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DICARBOXYLATE TRANSPORT ACROSS THE INNER MEMBRANE OF THE CHLOROPLAST ENVELOPE *

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Summary

The uptake of radioactively labeled dicarboxylates into the sorbitol-impermeable ^3H_2O space (the space surrounded by the inner envelope membrane) of spinach chloroplasts has been studied by means of silicone layer filtering centrifugation.

1. Malate, aspartate and a number of other dicarboxylates are rapidly transported across the envelope leading to an accumulation in the chloroplasts. This uptake proceeds mainly by a counterexchange with the dicarboxylates present there.
2. The dicarboxylate transport shows saturation characteristics allowing the determination of K_m and V.
3. All dicarboxylates transported act as competitive inhibitors of the transport.
4. The activation energy of the transport as determined from the temperature dependency is evaluated to be 7 kcal/mol.
5. The rate of dicarboxylate transport is influenced by illumination, the countertransported molecules and the pH in the medium. These changes effect the transport velocity, whereas the corresponding K_m values are not altered.
6. It is discussed whether there is more than one carrier involved in dicarboxylate transport in spinach chloroplasts.

Introduction

The chloroplast envelope consists of two membranes, of which the outer one has been found to be freely permeable to low molecular compounds such as sucrose, sorbitol, phosphate, nucleotides and dicarboxylates [2]. The func-

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* These results are part of a thesis (Medizinische Fakultät, Universität München) by K. Lehner. Some of the data have been included in a preliminary report [1].

tional barrier between the external space and the chloroplast stroma is the inner membrane. The space surrounded by the inner membrane will be defined here as the sorbitol-impermeable H_2O space. Evidence has been presented that there are a number of different translocators in the inner envelope membrane of spinach chloroplasts, enabling a specific transport of ATP [3], glucose and other hexoses [4], inorganic phosphate, 3-phosphoglycerate and dihydroxyacetone phosphate [5] and various dicarboxylates, e.g. malate, aspartate and glutamate [5]. In the present report the properties of the dicarboxylate transport will be dealt with in detail. The transport across the inner membrane of the chloroplast envelope will be measured as transport into the sorbitol-impermeable H_2O space of intact chloroplasts, and this will be called in short "transport into chloroplasts". The sorbitol-impermeable H_2O space of intact chloroplasts consists of the stroma and of the thylakoid space. It may be noted that the thylakoid space is not freely accessible from the external space. The observation of a large pH gradient between the thylakoid space and the external space of intact illuminated chloroplasts leads to this conclusion [6]. The thylakoid space appears to be only about 12% of the sorbitol-impermeable H_2O space [6]. Therefore the calculated metabolite concentration in the sorbitol-impermeable H_2O space will be in most cases very close to the corresponding concentrations in the stroma.

Materials and Methods

(a) Chloroplasts with intact envelopes were prepared from spinach obtained at the local market according to the method of Cockburn et al. [7] modified by Heldt and Sauer [2].

(b) The incubation was normally carried out in a medium containing 0.33 M sorbitol, 50 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethane sulphonic acid (HEPES) adjusted to pH 7.6 with NaOH, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 2 mM EDTA. Chlorophyll was assayed after the method of Whatley and Arnon [8]. If not stated otherwise, the temperature was 4°C.

(c) The measurement of the uptake of dicarboxylates was initiated by adding 10 μl of ^{14}C- or ^3H-labeled dicarboxylates to 300 μl of chloroplast suspension contained in a 400 μl capacity polypropylene microtube (Sarstedt, Nümbrecht, W. Germany). The uptake was terminated by rapid centrifugation of the chloroplasts through a layer of silicone oil into 20 μl of 1 M $HClO_4$. For details of silicone layer filtering centrifugation and evaluation of the uptake into the sorbitol-impermeable ^3H_2O space, which is the space surrounded by the inner envelope membrane, see ref. 6.

(d) When indicated, the chloroplasts were preincubated with 6—12 mM of dicarboxylates for 20 min at 4°C prior to transport measurement and washed afterwards by centrifugation. In this way the internal pool of dicarboxylates is exchanged by the desired species.

(e) The ^{14}C- and ^3H-labeled dicarboxylates were obtained from New England Nuclear Co., Boston, and Amersham, England. The specific activities employed were 1—4 Ci/mol (^{14}C) and 20—100 Ci/mol (^3H).

