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*Published in:*
Journal of Bacteriology



*Publication date:*
1993

JOURNAL OF BACTERIOLOGY, Feb. 1993, p. 1075–1080
0021-9193/93/041075-06$02.00/0

Vol. 175, No. 4

# Uptake of 4-Toluene Sulfonate by *Comamonas testosteroni* T-2

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Received 14 October 1992/Accepted 30 November 1992

**The mechanism of transport of the xenobiotic 4-toluene sulfonate (TS) in *Comamonas testosteroni* T-2 was investigated. Rapid uptake of TS was observed only in cells grown with TS or 4-methylbenzoate as a carbon and energy source. Initial uptake rates under aerobic conditions showed substrate saturation kinetics, with an apparent affinity constant ($K_t$) of 88 μM and a maximal velocity ($V_{max}$) of 26.5 nmol/min/mg of protein. Uptake of TS was inhibited completely by uncouplers and only marginally by ATPase inhibitors and the phosphate analogs arsenate and vanadate. TS uptake was also studied under anaerobic conditions, which prevented intracellular TS metabolism. TS was accumulated under anaerobic conditions in TS-grown cells upon imposition of an artificial transmembrane pH gradient (ΔpH, inside alkaline). Uptake of TS was inhibited by structurally related methylated and chlorinated benzenesulfonates and benzoates. The results provide evidence that the first step in the degradation of TS by *C. testosteroni* T-2 is uptake by an inducible secondary proton symport system.**

Sulfonated aromatic compounds can be released into the environment mainly as waste products from the manufacture of dyestuffs and detergents (3). A sulfonate group at an aromatic ring is a structure that is rarely found in nature (7) and is somewhat resistant to biodegradation (12). Bacteria that are able to degrade several substituted benzene- and naphthalenesulfonates have been isolated from industrial sewage treatment plants (26) or obtained by continuous adaptation of bacteria able to degrade natural aromatic compounds (1). The degradative pathways of a few naphthalenesulfonates (1) and benzenesulfonates (14, 16, 26) have been characterized in bacteria which are able to utilize these compounds as single sources of carbon and energy for growth. The role of transport across the cytoplasmic membrane in the biodegradation of aromatic sulfonates has not yet been studied in any detail, although some evidence that membrane transport is a limiting step has been reported (27). Permeabilization of bacterial cells increased the rate and expanded the substrate range for degradationl of sulfonated azodyes (18). In an *Alcaligenes* sp., seven different substituted benzenesulfonates are degraded in cell extracts, of which only three are degraded by whole cells (27). It has been concluded that the cell membrane of this strain is impermeable for aromatic sulfonates and that one or more specific transport systems play a role in the uptake of these compounds.

In general, transport of substituted aromatic compounds across bacterial cytoplasmic membranes is still poorly characterized. Uptake as the first step in the degradation of these compounds has often been ignored or considered to occur by passive diffusion. Mandelate has been claimed to enter the cell by passive diffusion (6), by facilitated diffusion (2), and by an active transport system (8). The uptake of 4-chlorobenzoate by a coryneform bacterium has been studied in more detail (4). Accumulation of 4-chlorobenzoate has been achieved under anaerobic conditions when nitrate is used as a terminal electron acceptor and when a proton motive force ($\Delta p$) is imposed artificially by ion-diffusion gradients, which indicates a $\Delta p$-driven transport system. Evidence for a carrier-mediated uptake system for benzoate in *Pseudomonas putida* has been obtained by use of a mutant strain that is blocked in benzoate metabolism and accumulates benzoate intracellularly (25). The benzoate uptake system is inducible and has been eliminated by mutation of a distinct gene. The transport mechanism has not been characterized, but some evidence for $\Delta p$-driven rather than ATP-driven uptake has been obtained (25). Benzoate is a weak acid which, like acetate, is often used for determination of the transmembrane pH gradient (ΔpH) in cells (10, 23). Intracellular accumulation of benzoate is proportional to the ΔpH, indicating that the membrane is sufficiently permeable only for the protonated form (benzoic acid). In contrast, aromatic sulfonates are rather strong acids, typically with $pK_a$ values of less than 1 (3). At physiological pH, hardly any of the protonated form is present, and passive diffusion is very low. Sulfonated instead of carboxylated reagents are often used in biochemical studies if no membrane permeation is desired (20).

