*nesota.* The presence of a single precipitation line was indicative of the purity of the product. Furthermore the determination of the content of 2-keto-3-deoxyoctonate (KDO)[5] gave values which were close to those reported by Youngner et al.[2] for purified *S. minnesota* Re glycolipid.

The purified GL was then subjected to electrodialysis according to Galanos and Lüderitz[4]. After the completion of the procedure, the pH of the solution was brought to neutrality by adding triethylamine. EDTA treatment of GL was carried out essentially as described by Rietschel et al.[7]. Prior to interferon induction, the concentration of the treated GL was precisely measured by KDO determination[5].

Interferon induction was tested in explanted mouse peritoneal leucocytes[8]. The cell suspension ($2.5 \times 10^6$ cells/ml) was treated with the GL at concentrations ranging from 0.01 ng to $10^4$ ng/ml and the incubation was allowed to proceed at 26 °C[9,10]. Samples were taken at the 20th h[11]. Interferon production was assayed in L-cells challenged with vesicular stomatitis virus.

A dose-response curve is presented in the figure. From it may be concluded that:

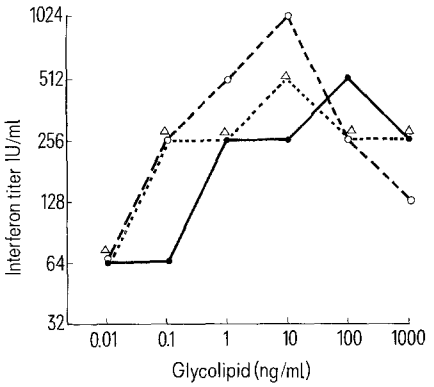
1. The minimal interferon-inducing concentration of the electrodialyzed and EDTA-treated GL comprised $^1/_{10}$ of the concentration of the original GL (0.1 ng vs 1.0 ng).
2. Both GL derivatives elicited peak levels of interferon (optimal interferon-inducing concentration) at concentrations which were 10-fold lower than that of the GL (10 ng and 100 ng, respectively).

When the interferon titer was plotted against the corresponding doses of the GL and its derivatives, a dose-response curve typical for endotoxins was obtained (figure). It should be emphasized that at higher GL concentrations the interferon-inducing activity tended to decrease, which is probably due to cytotoxicity[11].

In addition, it should be mentioned that the ratio of the optimal/minimal interferon-inducing concentration was approximately 100 for the 3 products examined.

The 10-fold increase of the interferon-inducing capacity of the electrodialyzed and EDTA-treated GL may be ascribed to the increased solubility of these 2 products.

Our results suggest that the solubility of the GL of *S. minnesota* Re exerts a marked effect on LPS-mediated interferon induction. This agrees favorably with the data reported by Youngner et al.[2] that the solubilization of GL resulting from partial alkaline hydrolysis or complexing with bovine serum albumine leads to enhanced interferon production.




*Salmonella minnesota* Re glycolipid-induced interferon production in mouse peritoneal leucocytes. ●——● Glycolipid, O– – –O electrodialyzed glycolipid, △·····△ glycolipid treated with 0.2 M EDTA, pH 7.0.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## Specific inhibition of formation of acid-fastness in mycobacteria by 3,3′-di-O-methylellagic acid


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*Summary.* 3,3′-Di-O-methylellagic acid obtained from *Euphorbia adenochlora* selectively inhibited the formation of acid-fastness in mycobacteria without retardation of their growth. Gross reductions in contents of wax D, cord factor and free mycolic acids were found in the nonacid-fast bacilli compared with the normal ones.


In general, acid-fastness of mycobacteria has been understood through the characteristic property of the outer cellular materials to form acid-stable complexes with aminoarylmethane dyes. Several investigators have reported that the mycolate residues were responsible for the acid-fastness of mycobacteria[1,2]. However, Murohashi et al. showed no correlation between amount of mycolic acids and strength of the acid-fastness[3]. On the other hand, another group reported that young mycobacteria revealed nonacid-fastness and exhibited resistance to degradation with lysozyme[4], glycine[4] and some chemical mutagens[5], and the mycobacterial acid-fastness might, therefore, be associated with the integrity of cell-walls.

As the first aim of our work was to investigate the mechanism of acid-fastness, we performed experiments to inhibit specifically the formation of acid-fastness in mycobacteria. We now wish to report that nonacid-fast mycobacteria were successfully obtained from acid-fast cultures after the application of the naturally occurring substance, 3,3′-di-O-methylellagic acid. Although the occurrence of inhibitors

on the development of acid-fastness in mycobacteria has been recognized widely in the plant kingdom[6], few works have been done on the active principle. Among them, the extract of *Euphorbia adenochlora* (Euphorbiaceae) has been shown to exhibit a potent inhibitory effect on the formation of acid-fastness in *Mycobacterium avium* and *M. bovis*[6]. The methanolic extract obtained from the fresh rhizoma of *E. adenochlora* gave crystalline materials, which exhibited inhibitory activity against several mycobacteria at $10^{-4}$ M concentrations. Purification of crystalline mixtures via acetate gave homogeneous pale yellow needles. The substance obtained has the following characteristics: $C_{16}H_{10}O_6$ ($M^+$ 298), mp > 360 °C, UV $\lambda_{max}$ nm (log $\varepsilon$): 248 (4.41), 287 (sh) (3.83), 359 (3.82), 372 (3.87). IR $\nu$ ($cm^{-1}$) (KBr): 3250 (OH), 1720 (lactone), 1610 (aromatic). Acetate (**II**), $C_{20}H_8$ ($M^+$ 382), m.p. 304–305 °C. NMR $\delta$ (ppm) ($CDCl_3$): 2.40 (s, 6H, $2\times CH_3CO$), 4.23 (s, 6H, $2\times CH_3O$), 7.84 (s, 2H, aromatic H). This substance was ascertained as 3,3′-di-O-methylellagic acid (**I**) by direct comparison with an authentic specimen, which was synthesized according to a reported procedure[7].

