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MALATE TRANSPORT IN BOVINE ADRENAL CORTEX MITOCHONDRIA

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SUMMARY

1. The kinetic properties of transport systems for Krebs-cycle anions in bovine adrenal cortex mitochondria have been studied in direct uptake and back-exchange assays.

2. At 0 °C and the optimal pH of 6.5, the uptake and the back-exchange of malate follow first-order kinetics. The K_m and V for malate uptake are 80 μM and 5 nmoles · min^{-1} · mg protein^{-1}, respectively.

3. The uptake of malate is accompanied by a release of phosphate. The phosphate/malate exchange is partially charge-compensated by an uptake of protons. Pyruvate arising from decarboxylation of malate is also released on uptake of malate.

4. The uptake of malate is inhibited by butylmalonate, a specific inhibitor of the dicarboxylate/phosphate exchange, but not by *N*-ethylmaleimide which is known to block the phosphate/OH^- exchange.

5. Low concentrations (< 0.1 mM) of phosphate stimulate the malate/malate exchange.

6. Succinate competitively inhibits the uptake of malate.

7. Succinate and malate uptakes occur at about the same rate. However, the K_m for malate is much lower than the K_m for succinate (500 μM). Furthermore the entry of malate is not inhibited by oncotic pressure as is the entry of succinate.

8. Bovine adrenal cortex mitochondria also transport 2-oxoglutarate; the oxoglutarate uptake is markedly stimulated by traces of phosphate and malate; $V = 5$ nmoles · min^{-1} · mg protein^{-1}; $K_m = 40$ μM. On the other hand, they display a low transport activity for citrate although citrate noncompetitively inhibits the malate uptake. Glutamate is apparently not transported.

INTRODUCTION

Adrenal cortex mitochondria are endowed with two electron-transport systems, the respiratory chain NADH–O_2 and the NADPH–cytochrome P_{450} chain, which

Abbreviations: MES, 2-*N*-morpholino ethane sulfonic acid; EGTA, ethylene glycol tetraacetic acid.

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are involved in oxidative phosphorylation and in steroid hydroxylation, respectively. The two chains are located in the inner mitochondrial membrane. The NADPH–cytochrome P_{450} chain can receive reducing equivalents either from NADH through the pyridine nucleotide transhydrogenase, or from substrates like isocitrate or malate* that can be dehydrogenated through specific NADP-dehydrogenases

In beef adrenal cortex mitochondria which exhibit a high malic enzyme activity, it has been shown that malate oxidation occurs via the ($NADP^+$)-malic enzyme [1, 2]. Malate occurs as part of the Krebs cycle within mitochondria, so that its oxidative decarboxylation into pyruvate through the mitochondrial malate enzyme tends to interrupt the Krebs cycle and result in a shortage of oxaloacetate. This situation may be obviated either by the ATP-dependent carboxylation of pyruvate into oxaloacetate, or by the supply of additional malate from the cytosol. This latter alternative has been discussed by Simpson and Estabrook [3] who reported some evidence for a malate–pyruvate shuttle whereby external malate enters mitochondria in exchange for internal pyruvate.

In a previous communication, the transport systems of the Krebs-cycle anions in bovine adrenal cortex mitochondria were identified by two classical techniques, the reduction of intramitochondrial pyridine nucleotides upon addition of penetrating anions, and the change in light scattering produced by isosmotic solutions of ammonium salts of the corresponding anions [4]. The data obtained with these methods suggested that beef adrenal cortex mitochondria are able to transport malate, succinate and oxoglutarate This paper is concerned with a determination of the kinetic parameters of the transport systems for Krebs-cycle substrates in bovine adrenal cortex mitochondria using ^{14}C-labeled anionic substrates Evidence is presented that the malate carrier is the most active of the Krebs-cycle anion carriers. The implication of malate transport in mitochondrial steroidogenesis is discussed.

EXPERIMENTAL PROCEDURES

Preparation of mitochondria

Beef adrenal glands were collected from freshly killed animals at a local slaughterhouse. The adrenal cortex tissue was scraped from the capsule and medulla and suspended in the homogenization medium. Two types of homogenization medium were used, one, "the bovine serum albumin medium", made of 0.27 M sucrose, 10 mM Tris–HCl and 0.2% bovine serum albumin, final pH 7.4, the other, the "EGTA medium", made of 0.27 M sucrose, 10 mM Tris–HCl and 0.5 mM ethylene glycol tetraacetic acid (EGTA), pH 7.4. After homogenization in a Potter–Elvehjem type glass homogenizer with a teflon pestle at 2000 rev./min, the large cell debris and nuclei were removed by centrifugation at $1000 \times g$ for 10 min; a crude mitochondrial fraction was obtained by centrifugation of the supernatant fluid at $8000 \times g$ for 15 min. The mitochondria were washed once with the homogenization medium, and finally suspended in 0.27 M sucrose, 2 mM Tris, pH 7.4.

Purification of radioactive substrates

Before use, all ^{14}C-labeled substrates were purified by chromatography ac-

* Unless stated, malate stands for L-malate.

cording to the method of Myers and Huang [5]. The ^{14}C-labeled compounds were located by autoradiography and eluted in water at 0 °C.

Measurement of anion transport

In kinetic studies of transport over short periods of incubation, the addition of a specific inhibitor provides a useful means to induce a sharply defined termination of the translocation of the substrate. In the case of mitochondria, this technique, first applied to the adenine nucleotide translocation using atractyloside as an inhibitor [6] has since been extensively used under the name of the inhibitor-stop method [7] for detailed kinetic studies of a number of transport systems [7–10]. For instance, phenylsuccinate, butylmalonate and benzylmalonate have been recommended as inhibitors to stop the entry of dicarboxylates into mitochondria [10]. After a number of attempts, we found that mersalyl at a final concentration of 10 mM could stop malate transport with a resolution time of about 1 s. Under our test conditions, the contact of mitochondria with mersalyl for a few seconds before centrifugation did not alter their morphology as shown by electron microscopy and did not alter the membrane permeability as assessed by the absence of leakage from test mitochondria that had been preloaded with [^{14}C]malate.