Results

Time course of dicarboxylate uptake

Fig. 1 shows the time course for the uptake of various dicarboxylates into chloroplasts. The uptake of aspartate, malate and succinate occurred very rapidly, whereas the uptake of malonate was extremely slow. Apparently, the uptake has a strong specificity. The rapid dicarboxylate uptake leads to an accumulation in the chloroplasts. Thus in the experiment of Fig. 1 the aspartate concentration in the chloroplasts was found after 400 s to be 30-fold higher than in the medium. As the kinetics of dicarboxylate uptake are very rapid, the rate measurements dealt with in the following have been obtained from measuring times of 10—15 s at 4°C. Thin-layer chromatography was used to check that under the conditions of the experiment (4°C, darkness, short measuring times) all the radioactive label was in the aspartate, malate and succinate. Similar results were also obtained with the other dicarboxylates dealt with in the following. These findings indicated that there was no significant metabolic interconversion of the dicarboxylates in the chloroplasts in our experiments.

Kinetic parameters

In the experiment of Fig. 2 the concentration dependence of the transport of a number of dicarboxylates was studied by varying the corresponding concentrations in the medium. The concentration dependence reveals hyperbolic saturation characteristics indicating substrate saturation of the transport. A double reciprocal plot of the data yielded a linear function, which enabled the determination of K_m (substrate concentration causing half maximal rate of transport) and V (maximal velocity of transport). In other experiments not shown here such linear curves were also obtained for the uptake of α-keto-

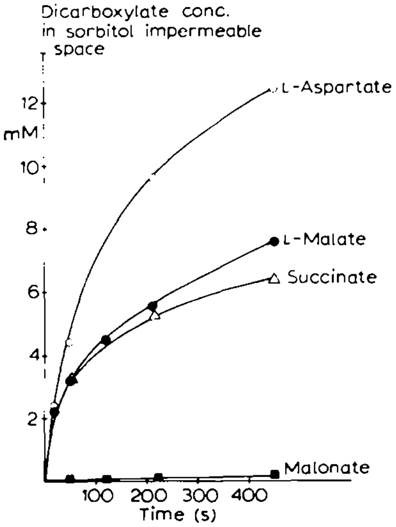

Fig. 1. Uptake of dicarboxylates (0.5 mM external concentration) into chloroplasts.

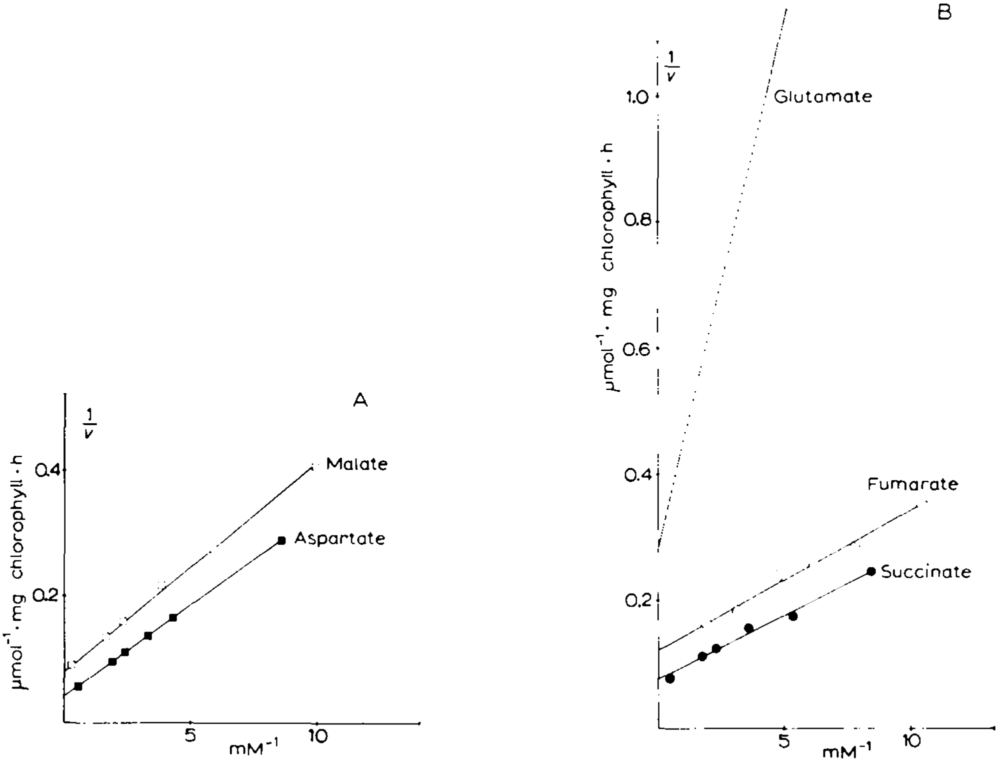

Fig. 2. Concentration dependence of the uptake of various dicarboxylates into the sorbitol-impermeable space of chloroplasts.

