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# PHOSPHATE TRANSPORT IN RAT-LIVER MITOCHONDRIA

J. B. HOEK, N. E. LOFRUMENTO*, A. J. MEYER AND J. M. TAGER

*Laboratory of Biochemistry, B. C. P. Jansen Institute**, University of Amterdam, Amsterdam (The Netherlands)*

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## SUMMARY

1. The kinetics of the efflux of $P_i$ and malate as well as the relationship between $P_i$ transport and intra- and extramitochondrial pH changes were studied in rat-liver mitochondria in the presence of rotenone and oligomycin at different pH's.

2. At high pH a fast efflux of $P_i$ from the mitochondria occurs in the first few seconds, followed by a slow re-entry of $P_i$ into the mitochondria. Under the same conditions the exit of malate shows a time lag of 2–4 sec. The exit of malate coincides with the re-entry of $P_i$.

3. In the presence of butylmalonate the exit of endogenous $P_i$ is coupled with a concomitant alkalinization of the mitochondrial matrix space, as calculated from the distribution of 5,5-[$^{14}$C]dimethyloxazolidine-2,4-dione.

4. The stoicheiometry of the $P_i$–hydroxyl exchange was found to be 1:1.

5. The kinetics of $P_i$ transport are consistent with previous observations that there is a direct exchange between $OH^-$ and $P_i$, but not between $OH^-$ and malate. The equilibrium distribution of $H_2PO_4^-$ and $OH^-$ deviates from the Donnan distribution. This may be explained by assuming a pH-dependent binding of $P_i$ in the mitochondria.

## INTRODUCTION

CHAPPELL AND CROFTS[1] and CHAPPELL AND HAARHOFF[2] have suggested that the transport of phosphate across the inner membrane of mitochondria is mediated by two specific translocators, one bringing about an exchange of $P_i$ with hydroxyl and the other an exchange with dicarboxylate ions like malate and succinate. Both translocators have been studied with the help of specific inhibitors such as butylmalonate (acting on the $P_i$–dicarboxylate exchange[3,4]) and sulphydryl-blocking reagents (acting on the $P_i$–hydroxyl exchange[5–9]). Some sulphydryl-blocking reagents, for instance mersalyl, inhibit the transport of $P_i$ *via* both carrier systems[9]. The inhibition by mersalyl can be relieved by addition of thiol compounds, like dithioerythritol.

Evidence of an exchange-diffusion reaction between $P_i$ and hydroxyl has been brought forward by PAPA *et al.*[10]. They found that the exit of $P_i$ (but not that of malate) is promoted by increasing the extramitochondrial pH. Using the bromthymol

Abbreviations: DMO, 5,5-dimethyloxazolidine-2,4-dione.

* Present address: Department of Biochemistry, University of Bari, Bari, Italy.

** Postal address: Plantage Muidergracht 12, Amsterdam, The Netherlands.

blue method of CHANCE AND MELA[11], they found that $P_i$ is able to discharge a pH gradient across the mitochondrial membrane that was built up by preincubation in the presence of valinomycin in a KCl medium. However, this method has been severely criticized[12–15].

In this study, more direct information on the relation between movements of $P_i$ and hydroxyl across the inner membrane of rat-liver mitochondria has been obtained. The stoicheiometry of the reaction was measured by studying changes in extramitochondrial pH, dependent upon the transport of $P_i$. Intramitochondrial pH changes were also studied, using the 5,5-dimethyloxazolidine-2,4-dione (DMO) method of WADDELL AND BUTLER[16] (see also refs. 17, 18).

By using the specific inhibitors of the movement of $P_i$ in exchange for malate and in exchange for hydroxyl, we have been able to separate in time an initial exit of $P_i$ (in exchange for hydroxyl) and a subsequent exchange of malate for $P_i$ initially extruded.

## METHODS

*Rat-liver mitochondria* were prepared exactly as described by MYERS AND SLATER[19].

*Experimental conditions*

(*a*) In the experiments at high buffer capacity the standard reaction mixture contained 15 mM KCl, 5 mM $MgCl_2$, 2 mM EDTA, 50 mM Tris–HCl, 2 μg/ml rotenone, 5 μg/ml oligomycin and 25 mM or 30 mM sucrose (derived from the mitochondrial suspension). Further additions, the reaction temperature, final volume and experimental procedure are indicated in the legends to the tables and figures.

In the experiments where the distribution of DMO was studied, the supernatant, obtained after rapid centrifugation of the reaction mixture in an Eppendorf Microfuge at maximal speed, was transferred to a temperature-controlled vessel connected with the pH meter (see (*b*)). The pH registered was taken as the value for external pH during the incubation. After determination of the pH, the supernatant and pellet were treated as described under (*b*), and samples of the supernatant and pellet were taken for the determination of $P_i$, and of $^3H$ and $^{14}C$ radioactivity.

