate followed by swelling methods was inhibited by mersalyl and NEM. Phosphate (2.5 mM) and nigericin (12.5 ng/mg protein) protected against NEM inhibition while mersalyl inhibition was not modified.

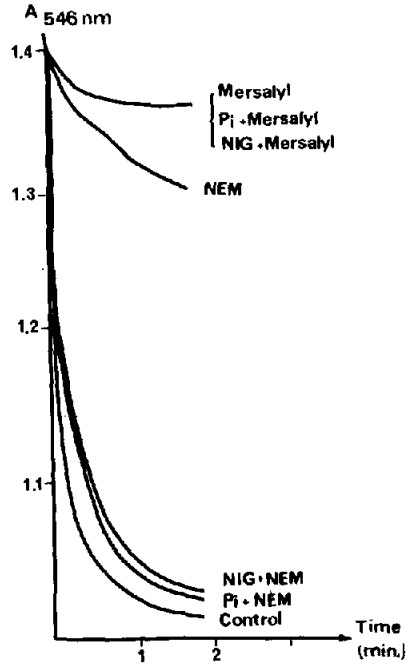


Fig.1. Phosphate and mersalyl effect on NEM inhibition of phosphate transport followed by swelling in 100 mM ammonium phosphate. 4 mg of mitochondrial protein were preincubated in 0.5 ml, 10 mM Tris-HCl buffer, 0.25 M sucrose, pH 7.4, 30°C with or without phosphate (2.5 mM) and nigericin (12.5 ng/mg protein). Then 30 sec after NEM (12.5 nmol/mg protein) or mersalyl (20 nmol/mg protein) were added. In the case of NEM, reactions were stopped by 10 mM cystein 1 min 30 sec later. Then 0.8 mg of protein were put in 1 ml 100 mM ammonium phosphate and decreased optical density monitored at 546 nm.

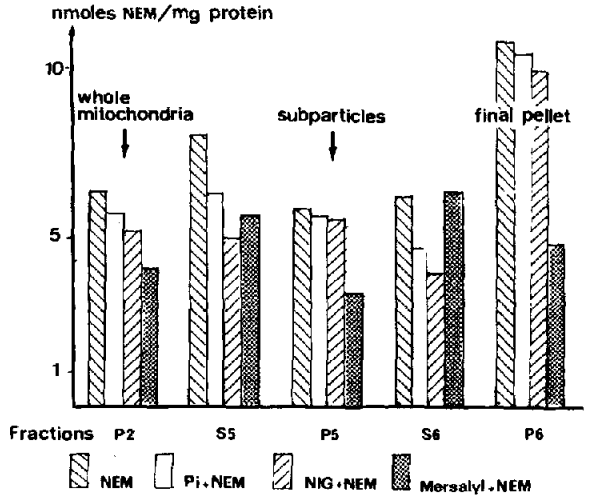


Fig.2. Phosphate, nigericin and mersalyl effect on NEM incorporation in different protein fractions resulting from fractionation of pig heart mitochondria. Mitochondria (80 mg) in 10 ml 10 mM Tris-HCl buffer, 0.25 M sucrose, pH 7.4, 30°C are treated by 1 μmol [^{3}H]NEM for 1 min. 30 sec. NEM reaction was stopped by added 10 mM cystein. The order of addition was: P_i (2.5 mM) or nigericin (12.5 ng/mg protein) or mersalyl (20 nmol/mg protein) and [^{3}H]NEM 30 sec later. Then two washings were made in Tris-HCl sucrose buffer and mitochondria centrifuged 39 000 *g*, 5 min. After washing, two hypotonic treatments were made by 6 ml bidistilled water 4°C, 8 min. In each case vesicle fractions were precipitated at 127 000 *g* for 30 min. Finally, insoluble fractions were submitted to sonication 6 X 10 sec, maximum power ultrasonic desintegrator MSE, in 6 ml Tris-HCl sucrose buffer. The subparticles fraction P_5 and the supernatant fraction S_5 were obtained by centrifugation at 127 000 *g* for 30 min. Supernatant fraction S_5 was and the supernatant fraction S_5 were obtained by centrifuged for 2 h at 237 000 *g* to obtain S_6 and P_6 fractions. In all the cases S is the supernatant fraction, P the insoluble fraction.