glutarate. The kinetic constants for the transport of oxaloacetate, which is apparently also taken up, could not be measured since this compound is not available radioactively labeled. Table I shows mean values for K_m and V obtained from several experiments. It appears from these data that the transport has a high affinity for fumarate and succinate, followed by malate and α-ketoglutarate and a much lower affinity for glutamate, whereas the V is highest with aspartate. The absolute values of V expressing the transport activity as related to the chlorophyll content of the chloroplasts showed considerable variations between different chloroplast preparations. The data of Table I were obtained with leaf material obtained at the local market or conventionally grown under artificial light. These chloroplasts usually showed a CO_2 fixation rate of 40—60 μmol/mg chlorophyll per h. At the end of our investigations we were able to use spinach grown in water culture according to Lilley and Walker [10]. In such chloroplasts, showing CO_2 fixation rates of about 100 μmol/mg chlorophyll per h, the V values were sometimes more than five times higher than in Table I.

Competition

Those dicarboxylates transported into the chloroplasts also act as competitive inhibitors of the transport. Fig. 3 shows that the uptake of malate was

TABLE I

KINETIC CONSTANTS OF DICARBOXYLATE TRANSPORT INTO THE SORBITOL-IMPERMEABLE SPACE OF SPINACH CHLOROPLASTS

Temperature 4°C. δ = standard deviation.

	V (μmol/mg chlorophyll per h)	δ	K_m (mM)	δ	n
L-Malate	18.6	7.0	0.39	0.06	11
Succinate	14.0	4.4	0.26	0.04	9
Fumarate	18.6	3.5	0.21	0.04	5
L-Aspartate	31.1	16.1	0.72	0.14	11
α-Ketoglutarate	26.4	—	0.19	—	3
L-Glutamate	7.9	3.2	1.17	0.23	5

competitively inhibited by the addition of fumarate, aspartate, glutamate, succinate, oxaloacetate and α-ketoglutarate. The corresponding K_i values, as determined in a number of different experiments, are presented in Tables II and III. Though in our experiments the absolute K_i values differed sometimes by a factor of 2, the relationship between the K_i of various substances and also between the K_m and K_i values was almost constant. As shown in Table II, the transport of malate was inhibited by fumarate and the transport of fumarate was inhibited by malate. Within the experimental error the K_m of malate was very similar to the K_i of malate for inhibition of fumarate and succinate trans-

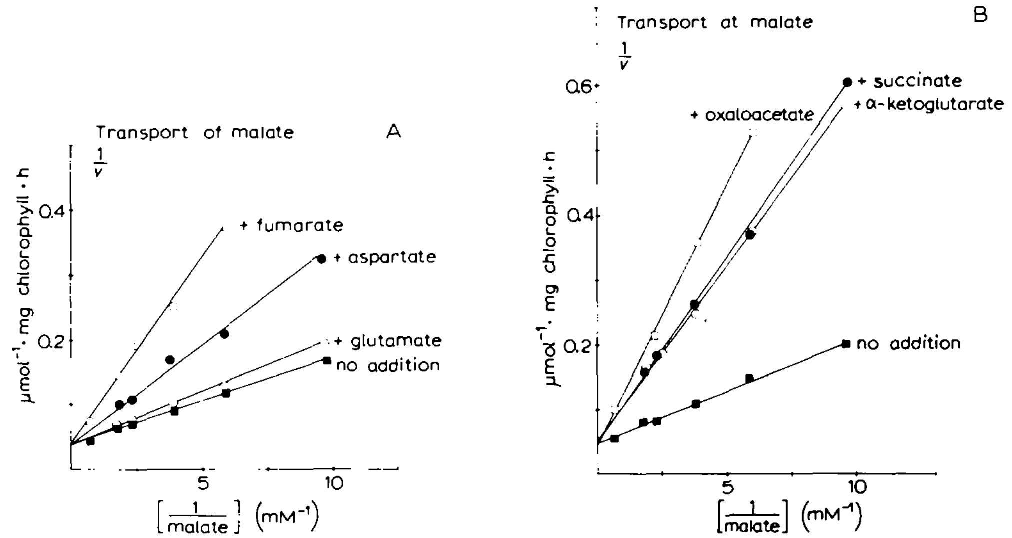

Fig. 3. Concentration dependence of L-malate transport. Inhibition by other dicarboxylates (1 mM). The data are the result of a series of experiments carried out with a single chloroplast preparation. In each set of experiments the concentration dependence of the malate uptake ± one inhibitor was measured. For the sake of simplicity, the different experiments were combined in the above figures by averaging the data for the uptake of malate in the absence of inhibitors. Since the K_m values for malate obtained in the different series varied between 0.30 and 0.40 mM, the simplified display of the data in the above figure does not allow an exact determination of the corresponding K_i values. For exact determination of these values see Tables II and III.