In the present study, we examined the transport of 4-toluene sulfonate (TS) in the gram-negative bacterium *Comamonas testosteroni* T-2, which can grow on TS as the only source of carbon and energy (14). Degradation of TS is initiated by oxidation of the methyl group (via the alcohol and the aldehyde), forming 4-sulfobenzoate, which is subsequently oxidized to protocatechuate, with simultaneous release of the sulfonate group as sulfite. Protocatechuate is further degraded via the *meta* cleavage pathway (Fig. 1) (14). The first step in TS degradation is catalyzed by a soluble, two-component monooxygenase (TS methyl-monooxygenase), which is NADH dependent (15). This indicates a cytoplasmic localization of the enzyme and emphasizes the need for rapid translocation of TS across the membrane. In this report, evidence is presented that this transport is mediated by an inducible secondary proton symport system.

## MATERIALS AND METHODS

**Organism and growth conditions.** *C. testosteroni* T-2 was grown in minimal medium containing TS or other carbon

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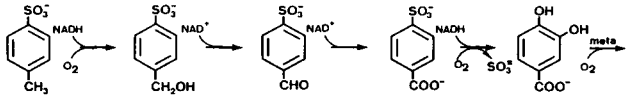

FIG. 1. Degradative pathway of TS in *C. testosteroni* T-2 (14).

sources as described before (14, 15). Cells were harvested at the mid-exponential growth phase by centrifugation (10 min at 15,000 × *g*), washed once, and resuspended in the desired buffer.

**Uptake assay under aerobic conditions.** Uptake of $^{14}$C-labeled compounds was routinely assayed by a filtration method (22). Cells were routinely suspended in 50 mM potassium phosphate, pH 7.0, buffer at concentrations of 0.5 to 1.0 mg of protein per ml. In some experiments, 50 mM potassium 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (potassium HEPES), pH 7.3, was used. The suspension was stirred magnetically and incubated for 5 to 10 min at 30°C in the presence or absence of any energy source (8 mM succinate or acetate) before the addition of the $^{14}$C-labeled compound to start uptake. At intervals, transport was terminated by diluting a 200-μl sample in 2 ml of ice-cold 100 mM LiCl and immediate filtration through a 0.45-μm cellulose-nitrate filter (BA 85; Schleicher & Schuell, Dassel, Germany). Filters were washed once with 2 ml of 100 mM LiCl and transferred to scintillation vials, and the radioactivity was determined by liquid scintillation spectrometry (Packard Tri-Carb-460 CD; Packard Instruments, Rockville, Md.). Kinetic parameters of TS uptake were determined from initial uptake rates (between 15 and 45 s) in cells incubated in the presence of 8 mM succinate. Concentrations of [$^{14}$C]TS were between 12 and 600 μM, and maximal velocity ($V_{max}$) and the apparent affinity constant ($K_t$) were calculated from Lineweaver-Burk plots.

**Uptake assays under anaerobic conditions and creation of artificial ion gradients.** Uptake assays under anaerobic conditions and the imposition of an electrical potential ($\Delta\psi$) or a $\Delta$pH by means of artificial ion gradients were done essentially as described elsewhere (4). Cells were incubated for 1 h at 4°C in 50 mM potassium phosphate–150 mM potassium acetate, pH 7.0, in the presence of 2 mM potassium EDTA and 50 μM valinomycin, centrifuged, and resuspended in 50 mM potassium phosphate–150 mM potassium acetate, pH 7.0, to a concentration of 40 mg of protein per ml. The cell suspension was transferred to a sealed bottle and flushed with oxygen-free nitrogen gas for about 10 min. To start uptake, 20 μl of the cell suspension was transferred anaerobically with a gas-tight Hamilton syringe into 1.2 ml of a buffer solution (pH 7.0), which was stirred magnetically at 30°C and continuously flushed with oxygen-free nitrogen gas. The buffer contained 25 μM $^{14}$C-labeled TS or 1.5 μM $^{14}$C-labeled L-alanine. The composition of the buffer varied according to the desired potential: 50 mM potassium phosphate–150 mM potassium HEPES for generation of a $\Delta$pH, interior alkaline; 50 mM sodium phosphate–150 mM sodium acetate for generation of a $\Delta\psi$, interior negative; 50 mM potassium phosphate–150 mM potassium acetate if no gradient was to be generated. At intervals, 100-μl samples were removed with a Hamilton syringe, diluted into 2 ml of ice-cold, $N_2$-saturated 100 mM LiCl, and rapidly filtered and rinsed, and the radioactivity was determined as described above.

