SOME INTERACTIONS OF REPRESENTATIVES OF THE ORAL FLORA WITH MERCURY

By

Helen Anne Lyttle B.D.S.

A Thesis submitted to the Faculty of Graduate Studies
in Partial Fulfilment of the Requirements for the Degree of

Master of Science

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DEDICATION

To my father David Ryding and to the memory of my mother Ellen.

My parents answered when I first asked "why" and they encouraged me to keep asking.

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SOME INTERACTIONS OF REPRESENTATIVES OF THE ORAL FLORA WITH MERCURY

ABSTRACT

Dental amalgam, which contains mercury, has been used extensively for over 1000 years as a restorative materal but the toxicity of mercury has brought into question the biocompatibility of amalgam. The present study explored some interactions between mercury in amalgam and oral bacteria.

Initially, mercury was shown to be present in dental plaque removed from amalgam and from tooth enamel in persons with amalgam restorations. In contrast, plaque from subjects without amalgam restorations was free of detectable mercury. These results suggested that plaque facilitated the release of mercury from amalgam that subsequently could be absorbed by plaque on enamel at sites remote from restored teeth. In order to explore this possibility an in vitro model was employed, using a biofilm composed of cells of S. mutans and extracellular carbohydrate matrix. Biofilms of S. mutans were grown, in sucrose broth, on amalgam cylinders supported on stainless steel wires. The amalgam was prepared at one time and divided into two aliquots, one was used in the experiments four days after preparation, the other was aged for two years before use. The biofilms on fresh amalgam liberated mercury into the broth around the amalgam and also bound mercury. Assay of the carbohydrate and protein content of biofilms from fresh amalgam relative to biofilms from control wires showed significantly lower amounts of both ($p<0.01$ and $p<0.02$, respectively)). Aged amalgam appeared unaffected by the biofilms, there was no release of mercury, mercury was not detected in the biofilms and there were no significant differences between the carbohydrate and protein levels of biofilms from aged amalgam and control wires. The survival of bacteria on amalgam in vitro and in vivo suggested that bacteria were resistant to mercury, although the degree or variation of resistance was not known. Ten selected strains from two common oral genera, Streptococcus and Actinomyces were tested for resistance to mercury and results showed that growth in the Streptococcus strains declined from 100% in medium without mercury to 11.5% in medium with 40 µg Hg/mL. The resistance of Actinomyces was less, with growth declining from 100% in

the control medium to 4% in medium with 30 μg Hg/mL and no growth in medium with 40μg Hg/mL. There was considerable variation in the degree of resistance shown by the strains of both genera. This variation in resistance was also shown in a further study where the time required for maximum growth, in the presence of mercury, ranged from 13 to 19 hours for Streptococcus strains and 18 to 39 hours for Actinomyces strains.

The resistance phenomenon was also investigated by attempting to adapt the plaque micro-organisms to increasing concentrations of mercury. The results showed that S. mitis 1 and S. oralis adapted to concentrations of mercury up to 65 μg/mL. It was also shown that mercury was bound in both the cell wall and cytoplasm of mercury-resistant and non-resistant Streptococcus strains.

Inhibition of bacterial metabolism by mercury, suggested by the results of analyses of in vitro biofilms was tested further. Results showed that growth, fermentation and hydrolysis were significantly lower ($p<0.04$) in media with 30 and 40 μg Hg/mL than in those with lower concentrations of mercury. Inhibition of enzymes was investigated as a possible cause of decreased metabolism. Enzymes tested in the Streptococcus strains gave a range of inhibition, in medium with 40 μg Hg/mL, of 28% to 100% and in the Actinomyces strains in the same mercury concentration there was 100% inhibition. In lower mercury concentrations some enzymes were unaffected and a few showed increased activity in comparison to the controls which were grown in medium without mercury.

It was concluded from these studies that mercury is found in plaque from dentitions restored with amalgam and the amount of mercury in 24 hr plaque is approximately 8% of the total daily intake, as estimated by the USEPA at 25 μg mercury per day. It was also concluded that biofilms absorb and facilitate the release of mercury from fresh amalgam, that the liberated mercury modifies the physiology of the micro-organisms in the biofilm, that oral bacteria vary in their degree of resistance and can adapt to mercury and that this element is bound in two cell compartments, the cell wall and cytoplasm. Aged amalgam was resistant to the activities of the biofilms, in that there was no detectable mercury and biofilm metabolism was not inhibited. This suggests that release of mercury from aged amalgam in vivo results from physical disruption of a passive surface layer.

SOME INTERACTIONS OF REPRESENTATIVES OF THE ORAL FLORA WITH MERCURY

CHAPTER 1

INTRODUCTION

1.1 AN HISTORICAL PERSPECTIVE

In the course of history, humans have been and continue to be creative in attempting to replace those parts of the body that have been lost or damaged through trauma and disease. The replacement of dental tissue is well known and has an interesting history. The use of ivory, bone, porcelain, precious and semi-precious metals and wood in the fabrication of dentures has been recorded (14, 51) and attempts to restore partially destroyed teeth, using metals and alloys have been described from ancient, through medieval to modern times (14, 52). The search for better materials and techniques continues.

One of the major considerations in the use of foreign materials in the body is that of biocompatibility. This is not only a recent concern because early records suggest that Rhazes, 865-923 A.D. (4), and later (1553) Paracelsus, (107) investigated the biocompatibility of the materials being used in their respective eras.

In the restoration of teeth the material that has caused the most controversy with regard to biocompatibility is dental amalgam (also referred to as silver amalgam), first mentioned in the Materia Medica of Su Kung, 659 A.D.(53). This controversy has waxed and waned since silver amalgam was re-introduced in Paris by M.Taveau in 1826 (14) and in North America in 1833 by the Crawcour brothers from England, leading to what is referred to in dental history as the "Amalgam War" (15, 52)). This conflict was the result of a public declaration by E. Parmley (100) that amalgam was "wholly inapplicable and unfit" for use in the mouth. As a result of the work initiated by Dr. G.V. Black (8) and continued under the auspices of the American Dental Association in conjunction with the National Bureau of Standards (26), the composition of amalgam has been specified and the improved material has been widely accepted by the dental profession.

1.2 THE PRESENT CONTROVERSY

The essence of the current controversy is the toxicity of mercury (4, 83, 100) and the fact that this element makes up approximately 50% of dental amalgam (103). Some investigators have shown that the presence of amalgam restorations causes an increase in the body burden of mercury (90, 92, 108), arguing that the origin of this mercury is that released from the set amalgam restorations.

If it is accepted that mercury is released from amalgam restorations, many questions arise regarding the release mechanism or mechanisms. To understand fully this release of mercury from amalgam restorations, it would be ideal if all the contributing aspects of the oral cavity, as an ecosystem, could be included in the investigations and one of these must surely be the presence of micro-organisms. Little attention has been paid to the possible role of bacterial colonization of amalgam restorations in the release of mercury (99, 84), whereas several authors have postulated the role of abrasion in mercury release (73, 121)

When the complicated process of corrosion of dental amalgam, with the associated release of mercury, is imposed on the the complex nature of the ecosystem of the oral cavity, many questions arise regarding the interactions of the resident bacterial oral flora with amalgam and in particular with mercury. Some parallels may be found in the behaviour of microorganisms and mercury in other ecosystems such as lakes and river estuaries.

1.3 THE ORAL CAVITY AS AN ECOSYSTEM

The oral cavity is a complex ecosystem with soft tissue, hard calcified tissue, secretions consisting primarily of saliva and to a lesser extent crevicular fluid, and micro-organisms. The dynamic interactions of the tissues and the normal oral flora are subject to other factors, for example, food intake, hygiene habits such as dental plaque removal and therapeutic interventions. The latter include antibiotic therapy and topical applications of fluoride and chlorhexidine, that can affect the oral flora, and drug or radiation treatments that can greatly influence salivary flow

In trying to understand a specific ecosystem such as the oral cavity, it often helps to visualize a more general environment and then apply the concepts to the particular. The sterile oral cavity of the newborn infant could be compared to a landscape of a newly

erupted volcanic island where no lifeforms exist. In time, this island will be "inoculated" with lifeforms by the sea, the wind and possibly by birds, but only those that can become established in the barren environment will survive and colonize the area. These original or pioneer populations, which will probably include micro-organisms, will change the environment to some extent, thus providing conditions favorable for colonization of the island by other populations. Over time the environment or ecosystem gradually changes with a resulting primary succession of lifeforms competing with each other to become established, each population being the best adapted to survive the prevailing conditions. These early species colonizing the island are designated as the pioneers. Eventually a balance is reached where the various populations inhabiting the island ecosystem co-exist, but no other populations can gain a foothold on the island. The role each population plays in maintaining its position in the stable community is referred to as its niche and the stable community made up of the various populations is referred to as the climax community. Hutchinson (55) has defined a niche as "an abstractly inhabited hypervolume" and Marsh and Martin (76) more simply, as the role of the population in the community.

If the conditions on the island were changed drastically, for example, by a volcanic eruption or a hurricane, the climax community would be upset and the populations would compete with each other to become established again, thus forming, through secondary succession, a new and possibly different climax community.

The oral cavity can now be visualized as a specific ecosystem that is inoculated with various populations of microorganisms that compete as colonizers and could eventually form a climax community. Ecological changes may occur that upset the balance causing competition and re-colonization by surviving populations or the establishment of new populations. Some factors that provide changes in the oral cavity are toothbrushing, dietary changes, a decrease in salivary flow and the introduction of foreign materials, some of which may be toxic.

The oral commensal resident or normal micro-organisms vary from one person to another and an individual's climax community will depend on the specific environmental pressures of that particular oral cavity. Bacterial populations with similar roles will compete for a niche in the community, with the phenotypic expression of the micro-organism being influenced by the environment (79).

It has been shown that an environmental stress can select a bacterial population with natural resistance. For example, aciduric organisms, such as Lactobacillus, will predominate as the environmental pH falls (81). A mutant may gain ascendancy over the

parent population because of its ability to withstand such stresses as a higher acid concentration or a lower oxygen potential and organisms can adapt to the environment by becoming resistant to the stress. Examples of the latter include resistance to fluoride (45, 47, 9) and mercury (123, 21, 41, 105, 16). The mechanisms for resistance can be phenotypic or genotypic, an example of the latter being the presence of an operon, on the genome, specifically for resistance to mercury (105).

TABLE 1

Predominant genera and species at various stages of tooth eruption

Before Tooth Eruption	Only After Tooth Eruption	Increase After Eruption
Streptococcus mitis	*Actinomyces naeslundii 1*	*Streptococcus sanguis*
Streptococcus salivarius		*Streptococcus mutans*
Lactobacillus		*Actinomyces naeslundii 2*
Actinomyces		
Veillonella		
Prevotella		
Fusobacterium		
Bacteroides		

Colonization of the oral cavity occurs soon after birth, with the infants probably being infected by their mothers and other closely involved care-givers (58, 38, 18) The microflora varies from the predentate to the dentate state, the teeth thus causing an environmental change by providing non-desquamating colonization sites (Table 1), (7, 35)

The distribution of bacteria in the oral cavity varies both qualitatively and quantitatively, with the largest masses of bacteria being found on the dorsum of the tongue and on the teeth. The latter provide sites such as pits, fissures, approximal surfaces and gingival crevices, that are conducive to the development of different communities (11, 12). These communities also differ from those found on the mucosal surfaces (39).

The environmental factors that influence colonization include the oxygen concentration, usually expressed as the redox potential (Eh), the pH of the specific site,

nutrients, the ability of the species to adhere as well as the retentive nature of the site and the effects of the immune system in the oral cavity (35, 79). Changes imposed on the teeth, such as a restoration, may, therefore, affect the composition of the bacterial community by changing the morphology and the physical and chemical characteristics of the site. Whether the shift in populations making up the bacterial community would be localized to the restored area or would affect the whole oral cavity is a matter for debate.

The genera and species of bacteria differ in proportion between those found on teeth and those in saliva, because of the selective abilities of bacteria to attach and thus colonize different surfaces. The retentive nature of the tooth surface will also influence colonization, with pits, fissures and approximal areas offering more protection than the vestibular and lingual smooth surfaces. The largest aggregates of micro-organisms are present on the teeth, embedded in an amorphous matrix, with the total mass being referred to as dental plaque.

1.4 DENTAL PLAQUE

Unlike the acquired pellicle on the teeth, which is almost invisible, dental plaque can be seen as a white or yellowish irregular deposit. This soft, nonmineralized deposit consists of densely packed layers of bacteria surrounded by a matrix made up of extracellular carbohydrate polymers produced by the bacteria and macromolecules and other elements derived from the saliva and crevicular fluid (29, 67, 106). Newbrun (88) records that in a personal communication, Mandel graphically describes dental plaque as "a bacterial aspic with millions of organisms standing shoulder to shoulder"!

Dental plaque, which is an etiological factor for both caries and periodontal disease, has been a topic of interest for many years. In the fourth century B.C. Aristotle related soft, adherent food deposits to tooth decay, in the 17th century A.D. Van Leeuwenhoek described the "animalcules" that he observed in dental deposits with the aid of his microscope, and many others, including G.V. Black, have written about dental plaque (88).

Plaque is tenacious and is not removed physiologically by tongue movements or salivary flow, but requires mechanical intervention, such as toothbrushing, for its removal. Deposits can be found not only on teeth but on artificial materials in the mouth, such as dentures and restorations. Boyar and Bowden (13) have shown that S. mutans, a species commonly found in dental plaque, can colonize amalgam restorations,

Ørstavik and Ørstavik (96) described the ability of S. sanguis to colonize a dental cement and Listgarten et al. (65) demonstrated the formation of dental plaque on epoxy resin crowns. Theilade et al. (113) characterized the plaque on the fitting surface of complete upper dentures and Macpherson et al. (70) compared the plaque microflora that colonized natural and appliance-borne human enamel as well as the acrylic of the appliance.

Although plaque consists principally of bacteria surrounded by a self-synthesized matrix, it is exposed to all the contents of the oral cavity, such as saliva, crevicular fluid, food and ingested liquids, and as such might be expected to absorb some or parts of these materials. This dynamic situation should be considered when attempting to define the constituents of plaque. Keeping this in mind, plaque has been described as being 60-70% bacterial, by volume, with the remaining 30-40% being the matrix (89). The organic components of plaque are mainly carbohydrates, proteins and lipids. The carbohydrate content of the matrix consists of polymers (glucans, fructans and heteropolysaccharides) manufactured by the bacteria. These polymers aid adhesion and cohesion of the cells and may act as a reservoir of fermentable carbohydrates during periods when the more simple dietary sugars are unavailable. Proteins and lipids are constituents of the bacterial cells and are also found in the matrix (106).

The inorganic constituents of dental plaque include calcium, phosphate, fluoride, potassium, sodium, magnesium, copper, lead, iron and strontium. Dawes and Jenkins (29) demonstated that the concentrations of calcium and phosphate in dental plaque are a magnitude higher than in saliva. They suggest that this may be due to salivary proteins, containing calcium and phosphate in bound form, infiltrating the plaque matrix. It is of interest to note that although some heavy metals were identified as constituents of plaque, mercury was not included. There is also a paucity, in the literature, regarding the concentration of mercury in saliva. Windeler (126) in determining the amount of mercury in parotid saliva suggested that the ratios of mercury in parotid saliva to that in blood and urine were 1:4 and 1:10 respectively.

Plaque develops through succession of bacterial populations that adhere to dental pellicle. The post-eruption, or acquired, dental pellicle is an invisible layer of proteins and glycoproteins adsorbed on to the tooth surface from saliva. Bacteria are not required for its formation although a few may be found in the early pellicle (80, 109). As well as the protective and reparative functions suggested for pellicle by Tinanoff (117) and Moreno and Zahradnik (85), that of being a substrate and source of nutrition for

colonizing micro-organisms has been suggested by Krasse (60). The initial adsorption of macro-molecules to clean enamel is one extreme of a continuum and mature plaque is the other with no clear demarcation between them (80).

The first micro-organisms to colonize the pellicle are pioneer populations. By the process of succession, these pioneers are followed by others until a stable or climax community is formed. The adsorbed bacteria are from the saliva and colonize the protected areas first, such as the fissures and the enamel close to the gingiva; hence, the number of available cells in the saliva will greatly influence colonization. In the area close to the gingiva clumps of bacteria are found while the less protected areas will initially only have single cells (64).

The adsorption is selective and not a matter of cells being entrapped by the pellicle; instead, the adherence is dependant on a specific interaction between the constituents of the cell walls, referred to as adhesins, and the macromolecules of the pellicle (42). Adhesion of bacteria is therefore a major factor in the ecology of tooth surfaces.

The pioneer bacteria are usually streptococci with the succeeding organisms being Actinomyces and Neisseria. Streptococcus sanguis is often an early colonizer and Streptococcus mitis and Actinomyces viscosus are also found in early plaque deposits (109, 114, 116). If the plaque is undisturbed, additional species join the community, including rods and filaments. Due to the changing metabolic (pH) and environmental conditions, such as the degree of anaerobiosis (redox potential) the proportions of the different populations change, with rods and filaments becoming more predominant. Preformed plaque encourages colonization by other bacteria. An example of this, is that Porphyromonas gingivalis adheres to the surface of Actinomyces and other Gram positive organisms by means of specific adhesins.

The different environments of habitats such as pits and fissures, approximal areas, exposed root surfaces and the smooth vestibular and lingual surfaces might be expected to have different climax communities (10). The protected approximal area, apical to the area of contact between teeth, would probably be more anaerobic than the vestibular surface and the depths of a fissure would benefit less from the buffering and cleansing effects of saliva than the exposed lingual surfaces. These differences add to the variety and compexity of the oral cavity as an ecosystem.

1.5 MICROFLORA OF THE ORAL CAVITY

Within the ecosystem of the oral cavity there exist various micro- or sub-ecosystems such as fissures of teeth, the approximal, supra- and sub-gingival surfaces of teeth and the mucosal areas including the dorsum of the tongue and the buccal mucosa. Investigations of the these ecosystems reveal similar flora but with differing proportions of the genera and species making up the climax communities. The principal bacterial representatives of the microflora of the oral cavity are shown in Table 2 and the dominant genera are Streptococcus and Actinomyces

Members of Streptococcus can be isolated from all sites in the mouth and represent the predominant genus. In early dento-gingival plaque the most frequently occurring species are Streptococcus mitis, Streptococcus sanguis and Streptococcus milleri with Streptococcus salivarius and Streptococcus mutans making up smaller proportions (116) In another study involving developing plaque in molar fissures, Streptococcus was the dominant genus with the most frequently isolated species, in descending order, being Streptococcus sanguis, Streptococcus salivarius and Streptococcus mutans (114). Streptococcus salivarius is a pioneer species with a preference for epithelial surfaces. The dominant species on the dorsum of the tongue are Streptococcus mitis biovar 2 and Streptococcus salivarius, the latter also being a dominant organism of the pharyngeal mucosa. Initial tooth colonization is primarily with Streptococcus sanguis, Streptococcus mitis biovar 1 and Streptococcus oralis; the former two organisms are also frequently isolated from the buccal mucosa (39). Streptococcus mutans, another common species in plaque, is associated with the aetiology of dental caries and has been shown to be transmitted from mothers to their children (58, 38,18), Streptococcus milleri has also been asociated with the initiation of caries (78).

Another dominant genus in the mouth is Actinomyces. Species of this genera can be isolated from most areas of the mouth particularly from supra-gingival and fissure plaque as well as sub-gingival plaque where the gingiva is healthy (115). The predominant species are Actinomyces israelii and Actinomyces naeslundii, genospecies 1 and 2 (56)

The genus Actinomyces and in particular the species Actinomyces naeslundii 2 (formerly A. viscosus serotype II) (56) is rarely isolated from the mouths of pre-

TABLE 2

The Major Genera and Species of Bacteria Found in Supra-gingival Dental Plaque

Genera	Species
Streptococcus	*mutans*
	sanguis
	oralis
	mitis biovars 1 and 2
	milleri
	salivarius
	gordonii
	angiosus
	vestibularis
Actinomyces	*naeslundii genosp 1 and 2**
	odontolyticus
Corynebacterium	*matruchotii*
Rothia	*dentocariosa*
Lactobacillus	*casei*
	fermenti
	plantarum
Haemophilus	
Veillonella	
Fusobacterium	
Prevotella	*melaninogenica*
	intermedia
Leptotrichia	
Eubacterium	
Bacteroides	
Neisseria	

*Previously A. viscosus serotype II

dentate infants; for like Streptococcus mutans and Streptococcus sanguis, it requires a non-desquamating surface for colonization and adherence (89, 78). Ellen (35) found that Actinomyces naeslundii genosp.1 colonizes the oral cavity of the predentate infant whereas Actinomyces naeslundii genosp. 2 is not established until after tooth eruption. He suggests that as well as adherence characteristics, these findings are related to the fact that Actinomyces naeslundii genosp. 1 colonizes the tongue and is found in relatively high concentrations in the saliva, thus being easily transmitted to the infant from the care-giver's saliva. In contrast Actinomyces naeslundii genosp. 2 colonizes the teeth and hence may not be so readily transmitted by the saliva.

Other Gram positive rods and filaments found in the oral cavity include Eubacterium, Proprionibacterium, Rothia and Lactobacillus (89). The latter, though able to grow at low pH and thus at one time thought to be associated with the initiation of dental caries, is not isolated in great numbers from plaque. Lactobacillus species are more often isolated from carious dentine (77). Theilade et al. (114) found that Lactobacillus was not a dominant genus in non-carious fissure plaque but with regard to root surfaces, Bowden et al. (10) demonstrated a marked association of Streptococcus mutans and Lactobacillus with carious lesions.

Gram negative cocci, such as Veillonella and Neisseria, are represented in the various micro-environments of the mouth, having been isolated from the dorsum of the tongue and supra- and sub-gingival as well as fissure plaque. The contribution of Gram-negative cocci is less in the sub-gingival plaque than in other areas and it decreases as Gram-negative rods begin to dominate in periodontal disease (115).

Gram-negative rods, mainly represented by Prevotella, Porphyromonas and Fusobacterium can be isolated from sub-gingival but not from fissure plaque and their contribution to plaque increases as periodontal health deteriorates (114, 115).

Micro-organisms other than bacteria are part of the oral ecosystem, and include yeasts, viruses, protozoa and mycoplasmas (77)

1.6 ACID PRODUCTION IN DENTAL PLAQUE

Acid production in plaque is reponsible for enamel demineralization and may also have a corrosive effect on restorative materials.

Bacteria derive energy from the breakdown of organic substrates and they also synthesize complex molecules, but one of the most important metabolic activities of many plaque bacteria is glycolysis. Glycolysis or fermentation is the anaerobic catabolism of carbohydrates and it occurs in plaques with reduced oxygen tensions. The end-products have the same empirical formula as the starting substrates but energy is released as a result of the conversion. An example of this reaction is provided by the breakdown of one molecule of glucose ($C_6H_{12}O_6$) to two molecules of lactic acid $2(C_3H_6O_3)$. Oral bacteria do not all produce the same end-products from carbohydrate catabolism because they use different metabolic pathways and environmental conditions, such as availability and specificity of substrates, pH and growth rate, will influence end-products. The products of heterofermentation include lactate, acetate, formate, propionate, butyrate, fumarate and succinate as well as ethanol.

With some streptococci, depending on the environment, 90% of the end-products of sugar fermentation will be lactate. This is an example of homofermentation whereas other organisms, referred to as heterofermentative, will produce a mixture of end-products. Streptococcus mutans is an example of a bacterium that is homofermentative, in plaque, using the Embden-Meyerhof pathway of glycolysis to produce lactate with pyruvate being an intermediary product. However, it is possible that the environment will influence the nature of the acid products even of normally homofermentative bacteria. Streptococcus mutans and Streptococcus sanguis, in glucose-limited cultures will produce acetate, formate and ethanol as their main end-products with only minor amounts of lactate. On the other hand, Streptococcus salivarius will continue to produce mainly lactate with only minor amounts of acetate, formate and ethanol in both glucose-rich and glucose-limited conditions (46).

The pH of the environment will also influence the nature of the end-products of bacterial glycolysis. Hamilton (46) has shown that at low pH, the inherent specific glycolytic activity tends to increase with a concomitant shift to homofermentation.

The acids produced as end-products have differerent properties as indicated by their different dissociation constants (pK). This value indicates the pH at which the acid is half-dissociated, the more dissociated acids providing more H^+ ions and thus being

classified as strong and the less dissociated as weak acids. Hence the tendency for plaque bacteria to shift to the production of the stronger acid, lactate, in an acid environment would be an important factor with regard to both enamel demineralization and metal and alloy corrosion.

Palaghias (97) has shown that different corrosion patterns of amalgam occur in various organic acids. In a six-month study, the greatest dissolution of mercury occurred in a lactic acid solution (0.5%), with a maximum value of 2.39 µg/mL being detected at four months. The maximum values in acetic, formic and succinic acids (0.5%) were 0.05, 0.04 and 0.02 µg/mL respectively, and these maximum levels were detected at six months. In general the dissolution patterns of mercury into the acid solutions appeared to be irregular with the exception of succinic acid, which only had a detectable level of mercury at six months.

1.7 DENTAL RESTORATIVE MATERIALS

In the second half of the twentieth century the materials used as dental restorations are pure and alloyed gold, polymers reinforced with silica-type particles, porcelain and silver amalgam. Gold, gold alloys, filled polymers and porcelain appear to be relatively chemically inert in the oral cavity and only dental (silver) amalgam is subject to varying degrees of tarnish and corrosion, with these processes being involved with mercury release (73, 74, 75, 82).

1.8 SILVER AMALGAM

Baum et al. (5) describe amalgam restorations as being "reasonably compatible with oral fluids" and Strickland and Wilder (110) claim that it is general knowledge that more posterior teeth are restored with amalgam than with any other material. The extensive use of amalgam is the result of the acceptance of this material as biocompatible, by the majority of the members of the dental profession.

According to Phillips (103) dental amalgam consists of a powdered alloy containing silver (70%), tin (17%), copper (12%) and zinc (0.8%) which is mixed with mercury in a 1:1 ratio to form a plastic mass which quickly sets to a solid form. Fairhurst (37) describes the setting reaction as follows,

$$16.78\ Ag_3Sn + Hg \text{ -----> } 12\ Ag_2Hg_3 + Sn_{7-8}Hg + 8.78\ Ag_3Sn$$

The matrix of the set material is the silver-mercury phase, containing approximately 67-70% mercury, and it is the main source of the mercury released from amalgam restorations. In the ternary silver-tin-mercury system, the matrix contains some dissolved tin which confers a degree of passivation to the set amalgam (75). It has been shown that mercury is evolved from freshly mixed and recently set amalgam, but with time, the amount of released mercury decreases and eventually reaches a plateau (2, 25, 74). However, amalgam is prone to corrosion and the oral cavity has an environment that favours this process of degradation. The factors contributing to corrosion include the nature of the amalgam alloy, moisture, temperature fluctuations, changes in the pH and the nature and solubility of the corrosion products. It may be significant that by reducing the pH, acid metabolic products from bacteria may promote corrosion.

1.9 TARNISH AND CORROSION

Tarnish must be mentioned in any discussion of corrosion because, although it is a deposition of oxides, sulphides and chlorides that can provide protection through passivity, it can also be the first step in the process of corrosion (102). It is the breakdown of the passivation provided by the tarnish layer that allows the dissolution of mercury, and the stability of the passive film of salts is dependant on the pH of the saliva remaining in the 4-9 range (36, 75). The passivation of amalgam might suggest some degree of stability but this is not supported by Hoar and Mears (50) who claim that ions can diffuse through the oxide layer of an apparently corrosion-resistant alloy.