All the experiments on malate transport were carried out with the following medium (standard saline medium): 100 mM KCl, 25 mM Tris–2-*N*-morpholino ethane sulfonic acid (MES), pH 6.5, 1 mM EGTA, 5 μM rotenone, 2 μg/ml antimycin and 2.5 μg/ml oligomycin. Malate transport was assayed either by direct uptake of [^{14}C]-malate, or by back exchange of unlabeled malate for intramitochondrial [^{14}C]malate In the direct-uptake procedure, adrenal cortex mitochondria were preincubated for 30 s in the standard medium; incubation was started by the addition of [^{14}C]malate and stopped by 10 mM mersalyl, immediately followed by rapid centrifugation in a 3200 Eppendorf centrifuge at 0 °C. The pellet was dissolved in 0.5 ml of a 4% sodium cholate solution and its radioactivity estimated by liquid scintillation counting in an Intertechnique SL30 counter. The scintillation fluid contained 100 g of naphthalene, 6 g of 2,5-diphenyloxazole and 300 mg of *p*-bis[2-(5′-phenyloxazolyl)]benzene per liter of 1,4-dioxane. The substrate uptake into the matrix space was calculated by correcting the amount of radioactivity in the mitochondrial extract for that present in the sucrose-permeable space plus adherent supernatant as determined by [^{14}C]-sucrose. In the back-exchange procedure, unlabeled malate was added to mitochondria that had been previously loaded with [^{14}C]malate. A significant loading, with a concentration factor amounting to 200, could be achieved by incubating the mitochondria for 10 min at 0 °C in the standard saline medium (20 mg protein/ml) with 10–20 μM [^{14}C]malate, followed by removing the external [^{14}C]malate by a ten-fold dilution and centrifugation. Less reproducible results were obtained by the technique of Meijer [11] in which rat liver mitochondria in sucrose were treated first with unlabeled malate, centrifuged and resuspended in sucrose before the addition of [^{14}C]-malate (carrier free). Adrenal cortex mitochondria loaded with [^{14}C]malate as described above were preincubated for 30 s in the standard saline medium; then incubation was initiated by the injection of unlabeled malate and stopped by rapid filtration (less than 2 s) through a 0.45 μm Millipore filter HAWP inserted in a filter holder fitted to a syringe. Spontaneous leakage in the absence of added malate was measured by the same method.

The uptake of 2-oxo[^{14}C]glutarate and of [^{14}C]citrate was measured in the same way as that of [^{14}C]malate, with malate-loaded mitochondria, using phenylsuccinate as an inhibitor to stop transport in the case of oxoglutarate, and 1,2,3-benzene tricarboxylate in the case of citrate. When oxo[^{14}C]glutarate was used, the standard saline medium was supplemented with 2 mM arsenite.

Determination of the mitochondrial space

[^{14}C]sucrose and $^{3}H_2O$ were added to the mitochondria under the same conditions as for the analysis of malate transport. The extramatrix space was deduced from the ^{14}C-radioactivity of the pellet obtained after centrifugation, and the total space from the ^{3}H radioactivity.

Analysis of products from malate metabolism

After incubation in the presence of [^{14}C]malate and centrifugation trichloroacetic acid was added to the mitochondrial pellets and supernatant to a final concentration of 5%. The trichloroacetic acid extracts were analyzed by thin-layer chromatography [5]. The chromatoplates were autoradiographed. The radioactive spots were scraped off; the powder was placed in scintillation vials, suspended in 1 ml water to which 10 ml of scintillation fluid was added, and counted.

Enzymatic assays

(NAD^+)-malate dehydrogenase, ($NADP^+$)-malic enzyme, ($NADP^+$)isocitrate dehydrogenase were assayed spectrophotometrically in a 3-ml cuvette at 20 °C after lysis of the mitochondria with 0.01% Triton ×100. The following assay media were used: (NAD^+)-malate dehydrogenase: 100 mM glycine–NaOH buffer, 10 mM malate and 3 mM NAD^+, final pH 10; ($NADP^+$)-malic enzyme: 100 mM Tris–HCl buffer, pH 7.4, 10 mM malate, 2 mM $MgCl_2$ and 0.6 mM $NADP^+$, final pH 7.4; ($NADP^+$)-isocitrate dehydrogenase: 100 mM Tris–HCl buffer, 4 mM isocitrate, 2 mM $MnSO_4$ and 0.3 mM $NADP^+$ final pH 7.4.

The citrate-cleavage enzyme was assayed in the adrenal cortex high-speed supernatant (100 000 × g, 30 min) as described by Srere [12]. The pH was 8.4 and temperature 20 °C.

Assay of malate

Endogenous malate was measured enzymatically in neutralized perchloric acid extracts of mitochondria with malate dehydrogenase and citrate synthase using the Aminco–Chance dual beam spectrophotometer at 350–375 nm according to Palmieri and Quagliariello [13].

Assay of P_i

Inorganic phosphate was determined by the method of Itaya and Ui [14].

RESULTS

1. *Optimal test conditions for measurement of malate transport in beef adrenal cortex mitochondria; EGTA and bovine serum albumin mitochondria*

The medium which consisted of 0.27 M sucrose, 10 mM Tris–HCl and 0.2% bovine serum albumin was routinely used for the homogenization of bovine adrenal

