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Uptake of C_4 Dicarboxylates and Pyruvate by *Rhodopseudomonas spheroides*

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The uptake of C_4 dicarboxylates by cells from exponential cultures of *Rhodopseudomonas spheroides* followed saturation kinetics at concentrations below 100 μM, with K_m values for succinate, malate, and fumarate of 2.7, 2.3, and 0.8, respectively. Corresponding V_{max} values of 50, 52, and 67.5 nmol/min per mg of protein at 20 C were obtained. Each of these compounds interfered competitively with uptake of the others, and a common transport system appears to be involved. Fructose-grown cells took up C_4 dicarboxylates only at very low rates, and pyruvate-grown cells took up C_4 dicarboxylates at one-third the rates found with succinate-grown cultures. Malonate and maleate inhibited uptake less severely, and aspartate and α-ketoglutarate had no effect at 100-fold excess. Divalent metals stimulated uptake. Light or respiration was required for uptake, and entered materials were rapidly converted to other metabolites, notably amino acids. Pyruvate entry appeared to be mediated by several systems, of which only one could be resolved kinetically. This system had a K_m of 13 μM and V_{max} of 5.6 nmol/min per mg of protein at 20 C. A number of related mono- and dicarboxylates interfered with pyruvate uptake. The pyruvate uptake system was distinguishable from the C_4 dicarboxylate system by the absence of divalent cation stimulation and by substrate and inhibitor specificity.

Non-sulfur photosynthetic bacteria require organic carbon compounds for most rapid growth, and mono- or dicarboxylic acids are generally the best substrates (15, 20). Such compounds are common microbial fermentation end products. Given light, the photosynthetic organisms are therefore able to use anaerobically a group of compounds which ordinarily tend to accumulate under such conditions and which are extensively used as substrates only under aerobic conditions. This advantage could make these photosynthetic organisms less dependent on the functioning of efficient transport systems such as have been most fully described in *Escherichia coli*, *Salmonella typhimurium*, and *Bacillus subtilis* (14), especially since photosynthetic organisms, through the use of light energy, could be expected to require smaller amounts of organic compounds for making a given amount of cell material than heterotrophs, which must also consume some organic material for energy generation.

Although early investigations showed that substrates were used efficiently (19), very little is known about the mechanisms involved in the uptake of organic materials by photosynthetic bacteria. *Rhodospirillum rubrum* and *Rhodopseudomonas spheroides*, both of which can grow on fructose, have phosphoenolpyruvate-dependent fructose phosphotransferase systems (18). *Chromatium* appears to lack systems for efficient uptake of amino acids (21). The transport of C_4 dicarboxylates has been studied extensively only in *E. coli* (6, 8) and *B. subtilis* (1, 3, 4), and the entry of monocarboxylic acids has received little attention in any organisms. Recently, Harold and Levin (5) have made a study of the exit mechanisms whereby lactate leaves cells of *Streptococcus faecalis*, and evidence for a group-translocation mechanism involving acetyl-coenzyme A:butyrate transferase for butyrate uptake by *E. coli* has been put forward (2). Such organisms as have been studied do not grow as rapidly on organic acids as on sugars and related compounds. This study was undertaken, therefore, to examine the entry of organic acids into a group of organisms for which they are preferred substrates, as well as to improve understanding of the ecological relationships of the non-sulfur photosynthetic bacteria. Metabolizable substrates were used in this investigation, and the term "uptake" as used here therefore makes no distinction between passage across the

cytoplasmic membrane and subsequent metabolic conversions undergone by the entered compound. Although there are obvious difficulties in detailed interpretation of experiments done under these conditions, there are also some advantages. The cells are carrying out a physiological process under normal metabolic conditions, and the diversification of entered material means that outflow of radioactive material due to an extensive rise in internal concentration is unlikely to become a complicating factor in the measurements. *R. spheroides* was chosen for this investigation because it grows well on a number of mono- and dicarboxylic acids.

MATERIALS AND METHODS

Culture and maintenance of organism. The culture of *R. spheroides* 1:2 used in this study was kindly supplied by W. R. Sistrom. Experimental cultures were grown in medium of the following composition: $(NH_4)_2SO_4$, 20 mM; KH_2PO_4, 1 mM; $MgCl_2$, 2.5 mM; $CaCl_2$, 0.07 mM; triethanolamine hydrochloride-potassium hydroxide, 10 mM: trace element solution (16), 10 ml/liter; nicotinic acid, 1 mg/liter; thiamine hydrochloride, 0.5 mg/liter; biotin, 0.1 mg/liter; organic carbon source, 10 mM. The pH was adjusted to 6.8, and the growth factors were added as a filter-sterilized solution after autoclaving.

Liquid cultures were grown in completely filled, screw-capped Pyrex tubes (16 by 125 mm) at 30 to 32 C and illuminated with two 30-W Lumiline incandescent strip lamps at a distance of 10 to 15 cm. The cultures were transferred daily and checked frequently for contamination. Optical densities of growing cultures were followed with a Fisher Electrophotometer II (red filter), which could accomodate the growth tubes. The optical density of all experimental cultures was measured in a Zeiss PMQ II spectrophotometer at 650 nm. Protein was measured by the procedure of Lowry et al. (9) on cell pellets that had been extracted with an acetone-methanol mixture (7:2, vol/vol) as described previously (11). A cell suspension having an optical density at 650 nm of 0.1 contained 20 μg of cell protein per ml.

