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L-CYCLOSERINE INHIBITION OF SPHINGOLIPID SYNTHESIS IN THE ANAEROBIC BACTERIUM BACTEROIDES LEVII

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Summary. L-cycloserine was found to significantly inhibit the activity of the first enzyme of the sphingolipid pathway when added to growing cultures of Bacteroides levii. The effect of cycloserine on the synthesis of the sphingolipids showed that ceramidephosphorylethanolamine was inhibited to a greater degree than ceramidephosphorylglycerol, although synthesis of both was significantly inhibited by cycloserine as determined by [^{32}P] incorporation and phosphorus determination. In contrast, synthesis of phosphatidylethanolamine and phosphatidylglycerol was not inhibited by L-cycloserine at 100 µg/ml. Peturbation of sphingolipid synthesis by L-cycloserine may therefore provide a useful tool for the study of the function of these membrane lipids.

Bacteroides levii, in common with certain other microorganisms, has the capacity to synthesize sphingolipids, although this property is somewhat rare among microorganisms (1). Sphingolipid biosynthesis has been studied in B. levii where vitamin K deprivation of the cells has been shown to result in a specific inhibition of these complex lipids (2). Further, deprivation of vitamin K has been shown to be related to a decrease in activity of the first enzyme of the sphingolipid pathway, 3KDS synthetase (3). This microorganism synthesizes two major sphingolipids, CPE and CPG, the content of which can comprise more than 50% of the phospholipid content of the cell. PS and PE are the other phospholipid components of these microorganisms (1).

The bacterial 3KDS synthetase has been solubilized and partially purified (4) and thiocyanate was shown to be an inhibitor of this enzyme. In a recent study (5) we have shown that D- and L-cycloserine (4-amino-3-isoxazolidinone), are potent inhibitors of both the bacterial and mouse brain microsomal 3KDS synthetases in vitro. The L- isomer is considerably more potent than the D-

ABBREVIATIONS: 3KDS, 3-ketodihydrosphingosine; CPE, ceramide phosphorylethanolamine; CPG ceramide phosphorylglycerol; PE, phosphatidylethanolamine; PS, phosphatidylserine.

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isomer. We have also shown that intraperitoneal administration of L-cycloserine in mice results in an inhibition of brain microsomal 3KDS synthestase and that this inhibition of the synthetase results in a significant decrease in ganglioside and cerebroside/sulfatide content of the brains of the cyloserine-treated animals. Inhibition of later steps in the glycolipid pathway has been described by Radin's group, which has studied inhibition of glycolipid synthesis using synthetic analogues of ceramide (6). In addition, tunicamycin has been shown to inhibit glycosylation of gangliosides (7,8).

In the present report we have determined the effect of L-cycloserine administration on the synthesis of sphingolipids and other phospholipids in growing cultures of B. levii in order to determine the specificty of cycloserine treatment and also to determine whether or not administration of this compound results in a coordinate reduction in synthesis of the two sphingolipids.

MATERIALS AND METHODS

The strain of B. levii utilized was that used in previous studies on vitamin K and sphingolipid metabolism (3,4). It was grown in trypticase, yeast extract medium supplemented with vitamin K_1 and a dilution of horse red cells under anaerobic conditions as described previously (3). Cultures for metabolic studies were grown in specially-adapted anaerobic jars which allow additions and samples to be taken with minimal disturbance of the E_h of the medium (9).

To determine phospholipid synthesis, cultures growing in the anaerobic jars were inoculated with [^{32}P] (500 µCi/200ml) and incubated for two doublings. The lipids were extracted and separated by two-dimensional thin layer chromatography (2). In experiments involving the effects of L-cycloserine on phospholipid synthesis, cycloserine at the concentrations indicated was added to the culture 30 min prior to [^{32}P] addition. In some experiments, phosphorus estimations on individual lipids were made by the method of Marinetti (10). Activity of 3KDS synthetase was determined by measuring the incorporation of [3-^{14}C]serine into 3KDS. Cells were centrifuged and washed twice with phosphate buffer (0.05M, pH 7.4) containing 1mM dithiothreitol. They were then suspended (1g wet weight in 9ml buffer) and sonicated three times for 15 sec., with a 1 min. interval between sonications. The supernatants of 100,000g, 90 min. of centrifugation were used for 3KDS synthetase assay. The reaction mixtures contained 0.5 µCi [L-3-^{14}C]serine (4mM), 0.2mM palmitoyl CoA in 125 µl and 200 µl of supernatant. The reactions were incubated (37°, 20 min.) and terminated by the addition of 5ml chloroform:methanol (2:1). The reaction mixtures were partitioned by the Folch procedure and the lipid phase was then dried and applied to a silica gel G plate. The plates were developed in chloroform:methanol:water:ammonia (280:70:6:1) and [^{14}C]-labeled 3KDS was detected by radioautography and counted in a scintillation counter.