cortex and the isolation of cortex mitochondria [15]. Mitochondria prepared by this procedure (bovine serum albumin mitochondria) were well coupled, and their respiratory control index was about 3, when assayed with malate as substrate. However, one major difficulty was encountered in attempts to characterize malate transport with these mitochondria, especially when using the back-exchange procedure. It was found that a large amount of ^{14}C radioactivity was evolved as $^{14}CO_2$ (50 and 80% after 20 min and 2 h standing at 2–4 °C) even although rotenone and antimycin were present at a concentration sufficiently high to stop the mitochondrial respiration. This behavior differs from that of rat liver mitochondria in which these respiratory inhibitors totally prevent the metabolism of malate [9]. The decarboxylation of malate by the very active ($NADP^+$)-malic enzyme present in bovine adrenal cortex mitochondria [1, 2] was probably responsible for the loss of CO_2, with the recycling of $NADP^+$ necessary for the continuous decarboxylation of malate being insured by side reactions. One side reaction which can play such a role is the hydroxylation of endogenous deoxycorticosterone through the NADPH–cytochrome P_{450} system. Metyrapone, a potent inhibitor of mitochondrial deoxycorticosterone hydroxylation [16], was, however, found ineffective in inhibiting malate metabolism even at a concentration as high as 50 μM, which totally inhibits the interaction between deoxycorticosterone and cytochrome P_{450} [17]. Another side reaction which is much less sensitive to metyrapone and which, therefore, may escape metyrapone inhibition is the side-chain cleavage of cholesterol. In experiments on malate transport, bovine serum albumin mitochondria could be used in assays on the initial kinetics of [^{14}C]-malate uptake which lasted for a few seconds; but they were unsuitable in assays on malate transport by back-exchange since this procedure requires a preloading of mitochondria with [^{14}C]malate, a process which takes at least 20 min.

Replacing bovine serum albumin by EGTA in the homogenization medium resulted in mitochondria (EGTA mitochondria) that had a much lower malate-metabolizing activity. The EGTA mitochondria loaded with [^{14}C]malate and incubated in the presence of respiratory inhibitors were able to retain the incorporated ^{14}C radioactivity with less than 10% of $^{14}CO_2$ loss in 2 h at 2° C.

Whereas the disappearance of [^{14}C]malate incorporated into EGTA mitochondria was negligible in the first 2 or 3 min of incubation at 2 °C, a number of ^{14}C labeled metabolites could be identified after 20 min, these being in decreasing order of accumulation, aspartate, glutamate, citrate, pyruvate and fumarate (Fig. 1). Since the operation of [^{14}C]malate loading, used in back-exchange assays required at least 20 min, only about 30–50% of the original [^{14}C]malate was left as such in EGTA mitochondria at the time of the assay. As shown in Fig. 2 some of the intramitochondrial [^{14}C]malate and [^{14}C]citrate in EGTA mitochondria loaded with [^{14}C]malate diffused spontaneously into the extramitochondrial space. However, when unlabeled malate was added to these mitochondria, a selective efflux of [^{14}C] malate occurred that was 3 times greater than in the absence of malate. This difference was sufficiently great for the kinetics of the induced efflux to be easily measured. By contrast the malate-induced efflux of citrate was negligible (10% of the spontaneous release).

In contrast to bovine serum albumin mitochondria, EGTA mitochondria did not appear to control the movement of protons in their membrane, which brought some limitation in their use especially in assays on phosphate/OH^- exchange. This

may be related to the fact that EGTA mitochondria exhibit a virtually uncoupled respiration. In a number of experiments described in this paper, EGTA mitochondria and bovine serum albumin mitochondria could be used interchangeably; however, there were special cases which required more specifically the use of one type of

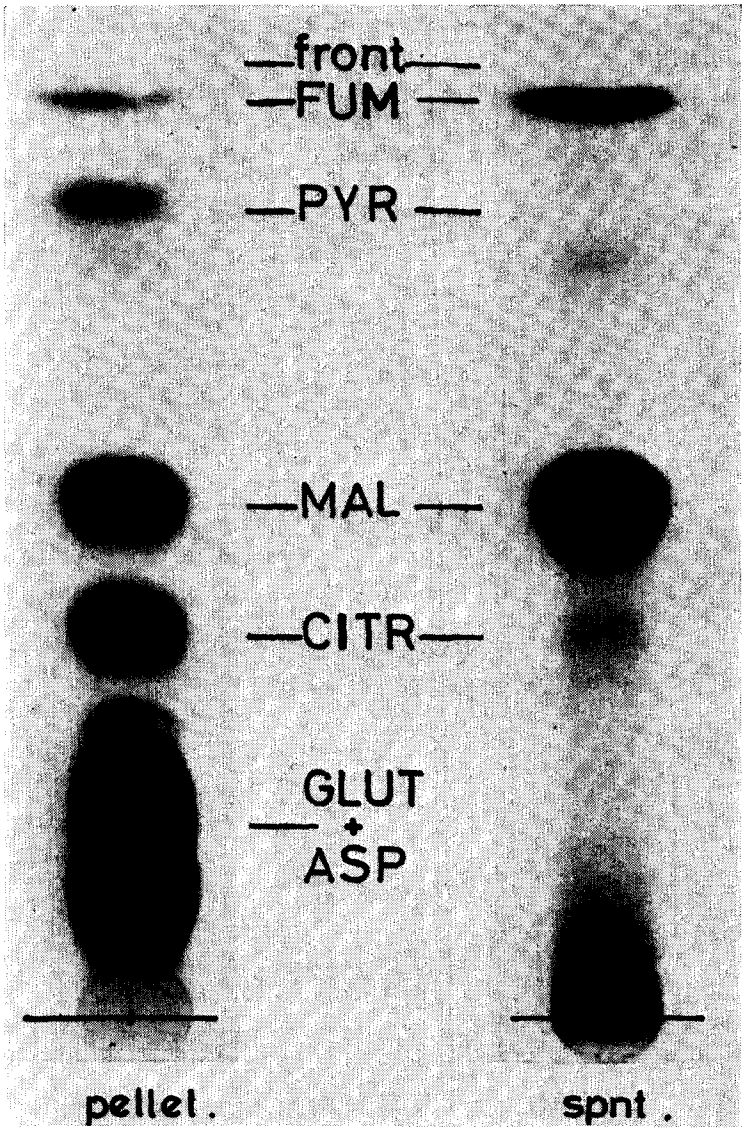


Fig. 1. Autoradiography of a chromatoplate showing metabolite products in [^{14}C]malate-loaded mitochondria. EGTA mitochondria (4.7 mg protein) were incubated for 20 min at 2 °C in 0.9 ml of the standard saline medium supplemented with 22 μM [^{14}C]malate. The incubation was stopped by the addition of 10 mM mersalyl, immediately followed by high-speed centrifugation. Trichloroacetic acid was added to the pellet and the supernatant to a final concentration of 5 %. The trichloroacetic acid extracts were chromatographed on a cellulose plate (see Methods). FUM, fumarate; PYR, pyruvate; CITR, citrate; GLUT, glutamate; ASP, aspartate.

mitochondrial preparation. For instance, EGTA mitochondria were preferred to bovine serum albumin mitochondria in kinetic studies involving the back-exchange procedure, while bovine serum albumin mitochondria were required for assays of the movement of protons associated with anion uptake. For the sake of clarity, all the results reported in this paper were obtained with EGTA mitochondria, except those bearing on the movement of protons where bovine serum albumin mitochondria were preferred because of their more rapid response.

