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Anion Carriers of Mitochondrial Membranes

With 147 Figures

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Preface

Almost a quarter of a century ago it became apparent that most of the important metabolites that circulate between cytosol and mitochondria do not cross the inner mitochondrial membrane freely but are transferred by specific carriers. During subsequent years, several carriers of this kind have been functionally identified. Their total number amounts, to our present knowledge, to 12-14. However, successful attempts to isolate some of them started only at the end of the last decade. Methods for isolation, purification, and functional reconstitution of mitochondrial carriers which have been developed during the last few years have opened a new and fascinating field. A few carriers have been sequenced, which enables the study of their arrangement within the membranes and elucidation of their mechanism of action. Genetic and comparative evolutionary studies also offer further possibilities. Finally, possible defects of particular carriers may help to understand the genesis of some inborn metabolic disorders.

In order to assemble the information available today on mitochondrial carriers and to set up projects for the future, an International Conference on Anion Carriers of Mitochondrial Membranes, was held on 5-9 July 1988 in Zakopane, Poland. Its program was formulated by the editors of this volume in collaboration with Pierre V. Vignais (Grenoble) and Attila Fonyó (Budapest) with the aim of bringing together practically all the scientists working in the field .

The Zakopane Conference, the first specifically devoted to mitochondrial transport proteins, was organized by the Nencki Institute of Experimental Biology in Warsaw to celebrate the 70th anniversary of its foundation. Named after Marceli Nencki (1847-1901), eminent physiologist and biochemist of Polish origin, active in Bern (Switzerland) and St. Petersburg (Russia), this institute was founded in 1918-1919, moved to Łódz after the Second World War, and in 1952 back to Warsaw as an establishment of the Polish Academy of Sciences.

One immediate result of the Conference was to demonstrate that the interest in mitochondrial carriers was not restricted to a small specialized group of scientists. Rather, due to the interactions that mitochondrial anion transport has with the rest of the cell, in catabolic and anabolic processes, and under physiological and pathological conditions, a wider echo was achieved among scientists operating in cell metabolism, organ and in vivo bioenergetics, as well as among researchers dedicated to medical problems.

The present book contains the full texts of most of the invited lectures presented at the Zakopane Conference.

Apart from the anion carriers of the inner mitochondrial membrane, the subject of the Conference was extended both to the proton-conducting protein of brown adipose tissue mitochondria, because of its structural similarity to the adenine nucleotide carrier, and also to the pore protein of the outer mitochondrial membrane, because of the methodological similarity of its isolation and its functional relationships with the inner mitochondrial membrane carriers.

Angelo Azzi Katarzyna A. Nałęcz Maciej J. Nałęcz Lech Wojtczak

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I. Isolation and Reconstitution of Carriers

Purification and Characterization of Three Mitochondrial Substrate Carriers: the Phosphate, the 2-Oxoglutarate and the Dicarboxylate Carriers

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Since several substrates of the mitochondrial enzymes must be transported from the cytosol into the matrix and several products have to leave the mitochondria, the inner mitochondrial membrane has to be equipped with transport systems which catalyze metabolic flows between the inner and the outer compartment. The existence of at least 9 transport systems for metabolites has been documented in some detail by studies performed in intact mitochondria (LaNoue & Schoolwerth, 1979; Meijer & Van Dam, 1981; Palmieri *et al.*, 1987). With the exception of the carrier for carnitine and acylcarnitine esters, the other carriers deal with the transport of anions: substrates of oxidative phosphorylation, ADP, ATP and phosphate, fuels of the tricarboxylic acid cycle, pyruvate, β-hydroxybutyrate and acetoacetate, dicarboxylates and tricarboxylates, and substrates of amino acid metabolism. This intense traffic of anions across the mitochondrial membrane is necessary, besides for oxidative phosphorylation, the tricarboxylic acid cycle and the amino acid metabolism, for the transfer of reducing equivalents in both directions and for important metabolic pathways, whose enzymes

Abbreviations: GOT, glutamate-oxaloacetate transamianse; SDS, sodium dodecylsulphate; EMA, eosin-5-maleimide; PTC, phenylisothiocyanate; p-sulpho-PTC, p-sulphophenylisothiocyanate; ANPP, 4-azido-2-nitrophenyl phosphate

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are partitioned between the cytosol and the mitochondria, such as gluconeogenesis, fatty acid synthesis, ketogenesis, β-oxidation of fatty acids and, in liver, urogenesis. Many of the properties of the proposed mitochondrial anion carriers, *i.e.* the high specificity, the existence of specific inhibitors, the saturation kinetics, the different distribution in various tissues and species and the inhibition by SH reagents have pointed to the protein nature of these transport systems. Their isolation, however, has been hindered for quite a long time.