3.2. *Protective effect of phosphate, nigericin and mersalyl against [^{3}H] NEM incorporation in different mitochondrial protein fractions*

Whole mitochondria were treated by [^{3}H] NEM under the conditions given in fig.1. Incorporation of [^{3}H] NEM was determined with [^{3}H] NEM alone, and in the presence of phosphate, nigericin or mersalyl. Then, in each case, whole mitochondria were submitted to 2 hypotonic treatments and sonication. The results are shown in fig.2.

6.4 nmol of [^{3}H] NEM/mg protein (15% of the total incorporation in pig heart mitochondria) were incorporated in whole mitochondria (fraction P_2). Phosphate, nigericin or mersalyl decreased [^{3}H] NEM incorporation by 0.6, 1.1 and 2.3 nmol/mg protein, respectively. There was no effect of phosphate, nigericin or mersalyl on [^{3}H] NEM incorporation in the supernatants resulting from the 2 hypotonic treatments (data not shown here). After sonication of mitochondrial vesicles, we obtained the sub-

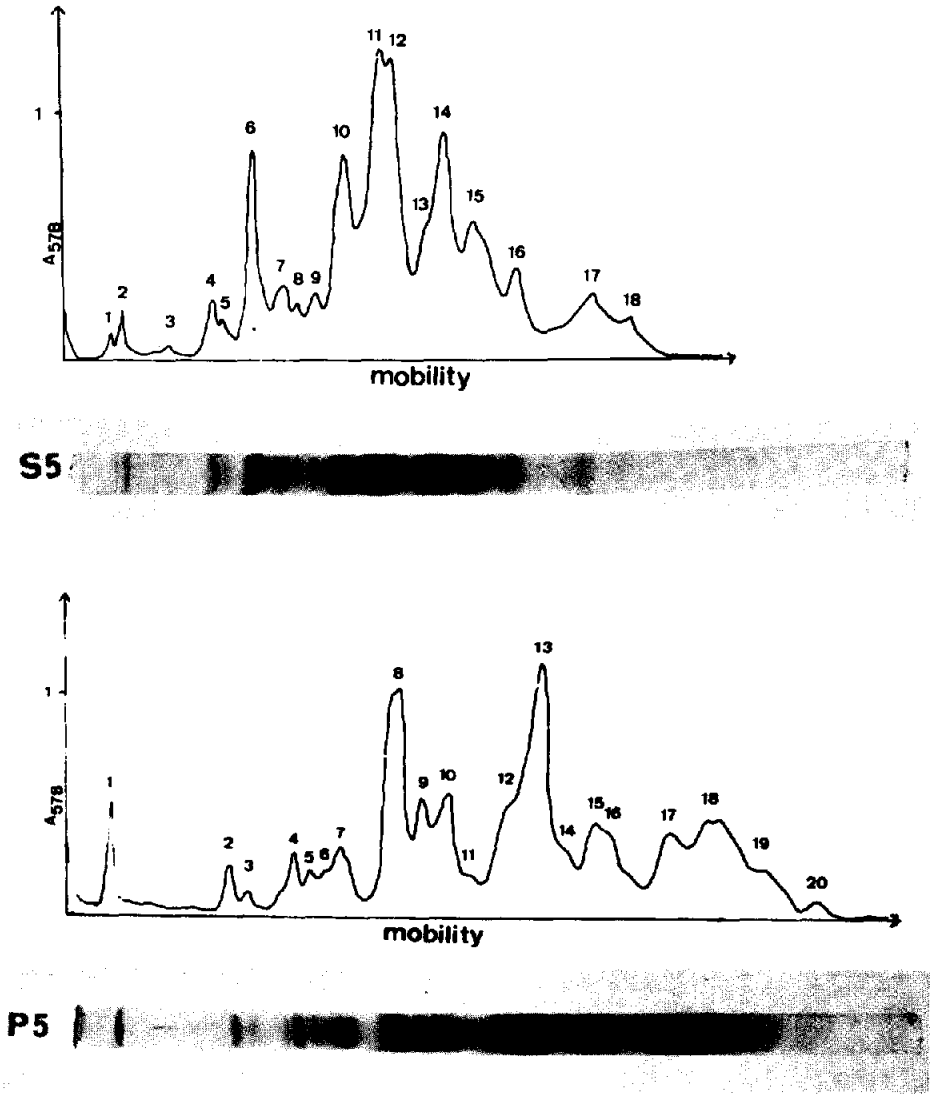


Fig.3. Densitometric traces of SDS-polyacrylamide gel electrophoresis and protein profiles of S_5 and P_5 fractions.

