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Uptake of *N*-(4′-pyridoxyl)amines and release of amines by renal cells: A model for transporter-enhanced delivery of bioactive compounds

(facilitated transport/transporter specificity/intracellular release)

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ABSTRACT **The importing of vitamin B_6 by renal proximal tubular cells from the rat is facilitated and Na^+-dependent and reflects specificity for the *meta*-phenolate pyridinium structure with a 5-hydroxymethyl function. This transporter can, however, accept competitively each of the natural nonphosphorylated vitamers (pyridoxine, pyridoxamine, and pyridoxal) and other B_6 analogues differing only in the groups at position 4. A series of *N*-(4′-pyridoxyl)amines was synthesized by sodium borohydride or boro[^{3}H]hydride reduction of aldimines formed by condensing the amines with pyridoxal. The unlabeled B_6-secondary amine compounds were found to competitively inhibit the uptake of [4′-^{3}H]pyridoxine by the renal cells. Moreover, the ^{3}H-labeled *N*-(4′-pyridoxyl)amines were shown to enter the cells by the process facilitated by the B_6 transporter. Upon entry the labeled compounds were converted to *N*-(5′-phospho-4′-pyridoxyl)amines in a reaction catalyzed by pyridoxal kinase, an enzyme that tolerates considerable functional variation in position 4 of the B_6 structure. The 5′-phosphates were subsequently converted within the cell to pyridoxal 5′-phosphate with liberation of the original amine in a reaction catalyzed by pyridoxamine (pyridoxine) 5′-phosphate oxidase, an enzyme with broad specificity for 4′-substituted amines on the 5′-phospho-B_6 structure. This system illustrates how knowledge of transporter specificity can permit design of a compound with potential biologic activity. A drug or other intracellular effector may be piggybacked onto a transported solute (e.g., vitamin or other nutrient) that gains facilitated entry to a cell and is, thereafter, metabolized to release the active compound.**

Though delivery of drugs and modulators to targets within cells has often relied on passive diffusion of a fraction of the pharmacologic levels of the compounds administered, increasing attention has been given to more effective means that utilize biologic mechanisms for enhancing entry of compounds into cells. One such theme has been the design of liposomes with encapsulated agents that may be endocytosed (1). Another means could conceivably utilize the ability of plasma membrane transporters to take in suitably modified solutes in carrier-mediated processes.

The transport of water-soluble vitamins by epithelial cells, such as intestinal enterocytes or renal proximal tubular cells, typically is by entry through the brush border (luminal) plasma membrane in a process that is facilitated at physiological concentration, reflects at least relative specificity for vitaminic structure, and is dependent upon Na^+ cotransport (2). Within the cell, metabolic alterations and associations with specific proteins often lead to metabolic trapping such that operational forms (e.g., coenzymes) are retained and only released through the basolateral (contraluminal) plasma membrane by reversion to the vitaminic form. The fate of vitamins with a nonepithelial cell such as the hepatocyte is somewhat similar in that transporters that contain binding proteins with relative specificity to facilitate entry are involved but Na^+ pumping may not be involved (3).

Our studies on uptake of vitamin B_6 by proximal tubular cells from rat kidneys revealed a facilitated process that has substrate specificity and may be modulated by sodium–hydrogen exchange and/or pH gradient effects (4). With the use of vesicles made from the brush-border membrane of these cells, the sodium ion dependence of the process was confirmed and the likelihood of mediation by sodium–hydrogen exchange was clarified (5). Characteristics of the B_6 transporter and the role of receptor/carrier proteins were clarified by the affinity isolation of specific B_6-binding proteins from the brush-border membrane.* From knowledge of the specificity of the transporter (4, *), which recognizes a 2-methyl-3-hydroxy-5-hydroxymethylpyridine but can accept analogues differing at position 4, it became clear one might compete with B_6 entry or even gain similar facilitated entry of *N*-(4′-pyridoxyl)amines, which are 4′ derivatives of pyridoxamine. Moreover, because of the specificity of pyridoxal kinase (7–9), the substituted pyridoxamines would likely be phosphorylated if they gained entry to the cytosol. Further, the resulting *N*-(5′-phospho-4′-pyridoxyl)amines would undoubtedly be good substrates for the pyridoxamine (pyridoxine) 5′-phosphate oxidase, which can act upon diverse 4′ secondary amine derivatives of B_6 phosphate (10–13). Hence, it seemed possible that compounds with amine functions could be covalently attached to vitamin B_6 at position 4 to make derivatives that would gain facilitated entry into cells by using the transporter for the vitamin and then be metabolically altered to release the original amine and pyridoxal phosphate. That this is indeed the case will be demonstrated in this report. Also the broader significance for the transporter-enhanced delivery and enzyme-catalyzed release of compounds into cells will be discussed.