**Osmotic shock procedure.** Cells grown in TS-minimal medium were exposed to the osmotic shock procedure described by Neu and Heppel (19). Cells (1 g, wet weight) were washed in 50 mM Tris-HCl (pH 7.8) containing 5 mM potassium EDTA and resuspended in 50 ml of the same buffer including 20% (vol/vol) sucrose. After 30 min of incubation at 20°C, cells were harvested by centrifugation, and the pellet was rapidly suspended in 100 ml of ice-cold 0.5 mM $MgSO_4$. After another 30 min of incubation, the cells were centrifuged, washed, resuspended in 50 mM potassium phosphate buffer, pH 7.0, and tested for aerobic TS uptake. Control cells were treated essentially in the same way but without sucrose.

**Determination of intracellular volume.** The specific intracellular volume of *C. testosteroni* T-2 was determined from the distribution of $^3H_2O$ and $^{14}$C-labeled sorbitol analyzed by a silicon oil centrifugation method (9) as described elsewhere (23). The silicon oil was made by mixing oils AR20 and AR200 (Wacker Chemie, Munich, Germany), resulting in a density of 1.03 g/ml. From the data, a specific internal cell volume of 3.2 ± 0.3 μl/mg of protein was calculated. This volume was used in all calculations.

**Assay of TS methyl-monooxygenase.** The activity of TS methyl-monooxygenase in whole cells and cell extracts of *C. testosteroni* T-2 was determined by monitoring the rate of oxygen consumption with a Clarke type oxygen electrode as described before (15).

**Analytical procedures.** The fate of intracellularly accumulated TS was analyzed by high-pressure liquid chromatography (HPLC). Aerobic uptake assays were carried out as described above except that 200 μM nonradioactive TS and samples of 3 to 5 mg of cell protein per filter were used. Cells on the filter were extracted with 200 μl of 10% (vol/vol) perchloric acid for 30 min. After neutralization with KOH and clarification by centrifugation, the supernatant was analyzed for TS and its degradation products by reversed-phase HPLC with a potassium phosphate-methanol solvent system as described elsewhere (14, 15).

For the determination of intracellular ATP levels, 200 μl of cell suspension was mixed with 200 μl of 20% (vol/vol) perchloric acid containing 5 mM potassium EDTA and stored on ice. After neutralization with KOH and centrifugation of the precipitate, the supernatant was diluted 10- to 40-fold in 40 mM Tris-$SO_4$–10 mM $MgSO_4$ (pH 7.7), and the ATP content was determined by the firefly luciferase assay (21).

Protein was determined by the method of Lowry et al. (17), with bovine serum albumin as the standard.

**Chemicals.** [*ring*-U-$^{14}$C]TS (455 GBq/mol) was obtained from Sigma Chemical Co., St. Louis, Mo., and [*ring*-U-$^{14}$C] benzoate (1.84 TBq/mol), $^3H_2O$ (37 MBq/ml), D-[U-$^{14}$C] sorbitol (1.23 TBq/mol), and L-[U-$^{14}$C]alanine (6.12 TBq/mol) were obtained from the Radiochemical Centre, Amersham; United Kindom. The Sources of difference sulfonated compounds are given elsewhere (14, 15). All other chemicals were of reagent grade and obtained from commercial sources.