Corrosion according to Phillips (102), is an "actual deterioration of the metal by reaction with its environment". The process becomes more convoluted with the increase in complexity of the metal alloy involved and the environment.

There are two general classifications of corrosion, chemical and electrolytic. Chemical corrosion is often associated with electrolytic corrosion in a moist environment and because of the nature of the oral cavity the latter type is predominant.

The electromotive force series is a classification of metals indicating, in order, their tendencies to go into solution. Electrolytic corrosion occurs when there are metals with different potentials immersed in an electrolyte and connected by a conductor. The polyphasic nature of dental amalgam provides the different metals and saliva and bacterial metabolic acids provide the electrolyte; with the bulk of the amalgam acting as the conductor, an electrolytic cell is formed.

Inhomogeneous surfaces of restorations, such as polished and unpolished areas, enhance corrosion, as do variations in the electrolyte (concentration cell corrosion). Electrolyte variations could occur with protected dental interproximal areas having a higher acid content than an occlusal area that is cleaned by salivary flow and food abrasion. Variations in oxygen potential, common in the oral cavity, can also influence corrosion

Amalgam consists of different phases with the silver and tin compound (Ag_3Sn) being referred to as the gamma phase. After reacting with mercury two new phases are formed, $gamma_1$ crystallizes as Ag_2Hg_3 and $gamma_2$ as $Sn_{7-8}Hg$, the latter being more prone to corrosion (103). It would seem then that corrosion resulting in the dissolution of the components of dental amalgam is one of the probable causes for the release of mercury from the set amalgam.

1.10 MERCURY

Mercury is a toxic element (4, 59, 61, 63, 127) which has caused concern to environmentalists and health care workers, with the Minamata Bay disaster probably being the most publicized incident (61). Canadian ecologists have expressed concern regarding pollution of the Great Lakes and major rivers with mercury from industrial plants, such as paper mills, and run-off from fields. The former source is from mercuric salts used in the paper industry as fungicides and the latter from the same salts used by grain agriculturalists as fungicides and pesticides (4)

This element, commonly known as quicksilver, is the only metal that exists as a liquid at room temperature. It occurs in several physical and chemical forms in three oxidation states:-

Hg^0	elemental mercury
Hg_2^{2+}	mercurous mercury and
Hg^{2+}	mercuric mercury

and forms a number of organo-metallic compounds. The short chain organo-mercurials, such as methylmercury, are stable and are toxic, whereas the longer chain compounds such as phenylmercury, are very unstable in mammalian tissues, breaking down to inorganic mercury (22).

1.11 THE ENVIRONMENTAL IMPACT OF MERCURY

Pollution of the environment is a common topic in the news media and public concern is directed towards contamination of water systems with the ensuing effects on forests, wildlife and humans. The contaminating chemicals, including heavy metals, affect the ecosystem, which could be the planet, a continent, a small defined water-system or the oral cavity with its community of micro-organisms. The latter is an ecosystem in which materials such as silver amalgam, gold and porcelain are placed as long-term dental restorations. Although all prosthetic materials used are supposedly biocompatible, they probably have some effect, e.g. physical or chemical, since they are not exact replicas of the natural materials, e.g. enamel, dentin and cementum that they are replacing.

Mercury occurs in the environment from both natural and anthropogenic sources, being found in rocks, soils, air, fresh and sea water (4). Concentrations of this element in the air above rocks, containing mercury, range from 1.6 - 16 parts per billion (p.p.b.) and in unpolluted air from 1.0 - 10 p.p.b.(104, 105).

In rocks mercury occurs naturally as ores, usually combined with sulphur, and in soils from the breakdown of rocks and from the use of mercury-containing pesticides and fungicides. Run-off from the land into rivers and lakes contributes to fresh-water contamination and eventually sea and ocean pollution, with acid rain increasing the amount of mercury in the run-off. Mercury is released by volcanic action as well as the burning of coal and crude oil. Other sources, connected to the activities of humans, include the agricultural and industrial use of mercury compounds as fungicides and disinfectants, an example of this being the pulp and paper industry. Health care systems have contributed to pollution with mercury by the use of mercury-containing cathartics, antiseptics, diuretics and dental amalgam (86, 104, 105). Approximately 1, 000, 000 tons of mercury enter the oceans each year with half the amount coming from natural and the other half from industrial sources (4)

Robinson and Tuovinem (105) report that the mercury deposition in the Greenland ice sheet as measured between 1962-1965, indicated an increase in the output from human sources. They did not make any reference to volcanic activity during that time period that could have contributed to the mercury deposition.

Organic mercury compounds are also pollutants with sewage treatment facilities being a source as well as for inorganic mercury (105). Phenyl mercury has been

detected in carpeted dental operatories and elevated blood levels of methyl mercury have been found in some dentists (27).

1.12 THE TOXICITY OF MERCURY AND MERCURY COMPOUNDS

The toxicity of mercury and organomercurials is described in texts on medicine (63) and toxicology (4) and in the literature of medical specialties (59, 61)

It is interesting to note that ingested mercury is not considered to be toxic because it is not absorbed to any significant degree by the gastro-intestinal tract. According to the USEPA (119) only 10% of the mercury ingested in food is retained. This has been a concern for many years as illustrated by the experiment performed on a monkey by Rhazes (A.D 865-923) to demonstrate the non-toxicity of ingested mercury (4). In contrast, inhaled mercury vapor is considered toxic because as well as being highly absorbed with 80% retention (87), it gains access to cells due to its solubility and lack of charge (4).

Based on the estimations of USEPA with regard to human daily intake, absorption and retention of mercury, it can be calculated that 5.6 µg of mercury is retained in the body (119). This is somewhat higher than the suggested retained value of 2.3 µg by Clarkson et al. (22), but these authors comment that the amount from food may be under-estimated since they only considered fish as a source of mercury.

Several investigators have estimated the amount of mercury that is obtained daily from that released from amalgam restorations. These estimations, ranging from 1.3 µg to 1.7 µg are based on the level of mercury vapor in the oral cavity and hence available for inhalation and ingestion. The amount of mercury thus retained from this source would be approximately 80% of that inhaled (1, 68, 101, 108, 112, 122) and 15% of that ingested (22).

Olsson et al. (94) in their investigation of mercury vapor transport from amalgam restorations in the oral cavity, concluded that mercury is dissolved as atoms (Hg^0) in saliva and is not bound to proteins. Berglund (6) found a positive correlation between the rate of release of mercury into the saliva and the occlusal ($p<0.01$) and total ($p<0.05$) surface areas of amalgam and Moberg (84) suggested that the emission of mercury was least from a buffered solution and was affected by salivary constituents and the acidogenicity of plaque.

The writings of the above authors imply that mercury is released from amalgam restorations in the oral cavity and the possible causes include the chemical and

metallurgical properties of dental amalgam, abrasion of amalgam restorations during mastication and the corrosion of amalgam in an acid milieu.

1.13 RATIONALE FOR THE STUDY

Since all factors, biotic and abiotic, have a role in any given ecosystem, any change in the system, such as the addition of a foreign material, especially one with known toxicity, would be expected to have some effect on the balance of the ecosystem. Thus, if dental amalgam is not inert when set, but continues to emit some mercury, it would be expected that the introduction of dental amalgam, as a restoration in the oral cavity, would have some effects on the balance of this particular environment, due to the toxicity of mercury.

Conversely, a dynamic ecosystem, such as the oral cavity, might be expected to have some effect on a foreign material placed in it.

Therefore, the purposes of the study described below were to investigate the effects of amalgam (mercury) on the oral flora and conversely, the effects of the oral flora on amalgam (mercury).

The study consisted of the following major components:-

1. Determination of the levels of mercury in plaque on:
   - a: amalgam restorations,
   - b: enamel of dentitions with amalgam restorations,
   - c: enamel of amalgam-free dentitions

2. A study of the release of mercury from freshly prepared and aged amalgam cylinders supporting a viable biofilm of Streptococcus mutans (2452).

3. The determination of the degree of mercury-resistance of some oral Streptococcus and Actinomyces.

4. The isolation and identification of the most mercury-resistant micro-organisms from plaque, using enrichment cultures.

5. The determination of the location of mercury within the cells of mercury-resistant and non-resistant bacteria.

6. The study of growth characteristics and some fermentation reactions and enzyme activities of mercury-resistant and non-resistant Streptococcus and Actinomyces strains in the presence and absence of mercury.

CHAPTER 2

MATERIALS AND METHODS

2.1 MICRO-ORGANISMS

The micro-organisms used in the various sections of the study are listed in Table 3.

2.2 CULTURE TECHNIQUES

Freeze-dried micro-organisms were reconstituted with BM (Appendix 1) and plated on to blood agar. Streptococcus were incubated in a chamber (Coy Man. Co., Ann Arbor, MI.) at 37°C for 16 hours in an anaerobic atmosphere (N_2 80%, CO_2 10%, H_2 10%) Actinomyces were placed in a carbon-dioxide-rich atmosphere (candle jar) and incubated at 37°C for 16 hours.

2.2.1 CONFIRMATION OF IDENTITY

The identities of the Streptococcus were confirmed by a series of tests based on those of Kilian et al. (57), as follows: the morphology of the colonies on blood agar and TYC plates and the microscopic appearance of the cells using Gram's stain. In addition to carbohydrate fermentation tests and the hydrolysis of aesculin and arginine (Table 4), the production of catalase, hydrogen peroxide and haemolysins were recorded. The API ZYM (API Lab. Prod. Ltd., St. Laurent, PQ) enzyme activity tests were also used (Table 5). The fluoride sensitivity of the organisms was tested by recording the presence or absence of growth at 10 and 50 ppm fluoride and fermentative activity as indicated by acid production. The latter was demonstrated using pH indicators with end-points of 5.5 and 6.5 respectively.

The identity of the Actinomyces isolates was checked by analysing acid end-products (3) and by serum agglutination (10)

TABLE 3

The Selected Micro-organisms Used in the Various Investigations

GENUS	SPECIES	STRAIN	ORIGIN	SOURCE
Streptococcus	*oralis*	NCTC 11427		National Collection of Type Cultures, U.K.
Streptococcus	*sanguis*	SK112	Oral cavity	Dr. M. Kilian
Streptococcus	*mitis I*	SK138		
Streptococcus	*sobrinus*	B13		
Streptococcus	*milleri*	NCTC 10709		National collection
Streptococcus	*salivarius*	IRH 1		Dr. I. Hamilton
Streptococcus	*mutans*	Pb1094A	Nursing caries	Dr. A. Milnes
Streptococcus	*mutans*	Pb 1094B	Nursing caries	Dr. A. Milnes
Streptococcus	*mitis 2*	SK 103		Dr. M. Kilian
Streptococcus	*mutans*	2452	Child's mouth	Dr. G. Bowden
Actinomyces	*naeslundii 1*	ATCC 12104		American Type Culture Collection
Actinomyces	*viscosus*	T6	Hamster	Dr. G. Bowden
Actinomyces	*viscosus*	NY1 (S)	Rats	Dr. J.S. Van der Hoeven
Actinomyces	intermediate	W752		Dr. G. Bowden
Actinomyces	intermediate	B102	Child's mouth	Dr. G. Bowden
Actinomyces	*naeslundii 2*	WVU627	Human mouth	Dr. M.A. Gerencser
Actinomyces	intermediate	B236	Child's mouth	Dr. G. Bowden
Actinomyces		IW34517	Root caries	Dr. G. Bowden
Actinomyces		IW32710	Root caries	Dr. G. Bowden
Actinomyces		IW36422	Root caries	Dr. G. Bowden

TABLE 4

SUBSTRATES FOR CARBOHYDRATE FERMENTATION

Mannitol	Arbutin
Sorbitol	Salicin
Raffinose	Dulcitol
Melibiose	D-Xylose
Trehalose	Inositol
Amygdalin	α-Methyl-D-Glucose
Inulin	

SUBSTRATES FOR HYDROLYSIS

Arginine

Esculin

2.2.2 MAINTENANCE OF CULTURES

(a) The isolates were maintained by subplating on to blood agar approximately every two days for Streptococcus and every four days for Actinomyces strains; the colonial morphology and Gram staining reactions were checked at each subplating.

(b) Inoculum Broth (Appendix 2) was used to provide inocula for the physiological tests and bulk culture. The broth was inoculated with organisms from the appropriate plate and incubated as described above.

2.2.3 CULTURE OF ORGANISMS AS BIOFILMS

Tryptone Soya Broth with 4% sucrose (Appendix 3) was used in the determination of the mercury released in vitro from amalgam cylinders suspended in sterile broth. Bacteria from the inoculated broth colonized the amalgam cylinders and the supporting stainless steel wires. The amalgam was transferred at 24-hour intervals to fresh tubes of media, and after the first transfer biofilms formed by the bacteria on the amalgam cylinders and supporting wires were visible (See Section 2.9)

2.3 API ZYM SYSTEM (API Lab. Prod. Ltd.)

The API ZYM system is a proprietary semi-quantitative method of detecting the activity of several enzymes by a bacterial stain, (Table 5). A series of microcupules containing dehydrated chromogenic enzyme substrates are rehydrated and the reactions initiated by the addition of an aqueous suspension of the test organisms. The inoculated strips are incubated at 37^{o}C in air for 4-6 hours, following which the detection reagents are added and the resulting colours compared with those on the colour chart provided.

The isolates to be compared are initially grown in Modified Actinomyces Defined Medium (Appendix 4B), with suspensions being prepared to a standardized optical density from centrifuged bacteria and deionized water.

TABLE 5

The API ZYM System for Detecting Enzyme Activity

MICROCUPULE	POSITIVE	NEGATIVE	ENZYME
1		colourless or colour of specimen	Control
2	violet	colourless	alkaline phosphatase
3	violet	colourless	esterase (C4)
4	violet	colourless	esterase lipase (C8)
5	violet	colourless	lipase (C14)
6	orange	colourless	leucine amino-peptidase
7	orange	colourless	valine amino-peptidase
8	orange	colourless	cystine amino-peptidase
9	orange	colourless	trypsin
10	orange	colourless	chymotrypsin
11	violet	colourless	acid phosphatase
12	blue	colourless	phospho-hydrolase
13	violet	colourless	α -galactosidase
14	violet	colourless	β galactosidase
15	blue	colourless	β-glucuronidase
16	violet	colourless	α glucosidase
17	violet	colourless	β glucosidase
18	brown	colourless	N acetyl-β glucosaminidase
19	violet	colourless	α mannosidase
20	violet	colourless	α fucosidase

Preparation, Inoculation, Incubation and Assessment of the Strips:

1. The incubation tray and lid are assembled and labelled.
2. Approximately 5 mL of tap water is placed in the tray to provide a humid atmosphere.
3. The API ZYM strip is removed from its sealed wrap and placed in the incubation tray.
4. A suspension is prepared from the pellet of cells centrifuged from a broth culture of the test organism and 0.5 mL of the suspension is dispensed into each microcupule with an Eppendorf Combi-tip, (Brinkman Indust. Co., Rexdale, ON)
5. After inoculation, the lids are replaced on the trays, which are incubated for four hours at 37°C in air and away from bright light.
6. After incubation, one drop of each of the reagents A and B are added to each microcupule and five minutes is allowed for the colour to develop. Exposing the tray to the light of a 1000 watt bulb for 10 seconds will ensure that the negative reactions become colourless.
7. The colours that develop are assigned a value from 0-5, with 0 being a negative reaction and 5 indicating maximum colour and hence maximum reactivity. The value is assessed by comparing the test colour with those on the provided colour chart.

2.4 VIABLE COUNTS OF THE NUMBER OF COLONY-FORMING UNITS (CFU) ON PLATES FOLLOWING INCUBATION

A series of dilutions of cell cultures in broth were plated onto blood agar which was incubated for 48 hours. The regular distribution of CFU's using the spiral plater (Spiral System, Clough Pike, Cincinnati, OH) allowed the number of CFU's in a sector of the plate to be counted and thus the total number on the plate calculated, using the formulae provided with the instrument.

2.5 DETERMINATION OF MERCURY CONCENTRATIONS

2.5.1 The mercury concentrations in the various experimental samples (see below) were determined by cold vapor atomic absorption spectrophotometry (CVAAS)

The apparatus used was a Perkin Elmer 4000 (Perkin-Elmer Corp., Norwalk, CT), with an attachment for mercury assays, and the method is based on the evolution of the mercury from a sample by reduction, using sodium borohydride,

The following is a simplified representation of the chemical reaction:-

$$Hg^{2+} + 2BH_4^- \text{ ------> } Hg + H_2 + B_2H_6$$

The test sample was prepared, ensuring that the mercury was in its ionic form in an acid solution. The addition of the reducing agent ($NaBH_4$) produces a reaction reducing mercury to the elemental state. Nitrogen (99.99% pure) is used in the pneumatic system of the apparatus to drive the $NaBH_4$ into the reaction flask.

Because of the volatility of mercury at ambient temperatures, the mercury vapour is driven along the apparatus by the hydrogen, produced during the reaction, to the quartz tube for the absorption measurement.

The source of light was a hollow cathode lamp and the wavelength of the light used for absorbance was 253.6 nm.

The details of the apparatus and the procedure are contained in the operator's manual of the MHS-10 Mercury/Hydride System (Bodenseewerk Perkin-Elmer & Co. GmbH, Ueberlingen, Germany).

In determining the standard curve, standard mercuric chloride solutions (Fisher Scientific, Fairlawn, NJ) containing 0 to 300 ng of mercury were used, because in this range, the absorption is linearly related to concentration. Likewise all test samples were prepared at the appropriate dilution to ensure that the mercury concentration was within this same range.

All the chemicals and reagents used were of reagent grade and were individually assayed for mercury before use.

The mercury concentrations appear as a digital representation on the Perkin-Elmer 4000 and are transcribed electronically to graph paper. The height of the peak produced represents the mercury concentration and the actual concentration can be calculated from the curve plotted using the standards and their equivalent peak heights.

2.5.2 RECOVERY OF A KNOWN CONCENTRATION OF MERCURY FROM BACTERIAL CELLS

Streptococcus mutans (2452) was grown for 24 hours in MADM without added mercury. The cells were separated by centrifuging (Sorvall Industries, Dupont, Mississauga, ON), washed three times with sterile, deionized water and freeze-dried.

The weight of an aliquot of the freeze-dried cells in a glass tube was determined, following which 400 µL of concentrated nitric acid and 50 µL of mercury standard, equivalent to 50 µg of mercury (Fisher Scientific, Fairlawn, NJ) were added. The glass tubes were sealed and placed in a heating block (Pierce Reacti-Therm Heating Module, Pierce Chem. Co.,Rockford, IL) at 80°C, for approximately 30 min until the cells had dissolved.

After being cooled, the solution of cells and mercury in concentrated nitric acid was placed in a 25 mL flask and made up to 25 mL with deionized water. Fifty µL, 100 µL, 150 µL and 200 µL aliquots of this solution were assayed for mercury by CVAAS.

The correlation between the amount of mercury added to the cell solution and the amount recovered was calculated from the regression curve. This curve was plotted using the actual results of the assays and expected results (standards) for the "x" and "y" axes respectively

The assays for the recovery of a known amount of mercury from the nitric acid extracts of cells are shown in Table 6 The mean peak heights represent the amount of mercury present in the standard solutions and the cell solutions to which a known amount of mercury had been added. A comparison shows very similar mean peak heights for the same mercury concentration in the standards and the cell solutions.

The peak heights for the solutions with 100 ng of mercury were identical at 3.8 cm and for the solutions containing 400 ng of mercury the two values were 13.1 and 12.5 cm. The percentage recovery of mercury from the cell solutions was 100,% 97.2,% 96.1% and 95.4% for the 100, 200, 300 and 400 ng of mercury solutions respectively. Thus the percentage recovery of mercury decreased as the total amount in the solution increased. The correlation, $r^2 = 1$, between the amounts recovered from the samples of the "spiked' cell solution and the amounts added to the cell solution was determined from the regression curve.

2.5.3 MEASUREMENT OF THE IMPACT OF WATER WASHES ON THE LEVELS OF BOUND MERCURY IN STRAINS OF STREPTOCOCCUS.

Two Streptococcus strains were used, a mercury-resistant strain, Streptococcus mitis I (Sk138) and a mercury-sensitive strain Streptococcus mutans (PB1094 A), as determined below (Section 2.13).

Modified Actinomyces Defined Medium (MADM) was used with the addition of 40 µg/mL and 5 µg/mL of mercury for S. mitis and S. mutans respectively.

Each test strain was grown in duplicate in 1 L of MADM without mercury for 24 hours and 1 mL of this suspension was used for inoculation of the mercury-containing MADM. The remaining cell suspension was centrifuged (Sorvall Industries, Dupont, Mississauga, ON) to separate the cells which were then washed three times with deionized water and freeze-dried. These cells provided the mercury-free control.

This procedure was repeated with the cell suspensions in mercury-containing MADM, but the separated cells were divided into two aliquots, one being washed 3 times and the other 6 times, with deionized water, after which the cells were freeze-dried.

The cells were prepared as in Section 2.5.2., omitting the addition of the mercury standard solution. The concentrated nitric acid cell solutions were cooled and then made up to 10 mL and assayed for mercury as described in Section 2.5.1.

The results of the mercury assays for the control cells (medium without mercury) and those grown in a mercury-containing medium and receiving either three or six washes with deionized water, upon removal from the medium, are shown in Table 7. The controls for both strains, Streptococcus mutans (Pb1094A), a non-resistant strain, and Streptococcus mitis I (SK138), a mercury-resistant strain did not contain any detectable mercury.

For the non-resistant strain, Streptococcus mutans (Pb1094A), the bound mercury concentrations after three washes were 1.44 and 1.47 µg /mg and after six washes, 1.54 and 1.55 µg/mg. For the mercury-resistant strain, Streptococcus mitis (SK138), the bound mercury concentrations after three washes were 3.85 and 3.21 µg/mg and after six washes 2.93 and 4.25 µg Hg/mg. These results indicated that the mercury content of the cells was stable.

TABLE 6

The Mercury Assays of the Standard Solution
and the Cell Solution with a Known Amount of Added Mercury

Solution		Total Hg Content: ng	Peak Ht. cm Mean (sd)	% Recovery of Hg From the Cell Soln.
Blank		0	0.3 (0.06)	
Standard	1	100	3.8 (0.10)	
	2	200	7.1 (0.15)	
	3	300	10.4 (0.15)	
	4	400	13.1 (0.23)	
Cell Soln and Hg	1	100	3.8 (0.06)	100.0
	2	200	6.9 (0.11)	97.2
	3	300	10.0 (0.25)	96.1
	4	400	12.5 (0.10)	95.4

TABLE 7

The Mercury Assays of Streptococcus mutans (Pb1094A) and Streptococcus mitis I (SK138) Grown in Medium Containing Mercury.

The Cells Received either Three or Six Washes before being Freeze-Dried

Test		S. mutans (Pb1094A) Hg conc: µg/mg	S. mitis (SK138) Hg conc: µg/mg
Control	1	0.00	0.00
	2	0.00	0.00
3 Washes	4	1.47	3.85
	5	1.44	3.21
6 Washes	6	1.54	2.93
	7	1.55	4.25

2.6 IDENTIFICATION OF A MEDIUM WITH A LOW POTENTIAL FOR BINDING MERCURY, THAT WOULD SUPPORT THE GROWTH OF STREPTOCOCCUS AND ACTINOMYCES STRAINS

Since the studies involved testing the effects of various concentrations of mercury, in the medium, on the bacteria, it was necessary to use media that would not bind mercury to a significant extent. Bound mercury would not be available to interact with the organism and this could give inaccurately high levels for mercury resistance.

In order to design a medium that would support adequate growth, yet not bind mercury, a comparison was made of the growth of test micro-organisms on blood agar plates with that on Actinomyces Defined Medium (Appendix 4A) agar plates. The latter was chosen because of its low peptide content and thus its low potential for binding mercury.

One hundred µL of the test inoculum was placed in 1 mL of Reduced Transport Fluid (RTF), (Appendix 5) and vortexed to obtain an even distribution of organisms in the suspension. Dilutions of 1:10, 1:100, 1:1000, 1:5000 and 1:10,000 were prepared in RTF and 50 µl-aliquots were plated on to the two media using a spiral plater (Spiral System).

The plates were incubated anaerobically for 24 hours, following which the CFU on the plates were counted and the total number of CFU's on the plates calculated (Table 8).

TABLE 8

Mean CFU Count on Blood Agar and Actinomyces Defined Medium(ADM)

Medium	Colony-forming Units
Blood agar	10.55×10^6
ADM	4.11×10^6

Although the growth on ADM was less than 50% of that on blood agar, it was considered satisfactory because of the medium's ability to support the test strains. Thus

ADM was selected as a low-mercury-binding medium for use in mercury sensitivity assays.

When a solution of mercuric chloride, with 5% mercury (6.765 g in 100 mL water) was added to ADM to provide 5, 10, 20, 30 and 40 µg/mL, a black precipitate occurred. However, when ferrous sulphate ($FeSO_4$) was eliminated from ADM the addition of mercuric chloride did not cause a precipitate. It was decided to use ADM without the addition of $FeSO_4$ and this medium is referred to as Modified Actinomyces Defined Medium (MADM), (Appendix 4B).

2.7 A COMPARISON OF THE MERCURY-BINDING PROPERTIES OF BLOOD AGAR AND MADM AGAR

Two test agars, MADM agar and blood agar, were prepared with the addition of 5% Hg, as $HgCl_2$, to provide a concentration of 25 µg of mercury /mL. Control agars without mercury were also prepared.

Ten dialysis cells were prepared by tying a knot at one end of 30-cm lengths of No. 6 wet cellulose dialysis tubing with a molecular weight cut-off of 1000 (Spectrum Medical Ind. Inc., Los Angeles, CA), with four cells for each of the test agars and one cell for each control.

Two g of the prepared agars was placed in each of the dialysis cells with 5 mL of deionized water and, after the open end was tied off, each cell was suspended in 12 mL of deionized water in a test-tube.

After 5 hours 1-mL aliquots were removed and stored at 4^0C and an equal volume of deionized water was added to each test-tube to maintain the original volume of the test system. Aliqots were removed in a similar fashion after 24 and 48 hours.

The samples were assayed for mercury by CVAAS and the concentrations of mercury in the fluid surrounding the dialysis membrane were expressed as percentages of the available mercury. The percentage availabilities at 5, 24 and 48 hours are shown in Figure 1.

2.8 DETERMINATION OF THE MERCURY CONTENT OF PLAQUE ON AMALGAM AND ENAMEL

Fifteen volunteer subjects were included for this study, 10 of whom had amalgam restorations that had been in place for at least two years. This ensured that any mercury detected was not part of that typically released from freshly mixed amalgam. The other five subjects had no dental restorations.

The nature of the study was explained and the volunteers signed a consent form, indicating that they understood the possible consequences of refraining from oral hygiene for 24 hours (Appendix 6).

The subjects refrained from flossing and brushing their teeth for 24 hours, following which the plaque was removed from amalgam and enamel surfaces with sterile teflon-coated plastic-filling instruments (American Dental Manufacturing Co., Missoula, MT). It was considered that this type of instrument was less likely to remove particles of amalgam than a similar instrument made of steel. The subjects were then encouraged to floss and brush their teeth.

The plaque samples from each subject were placed separately in 5-mL aliquots of sterile deionized water and stored at -10^{o}C.

This procedure was repeated four, five or six times, for different subjects, in order to obtain adequate amounts of plaque, and subsequent to thawing, the amalgam plaque samples for each subject were pooled, as were those from enamel.

The pooled suspensions, approximately 30 mL each, were centrifuged and the pellet was resuspended in 1 mL of sterile deionized water to facilitate transference into previously weighed and labelled tubes for freeze-drying, following which the weight of the freeze-dried plaque was calculated. It had been previously determined that repeated washings did not remove mercury from the cells.

