Morris, D. R. (1982) *Biochim. Biophys. Acta 716*, 169–177.
Shain, S. A., Hilliard, J. K., & de Leon, C. (1983) *Endocrinology (Baltimore) 113*, 1292–1297.
Tabor, C. W., & Tabor, H. (1984a) *Annu. Rev. Biochem. 536*, 749–790.
Tabor, C. W., & Tabor, H. (1984b) *Adv. Enzymol. Relat. Areas Mol. Biol. 56*, 251–282.
Tang, K.-C., Pegg, A. E., & Coward, J. K. (1980) *Biochem. Biophys. Res. Commun. 96*, 1371–1377.
Williams-Ashman, H. G., & Pegg, A. E. (1981) *Polyamines in Biology and Medicine* (Morris, D. R., & Marton, L. J., Eds.) pp 43–73, Marcel Dekker, New York.

Kinetics of P_i–P_i Exchange in Rat Liver Mitochondria. Rapid Filtration Experiments in the Millisecond Time Range†

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ABSTRACT: Phosphate–phosphate exchange through the inorganic phosphate (P_i) carrier of rat liver mitochondria was investigated by a new rapid filtration technique, which does not require the use of transport inhibitors to stop the reaction and offers high time resolution (starting from 10 ms), thus allowing kinetic measurements on a fine time scale even at room temperature. At approximately 22 °C, isotopic equilibrium of [^{32}P]P_i is achieved within 0.8–2.5 s—depending on the P_i concentration—and an initial linear phase, lasting for 400–500 ms, is observed. Complete inhibition of P_i exchange by an excess (33 nmol/mg) of mersalyl, a well-known organomercurial inhibitor, required 200 ms, pointing to the insufficiency of this reagent for effective inhibitor stop. On the other hand, investigation of the effect of mersalyl (allowed to react with mitochondria for at least 20 s) on the initial rate of P_i exchange supports earlier observations on the protective effect of this inhibitor; i.e., up to 3 nmol of mersalyl/mg of protein does not decrease the transport rate whereas these low concentrations protect approximately 50% of the transport capacity from irreversible inactivation by *N*-ethylmaleimide. In nonrespiring mitochondria, at pH 7.3, P_i exchange exhibited a K_m of 1.6 mM and a V_{max} of 3.0 μmol min^{-1} (mg of mitochondrial protein)$^{-1}$. The increase of the membrane potential without any concomitant change of ΔpH had no significant influence on the kinetic parameters. The maximal velocity of P_i transport is significantly higher than the maximal velocity of all the other components of oxidative phosphorylation at comparable temperatures. The possible physiological significance of this excess capacity is discussed.

The mitochondrial phosphate carrier brings about both P_i–P_i exchange and net movement of the anion across the inner membrane, the latter process being accompanied by the cotransport of H^+ (or the countertransport of OH^-) [for a review, see Fonyó (1979)]. The carrier protein has been partially purified (Kolbe et al., 1982, 1984), and its kinetic properties have been investigated in reconstituted proteoliposomes (Wohlrab & Flowers, 1982; Wohlrab et al., 1984). The exceptional activity of this transport protein explains the scarcity of comparable data obtained in intact mitochondria. In fact, the only published study (Coty & Pedersen, 1974) was carried out at 0 °C with pCMB,[1] an organomercurial SH reagent in an "inhibitor-stop" assay.

In this paper we report a new approach to kinetic investigations that does not require the use of inhibitor substances to stop the transport process and exhibits significantly higher time resolution than previous techniques. The new method has allowed us to determine kinetic parameters at room temperature and under different metabolic conditions.

Experimental Procedures

Materials. For filtration 1.2-μm pore size cellulose nitrate filters (Millipore RAWP) were used. Carrier-free [^{32}P]-orthophosphoric acid, [^{14}C]methylamine, and [^{14}C]acetate were produced in Saclay, France. The supplied [^{32}P]P_i source was purified by filtration through Millex-HA sterile 0.45-μm filters (Millipore). All the other reagents were of the highest purity commercially available.

Theoretical Aspects of Rapid Filtration. The essential idea of the rapid filtration technique resides in the fact that the transport process is stopped by the cessation of substrate supply and not by the addition of a specific inhibitor. In this way there is no complication due to the evaluation of the time of mixing and action of the blocking agent. The scheme of Figure 1 shows the principle of the rapid filtration device. By means of a specifically designed filter holder, mitochondria are im-

