The use of a potential-sensitive cyanine dye for studying ion-dependent electrogenic renal transport of organic solutes

Uptake of L-malate and D-malate by luminal-membrane vesicles

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The mechanisms of uptake of dicarboxylic acids by rabbit renal luminal-membrane vesicles were studied by the use of filtration and spectrophotometric techniques as described in an accompanying paper [Kragh-Hansen, Jørgensen & Sheikh (1982) *Biochem. J.* **208**, 359–368]. Addition of L- or D-malate to dye-membrane-vesicle suspensions in the presence of Na⁺ gradients (extravesicular > intravesicular) resulted in spectral curves indicative of depolarization events. The renal uptake of dicarboxylic acids was dependent on the type of Na⁺-salt anion present and could be correlated with the ability of the anions to penetrate biological membranes (i.e. Cl⁻ > SO₄²⁻ > gluconate). Identical results were obtained by a filtration technique with Sartorius membrane filters. The results indicate that the dicarboxylic acids are taken up by the membrane vesicles in an electrically positive form (i.e. Na⁺/substrate coupling ratio 3:1) by an Na⁺-dependent transport system. This proposal was further supported by spectrophotometric experiments with various ionophores such as valinomycin, gramicidin and nigericin. The absorbance changes associated with simultaneous addition of L- and D-malate and spectrophotometric competition studies revealed that the two isomers are taken up by a common transport system. Spectral changes of the dye induced by addition of increasing concentrations of L- or D-malate indicated that the transport system favours the unphysiological D-form rather than the L-form of malate. Furthermore, it was observed that the affinity of both isomers for the transport system was dependent on the concentration of Na⁺ in the medium.

Lotspeich and co-workers originally studied the mechanism of renal excretion of tricarboxylic acid-cycle intermediates by the use of a clearance technique (Lotspeich & Woronkow, 1964; Runeburg & Lotspeich, 1966; Kook & Lotspeich, 1968). They found that both di- and tri-carboxylic acids are re-absorbed by the renal proximal tubule and readily converted into other carboxylic acids, a proportion of which is excreted in the urine. There was no evidence for renal uptake of the naturally occurring L-isomers of these compounds across the basolateral membrane.

Inherent, however, in the use of the clearance technique is the difficulty in the interpretation of transport data, because of partial degradation of the substrates, failure to dissociate metabolic from transport processes and inability to establish well-defined ionic gradients to drive the transport. The use of renal membrane vesicles to study the mechanism of transport of organic substrates offers

many advantages in these respects (Kragh-Hansen *et al.*, 1982). Wright and co-workers (Kippen *et al.*, 1979, 1980; Wright *et al.*, 1980) demonstrated, by using a Millipore-filtration technique, that luminal-membrane vesicles possess a stereospecific, electro-neutral and Na⁺-dependent transport system for di- and tri-carboxylic acids. This conclusion was based on the following observations. (1) L-Aspartate, but not D-aspartate, efficiently inhibited the uptake of [¹⁴C]succinate, indicating that the carrier-mediated transport system is sensitive to the stereoconfiguration of substrates. (2) The uptake of citrate was unaffected by the K⁺ diffusion potential created by addition of valinomycin to KCl-loaded vesicles. (3) The uptake of 2-oxoglutarate and citrate was lower in the presence of an Na⁺-salt gradient (extravesicular > intravesicular) of the more permeant anion SCN⁻ as compared with Cl⁻. (4) Kinetic studies on the stoichiometric relationship between the fluxes of ²²Na⁺ and organic anion revealed an

Na⁺/substrate coupling ratio of approx. 2:1, indicating electroneutral transport of succinate and other dicarboxylic acids.

The above-mentioned findings are in contradiction to electrophysiological studies performed by Frömter and co-workers. By means of electrical measurements they found that renal transport of doubly charged anions such as 2-oxoglutarate or malonate caused depolarization, suggesting an Na⁺/dicarboxylic acid coupling ratio greater than 2:1, possibly 3:1 (Samarzija *et al.*, 1980). In the preceding paper we reported that addition of succinate, in the presence of an Na⁺ gradient, to dye-luminal-membrane suspensions produced transient depolarization, indicative of an Na⁺-dependent electrogenic transport system for dicarboxylic acids (Kragh-Hansen *et al.*, 1982). This apparent discrepancy in the results obtained by different techniques for tricarboxylic acid-cycle intermediates led us to re-investigate the mechanism of these compounds both by a filtration technique and by spectrophotometric measurements. For this purpose, in addition to succinate, we have studied the uptake of L- and D-malate as representatives of stereoisomers of tricarboxylic acid-cyclic intermediates. In the present paper we demonstrate the existence of an Na⁺-dependent electrogenic transport system for dicarboxylic acids on the luminal-membrane vesicles prepared from rabbit kidney cortex. Furthermore, we show that the unphysiological stereoisomer, D-malate, has higher affinity for the transport system than does L-malate. Thus our findings rule out the possibility that the transport system is strictly stereospecific.