Since the optimal pH for malate transport is 6.5 (Fig. 3), most of the experiments were carried out at this pH in a saline medium made of 100 mM KCl, 25 mM Tris–MES, 1 mM EGTA, 5 μM rotenone, 2 μg/ml antimycin and 5 μg/ml oligomycin.

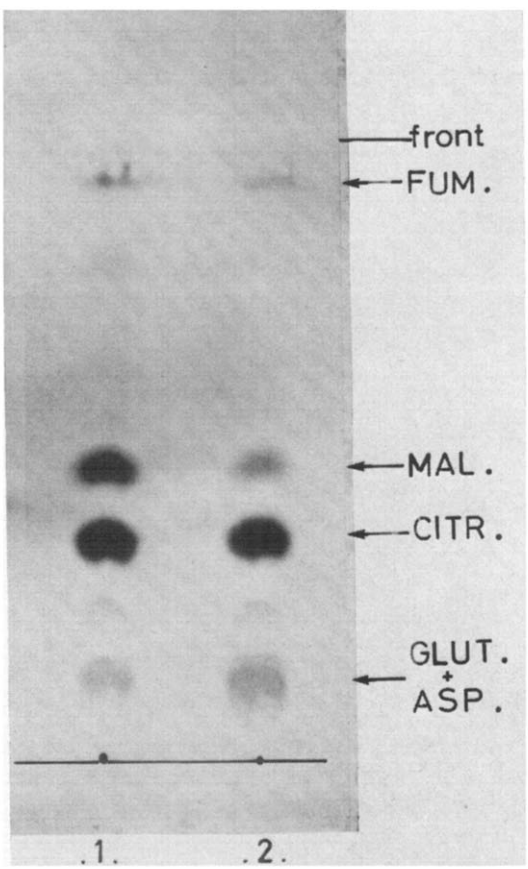


Fig. 2. Autoradiography of a chromatoplate showing the specific exchange of intramitochondrial [^{14}C]malate, with added malate. EGTA [^{14}C]malate-loaded mitochondria (4 mg protein) were preincubated for 30 s in 2.5 ml of standard saline medium, final pH 6.5, temperature 2 °C (see Methods). The exchange was started by adding 2 mM malate and stopped after 1 min by rapid filtration. An aliquot (10 μl) of filtrate acidified with trichloroacetic acid was directly chromatographed on a cellulose chromatoplate as described in Methods. After autoradiography the different spots were scraped off and counted. The counts (dpm) were the following, Track 1 (malate added): GLUT+ASP 36, CITR 180, MAL 113, FUM 17. Track 2 (malate omitted): GLUT+ASP 50, CITR 171, MAL 30, FUM 11.

Replacing KCl by sucrose and keeping constant the osmolarity of the medium led to a 2-fold decrease of the rate of malate uptake, in agreement with a report by Meisner et al. on the stimulating effect of monovalent cations on anion transport in rat liver mitochondria [18].

A recent report in the literature [19] has focused attention on a possible artifactual binding of metabolic anions to mitochondria. To ascertain that the [^{14}C]-malate uptake by adrenal cortex mitochondria was related to the activity of a specific transport system, we have compared the rates of [^{14}C]malate uptake in intact and in denatured mitochondria. The mitochondria were denatured by three procedures: (a) boiling for 15 min; (b) rapid freezing at −30 °C and thawing for 3 times; (c) treatment for 10 min at 0 °C with 0.05% and 1% glutaraldehyde. Mitochondria treated as in (a) and (c) above were centrifuged and resuspended in 0.27 M sucrose, while the freeze-thawed mitochondria were only rehomogenized. The denatured mitochondria were then incubated with [^{14}C]malate as in the uptake assays for 1 min at 0 °C. In no case was any significant binding of [^{14}C]malate observed.

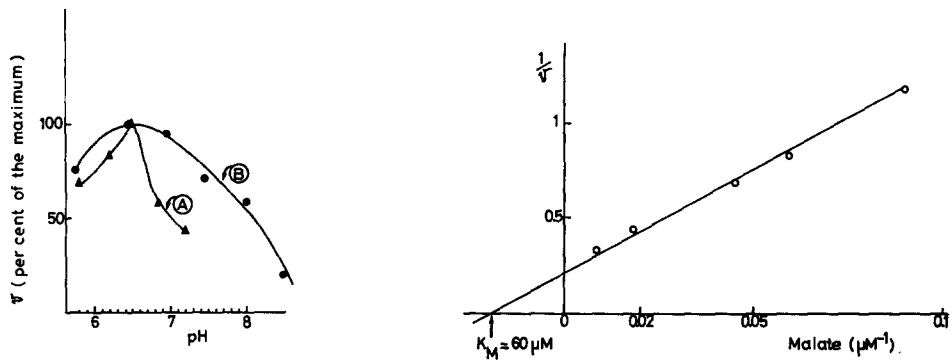
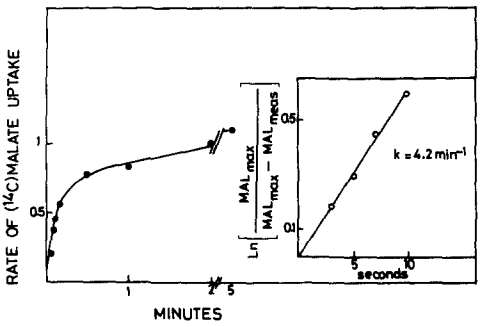


Fig. 3. pH effect. (A) [^{14}C]malate uptake. EGTA mitochondria (5 mg protein) were preincubated for 30 s in 0.9 ml of standard saline medium (see Methods) at 0 °C. The uptake of [^{14}C]malate was started by the addition of 22 μM [^{14}C]malate and stopped after 5 s by the addition of 10 mM mersalyl followed by rapid centrifugation of the mitochondria (see Methods). (B) [^{14}C]malate efflux. [^{14}C]-malate-loaded mitochondria, 4 mg protein, were preincubated for 30 s at 0 °C in the standard saline medium. Final volume 2.5 ml. The back-exchange was initiated by adding 2 mM [^{12}C]malate and terminated after 1 min by a Millipore filtration.