The fraction of the pellet volume occupied by the mitochondrial matrix space was determined in parallel incubations in which [$^{14}C$]sucrose was added instead of DMO. In these incubations the same amount of ethyl acetate was added as in the DMO experiments.

The intramitochondrial pH was calculated from the distribution of DMO. The formula used by IRVINE *et al.*[20] and by ADDANKI *et al.*[18] which is derived from the Henderson–Hasselbach relation, was modified as follows:

$$pH_{in} = pK_a + \log\left[\left\{\frac{1}{V_m}(C-1)+1\right\}\{1+10^{pH_{out}-pK_a}\}-1\right]$$

where $pH_{in}$ = pH of the matrix; $pK_a$ = $pK$ of DMO; $C$ = ratio of specific activity of [$^{14}C$]DMO in total pellet water to specific activity of [$^{14}C$]DMO in supernatant; $V_m$ = fraction of the total pellet water occupied by the mitochondrial matrix space; $pH_{out}$ = pH of the extramitochondrial water.

The accuracy of the determinations in the experiments was estimated to be about 0.06 pH unit (*cf.* ref. 21).

(*b*) Experiments at low external buffer capacity were performed at 25° in a vessel (volume 1.2 ml) in which a glass electrode (Electrofact, Type 7GR 241) and a reference electrode were atttached. The electrodes were connected with an electrometer (Electronic Instruments Ltd. pH unit, Types C33B2 *plus* 33B2), for registration of the pH of the medium. The pH meter was calibrated with a standard phosphate buffer of pH 6.99 at 25°.

The reaction mixture contained 75 mM KCl, 5 mM $MgCl_2$, 2 mM EDTA, 3 mM Tris–HCl, 2 μg/ml rotenone, 5 μg/ml oligomycin, 3 mM butylmalonate and 25 mM sucrose (derived from the mitochondrial suspension) in a final volume of 1.21 ml. Other additions are indicated in the legends to Fig. 7 and Table II.

After all the additions had been made and the pH changes due to these additions had been completed, a 1-ml sample of the reaction mixture was transferred to a centrifuge tube. The mitochondria were rapidly spun down in an Eppendorf Microfuge at full speed. A sample of the supernatant was acidified with $HClO_4$ to a final concentration of 3.5%. The centrifuge tube was carefully blotted with filter paper and the protein was denatured by addition of 0.5 ml 3.5% $HClO_4$. After removal of protein, $P_i$ was determined in the supernatant and in the mitochondrial extract.

The buffering capacity of the reaction media was determined in parallel incubations by the addition of known amounts of HCl.

*Malate* was determined with citrate synthase, malate dehydrogenase, $NAD^+$ and acetyl-CoA (see ref. 22).

*Phosphate* was determined according to the method of WAHLER AND WOLLENBERGER[23].

*Radioactivity* was measured after dissolving 50-μl samples in 10 ml toluene–ethanol (3:1, by vol.) containing 2 g 2,5-diphenyloxazole *plus* 30 mg 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)-benzene per l.

*Protein* was determined with the biuret method according to CLELAND AND SLATER[24].

[2-$^{14}$C]DMO was obtained from New England Nuclear as a solution in ethyl acetate (specific activity 7.96 mC/nmole). $^{14}$C-labelled sucrose and $^3H_2O$ were obtained from the Radio-Chemical Centre, Amersham, England.

## RESULTS

### *Kinetics of the efflux of $P_i$ and malate from rat-liver mitochondria*

In the experiment of Fig. 1, the efflux of $P_i$ and malate from freshly isolated rat-liver mitochondria, suspended in media of different pH, was followed at 25°. At the times indicated, mersalyl was added to inhibit the $P_i$–hydroxyl and $P_i$–malate exchanges (see ref. 4). At pH 6.5, little if any movement of $P_i$ or malate occurred. The efflux of both anions was markedly enhanced by increasing the pH of the suspending medium. At pH 7.5 or 8.5, there was a rapid efflux of $P_i$ during the first 1–2 sec, followed by a slow re-entry. On the other hand, there was no exit of malate during the first 2–4 sec. The efflux of malate coincided approximately in time with the re-entry of $P_i$. The kinetics show clearly that an exit of $P_i$ precedes an exchange of external $P_i$ for internal malate.

The experiment of Fig. 2A shows that butylmalonate, which inhibits the $P_i$–malate exchange[3,4], largely abolished the re-entry of $P_i$ into the mitochondria. This influx of $P_i$ could also be prevented by adding malate to the incubation medium (Fig. 2B). With added malate, an exchange of intramitochondrial $P_i$ for extramitochondrial malate must have occurred.