The ADP/ATP transport system has been the first mitochondrial carrier to be isolated (Klingenberg *et al.*, 1978). It was purified by solubilization of the mitochondrial membranes with Triton and chromatography on hydroxyapatite. Later, around the end of 1980, the phosphate carrier has also been purified by chromatography on hydroxyapatite (Wohlrab, 1980; Kolbe *et al.*, 1981; Palmieri *et al.*, 1981; Touraille *et al.*, 1981). Until 1984 the hydroxyapatite eluate of Triton-solubilized heart mitochondria was generally considered to contain only the ADP/ATP carrier and the phosphate carrier. However, when high resolution SDS gel electrophoresis is applied, the pass-through of hydroxyapatite of Triton-solubilized heart mitochondria can be shown to contain 5 protein bands in the molecular weight region of 30-36 kDa (Bisaccia & Palmieri, 1984). The first fraction contains four protein bands called 2-5, since band 1 appears only in later fractions. With the exception of the protein band with the lowest M_r (band 5) which is the ADP/ATP carrier, the identity of the other bands was unknown.

In the last few years we have identified three of the four remaining bands of the hydroxyapatite pass-through of Triton-solubilized heart mitochondria as carriers or pore proteins. Band 2 represents the mitochondrial porin (De Pinto *et al.*, 1985), band 3 the phosphate carrier (Bisaccia &P almieri, 1984) and band 4 the oxoglutarate carrier (Bisaccia *et al.*, 1985). This identification was based on the purification of each protein band and the use of functional reconstitution as a monitor of the transport activity during isolation. Using the same strategy we have also identified the dicarboxylate carrier and the oxoglutarate carrier in the hydroxyapatite pass-through of Triton-solubilized liver mitochondria (Bisaccia *et al.*, 1988).

The aim of this paper is to give an overview on the state of the art about identification and characterization of the mitochondrial substrate carriers we have purified from heart and/or liver mitochondria.

METHODS

The procedures for the isolation of carrier proteins have been reported previously. Essentially, the phosphate carrier was purified by chromatography on hydroxyapatite of Triton X-114 solubilized heart mitochondria supplemented with

4 mg/ml cardiolipin (Bisaccia & Palmieri, 1984). The oxoglutarate carrier was purified by chromatography on hydroxyapatite and celite of Triton X-114 solubilized heart mitochondria in the presence of 2 mg/ml cardiolipin (Bisaccia *et al.*, 1985). Porin was purified from Triton X-100 solubilized mitochondria by chromatography on hydroxyapatite and celite in the absence of cardiolipin (De Pinto *et al.*, 1985; De Pinto *et al.*, 1987). The dicarboxylate carrier and the oxoglutarate carrier from rat liver mitochondria were purified by chromatography on hydroxyapatite after partial or extensive removal of Triton X-114 from the mitochondrial extract (Bisaccia *et al.*, 1988).

Reconstitution of carrier proteins into liposomes was carried out by the freeze-thaw-sonication procedure (Kasahara & Hinkle, 1977) or by removing the detergent by chromatography on Amberlite (Krämer & Heberger, 1986; Indiveri *et al.*, 1987a; Bisaccia *et al.*, 1988).

Transport of labelled substrates in reconstituted liposomes was measured by the inhibitor stop method (Palmieri & Klingenberg, 1979). Polyacrylamide slab gel electrophoresis, staining and protein determination were performed as described previously (Bisaccia & Palmieri, 1984).

For immunoblotting of the oxoglutarate carrier, the proteins separated on 17.5% acrylamide slab gels were electro-blotted onto nitrocellulose. The transferred proteins were incubated with an antiserum raised against the oxoglutarate carrier purified from bovine heart mitochondria. The antibody-antigen complexes were decorated with peroxidase-conjugated antirabbit IgG. The peroxidase reaction was performed in a mixture containing 4-chloro-1-naphtol and H_2O_2.

