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Inhibition of Serine Palmitoyltransferase in Vitro and Long-Chain Base Biosynthesis in Intact Chinese Hamster Ovary Cells by β-Chloroalanine†

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ABSTRACT: The effects of β-chloroalanine (β-Cl-alanine) on serine palmitoyltransferase activity and the de novo biosynthesis of sphinganine and sphingenine were investigated in vitro with rat liver microsomes and in vivo with intact Chinese hamster ovary (CHO) cells. The inhibition in vitro was rapid (5 mM β-Cl-alanine caused complete inactivation in 10 min), irreversible, and concentration and time dependent and apparently involved the active site because inactivation only occurred with β-Cl-L-alanine (not β-Cl-D-alanine) and was blocked by L-serine. These are characteristics of mechanism-based ("suicide") inhibition. Serine palmitoyltransferase (SPT) was also inhibited when intact CHO cells were incubated with β-Cl-alanine (complete inhibition occurred in 15 min with 5 mM), and this treatment inhibited [^{14}C]serine incorporation into long-chain bases by intact cells. The concentration dependence of the loss of SPT activity and of long-chain base synthesis was identical. The effects of β-Cl-L-alanine appeared to occur with little perturbation of other cell functions: the cells exhibited no loss in cell viability, [^{14}C]serine uptake was not blocked, total lipid biosynthesis from [^{14}C]acetic acid was not decreased (nor was the appearance of radiolabel in cholesterol and phosphatidylcholine), and [^{3}H]thymidine incorporation into DNA was not affected. There appeared to be little effect on protein synthesis based on the incorporation of [^{3}H]leucine, which was only decreased by 14%. Although β-Cl-L-alanine is known to inhibit other pyridoxal 5′-phosphate dependent enzymes, alanine and aspartate transaminases were not inhibited under these conditions. These results establish the close association between the activity of serine palmitoyltransferase and the cellular rate of long-chain base formation and indicate that β-Cl-alanine and other mechanism-based inhibitors might be useful to study alterations in cellular long-chain base synthesis.

Serine palmitoyltransferase (SPT) (EC 2.3.1.50) is a pyridoxal 5′-phosphate dependent enzyme that catalyzes the first committed step of sphingolipid biosynthesis, the condensation of palmitoyl-CoA and serine to form 3-ketosphinganine (Snell et al., 1970). Several lines of evidence suggest that SPT catalyzes a rate-limiting step in sphingolipid biosynthesis (Braun et al., 1970; Williams et al., 1984b). SPT appears to

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have a lower specific activity than the other enzymes of ceramide biosynthesis (Braun et al., 1970; Morell & Radin, 1970; Stoffel et al., 1968), and the activity correlates well with the amount of tissue sphingolipids (Merrill et al., 1985). In microsomes, which contain SPT and the next enzyme of the pathway, an NADPH-dependent reductase, 3-ketosphinganine does not accumulate (Braun et al., 1970; Snell et al., 1970; Stoffel et al., 1968; Williams et al., 1984b). Furthermore, studies of the incorporation of [^{14}C]serine into sphingolipids using intact LM cells detected no free long-chain base intermediates; only ceramides and other complex sphingolipids contained significant radiolabel (Merrill & Wang, 1986). Other studies with these cells have found that long-chain base formation is substantially influenced by the availability of the substrates for serine palmitoyltransferase (Merrill et al., 1988).

If SPT catalyzes a kinetically important step in this pathway, then inhibition of this enzyme will result in an equivalent reduction in long-chain base synthesis. Pyridoxal 5′-phosphate dependent enzymes have often been studied using mechanism-based ("suicide") inhibitors (Badet et al., 1984; Walsh, 1984). For examples, β-haloalanines have been shown to be effective mechanism-inhibitors of pig heart aspartate aminotransferase (Morino & Okamoto, 1973, 1978), alanine aminotransferase (Golichowski & Jenkins, 1978), L-aspartate-β-decarboxylase (Relyea et al., 1974), and serine transhydroxymethylase (Wang et al., 1981). Serine palmitoyltransferase is likely to be affected by such compounds on the basis of its postulated mechanism (Krisnangkura & Sweeley, 1976). In fact, Sundaram and Lev (1984a–c) have shown that cycloserine, another mechanism-based inhibitor of pyridoxal 5′-phosphate dependent enzymes, inhibits SPT in *Bacteroides levii* and rat brain microsomes with a subsequent lowering of some complex sphingolipid pools.