In Fig. 3 it can be seen that the addition of small amounts of $P_i$ to the incubation medium reduced the extent of the initial $P_i$ efflux, without significantly affecting the entry of $P_i$ into the mitochondria during the second phase.

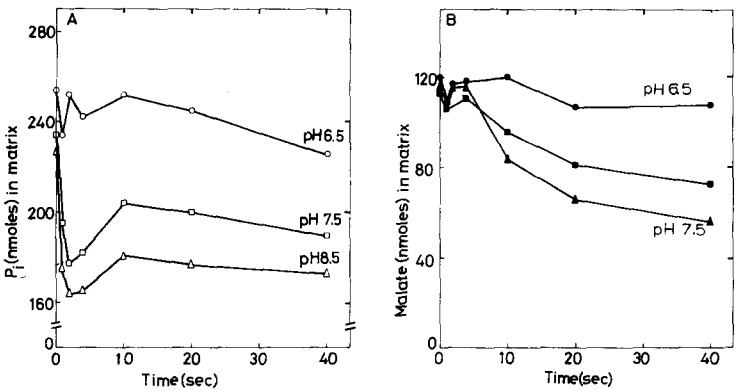


Fig. 1. Time-course of the efflux of $P_i$ and malate from freshly isolated rat-liver mitochondria at different pH values. Mitochondria (11.8 mg protein) were suspended in a medium containing the standard components *plus* 1 mM arsenite. Final volume, 1.5 ml. Temperature, 25°. At the times indicated 1 $\mu$mole mersalyl was added and immediately afterwards the mitochondria were separated from the incubation medium by rapid centrifugation in an Eppendorf microcentrifuge (Model 3200). $P_i$ and malate were determined in the neutralized mitochondrial extracts as described under METHODS.

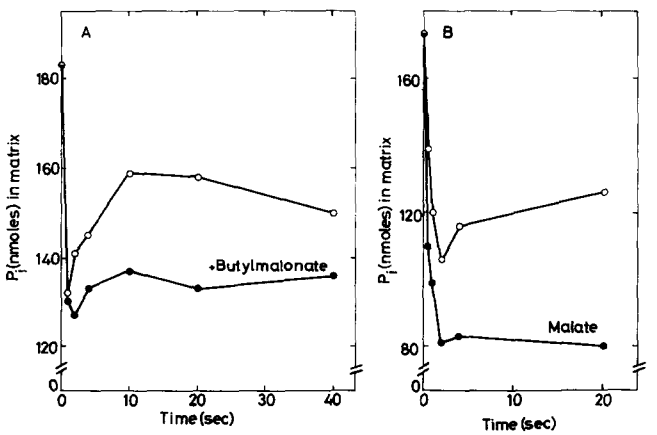


Fig. 2. Effect of butylmalonate and malate on the efflux of $P_i$ from freshly isolated rat-liver mitochondria. Mitochondria (Expt. A, 9.8 mg; Expt. B, 7.5 mg) were incubated in the same medium as described for Fig. 1. Final pH, 7.5. Where indicated, 2 mM butylmalonate (Expt. A) or 2 mM malate (Expt. B) were also present. For other experimental details, see Fig. 1.

*$P_i$ transport and intramitochondrial pH changes*

The movement of $P_i$ across the mitochondrial membrane in exchange for $OH^-$ should cause a change in internal pH of the mitochondrion. Changes in intramitochondrial pH were studied by determination of the distribution of [2-$^{14}$C]DMO across the mitochondrial membrane[16–18].

In Table I, lines 1–4, the results are shown of an experiment in which mitochondria were incubated in the presence of rotenone, oligomycin, butylmalonate and different concentrations of [$^{14}$C]DMO. The extramitochondrial pH was 7.48. An

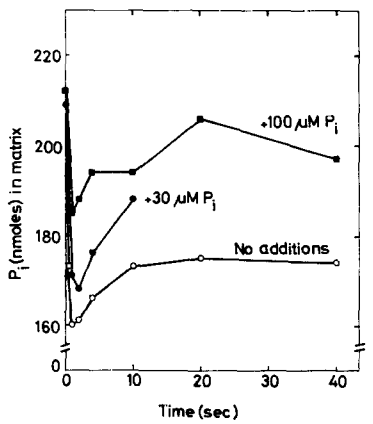


Fig. 3. Effect of small amounts of added $P_i$ on the efflux of $P_i$ from freshly isolated rat-liver mitochondria. Mitochondria (10.7 mg protein) were incubated in the same medium as described for Fig. 1. Final pH, 7.5. Where indicated, 45 or 150 nmoles $P_i$ were also present. For other experimental details, see Fig. 1.