Uptake of radioactive compounds. Overnight cultures were diluted into fresh medium to a concentration of 20 to 30 μg of cell protein per ml, and growth was followed for 1 to 2 doublings at 30 C. The culture tube was then centrifuged for 6 min at 4,000 $\times$ *g* at room temperature, and the pelleted cells were washed once, as before, in the appropriate medium which had been deaerated by boiling vigorously for 15 s under a stream of prepurified nitrogen, or of 95% N_2-5% H_2 passed through a Deoxo catalyst cartridge (Engelhart Industries, East Newark, N.J.) and cooled to room temperature under the gas stream. Residual oxygen in buffers prepared in this way was measured with an oxygen electrode and amounted to 4 to 6 μM. Suspensions were prepared under these same conditions so that they contained about 20 μg of cell protein per ml.

Portions of this suspension (usually 6.5 ml) were dispensed into a series of up to six 15-ml Corex centrifuge tubes and equilibrated anaerobically in light for 15 min at 20 C in a glass-sided water bath illuminated with two 30-W incandescent light strips, while a stream of 95% N_2-5% H_2 was passed through each tube. For some survey experiments, the volume of suspension was reduced to 3.5 ml, and for extraction of pools the cell concentration was increased to about 40 μg of protein per ml. Uptake was initiated by addition of labeled substrate at the desired concentration and specific activities ranging from 0.5 to 3 μCi/μmol. The suspension was thoroughly mixed by increasing the gas flow for about 5 s before turning it off. Samples of 1 ml were withdrawn with an automatic pipette at intervals of about 15 s, filtered through 25-mm, 0.45-μm pore membrane filters, and washed once with 5 ml of 20 mM triethanolamine buffer, pH 6.8. Filtering and washing took about 5 s after the removal of the sample from the experimental tube; the time of addition of the wash fluid was taken as the time at which uptake was stopped. Filters were dried for 30 min at 70 C and counted in a Beckman LS 100 liquid scintillation spectrometer, with 0.4% 2,5-bis(5'-tertiary butyl 2-benzoxazolyl)thiophene in toluene as the scintillation fluid. Further washes did not reduce radioactivity associated with the filtered cells. Usually, six 1-ml samples were taken from each experimental tube over an interval of 90 to 120 s, although the sampling time was extended in some of the inhibition experiments. In kinetic experiments, up to six different concentrations of substrate were run sequentially with the same experimental cell suspension. Where more concentrations were required and two different suspensions had to be used to cover the full range, at least one concentration was duplicated in the second half of the experiment. Such duplicates generally agreed within 10%, and the experiment was repeated if this difference was exceeded.

Extraction and identification of labeled material from suspensions. Filtered samples, generally 2 ml, were washed as usual. The suction was stopped, and the filters were then flooded with 2 ml of 0.4 N formic acid. The acid was allowed to drip through the filter slowly at room temperature. At the end of 10 min, the filter was sucked dry and washed with 2 ml more of formic acid. The combined filtrates were dried under a stream of air at 35 to 40 C and dissolved in a small volume of water, usually 40 μl. A small volume was used for radioactivity determination, and a known quantity was chromatographed on plastic-backed MN CEL-300 cellulose thin-layer plates (Brinkmann Instruments), with ethyl ether-formic acid-water (7:2:1, vol/vol/vol) (13) as solvent. Radioactive areas were located by using X-ray film, cut out, and counted. The use of boiling water or phenol-saturated water (5) for pool extraction gave lower recoveries of labeled material.

Radioactive materials. [2,3-^{14}C]succinate, [2,3-^{14}C]fumarate, and [U-^{14}C]pyruvate were obtained from International Chemical and Nuclear; [U-^{14}C]malate was obtained from Amersham-Searle. All radioactive chemicals were checked chromatographically for purity when received and at intervals during

their use in experiments. Pyruvate showed significant decomposition within 3 weeks of making solutions, even if kept frozen.

RESULTS

Uptake of C_4 dicarboxylates. Cells from experimental cultures took up succinate, fumarate, and malate at fairly reproducible rates, when washed and suspended in salts of the same composition as the growth medium, with a broad pH optimum. Uptake was most rapid at pH 7.5, and the rates at pH 6.6 and 9.2 were 45 and 73% of the maximum. Uptake was linear for several minutes, provided that concentrations were not lower than 1 μM. Suspensions in a number of different buffer solutions gave lower and less-consistent uptake rates (Table 1), and addition of divalent cations caused considerable stimulation. Mn^{2+} and Mg^{2+} were the most active of those tested, and stimulation with Mn^{2+} was concentration dependent up to 20 μM, which gave the highest rates in a number of experiments. Cell suspensions in 10 mM *N*-2-hydroxyethyl-piperazine-*N'*-2'-ethanesulfonic acid (HEPES)-Na^+ (pH 7.5) took up dicarboxylates at rates close to those obtained in the medium salts, and this buffer was therefore used for most experiments. HEPES neutralized with KOH or tris(hydroxymethyl)aminomethane base and especially NH_4OH supported only lower uptake rates, and an effect of monovalent cations is thus also apparent. Addition of ethylenediaminetetraacetate (EDTA) was strongly inhibitory to uptake, and this inhibition was not relieved by increasing the concentration of dicarboxylate (Table 2).