In this paper, we have studied the inactivation of SPT in vitro by β-chloro-L-alanine (β-Cl-L-alanine) and the subsequent inhibition of long-chain base synthesis in intact Chinese hamster ovary (CHO) cells. These investigations illustrate the utility of such reagents in studying long-chain base synthesis and further support the hypothesis that SPT catalyzes one of the key rate-limiting steps in sphingolipid biosynthesis.

Experimental Procedures

Materials. Ham's F12 medium MCDB301 medium (a formulation similar to F12) minus serine, and fetal calf serum were obtained from Gibco. L-[3-^{14}C]Serine and [*methyl*-^{3}H]thymidine were from Amersham, and L-[^{3}H]serine and [1-^{14}C]acetate were from ICN. L-[3,4,5-^{3}H(N)]Leucine was obtained from New England Nuclear. β-Cl-[^{14}C]alanine was synthesized by the procedure of Relyea et al. (1977) and was pure on the basis of paper chromatography. Sphinganine was purchased from Sigma and U.S. Biochemicals, and sphingosine (sphingenine) and β-Cl-L-alanine were from Sigma. Tissue culture dishes (100 mm) were obtained from Falcon.

Isolation of Rat Liver Microsomes. Microsomes were isolated from the livers of rats killed by CO_2 inhalation as described by Williams et al. (1984a).

Cell Culture. Chinese hamster ovary cells (CHO-WT5) were originally obtained from Louis Simonovitch, Hospital for Sick Children, Toronto, Canada. The cells were maintained at 37 °C in 5% CO_2 and 100% humidity in Ham's F12 medium with 5% fetal calf serum supplemented with penicillin G (100 units/mL), streptomycin sulfate (100 μg/mL), and sodium bicarbonate (1.176 g/L). For subculturing, the cells were removed from the dish with 0.5 mM EDTA in Puck's saline (0.14 M NaCl, 5.4 mM KCl, 5.5 mM glucose, and 4.3 mM sodium bicarbonate). Unless otherwise noted, intact cells were incubated with Ham's F12 (without fetal calf serum) 24 h prior to the experiment.

Serine Palmitoyltransferase Activity Assay. Serine palmitoyltransferase was assayed with whole cells disrupted by sonication as described by Merrill (1983) and in rat liver microsomes as described by Williams et al. (1984a) using 1 mM [^{3}H]serine (34 mCi/mmol) and 50 μM pyridoxal 5′-phosphate.

Analysis of Long-Chain Base Synthesis. The medium was removed and replaced by 2 mL of MCDB301 medium minus serine containing 0.023 mM [^{14}C]serine (55 mCi/mmol) for 2 h. Long-chain bases were analyzed as described by Merrill and Wang (1986). In brief, cells were scraped in 0.5 mL of water and extracted with chloroform and methanol by the Folch procedure. The chloroform extract was dried over sodium sulfate, and the solvent was evaporated under nitrogen. Samples were hydrolyzed in acid for 18 h at 70 °C and then neutralized and reextracted. Long-chain bases were separated by thin-layer chromatography using silica gel H (Brinkmann) plates developed with chloroform/methanol/2 N NH_4OH (40:10:1), visualized by autoradiography, and quantitated by liquid scintillation counting as described previously (Merrill & Wang, 1986).

