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Stickland Reactions of Dental Plaque

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Dental plaque samples from monkeys (*Macaca fascicularis*) were shown to contain proline reduction activity in coupled Stickland reactions with other amino acids and also with certain end products of bacterial glucose metabolism. The unusually high concentration of bound and free proline in the oral environment may be of importance in both the production of base and in the removal of acid from the tooth surface after dietary carbohydrate ingestion.

Proteins of the salivary secretions are unusually rich in the amino acid proline. In human parotid saliva, for instance, proline makes up over 28% of the total amino acid residues (12). This high concentration of bound proline in the oral environment is reflected in the levels of the free amino acid at the tooth surface within the extracellular aqueous phase of dental plaque (7), presumably as a result of proteolysis by members of the oral microbiota (13). Thus, proline occupies a prominent position in the free amino acid pool available for the growth and metabolism of dental plaque bacteria.

We recently reported the presence of a high concentration of δ-NH_2 valeric acid in the aqueous phase of plaque (8). Indeed, in some cases this compound made up 25% of the total extracellular free amino acid pool. δ-NH_2 valeric acid is produced in bacterial systems by either the reductive deamination of ornithine or the reductive ring cleavage of proline (3). Microbial proline reduction is classically associated with the genus *Clostridium*, which utilizes a novel mechanism that takes its name from the author who first described its operation: the Stickland reaction (18, 19). More recently, other amino acid-fermenting anaerobes, notably the peptostreptococci, have been shown to possess similar activity (5). These last organisms are known to form a substantial fraction of the plaque microbial population (14, 16).

The Stickland reaction involves the coupled oxidation-reduction of suitable pairs of amino acids. The reaction may be thought of as three distinct processes: first, the NAD^+-linked deamination of the oxidizable component of the pair to its corresponding keto acid and ammonia; second, the dehydrogenation, again NAD^+-linked, of the keto acid to an acyl coenzyme A and CO_2; third, the oxidation of the NADH generated from the first two reactions by means of an amino acid reductase system (15). Keto acids may participate directly in the reaction provided that they are structurally related to an amino acid. In addition, it is suggested that molecular hydrogen may replace the oxidizable amino acid in the reaction, provided that a hydrogenase system is present (11). Our aim was to examine the possible sources of the reducing power used in the conversion of proline to δ-NH_2 valeric acid in dental plaque.

Whole mouth plaque was removed from monkeys (*Macaca fascicularis*) fed a cariogenic diet (4). The plaque was pooled and stored on ice for no longer than 2 h before use. It was then homogenized in prereduced saline, and samples were incubated with the appropriate substrates at 37°C in capped microcentrifuge tubes. In a preliminary series of experiments, the suspensions were incubated in phosphate-buffered saline, pH 7.0, in the presence of proline (20 mM) and one of a variety of the possible oxidizable substrates commonly found in dental plaque. These included the full range of amino acids, the organic acids produced during the mixed fermentation of dietary carbohydrate by dental plaque organisms, pyruvate, and glucose. After incubation for 4 h, the homogenates were centrifuged at 10,000 × *g* for 15 min at 4°C, and the supernatants were removed. Semi-quantitative analyses of δ-NH_2 valeric acid were performed directly on the supernatants with a Hewlett Packard 5840 gas chromatograph fitted with a Carbowax 20M capillary column equipped with an N/P FID detector specific for the detection of compounds containing nitrogen or phosphorus. Although δ-NH_2 valeric acid is itself nonvolatile, if a sufficiently high injection port temperature is used (270°C), the molecule cyclizes to form its volatile lactam, 2-piperidinone, which can be chromatographically separated in the usual manner. The identity of the 2-piperidinone produced

from both standard solutions of δ-NH_2 valeric acid and sample supernatants was confirmed by GC/MS (manuscript in preparation). This procedure offered a rapid means of screening a wide range of compounds for proline reduction capacity. The rates of proline reduction by alanine, glucose, and lactic acid were examined in a series of timed plaque incubations. Equal volumes (50 μl) of the plaque homogenate (150 mg/ml of saline) were incubated in phosphate-buffered saline, pH 7.0 and 5.5, with proline alone, proline and alanine, lithium-L-lactate, or glucose. All final concentrations were 20 mM except that of glucose, which was 10 mM. The final volume in all cases was 350 μl. At intervals up to 24 h, one tube from each series was chilled on crushed ice and centrifuged as described earlier, and the supernatant was removed. Analysis of the δ-NH_2 valeric acid content was performed on a Beckman 121 MB automatic analyzer with modified citrate buffers for elution (1) and with Trione (Pickering Laboratories, Mountain View, Calif.) for detection.

The amino acids alanine, tyrosine, and arginine, the organic acids pyruvic and lactic acids, and glucose all stimulated the reduction of proline. Plaque incubations containing proline alone showed a low level of δ-NH_2 valeric acid production, presumably as a result of the oxidation of substrates within the plaque sample (Fig. 1). At pH 7.0, glucose (10 mM) and lactate (20 mM) gave identical initial rates of proline reduction (27.3 μmol of proline reduced per g [wet wt] of plaque per h). Since a large fraction of the saccharolytic organisms of plaque in these incubations would be expected to perform a homofermentative metabolism of the added glucose, the proline reduction by glucose may be attributed to the effect of lactate.