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[1] Abbreviations: NADH, reduced nicotinamide adenine dinucleotide; NEM, *N*-ethylmaleimide; pCMB, *p*-(chloromercuri)benzoic acid; POPOP, 1,4-bis[5-phenyl-2-oxazolyl]benzene; PPO, 2,5-diphenyloxazole; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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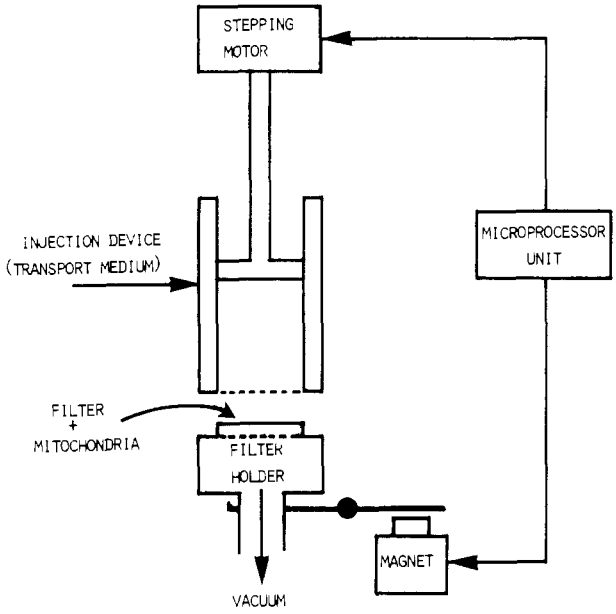

FIGURE 1: Scheme of the rapid filtration device.

mobilized on a constant surface ($\simeq$400 mm^2) of a Millipore nitrocellulose filter. Excess liquid is sucked off through the filter by a vacuum pump. The filter holder is then placed below an injection device and the transport medium is pushed through the filter (and thus through the mitochondrial layer) for a precisely controlled period of time. The constant vacuum allows the instantaneous linear passage of the fluid. The transport process is terminated by interruption of the substrate flow due to the synchronous stop of the piston movement and the spatial separation of the filter holder from the injection device. The movements of both piston and filter holder are controlled by a microprocessor unit. Filtration time can be varied between 10 ms and 10 s; the flow rate can be varied between 10 μL/s and 4 mL/s. The rapid filtration apparatus is produced by Bio-Logic Co. (Zirst, 38240 Meylan, France). A complete description of the equipment has been reported by Dupont (1984).

Practical Determination of $P_i \rightleftharpoons [^{32}P]P_i$ Exchange by Rapid Filtration. Mitochondria were routinely diluted to a final concentration of 2.5–3.5 mg/mL in a medium composed of 270 mM sucrose, 12 mM Tris-HCl, pH 7.3, 1 mM $MgCl_2$, 5 mM *n*-butyl malonate (potassium salt), 12 μM carboxyatractyloside, 2 μM rotenone, 2 μg of oligomycin/mL, and varying concentrations of P_i (potassium salt), pH 7.3 (preincubation medium). After a period of equilibration of 1–2 min, 1 mL of this suspension was placed in the filter holder of the rapid filtration apparatus and the excess fluid removed by the vacuum pump. The preincubation medium supplemented with tracer amounts of $[^{32}P]P_i$ was then passed through the immobilized mitochondria for a preset time. At the end of the transport step the filters were solubilized in the scintillation cocktail (PPO + POPOP + Triton X-100 + toluene) (Patterson & Greene, 1965), and radioactivity was counted in an Intertechnique SL30 scintillation spectrometer. In experiments where $[^{32}P]P_i$ efflux was followed, the preincubation medium contained $[^{32}P]P_i$, and the radioisotope was omitted from the transport medium.

Millipore filters take up 30–40 μL of the filtered solution. As this dead volume depends on both the amount of mitochondrial protein layered on them and the flow parameters, background radioactivity was determined in the presence of mitochondria in which the P_i carrier has been previously inhibited. In these cases 30 nmol of mersalyl or 150 nmol of NEM per milligram of protein was added to the preincubation medium, and the transport assay was carried out for the same period of time with the same flow rate as with the uninhibited samples. In $[^{32}P]P_i$ efflux experiments, during the preincubation period the inhibitor was added first, followed by $[^{32}P]P_i$. Intramitochondrial $[^{32}P]P_i$ content was calculated from the difference of counts obtained in the untreated and inhibitor-treated samples. As the presence of 5 mM *n*-butyl malonate excludes P_i movements through the dicarboxylate carrier, changes of intramitochondrial $[^{32}P]P_i$ content represent exclusively the activity of the P_i carrier (P_i^-/OH^- translocation). In dilute suspensions the intramitochondrial phosphate content changes rapidly (after 4–6 min), so care was taken not to dilute at once more than 5 mL of the suspension and to use it within 3 min.

Determination of ^{32}P Uptake by Centrifugation. In these experiments, rat liver mitochondria corresponding to 3 mg of protein were diluted in 1 mL of preincubation medium (the composition being the same as in the filtration experiments). After 1-min equilibration, a tracer amount of $[^{32}P]P_i$ was added in a small volume ($\simeq$10 μL). After a further 90-s incubation, mitochondria were spun down in an Eppendorf bench centrifuge, the supernatant was sucked off, and the tubes were rinsed with chilled 0.27 M sucrose. The pellets were dissolved in 4% sodium dodecyl sulfate, and radioactivity was counted in the same way as for the filters. Phosphate transport was again considered as the difference of noninhibited and mersalyl-treated samples (in the latter case mersalyl was added 60 s before $[^{32}P]P_i$).