Analysis of Cholesterol, Phosphatidylcholine, and Total Lipid Synthesis. As in the analysis of long-chain base synthesis, the medium was removed and replaced with 2 mL of MCDB301 medium and 0.052 mM [^{14}C]acetate (9.6 mCi/mmol) for 2 h. The medium was removed, the cells were extracted as described above, and separate aliquots were used for the analyses of cholesterol, phosphatidylcholine, and total lipids. The samples for cholesterol analysis were incubated with 0.2 mL of 0.5 M KOH in methanol for 1 h at 37 °C and extracted as described above. After the samples were dried, they were redissolved in chloroform/methanol (2:1) and applied to silica gel thin-layer chromatography plates. The plates were developed in chloroform/methanol (30:1) for cholesterol analyses, and in chloroform/methanol/acetic acid/water (56:30:4:2) for phospholipids. The spots were visualized with iodine, and the regions corresponding to the position of the cholesterol and phosphatidylcholine standards were scraped and put in scintillation vials. They were then solubilized in 0.1 mL of Protosol for 1 h, and radioactivity was quantitated by scintillation counting in 4 mL of Econofluor. Total lipid synthesis was analyzed by evaporating the 0.2 mL of the extract in a scintillation vial and then measuring radioactivity by scintillation counting.

Uptake Experiments. Transport experiments were conducted following a modification of the procedure of Englesburg et al. (1976). The medium was removed and replaced by 2 mL of modified MCDB301 medium containing 0.01 mM [^{14}C]serine or 1 mM β-Cl-[^{14}C]alanine for various times. The medium was removed, and each vial was rapidly washed 3 times with cold Puck's saline. The vials were inverted to dry for 1 h. Cells were solubilized in 0.2 mL of Soluene or Protosol for 1 h, and radioactivity was quantitated by scintillation counting in Econofluor with correction for quenching.

Alanine and Aspartate Transaminase Assays. Intact CHO cells were incubated in F12 minus serine and fetal calf serum 24 h prior to the experiment. The cells were removed from each dish with 0.5 mM EDTA in Puck's saline, centrifuged, and resuspended in Puck's saline. The cells were transferred to test tubes, sonicated, and incubated at 37 °C for 10 min with a final concentration of 0, 1, 5, or 100 mM β-Cl-alanine. Alanine and aspartate transaminase activities were measured as described by Bergmeyer and Brent (1974a,b).

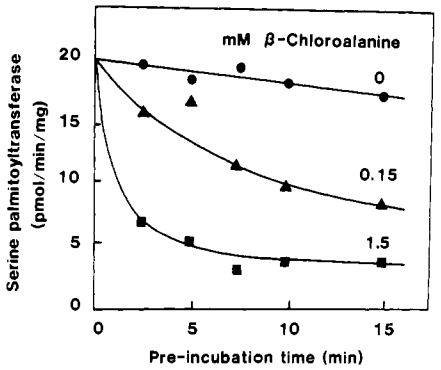

FIGURE 1: Time dependence of inhibition of SPT activity by β-Cl-alanine in rat liver microsomes. Microsomes were incubated at 37 °C for the times indicated with β-Cl-alanine to give final concentrations of 0, 150 μM, or 1.5 mM and then diluted during assays of serine palmitoyltransferase to a concentration that yielded no further inactivation as described under Experimental Procedures. The results are given for quadruplicate assays.

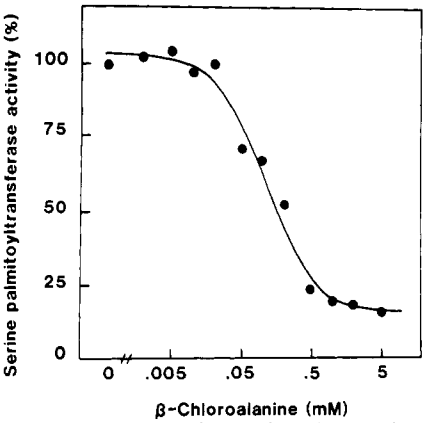

FIGURE 2: Concentration dependence of inhibition of serine palmitoyltransferase activity by β-Cl-alanine in rat liver microsomes. The conditions were the same as in Figure 1 except that a single preincubation time (10 min) was used and the concentration of β-Cl-alanine was varied as shown.