particle fraction (P_5) and the supernatant fraction (S_5). Phosphate and nigericin effects were just observed in S_5 ($\Delta = -1.7$ nmol [^{3}H]NEM/mg protein and -3 nmol, respectively) whereas mersalyl decreased by 2.5 nmol/mg protein and 2.4 nmol [^{3}H]NEM incorporation in P_5 and S_5 respectively. When S_5 was centrifuged again at 237 000 *g* the typical effect of mersalyl was completely recovered in the final pellet P_6 ($\Delta = -5$ nmol [^{3}H]NEM/mg protein). Supernatant fraction S_6 did not exhibit a mersalyl effect, whereas phosphate and nigericin effects were maintained.

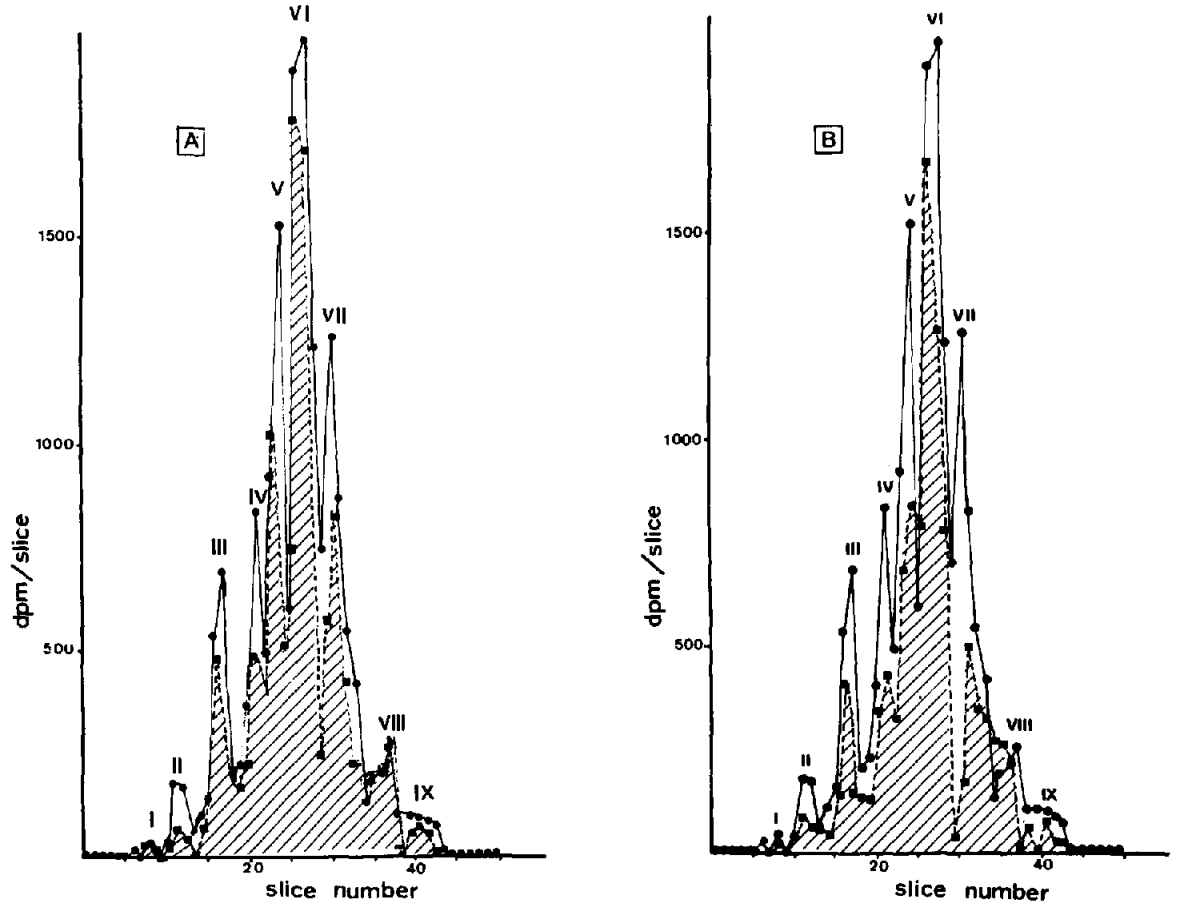
The results described in this experiment suggested that protective effect against NEM inhibition either by phosphate or nigericin, or by mersalyl, involves two kinds of protein: a rather soluble system (phosphate and nigericin), a rather insoluble system localised at the level of the subparticle fraction (mersalyl).