Other Methods. Rat liver mitochondria were prepared according to Johnson & Lardy (1967). Protein was determined in the dilute mitochondrial suspension by the procedure of Lowry et al. (1951) using bovine serum albumin as standard. Chemical phosphate determination was carried out in trichloroacetic acid extracts with the method of Fiske & Subbarow (1925). Incorporation of $[^{32}P]P_i$ into adenine nucleotides was determined by the paper chromatographic method of Duée (1968) using a perchloric acid extract of mitochondria neutralized by KOH. The transmembrane pH difference was estimated from the distribution of isotopically labeled acetate and methylamine, as described by Nicholls (1974). Intramitochondrial NADH content was determined fluorometrically. Reduction of the nicotinamide nucleotides was achieved by preincubation of mitochondria for 5 min in the presence of 2 μM rotenone. The mitochondria were separated by centrifugation or by filtration, and in the latter case the transport process was imitated by passing 3 mL of preincubation fluid through the filters. Both the filters and the pellets were then solubilized in 4% sodium dodecyl sulfate, and the intensity of emitted light was recorded in a Perkin-Elmer MPF-2A spectrofluorometer at 440 nm, the excitation wavelength being 350 nm. Small volumes (<20 μL) of calibrated NADH solution were then added, and calculations were carried out on the basis of these internal standards.

RESULTS

Control of the Rapid Filtration Method. The high background radioactivity of the filters—arising from P_i concentrations in the millimolar range—forced us to increase the amount of mitochondrial protein up to 3.5 mg per assay. The rapid passage of the transport solution through this layer of particles could only be obtained by utilization of large pore size filters (1.2 μm). This condition and the exposure of mitochondria to important mechanical forces (constant vacuum combined with liquid pressure) prompted us to test the intactness of the particles in several ways.

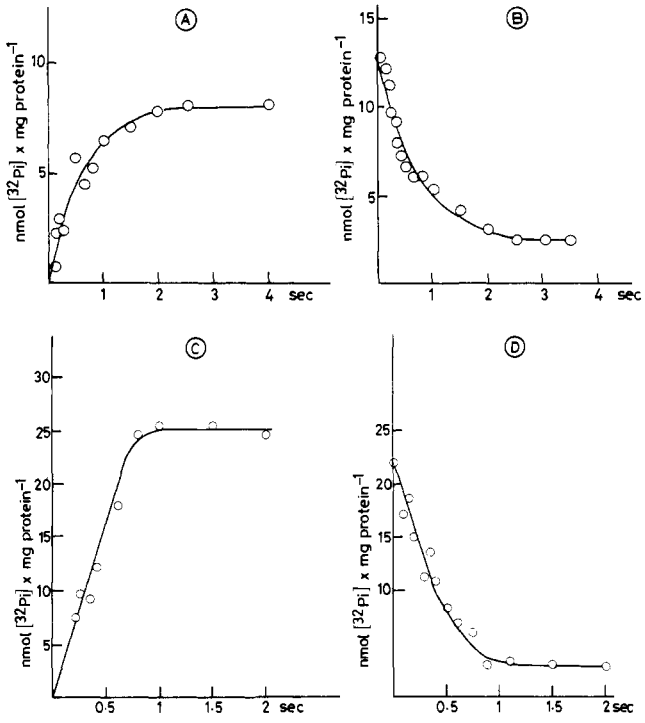

FIGURE 2: Time course of $[^{32}P]P_i$ influx (A and C) and efflux (B and D). The concentration of P_i during preincubation and transport was 0.5 (A and B) or 4 mM (C and D). The applied flow rate varied between 4 and 0.8 mL/s. The amount of mitochondrial protein layered on the filters was 3.3 (A and C) or 2.9 mg (B and D).

First, the rapid filtration technique was compared with the more classic centrifugation procedure. In these experiments both the mitochondria immobilized on filters and the centrifugation pellets were dissolved in 4% sodium dodecyl sulfate, and further determinations were done from these solutions. Following the filtration step, 96% of the deposited protein was recovered from the filters; thus, an extensive loss of the particles can be excluded.

The rationale of NADH determinations was to follow a low molecular weight compound present in the matrix space, the content of which should be a more sensitive indicator of membrane leakiness than the loss of high molecular weight enzymes. We found 14.8 nmol of NADH/mg of protein on the filters and 15.3 nmol/mg in the centrifugation pellet. As this difference represents only 3.5% of the total content, we feel that the possibility of serious disruption of mitochondrial membranes can be disregarded.

The only parameter of P_i transport that can be estimated at room temperature by the centrifugation method is the amount of intramitochondrial $[^{32}P]P_i$ in the state of isotopic equilibrium. We obtained 17.0 nmol of $[^{32}P]P_i$/mg of protein by filtration and 17.9 nmol/mg by centrifugation. The good correlation of the two techniques indicates that the flow rate was sufficient to provide adequate substrate supply on the filters. In addition, the rate of P_i transport was found to be independent of the flow rate in the range of 1–4 mL/s. Comparison of electron micrographs of mitochondria in the original suspension and after the rapid filtration process shows that the highly electron dense, "condensed" structure remained well preserved (data not shown).

Finally, the amount of $[^{32}P]P_i$ taken up in mitochondria for a period of time of 400 ms was linearly related to the amount of mitochondrial protein deposited on the filters within a range of 1–5 mg of protein (not shown).