Kinetics. Succinate uptake at low concentrations showed saturation kinetics (Fig. 1). At higher concentrations, between 0.2 and 1 mM, the uptake rate increased again. No clear second saturation was observed in the range of concentrations tested. A plot of the rate of malate uptake at differing concentrations had a similar form (data not shown). Fumarate was also taken up rapidly from low external concentrations, showing saturation (Fig. 2). From such experiments, K_m values of 2.7, 2.3, and 0.8 μM were calculated for uptake of succinate, malate, and fumarate, respectively. Corresponding V_{max} values were 50, 52, and 67 nmol/min per mg of protein at 20 C. Any one of these compounds interfered with the uptake of another at comparable concentration (Fig. 3), and double-reciprocal plots indicated that the inhibition was competitive (Fig. 4). This suggests that a single uptake system was mediating the entry of these

TABLE 1. *Rates of fumarate uptake in different suspending media*

Suspending medium	Addition	Uptake rate[a]	Stimulation[b]
C-free medium	None	45.2	
	Mn^{2+}, 20 μM	45.2	0
Triethanolamine,[c] 10 mM	None	2.7	
	Mg^{2+}, 0.1 mM	18	6.9
	1 mM	21.6	8.0
	10 mM	22	8.1
	None	1.53	
	Ca^{2+}, 10 μM	4.6	3.0
	100 μM	22.8	14.9
	Co^{2+}, 1 μM	3.6	2.4
	10 μM	9.1	5.9
	Mn^{2+}, 1 μM	3.2	2.1
	10 μM	14.6	9.5
Tris-hydrochloride,[d] 10 mM	None	13.6	
	Mn^{2+}, 20 μM	23.1	1.7
HEPES-Na^+, 10 mM	None	39.2	
	Mn^{2+}, 20 μM	53.1	1.4
HEPES-K^+	None	17.5	
	Mn^{2+}, 20 μM	15.2	
HEPES–Tris-hydrochloride	None	26	
	Mn^{2+}, 20 μM	26.4	0
HEPES-NH_4^+	None	9.9	
	Mn^{2+}, 20 μM	11.7	1.2

[a] Nanomoles per minute per milligram of protein at 20 C; pH 7.5; 5 μM fumarate.

[b] (Rate + addition)/rate in unsupplemented medium.

[c] Triethanolamine buffers were prepared either from free base with HCl or from the crystalline hydrochloride with NaOH.

[d] Tris, tris(hydroxymethyl)aminomethane.

three compounds. A number of structurally related compounds were tested for inhibition of C_4 dicarboxylate uptake; aspartate, phthalate, citrate, α-ketoglutarate, pyruvate, alanine, and acetate had no effect on uptake of succinate or fumarate when added at 5 to 100 times the concentration of test substance. Malonate inhibited uptake of fumarate (5 μM) by 30% and maleate by 70% when tested in 100-fold excess. It is thus evident that uptake is highly selective.

Energy requirements. Anaerobic suspensions in foil-wrapped tubes usually took up radioactive dicarboxylates at less than 10% of the rate found for illuminated suspensions. Aerobic suspensions were much less affected by illumination (Table 3), and in some experiments the rates were almost identical. It seems probable that either light or oxygen is required for maximal uptake rates and that occasional elevated anaerobic dark uptake rates were due to residual traces of oxygen in the medium. This suggestion is supported by the finding that dark anaerobic uptake controls were consistently low when twofold or higher cell concentrations were employed; under these conditions, cell respiration would be expected to aid in the establishment of anaerobic conditions. The uncouplers *m*-chlorocarbonylcyanide phenyl hydrazone (CCCP) and dibromophenol effectively prevented any uptake when present in concentrations of 10 and 20 μM, respectively. CCCP inhibition took effect rapidly (Fig. 5), and at higher concentrations caused release of some previously accumulated radioactivity. Chromatography of this released material indicated the presence of many radioactive compounds, suggesting general membrane damage. Dibromophenol, on the other hand, was slow to take effect, and maximal inhibition was only obtained after 10 to 15 min of preincubation. These findings taken together suggest that energy is required for dicarboxylate uptake and that this requirement can be met either by illumination or by respiration. Whether this requirement is for the passage across the cell membrane or for subsequent metabolism cannot be assessed from these experiments.

TABLE 2. *Effect of Mn^{2+} and EDTA on fumarate uptake*[a]

Fumarate (μM)	Addition (μM)	Rate[b]	% Control
5	None	20.3	
	Mn^{2+}, 20	41.5	204
	EDTA, 1	17.6	87
	10	2.0	10
	100	0.8	4
20	None	24.4	
	EDTA, 5	11.7	48

[a] Cells were suspended in 10 mM HEPES-Na^+, pH 7.5; measurements at 20 C.

[b] Nanomoles per minute per milligram of protein.