MATERIALS AND METHODS

Chemicals. Bovine serum albumin; collagenases type Ia and type IV; deoxyribonuclease I; heparin; Hepes; hydrochloride salts of pyridoxine, pyridoxal, and pyridoxamine; the 5′-phosphates of these vitamers; β-alanine; and Triton X-100 were purchased from Sigma. Methylamine hydrochloride, benzylamine, tryptamine, and ethylenediamine were obtained from Aldrich. Sodium boro[^{3}H]hydride (5–20 Ci/mmol in 0.1 M NaOH; 1 Ci = 37 GBq) was purchased from Amersham. All other chemicals were reagent grade.

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*McCormick, D. B., Bowers-Komro, D. M., Bonkovsky, J. L., Larsen, C. & Zhang, Z., Eighth International Symposium on Vitamin B_6 and Carbonyl Catalysis, Oct. 15–19, 1990, Osaka.

Synthesis of [4′-^{3}H]Pyridoxine and Analogues. [4′-^{3}H]-Pyridoxine was synthesized by sodium boro[^{3}H]hydride reduction of pyridoxal, and the purity was checked by TLC and a radioautogram. The final specific radioactivity was 1.17 Ci/mmol.

Unlabeled and ^{3}H-labeled *N*-(4′-pyridoxyl)amines were synthesized by sodium borohydride or boro[^{3}H]hydride reduction of aldimines formed by condensing the amines (methylamine, benzylamine, tryptamine, ethylenediamine, and β-alanine) with pyridoxal according to the procedure described earlier (13). The crude *N*-(4′-pyridoxyl)-β-alanine was applied to a Dowex 50W (H^+) column, washed with water, and eluted with 1% ammonium hydroxide. The other crude *N*-pyridoxylamines were separately applied to Amberite CG-50 (H^+) and eluted with 2% (vol/vol) ammonium hydroxide. Ultraviolet spectra of the compounds were checked in acidic, basic, and neutral buffer systems, and all were comparable to a pyridoxamine standard (14). The purity of the compounds was additionally monitored by TLC using Kodak cellulose chromagram sheets in two solvent systems: (*i*) methylethyl ketone/ammonia/ethanol/water, 3:1:1:1 (vol/vol), and (*ii*) *tert*-amyl alcohol/acetone/water/diethylamine, 8:7:4:1 (vol/vol), with visualization by Gibb's reagent (2,6-dichloroquinone-4-chlorimide) or ninhydrin spray.

Specific radioactivities of the ^{3}H-labeled *N*-(4′-pyridoxyl)amines were 4.10, 1.01, 0.51, 0.81, and 0.67 Ci/mmol for analogues from methylamine, benzylamine, tryptamine, ethylenediamine, and β-alanine, respectively.

Renal Cell Isolation. Renal proximal tubular epithelial cells were isolated as described (4) from male Sprague–Dawley rats (Sasco, Omaha, NB) weighing between 220 and 320 g. For this the kidneys were perfused *in situ* using the recirculating collagenase method described by Jones and coworkers (15, 16). The concentration and viability of the isolated cells were determined using a hemocytometer and noting the ability of the cells to exclude 0.2% trypan blue. The usual yield was from 90×10^6 to 130×10^6 cells per rat; viability in these experiments averaged 93%. Protein concentration, estimated using the Bradford method (17), averaged 0.23 mg per 10^6 cells. Cells were maintained at room temperature with gentle swirling in a Krebs–Henseleit buffer containing 12.5 mM Hepes, 12 mM glucose, and 7 mM glutamate (pH 7.4) preequilibrated with 95% O_2/5% CO_2. All studies were completed within 2 h of cell isolation.

Uptake Studies. Samples (10^6 cells per ml) were preincubated for 5 min in open plastic vials secured in a shaking water bath at 37°C. The incubation solution was the modified Krebs–Henseleit buffer (pH 7.4). Special conditions for each set of experiments are detailed in the figures and table.