Nine hundred µL of concentrated nitric acid was added to each weighed sample of the freeze-dried plaque, the glass tubes were sealed and placed in a heating block (Pierce Reacti-Therm Heating Module, Pierce Chem. Co., Rochford, IL) at 80^{o}C until the plaque was completely dissolved, following which the solutions were made up to 1000 µL When necessary, the sample solutions were further prepared in known dilutions so that the concentrations were in the same range as the standard mercury samples. The total recovery of mercury by this method had been previously verified, as described in Sections 2.5.2 and 2.5.3.

The mercury contents of known volumes of the plaque solutions were determined by CVAAS, as described in Section 2.7 and the mercury contents of the freeze-dried plaques calculated, values being expressed as ng of mercury per mg of dry plaque.

2.9 RELEASE OF MERCURY FROM FRESH AMALGAM BY BIOFILMS

Streptococcus mutans (2452) was grown anaerobically in Tryptone-Soy Broth (Oxoid, Canada) for 16 hours at 37°C as described in Section 2.2. The experimental and control tubes were inoculated with 100 µL of this broth culture.

Amalgam (Dispersalloy Lot 5113, Johnson and Johnson Inc. Montreal,.PQ) was prepared according to the manufacturer's instructions. The test cylinders were formed by condensing the amalgam around the serrated end of a stainless steel orthodontic wire (size 028) placed centrally in a stainless steel mold. All the specimens were condensed by the same operator within a two-hour time period, and the amalgam cylinders and support wires were sterilized by ethylene oxide.

The tests were carried out on a series of 12 tubes. Four were designated experimental and labelled E1, E2, E3 and E4 and eight tubes formed duplicate sets of four controls. The latter were labelled C1A and B, C2A and B, C3A and B and C4A and B. The contents of the tubes are described in Table 9 Briefly, E1-4 were identical, each containing 15 mL of medium inoculated with 100 µL of broth culture and the amalgam cylinder attached to the stainless steel wire which was suspended over the lip of the tube. Control tubes all contained 15 mL of medium with the following additions. C1 contained amalgam on the stainless steel wire but was left uninoculated. C2 contained only the stainless steel wire (no amalgam) and was inoculated. The third control, C3, contained the amalgam and stainless steel wire, and was uninoculated but had its pH adjusted to pH 4.0 by the addition of 1 M hydrochloric acid. The fourth control, C4, consisted of inoculated medium without amalgam or stainless steel.

After 24 hours growth at 37°C in an anaerobic chamber, the amalgam specimens and wires including any biofilms (adherent bacteria and associated glucans) were transferred aseptically to tubes containing fresh sterile medium. Daily transfers were completed in this manner over the course of the study.

TABLE 9

Contents of the Experimental and Control Tubes for the Determination of the Effect of a Biofilm on Amalgam

TUBE	MEDIUM	AMALGAM	S.S. WIRE	INOCULUM	HCl
E 1,2,3,4	+	+	+	+	-
C1 A and B	+	+	+	-	-
C2 A and B	+	-	+	+	-
C3 A and B	+	+	+	-	+
C4 A and B	+	-	-	+	-

The numbers of viable bacteria in the inoculating broth and the spent media were determined as described in Section 2.6 Spent media from the uninoculated control tubes (C1 and C3) were omitted from this treatment, after the absence of any bacteria had been confirmed.

Spent media from the tubes were centrifuged at 8,000 *g* for 20 minutes (Dynac Centrifuge: Clay Adams, Div. of Becton, Dickinson and Co., Parsipany, NJ) and the cell pellets and supernatants were stored frozen (-20^{o}C) in glass to avoid adsorption of mercury by plastic (44). The pH of the stored supernatant medium samples was recorded before freezing. The viability, identity and purity of the micro-organisms in the media were confirmed at the end of the experiment.

The bacterial deposits from the wires and the amalgam cylinders were removed, suspended in 2.0 mL of deionized water and stored frozen. The biofilms were removed using a Teflon-coated instrument (American Dental Manufacturing Co.) to avoid scratching the surfaces of the amalgam and wire. The biofilms from the wires and amalgams and the bacterial cell pellets and supernatants were analyzed for mercury by CVAAS (Section 2.7).

The viability, identity and purity of the bacteria in the biofilms were confirmed at the end of the experiment.

2.10 CARBOHYDRATE ASSAY OF THE BIOFILMS:

The dry weight of biomass in 100 µL of the suspensions (amalgam and wire biofilms in deionized water) was determined and the cell pellets from control C4 were weighed. Carbohydrate content was assayed by the phenol, sulfuric acid method of Dubois et al. (33), as described below, with the colorimetric measurements being made at 490 nm on a LKB-Biochrom, Ultraspec 4050 (Cambridge, England).

PHENOL-SULFURIC ACID - CARBOHYDRATE ASSAY

Standard: 100 µg/mL (10 mg glucose in 100 mL deionized water)

Standard Curve:

CARBOHYDRATE CONTENT	VOLUME OF STANDARD SOLN.	VOLUME OF WATER
blank	0 µL	1000 µL
20 µg	200 µL	800 µL
40 µg	400 mL	600 µL
60 µg	600 µL	400 µL
80 µg	800 µL	200 µL
100 µg	1000 µL	0 µL

All samples were prepared in duplicate

One mL of a 5% phenol solution was added to each sample, followed by 5 mL of concentrated sulfuric acid. The samples were allowed to stand for 20 minutes during which time an exothermic reaction occurred producing a yellow-brown colour. The colorimetric readings (absorbance) were made at a wavelength of 490 nm and the standard curve was obtained by plotting the carbohydrate concentrations against the absorbance readings.

The dry weight of biomass in 100 µL of suspension was measured and the cells re-suspended in 1 mL of deionized water. These suspensions were used for the carbohydrate assays by treating them in the same way as the standards. If necessary,

appropriate dilutions of the test samples were made to ensure that the concentrations fell within the standard curve.

2.11 PROTEIN ASSAY OF THE BIOFILMS:

Disruption of the biofilms was accomplished by lysing the cells with sodium hydroxide, and the protein content from a known weight of biofilm was assayed using the Bio-Rad Protein Assay dye reagent (Bio-Rad Laboratories, Richmond, CA), as described below. The colorimetric measurements were made at 595 nm on a LKB-Biochrom, Ultraspec 4050.

PROTEIN ASSAY WITH BIO-RAD DYE REAGENT

Standard: 1 μg/μL (10 mg albumin in 10 mL of deionized water)

Standard Curve:

PROTEIN CONTENT	VOLUME OF STANDARD SOLN.	VOLUME OF WATER
blank	0 μL	100 μL
20 μg	20 μL	80 μL
40 μg	40 μL	60 μL
60 μg	60 μL	40 μL
80 μg	80 μL	20 μL
100 μg	100 μL	0 μL

All samples were prepared in duplicate.

Two hundred mL of a 1:4 dilution of the Bio-Rad dye reagent was prepared and 5 mL of this solution was added to each sample. The reaction with protein produces a blue colour and the colorimetric readings (absorbance) readings were made at a wavelength of 595 nm.

The dry weight of biomass in 50 μL of suspension was measured and the cells were re-suspended in 50 μL of deionized water. One hundred μL of 0.1 N NaOH was added and the solution was heated at 80^{o}C to lyse the cells, at which time the solution became translucent. The pH values of the solutions were adjusted to 7.0 and the volumes made

up to 1 mL with deionized water. These solutions were used for the protein assays by treating them in the same way as the standard samples for the absorbance readings. If necessary, appropriate dilutions of the test samples were made to ensure that the concentrations fell within the standard curve.

2.12 RELEASE OF MERCURY FROM AGED AMALGAM BY BIOFILMS

The investigation as decribed in Section 2.9, was repeated after 30 months, during which time the unused amalgam cylinders were stored in the sterile wrappings in air at room temperature. This time period allowed the amalgam to "age" and thus develop a passive oxide layer on the surface. The experimental protocol was refined as follows.

The experimental tubes (E) were prepared in duplicate, as were the control tubes (Table 9), and the cell pellets were freeze-dried (f.d.) so that the mercury concentration of all the cells could be calculated instead of only the viable cells. This latter refinement was based on the idea that some mercury was probably present in the dead cells of the assayed mass.

Other than the above noted refinements, the materials and method of the repeated study were as described in Section 2.9.

2.13 DETERMINATION OF THE SENSITIVITY OF SELECTED ORAL ORGANISMS TO MERCURY

Micro-organisms: The bacteria used in this investigation are listed in Table 10:

Culture Technique: As described in Section 2.2.1 The initial growth of each organism was used for verification of its identity and purity:

Preparation of the MADM agar Plates: The sterile modified Actinomyces defined medium was prepared and divided into six aliquots. Mercury was added, as $HgCl_2$, to achieve the following concentrations: 0, 5, 10, 20, 30 and 40 µg/mL, and sufficient plates were poured for each strain to be plated in two dilutions in duplicate for each concentration of mercury.

Inoculation and Incubation of the Plates : After 16 hours of incubation, dilutions of the culture were prepared and distributed on the plates using the spiral plating technique (Section 2.4):

MERCURY CONC IN MADM	DILUTIONS FOR PLATING
0 µg/mL	10^{-3}
	10^{-4}
5 µg/mL	10^{-3}
	10^{-4}
10 µg/mL	10^{-3}
	10^{-2}
20 µg/mL	10^{-3}
	10^{-2}
30 µg/mL	10^{-2}
	10^{-1}
40 µg/mL	10^{-2}
	10^{-1}

The plates were incubated for 24 hours anaerobically at 37°C and the resulting growth recorded by counting the CFU (Section 2.4)

For each strain, the mean count of CFU's for the two dilutions for each mercury concentration was calculated and the growth on the mercury-containing plates was expressed as a percentage of that on the control plates without mercury.

TABLE 10
The Bacteria Used For Assessment of Resistance and Non-Resistance to Mercury

SPECIES	STRAIN or SOURCE	STRAIN NUMBER
Streptococcus	*sanguis*	SK112
Streptococcus	*oralis*	NCTC11427
Streptococcus	*mitis I*	SK138
Streptococcus	*sobrinus*	B13
Streptococcus	*milleri*	NCTC10709
Streptococcus	*salivarius*	IRH 1
Streptococcus	*mutans*	Pb1094A
Streptococcus	*mutans*	Pb1094B
Streptococcus	*mitis II*	SK103
Streptococcus	*mutans*	2452
Actinomyces	*naeslundii I*	ATCC12104
Actinomyces	*viscosus*	T6
Actinomyces	*viscosus*	NYI (S)
Actinomyces	(intermediate)	W752
Actinomyces	(intermediate)	B102
Actinomyces	*naeslundii II*	WVU627
Actinomyces	(intermediate)	B236
Actinomyces	from root caries	IW4517
Actinomyces	from root caries	IW32710
Actinomyces	from root caries	IW36422

2.14 ISOLATION OF THE MOST MERCURY-RESISTANT MICRO-ORGANISM(S) FROM A SAMPLE OF PLAQUE USING AN ENRICHMENT CULTURE

Plaque from the lingual enamel of a lower right first permanent molar, of a dentition with some amalgam restorations, was removed and placed in 5 mL of Reduced Transport Fluid (Appendix 5) The sample was sonicated (Kontes Scientific Glassware, Vineland, NJ) for approximately one minute to produce an even dispersion of the organisms throughout the suspension. The modified Actinomyces defined medium (Appendix 4B) was used for the enrichment culture in bottles and the same medium with the addition of agar was used for the plates. The latter, along with blood agar plates, were inoculated with the micro-organism(s) for identification. Before dispensing the medium and the agar-medium into the bottles and plates respectively, 5% Hg, as $HgCl_2$, was added to achieve the following concentrations:- 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65 and 70 µg of Hg/mL.

Initially, the bottles containing medium with 1 to 25 µg Hg/mL were inoculated with 20 µL of the plaque suspension. These tests were labelled A to F and were cultured anaerobically for 24 hours, as described in Section 2.2. The presence of growth, determined visually, was noted and 20 µL from each bottle was used for inoculation of the fresh medium with the next higher mercury concentration, e.g. 20 µL from the bottle containing 1 µg Hg/mL was used for inoculation of the bottle of fresh medium containing 5 µg Hg/mL. This pattern was repeated every 24 hours. When growth did not appear to have occurred, the bottles were incubated for a further 24 hours and in some cases growth was then obvious. Since it was not known whether this was due to adaptation of the organism(s) or the loss of mercury from the medium, it was decided to record only the 24-hours growth experience for this study.

When growth was not apparent after 24 hours, a sample from that particular bottle was plated undiluted on to a plate with the same mercury concentration as the medium of the bottle and a sample from the medium with the highest mercury concentration that sustained growth was plated at 1:100, 1:1000 and 1:10,000 dilutions. These plates were incubated anaerobically, as before, and any colonies present were noted and subplated on to blood agar plates for later identication.

Media containing the higher concentrations of mercury, 30 to 70 µg Hg/mL were used as the study progressed and when no further growth occurred, the enrichment culture was stopped. The micro-organism that grew in the highest concentration of mercury in the medium was identified by the tests described in Section 2.2.1.

2.15 A COMPARISON OF THE LOCATION OF MERCURY WITHIN THE BACTERIAL CELL IN SELECTED MERCURY-RESISTANT AND MERCURY-SENSITIVE BACTERIA.

Modified Actinomyces Defined Medium (MADM) was used with the addition of 40 µg/mL and 5 µg/mL of mercury for S. mitis and S. mutans respectively.

Each strain was grown in duplicate in MADM for 24 hours in 1 L of the medium without the addition of mercury. This suspension was used for inoculation of the mercury-containing medium. The remaining suspension was centrifuged to separate the cells which were then washed three times with deionized water and freeze-dried. These cells provided the mercury-free control.

This procedure was repeated with the suspensions in the mercury-containing medium and the separated cells were washed three times as before with deionized water and freeze-fried.

A suspension in deionized water was prepared from a known weight of the freeze-dried cells.

Cells were disrupted using a Mickle Tissue Disintegrator (Mickle Engineering Co., Surrey, U.K.). Glass beads (40/60 mesh) were placed in each bottle of the disintegrator to the level of the second indentation from the bottom and the cells in deionized water were added leaving a space of 1.5-2.0 mm below the stopper. Approximately 3 drops of octanol were added to prevent frothing. The bottles were placed in the machine which was run at the maximum amplitude.

When the breakup of the cells had been achieved, as indicated by a negative staining by Gram's stain, the beads were allowed to settle and the supernatant was poured off and retained in a graduated cylinder. The beads were washed free of cell fragments by mixing with deionized water, shaking and allowing to settle, the supernatant being added to the original.

The supernatant suspension was centrifuged at 2,000 *g* for 10 min to remove any remaining whole cells and beads and was then centrifuged again at 13,000 *g* for 20 min to separate the walls and the adherent membranes from the cytoplasm. The pellet of walls and the cytoplamic supernatant were both saved and the volume of the cytoplasmic supernatant was recorded before it was stored at 4°C.

The pellet of cell walls was suspended in 0.2 M phosphate buffer (pH 8.0) and treated with 0.5 mg/mL of Pronase (B.D.H. Toronto, ON), with 3-4 drops of toluene being added as a preservative The suspension of cell fragments and the enzyme was

incubated at 37°C for 16 hours and then centrifuged at 13,000 *g* for 30 min. The pellet containing the cell walls was washed with deionized water and centrifuged as before and the volume of the supernatant was recorded before it was stored at 4°C. The pellet of cell walls was freeze-dried.

Solutions of known weights of freeze-dried cell walls were dissolved in nitric acid as described in Section 2.5.3 and appropriate dilutions of these and the cytoplasm solutions were assayed for mercury as in Section 2.5 All samples were assayed in duplicate.

2.16 THE IMPACT OF VARIOUS CONCENTRATIONS OF MERCURY IN THE MEDIUM ON THE GROWTH CHARACTERISTICS OF SOME MERCURY-RESISTANT AND NON-RESISTANT STRAINS.

The strains of bacteria (Table 11) were cultured as described in Section 2.2, and 100 µL of inoculum broth for each organism (Section 2.2.2(b)) was added to 15-mL aliquots of MADM without mercury. After 24 hours growth, 10 µL of the MADM inoculum for each strain was added to the 15-mL aliquots of medium containing 0, 5, 10, 20, 30, and 40 µg Hg/mL

All the tests were performed in duplicate and the identity and purity of each strain was confirmed at the beginning and end of the growth periods.

Growth curves for each micro-organism were prepared by measuring growth by the change in optical density.

Optical density readings (turbidity) were recorded in a Klett-Summerson photoelectric colorimeter with a red filter (640-700 nm) (Klett Manufacturing Co., Inc., NY). The readings for the freshly inoculated tubes were recorded and again after 8 hours when growth was apparent. Readings were then recorded every two hours until no further growth occurred. The rate of growth of each test organism, for each mercury concentration, was determined by plotting the optical density against time in hours.

TABLE 11

The Bacteria Used for Assessment of Growth and Enzyme Activity in the Presence of Mercury.

GENUS	SPECIES	STRAIN	Hg RESISTANCE
Streptococcus	*mitis 1*	SK138	Resistant
Streptococcus	*mutans*	2452	Resistant
Streptococcus	*milleri*	NCTC 10709	Non-resistant
Streptococcus	*mutans*	PB1094A	Non-resistant
Actinomyces	*viscosus*	T6	Resistant
Actinomyces	*naeslundii 1*	ATCC 12104	Resistant
Actinomyces	(intermediate)	B236	Non-resistant
Actinomyces	*naeslundii 2*	WVU627	Non-resistant

2.17 THE IMPACT OF VARIOUS CONCENTRATIONS OF MERCURY IN THE MEDIUM ON THE FERMENTATION AND HYDROLYSIS CHARACTERISTICS OF SOME MERCURY-RESISTANT AND NON-RESISTANT STRAINS.

The same strains were used for this investigation (Table 11) as for that described in Section 2.16.

The media used for the fermentation plates were modified Actinomyces defined medium agar (Appendix 4B) and Streptococcus Sugar Base agar (Appendix 7). The sterile media, with bromo-cresol purple as the indicator, were prepared with agar, and the various sterile sugar solutions (0.5%) and 5% Hg were added aseptically before pouring the plates. The concentrations of mercury were, 0, 10, 20, 30, and 40 µg Hg/mL

The inoculum (Section 2.2.2 (b)) of each test micro-organism was streaked onto the plates and incubated as before (Section 2. 2) for 24 hours, after which time the presence or absence of a colour change indicating the production of metabolic acids was recorded. The presence or absence of growth on the plates was also noted.

2.18 THE IMPACT OF VARIOUS CONCENTRATIONS OF MERCURY IN THE MEDIUM ON THE ENZYME PROPERTIES OF SOME MERCURY-RESISTANT AND NON-RESISTANT STRAINS.

The API ZYM method of assessing the activities of constitutive and inducible enzymes was used in these tests (Section 2.3). Four Streptococcus and four Actinomyces strains (Table 11) were grown in MADM, with mercury present as the inducer. The concentrations of mercury were 0, 10, 20, 30, and 40 µg Hg/mL. Three tubes of each mercury concentration per strain were incubated so that sufficient organisms were available to produce a sufficiently turbid suspension for the API ZYM tests. The API ZYM strips were prepared and inoculated with the bacterial suspensions for each mercury concentration. The enzyme activities, as evidenced by the colour development, were recorded numerically, following the colour chart provided by the manufacturer.

CHAPTER 3

RESULTS

3.1 A COMPARISON OF THE MERCURY-BINDING PROPERTIES OF BLOOD AGAR AND MADM AGAR.

The results of the dialysis cell experiment for assessment of the mercury-binding properties of blood agar and MADM agar are shown in Figure 1. When the mercury-containing agar was added to the deionized water in the cell, the mercury concentration within the cell was 2.6 µg Hg/mL (100%).

After five hours, 1.04 and 0.0 µg Hg/mL were present in the fluid outside the dialysis cells for MADM and blood agar respectively. The media concentrations of mercury were similar after 24 and 48 hours, both being 1.3 µg Hg/mL for MADM and 0.09 µg Hg/mL and 0.08 µg Hg/mL, respectively, for blood agar. These values are expressed as percentage availabilities in Figure 1, with MADM having 40%, 50% and 50 % at 5, 24 and 48 hours, respectively, and blood agar having 0%, 3.5% and 3.1% availabilities after the same time periods.

3.2 THE MERCURY CONTENT OF PLAQUE ON AMALGAM AND ENAMEL

The amount of plaque collected from both amalgam and enamel sites varied considerably among the subjects and more plaque was present on enamel than on amalgam surfaces (Table 12). The ranges for enamel and amalgam were 1.2-10.8 mg (mean 4.0 mg) and 1.1-7.1 mg (mean 2.5 mg), respectively, from the subjects with amalgam restorations. The range of the much higher weights of plaque collected from the entire dentitions of subjects without restorations was 27.6-47.0 mg (mean 37.0 mg).

The mercury content of the plaque from enamel and amalgam sites of the restored dentitions (Table 12) also presented a wide range of values, 0.01 - 0.54 µg Hg/mg plaque from enamel and 0.19 - 1.31 µg Hg/mg plaque from amalgam, with the means (SD) being 0.20 (0.19) and 0.72 (0.34) µg Hg/mg plaque, respectively. No mercury was detected in the plaque from the unrestored dentitions.

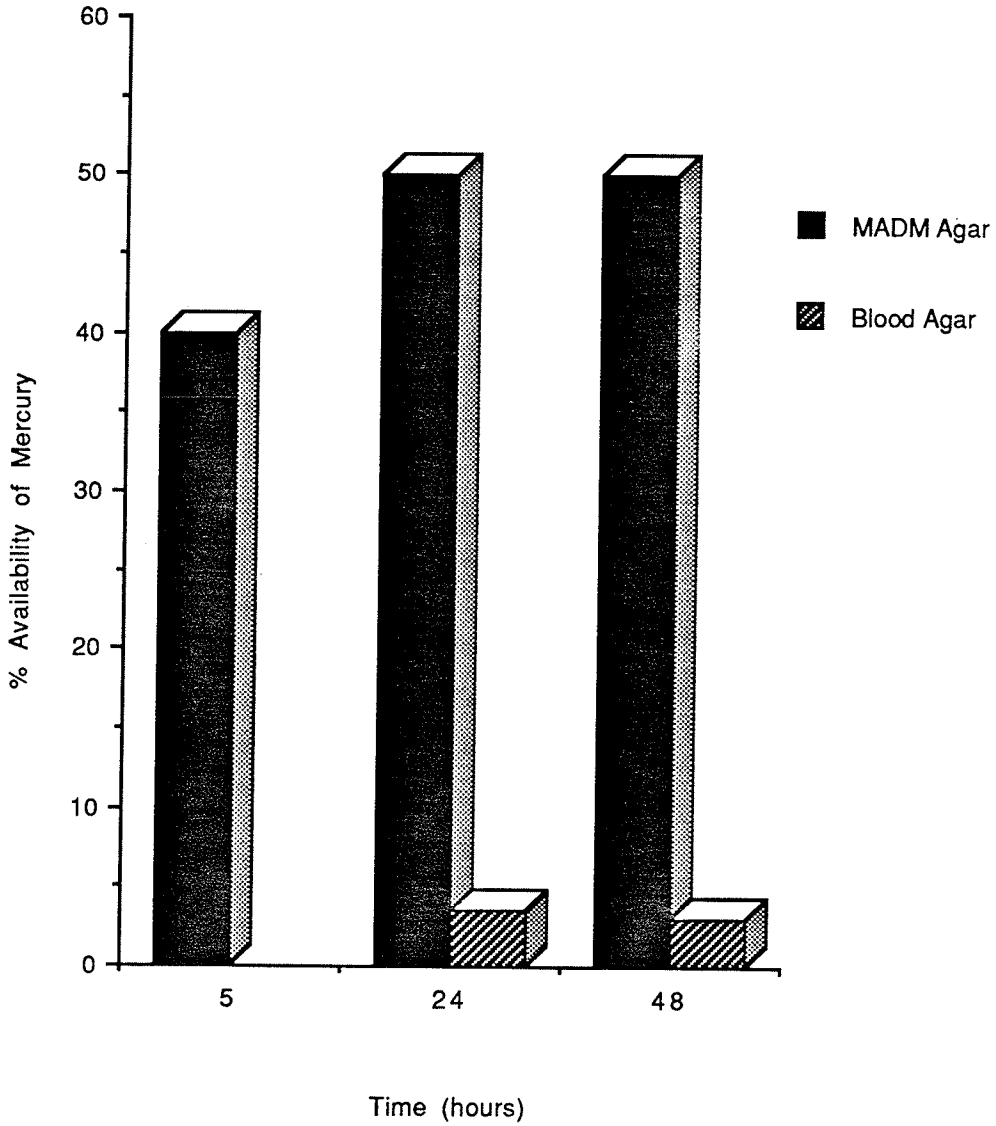

FIGURE 1

Mercury Binding by MADM and Blood Agars

TABLE 12

Mercury Content of Plaque From Amalgam and Enamel Sites

Subjects 1-10 Amalgam restorations present
Subjects 11-15 No amalgam restorations

SUBJECT	SOURCE	PLAQUE WT mg	Hg CONTENT µg	µg Hg/mg PLAQUE
1	amalgam	1.1	1.08	0.98
2	amalgam	2.5	1.71	0.68
3	amalgam	3.5	2.72	0.78
4	amalgam	1.8	0.98	0.54
5	amalgam	7.1	1.37	0.19
6	amalgam	1.1	1.20	1.09
7	amalgam	2.2	0.72	0.33
8	amalgam	1.6	1.31	0.82
9	amalgam	1.1	1.44	1.31
10	amalgam	2.9	1.44	0.50
			Mean (SD)	0.72 (0.34)
1	enamel	1.8	0.49	0.27
2	enamel	3.2	0.17	0.05
3	enamel	4.1	2.20	0.54
4	enamel	1.2	0.62	0.52
5	enamel	10.8	0.63	0.06
6	enamel	1.5	0.25	0.17
7	enamel	3.8	0.58	0.15
8	enamel	3.0	0.30	0.10
9	enamel	4.1	0.40	0.10
10	enamel	6.6	0.10	0.01
			Mean (SD)	0.20 (0.19)
11	enamel	27.6	0	0
12	enamel	34.3	0	0
13	enamel	40.2	0	0
14	enamel	47.0	0	0
15	enamel	35.7	0	0
			Mean (SD)	0 (0)

TABLE 13

The Mercury Concentrations of the Enamel and Amalgam Plaque of the Subjects with Amalgam Restorations, Expressed as a Ratio

SUBJECT	RATIO
1	1 : 3.6
2	1 : 13
3	1 : 1.5
4	1 : 1.5
5	1 : 3.3
6	1 : 6.6
7	1 : 2.1
8	1 : 8.2
9	1 : 13.4
10	1 : 33.3

A comparison of the mercury levels of the plaque from both sites for each subject with restorations is shown in Table 13 as ratios, the range being 1:1.1 - 1: 33.3.

The statistical analysis of the results showed that there was significantly more mercury content in the plaque from amalgam than in plaque from enamel surfaces ($p < 0.001$). The results for the unrestored dentitions were not included in this analysis after it was shown by a one-sample t-test (two-tailed) that the zero values were not within the distribution of the enamel results from the restored dentitions. This part of the test consisted of comparing a constant with a distribution.

