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Growth Inhibition of *Mycoplasma* by Inhibitors of Polyterpene Biosynthesis and Its Reversal by Cholesterol

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ABSTRACT

SMITH, PAUL F. (University of South Dakota, Vermillion), AND CARL V. HENRIKSON. Growth inhibition of *Mycoplasma* by inhibitors of polyterpene biosynthesis and its reversal by cholesterol. J. Bacteriol. 91:1854–1858. 1966.—Compounds which inhibit enzymatic reactions in the biosynthetic pathway to carotenoids inhibited growth of a sterol-nonrequiring species, *Mycoplasma laidlawii*, strain B, and *M. hominis*, strain 07. Since *M. hominis* lacks the enzymes for polyterpene biosynthesis, the inhibitory compounds must act also at other sites. Most inhibitors exerted a lytic effect at bactericidal levels. The inhibition of *M. laidlawii* is reversed by exogenous cholesterol. *M. laidlawii* exhibited a greatly increased content of cholesterol and a greatly decreased content of carotenoids when grown in the presence of phenethylbiguanide and cholesterol. These results are considered as further evidence for a common function for sterols and carotenols in *Mycoplasma*.

Analogous functions have been postulated for sterols and carotenols in *Mycoplasma* (13). This hypothesis is based on (i) the sparing action of cholesterol on carotenoid synthesis by *M. laidlawii* (14); (ii) the ability of certain dihydroxy carotenoids or carotenoid precursors to replace the sterol requirement of certain species of *Mycoplasma* (Henrikson and Smith, *unpublished data*); (iii) the elicitation of sensitivity to digitonin-induced lysis by growth of sterol nonrequiring *Mycoplasma* in the presence of cholesterol (10); (iv) the similarity of molecular dimensions, conformation, and configuration of sterols and carotenols required for support of growth or found in the organisms (Henrikson and Smith, *unpublished data*); (v) the synthesis of glucosides and esters of sterols and carotenols by *Mycoplasma* (11, 14); and (vi) the location of sterols and carotenoids solely in the cell membranes (8, 15). Furthermore, the demonstration of enzymatic blocks in the biosynthetic pathways to polyterpenes in sterol-requiring *Mycoplasma* and the biosynthetic sufficiency for this pathway in sterol nonrequiring *Mycoplasma* indirectly supports this hypothesis (Henrikson and Smith, Bacteriol. Proc., p. 93, 1965; 16).

Contrarily, Razin and Cleverdon (Bacteriol. Proc., p. 92, 1965) concluded that carotenoids do not occupy similar sites in the membranes, since neither increased carotenoid synthesis stimulated by exogenous acetate nor inhibition of carotenogenesis by diphenylamine or thallium acetate affected the sterol content of *M. laidlawii* grown in the presence of cholesterol. Alternate explanations compatible with the hypothesis of analogous function of carotenols and sterols are that all the sites for sterol or carotenol were not saturated under their conditions, as exemplified by the ability of resting cells to incorporate additional cholesterol (15) and that the inhibitors employed by Razin and Cleverdon only prevent the stepwise oxidation of colorless polyterpenes (e.g., phytoene and phytofluene) to the colored polyterpenes. Thus the organisms may hydroxylate the colorless compounds which would satisfy the polyterpenol requirement but which would not have been detected spectrophotometrically.

However, compounds which inhibit enzymatic steps in the biosynthetic pathway to polyterpenes prior to the formation of the first C40 compound should inhibit growth, and this inhibition should be reversed by supplying exogenous cholesterol. The present communication presents the results of such a study.

MATERIALS AND METHODS

Organisms. M. laidlawii, strain B, a sterol-nonrequiring organism which synthesizes carotenoid pig-

ments, was used as the test organism. *M. hominis*, type II, strain 07, a sterol-requiring organism, was employed as a control on the specificity of the inhibitory compounds.

Assay system. M. laidlawii was tested in a medium composed of 2% Difco tryptose, 0.5% glucose, 0.5% sodium acetate, 0.5% sodium chloride, 0.05% lipid free Difco PPLO Serum Fraction, and 0.0003% sodium oleate. The Serum Fraction and sodium oleate were added to permit sterol uptake (15). Varying levels of inhibitor or constant inhibitor and varying amounts of cholesterol, purified through the dibromide, were added as ethyl alcohol solutions. The amount of ethyl alcohol was kept constant at 0.1 ml/10 ml of culture medium. *M. hominis* was tested in the culture medium of Morton et al. (7) supplemented with 1% Difco PPLO Serum Fraction. A 100-ml amount of culture medium inoculated with about 10^8 organisms from 24-hr-old cultures was dispensed in 10-ml amounts and was supplemented appropriately. After 24 hr of incubation, appropriate dilutions from the duplicate 10-ml test cultures were made in phosphate-buffered saline (*p*H 7.4). Duplicate 0.01-ml samples were plated on agar plates without spreading and were counted after 3 days at 37 C (12).