Time Course of $[^{32}P]P_i$–P_i Exchange. The exchange reaction could be estimated by following either the influx or the efflux of the radiolabeled species. As the two approaches are subject to different kinds of experimental error, we preferred to carry out the exchange reaction in both directions. In Figure 2, typical time courses are shown in the presence of the highest and lowest P_i concentrations used in this study. It should be noted that the $[^{32}P]P_i$ influx and efflux curves are the mirror images of each other. In the rapid filtration technique, because of the continuous substrate flow during the transport process, the specific activity of the substrate remains constant in the external space, and isotopic dilution only occurs in the intramitochondrial space.

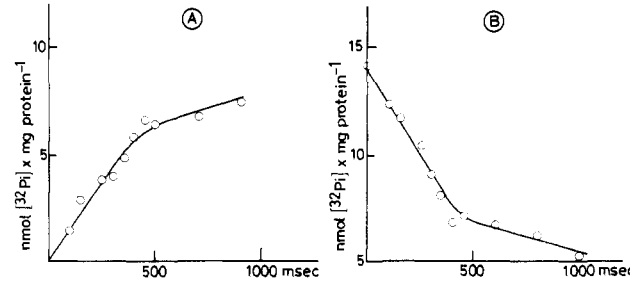

FIGURE 3: Detailed time course of $[^{32}P]P_i$ influx (A) and efflux (B). The concentration of P_i was 1 mM, and 3.2 (A) or 2.5 (B) mg of mitochondrial protein was layered on the filters. The flow rate varied between 1.5 and 4 mL/s.

The kinetics of $[^{32}P]P_i$ influx and efflux follows an exponential curve (Figure 2). In the case of 0.5 mM P_i the movements of $[^{32}P]P_i$ are essentially complete by 2.5 s (Figure 2A,B), whereas in the presence of 4 mM P_i less than 1 s is sufficient to achieve isotopic equilibrium (Figure 2C,D). The equilibration time proved to be rather constant and well reproducible in the individual experiments. In contrast to this, the amount of $[^{32}P]P_i$ in equilibrium showed some variations in the different preparations. For example, in 16 experiments carried out in nonrespiring mitochondria in the presence of 1 mM P_i, the rapidly exchangeable P_i pool varied between 12.3 and 17.8 nmol/mg of protein, the mean (±SD) being 14.8 ± 1.77 nmol/mg. This value accounts for 80% of the chemically detectable P_i content. The difference between the rapidly exchangeable and the total P_i pools increased if mitochondria were allowed to respire but virtually disappeared upon treatment with uncoupler or the Ca^{2+} ionophore A23187. This dependence of the nonexchangeable (or slowly exchangeable) P_i pool on the metabolic conditions suggests that it represents the fraction of phosphate chelated by Ca^{2+}.

The rapid phase of $[^{32}P]P_i$ influx shown in Figure 2 was followed by a slow increase in intramitochondrial $[^{32}P]P_i$ content, amounting to 2 nmol of P_i/mg of protein at the end of 120 s. This process might represent equilibration with a slowly exchangeable P_i pool, like the Ca–P_i complex discussed above. On the other hand, even in the presence of rotenone and oligomycin, we detected a residual incorporation of approximately 1 nmol of $[^{32}P]P_i$ into ATP in 2 min. These observations provide the explanation for the fact that, in the back-exchange experiments, 2–3 nmol of $[^{32}P]P_i$ remained attached to the mitochondria (see Figure 2B,D). It should be recalled that in these experiments the particles were preincubated for 2 min in the presence of labeled phosphate (see Experimental Procedures).

Investigation of $[^{32}P]P_i$ exchange on a more expanded time scale revealed an initial, virtually linear phase, lasting for approximately 400 ms (Figure 3). Within the limits of detection of our technique no indication of either a lag phase or an initial jump in the $[^{32}P]P_i$ content was observed. Thus, the present resolution does not allow distinction between the

Table I: Estimation of the Time of Action of Mersalyl[a]

phosphate concn (mM)	$[^{32}P]P_i$ in pellet (nmol/mg): no mersalyl (1)	mersalyl + $[^{32}P]P_i$ together (2)	mersalyl pretreatment (3)	total $[^{32}P]P_i$ uptake (4)	$[^{32}P]P_i$ uptake before action of mersalyl (5)	V_{in} of $[^{32}P]P_i$ transport (nmol mg^{-1} s^{-1}) (6)	time of action of mersalyl (ms) (7)
0.5	19.1	4.2	2.4	16.7	1.8	11.8	152
1	23.9	7.8	3.8	20.1	4.0	18.9	210
2	34.3	14.2	7.8	26.5	6.4	27.8	230
4	45.9	22.8	15.3	30.6	7.5	35.7	210

[a] Mitochondria (2.9 mg of protein) were diluted into 1 mL of preincubation medium (see Experimental Procedures) containing the indicated concentrations of potassium phosphate. Mersalyl (33 nmol/mg of protein) was added either 60 s before or simultaneously to $[^{32}P]P_i$. For further details see Experimental Procedures. Numbers in parentheses are column numbers. The values in column 4 are the differences between the values in columns 1 and 3; likewise, column 5 represents the difference between columns 2 and 3. Data in column 6 are obtained from Figure 4.

