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# UPTAKE OF OXIDIZED FOLATES BY RAT LIVER MITOCHONDRIA

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**Folate, dihydrofolate, and methotrexate are rapidly taken up by rat liver mitochondria. The apparent maximal matrix folate concentration is about 2.5-fold that of the suspending medium, whereas dihydrofolate and methotrexate equilibrate across the inner membrane. Fully reduced folates, including tetrahydrofolate, 5-methyltetrahydrofolate, and 5,10-methylenetetrahydrofolate penetrate only the intermembrane space. Addition of dihydrofolate or methotrexate effects a rapid release of pre-loaded folate, and external methotrexate promotes the release of pre-loaded dihydrofolate. The extent of dihydrofolate uptake is enhanced by addition of folate. These results suggest that oxidized folates are transported to the matrix by a carrier-mediated mechanism.**

## Introduction

The presence of folate derivatives in the mitochondrial fraction of rat liver was first reported by Wang et al. [1], with 10-formyltetrahydrofolate being the major constituent, while smaller amounts of 5-methyltetrahydrofolate, and possibly tetrahydrofolate, were also detected. Although the mitochondrial localization of these compounds was not determined, their presence in the matrix is indicated by the coincident release of pyridine nucleotides and folates during mitochondrial swelling [2] and by the matrix localization of tetrahydrofolate-dependent serine transhydroxymethylase [3]. The in vivo uptake of folate by mitochondria is suggested by the work of Shin et al. [4], who reported a rapid incorporation of label into reduced and oxidized polyglutamylfolates of various subcellular fractions of rat liver, including mitochondria, after intraperitoneal injection of [$^3$H]folate. However, there have been no previous reports of folate uptake by isolated mitochondria. That tetrahydrofolate is not transported across the mitochondrial inner membrane is indicated by the lack of mitochondrial swelling in isotonic ammonium tetrahydrofolate and by the latency of serine transhydroxymethylase [3], even though serine rapidly enters the matrix by a carrier-mediated process [5]. In this communication we report the uptake of folate, dihydrofolate, and methotrexate, and confirm the lack of transport of reduced folates, by rat liver mitochondria.

## Experimental

Rat liver mitochondria were prepared from Sprague-Dawley male rats according to a modification of the method of Schnaitman and Greenawalt [6], using 0.25 M sucrose as the isolation medium. In experiments where lysosomes were removed from the mitochondrial fraction, the digitonin treatment procedure as described by Kun [7] was utilized. Lysosomal contamination of treated mitochondria, determined by monitoring the activity of β-*N*-acetylhexosaminidase using 4-methylumbelliferyl-2-acetamido-2-

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Mops, 3-(*N*-morpholino)propanesulfonic acid.

deoxy-β-D-glycopyranoside as substrate [8], was decreased by approx. 90%. Protein determinations were performed by the biuret method [9]. NADPH, folic acid, 5-methyltetrahydrofolate, and methotrexate were obtained from Sigma Chemical Company. [3′,5′,9(*n*)-$^3$H]Folate, [2-$^{14}$C]folate, [5-$^{14}$C]methyltetrahydrofolate, and [3′,5′,9(*n*)-$^3$H]methotrexate were obtained from Amersham/Searle Corporation. [$^{14}$C]Sucrose and $^3$H-labeled water were purchased from New England Nuclear. Thymidylate synthetase and dihydrofolate reductase, isolated from *Lactobacillus casei*, were generous gifts from Dr. R.B. Dunlap.