Experimental

Materials

Valinomycin, gramicidin, L-malic acid, D-malic acid, succinic acid, Trizma base and Trizma hydrochloride were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Radioactive [1,4-¹⁴C]-succinic acid (sp. radioactivity 116.8 mCi/mmol) and L-[U-¹⁴C]malic acid (sp. radioactivity 59.9 mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. 3,3'-Diethyloxadycarbocyanine iodide was bought from Eastman Kodak Co., Rochester, NY, U.S.A. Nigericin was supplied by Calbiochem-Behring Corp., La Jolla, CA, U.S.A. Sodium and potassium salts of D-gluconate were products of Fluka A.G., Buchs, Switzerland. All other reagents were of A.R. grade.

Membrane purification

The general experimental procedures were similar to those described by Kippen *et al.* (1979), with the following modifications. Adult rabbits (body wt. 2–4 kg) of either sex were anaesthetized with sodium

pentobarbitone (Nembutal; Abbott), and exsanguinated. To remove plasma albumin the kidneys were perfused with a solution of 320 mM-sorbitol in 1 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/Tris buffer, pH 7.5, via a needle inserted in the renal artery. The cortex was dissected, minced and homogenized in sorbitol/Hepes/Tris buffer to make a 10% (w/v) homogenate, in a Potter-Elvehjem homogenizer (45 strokes at maximum speed). The homogenate was centrifuged at 1500 g for 15 min in a Sorvall RC-2B centrifuge, and the supernatant was pipetted out and re-centrifuged at 110 000 g for 30 min in a Beckman L2-65 ultracentrifuge. The supernatant was decanted, and the light fluffy layer was separated from the dark pellet and suspended in sorbitol/Hepes/Tris buffer. To 9 parts of the renal suspension was added 1 part of a solution containing 100 mM-CaCl₂ dissolved in 320 mM-sorbitol/1 mM-Hepes/Tris buffer, pH 7.5. The suspension was stirred for 20 min at 4°C and then centrifuged at 1500 g for 15 min in a Sorvall RC-2B centrifuge. The supernatant was collected and re-centrifuged at 25 000 g for 30 min in a Beckman L2-65 ultracentrifuge. The pellet thus formed was resuspended in sorbitol/Hepes/Tris buffer to a final protein concentration of 10 mg/ml, consisting of highly purified luminal-membrane vesicles. The vesicle suspensions were stored at 0–4°C until use.

Criteria of purity

The fractions were assayed for various enzymic activities. Alkaline phosphatase activity was assayed by the method of Bessey *et al.* (1946) and used as a marker for luminal-membrane vesicles. Na⁺ + K⁺-stimulated ATPase activity was assayed as described by Ottolenghi (1975). Succinate dehydrogenase and glucose 6-phosphatase activities were measured as described elsewhere (Sheikh, 1972, 1976). The amount of protein in various fractions was determined by the method of Lowry *et al.* (1951) as modified by Peterson (1977), with human serum albumin (Sigma Chemical Co.) as standard.

Transport studies

The renal uptake of succinate and L-malate was studied both by spectrophotometry and by a filtration technique. Transport of D-malate was examined only by the spectrophotometric method, since the radioactive form of this compound is unavailable. Spectrophotometric measurements were performed as described in the preceding paper (Kragh-Hansen *et al.*, 1982). Uptake of ¹⁴C-labelled substrates by luminal-membrane vesicles was followed by the filtration technique originally described by Hopfer *et al.* (1973) and modified by Kippen *et al.* (1979). The details of the individual experiments are given in the legends to the Figures.

Results and discussion

Effect of various salts on the renal uptake of dicarboxylic acids

Fig. 1(a) shows the time course of uptake of L-malate into luminal-membrane vesicles under various incubation conditions as measured by a filtration technique with Sartorius membrane filters. It is seen that in the presence of extravesicular > intravesicular Na^+ gradients the initial rate of L-malate uptake is greater than that in the presence of K^+ or mannitol. Furthermore, transient overshoots are observed only in the presence of Na^+ . These results are in accordance with the proposal of Na^+ -dependent transport of dicarboxylic acids (Wright *et al.*, 1980). From Fig. 1(a) it can also be seen that the magnitude of the overshoots is dependent on the type of sodium salt present. The maximal L-malate uptake in the presence of the more permeant anion, Cl^- (1 nmol/mg of protein), is significantly higher than that measured in the

presence of SO_4^{2-} (0.6 nmol/mg of protein), and is almost double that found when the impermeant anion D-gluconate was present (0.5 nmol/mg of protein).

The uptake of L-malate was also studied by spectrophotometry, with the potential-sensitive dye 3,3'-diethyloxadadicarbocyanine iodide (Kragh-Hansen *et al.*, 1982). It is apparent from Fig. 1(b) that addition of L-malate to the membrane vesicles in the presence of extravesicular > intravesicular Na^+ -salt gradients of various anions results in spectral curves of a form resembling the membrane filtration curves shown in Fig. 1(a). The magnitude of the spectral changes ('overshoot') varies with the type of Na^+ -salt anion in the same direction as seen in the filtration experiments.

As mentioned in the introduction, Kippen *et al.* (1979) have measured the uptake of 2-oxoglutarate and citrate by luminal-membrane vesicles, and found greater uptake of these compounds in the presence of the less permeant anion Cl^- as compared with

Fig. 1. Uptake of L-malate by renal luminal-membrane vesicles

(a) Uptake of 100 μM-L-malate (including tracer amounts of ^{14}C -labelled L-malate) by membrane vesicles, as studied by the filtration technique with Sartorius membrane filters. The pH was 7.5, and the temperature 20°C. The intravesicular medium was 320 mM-sorbitol/1 mM-Hepes/Tris buffer, whereas the external medium was 155 mM- NaCl /15 mM-Tris/HCl buffer (●), 103 mM- Na_2SO_4 /15 mM-Tris/HCl buffer (▲), 155 mM-sodium gluconate/15 mM-Tris/HCl buffer (□), 155 mM-KCl/15 mM-Tris/HCl buffer (◇) or 310 mM-mannitol/15 mM-Tris/HCl buffer (○). The data are from a representative experiment performed in triplicate. (b) Uptake of 5 mM-L-malate by membrane vesicles (protein concentration 0.25 mg/ml), as studied by spectrophotometry. The pH was 7.5, and the temperature 20°C. The intravesicular medium was 320 mM-sorbitol/1 mM-Hepes/Tris buffer, whereas the external medium was 155 mM- NaCl /15 mM-Tris/HCl buffer (curve 1), 103 mM- Na_2SO_4 /15 mM-Tris/HCl buffer (curve 2), 155 mM-sodium gluconate/15 mM-Tris/HCl buffer (curve 3) or 155 mM-KCl/15 mM-Tris/HCl buffer (curve 4). The dye concentration was 15 μM. The break in the curves, at 0 min, indicates the addition of solute. All the spectral curves were corrected for the effect of adding a small volume of 15 mM-Tris/HCl buffer alone (the medium of the solute's stock solutions). The spectrophotometer was operated in the dual-wavelength mode with 580 nm and 610 nm (reference wavelength).

SCN⁻ (Berner & Kinne, 1976). This finding is in contradiction to the present results obtained with L-malate (Figs. 1*a* and 1*b*). The possibility that the results with L-malate are not representative for the tricarboxylic acid-cycle intermediates has been examined by studying the uptake of another carboxylic acid, namely succinate, by the membrane vesicles. Succinate was chosen because it is shown to be transported by the same system as that of citrate and 2-oxoglutarate (Wright *et al.*, 1980). Fig. 2 shows that the results obtained with this compound are effectively the same as those obtained with L-malate (Fig. 1*a*). Thus the results in Figs. 1 and 2 show that both L-malate and succinate are taken up by an electrogenic Na⁺-dependent mechanism, resulting in depolarization of the membrane vesicles.

Fig. 2. Time course of succinate uptake by renal luminal-membrane vesicles

Uptake of 100 μ M-succinate (including tracer amounts of ¹⁴C-labelled succinate) by membrane vesicles, as studied by a filtration technique. The intravesicular medium was 320 mM-sorbitol/1 mM-Hepes/Tris buffer, whereas the external medium was 155 mM-NaCl/15 mM-Tris/HCl buffer (●), 103 mM-Na₂SO₄/15 mM-Tris/HCl buffer (▲), 155 mM-sodium gluconate/15 mM-Tris/HCl buffer (□), 155 mM-KCl/15 mM-Tris/HCl buffer (◇) or 310 mM-mannitol/15 mM-Tris/HCl buffer (○). The data are from a representative experiment performed in triplicate.

The reason for the discrepancy between the results shown in Figs. 1 and 2 and those reported by Kippen *et al.* (1979) is difficult to explain. However, it should be noted that in a later study that group of workers, using a cyanine dye containing heterocyclic sulphur, observed that addition of succinate or citrate to dye-membrane suspension produced transient increase in fluorescence, indicative of a depolarizing event(s) associated with the transport of these substrates (Wright *et al.*, 1981), in agreement with the experimental results given in Figs. 1 and 2 of the present paper.

The spectral changes of 3,3'-diethyloxadecarboxyanine iodide accompanying addition of D-malate to luminal-membrane vesicles under different conditions are shown in Fig. 3. It is seen that also this isomeric form of malate is taken up by an Na⁺-dependent electrogenic process. Furthermore, the magnitude of the 'overshoot' curves are almost the same as those measured with L-malate (Fig. 1*b*).

Fig. 3. Uptake of D-malate by renal luminal-membrane vesicles

Uptake of 5 mM-D-malate by membrane vesicles, as studied by spectrophotometry. The experimental conditions were the same as those described in Fig. 1*b* legend.

Effect of ionophores on the uptake of L- and D-malate

The proposal of Na^+ -dependent electrogenic transport of L- and D-malate by luminal-membrane

vesicles was supported further by experiments with various ionophores. The effect of valinomycin, which mediates electrogenic K^+ movements, on the transport of the two isomers is illustrated in Figs.

Fig. 4. Effect of valinomycin on renal transport of L- and D-malate

(a) Absorbance changes caused by addition of 5 mM-L-malate to luminal-membrane vesicles. (b) Absorbance changes caused by addition of 5 mM-D-malate. Internal medium was 155 mM-KCl/15 mM-Tris/HCl buffer, and external medium 155 mM-NaCl/15 mM-Tris/HCl buffer. Curves 1, addition of 5 μg of valinomycin/mg of protein; curves 2, addition of valinomycin and, about 1½ min later, L- or D-malate; curves 3, addition of solute in the absence of valinomycin. For further details see Fig. 1(b) legend. (c) Uptake of 100 μM -L-malate (including tracer amounts of ^{14}C -labelled L-malate) by membrane vesicles, as studied by a filtration technique. Internal medium was 103 mM- K_2SO_4 /15 mM-Tris/HCl buffer, and external medium 103 mM- Na_2SO_4 /15 mM-Tris/HCl buffer. The results were obtained in the presence (●) or in the absence (○) of valinomycin. The data are from a representative experiment performed in triplicate.

4(a), 4(b) and 4(c). The experiments were performed with both an intravesicular > extravesicular K^+ gradient and an extravesicular > intravesicular Na^+ gradient. Figs. 4(a) and 4(b) depict the rate of uptake of L- and D-malate with and without addition of valinomycin respectively. It is apparent, by comparing curves 2 with curves 3, that pretreatment of the membrane vesicles with valinomycin increases the rate of uptake of both L- and D-malate. To test this finding the uptake of L- [^{14}C]malate was studied by the filtration technique. Fig. 4(c) shows that addition of valinomycin increases the maximal uptake of malate by approx. 50%. In the presence of valinomycin K^+ diffuses out of the vesicles, rendering the interior of the vesicles more negative, which in turn favours electrogenic uptake of L- and D-malate.

The effect of gramicidin, which mediates electrogenic Na^+ movements, on the optical response associated with addition of L- or D-malate to membrane vesicles was also studied. The experiments were performed with the same initial concentration of Na^+ inside and outside the vesicles. It was observed that pretreatment of the membrane vesicles with gramicidin greatly diminished the

optical response both with L-malate and with D-malate. The effect of simultaneous addition of valinomycin and nigericin, the latter ionophore mediating electroneutral exchange of Na^+ and K^+ , on the optical response associated with uptake of L- and D-malate by membrane vesicles was also studied. The experiments were performed with the same initial concentrations of Na^+ and K^+ inside and outside the vesicles. We found that pretreatment of the membrane vesicles with the ionophores greatly diminished the optical response both with L-malate and with D-malate. The rationale for the small optical responses in the presence of the above-mentioned ionophores has been thoroughly discussed in the preceding paper (Kragh-Hansen *et al.*, 1982).