3.3 RELEASE OF MERCURY FROM FRESH AMALGAM BY BIOFILMS

The Composition of the Biofilms

Carbohydrate Content:

The carbohydrate contents of the biofilms removed from the fresh amalgam cylinders and the wires are shown in Table 14. The statistical analysis of the results using two-way analysis of variance (ANOVA) showed that there were no significant differences in carbohydrate content between biofilms from the fresh experimental amalgam and its supporting wire, 34.9 (SD 6.6) and 69.4 (SD 30.3) µg /mg, respectively. In contrast, the carbohydrate content of the biofilm from the control wire, 460.1 (SD 182.6) µg/mg was significantly higher ($p<0.01$) than those of fresh experimental amalgam and wire biofilms, 34.9 (SD 6.6) and 69.4 (SD 30.3) µg/mg, respectively.

Protein Content:

The results of the protein assays, as shown in Table 15 have a similar pattern to those for carbohydrate. The statistical analysis of the results using two-way analysis of variance (ANOVA) suggests that there was no significant difference between the protein content of the biofilms from the fresh experimental amalgam cylinders and supporting wires, 3.7 (SD 0.8) and 8.2 (SD 3.5) µg/mg respectively. The protein content of the biofilm from the control wire, 56.9 (SD 30.6) µg/mg , was significantly higher ($p<0.02$) than those of the fresh experimental amalgam and wire biofilms, 3.7 (SD 0.8) and 8.2 (SD 3.5) µg/mg, respectively.

TABLE 14

The Mean Carbohydrate Content of the Biofilms from the Amalgam Cylinders and the Suspension Wires. Expressed as µg of Carbohydrate per mg of Biofilm

Investigation	Amalgam µg/mg	Supporting Wire µg/mg	Control Wire µg/mg
Fresh amalgam	34.9 (6.6)	69.4 (30.3)	460.1 (182.6)
Aged amalgam	409.9 (172.3)	395.4 (180.5)	222.7 (8.2)

Standard deviations are shown in parentheses (n = 4)

TABLE 15

The Mean Protein Content of the Biofilms from the Amalgam Cylinders and the Suspension Wires. Expressed as µg of Protein per mg of Biofilm

Investigation	Amalgam µg/mg	Supporting Wire µg/mg	Control Wire µg/mg
Fresh amalgam	3.7 (0.8)	8.2 (3.5)	56.9 (30.6)
Aged amalgam	66.8 (34.5)	55.3 (36.1)	24.0 (6.8)

Standard deviations are shown in parentheses (n = 4)

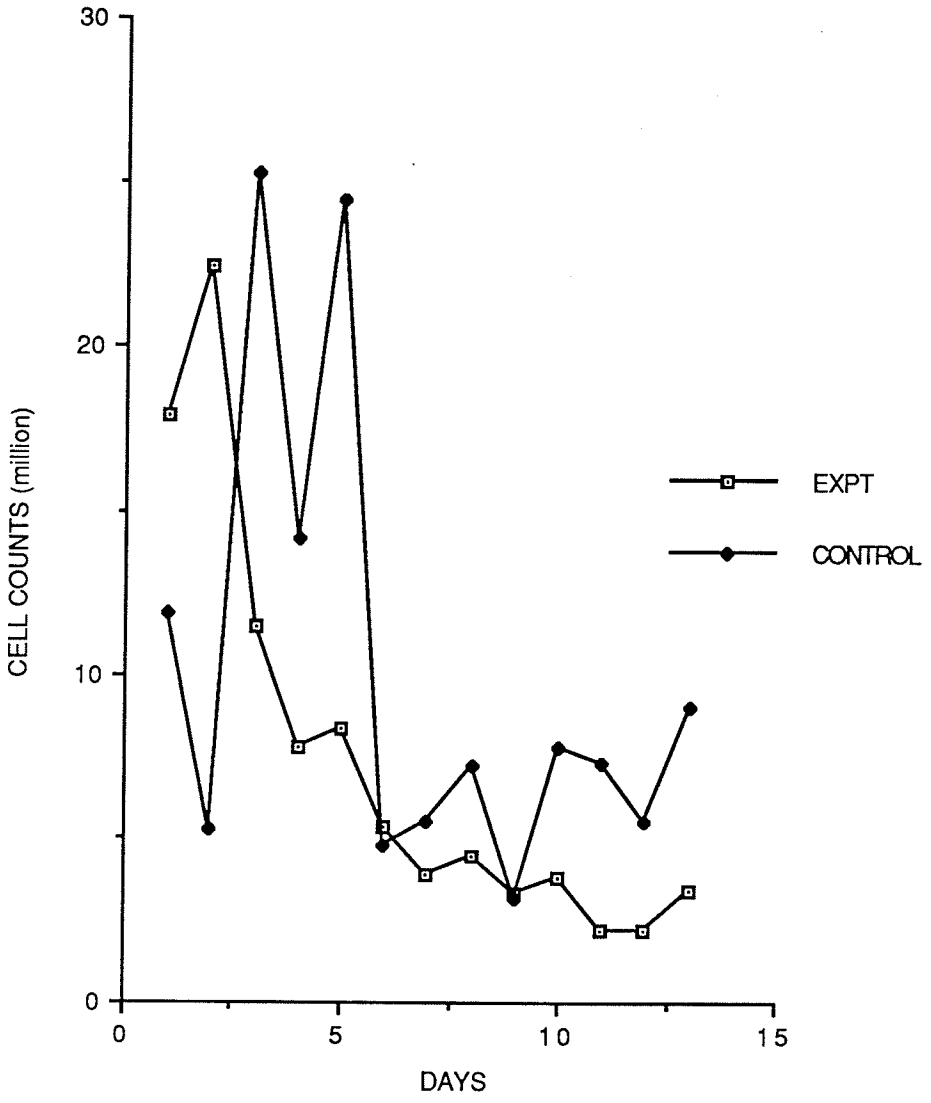

FIGURE 2

Counts of Free Cells From Fresh Amalgam (Expt) and Wire (Control)

The Liberation of Cells From the Biofilms

The counts of free cells, originating from the biofilms, in the medium of the experimental (E) and control groups (C2) are shown in Figure 2, which shows little difference between the two groups The experimental group cell count was 17.9 X 10^6/mL on day 1 and with minor fluctuations decreased to 3.4 X 10^6/mL on day 13.

The growth pattern for the wire control (C2) showed less change over the experimental time, being 11.9 x 10^6/mL on day 1 and 9.0 x 10^6/mL on day 13.

The cell counts in the media of the controls without amalgam and wire confirmed growth over the experimental period of time with a mean cell count of 122 (108.7) X 10^6/mL

Liberation of Mercury From the Amalgam

Mercury content of the Biofilms from Amalgams and Wires:

The concentrations of mercury in the biofilms removed from the fresh amalgam cylinders and wires are shown in Table 16. Statistical analysis by the Student t-test (two-tailed) showed that the mercury content of the biofilm from the amalgam cylinder was significantly higher ($p<0.1$) than that from the supporting wire but there was no significant difference between those from the supporting wire and the control wire. This suggests a lack of uptake of mercury from the supernatant by the wire biofilm.

Mercury Content of the Supernatant:

The mercury concentrations in the supernatant media are shown in Table 17. There appeared to be a fluctuating pattern in the experimental group (E) but the uninoculated amalgam control (C1) showed a high initial mercury content that rapidly decreased to a low plateau. The controls without a mercury source, (C2 and C4) had low or undetectable levels and the control C3 (pH 4.0) had fairly low, stable levels with a slight decrease between Days 1 and 5 and Days 16 and 20.

Within the experimental group, E, the mercury content of the supernatant fluids were significantly higher ($p<0.05$) on Days 1 and 20 than on Days 5 and 16 and the experimental group, E, was significantly higher than the controls ($p<0.003$) only on Day 20.

Spent Media pH:

The pH values for the spent media are shown in Table 18. The acid production by the bacteria caused a uniform drop in pH in the inoculated tubes, E, C2, C3 and C4, from

TABLE 16

The Concentration of Mercury in the Biofilms from Fresh Amalgam Cylinders and Suspension Wires Expressed as ng of Mercury per mg of Biofilm.

Test	Biofilm from Amalgam ng Hg/mg biofilm	Biofilm from Wire ng Hg/mg biofilm
E	3.2 (5.8)	0.28 (0.29)
C2		0.44 (0.47)

E experimental
C control

Standard deviations are shown in parentheses (n = 4)

TABLE 17

Mercury Content of the Supernatant Fluid from the Fresh Amalgam Investigation, Expressed as ng of Mercury per mL of Supernatant Fluid (SNF)

TEST	DAY 1 ng Hg/mL SNF	DAY 5 ng Hg/mL SNF	DAY 16 ng Hg/mL SNF	DAY 20 ng Hg/mL SNF
E	36.25(11.8)	12.0(0.8)	17.7(4.2)	33.5(4.7)
C1	283	6	6	6
C2	10	8	5	u/d
C3	23	27	15	15
C4	15	u/d	9	u/d

E experimental
C control
u/d undetected

Standard deviations are shown in parentheses (n = 4)

pH 7.0 to 4.33, the latter value being similar to the uninoculated but experimentally adjusted pH control, C3, pH 4.0. The pH of the uninoculated control C1 remained neutral (pH 7.0).

Mercury Content of the Free Cells in the Medium:

The mercury content of the free cells is shown in Table 19. Again, there appeared to be a fluctuating pattern to the mercury content in the experimental group but statistically there were no significant differences within the experimental group over time. Surprisingly, the wire control, C2, with no mercury source had a high mercury content on Day 1 but very low values on days 5, 16 and 20. The control without amalgam and wire, C4, had low mercury values, comparable with those on Days 5, 16 and 20 of the wire control, C2. The mercury contents of the cells of the experimental group, E, were significantly higher ($p<0.01$) than those of the controls on Days 5, 16 and 20 but not on Day 1.. The results were analysed using a two-way analysis of variance (ANOVA), with multiple comparisons adjusted by the Bonferroni correction factor. This correction ensured a very conservative treatment of the results.

3.4 RELEASE OF MERCURY FROM AGED AMALGAM BY BIOFILMS

The Composition of the Biofilms

Carbohydrate Content:

The carbohydrate contents of the biofilms from the aged amalgam cylinders, their supporting wires and the control wires are also shown in Table 14. In the aged amalgam investigation, there was no significant difference between the carbohydrate contents of the biofilms from the amalgam and its supporting wire nor between either of these and the carbohydrate content of the biofilm from the control wire.

Statistical comparison of the results showed that there was a significantly lower ($p<0.001$) carbohydrate content in biofilms from fresh than in those from aged amalgam cylinders. There was also a significantly lower ($p<0.001$) carbohydrate content in the biofilm from the supporting wire of the "fresh' amalgam that in that of the 'aged' amalgam but the difference in the results forthe two control wires was not significant. These results were analysed by a two-way analysis of variance (ANOVA).

TABLE 18

Mean (SD) pH of the Spent Media Over the Experimental Period

Biofilm investigation with fresh amalgam			Biofilm investigation with aged amalgam	
Test	pH		Test	pH
E1	4.35 (0.25)		E1	4.34 (0.05)
E2	4.30 (0.10)		E2	4.29 (0.79)
E3	4.30 (0.10)			
E4	4.30 (0.10)			
C1A	6.90 (0.10)		CIA	7.14 (0.81)
C1B	7.00 (0.10)		C1B	6.79 (0.03)
C2A	4.4 0(0.10)		C2A	4.29 (0.09)
C2B	4.4 0(0.10)		C2B	4.30 (0.13)
C3A	4.00 (0.05)		C3A	4.34 (0.27)
C3B	4.00 (0.05)		C3B	4.25 (0.29)
C4A	4.35 (0.05)		C4A	4.25 (0.08)
C4B	4.30 (0.05)		C4B	4.23 (0.10)

Standard deviations are shown in parentheses

Fresh amalgam (n = 20)

aged amalgam (n = 13)

TABLE 19

Mercury content of the Free Cells Released into the Medium from Fresh Amalgam Biofilms Expressed as ng of Mercury per 10^6 Viable Cells

TEST	DAY 1 ng Hg/10^6 cells	DAY 5 ng Hg/10^6 cells	DAY 16 ng Hg/10^6 cells	DAY 20 ng Hg/10^6 cells
E	211.6(48.2)	15.3(8.7)	187.7(89.4)	87.6(141.7)
C2	387.4	0.65	2.4	2.7
C4	2.0	0.3	0.2	0.1

E experimental
C control

Standard deviations are shown in parentheses (n = 4)

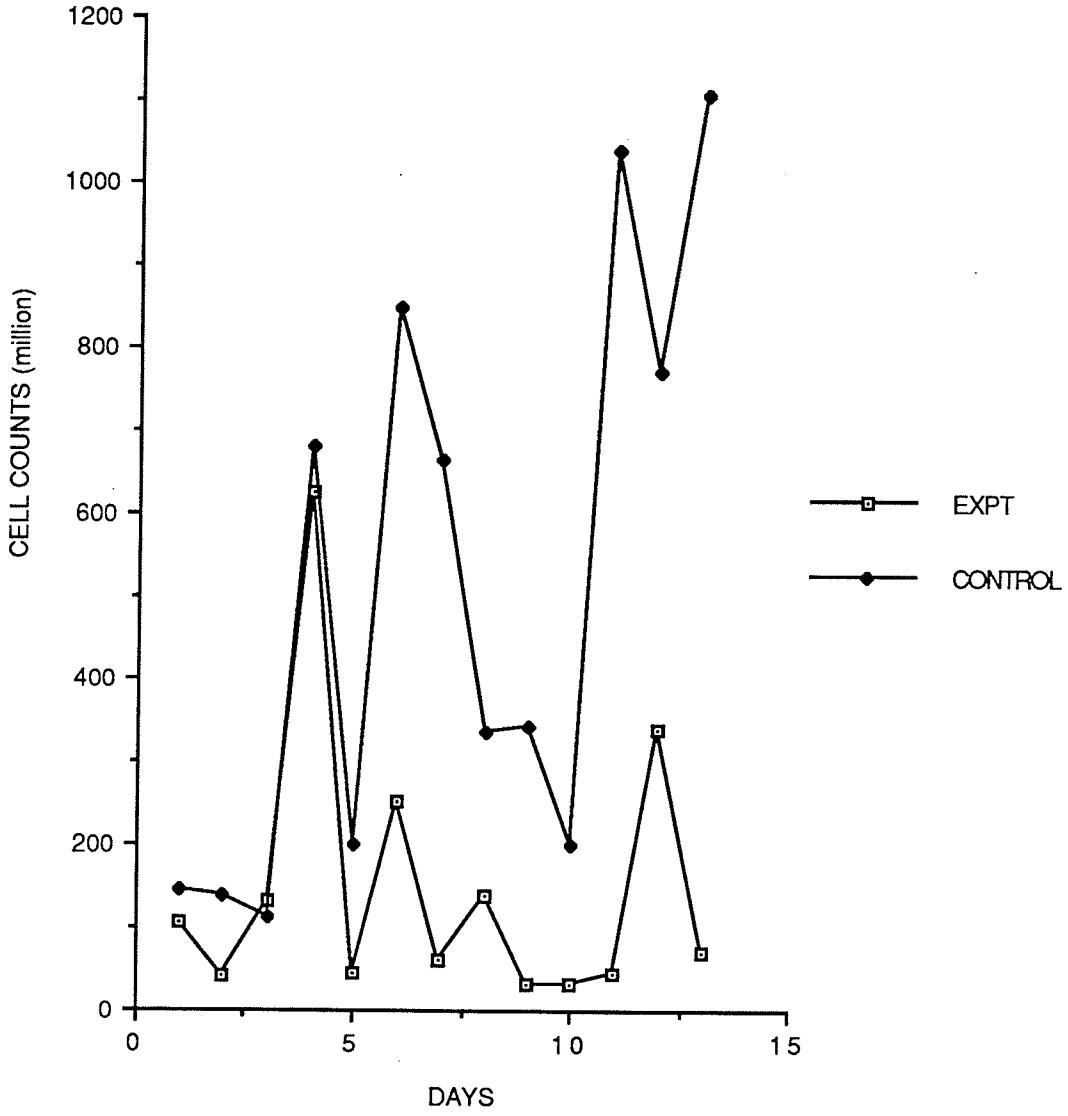

FIGURE 3

Counts of Free Cells From Aged Amalgam (Expt) and Wire (Control)

Protein content:

Table 15 contains the results of the protein analyses of the biofilms from the aged amalgam study. By using the same method of statistical analysis as for the carbohydrate results (ANOVA), it was shown that there were no significant differences between the protein contents of the biofilms from the experimental amalgams and wires and the control wires.

Statistical comparison of the results of the "fresh" and "aged" studies showed that the protein content of the biofilm from the "fresh" amalgam cylinder was significantly lower ($p<0.001$) that that from the "aged" amalgam. Similarly, the protein content of the biofilm from the supporting wire of the "fresh" amalgam was significantly lower ($p<0.001$) than that from the "aged" amalgam cylinder. The difference between the protein contents of the control wires from the fresh and aged amalgam was not significant.

The Liberation of Cells From the Biofilms:

The counts of free cells, originating from the biofilms, in the medium of the experimental (E) and control group (C2) are shown in Figure 4 where it can be seen that the experimental (E) cell counts remained slightly lower than the control.

For the experimental group there was a decrease from 105 x 10^6/mL on day 1 to 70 x 10^6/mL on day 13 but there were periods of growth during the experimental time giving counts of 627 x 10^6/mL, 251 x 10^6/mL, 139 x 10^6/mL and 338 x 10^6/mL on days 4, 6, 8 and 12, respectively.

Over the experimental period, the growth pattern for the wire control (C2) showed an irregular increase with the cell count for day 1 being 144 x 10^6/mL and for day 13, 1108 x 10^6/mL.

The cell counts in the media of the controls without amalgam and wire confirmed growth over the experimental period with a mean cell count of 815.9 (536.9) x 10^6/mL.

<u>Liberation of Mercury From the Amalgam.</u>

Mercury content of the Biofilms from Amalgams and Wires:

There was no detectable mercury in the biofilms removed from the aged amalgam cylinders and wires.

Mercury Content of the Supernatant:

There was no detectable mercury in the supernatant media.

Spent Media pH:

The pH values for the spent media in the aged amalgam investigation are also shown in Table 18 The acid production by the bacteria caused a uniform drop in pH in the inoculated tubes, E, C2, C3 and C4, from pH 6.96 to 4.28, the latter value being similar to the uninoculated but experimentally adjusted pH control, C3, pH 4.3. The pH of the uninoculated control C1 remained neutral (7.0).

Mercury Content of the Free Cells in the Medium:

There was no detectable mercury in the free cells in the medium.

3.5 THE SENSITIVITY OF SELECTED ORAL ORGANISMS TO MERCURY

The sensitivity of the selected Streptococcus strains to mercury in the medium is shown in Table 20 where the growth of the strains is expressed as a percentage of that in the medium without mercury. The strains showed a wide variation in their response to mercury, from the very sensitive strain Streptococcus milleri (NCTC 10709) to the least sensitive Streptococcus mitis I (SK138). All the strains grew in MADM with 5 µg Hg/mL but only eight of the 10 strains grew in the presence of 10 µg Hg/mL, with Streptococcus milleri and Streptococcus mutans (Pb1094A) showing no growth. Only seven strains grew when the concentration of mercury was 20 µg Hg/mL, five at 30 µg Hg/mL and at the highest test concentration of 40 µg Hg/mL only four strains showed any growth. Among those growing in the highest concentration there was a considerable range with Streptococcus mitis I (SK138) having growth equivalent to 74.6% of that in the control whereas Streptococcus salivarius (IRH 1) showed only 1.3% of the control growth. The means showed a gradual decline as the mercury concentration increased and this relationship is illustrated in Table 20.

The sensitivity of the Actinomyces strains is shown in Table 21. There was considerable variation in the sensitivity to mercury even at the lowest test concentration of 5 µg Hg/mL, with Actinomyces naeslundii (ATCC12104) showing 154

TABLE 20

Growth of Selected Streptococcus Strains in MADM with Various Mercury Concentrations Expressed as a Percentage of the Growth in MADM Without Mercury

STRAIN	STRAIN NUMBER	0 µg Hg/mL	5 µg Hg/mL	10 µg Hg/mL	20 µg Hg/mL	30 µg Hg/mL	40 µg Hg/mL
S. sanguis	SK112	100	46.0	73.1	64.5	0	0
S. oralis	NCTC 11427	100	103.5	62.6	17.9	0	0
S. mitis I	SK138	100	100.0	104.0	113.0	77.6	74.6
S. sobrinus	B13	100	103.7	50.0	43.8	32.6	25.4
S. milleri	NCTC10709	100	0.3	0.0	0.0	0.0	0.0
S. salivarius	IRH 1	100	81.5	27.4	31.4	23.1	1.3
S. mutans	Pb1094A	100	12.3	0.0	0.0	0.0	0.0
S. mutans	Pb1094B	100	20.4	40.8	0.0	0.0	0.0
S. mitis II	SK103	100	88.4	56.5	49.6	1.6	0.0
S. mutans	2452	100	56.8	43.4	44.2	23.8	14.1
MEAN (SD)		100 (0)	61.29 (39.82)	45.78 (31.82)	36.44 (35.42)	15.87 (25.10)	11.54 (23.76)

TABLE 21

Growth of Selected Actinomyces Strains in MADM with Various Mercury Concentrations Expressed as a Percentage of the Growth in MADM Without Mercury

STRAIN	STRAIN NUMBER	0 µg Hg/mL	5 µg Hg/mL	10 µg Hg/mL	20 µg Hg/mL	30 µg Hg/mL	40 µg Hg/mL
A. naeslundii	ATCC12104	100	154.0	97.0	101.0	39.5	0.0
A. viscosus	T6	100	106.0	34.9	9.0	3.0	0.0
A. viscosus	NYI (S)	100	71.5	24.7	0.0	0.0	0.0
intermediate	W752	100	98.0	40.6	0.0	0.0	0.0
intermediate	B102	100	100.0	93.0	31.4	0.0	0.0
A naeslundii 2	WVU627	100	0.0	0.0	0.0	0.0	0.0
intermediate	B236	100	0.0	0.0	0.0	0.0	0.0
Root caries	IW4517	100	*	70.8	0.0	0.0	0.0
Root caries	IW32710	100	*	70.6	0.0	0.0	0.0
Root caries	IW36422	100	*	28.3	0.0	0.0	0.0
MEAN (SD)		100 (0)	* 75.64 (57.18)	45.99 (35.25)	14.14 (32.09)	4.25 (12.42)	0.0 (0.0)

* Plating error. The mean for growth in medium with 5 µg Hg/mL was calculated for seven strains.

% of the control growth and two strains, Actinomyces viscosus 2 and an intermediate strain (B236) showing no growth. Five of the selected strains showed growth in MADM with 5 µg Hg/mL and two strains had no growth. The growth for the three strains isolated from root caries, IW4517, IW32710 and IW36422, in 5 µg Hg/mL, could not be calculated due to a plating error, but these three strains grew in 10 µg Hg/mL as did the other strains with the exception of Actinomyces naeslundii 2 (WVU627) and an intermediate strain (B236). Only Actinomyces naeslundii 1 (ATCC12104),

Actinomyces viscosus (T6) and an intermediate strain (B102) showed growth at 20 µg Hg/mL. The former two strains also grew at 30 mg Hg/mL but not the latter strain and none showed growth at the highest test concentration of 40 µg Hg/mL. As with the Streptococcus strains, the means for the growth percentages decreased as the mercury concentration in the medium increased (Table 21).

A statistical comparison of the results of the Streptococcus and Actinomyces by ANCOVA (covariance analysis) showed a significant difference ($p < 0.05$) between the resistance of the two genera tested, with Streptococcus being the more resistant to mercury.

3.6 THE ISOLATION OF THE MOST MERCURY-RESISTANT MICRO-ORGANISMS FROM A SAMPLE OF PLAQUE, USING AN ENRICHMENT CULTURE

The results of the enrichment culture test (Table 22), show that test group A, which was initially transferred into medium with 1 µg Hg/mL survived 13 subcultures into media with mercury concentrations increasing by 5 µg/mL increments. No growth was apparent after the 14^{th} transfer into medium containing 70 µg Hg/mL. Test group F, which was first inoculated into medium with 25 µg Hg/mL, did not survive when transferred into a mercury concentration of 30 µg /mL. The test groups B, C, D and E showed adaptation characteristics between the two extremes of A and F, initially being inoculated into media with 5, 10, 15 and 20 µg Hg/mL, respectively, and tolerating mercury concentrations up to and including, 60, 50, 55 and 25 µg/mL respectively.

The results with the samples plated from the highest mercury concentrations with viable cells for each test indicated that Streptococcus strains were selected at an early stage and the surviving species are named in the last column of Table 22 The results of the identification tests are shown in Table 23.

TABLE 22

Growth Patterns of Plaque Using an Enrichment Culture.

Each day 20 µL of the growth suspension was inoculated into medium with the next higher concentration of mercury.

Test Group	Number of Subcultures	Initial Hg Conc. µg/mL	Max. Hg Conc. Sustaining Growth µg/mL	Surviving Species
A	14	1	65	*Streptococcus mitis 1* *Streptococcus oralis*
B	12	5	60	*Streptococcus oralis*
C	10	10	50	*Streptococcus oralis*
D	8	15	55	*Streptococcus oralis*
E	2	20	25	*Streptococcus sanguis*
F	1	25	(no growth)	

TABLE 23

Identification Tests for the Viable Organisms in the Enrichment Culture Tests

adher = adherent

Enrichment Culture	Colony Shape	Gram Stain	Haemolysis	Catalase	H_2O_2	TYC Colonies
A 1	coccus	+	alpha	-	+	hard/adher
A 2	coccus	+	alpha	-	+	hard/adher
B	coccus	+	alpha	-	+	hard/adher
C	coccus	+	alpha	-	+	hard/adher
D	coccus	+	alpha	-	+	hard/adher
E	coccus	+	alpha	-	+	hard/adher

Enrichment Culture	Mannitol	Sorbitol	Raffinose	Melibiose	Trehalose	Amygdalin	Inulin	Arginine	Aesculin
A 1	-	-	+	+	+	-	-	-	-
A 2	-	-	+	+	-	-	-	-	-
B	-	-	+	+	-	-	-	-	-
C	-	-	+	+	-	-	-	-	-
D	-	-	+	+	-	-	-	-	-
E	-	+	+	+	+	-	+	-	-

The results contained in Table 22 were statistically analysed with the Spearman Correlation Coefficients test, which indicated a very strong negative correlation (r = -1) between the number of subcultures in which growth was sustained and the initial concentration of mercury. In essence, the lower the initial mercury concentration, the higher the number of subcultures sustained and thus the higher the final mercury concentration in which the micro-organisms remained viable.

3.7 THE LOCATION OF MERCURY WITHIN THE BACTERIAL CELL IN THE SELECTED MERCURY-RESISTANT AND MERCURY-SENSITIVE BACTERIA.

The results of the tests of the location of mercury within the two selected strains are shown in Table 24. For the mercury-resistant strain Streptococcus mitis I (SK138) the mercury concentration in the cell wall was 3.49 µg/mg cell wall and in the cytoplasm, 5.86 µg/mg cell (this latter measurement represents the mercury concentration in the cytoplasm extracted from the stated weight of cells).

For the non-resistant strain, Streptococcus mutans (Pb1094A), the mercury concentrations in the wall and cytoplasm were 1.92 µg/mg cell wall and 0.8 µg/mg cell respectively.

Thus the ratios of the mercury concentrations for the wall and cytoplasm for the resistant strain (SK138), was 1:1.68 (approximately 1:2) and the equivalent ratio for the non-resistant strain (Pb1094A) was 1:0.4.

3.8 THE IMPACT OF VARIOUS CONCENTRATIONS OF MERCURY IN THE MEDIUM ON THE GROWTH CHARACTERISTICS OF SOME MERCURY-RESISTANT AND NON-RESISTANT STRAINS.