TABLE II

COMPARISON OF K_m AND K_i OF DICARBOXYLATE TRANSPORT INTO THE SORBITOL-IMPERMEABLE SPACE OF SPINACH CHLOROPLASTS

Exptl.	Transport of	K_m (mM)	Inhibitions by	K_i (mM)
A	L-Malate	0.42	Fumarate	0.31
	Fumarate	0.27	L-Malate	0.37
	Succinate	0.29	Fumarate	0.30
	Fumarate	0.29	Succinate	0.30
B	L-Malate	0.39	Succinate	0.25
		0.39	Oxaloacetate	0.26
		0.39	Fumarate	0.33
		0.39	α-Ketoglutarate	0.41
		0.39	L-Aspartate	0.52
	Succinate	0.26	L-Malate	0.32
		0.26	Oxaloacetate	0.22
		0.26	Fumarate	0.26
		0.26	α-Ketoglutarate	0.35
		0.26	L-Asparate	0.47
	Aspartate	0.72	L-Malate	0.72
		0.72	Succinate	0.50
		0.72	Oxaloacetate	0.70
		0.72	Fumarate	0.45
		0.72	α-Ketoglutarate	0.86

port. Comparable results were also obtained for the K_m and K_i of fumarate and succinate. Furthermore, the K_i of various dicarboxylates for the inhibition of malate and succinate transport were very similar. It appears from these data that malate and succinate are bound to the same site of the same carrier. In the case of aspartate the situation is not quite as clear. The K_i of aspartate for the inhibition of malate and succinate transport was markedly lower than the K_m

TABLE III

COMPETITIVE INHIBITION OF THE TRANSPORT OF L-MALATE AND SUCCINATE INTO THE SORBITOL-IMPERMEABLE SPACE OF SPINACH CHLOROPLASTS

For the determination of each inhibition constant K_i the corresponding K_m values were assayed simultaneously. Malate: K_m 0.34 (0.31—0.43); succinate: K_m 0.20 (0.19—0.21). The K_i values were normalized to the average K_m values.

Transport of	Inhibitor	K_i (mM)
A. L-Malate	Succinate	0.35
	Fumarate	0.32
	Oxaloacetate	0.26
	L-Aspartate	0.58
	meso-Tartrate	0.83
	L-Tartrate	1.08
	D-Tartrate	2.71
	Thiomalate	0.91
	α-Ketoglutarate	0.40
	L-Glutamate	1.00
	Glutarate	1.97
B. Succinate	L-Malate	0.19
	D-Malate	0.17

of aspartate transport, whereas the K_i of malate, oxaloacetate, fumarate and α-ketoglutarate for the inhibition of aspartate transport were about twice as high as for the inhibition of malate, fumarate and succinate transport. These data could be explained by assuming that there are different dicarboxylate carriers with overlapping specificity which are interacting with all dicarboxylates studied here. One carrier may preferentially transport aspartate, and one the rest of the dicarboxylates mentioned. The corresponding properties of glutamate transport have not been elaborated yet.

Specificity of the carrier

In spite of the complications concerning aspartate transport, the measurement of K_i gives information on the specificity of the dicarboxylate transport. In Table III the K_i of various dicarboxylates for the inhibition of malate transport have been compiled. In Table IV the interaction of some weak inhibitors has been determined. The structural requirements for the binding of substrates by the dicarboxylate carrier involved in malate transport can be deduced from these data.

It seems to be essential that the carbon atoms of the two carboxylic groups are at a certain distance from each other. If this distance is reduced, as for instance in the C_3 compound malonate, or the C_4 *cis*-isomer maleate or orthophthalate, there appears to be little or no interaction with the carrier. The same holds if the length of the carbon chain is increased to C_6, as in adipate. In C_4 dicarboxylates the binding to the carrier is only little affected by the presence of an oxo or an hydroxyl group in position 2. There is also no difference between the two stereoisomers of malate. The presence of an amino group in position 2 (aspartate) slightly lowers the binding of the carrier. However, the interaction with the carrier is completely inhibited if there is a phenyl group attached to position 2 (phenylsuccinate). A further substitution at position 3,

TABLE IV

INHIBITION OF THE TRANSPORT OF MALATE (0.33 mM) INTO THE SORBITOL · IMPERMEABLE SPACE OF SPINACH CHLOROPLASTS

Rate of malate transport in the absence of inhibitor: 20 μmol/mg chlorophyll per h.