Results and Discussion

Isolation of carrier proteins from heart mitochondria

A prerequisite in the elucidation of the function of a protein is its isolation. We have indeed isolated band 2, band 3 and band 4 in a pure form, *i.e.* each of the protein bands present in the first fraction of the hydroxyapatite eluate of Triton-solubilized heart mitochondria. For identification we tested each isolated protein for several transport activities after their reconstitution into liposomes. In this way we found that band 3 of M_r 33 kDa is the phosphate carrier (Bisaccia & Palmieri, 1984) and band 4 of M_r 31.5 kDa is the oxoglutarate carrier (Bisaccia *et al.*, 1985). The isolated band 2 of M_r 35 kDa did not show any transport activity for substrates of mitochondrial anion carriers. However, we thought that it could be another very hydrophobic protein which forms channels, *i.e.* the porin of the outer mitochondrial membrane. This was confirmed by reconstitution experiments in planar bilayer membranes in which band 2

induces a step-wise increase of the membrane conductance due to the formation of single channels or pores, which have characteristics similar to those produced by porins previously isolated from bacteria and *Neurospora crassa* (De Pinto *et al.*, 1985).

Phosphate carrier

The isolated band 3 protein, when reconstituted into liposomes, exhibits transport properties very similar to those described for the phosphate transport system in mitochondria. Thus, it catalyzes both the unidirectional transport of phosphate (uptake or efflux) and the exchange between the internal and the external phosphate. The transport of phosphate in the reconstituted system is inhibited by SH-blocking reagents. The reconstituted phosphate exchange follows a first order kinetics and is highly temperature dependent with an E_A of 64 kJ/mol in the lower temperature range. The affinity of the carrier for phosphate is low (K_m for external phosphate 2.2 mM) and the maximum exchange rate at 25°C is 71,000 μmol/min x g protein. Furthermore, the movement of phosphate in proteoliposomes is regulated by the pH gradient across the membrane, as already shown in mitochondria (Palmieri *et al.*, 1970). Based on functional reconstitution the band 3 protein, *i.e.* the phosphate carrier, has been purified 290 fold with a protein yield of 0.22%. This purification factor gives an estimation for the enrichment of the carrier protein. However, purification factors based on reconstituted activities are in general not very accurate due to possible inactivation during purification and incorporation into liposomes.

The isolated phosphate carrier consists of a single band of M_r 33 kDa upon high resolution SDS gel electrophoresis. Kolbe *et al.* (1984) published that the isolated phosphate carrier consists of two bands. We however found that the appearance of these two bands depends on the experimental conditions of how the gel electrophoresis is run. In our hands, under appropriate conditions *i.e.* in the presence of 5% 2-mercaptoethanol, we reproducebly find only one band. In collaboration with Aquila and Klingenberg we have determined more than 90% of the amino acid sequence of the phosphate carrier by Edman degradation (unpublished results). In the meantime (Runswick *et al.*, 1987) have published the entire sequence by cDNA. From the sequence data it appears that the phosphate carrier is homologous to the ADP/ATP carrier and to the uncoupling protein (Runswick *et al.*, 1987; Aquila & Klingenberg, 1987).

Although these results have greatly improved our understanding of the structure of the phosphate carrier, the mechanism of transport remains unknown. To gain more information about the mechanism, it is important to elucidate the phosphate-binding site and to find out which aminoacid residues play a key role in phosphate translocation. So far, SH groups represent the only well-defined functional groups of the phosphate carrier (Fonyo, 1978).

