THE EFFECT OF BACITRACIN AND Ca^{++} ON THE FORMATION OF POLYPRENYLPYROPHOSPHATES AND THEIR INCORPORATION INTO UBIQUINONE PRECURSORS IN MAMMALIAN AND BACTERIAL SYSTEMS

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SUMMARY. Formation of ubiquinone precursors, polyprenylpyrophosphate, and 3-polyprenyl-4-hydroxybenzoic acid is greatly inhibited by bacitracin. The antibiotic prevents the transfer of polyprenylpyrophosphate to p-hydroxybenzoic acid in rat liver mitochondria and in cell-free extracts of Rhodospirillum rubrum. In extracts of Micrococcus lysodeikticus bacitracin also inhibits the polymerization of isopentenylpyrophosphate to the longer chain polyprenylpyrophosphates. Ca^{++} also inhibits the formation of polyprenylpyrophosphate and 3-polyprenyl-4-hydroxybenzoate in rat liver mitochondria. The significance of agents which influence the availability of polyprenylpyrophosphate in synthetic pathways is discussed.

Bacitracin is a cyclic polypeptide antibiotic produced by Bacillus licheniformis. Bacitracin has been shown to have a variety of effects in that it inhibits some hydrolytic enzymes (1), interferes with cell wall biosynthesis (2) and inhibits sterol biosynthesis (3).

Stone and Strominger (3) proposed that the action of bacitracin in cell wall biosynthesis is due to inhibition of the enzyme C_{55}-isoprenylpyrophosphate phosphatase, which results from the formation of a tripartite complex between bacitracin, the C_{55}-isoprenylpyrophosphate and a divalent cation. Recently the same authors (4) found that bacitracin inhibited sterol biosynthesis from mevalonate and demonstrated that it occurred by a similar mechanism, i.e., they could demonstrate formation of a complex between bacitracin and farnesylpyrophosphate and a metal ion.

Abbreviations: PHB, p-hydroxybenzoate; MLE, Micrococcus lysodeikticus extract; PPHB, 3-polyprenyl-4-hydroxybenzoate; IPP, isopentenylpyrophosphate; EGTA, ethylene glycol-bis-(β-aminoethylether)N,N'tetraacetic acid; PPP, 2-polyprenylphenol.

Our laboratory has been engaged in a study of the mechanism of ubiquinone biosynthesis (5,6,7). Momose and Rudney (7) have shown that the formation of 3-nonaprenyl-4-hydroxybenzoic acid and 3-decaprenyl-4-hydroxybenzoic acid, the first prenylated precursors in the synthesis of UQ-9 and UQ-10 in rat liver, takes place in mitochondria from p-hydroxybenzoate and IPP. The enzymes isopentenylpyrophosphate isomerase and diprenyltransferase catalyze the formation of longer chain polyprenylpyrophosphate chains from IPP. The enzyme PHB:polyprenyltransferase transfers the polyprenyl chain to PHB to form PPHB. All of these enzymes are present in the inner membrane of rat liver mitochondria. During the course of these studies we reported the profound inhibitory effect of bacitracin on PPHB synthesis (7). In this report we detail the mechanism of this effect, and also describe the inhibition of PPHB formation by Ca^{++}.

Methods and Materials - Rat liver mitochondria were prepared according to the method of Parsons and Williams (8) as modified by Momose and Rudney (7).

Rhodospirillum rubrum was grown photosynthetically in the medium of Ormerod et al. (9) with minor modifications according to Raman et al. (5). A sonicate of R. rubrum was prepared with 0.015 M tricine-KOH buffer, pH 8.0, according to procedures described (5). The sonicate was centrifuged at 48,200 x g for one hour and the supernatant fraction was used.

Micrococcus lysodeikticus cells were stored and grown as previously described (10) and harvested in late log phase of growth. The cells were washed two times with cold distilled water, lyophilized and stored at -20° C. Micrococcus lysodeikticus extract was preincubated with isopentenylpyrophosphate as previously described, as a source of polyprenylpyrophosphate derivatives (5). Momose and Rudney (7) and Allen et al. (11) have shown that these extracts can supply polyprenylpyrophosphate chains ranging from C_{35} to C_{50}.