Inhibitor (1 mM)	Inhibiton of malate tranport (%)
Malonate	0
Maleate	4
Orthophthalate	10
Fumarate	63
Succinate	58
Phenylsuccinate	0
Isophthalate	20
Glutarate	12
Adipate	0
Terephthalate	6
Acetate	1
Phosphate	0
Citrate	0

as in thiomalate or in the tartrates lowers the binding with the carrier. For the binding of C_5 dicarboxylates it appears essential that the hydrogen in position 2 is substituted. Substitution by an oxo group (α-ketoglutarate) leads to stronger binding than substitution by an amino group (glutamate). There is also some interaction of the carrier with isophthalate. It may be noted that citrate, acetate and phosphate do not interact with the carrier.

From its structural requirements the dicarboxylate transport in chloroplasts seems to be entirely different from the dicarboxylate and α-ketoglutarate transport in mitochondria [11,12]. Malonate and maleate, which do not bind to the chloroplast carrier, are transported very well by the mitochondrial systems. Fumarate, on the other hand, which is rapidly transported into chloroplasts, is not transported by the mitochondrial carrier. The known inhibitors of dicarboxylate transport in mitochondria, butylmalonate and phenylsuccinate, did not inhibit malate or aspartate transport in chloroplasts (experiments not shown here).

Counter exchange

Evidence has been presented that the dicarboxylate transport in chloroplasts involves a counterexchange [5]. The experiments of Fig. 4 and Table V show the balance of this counterexchange. In the experiment of Fig. 4A the chloroplasts were preincubated with [^{3}H]aspartate in the dark and washed afterwards. [^{14}C]Malate was added and the uptake and the release of these compounds was measured simultaneously by a double label technique using a liquid scintillation counter. In an analogous experiment (Fig. 4B) the chloroplasts were preincubated with [^{14}C]malate, and [^{3}H]aspartate was added afterwards. In both experiments thin-layer chromatography was used to check that there was no interconversion of the labeled malate or aspartate during the preincubation. The data of both experiments show that in the initial phase of the exchange the amounts of dicarboxylates taken up were about equal to the amounts of dicarboxylates released. Very similar results have been obtained in the experiments of Table V. Here the chloroplasts were preloaded with ^{3}H-

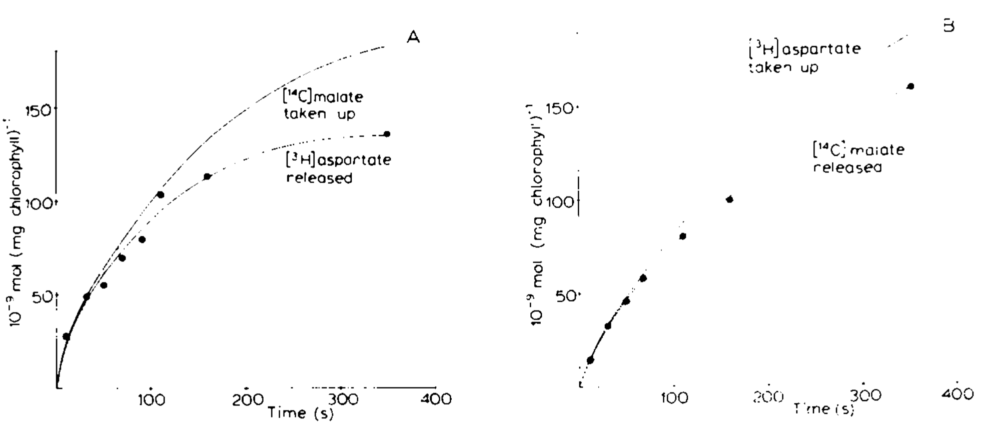

Fig. 4. Simultaneous measurement of the uptake and release of dicarboxylates. (A) Chloroplasts preincubated with L-[^{3}H]aspartate, addition of L-[^{14}C]malate (1 mM). (B) Chloroplasts preincubated with L-[^{14}C]malate, addition of L-[^{3}H]aspartate (0.2 mM).