Inhibitory compounds. The following compounds which exhibit inhibitory activity at specific sites in the biosynthetic pathway to polyterpenes or prevent acetate-C^{14} incorporation into cholesterol in mammalian systems were tested: α(*p*-chlorophenoxy) isobutyric acid (1); farnesenic acid (9); vanadium trichloride (2, 18); chlorpropamide (*N*-propyl-*N'*-*p*-chlorobenzenesulfonylurea), tolbutamide (*N*-butyl-*N'*-*p*-toluenesulfonylurea), and phenethylbiguanide (*N'*-β-phenethylformanidinyliminourea) (3); β-diethylaminoethyldiphenyl propyl acetate hydrochloride (SKF 525A) (4); diphenylamine (6); geraniol, farnesol, nerolidol, citronellol, and citronellal (5). All compounds were purchased from K and K Laboratories, Inc., Plainview, N.Y., except phenethylbiguanide, which was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis., and diphenylamine, from Fisher Scientific Co., Pittsburgh Pa. SKF 525A was a generous gift from W. L. Holmes, Smith Kline & French Laboratories, Philadelphia, Pa.

RESULTS AND DISCUSSION

The bacteriostatic and bactericidal levels of each compound for the two species examined together with the reported site of inhibition are given in Table 1. Examples of actual titrations of growth inhibition are shown in Table 2. A very narrow range between the concentration preventing growth and the concentration causing death of the organisms can be noted for many compounds. This, coupled with the experimental error in the method, produced difficulties in demonstrating reversal of inhibition by cholesterol, particularly since the bactericidal level was lytic also (Table 3). Lysis of *M. hominis* also occurs at high concentrations of inhibitors (Table 2). The inability of chlorpropamide and tolbutamide to inhibit growth significantly is compatible with the site of their action, i.e., cyclization of squalene and the nonparticipation of this step in carotenoid biosynthesis. Although α(*p*-chlorophenoxy) isobutyric acid inhibits the conversion of acetate to mevalonate in the intact rat and liver slices, it is ineffective in liver ho-

TABLE 1. *Growth inhibition of Mycoplasma by compounds interfering with polyterpene biosynthesis**

Inhibitor	Site of inhibition	*M. laidlawii*		*M. hominis*	
		Static level	Cidal level	Static level	Cidal level
		M	*M*	*M*	*M*
α(*p*-Chlorophenoxy) Isobutyric acid	Acetate → MVA	$>8 \times 10^{-3}$	—	ND	ND
Farnesenic acid	MVA → MVAP	2.5×10^{-4}	7.5×10^{-4}	ND	ND
Vanadium trichloride	MVA → MVAP	1×10^{-3}	—	5×10^{-5}	1×10^{-4}
SKF 525A†	IPP → DMAPP → GPP	1×10^{-5}	5×10^{-5}	1×10^{-4}	—
Phenethylbiguanide	FPP →	4×10^{-3}	8×10^{-3}	$>4 \times 10^{-3}$	8×10^{-3}
Chlorpropamide	Cyclization of squalene	$>8 \times 10^{-3}$	—	ND	ND
Tolbutamide	Cyclization of squalene	$>8 \times 10^{-3}$	—	ND	ND
Diphenylamine	Oxidation of polyterpenes	10^{-4}	—	ND	ND
Geraniol	Acetate → NSL	3×10^{-5}	1×10^{-4}	7.5×10^{-4}	1×10^{-3}
Farnesol	Acetate NSL	3×10^{-5}	1×10^{-4}	ND	ND
Nerolidol	Acetate → NSL	$>1 \times 10^{-5}$	3×10^{-5}	ND	ND
Citronellol	Acetate → sterol	5×10^{-5}	1×10^{-4}	ND	ND
Citronellal	MVA → sterol	2.5×10^{-4}	7.5×10^{-4}	5×10^{-4}	7.5×10^{-4}

* Abbreviations: MVA = mevalonic acid; MVAP = 5-phosphomevalonic acid; IPP = isopentenyl pyrophosphate; DMAPP = dimethylallyl pyrophosphate; GPP = geranyl pyrophosphate; FPP = farnesyl pyrophosphate; NSL = unsaponifiable lipid; and ND = not done.

