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Structural Analogues of Pyrroline 5-Carboxylate Specifically Inhibit Its Uptake into Cells

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Summary. Pyrroline 5-carboxylate, a naturally occurring intermediate, is a potent activator of redox-dependent metabolic pathways. This effect of pyrroline 5-carboxylate is due, at least in part, to the special mechanism mediating its entry into cells. Using Chinese hamster ovary cells we recently characterized the cellular uptake of pyrroline 5-carboxylate as a process transferring oxidizing potential *pari passu* with cell entry, a process consistent with group translocation. We sought to identify specific inhibitors to probe this unique uptake mechanism, to blockade the metabolic effects of pyrroline 5-carboxylate, and to provide strategies to identify the putative carrier protein. Because pyrroline 5-carboxylate, a ring structure with a tertiary nitrogen, is in spontaneous equilibrium with glutamic-γ-semialdehyde, an open-chain structure, we tested analogues of both. Most open-chain aldehydes at 10 mM had little effect on the uptake of pyrroline 5-carboxylate. Although succinic semialdehyde did inhibit, its effect was nonspecific in that the uptake of α(methylamino) isobutyric acid was inhibited as much as the uptake of pyrroline 5-carboxylate. In contrast, pyrroline 2-carboxylate and other cyclic compounds with tertiary nitrogens, e.g., pyridines, were specific inhibitors of pyrroline 5-carboxylate uptake. Respective potencies of pyridine derivatives depended on the nature and location of constituent groups. Kinetics studies showed that these inhibitors were competitive with pyrroline 5-carboxylate and the most potent inhibitor, 2,6-pyridinedicarboxaldehyde, exhibited a K_{i2} of 0.27 ± 0.05 mM. In the face of their effect on P5C uptake, the most potent of these analogues, 2-pyridinecarboxaldehyde and 2,6-pyridinedicarboxaldehyde, did not inhibit the activity of pyrroline 5-carboxylate reductase, the enzyme that converts pyrroline 5-carboxylate to proline. Nevertheless, the analogues markedly inhibited the stimulatory effect of P5C on the pentose phosphate shunt. Importantly, not only did 2-pyridinecarboxaldehyde protect the pyrroline 5-carboxylate uptake mechanism from the inhibitory effects of a sulfhydryl-reactive agent, but also its inhibitory effect became irreversible in the presence of sodium cyanoborohydride. These inhibitors may help discriminate events mediated by the transport carrier from those mediated by intracellular metabolism and may provide a method for identifying and characterizing the putative carrier for P5C.

Key Words proline · transport · redox · amino acids · membranes · transport carriers

Introduction

Pyrroline 5-carboxylate (P5C)[1], traditionally considered an intracellular intermediate in the interconversions of proline, ornithine, and glutamate, is now recognized as a regulator of cellular reducing-oxidizing (redox) potential. It circulates as a constitutent of human plasma, and levels undergo nutrition-dependent diurnal fluctuation consistent with it being an intercellular communicator (Fleming et al., 1989). Effector functions demonstrated in vitro include stimulation of the activity of the hexose monophosphate shunt and increased production of 5-phosphoribosyl 1-pyrophosphate and purine nucleotides (Phang et al., 1982; Yeh & Phang, 1988). These redox-dependent effects of P5C accompany its conversion to proline with the concomitant oxidation of NADPH to $NADP^+$.

It is now evident that the cellular uptake of P5C plays an important role in the metabolic effects of P5C. It is rapidly reduced to proline as it traverses the plasma membrane (Mixson & Phang, 1988). In fact, no extracellular P5C can be found inside the cell. Thus, the uptake mechanism is consistent with group translocation. But unlike most group translo-

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[1] *Abbreviations:* P5C, pyrroline 5-carboxylic acid; P2C, pyrroline 2-carboxylic acid; MeAIB, α(methylamino) isobutyric acid; 2PC, 2-pyridinecarboxaldehyde; 2,6PC, 2,6-pyridinedicarboxaldehyde; 2PCA, 2-pyridinecarboxylic acid; and PCMBS, *p*-chloromercuribenzosulfonate.

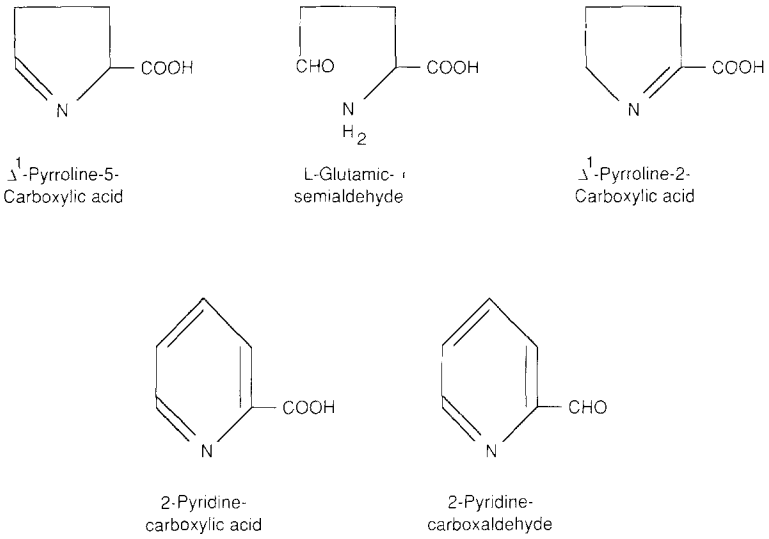

Fig. 1. Structural formulas of P5C, glutamic-γ-semialdehyde, and selected analogues. P5C and glutamic-γ-semialdehyde are tautomers in spontaneous equilibrium

cation systems in which phosphate groups are transferred to the entering substrate (Kundig & Roseman, 1964, 1966), P5C upon cell entry accepts electrons from NADPH. In addition, this special, saturable uptake mechanism is temperature dependent, sodium independent and unshared by other amino acids or imino acids. The mechanism has been identified functionally in several cultured cell lines including Chinese hamster ovary cells (Mixson & Phang, 1988), human skin fibroblasts and adriamycin-sensitive and -resistant human breast tumor cell lines (A.J. Mixson, G.C. Yeh & J.M. Phang, *unpublished observations*). Taken together, these findings led us to propose that P5C uptake is mediated by a novel membrane carrier protein.