Streptococcus Strains

The growth patterns for the four Streptococci strains are shown in Figures 4-7 The two mercury-resistant strains were S. mitis I (SK 138) and S. mutans (2452) and the non-resistant strains were S. mutans (Pb1094A) and S. milleri (NCTC 10709). These results are summarized in Table 25.

The mercury-resistant strain S. mitis I (SK 138) (Figure 4) showed similar lag periods in all mercury concentrations and the control. Maximum growth in the test

TABLE 24

Mercury Concentration in the Cell Wall and Cytoplasm
of Streptococcus mitis I (SK138) and Streptococcus mutans (Pb1094A)

The mercury concentrations in the two cell compartments (cell wall and cytoplasm) are expressed as a ratio for each strain

STRAIN	COMPARTMENT	µg Hg/mg cell wall	µg Hg/mg cell	RATIO W : C
SK138	wall W	3.49		
SK138	cytoplasm C		5.86	1 : 1.68
Pb1094A	wall W	1.92		
Pb1094A	cytoplasm C		0.8	1 : 0.4

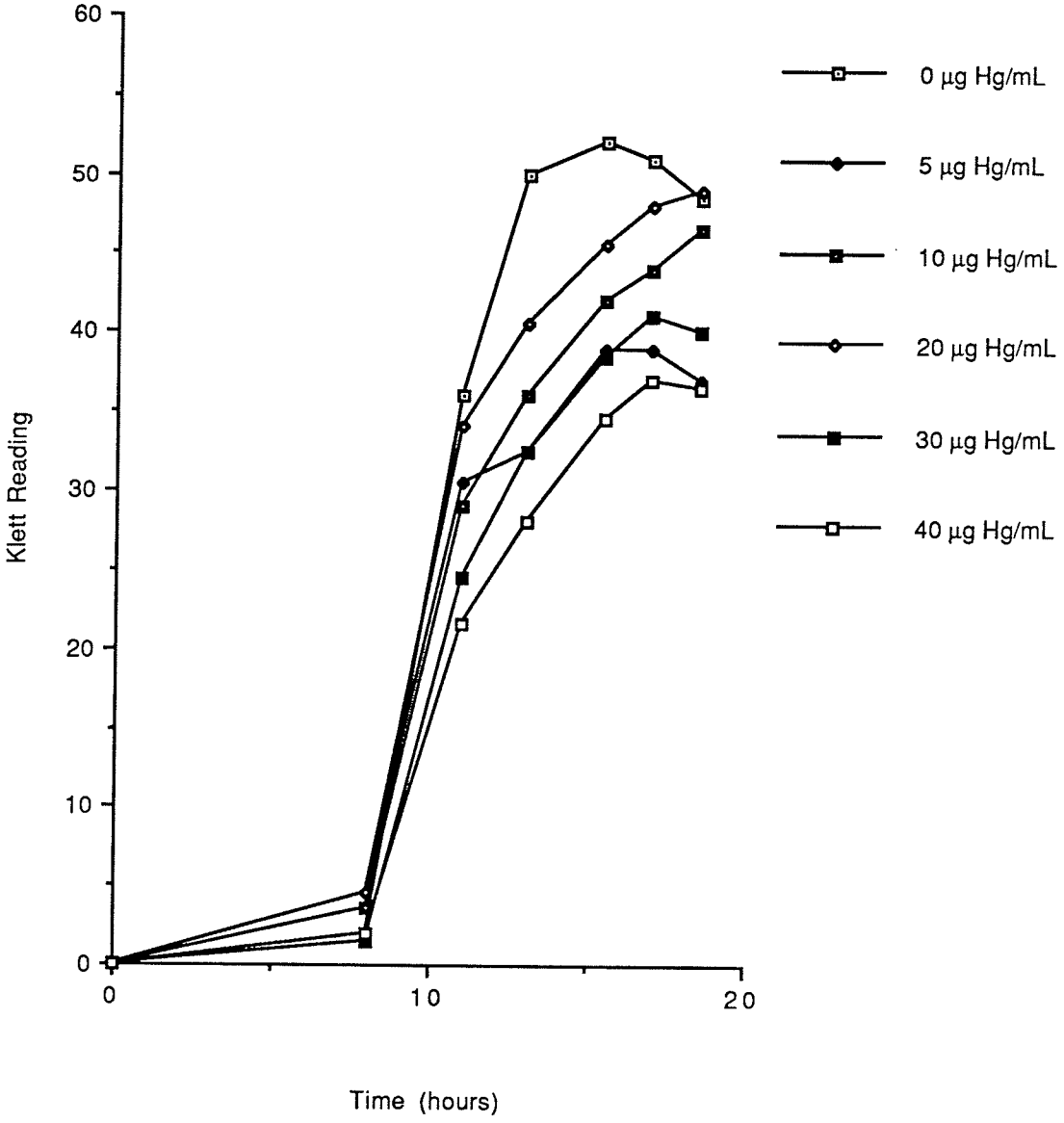

FIGURE 4

Effect of Mercury Concentration on Growth of S. mitis 1 (SK138)

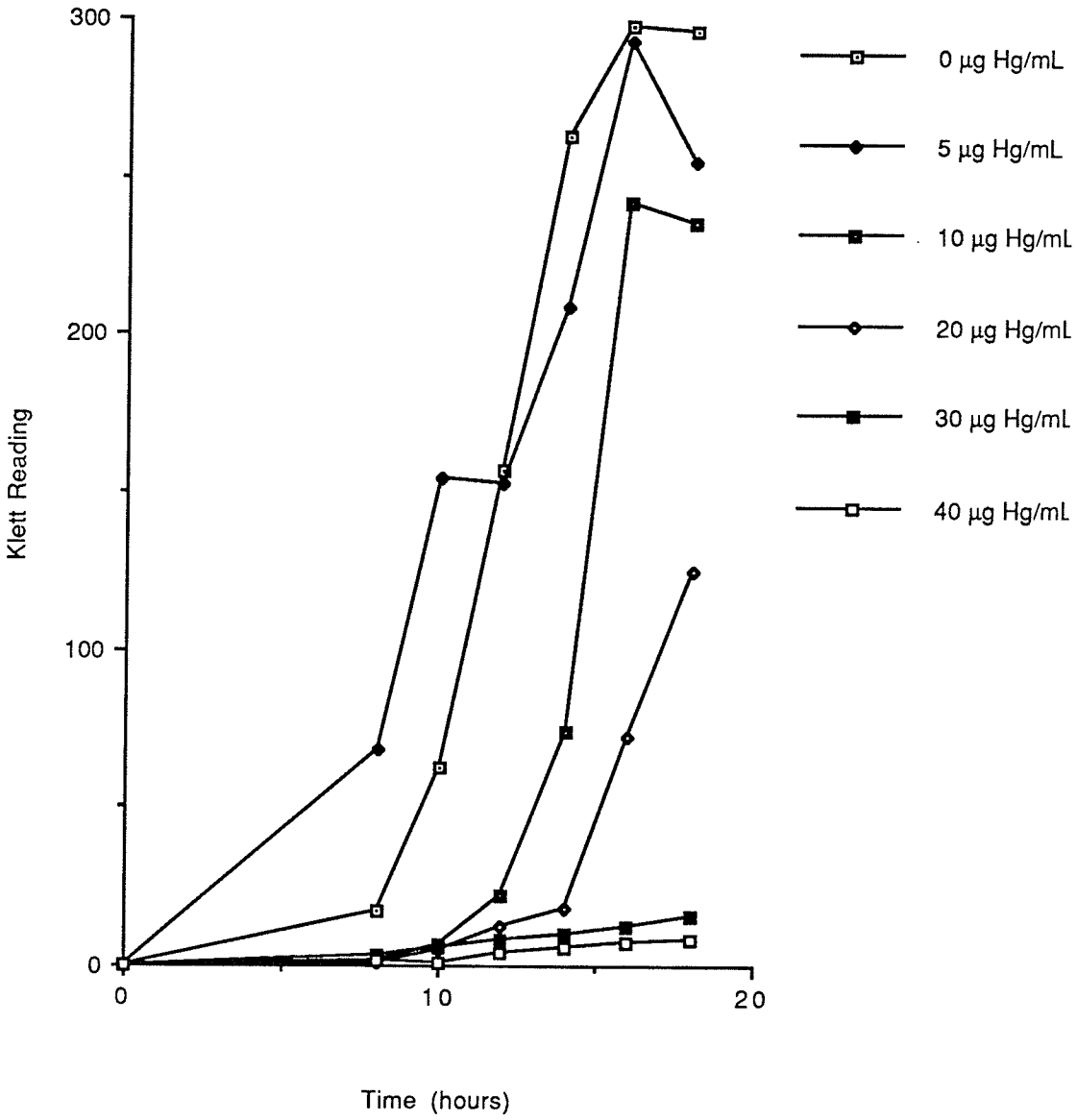

FIGURE 5

Effect of Mercury Concentration on Growth of S. mutans (2452)

period occurred in the control and the least growth in the highest mercury concentration. It was of interest that initial growth in the 20 µg Hg/mL concentration was greater than that in the 5 and 10 µg Hg/mL tests but with the former tapering off considerably after approximately 13 hours.

The other mercury-resistant strain Streptococcus mutans (2452) (Figure 5, showed maximum growth at the end of the test period (19 hours) for the control (0 µg Hg/mL) although the lag period, in the control, was longer than that in the 5 µg Hg/mL medium. The lag periods of the organism in the other media increased as the concentration of mercury in the medium increased.

These results measured by optical density (Figures 4 and 5), for the two mercury-resistant Streptococcus strains are consistent with those from mercury-containing MADM agar plates (Table 20). Although both strains adapted to a mercury concentration of 40 µg/mL, Streptococcus mitis (SK138) gave 74.6% of its control growth in the presence of mercury, whereas Streptococcus mutans (1452) gave only 14.1% (Table 20).

Tests of Streptococcus mutans (Pb1094A) (Figure 6) a non-resistant strain, showed similar lag periods for the control and the 5, 10 and 20 µg Hg/mL tests and comparable lag periods for the two highest concentrations. Although growth was slower in the 30 and 40 µg Hg/mL tests, after 20 hours there was little difference in the maximum growth among the six tests (Table 25).

The other non-resistant strain, Streptococcus milleri (NCTC 10709) (Figure 7) showed similar growth patterns over the test period for the control and the 5 µg Hg/mL tests with the latter having the slightly shorter lag period. The tests with higher mercury concentrations all showed decreasing growth and increasing lag periods with increasing mercury concentrations. The tests fell into two distinct groups with the control and 5 µg Hg/mL having a greater maximum growth (19 hours) than the 10, 20, 30 and 40 µg Hg/mL group (Table 25).

The growth patterns for the non-resistant strain Streptococcus milleri (NCTC10709) also agreed with the results shown in Table 20, with growth in the media with 0 and 5 µg Hg/mL but little or no growth in the higher mercury concentrations.

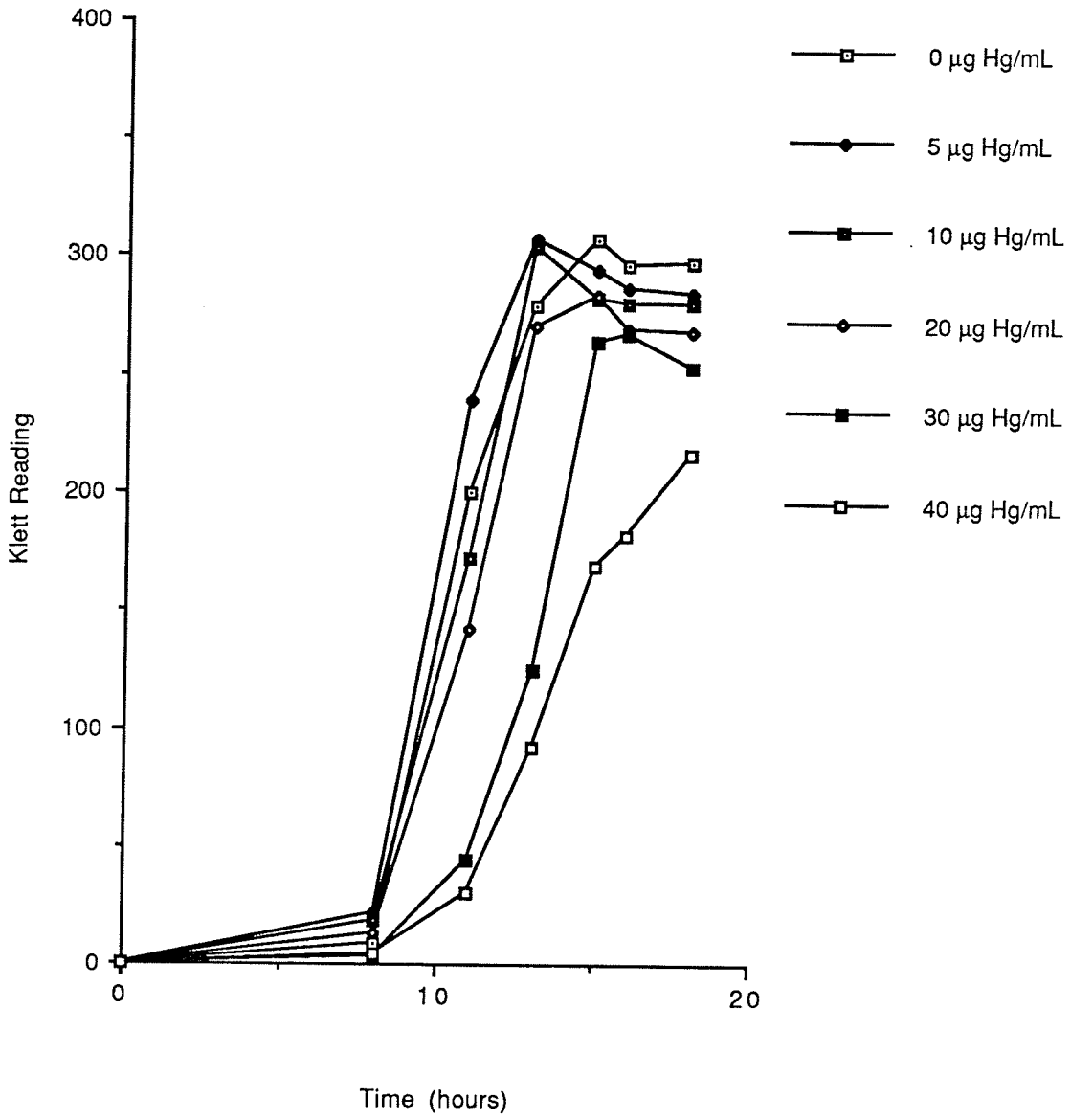

FIGURE 6

Effect of Mercury Concentration on Growth of S. mutans (Pb1094A)

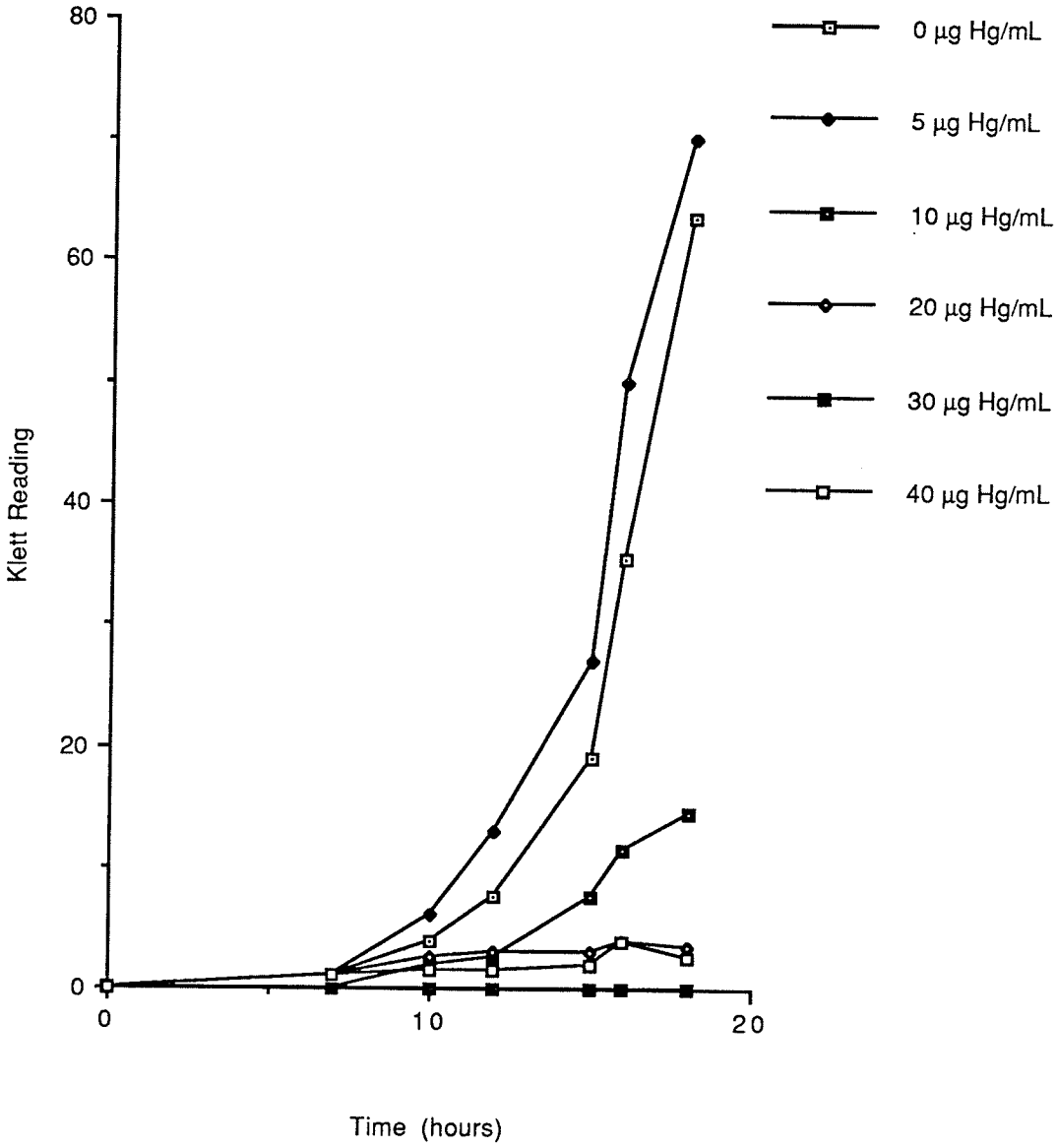

FIGURE 7

Effect of Mercury Concentration on Growth of S. milleri (NCTC 10709)

TABLE 25

Characteristics of Growth of Streptococcus Strains in Media with Various Concentrations of Mercury

Organism	Mercury conc. µg/mL	Lag Period Hours	Max.Klett Reading	Time for Max OD Hours
S. mitis SK138	0	8	52	16
	5	8	39	17
	10	8	46	19
	20	8	46	19
	30	9	41	17
	40	9	37	17
S. mutans 2452	0	8	298	16
	5	8	293	16
	10	10	241	16
	20	11	124	18
	30	11	15	18
	40	14	9	18
S. mutans Pb1094A	0	8	297	19
	5	8	307	13
	10	8	304	13
	20	8	283	15
	30	10	267	16
	40	10	267	16
S. milleri NCTC 10709	0	9	64	18
	5	9	70	18
	10	12	15	18
	20	12	4	16
	30	0	0	0
	40	15	4	16

The growth characteristics, as measured by optical density, of the other non-resistant strain Streptococcus mutans (Pb1094A) (Figure 6) were not consistent with the growth patterns on mercury-containing MADM plates (Table 20). In the former, growth was apparent in all the test concentrations of mercury, whereas in the latter no growth occurred on the plates with 10, 20, 30 and 40 µg Hg/mL.

There was an obvious difference in the lag periods in the higher mercury concentrations, between the mercury-resistant Streptococcus mitis (SK138) (Figure 4) and non-resistant Streptococcus milleri (Figure 7) (Table 25), with the former being approximately four hours shorter than the latter in medium with 40 µg Hg/mL. However, the difference in lag time between Streptococcus mitis (SK138) (Figure 4) and the non-resistant Streptococcus mutans (Pb1094A) (Figure 6), (Table 25) was very slight except at the concentrations of 30 and 40 µg Hg/mL, in which the lag period of Streptococcus mutans (Pb1094A) was two hours longer than that of Streptococcus mitis (SK138) (Table 25).

A comparison of the lag periods of the mercury-resistant strain Streptococcus mutans (2452), (Figure 5), with those of the non-resistant strain Streptococcus mutans (Pb1094A), (Figure 6), showed a shorter lag period in 0 and 5 µg Hg/mL for the resistant strain, but longer lag periods in 10, 20, 30 and 40 µg Hg/mL. A comparison with the other non-resistant strain Streptococcus milleri (NCTC10709), (Figure 7), also showed shorter lag periods for the resistant strain in 0, 5, 10 and 20 µg Hg/mL but the lag times for the two highest mercury concentrations, 30 and 40 µg/mL were similar (Table 25)

Actinomyces Strains

The results of the growth tests for the two mercury-resistant Actinomyces strains and the two non-resistant strains are shown in Figures 8 and 9, and 10 and 11 respectively. The resistant strains were Actinomyces viscosus (T6) and Actinomyces naeslundii 1 (ATCC 12104) and the non-resistant strains were an intermediate Actinomyces (B236) and Actinomyces naeslundii 2 (WVU 627). These results are summarized in Table 26.

In the Actinomyces viscosus (T6) tests (Figure 8), the lag periods for the 0, 5 and 10 µg Hg/mL concentrations were similar. Overall the growth rate decreased as the

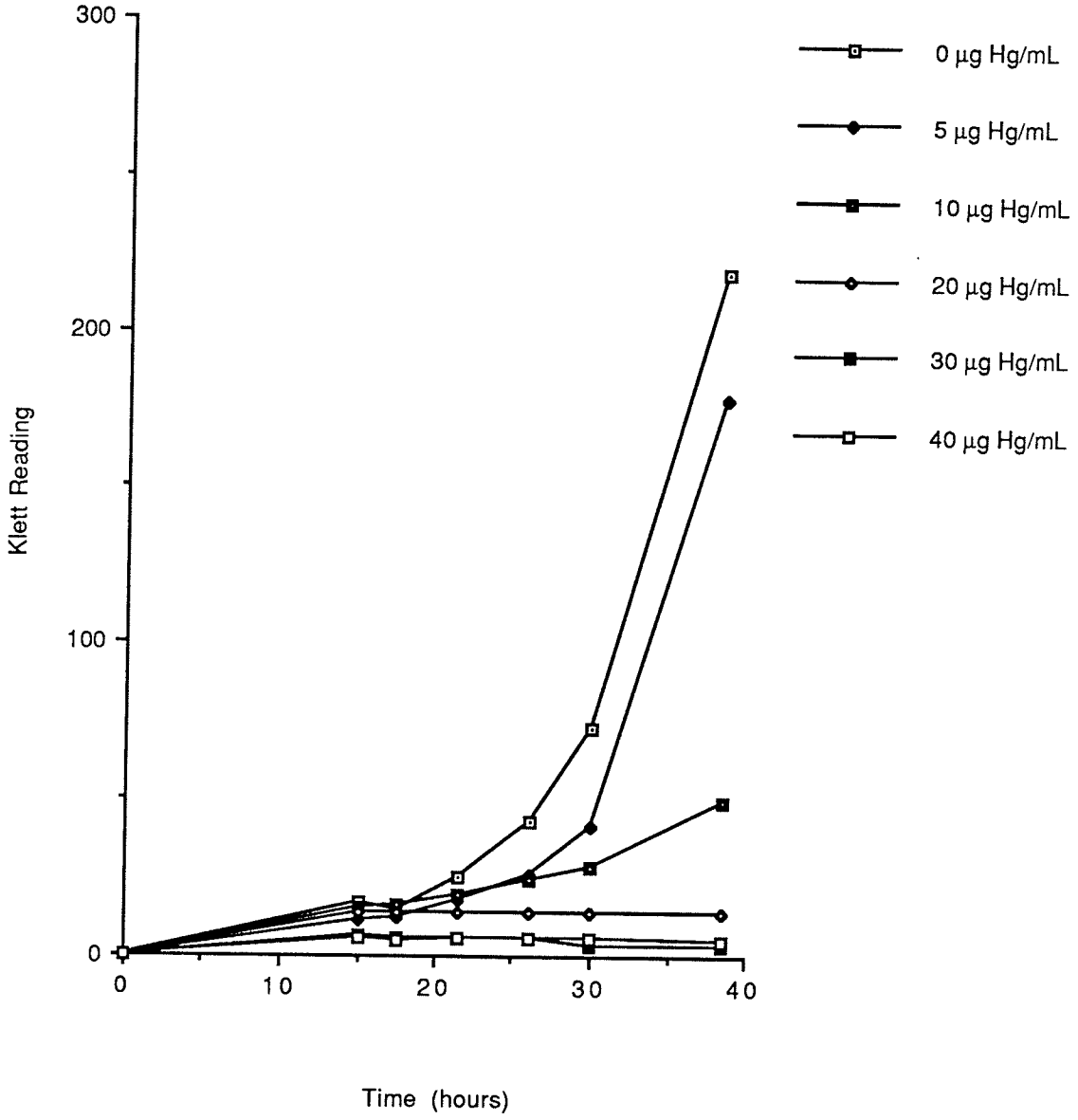

FIGURE 8

Effect of Mercury Concentration on Growth of A. viscosus (T6)

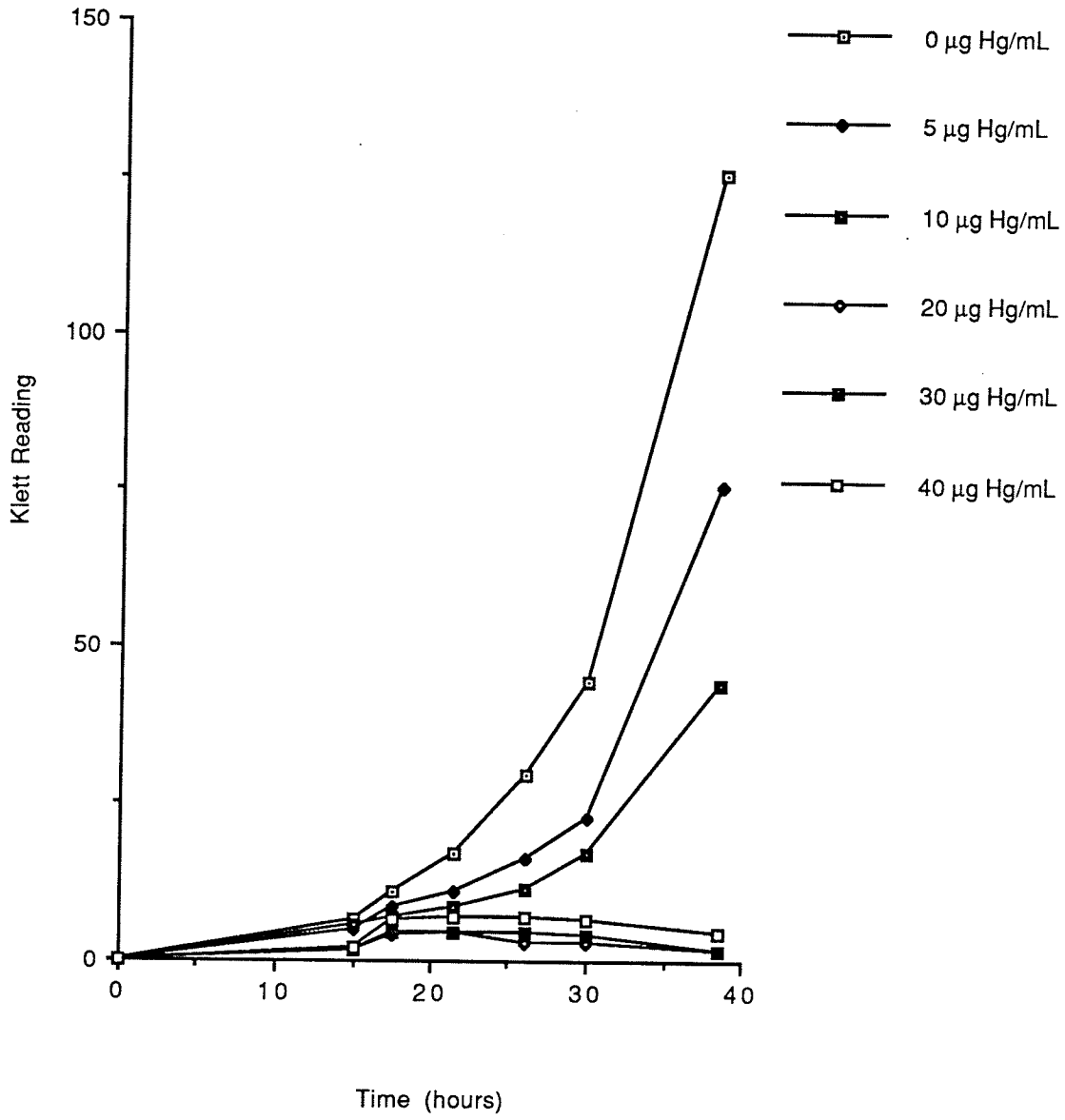

FIGURE 9

Effect of Mercury Concentration on Growth of A. naeslundii 1 (ATCC 12104)

mercury concentration increased, with the rates for the 0 and 5 µg Hg/mL being comparable. The control (0 µg Hg/mL) had the greatest maximum growth at the end of the test period (39 hours) and the growth in each of the 20, 30 and 40 µg Hg/mL tests was barely detectable.

