Activity of Sodium Ricinoleate Against *In Vitro* Plaque

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Abstract □ The antiplaque activity of sodium ricinoleate was studied *in vitro* against intact preformed plaques of *Streptococcus mutans*. The data obtained suggest that sodium ricinoleate may not interfere with *in vivo* plaque formation by *S. mutans*; however, there is some evidence that sodium ricinoleate may render these plaques less acidogenic.

Keyphrases □ Sodium ricinoleate—activity against *in vitro* dental plaque, bacterial formation □ Dental plaque—activity of sodium ricinoleate against bacterial formation, *in vitro* □ Bacterial formation—activity of sodium ricinoleate against *in vitro* dental plaque

The relationship between structure and bactericidal properties for numerous straight-chain fatty acids and derivatives has been studied (1, 2). The antimicrobial activity of fatty acids was shown to be a function of chain length, unsaturation, conformation, and functional groups. *Cis* monoenoic and dienoic C_{18} fatty acids were the most inhibitory (1), and unsaturation at the 2, 7, or 8 position gave the greatest biological activity (2). Long-chain fatty acids have limited solubility in water, and they are difficult to manipulate in most test situations; therefore, the aqueous soluble salt of a fatty acid which had several of the desirable antimicrobial features identified previously (1, 2) was examined as a novel antiplaque agent. Sodium ricinoleate [$CH_3(CH_2)_5CH(OH)CH_2CH{=}CH(CH_2)_7COO^-Na^+$], a *cis*-monoenoic C_{18} fatty acid salt, but with unsaturation at the 9 position, has attracted considerable attention due to its detoxifying, bactericidal, and anticalculus properties (3–15). Considering these studies and that sodium ricinoleate is extremely surface active, it was hypothesized that sodium ricinoleate could inhibit plaque formation and retention in the oral cavity through three possible modes of action: (*a*) antiadherent effect (coating the teeth with a repellent monolayer), (b) antibacterial effect (attaining bactericidal concentrations at the tooth surface, in the gingival sulcus, and in the intact plaque), and (*c*) detergent effect (breaking up the plaque for mechanical removal).

Although plaque has a mixed bacterial composition *in vivo*, there is a strong association between heavy infection by *S. mutans* and caries in humans and experimental animals (16–20), between the topographical localization of lesions on the enamel with overlying heavy colonization of *S. mutans* on that tooth site (21, 22), and between that colonization event and the subsequent inception of a lesion below the site of colonization (21, 23). Consequently, sodium ricinoleate's antiplaque activity was tested against *S. mutans*—the organism commonly associated with dental caries.

An *in vitro* assay had to be chosen which would reasonably simulate oral conditions and which would allow determination of dose–response relationships. In addition, the assay had to be reproducible, easily executed, inexpensive, and produce results in a minimum of time. *In vitro* tests have been developed in which model plaque specimens are formed on glass, wire, agar, bovine enamel, ceramic hydroxyapatite, and human teeth (24). After assessing the merits of each technique, the wire method (25–28) was chosen as the *in vitro* assay system to be used in this study. Growth of plaque on wires has received criticism in the past because wire is neither tooth enamel nor is it pellicle-coated (29). However, it has been shown (30) that plaque formation by *S. mutans* on enamel correlates closely (with and without salivary pellicle) with that on nichrome wires at both electron microscopic and biochemical levels. The wire model for bacterial attachment is a good predictor of bacterial–enamel interactions with this organism.

EXPERIMENTAL

Materials and Methods—Sodium ricinoleate[1] and chlorhexidine digluconate[2] were studied. *S. mutans* 6715-13 was studied; its properties with respect to a variety of antiseptics is typical of strains of *S. mutans* (26–28). The organisms were maintained by monthly transfer to fresh fluid thioglycollate medium containing 20% (v/v) meat extract[3] and excess calcium carbonate. Cultures were stored at 4°.