Fig. 4. Dependence of the rate of [^{14}C]malate uptake on malate concentration. Experimental conditions as in Fig. 3A except that the malate concentration was varied. Mitochondria, 6.1 mg protein; temperature, 0 °C, pH 6.5. The rate of malate uptake, v, is expressed in nmoles · min^{-1} · mg protein^{-1}.

2. *Kinetics of* [^{14}C]*malate uptake*

Results reported in this section were obtained with EGTA mitochondria. Control experiments carried out with bovine serum albumin mitochondria yielded essentially similar results. The effect of external malate concentration on the rate of malate uptake is shown in Fig. 4. In this experiment, V was 4.8 nmoles · min^{-1} · mg protein^{-1} at 0 °C, and K_m was 60 μM. The values of K_m found in six similar experiments ranged from 60 to 100 μM. The kinetics of uptake were first-order ($k = 4.2$ min^{-1} at 0 °C), at least for the first 30 s of incubation (Fig. 5).



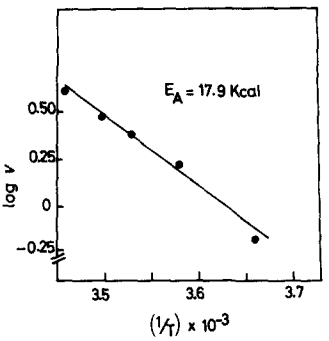


Fig. 5. Kinetics of [^{14}C]malate uptake. Experimental conditions as in Fig. 3A except that the concentration of [^{14}C]malate was saturating (217 μM). Mitochondrial protein, 4.4 mg. v is expressed in nmoles · min^{-1} · mg protein^{-1}. Insert: Logarithmic plot of [^{14}C]malate uptake. MAL_{max} refers to malate accumulated after the equilibrium was reached (5 min).

Fig. 6. Arrhenius plot of the temperature dependence of the rate of [^{14}C]malate uptake. Experimental conditions as in Fig. 3A except that temperature was varied. Mitochondrial protein 4.9 mg. The rate of malate uptake, v, is expressed in nmoles · min^{-1} · mg protein^{-1}.

The effect of temperature was analyzed between 1 °C and 16 °C. (Fig. 6). In an Arrhenius plot, a straight line was obtained with an activation energy equal to 17.9 kcal/mole.

3. *Kinetics of* [^{14}C]*malate efflux*

As previously stated [^{14}C]malate is selectively released from [^{14}C]malate-loaded mitochondria upon addition of malate. For five experiments, the values of K_m obtained for the external malate concentration ranged from 70 to 160 μM. The kinetics of [^{14}C]malate efflux induced by addition of a saturating concentration of malate (2 mM) were first-order (Fig. 7, Insert A). A rate of malate efflux of 1.1

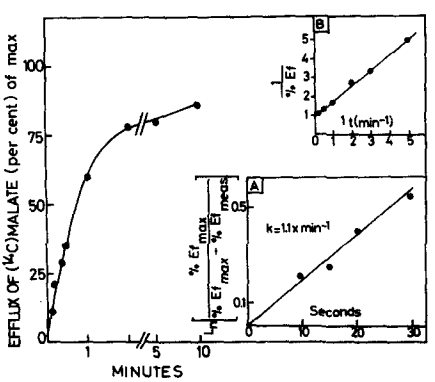


Fig. 7. Kinetics of [^{14}C]malate efflux. EGTA [^{14}C]malate-loaded mitochondria, 4 mg protein, were preincubated for 30 s in 2.5 ml of standard saline medium at 0 °C. The back exchange was initiated by adding 2 mM [^{12}C]malate and terminated by rapid Millipore filtration. Values are corrected for spontaneous release of [^{14}C]malate. Insert: Logarithmic plot of the exchange. $Efflux_{max}$ (Ef_{max}) refers to the efflux obtained after the equilibrium was reached (10 min).

nmole · min^{-1} · mg $protein^{-1}$ at 0 °C could be determined on the basis of a calculated concentration of internal [^{14}C]malate of about 1 nmole/mg protein (corrected for metabolization of [^{14}C]malate at the time of assay). The release of [^{14}C]malate induced by external malate, therefore, appeared to be about 4 times slower than the uptake of [^{14}C]malate.

To calculate the percentage of internal [^{14}C]malate exchanged at equilibrium, the time course of efflux was represented in a double-reciprocal plot system, where the reciprocal of the percentage of efflux (1/Ef) was plotted as a function of the reciprocal of the incubation period (Insert B). In this representation, the intercept on the 1/Ef axis gives the reciprocal of the percentage of [^{14}C]malate released at equilibrium. By this method virtually 100% of the internal [^{14}C]malate was found to be exchangeable. 2 mM succinate or 2 mM malonate added to [^{14}C]malate-loaded mitochondria were also able to induce a release of [^{14}C]malate, but the rate was 3–4 times slower than malate. D-Malate, citrate and oxoglutarate were ineffective.