## RESULTS

**Uptake of TS under aerobic conditions.** Under aerobic conditions, cells of *C. testosteroni* T-2 grown on TS rapidly accumulated radioactivity from $^{14}$C-labeled TS (Fig. 2). The uptake was linear for almost 1 min and did not reach a steady state within 10 min. Preincubation of the cells for 10 min with 8 mM succinate or acetate increased the initial uptake rate two- to threefold (not shown). Analysis of initial uptake rates versus TS concentration resulted in monophasic saturation

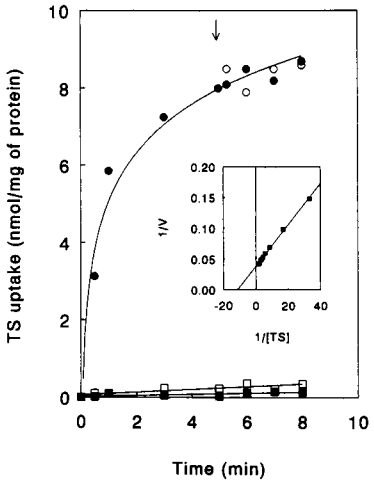


FIG. 2. Uptake of TS by *C. testosteroni* T-2 under aerobic conditions. Cells grown with TS (●) or succinate (□) as the carbon source were incubated in 50 mM potassium phosphate, pH 7.0 (0.5 mg of protein per ml), and uptake was started by the addition of $^{14}$C-labeled TS (52 μM, final concentration). ○, effect of the addition (at time marked by arrow) of 2 mM (final concentration) unlabeled TS after 5 min of uptake of [$^{14}$C]TS in TS-grown cells; ■, uptake in TS-grown cells in the presence of 50 μM CCCP. Inset: Double-reciprocal plot of initial TS uptake rates ($V$) (nmol (nanomoles of TS per minute per milligram of protein) versus TS concentration (millimolar).

kinetics (Fig. 2, inset). At pH 7, a $V_{max}$ of 26.5 nmol of TS per min per mg of protein and an apparent $K_t$ of 88 ± 7 μM were obtained. Kinetic parameters were not strongly influenced by external pH in the range from 6 to 8. At pH 6.0, the $V_{max}$ and apparent $K_t$ were 24.3 nmol/min/mg of protein and 85.6 μM, respectively, and at pH 8.0, there were 22.0 nmol/min/mg of protein and 79.5 μM, respectively. Cells grown on succinate showed very low rates of TS uptake (Fig. 2).

Radioactivity accumulated from $^{14}$C-labeled TS could not be released after the addition of excess unlabeled TS (Fig. 2). This indicates that [$^{14}$C]TS cannot be exchanged for unlabeled TS and suggests that most of the incorporated [$^{14}$C]TS is rapidly converted into degradation products or incorporated into cell material. To analyze the fats of incorporated TS, perchloric acid extracts of cells incubated in the presence of 0.2 mM TS for 20 s and 5 min were analyzed by HPLC. The detection limit of the assay corresponded to an intracellular TS concentration of 0.1 mM (0.3 nmol/mg of protein). At least one degradation product, 4-sulfobenzoate, could be detected at internal concentrations of up to 0.5 mM, whereas no TS was detected (data not shown).

To study the energy-coupling mechanism of TS transport, the effects of different inhibitors of energy metabolism on TS uptake were tested. The uncouplers carbonylcyanide *m*-chlorophenylhydrazone (CCCP) and carbonylcyanide *p*-trifluoromethoxyphenylhydrazone, which can dissipate the $\Delta p$ (i.e., both $\Delta\psi$ and $\Delta$pH) (11), inhibited TS uptake completely (Table 1; see also Fig. 2). On the other hand, neither protonophore (at the same concentrations) inhibited TS oxidation in cell extracts or in cells treated with 0.1% (vol/vol) Triton X-100, whereas 95% inhibition was observed in untreated cells (data not shown). This indicates that only the uptake of TS and not its metabolism was affected by these uncouplers. Valinomycin and nigericin, ionophores

TABLE 1. Effect of different inhibitors on TS uptake and intracellular ATP concentration in cells of *C. testosteroni* T-2[a]

| Addition (concn) | TS uptake (% of control) | Internal ATP concn (% of control) |
|---|---|---|
| None[b] (control) | 100 | 100 |
| CCCP (50 μM) | 1 | 35 |
| Trifluoromethoxyphenylhydrazone (50 μM) | 1 | ND[c] |
| Valinomycin plus nigericin (50 μM each) | 69 | ND |
| *N,N'*-Dicyclohexylcarbodiimide (0.5 mM) | 88 | 55 |
| Vanadate (1 mM) | 83 | 105 |
| Arsenate (1 mM) | 80 | 61 |

[a] TS-grown cells were washed and resuspended in 50 mM potassium HEPES–4 mM potassium EDTA, pH 7.3. After incubation for 10 min at 30°C in the presence or absence of an inhibitor, initial uptake rates (with 52 μM $^{14}$C-labeled TS) and intracellular ATP concentrations were determined.