[^{3}H]Thymidine Incorporation into DNA and [^{3}H]Leucine Incorporation into Protein. Intact CHO cells were incubated in F12 minus thymidine and fetal calf serum or F12 minus fetal calf serum 24 h prior to the experiment for DNA and protein synthesis, respectively. The medium was removed and replaced with fresh medium containing 0 or 5 mM β-Cl-alanine and incubated with 1 μCi of [^{3}H]thymidine or [^{3}H]leucine for 2 h. Another group of cells was only incubated in medium containing 0 or 5 mM β-Cl-alanine for 15 min, after which the medium was removed, the cells were washed, and new medium with 1 μCi of [^{3}H]thymidine (77 Ci/mmol) or [^{3}H]leucine (141 Ci/mmol) was added for 2 h. Incorporation of [^{3}H]thymidine into DNA was measured as described by Bremer et al. (1984). The amount of [^{3}H]leucine in protein was measured by removing the cells from the dishes with EDTA, recovering the cells by centrifugation, and adding 100 μL of 0.65% bovine serum albumin and 2 mL of cold 5% trichloroacetic acid. After addition of another 2 mL of cold 5% TCA, the suspension was centrifuged at 2000 rpm for 2 min. The pellet was solubilized in 100 μL of 0.5 N NaOH, 2 mL of 5% TCA was added, and the suspension was centrifuged again. This procedure was repeated, the pellet was resolubilized in 100 μL of 0.5 N NaOH, and the radioactivity was quantitated by scintillation counting in 4 mL of Universol counting cocktail.

Results

Inhibition of SPT Activity in Rat Liver Microsomes. The inhibition of SPT by β-Cl-alanine was first examined with rat liver microsomes because they are a well-characterized source of activity. SPT was inhibited by β-Cl-alanine in a time-dependent manner as shown in Figure 1. In microsomes treated with 1 mM β-Cl-alanine, 50% inhibition occurred in 1.5 min, and essentially complete inhibition occurred within 7.5 min. With 150 μM β-Cl-alanine, 50% inhibition occurred in 9.5 min, but complete inhibition was not observed during the 15-min incubation.

The inhibition by β-Cl-alanine during a 10-min incubation exhibited the concentration dependence shown in Figure 2. In agreement with the data in Figure 1, approximately 50% inhibition was observed with 150 μM β-Cl-alanine. Essentially complete inhibition was achieved with 1–5 mM β-Cl-alanine. The small amount of activity remaining is probably due to the incorporation of radiolabeled serine into other lipid products.

These data are consistent with β-Cl-alanine acting as a mechanism-based inhibitor. Another criterion for this mode of inactivation is that it is irreversible. This was tested by assaying microsomes after removal of the free β-Cl-alanine using a Sephadex G-10 column (Table I). Little or no activity was restored by this treatment (the removal of all but traces of the β-Cl-alanine was documented using radiolabeled inhibitor). Slight increases in some of the samples (which include both of the controls) may reflect changes in activity due to the incubation at 37 °C per se.

If the inactivation is occurring at the active site of SPT, as is required for suicide inhibition, L-serine should protect the enzyme from inactivation by β-Cl-alanine. Microsomes were incubated with 1 mM β-Cl-alanine and various concentrations of serine and assayed for SPT activity as shown in Table II. L-Serine prevented the inhibition in a concentration-dependent manner: 1 mM L-serine blocked the inactivation by nearly 50%, and this is approximately the K_m for this substrate (Williams et al., 1984). Complete activity was restored by

Table I: Irreversibility of the Inhibition of Serine Palmitoyltransferase Activity by β-Cl-alanine in Rat Liver Microsomes

	activity before[a]		activity after[a]	
concn (mM)	pmol min^{-1} mg^{-1}	%	pmol min^{-1} mg^{-1}	%
0[b]	16.8 ± 3.6		17.1 ± 1.7	
0[c]	17.3 ± 3.6	100	24.7 ± 5.3	100
0.15	10.7 ± 1.1	62	8.7 ± 0.5	35
1.5	2.8 ± 0.6	16	5.4 ± 0.8	21

[a] Samples were incubated with β-Cl-alanine for 10 min at 37 °C and then assayed for SPT activity before and after the sample was chromatographed on a Sephadex G-10 column to remove free β-Cl-alanine. The percent refers to the percent activity compared to the sample treated identically, except that the inhibitor was omitted during the incubation. [b] A control without β-Cl-analine and no incubation at 37 °C. [c] A control without β-Cl-alanine but with incubation for 10 min at 37 °C.