TABLE I

EFFECT OF DMO CONCENTRATION AND ADDITION OF DINITROPHENOL AND NIGERICIN ON $P_i$ DISTRIBUTION AND pH GRADIENT

The reaction mixture contained the standard components (except for the DMO concentration, which was varied in the first four lines) and 5.0 mg protein. DMO, dinitrophenol (100 μM) and nigericin (2 μg/ml) were added as indicated. The reactions were carried out in small centrifuge tubes in a final volume of 1.3 ml. The final pH was 7.48. The reaction temperature was 25° ($pK_a$ of DMO at 25° is 6.33). The reactions were started by the addition of mitochondria and stopped after 1 min by rapid centrifugation. Further treatment as described under METHODS. *C* is the ratio of specific activity of [$^{14}$C]DMO in total pellet water to that in the supernatant (see METHODS).

| *Additions* | $[P_i]_{in}$ (*mM*) | $[P_i]_{out}$ (*mM*) | *C* | $pH_{in}$ | $pH_{in}-pH_{out}$ |
|---|---|---|---|---|---|
| DMO (3.0 μM) | — | 0.19 | 2.08 | 8.17 | 0.69 |
| DMO (7.5 μM) | 9.2 | 0.12 | 2.38 | 8.26 | 0.78 |
| DMO (15 μM) | 9.7 | 0.15 | 2.03 | 8.16 | 0.68 |
| DMO (30 μM) | 10.0 | 0.15 | 2.13 | 8.19 | 0.71 |
| None* | 8.7 | 0.17 | | | |
| DMO (15 μM) + dinitrophenol* | 4.4 | 0.24 | 1.16 | 7.68 | 0.20 |
| DMO (15 μM) + nigericin* | 4.1 | 0.23 | 0.89 | 7.26 | −0.22 |

* Two incubations

average value for the intramitochondrial pH of 8.19 was found, which is 0.71 pH unit higher than the extramitochondrial pH. This pH gradient is higher than those reported by ADDANKI *et al.*[18] (see DISCUSSION). The distribution of $P_i$ across the inner mitochondrial membrane is not significantly affected by the addition of DMO (Table I, line 5).

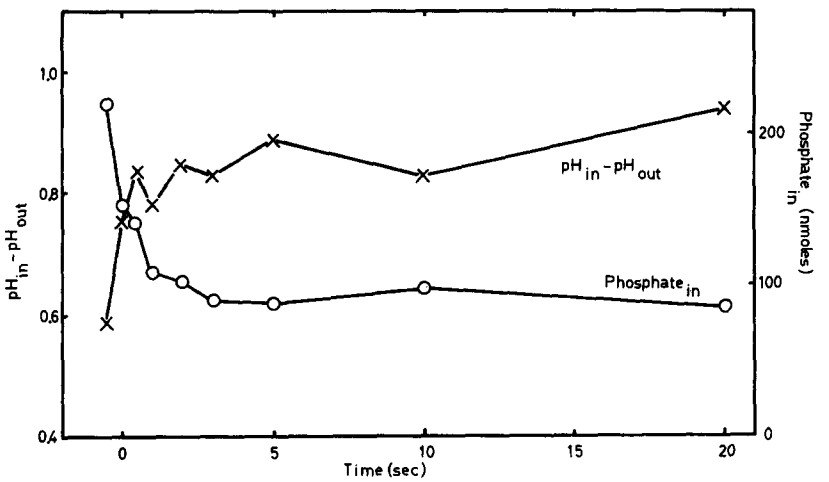


Fig. 4. Time-course of $P_i$ extrusion and change in pH gradient across the mitochondrial membrane. The incubation medium contained the standard components *plus* 6 mM butylmalonate, 15 $\mu$M [2-$^{14}$C]DMO, $^3H_2O$, 13 mM ethylacetate (derived from the DMO solution) and 6.5 mg protein. Final volume, 1.40 ml; final pH, 7.34. Temperature, 20°. Further experimental details, see Fig. 1 and METHODS. The experimental point indicated at $t = -0.5$ sec was obtained by pretreating the mitochondria with mersalyl for 30 sec, and starting the reaction by addition of the mixture of mersalyl and mitochondria to the same final concentrations of protein and mersalyl as in the other incubations.

Uncouplers promote an exit of $P_i$ from mitochondria[25]. Nigericin induces an exchange of protons for $K^+$ (ref. 26), so that it can be expected to affect an existing pH gradient. Moreover, this compound stimulates the exit of endogenous $P_i$ from the mitochondria[27]. Table I shows that both compounds decreased the intramitochondrial concentration of $P_i$, at the same time drastically diminishing the pH gradient across the mitochondrial membrane (see also ref. 28).