[b] In the absence of an inhibitor, the initial TS uptake rate was 4.5 ± 0.2 nmol/mg of protein and the internal ATP concentration was 1.50 ± 0.2 mM; these values were taken as 100%.

[c] ND, not determined.

which can dissipate the $\Delta\psi$ and the $\Delta$pH, respectively, in the presence of $K^+$ (11), in combination inhibited TS uptake only partially (Table 1). Preincubation of the cells in phosphate-free buffer in the presence of the phosphate analogs arsenate and vanadate or of the ATPase inhibitor *N,N'*-dicyclohexylcarbodiimide did not decrease initial rates of TS uptake by more than 20% (Table 1). Intracellular ATP levels were also measured after incubation with different inhibitors (Table 1). *N,N'*-Dicylohexylcarbodiimide arsenate, and vanadate decreased ATP pools by 45, 39, and 0%, respectively. CCP reduced the intracellular ATP level only to 65% of the untreated level. These observations indicate that there is no correlation between TS uptake and intracellular ATP concentration.

To investigate whether an ATP-driven periplasmic-binding protein-dependent transport system was responsible for TS uptake, TS uptake was studied in cells which were exposed to an osmotic shock. Such treatment did not affect the TS uptake rate significantly; the rates were 3.5 and 3.3 nmol/min/mg of protein for untreated and osmotically shocked cells, respectively. These results suggest that TS is transported by an inducible transport system and that the $\Delta p$ or one of its components rather than ATP or other high-energy phosphate bonds function as the driving force.

**Membrane permeability of TS.** To study passive diffusion of *C. testosteroni* T-2 through the cell membrane, transport of $^{14}$C-labeled TS and of $^{14}$C-labeled benzoate was compared in uninduced (succinate-grown) cells (Fig. 3). TS and benzoate are not metabolized by succinate-grown cells of *C. testosteroni* T-2 (14). Benzoate was rapidly taken up and reached a steady-state level of accumulation of about 4 (in/out) after approximately 5 min. In the presence of CCCP, an accumulation ratio (in/out) of about 1 was reached. These observations are consistent with uptake of benzoate being a passive process in response to a $\Delta$pH. In contrast, uptake of TS proceeded very slowly irrespective of the presence or absence of CCCP. Equilibration with the external concentration was not reached after 20 min of incubation, indicating that the passive membrane permeability of TS is very low.

**Uptake of TS under anaerobic conditions.** Attempts to obtain TS uptake in isolated membrane vesciles of *C. testosteroni* T-2 were not successful (13), which hindered

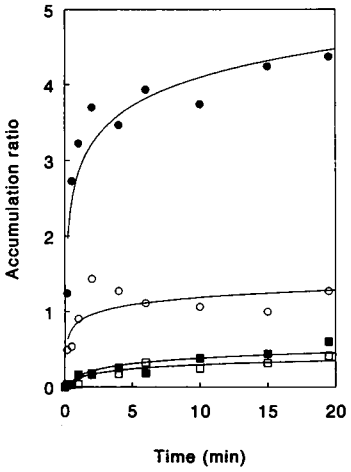


FIG. 3. Uptake of [$^{14}$C]benzoate and [$^{14}$C]TS in succinate-grown cells of *C. testosteroni* T-2. Cells were incubated in 50 mM potassium phosphate, pH 7.0, without (solid symbols) or with (open symbols) 50 μM CCCCP. Uptake was started by the addition of 52 μM $^{14}$C-labeled TS (■, □) or 46 μM $^{14}$C-labeled benzoate (●, ○). For calculation of the accumulation ratio (internal concentration/external concentration), a specific internal volume of 3.2 μl/mg of protein was used.