The lag periods for the other mercury-resistant strain, Actinomyces naeslundii 1 (ATCC 12104) (Figure 9), showed a gradual increase with increasing mercury concentrations in the medium with the shortest lag period being that of the control. The control had the greatest maximum growth at the end of the test period (39 hours), with a gradual decrease in the 5, 10, and 20 µg Hg/mL tests and barely detectable growth in the 30 and 40 µg Hg/mL tests.

The growth patterns, as measured by optical density, (Figures 8 and 9) for the two mercury-resistant strains were comparable with the growth patterns on mercury-containing MADM agar plates (Table 21). Growth gradually decreased with higher mercury concentrations until there was no growth in medium with 40 µg Hg/mL.

Figure 10 shows the growth patterns for the non-resistant intermediate Actinomyces strain (B236). The lag periods of the tests with 5, 10, 20, 30 and 40 µg Hg/mL were much greater than that for the control (0 µg Hg/mL) and in comparing the maximum growth at the end of the test period (39 hours), that in the 5 µg Hg/mL concentration was approximately one third of that of the control and the growth levels in the remaining concentrations (10, 20, 30 and 40 µg Hg/mL) were barely detectable (Table 26).

The results for the other non-resistant Actinomyces strain, A. naeslundii 2 (WVU 627) are shown in Figure 11. The lag periods in the 0, 5 and 10 µg Hg/mL tests were similar but the rate of growth decreased as the mercury concentration increased. At the end of the test period (39 hours) the maximum growth had occurred in the control with the value in the 5 µg Hg/mL test being approximately one third of the control. The maximum growth in the 5 µg Hg/mL test occurred at 30 hours but was still less than that of the control. The maximum growth for the 10 µg Hg/mL test was much lower than that of the 5 µg Hg/mL concentration and the growth in the 20, 30 and 40 mg Hg/mL tests was barely detectable (Table 26)

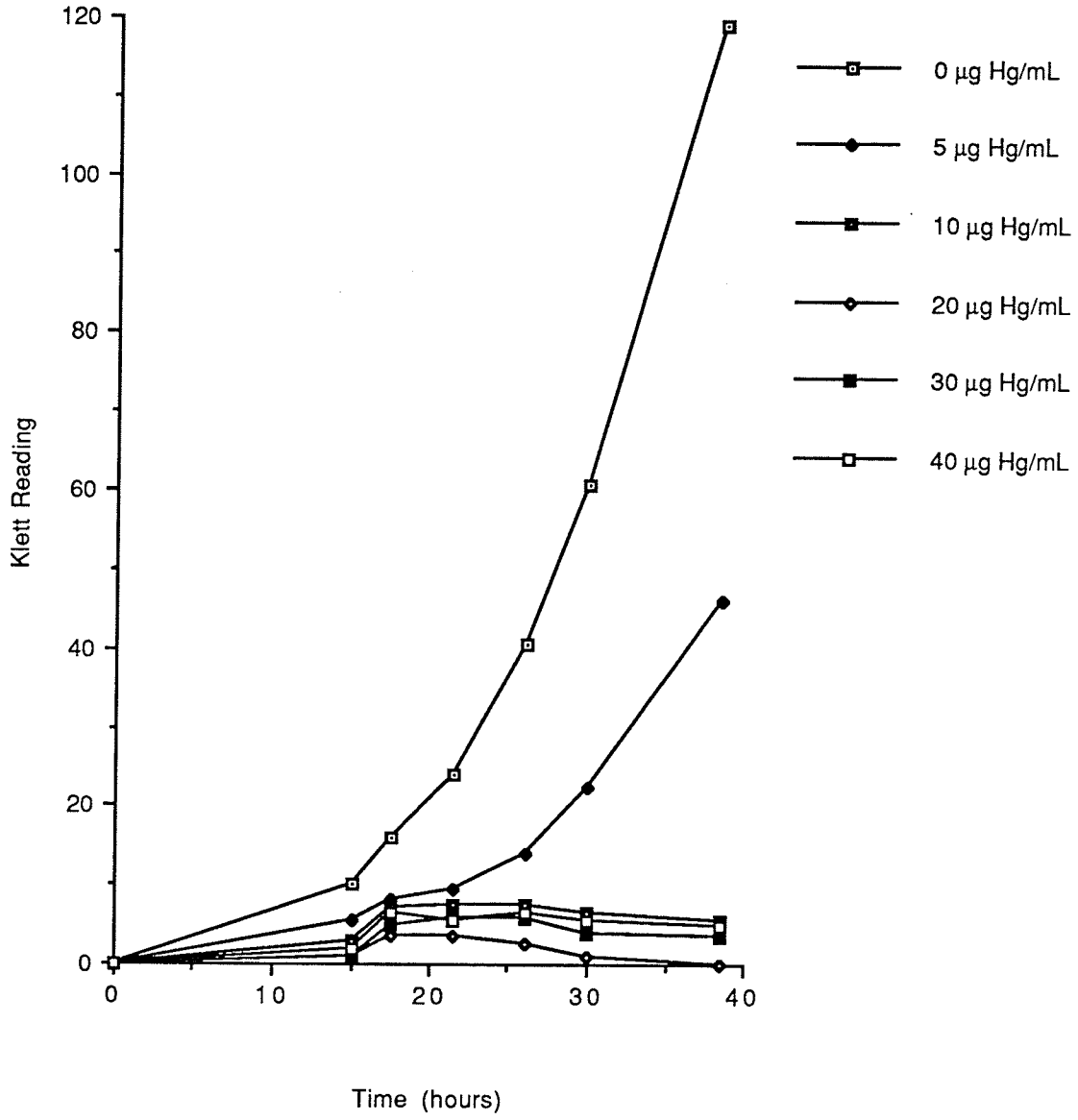

FIGURE 10

Effect of Mercury Concentration on Growth of an intermediate Actinomyces (B236)

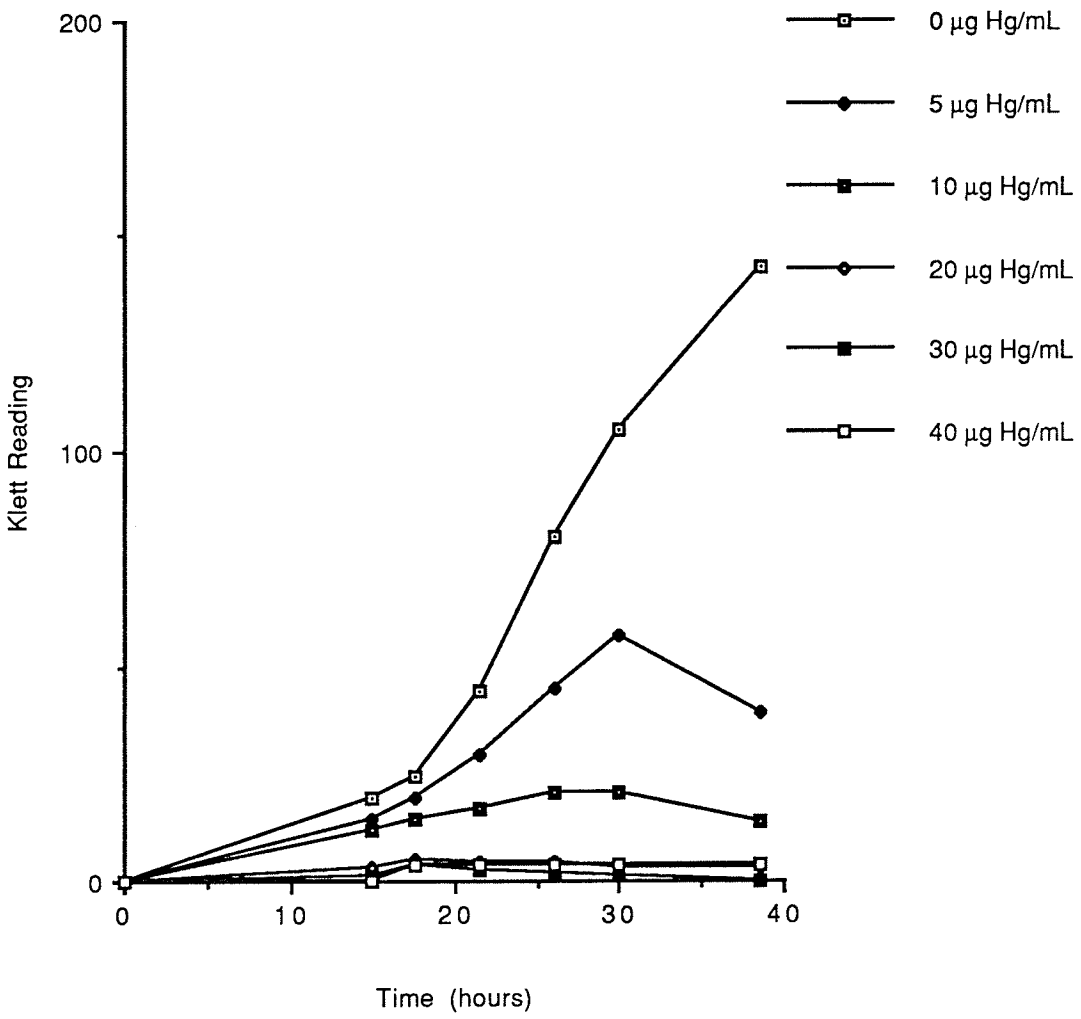

FIGURE 11

Effect of Mercury Concentration on Growth of A. naeslundii 2 (WVU 627)

TABLE 26

Characteristics of Growth of Actinomyces Strains
in Media with Various Concentrations of Mercury

Organism	Mercury conc. µg/mL	Lag Period Hours	Max. Klett Reading	Time for Max OD Hours
A. viscosus T6	0	15	218	39
	5	15	178	39
	10	15	49	39
	20	15	14	18
	30	15	6	22
	40	15	6	22
A. naeslundii 1 ATCC 12104	0	15	125	39
	5	15	76	39
	10	15	44	39
	20	16	5	22
	30	15	5	18
	40	15	7	22
Actinomyces (intermed) B236	0	15	119	39
	5	15	46	39
	10	16	8	22
	20	16	4	18
	30	16	6	22
	40	16	7	18
A. naeslundii 2 WVU 627	0	15	143	39
	5	15	57	30
	10	15	21	26
	20	16	5	22
	30	18	4	18
	40	18	4	18

The growth patterns of the non-resistant Actinomyces strains (Figures 10 and 11) were not comparable with those on mercury-containing MADM agar plates (Table 21). In the latter, growth was only apparent on the control plates (0 µg Hg/mL) whereas in the former, growth was detected in medium with 5 µg Hg/mL for the intermediate Actinomyces (B236) and with 5 and 10 µg Hg/mL for Actinomyces naeslundii 2 (WVU627).

A comparison of the lag periods for the mercury-resistant and non-resistant Actinomyces test strains shows very little difference (Table 26).

3.9 THE IMPACT OF VARIOUS CONCENTRATIONS OF MERCURY IN THE MEDIUM ON THE FERMENTATION AND HYDROLYSIS CHARACTERISTICS OF SOME MERCURY-RESISTANT AND NON-RESISTANT STRAINS.

The results for the growth, fermentation and hydrolysis tests for the Streptococcus strains are shown in Table 27 where these characteristics of the strains in the various mercury concentrations in the medium can be compared with those of the control (0 µg Hg/mL). The growth, fermentation and hydrolysis activities were suppressed even in the low mercury concentrations with a few exceptions. The latter include growth but no fermentation by Streptococcus mitis I (SK138) and Streptococcus mutans (Pb1094A) on trehalose agar containing 10 µg Hg/mL and growth but no hydrolysis by Streptococcus mutans (Pb1094A) in aesculin broth with 10, 20 and 30 µg Hg/mL. In arginine broth, Streptococcus mitis 1 (SK138), Streptococcus mutans (2452), and Streptococcus milleri (NCTC10709) grew without causing hydrolysis, in mercury concentrations of 0, 10, and 20 µg/mL. Streptococcus mutans (Pb1094A) demonstrated growth but no hydrolysis in the control and all test concentrations of mercury

On TYC agar, S. mitis I (SK138) grew in the presence of 0, 10 and 20 µg Hg/mL and the colonies were soft and non-adherent to the agar at all these concentrations.

S. mutans (2452) grew in the presence of mercury at 0, 10, 20 and 30 µg Hg/mL and the colonies at all these concentration were hard and adherent to the agar.

S. mutans (Pb1094A) grew on TYC agar containing 0, 10, 20, 30 and 40 µg Hg/mL with the colonies being hard and adherent on the agars with 0, 10 and 20 µg Hg/mL but these colony characteristics were lost at the higher mercury concentrations, at which the colonies were soft and non-adherent.

TABLE 27

Growth, Fermentation and Hydrolysis Activities for Streptococci

on Plates with Streptococcus - Sugar Base with Various Concentrations of Mercury

ad = colonies adherent to TYC agar

n-ad = colonies non-adherent to TYC agar

STRAIN	SKI38											
Substrate	Glucose	Mannitol	Sorbitol	Raffinose	Trehelose	Meliblose	Sucrose	Lactose	Aesculin	Arginine	TYC	TYC
Hg Conc	Gth/Ferm	Gth/Ferm	Gth/Ferm	Gth/Ferm	Gth/Ferm	Gth/Ferm	Gth/Ferm	Gth/Ferm	Gth/Hyds	Gth/Hyds	Growth	Descript.
0	+ / +	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -	+	soft/n-ad
10	- / -	- / -	- / -	- / -	+ / -	- / -	- / -	- / -	- / -	+ / -	+	soft/n-ad
20	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	+ / -	-	
30	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	-	
40	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	-	
STRAIN	2452											
0	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / -	+	hard/ad
10	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	+ / -	+	hard/ad
20	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	+ / -	+	hard/ad
30	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	+	hard/ad
40	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	-	
Strain	Pb1094A											
0	+ / +	+ / +	+ / +	+ / +	+ / +	+ / -	+ / +	+ / -	+ / +	+ / -	+	hard/ad
10	- / -	- / -	- / -	+ / -	+ / -	- / -	- / -	- / -	+ / -	+ / -	+	hard/ad
20	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	+ / -	+ / -	+	hard/ad
30	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	+ / -	+ / -	+	soft/n-ad
40	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	+ / -	+	soft/n-ad
Strain	NCTC 10709											
0	+ / +	+ / -	+ / -	+ / -	+ / +	+ / -	+ / +	+ / +	+ / -	+ / -	+	soft/n-ad
10	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	+ / -	+	soft/n-ad
20	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	+ / -	+	soft/n-ad
30	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	+	soft/n-ad
40	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	-	

TABLE 28

Growth, Fermentation and Hydrolysis for Actinomyces

on Plates with Streptococcus - Sugar Base with various Concentrations of Mercury

ad = colonies adherent to TYC agar

n-ad = colonies non-adherent to TYC agar

STRAIN	T6											
Substrate	Glucose	Mannitol	Sorbitol	Raffinose	Trehelose	Melibiose	Sucrose	Lactose	Aesculin	Arginine	TYC	TYC
Hg Conc	Gth/Ferm	Gth/Ferm	Gth/Ferm	Gth/Ferm	Gth/Ferm	Gth/Ferm	Gth/Ferm	Gth/Ferm	Gth/Hyds	Gth/Hyds	Growth	Descript.
0	+/+	+/-	+/-	+/+	+/-	+/+	+/+	+/-	+/+	+/-	+	hard/ad
10	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	+/-	+	hard/ad
20	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-	
30	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-	
40	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-	
STRAIN	ATCC 12104											
0	+/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/-	+	soft/n-ad
10	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	+/-	+/-	+	soft/n-ad
20	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	+/-	-/-	+	soft/n-ad
30	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	+	soft/n-ad
40	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-	
STRAIN	B236											
0	+/+	+/-	+/-	+/+	+/+	+/-	+/+	+/-	+/-	-/-	+	hard/ad
10	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-	
20	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-	
30	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-	
40	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-	
STRAIN	WVU627											
0	+/+	+/-	+/-	+/+	+/+	+/+	+/+	+/-	+/-	-/-	+	soft/ad
10	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	+/-	-/-	+	soft/ad
20	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	+/-	-/-	-	
30	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-	
40	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-	

S. milleri (NCTC 10709) grew on TYC agar with 0, 10, 20 and 30 µg Hg/mL and the colonies at these concentrations were all soft and non-adherent to the agar.

The identities of the organisms were verified from growth on blood-agar plates.

The results of the growth, fermentation and hydrolysis tests with the Actinomyces strains are recorded in Table 28 where these characteristics of the organisms in the various sugar media with 10, 20, 30 and 40 µg Hg/mL can be compared with the controls without mercury.

As with the Streptococcus strains, growth, fermentation and hydrolysis were generally suppressed by the presence of mercury even in the lowest concentration (10 µg Hg/mL) but as before there were some exceptions.

Actinomyces viscosus 1 (T6) demonstrated growth in arginine broth with 10 µg Hg/mL and on TYC agar with the same mercury concentration with the colonies being hard and adherent to the agar.

Actinomyces naeslundi (ATCC12104) demonstrated growth in aesculin broth with 0, 10 and 20 µg Hg/mL. The aesculin control broth was hydrolysed but this did not occur in those containing mercury. In the arginine broth growth occurred in the control and at 10 µg Hg/ml but arginine was not hydrolysed. Growth occurred on the control TYC agar and on those with 10, 20 and 30 µg Hg/mL with the colonies being soft and non-adherent.

The intermediate Actinomyces strain (B236) did not grow in any of the mercury-containing media.

Actinomyces viscosus 2 (WVU 627) grew in the control aesculin broth and in the broths with 10 and 20 µg Hg/mL but growth on the TYC agars was observed only on the control and that with 10 µg Hg/mL. The colonies on the former and the latter agars were soft and adherent.

The identities of the organisms were verified from growth on blood-agar plates.

Analysis of the results by the McNemars test (48) for correlated proportions showed significantly lower ($p<0.04$) metabolic activities of the organisms on plates with 30 and 40 µg Hg/mL than those on plates with lower mercury concentrations.

3.10 THE IMPACT OF VARIOUS CONCENTRATIONS OF MERCURY IN THE MEDIUM ON THE PROPERTIES OF SOME ENZYMES OF MERCURY-RESISTANT AND NON-RESISTANT STRAINS.

The results of the enzyme activity tests for the Streptococcus strains are shown in Table 29. Appendices 8 and 9 contain the raw data for the mercury-resistant strains, Streptococcus mitis I (SK138) and Streptococcus mutans (2452), respectively, and Appendices 10 and 11 for the non-resistant strains, Streptococcus mutans (Pb1094A) and Streptococcus milleri (NCTC 10709), respectively.

In the medium without mercury, six enzymes were detected for Streptococcus mitis I (SK138), alkaline phosphatase, esterase, esterase lipase, acid phosphatase and phospho-hydrolase. Of these only phospho-hydrolase appeared to be unaffected by mercury. The alkaline and acid phosphatases had reduced activity in 40 μg Hg/mL but the others had no detectable activity at this mercury concentration.

Streptococcus mutans (2452) had 12 detectable enzymes in the control, alkaline phosphatase, esterase, esterase lipase, lipase, leucine amino-peptidase, valine amino-peptidase, cystine amino-peptidase, trypsin, chymotrypsin, acid phosphatase, phospho-hydrolase and α-glucosidase. Of these, only leucine amino-peptidase appeared to be unaffected by mercury although α-glucosidase may also fall into the same category. Esterase lipase and phospho-hydrolase maintained some activity and the remaining enzymes that were detected in the control test had no detectable activity in medium containing 40 μg Hg/mL

The detectable enzymes in the control for Streptococcus mutans (Pb1094A) were similar to those for Streptococcus mutans (2452) with the addition of β-glucosidase. The thirteen detectable enzymes were alkaline phosphatase, esterase, esterase lipase, lipase, leucine amino-peptidase, valine amino-peptidase, cystine amino-peptidase, trypsin, chymotrypsin, acid phosphatase, phospho-hydrolase, α-glucosidase and β-glucosidase.

There was little effect on the enzyme activity in medium containing 10 μg Hg/mL but this was the highest mercury concentration in which growth of this particular strain was maintained.

The results for the other non-resistant strain Streptococcus milleri (NCTC 10709) showed 10 detectable enzymes in the control, alkaline phosphatase, esterase,

esterase lipase, lipase, leucine amino-peptidase, valine amino-peptidase, cystine amino-peptidase, acid phosphatase, phospho-hydrolase, β-galactosidase and α-glucosidase. Of these, approximately one third, esterase lipase, acid phosphatase and phospho-hydrolase were unaffected by mercury, one third, alkaline phosphatase, esterase, leucine amino-peptidase and α-glucosidase were partially suppressed by mercury and the remaining third, valine amino-peptidase, cystine amino-peptidase and β-galactosidase were totally suppressed in medium containing 40 μg Hg/mL.

The enzyme activities of the Streptococcus strains are summarized in the upper part of Table 30 where the number of detectable enzymes for each mercury concentration is expressed as a percentage of those detected in the control medium.

The control tests were those without mercury in the medium; therefore, for each micro-organism, the readings for each mercury concentration can be compared with that for the 0 μg Hg/mL test to assess the effect of a particular concentration of mercury in the medium on the enzyme activity.

For all four Streptococcus strains, there were fewer detectable enzymes when mercury was present at concentrations of 20, 30 and 40 μg/mL than in the control but the decrease occured in an irregular manner as the mercury concentration increased.

Streptococcus mutans (2452) and Streptococcus milleri (NCTC 10709) had a decrease in enzyme activity in 10 μg Hg/mL yet activity was present in all the test concentrations. The other Streptococcus mutans strain (Pb1094A) had the same enzyme activity in 10 μg Hg/mL as in the control but this was the highest concentration in which growth was maintained.

The results of the enzyme activity tests for the Actinomyces strains are also shown in Table 29. Appendices 12 and 13 contain the raw data for mercury-resistant strains, Actinomyces viscosus (T6) and Actinomyces naeslundii 1 (ATCC 12104), respectively, and Appendices 14 and 15 for the non-resistant intermediate strain (B236) and non-resistant Actinomyces naeslundii 2 (WVU627), respectively.

In Actinomyces viscosus (T6) there were 12 detectable enzymes in the control, alkaline phosphatase, esterase, esterase lipase, leucine amino-peptidase, valine amino-peptidase, cystine amino-peptidase, acid phosphatase, α-galactosidase, β-galactosidase, α- and β-glucosidase and α-fucosidase and one enzyme, N-acetyl-β-glucosaminidase, that was not detected in the control and yet was active in the medium with 10 μg Hg/mL.

Three effects from mercury can be seen, no effect, partial, and total suppression, with one third of the control enzymes being found in each category. Those with no

TABLE 29

The Change in Enzyme Activity (0-5), in the Various Test Micro-Organisms
from the Control (0 µg Hg/mL) to the Highest Mercury Concentration that sustained Growth.
The numbers are roughly equivalent to the activity of the enzyme.

Enzyme	S. mitis SK138 µg Hg/mL 0 - 40	S. mutans 2452 µg Hg/mL 0 - 40	S. mutans Pb1094A µg Hg/mL 0 - 10	S. milleri NCTC 10709 µg Hg/mL 0 - 40	A. viscosus 1 T6 µg Hg/mL 0 - 10	A. naes.1 ATCC 12104 µg Hg/mL 0 - 30	Interm. Actin B236 µg Hg/mL 0 - 10	A. naes. 2 WVU 627 µg Hg/mL 0 - 10
alkaline phosphatase	3 - 1	2 - 0	1 - 1	5 - 4	5 - 4	2 - 5	1 - 0	2 - 0
esterase	2 - 0	2 - 0	2 - 2	2- 1	1 - 0	1 - 0	1 - 0	1 - 0
esterase lipase	3 - 0	2 - 1	2 - 2	3 - 3	2 - 2	1 - 3	1 - 0	1 - 0
lipase	1 - 0	1 - 0	1 - 1	0 - 0	0 - 0	0 - 0	0 - 0	0 - 0
leucine amino-peptidase	0 - 0	5 - 5	3 - 3	5 - 2	3 - 3	4 - 5	5 - 5	4 - 4
valine amino-peptidase	0 - 0	5 - 0	3 - 2	1 - 0	2 - 1	3 - 3	4 - 1	3 - 3
cystine amino-peptidase	0 - 0	2 - 0	2 - 2	1 - 0	1 - 0	1 - 0	1 - 0	1 - 0
trypsin	0 - 0	2 - 0	1 - 1	0 - 0	0 - 0	0 - 1	0 - 0	0 - 0
chymotrypsin	0 - 0	5 - 0	3 - 3	0 - 0	0 - 0	0 - 0	0 - 0	0 - 0
acid phosphatase	5 - 3	3 - 0	2 - 2	5 - 5	5 - 4	5 - 5	4 - 4	3 - 3
phospho-hydrolase	1 - 1	2 - 1	2 - 1	1 - 1	0 - 0	1 - 3	1 - 1	1 - 1
α-galactosidase	0 - 0	0 - 0	0 - 0	0 - 0	2 - 1	2 - 0	1 - 0	0 - 0
β-galactosidase	0 - 0	0 - 0	0 - 0	5 - 0	5 - 5	3 - 0	5 - 5	5 - 5
β-glucuronidase	0 - 0	0 - 0	0 - 0	0 - 0	0 - 0	0 - 0	0 - 0	0 - 0
α-glucosidase	0 - 0	4- -5	1 - 2	5 - 1	2 - 0	3 - 0	2 - 1	1 - 1
β-glucosidase	0 - 0	0 - 0	1 - 2	0 - 0	2 - 2	1 - 1	1 - 1	1 - 0
N-acetyl-β-glucosaminidase	0 - 0	0 - 0	0 - 0	0 - 0	0 - 3	0 - 0	0 - 0	0 - 2
α-mannosidase	0 - 0	0 - 0	0 - 0	0 - 0	0 - 0	0 - 0	0 - 0	0 - 0
α-fucosidase	0 - 0	0 - 0	0 - 0	0 - 0	1 - 0	0 - 0	0 - 0	0 - 0

TABLE 30

The Enzyme Activity of the Selected Streptococcus and Actinomyces Strains Expressed as a Percentage of the Number of Detectable Enzymes in the Control (Actual numbers are shown in parentheses)

Hg Conc µg/mL	0	10	20	30	40
S. mitis I (SK138)	100 (6)	100 (6)	66.7 (4)	66.7 (4)	50 (3)
S. mutans (2452)	100 (12)	83.3 (10)	33.3 (4)	33.3 (4)	33.3 (4)
S. mutans (Pb1094A)	100 (13)	100 (13)	0 (0)	0 (0)	0 (0)
S. milleri (NCTC 10709)	100 (9)	100 (9)	77.7 (7)	77.7 (7)	77.7 (7)

Hg Conc µg/mL	0	10	20	30	40
A. viscosus (T6)	100 (12)	75 (9)	0 (0)	0 (0)	0 (0)
A. naeslundii 1 (ATCC 12104)	100 (12)	66.7 (8)	33.3 (4)	66.7 (8)	0 (0)
Actinomyces (B236) (intermediate strain)	100 (12)	58.3 (7)	0 (0)	0 (0)	0 (0)
A. naeslundii 2 (WVU 627)	100 (11)	58.3 (7)	0 (0)	0 (0)	0 (0)

apparent effect from mercury were esterase lipase, leucine amino-peptidase, β-galactosidase and β-glucosidase. The enzymes suffering partial suppression were alkaline phosphatase, valine amino-peptidase, acid phosphatase and α-galactosidase and the control enzymes that were undetectable in 40 μg Hg/mL were esterase, cystine amino-peptidase, α-glucosidase and α-fucosidase.