† SKF 525A = β-diethylaminoethyldiphenyl propyl acetate HCl.

TABLE 2. *Titration of inhibition of Mycoplasma by selected compounds*

Phenethylbiguanide			SKF525A			Vanadium trichloride		
Molarity of inhibitor	Colony counts/ml*		Molarity of inhibitor	Colony counts/ml		Molarity of inhibitor	Colony counts/ml	
	L-B	07		L-B	07		L-B	07
0	1.5×10^{11}	6.0×10^{9}	0	1.4×10^{10}	9×10^{9}	0	4.4×10^{10}	2.5×10^{9}
4×10^{-6}	1.0×10^{11}	7.9×10^{9}	5×10^{-6}	7.7×10^{9}	5.1×10^{9}	5×10^{-5}	3.5×10^{10}	3.9×10^{8}
4×10^{-5}	9.4×10^{10}	3.7×10^{9}	7.5×10^{-6}	6.6×10^{7}	6.7×10^{9}	7.5×10^{-5}	4.0×10^{10}	2.0×10^{2}
4×10^{-4}	1.3×10^{11}	3.9×10^{9}	1×10^{-5}	1.2×10^{7}	5.9×10^{9}	1×10^{-4}	5.3×10^{10}	0
8×10^{-4}	8.5×10^{10}	4.6×10^{9}	2.5×10^{-5}	2.2×10^{4}	2.2×10^{9}	2.5×10^{-4}	4.0×10^{10}	0
4×10^{-3}	7.8×10^{7}	2.6×10^{8}	5×10^{-5}	0	2.9×10^{6}	5×10^{-4}	2.3×10^{9}	0
8×10^{-3}	0	0	7.5×10^{-5}	0	4.4×10^{5}	$7.5 \times 10^{4-}$	6.3×10^{8}	0

* Colony counts are means of eight determinations. Abbreviations: L-B = *M. laidlawii*, strain B; and 07 = *M. hominis*, strain 07.

TABLE 3. *Lytic action of selected inhibitors on Mycoplasma laidlawii, strain B*

Inhibitor	At zero min	At 10 min and 22 C	At 20 min and 37 C	At 60 min and 37 C
β-Diethylaminoethyl diphenylpropyl acetate HCl (10^{-4} M)	0.525	*0.505	0.362	0.320
Phenethylbiguanide (8×10^{-3} M)	0.525	0.525	0.505	0.445
Geraniol (3×10^{-4} M)	0.525	0.440	0.320	0.320
Farnesol (3×10^{-4} M)	0.525	0.415	0.370	0.360
Citronellal (7.5×10^{-4} M)	0.525	0.420	0.340	0.330
Control with ethyl alcohol	0.525	0.525	0.525	0.525
Control	0.525	0.525	0.525	0.525

* OD at 420 mμ.

TABLE 4. *Reversal by cholesterol of growth inhibition induced with inhibitors of polyterpene biosynthesis (Mycoplasma laidlawii, strain B)*

Inhibitor	Concn of inhibitor	Concn of cholesterol giving complete reversal
	M	M
Farnesenic acid	2.5×10^{-4}	8×10^{-6}
Vanadium trichloride	$\times 10^{-3}$	8×10^{-5} (partial reversal)
β-Diethylaminoethyl-diphenylpropylacetate HCl	$\times 10^{-5}$	4×10^{-6}
Phenethylbiguanide	$\times 10^{-3}$	8×10^{-5}
Geraniol	$\times 10^{-4}$	4×10^{-4} (partial reversal)
Farnesol	$\times 10^{-4}$	4×10^{-4}
	3×10^{-5}	8×10^{-6}
Citronellal	2.5×10^{-4}	4×10^{-6}

TABLE 5. *Titration of reversal by cholesterol of geraniol, farnesol, and phenethylbiguanide induced inhibition of growth of Mycoplasma laidlawii*