Evidence for a common transport system for L- and D-malate

The results presented in the preceding sections show that the characteristics of the transport of L-malate and that of D-malate are very much alike. In this section we examine whether the isomers are transported by two different transport systems or by a common system. Figs. 5(a) and 5(b) show the

Fig. 5. Absorbance changes of 3,3'-diethyloxadibocyanine iodide induced by adding L- and D-malate. Absorbance changes caused by addition of 20 mM-L-malate (a), 20 mM-D-malate (b) or 10 mM-L-malate plus 10 mM-D-malate (c) to luminal-membrane vesicles. The intravesicular medium was 320 mM-sorbitol/1 mM-Hepes/Tris buffer, whereas the external medium was 155 mM-NaCl/15 mM-Tris/HCl buffer. For further details see Fig. 1(b) legend.

optical curves obtained after the addition of L-malate and D-malate respectively. If L- and D-malate are added simultaneously to the membrane-vesicle suspension in concentrations sufficient to saturate their respective transport systems, the optical response should be the sum of those shown by Figs. 5(a) and 5(b) if the solutes are taken up by two different systems, whereas the result will be the same as Figs. 5(a) and 5(b) if the isomers are transported by a common system. It is seen (Fig. 5c) that the result of adding L- and D-malate simultaneously to the membrane vesicles is in full accordance with the latter possibility. Thus the results presented in Fig. 5 unambiguously show that L- and D-malate are taken up by a common transport system.

Uptake of L- and D-malate at various concentrations

Fig. 6(a) shows the total absorbance changes induced by the addition of increasing concentrations of L- and D-malate to suspensions of luminal membrane vesicles. Curves 1 and 2 depict the uptake of D- and L-malate respectively under a 155 mM- Na^+ -gradient. The relationship between increases in total absorbance change and increases in the concentrations of organic anions can be described by an equation identical in form with the Michaelis-Menten equation:

$$\Delta A = \frac{\Delta A_{\max} [S]}{K_A + [S]}$$

where ΔA is the absorbance change measured by addition of malate at concentration $[S]$, $\Delta A_{\max}$ is the maximal absorbance change and K_A is the malate concentration producing a half-maximal absorbance change. In order to calculate apparent K_A values for D- and L-malate we have made a plot of reciprocal values of concentrations of organic anions versus reciprocal values of absorbance changes (Fig. 6b). It can be seen that both curves are rectilinear and intersect one another on the ordinate. This analysis of the experimental data thus demonstrates that the $\Delta A_{\max}$ values for D- and L-malate are the same, whereas apparent K_A values of these isomers significantly differ (apparent K_A values for D- and L-malate are calculated to be approx. 0.15 mM and 0.24 mM respectively). Curves 3 and 4 of Fig. 6(a) show the uptake of D- and L-malate in the presence of 15 mM- NaCl and 140 mM-choline chloride. It appears that the $\Delta A_{\max}$ for the organic anion isomers is unaltered under these conditions, whereas the apparent K_A values for both D- and L-malate are increased more than 20-fold. These results revealed that Na^+ ions influenced the affinity for transport of tricarboxylic acid-cycle intermediates. Furthermore, the transport system favours the D-form rather than the L-form of malate.

Fig. 6. Effect of Na^+ concentration on the kinetics of L- and D-malate uptake

(a) Effect of substrate concentration on the L-malate ($\circ$ and $\bullet$) and D-malate (Δ and $\blacktriangle$)-induced absorbance changes of 3,3'-diethyloxadycarbocyanine iodide. $\circ$ and Δ , External medium was 155 mM- NaCl /15 mM-Tris/HCl buffer; $\bullet$ and $\blacktriangle$, external medium was 15 mM- NaCl /140 mM-choline chloride/15 mM-Tris/HCl buffer. The intravesicular medium was in all cases 320 mM-sorbitol/1 mM-Hepes/Tris buffer. For further details see Fig. 1(b) legend. (b) Results obtained in the presence of 155 mM- NaCl represented in a double-reciprocal plot.

Concluding remarks

The spectrophotometric method described in the preceding paper (Kragh-Hansen *et al.*, 1982) has enabled us to study in detail the mechanisms of uptake of D-malate by luminal-membrane vesicles, and the results have revealed that the dicarboxylic acid-transport system is not strictly stereospecific. Moreover, preliminary spectrophotometric experiments (results not shown) with other tricarboxylic acid-cycle intermediates confirmed the previous findings that the di- and tri-carboxylic acids share

the same transport system. However, the question whether this transport system can accommodate acidic amino acids such as aspartic acid and glutamic acid remains to be solved. It should be noted that the results on renal uptake of organic anions observed by the use of the filtration technique and the spectrophotometric method are in accordance. By contrast with previous findings, the results given in the present paper demonstrate unequivocally that the uptake of dicarboxylic acids by luminal-membrane vesicles is an electrogenic Na^+ -dependent process.

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