The initial rate of proline reduction by alanine was somewhat lower at 17.1 μmol of proline reduced per g (wet wt) of plaque per h. At pH 5.5, the rates of proline reduction by lactate and alanine were 88 and 77%, respectively, of the rates at pH 7.0. Glucose was ineffective at pH 5.5. This may have been a result of the reduced lactate concentrations that were found in these low pH incubations.

In terms of the pathogenesis of the carious lesion, the activity of organisms performing Stickland reactions may have two direct effects. First, the coupled oxidation-reduction of pairs of amino acids leads to the formation of ammonia. A high concentration of ammonia would act as a buffer against the pH fall produced by the microbial fermentation of dietary carbohydrate and hence serve to protect against the demineralization of the tooth enamel. In the case of Stickland reactions with proline as the hydrogen acceptor, one molecule of ammonia is produced per mole-

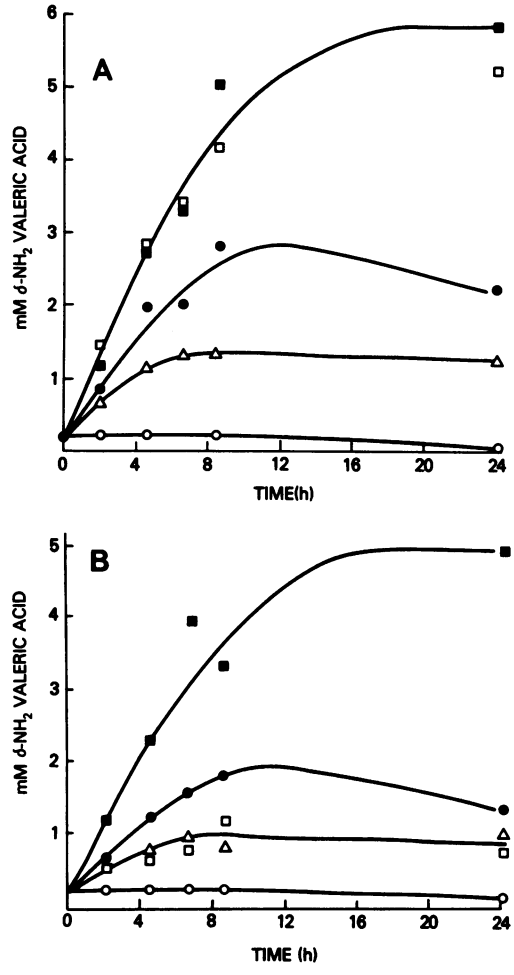

FIG. 1. Rates of production of δ-NH_2 valeric acid in incubations of dental plaque at pH 7.0 (A) and pH 5.5 (B) with no added substrates (○), with proline alone (△), and with proline and alanine (●), lithium L-lactate (■), or glucose (□).

cule of amino acid oxidized. Although it is likely that proline, given its abundance in the oral environment, functions as the main hydrogen-accepting amino acid in dental plaque, other amino acids may also function in this capacity. In such cases, a higher ammonia yield would result. The expected products of such reactions, the straight- and branched-chain volatile fatty acids, have been found in the free fluid phase of plaque (6).

The second effect is that the reduction of proline in dental plaque is stimulated by the presence of lactic acid. This acid is generally considered to be the most cariogenic of the organic acids produced by the oral microbiota because of its frequent high concentration and low pK_a (9). In the mixed microbiota of dental

plaque, certain bacteria, notably the veillonella, are capable of the oxidation of lactate to acetate, propionate, and hydrogen. The molecular hydrogen evolved during such a reaction could be responsible for the proline reduction in the lactate incubations seen in this study. Another and more intriguing possibility, however, is that lactate may participate directly in a Stickland reaction with proline, the requirements for such activity being amino acid-fermenting organisms possessing lactate dehydrogenase. Although organisms of the genus *Clostridium* (2) and at least one member of the genus *Peptostreptococcus* (17) are known to contain lactate dehydrogenase activity, Stickland reactions involving lactate as the hydrogen donor have thus far not been reported. We are currently examining oral isolates for their ability to utilize lactate in Stickland reactions. Whether lactate is used directly or whether the molecular hydrogen from lactate oxidation by the veillonella is the source of the proline reducing activity, the formation of δ-NH_2 valeric acid via the Stickland reaction will lead to a decreased lactate concentration at the tooth surface. This will occur either by a direct action on the lactate or by the removal of hydrogen, causing a shift in the equilibrium of the veillonella reaction.

An abundant supply of proline in plaque has implications beyond those of pH regulation alone. In an anaerobic mixed microbial system such as dental plaque, oxidation of complex organic molecules such as dietary sugars ultimately requires a means of removing electrons from the system. In comparable systems, this is achieved by a variety of mechanisms, including the reduction of nitrate eventually to nitrite or ammonia, the evolution of molecular hydrogen, and methane formation (10). The oral environment may be unique in that it offers another means of hydrogen transfer through the system, namely a high concentration of a readily reducible substrate, proline, and a population equipped to perform the reaction.

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