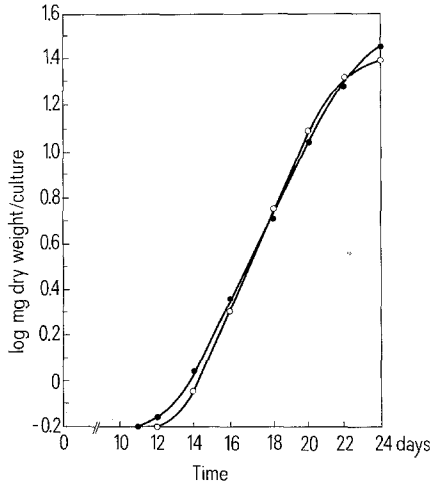



Fig. 1. Growth of *Mycobacterium avium* strain 4121. *M. avium* was cultivated as a surface pellicle on Sauton medium at 37 °C with or without addition of 3,3′-di-O-methylellagic acid. Rates of growth are indicated as dry weight of the bacilli per 5 ml of cultures. Symbols: ●: Control culture; ○: cultivated on a Sauton medium with addition of $6\times 10^{-5}$ M of 3,3-di-O-methylellagic acid.

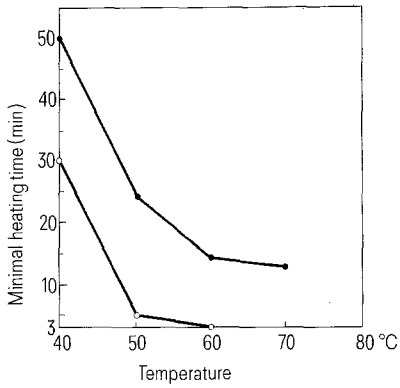
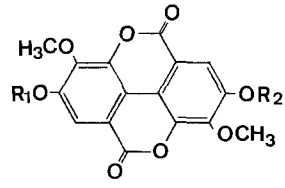



Fig. 2. Heat sensitivities of acid-fast and nonacid-fast *Mycobacterium avium* strain 4121. After cultivation for 4 weeks as described in the text, $3\times 10^8$ bacilli were resuspended in 1.0 ml of steril saline solution and suspension was kept at various temperatures. The heat-treated bacilli were cultivated on a Sauton medium at 37 °C. The minimal heating time on the ordinate shows the minimal incubation time at the temperature indicated until growth of bacilli is not observed by cultivation for 3 weeks. Symbols: ●: Normal *M. avium*; ○: nonacid-fast *M. avium*, which was cultivated on a Sauton medium with addition of $6\times 10^{-5}$ M of 3,3′-diO-methylellagic acid.

When *M. avium* strain 4121 or *M. bovis* strain BCG were cultivated at 37 °C as a surface pellicle on a Sauton medium containing $6\times 10^{-5}$ M of **I** for 4–6 weeks, the bacilli lost their acid-fastness completely. Similarly, 3,3′,4-tri-O-methylellagic acid (**III**) inhibited the formation of acid-fastness, whereas ellagic acid, 3,3′,4,4′-tetra-O-methylellagic acid (**IV**) and the corresponding acetate (**II**) showed no inhibitory activity. Growth curves were investigated under various conditions. As shown in figure 1, there was no difference between growth rates of *M. avium* on the Sauton media with and without addition of **I**. Furthermore, **I** has almost no inhibitory effect on growth of the organisms up to a concentration of $5\times 10^{-4}$ M. The nonacid-fast *M. avium* did not exhibit the cord-like-growth of the normal mycobacteria but showed polymorphous shapes. An inhibitory effect on the formation of acid-fastness was not observed when **I** was added to the medium after the exponential growth.




I: $R_1 = R_2 = H$
II: $R_1 = R_2 = Ac$
III: $R_1 = H$, $R_2 = CH_3$
IV: $R_1 = R_2 = CH_3$

Nonacid-fastness of mycobacteria is well-reflected in the higher heat sensitivity (figure 2). In the nonacid-fast bacilli, the contents of wax D (glycolipids), cord factor and free mycolic acids were markedly reduced[8]. Since mycobacteria show nonacid-fastness during exponential growth, formation of the layer responsible for acid-fastness should occur during the stationary phase. Furthermore, it has been shown that wax D is derived from peptidoglycolipids of cell walls[9]. Reduction of the substances responsible for acid-fastness may be explained by an inhibitory action of **I** on the production of lipids, waxes and mycolic acids. **I** might presumably suppress hydrolysis of peptidoglycolipids of the cell walls without inhibiting cell growth. The absence of detectable substances responsible for acid-fastness in the nonacid-fast mycobacteria shows that the formation of the substances responsible for acid-fastness is not an essential feature of cell growth. From these facts, the mode of action of **I** is restrictive and is distinct from that of isoniazid[10].

The discovery of **I** paves a new approach to the comprehensive understanding of the molecular basis on the formation of acid-fastness in the mycobacterial organisms.

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