Fate of accumulated intracellular com-

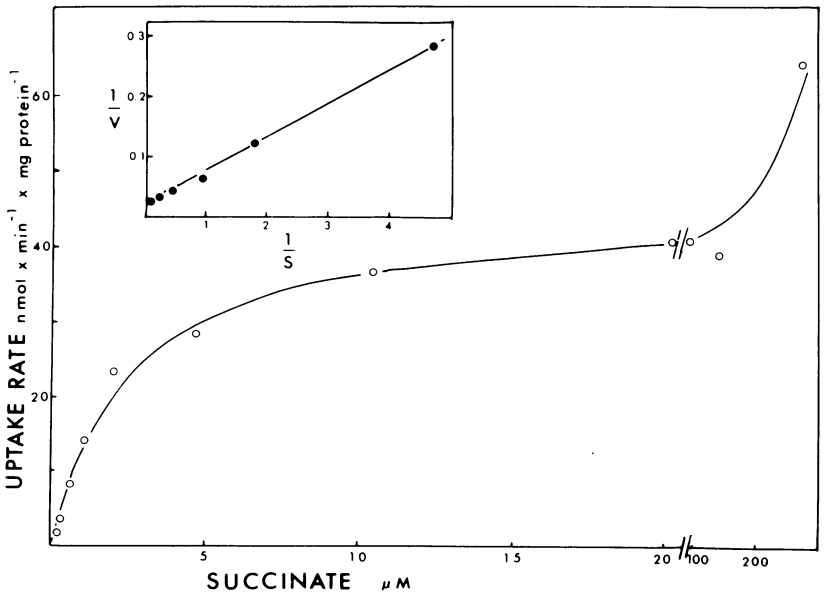

FIG. 1. *Succinate uptake kinetics. Succinate-grown cells were suspended in 10 mM HEPES-20 μM Mn^{2+}, and uptake rates at each concentration were measured at 20 C. The insert shows a double-reciprocal plot of the lower-concentration data.*

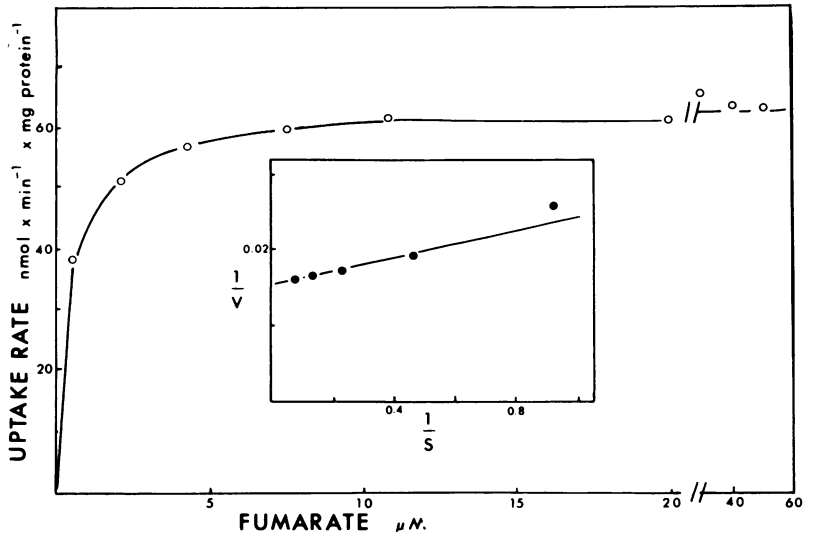

FIG. 2. *Fumarate uptake kinetics. Succinate-grown cells were suspended in 10 mM HEPES-20 μM Mn^{2+}. Rates were measured at 20 C. The insert shows a double-reciprocal plot of the lower-concentration data.*

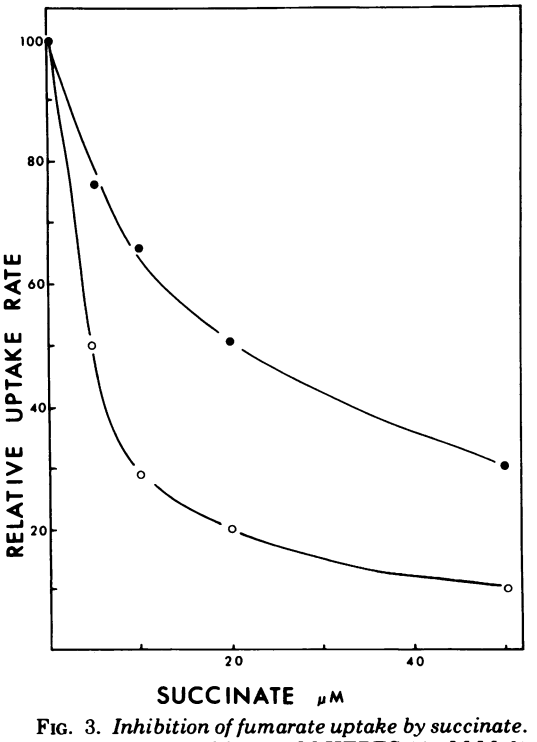

FIG. 3. *Inhibition of fumarate uptake by succinate. Rates were measured in 10 mM HEPES-20 μM Mn^{2+} at 20 C. Symbols: ○, 5 μM fumarate; ●, 20 μM fumarate.*