Fig. 4 shows the relationship between $P_i$ extrusion and the intramitochondrial pH change as a function of time in the presence of butylmalonate (*cf.* Fig. 2A).

It was observed that even when mersalyl was present from the start of the incubation, a considerable part of the total $P_i$ was found in the extramitochondrial space. This suggests that the inhibition by mersalyl is not instantaneous. This was tested by preincubating the mitochondria with 1 $\mu$mole mersalyl. After 30 sec, the mitochondria *plus* mersalyl were added to the reaction medium. Indeed, in this incubation, 220 nmoles $P_i$ were found inside the mitochondria and the pH gradient was 0.59 unit. These values are plotted at $-0.5$ sec, it being arbitrarily assumed that 0.5 sec was necessary for mersalyl inhibition to set in. The increase in intramitochondrial pH followed the same time-course as the $P_i$ movement. The largest change was observed in the period before mersalyl inhibition sets in.

A quantitative comparison of the relative rates of the $P_i$ movements and the pH change in this experiment is shown in Fig. 5. The changes in intramitochondrial pH and logarithms of the changes in $P_i$ concentration at the different times relative to the initial values are plotted in Figs. 5A and 5B, respectively. This illustrates again the close correlation between $P_i$ movement and the pH change, especially in the

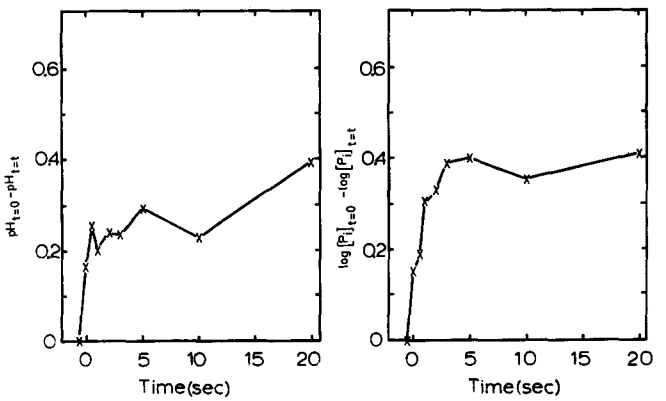


Fig. 5. Comparison of the rates of $P_i$ transport and intramitochondrial pH change. The plotted values are obtained from the points of Fig. 4.

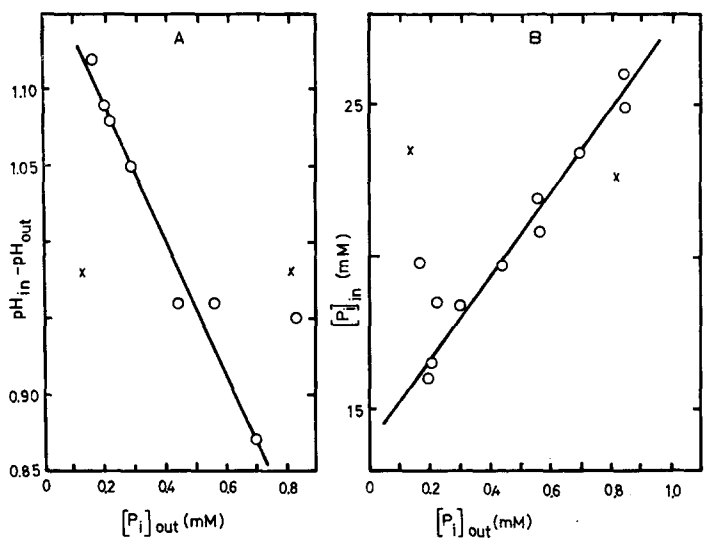


Fig. 6. Variation of intramitochondrial $P_i$ concentration and pH gradient across the mitochondrial membrane at different extramitochondrial $P_i$ concentrations. The reaction mixture contained the standard components *plus* 6 mM butylmalonate, 15 $\mu$M [2-$^{14}$C]DMO, $^3H_2O$, 13 mM ethyl acetate, 8.6 mg protein and different concentrations of added $P_i$ from 0 to 0.77 mM. The incubations were carried out in small centrifuge tubes in a final volume of 1.30 ml. The final pH was 6.56; the reaction temperature was 22° ($pK_a$ of DMO at 22° = 6.37). After a reaction time of 1 min mitochondria were separated from the incubation medium by rapid centrifugation. Further treatment was as described under METHODS. The final $P_i$ concentrations in the extramitochondrial space were plotted against the pH gradient across the mitochondrial membrane (A) and against the intramitochondrial $P_i$ concentration (B). O—O, experiments in the absence of mersalyl; X, experiments in the presence of mersalyl.

initial, rapid phase. The addition of small amounts of $P_i$, that partly prevents the exit of $P_i$ from the mitochondrial matrix space (*cf.* Fig. 3) also affects the pH gradient. This is shown in Fig. 6, where the final extramitochondrial $P_i$ concentration is plotted against the difference in intra- and extramitochondrial pH (Fig. 6A) and against the $P_i$ concentration in the matrix space (Fig. 6B).