detailed characterization of the energetics of TS transport. To study transport in TS-grown cells in the absence of TS metabolism, transport experiments were performed under anaerobic conditions. Under these conditions, the first step of TS degradation, the reaction catalyzed by an oxygen-dependent monooxygenase, is inhibited (14, 15). In cells grown under anaerobic conditions, a ΔpH and a Δψ were created by means of an acetate diffusion potential and a valinomycin-induced potassium diffusion potential, respectively. Uptake of $^{14}$C-labeled TS was observed upon imposition of a ΔpH (inside alkaline), whereas imposition of a Δψ resulted in an uptake similar to that in nonenergized cells (Fig. 4A). In a control experiment, an artificially imposed ΔpH and a valinomycin-induced potassium diffusion potential could drive anaerobic uptake of $^{14}$C-labeled L-alanine (Fig. 4B). Given a specific intracellular volume of 3.2 μl/mg of protein, an eightfold accumulation of TS was maximally obtained with the artificially imposed ΔpH. Determination of the intracellular ATP concentrations in parallel experiments showed that an artificially imposed ΔpH and Δψ increased the intracellular ATP concentration (after 30 s) from 0.22 ± 0.04 to 0.39 ± 0.07 and 0.88 ± 0.1 mM, respectively. These observations indicate that the ΔpH-driven accumulation of TS is not the result of an increased intracellular level of ATP.

In contrast to TS uptake under aerobic conditions, most of the radioactivity accumulated from $^{14}$C-labeled TS under anaerobic conditions was released upon addition of excess unlabeled TS (Fig. 4C), indicating that TS is not metabolized and that [$^{14}$C]TS exchanges for unlabeled TS. Addition of benzenesulfonate or 4-sulfobenzoate did not result in a significant efflux of [$^{14}$C]TS (Fig. 4C). The addition of 50 μM CCCP (final concentration) also resulted in a release of accumulated [$^{14}$C]TS (Fig. 4C). Cells grown on succinate did not show any significant accumulation of [$^{14}$C]TS under anaerobic conditions, whether a ΔpH was imposed or not (data not shown). These results indicate that TS is taken up by an inducible transport system which can be energized by the ΔpH.

**Substrate and inducer specificity.** The effect of structural analogs of TS on [$^{14}$C]TS uptake was tested. In the aerobic uptake assay, inhibition of uptake could also be caused by inhibition of TS metabolism. Therefore, the effect of the TS analogs on ΔpH-driven anaerobic uptake of [$^{14}$C]TS was also analyzed (Table 2). Strong inhibition of aerobic and anaerobic TS uptake was observed with unlabeled TS, 4-chlorobenzenesulfonate, 4-chlorobenzoate, 3- and 4-methylbenzoate, 4-methoxybenzoate, and benzoate, suggesting that the transport system has affinity for TS as well as for some methylated and chlorinated benzenesulfonates and benzoates. However, only TS and 4-chlorobenzenesulfonate elicited a rapid exchange of anaerobically accumulated [$^{14}$C]TS (Fig. 4C), indicating that these compounds are transported by the same transport system.

Induction of TS uptake was studied by growing cells with 8 mM acetate in the presence of a potential inducer at a concentration of 2 mM. Only TS and 4-methylbenzoate could significantly induce uptake of $^{14}$C-labeled TS (with

