# ETHYLMERCURITHIOSALICYLATE – A NEW REAGENT FOR THE STUDY OF PHOSPHATE TRANSPORT IN MITOCHONDRIA

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## 1. Introduction

The physiological function of mitochondria requires the transport of $P_i$ across the inner mitochondrial membrane for 3 main metabolic reactions: (1) Uptake of phosphate together with ADP for the synthesis of ATP within the matrix; (2) Exchange of phosphate against dicarboxylates for metabolite fluxes between cytosol and matrix; (3) Uptake and release of phosphate together with $Ca^{2+}$ for calcium homeostasis in the cytoplasm [1]. For these functions 4 separate phosphate transport systems have been described in mitochondria which could only be differentiated by their sensitivity against SH-group inhibitors: (1) The electroneutral phosphate/proton symporter is inhibited by MalNEt and low concentrations of *p*-chloromercuribenzoate and mersalyl [2–4]; (2) The electroneutral dicarboxylate antiporter, exchanging dicarboxylates or phosphate against each other, is inhibited by *p*-chloromercuribenzoate, mersalyl and the dicarboxylate analogon butylmalonate, but not by MalNEt [5–7]; (3) The electrogenic phosphate uniporter is inhibited by mersalyl [8] and with inverted inner membrane vesicles by *p*-chloromercuribenzoate and MalNEt [9]; (4) The electrogenic calcium/phosphate symporter was found insensitive against MalNEt and mersaylyl [10,11]. The latter transport system, however, does not seem to represent a separate transporter, because the insensitivity against MalNEt and *p*-chloromercuribenzoate could not be corroborated [12,13].

The simultaneous occurrence of the electroneutral phosphate/proton symporter (phosphate uptake) and the electrogenic phosphate uniporter (phosphate release) in mitochondria, would result in a futile cycle, driven by the mitochondrial proton pump. Therefore a strong regulation of the phosphate transport system has to be assumed. The existence of only one regulated transport system which functions either as proton/phosphate symporter or as phosphate/dicarboxylate antiporter has also been suggested [14–16]. Here the effect of the antiseptic ethylmercurithiosalicylate (thiomersal), which contains sulfur and mercury in a covalent linkage, on the phosphate uptake of mitochondria is described. The data suggest a regulated sensitivity of the phosphate/proton symport against SH-inhibitors.

*Abbreviations:* PMS, *p*-chloromercuri phenylsulfonate; MalNEt, *N*-ethylmaleimide; SDS, sodium dodecylsulfate

## 2. Materials and methods

MalNEt, thiomersal and thiosalicylic acid were purchased from Serva (Heidelberg), PMS and rotenone from Sigma (St Louis). [$^{32}$P]Phosphate and [$^{3}$H]-sucrose (3 Ci/mmol) were obtained from Amersham Buchler. All other chemicals were of analytical grade.

Rat liver mitochondria were isolated by standard procedures [17]. Swelling of mitochondria was done as in [16], either in 100 mM ammonium phosphate (pH 7.3), 2 mM EDTA and 1 $\mu$M rotenone (A), or in 80 mM potassium phosphate (pH 7.3), 40 mM ammonium chloride, 1 $\mu$M rotenone (B). The uptake of [$^{32}$P]phosphate was measured in aliquots (800 $\mu$l) taken from cuvettes containing 2 ml swelling medium (B) and 0.2 $\mu$Ci [$^{32}$P]phosphate plus 2 $\mu$Ci [$^{3}$H]-sucrose, at the indicated times after addition of mitochondria. After centrifugation for 30 s in an Eppendorf centrifuge the supernatant was immediately removed and the pellet dissolved in 300 $\mu$l 2% SDS and counted in 10 ml scintillation fluid. Protein was determined by the biuret method [18].

## 3. Results

Thiomersal alone has almost no influence on the uptake of phosphate into rat liver mitochondria, as measured by the swelling rate in isotonic ammonium phosphate. Only 10% inhibition is found with 110 nmol thiomersal/mg mitochondrial protein and 16% with 220 nmol/mg (not shown). Preincubation of mitochondria with PMS (27 nmol/mg) leads to complete inhibition of swelling, which can be abolished by addition of thiomersal (32 nmol/mg) as shown in fig.1B. After a short lag-phase the final swelling rate is even higher than in the control (fig.1A). To exclude the possibility that thiomersal might have displaced PMS from the functional SH-group by forming a complex with it, excess PMS was added to the thiomersal activated mitochondria (fig.1C). No change of the activated swelling rate was observed, although the amount of PMS (42 nmol/mg) exceeded the amount of thiomersal (8.5 nmol/mg). This result also excludes the possibility that thiosalicylate, which might have been formed from thiomersal by transmercaptization, released the inhibition by reaction with PMS. Excess thiosalicylate, like many other SH-compounds, can release the inhibition of swelling by PMS (fig.1D).

Since PMS reacts reversibly with the phosphate carrier SH-group, it could not be excluded that thiomersal might have influenced the binding of PMS to the SH-group in an unknown way. We therefore investigated the effect of thiomersal on MalNEt inhibited mitochondria. MalNEt is known to react irreversibly with SH-groups of proteins. In fig.2 the effect of thiomersal on the swelling of MalNEt inhibited mitochondria is shown. Similar to PMS-inhibited mitochondria thiomersal restores swelling to a similar rate (fig.2B) to that of the control (fig.2A). With 27 nmol/mg (fig.2C) the swelling rate is even higher than that obtained with 11 nmol thiomersal/mg (fig.2B). If after the addition of thiomersal (11 nmol/mg) excess thiosalicylate (57 nmol/mg) is added, the reactivated swelling is inhibited again (fig.2C). This result may be explained by formation of a complex between thiomersal and thiosalicylate, which relieves the effect of thiomersal. In addition it clearly demonstrates that thiomersal did not remove the blockade of the functional SH-group by MalNEt, but instead reactivated the phosphate transport by a reversible change.

The measurement of phosphate uptake in mitochondria by the swelling method, although well established, is only an indirect method. In order to exclude that thiomersal induced an unspecific change of the absorbance of mitochondria, the uptake of

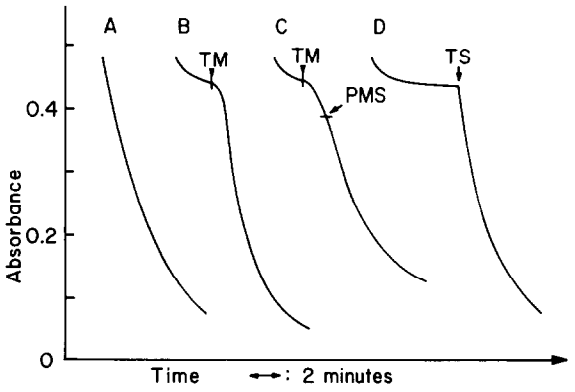


Fig.1. Effect of thiomersal (TM) and thiosalicylate (TS) on the swelling of PMS-inhibited mitochondria in ammonium phosphate. Swelling was measured in medium B (see section 2). (A) Control without inhibitor. (B) Mitochondria were preincubated for 1 min at 0°C with 27 nmol PMS/mg; addition, 36 nmol thiomersal/mg. (C) Preincubation as in (B) with 21 nmol PMS/mg; additions, 8.5 nmol thiomersal/mg and 21 nmol PMS/mg. (D) Preincubation as in (C); additions, 61 nmol thiosalicylate/mg.

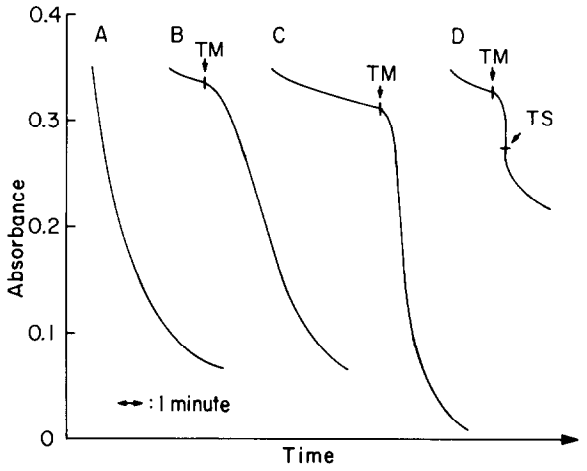


Fig.2. Effect of thiomersal (TM) and thiosalicylate (TS) on the swelling of MalNEt-inhibited mitochondria in ammonium phosphate. Swelling was measured in medium B (see section 2). (A) Control without inhibitor. (B) Mitochondria were preincubated for 1 min at 0°C with 32 nmol MalNEt/mg; addition, 11 nmol thiomersal/mg. (C) Preincubation as in (B); addition, 27 nmol thiomersal/mg. (D) Preincubation as in (B); addition, 32 nmol thiomersal/mg and 57 nmol thiosalicylate/mg.

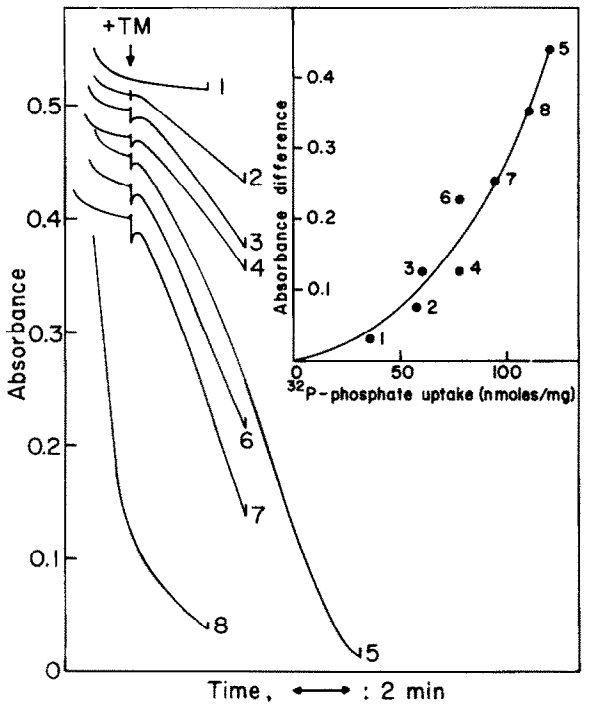


Fig.3. Correlation of swelling in ammonium phosphate with the uptake of [$^{32}$P]phosphate in mitochondria. The swelling medium (B) contained in addition [$^{32}$P]phosphate and [$^{3}$H]-sucrose. At the end of the swelling, as indicated in the figure, aliquots were taken and the [$^{32}$P]phosphate uptake was estimated as in section 2 (see insert). (1–7) Mitochondria were preincubated for 1 min at 0°C with 32 nmol MalNEt/mg; addition of thiomersal (TM) (nmol/mg) where indicated: 2 (12.4), 3 (17.8), 4 (20.9), 5 (23.3), 6 (31.0), 7 (46.5). (8) Control without inhibitor.

phosphate was controlled with [$^{32}$P]phosphate. In fig.3 the amount of [$^{32}$P]phosphate, taken up by the mitochondria during the swelling, is correlated with the change of absorbance at various concentrations of thiomersal. Both the swelling of the control and that of MalNEt inhibited thiomersal-activated mitochondria, correspond to an equivalent amount of [$^{32}$P]-phosphate taken up into the mitochondria.

The experiments of fig.1–3 were performed with swelling medium B containing high concentrations of potassium. Some organic mercurials are known, however, to induce a potassium permeability in mitochondria [19]. In order to exclude that phosphate might have been taken up into mitochondria via an electrogenic phosphate transporter [9] together with potassium, the experiments were repeated in potassium-free swelling medium A. The results (not shown) were almost identical: thiomersal could restore the swelling of MalNEt-, PMB- or mersalyl-inhibited mitochondria.

## 4. Discussion

It is generally accepted that the phosphate/proton symporter of mitochondria contains one or two functional SH-groups, which are sensitive against MalNEt and some organic mercury compounds (reviewed [20]). The inhibition of phosphate transport by MalNEt is suggested to be irreversible, whereas that obtained with organic mercury compounds can be totally reversed by addition of SH-compounds like cysteine or mercaptoethanol. These data show a reactivation of MalNEt or PMS-inhibited phosphate transport by thiomersal. The inhibition of thiomersal-reactivated phosphate transport by thiosalicylate (fig.2D) excludes the possibility that thiomersal might have reversed the inhibition by reaction with MalNEt. Another possibility could be the induction of an unspecific anion permeability in mitochondria by thiomersal. This, however, seems rather improbable since pretreatment of mitochondria with 30 nmol thiomersal/mg inhibits the uptake of malate, as measured by the swelling in isotonic malate [21]. The amount of phosphate taken up under swelling conditions in the presence of thiomersal (~120 nmol/mg) excludes the uptake of phosphate via the MalNEt-insensitive phosphate/dicarboxylate antiporter, because there is no equivalent amount of dicarboxylate anions in the mitochondria [22]. There are at least two possibilities to explain the uptake of phosphate in the presence of PMS or MalNEt and thiomersal.

(1) Thiomersal activates a latent phosphate transport system of mitochondria which is different from the known phosphate transport systems.
(2) It could be suggested that MalNEt and organic mercurials react with a SH-group of the phosphate/proton symporter which is not directly involved in the transport process.

The binding of the inhibitors causes some changes in the carrier protein, resulting in inhibition of phosphate transport. This change might be reversed by thiomersal without removing the SH-inhibitors. At present we cannot decide between the two possibilities.

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