The internal $P_i$ concentration increased linearly with the external $P_i$ concentration in the range studied. At the same time a decrease in intramitochondrial pH was observed, that also showed a linear relationship with the external $P_i$ concentration. The control incubations in the presence of mersalyl show that the pH changes were indeed due to the movement of $P_i$.

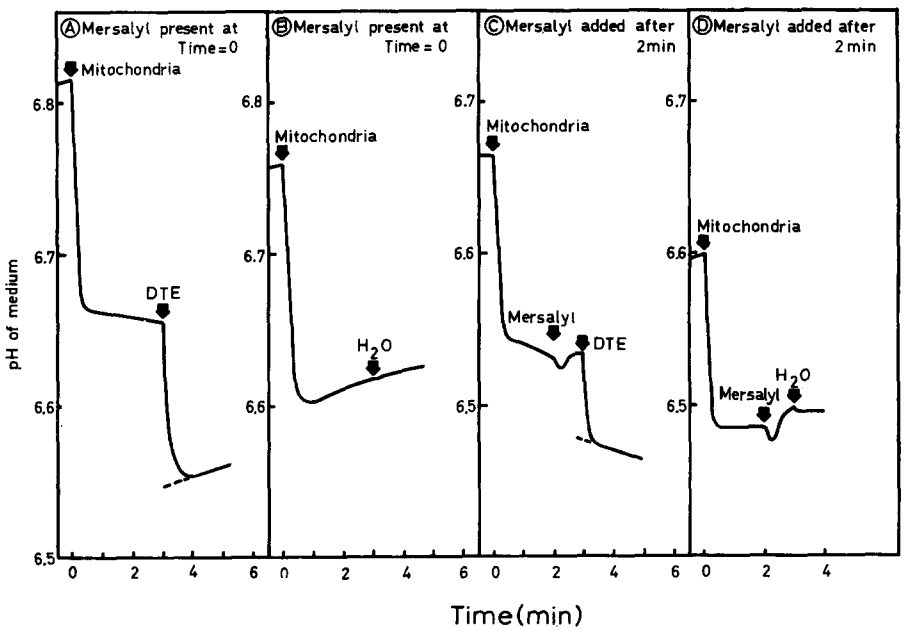


Fig. 7. Effect of mersalyl on dithioerythritol-induced pH changes. Experimental conditions as described under METHODS. In A and B the incubation medium contained the standard components *plus* 0.2 mM mersalyl. The reaction was started at $t = 0$ by addition of mitochondria (6.2 mg protein). At $t = 3$ min either dithioerythritol (DTE) was added to a final concentration of 0.3 mM, or an equivalent volume of water. At $t = 5$ min 1.0 ml of the incubation was removed for rapid centrifugation. In C and D the same procedure was followed, except that mersalyl was absent at the start of the reaction but was instead added at $t = 2$ min.

*Stoicheiometry of the $P_i$–hydroxyl exchange*

In order to confirm the direct relationship between movements of $P_i$ and $OH^-$, the stoicheiometry of this exchange was determined. In order to do this accurately, it was essential to separate the pH changes due to $P_i$ movement from possible pH changes due to other processes. Moreover the $P_i$–dicarboxylate exchange had to be inhibited. To fulfil these requirements rat-liver mitochondria were incubated in a medium of low buffer capacity in the presence of rotenone, oligomycin, and butylmalonate.

In the experiments of Fig. 7A, mitochondria were preincubated in the presence

TABLE II

STOICHEIOMETRY OF $P_i$–HYDROXYL EXCHANGE

Experimental details are described under METHODS and in the legend to Fig. 7. The buffer capacity of the medium in Expts. A and B was 1.63 mM $H^+$ per pH unit, in Expts. C and D 1.8 mM $H^+$ per pH unit. The concentration of mersalyl added was 0.2 mM and that of dithioerythritol 0.3 mM. $P_i$ extrusion was calculated as the difference between the amounts of $P_i$ in Expts. A and B, and in Expts. C and D (see Fig. 7). Results obtained from $P_i$ determinations in pellet and supernatant were averaged. The expected stoicheiometry was calculated on the basis of an extramitochondrial pH of 6.55.