RESULTS AND DISCUSSION

L-cycloserine causes the inhibition of growth of B. levii at relatively high concentrations, between 100 and 200 µg/ml (data not shown). Accordingly, these two concentrations were used to determine a possible inhibitory effect on sphingolipid and other phospholipid synthesis. The administration of either concentration to a growing culture caused a dramatic inhibition of the activity of 3KDS synthetase (Fig. 1). No increase in enzyme activity occured in the presence of cycloserine during the subsequent doubling of the cells. The activity of the enzyme after 60 min. incubation with cycloserine was 70% and 80% inhibited at concentrations of 100 and 200 µg/ml. The inclusion of 100 µg/ml L-cycloserine in the medium caused a reduction in [^{32}P] incorporation of 65% for CPE and 24% for CPG whereas there were negligible effects on the incorporation of [^{32}P] into PE and PS. With 200 µg/ml L-cycloserine, a greater inhibition of incorporation into both sphingolipids was seen, that is, an 83% and 76% inhibtion into CPE and CPG respectively. At this concentration, a reduction of [^{32}P] incorporation of 16% and 28% into PE and PS was found (Figs. 2 and 3).

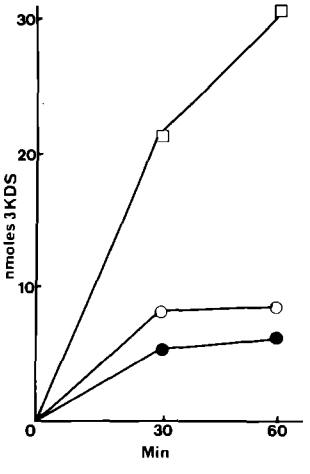

Fig. 1. Inhibition of 3KDS synthetase activity in vivo. A culture of B. levii was grown to 62 Klett units and L-cycloserine added at the concentrations indicated. 50 ml was withdrawn and the cells washed. Enzyme activity was determined on the supernatant of a sonicated extract of the cells as described previously (3). □ Control, ○ 100 µg/ml L-cycloserine, ● 200 µg/ml L-cycloserine.