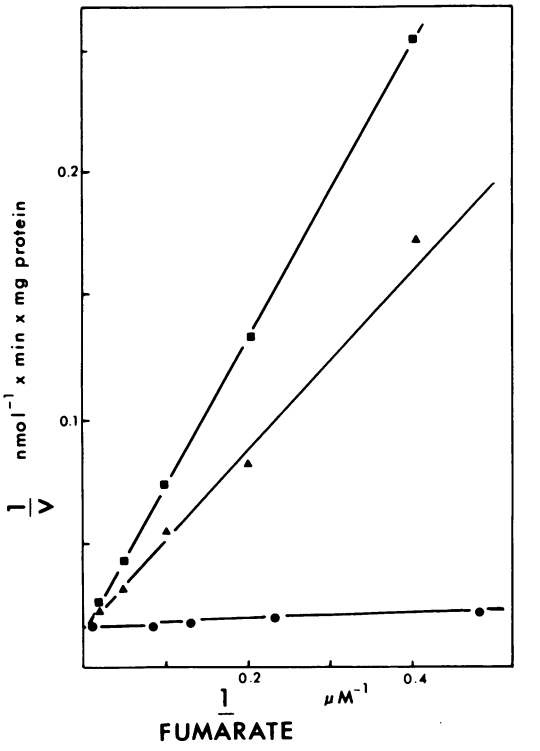

FIG. 4. *Inhibition of fumarate uptake by malate. Double-reciprocal plot of rates measured in 10 mM HEPES-20 μM Mn^{2+} at 20 C. Symbols: ●, No malate; ▲, 10 μM malate; ■, 20 μM malate.*

pounds. Extraction of cells that had taken up radioactive succinate, fumarate, or malate for short periods with 0.4 N formic acid resulted in 70 to 90% solubilization of the total radioactivity after 25 to 40 s; soluble material accounted for 50 to 70% of the total after periods of 2 to 3 min. Chromatography of the extracted material even

TABLE 3. *Effect of aeration and light on fumarate uptake*[a]

Conditions	Uptake rate[b]
Anaerobic, light	42.8
Anaerobic, dark	0.7
Aerobic, light	34.8
Aerobic, dark	25..5

[a] Measurements of uptake were made in 10 mM HEPES-20 μM Mn^{2+} (pH 7.5) at 20 C. Fumarate concentration, 5 μM.

[b] Nanomoles per minute per milligram of protein.

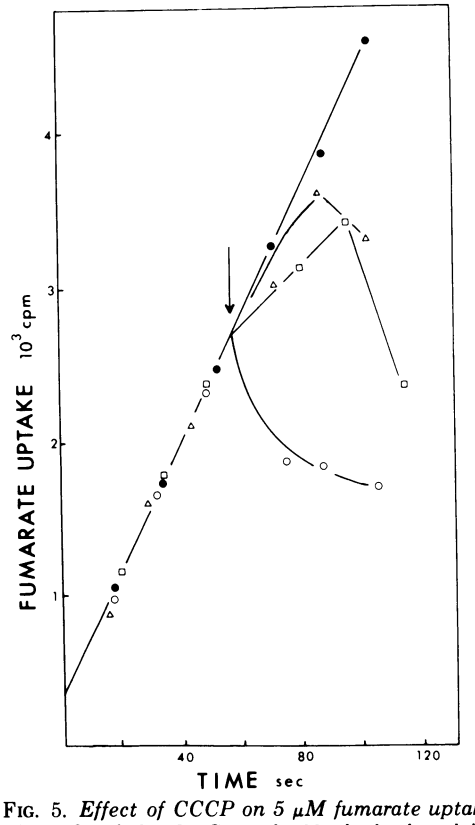

FIG. 5. *Effect of CCCP on 5 μM fumarate uptake in light. Symbols: ●, Control—30 μl of ethanol injected at time marked by arrow; △, 2 μM CCCP injected at arrow; □, 10 μM CCCP injected at arrow; ○, 20 μM CCCP injected at arrow.*

after the shortest time periods showed that, although about 20% of the entered succinate was recovered unchanged after a 15-s uptake eight or nine other radioactive spots were also evident, whereas almost 30% of the total counts on the chromatogram was in material that did not move significantly from the origin. Malate could be identified among the metabolic products when either succinate or fumarate had been taken up, but fumarate could be identified only when that compound itself had been used. In all cases the greatest accumulation of radioactivity was in the region of the chromatogram where a number of amino acids are poorly resolved in the system used here; only glutamate was clearly identified as a major component. Calculations of internal concentrations in cells that had taken up succinate, using a value of 5-μl internal water space per mg of protein (11), indicated that the internal concentration of succinate was two to three times the external concentration, a concentration difference that is probably within the experimental error. A major difference between internal and external dicarboxylate concentration is thus not established under these conditions.

Effect of growth substrate. The rate of C_4 dicarboxylate uptake was significantly greater if the cells had been grown on succinate than if they had been grown on pyruvate, whereas uptake was dramatically reduced after growth in fructose (Table 4). Substitution of fumarate or malate for succinate as growth substrate did not affect the rate of uptake of the other dicarboxylates. A pyruvate-grown culture was transferred to individual tubes containing 10 mM pyruvate, succinate plus 50 μg of chloramphenicol per ml, or succinate alone, and the cultures were incubated for 2 h in light before centrifuging and measuring fumarate uptake in the usual way. Rates of 11.6, 21.2, and 51.6 nmol taken up per min per mg of protein were calculated; this indicates that protein synthesis

TABLE 4. *Effect of growth substrate on uptake rates*[a]

Growth substrate	Test substrate[b]	Uptake rate[c]
Succinate	Succinate (5-100)	31 ± 9.4
	Fumarate (5-50)	53.3 ± 7.1
	Malate (5-20)	25.9 ± 3.5
	Pyruvate (25-50)	14.6[d]
Fumarate	Succinate (20)	26.8[e]
	Fumarate (5)	51.5[e]
Pyruvate	Succinate (5-20)	11.7[e]
	Fumarate (5-20)	11.5[d]
	Pyruvate (25-50)	11.9 ± 5.4
Fructose	Succinate (20)	0.9
	Fumarate (5)	1.9[e]

[a] All measurements were carried out in 10 mM HEPES-20 μM Mn^{2+} (pH 7.5) at 20 C.