Table I. The Effect of Bacitracin on the Formation of PPHB in Rat Liver Mitochondria

Bacitracin mg	Radioactivity in PPHB dpm	Inhibition %
Expt. 1		
0	3112	0
0.05	344	89.0
0.10	352	88.5
0.50	334	89.5
1.00	0	100
Expt. 2		
0	1216	0
5.00	141	89
Expt. 3		
0	127,000	0
0.25	2,190	98

Expt. 1. To 1 ml of mitochondrial suspension in 0.1 M phosphate buffer, pH 7.3, prepared as described (7) were added: 10 µl of 1.0 M $MgCl_2$, 10 µl of 0.1 M $MnCl_2$, 10 µl of 1.0 M KF, 0.1 ml of 10 mM IPP, 10 µl of 0.1 mM carboxy-C^{14}-PHB, 55 µcurie/µmole, and various aliquots of a 0.2% bacitracin solution. Incubation time, 2 hours at 37°.

Expt. 2. Endogenous transfer of polyprenyl side chain to PHB. 20 ml of mitochondrial suspension incubated as in Expt. 1 without added IPP. The concentrations of other components were the same as in Expt. 1.

Expt. 3. MLE and IPP incubated for 2 hours as described (5) and 1 ml of this incubation mixture was added to 1 ml of a rat liver mitochondrial suspension containing the same components as in Expt. 1, except no IPP was present.

The incubation conditions in experiments utilizing rat liver mitochondria and the isolation and detection of polyprenyl derivatives of PHB by reverse phase chromatography have also been described previously (7).

Bacitracin was purchased from Sigma Chemical Company and had an activity of 71,400 units/gram.

Results and Discussion - When bacitracin is present in rat liver mitochondrial preparations, which are able to form PPHB from PHB and IPP, the formation of PPHB is inhibited (Table I, Expt. 1). Momose and Rudney (7) noted that PPHB could be formed from PHB and endogenous polyprenyl-

pyrophosphate which was found to be present in rat liver mitochondria. The formation of PPHB from PHB and the endogenous polyprenylpyrophosphate was also strongly inhibited by bacitracin (Table I, Expt. 2). When MLE and IPP are incubated, polyprenylpyrophosphate chains ranging from C_{35} to C_{50} are produced (7,10). When mitochondria are supplemented with a preparation of MLE and IPP preincubated as described (5,7), a large incorporation of PHB into PPHB is observed. The formation of PPHB under these circumstances is primarily reflective of the activity of the PHB:polyprenyltransferase. This activity is also almost completely inhibited by bacitracin (Table I, Expt. 3). These results show that not only is the synthesis of nonaprenyl- and decaprenylpyrophosphate from IPP inhibited (Expt. 1), but the activity of PHB:polyprenyltransferase is also severely reduced (Expt. 2 and 3).

Similar effects are observed when bacitracin is added to R. rubrum preparations capable of forming PPHB, using the system previously described by Raman et al. (Table II). In Expt. 2 it is shown that preincubation of the MLE with bacitracin and IPP prevents PPHB formation. Expt. 3 shows that the PHB:polyprenyltransferase of R. rubrum is inhibited since polyprenyl-pyrophosphate chains are already present. The results of Expt. 2 are explained by the demonstration that bacitracin completely inhibits the synthesis of polyprenylpyrophosphate from IPP in a MLE and in a rat liver mitochondrial preparation (Fig. 1, A and B). In other experiments we have observed that divalent cation chelators such as EDTA and EGTA can prevent the inhibitory effects of bacitracin on polyprenylpyrophosphate formation and PPHB synthesis.

These results agree with and extend to ubiquinone synthesis the observations which Stone and Strominger (3,4) obtained in analogous systems, i.e., the effect of bacitracin on the C_{55}-isoprenylpyrophosphate phosphatase and on the synthesis of isoprenoid precursors of sterols. These data support their postulated formation of a complex between bacitracin, a divalent cation and the polyprenylpyrophosphate.