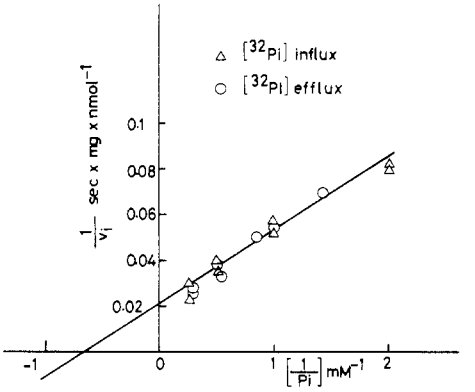

FIGURE 4: Double-reciprocal plot of the dependence of P_i–P_i exchange on the medium P_i concentration in nonrespiring mitochondria. Data were obtained from $[^{32}P]P_i$ influx (△) and $[^{32}P]P_i$ efflux (○) experiments. A straight line was fitted to the data points by the least-squares analysis to calculate the K_m and V_{max} values.

binding and the true translocation steps. We have limited our evaluation to a measurement of the initial rate of the transport curves. This was performed by fitting the experimental points up to 350 ms by a straight line.

Dependence of Initial Rate of P_i Transport on Phosphate Concentration. The experimental conditions allowed sufficient precision only in the concentration range of 0.5–4 mM P_i. The data of three individual experiments carried out on nonrespiring mitochondria are summarized in Figure 4. The double-reciprocal plot revealed a K_m of 1.6 mM and a V_{max} of 50 nmol mg^{-1} s^{-1} (3.0 μmol mg^{-1} min^{-1}). The observed K_m value is in good agreement with previous data obtained either by direct method at 0 °C (Coty & Pedersen, 1974) or by an indirect method at room temperature (Fonyó et al., 1974). The V_{max} of P_i transport is however 15 times higher at room temperature than at 0 °C.

The effect of various metabolic conditions on the kinetic parameters of P_i transport had not been investigated previously. We carried out a study on mitochondria respiring on endogenous substrates. In the rapid filtration procedure, the high flow rate of the transport medium equilibrated with air, 2–4 mL/s, ensured abundant oxygen supply of mitochondria immobilized on the filters. In spite of its slow rate, endogenous respiration was efficient enough to allow phosphorylation of endogenous ADP or accumulation of cations. On the basis of distribution of [^{14}C]methylamine and [^{14}C]acetate (Nicholls, 1974), it was verified that under the applied experimental conditions (at least 0.5 mM P_i present) the change of intramitochondrial pH induced by respiration was negligible (<0.1 unit). It should be remarked that the reproducibility of $[^{32}P]P_i$ transport rate measurements in respiring mitochondria was poorer than in nonrespiring ones. In five experiments carried out with respiring mitochondria, we found the following values: K_m = 1.85 ± 0.65 mM and V_{max} = 2.7 ± 0.6 μmol mg^{-1} min^{-1}. These values are rather close to those found in rotenone-blocked mitochondria.

Estimation of Reaction Time of Mersalyl. Organic mercurial compounds have been used previously in inhibitor-stop experiments as agents that block P_i movements (Coty & Pedersen, 1974). In view of the unexpectedly rapid initial phase of P_i transport the question arises as to whether organic mercurials really act as fast as it was believed.

The problem was examined in the experiment of Table I. The influx of $[^{32}P]P_i$ was measured at different P_i concentrations by the centrifugation method. Mitochondria were preincubated for 1 min in a medium containing the indicated concentration of unlabeled P_i. Subsequently, a tracer amount of $[^{32}P]P_i$ was added (1) in the absence of mersalyl, (2) together with mersalyl, or (3) 60 s after mersalyl (mersalyl was applied in large excess). $[^{32}P]P_i$ in the pellets of mersalyl-pretreated samples (column 3) represents the amount of P_i outside the matrix space. However, if the inhibitor was added at the same time as the transported substrate (column 2), more $[^{32}P]P_i$ was found in the pellets than in the mersalyl-pretreated samples. Thus, $[^{32}P]P_i$ influx began before the blocking effect of mersalyl set in. The amount of $[^{32}P]P_i$ taken up in this initial, "not yet inhibited" period (column 5) increased with the P_i concentration and accounted for approximately 25% of the total $[^{32}P]P_i$ transport in the presence of 4 mM P_i. Dividing the data of column 5 by the initial rates of transport obtained at the corresponding P_i concentrations gives a value of 150–230 ms (column 7) as the minimum time required for the development of full inhibition by mersalyl. The temperature dependence of P_i transport is presumably more pronounced than that of the reaction time of mersalyl; thus at low temperatures the relation of these two factors should be more favorable. Indeed, at 4 °C $[^{32}P]P_i$ uptake before the mersalyl block (calculated as in Table I) was significantly diminished, but it did not disappear completely (e.g., a value of 0.9 nmol of P_i/mg of protein was found in the presence of 1 mM P_i). Thus, even mersalyl, which is considered the most rapid inhibitor of the mitochondrial phosphate carrier, proves to be too slow with respect to the speed of P_i transport; its application in inhibitor-stop assays may lead to serious overestimation of the transport rate.