Table II: L-Serine Protection of β-Cl-alanine Inactivation of Serine Palmitoyltransferase in Rat Liver Microsomes[a]

[serine] (mM)	[β-Cl-alanine] (mM)	% act.
0	0	100 ± 16
0	1	24 ± 4
1	1	48 ± 10
2	1	68 ± 7
5	1	109 ± 10
10	1	136 ± 6
10	0	144 ± 34

[a] Samples were incubated for 10 min at 37 °C with plus or minus cold serine and β-Cl-alanine and assayed for SPT as described under Experimental Procedures.

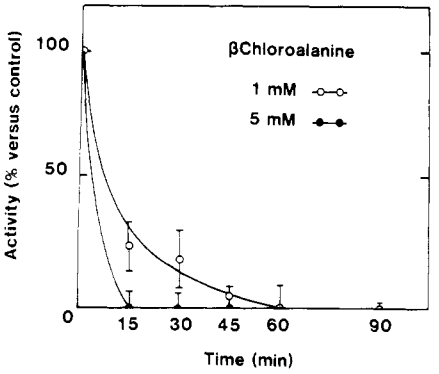

FIGURE 3: Concentration and time dependence of inhibition by β-Cl-alanine in Chinese hamster ovary cells. Cells were incubated at 37 °C for the times indicated with 1 or 5 mM β-Cl-alanine and then washed, and serine palmitoyltransferase was assayed in vitro. The results are reported as a percentage of the total activity in the control. Results represent means ± SE of quadruplicate assays.

5 mM; an increase in activity over control values was, again, observed (e.g., with 5 and 10 mM L-serine in the presence and absence of β-Cl-alanine).

Stereospecificity of Inhibition in Rat Liver Microsomes. To investigate the stereospecificity of inhibition of SPT, inhibition by β-Cl-D-alanine was tested by using up to 10 times the concentration of β-Cl-L-alanine that affected 50% inhibition. Even at 2 mM, β-Cl-D-alanine did not inhibit SPT activity detectably (data not shown); hence, the L isomer is strongly preferred.

Inhibition of SPT Activity in CHO Cells. Since β-Cl-alanine acted as a potent inhibitor in vitro, its ability to inhibit SPT in a concentration- and time-dependent manner in intact CHO cells was then tested. Cells were incubated with up to 5 mM β-Cl-alanine for various times, washed, then disrupted, and assayed for SPT in vitro (Figure 3). Cells incubated alone exhibited no loss of activity over this time course, whereas both 1 and 5 mM β-Cl-alanine caused a rapid loss in SPT activity with no loss in cell viability. Within 15 min, 5 mM β-Cl-alanine completely inhibited SPT, and both 1 and 5 mM β-Cl-alanine completely inhibited SPT for at least 90 min with no loss in cell viability. Furthermore, cell viability remained within control values for at least 6 h (data not shown). To determine if any recovery of activity occurred during over the 2-h time course that was necessary for the measurement of long-chain base synthesis (i.e., to obtain enough cpm for reliable measurement of the long-chain bases), SPT activity was assayed after 3 h, and none was observed. We have also found that the turnover of newly synthesized long-chain bases probably does not complicate these measurements because the rate is slow compared to the incorporation of radiolabel (Merrill et al., 1986; Medlock and Merrill, unpublished results).

Inhibition of Long-Chain Base Biosynthesis in CHO Cells. To delineate the relationship between inhibition of SPT and long-chain base synthesis, intact cells were incubated with up to 5 mM β-Cl-alanine for 15 min, washed, and incubated with [^{14}C]serine, and the amount of radiolabel incorporated into sphinganine plus sphingenine was determined. Concentration-dependent inhibition was observed, with complete inhibition at 5 mM as shown in Table III.