Uptake was initiated by addition of [^{3}H]pyridoxine, a mixture of [^{3}H]pyridoxine and *N*-(4′-pyridoxyl)amine, or ^{3}H-labeled *N*-(4′-pyridoxyl)amine. Radioactivity was assayed at various time points by applying 0.8 ml of the cell suspension to 8-μm (pore size) membrane filters (Millipore) under suction and immediately washing the cells twice with 4 ml of ice-cold Krebs–Henseleit buffer containing 12.5 mM Hepes (pH 7.4). Filtration and washing were usually completed within 12 sec. The cell blank was an identical sample kept on ice that was similarly filtered and washed immediately after adding the ^{3}H-labeled compound. In most experiments, 0.5-min or 1-min uptake values that reflect apparent initial rates were used.

Filters with retained cells were transferred to scintillation vials, 0.5 ml of 0.5% Triton X-100 was added to lyse the cells for 10 min, and then 10 ml of Ultima-Gold scintillation fluid (Packard Instrument) was added. Radioactivity was determined using a Packard Tri-Carb 1900-TR scintillation counter.

Statistics. Uptake was measured with triplicate or quadruplicate samples from at least three cell preparations for each experiment. Data are expressed as the mean ± SEM.

Metabolism. To examine cellular metabolism of the transported *N*-(4′-pyridoxyl)amines, cells were incubated for 50 min at 37°C in 0.5 μM [^{3}H]pyridoxine or ^{3}H-labeled *N*-(4′-pyridoxyl)amine. The uptake was stopped by adding twice the volume of ice-cold Krebs–Henseleit buffer containing 12.5 mM Hepes (pH 7.4). Excess [^{3}H]pyridoxine or ^{3}H-labeled *N*-(4′-pyridoxyl)amine was removed immediately by centrifugation followed by aspirating the supernatant. The cells were washed once more by repeating the above procedure, and the pellet was resuspended in Krebs–Henseleit buffer containing 12.5 mM Hepes (pH 7.4). The final pellet of cells was homogenized in 5% (wt/vol, final concentration) trichloroacetic acid. The precipitate was removed by centrifugation and the supernatant was extracted twice with diethyl ether to remove trichloroacetic acid.

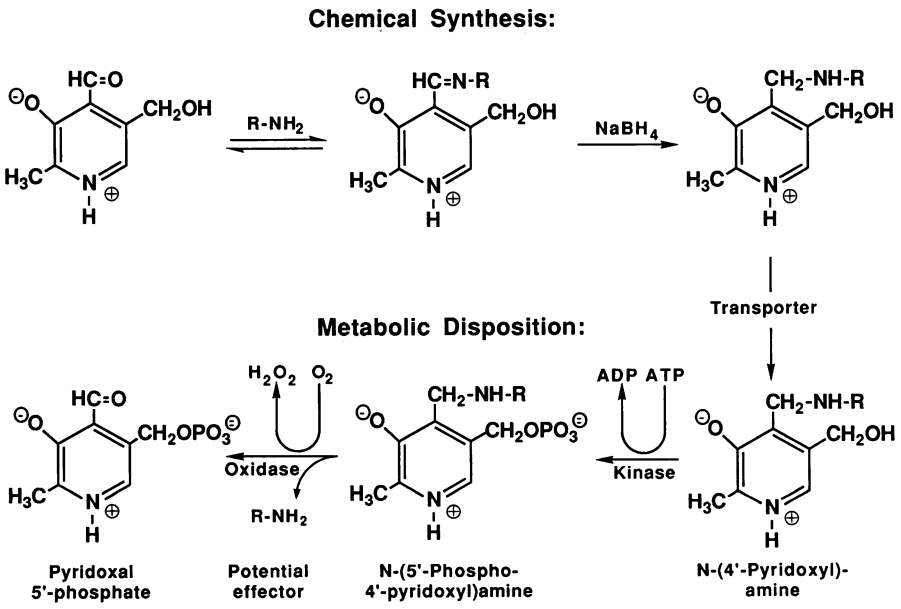

FIG. 1. Synthesis and metabolism of *N*-(4′-pyridoxyl)amines where R is a methyl, benzyl, tryptophyl, β-aminoethyl, or β-alanyl group.