TABLE V

COUNTEREXCHANGE OF DICARBOXYLATES

Results are expressed in μmol/mg chlorophyll per 30 s.

Chloroplasts preloaded with	[^{14}C]dicarboxylate added (0.25 mM)	[^{14}C]dicarboxylate uptake	[^{3}H]dicarboxylate release
A. [^{3}H]Succinate	L-Malate	102	94
	Fumarate	116	96
	Succinate	116	100
	α-Ketoglutarate	75	72
	L-Aspartate	79	60
	L-Glutamate	21	14
B. [^{3}H]Aspartate	L-Malate	81	74
	Fumarate	71	64
	Succinate	71	72
	α-Ketoglutarate	70	67
	L-Glutamate	41	35
	L-Aspartate	117	116

labeled succinate or aspartate and several ^{14}C-labeled dicarboxylates were added. The reactions were terminated after 30 s and the balance of counter transport was evaluated. In all cases the amounts of either succinate or aspartate released were equal to or only slightly lower than the amounts of dicarboxylates taken up. The relatively larger difference between the uptake of glutamate and the release of succinate may be not real, since the error involved in measuring the very low rates is relatively high. These data demonstrate that the transport of all dicarboxylates tested involves a countertransport. All combinations of dicarboxylates for countertransport seem to be possible. In the later phase of the time courses of counter transport in Fig. 4 there are more labeled dicarboxylates taken up than released. This additional uptake could of course be connected with the undetected release of some unlabeled dicarboxylate. This would have to be a compound which is transported more slowly than succinate, malate or aspartate, perhaps glutamate. It is also possible, on the other hand, that the countertransport is not strictly coupled, allowing net transport of dicarboxylates in one direction. The rate of such an unidirectional transport would be directed against a concentration gradient, since in our experiments the dicarboxylate concentration in the chloroplasts was always much higher than in the medium.

The rate of additional dicarboxylate uptake without apparent release was found to be decreased by illumination. In one experiment employing chloroplasts preincubated with succinate, the rate of aspartate uptake minus succinate release at 18°C was 0.8 μmol per mg chlorophyll per h in the light and 1.0 in the dark. If there would be indeed an unidirectional transport involved in the additional aspartate uptake, one may ask how this transport is energy driven. Further studies will be required to answer this question.

Efflux of dicarboxylates

The possibility of a slow unidirectional influx of dicarboxylates into chloroplasts, as discussed before, raises the question whether there is also an unidirectional efflux of dicarboxylates occurring. Such an efflux in the absence of added dicarboxylates in the medium can be in fact observed, as shown in Table VI, though its rate is about two orders of magnitude lower than the rate of countertransport. It may be noted that such a leakage is not observed with the metabolites transported by the phosphate translocator, e.g. inorganic phosphate (Table VI). This result concurs with the observation that with phosphate transport there is also no additional uptake without an accompanying efflux [1]. Apparently the countertransport facilitated by the phosphate translocator is more strictly coupled than the dicarboxylate transport, preventing the loss of phosphate and phosphorylated intermediates from the stroma. It is interesting that the efflux of dicarboxylates is increased by illumination of the chloroplasts. This observation makes it unlikely that the observed efflux is due to an exchange with traces of dicarboxylates present in the medium, since the transport of dicarboxylates into the chloroplasts does not show such a high stimulation by light (Table IX). Furthermore, the light stimulation of the efflux concurs with a light inhibition of the influx. The cause for this effect shall have to be investigated.

Dependency of transport on pH, countertransported molecules and illumination

A number of experiments yielded pH optima for the rates of malate and aspartate uptake between 7.0 and 7.5 (experiments not shown here). As shown in Table VII the pH of the medium from pH 7.0 to pH 8.0 did not alter the K_m values for the transport of malate and aspartate. When considering the pK values of these substances (malate: 3.4, 5.2; aspartate: 2.0, 3.9, 9.8 [13]) it seems very likely that malate binds to the carrier as a divalent and aspartate as a monovalent anion. If aspartate would be also transported as a divalent anion, the K_m for aspartate should be decreased with the increasing pH. Thus the pH optimum of the transport appears not to be due to alterations of the affinity of the carrier, but apparently reflects an optimum of the transport velocity. As malate and aspartate appear to be transported as differently charged anions, the rate of uptake should be influenced by the countertransported substance. In

TABLE VI

EFFLUX OF DICARBOXYLATES FROM THE SUCROSE-IMPERMEABLE SPACE OF SPINACH CHLOROPLASTS IN THE ABSENCE OF DICARBOXYLATES IN THE MEDIUM

The chloroplasts were preincubated with ^{3}H-labeled dicarboxylates or inorganic [^{32}P]phosphate (see Materials and Methods).