Effect of Low Mersalyl Concentrations on Initial Rate of P_i Transport. Mersalyl has an interesting effect on the P_i carrier of mitochondria. Low amounts of mersalyl do not inhibit P_i transport, but they protect the carrier from irreversible blocking agents, like NEM. When mitochondria are treated sequentially by mersalyl, followed by excess NEM, their P_i transport can be reactivated by mercaptoethanol or dithiothreitol, due to the displacement of bound mersalyl and

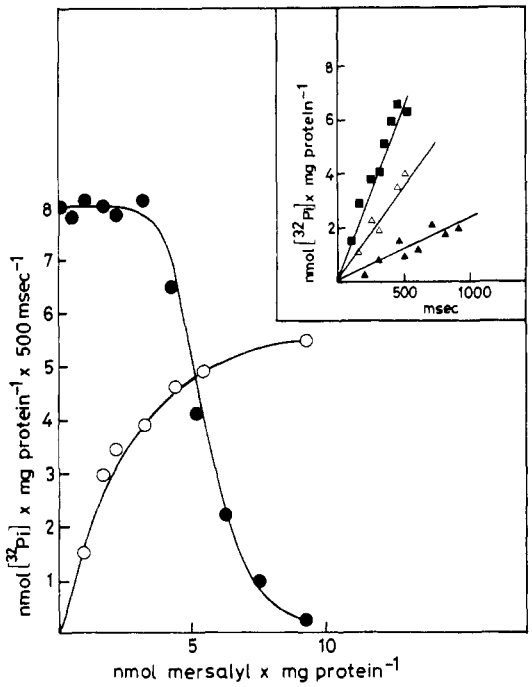

FIGURE 5: Effect of mersalyl on the initial velocity of P_i transport: demonstration of inhibition and protection in [^{32}P]P_i influx experiment in the presence of 1 mM P_i. (●) The indicated amounts of mersalyl reacted for 60 s with mitochondria. (○) Addition of the indicated amounts of mersalyl was followed after 60 s by 170 nmol of NEM/mg of protein and after a further 60 s by 2.8 mM mercaptoethanol. [^{32}P]P_i influx was measured for 500 ms, the flow rate being 2 mL/s; 3.4 mg of mitochondrial protein was layered on the filter. Insert: Effect of 4.1 (△) or 6.5 (▲) nmol of mersalyl/mg of protein on the initial rate of [^{32}P]P_i influx; (■) control without mersalyl. P_i concentration, 1 mM; protein, 3.3 mg/filter; flow rate, between 2 and 4 mL/s.

the consequent recovery of a fraction of SH groups belonging to the P_i carrier. So far, this well-known phenomenon has only been demonstrated by indirect techniques based on osmotic swelling of mitochondria (Fonyó et al., 1974, 1975) or by measuring [^{32}P]P_i uptake at 10 s, i.e., under equilibrium conditions (Touraille et al., 1980). It was therefore interesting to verify whether the "not inhibiting but nevertheless protecting effect" of low mersalyl concentrations could also be demonstrated under conditions where the true initial rate was measured.

Partially inhibitory concentrations of mersalyl decreased the rate of the initial phase of [^{32}P]P_i influx but did not alter the linear character of it (see insert of Figure 5). Thus, in the decisive experiment, [^{32}P]P_i influx was only measured at 500 ms. Two parallel series of incubations were carried out: one series was in the presence of various amounts of mersalyl whereas in the other series mersalyl was followed by NEM and finally by an excess of mercaptoethanol. It was also controlled so that either NEM alone or NEM followed by mercaptoethanol brought about complete inhibition of P_i exchange. As shown in Figure 5, up to 3 nmol of mersalyl/mg of protein did not influence the initial velocity whereas the protecting effect of these low concentrations against NEM inhibition was evident. Thus, direct initial rate measurements confirm the results obtained previously by indirect techniques.

Discussion

Technical Aspects. In this paper we report the application of a new technique suitable for measurements on various transport systems or immobilized enzymes. In principle, the rapid filtration device allows measurements as short as 10 ms (Dupont, 1984). So its time resolution is at least 1 order of magnitude better than that of previously available apparatuses (Palmieri & Klingenberg, 1979). Due to the low substrate affinity of the mitochondrial P_i carrier, we were obliged to work under very unfavorable experimental conditions (high radioactivity, significant background, high concentration of mitochondria) that rendered impossible the acquisition of reproducible data below 100 ms and the detailed analysis of slow processes. In spite of these limitations the applied method proved to be valuable in determining the initial rate of P_i transport at room temperature, a parameter that was unobtainable in earlier studies.