3.3. *Electrophoretic analysis of S_5 and P_5 mitochondrial protein fractions obtained after sonication*

Electrophoresis patterns of S_5 and P_5 protein fractions are given in fig.3. S_5 which contains 13% of the total mitochondrial protein content [13] exhibited 18 bands while P_5 (65% of the total mitochondrial protein content) exhibited 20 bands.

We can see in fig.4 the [^{3}H]NEM-labelled mitochondrial proteins S_5. Fig.4A shows that phosphate decreased the [^{3}H]NEM incorporation in peaks II, IV, V and VII. Experiments were repeated 4 times and differences observed for peaks II and VI cannot be considered significant. The effect of nigericin (fig.4B) was observed in the same peaks as for phosphate. Mersalyl just affects peak VI (fig.4C).

When an electrophoresis was made with protein fraction P_5 (fig.5) we found an intensive decreasing



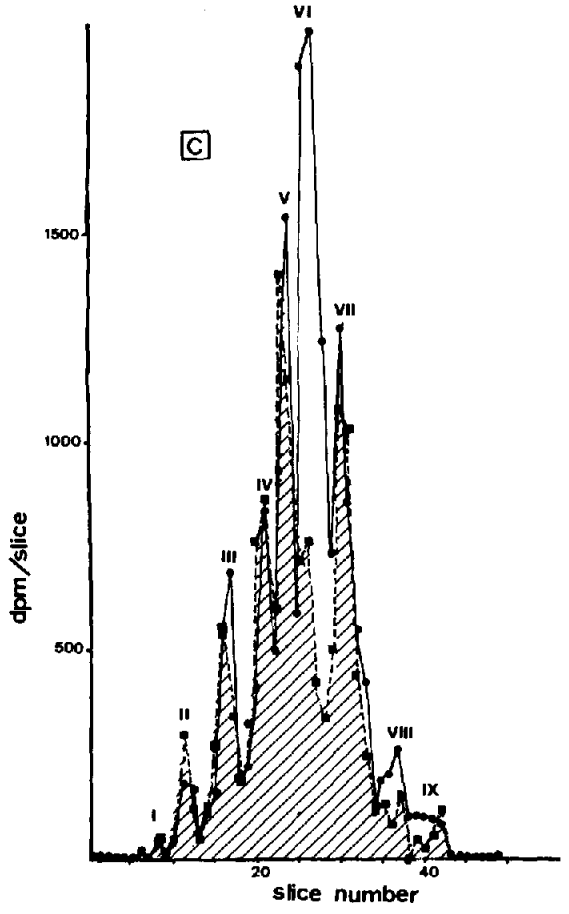


Fig.4. Gel electrophoretic analysis of [^{3}H] NEM-labeled proteins of supernatant fraction S_5. Labeled of mitochondria with [^{3}H]NEM and fractionation were carried out as explained in fig.2. Samples were treated and analysed on SDS-polyacrylamide gel electrophoresis as explained in Material and methods. After electrophoresis, gels were cut into 2 mm slices and assayed for radioactivity. (A) P_i protective effect on NEM incorporation (●——● NEM; ■——■ P_i + NEM). (B) Nigericin protective effect on NEM incorporation (●——● NEM; ■——■ Nigericin + NEM). (C) Mersalyl protective effect on NEM incorporation (●——● NEM; ■——■ Mersalyl + NEM).

effect of mersalyl on [^{3}H] NEM incorporation (peaks V, VI and VII).