Since these results were strikingly similar to those for the inhibition of SPT activity (Figure 3), a series of experiments were conducted in which both the SPT activity (measured in vitro after breaking the cells) and the long-chain base synthesis (measured in intact cells) were determined (Figure 4). The inhibition curves are essentially parallel, which strongly suggests that SPT activity is comparable to the overall flux through this pathway.

Table III: Concentration Dependence of Inhibition of Long-Chain Base Synthesis by β-Cl-alanine in CHO Cells[a]

concn (mM)	% act. (pmol min^{-1} mg^{-1})
0	100 ± 8
1	44 ± 20
3	24 ± 15
5	8 ± 3

[a] Intact cells were incubated at 37 °C with β-Cl-alanine for 15 min, washed, and incubated with [^{14}C]serine for 2 h, and long-chain bases were assayed as described under Experimental Procedures.

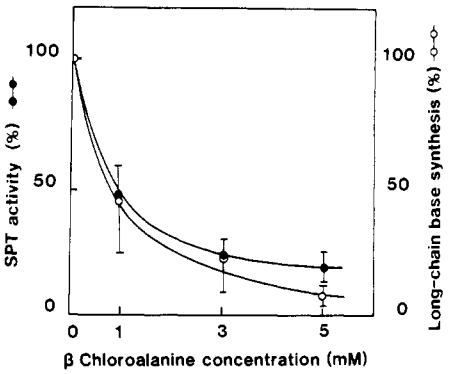

FIGURE 4: Comparison of β-Cl-alanine inhibition of serine palmitoyltransferase activity and long-chain base synthesis in intact cells. Cells were incubated with different concentrations of β-Cl-alanine for 15 min and then washed with Puck's saline. The cells were then incubated for 2 h with [^{14}C]serine for the determination of long-chain base biosynthesis, or SPT activity was measured in vitro with sonicated cells as described under Experimental Procedures. Results represent the mean ± SE of two experiments for long-chain base synthesis (n = 8) and six experiments (n = 18) for serine palmitoyltransferase activities. The results are reported as the percentages of the control cells that were incubated without β-Cl-alanine.

Uptake of [^{14}C]Serine and [^{14}C]-β-Cl-alanine. Since the inhibition of [^{14}C]serine incorporation into long-chain bases would also occur if β-Cl-alanine affected serine transport, the uptake of [^{14}C]serine was measured. The rate in untreated cells was 1.44 ± 0.11 nmol h^{-1} (10^6 cells)$^{-1}$, compared to 1.95 ± 0.16 nmol h^{-1} (10^6 cells)$^{-1}$ after treatment with 5 mM β-Cl-alanine for 15 min. Hence, there is no inhibition of serine uptake in the presence of β-Cl-alanine; instead, there was a slight increase. This increase was probably due to trans stimulation of serine uptake since CHO cells contain an ASC transport system (Shotwell et al., 1981).

The cellular uptake of β-Cl-alanine was also established by incubating CHO cells with radiolabeled β-Cl-alanine. After a 15-min incubation with 0.5 mM [^{14}C]-β-Cl-alanine, the cells contained approximately 0.1 mM β-Cl-alanine, assuming that the volume of 10^6 cells is approximately 5 μL.

Analysis of the Synthesis of Other Lipids after Treatment with β-Cl-alanine. To assess whether β-Cl-alanine is generally inhibiting lipid synthesis, the effects of 5 mM β-Cl-alanine on other lipid biosynthetic pathways were examined by following the incorporation of [^{14}C]acetate into total lipids, cholesterol, and phosphatidylcholine (Table IV). Neither cholesterol nor phosphatidylcholine synthesis was significantly affected by β-Cl-alanine treatment, and furthermore, the synthesis of total lipid pools was not affected.