Samples of the aqueous cell extract were chromatographed using a 0.5 × 4 cm Dowex 1-acetate column as described by Voet *et al.* (18). The cell extract and 10 μg of pyridoxal 5′-phosphate were loaded on the column in 0.1 M ammonium acetate (pH 4.5). Pyridoxine and *N*-(4′-pyridoxyl)amines are not retained by the column under these conditions. The column was then washed with 0.1 M acetic acid to remove any other trace impurities and the [^{3}H]pyridoxal 5′-phosphate was eluted with 0.1 M chloroacetic acid. Elution of pyridoxal 5′-phosphate was monitored by UV absorption (296 nm). Fractions (2.5–4 ml) were collected and radioactivity was measured in 10 ml of Ultima-Gold scintillation fluid. The specific activity of pyridoxal 5′-phosphate was assumed to be half the [^{3}H]pyridoxine or ^{3}H-labeled *N*-(4′-pyridoxyl)amine since half of the tritium is lost upon oxidation at the 4′-methylene (19). Renal proximal tubular cells that were treated immediately after mixing with 0.5 μM [^{3}H]pyridoxine or ^{3}H-labeled *N*-(4′-pyridoxyl)amine after the above procedure formed only trace amounts of pyridoxal 5′-phosphate.

The overall fate of the compounds from synthesis through uptake and metabolism is shown in Fig. 1.

RESULTS

The uptake of [^{3}H]pyridoxine alone compared to uptake of the same amount in an equimolar mixture of each *N*-(4′-pyridoxyl)amine is shown in Fig. 2. Each of the unlabeled *N*-(4′-pyridoxyl)amines inhibits [^{3}H]pyridoxine uptake by renal proximal tubular cells over the course of the experiment. The extents of inhibition of initial uptake (1 min) of [^{3}H]pyridoxine at physiologic level (0.5 μM) by 0.5 μM *N*-(4′-pyridoxyl)amines are given in Table 1. The inhibition of [^{3}H]pyridoxine uptake by *N*-(4′-pyridoxyl)amines was comparable to that of unlabeled pyridoxine and pyridoxamine. Of the five derivatives tested, the negatively charged *N*-(4′-pyridoxyl)-β-alanine caused the least inhibition, whereas the positively charged *N*-(4′-pyridoxyl)ethylenediamine elicited the most inhibition. The free amines used to make the derivatives had no significant effect on uptake, except for methylamine, which caused about 14% inhibition. The competitive nature of the inhibition of the *N*-(4′-pyridoxyl)amines against uptake of [^{3}H]pyridoxine was further reflected by double-reciprocal plots of values for 1/uptake versus 1/[^{3}H]pyridoxine concentration (data not shown), which generated lines with a common intercept on the 1/uptake (ordinate) axis.

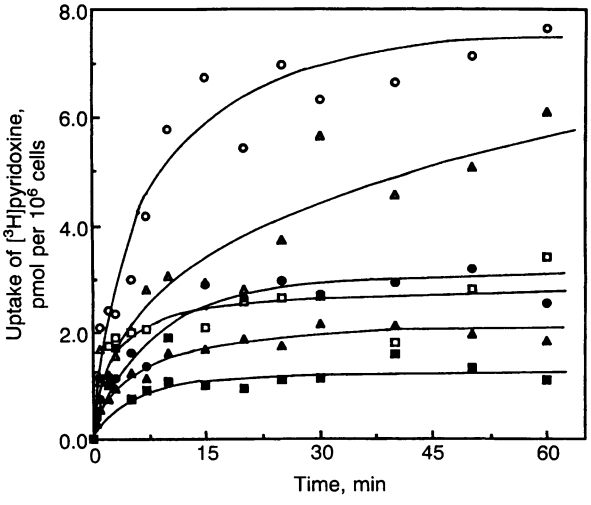

FIG. 2. Rates of uptake of [^{3}H]pyridoxine in the absence and presence of *N*-(4′-pyridoxyl)amines. Compounds tested were 1 μM ^{3}H-labeled vitamin alone (○) or mixtures of 1 μM [^{3}H]pyridoxine with 1 μM *N*-(4′-pyridoxyl)amine derivatives made from ethylenediamine (△), β-alanine (□), benzylamine (●), methylamine (▲), and tryptamine (■).