[b] Numbers in parentheses indicate the range of concentrations used (micromolar).

[c] Nanomoles per minute per milligram of protein.

[d] Average of four determinations.

[e] Average of two determinations. Standard deviations were calculated from 6 determinations for malate uptake and from 14 to 16 determinations for other compounds.

is required for the increase in rate seen in dicarboxylate-grown cells and that this increase can be achieved within a fraction of a doubling time.

Pyruvate uptake. In contrast to the uptake of dicarboxylates, it proved difficult to obtain a satisfactory kinetic definition of systems concerned with pyruvate entry. Five experiments with pyruvate-grown cells agreed in showing a plateau in the plot relating uptake rate to external concentration at about 50 μM. Higher concentrations gave higher uptake rates, with indications of inflections at about 0.1 and 0.5 mM in two experiments. Figure 6 shows one of these from which it is possible to calculate a K_m of 13 μM and a V_{max} of 5.6 nmol/min per mg of protein at 20 C for the system functioning at low pyruvate concentrations. Although pyruvate was taken up as rapidly by cells grown in succinate as those in pyruvate (Table 4), similar plots showed no signs of saturation of individual uptake systems. As was the case after dicarboxylate uptake, labeled material spread very rapidly into other small molecule pools and, after no more than 30 s, less than 10% of the labeled material was recovered as pyruvate, while the bulk of the counts appeared in the amino acid region of the chromatogram.

Further experiments were carried out only with a concentration of pyruvate between 25 and 50 μM and with pyruvate-grown cells. A number of compounds with some molecular similarity to pyruvate inhibited uptake (Table 5). Alanine, propanol, and α-ketoglutarate produced less than 10% inhibition at concentrations up to 20 times that of pyruvate, whereas fumarate or succinate inhibited by about 20% at 2 mM. Acetate and butyrate were both inhibitory at high concentrations; propionate was among the most effective of compounds tested at preventing pyruvate entry. Separate experiments showed that it was itself taken up only slowly, at a concentration-independent rate of 0.6 to 0.8 nmol/min per mg of protein, suggesting that it was truly blocking entry of pyruvate

TABLE 5. *Inhibition of pyruvate uptake by related compounds*[a]

Addition		Rate[b]	% Control
None		7.9	100
Lactate,	2 μM	5.6	71
	20 μM	2.8	36
	200 μM	3.06	39
Acetate,	2 mM	1.8	22
Butyrate,	2 mM	1.4	18
None		11.7	100
Propionate,	20 μM	3.5	30
	50 μM	2.1	17
	150 μM	1.6	14
	500 μM	0.7	6

[a] Uptake was measured in 10 mM HEPES (pH 7.5) at 20 C with 50 μM pyruvate.

[b] Nanomoles per minute per milligram of protein.

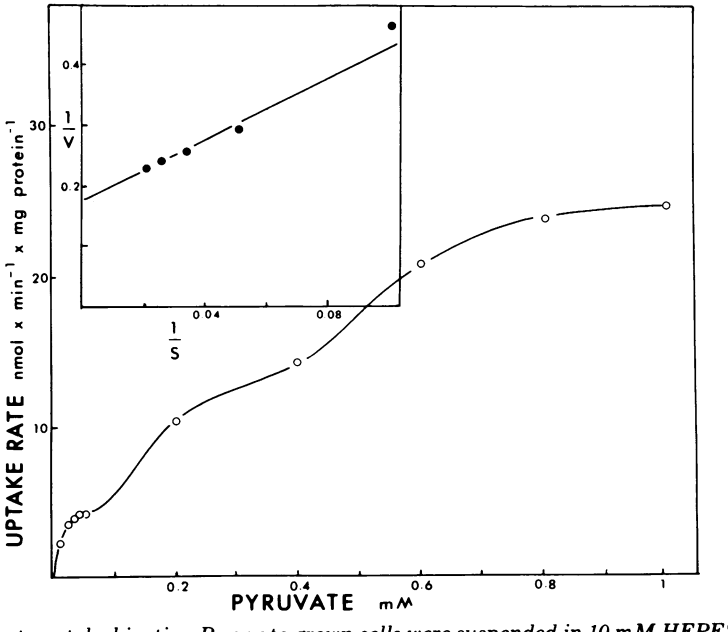

FIG. 6. *Pyruvate uptake kinetics. Pyruvate-grown cells were suspended in 10 mM HEPES-20 μM Mn^{2+}, and uptake rates were measured at 20 C. The insert shows a double-reciprocal plot of the lower-concentration data.*

rather than affecting metabolic conversion of entered material.

The entry of pyruvate differed from that of C_4 dicarboxylates in that its rate was as great in triethanolamine or HEPES buffer as in the medium salts, and in that the addition of divalent cations gave at most a 20% stimulation of uptake rate and EDTA was not inhibitory. An energy source was required, however, and dark controls usually accumulated not more than 10% of the radioactivity taken up by illuminated anaerobic suspensions. Aerated suspensions took up pyruvate as rapidly in dark as in light.