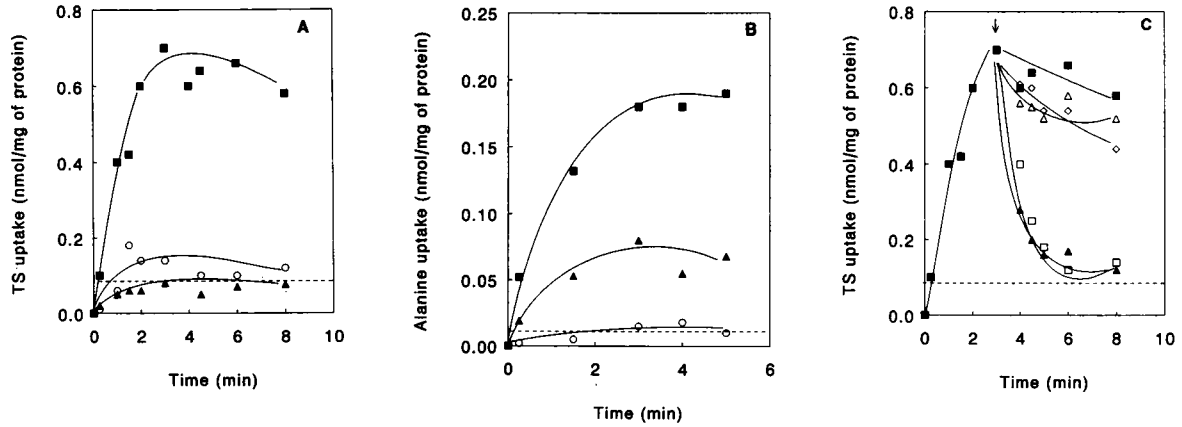


FIG. 4. Uptake of TS (A and C) and L-alanine (B) in cells of *C. testosteroni* T-2 under anaerobic conditions in the presence of an artificially imposed ΔpH or Δψ, created by means of artificial ion diffusion potential (see test for details). (A and B) Symbols: ■, acetate gradient (ΔpH); ▲, potassium gradient (Δψ); ○, no gradient. The dotted lines indicate equilibration with the external concentration. (C) Effect of addition (at time marked by the arrow) of 50 μM CCCP (▲), 2 mM unlabeled TS (□), 2 mM benzenesulfonate (△), or 2 mM 4-sulfobenzoate (◇) after 3 min of [$^{14}$C]TS uptake in the presence of a ΔpH. Sulfonates were added from anaerobic stock solutions neutralized to pH 7.0 with KOH.

TABLE 2. Inhibition of [$^{14}$C]TS uptake by structurally related compounds[a]

| Addition | Residueal [$^{14}$C]TS uptake (% of control) | |
|---|---|---|
| | Aerobic[b] | Anaerobic[c] |
| None (control) | 100 | 100 |
| TS | 6 | 8 |
| 4-Chlorobenzenesulfonate | 8 | 7 |
| 4-Hydroxybenzenesulfonate | 38 | 42 |
| 4-Aminobenzenesulfonate | 49 | 77 |
| 4-Sulfobenzoate | 68 | 48 |
| Benzenesulfonate | 73 | 95 |
| Sulfate | 97 | ND[d] |
| Sulfite | 110 | ND |
| 4-Chlorobenzoate | 5 | 5 |
| 4-Methylbenzoate | 18 | 16 |
| 3-Methylbenzoate | 22 | 18 |
| 2-Methylbenzoate | 52 | 59 |
| Benzoate | 27 | 10 |
| 4-Methoxybenzoate | 31 | 29 |
| 4-Hydroxybenzoate | 76 | 92 |
| Protocatechuate | 105 | ND |
| Terephthalate | 89 | 100 |
| Phenylalanine | 98 | ND |

[a] Benzoates and sulfonates were added from neutralized stock solutions as the potassium salts.

[b] Aerobic uptake was started by simultaneous addition of 25 μM $^{14}$C-labeled TS and 1 mM unlabeled compound to cell suspensions in 50 mM potassium phosphate, pH 7.0. Initial uptake rates were recorded between 15 and 45 s after additions. The 100% value correspond to 3.1 nmol of TS taken up per min per mg of protein.

[c] Uptake of $^{14}$C-labeled TS under anerobic conditions in the presence of a ΔpH was determined as described in Materials and Methods. The uptake rates were estimated from the uptake in the first 60 s; 100% corresponds to 0.40 nmol of TS taken up per mg of protein. $^{14}$C-labeled TS and unlabeled analogs were present at 25 μM and 1 mM, respectively.

[d] ND, not determined.

initial uptake rates of 68 and 51%, respectively, of those of cells grown on TS only). 4-Chlorobenzenesulfonate, 4-sulfobenzoate, 4-methoxybenzoate, terephthalate, benzoate, 4-hydroxybenzoate, and protocatechuate did not induce TS uptake. 4-Chlorobenzoate was growth inhibiting at the concentration used. Thus, the TS transport system showed a rather high substrate and inducer specificity.