4. *Nature of the internal anions exchanged with external malate*

The exchange–diffusion reaction between phosphate and dicarboxylates in rat liver mitochondria, first postulated by Chappell and Haarhoff [20], has been demonstrated by Papa et al. [21]. Further McGivan and Klingenberg [22] have shown that in rat liver mitochondria, the uptake of malate and protons is charge compensated by an efflux of phosphate. Data in Fig. 8 indicate that bovine adrenal cortex mitochondria also possess a mersalyl-sensitive phosphate/dicarboxylate carrier which is able to exchange internal phosphate for external malate and protons. However, it is clear that the net uptake of negative charge (2 malate, 1 H^+) is far from compensated by the efflux of the negatively charged phosphate. Even allowing for the fact that part of the [^{14}C]malate is evolved as $^{14}CO_2$, it seems likely that other internal anions in addition to phosphate are exchanged against external malate. In line with this idea the following result, which shows that internal pyruvate is released upon addition of malate, is noteworthy. When adrenal cortex mitochondria were incubated with [^{14}C]malate and 1 mM arsenite, the metabolism of [^{14}C]malate was partially blocked at the level of pyruvate and [^{14}C]pyruvate accumulated, together with traces of [^{14}C]citrate, [^{14}C]glutamate and [^{14}C]aspartate. A large fraction (70–80%)

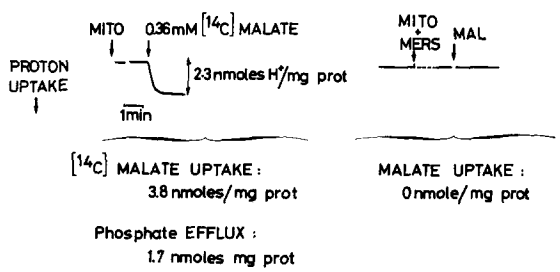


Fig. 8. Proton movement during the uptake of malate. Bovine serum albumin mitochondria (28 mg of protein) were preincubated for 4 min in 5.5 ml of standard saline medium at 0 °C. The uptake was initiated by adding 0.36 mM of [^{14}C]malate. When present, mersalyl (Mers) was 0.1 mM. After the pH change had ceased a sample was withdrawn for estimation of [^{14}C]malate and endogenous phosphate (as described in Methods). The phosphate value recorded on the Figure is corrected from a control without added malate.

of the accumulated [^{14}C]pyruvate was released in the external medium in contrast to the total retention of [^{14}C]glutamate and [^{14}C]aspartate within the mitochondria. The release of internal pyruvate upon addition of malate confirms a previous report by Simpson and Frenkel [23], on the existence of a malate/pyruvate shuttle first postulated by Simpson and Estabrook [1, 3]. Our observations furthermore suggests that glutamate and aspartate are transported only slowly, if at all, in agreement with data reported below.

5. *Effect of inhibitors of phosphate transport on* [^{14}C]*malate efflux*

The effect of various inhibitors of phosphate transport on the [^{14}C]malate efflux induced by the addition of malate is summarized in Table I. 20–40 mM butylmalonate which totally inhibit the phosphate/dicarboxylate translocator [24] inhibited the efflux of [^{14}C]malate by 55–75%. *N*-Ethylmaleimide, which blocks the phosphate/OH^- translocator but not the phosphate/dicarboxylate translocator [25] did not inhibit the [^{14}C]malate efflux. These data suggest that on addition of malate to [^{14}C]malate-loaded mitochondria, some internal phosphate is released, which exchanges back with internal [^{14}C]malate. This exchange is selectively blocked by butylmalonate. In short, a large part of the release of [^{14}C]malate would be due to a phosphate/malate exchange.

TABLE I

EFFECT OF INHIBITORS OF PHOSPHATE TRANSPORT ON [^{14}C]MALATE EFFLUX FROM [^{14}C]MALATE-LOADED MITOCHONDRIA

EGTA mitochondria preloaded with [^{14}C]malate (2 mg protein) were preincubated during 30 s in the presence of inhibitors in 2.5 ml of standard saline medium. The exchange was started by the addition of 2 mM malate at 0 °C and stopped after 20 s by Millipore filtration.

Inhibitor	Concn (mM)	Percent inhibition
N-ethylmaleimide	1	0
Butylmalonate	20	55
Butylmalonate	40	75

6. *Effect of external phosphate on* [^{14}C]*malate efflux*

In line with experiments dealing with the phosphate/dicarboxylate carrier, we have tested the effect of phosphate on the [^{14}C]malate efflux from [^{14}C]malate-loaded mitochondria incubated in the presence of malate or succinate. The concentrations of phosphate used in these experiments (0.01–0.1 mM) were sufficiently low to not compete with the malate (or succinate)/malate exchange. As shown in Table II, addition of phosphate together with malate or succinate induced an increased efflux of [^{14}C]malate which was roughly similar in both cases (malate and succinate), assuming first-order kinetics of efflux. Phosphate alone in the absence of added malate or succinate had no effect. When phosphate was used at a concentration higher than 1 mM and the concentration of malate was kept low (50 μM), an inhibition of the malate uptake was found, in agreement with the report of Palmieri et al. [9] showing that phosphate competes with malate for entry into rat liver mitochondria.

TABLE II

EFFECT OF PHOSPHATE ON THE EFFLUX OF [^{14}C]MALATE FROM [^{14}C]MALATE-LOADED MITOCHONDRIA

3.8 mg of EGTA mitochondria loaded with [^{14}C]malate were preincubated for 30 s in 2.5 ml of standard saline medium at 0 °C. The efflux of [^{14}C]malate was started by adding [^{12}C]malate or [^{12}C]succinate or phosphate and terminated after 20 s by Millipore filtration (see Methods).

Malate (mM)	Succinate (mM)	Phosphate (mM)	Percent of efflux after 20 s
—	—	0.1	< 5
0.1	—	—	17
0.1	—	0.05	21
2	—	—	30
2	—	0.01	35
2	—	0.05	41
2	—	0.1	44
—	2	—	8
—	2	0.1	23

The EGTA mitochondria which were used in these experiments presumably transport phosphate mainly by a phosphate/dicarboxylate carrier, for their phosphate/OH^- carrier is not very efficient as shown by the small proton movement accompanying the uptake of malate (as compared to the rapid and significant proton uptake in the case of bovine serum albumin mitochondria (see Section 4). This is consistent with results (not reported here) showing that the uptake of ^{32}P-labeled phosphate by EGTA mitochondria was sensitive to 20 mM butylmalonate, but not sensitive to 0.2 mM *N*-ethylmaleimide.