DISCUSSION

This investigation makes it plain that *R. spheroides* is indeed able to take up carbon substrates such as dicarboxylates and pyruvate when their external concentration is low. The relatively simple kinetics of C_4 dicarboxylate uptake from low external concentrations suggests that a single, common transport system may be involved in the movement of succinate, fumarate, and malate. Indirect support for this comes from the effects of growth substrates on uptake rates, which suggest that the system is inducible, as it is in other organisms. It is of course possible that increased uptake is the result of induction of enzymatic machinery unconnected with the actual entry into the cell. However, there was no difference in succinic dehydrogenase and malic dehydrogenase and, at most, a twofold difference in fumarase activity in cell-free extracts prepared from fructose-grown and succinate-grown cells, which showed a 30-fold difference in uptake rate (data not shown). Initial steps in C_4 dicarboxylate metabolism are, thus, little affected by growth substrate. One further piece of indirect evidence for the functioning of a single system in the entry of these compounds lies in the competitive effects of each on the entry of other. Succinate is as effective in preventing malate entry as malate itself is in preventing uptake of succinate, although malate is metabolically downstream and might, therefore, be expected to be relatively insensitive if competition occurred at some point after entry into the cell. The investigations of Fournier et al. (1) with *B. subtilis* suggest that more specific systems may be involved, for instance in the transport of malate, when higher concentrations of substrates are employed. Whether this is true also in *R. spheroides* has not yet been resolved, although there are indications of more rapid uptake at concentrations of about 1 mM and above. There are a number of differences in detail between the uptake C_4 dicarboxylates described here and systems from other organisms. The affinities in *R. spheroides* lie in a different order, namely fumarate > succinate = malate ≫ malonate and maleate. The introduction of an amino group, as in aspartate, appears to prevent recognition of the molecule, as is the case in *B. subtilis* (1), although aspartate is well transported by the *E. coli* system (6).

The K_m values measured for the uptake of dicarboxylates in these experiments are an order of magnitude or more lower than those reported for other organisms, whereas the V_{max} values are at least as high or higher when allowance is made for the different temperature and units used. It can be calculated that, for the doubling time of 6.5 to 7 h measured for this strain at 20 C, substrate must be taken into the cells at a minimal rate of 35 to 40 nmol/min per mg of protein for dicarboxylate and 45 to 50 nmol/min per mg of protein for pyruvate, provided that all entering carbon is incorporated into cell material. In practice, some CO_2 is released by photosynthetic cells metabolizing compounds more oxidized than the average state of reduction for cell material (12, 19), so that a rather higher entry rate must, in fact, be maintained at least for fumarate and malate. The rates of uptake of C_4 dicarboxylates observed in the present study, even at low concentrations, come fairly close to these estimated rates and suggest that the system most fully investigated here can supply cellular needs for near maximal growth rates, although indications of more rapid uptake were indeed obtained at higher external concentrations. Support for the suggestions that a low-concentration system does indeed function during growth comes from malate-limited continuous cultures, in which residual malate concentrations in the steady state were found to be only 0.1 to 0.3 μM, $\frac{1}{10}$ or less of the K_m for malate uptake measured in short-term experiments (unpublished data). There is a substantial gap between the maximal observed rates at which pyruvate was taken up by the only system that could be defined kinetically in these suspensions and the calculated requirement, and the increased uptake rates observed at higher concentrations must be of importance in mediating entry in organisms growing at maximal rates in excess substrate. This would seem also to be the case with *E. coli* and *B. subtilis* using carboxylates. The published V_{max} values for entry of 20 to 30 nmol/min per mg of protein at 37 C for low-concentration uptake systems (3, 8) fall substantially short of the approaching 200 nmol/min per mg of protein needed to maintain a doubling time

of about 90 to 120 min, allowing for the fact that part of the entering material must be oxidized to permit macromolecular synthesis with the remainder. Adequate rates are possible with higher concentrations systems in *B. subtilis* (1). From the experiments described here, *R. spheroides* could be expected to maintain a close-to-maximal growth rate at substantially lower dicarboxylate concentrations than these other organisms and should be able to outgrow them under such conditions. It would be of interest to extend these studies to other aerobic organisms that use C_4 dicarboxylates preferentially to find out whether high capacity, as well as high affinity, of the uptake system is generally found.

The uptakes of pyruvate and C_4 dicarboxylates are quite differently affected by alteration in chemical environments, at least from external concentrations of 50 μM or less. The C_4 dicarboxylate system was affected by a very restricted range of compounds, the absence of other charged groups being apparently more important than either chain length or stereoisometry; monocarboxylates tested, pyruvate among them, were without effect. The entry of pyruvate at low concentrations was affected by a wider range of compounds, of which unsubstituted monocarboxylates were the most active, although dicarboxylates were also somewhat inhibitory. Over the limited range tested, chain length did not appear very important, but introduction of an amino group again made the C_3 analogue unrecognizable. Divalent cations were required for entry of dicarboxylates but not for pyruvate. Because the effect of Mn^{2+} is not altered by changing dicarboxylate concentration, it seems probable that its effect is related to cell surface organization, rather than to a requirement for symport. A similar conclusion was reached by Rayman et al. (17) for the much higher Mg^{2+} requirement found for dicarboxylate transport in *E. coli* membrane vesicles. A generalized alteration of surface conformation is however improbable in view of the lack of effect of EDTA on the entry of pyruvate.