## DISCUSSION

Several lines of evidence for an inducible secondary transport system for TS in *C. testosteroni* T-2 have been obtained. (i) The TS transport system is only active in cells grown in the presence of TS or 4-methylbenzoate. Transport rates of TS in succinate-grown cells are very slow (1 to 2 pmol/min/mg of protein), whereas high initial uptake rates (5.2 nmol/min/mg of protein) are found in induced cells under similar conditions (Fig. 2). ΔpH-driven TS accumulation under anaerobic conditions is observed in induced cells only. (ii) The membrane is barely permeable to the negatively charged form of TS, and unlike benzoate, passive diffusion of the protonated form across the membrane is negligible because of the low $pK_a$. The $pK_a$ of TS is −1.3 (3), whereas the $pK_a$ of the carboxyl group of benzoate is 4.19 (28). Furthermore, the membrane-phosphate buffer partition coefficient of TS, studied in liposomes prepared from *Escherichia coli* phospholipids, is 0.96 ± 0.11 (24). This coefficient is approximately 8 and 80 times smaller than the corresponding values for benzoate and toluene, respectively. (iii) TS uptake is inhibited by a few structurally related compounds. (iv) Different mechanisms for carrier-mediated transport of the anionic form of TS have been considered (11): an ATP-driven TS transport system; a symport system with $H^+$ or $Na^+$ as the coupling ion; and a TS-uniport system with no coupling ion (facilitated diffusion).

Evidence in favor of an ATP-driven transport mechanism has not been obtained. In fact, the inhibitor studies and the lack of correlation between transport activity and ATP pools argue against such a mechanism. The driving force for a TS-uniport system would be the concentration gradient maintained by the rapid metabolism, as has been suggested for benzoate uptake in *Rhodopseudomonas palustris* (5). Such a mechanism is unlikely, since a membrane potential (inside negative) would form a counterforce for the uptake process. Moreover, inhibition of TS uptake by uncouplers and the stimulation of transport by an acetate diffusion potential under anaerobic conditions indicate coupling to the $\Delta p$. Although the effects of CCCP and trifluoromethoxyphenylhydrazone could also be the result of a direct effect on the carrier, the CCCP-induced release of accumulated [$^{14}$C]TS under anaerobic conditions strongly indicates that these uncouplers affect TS transport through dissipation of the (electro)chemical $H^+$ gradient.

Taken together, the results support a $TS^-/H^+$ symport mechanism. The driving force for TS uptake would be $\Delta\bar{\mu}_{TS}/F + nZ\Delta pH - (n - 1)\Delta\psi$, where $n$ represents the number of protons symported with TS. If TS is symported with 1 proton, the contribution of the $\Delta p$ to the driving force will be the ΔpH only, whereas if TS is symported with 2 proteins, the $\Delta\psi$ would also be involved. No evidence that a $\Delta\psi$ is involved in TS transport has been obtained. However, one should be cautious in interpreting the effects of metabolic inhibitors because these compounds are not always effective in intact cells, e.g., valinomycin and nigericin inhibited TS uptake only partially, whereas CCCP inhibited it completely. More detailed investigation of the energetics of TS transport would require studies with isolated membrane vesicles. Preliminary attempts to study TS uptake in right-side-out membrane vesicles prepared from cells of *C. testosteroni* T-2 were not successful (13).

The first step in the degradation of TS by *C. testosteroni* T-2 is uptake by an inducible carrier. In actively metabolizing cells, TS is not accumulated intracellularly but immediately hydroxylated by the TS methyl-monooxygenase, which has a high affinity for TS (15). The TS uptake system is induced by TS and 4-methylbenzoate but not by 4-sulfobenzoate or terephthalate. The expression of the transport system is apparently coregulated with that of the enzymes of the "TS upper pathway," catalyzing the oxidation of the methyl group of TS, and of 4-methylbenzoate (16), suggesting that the corresponding genes are clustered in an operon. The inhibition studies with structural analogs show that the uptake system is inhibited by chlorinated and methylated benzoates and benzenesulfonates. However, so far we have no conclusive evidence that all these compounds are really taken up by the TS uptake system. No inhibition of TS uptake by phenylalanine, sulfate, or sulfite was found, indicating that TS is not taken up by a transport system for sulfate or aromatic amino acids. 4-Sulfobenzoate and protocatechuate, which are intermediates in the degradative pathway of TS in *C. testosteroni* T-2 and which can also serve as growth substrates, do not effectively inhibit or induce the TS

transport system. The TS uptake system is therefore a rather specific system which might have evolved from a transport system for natural carboxylated aromatic compounds. An analogous evolution has been postulated for the enzymes which degrade aromatic sulfonates (1, 16).

## ACKNOWLEDGMENT

This work was supported by a grant from the Swiss National Science Foundation.

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