Minimum Inhibitory Concentration and Minimum Bactericidal Concentration for Bacterial Suspension—The minimum inhibitory concentration of sodium ricinoleate against *S. mutans* in complex medium (31) with 5% (w/v) sucrose, 0.005% (w/v) sodium carbonate, and bromocresol purple pH indicator (2 mg/liter) was determined by the macrobroth dilution method (32). (The pH indicator was incorporated into the culture fluid to visually detect acid production: a color change from purple (pH > 6.8) to yellow (pH < 5.2) denoted acid production.) The test was then repeated to measure the minimum bactericidal concentration as a function of time for various concentrations of sodium ricinoleate. At hourly intervals, the color of each solution was noted, and one loopful of each solution was plated on blood agar to detect viable organisms.

Minimum Bactericidal Concentration and Minimum Killing Time for Intact Plaque—Plaque was grown on #20 nichrome wires using a previous method (26). In that method 0.1 ml of an overnight culture of *S. mutans* in fluid thioglycollate was transferred to 150 × 18-mm culture tubes containing 10 ml of sterile complex medium (31) with 5% sucrose and 0.005% sodium carbonate. The nichrome wires, fixed to culture tube closures, were suspended in this bacterial broth for 24 hr at 37°. The wires were transferred daily to fresh medium until the wire-adherent microorganisms (*in vitro* plaques) had grown to a McCabe rating of 4 (25). Then the wires were transferred for 2 hr to fresh broth, rinsed free of broth in sterile distilled water, and immersed for varying times in 10 ml of aqueous sodium ricinoleate (1–10%) or control agents (0.2% chlorhexidine[4] and sterile distilled water). After treatment, the plaque samples were rinsed twice in 15 ml of sterile distilled water and transferred to fresh complex medium containing 5% sucrose, 0.005% sodium carbonate, and bromocresol purple pH indicator. The plaque samples were judged to have been killed when culture acid production

[1] N. L. Industries, Bayonne, NJ 07002.
[2] Hibitane, Imperial Chemical Ind., Macclesfield, England.
[3] Difco Laboratories, Detroit, MI 48232.
[4] Chlorhexidine (0.2%) was used as a positive control throughout these studies because the interaction of chlorhexidine with *S. mutans* has been described extensively (26, 33).

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Table I—Effects of Various Concentrations of Sodium Ricinoleate upon Cell Growth, Survival Time, and Acid Production of *Streptococcus mutans*

Concentration, Sodium Ricinoleate, %	Cell Growth[a]	Maximum Survival time, Hr	pH[b]
10	No	0.1	>6.8
5	No	1	>6.8
4	No	2	>6.8
3	No	3	>6.8
2	No	5	>6.8
1	No	7	>6.8
5×10^{-1}	Yes	10	>6.8
2.5×10^{-1}	Yes	12	>6.8
1.25×10^{-1}	Yes	15	>6.8
6.25×10^{-2}	Yes	18	5.2–6.8
3.12×10^{-2}	Yes	22	5.2–6.8
1.56×10^{-2}	Yes	>24	<5.2
0	Yes	>24	<5.2

[a] Detected as an increase in turbidity. [b] As determined by the bromocresol purple pH indicator that was incorporated in the culture fluid.

stopped [*i.e.*, lack of a color change from purple (pH > 6.8) to yellow (pH < 5.2)], by a lack of increase in culture turbidity, and by the failure of 24-hr posttreatment plaque samples to grow when plated on either blood agar or *mitis salivarius* agar.

Penetration Through the Plaque Barrier—Immature wire-adherent plaques were grown to a McCabe rating of 1. The wires were immersed for 10 min in either 10% sodium ricinoleate or control agents (sterile distilled water and 0.2% chlorhexidine[4]), and the antibacterial effects of the agents were tested as detailed above.