Efflux of	Temperature (°C)	Efflux rate (μmol/mg chlorophyll per h)	
		Dark	Light
L-Aspartate	0	0.05	0.17
	20	0.49	1.25
Fumarate	0	0.11	0.20
	20	0.27	0.82
Phosphate	0	0.02	0.02
	20	0.06	0.07

TABLE VII

DEPENDENCY OF DICARBOXYLATE TRANSPORT INTO THE SORBITOL-IMPERMEABLE SPACE OF SPINACH CHLOROPLASTS ON THE pH IN THE MEDIUM

Temperature 4°C.

Dicarboxylate transported	pH in medium	K_m (mM)	V (μmol/mg chlorophyll per h)
L-Malate	7.0	0.28	31
	8.1	0.28	11
L-Aspartate	7.0	0.56	57
	8.1	0.56	14

the experiment of Table VIII, transport of malate and aspartate into chloroplasts, which had been preincubated with either of these substances, was measured. It appears that preloading of the chloroplasts with aspartate favours aspartate transport into the chloroplasts, whereas preloading with malate increases the uptake of malate (or succinate and fumarate (not shown here)). This is specially noticable when malate and aspartate are added together. Preloading of the chloroplasts with different dicarboxylates did not markedly change the K_m for dicarboxylate transport into the chloroplasts (experiments not shown). Again, the differences of the transport rates appear to be entirely due to changes of the transport velocity, probably caused by the charge of the counterion.

The rates of dicarboxylate uptake can be also altered to some extent by illumination of the chloroplasts. In the experiment of Table IX, illumination caused an increase of the transport of malate and a decrease of the transport of aspartate. Since the chloroplasts were preloaded with aspartate, an exchange with malate would involve a transfer of a negative charge into the stroma. This would require a light-dependent transport of a compensating ion across the envelope. Further investigations of this subject, including the study of cation

TABLE VIII

DEPENDENCY OF THE UPTAKE OF ASPARTATE AND MALATE INTO THE SORBITOL-IMPERMEABLE SPACE ON THE PREINCUBATION OF THE CHLOROPLASTS WITH EITHER ASPARTATE OR MALATE

(See Materials and Methods). The dicarboxylate concentrations for the uptake measurements were 0.25 mM.

Preincubation with	Addition of	Uptake (10^{-9} mol/mg chlorphyll per 15 s)			
		Aspartate	Malate	Total	$\frac{\text{Aspartate}}{\text{Malate}}$
L-Aspartate	L-Aspartate	61	—		
	L-Malate	—	37		
	L-Aspartate + L-Malate	54	20	74	2.7
L-Malate	L-Aspartate	56	—		
	L-Malate	—	66		
	L-Aspartate + L-Malate	40	48	88	0.8

TABLE IX

DEPENDENCY OF THE SIMULTANEOUS UPTAKE OF ASPARTATE AND SUCCINATE INTO THE SORBITOL-IMPERMEABLE SPACE OF SPINACH CHLOROPLASTS ON ILLUMINATION

(80 W/m^2, Temperature 4°C). The chloroplasts were preincubated with unlabeled aspartate (see Materials and Methods). The dicarboxylate concentration for the uptake measurements was 0.25 mM.

	Uptake (10^{-9} mol/mg chlorophyll per 15 s)			
	L-Aspartate	Succinate	Total	$\frac{\text{Aspartate}}{\text{Succinate}}$
Dark	54	21	75	2.5
Light	32	32	64	1.0

movements across the envelope, will be required to verify this speculation. Illumination had no effect on the K_m values for aspartate and malate, as was found in other experiments not shown.

Temperature dependency

Fig. 5 investigates the temperature dependency of the transport of succinate and aspartate. The Arrhenius plot of the data yielded a linear function. The activation energy as derived from the slope is about 7 kcal/mol for both substances. Similar values have been also obtained for malate in other experiments. This value is much lower than the activation energy of phosphate transport in chloroplasts (16 kcal/mol, Fliege, R. and Heldt, H.W., unpublished results). Thus a change of the temperature from 4 to 20°C only leads to a 2-fold increase of the transport rate. In other experiments not shown here it was found in the case of aspartate and malate that a change of the temperature from 3 to 20°C did not affect the corresponding K_m values but resulted in an about 2-fold increase of the V values.