Molarity of cholesterol	Colony count/per ml		
	Geraniol (10^{-4} M)	Farnesol (10^{-4} M)	Phenethylbiguanide (10^{-3} M)
0	0	0	5.3×10^{7}
8×10^{-7}	2.0×10^{2}	0	1.2×10^{9}
4×10^{-6}	5.0×10^{2}	0	1.6×10^{9}
8×10^{-6}	1.0×10^{2}	0	7.2×10^{9}
4×10^{-5}	4.7×10^{2}	0	1.1×10^{9}
8×10^{-5}	1.7×10^{4}	0	9.9×10^{10}
4×10^{-4}	1.4×10^{8}	5.7×10^{10}	6.4×10^{10}
No inhibitor	1.2×10^{11}	3.8×10^{10}	9.5×10^{10}

mogenates (1). Its effect on *M. laidlawii* appears to mimic the condition found in liver homogenates. Vanadium trichloride, which is presumed to inhibit cholesterol biosynthesis by prevention of maintenance of adenosine triphosphate (ATP) levels necessary for phosphorylation of mevalonic acid, is more effective against *M. hominis* which lacks all of the enzymes in the pathway to polyterpenes (Henrikson and Smith, Bacteriol. Proc., p. 93, 1965; 16). Thus the specificity of its inhibition is questionable. This qualification can be made for the other compounds tested, for selected types also inhibit *M. hominis*, a sterol-requiring species, albeit at slightly higher levels. But, since *M. hominis* lacks the enzymes reportedly inhibited by the compounds tested, growth inhibition of this organism obviously is not directed toward enzymes in the biosynthetic pathway to polyterpenes. What other sites are involved is not known. One would anticipate that multiple inhibitory sites are involved in *M. laidlawii*, including those concerned with

TABLE 6. *Nature of the unsaponifiable lipid in Mycoplasma laidlawii grown with phenethylbiguanide plus cholesterol*

M. laidlawii with	Dry wt (μg/ml) of culture	Cholesterol (μg/mg, dry wt)	Pigment (OD_{438}/mg, dry wt)
No additions	27.2 ± 0.36	<0.003	0.013
Cholesterol (2.8×10^{-5} M)	37.8 ± 0.06	53.3 ± 1.3	0.010
Cholesterol (2.8×10^{-5} M) + phenethylbiguanide (10^{-3} M)	30.4 ± 1.14	70.9 ± 1.7	0.002

polyterpene biosynthesis. Furthermore, it is possible that different sites of inhibition may exist in the two organisms, since marked differences in their metabolic pathways are known. Thus, *M. laidlawii* metabolizes glucose and has a flavin-terminated respiratory pathway, whereas *M. hominis* is incapable of hexose fermentation but possesses oxidative metabolic pathways and a cytochrome-terminated respiratory pathway (15). Therefore, reversal of inhibition by cholesterol would be a conclusive result, whereas nonreversal would not differentiate between a conclusion that sterol does not substitute for carotenol and a conclusion that some additional effect other than inhibition of carotenoid synthesis is occurring.

Tables 4 and 5 present data on the reversal of growth inhibition by exogenous cholesterol. Only partial reversal was achieved when vanadium trichloride and geraniol were employed as inhibitors. This effect can be explained by the inhibition of ATP formation in the case of vanadium trichloride and by the inability to supply enough solubilized cholesterol in the case of geraniol. Inhibition by farnesol, a compound analogous to geraniol, was completely reversed. Compounds which did not produce significant inhibition of growth were not tested in this system. Hence, it would appear from growth studies that cholesterol effectively replaces the end product of polyterpene biosynthesis in *M. laidlawii*.

An alternative explanation for the reversal by cholesterol of growth inhibition could be merely the prevention of lysis of the organisms, thereby permitting growth to occur. Obviously, cholesterol exerts an antagonism toward the lytic effect as can be noted with geraniol inhibited *M. laidlawii* (Table 5). However, the amount of cholesterol necessary to permit growth exceeds the amount required to prevent lysis.

Resolution of these two possibilities was sought by determination of the amounts of carotenoid and cholesterol in *M. laidlawii* grown under conditions in which phenethylbiguanide-induced growth inhibition was reversed by exogenous cholesterol. Methods used for extraction and analyses of lipids were reported previously (11). As shown by Table 6, the carotenoid content is markedly reduced and the cholesterol level markedly increased in the presence of phenethylbiguanide and cholesterol when compared with the controls. Thus, prevention of biosynthesis of carotenoids in *M. laidlawii* is circumvented by its utilization of cholesterol, a result supporting a hypothesis of analogous functions for sterol and carotenol.

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