Since positive charges are likely to be present at the substrate-binding site we have investigated the effect of phenylisothiocyanate (PITC) and p-sulphophenyl-isothiocyanate (p-sulphoPITC) on the activity of the phosphate carrier. PITC and p-sulphoPITC were chosen as reagents for the identification of essential lysine residues, since the hydrophobic PITC is expected to modify preferably lysine residues within the core of the membrane (Sigrist & Zahler, 1978) and the polar p-sulphoPITC lysines located at the surface of the membrane. In these experiments, mitochondria were incubated with 2 mM p-sulphoPITC or 2 mM PITC at pH 7.5 and 9, respectively. After washing the mitochondria, the phosphate carrier was solubilized, purified and tested for transport activity in the reconstituted system. Both PITC and p-sulphoPITC cause a substantial decrease of the phosphate-phosphate exchange activity either at pH 7.5 or at pH 9.0. Since isothiocyanates react with nucleophiles in their unprotonated form and may therefore react also with cysteines, it was necessary to check whether the inhibition can be accounted for by an interaction of p-sulphoPITC and PITC with SH groups. This was done by including an excess of DTE in the washing medium at pH 8.5, since these conditions reverse the binding of these inhibitors with SH groups, but not with NH_2 groups. Under these conditions, therefore, the remaining inhibition can be attributed to the reaction with NH_2 groups. In the presence of p-sulphoPITC, DTE restores the activity but only partially; a significant portion of the total inhibition caused by p-sulphoPITC is still present after the treatment with DTE. In the case of PITC, on the other hand, DTE does not cause any appreciable reactivation presumably because in the lipid core of the membrane cysteines are protonated and therefore not reactive. Furthermore, the DTE-insensitive inhibition by p-sulphoPITC is pH-dependent, *i.e.* is more evident at pH 9.0 than at pH 7.5 (Genchi *et al.*, 1988), since this reagent interacts with lysine(s) located at the surface of the membrane which undergo protonation and deprotonation according the pH of the medium. In contrast, the inhibition by PITC is not affected by changing the external pH (Genchi *et al.*, 1988), since this apolar reagent interacts with the buried lysines of the hydrophobic phase of the membrane which should be independent from the bulk-phase pH. On this basis we suggest the presence of a least two types of essential lysine residues in the phosphate carrier, one type located in the hydrophobic moiety of the protein and the other at the surface of the protein. Both kinds of lysines seem to be important for the transport of phosphate because their modification leads to the inhibition of the phosphate carrier.

In order to have a tool for the characterization of the substrate-binding site of the phosphate carrier, we have investigated the reactivity of the carrier with the photoreactive substrate analogue 4-azido-2-nitrophenyl phosphate (ANPP), which was introduced by Lauquin *et al.* (1980) to label the phosphate-binding site of the F_1-ATPase. In the dark ANPP inhibits the transport of phosphate in a competitive manner. Upon illumination however the inhibition becomes irreversible indicating

covalent binding of the inhibitor (Tommasino *et al.*, 1987). In contrast, the non phosphorylated analogue, 4-azido-2-nitrophenol, does not cause any inhibition of phosphate transport even after illumination. Thus the presence of the phosphate group in the ANPP molecule is an essential requirement for the inhibition of phosphate transport. This also suggests that ANPP reacts at the substrate-binding site of the phosphate carrier. This suggestion is supported by the finding that phosphate protects the carrier against inactivation by ANPP and that this effect is specific, since other anions, like sulphate, do not show any protection (Tommasino *et al.*, 1987). In other experiments we have also found that the radioactivity bound to the purified phosphate carrier is drastically reduced by the presence of phosphate during the incubation of the mitochondria with ^{32}P-ANPP. On this basis we conclude that ANPP can be used as a label of the substrate-binding site of the phosphate carrier.

2-Oxoglutarate carrier

The functional properties of the isolated band 4 protein of M_r 31.5 kDa reconstituted into liposomes closely resemble those of the oxoglutarate transport system as characterized in mitochondria (Bisaccia *et al.*, 1985). The purified and reconstituted protein catalyzes an exchange between 2-oxoglutarate and L-malate. As the transport system in mitochondria (Palmieri *et al.*, 1972), the purified protein accepts also other dicarboxylates, although with a lower affinity. It is inhibited by phthalonate and, less specifically, by some dicarboxylate analogues. It is also inhibited by certain SH reagents (mersalyl and p-chloromercuribenzoate) but not by N-ethylmaleimide. The substrate affinity for oxoglutarate is 65 μM and the maximum exchange rate at 25°C reaches 4,000-22,000 μmol/min • g protein, in dependence of the particular reconstitution conditions (Indiveri *et al.*, 1987b). The activation energy of the exchange reaction is 54 kJ/mol. The transport is independent of pH in the range between 6 and 8. The oxoglutarate carrier purified from bovine heart and reconstituted into liposomes catalyzes also some unidirectional uptake of oxoglutarate (Indiveri *et al.*, 1987b). This uptake is completely sensitive to the specific inhibitor phthalonate and is only confined to the first fraction of the oxoglutarate preparation. The main indications for an unidirectional transport of oxoglutarate are (1) the uptake of oxoglutarate into unloaded liposomes and (2) the lack of efflux of labelled oxoglutarate after addition of cold substrate to unloaded liposomes. The possibilities of a binding to the protein or a half cycle reaction, *i.e.* a single inward translocation event, are ruled out by the fact that the number of substrate molecules transported exceeds the amount of the carrier protein by 3 orders of magnitude. It is likely that the solubilized and reconstituted oxoglutarate carrier can exist in two states of conformation, which catalyze different mechanisms of ttransport. This explained, for exam'ple, the interaction with the dry