7. *Uptake of other anions than malate*

Table III presents data comparing the rates of uptake and the values of K_m of various di- and tricarboxylate anions with those for rat liver mitochondria [9, 26–28]. Similar values were obtained with bovine serum albumin mitochondria, except in the case of citrate transport. Oxo[^{14}C]glutarate uptake required a preloading of mitochondria with malate. The K_m value for oxoglutarate was about 40 μM. In contrast to rat liver mitochondria, the entry of citrate in beef adrenal cortex mitochondria was slow; it was not substantially increased by preloading mitochondria with malate or by incubating simultaneously [^{14}C]citrate with a small amount of malate (0.1 mM) and/or phosphate (0.5 mM). Using bovine serum albumin mitochondria, the rate of citrate uptake in the presence of malate and phosphate amounted to 0.5 nmole · min^{-1} · mg protein^{-1}. The low rate of citrate uptake shown by the isotopic method is in disagreement with results obtained by the osmotic method which showed a swelling of adrenal cortex mitochondria in ammonium citrate in the presence of traces of malate [4, 29] or of succinate [29]. However, we observed that the citrate-induced swelling in the presence of malate [4] or of succinate (unpublished results) was not sensitive to 1,2,3-benzene tricarboxylate, a specific inhibitor of citrate transport in rat liver mitochondria [30]; it may, therefore, be artefactual.

TABLE III

COMPARED ACTIVITIES OF TRANSPORT OF VARIOUS ANIONS IN MITOCHONDRIA OF BEEF ADRENAL CORTEX AND OF RAT LIVER MITOCHONDRIA

Experiments with adrenal cortex mitochondria refer to standard conditions of uptake in saline medium, temperature 0 °C (except for glutamate), pH 6.5, mitochondrial protein ranging between 3.6 and 5.1 mg.

Substrate	EGTA adrenal cortex mitochondria, 0 °C		Rat liver mitochondria, 9 °C [9, 26–28]	
	V (nmoles · min^{-1} · mg protein^{-1})	K_m (mM)	V (nmoles · min^{-1} · mg protein^{-1})	K_m (mM)
Malate	5	0.08	69	0.23
Succinate	8	0.5	64	1.17
Malonate	4	0.13	77	0.37
Oxoglutarate	5*	0.04	43	0.046
Citrate**	0.1*	—	23	0.12
Glutamate	<0.1	—	5	4

* In the presence of 0.1 mM malate and 0.5 mM phosphate.

** With bovine serum albumin mitochondria. V was 0.5 nmole · min^{-1} · mg protein^{-1}.

Whereas citrate did not readily enter beef adrenal cortex mitochondria, it noncompetitively inhibited the entry of [^{14}C]malate. Oxoglutarate also inhibited malate uptake noncompetitively. The K_i values relative to the inhibition of malate transport were 280 μM for citrate, and 220 μM for oxoglutarate (Figs 9A and B).

In other experiments carried out with succinate and malonate at 0 °C with EGTA mitochondria, it was found that these anions are taken up with a K_m of 0.5 mM and 0.13 mM; and a V of 8 and 4 μmoles · min^{-1} · mg protein^{-1}, respectively (Table III). Succinate competitively inhibited the malate/malate exchange, in agree-

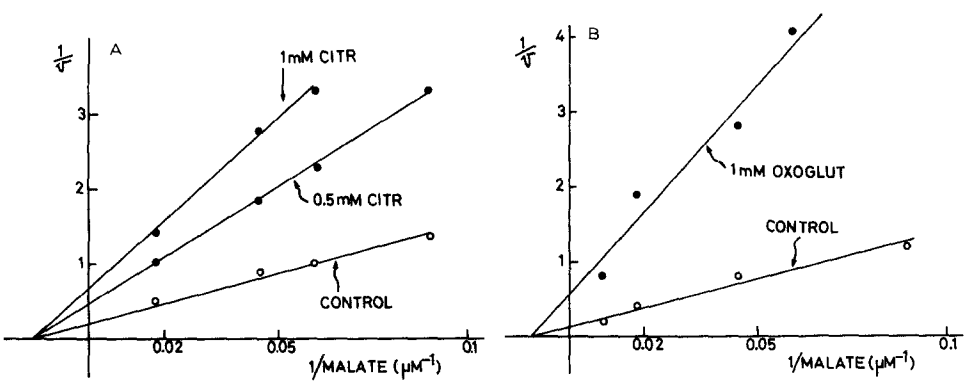


Fig. 9. Noncompetitive inhibition of malate uptake by citrate and oxoglutarate. (A) Citrate. (B) Oxoglutarate. Experimental conditions as in Fig. 3A except that [^{14}C]malate was added to the concentrations indicated. Where present citrate and oxoglutarate were added simultaneously with [^{14}C]-malate. Mitochondrial protein: 3.1 mg in A, 5.6 mg in B. The rate of malate uptake, v, is expressed in nmoles · min^{-1} · mg protein^{-1}.

ment with the suggestion that succinate is translocated by the same carrier as malate [9, 20].

Assays carried out with [^{14}C]glutamate at 25 °C in the standard saline medium did not reveal any significant uptake of radioactivity in beef adrenal cortex mitochondria. For comparison, glutamate enters rat liver mitochondria in exchange for OH^- [31] with a rate of 23 nmoles · min^{-1} · mg protein^{-1} at 25 °C and a K_m of 5 mM [32]. This result corroborates observations (see above) showing that the [^{14}C]glutamate which accumulates in adrenal cortex mitochondria, due to a metabolism of [^{14}C]malate, is not released outside the matrix space. It confirms the finding that NAD(P)$^+$ in adrenal cortex mitochondria is not reduced by added glutamate (unpublished result).