RESULTS AND DISCUSSION

Minimum Inhibitory Concentration and Minimum Bactericidal Concentration for Bacteria in Suspension—*S. mutans* was selected as the test organism because it has been identified as a plaque producer associated with lesions (16–23). The minimum inhibitory concentration against *S. mutans* was determined to be 1% (or 3.12×10^{-2} *M*) sodium ricinoleate. During the minimum inhibitory concentration testing, four distinct concentration-dependent cell growth and acid production phenomena were identified: (*a*) no cell growth, medium pH[5] > 6.8; (*b*) cell growth[6], medium pH > 6.8; (*c*) cell growth, medium pH 5.2–6.8; and (*d*) cell growth, medium pH < 5.2. To better characterize these phenomena and to determine the minimum bactericidal concentration as a function of time, another experiment was conducted in which the pH of each culture and the viability of the organisms were examined over a 24-hr period (Table I).

Minimum Bactericidal Concentration and Minimum Killing Time for Intact Plaque—*S. mutans* formed uniform wire-adherent plaque specimens. When the plaque specimens had reached a McCabe rating of 4, they were placed in treatment solutions for 10 min according to the method described in *Experimental*. Although 0.2% chlorhexidine killed the plaque specimens, the sodium ricinoleate (1–10%) treatments and the distilled water controls were unable to do so. The plaques that had been in the sodium ricinoleate treatment group showed a sizable lag time in detectable acid production when compared with the distilled water control group. This lag time increased with increasing concentrations of sodium ricinoleate.

To test the effects of increased treatment time upon plaque viability, those that had reached a McCabe rating of 4 were submerged for varying times in 1% sodium ricinoleate, 0.2% chlorhexidine, or distilled water. Even after 18 hr of treatment, the plaques remained viable in both the sodium ricinoleate and distilled water groups; however, the 1% sodium ricinoleate group showed a marked delay in reduction of medium pH when compared with the distilled water controls (Table II).

The minimum bactericidal concentration of sodium ricinoleate against *in vitro* plaques of *S. mutans* was not established because concentrations of sodium ricinoleate >10% and treatment times >10 min are beyond the conditions that would be clinically acceptable.

[5] As determined by the bromocresol purple pH indicator incorporated in the culture fluid.

[6] Detected as an increase in culture turbidity.

Table II—Effects of Varying Duration of Treatment Upon the Ability of *Streptococcus mutans* in Intact Plaque to Lower the Culture Fluid pH < 5.2[a]

Length of Treatment, Hr	Hours Between Treatment and Detection of Acid: 1% Sodium Ricinoleate	Distilled Water	0.2% Chlorhexidine
1	24	24	—[b]
7	48	24	—
18	72	36	—

[a] As determined by the bromocresol purple pH indicator that was incorporated in the culture fluid. [b] Chlorhexidine (0.2%) kills the plaque within 10 min; therefore, the plaque specimens were not viable and did not produce acid.

It is possible that agent adsorption to saliva-coated surfaces *in vivo* could play a significant role in the pharmacodynamics of any plaque antiseptic. However, this is of significant interest only if the agent shows appreciable potency. The test conditions used (≤7 hr of direct plaque exposure to 1% ricinoleate being required to evidence any antimicrobial effect) strongly suggest that even substantial adsorption–desorption would only trivially affect potency. Thus, no assessment was made of agent binding *per se*.

When the experiments were completed, the ease of plaque removal was examined in the sodium ricinoleate and distilled water treatment groups; in both groups the plaque remained firmly attached to the wires. A qualitative difference was not evident between the two groups.

Penetration Through the Plaque Barrier—Wires with only minimal plaque accumulation (McCabe rating of 1) were immersed for 10 min in 10% sodium ricinoleate, distilled water, or 0.2% chlorhexidine. At the end of the testing, the plaque specimens treated with 10% sodium ricinoleate or distilled water remained viable. Again, a sizable delay in detectable acid production, in some instances as great as 3 days, was observed following the sodium ricinoleate treatment. These data suggest that the outer layer of cells and glucan matrix of *S. mutans* plaque constitute a diffusion barrier to sodium ricinoleate, allowing a small number of *S. mutans* to survive.

CONCLUSIONS

The recognition of the important role of dental plaque in the pathogenesis of caries, gingivitis, and periodontal disease has prompted many investigators to search for chemical agents capable of either inhibiting or removing dental plaque. Useful antiplaque agents may be ones that quickly inhibit bacterial attachment to the tooth surface, kill intact plaque, and/or assist in the removal of attached plaque and matrix. The ideal agent must be effective at a concentration that is nontoxic to humans and nonirritating to the oral mucosa.