hydroxyapatite/celite in the course of the adsorption chromatography, which would predominantly affect the first eluted fraction.

Apart from the inhibition by mersalyl and p-chloromercuribenzoate, nothing is known about the SH groups or other functional groups of the oxoglutarate carrier. The studies on the oxoglutarate carrier's sulphydryl groups have been hindered by their relatively low reactivity with mercurials and also by the impossibility to label the protein with radioactive N-ethylmaleimide. However, we have now found that a fluorescent maleimide, *i.e.* eosin-5-maleimide (EMA), strongly inhibits the oxoglutarate carrier (Zara & Palmieri, 1988). Thus half maximal inhibition is achieved with 26 μM EMA. Since EMA is impermeable and reacts covalently, we have employed this reagent for investigating the localization of the SH groups of the membrane-bound oxoglutarate carrier. This was done both in mitochondria and submitochondrial particles, which are inside-out vesicles with respect to mitochondria. In these experiments mitochondria and submitochondrial particles were incubated with 250 μM EMA for 45 min at 0^{o}C and in the dark. After washing, the oxoglutarate carrier was purified, tested for transport activity in the reconstituted system and subjected to gel electrophoresis. As revealed by fluorography, the oxoglutarate carrier isolated from mitochondria is labelled by EMA, but the carrier isolated from submitochondrial particles is not (Zara & Palmieri, 1988). Correspondingly the activity of the reconstituted oxoglutarate carrier isolated from EMA-labelled mitochondria is inhibited by 75%, whereas that of the carrier derived from EMA-labelled submitochondrial particles is not affected. These results clearly indicate that the essential EMA-reacting SH group(s) of the oxoglutarate carrier are located at the cytosolic face of the inner mitochondrial membrane. This asymmetry of the oxoglutarate carrier seems to be preserved even after reconstitution of the protein into the liposomes, since EMA also almost completely inhibits the reconstituted carrier (Zara & Palmieri, 1988). Experiments are now in progress in our laboratory to locate these essential SH groups within the amino acid sequence of the carrier.

We have started to determine the primary structure of the oxoglutarate carrier by cDNA in collaboration with Dr. J. Walker. The results so far obtained show a high degree of homology with the ADP/ATP carrier and the phosphate carrier. It has been suggested that the ADP/ATP carrier and the phosphate carrier can be considered to be formed by three fragments of about 100 amino acids, which are homologous one to another (Runswick *et al.*, 1987; Aquila *et al.*, 1987). It is interesting that we have found three pieces in the oxoglutarate carrier, which are homologous to 3 pieces of the ADP/ATP carrier and the phosphate carrier, which are present in the first, the second and the third fragment of their structures. This suggests that also the oxoglutarate carrier may consist of three repeating units.

In other experiments, the reactivity of an antiserum raised against the oxoglutarate carrier from bovine heart with the same protein from other sources was investigated by immunoblot. The antiserum cross-reacts with the oxoglutarate carrier present in heart mitochondria of all the species tested (beef, pig, rat and rabbit). In contrast, there is no cross reactivity of the antiserum raised against the oxoglutarate carrier purified from bovine heart mitochondria with the same carrier of other tissues such as liver and kidney even from the same species. This indicates an organ specificity of the antigenic properties of the oxoglutarate carrier. The antiserum was also employed to estimate the amount of the oxoglutarate carrier in heart mitochondria with the enzyme-linked immunosorbant assay. In this way we have found that the oxoglutarate carrier represents about 0.2% of the total mitochondrial proteins in beef-, pig- and rat-heart. This value is much lower than the content of the adenine nucleotide carrier and close to that of the phosphate carrier in heart mitochondria.