There are several intriguing aspects of this uptake mechanism which deserve exploration. First, it is unclear whether P5C, a 5-member cyclic structure containing a tertiary nitrogen, or its open-chain tautomer, glutamic-γ-semialdehyde, initially binds to the putative transport carrier (Fig. 1). The identification of related molecules which may share the P5C uptake mechanism for cell entry is also important. Additionally, the functional distinction of the uptake mechanism for P5C from its conversion to proline remains unresolved. Thus, we examined a number of analogues of P5C and glutamic-γ-semialdehyde to identify possible inhibitors of P5C uptake. We identified several specific inhibitors and characterized their effects. These inhibitors were helpful in answering the aforementioned questions. In addition, they may be useful in our ongoing attempts to isolate the putative carrier protein for P5C. By understanding the properties of these inhibitors, we may gain further insight into P5C-mediated activation of metabolic pathways.

Materials and Methods

Chemicals

DL-P5C dinitrophenylhydrazone was obtained from Sigma and was hydrolyzed to DL P5C according to the method of Mezl and Knox (1976). Where indicated, concentrations of P5C were for the L form. Δ^1-Pyrroline 2-carboxylate was synthesized enzymatically from D-proline using purified hog kidney D-amino acid oxidase (Boehringer-Mannheim) and purified by cation-exchange chromatography (Meister, 1954). 2,6-Pyridinedicarboxaldehyde, 2-pyridinecarboxylate, 2,3-pyridinedicarboxylic acid, 2,4-pyridinedicarboxylic acid, 4,8-dihydroxyquinoline, thymine, uracil, adenosine and pyridine were purchased from Aldrich Chemical. Other analogues and chemicals were purchased from Sigma Chemical. (U-^{14}C) α(Methylamino) isobutyric acid (MeAIB; 53.5 mCi/mmol) and L-(U-^{14}C) serine (169 mCi/mmol) were purchased from Dupont-New England Nuclear; L-(U^{14}C) leucine (348 mCi/mol) was obtained from Amersham. L-(U^{14}C) P5C was prepared as described by Smith, Downing and Phang (1977).

Cells

Chinese Hamster ovary cells (CHO-K1) were maintained as previously described (Mixson & Phang, 1988). Cells were inoculated at a density of 5×10^5 cells in 1 ml of Eagle's minimum essential medium supplemented with 10% fetal bovine serum and allowed to grow for 3 days. Prior to uptake studies, cells were equilibrated with Krebs-Ringer phosphate (KRP) buffer for 1 hr.

Measurement of Uptake

We measured P5C uptake using our adaptation (Mixson & Phang, 1988) of the cluster tray method of Gazzola et al. (1981). The analogues of P5C were coincubated with the radiolabeled amino acid being studied for uptake. Cells were incubated for 1 min at 37°C in 0.3 ml of KRP buffer with glucose (5.5 mM) and $CaCl_2$ (0.2 mM). After the incubation period, the medium was removed

by inverting and shaking the plate, and each well was washed twice with 2 ml of cold phosphate-buffered saline. We then added 250 μl of 10% trichloroacetic acid to each well, and after 30 min of equilibration, an aliquot from each well was transferred to a scintillation vial to measure the radioactivity by scintillation spectrometry.

Metabolism of P5C

P5C and its metabolic derivatives were recovered by cation-exchange chromatography as previously described (Phang et al., 1975; Mixson & Phang, 1988). After incubation and washing with phosphate-buffered saline, the monolayer was extracted with 0.3 ml of 10% trichloroacetic acid. An aliquot was treated with *O*-aminobenzaldehyde and then applied to a 1-ml bed volume column of cation exchange resin and eluted sequentially with 1 N HCl, 2 N HCl, and 2 N NaOH.

Assay of P5C Reductase Activity

P5C reductase activity of Chinese hamster ovary cells was assayed as previously described (Phang, Downing & Valle, 1973). Activity in the presence or absence of NADH or NADPH was measured by recovering product proline from substrate ^{14}C-P5C.

Pentose Phosphate Shunt

Chinese hamster ovary cells were plated at a density of 2×10^6 cells/cm^2 in Coastar 25-cm^2 flasks. After 3 days, growth medium was replaced with KRP buffer for 45 min before the activity of the pentose phosphate shunt was measured by a previously described method (Yeh & Phang, 1983; Mixson & Phang, 1988). The activity of the pentose phosphate shunt was measured by oxidation of D-(1-^{14}C) glucose. The concentration of glucose was 5.5 mM, and the duration of incubation was 10 min.

The Effect of *p*-Chloromercuribenzosulfonate on P5C Uptake

Chinese hamster ovary cells were first incubated for 10 min with various concentrations of PCMBS. This medium was aspirated, the monolayer was washed with KRP buffer and the uptake of P5C or MeAIB was assessed in an additional 5-min incubation. In experiments showing protection against the effect of PCMBS, P5C or an analogue was present during the first incubation with PCMBS.

Treatment of Cells with Sodium Cyanoborohydride

Chinese hamster ovary cells were preincubated for 1 hr with various inhibitors in the presence or absence of 5 mM sodium cyanoborohydride. Following preincubation, the medium was removed and 2 ml of PBS was applied to the monolayer. After 2 min, the PBS was removed and this wash procedure was repeated ×4. We then determined 1-min uptakes for either P5C or alanine (0.08 mM during uptake).