[3′,5′,9(*n*)-$^3$H]Folate, [2-$^{14}$C]folate, and [3′,5′,9-(*n*)-$^3$H]methotrexate were purified by the procedure of Goldman et al. [10] followed by chromatography on Bio-Rad P-10 to ensure removal of ammonium bicarbonate. Unlabeled and dihydro[3′,5′,9(*n*)-$^3$H]-folate were prepared from folate and [3′,5′,9(*n*)-$^3$H]folate, respectively, by a modification of the procedure of Blakley [11]. Dihydrofolate concentration was estimated using either $\epsilon_{225} = 24\,100$, $\epsilon_{282} = 22\,400$ [12] or by dihydrofolate reductase assays [13]. Similar results were obtained with either method. (+)-L-Tetrahydro[3′,5′,9(*n*)-$^3$H]folate was prepared by dihydrofolate reductase catalyzed reduction of [3′,5′,9(*n*)-$^3$H]folate and purified by the method of Zakrzewski and Sansone [14]. Tetrahydrofolate was quantitated either by thymidylate synthetase assay [15] or spectrophotometrically using $\epsilon_{297} = 29\,100$ [16]. All purified folates, as well as 5-[$^{14}$C]methyltetrahydrofolate, were radiochemically pure when chromatographed on Whatman No. 3 MM paper in either butanol/pyridine/water (1 : 1 : 1, v/v) or 0.5% aqueous sodium carbonate. (−)-L-5,10-Methylenetetrahydro[2-$^{14}$C]folate was produced by incubating 290 μM (+)-L-tetrahydro[2-$^{14}$C]folate with 1 mM formaldehyde [17] for at least 5 min before initiating the uptake experiment.

Uptake studies were performed by the procedure of Harris and Van Dam [18]. The incubation medium (pH 7.4) at 4°C contained 112 mM KCl, 10 mM $MgCl_2$, 1 mM KCN, 1.5 mM EDTA, 7.5 mM Mops buffer, the appropriate concentration of $^3$H- or $^{14}$C-labelled compounds, and mitochondrial protein (5 mg/ml). Ascorbate (10 mM) was added to suspensions containing either dihydrofolate or tetrahydrofolate. At indicated times, 200-μl aliquots were removed and centrifuged through silicon oil. Silicon oil was either General Electric Versilube (R) F-50 or a mixture (183 : 17) of Dow Corning DC550 : DC200. Mixtures containing [$^3$H]folates also contained 0.1 μCi [U-$^{14}$C]sucrose to assess the intermembrane space; while 0.1 μCi $^3$H-labeled water was added to mixtures containing [$^{14}$C]folates to determine total mitochondrial volume. Intermembrane space and total mitochondrial volumes were also determined in parallel experiments containing both 0.1 μCi $^3$H-labeled water and 0.1 mCi [U-$^{14}$C]-sucrose. For 1 mg of mitochondrial protein the average [$^3$H]labeled water volume was 3.10 ± 0.45 μl and the average [U-$^{14}$C]sucrose volume was 2.25 ± 0.35 μl. All samples were counted in glass scintillation vials containing 10 ml of Beckman Ready Solv HP liquid.

In counter-transport experiments, mitochondria (10 mg protein) were incubated in 1.8 ml of solution (pH 7.4) containing 125 mM KCl, 11 mM $MgCl_2$, 1.1 mM KCN, 1.7 mM EDTA, 8.3 mM Mops buffer, 0.1 μCi [U-$^{14}$C]sucrose, and 0.1 μCi $^3$H in either 80 μM dihydrofolate or 35 μM folate. Intermembrane space and total mitochondrial volumes were determined as described above. At indicated times, aliquots containing 1 mg protein were removed and spun through silicon oil. At 6 min, 140 μl of solution of 10 mM potassium phosphate (pH 7.4), or 5 mM of the appropriate unlabeled folate was added. Subsequently, aliquots containing 1 mg protein were removed at indicated times and spun through silicon oil to determine the apparent influx or efflux of the radiolabeled folate.

## Results

The uptake of several folates by rat liver mitochondria is shown in Fig. 1. Tetrahydrofolate, 5-methyltetrahydrofolate, and 5,10-methylenetetrahydrofolate uptake is limited to the intermembrane space. By contrast, folate is accumulated by mitochondria giving, in fourteen experiments, an apparent maximal matrix concentration of 2.57 ± 0.46 times that of the external medium. An initial very rapid uptake of folate, corresponding to equilibration across the inner membrane, is followed by an accumulation phase that is variable in rate and often difficult to discern. Folate uptake was unaffected by succinate-supported respiration, 10 mM cyanide, or $10^{-5}$