Isolation of carrier proteins from liver mitochondria

Some mitochondrial anion-transporting systems, such as the dicarboxylate carrier and the citrate carrier, are virtually absent in heart (Sluse *et al.*, 1971; Robinson & Oei, 1975), but have high activity in liver (Palmieri *et al.*, 1971; Palmieri *et al.*, 1972). The isolation of mitochondrial substrate carriers from liver is more difficult than from heart. Until recently, only the phosphate carrier has been purified from rat liver (Kaplan *et al.*, 1986). Its isolation, however, has needed a much more elaborate procedure than the corresponding heart carrier. Similarly for the oxoglutarate carrier, the procedure developed for its isolation from heart (Bisaccia *et al.*, 1985) does not result in a pure preparation when applied to liver (unpublished data). Although our standard procedure of hydroxyapatite chromatography (Bisaccia & Palmieri, 1984) already results in a considerable enrichment of the activity of the oxoglutarate carrier and the dicarboxylate carrier, these proteins are by no means pure. In fact, the "standard" hydroxyapatite eluate of Triton-solubilized liver mitochondria contains many more bands than the corresponding eluate from heart. The first step of improvement was achieved by varying the amount of hydroxyapatite and of the concentration of Triton X-114 in the elution buffer. The breakthrough in the purification of single carriers, however, was achieved by extensive removal of the detergent by hydrophobic chromatography before application onto the hydroxyapatite columns. This led to adsorption of the carrier proteins to the hydroxyapatite and made possible the specific elution of these translocators.

Oxoglutarate carrier from liver

Once bound to the column according to this procedure, the oxoglutarate carrier can be easily eluted in pure form by application of the appropriate buffer and detergent concentration (Bisaccia *et al.*, 1988). The isolated protein consists of one single band in SDS gel chromatography, showing a slightly higher molecular weight as compared to that of the oxoglutarate carrier isolated from heart mitochondria (Bisaccia *et al.*, 1985). If reconstituted into liposomes the purified protein catalyzes a phthalonate-sensitive oxoglutarate/oxoglutarate exchange. Uptake of oxoglutarate is negligible when unloaded liposomes are used. Further, the purified oxoglutarate carrier transports L-malate and other dicarboxylates besides oxoglutarate and is inhibited by certain substrate analogues and by certain SH reagents, as found in mitochondria (Palmieri *et al.*, 1972).

Dicarboxylate carrier from liver

The procedure described above for the purification of the oxoglutarate carrier from liver does not lead to purification of the dicarboxylate carrier, since this protein binds more strongly to hydroxyapatite and could be eluted from the column only in small amounts and in the presence of the oxoglutarate carrier. The experimental consequence was to optimize the concentration of detergent which should be high enough on the one hand to avoid strong interaction of the dicarboxylate carrier with the hydroxyapatite during column chromatography and low enough on the other hand to retain the contaminating proteins. By hydroxyapatite chromatography of the mitochondrial extract in which the Triton X-114 was decreased to 1.5%, we have achieved to obtain a preparation which exhibits a high dicarboxylate carrier activity after reconstitution into liposomes. On SDS gel electrophoresis this preparation consists mainly of one protein band with an M_r of 28 kDa (Bisaccia *et al.*, 1988).

The 28 kDa protein isolated from rat liver mitochondria, when reconstituted into liposomes, shows transport properties very similar to those of the dicarboxylate carrier characterized in mitochondria. It catalyzes a very active butylmalonate-sensitive malate/phosphate exchange, and in unloaded liposomes the uptake of both malate and phosphate is negligible. As the transport system in mitochondria (Palmieri *et al.*, 1971; Crompton *et al.*, 1974a and 1974b), the purified and reconstituted protein transports not only dicarboxylates (but not oxoglutarate), but also phosphate and some sulphur containing compounds, *i.e.* sulphate and thiosulphate. It is inhibited by some dicarboxylate analogues (but very slightly by phthalonate) and by SH reagents including eosin-5-maleimide (but not by N-ethylmaleimide). In more recent experiments we have found that phosphate appears to bind to the carrier at a site different from that for dicarboxylates. The main indications are a) the inhibition of malate uptake by external phosphate is non-competitive, b) the inhibition of phosphate uptake by external malate