Table 1. Effect of analogues on P5C uptake[a]

	P5C uptake (percent of control)	
	[*I*] = 1 mM	[*I*] = 10 mM
Proline	ND	87.6
Δ^1Pyrroline 2-carboxylate	89.6	38.4
Pyroglutamic acid	ND	99.5
Pyridine	100.4	71.9
2-Pyridinecarboxylic acid	87.1	44.3
3-Pyridinecarboxylic acid (nicotinate)	94.8	71.9
Nicotinamide	94.6	88.5
2,3-Pyridinedicarboxylic acid	90.2	41.7
2,4-Pyridinedicarboxylic acid	84.7	44.7
2-Pyridinecarboxaldehyde	40.5	8.5
3-Pyridinecarboxaldehyde	68.7	13.3
4-Pyridinecarboxaldehyde	72.4	12.1
2,6-Pyridinedicarboxaldehyde	21.4	7.6
Glyceraldehyde	ND	102.3
Succinic semialdehyde	ND	65.8
Thymine	86.0	80.1
Uracil	92.0	ND
Adenosine	88.1	87.1
NADPH	93.3	86.6

[a] One-min uptakes of P5C were measured in the presence of various analogues at the indicated concentrations. The concentration of P5C was 0.08 mM. The control value for P5C uptake in the absence of inhibitors was 6.9 ± 0.8 nmol/min · mg protein. Inhibitor data are expressed as percent of control values and represent the average of four determinations.

Results

To find potential inhibitors of P5C uptake, we tested a series of compounds which were structural analogues of either the ring or chain form of pyrroline 5-carboxylate (glutamic-γ-semialdehyde). As shown in Table 1, proline was without effect and P2C, a close analogue to P5C, was a weak inhibitor. Interestingly, compounds derived from pyridine were inhibitors of P5C uptake. Even at 1-mM concentration, greater than 50% inhibition was observed with several pyridine derivatives. Other cyclic compounds containing secondary rather than tertiary nitrogens were without effect. Straight-chain aldehydes did not inhibit P5C uptake with the exception of succinic semialdehyde which at 10 mM inhibited P5C uptake by 34.2%, a potency distinctly less than that of the pyridine derivatives.

More significant still, the inhibitory effect of the pyridine derivatives was highly specific for P5C uptake, whereas that of succinic semialdehyde was

Table 2. Effect of P5C analogues on the A, ASC, and the L transport systems[a]

	Amino acid uptake (% control)		
	MeAIB	SER	LEU
Δ^1Pyrroline 2-carboxylate	97.2	100.8	112.7
2-Pyridinecarboxaldehyde	104.7	92.4	114.6
2-Pyridinecarboxylic acid	87.1	84.3	111.2
2,6-Pyridinedicarboxaldehyde	48.7	102.0	65.3
Succinic semialdehyde	61.8	101.3	82.5

[a] One-min uptakes of MeAIB, serine, and leucine were determined in the presence of the indicated analogues at a concentration of 10 mM. The concentration of MeAIB, serine, or leucine was 0.08 mM. Control uptake values without inhibitors for MeAIB, serine and leucine were 3.43 ± 0.85, 5.47 ± 0.38 and 15.8 ± 1.3 nmol/min · mg protein, respectively. Values with inhibitors are expressed as percent of control uptake and represent the average of six determinations.

not. We compared their relative effects on P5C uptake with those on other amino acid systems. MeAIB, serine and leucine were chosen because their uptakes are preferentially or exclusively mediated by systems A, ASC and L, respectively. As shown in Table 2, 2-pyridinecarboxaldehyde was not only potent in its inhibition of P5C uptake but also highly specific. At a concentration of 10 mM, which inhibited P5C uptake by 92%, this compound had little effect on uptake by systems A, ASC or L. 2-Pyridinecarboxylate and pyrroline 2-carboxylate were less potent than 2-pyridinecarboxaldehyde but they were both highly specific. Although 2,6-pyridinedicarboxaldehyde was highly potent in inhibiting P5C uptake, it was less specific in that it also inhibited the uptake of other amino acids albeit much less than that of P5C. In marked contrast to these pyridine derivatives, succinic semialdehyde was equipotent in its inhibition of uptake by system A as it was for uptake of P5C. Thus, specific inhibition of P5C uptake was observed only with ring compounds with a tertiary nitrogen.

Superimposed on the specificity bestowed by the basic pyridine ring structure, the potency of a given compound was affected by the nature and location of constituent groups on the ring. Full exploration of compounds with these constituent groups was limited by their availability. Nevertheless, an interesting pattern emerged. A carbonyl constituent on the pyridine ring bestowed greater potency and, in general, a carboxaldehyde was more potent than a carboxylate group. For the pyridine carboxaldehydes, potency was as follows: position 2 > 3 > 4. Furthermore, the addition of a second carboxaldehyde at position 6 increased the potency over a single carboxaldehyde at position 2. In contrast, effects of additions of a second carboxyl group were no more potent than that of a single carboxyl group at position 2.

Having identified compounds which specifically inhibited P5C uptake, we carefully quantitated their respective potencies. Using the uptake of P5C at 0.04 mM as the endpoint, we varied the concentration of the inhibitors from 0.02 to 5.0 mM. As seen in Fig. 2, each compound inhibited P5C uptake in a concentration-dependent fashion. The concentrations resulting in 50% inhibition ($K_{1/2}$) were 0.62, 0.27, 5.8 and 6.1 mM for 2-pyridinecarboxaldehyde, 2,6-pyridinedicarboxaldehyde, 2-pyridinecarboxylic acid, and pyrroline 2-carboxylate, respectively.

Although these compounds showed specificity in inhibiting P5C uptake relative to other amino acid transport systems, the mechanism of the inhibitory effect remained unelucidated. To obtain additional insight into this effect, we performed Michaelis-Menten kinetics studies with and without the inhibitors. The concentration of P5C was varied between 0.02 and 1 mM, and inhibitors were present at several specified concentrations (Fig. 3). It is clear that all these inhibitors, 2-pyridinecarboxaldehyde, 2,6-pyridinedicarboxaldehyde, 2-pyridinecarboxylate and pyrroline 2-carboxylate inhibited P5C uptake competitively. The apparent affinity, K_m, was altered, whereas the maximal rate of uptake, V_{max}, remained unchanged. These data suggest that the aforementioned inhibitors produced their effect by interacting at the binding site for P5C.