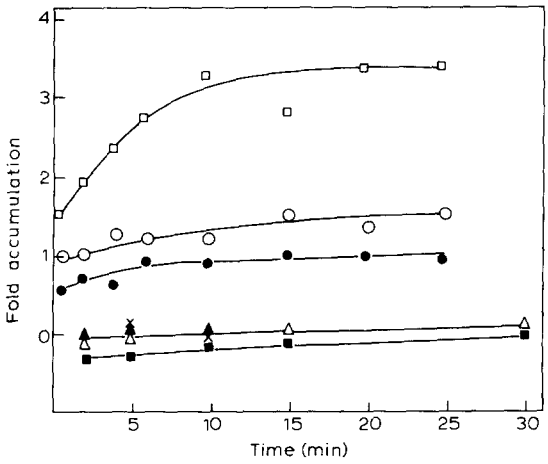


Fig. 1. Uptake of folates by rat liver mitochondria. Incubation mixtures contained 50 μM [$^3$H]folate (□), 210 μM dihydro[$^3$H]folate (●), 100 μM [$^3$H]methotrexate (○), 400 μM tetrahydro[$^3$H]folate (■), 290 μM 5,10-methylenetetrahydro[$^3$H]folate (×), or 5-[$^{14}$C]methyltetrahydrofolate at 18 μM (△) or 520 μM (▲). Fold accumulation values are such that 0 indicates no entry into the matrix, 1 represents complete equilibration across the inner membrane, and values greater than 1 indicate accumulation in the matrix.

M carbonyl cyanide *m*-chlorophenylhydrazone, indicating that the accumulation of this compound may not be directly linked to the oxidative phosphorylation system. A mitochondrial folate binding protein reported by Zamierowski and Wagner [20] could account for folate accumulation. However, no evidence for a binding protein was found when mitochondria were incubated with [$^3$H]folic acid for 10 min, disrupted by sonication, and the sonicate chromatographed on a Bio-Rad P-2 column. The uptakes of dihydrofolate and the folate analog, methotrexate, are consistent with the rapid equilibration of the compounds across the inner membrane with little or no accumulation in the matrix. The apparent maximal matrix concentrations, in nine experiments, for dihydrofolate and methotrexate were 0.98 ± 0.19 and 1.25 ± 0.26 times that of the external solution, respectively. Treatment of mitochondria with low digitonin concentrations to disrupt contaminating lysosomes, while maintaining mitochondrial structural integrity [7] did not alter any of the typical folate uptake patterns.

These data suggested the presence in the inner membrane of a carrier system specific for the transport of oxidized folates. To test this hypothesis,

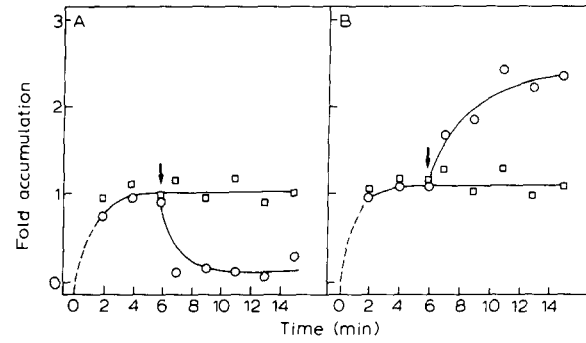


Fig. 2. Effect of methotrexate and folate on dihydrofolate uptake. Mitochondria were preincubated with dihydro-[$^3$H]folate. At 6 min, indicated by arrow, the mixtures were made 500 μM in methotrexate (○), Panel A, or folate (○), Panel B. The control experiments (□) 1 mM potassium phosphate (pH 7.4) was added.

counter-transport type experiments were performed. Fig. 2A shows that addition of methotrexate to mitochondria pre-equilibrated with dihydrofolate effects a rapid release of dihydrofolate to the medium However, addition of folate (Fig. 2B) promotes an accumulation of dihydrofolate such that its apparent matrix concentration is about 2.5 times that of the external medium. Both dihydrofolate and methotrexate promote the release of pre-loaded folate (Fig. 3). In other experiments, neither tetrahydrofolate nor 5-methyltetrahydrofolate affected folate or dihydrofolate uptake, and neither folate nor dihydrofolate stimulated an uptake of reduced folates.