Table 1. Inhibition of initial (1.0 min) uptake of 0.5 μM [^{3}H]pyridoxine by 0.5 μM *N*-(4′-pyridoxyl)amines

Vitamer/analogue added	*n*	Uptake, % of control
None	6	100 ± 6
Pyridoxine	3	56 ± 4
Pyridoxamine	3	52 ± 1
N-(4′-Pyridoxyl)methylamine	3	70 ± 15
N-(4′-Pyridoxyl)benzylamine	3	62 ± 8
N-(4′-Pyridoxyl)tryptamine	3	64 ± 8
N-(4′-Pyridoxyl)ethylenediamine	3	55 ± 8
N-(4′-Pyridoxyl)-β-alanine	3	79 ± 4

All cultures contained 0.5 μM [^{3}H]pyridoxine; vitamers or analogues were added at 0.5 μM. Radioactive pyridoxine and unlabeled vitamer, analogue, or amine were mixed before addition to cell suspensions. Uptake values are given as the mean ± SEM.

Since vitamin B_6 is carried into cell by a transport system with relative specificity that tolerates variations at position 4′ of the B_6 structure, the possibility of direct uptake of *N*-(4′-pyridoxyl)amines was examined using the radioactive derivatives. The time courses for uptake of ^{3}H-labeled *N*-(4′-pyridoxyl)amines compared to [^{3}H]pyridoxine are shown in Fig. 3. Uptake for the analogues was very similar to that for pyridoxine. For pyridoxine the velocities at initial (v_o) and maximal (V_{max}) uptake were calculated as 1.2 and 6.4 pmol per 10^6 cells per min, respectively. The K_t was determined to be 4.3 μM. Values for the *N*-(4′-pyridoxyl)amines ranged from 0.1 to 0.9 pmol per 10^6 cells per min for v_o, 5.6 to 7.6 pmol per 10^6 cells per min for V_{max}, and 4 to 10 μM for K_t. The K_t values for the analogues corresponded well to their abilities to compete with pyridoxine for initial uptake.

Studies of cellular metabolism showed that the transported ^{3}H-labeled *N*-(4′-pyridoxyl)amines were at least partially converted to pyridoxal 5′-phosphate (see Fig. 1). The anion-exchange chromatographic profiles of ^{3}H-labeled compounds extracted from cells incubated for 50 min with 0.5 μM [^{3}H]pyridoxine or ^{3}H-labeled *N*-(4′-pyridoxyl)amine are given in Fig. 4. Maximal radioactivity was eluted at exactly the same position as standard pyridoxal 5′-phosphate. Metabolic conversion of [^{3}H]pyridoxine resulted in 1.9 pmol of [^{3}H]pyridoxal 5′-phosphate in the extract from 10^6 cells incubated with 0.5 μM vitamin for 50 min. Incubation of the

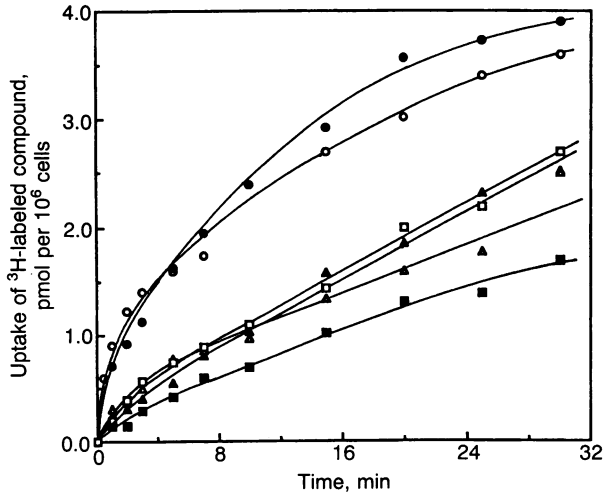

FIG. 3. Rates for uptake of [^{3}H]pyridoxine compared to ^{3}H-labeled *N*-(4′-pyridoxyl)amines. Compounds (0.5 μM) were vitamin (○) or derivatives made from ethylenediamine (△), β-alanine (□), benzylamine (●), methylamine (▲), and tryptamine (■).