Having identified specific, competitive inhibitors of P5C uptake, we considered whether they would inhibit the transfer of oxidizing potential mediated by P5C. Using the stimulation of pentose phosphate shunt as the endpoint, we found that these inhibitors of P5C uptake attenuated the effect of P5C on the shunt (Table 3). Both pyridine aldehydes had a modest effect on stimulating the pentose phosphate shunt. However, the magnitude of their effect at saturating concentrations was only 25–30% that produced by P5C. In contrast pyrroline 2-carboxylate had a marked inhibitory effect on pentose phosphate shunt activity. Strikingly, all four analogues markedly attenuated the stimulatory effect of 0.3 mM P5C. Especially noteworthy, 2-pyridinecarboxylate which, itself, had minimal effect on shunt activity, completely abolished the stimulatory effect of P5C.

Of course the transfer of oxidizing potential by P5C depends not only on its cellular entry but also on its conversion to proline with concomitant oxidation of NADPH to $NADP^+$. Therefore, the blockade of the stimulatory effect of P5C could be at the level of its conversion to proline. However, this was not

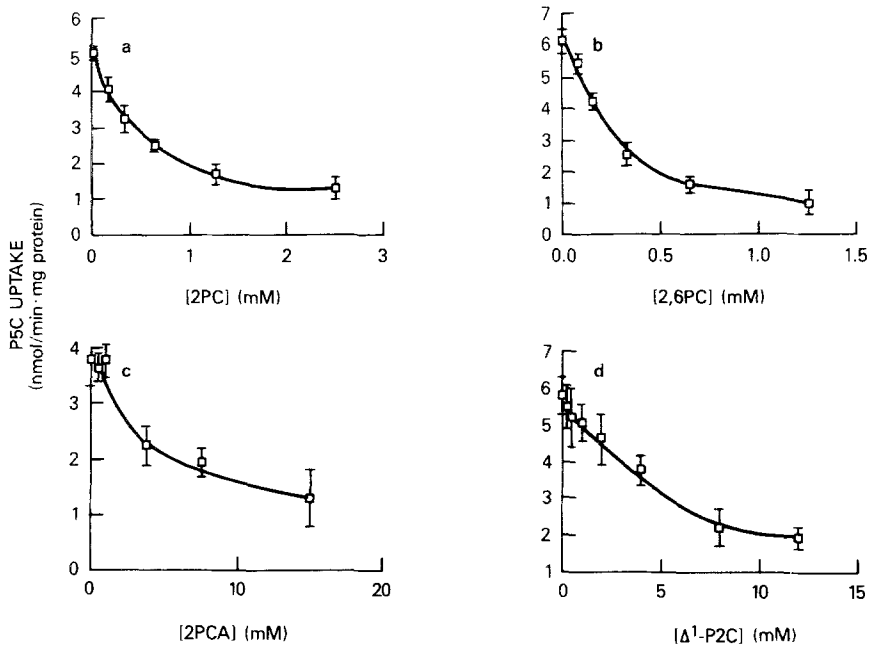

Fig. 2. Inhibition of P5C uptake by analogues. One-min P5C uptakes were measured with various concentrations of inhibitors. The concentration of P5C was 0.04 mM. The values shown represent the mean and SD of six determinations. The $K_{1/2}$ values are the concentrations of inhibitors at which uptake values are 50% of control. (*a*) Inhibition by 2-pyridinecarboxaldehyde; $K_{1/2}$ = 0.62 mM. (*b*) Inhibition by 2,6-pyridinedicarboxaldehyde; $K_{1/2}$ = 0.27 mM. (*c*) Inhibition by 2-pyridinecarboxylate; $K_{1/2}$ = 5.8 mM. (*d*) Inhibition by Δ^1-Pyrroline-2-carboxylate; $K_{1/2}$ = 6.1 mM

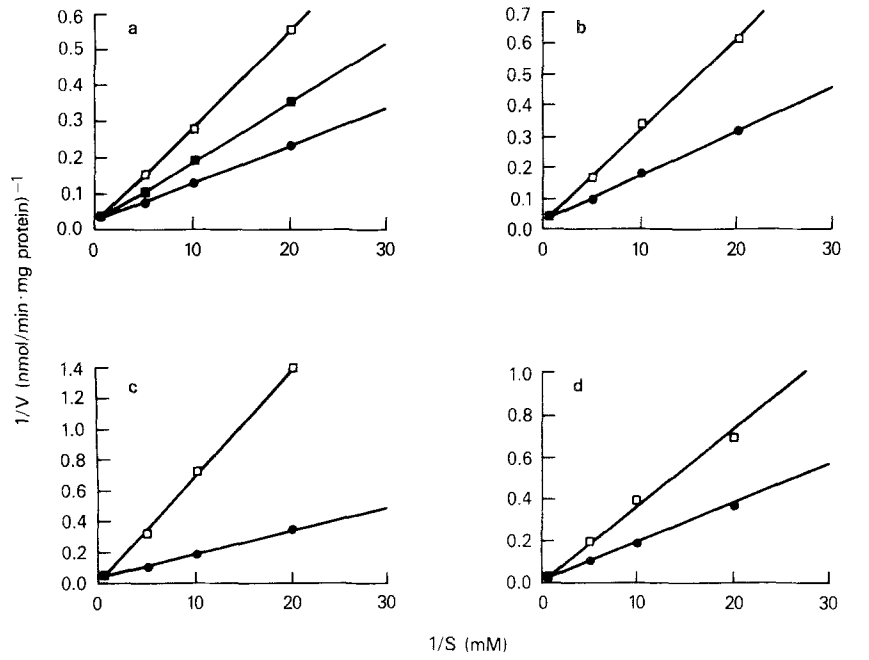

Fig. 3. Kinetics studies on the inhibition of P5C uptake. Michaelis-Menten kinetics studies were based on 1-min P5C uptakes with P5C ranging from 0.05 to 2.0 mM and in the presence or absence of the indicated inhibitors. Lineweaver-Burk transformations of the data are shown. Each values represents the average of six determinations. (*a*) Double-reciprocal plots of P5C uptake values in the presence of 0 (●—●); 0.5 mM (■—■); and 1.0 mM (□—□) 2-pyridinecarboxaldehyde. (*b*) Double-reciprocal plots of P5C uptake values in the presence of 0 (●—●) or 0.25 mM (□—□) 2,6-pyridinedicarboxaldehyde. (*c*) Double-reciprocal plots of P5C uptake values in the presence of 0 (●—●) and 7.5 mM (□—□) 2-pyridinecarboxylic acid. (*d*) Double-reciprocal plots of P5C uptake values in the presence of 0 (●—●) and 5 mM (□—□) Δ^1pyrroline-2-carboxylate

the case as inhibitors at concentrations sufficient to limit P5C entry had no effect on the conversion of P5C to proline. Exogenous-labeled P5C was recovered only as intracellular proline (*data not shown*), a finding similar to our previously reported studies characterizing P5C uptake in the absence of inhibitors (Mixson & Phang, 1988).

Additional evidence that 2,6PC, 2PC, and 2PCA blocked the effector functions of P5C at its cellular uptake rather than its conversion to proline was provided by direct studies on pyrroline 5-carboxylate reductase activity. The activity in extracts of Chinese hamster ovary cells was only minimally affected by the aforementioned inhibitors (Table 4). Furthermore, these findings suggest that the interaction of P5C with its putative membrane carrier is different than that with P5C reductase. In contrast, Δ^1-pyrroline 2-carboxylate, which unlike proline, in-

Table 3. Inhibition of P5C stimulation of pentose phosphate shunt activity[a]

Addends	Pentose phosphate shunt activity		
	Δ Over control (nmol/mg)	Δ Over respective control (nmol/mg)	% of P5C effect
P5C	9.00	9.00	100
2-PC	4.94		
2-PC + P5C	7.13	2.19	24
2,6-PC	2.50		
2,6-PC + P5C	3.26	0.76	8
2-PCA	−0.34		
2-PCA + P5C	0.30	0.64	7
P2C	−1.60		
P2C + P5C	1.40	3.00	33

[a] Pentose phosphate shunt activity was assessed by measuring $^{14}CO_2$ production from [1-^{14}C]-D-glucose as described in Materials and Methods. The concentration of glucose was 5.5 mM, and the duration of incubation was 10 min. Activity was determined in the presence of the indicated addends. The concentrations were as follows: P5C, 0.3 mM; 2-PC, 5 mM; 2,6-PC, 5 mM; and 2-PCA, 12.5 mM. Data are expressed as the increase over the control value (2.16 ± .07 nmol/mg protein) and were derived from the mean of six determinations from two experiments.

Table 4. Effect of uptake inhibitors on P5C reductase activity[a]

Inhibitor	P5C reductase activity	
	[I] (mM)	(% of control)
2-Pyridinecarboxaldehyde	0.5	102.3
	1.0	106.6
	2.0	102.3
2,6-Pyridinedicarboxaldehyde	0.25	114.1
	0.5	112.8
	1.0	98.9
2-Pyridinecarboxylic acid	1.0	91.0
	2.0	91.7
	5.0	82.1
Δ^1Pyrroline-2-carboxylic acid	1.0	86.3
	2.0	75.0
	5.0	53.4
Proline	1.0	79.5
	2.0	63.6
	5.0	55.0

[a] P5C reductase activity in extracts of Chinese hamster ovary cells was measured with NADPH as cofactor. The indicated inhibitors of P5C uptake were present at various concentrations. The concentration of P5C was 0.05 mM in L and the concentration of NADPH was 0.30 mM. The duration of incubation was 20 min and product [^{14}C]proline was recovered by cation-exchange chromatography. Data are expressed as percent of control, and values shown represent the average of duplicate determinations.

hibits the cellular entry of P5C, markedly inhibited P5C reductase activity. Thus, Δ^1-pyrroline 2-carboxylate inhibited the effect of P5C on pentose phosphate shunt activity at both P5C uptake and its conversion by P5C reductase.

These structural analogues may prove useful in attempts to identify the putative carrier protein for P5C. On the functional level, they offer protection from the inhibitory effect of PCMBS, a compound which reacts with free sulfhydryl groups of cell surface proteins. Similar to the its effects on other amino acid transport system (Hare, 1975; Batt, Abbot & Schachter, 1976; Goto, Hanamura & Tamemasa, 1982; Chiles & Kilberg, 1986), PCMBS markedly inhibited P5C uptake. The magnitude of the inhibition increased with increasing concentrations of PCMBS and strikingly, it was more potent (100-fold) in inhibiting P5C uptake than MeAIB uptake (Fig. 4). Of special interest, the presence of P5C or 2-PC during the exposure to PCMBS specifically protected P5C uptake from the inhibitory effect of PCMBS (Fig. 5). Clearly, the presence of P5C or its analogue was protecting a free sulfhydryl group on a cell surface protein critical in P5C uptake. Whether this sulfhydryl group resides on the putative carrier protein remains to be shown.

Although 2-PC inhibited P5C uptake, the effect was readily reversible. In cells preincubated with 2-PC washing with KRP buffer reversed the inhibitory effect. To explore the nature of the interaction between 2-PC and the putative P5C carrier we used $NaCNBH_3$, a reducing agent which reacts specifically with Schiff bases at pH 7.4 (Borch, Bernstein & Durst, 1971; Jentoft & Dearborn, 1979; Jentoft et al., 1979). Treatment with $NaCNBH_3$ by itself had no effect on the uptake of either P5C or alanine (Table 5). However, the presence of $NaCNBH_3$ during the exposure to 2-PC made its inhibition of P5C uptake irreversible. By contrast, irreversible inhibition was not seen when cells were preincubated with P5C in the presence of $NaCNBH_3$; presumably the inability to produce irreversible inhibition was because P5C (in its ring form) is without an aldehydic moiety. Furthermore, $NaCNBH_3$ had no effect on the interaction between alanine and its carriers.

Discussion

The cellular uptake of P5C is unusual in several aspects. It is not mediated by the major amino acid transport systems but only by its own saturable, sodium-independent system. Furthermore, P5C is

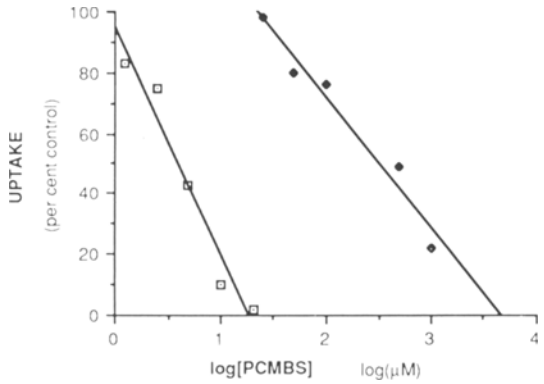

Fig. 4. Inhibition of P5C and MeAIB uptake by *p*-chloromercuribenzosulfonate. Cells were treated without or with various concentrations of PCMBS for 10 min after which the treatment medium was aspirated and the monolayer washed with Krebs-Ringer phosphate buffer. Uptake of either P5C (0.04 mM) (□—□) or MeAIB (0.04 mM) (■—■) were then determined in a 5-min incubation. Data are expressed as percent of control and represent the mean of four determinations

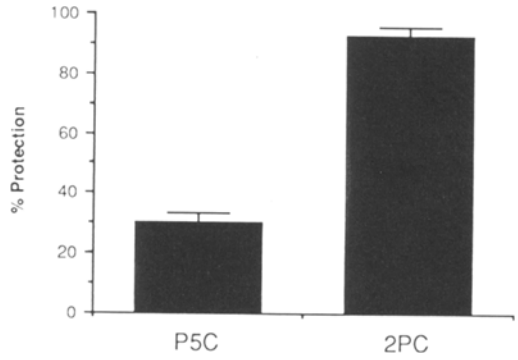

Fig. 5. P5C or 2-PC protects against the inhibitory effect of PCMBS. Cells were preincubated for 10 min in the presence or absence of 12 μM PCMBS with or without 10 mM P5C or 2-PC. Following treatment, the medium was aspirated and the monolayer washed with Krebs-Ringer phosphate buffer. The uptake of P5C (0.04 mM) was then determined in a 5-min incubation. Percent protection was calculated by using the expression, $[V_{p+i} + V_p - V_i)/(V - V_i)] \times 100$, where V is the uptake value in preparations preincubated without addends; V_i, the value after treatment with PCMBS; V_{p+i}, the value obtained after treatment with PCMBS but in the presence of either P5C or 2-PC; and V_p, the value with P5C or 2-PC treatment without PCMBS

not accumulated against a concentration gradient but, instead, P5C is converted to proline *pari passu* with cell entry in a process consistent with group translocation (Mixson & Phang, 1988). These characteristics of pyrroline 5-carboxylate uptake have been generalized to a number of cell lines and has led to the proposal that the process is mediated by a special "carrier protein" linked, at least functionally, to P5C reductase which converts P5C to proline. Specific inhibitors of P5C uptake would help to elucidate the components of this uptake mechanism and to provide approaches for identification and purification of the putative carrier protein.

P5C is an unusual molecule in that the cyclic tertiary nitrogen structure is in spontaneous equilibrium with the open-chain glutamic-γ-semialdehyde (Fig. 1). Which of these structures is recognized by the putative carrier may be revealed by the structure of specific inhibitors of P5C uptake. We found that the most potent inhibitors of P5C uptake were compounds with cyclic structures containing a tertiary nitrogen. These compounds, 2-pyridinecarboxaldehyde, 2,6-pyridinedicarboxaldehyde and 2-pyridinecarboxylic acid had marked inhibitory effects on the uptake of P5C. In contrast, analogues with open-chain aldehydes produced no specific inhibition. Even those compounds with a cyclic structure and secondary nitrogens such as proline and pyroglutamic acid have little inhibitory effect. The pyridine compounds which are competitive inhibitors of P5C uptake do not exist as open-chain tautomers. Taken together, these findings suggest that it is P5C, the cyclic structure with a tertiary nitrogen, rather than the open-chain glutamic-γ-semialdehyde which is recognized by the transport carrier.

In terms of inhibition of P5C uptake, 2-pyridinecarboxylic acid and 2-pyridinecarboxaldehyde possess excellent discriminatory properties. Even at 10 mM, a concentration which completely inhibited the uptake of P5C, these analogues only minimally inhibited the uptake of amino acids representing the A, ASC and L systems. This level of discrimination is rarely seen except for commonly accepted model analogues such as α(methylamino) isobutyric acid and 2-aminobicyclo-(2,2,1) heptane-2-carboxylic acid for the A and L systems, respectively (Christensen, 1988). In fact, at high concentrations, 2-pyridinecarboxaldehyde and 2-pyridinecarboxylic acid produced less inhibition of transport by systems A, ASC and L than P5C, itself (*data not shown*). These inhibitors with their high degree of specificity may be helpful, indeed, in isolating the putative carrier for P5C.

These specific inhibitors also provided strong evidence supporting the existence of a membrane carrier protein for P5C. Our previously published studies did not rule out the possibility that P5C entered the cell by diffusion and then was trapped by its conversion to proline by P5C reductase. Our current work makes this highly unlikely; concentrations of inhibitor far exceeding the levels necessary to inhibit the uptake of P5C were without any effect on P5C reductase. Furthermore, in the face of inhibition of P5C uptake, the fate of P5C entering the cell,

Table 5. Evidence for covalent modification of P5C transport carrier[a]

Addends during preincubation	P5C uptake (% control)		ALA uptake (% control)	
Sodium cyanoborohydride	(−)	(+)	(−)	(+)
2-PC	114	15	104	97
2-PC + 2-PCA	109	38	93	94
2-PC + P5C	106	61	102	98
2-PC + P5C + 2-PCA	102	82	95	98
P5C	84	90	98	103

[a] Cells were preincubated for 1 hr with the indicated compounds in the presence (+) or absence (−) of 5 mM sodium cyanoborohydride. The following concentration were used; 2-pyridinecarboxaldehyde (2-PC), 0.5 mM; P5C, 5 mM; and 2-pyridinecarboxylic acid (2-PCA), 20 mM. Following preincubation, the medium was removed and the monolayer was washed ×4 with 2 ml of PBS. We then determined 1-min uptakes for either P5C or alanine (0.08 mM during uptake) and found control values to be 1.74 ± 0.21 and 1.26 ± 0.07 nmol/min · mg protein, respectively. Data are expressed as percent of respective controls and represent the average of at least six determinations.

i.e., conversion to proline, was unaffected; all the P5C entering the cell was recovered as proline. Thus, the mechanism of inhibition was not at the level of P5C reductase but most likely at the level of the carrier protein.

The consequences of this inhibition of P5C uptake by specific inhibitors include the blockade of the transfer of oxidizing potential mediated by P5C. Since P5C can initiate a sequence of metabolic events including the stimulation of the pentose phosphate shunt, the increased production of phosphoribosyl pyrophosphate and the increased synthesis of purine ribonucleotides (Yeh & Phang, 1988), the discovery of inhibitors which can block this sequence will be invaluable in future studies. 2-Pyridinecarboxylic acid may be especially useful because this compound, itself, had little effect on the pentose phosphate shunt but it completely abolished the stimulatory effect of P5C.

Moreover, these inhibitory analogues may provide additional insight into effector functions of P5C. Although the transfer of oxidizing potential mediated by P5C has been related stoichiometrically to the generation of $NADP^+$ and activation of the pentose phosphate shunt, this mechanism cannot explain all the effector functions of P5C. In this context, it is significant that several of these inhibitory analogues are potent chelators of iron and zinc (Testa et al., 1985; Lannon, Lappin & Segal, 1986; Johnson & Mayer, 1987; Kazakov et al., 1988). P5C and the aforementioned analogues have in common a cyclic structure containing a tertiary nitrogen and a carbonyl group in position 2 or 5. It is increasingly clear that chelators play an important role in biological reducing-oxidizing reactions (Mauk et al., 1979; Mauk, Borgdignon & Gray, 1982; Chapman et al., 1984). Whether P5C is also a chelator is currently being investigated. Thus, the identification of 2-pyridinecarboxylic acid and 2-pyridine carboxaldehyde as specific analogues may provide additional insight into the effector functions of P5C.

It has not escaped our notice that some of these inhibitors of P5C uptake are naturally occurring metabolic intermediates. 2-Pyridinecarboxylic acid is an intermediate in tryptophan metabolism (Burrow 1969), whereas quinolinic acid (2,3-pyridinedicarboxylic acid) is an excitatory neurotransmittor (Lehmann et al., 1985; Addae & Stone, 1986; Peters & Choi, 1987). However, little is known about the distribution of these compounds in tissues or biologic fluids; whether they physiologically affect P5C uptake and attenuate the effector functions of P5C in vivo is of considerable interest. We also considered that the uptake mechanism defined for P5C may mediate the uptake of a family of cyclic compounds with tertiary nitrogens. Additional studies are necessary to elucidate the possible sharing of transport systems between P5C and other cyclic structures with tertiary nitrogens.

In addition, since sulfhydryl-reactive agents inhibit amino acid and glucose transport (Hare, 1975; Bett et al., 1976; Goto et al., 1982; Chiles & Kilberg, 1986), we examined their effects on P5C uptake. Several sulfhydryl-reactive agents, N-ethylmaleimide, iodoacetate, and PCMBS all were inhibitory (*data not shown*) but the most potent was PCMBS. This was of special interest because PCMBS enters cells slowly if at all and its effects may be due to interaction with sulfhydryl groups on cell surface proteins. These studies showed that P5C uptake is blocked by the inactivation of a cell surface protein which may be the putative transport carrier for P5C. Not only does PCMBS interact with the P5C carrier, it mostly likely interacts at or near the binding site for P5C. This view is supported by the finding that the presence of P5C or its analogue, 2-PC, during treatment by PCMBS markedly attenuated its inhibitory effect on P5C uptake. These findings suggest that radioactively labeled PCMBS may be useful in attempts to isolate the putative carrier for P5C.

Finally, the interaction of these analogues suggest another promising approach in the isolation of the P5C carrier. The observation that P5C and 2PCA were less effective than the pyridine carboxaldehydes in protecting against PCMBS suggested the involvement of a Schiff's base perhaps between carboxaldehyde and amino groups on the P5C carrier. This was, indeed, the case. The presence of $NaCNBH_3$, a reducing reagent highly specific for

Schiff's base (Borch et al., 1971; Jentoft & Dearborn, 1979; Jentoft et al., 1979), made 2-PC an irreversible inhibitor of P5C uptake, presumably by stabilizing the interaction between this carboxaldehyde and the P5C carrier. Importantly, P5C attenuated this irreversible inhibition. Under the conditions of these experiments, $NaCNBH_3$ even at concentrations up to 10 mM did not inhibit the uptake of alanine nor did it produce any other apparent toxic effect. Thus, the use of tritiated $NaCNBH_3$ in the presence of 2-PC may provide another methodologic approach for identifying and isolating the putative carrier for P5C. P5C blockade of the incorporation of tritium would be limited to its interaction with membrane proteins with extracellular accessibility since the entry of P5C is accompanied by its conversion to proline.

References

Addae, J.I., Stone, T.W. 1986. Effects of topically applied excitatory amino acids on evoked potentials and single cell activity in rat cerebral cortex. *Eur. J. Pharmacol.* **121**:337–343

Batt, E.R., Abbott, R.E., Schachter, D. 1976. Impermeant maleimides. *J. Biol. Chem.* **251**:7184–7190

Borch, R.F., Bernstein, M.D., Durst, H.D. 1971. The cyanohydridoborate anion as a selective reducing agent. *J. Am. Chem. Soc.* **93**:2897–2904

Burrow, B.F. 1969. Metabolism of porphyrins and corrinoids. *In:* Metabolic Pathways. D.M. Greenberg, editor. pp. 404–522. Academic, New York

Chapman, S.K., Knox, C.B., Kathigamanathan, P., Sykes, A.G. 1984. Kinetic studies on 1 : 1 electron-transfer reactions involving blue copper proteins. *J. Chem. Soc. Dalton Trans.* **12**:2769–2774