Table II. The Effect of Bacitracin on PPHB Formation on Cell-Free Extracts of Rhodospirillum rubrum supplemented with Micrococcus lysodeikticus Extracts Preincubated with Isopentenylpyrophosphate

Expt.	Supplement MLE (A)	MLE (B)	R. rubrum Sonicate (A)	R. rubrum Sonicate (B)	Radioactivity in PPHB & PPP dpm
1	1.0 ml	0	1.0 ml	0	7230
2	0	1.0 ml	1.0 ml	0	73
3	1.0 ml	0	0	1.0 ml	72

MLE (A). M. lysodeikticus preincubated with IPP as previously described (5).
MLE (B). Same as MLE (A) except 1.0 mg bacitracin present during preincubation with IPP.
R. rubrum sonicate (A). Prepared as previously described (5).
R. rubrum sonicate (B). Same as R. rubrum sonicate (A) except 1.0 mg bacitracin added.
All other conditions as previously described (5).

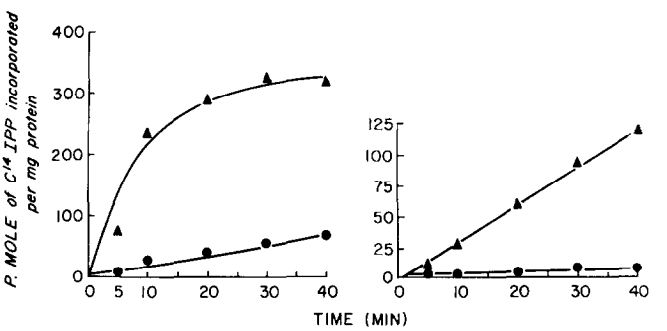

Fig. 1. Effect of bacitracin on the synthesis of longer chain polyprenylpyrophosphate. 0.1 ml of C^{14}-IPP, 60 μcurie/μmole, was added to 5 ml of mitochondrial preparation as described in Table I, experiment 1, omitting the non-radioactive IPP and radioactive PHB. 0.025 ml of the same C^{14}-IPP solution was added to a 5ml preparation of MLE (5). The determination of the extent of polyprenylpyrophosphate synthesis has been described (7). ▲—▲ without and ●—● with bacitracin 0.5 mg/ml.

Mg^{++} is required for the synthesis of polyprenylpyrophosphate chains in rat liver mitochondrial preparations (7). We have observed that Ca^{++} exerts a strong inhibitory effect on PPHB formation in mitochondria even in the presence of Mg^{++} (Table III, Expt. 1), i.e., the concentration

Table III The Inhibition of PPHB Synthesis by Ca^{++}

Experiment	Radioactivity in PPHB dpm
Expt. 1	
Control	6,102
0.2 M Ca^{++}	2,361
0.05 M Ca^{++}	308
0.03 M Mg^{++}	5,454
0.06 M Mg^{++}	4,635
Expt. 2	
Control	2,450
0.0001 M Ca^{++}	2,200
0.001 M Ca^{++}	2,150
0.01 M Ca^{++}	242

Expt. 1. Control consisted of 1.0 ml of rat liver mitochondrial suspension prepared as for Table I, Expt. 1. MLE and IPP were preincubated for 2 hours as previously described (5). 0.1 ml of this mixture and 10 µl of 0.1 mM carboxy-C^{14}-PHB, 55 µcuries/µmole were added and incubated for 30 minutes at 37°.

Expt. 2. 1 ml of rat liver mitochondria incubated in medium and conditions as outlined in Table I, Expt. 1, except that U-C^{14}-PHB was present, 10.9 µcuries/µmole.

In each experiment Mg^{++} and Ca^{++} concentrations do not include endogenous ions present in the mitochondrial preparations.

of Mg^{++} in the control and tubes containing added Ca^{++} is 0.01 molar. The inhibition by Ca^{++} is evident when polyprenylpyrophosphate chains are preformed and added via the preincubated MLE and IPP preparations, or when polyprenylpyrophosphate chains are synthesized in situ from IPP added to the mitochondria (Table III, Expt. 2). This implies that the PHB:polyprenyl transferase is inhibited as well as the synthesis of polyprenylpyrophosphate chains.

We have also observed that a complex relationship exists between the effects of bacitracin and chelators relatively specific for Ca^{++} and Mn^{++}, such as EGTA. These experiments will be reported elsewhere.

In general, it may be expected that a variety of agents which

complex with polyprenylpyrophosphates will exert a profound control on the biosynthesis of a host of biosynthetic pathways which involve polyprenylpyrophosphates as intermediates, e.g., sterols, quinones with isoprenoid substituents, carotenoids, rubber, terpenes, glycoproteins, and cell wall polysaccharides. The nature of this control warrants further investigation.

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