An indisputable merit of the rapid filtration technique is that the transport process is terminated by the cessation of substrate supply and not by an inhibitor substance. This fact is especially important in the case of mitochondrial P_i transport. As we show in Table I, complete inhibition by mersalyl is only achieved in approximately 200 ms; this interval allows the translocation of 10–25% of the total exchangeable P_i pool. In addition, the reaction time of the inhibitor might depend on the metabolic conditions (Fonyó & Vignais, 1980; Ligeti & Fonyó, 1984). Thus, mersalyl does not satisfy the criteria of an inhibitor appropriate for inhibitor-stop or quench-flow experiments at room temperature. At 0 °C, the use of mersalyl might be less subject to criticism than its use at room temperature because of the lower rate of P_i transport. As the other inhibitors of the mitochondrial P_i carrier are known to act more slowly, for the time being we do not see any alternative way to study kinetic aspects of P_i transport.

The rather unusual conditions utilized in rapid filtration experiments might give rise to some criticism. However, both the results of control measurements and the good agreement between [^{32}P]P_i influx and efflux data (Figures 2 and 3) suggest that mitochondria preserved their structural and functional integrity.

Transport Capacity of the P_i Carrier. In nonrespiring mitochondria we found the K_m of P_i–P_i exchange to be 1.6 mM, in good agreement with previously published data (Coty & Pedersen, 1974; Fonyó et al., 1974). On the other hand, at approximately 22 °C the maximal transport rate was 3000 nmol mg^{-1} min^{-1}, i.e., 15 times higher than at 0 °C (Coty & Pedersen, 1974). The maximal rates of oxidative phosphorylation observed in respiring liver mitochondria with succinate and pyruvate, respectively, as substrates at 25 °C are about 100 [e.g., Groen et al. (1982)] and 150–175 nmol of O_2 min^{-1} mg^{-1} [e.g., Doussière et al. (1984)], which allow the production of 400 and 900–1050 nmol of ATP min^{-1} mg^{-1}, respectively. The complex of substrate carrier–dehydrogenase–respiratory chain has no excess capacity, as the rate of O_2 consumption stimulated by different means (e.g., uncoupler, Ca^{2+}, valinomycin plus K^+) does not exceed the above-mentioned values. The maximal rate of ATPase (ATP synthase) in situ is hard to estimate. Under optimal conditions (pH 8, high HCO_3^- concentration, presumably released endogenous inhibitor) the specific activity was reported to be 1.3–3.9 μmol min^{-1} (mg of total mitochondrial protein)$^{-1}$ at 25 °C (Soper & Pedersen, 1976; Lambeth & Lardy, 1971). Under natural conditions this value should be less. It is noteworthy that the observed V_{max} for P_i transport exceeds greatly the overall rate of oxidative phosphorylation. One is tempted to search for the rationale of this remarkable property. The fact that net P_i transport is coupled to H^+ movement could provide a possible explanation. In this aspect the function of the P_i carrier would be not only to supply one of the elementary substrates of oxidative phosphorylation into the matrix space but also to equilibrate rapidly transmembrane pH differences, and thereby

to maintain the H^+ electrochemical gradient almost completely in the form of membrane potential.

In the following, the turnover of the P_i carrier is compared to that of a selected number of transport systems. In three different studies the amount of the P_i carrier of rat liver mitochondria was estimated to be 50–60 pmol/mg of protein (Coty & Pedersen, 1975; Hadvary & Kadenbach, 1976; Fonyő & Vignais, 1980). From these data we arrive at a turnover number of 50 000–60 000 min^{-1}, at a temperature of 20–25 °C. This value is significantly higher than the corresponding parameters of other membrane transport systems studied in detail: mitochondrial adenine nucleotide carrier at 18 °C, 500 min^{-1} (Klingenberg, 1976); lactose permease of *Escherichia coli* at 25 °C, 2900 min^{-1} (Wright et al., 1983); brush border membrane Na^+–glucose transporter at 20 °C, 1200 min^{-1} (Semenza et al., 1984); P_i carrier of chloroplast membrane at 20 °C, 5000 min^{-1} (Flügge & Heldt, 1979); sarcoplasmic reticulum Ca-ATPase at 25 °C, 600–1200 min^{-1} (Inesi, 1972); plasma membrane Na,K-ATPase, 4400–16 700 min^{-1} (Bader et al., 1968). In contrast to this, the band 3 protein of erythrocytes responsible for Cl^-/HCO_3^- exchange has at 38 °C a turnover number of 50 000 s^{-1} (Lowe & Lambert, 1983). The calculated turnover number of the P_i carrier in intact rat liver mitochondria is also approximately 1 order of magnitude higher than in reconstituted proteoliposomes containing the purified beef heart protein [2000 min^{-1}, Wohlrab & Flowers (1982); 7000 min^{-1}, Wohlrab et al. (1984)]. Thus, either the purity and the functional integrity of the isolated protein or eventually the amount of the P_i translocator in the intact mitochondria has to be reevaluated.

Kinetic Parameters under Various Conditions. This is the first time that the effect of mersalyl on the initial rate of P_i transport could be investigated directly, excluding any kind of artifacts due to limitations by compensating ion movements or to insufficient time resolution. As shown in Figure 5, up to 3 nmol of mersalyl/mg of protein did not diminish the initial velocity of P_i translocation whereas it protected the P_i carrier effectively from the effect of irreversible blocking agents. Thus, direct rate measurements do support the hypothesis about two equivalent SH groups on the external surface of the transport protein, a hypothesis originally based on indirect measurements (Fonyő et al., 1974).