8. *Effect of oncotic pressure on the transport of malate*

In an attempt to simulate physiological conditions some effects of oncotic pressure on mitochondrial structure and functions have been recently studied. Harris et al. [33] and Bakeeva et al. [34] showed that high-molecular weight polymers (bovine serum albumin, polyvinyl pyrrolidone, dextran, Ficoll) added to liver or heart mitochondria induce an enlargement of the matrix and a decrease in the intermembrane space. These morphological changes were accompanied by a decreased ability of the mitochondria to oxidize a number of Krebs-cycle intermediates, including succinate, pyruvate, oxoglutarate, isocitrate, although oxidation of malate was not affected.

We have been able to confirm these effects on the morphology of EGTA adrenal cortex mitochondria and their ability to transport malate and succinate. Whereas 4% polyvinyl pyrrolidone, 5% Ficoll and 1.5% bovine serum albumin did not alter the rate of [^{14}C]malate uptake, they inhibited the uptake of [^{14}C]succinate by up to 40%. These data point to the prominent role of the malate carrier in the economy of adrenal cortex.

9. *Enzyme activities responsible for NADP$^+$ reduction in adrenal cortex mitochondria*

Data reported in the above sections have shown that the rate of transport of malate in beef adrenal cortex mitochondria is significantly higher than that of citrate. Since the probable role of malate and citrate is to provide reducing equivalents to internal NADP$^+$ through the specific NADP$^+$ dehydrogenases, it was interesting to test the respective activity of these dehydrogenases in beef adrenal cortex mitochondria. The specific activity of (NADP$^+$)-malic enzyme (Table IV) was

TABLE IV

ISOCITRATE AND MALATE DEHYDROGENASE ACTIVITIES IN BEEF ADRENAL CORTEX MITOCHONDRIA

Enzyme	Activity (nmoles · min^{-1} · mg protein $^{-1}$)	
	Own data	Data of Simpson and Boyd [35]
Malic enzyme (NADP$^+$)	31	10.2
Malate dehydrogenase (NAD$^+$)	2454	400
Isocitrate dehydrogenase (NADP$^+$)	11.5	6.3

3-times higher than that of ($NADP^+$)-isocitrate dehydrogenase. These data can be compared to those reported by Simpson and Boyd [35]. Although the absolute values of enzyme activities are different, their ratio is similar. It is noteworthy that the specific activity of the mitochondrial ($NADP^+$)-malic enzyme is much higher in adrenal cortex than in any other tissue; for instance beef liver seems to be devoid of malic enzyme activity [2]. The high malic enzyme activity in beef adrenal cortex mitochondria may be related to the relatively rapid transport of malate ions.

DISCUSSION

The present kinetic studies based on the use of ^{14}C-labeled anions confirm and extend previous data [4] where it was shown by spectrophotometry (reduction of endogenous pyridine nucleotides) and by the swelling technique (swelling in the ammonium salts of anions) (1) that malate and succinate readily enter bovine adrenal cortex mitochondria by a phosphate-stimulated process, (see also Sauer and Park [36]), (2) that oxoglutarate is exchanged for malate, (3) that citrate does not readily enter mitochondria. The swelling initiated by ammonium citrate in the presence of traces of malate (or succinate) was independently reported by Tsang and Johnstone [29] and ourselves [4]. However, we found [4] that 1,2,3-benzene tricarboxylate, an inhibitor of citrate entry in rat liver mitochondria, had no inhibitory effect on the citrate-induced swelling of beef adrenal cortex mitochondria. Besides, the data on citrate swelling were not corroborated by spectrophotometric data (4) nor isotopic data (this paper). It must be noted that Tsang and Johnstone ([29] have observed an uptake of [^{14}C]citrate in adrenal cortex mitochondria incubated in the presence of succinate. However, their data cannot be easily compared to ours since they measured citrate uptake under equilibrium conditions (10 min of incubation at 20 °C) while the kinetic experiments described in this paper required an incubation period of a few seconds at 0 °C.

In the present paper the properties of malate transport in adrenal cortex mitochondria (kinetics, specificity, pH and temperature dependence, effect of specific inhibitors) have been analyzed in detail. They are similar to those described in the case of rat liver [9]. The release of endogenous phosphate upon addition of malate as well as the effect on malate uptake of specific inhibitors of phosphate transport lead us to conclude that, similarly to rat liver [22] the transport of malate in bovine adrenal cortex mitochondria mainly occurs by a phosphate/malate exchange.

There are a number of features which distinguish Krebs-cycle anion transport in adrenal cortex mitochondria and in liver mitochondria. The low transport activity of citrate and the apparent lack of glutamate transport in adrenal cortex mitochondria contrasts with the high efficiency of these anion carriers in liver mitochondria. Adrenal cortex mitochondria behave like heart mitochondria as far as citrate transport is concerned [37]. Sluse et al. [37] have suggested that the low activity of citrate transport in heart mitochondria is related to the absence of fatty acid synthesis in heart cytosol, this synthesis occurring exclusively in mitochondria. A totally different situation holds for liver where fatty acid synthesis takes place in the cytosol and requires acetylCoA. AcetylCoA arises by enzymatic cleavage of citrate in cytosol; thus a likely function of the citrate carrier is to deliver mitochondrial citrate to cytosol. In the case of beef adrenal cortex cytosol, the specific activity of the citrate-

cleavage enzyme is of the same order as in liver (5.2 nmoles · min^{-1} · mg $protein^{-1}$ at 20 °C vs 8.4 in the case of liver) (unpublished results); thus the slow release of citrate from mitochondria to cytosol in the adrenal cortex cell could be a limiting factor in the rate of formation of acetylCoA and thereby in steroidogenesis.

Although bovine adrenal cortex mitochondria apparently do not transport glutamate, they contain a pool of glutamate and aspartate which become readily labeled on the addition of [^{14}C]malate. This rapid labeling is obviously due to the aspartate aminotransferase activity which is reported to be very high by Simpson and Boyd [35].

Succinate and malate are transported at about the same rate in adrenal cortex mitochondria; however, the K_m for malate is much lower than the K_m for succinate; furthermore, the entry of malate is not inhibited by oncotic pressure as the succinate uptake is. These results emphasize the relatively important role of the mitochondrial malate transport in the economy of the adrenal cortex cell.

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