Chiles, T.C., Kilberg, M.S. 1986. System A transport activity in normal rat hepatocytes and transformed liver cells: Substrate protection from inactivation by sulfhydryl-modifying reagents. *J. Cell. Physiol.* **129**:321–328

Christensen, H.N. 1988. What are the requisites for a model transport analog? *Trends Biochem. Sci.* **13**:40–42

Fleming, G.A., Granger, A., Rogers, Q.R., Prosser, M., Ford, D.B., Phang, J.M. 1989. Fluctuations in plasma pyrroline-5-carboxylate concentrations during fasting and feeding. *J. Clin. Endocrinol. Metab.* **69**:448–456

Gazzola, G.C., Dall'Asta, V., Franchi-Gazzola, R., White, M.F. 1981. The cluster-tray method for rapid measurement of solute fluxes in adherent cultured cells. *Anal. Biochem.* **115**:368–374

Goto, R., Hanamura, S., Tamemasa, O. 1982. Effects of *p*-chloromercuribenzene sulfonate on the uptake of D- and L-leucine in Ehrlich ascites tumor cells. *J. Pharm. Dyn.* **5**:780–782

Hare, J.D. 1975. Distinctive alterations of nucleoside, sugar, and amino acid uptake by sulfhydryl reagents in cultured mouse cells. *Arch. Biochem. Biophys.* **170**:347–352

Jentoft, N., Dearborn, D.G. 1979. Labeling of proteins by reductive methylation using sodium cyanoborohydride. *J. Biol. Chem.* **254**:4359–4365

Jentoft, J.E., Jentoft, N., Gerken, T.A., Dearborn, D.G. 1979. ^{13}C NMR studies of ribonuclease A methylated with [^{13}C] formaldehyde. *J. Biol. Chem.* **254**:4366–4370

Johnson, D., Mayer, H.K. 1987. Synthesis of some copper (II) complexes with ligands derived from the Schiff base reaction between 2-pyridine carboxaldehydes and aliphatic amines. *Inorg. Chim. Acta* **126**:L1–L2

Kazakov, S.A., Astashkina, T.G., Mamaev, S.V., Vlassov, V.V. 1988. Site-specific cleavage of single-stranded DNAs at unique sites by a copper-dependent redox reaction. *Nature* **335**:186–188

Kundig, W., Roseman, S. 1964. Phosphate bound to histidine in a protein as an intermediate in a novel phosphotransferase system. *Proc. Natl. Acad. Sci. USA* **52**:2067–2074

Kundig, W., Roseman, S. 1966. A phosphoenolpyruvate-hexose phosphotransferase system from *Escherichia coli*. *Methods Enzymol.* **9**:396–403

Lannon, A.M., Lappin, A.G., Segal, M.G. 1986. Redox reactions of some iron (II), iron (III), and cobalt (III) picolinate complexes. *J. Chem. Soc. Dalton Trans.* **3**:619–624

Lehmann, J., Ferkany, J.W., Schaeffer, P., Coyle, T. 1985. Dissociation between the excitatory and "excitotoxic" effects of quinolinic acid analogs on the striatal cholinergic interneuron. *J. Pharmacol. Exp. Ther.* **232**:873–882

Mauk, A.G., Borgdignon, E., Gray, H.B. 1982. Proteins and inorganic redox agents. Reactions involving bis(dipipecolate) complexes of cobalt (III) and iron (II) and stellacyanin, plastocyanin, and azurin. *J. Am. Chem. Soc.* **104**:7665–7657

Mauk, A.G., Coyle, C.L., Bordignon, E., and Gray, H.B. 1979. Bis(dipicolinate) complexes of cobalt (III) and iron (II) as new probes of metalloprotein electron-transfer reactivity. *J. Am. Chem. Soc.* **101**:5054–5056

Meister, A. 1954. The keto analogues of arginine, ornithine and lysine. *J. Biol. Chem.* **196**:577–585

Mezl, V.A., Knox, W.E. 1976. Properties and analysis of a stable derivative of pyrroline-5-carboxylic acid for use in metabolic studies. *Anal. Biochem.* **74**:430–440

Mixson, A.J., Phang, J.M. 1988. The uptake of pyrroline 5-carboxylate. *J. Biol. Chem.* **263**:10720–10724

Peters, S., Choi, D.W. 1987. Quinolinate is a weak excitant of cortical neurons in cell culture. *Brain Res.* **420**:1–10

Phang, J.M., Downing, S.J., Valle, D. 1973. A radioisotopic assay for Δ^1-pyrroline-5-carboxylate reductase. *Anal. Biochem.* **55**:266–271

Phang, J.M., Downing, S.J., Valle, D.L., Kowaloff, E.M. 1975. A radioisotopoic assay for proline oxidase activity. *J. Lab. Clin. Med.* **85**:312–317

Phang, J.M., Downing, S.J., Yeh, G.C., Smith, R.J., Williams, J.A., Hagedorn, C.H. 1982. Stimulation of the hexosemonophosphate-pentose pathway by pyrroline-5-carboxylate in cultured cells. *J. Cell. Physiol.* **110**:255–261

Smith, R.J., Downing, S.J., Phang, J.M. 1977. Enzymatic synthesis and purification of L-pyrroline-5-carboxylic acid. *Anal. Biochem.* **82**:170–176

Testa, U.K., Louache, F., Titeux, M., Thompoulos, P., Rouchant, H. 1985. The iron-chelating agent, picolinic acid, enhanced transferrin receptor expresssion in erythroleukaemic cell lines. *Br. J. Haematol.* **60**:491–502

Yeh, G.C., Phang, J.M. 1983. Pyrroline-5-carboxylate stimulates the conversion of purine antimetabolites to their nucleotide forms by a redox-dependent mechanism. *J. Biol. Chem.* **258**:347–352

Yeh, G.C., Phang, J.M. 1988. Stimulation of phosphoribosylpyrophosphate and purine nucleotide production by pyrroline 5-carboxylate in human erythrocytes. *J. Biol. Chem.* **263**:13083–13089

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