The rapid filtration device is suitable for investigation of kinetic properties under different metabolic conditions. We examined the effect of respiration. An increase in the membrane potential, without any concomitant change in the transmembrane pH difference, had no significant influence either on the affinity or on the maximal transport rate of the P_i carrier. More exciting and probably more informative would be to discern the effect of pH on the kinetic parameters. It should be recalled that modifications of the intramitochondrial (transmembrane?) pH alter the position of SH groups situated on the external surface, suggesting a conformational change in the protein structure (Ligeti & Fonyő, 1984). Unfortunately, a significant pH difference across the internal mitochondrial membrane can only be created in the absence of external phosphate, excluding the possibility of $[^{32}P]P_i$ exchange experiments. To find adequate conditions where the kinetics of net P_i transport can be followed without the limitations of H^+-compensating processes is our future project.

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Registry No. Phosphate, 14265-44-2; mersalyl, 492-18-2.

References

Bader, H., Post, R. L., & Bond, G. H. (1968) *Biochim. Biophys. Acta 150*, 41–46.

Coty, W. A., & Pedersen, P. L. (1974) *J. Biol. Chem. 249*, 2593–2598.

Coty, W. A., & Pedersen, P. L. (1975) *J. Biol. Chem. 250*, 3515–3521.

Doussière, J., Ligeti, E., Brandolin, G., & Vignais, P. V. (1984) *Biochim. Biophys. Acta 766*, 492–500.

Duée, E. D. (1968) *Bull. Soc. Chim. Biol. 50*, 1215–1219.

Dupont, Y. (1984) *Anal. Biochem. 142*, 504–510.

Fiske, C. H., & Subbarow, Y. (1925) *J. Biol. Chem. 66*, 375–400.

Flügge, U. I., & Heldt, H. W. (1979) in *Function and Molecular Aspects of Biomembranes Transport* (Quagliariello, E., Palmieri, F., Papa, S., & Klingenberg, M., Eds.) pp 373–382, Elsevier/North-Holland, Amsterdam.

Fonyő, A. (1979) *Pharmacol. Ther. 7*, 627–645.

Fonyő, A., & Vignais, P. V. (1980) *J. Bioenerg. Biomembr. 12*, 137–149.

Fonyő, A., Palmieri, F., Ritvay, J., & Quagliariello, E. (1974) in *Membrane Proteins in Transport and Phosphorylation* (Azzone, G. F., Klingenberg, M. E., Quagliariello, E., & Siliprandi, N., Eds.) pp 283–286, North-Holland, Amsterdam.

Fonyő, A., Ligeti, E., Palmieri, F., & Quagliariello, E. (1975) *Proc. FEBS Meet. 35*, 287–306.

Groen, A. K., Wanders, R. J. A., Westerhoff, H. V., Van der Meer, R., & Tager, J. M. (1982) *J. Biol. Chem. 257*, 2754–2757.

Hadvary, P., & Kadenbach, B. (1976) *Eur. J. Biochem. 67*, 573–581.

Inesi, J. (1972) *Annu. Rev. Biophys. Bioeng. 1*, 191–210.

Johnson, D., & Lardy, H. (1967) *Methods Enzymol. 10*, 94–96.

Klingenberg, M. (1976) in *The Enzymes of Biological Membranes: Membrane Transport* (Martonosi, A. N., Ed.) Vol. 3, pp 383–438, Plenum Press, New York.

Kolbe, H. V. J., Costello, D., Wong, A., Lu, R. C., & Wohlrab, H. (1984) *J. Biol. Chem. 259*, 9115–9120.

Lambeth, D. O., & Lardy, H. A. (1971) *Eur. J. Biochem. 22*, 355–363.

Ligeti, E., & Fonyő, A. (1984) *Eur. J. Biochem. 139*, 279–285.

Lowe, A. G., & Lambert, A. (1983) *Biochim. Biophys. Acta 694*, 353–374.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Nicholls, D. G. (1974) *Eur. J. Biochem. 50*, 305–315.

Palmieri, F., & Klingenberg, M. (1979) *Methods Enzymol. 56*, 279–301.

Patterson, M. S., & Greene, R. C. (1965) *Anal. Chem. 37*, 854–857.

Semenza, G., Kessler, M., Hosang, M., Weber, J., & Schmidt, U. (1984) *Biochim. Biophys. Acta 779*, 343–379.

Soper, J. W., & Pedersen, P. L. (1976) *Biochemistry 15*, 2682–2690.

Touraille, S., Briand, Y., Alziari, S., & Durand, R. (1980) *FEBS Lett. 121*, 230–234.

Wohlrab, H., & Flowers, N. (1982) *J. Biol. Chem. 257*, 28–31.

Wohlrab, H., Collins, A., & Costello, D. (1984) *Biochemistry 23*, 1057–1064.

Wright, J. K., Riede, I., & Overath, P. (1981) *Biochemistry 20*, 6404–6415.