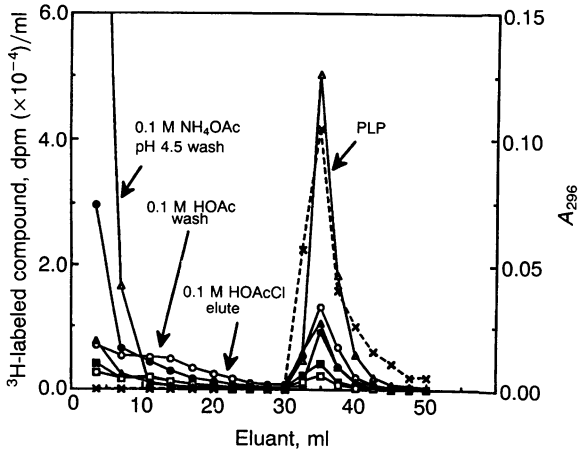

FIG. 4. Anion-exchange chromatographic profiles of ^{3}H-labeled compounds extracted from cells incubated with [^{3}H]pyridoxine or ^{3}H-labeled *N*-(4′-pyridoxyl)amine. Compounds (0.5 μM) were vitamin (○) or derivatives made from ethylenediamine (△), β-alanine (□), benzylamine (●), methylamine (▲), and tryptamine (■). Pyridoxal 5′-phosphate concentration is shown as A_{296} (dashed line). PLP, pyridoxal 5′-phosphate.

^{3}H-labeled *N*-(4′-pyridoxyl)amines resulted in a similar conversion, 1.8–1.9 pmol of [^{3}H]pyridoxal 5′-phosphate, except for the lower conversion (0.7 pmol) observed for the β-alanine derivative.

DISCUSSION

The competitive inhibition of [^{3}H]pyridoxine uptake by *N*-(4′-pyridoxyl)amines and the direct uptake of the ^{3}H-labeled analogues suggest that they utilize the same vitamin B_6 transport system in rat renal proximal tubular cells. The study on their subsequent metabolism within the cells demonstrated that pyridoxal kinase and pyridoxamine (pyridoxine) 5′-phosphate oxidase can catalyze the conversion of *N*-(4′-pyridoxyl)amines to pyridoxal 5′-phosphate and release the corresponding free amines (see Fig. 1).

As in some other transport systems, negatively charged solutes (e.g., ATP) are less favorably imported. This may be the reason for poorer uptake of the β-alanyl derivative. On the other hand, it seems that positively charged functions, as with the derivative made from ethylenediamine, and bulky side groups (e.g., benzylamine and tryptamine) do not significantly impair uptake or metabolism.

The broader applicability of *N*-(4′-pyridoxyl)amines for delivery of biologically active amines within cells can be appreciated when it is realized that cells other than renal cells facilitate entry of vitamin B_6, and almost all facultative and aerobic cells have both pyridoxal kinase and pyridoxamine (pyridoxine) 5′-phosphate oxidase. For example, the hepatocyte has similar characteristics with regard to specificity for the facilitated transport of B_6 (20). Prokaryotic and eukaryotic cells have kinases (8) and oxidases (21) competent to release the amine. Hence, the present example with *N*-(4′-pyridoxyl)amines could be used where delivery to several tissues (e.g., liver, kidney, and brain) will result in significant release of the amine. Generally, there is facilitated entry with relative specificity for other vitamins with most cells. Studies with renal cells and riboflavin (22) or L-ascorbic acid (23) and studies with liver cells and riboflavin (24) or biotin (6) suggest transporter systems that may be utilized. In many cases, it can be anticipated that functional groups other than amines could link a vitamin or other cellular nutrient to a considerable range of drugs or intracellular effectors. This generalization is illustrated in Fig. 5. The enhancement of effector transport by chemical linkage to solutes that gain facilitated entry followed by cleavage to release the effector may be widely applicable and is worthy of further investigation. By selection of the types of transporters and levels of activities of cleavage enzymes within various cells, it should be possible to design compounds that are somewhat selective in their ultimate targets.

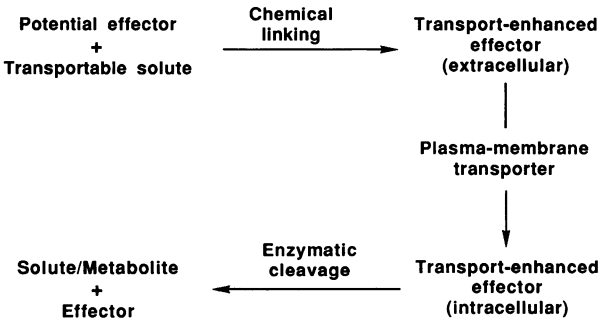

FIG. 5. Generalized delivery of effectors by using transporters with subsequent metabolic release.

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