In the preliminary minimum inhibitory concentration and minimum bactericidal concentration experiments, sodium ricinoleate was shown to possess bactericidal activity against the representative *S. mutans* strain 6715-13. In later experiments, wire-adherent plaque specimens were treated with various concentrations of sodium ricinoleate for varying lengths of time. In all tests, the intact plaque samples survived. The ability of sodium ricinoleate to kill bacteria in suspension, but not in intact plaque, clearly demonstrates the lack of a correlation between antibacterial activity against cells in suspension and antiplaque activity (9, 26, 29).

The treatment of intact plaque with sodium ricinoleate brought about a noticeable decrease in detectable acid production; the effect became more pronounced with increasing concentrations of sodium ricinoleate. This reduced acidogenicity can be due to the buffering of acidic bacterial end-products by sodium ricinoleate; the increased bacterial killing with increased concentrations of sodium ricinoleate; and/or the reversible effects such as inhibition of metabolic processes, as has been shown for a number of long-chain fatty acids (34). By reducing the acidogenicity of established plaque, sodium ricinoleate may exert a clinical effect upon the cariogenicity of plaque caused by *S. mutans* even if plaque formation by this organism is not totally prevented.

REFERENCES

(1) J. J. Kabara, D. M. Swieczkowski, A. J. Conley, and J. P. Truant, *Antimicrob. Agents Chemother.*, **2**, 23 (1972).

(2) J. J. Kabara, A. J. Conley, and D. M. Swieczkowski, *J. Med. Chem.*, **16**, 1060 (1973).

(3) W. P. Larson, *Proc. Soc. Exp. Biol. Med.*, **19**, 62 (1921).

(4) W. P. Larson and E. Nelson, *ibid.*, **21**, 278 (1924).
(5) W. P. Larson R. D. Evans, and E. Nelson, *ibid.*, **22**, 194 (1924).
(6) T. B. Hartzell, *J. Am. Dent. Assoc.*, **12**, 1452 (1925).
(7) W. P. Larson and H. Eder, *J. Am. Med. Assoc.*, **86**, 998 (1926).
(8) H. E. Jones, *Br. J. Dent. Sci.*, **70**, 171 (1927).
(9) A. S. Hopkins, *Dent. Cosmos*, **72**, 830 (1930).
(10) S. F. Seeley, *Ann. Surg.*, **96**, 350 (1932).
(11) S. V. Mead, *J. Dent. Res.*, **16**, 41 (1937).
(12) W. F. Dossenback and H. R. Muhlemann, *Helv. Odontol. Acta*, **5**, 25 (1961).
(13) H. E. Schroeder, T. M. Marthaler, and H. R. Muhlemann, *ibid.*, **6**, 6 (1962).
(14) D. G. Demmers and C. M. Belting, *J. Periodontol.*, **38**, 294 (1967).
(15) W. van Betteray and P. Riethe, *Caries Res.*, **7**, 85 (1973).
(16) R. J. Fitzgerald and P. H. Keyes, *J. Am. Dent. Assoc.*, **61**, 9 (1960).
(17) R. J. Fitzgerald, H. V. Jordan, and H. R. Stanley, *J. Dent. Res.*, **39**, 923 (1960).
(18) B. Krasse, *Arch. Oral Biol.*, **11**, 429 (1966).
(19) N. W. Littleton, S. Kakehashi, and R. J. Fitzgerald, *ibid.*, **15**, 461 (1970).
(20) J. D. de Stoppelaar, J. van Houte, and O. Backer Dirks, *Caries Res.*, **3**, 190 (1969).
(21) T. Ikeda, H. J. Sandham, and E. L. Bradley, *Arch. Oral Biol.*, **18**, 555 (1973).
(22) S. Duchin and J. van Houte, *ibid.*, **23**, 779 (1978).
(23) B. Kohler, B. M. Pettersson, and D. Bratthall, *Scand. J. Dent. Res.*, **89**, 19 (1981).
(24) R. A. Coburn, *J. Dent. Res.*, **58**, 2396 (1979).
(25) R. M. McCabe, P. H. Keyes, and A. Howell, Jr., *Arch. Oral Biol.*, **12**, 1653 (1967).
(26) J. M. Tanzer, Y. Reid, and W. Reid, *Antimicrob. Agents Chemother.*, **1**, 376 (1972).
(27) J. M. Tanzer, A. M. Slee, B. Kamay, and E. Scheer, *ibid.*, **12**, 107 (1977).
(28) *Idem.*, **15**, 408 (1979).
(29) R. T. Evans, P. J. Baker, R. A. Coburn, and R. J. Genco, *J. Dent. Res.*, **56**, 559 (1977).
(30) N. Tinanoff, J. M. Tanzer, and M. L. Freedman, *Infect. Immun.*, **21**, 1010 (1978).
(31) H. V. Jordan, R. J. Fitzgerald, and A. E. Bowler, *J. Dent. Res.*, **39**, 116 (1960).
(32) J. A. Washington, II., and A. L. Barry, in "Manual of Clinical Microbiology," 2nd ed., E. H. Lennette, E. H. Spaulding, and J. P. Truant, Eds., American Society for Microbilogy, Washington, D.C., 1974, p. 414.
(33) J. M. Tanzer, A. M. Slee, and B. A. Kamay, *Antimicrob. Agents Chemother.*, **12**, 721 (1977).
(34) E. Freese, C. W. Sheu, and E. Galliers, *Nature (London)*, **241**, 321 (1973).