| *Expt.* | *Additions at time (min)* | | | *Δ pH* | *Dithioerythritol-induced $H^+$ formation (nmoles)* | *Dithioerythritol-induced $P_i$ extrusion (nmoles)* |
|---|---|---|---|---|---|---|
| | *0* | *2* | *3* | | | |
| A | Mersalyl | — | Dithioerythritol | 0.109 | 215 | 95 |
| B | Mersalyl | — | Water | 0.000 | | |
| C | — | Mersalyl | Dithioerythritol | 0.058 | 123 | 23 |
| D | — | Mersalyl | Water | 0.002 | | |

Mersalyl-sensitive $H^+$ formation: 215 − 123 = 92 nmoles.
Mersalyl-sensitive $P_i$ transport: 95 − 23 = 72 nmoles.
Stoicheiometry found: 92/72 = 1.28.
Stoicheiometry expected: 1.18.

of mersalyl, so that the efflux of intramitochondrial $P_i$ was inhibited[5–8]. $P_i$ extrusion was induced by added dithioerythritol which relieves the inhibition by mersalyl. A sharp decrease in pH occurred on the addition of dithioerythritol. This pH change was considerably less if mersalyl was added to the incubation medium 2 min after the addition of mitochondria (Fig. 7C). This residual pH change is probably due mainly to binding of dithioerythritol to excess mersalyl. The controls (Figs. 7B and 7D) show that no pH change occurred when water was added instead of dithioerythritol. The addition of dithioerythritol in the absence of mersalyl did not give rise to a significant pH change, nor did the addition of an excess of dithioerythritol (not shown). The slight drift in pH which was found in most of the incubations was corrected for by extrapolation to the time of addition of dithioerythritol or water*.

The differences in $P_i$ extrusion found in the presence and in the absence of dithioerythritol (Figs. 7A and 7B, and Figs. 7C and 7D, respectively) were used to calculate the dithioerythritol-induced $P_i$ efflux (Table II). The dithioerythritol induced pH changes were converted into nmoles $H^+$ with the known buffer capacity of the incubation mixtures, determined in parallel experiments.

The difference in dithioerythritol-induced $P_i$ extrusion and $H^+$ formation between Expts. A and C is due to the mersalyl-sensitive $P_i$–hydroxyl exchange occurring in the first 2 min. The stoicheiometry of this reaction was calculated to be 1.28 (see Table II). Theoretically, in the case of an exchange of $H_2PO_4^-$ against $OH^-$, a stoicheiometry different from 1 is to be expected, because the $H_2PO_4^-$ will dissociate to a degree determined by the extramitochondrial pH, thus giving rise to an extra $H^+$ formation. At the pH of the experiment (6.55) the theoretical stoicheiometry was calculated to be 1.18.

* This drift may be due to instrumental artifacts, or to diffusion of $CO_2$.

DISCUSSION

*Calculation of the intramitochondrial pH from the distribution of DMO*

The DMO method, introduced in 1959 by WADDELL AND BUTLER[16], and applied to isolated heart mitochondria by ADDANKI *et al.*[17,18], is based on several assumptions.

(a) The concentration of the uncharged DMO in the mitochondrial matrix space is rapidly equilibrated with that in the extramitochondrial space.

(b) The rate of transport of the DMO anion is low compared to that of the uncharged acid. (An exchange mechanism between the DMO anion and hydroxyl, as suggested by GHOSH AND CHANCE[29], is formally equivalent to a transport of DMO in the uncharged form.)

(c) DMO is concentrated in the water phase, and does not bind to mitochondrial constituents.

(d) The $pK_a$ of DMO is the same inside and outside the mitochondrion.

(e) The distribution is not affected by a membrane potential or by any other form of energy.

(f) DMO does not affect mitochondrial processes.

ADDANKI *et al.*[17,18] have performed several experiments to test these assumptions for mitochondrial systems. They conclude that DMO meets all the requirements satisfactorily. ADDANKI *et al.*[18] used [$^{14}$C]dextran for the estimation of the intramitochondrial volume. It is known that dextran cannot penetrate the outer membrane of mitochondria, whereas it is the inner mitochondrial membrane that is supposed to be impermeable to $H^+$ or $OH^-$. This overestimation of the intramitochondrial volume causes an underestimation of the pH of the matrix space. We determined the intramitochondrial volume with [$^{14}$C]sucrose in parallel incubations. This gives values of about 1 $\mu$l matrix water per mg protein (*cf.* ref. 30) as opposed to a value of 2.25 $\mu$l water per mg protein obtained by ADDANKI *et al.*[17,18]. The concentration factors for the accumulation of DMO in the total pellet water found by ADDANKI *et al.*[18] are similar to those found in our experiments. It can be calculated from their data, assuming a matrix volume of 1 $\mu$l/mg protein, that the pH gradients are of the same order of magnitude (0.6–0.9 pH unit) as those calculated in our experiments, in spite of the differences in reaction conditions.

*Relationship between phosphate and malate movements and pH changes*

The stoicheiometry between $P_i$ movement and the amount of $H^+$ appearing in the external medium, as shown in Fig. 7 and Table II, is in accordance with a 1:1 exchange diffusion of $H_2PO_4^-$ and $OH^-$, as proposed by CHAPPELL AND CROFTS[1] and CHAPPELL AND HAARHOFF[2], and is formally equivalent to transport of $P_i$ as the uncharged acid, as proposed by MITCHELL AND MOYLE[31].

The experiments of Figs. 1–4 show that the initial, rapid extrusion of $P_i$ from the mitochondria is closely linked to an increase in the pH of the matrix space. The exit of malate is dependent on this prior efflux of $P_i$ in exchange for hydroxyl, indicating an exchange of intramitochondrial malate for extramitochondrial $P_i$. The extrusion of $P_i$ can be reduced by addition of small amounts of $P_i$; this results in a decrease of the pH gradient across the inner membrane. The exit of $P_i$ can also be decreased by lowering the concentration of $OH^-$ in the medium. Then the efflux of malate is decreased as well, even though malate does not exchange directly with

hydroxyl. These observations are in accordance with the existence of two translocators for $P_i$ in the inner mitochondrial membrane, one exchanging $P_i$ for hydroxyl and the other one exchanging $P_i$ for malate[1,2].

The experiments suggest that the high intramitochondrial pH is caused partly by the exit of $P_i$. An estimation of the pH gradient that would remain if no $P_i$ at all were transported can be made by extrapolation from the experiment of Figs. 4 and 5, if it is assumed that all the phosphate found in this experiment (305 nmoles) was originally present in the matrix space. The value of $\log[P_i]_{t=-0.5} - \log[P_i]_{t=t}$ is then $-0.14$. Applying this value to the correlated pH changes (assuming a constant buffer capacity) a minimal value of 0.45 pH unit can be calculated for the remaining pH gradient. The source of the gradient is unknown; it may be related to $P_i$ exit during preparation of the mitochondria. Furthermore, the incubation conditions may play a role in this respect. It was shown by ADDANKI *et al.*[18] that omission of $Mg^{2+}$ from the medium causes a significant decrease of the pH gradient.

The equilibrium relation between the distribution of $P_i$ and $OH^-$ can be quantitatively described by comparing the pH gradient with that of the $H_2PO_4^-$. The latter can be calculated from the experiments of Figs. 4 and 5, and Table I, by dividing the total $P_i$ by the factor $(1 + 10^{pH-pK_a})$. If a Donnan-type equilibrium distribution exists, and if the activity coefficient of $P_i$ is identical on both sides of the membrane, the Gibbs–Donnan ratio $([H_2PO_4^-]]_{in}/[H_2PO_4^-]_{out})/(10^{pH_{in}-pH_{out}})$ should be equal to 1. It was found that for all the experimental points the concentration gradient of $H_2PO_4^-$ is significantly higher than the activity gradient of $OH^-$. An average value for the Gibbs–Donnan ratio of $3.3 \pm 0.7$ (standard deviation) was found for the experiments performed at pH 7.5 and one of $1.9 \pm 0.3$ (standard deviation) for the experiments at pH 6.5. This would indicate that the factor causing the distribution of $H_2PO_4^-$ and $OH^-$ to deviate from the Donnan distribution is not constant at different pH values. One possible explanation may be a pH-dependent reversible binding of $P_i$ in the mitochondrion. Nevertheless, given a certain difference in activity coefficient of $P_i$ inside and outside the mitochondria, these results confirm earlier reports[28,32] in that the distribution of $P_i$ is determined by the pH gradient and *vice versa*.

If it is assumed that a difference in activity of $P_i$ in the intra- and extramitochondrial space causes the equilibrium distribution to deviate from the value expected, the pH gradient can be used to calculate the Donnan potential across the mitochondrial membrane due to this gradient:

$$V = \frac{RT}{F} \ln \frac{[H^+]_{out}}{[H^+]_{in}} = \frac{RT}{F} 2.30 \, (pH_{in} - pH_{out})$$

A pH gradient of 0.91 unit (as observed in the experiment of Fig. 4) would give a Donnan potential of 53 mV, positive inside. However, this value does not take into account possible gradients of other transportable ions.

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