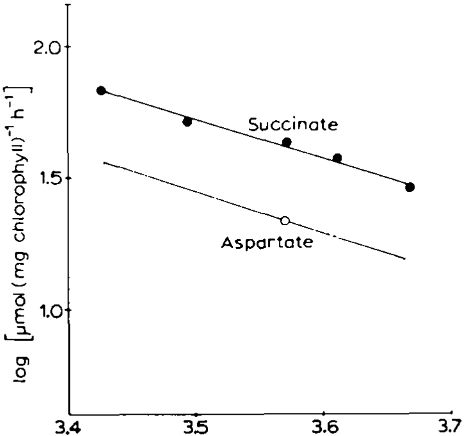

Fig. 5. Temperature dependency of the rates of dicarboxylate uptake into the sorbitol-impermeable space of chloroplasts as presented in an Arrhenius plot. The concentrations of succinate and L-aspartate where 60 µM. The activation energy derived from the graph is 7.2 kcal/mol for succinate and 7.5 kcal/mol for L-aspartate.

TABLE X

DICARBOXYLATE CONTENT OF FRESHLY PREPARED ISOLATED CHLOROPLASTS AS MEASURED IN THREE DIFFERENT PREPARATIONS (A, B, C)

The chloroplasts, kept in the dark, were deproteinized in 0.6 M $HClO_4$, and the dicarboxylates measured in the neutralized extract by enzymatic assay. For methods see ref. 15. Results are expressed in nmol/mg chlorophyll.

Preparation	A	B	C
L-Malate	129	148	104
α-Ketoglutarate	3.8	6.5	4.3
L-Aspartate	245	273	235
L-Glutamate	491	532	366

Dicarboxylate content in chloroplasts

As the transport into the chloroplasts depends on the dicarboxylate pool in the stroma, the dicarboxylate content of freshly prepared spinach chloroplasts was determined by enzymatic assay (Table X). Large amounts of glutamate and aspartate, and also of malate were found. Heber and Krause [14] have analyzed similar amounts of malate in spinach chloroplasts isolated in non-aqueous media.

Discussion

Our data clearly show that those dicarboxylates transported across the envelope also act as competitive inhibitors of dicarboxylate transport. Malonate, which is not transported, also does not inhibit transport. Apparently, the carrier facilitating the transport of a single dicarboxylate also interacts with all other dicarboxylates transported across the envelope. In the case of malate, fumarate and succinate the corresponding K_m and K_i values were found to be very similar. These results indicate that these substances, and probably also oxaloacetate, of which the direct uptake could not be measured for practical reasons, are transported by the same carrier. This argument does not hold in the case of aspartate, since here the K_m and K_i values were found to differ considerably. Furthermore the V for the transport of aspartate appeared to be much higher than for the other dicarboxylates. Provided that these differences are not due to the complexity of the system, e.g. the fact that the transport rate is also dependent on the internal dicarboxylate concentrations, our results may indicate the existence of different carriers with overlapping specificity. One carrier might have a relatively high V for malate, and another one for aspartate. Further studies employing transport inhibitors will be required to test this possibility. Unfortunately, specific inhibitors of dicarboxylate transport in chloroplasts have not been discovered yet.

The dicarboxylate transport involves a counterexchange. The unidirectional transport, if no artifact, is almost two orders of magnitude slower. For this reason the dicarboxylate transport in chloroplasts appears not to be suited for a major net carbon transport across the envelope. However, it may have an important function for the transfer of reducing equivalents. In spinach leaves large activities of NAD-dependent malate dehydrogenase have been found in the cytosol as well as in the stroma [16] and furthermore NADP-dependent malate dehydrogenase in the stroma [17]. As the envelope is impermeable for pyridine nucleotides, it was proposed that a malate-oxaloacetate shuttle may enable a transfer of reducing equivalents. Such a shuttle has been demonstrated to operate in vitro [14], but the physiological function of this shuttle has been questioned on the ground that the oxaloacetate concentration being in equilibrium with malate is very low [18]. There are also high activities of glutamate-oxaloacetate transaminase found in the stroma and the cytosol of spinach leaves [16,19]. It has been therefore suggested that oxaloacetate might undergo transamination with glutamate leading to the formation of aspartate and α-ketoglutarate, the latter two compounds being transported across the envelope in exchange with glutamate and malate, and this malate-aspartate cycle was demonstrated to occur in vitro [18]. As in mitochondria (for references see ref. 20) also in chloroplasts this cycle may be important for the transfer of reducing equivalents.

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