DNA and Protein Synthesis after Treatment with β-Cl-alanine. The incorporation of [^{3}H]thymidine into DNA and [^{3}H]leucine into protein was determined in order to further define the specificity of inhibition of SPT by β-Cl-alanine. Intact cells were incubated with 0 and 5 mM β-Cl-alanine for

Table IV: Effects of β-Cl-alanine on Synthesis of Total Lipid, Cholesterol, and Phosphatidylcholine[a]

	β-Cl-alanine addition	cpm [^{14}C]acetate[b]
total lipid	−	44620 ± 2790
	+	43660 ± 4900
cholesterol	−	4200 ± 330
	+	4980 ± 1030
phosphatidylcholine	−	14920 ± 2220
	+	11580 ± 1780

[a] Intact cells were incubated at 37 °C with 5 mM β-Cl-alanine for 15 min, washed, and then incubated with [^{14}C]serine for 2 h. The cells were assayed for incorporation of [^{14}C]acetate into total lipid, cholesterol, or phosphatidylcholine as described under Experimental Procedures. [b] cpm incorporated per hour per 10^6 cells.

Table V: Incorporation of Radiolabeled Precursors into DNA and Protein after Treatment with β-Cl-alanine[a]

[^{3}H]Thymidine Incorporation into DNA		
[β-Cl-alanine] (mM)	time[b]	rate of incorporation[c]
0	15 min	1.15 ± 0.01
0	2 h	1.49 ± 0.11
5	15 min	1.25 ± 0
5	2 h	1.32 ± 0.05
[^{3}H]Leucine Incorporation into Protein		
[β-Cl-alanine] (mM)	time[b]	cpm incorporated
0	15 min	13470 ± 1310
0	2 h	18000 ± 320
5	15 min	11530 ± 380
5	2 h	13760 ± 1810

[a] Incorporation of [^{3}H]leucine into protein and [^{3}H]thymidine into DNA was done as described under Experimental Procedures. [b] Incubation time in β-Cl-alanine; total incubation time with radiolabel is 2 h. [c] Picomoles per minute per 10^6 cells.

various times, and as shown in Table V, there was no change in incorporation of [^{3}H]thymidine into DNA. [^{3}H]Leucine incorporation into protein was inhibited by 14% after treatment for 15 min with 5 mM β-Cl-alanine and by 24% after 2 h. There was no loss in cell viability (data not shown).

Alanine and Aspartate Transaminase Activities in CHO Cells. To also evaluate the extent to which other pyridoxal 5′-phosphate dependent enzymes may have been affected, alanine and aspartate transaminase, previously found to be inactivated by β-Cl-alanine (Golichowski & Jenkins, 1978; Morino & Okamoto, 1973, 1978), were assayed under the same conditions that resulted in complete inactivation of SPT in CHO cells. As shown in Table VI, there was no loss in either activity after a 10-min incubation with 5 mM β-Cl-alanine in broken CHO cells (similar results were found when intact cells were used in the incubation with the inhibitor; not shown). These findings were not inconsistent with the previous reports because the conditions used for our assays (in vitro and in vivo) differ significantly with respect to the buffers, pH, and specific cofactors that have been used to achieve rapid inactivation of these other enzymes. To confirm that these enzymes could be inhibited by β-Cl-alanine, the experiment was conducted using 100 mM β-Cl-alanine, which resulted in complete inhibition of both transaminases. Thus, inactivation did occur in the presence of β-Cl-alanine, but at much higher concentrations than those needed for complete inhibition of SPT.

Table VI: Aminotransaminase Activities in Chinese Hamster Ovary Cells after Treatment with β-Cl-alanine[a]

	activity [nmol min^{-1} (mg of protein)$^{-1}$]	
[β-Cl-alanine] (mM)	aspartate amino-transferase	alanine aminotransferase
0	36 ± 2	6.7 ± 0.9
1	44 ± 3	7.2 ± 2.2
5	41 ± 1	6.7 ± 1.4
100	0 ± 0.4	0.5 ± 0.5

[a] Sonicated cells were incubated at 37 °C at varying concentrations of β-Cl-alanine for 10 min. The cells were assayed for these transaminases as described by Bergmeyer and Bernt (1974a,b).