This organism did not grow in media with 20, 30 and 40 μg/mL of mercury.

The other relatively mercury-resistant Actinomyces strain was Actinomyces naeslundii 1 (ATCC 12104), which had 12 detectable enzymes in the control, alkaline phosphatase, esterase, esterase lipase, leucine amino-peptidase, valine amino-peptidase, cystine amino-peptidase, acid phosphatase, phospho-hydrolase, α-galactosidase, β-galactosidase, α- and β-glucosidase and one enzyme, trypsin, which, though not detectable in the control, showed some weak activity in the medium containing 30 μg Hg/mL. The three enzymes that were unaffected by mercury were valine amino-peptidase, acid phosphatase and β-glucosidase. There were no enzymes with partial suppression but those that were totally suppressed in 30 μg Hg/mL were esterase, cystine amino-peptidase, α- and β-galactosidase and α-glucosidase. There were five enzymes in this micro-organism which demonstrated greater activity in the presence of mercury than in the control; these were alkaline phosphatase, esterase lipase, leucine amino-peptidase, trypsin and phospho-hydrolase.

This organism did not grow in the medium containing 40 μg Hg/mL.

As shown in Table 29, the non-resistant intermediate Actinomyces strain (B236) had the same detectable enzymes in the control as did Actinomyces naeslundii 1 (ATCC 12104) although the activity of the enzymes in the presence of mercury was not identical for the two micro-organisms. Mercury had no apparent effect on leucine amino-peptidase, acid phosphatase, phospho-hydrolase, β-galactosidase and β-glucosidase. Valine amino-peptidase and α-glucosidase suffered partial suppression and alkaline phosphatase, esterase, esterase lipase, cystine amino-peptidase and α-galactosidase were totally suppressed in 10 μg Hg/mL. Unlike Actinomyces naeslundii 1 (ATCC 12104), none of the enzymes of this intermediate Actinomyces strain (B236) showed increased activity in mercury in comparison with that in the control.

The organism did not grow in media with mercury concentrations of 20, 30 and 40 μg/mL.

The other relatively non-resistant Actinomyces strain, Actinomyces naeslundii 2 (WVU627), was similar to the previous two Actinomyces strains. The 11 enzymes

detected in the control test were alkaline phosphatase, esterase, esterase lipase, leucine amino-peptidase, valine amino-peptidase, cystine amino-peptidase, acid phosphatase, phospho-hydrolase, β-galactosidase, α– and β-glucosidase. N-acetyl-β-glucosaminidase though not detected in the control showed some activity in the medium with 10 μg Hg/mL, similar to that in Actinomyces viscosus (T6). Just over 50% of the enzymes maintained the same level of activity in medium with 10 μg Hg/mL as in the control medium; these were leucine amino-peptidase, valine amino-peptidase, acid phosphatase, β-galactosidase and α-glucosidase. The other control enzymes were totally suppressed in 10 μg Hg/mL.

This organism did not grow in the media with 20, 30 and 40 μg/mL of mercury.

The enzyme activities of the Actinomyces strains are summarized in the lower part of Table 30, where the number of detectable enzymes for each mercury concentration is expressed as a percentage of those detected in the control medium. The control tests were those without mercury in the medium; therefore, for each micro-organism, the readings for each mercury concentration can be compared with that for the 0 μg Hg/mL test to assess the effect of a particular concentration of mercury in the medium on the enzyme activity.

For all four Actinomyces strains, there were fewer detectable enzymes than in the control when mercury was present at a concentration of 10 μg/mL. There was no growth in media with mercury concentration greater than 10 μg/mL with the exception of Actinomyces naeslundii (ATCC 12104). This strain showed a further decrease of enzyme activity in the medium with 20 μg Hg/mL but the number of detected enzymes in the medium with 30 μg hg/mL was the same as that in 10 μg Hg/mL. None was detected in the medium with 40 μg Hg/mL.

The results in Table 30 show that, with the exceptions of Streptococcus mutans (Pb1094A) and Actinomyces naeslundi (ATCC 12104), the enzymes of the Streptococcus strains maintained activity in the higher mercury concentrations to a greater degree than the enzymes of the Actinomyces strains.

The results in Table 29 have been described as they relate to the micro-organisms but some patterns can also be discerned by considering the individual enzymes.

Of the 19 enzymes tested, only four were detected in the controls of all the test micro-organisms; these four were, alkaline phosphatase, esterase, esterase lipase and

acid phosphatase. Lipase was present in all the Streptococcus strains but none of the Actinomyces and β-galactosidase was detected in all the Actinomyces grown in medium without mercury but in only one of the Streptococcus strains (Streptococcus milleri NCTC 10709). Leucine amino-peptidase, valine amino-peptidase and cystine amino-peptidase were detected in all the strains tested except Streptococcus mitis (SK138).

All of the enzymes detected in the controls were affected by mercury in at least one of the test strains, although some appeared to suffer only minor suppression. Leucine amino-peptidase was partially suppressed (5 to 2) only in Streptococcus milleri (NCTC10709), and although acid phosphatase was totally suppressed (3 to 0) in Streptococcus mutans (2452), it was only partially suppressed in Streptococcus mitis (SK138), (5 to 3) and Actinomyces viscosus (T6), (5 to 4). Mercury also appeared to have little effect on the activity of phospho-hydrolase. This enzyme was partially suppressed (2 to 1) in both the Streptococcus mutans strains (2452 and Pb1094A).

The opposite effect, that of increased activity (1 to 3) was observed for phospho-hydrolase in Actinomyces naeslundii 1 (ATCC 12104). It is interesting to note that this organism had several other enzymes that showed increased activity from that observed in the control; these were alkaline phosphatase (2 to 5), esterase lipase (1 to 3), leucine amino-peptidase (4 to 5) and trypsin (0 to 1). Other enzymes that demonstrated increased activity in the presence of mercury were, α-glucosidase (1 to 2), in Streptococcus mutans, (2452 and Pb1094A), β-glucosidase (1 to 2), in Streptococcus mutans (Pb1094A) and N-acetyl-β-glucosaminidase (0 to 3 and 0 to 2), in Actinomyces viscosus (T6) and Actinomyces naeslundii 2, (WVU627), respectively.

The enzymes that appeared to be most susceptible to mercury were esterase, with total suppression in six of the eight strains; lipase, with two of three totally suppressed; cystine amino-peptidase, with six of seven totally suppressed and α-galactosidase, with two of three being totally suppressed.

Although there were some consistent patterns seen, such as those described above, a few enzymes demonstrated the complete range of no effect, partial or total suppression and increased activity in different micro-organisms. A good example of this is esterase lipase, which suffered no effect in Streptococcus mutans (Pb1094A), Streptococcus milleri (NCTC10709) and Actinomyces viscocus (T6). The same enzyme was partially suppressed in Streptococcus mutans (2452), totally suppressed in Streptococcus mitis (SK138), the intermediate Actinomyces strain (B236) and Actinomyces naeslundii 2 (WVU 627) and had increased activity in Actinomyces naeslundii 1 (ATCC12104).

Statistical analysis by the Chi-Square test showed significantly lower enzyme activity of the Streptococcus strains in media with 20, 30 and 40 mg Hg/mL than in media with lower mercury concentrations. For Streptococcus mitis (SK138) and Streptococcus milleri (NCTC 10709) $p<0.05$, and for Streptococcus mutans (2452) and (Pb1094A) $p<0.01$.

There was also significantly lower enzyme activity in the Actinomyces strains grown with mercury, with $p<0.01$ for all strains at 20, 30 and 40 µg Hg/mL with the exception of Actinomyces naeslundii (ATCC 12104) at 30 µg Hg/mL ($p<0.05$) and $p<0.05$ for all strains at 10 µg Hg/mL.

CHAPTER 4

DISCUSSION

Mercury has been an enigmatic element from the time of the alchemists to the present day with the third "Amalgam War" still unsettled (62)

It is generally agreed that dental amalgam does continue to release mercury after the initial setting reaction is complete. In vitro testing of mercury dissolution from amalgam showed that the dissolution rates decreased rapidly with time (111, 17, 32) and Marek (73) concluded that the long-term loss of mercury from amalgam is not sufficient to cause concern.

Amalgam and its reactions have been studied intensively and the oral flora has also received thorough scrutiny but a search of the literature over the past decade did not reveal any studies involving the mercury content of dental plaque. The in vivo or real life situation is one in which amalgam restorations are an abiotic part of an ecosystem that includes plaque. It could therefore be expected that some interplay occurs between these two.

Determining the mercury content of plaque was the starting point for the investigations described above. No mercury was detected in the plaque from unrestored dentitions, even though mercury occurs in the environment, in air, water and food (127). Perhaps the ingested material is not retained in the oral cavity long enough for mercury to be released or there is no mechanism which facilitates the release in the mouth. Mercury in food could be bound to such radicles as bisulphide and sulphydryl, and therefore would not diffuse into the saliva, from which it could be taken up by plaque. Written records of the subjects' diets were not kept for the experimental period, but on questioning at the time of plaque collection, no subject had eaten food with a known high mercury content, such as seafood, during the 24 hours of plaque growth.

The presence of mercury in the plaque obtained from dentitions restored with amalgam suggests that this restorative material was the source of mercury. This is supported by the significant difference between the mercury content of the plaque from the surface of amalgam, the mean being 0.7 μg Hg/mg plaque, and that from enamel of restored dentitions, 0.2 μg Hg/mg plaque.

Another possible source of mercury could be saliva. Windeler (126) determined that the ratio of mercury in parotid saliva to that in blood was 1:4, thus implying a possible source for mercury in the mouth If the source was endogenous, an even distribution between the plaque on enamel and on restorations would be expected, whereas in the present study there was a significant difference in the mercury content of the plaque from the two sources. Moreover, an endogenous mercury source would be indicated by the presence of mercury in the plaque from the unrestored dentitions. These considerations support the suggestion that amalgam is the source of the mercury found in plaque from the restored dentitions.

The complex nature of the ecosystem of the oral cavity is illustrated by the different distributions of mercury-containing plaque in the subjects. Table 13 shows the ratios of the mercury concentrations of the plaque from enamel, to that of amalgam for each subject, and these ranged from 1:33 for subject "10" to 1:1.5 for subjects "3" and "4". Subject "10" had amalgam restorations only in a single quadrant of the dentition, whereas subjects "3" and "4" had amalgam restorations in all four quadrants. If mercury were being released into the oral cavity and was circulating either through saliva or air, it might be expected that an even distribution in plaque would occur. Therefore the results in Table 12 suggest that the release of mercury from amalgam, and the subsequent uptake by the micro-organisms of the plaque is principally a localized activity, but the fact that some mercury was present in the plaque from enamel shows that the released mercury is also being distributed, to some degree, within the oral cavity. This availability may be a function of the amount released but may also be influenced by the movement of saliva over the teeth and mucosal surfaces and air flow within the oral cavity. Dawes et al. (30) suggested that the velocity of the salivary film varies in different locations in the mouth, and estimated the range to be 0.8 mm/min in the upper anterior vestibular region to 7.6 mm/min in the lower anterior lingual area for unstimulated saliva. A region that would likely be restored with dental amalgam, such as the upper posterior, had an estimated velocity of 6.8 mm/min. Dawes et al. (30), Dawes (28) and Macpherson and Dawes (69) have discussed the implication of the varied velocities of salivary films on acid clearance from plaque and hence on caries experience. A parallel implication could also be suggested on the effect acid clearance would have on corrosion patterns of amalgam and hence mercury release. The velocity of the salivary film might be expected to have some influence on the rate of diffusion of mercury from plaque into saliva and thus its availability to plaque on enamel, but if mercury were bound to one or more components of plaque, the velocity of the salivary film would not be the only influence on mercury distribution by saliva. The mean of

the total amounts of mercury contained in a 24-hour collection of plaque was 2.0 µg with the median being 1.8 µg. These values are close to the daily amounts calculated as being obtained from amalgam restorations by Berglund (6) (1.7 µg/day), Mackert (68) (1.24 µg/day) and Snapp et al. (108) (1.3 µg/day). Mackert calculated his value from the data published by Vimy and Lorscheider (122). Olsson and Bergman (93) recalculated the results of several investigators and concluded that the mean amount of swallowed mercury from intra-oral vapour was approximately 10 µg/day with 1 µg Hg/day being absorbed from the gastro-intestinal tract.

It is tempting to speculate from the present investigation, that the plaque was acting as a mercury trap or sink and if brushing and flossing had occurred during the 24 hours, presumably the plaque-bound mercury would have been removed from the mouth. On the other hand, without uptake by plaque, the mercury could have been ingested with food and saliva or been inhaled.

Since methyl-mercury is more toxic than elemental or ionic mercury, there may be a concern regarding the possible methylation of mercury by some populations of the plaque. Edwards and McBride (34) showed that intestinal bacterial populations could methylate mercury and Heintze et al. (49) demonstrated that some oral Streptococcus species and strains could, in vitro, methylate mercury, but they warned against extrapolating this result to the more complex in vivo situation. More recently, Chang et al. (19), in investigating the factors affecting the blood mercury concentrations of dentists, argued that in vivo chemical or enzymatic biotransformation of inorganic mercury to organomercury, probably does not occur. The presence of methylmercury in plaque or the production of methyl mercury by whole plaque, rather than by pure strains in vitro, merits investigation.

The initial finding that plaque in contact with a mercury source contained more mercury than plaque distant from the source led to speculation that the micro-organisms involved might have an active role in the release of mercury from the amalgam. Based on in vitro studies, it has been proposed by Moberg (84) that release of mercury from amalgam may be influenced by factors determining the pH at the surface of the amalgam, such as the acidogenicity of plaque. Thus, the metabolic acids produced by the organisms of the plaque might facilitate the release of mercury from amalgam through corrosion and an in vitro study by Palaghias (97) showed that amalgam was corroded by acetic, formic, lactic and succinic acids, all of which are end-products of plaque carbohydrate catabolism (23, 46).

Acid-mediated corrosion could explain the effect of plaque on amalgam but there is another aspect to be considered and that is the effect of mercury on the plaque bacteria, since mercury is toxic. Studies by ecologists concerned with the pollution of natural bodies of water have revealed many complex interactions between bacteria and mercury. These interactions, considered as mechanisms of resistance to the toxicity of mercury, include methylation and demethylation, the production of hydrogen sulphide and the binding of the element to cell walls (24, 34, 41, 44, 54, 71, 104, 105). Since mercury was detected in the micro-organisms of the plaque, it could be speculated that these organisms had some resistance to mercury such as that described, in the literature quoted above, for the bacteria of other ecosystems.

The mean concentration of mercury in the freeze-dried plaque from amalgam restorations was 0.72 µg/mg; thus it can be estimated that the concentration in wet plaque would be 0.14 µg/mL. This estimation is made on the basis of plaque being 80% water (29, 106) and the assumption that the mercury was all in solution, but does not allow for the difference in density of wet plaque and water. This concentration is well below the maximum mercury concentration of 2.5 µg/mL (50% of 5 µg/mL was available), in which the more sensitive organisms of the study, such as the intermediate strain of Actinomyces (B236) and Actinomyces naeslundii 2 (WVU 627) survived. The low concentration of mercury in plaque could have some inhibitory effect on the metabolism of the micro-organisms without being bacteriocidal. Evidence for this inhibition is discussed below but the effect of non-bacteriocidal concentrations of mercury on the metabolism of sensitive and non-sensitive oral micro-organisms could be a topic for further investigation.

The in vitro study, involving a biofilm of a pure strain of Streptococcus mutans on amalgam, supported the thesis that the organisms colonizing amalgam facilitated the release of mercury and in turn, were affected by the toxicity of the mercury released. The organism, Streptococcus mutans (2452), successfully colonized both the fresh and aged amalgam cylinders, their supporting wires and the control wires, producing a thick biofilm. The cells of this biofilm provided the inoculum for the daily transfers into fresh media and testing at the end of the experimental periods for the fresh and aged amalgams confirmed the presence of viable cells within the biofilms.

There was significantly less carbohydrate and protein in the biofilms on the fresh amalgams and their supporting wires than in those from the control wires and from the aged amalgam. This suggests an inhibitory effect on metabolism from some component of the fresh amalgam and the metabolic inhibition in the biofilms of the

supporting wires implies that the inhibitory factor was being released into the environment.

The "aging" process had consisted of leaving the sterilized amalgam cylinders, wrapped in paper, in air for almost two years. This period would have allowed a passive, protective tarnish layer to form on the amalgam, thus inhibiting the release of mercury. Marek (73) noted that the in vitro corrosion rate of amalgam was increased if the amalgam surface was abraded and this would be in keeping with the suggestion that the body-burden of mercury correlates with the number of occlusal surfaces restored with amalgam (108). The increase in the corrosion rate and thus in mercury release, was attributed to the disruption of the passive layer, but in the present study the protective layer was not removed and thus could have prevented or reduced the loss of mercury from the amalgam. This could explain the observed difference in the metabolic activities of the Streptococcus in the biofilms on the fresh and aged amalgams. This explanation is supported by the similarity of metabolic activities in the organisms grown on the aged amalgams, their supporting wires and the control wires. Patterson et al. (101) and Berglund (6) have shown that activities such as tooth brushing and eating abrasive foods increases the rate of release of mercury from amalgam restorations. This observation may be due to the disruption of the passive layer formed on the amalgam surface.

The presence of mercury in the biofilms from the fresh amalgam cylinders shows that some mercury was being released from the amalgam and this toxic element could be the inhibiting factor. This would agree with the findings of Choi and Kim (20), Nourollahi and Meryon (91) and Orstavik (95) that amalgam has anti-bacterial properties and that mercury is one of the causative elements. Although the metabolism of the bacteria of the biofilms was inhibited, the organisms remained viable to the end of the experimental period thus indicating that they possessed some form of resistance to mercury.

A comparison of the growth of cells free in the medium also suggests that the metabolism, as measured by cell counts, of the bacteria from the fresh amalgam was inhibited. There was an 80% decrease in the cell count, compared with a 25% decrease in the control. The decrease in the cell count from the aged amalgam was 33% and the control showed an increased cell count over the experimental period.

The uninhibited metabolism of biofilms from aged amalgam and their lack of detectable mercury suggests that mercury was not being released from the amalgam cylinders. The evaporation rate of mercury from amalgam decreases with time and is

much lower after oxidation (31, 111); therefore, in the present investigation a very low rate would be expected from the amalgam after two years. This in vitro situation is not applicable to the clinical situation where the passive layer would be disturbed by mastication, tooth brushing and flossing, but the low release of mercury after the initial setting time would apply.

The fluctuating pattern of the mercury content of the free cells from the experimental fresh amalgam biofilms, and the supernatant fluids could have been due to the formation and removal of passive layers on the amalgam. Initially a high mercury release would be expected and this was seen on Day 1, in the mercury concentration of the supernatant fluids as well as the free cells (Tables 17 and 19). With time the rate of release would be expected to decrease and this occurred in the uninoculated control, C1 (Table 17) but in the experimental group, E, the mercury release fluctuated in both the cells and the supernatant. Perhaps a passive layer formed but was disrupted by the metabolic acids, probably lactate, produced by the Streptococcus. The deposition of corrosion products would prevent mercury release but further corrosive activity would be disruptive, allowing the escape of mercury from the amalgam surface. Thus a cycle of passivation and corrosion would produce a cyclic release of mercury. This pattern of corrosion may have a parallel in vivo, particularly in an area protected from masticatory friction, such as a protected approximal surface under the contact between two teeth. The in vivo corrosion pattern would be more complex than that in vitro with the former having a community of many bacterial populations producing varied organic acids as metabolic end-products.

The corrosive environment of the experimental group would have fluctuated, with the initial pH being 7.0 after transfer into fresh medium, but within 24 hours dropping to approximately 4.0 (Table 14). However, the corrosive environment of the acidified control, C3 would have been somewhat different with the pH of 4.0 being constant. Palaghias' (97, 98) investigations regarding the corrosion of amalgam by different organic acids and sodium chloride, sodium sulphide and ammonia demonstrated that the release of mercury varied with different electrolytes. A more complex corrosive environment would be produced by the biofilm than in the acidified control and these differences in the environments of control group, C3, and the experimental group, E, may account for their different rates of mercury release. The presence of mercury in the controls for the fresh amalgam investigation, with no known mercury source, is surprising but the values are at a very low level.

The in vivo evidence that plaque could assimilate mercury and the in vitro evidence that a single pure strain, Streptococcus mutans (2452), could facilitate the

release of mercury raised questions regarding the relative resistance or sensitivity of other strains and species of oral flora. The strain of Streptococcus used in the in vitro study on biofilm study had been chosen because of its known ability to colonize amalgam (13).

The growth study involving 10 Streptococcus and 10 Actinomyces strains revealed a range of resistance to mercury with the greater overall resistance being in the Streptococcus strains tested. The resistance may not be due to the inherent characteristics of the organism but may be acquired through infection by a plasmid. A plasmid consists of a closed circle of double-stranded DNA which is not part of the genome of the organism but which can confer an advantage for competition in the ecosystem (16, 21, 105, 124). This could explain the difference between the Streptococcus mutans strains (Pb1094A and 2452), with one of the strains having acquired the resistance through infection by a plasmid which was carrying the gene(s) for mercury resistance. The non-resistant strain (Pb1094A) was isolated from the mouth of a young child from an area with a poor dental service, whereas the resistant strain (2452) was from a city child. It could be speculated that the city child was more likely to have been infected with a Streptococcus mutans strain from a care giver who had amalgam restorations. In this case, the organisms populating an environment with a mercury source would probably be those with resistance to mercury. The caregiver in the area with no dentist may have had an unrestored dentition and thus the oral flora may have consisted of populations without mercury resistance. This hypothesis could be tested by determining the degree of resistance to mercury in oral micro-organisms isolated from children with unrestored dentitions, whose care-givers had amalgam restorations. The control group would consist of children with unrestored dentitions whose care-givers also had unrestored teeth.

The observed greater resistance of Streptococcus than that of Actinomyces is consistent with the results of the enrichment culture study (Table 22), where the organisms that survived in mercury concentrations from 25 to 65 µg/mL were identified as Streptococcus. Because any adaptation could have affected the characteristics of the micro-organisms, the surviving organisms were maintained on blood agar after isolation, thus allowing for reversal of phenotypic adaptation before identification. This reversal was not measured in the present investigation and it should be noted that Hamilton (45) found that strains of Streptococcus salivarius with acquired resistance to fluoride, by adaptation or mutation, retained their resistance after passage for 500 generations in fluoride-free medium. This was in contrast to the findings of

Williams (125) that Streptococcus strains isolated from dental plaque and "trained " to grow in the presence of high fluoride concentrations, lost this ability after two to three days, when grown in medium free of fluoride.

The two species that survived up to 65 µg Hg/mL in the enrichment culture were Streptococcus mitis 1 and Streptococcus oralis; the former also showed a high level of resistance on the MADM plate study whereas the latter did not grow on plates with a mercury concentration higher than 20 µg/mL. The results for Streptococcus sanguis are consistent for both studies with this species surviving in 25 µg Hg/mL in the enrichment cullture and 20 µg/mL in the MADM plate study.

These irregularities could be explained in a similar manner to that given above for the difference in resistance between the two Streptococcus mutans strains. Organisms of the same genus and species, but different strains, and thus originating from different sources may have been subjected to different environmental stresses. The Streptococcus oralis strain from the enrichment culture originated from fresh plaque whereas the strain used for the growth study on the MADM plates was a freeze-dried laboratory strain; therefore a difference in their mercury-resistance properties should not be unexpected.

The inverse relationship, demonstrated in the enrichment culture study, between the initial mercury concentration and the number of subcultures sustaining growth, strongly suggests that some of the plaque organisms were adapting to mercury. Another possible explanation would be that of selection. In this scenario, the selected mercury-resistant strains would have been present in all the inoculum aliquots; thus growth would have been observed in all the test groups (Table 22) up to the same mercury concentration. On the contrary, this growth in all the test groups was not observed and this counts against the selection explanation. Therefore the results suggest that being subjected to the pressure of mercury gradually, i.e. starting in a low concentration and moving into increasingly higher concentrations, induced mercury resistance, thus adapting the organism to the environment. This study could be expanded by maintaining the surviving strains in a medium without mercury and then subjecting aliqots of the same inoculum to the various mercury concentrations. This follow-up would help to confirm that the observed results were due to adaptation.

With the data available from the study on resistance to mercury, as measured by cell counts on agar plates, further growth studies measured by optical density were confined to resistant and sensitive examples of Streptococcus and Actinomyces.

The greater lag periods of growth of the organisms in the mercury-containing medium is consistent with the presence of a growth inhibitor, as is the overall lower density of growth in comparison with that of the controls. The increased lag periods in the presence of mercury might suggest adaptation in that the mercury could have induced the mercury-metabolizing enzymes. On the other hand it may suggest selection, whereby the resistant cells would survive while non-resistant cells would lyse and a time lapse would occur until the few resistant cells reached a concentration detectable by optical density. Another possible explanation is that during the lag period mercury was removed by some resistance mechanism, possibly enzyme-based reactions producing the more volatile methyl-mercury (41, 105); thus when the mercury concentration had been reduced to below the toxic level, growth occurred. The lag periods could be further investigated by assaying the media at various time intervals during the experimental period for loss of mercury..

There were some slight differences between the degree of resistance of the test strains, as measured by optical density, (Figures 4 to 11) and counts of colony-forming units (Tables 20 and 21). Growth occurred in higher mercury concentrations in the fluid medium (Figures 4 to 11) than on the agar plates (Tables 21 and 22). The greatest discrepancy occurred with Streptococcus mutans (Pb1094A) which grew in mercury concentrations in the optical density study that appeared to inhibit totally growth on the agar plates. These differences could be due to the different consistencies of the test media, with one being fluid and the other semi-solid. The binding of mercury had been tested for agar; however, diffusion and loss of mercury from the aqueous medium was not tested. If mercury was being lost from the less viscous medium it would account for the apparent increase in resistance of the organisms in this form of the medium.