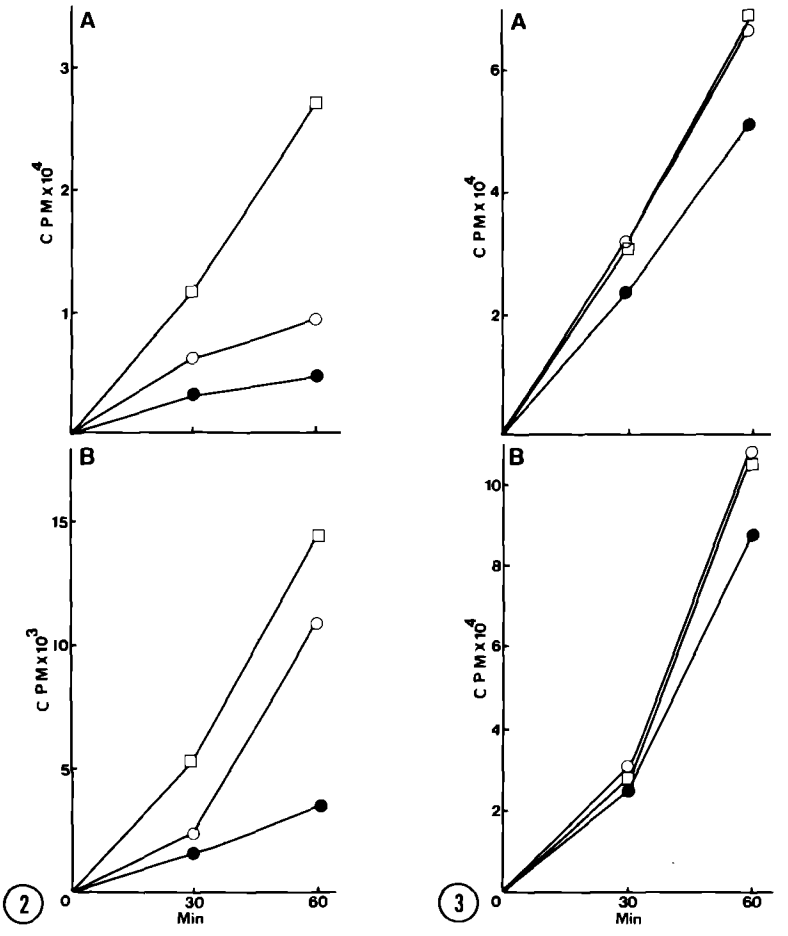

Fig. 2. Effect of L-cycloserine on in vivo sphingolipid synthesis. Cultures grown to 95 Klett units were inoculated with L-cycloserine. Thirty minutes later, [^{32}P] (500 µCi) was added. Samples were then withdrawn at the times indicated and lipids were extracted, chromatographed and [^{32}P] incorporation into individual lipids determined (2). □ Control, ○ 100 µg/ml, ● 200 µg/ml L-cycloserine. A, ceramidephosphorylethanolamine; B, ceramidephosphoryl-glycerol.

Fig. 3. Effect of L-cycloserine on phospholipid synthesis. [^{32}P] incorporation into phosphatidylserine and phosphatidylethanolamine was determined as described in Fig. 2. A, phosphatidylserine; B, phosphatidylethanolamine.

Phosphorus determinations performed on lipid extracts of cultures incubated in the presence of 100 and 200 µg/ml cycloserine for 90 min showed reductions of 43% and 65% respectively for CPE and 50% and 48% for CPG. In contrast, reductions of 13% and 20% where found for PE and 3% and 27% for PS following incubation of the culture with 100 and 200 µg/ml cycloserine respectively.

The inhibition of the enzyme controlling the first step in the sphingolipid pathway should therefore result in a decrease in the subsequent steps in

biosynthesis. This was found in previous studies on the inhibition of ganglioside and cerebroside/sulfatide synthesis in mouse brain (5). The effect of L-cycloserine on sphingolipid synthesis in B. levii closely resembles that obtained in vivo in mice where a profound effect (a 70% reduction) was observed on 3KDS synthetase activity, and which, over the course of administration for a week, resulted in a reduced accumulation of total gangliosides of 16% (5). Past experiments have shown that [^{32}P] incorporation is a valid index of sphingolipid and other phospholipid synthesis in the Bacteroides (2). In the present experiments, a 70% inhibition in synthetase activity was observed at 100 µg/ml, whereas an 80% inhibition occurred at 200 µg/ml L-cycloserine. This corresponded respectively to a 65% and 83% inhibition in [^{32}P] incorporation into CPE. With CPG, 100 µg/ml produced only a 24% inhibition in [^{32}P] incorporation; that is, CPG synthesis is less sensitive to the action of cycloserine than is CPE synthesis. This may be of significance since CPE is the major sphingolipid in this bacterium. With respect to the phospholipids PE and PS, there was little difference in [^{32}P] incorporation in cultures incubated with and without 100 µg/ml L-cycloserine.

It would therefore appear that while the primary effect of cycloserine administration is on the synthesis of sphingolipids, a smaller effect is found affecting both PE and PS synthesis but at a higher (200 µg/ml) concentration only. This could be expected since serine plays a role in the synthesis of these lipids. The results obtained on phosphorus estimations generally reflect those obtained with [^{32}P] incorporation.

The specific effects of inhibition of the initial steps in sphingolipid biosynthesis and hence the whole pathway have not so far been investigated. For example, the function of the phosphosphingolipids CPE and CPG in the membranes of this microorganism is unknown. L-cycloserine may therefore provide a valuable tool with which to probe the role of these complex lipids in membrane metabolism. L-cycloserine may also prove useful in the derivation of mutants of B. levii, in which overproduction of sphingolipids or intermediates may provide models for certain types of the sphingolipidoses.

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