Discussion

These experiments establish that β-Cl-alanine inhibits SPT in vitro and in intact cells. This inhibition is concentration and time dependent and irreversible and apparently occurs at the active site, which are characteristics of the mechanism-based inhibition by β-haloalanines of numerous other pyridoxal 5′-phosphate dependent enzymes. This behavior is consistent with the proposed mechanism for SPT (Krisnangkura & Sweeley, 1976), in which the α-hydrogen of L-serine (enzyme bound as a Schiff base with pyridoxal 5′-phosphate) is removed to generate a carbanion that attacks the carbonyl group of the cosubstrate palmitoyl-CoA. In the case of the β-haloalanines, the halide ion is probably eliminated to form a reactive electrophile that inactivates the enzyme by any of a variety of possible mechanisms (Likos et al., 1982; Ueno et al., 1982; Walsh, 1984).

The susceptibility of SPT to this type of inactivation in vitro suggests that inhibition might also be possible in vivo. This was demonstrated with Chinese hamster ovary cells, which exhibited both a loss of SPT activity and a loss of long-chain base biosynthesis. Since these occurred in parallel, this gives further evidence for the hypothesis that SPT catalyzes a rate-determining step in sphingolipid synthesis (Merrill et al., 1988).

Although β-Cl-alanine might affect sphingolipid synthesis by a variety of other ways in intact cells, the effects of this compound appeared to be primarily via inhibition of SPT on the basis of several lines of evidence. It caused no inhibition of the uptake of [^{14}C]serine and did not interfere with lipid synthesis in general. Fatty acyl-CoA's are common components of the sphingolipid and glycerolipid pathways; therefore, if β-Cl-alanine was affecting palmitoyl-CoA formation significantly, it would probably also affect glycerolipid biosynthesis from [^{14}C]acetic acid.

Since it is well-known that β-Cl-alanine can inhibit several other enzymes, it was somewhat surprising to us that it had few deleterious effects on these cells under the conditions used. It was not cytotoxic, and cell growth continued within control values after removal of β-Cl-alanine. Although there appeared to be some inhibition of protein synthesis based on [^{3}H]leucine incorporation into protein, this had no apparent effect on the complex processes reflected in [^{3}H]thymidine incorporation into DNA and [^{14}C]acetic acid incorporation into other lipids.

Serine palmitoyltransferase appears to be more sensitive to inactivation by β-Cl-alanine than several other pyridoxal 5′-phosphate dependent enzymes. Hence, sphingolipid synthesis might be altered without affecting many of the other cellular processes that are theoretically inhibited by β-Cl-alanine. This raises interesting questions about whether or not sphingolipid synthesis is inhibited when fluoroalanines, which have been studied as potential antimicrobial agents, are used with mammalian systems. In preliminary studies, we have observed that β-F-L-alanine is more potent than β-Cl-L-alanine in inhibiting SPT in vitro and that some inhibition is observed with β-F-D-alanine (Medlock and Merrill, unpublished results).

In other investigations of the inhibition of SPT by cycloserine, Sundaram and Lev (1984c) observed decreases in ceramide phosphorylglycerol and ceramide phosphoryl-

ethanolamine synthesis in *Bacteroides levii* without significant inhibition of phosphatidylglycerol or phosphatidylethanolamine formation. Cycloserine also inhibits SPT in rat brain microsomes and results in lowered levels of gangliosides and cerebrosides (Sundaram & Lev, 1984b). Williams et al. (1987) have reported that cycloserine decreases SPT activity in aorta from rabbits fed diets high in cholesterol. If this were to affect sphingomyelin levels, it may also have an effect on cholesterol metabolism, since there is thought to be a link between these two lipids [see Stevens et al. (1986) for a discussion of this point]. These findings illustrate the broader potential for inhibitors of SPT. Further studies should be done to determine in more detail which complex sphingolipid pools are affected by the inhibition with β-Cl-L-alanine, and to develop more specific inhibitors based on this model.

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Registry No. SPT, 62213-50-7; β-Cl-L-alanine, 2731-73-9; sphingosine, 123-78-4; sphinganine, 764-22-7.

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