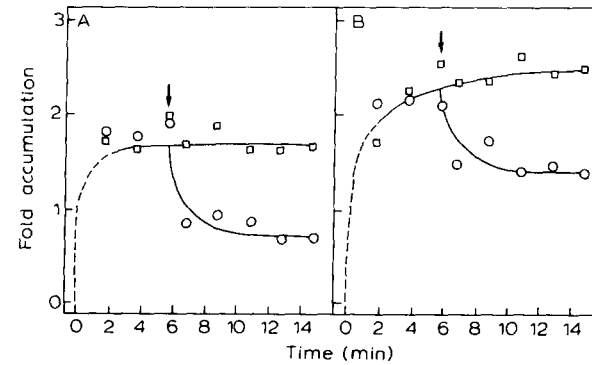


Fig. 3. Effect of dihydrofolate and methotrexate on folate uptake. Conditions were as described under Fig. 2 except mitochondria were preincubated with [$^3$H]folate. Panel A, 500 μM dihydrofolate added; Panel B, 500 μM methotrexate added.

## Discussion

In contrast to L1210 cells which concentrate reduced folates and methotrexate, but not folic acid [21,22], the data presented indicates that rat liver mitochondria possess a carrier system(s) that is selective for oxidized folates. The lack of tetrahydrofolate, 5-methyltetrahydrofolate, and 5,10-methylenetetrahydrofolate uptake by mitochondria, coupled with the metabolic requirement for reduced folates in the matrix [3] indicates the existence of separate non-rapidly exchanging cytosolic and mitosolic reduced folate pools. This observation is consistent with the operation of a substrate shuttle system proposed previously [3], comprised of cytosolic and mitochondrial isoenzymes of serine transhydroxymethylase, functioning in the transfer of one-carbon units between the two cellular compartments. Since reduced folates do not enter the matrix, the mitochondrial folate pool may be derived from the uptake of cytosolic oxidized folates, possibly dihydrofolate formed in the thymidylate synthesis cycle (cf. Ref. 23). The uptake of folate and dihydrofolate requires their subsequent reduction to tetrahydrofolate if they are to function in folate-linked mitochondrial metabolic pathways. Evidence for the presence of dihydrofolate reductase in the matrix of rat liver mitochondria has not appeared. Reports that the enzyme is distributed in the mitochondrial as well as the cytosolic fractions of liver [24,25] could not be confirmed [26]. In this regard, it is of interest that distinct mitochondrial and cytoplasmic isoenzymes of dihydrofolate reductase and serine transhydroxymethylase have been identified in *Saccharomyces cerevisiae* [27].

The uptake of oxidized folates is not readily explained by simple diffusion of the compounds across the inner membrane based on differences in hydrophobicity when compared to reduced folates, nor is it likely the result of non-specific binding, since all the folates are structurally similar. The rapid release from mitochondria of folate on addition of dihydrofolate or methotrexate and of dihydrofolate on addition of methotrexate indicates that a single transport system promotes the uptake of these folates. The existence of a second folate-specific carrier is suggested by its apparent concentration in the matrix. Folate accumulation might result either from its antiport with an unknown matrix anion or by metabolic conversion. The latter is eliminated, since accumulated [$^3$H]folate remains radiochemically pure during the course of the experiment. Alternatively, the apparent concentration of folate above equilibration may be an artifact accounted for by binding to mitochondrial components, although no evidence for such binding was found in the present study. The strongest evidence for folate and dihydrofolate transport is provided by the observation that dihydrofolate accumulation is enhanced by folate. This stimulation could result from the concentrative uptake of folate followed by counter-transport with external dihydrofolate, or by an increased sensitivity of dihydrofolate efflux to inhibition by folate as compared to influx. However, these results are inconsistent with uptake involving simple binding to the mitochondrial exterior.

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