In general, the difference in the lag periods and the degree of growth supported the concept that the test micro-organisms had different abilities to cope with mercury and classifying them as more-resistant and less-resistant is probably more accurate than as resistant or sensitive.

The results of the optical density growth study also confirmed the finding in the earlier study that of the genera tested, Streptococcus were more resistant to mercury than Actinomyces.

The differences between species and strains within a genus in their ability to cope with the environmental stress of mercury and the observation that the freeze-dried organisms that had been grown in mercury-containing medium were greyer in colour

than those grown without mercury, prompted further investigation regarding the uptake of mercury by bacteria.

It was established that the recovery of mercury from solutions of bacteria was in the range of 95% to 100% (Table 6) and that multiple washes of the cells did not change the results of the mercury assays (Table 7). The inability to remove the mercury by washing the cells and the grey colour of the freeze-dried cells as described above suggests that the mercury is bound to the cell in an inorganic form and this would be in keeping with suggested methods of resistance. Several methods of resistance to mercury have been described (41, 54, 105) and those that do not involve the formation of a volatile organic mercury compound include binding to the cell wall and the formation of mercuric sulphide. The latter method, in effect, removes mercury from the system since mercuric sulphide has a low solubility in an aqueous medium. If mercuric sulphide was being formed within the cell it would explain the noted grey colour of the cells.

The speculations with regard to methods of resistance and the colour of the cells led to the analysis of the mercury content of components of the cells. The results showed that both the resistant strain Streptococcus mitis 1 (SK138) and the less-resistant Streptococcus mutans (Pb1094A) bound mercury in the cell wall and in the remaining parts of the cell; therefore the exclusion of mercury was not the basis for resistance. Vaitzuis et al. (120) state that most of the mercury bound to bacterial cells is associated with either the cell wall or the cytoplasmic membrane and that the changes observed in cell structure are probably due to the interference by mercury with cytoplasmic membrane synthesis and function. This would account for the earlier observation that the protein content of the biofilm exposed to mercury was less that that of the mercury-free control.

The two species tested cannot be compared with regard to the absolute amounts in each component or to the total bound mercury because they were grown in different mercury concentrations. Streptococcus mitis 1 (SK138) was grown in medium containing 40 µg Hg/mL whereas the medium for Streptococcus mutans (Pb1094A) contained only 5 µg Hg/mL, these being the maximum concentrations in which the two bacteria had survived in a previous test (Table 20). However, a comparison can be made between the two bacteria, of the percentage of the total bound mercury that was found in the cell wall, and these were 37% and 76% for Streptococcus mitis 1 (SK138) and Streptococcus mutans (Pb1094A) respectively.

The organism grown in the lower mercury concentration had the higher percentage of mercury in the wall. Perhaps the initial activity of the cell was to bind

the mercury in the wall thus protecting the intracellular components. After saturation of all the binding sites, the mercury would pass into the cell, thus changing the ratio of the mercury content of the wall to the remainder of the cell. Since the cell walls had been treated with pronase for removal of protein, the presence of mercury indicates that it may have been bound to mucopeptides and carbohydrates.

Further investigation, using individual strains, is required to determine whether the mercury in the wall, as a percentage of the total bound mercury, is influenced by the concentration of mercury in the medium, after which comparisons between resistant and less-resistant strains, species and genera could be made.

The results of the metabolic activity tests in the presence of mercury illustrate the inhibiting effect of this toxic element. Fermentation of sugars and the hydrolysis of aesculin and arginine were significantly suppressed. The morphological characteristics of the individual colonies growing on TYC were not affected with the exception of Streptococcus mutans (Pb1094A), the colonies of which changed from being hard and adherent to soft and non-adherent when the mercury concentration reached 30 and 40 µg/mL.

This suppression of enzyme-dependant metabolic activities was supported by the results of the API ZYM tests for the detection of specific enzymes. In general, the loss or suppression of enzyme activity in the test strains, agreed with the earlier classification into resistant and less-resistant strains and also with the finding that of the two genera tested, Streptococcus appeared to be the more resistant (Tables 20, 21, 30). Therefore it might be expected that a comparison of the bacterial populations of plaque, from an amalgam restoration and an unrestored equivalent tooth in the same mouth, would show different ratios of Streptococcus and Actinomyces and perhaps different species and strains of these genera; this would be worth investigation.

The suppression of enzyme activity would also explain the earlier finding that in the biofilms grown in the presence of mercury, the protein and carbohydrate contents were reduced. Consistent with this was the lack of fermentation and hydrolysis for specific substrates (Table 24).

The suppression of enzymes was not enzyme-specific in that an enzyme that was totally suppressed in a specific mercury concentration in one strain was only partially suppressed in another and was unaffected in yet another strain. Perhaps the enzymes within a bacterium are interdependant and can compensate for each other; this process would contribute to the characteristics specific to a micro-organism.

An intriguing finding was that some of the enzymes appeared to increase in activity in the presence of mercury and, in Actinomyces viscosus (T6) and Actinomyces naeslundii 2 (WVU 627), the enzyme N-acetyl β glucosaminidase was only detected when mercury was present, thus implying an induction of the enzyme by mercury. This induction could be part of the organisms' resistance mechanisms. The biochemical basis for enzyme induction and suppression is beyond the scope of this study but it is interesting to note that the enzyme, cystine amino-peptidase appeared to be particularly susceptible to suppression by mercury. This enzyme contains a disulphide radicle to which mercury has a propensity to bind, so this could be a possible mechanism of suppression.

Two approaches were used to investigate the adaptation of some Streptococcus strains to mercury. The results for enzyme activity are recorded in Table 30 and those for growth, as measured by the count of colony-forming units on agar plates, are shown in Table 20; these data can be compared.

The results of the two investigations were comparable for Streptococcus mitis 1 (SK138) and Streptococcus mutans (2452) in that both showed that these strains had some resistance to mercury.

Streptococcus mutans (Pb1094A) appears from Table 30 to have resistance in 10 µg Hg/mL, whereas Table 20 shows no resistance at this mercury concentration. The results for the higher levels of mercury are in agreement.

The results for Streptococcus milleri (NCTC 10709) were quite different for the two investigations. Table 30 indicates that this strain had considerable resistance to mercury, but the growth results in Table 20 suggest that it was a non-resistant strain of Streptococcus.

A similar comparison can be made for some Actinomyces strains. The enzyme investigation results in Table 30 suggest that Actinomyces viscosus (T6) was resistant at 10 µg Hg/mL and the growth results (Table 21) were similar with some resistance also evident in 20 and 30 µg Hg/mL.

The two sets of results (Tables 21 and 30) for Actinomyces naeslundii (ATCC 12104) suggest a similar pattern of resistance to mercury for this strain.

The results in Tables 21 and 30 are in agreement for the lack of resistance to mercury of the intermediate Actinomyces strain (B236) and Actinomyces naeslundii 2 (WVU627) except at the 10 µg/mL concentration of mercury. At this mercury level

there was some enzyme activity for both strains but neither demonstrated growth on plates.

With the exception of Streptococcus milleri (NCTC 10709) the results of the two investigations are comparable for the Streptococcus and Actinomyces strains included in both studies.

The therapeutic use of mercury in inhibiting bacterial growth is not new. Mercury-based ointments for treatment of eye and skin infections have been used up to the present time and the treatment of syphilis with mercury is expounded by Lloyd (66) in a medical text in 1945, although by 1959 French and McLeod (40) state that "In the last decade penicillin has revolutionized the treatment of syphilis". Unfortunately, the growth of the organisms responsible for dental caries has not been inhibited to the point where amalgam restorations could be considered a preventive as well as a restorative measure; clinicians are only too aware of recurrent caries around amalgam restorations. As with other bacteriostatic and bacteriocidal materials, such as penicillin, the bacteria seem to gain the upper hand through their ability to express resistance, either through the process of selection or adaptation.

The studies described above have shown that some interactions occur in the oral ecosystem between the oral flora and the mercury of dental amalgam. Release of mercury from the amalgam is facilitated, probably by the metabolic activity of those organisms resistant to the toxic effects of mercury. The released mercury is taken up to various degrees by the bacteria which appear to store mercury in the cell wall and in the remaining parts of the cell. An estimate of the total mercury taken up by plaque over a 24 hour period is 1.8 µg, which is well below the value of 43 µg, the maximum daily intake of mercury recommended by the World Health Organization. The amounts of mercury released by the biofilms are not of an order considered to be a health hazard if ingested or inhaled.

Some bacteria appear to be more resistant than others to mercury and this would cause a change in the populations of micro-organisms making up the community of oral flora; thus the presence of amalgam restorations probably has an effect on the ecosystem of the oral cavity. The effect may be difficult to detect clinically because of the complex nature of plaque and the fact that caries and periodontal disease are not dependant on specific organisms.

Several authors have noted the co-existence and possible relationship of resistance to mercury and to antibiotics (41, 86, 118) and this might pose a problem. If the presence of amalgam restorations and thus mercury, selects micro-organisms with a resistance to mercury, and this resistance is associated with resistance to one or more antibiotics (the resistance having been conferred by a plasmid), then the effective treatment of the patient with antibiotics could be compromised. This could have a particular application where an opportunist population from the oral cavity was the cause of a systemic infection, and of particular concern would be the immunocompromised patient. The normal hospital practice of culture and sensitivity tests before prescribing an antibiotic would circumvent the problem of prescribing an inappropriate antibiotic but it is noteworthy that penicillin-type antibiotics are usually effective against most oral infections. The possible association of mercury and antibiotic resistance in the oral flora merits investigation.

The entrapment of mercury by plaque might encourage the facetious suggestion that lack of oral hygiene would prevent body-uptake of mercury, but the demonstrated cyclical release shows that eventually more mercury is released; therefore the removal of acid-producing plaque should still be recommended. Other clinical practices such as burnishing and polishing amalgam restorations should also be encouraged because the finished surfaces are more resistant to corrosion (72, 102).

The results of these studies confirm that although mercury is released from dental amalgam intra-orally, the amount is approximately 4% of the allowable human daily intake of mercury (127). The advantages of having an economically feasible restorative material, such as dental amalgam, that has withstood the test of time, outweighs the, as yet unproven, risk of inhaling or ingesting mercury released from amalgam restorations.

The relationship between the mercury content of plaque and blood and urine levels of mercury has not been defined. The total body burden of mercury, indicated by blood and urine levels, is dependant on dietary sources as well as the amalgam source but the level in plaque appears, from these studies, to be entirely dependant on the release of mercury from amalgam restorations. It would therefore be interesting to determine whether there is a correlation between the mercury level in plaque and the total body-burden of mercury as defined by blood and urine values, as this could be used as a indicator of the mercury contribution from amalgam restorations.

These studies confirm the complex nature of an ecosystem consisting of biotic and abiotic components, specifically that of the oral cavity, its microflora and their resistance to mercury.

CONCLUSIONS

From the results of the studies described above, it was concluded that mercury is found in plaque from dentitions restored with amalgam and the amount of mercury in 24 hr plaque is approximately 8% of the total daily intake, as estimated by the USEPA at 25 µg mercury per day. It was also concluded that biofilms absorb and facilitate the release of mercury from fresh amalgam and that the liberated mercury modifies the physiology of the micro-organisms in the biofilm. From the studies on resistance it was concluded that oral bacteria vary in their degree of resistance and can adapt to mercury and that this element is bound in two cell compartments, the cell wall and cytoplasm. Aged amalgam was resistant to the activities of the biofilms, in that there was no detectable mercury released and biofilm metabolism was not inhibited. This suggests that release of mercury from aged amalgam in vivo results from physical disruption of a passive surface layer.

Although these studies answered some questions they also raised others that warrant further investigation. The question of methylation of mercury by plaque remains unanswered and since methylmercury is more toxic than elemental mercury, this question is worthy of investigation. The study of the relationship between mercury-resistance and the distribution of bound mercury in the cell compartments needs to be expanded to include more mercury-resistant and non-resistant strains. The effect of various concentrations of mercury in the medium on the distribution in the cell compartments should also be included in the study. Another question is that of the relationship between resistance to mercury and resistance to other inhibitory agents such as antibiotics.

The study of the interactions of biotic and abiotic components of an ecosystem such as the oral cavity is an ongoing one and as questions are answered, the answers point to more questions; - such is the nature of research.

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APPENDICES

1. BM Medium
2. Inoculum Broth
3. Tryptone Soya Broth (4% sucrose)
4. A: Actinomyces Defined Medium (ADM)
   B: Modified Actinomyces Defined Medium (MADM)
5. Reduced Transport Fluid (RTF)
6. Consent Form for Human Plaque Study
7. Streptococcus Sugar-Base Agar
8. Effect of Mercury on Enzyme Activity of Streptococcus mitis I (SK138)
9. Effect of Mercury on Enzyme Activity of Streptococcus mutans (2452)
10. Effect of Mercury on Enzyme Activity of Streptococcus mutans (Pb1094A)
11. Effect of Mercury on Enzyme Activity of Streptococcus milleri (NCTC 10709)
12. Effect of Mercury on Enzyme Activity of Actinomyces viscosus (T6)
13. Effect of Mercury on Enzyme Activity of Actinomyces naeslundii (ATCC 12104)
14. Effect of Mercury on Enzyme Activity of an intermediate Actinomyces strain (B236)
15. Effect of Mercury on Enzyme Activity of Actinomyces naeslundii II (WVU627)

Appendix 1

B.M. MEDIUM

Tryptone	1.0%
Tryptose	1.0%
Yeast Extract	0.5%
Glucose	0.5%
Sodium chloride	0.5%
L-cysteine HCl	0.075%

Dissolve ingredients in deionized water and adjust pH to 7.4

Add haemin as required

Add Menadione/Vitamin K_1 as required

Dispense as required

Autoclave at 121^{o} C for 30 minutes

Appendix 2

INOCULUM BROTH

Tryptose	2.0%
Yeast Extract	0.5%
Sodium chloride	0.5%
Sodium phosphate (dibasic)	0.1%
Bromocresol purple	0.001%
Dextrose	0.1%

Dissolve ingredients in deionized water and adjust pH to 7.6.

Dispense as required

Autoclave at 121^{o} C for 30 minutes

Appendix 3

TRYPTONE SOYA BROTH (4% SUCROSE)

Tryptone Soya Broth	3%
Sucrose	4%

Dissolve ingredients in deionized water and adjust the pH to 7.0

Dispense as required

Autoclave at 121° C for 30 minutes

Appendix 4A

ACTINOMYCES DEFINED MEDIUM (ADM)

1. Basic Solution

Potassium phosphate (monobasic)	0.6%
Potassium phosphate (dibasic)	0.9%
Calcium chloride	0.002%
Magnesium sulphate	0.02%
Sodium acetate	0.03%

add

2.

Glucose (dextrose)	0.5%
L-cysteine HCl	0.02%
Glutathione	0.005%
L-asparagine	0.01%
L-tryptophane	0.004%
Vitamin Casamino acid	0.2%
L-Glutamic acid	0.05%

3. Solution 1

P.A.B.	0.02%
Thiamine (Aneurine)	0.02%
Riboflavin	0.02%
Nicotinic acid	0.02%
Pyridoxal HCl	0.02%
Inositol	0.02%
Calcium pantothenate	0.02%

Dissolve ingredients in deionized water and adjust pH to 7.0
Add 10 mL to 1 litre of the Basic solution.
(Store frozen)

Appendix 4A (cont.)

Solution 2

DL Thioctic acid	0.01%
Biotin	0.01%
Haemin	0.01%
Folic acid	0.02%

Dissolve haemin first in 1 drop of d. H_2O and 1 drop of ammonium hydroxide.
Add 1 mL to 1 litre of the Basic solution
(Store frozen)

Solution 3

Ferrous sulphate	0.400%
Manganous sulphate	0.015%
Sodium molybdate	0.015%

Dissolve ingredients in 100 mL of deionized water
Add 1 mL to 1 litre of the Basic solution
(Store frozen)

Appendix 4B

MODIFIED ACTINOMYCES DEFINED MEDIUM (MADM)

1. Basic Solution

Potassium phosphate (monobasic)	0.6%
Potassium phosphate (dibasic)	0.9%
Calcium chloride	0.002%
Magnesium sulphate	0.02%
Sodium acetate	0.03%

add

2.

Glucose (dextrose)	0.5%
L-cysteine HCl	0.02%
Glutathione	0.005%
L-Asparagine	0.01%
L-Tryptophane	0.004%
Vitamin Casamino acid	0.2%
L-Glutamic acid	0.05%

3. Solution 1

P.A.B.	0.02%
Thiamine (Aneurine)	0.02%
Riboflavin	0.02%
Nicotinic acid	0.02%
Pyridoxal HCl	0.02%
Inositol	0.02%
Calcium pantothenate	0.02%

Dissolve ingredients in deionized water and adjust pH to 7.0
Add 10 mL to 1 litre of the Basic solution.
(Store frozen)

Appendix 4B (cont.)

Solution 2

DL Thioctic acid	0.01%
Biotin	0.01%
Haemin	0.01%
Folic acid	0.02%

Dissolve haemin first in i drop of d. H_2O and 1 drop of ammonium hydroxide.
Add 1 mL to 1 litre of the Basic solution
(Store frozen)

Solution 3

Manganous sulphate	0.015%
Sodium molybdate	0.015%

Dissolve ingredients in 100 mL of deionized water
Add 1 mL to 1 litre of the basic solution
(Store frozen)

Mercuric chloride (when required)

Add an appropriate volume of 5% $HgCl_2$ to make the required mercury concentration in the medium.

Appendix 5

REDUCED TRANSPORT FLUID

Potassium phosphate (monobasic)	0.045%
Potassium phosphate (dibasic)	0.045%
Sodium chloride	0.09%
Ammonium sulphate	0.09%
Magnesium sulphate	0.018%
Ethylene diamine tetracetic acid (EDTA)	0.038%
Sodium carbonate	0.04%
Dithiothreitol	0.02%

Dissolve ingredients in deionized water

Dispense as required

Autoclave at 121^0C for 30 minutes

Appendix 6

INFORMED CONSENT FORM

STUDY: Acquisition of plaque to determine if there is a difference in the mercury content of plaque from enamel and amalgam restorations.

I.....agree to avoid oral hygiene activities for 24 hours and to allow Dr. H.A. Lyttle or her designate to remove plaque, from my teeth, to be used in a subsequent laboratory investigation. I understand that I may be requested to do so several times but not more than once in any 7 day period.

I understand the possible results of refraining from oral hygiene (caries, gingivitis and periodontitis) but accept that there is little likelihood of these diseases occurring as a result of participation in this study.

I understand that my particioation in this study is voluntary and that I can withdraw at any time with no penalty.

NAME (please print) .....

SIGNATURE .....

WITNESS

NAME (please print) .....

SIGNATURE .....

cnst/plq
hal/90

Appendix 7

STREPTOCOCCUS SUGAR-BASED AGAR

Proteose peptone	2.0%
Yeast extract	0.5%
Sodium chloride	0.5%
Di sodium phosphate (sodium phosphate dibasic)	0.1%
Phenol red	0.002%

Add following for specific sugars:

Melibiose (O)	0.5%
Raffinose (F)	0.5%
Mannitol (M)	0.5%
Sorbitol (S)	0.5%

The Enzyme Activity of Streptococcus mitis I (SK 138)
With Various Concentrations of Mercury in the Medium

Hg Conc. µg/mL	Control	alkaline phospha -tase	esterase	esterase lipase (C8)	lipase (C14)	leucine amino-peptidase	valine amino-peptidase	cystine amino-peptidase	trypsin	chymo-trypsin
0	0	3	2	3	1	0	0	0	0	0
10	0	3	1	3	1	0	0	0	0	0
20	0	4	0	1	0	0	0	0	0	0
30	0	2	0	1	0	0	0	0	0	0
40	0	1	0	0	0	0	0	0	0	0

Hg Conc. µg/mL	acid phosphat -ase	phospho hydrol-ase	α-galacto-sidase	β-galacto-sidase	β-glucuron -idase	α-gluco-sidase	β-gluco-sidase	N acetyl-β glucosa-minidase	α manno-sidase	α fucosid-ase
0	5	1	0	0	0	0	0	0	0	0
10	5	1	0	0	0	0	0	0	0	0
20	5	1	0	0	0	0	0	0	0	0
30	4	1	0	0	0	0	0	0	0	0
40	3	1	0	0	0	0	0	0	0	0

The Enzyme Activity of Streptococcus mutans (2452)
With Various Concentrations of Mercury in the Medium

Hg Conc. µg/mL	Control	alkaline phospha -tase	esterase	esterase lipase (C8)	lipase (C14)	leucine amino-peptidase	valine amino-peptidase	cystine amino-peptidase	trypsin	chymo-trypsin
0	0	2	2	2	1	5	5	2	2	5
10	0	0	2	0	1	5	5	1	0	5
20	0	0	0	1	0	5	0	0	0	0
30	0	0	0	1	0	5	0	0	0	0
40	0	0	0	1	0	5	0	0	0	0

Hg Conc. µg/mL	acid phosphat -ase	phospho hydrol-ase	α-galacto-sidase	β-galacto-sidase	β-glucuron -idase	α-gluco-sidase	β-gluco-sidase	N acetyl-β glucosa-minidase	α manno-sidase	α fucosid-ase
0	3	2	0	0	0	4	0	0	0	0
10	1	2	0	0	1	0	1	0	0	0
20	0	1	0	0	0	4	0	0	0	0
30	0	1	0	0	0	3	0	0	0	0
40	0	1	0	0	0	5	0	0	0	0

The Enzyme Activity of Streptococcus mutans (Pb1094 A)
With Various Concentrations of Mercury in the Medium

Hg Conc. µg/mL	Control	alkaline phospha -tase	esterase	esterase lipase (C8)	lipase (C14)	leucine amino-peptidase	valine amino-peptidase	cystine amino-peptidase	trypsin	chymo-trypsin
0	0	1	2	2	1	3	3	2	1	3
10	0	1	2	2	1	3	2	2	1	3
20										
30										
40										

Hg Conc. µg/mL	acid phosphat -ase	phospho hydrol-ase	α-galacto-sidase	β-galacto-sidase	β-glucuron -idase	α-gluco-sidase	β-gluco-sidase	N acetyl-β glucosa-minidase	α manno-sidase	α fucosid-ase
0	2	2	0	0	0	1	1	0	0	0
10	2	1	0	0	0	2	2	0	0	0
20										
30										
40										

No growth occurred in the media with mercury concentrations of 20, 30 and 40 µg/mL

The Enzyme Activity of Streptococcus milleri (NCTC 10709)
With Various Concentrations of Mercury in the Medium

Hg Conc. µg/mL	Control	alkaline phospha -tase	esterase	esterase lipase (C8)	lipase (C14)	leucine amino-peptidase	valine amino-peptidase	cystine amino-peptidase	trypsin	chymo-trypsin
0	0	5	2	3	0	5	1	1	0	0
10	0	5	2	3	0	5	0	1	0	0
20	0	3	1	2	0	1	0	0	0	0
30	0	4	1	2	0	1	0	0	0	0
40	0	4	1	3	0	2	0	0	0	0

Hg Conc. µg/mL	acid phosphat -ase	phospho hydrol-ase	α-galacto-sidase	β-galacto-sidase	β-glucuron -idase	α-gluco-sidase	β-gluco-sidase	N acetyl-β glucosa-minidase	α manno-sidase	α fucosid-ase
0	5	1	0	5	0	5	0	0	0	0
10	5	1	0	5	1	5	0	0	0	0
20	5	1	0	0	0	1	0	0	0	0
30	5	1	0	0	0	1	0	0	0	0
40	5	1	0	0	0	1	0	0	0	0

The Enzyme Activity of Actinomyces viscosus (T6)
With Various Concentrations of Mercury in the Medium

Hg Conc. µg/mL	Control	alkaline phospha -tase	esterase	esterase lipase (C8)	lipase (C14)	leucine amino-peptidase	valine amino-peptidase	cystine amino-peptidase	trypsin	chymo-trypsin
0	0	5	1	2	0	3	2	1	0	0
10	0	4	0	2	0	3	1	0	0	0
20										
30										
40										

Hg Conc. µg/mL	acid phosphat -ase	phospho hydrol-ase	α-galacto-sidase	β-galacto-sidase	β-glucuron -idase	α-gluco-sidase	β-gluco-sidase	N acetyl-β glucosa-minidase	α manno-sidase	α fucosid-ase
0	5	0	2	5	0	2	2	0	0	1
10	4	0	1	5	0	0	2	3	0	0
20										
30										
40										

No growth occurred in the media with mercury concentrations of 20, 30 and 40 µg/mL

The Enzyme Activity of Actinomyces naeslundii 1 (ATCC 12104)
With Various Concentrations of Mercury in the Medium

Hg Conc. µg/mL	Control	alkaline phospha -tase	esterase	esterase lipase (C8)	lipase (C14)	leucine amino-peptidase	valine amino-peptidase	cystine amino-peptidase	trypsin	chymo-trypsin
0	0	2	1	1	0	4	3	1	0	0
10	0	2	0	0	0	5	2	0	0	0
20	0	0	0	0	0	5	1	0	0	0
30	0	5	0	3	0	5	3	0	1	0
40										

Hg Conc. µg/mL	acid phosphat -ase	phospho hydrol-ase	α-galacto-sidase	β-galacto-sidase	β-glucuron -idase	α-gluco-sidase	β-gluco-sidase	N acetyl-β glucosa-minidase	α manno-sidase	α fucosid-ase
0	5	1	2	3	0	3	1	0	0	0
10	4	0	1	5	0	3	3	0	0	0
20	0	1	0	0	0	1	0	0	0	0
30	5	3	0	0	0	0	1	0	0	0
40										

No growth occurred in the medium with a mercury concentration of 40 µg/mL

The Enzyme Activity of an intermediate Actinomyces strain (B 236)
With Various Concentrations of Mercury in the Medium

Hg Conc. µg/mL	Control	alkaline phospha -tase	esterase	esterase lipase (C8)	lipase (C14)	leucine amino-peptidase	valine amino-peptidase	cystine amino-peptidase	trypsin	chymo-trypsin
0	0	1	1	1	0	5	4	1	0	0
10	0	0	0	0	0	5	1	0	0	0
20										
30										
40										

Hg Conc. µg/mL	acid phosphat -ase	phospho hydrol-ase	α-galacto-sidase	β-galacto-sidase	β-glucuron -idase	α-gluco-sidase	β-gluco-sidase	N acetyl-β glucosa-minidase	α manno-sidase	α fucosid-ase
0	4	1	1	5	0	2	1	0	0	0
10	4	1	0	5	0	1	1	0	0	0
20										
30										
40										

No growth occurred in the media with mercury concentrations of 20, 30 and 40 µg/mL

The Enzyme Activity of Actinomyces naeslundii 2 (WVU 627)
With Various Concentrations of Mercury in the Medium

Hg Conc. µg/mL	Control	alkaline phospha -tase	esterase	esterase lipase (C8)	lipase (C14)	leucine amino-peptidase	valine amino-peptidase	cystine amino-peptidase	trypsin	chymo-trypsin
0	0	2	1	1	0	4	3	1	0	0
10	0	0	0	0	0	4	3	0	0	0
20										
30										
40										

Hg Conc. µg/mL	acid phosphat -ase	phospho hydrol-ase	α-galacto-sidase	β-galacto-sidase	β-glucuron -idase	α-gluco-sidase	β-gluco-sidase	N acetyl-β glucosa-minidase	α manno-sidase	α fucosid-ase
0	3	1	0	5	0	1	1	0	0	0
10	3	1	0	5	0	1	0	2	0	0
20										
30										
40										

No growth occurred in the media with mercury concentrations of 20, 30 and 40 µg/mL