Correlations between densitometric peaks and radioactive peaks are given in table 1. Furthermore approximate mol. wts. of each protected protein were estimated and the approximate protected nmoles SH/nmole protein were calculated. We can see that phosphate and nigericin give a maximal protection at the level of the same proteins (mol. wts. of 80 000 and 30 000 respectively). These two major components were rather soluble protein systems removed by sonication of mitochondrial ghosts. The mersalyl effect was observed at the level of two insoluble major protein components of 39 000 and 34 000 mol. wt., respectively. Protein component (mol. wt. 39 000) was both found in S_5 and P_5 fractions. When supernatant S_5 fraction was centrifuged again mersalyl-sensitive protein was collected in the final pellet P_6 whereas phosphate and nigericin-sensitive protein was removed in the supernatant fraction S_6.

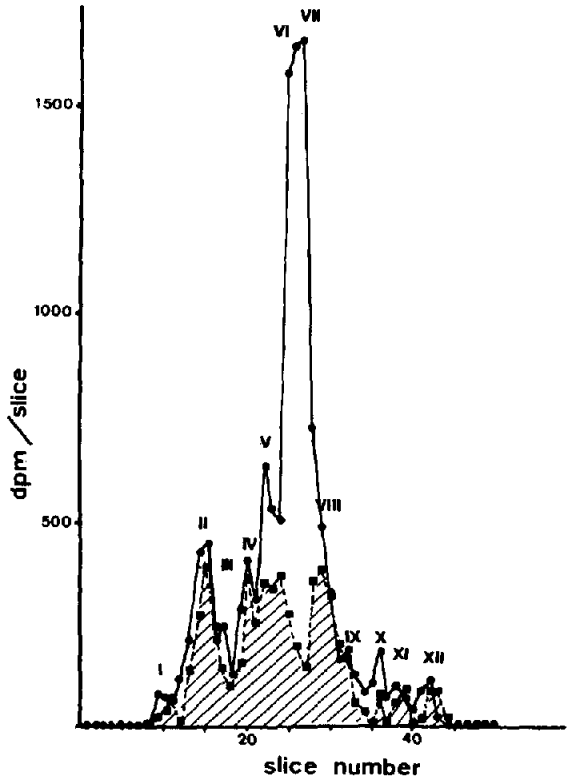


Fig.5. Gel electrophoretic analysis of [^{3}H] NEM-labeled proteins of fraction P_5. Mersalyl protective effect on NEM incorporation. (●——● NEM; ■——■ Mersalyl + NEM). For details see fig.2 and Material and methods.

Table 1
Correlations between densitometric and radioactive peaks

Fractions	Protective effect	Densitometric peak	Approximate mol. wt.	% of total mitochondrial protein	Radioactive peak	Protected nmoles[a] (SH groups/ nmole protein)
	P_i	7 fig.3	80 000	0.3	III fig.4A	0.4
		10	59 000	1.2	IV	0.07
		12	46 000	1	V	0.2
		16	30 000	0.4	VII	0.35
S_s	NIG	7	80 000	0.3	III fig.4B	0.4
		10	59 000	1.2	IV	0.1
		12	46 000	1	V	0.2
		16	30 000	0.4	VII	0.5
	Mersalyl	14	39 000	1.15	VI fig.4C	0.5
P_s	Mersalyl	12 fig.3	49 000	3.1	V fig.5	0.2
		13	39 000	4.2	VI	0.5 – 0.8
		14	34 000	1	VII	0.5 – 1.7

[a]Protected nmoles SH groups/nmoles protein were estimated by difference of nmoles [^{3}H] NEM bound for each peak of [^{3}H] NEM-labeled protein. These values are minimum values because we cannot exclude that one peak contains one or more proteins of same mol.wt.

4. Conclusion

Our data clearly demonstrate that protective effect of phosphate and protective effect of mersalyl against NEM-inhibition of phosphate transport act at the level of two kinds of proteins.

(1) Two major components are phosphate and nigericin NEM sensitive. According to our previous data [13] it has been also demonstrated that these two proteins components are valinomycin NEM sensitive (results not shown here) suggesting a relationship between these proteins and the energy linked proton translocation process.

Relationships between these proteins and the phosphate translocation process are not evident and are under further investigations.

(2) Two other insoluble major components localised at the level of the subparticular fraction are mersalyl NEM sensitive.

We can suggest that these proteins are implicated in the translocation of phosphate in pig heart mitochondria.

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