C_4 dicarboxylates and pyruvate are rapidly converted into other metabolites after entry into the cell. The succinate pool in *R. spheroides* is 6 to 8 nmol/mg (dry weight) (7) or about 14 nmol/mg of protein, meaning that the whole must turn over about four to five times per minute during growth. Rapid conversion of entered dicarboxylates occurs also in *E. coli*, and it has been necessary to use mutants defective in dicarboxylate-converting enzymes (6, 8) or membrane vesicles (10, 17) for unequivocal demonstration that a substantial concentration difference can, in fact, be established between the cell and the environment. In the gram-positive *B. subtilis*, it appears easier to demonstrate such a concentration difference (3), although mutants (1) and non-metabolizable substrates (21) have also been used extensively in investigation with this organism. Appropriate mutants of *R. spheroides* are currently being sought and should help to establish whether the demonstrated energy requirement for uptake in wild-type cells is the result of energy-dependent entry or is needed for maintenance of metabolic flux which, in turn, permits continued entry by a facilitated diffusion system. A longer-term objective of these studies is to examine light-driven movements of molecules in membrane preparations. The similarities uncovered between the processes involved in dicarboxylate transport in this photosynthetic organism and those previously described in heterotrophs suggest that the system may indeed require energy directly and, therefore, be suitable for such investigations.

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LITERATURE CITED

1. Fournier, R. E., M. N. McKillen, A. B. Pardee, and K. Willeke. 1972. Transport of dicarboxylic acids in *Bacillus subtilis*. J. Biol. Chem. **247**:5587-5595.
2. Frerman, F. E. 1973. The role of acetyl coenzyme A:butyrate coenzyme A transferase in the uptake of butyrate by isolated membrane vesicles of *Escherichia coli*. Arch. Biochem. Biophys. **159**:444-452.
3. Ghei, O. K., and W. W. Kay. 1972. A dicarboxylic acid transport system in *Bacillus subtilis*. FEBS Lett. **20**: 137-140.
4. Ghei, O. K., and W. W. Kay. 1973. Properties of an inducible C_4 dicarboxylic acid transport system in *Bacillus subtilis* J. Bacteriol. **114**:65-79.
5. Harold, F. C., and E. Levin. 1974. Lactic acid translocation: terminal step in glycolysis by *Streptococcus faecalis*. J. Bacteriol. **117**:1141-1148.
6. Kay, W. W., and H. L. Kornberg. 1971. The uptake of C_4 dicarboxylic acids by *Escherichia coli*. Eur. J. Biochem. **18**:274-281.
7. Lascelles, J., and K. Rodgers. 1961. The intracellular concentration of succinic acid in photosynthetic bacteria. Biochem. J. **80**:244-245.
8. Lo, T. C. Y., M. K. Rayman, and B. D. Sanwal. 1972. Transport of succinate by *Escherichia coli*. I. Biochemical and genetic studies of transport in whole cells. J. Biol. Chem. **247**:6323-6331.
9. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
10. Matin, A., and W. N. Konings. 1973. Transport of lactate and succinate by membrane vesicles of *Escherichia coli*, *Bacillus subtilis* and a Pseudomonas sp. Eur. J. Biochem. **34**:58-67.
11. Miović, M. L., and J. Gibson. 1973. Nucleotide pools and

adenylate energy charge in balanced and unbalanced growth of *Chromatium*. J. Bacteriol. **114**:86–95.
12. Muller, F. M. 1933. On the metabolism of the purple sulfur photosynthetic bacteria in organic media. Arch. Mikrobiol. **4**:131–166.
13. Myers, W. F., and K. Y. Huang. 1966. Separation of intermediates of the citric acid cycle and related compounds by thin layer chromatography. Anal. Biochem. **17**:210–213.
14. Oxender, D. L. 1972. Membrane transport. Annu. Rev. Biochem. **41**:277–314.
15. Pfennig, N. 1967. Photosynthetic bacteria. Annu. Rev. Microbiol. **21**:285–324.
16. Pfennig, N., and K. D. Lippert. 1966. Über das Vitamin B_{12}-bedürfnis phototropher Schwefelbakterien. Arch. Mikrobiol. **55**:245–256.
17. Rayman, M. K., T. C. Y. Lo, and B. D. Sanwal. 1972. Transport of succinate in *Escherichia coli*. II. Characteristics of uptake and energy coupling with transport in membrane preparations. J. Biol. Chem. **247**:6332–6339.
18. Saier, M. H., B. U. Feucht, and S. Roseman. 1971. Phosphoenolpyruvate-dependent fructose phosphorylation in photosynthetic bacteria. J. Biol. Chem. **246**:7819–7821.
19. van Niel, C. B. 1941. The bacterial photosyntheses, p. 263–328. *In* F. F. Nord and C. H. Werkman (ed.), Advances in enzymology, vol. 1. Interscience, New York.
20. van Niel, C. B. 1944. The culture, general physiology, morphology, and classification of the non-sulfur purple and brown bacteria. Bacteriol. Rev. **8**:1–118.
21. Wagner, B. J., M. L. Miović, and J. Gibson. 1973. Utilization of amino acids by *Chromatium* sp., Strain D. Arch. Mikrobiol. **91**:255–272.
22. Willeke, K., and R. Lange. 1974. C_4-dicarboxylate transport in *Bacillus subtilis* studied with 3-fluoro-L-erythromalate as substrate. J. Bacteriol. **117**:373–378.