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COMMUNICATIONS

Use of Continuous Withdrawal Technique to Estimate the Initial Area Under the Curve

Keyphrases □ Continuous withdrawal technique—estimation of the initial area under the plasma concentration–time curve □ Pharmacokinetics—use of the continuous withdrawal technique to estimate the initial area under the plasma concentration–time curve □ Clearance—use of the continuous withdrawal technique to estimate the plasma concentration–time curve

To the Editor:

Recently it was pointed out (1) that the instantaneous input hypothesis of the conventional compartmental models may overestimate the area under the plasma concentration *versus* time curve (*AUC*). It was shown that the *AUC* of furosemide could be overestimated, using extrapolation to time zero, by as much as 20% in the dog. This is consistent with data found for Evans blue when used to measure cardiac output (2). In the first 12 sec after an intravenous bolus injection, no dye could be found in the arterial blood; then, the concentration rose gradually, not reaching a peak for several more seconds. Thereafter, it declined for 10 sec before it rose again to a second, lower peak at ~38 sec.

Based on physiological considerations, the delay could be explained as the time needed to reach the sampling site from the administration site, with the second peak perhaps resulting from recirculation of the dye. This phenomenon is important for drugs with a fast initial decline, particularly where the initial phase potentially contains a significant portion of the *AUC*. It is the purpose of this communication to illustrate an ideal method for determination of the *AUC* in the initial period which reflects accurate values, regardless of distribution and elimination rates and sampling sites. This method is based upon the continuous sampling technique described previously (3) to determine various pharmacokinetic parameters, and has been used in our laboratory for several years with good success (4).

A peripheral vein was cannulated prior to the start of the study to facilitate constant blood withdrawal. The drug was then administered in another vein after initiating the constant withdrawal.

The amount of drug in collected plasma, A_w, withdrawn over time t, when a constant blood withdrawal at rate $\dot{V}$ is carried out, can be obtained from:

$$A_w = (1 - H)\dot{V} \int_0^t C dt \qquad \text{(Eq. 1)}$$

where C is the concentration of drug in the plasma at any time, and H is the hematocrit. The area under the curve, $\int_0^t C dt$, in the time period, t, therefore, can be obtained by the ratio $A_w/[\dot{V}(1 - H)]$. Because the plasma drug amount withdrawn is equal to the total volume withdrawn, V_w, multiplied by the plasma concentration in the withdrawn sample, C_w, and because: