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THE EFFECTS OF FLUORIDES ON ORAL MICROORGANISMS.

by

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A thesis submitted in partial fulfilment of
the requirements for the degree of
Master of Dental Science.

DEPARTMENT OF PREVENTIVE DENTISTRY.

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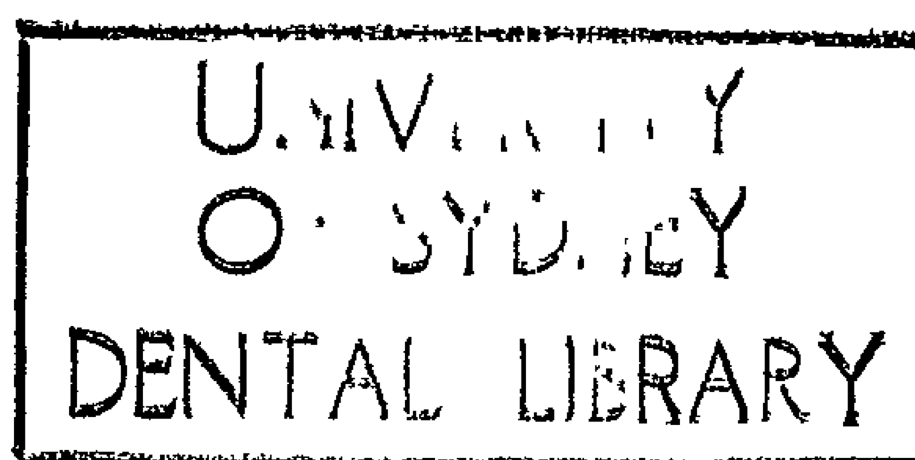

Abstract.

This study is an in vitro examination of the effect of sodium fluoride, stannous fluoride and silver fluoride on oral microorganisms involved in periodontal disease. The microorganisms Streptococcus sanguis G9B, Actinomyces viscosus T14 and Bacteroides intermedius NCTC 9336 of known numbers were inoculated into a test solution of different fluoride concentrations. At exposure intervals 0, 1-5 and 15 minutes samples were plated on agar. After incubation the number of colony forming units were countered.

In procedure one and two, sterile deionized filtered water was the test solution. Silver fluoride and stannous fluoride at 10ppmF were equally effective against the microorganisms, allowing no growth at 1-5 and 15 minutes. Sodium fluoride had minimal effect at 100ppmF at 1-5 minutes. Acidulating the sodium fluoride enhanced sodium fluorides' effect.

In procedure three a 6% serum albumin solution was the test solution. At 1-5 minutes 100ppmF stannous fluoride is the most effective against S.sanguis G9B, and 100ppmF silver fluoride is the most effective against A.viscosus T14 and B.intermedius NCTC 9336. Both silver fluoride and stannous fluoride were effective after 15 minutes.

The results indicate that silver fluoride is potentially a more effect antimicrobial agent than stannous fluoride. Further research using silver fluoride is indicated.

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Introduction and Literature Review.

Gingivitis and periodontitis are diseases caused by the microbial colonization of the gingival sulcus (Socransky 1970, Page and Schroeder 1982). Treatment has traditionally consisted of the removal of microorganisms and local factors by mechanical means. The adjunctive use of chemotherapeutic agents has gained support as a treatment regime. One such agent that has been used to aid treatment is fluoride. Both sodium fluoride and stannous fluoride have been studied (Perry 1982). The compound, silver fluoride has been topically applied in one clinical trial (Oppermann and Johansen 1980), however no in vitro studies have been reported. This prompted the present study to be undertaken to compare the effects of three fluoride compounds, sodium fluoride, stannous fluoride and silver fluoride, on three oral microorganisms involved in periodontal disease.

1.1 Definition.

Periodontal diseases consist of a large number disorders of which gingivitis and periodontitis are the most common. Gingivitis is an inflammatory disorder that is characterized by redness, bleeding on gentle probing, oedema and involves only the marginal gingival tissues. Periodontitis is an inflammatory disorder of the supporting tissues of the teeth, with a destructive change leading to loss of alveolar bone and loss of the connective tissue attachment to the root surface. For the purposes of this review the term 'periodontal disease' will imply gingivitis and

periodontitis. Historically the presence of this disease has been known for centuries.

1.2 Historical.

Recently Gold (1985 a,b,c) has reviewed the history of periodontics, a brief outline follows.

Early references to dental and gingival problems have been recorded on ancient Egyptian papyrus, circa 1550 BC. Reference to dental cures and oral hygiene practices in other ancient cultures, such as the Chinese and Arabic have been recorded.

It was Van Leeuwenhoek in 1683 who first described the appearance of oral microorganisms. He scraped 'white matter' from his teeth and examined it using an early microscope. He found living 'animicules' of various shapes and sizes both stationary and moving.

Pierre Fauchard 1728 has been credited for noting the important relationship between oral hygiene and the aetiology of periodontal disease. He advised the seeking of regular removal of dental calculus with instruments and the need for excellent oral hygiene.

John Hunter in 1803 described the use of chemical adjuncts, both alkaline and acid solvents in the scaling of teeth to remove dental calculus.

John Riggs in 1876 stated that periodontal disease was a local problem and not a systemic problem as accepted at that time. The disease came to be known as "Riggs disease".

In 1890 Miller described and classified oral microorganisms. He felt that the disease was a mixed infection of a non-specific normal oral flora. He postulated the so called 'Non-Specific Plaque Hypothesis'. This implied that the periodontal disease was a result of the entire plaque flora.

Löe, Theilade and Jensen (1965) carried out their experimental gingivitis study which provided scientific evidence to implicate oral microorganisms in the aetiology of gingivitis in humans.

Loesche (1976) and Socransky (Page and Schroeder 1982) have been credited for proposing the 'Specific Plaque Hypothesis'. This states that

> only certain plaques cause infections because of the presence of a pathogen(s) and/or a relative increase in the levels of certain indigenous plaque organisms (Loesche 1976).

In 1986 Theilade questioned the strict concepts of the Specific Plaque Hypothesis and indicated that a compromise between the two hypotheses may be more appropriate.

Present day research has been focused on producing evidence for these hypotheses and finding successful efficient treatment modalities for the control of periodontal disease.

The following literature review will outline

- the evidence for the microbiological aetiology of gingivitis and periodontitis
- the stages in the development of dental plaque

• the microorganisms associated with health, gingivitis and periodontitis and

• the treatment modalities used with special reference to adjunctive chemotherapy, especially the use of fluorides.

1.3 Aetiology of Periodontal disease.

Dental plaque may be defined as any organized mass, consisting mainly of microorganisms that adhere to or occupy the gingival crevice. Consisting also of an organic, polysaccharide protein matrix, bacterial by-products and inorganic components (Glossary of Periodontic Terms 1986). Evidence for the involvement of dental plaque in the initiation and progression of periodontal disease comes from a number of sources.

1.31 Epidemiolgical studies.

A relationship between age and the increase in frequency and severity of periodontal disease has been noted (Belting et al. 1953, Marshall-Day et al. 1955) The prevalence of gingivitis is high during the teenage years with adulthood producing an increase in the prevalence of periodontitis. The older the population the greater the number of periodontits cases that are observed to occur. Hugoson and Jordan (1982) studied 600 Swedish individuals aged 20 to 70 years and found 30-50% of tooth surfaces were covered with plaque. In the 20 year olds, 28% were disease free and 4% had evidence of bone destruction. In the older age groups, 50-70 years, none were disease free, evidence of early bone destruction was high and severe periodontal breakdown was present in 3-8% of dentulous individuals. Despite criticism of the indices used in epidemiological studies, these studies frequently

show a relationship between the presence of the disease and oral uncleanliness.

1.32 Human autopsy material.

A close relationship has been shown between the location of the microbial deposits and the extension of the inflammatory connective tissue lesion. The distance of the inflammatory lesion from the dental plaque is 1-2mm with normal connective tissue (1mm) between the infiltrate and alveolar bone (Waerhaug 1979).

1.33 Clinical Trials.

The development of gingivitis in humans was demonstrated by dental plaque accumulation over 21 days by Löe et al. (1965). The effective mechanical removal of dental plaque can reduce and eliminate inflammation (Lovdal et al. 1961, Löe et al 1965., Axelsson and Lindhe 1974). The use of chemical agents, such as Chlorhexidine (Löe and Schiott 1970) and antibiotics (Löe et al. 1967) have been shown to reduce experimental gingivitis.

The evidence for the progression of gingivitis to periodontitis in humans is lacking. The presence of gingivitis is not a prognostic indicator of future breakdown in humans (Listgarten et al. 1985). In a population not influenced by dental treatment 11% of individuals exhibited no progression of the disease beyond gingivitis, while 81% showed moderate attachment loss and 8% rapid attachment loss (Löe et al. 1986).

The progression of periodontitis has been shown to be retarded through professional and patient maintenance (Ramfjord et al. 1968,1975, Knowles et al. 1979, Axelsson and Lindhe 1978,1981, Lindhe

and Nyman 1984 and Lindhe et al. 1984). A proportion of those patients left untreated continue to show sighs of further loss at some sites (Lindhe et al. 1983a).

1.34 Animal Models.

The development of gingivitis and progression to periodontitis has been shown to occur naturally in dogs (Saxe et al. 1967,. Lindhe et al. 1973). The continued mechanical removal of dental plaque has prevented the disease initiation and progression (Lindhe et al. 1973,1975).

In gnotobiotic animals the presence of silk ligatures does not cause any tissue breakdown unless microorganisms are present (Rovin et al. 1966). Both Gram positive and Gram negative microorganisms isolated from human dental plaque which are monoinfected into germ free rodents cause considerable tissue breakdown and bone loss (Jordan et al. 1972).

1.4 Dental plaque formation.

Studies examining the development of dental plaque on cleaned tooth surfaces have shown the rapid formation of a acquired pellicle (Gibbon and Van Houte 1973, Theilade 1977, Lie 1979). The pellicle is derived predominantly from salivary glycoproteins and has a complex structure (Lie 1979). Initial colonization occurs via direct absorption to the acquired pellicle, via surface threads, to irregularities of pellicle or hydroxyapatite crystal or entrapment in organic material (Gibbon and Van Houte 1973, Lie 1979). This absorption occurs in two stages, the first is a reversible phase thought to be due to Van der Waals forces between the negatively

charged pellicle and microorganisms. In the second phase bridging occurs between macromolecules producing an irreversible binding of microorganisms (Gibbon and Van Houte 1973). The time of colonization varies from minutes to several hours (Socransky et al. 1977, Theilade 1977). The colonization and development is also influenced by state of health of the gingival tissues and oral hygiene (Hillam and Hull 1977, Goh et al. 1986).

The early colonizers are mainly Gram positive cocci and rods, especially Streptococcus sanguis and Actinomyces viscosus. After 30-40 hours the supragingival bacterial numbers increase 100-1000 fold, due mainly to the rapid proliferation of S.sanguis (Socransky et al. 1977). After three days Gram negative species appear, e.g. Veillonella (Theilade et al. 1982). This increase is due to multiplication of bacteria present on the surface not from continued absorption from saliva (Brecx et al. 1983). The presence of subgingival plaque has been shown to occur after 7 days in dogs and in usually continuous with supragingival deposits (Ten Napal et al 1985). Van Palenstein Helderman (1981) found no significant difference in the composition of supragingival and subgingival plaque samples up to 2 weeks in humans. With time, the microbial flora changes from a predominate Gram positive to a Gram negative flora. Evidence indicates that species of microorganisms colonized in a sequential manner (Moore et al. 1982a, 1984a) and with specific coaggregation reactions between microorganisms (Cisar 1982). Cisar showed the importance of S.sanguis to allow colonization by the Actinomyces species, especially A.viscosus and the ability of these Gram positive microorganisms to enhance binding with Gram negative

microorganisms, such as the Bacteroides sp. and Fusobacterium nucleatum.

Dental plaque, allowed to accumulate on epoxy crowns shows an initial pellicle formation with no adherent microorganisms. After one day the plaque consists of coccoid and filamentous microorganisms with an intercellular matrix. By one week the cocci have been replaced with filamentous forms and at 3 weeks there is a loose covering of "corn cob" formations and some spirochaetes. After two months the bulk of the plaque is densely packed filamentous microorganisms orientated perpendicular to the crown, with "bristle brush" formations and a loose plaque mainly spirochaetes and flagellated microorganisms (Listgarten et. al. 1975).

The subgingival plaque associated with disease types has been studied using electron microscopy. In clinically healthy patients there was a thin layer of adherent bacterial cells up to 60μm thick, mainly consisting of Gram positive cocci with a few filamentous and Gram negative microorganisms. In gingivitis cases the flora was densely packed with a wide variety of coccoid and filamentous forms and in the layer closest to the tooth, cell lysis and mineralization were seen. Flagellated microorganisms and spirochaetes were frequently present. In the periodontitis samples, the tooth surface was mainly covered with filamentous forms, next a transitional zone of flagellated microorganisms and a surface zone of mostly spirochaetes, "bristle brush" and "test-tube" formations covered with polymorphonuclear cells and macrophages. The flora is predominantly Gram negative (Listgarten 1976).

1.5 Associated microorganisms.

Investigations into the aetiological role of microorganisms in infectious diseases have used Koch's postulates. The pathogenic microorganism should

1. regularly be found in lesions of the disease

2. be grown in pure culture on artificial media

3. the inoculation of this culture produces a similar disease in experimental animals

4. the microorganism can be recovered from the lesions in these animals.

Problems existed with dental infections as many of the isolates could not be inoculated into an animal model and the induced disease may not be the same as in the human. Therefore Socransky (1979) proposed five criteria to determine oral pathogens,

1. Association with the disease.

2. Elimination or suppression of the organism with treatment.

3. Host Response.

4. Animal pathogenicity.

5. Mechanisms of pathogenicity.

This review will concentrate on evidence obtained from cultural and microscopic techniques.

1.51 Clinical Health.

Gram positive microorganisms dominate the dental plaque, especially S.sanguis, Streptococcus mitis, A.viscosus and Actinomyces naeslundii (Socransky et al. 1977, Slots 1979). Gram negative microorganisms belonging to the following genera have been isolated in low numbers, Neisseria, Veillonella, Bacteroides and Fusobacterium (Van Palenstein Helderman 1981, Savitt and Socransky 1984). Darkfield studies confirm the high coccoid cell counts (75% of the total count) and the low motile to non-motile ratio, 1:49 (Listgarten and Hellden 1978).

Page and Schroeder (1982) have proposed that the presence and activity of the Gram positive flora is essential for the disease to develop.

1.52 Gingivitis.

Löe et al. (1965) in their experimental gingivitis study showed a change in the flora from a predominantly Gram positive to a predominantly Gram negative flora over 21 days. Syed and Loesche (1978) noted that prior to the onset of clinical gingivitis there was a shift from a Streptococcus dominated plaque to an Actinomyces dominated plaque.

Over 166 bacterial species have been isolated from young adults with experimental gingivitis, A.naeslundii, A.viscosus, F.nucleatum, Veillonella parvula, Lactobacilus sp. and Treponema sp. being the most prominent (Moore et al. 1982a). In young children (4-6 years) the flora was different to the young adults

due to larger proportions of Leptotrichia sp., Capnocytophaga sp., Selanomonas sp. and Bacteroides sp. (Moore et al. 1984b).

In cross sectional studies on gingivitis the microorganisms present are A.viscosus, S.sanguis, F.nucleatum, Bacteroides intermedius, Haemophilus sp. and a few motile rods and spirochaetes (Listgarten 1976, Slots 1979, Savitt and Socransky 1984).

Gingivitis associated with pregnancy showed a marked increase in the microorganism B.intermedius during the second trimester (Kornman 1980). Acute necrotizing ulcerative gingivitis (ANUG) has a constant flora of B.intermedius, Fusobacterium sp. and Treponema sp. (Loesche et al. 1982).

There appears to be a large variation in the microorganisms present in gingivitis and it is now generally agreed that gingivitis represents a non-specific infection (Page 1986).

1.53 Periodontitis.

Page and Schroeder in 1982 sub-classified periodontitis into four clinical entities. The review will provide information on Rapidly Progressive periodontitis and Adult periodontitis.

The composition of the supragingival plaque in relation to periodontitis is similar to that observed in gingivitis (Listgarten 1976), darkfield studies of subgingival diseased sites indicates a more complex flora with increased numbers of motiles, curved rods and small, medium and large spirochaetes. The ratio of motile to non-motile is 1:1 (Listgarten and Hellden 1978).

Slots (1979) found Bacteroides gingivalis and F.nucleatum to be present in advanced periodontitis. Tanner et al. (1979) found in young adults (Rapidly Progressive periodontitis), that B.gingivalis and Actinobacillus actinomycetemcomitans were the predominant cultured microorganisms. In studies of adult periodontitis patients active sites have elevated numbers of the species B.intermedius, B.gingivalis, A.actinomycetemcomitans, Eikenella corrodens, F.nucleatum, increased motiles and large numbers of spirochaetes (Tanner et al. 1979, Savitt and Socransky 1984, Loesche et al. 1985, Dzink et al. 1985).

Moore et al. (1982b, 1983) found the subgingival flora of moderate adult periodontitis and severe periodontitis in young adults shared many of the predominant bacterial species. Slots (1986) reviewed the bacterial specificity of adult periodontitis, A.actinomycetemcomitans was detected in 50% of progressing sites, B.gingivalis in 42-52% of progressing sites and B.intermedius in 59-89% of progressing sites. Loesche et al (1985) found that periodontitis was due to a 'specific anaerobic infection involving spirochaetes and to a lesser extent B.gingivalis and B.intermedius.'

The progress of periodontitis is thought to be episodic in nature (Socransky et al. 1984) with periods of disease activity and quiescence. Studies have attempted to relate the sampled microorganisms to the presence or absence of disease activity. However, the problem of determining disease activity makes it difficult to relate the microorganisms to periods of disease activity or quiescence. At present no single microorganism has proven to be the main pathogen.

1.6 Treatment.

The treatment of periodontal disease has involved the effective removal of microorganisms by personal oral hygiene and professional care. Mechanical removal of microorganisms both supragingivally and subgingivally, possible surgical intervention and the long term maintenance of patients have been the corner stones of periodontal therapy.

The mechanical removal of supragingival dental plaque by the individual has been shown to produce resolution of gingivitis and restitution of gingival health (Löe et al. 1965). The composition of the dental plaque becomes dominated with Gram positive microorganisms (Theilade et al 1966). The removal of supragingival dental plaque alone has little effect on the clinical status of patients with periodontitis and has no effect on the subgingival flora (Listgarten et al. 1978, Cercek et al. 1983, Beltrami et al. 1987). Controlled oral hygiene by regular professional care is effective in slowing the disease progression (Axelsson and Lindhe 1978, 1981). However, closer analysis of individual site data indicated a small percentage of sites and patients continued to show disease progression (Lindhe et al. 1983a).

Studies using scaling, root planing and supragingival plaque removal in patients with periodontitis have shown marked improvement in clinical and microbiological effects (Listgarten et al. 1978, Baderstein et al. 1981, Cercek et al. 1983, Pihlstrom et al. 1983, Isidor and Karring 1986). Successful treatment of patients with periodontitis in general produces a subgingival dental plaque with higher proportions of S.sanguis, A.viscosus and lower

proportions of B.gingivalis (Loesche et al. 1985). Studies that have monitored the flora after scaling and root planing by darkfield microscopy have noted a recolonization of deep periodontal pockets. Mousques et al (1980) found coccoid microorganisms returned to baseline in 21 days and spirochaetes returned in 42 days. Magnusson et al. (1984) and Lavanchy et al. (1987) found recolonization to baseline counts took 4-8 weeks to occur even with professional cleaning. This would imply that the

> proliferation of the bacteria remaining in the pocket after instrumentation is a very important determinant in the reestablishment of a periodontopathic microbiota. (Lavanchy et al. 1987)

The importance of adequate continued removal of supragingival and subgingival dental plaque in both surgical and non-surgical treatments to ensue the long term prevention of disease progression is evident from numerous studies (Nyman et al. 1977, Ramfjord et al. 1982, Lindhe et al. 1984, Lindhe and Nyman 1984).

Chemotherapeutic agents have been utilized as adjunctive therapy in the prevention and treatment of periodontal disease.

1.7 Chemotherapy.

Antimicrobial agents have been used since the early 1800's to aid periodontal treatment (see section 1.2). These agents have been used systemically and locally to enhance local treatment. With the increased knowledge into the aetiology and clinical diagnosis of periodontal disease entities a selective use of antimicrobial agents is warranted.

1.71 Rationale.

Antimicrobial agents are used to enhance the removal of dental plaque or specific microorganisms in the prevention, treatment and maintenance of periodontal disease.

1.72 Efficacy.

Studies have shown that the use of antimicrobial agents alone can produce similar effects to conventional therapy.(Löe et al. 1967). Löe and Schiott (1970), found a mouthwash of tetracycline or chlorhexidine effective in the resolution and halting the development of gingivitis. Antibiotics used in the treatment of periodontitis with effective oral hygiene control have shown similar microbiological improvement to scaling and root planing (Listgarten et al. 1978, Lindhe et al. 1983b, Radinovich 1985). Lindhe at al. (1983b) found that the clinical and microbiolgical results, after 50 weeks, of a long-term, low dose use of systemic tetracycline without scaling were similar to the therapy of scaling and root planing. The subgingival irrigation with stannous fluoride, two applications (Mazza et al. 1981) or chlorhexidine, one application (Lander et al. 1986) without scaling and root planing altered the subgingival flora to one associated with health. Recolonization occurred in 4 to 10 weeks.

The additional benefits of adjunctive chemotherapy with scaling and root planing compared to mechanical debridement in adult periodontitis have shown no added effects (Listgarten et al. 1978, Lindhe et al. 1983c, Radinovich 1985). However, Radinovich (1985) noted a decrease in the number of pockets probing 7mm or more in the group that received tinidazole plus mechanical debridement.

Improvement in the clinical results of patients with localized juvenile periodontitis was noted with the use of tetracyclines (Slots and Rosling 1983, Lindhe and Liljenberg 1984, Haffajee et al. 1984). Lundström et al. (1984) found tetracyline and metronidazole effective in the retreatment of patients with recurrent periodontitis. The treatment of ANUG with metonidazole was shown to be an effective treatment with minimal recurrence of the symptoms after 12 months (Duckworth et al. 1966). Adolescents and young adults with rapid destructive forms of periodontitis have benefited from the additional use of chemotherapy (Davies et al. 1985). Mouthwashes used following surgery are effective in assisting healing (Westfelt et al. 1983).

Most of the studies have relied upon excellent self performed oral hygiene practices and frequent professional care. The use of chemotherapy in every patient in not advocated, nor is its use alone instead of mechanical debridement. Patients in certain situations, such as problems of manual dexterity, following surgery and certain systemic and local condition may benefit from adjunctive chemotherapy.

1.73 Antimicrobial agents used.

Antibiotics:e.g. Penicillin, Tetracyclines, Metronidazole, Tinidazole

Enzymes:e.g. amyloglucosidase, dextranase

Bisbiguanides:e.g. chlorhexidine, alexidine, octenidine

Quaternary ammonium compounds:e.g. benzethonium chloride, cetylpyridinium chloride

Phenolic compounds:e.g. Listerine with essential oils

Sanguinarine:

Fluorides:e.g. sodium fluoride, stannous fluoride

Heavy metals:e.g tin, copper, silver

Peroxides:

The above antimicrobial agents have been used either systemically or locally or both to aid in the removal of both supragingival and subgingival dental plaque. The comparison of these antimicrobial agents has recently been reviewed (Loesche 1967, Addy 1986, Kornman 1986).

The local delivery supragingivally has been by mouthwash and by irrigating devices such as a Broxojet (Lang and Räber 1981). The subgingival application has been by direct irrigation and by slow release devices such as hollow fibres (Goodson et al. 1979), acrylic strips and dialysis tubing (Addy et al. 1982) and ethyl cellulose with polyethyline glycol (Golomb et al. 1984). See Newman (1986) for a review of types of antimicrobial delivery available.

To date, chlorhexidine has proved to be the most effective agent, however, due a number of side effects other agents are still sort (Tonelli et al. 1983, Kornman 1986). Fluoride has been one of these agents and a review of its effects will follow.

1.8 Fluoride.

Fluorides have been used extensively in dentistry for the treatment of dental caries. In the early 1900's the occurrence of

mottled teeth was observed in Colorado. In 1931, Churchill a chemists in Bauxite, Arkansas noted an association between the high fluoride content in the drinking water and mottled teeth. McKay and then Dean observed the relationship of fluoride and dental caries. From the studies of Dean in the 1940's arose the concept of water fluoridation (Dunning 1975). Bibby and Van Kesteren (1940) found that sodium fluoride concentrations of less than 1 part per million fluoride (ppmF) limited acid production in Streptococci sp. and inhibited growth in concentrations in excess of 250ppmF. The possibility that locally applied fluorides may reduce dental plaque formation has prompted further research into fluorides.

1.81 The action of fluoride.

Fluoride is present in saliva in two forms, free fluoride ions or bound to microorganism, epithelial cells or inorganic substances at neutral pH . The bound form is readily converted into the free form by an acidic solution or chelating agent. The amount of fluoride found in saliva is 0.01 to 0.29ppmF and this value increases with fluoride ingestion (Jenkins and Edgar 1977).

The level of fluoride found in dental plaque is influenced by the level in the drinking water and the use of fluoride containing dentifrices. Birkeland and Speirs (1977) found plaque to have 5 to 10ppmF and at the pH of 6.0 to contain a free fluoride content similar to saliva. The fluoride binds to cell walls, albumin, and can inhibit the release of calcium and phosphate (Kleinberg et al. 1977). With a decrease of pH, for example due to production of

acids by microorganisms, fluoride ions are released and the pH change is inhibited (Birkeland and Speirs 1977).

Fluoride binds to the enamel surface, mostly as fluorapatite or calcium fluoride depending on the available fluoride ion concentration (Grφn 1977). This binding decreases the solubility of the enamel. Stannous fluoride decreases the free surface energy of the enamel surface and gives surfactant properties to the surface (Glantz and Nyquist 1966). Periodontally diseased root surfaces, cementum and dentine, have a higher fluoride content than healthy root surfaces (Wirthlin et al. 1979).

Fluoride effects the formation of dental plaque by inhibiting the binding of proteins to hydroxyapatite. The bridging between microorganisms and the acquired pellicle is inhibited by the competitive binding of calcium ions (Rölla 1977, Yotis and Brenman 1983).

The interaction of fluoride and microorganisms in mainly through the inhibition of carbohydrate metabolism. Fluoride binds to the cell membrane causing a greater leakage of potassium, similar action as produced by chlorhexidine (Hamilton 1977). A number of enzymes involved in the microbial metabolism are inhibited, for example enolase, phosphatases, succinate dehydrogenase and catalases (Hamilton 1977, Silverstone et al. 1981). Enolase is sensitive to 0.5 to 1.0ppmF in the presence of phosphate (Birkeland and Speirs 1977). Camosci and Tinanoff (1984) found that stannous fluoride altered the growth and metabolism of Streptococcus mutans by the large uptake of tin into the cell wall.

Stannous fluoride applied either topically (20,000ppmF) or as a mouthwash (500ppmF) has been shown to have a prolonged effect on dental plaque for 4 and 2 weeks respectively (Svanberg and Westergren 1983).

1.82 Side effects.

A recent report by the National Health and Medical Research Council (NHMRC 1985) on the safety of water fluoridation, fluoride supplements and fluoride dentifrices, found no scientific evidence to support the allegations of adverse side effects with long term use of fluorides at the the present recommended levels. The fluoride content of toothpastes on the Australian market is approximately 1000ppmF (NHMRC 1985).

A single dose of about 2.5g of fluoride is lethal for adults and sub-lethal doses cause non-specific effects such as vomiting and diarrhoea (Holland 1980, Silverstone et al. 1981).

Tooth staining has been reported as a side effect in some clinical clinical trials (Svantum et al. 1977, Yankell et al. 1982). Svantum et al. (1977) found that dryness, a strong metallic taste and in one subject the development of a desquamative lesion were possible side effects. No other detrimental effects to periodontal tissue have been reported (Perry 1982).

In vitro cytotoxic effects on cell culture systems, neutrophils and fibroblasts, have been reported (Gabler and Leong 1979,1980, Holland 1980). The development of resistant microorganisms and cell cultures to fluoride is possible (Repaske and Suttie 1979, Holland 1980).

1.83 Associated heavy metals.

The most commonly associated metal with fluoride that is used in dentistry is tin, as stannous fluoride (SnF_2). Copper and silver fluorides have also been used. An outline of the properties of tin and silver will be discussed.

1.831 Tin.

Tin has been used as a fungicide, a bactericide and as a textile and wood preservative. It leaves no toxic residues in the environment and is intrinsically less toxic than silver (Russell et al. 1981). Stannous fluoride undergos hydrolysis in an aqueous environment, with stannous ion conversion to stannous hydroxide. The stability of the solution can be maintained by not altering its native pH (pH 3.2 for 100ppmF) (Tinanoff 1985).

1.832 Silver.

Silver vessels were used to maintain water, as the silver is slightly soluble in water and the ion concentration achieved inhibits microbial growth. Silver compounds have been used in medicine in eye infections and in prevention of infections in burns. Silver nitrate is an astringent compound and a protein precipitant. Its combination with albumin or gelatin removes its astringency and allows a slow release of silver ions (Russell et al. 1981). Silver nitrate was use by Luton in 1924 for the lavage of periodontal pockets (Gold 1985c). Hanke (1940) noted the effectiveness of silver on inhibiting dental plaque and the common practice of applying a saturated solution to carious lesions. He warned against the use as a mouthrinse due to the taste,

causticity and discolouring properties. Silver fluoride has been topically applied in one clinical trial (using 95ppmF), no side effects were reported (Oppermann and Johansen 1980). Oppermann and Johansen (1980) found silver was retained in dental plaque at high levels 6 hours after silver fluoride application.

1.84 Comparison and effectiveness of fluorides.

1.841 Laboratory studies.

Bibby and Van Kesteren (1940) found sodium fluoride (NaF) affected acid production by oral microorganisms at less than 1ppmF and affected growth in concentrations in excess of 250ppmF.

Newman et al. (1979) tested a number of oral microorganisms, such as A.viscosus, S.sanguis, F.nucleatum, Bacteroides sp., against sodium fluoride using a broth and agar dilution technique. The growth of all microorganisms was affected with 1500ppmF. The fluoride solution remained in contact with the microorganism throughout the experiment, at least 48 hours. The authors noted that the fluoride solution affected the trypticase soy broth resulting in turbidity before inoculation with the microorganism.

Yoon and Berry (1979) examined the effect of acidulated phosphate fluoride (APF), sodium fluoride and stannous fluoride (SnF_2) on five strains of A.viscosus. The microorganism was exposed to various fluoride concentration for 1 hour and 24 hours in brain heart infusion broth. At 1 hour, stannous fluoride was the most effective in suppressing growth at 500ppmF. Sodium fluoride

(250ppmF) and APF (1000ppmF) had no affect at 1 hour. After 24 hours stannous fluoride suppressed growth with 100ppmF and APF and NaF suppressed growth at 200ppmF. The pH readings for the SnF_2 solutions ranged from 6.0 to 7.0, indicating the broth solution altered the inherent low pH (see section 1.83).

Using a similar method Tsao et al. (1982) found SnF_2 was effective against S.sanguis, A.viscosus, B.gingivalis, B.intermedius and F.nucleatum at 250ppmF after 24 hours in broth.

Yoon and Newman (1980) tested the antimicrobial effect of SnF_2, APF and NaF against the microorganisms, Bacteroides melaninogenicus, B.intermedius and B.gingivalis. At 1 hour exposure, SnF_2 killed B.melaninogenicus with 500ppmF, B.intermedius and B.gingivalis with 200ppmF. NaF was the least effective and APF was intermediate in its effect. After 8 hours exposure, SnF_2 was effective at 100ppmF, NaF effective at 700ppmF and APF at 500ppmF. Increased acidity was shown to enhance killing, however, acidity alone did not account for the greater effectiveness of SnF_2.

The effect of tin was examined by Yoon et al. (1980) by comparing SnF_2 against stannous chloride ($SnCl_2$). At 1 hour B.intermedius was killed by a 300ppmF solution of SnF_2 and $SnCl_2$ killed the microorganism at a Sn concentration equivalent to that in 600ppmF (SnF_2). This indicated that tin has antimicrobial properties, however, its the combination of tin and fluoride that provides its added effect. Similar finding were noted by Mayhew and Brown (1980) on the growth of S.mutans. They found SnF_2 at

600ppmF was bactericidal after 4 hours at pH 5.9, however, at pH 7.2 viability was still present at 4 hours.

Caufield and Wannemuehler (1983) addressed the problem of the possible inactivation of the antimicrobial agent in culture media. Microorganisms were grown on filter membranes, then a drop of the test solution was spotted on the filter and left for 5 minutes. The filter was incubated in broth for 48 hours. An 8% (20,000ppmF) solution of SnF_2 was effective against B.gingivalis and A.actinomycetemcomitans, and not effective against A.viscosus. A solution of 0.5% chlorhexidine was effective against all tested microorganisms.

Mandell (1983) tested sodium fluoride against 22 oral microorganisms using an agar dilution technique. The microorganisms were susceptible to a range of fluoride concentration (128 to 2048ppmF). A.viscosus and A.actinomycetemcomitans were the most susceptible. Iida et al. (1986) also using an agar dilution technique found the Bacteroides sp. and Fusobacterium sp. to be the most susceptible, range 22.5 to 1440ppmF. The Actinomyces sp. were susceptible in the range of 720 to 2880ppmF.

The in vitro studies have shown that stannous fluoride is the most effective fluoride agent. Different microorganisms are susceptible to different fluoride concentrations and the pH of the fluoride solution is very important. It is difficult to relate the results obtained to a clinical situation, due to the long exposure times used by most studies. The problems with the use of broth solutions for testing has been noted by a number of authors

(Newman et al. 1979, Caufield and Wannemuehler 1983). As fluoride binds to protein, calcium and phosphate compounds, it would seem inappropriate to use broth solutions in comparison tests of fluoride compounds.

1.842 Clinical studies.

Most of the clinical studies have involved the use of stannous fluoride as a mouthrinse and in localized irrigation.

Andres et al. (1974) compared the effects of a 1200ppmF mouthrinse of NaF and SnF_2, and saline. Each of the seven subjects rinsed with 40ml of the test solution in the following manner. One third of the solution was rinsed vigorously for 10 seconds, then one third held in the mouth for 1 minute and this was repeated, and then this expectorated fluid was plated on agar to provide bacterial counts. Similar expectorated samples were taken for the next 5 hours. Five days were allowed before the experiment was repeated using a different agent. Stannous fluoride significantly reduce the numbers of microorganisms over the 5 hours. A 99% reduction of pre-rinse microbial numbers occured immediately following the mouthrinse and a 93% reduction after 5 hours. The effect of sodium fluoride was minimal.

Tinanoff et al. (1976) found that rinsing once or twice a day for seven days with 100ppmF SnF_2 caused a marked reduction in the numbers of microorganisms in early plaque formation. There was inhibition of binding to the enamel surface and binding between organisms. The mouthrinse consisted of 10ml of solution rinsed for one minute. Twice daily rinse with 100ppmF NaF had some

effect in altering the microorganisms' attachment to the enamel surface.

White and Taylor (1979) found that rinsing (1000ppmF) and brushing with stannous fluoride reduced the plaque formation compared to a placebo. During the first six days (rinsing and brushing) there was no difference in plaque scores, the next five days only rinsing was performed and there was less plaque present using the SnF_2 rinse. No other clinical parameters were reported.

Tinanoff et al. (1980) used a double blind cross over design study to test the effect of SnF_2, using a mouthrinse of 250ppmF twice a day for five days with regular oral hygiene. There was a significant reduction in plaque wet weight and in the number of microorganisms in the plaque. There was no difference in the gingival inflammation. Similar finding were shown by Svanberg and Rölla (1982) using a 100ppmF rinse of SnF_2. Yankell et al. (1982) in a 5 day study using only a SnF_2 mouthrinse (250 or 1000ppmF) found a significant plaque reduction but alteration in other clinical parameters.

Larson et al. (1985) used a similar experimental design to Tinanoff et al. (1980) with 60 days of rinsing with SnF_2 (500ppmF). They found no change in the gingival index and motility counts of the sampled dental plaque. Leverett et al. (1984) studied two groups of children (12 to 15 years) over 28 months. A daily rinse of NaF or SnF_2 at a concentration of 250ppmF was used. After the first 4 months there was less plaque accumulation with the SnF_2 rinse. This difference was no longer present after 16 months.

Mazza et al. (1981) monitored 10 male patients with advanced periodontal disease (6mm or greater probing in at least 4 sites) subgingivally irrigated with SnF_2 (4100ppmF or 1000ppmF) or sterile saline. A second subgingival irrigation was carried out two days after the first and no other treatment was performed during the 10weeks. By day four a significant improvement in the bleeding index was achieved with the SnF_2. Irrigation with 4100ppmF produced a total elimination of spirochaetes and motiles after 4 days. By 6 weeks spirochaetes and motiles had returned to 50% of base line figures and 8th week counts were similar to baseline. The 1000ppmF solutions showed similar trend, with a return to base line counts at 4 weeks. Saline irrigation caused a reduction of spirochaetes and motiles, with a return to base line after 1 week.

Self performed daily irrigation (50ppmF SnF_2) using a Water Pik and mechanical oral hygiene in patients with periodontitis has been shown to improve clinical parameters (Boyd et al. 1985). During the 18 week study those subjects irrigating with SnF_2 showed significant improvement. Those subjects who irrigated with water for 10 weeks and then changed to SnF_2 showed improvement in clinical health. The subjects who changed from SnF_2 to water showed a worsening of their condition.

Oppermann et al. (1980) topically applied silver nitrate and stannous fluoride to nine subjects. The silver nitrate was the most effective at inhibiting acid production in dental plaque. Oppermann and Johansen (1980) topically applied silver fluoride, copper fluoride and stannous fluoride (all at 95ppmF) to patients

with 3 day old sucrose induced plaque. Copper fluoride and silver fluoride were the most effective at inhibiting acid production.

Svantun et al. (1977) compared a 0.1% solution of chlorhexidine to SnF_2 solution with concentrations of 500 and 750ppmF. After four days of mouthrinsing they found very little difference in their plaque index. A further trial of three weeks on 750ppmF SnF_2 showed that these clinical findings could be maintained. Helldén et al. (1981) comparing 0.2% chlorhexidine to 250ppmF concentration of SnF_2 found chlorhexidine to be more effective in reducing bacterial counts, gingival index and plaque index. This was a cross over trial with a mouthrinse of SnF_2 used for 5 days, two days of no rinsing then five days rinsing with chlorhexidine. Waler and Rölla (1982) compared 0.1% chlorhexidine to copper sulphate and silver nitrate as a mouthrinse over 4 days without mechanical oral hygiene and with sucrose containing chewing gum. All three showed significant reduction of plaque formation compared to the placebo rinse (Sucrose). Silver nitrate was not as effective as chlorhexidine and copper sulphate.

There has been one report of the use of SnF_2 after periodontal surgery to reduce plaque and inflammation (Frankel et al. 1982). A twice daily rinse with a 250ppmF solution showed significant reduction of plaque accumulation, gingival inflammation and crevicular fluid flow compared to a placebo mouthrinse.

The above review would tend to indicate that fluorides have uses in certain situations in periodontics. They may assist in plaque removal and have some benefit post surgery. Their long term use has doubtful effect on gingival health and plaque removal. Their

use as adjunctive aids in subgingival irrigation in patients with periodontitis is promising and deserves further research. To date SnF_2 has been the agent most studied. However, limited evidence on the use of other fluoride compounds, such as silver fluoride, tends to indicate their potential as more effective agents.

1.9 Aim.

The aim of the present study was to test, in vitro, the effects of three fluoride compounds, sodium fluoride, stannous fluoride and silver fluoride, on three oral microorganisms related to periodontal disease, S.sanguis, A.viscosus and B.intermedius. Sodium fluoride and stannous fluoride were chosen for their previous know effects on oral microorganisms. Silver fluoride has been shown clinically to reduce dental plaque acid production (Oppermann and Johansen 1980) and silver is a known antimicrobial. The microorganisms were chosen as they are representatives of dental plaque associated with clinical health, gingivitis and periodontitis.

MATERIALS AND METHOD.

2.1 Culture and culture conditions.

In this study freeze dried vials of the following microorganisms were provided from the type culture collection of the Institute of Dental Research.

The selected Gram-positive coccus obtained was Streptococcus sanguis G9B. This microorganism was revived in Trypticase Soy Broth (TSB, Oxoid, England) and maintained by weekly subculture on Trypticase Soy Blood Agar (TSBA, Oxoid, England) which contained 3% defibrinated sheep blood (Commonwealth Serum Laboratories). The purity of the S.sanguis G9B was checked by Gram staining and the use of RapID STR System for biochemical identification of Streptococci (see Plate 2.1 and 2.2). The following results were obtained providing identification as S.sanguis ,

L-Arginine	Esculin	Mannitol	Sorbitol	Raffinose
-	+	-	-	-

Inulin	D-galactoside	D-glucoside	D-N-acetylglucosaminide
+	-	-	-

p-nitrophenyl phosphate	Tyrosine	Hydroxyproline	Lysine
+	+	-	+

Pyrrolidine	Haemolysis
-	non-beta

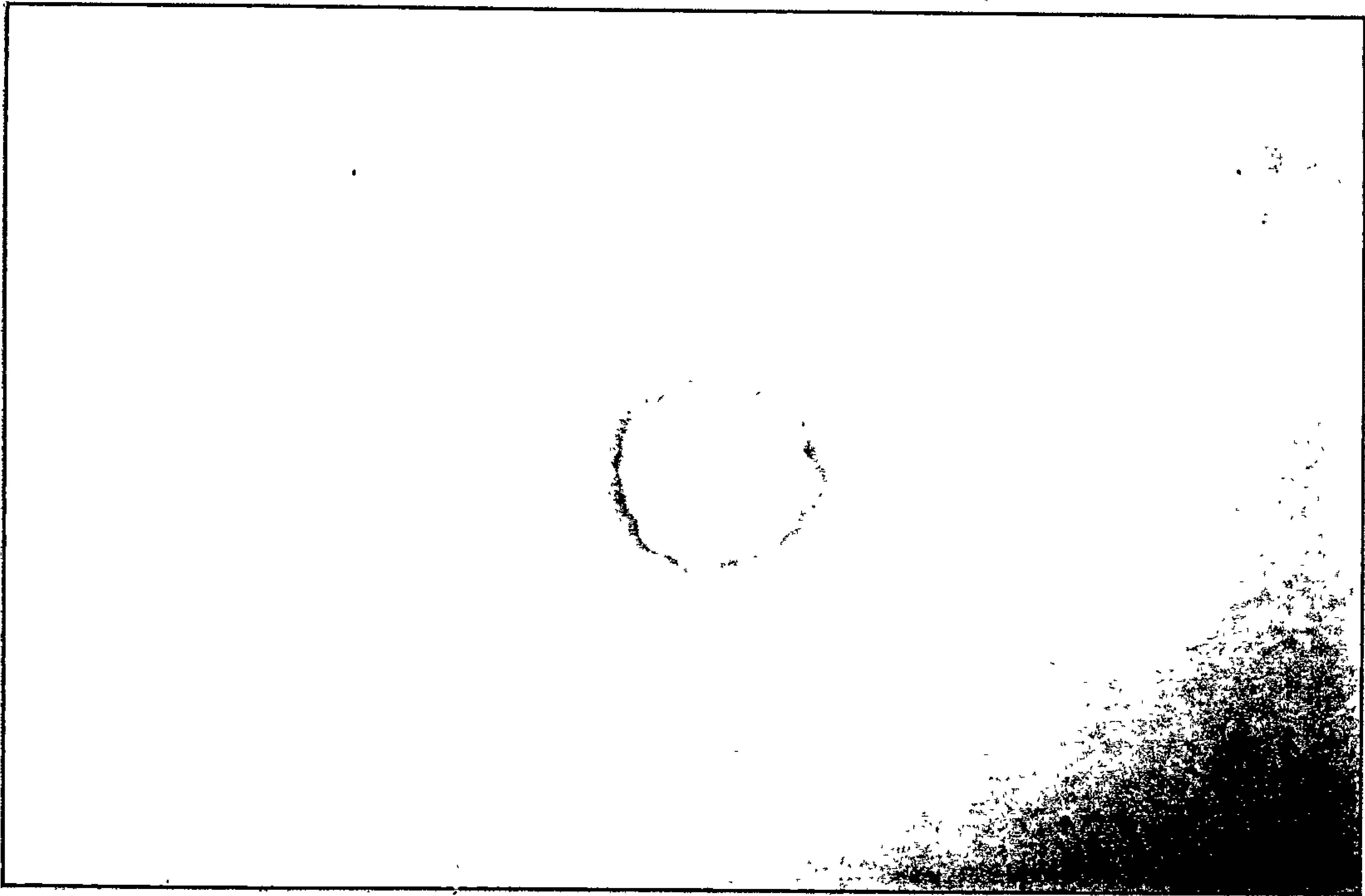

Plate 2.1. S.sanguis G9B colonies grown on T.S.B.A.

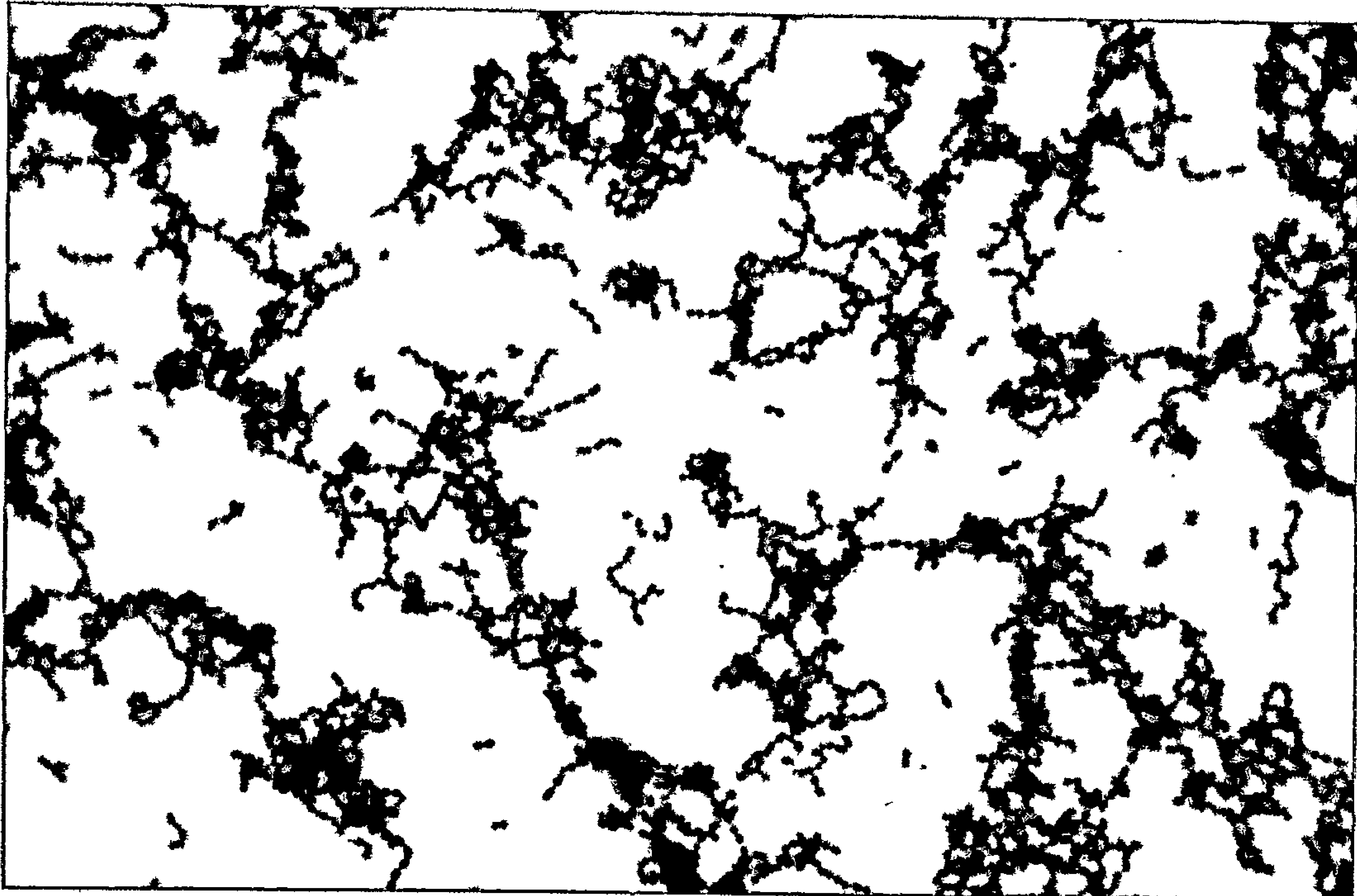

Plate 2.2. Gram stain of S.sanguis G9B showing Gram positive coccal microorganisms. x 1200.

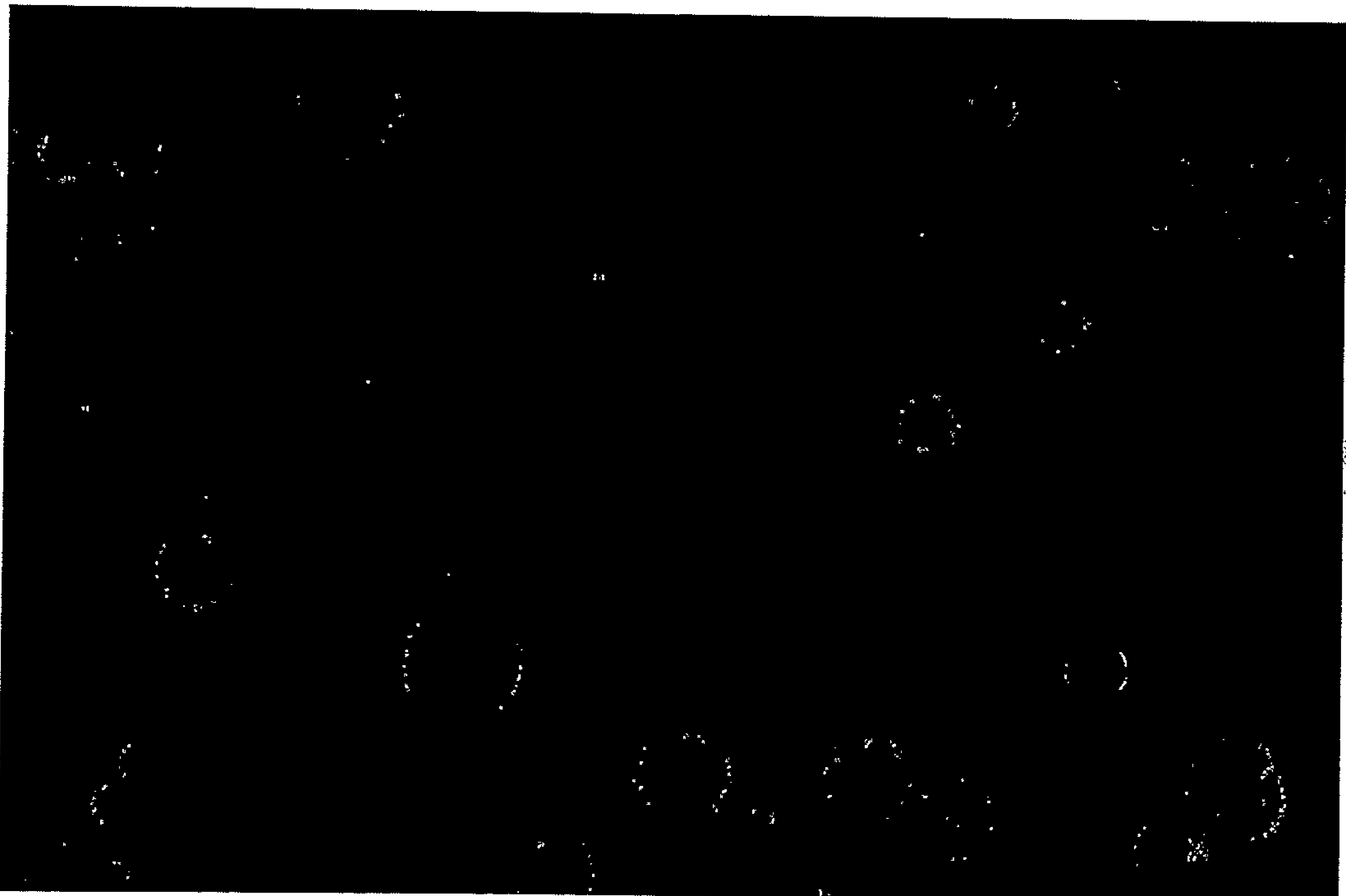

Plate 2.3. A.viscosus T14 colonies grown on T.S.B.A.

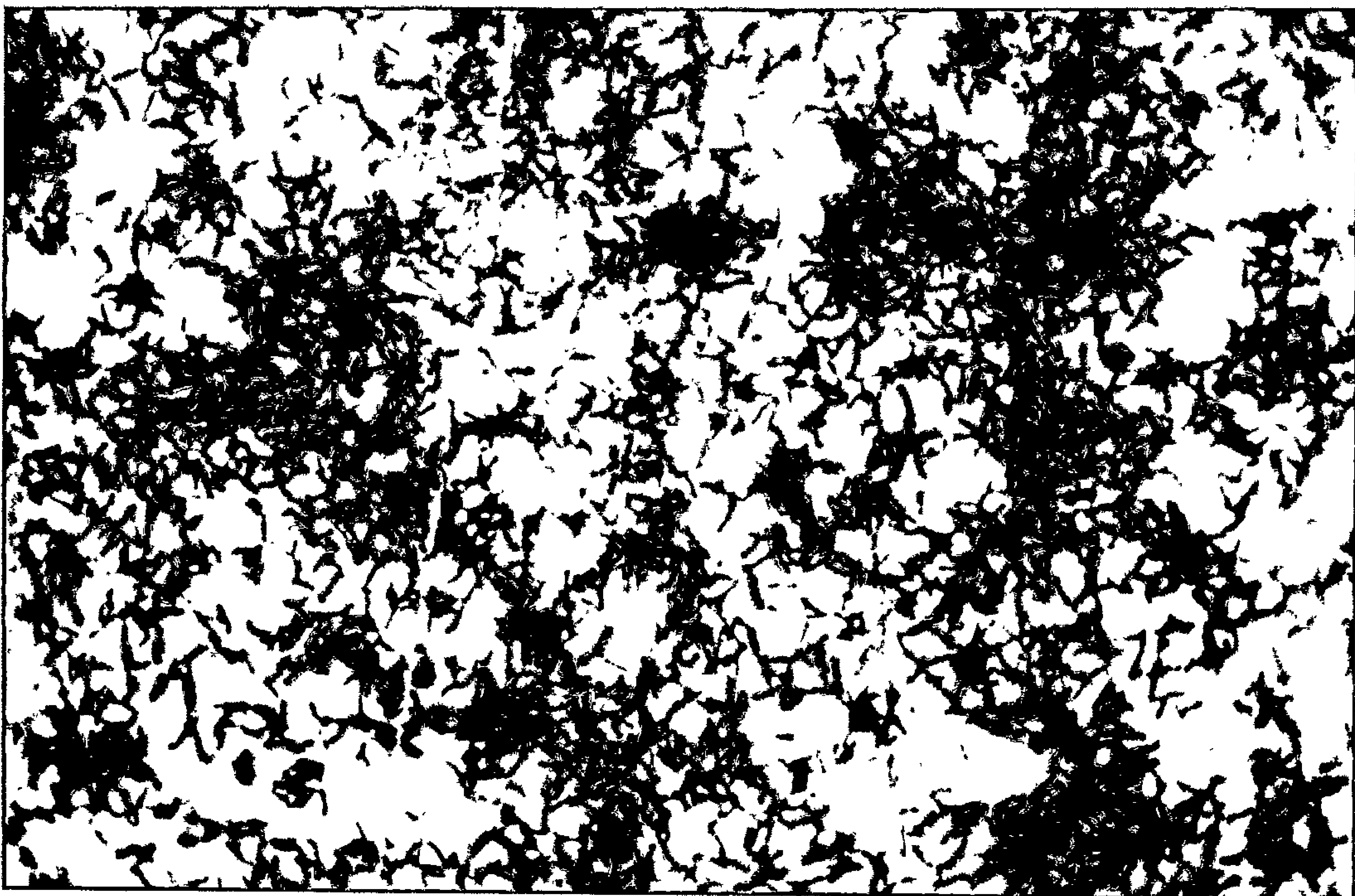

Plate 2.4. Gram stain of A.viscosus T14 showing Gram positive filamentous branching microorganisms. x 1200.

The selected Gram-positive rod or branching filament of the oral cavity was Actinomyces viscosus T14. This microorganism was revived in TSB and weekly subcultured on TSBA with 3% defibrinated sheep blood. The purity of the A.viscosus T14 was constantly checked by Gram-staining, by the catalase test for which this microorganism is positive and by the study of the colony form on the blood agar (see Plate 2.3 and 2.4).

The selected Gram-negative microorganism of the oral cavity and in particular dental plaques was Bacteroides intermedius NCTC 9336. This microorganism was revived in Basal Media (BM, McKee, McDermid, Baskerville, Dorsett, Ellwood and Marsh 1986) and maintained by weekly subculture on Enriched Trypticase Soy Agar (ETSA, Syed, Svanberg and Svanberg 1980) which contained 0.5μg/ml haemin, 1μg/ml menadione and 3% defibrinated sheep blood. The purity of the B. intermedius NCTC 9336 was checked continuously throughout the study by Gram staining, the production of black pigment in the colony forming units (CFU) on ETSA and the following biochemical tests (see Plate 2.5 and 2.6).

Lactose	Glucose	Trypsin-like Enzyme	Indole	Esculin
-	+	-	+	-

2.2 Growth Conditions:

In this study the S.sanguis G9B was incubated for each experiment at 37°C in aerobic conditions and the plates used for repeated subculture were also incubated aerobically.

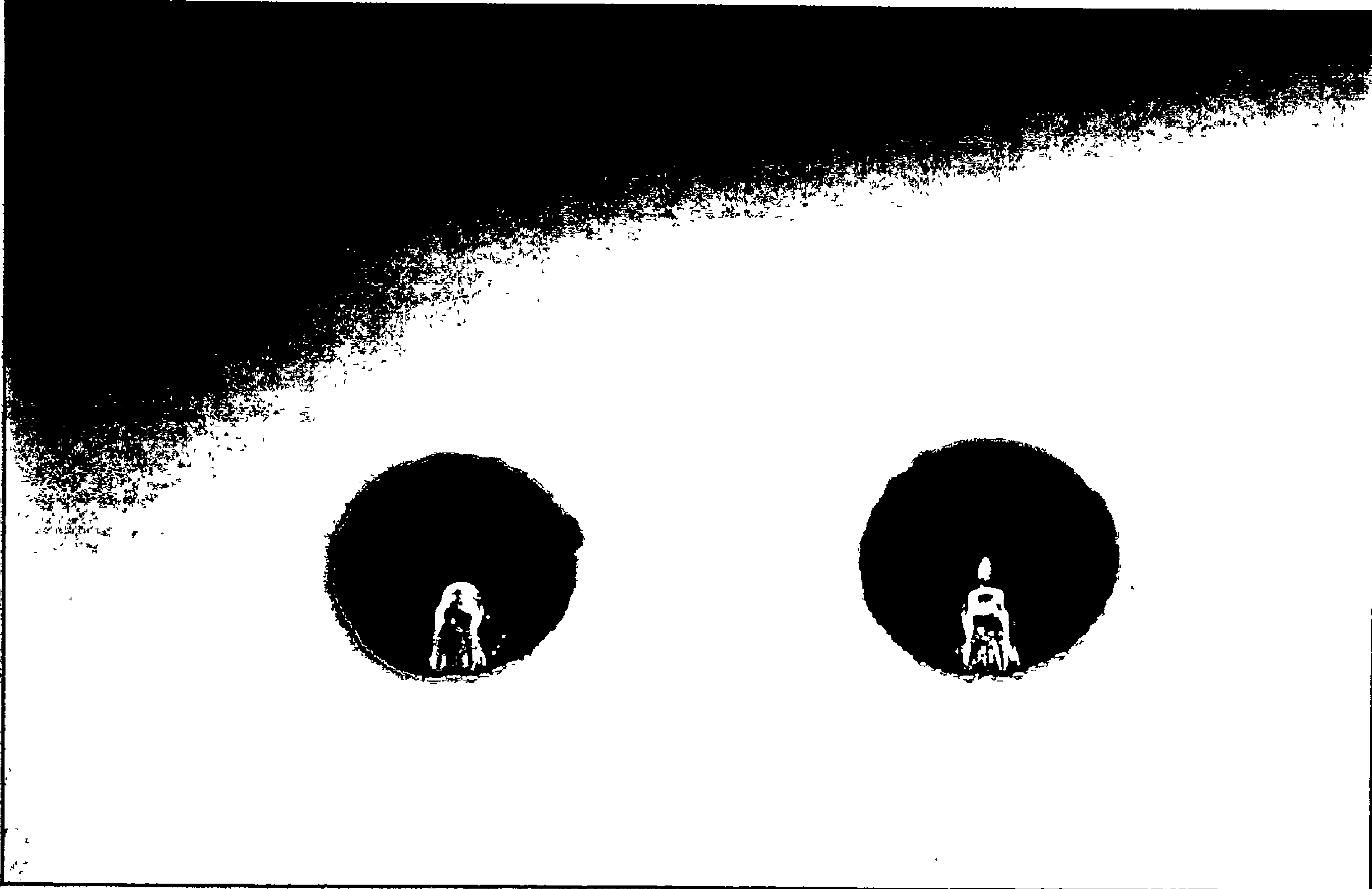

Plate 2.5. B.intermedius NCTC 9336 colonies grown on E.T.S.A.

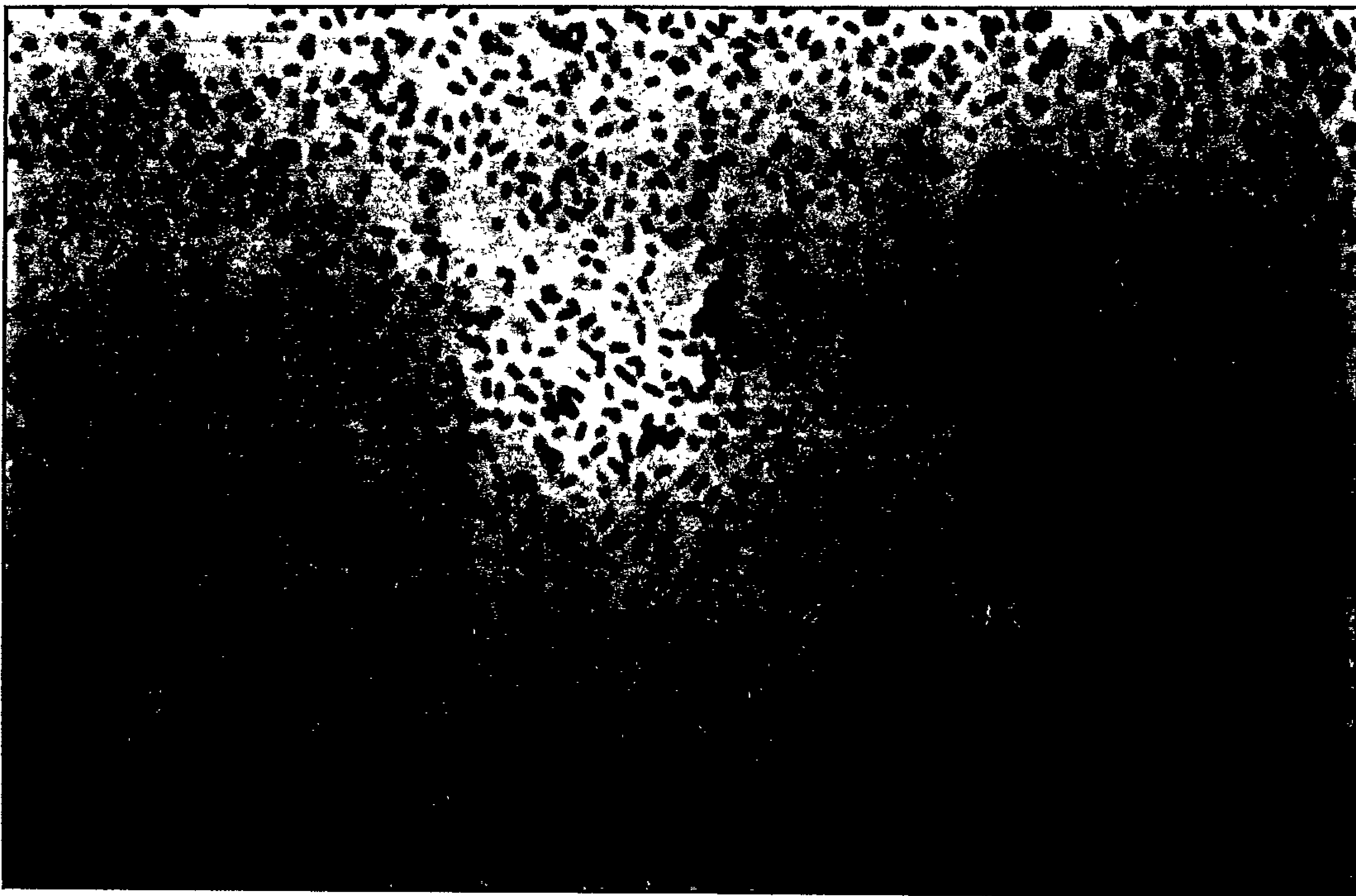

Plate 2.6. Gram stain of B.intermedius NCTC 9336 showing Gram negative bacilli microorganisms. x 1200.

To determine the growth curve and in particular the various growth phases, freshly grown colonies of S.sanguis G9B from the TSBA were inoculated into TSB and incubated for 19 hours. After 19 hours of growth a 0.1ml aliquot of this broth was placed into a 16 x 150mm glass tube (Pyrex No. 9825) containing 10mls of sterile TSB. This tube was used to obtain the growth curve. At selected time intervals during the next 12 hours the inoculated tube was compared with an uninoculated tube to obtain a spectrophotometric reading at a wavelength of 600nm (Spectronic 20, Bausch and Lomb, New York). Both tubes were removed from the incubator, vortexed for 15 seconds and placed in the spectrophotometer using the same reference mark on the tube. The absorbance value was read within 15 seconds of insertion into the machine. By plotting absorbance against time a graph of growth rate was obtained. In addition at selected absorbance values an aliquot of the broth was obtained and serially diluted 1 in 10 in sterile deionised water. A 0.1ml aliquot of these dilutions was spread in the normal manner onto duplicate TSBA plates. These plates were incubated as previously described. The plates were removed after 48 hours and the total colony forming units (CFU) determined. Plates containing between 60 and 300 CFU were used for this procedure and the plates were examined under a magnifying loop.

For all experimental procedures using the microorganism S.sanguis G9B, growth to the absorbance reading of 0.1 was used. This reading relates to the early portion of the log growth phase (see Figure 2.1 and 2.2).

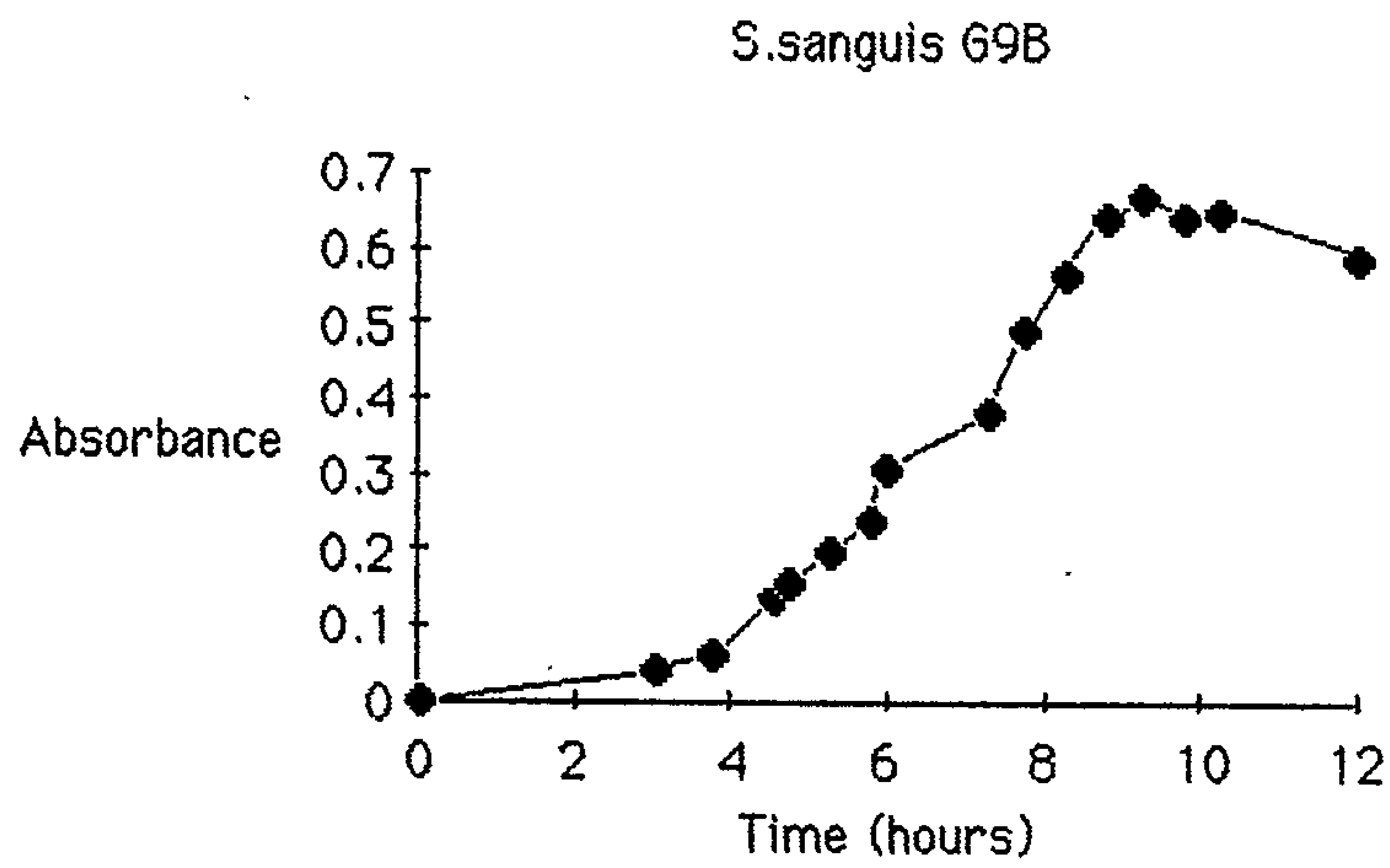

Figure 2.1. Growth of S.sanguis G9B over 12 hours. An absorbance reading of 0.1 was gained after approximately 4 hours of growth.

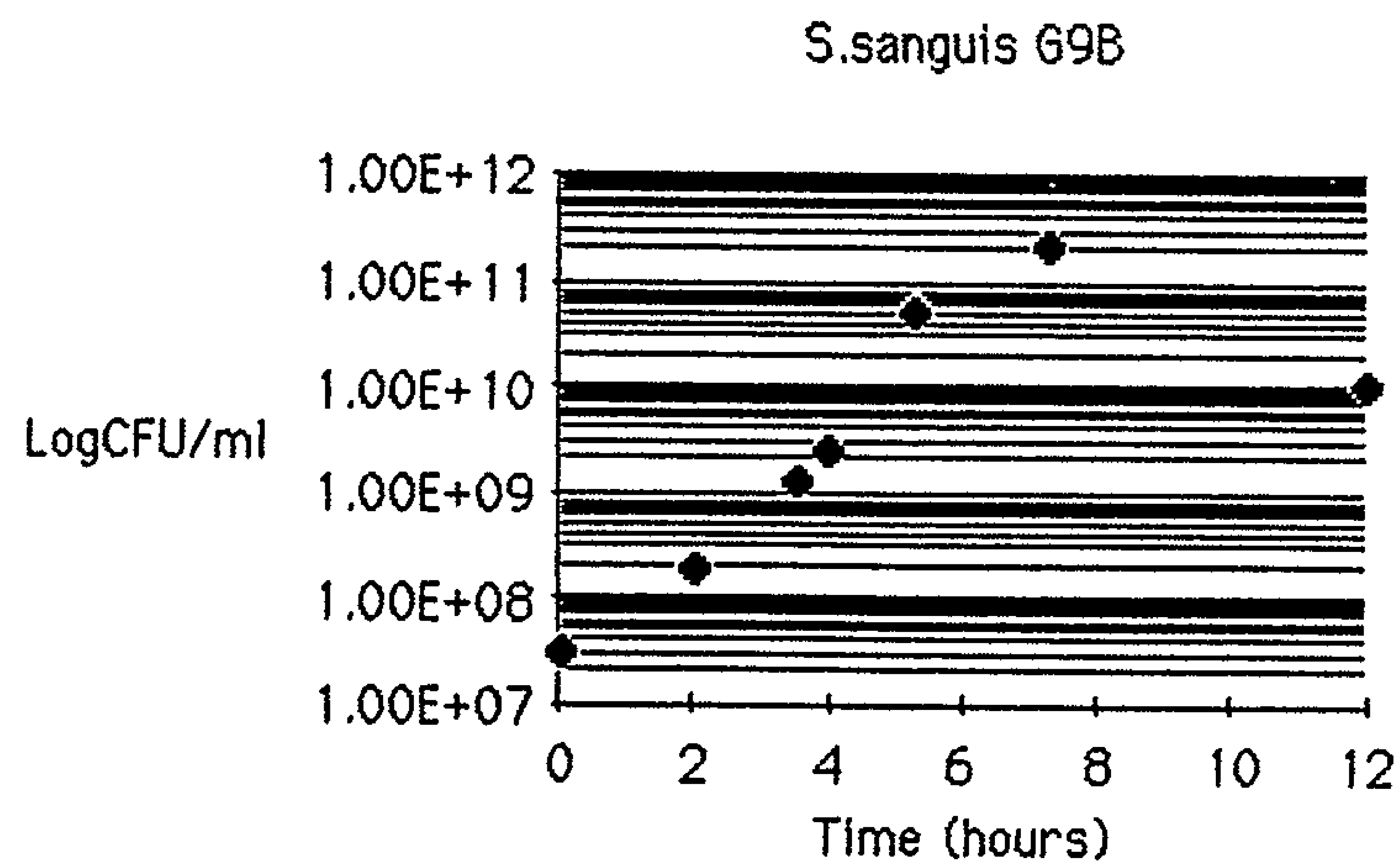

Figure 2.2. Growth curve of the Log CFU/ml over a 12 hour period. Four hours growth gives 10^9 CFU/ml. This occurs in the early portion of the log growth phase.

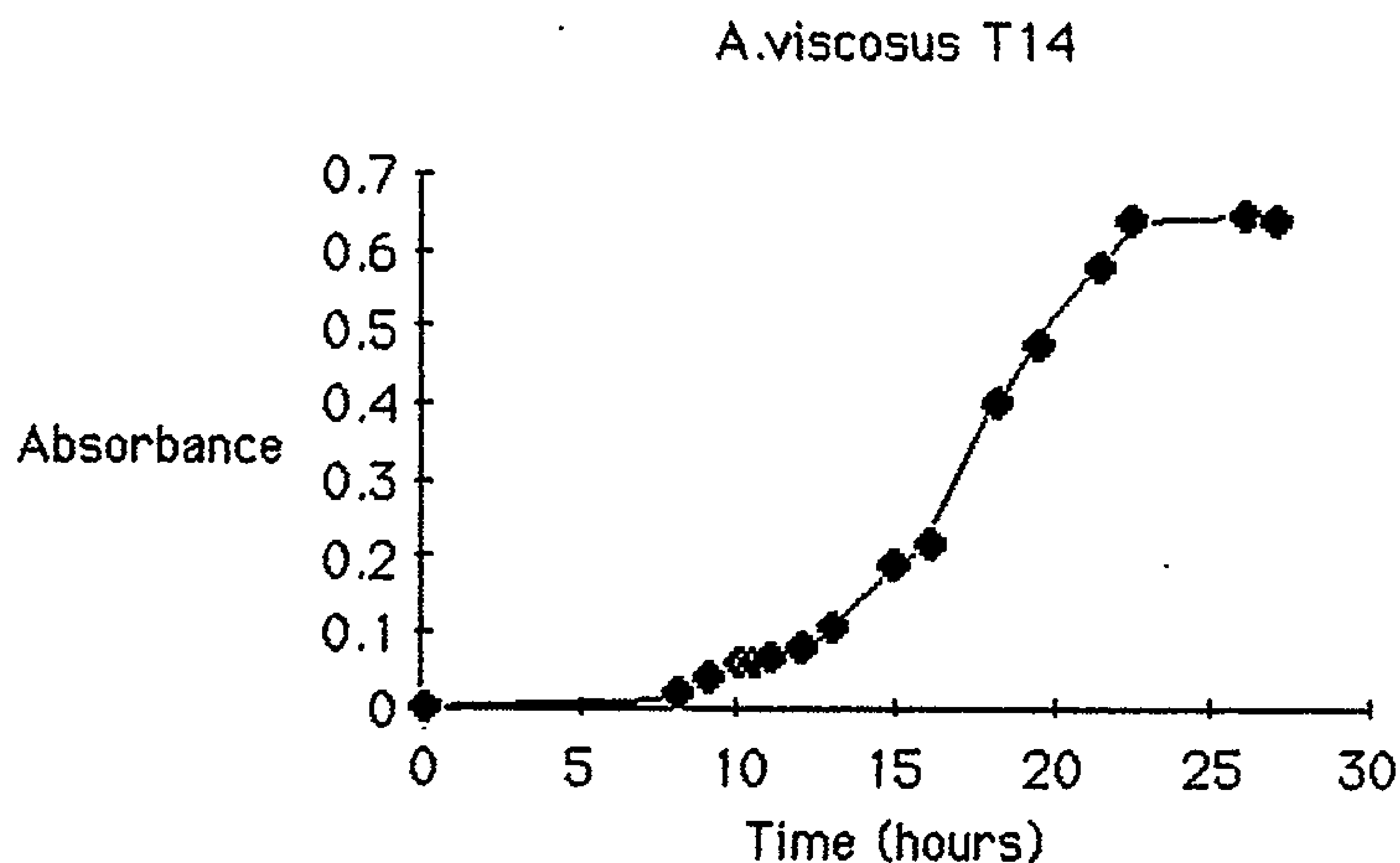

Figure 2.3. Growth of A.viscosus T14 over 27 hours. An absorbance reading of 0.1 was gained after 13 hours of growth.

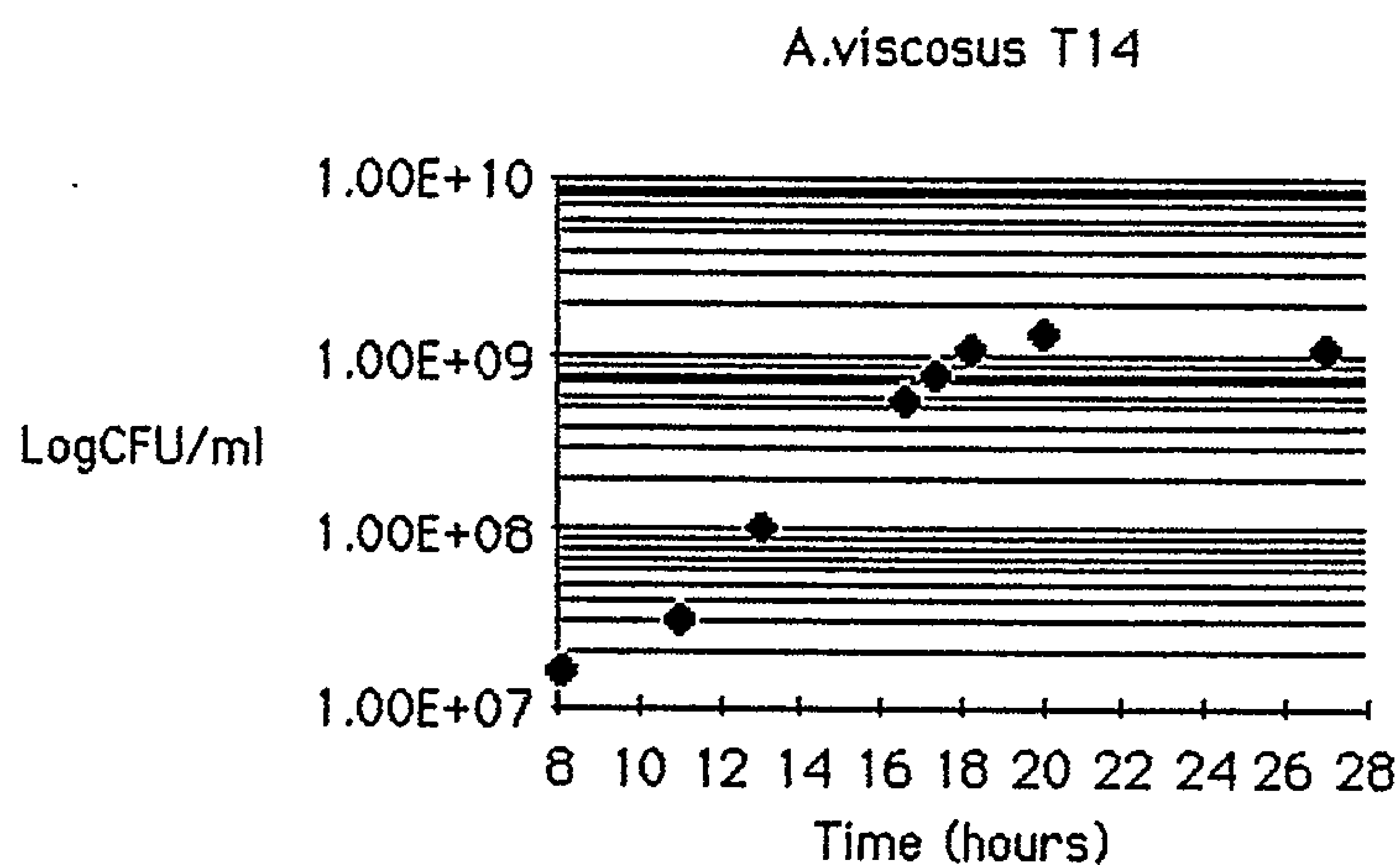

Figure 2.4. Growth curve of the Log of CFU/ml over a 27 hour period. Thirteen hours growth gives 10^8 CFU/ml. This occurs in the early portion of the log growth phase.

A.viscosus T14 was incubated for each experiment at 37°C in anaerobic conditions. This was carried out in an anaerobic glove box (Coy Laboratory Products, Inc., Ann Arbor, Michigan) in an atmosphere of 10% carbon dioxide, 5% hydrogen and 85% nitrogen. Both the broth tubes and plates were prereduced in the anaerobic glove box for 24 hours before use. The plates used for repeated subculture were incubated anaerobically.

To determine the growth curve, freshly grown colonies of A.viscosus T14 from the TSBA were inoculated into TSB and incubated for 24 to 26 hours. After 24-26 hours growth, a 0.1ml aliquot of the broth was placed into 16 x 150mm glass tube (Pyrex No. 9825) containing 10ml of sterile TSB. At selected time intervals during the next 27 hours the inoculated tube was compared with an uninoculated tube using the spectrophotometer in the same manner as previously described. The plotting of absorbance against time provided a growth rate graph. At selected absorbance values an aliquot of broth was serially diluted as with S.sanguis G9B and spread onto duplicate TSBA plates. These plates were incubated anaerobically and at 48 hours were removed for counting. Plates with 60 to 300 CFU were use for this procedure and the plates were examined under a magnifying loop.

For experimental procedures using the microorganism A.viscosus T14 growth to the absorbance reading of 0.1 was used. This is in the early portion of the log phase of growth (Figure 2.3 and 2.4).

B.intermedius NCTC 9336 was incubated for each experiment at 37°C in anaerobic conditions. This was performed in an anaerobic glove box (Coy Laboratory Products) in an atmosphere of 10%

carbon dioxide, 5% hydrogen and 85% nitrogen. Broth tubes and plates were prereduced for 24 hours in the glove box before use. The plates used for subculture were incubated anaerobically.

To determine the growth curve freshly grown colonies of B.intermedius NCTC 9336 from the ETSA were inoculated into BM and incubated for 30 to 48 hours. After 30-48 hours growth a 0.1ml aliquot of the broth was placed into 16 x 150mm glass tubes (Pyrex No.9825) containing 10ml of sterile BM. At selected time intervals during the next 55 hours the inoculated tube was compared with an uninoculated tube using the spectrophotometer in the same manner as described for S.sanguis G9B. The plotting of absorbance against time provided a growth rate graph. In addition at selected absorbance values an aliquot of broth was obtained and serially diluted 1 in 10 in sterile deionised water. A 0.1ml aliquot of these dilutions was spread onto duplicate ETSA plates. The plates were incubated anaerobically and removed after 7 to 10 days to determine the CFU. Plates containing 60 to 300 CFU were used and the plates were examined under a magnifying loop.

For all experimental procedures using the microorganism B.intermedius NCTC 9336, growth to the absorbance reading of 0.12 was used. This relates to the early portion of the log phase of growth (Figure 2.5 and 2.6).

2.3 Fluorides.

In these experiments three fluoride compounds were compared for their effects on the growth of the microorganisms.

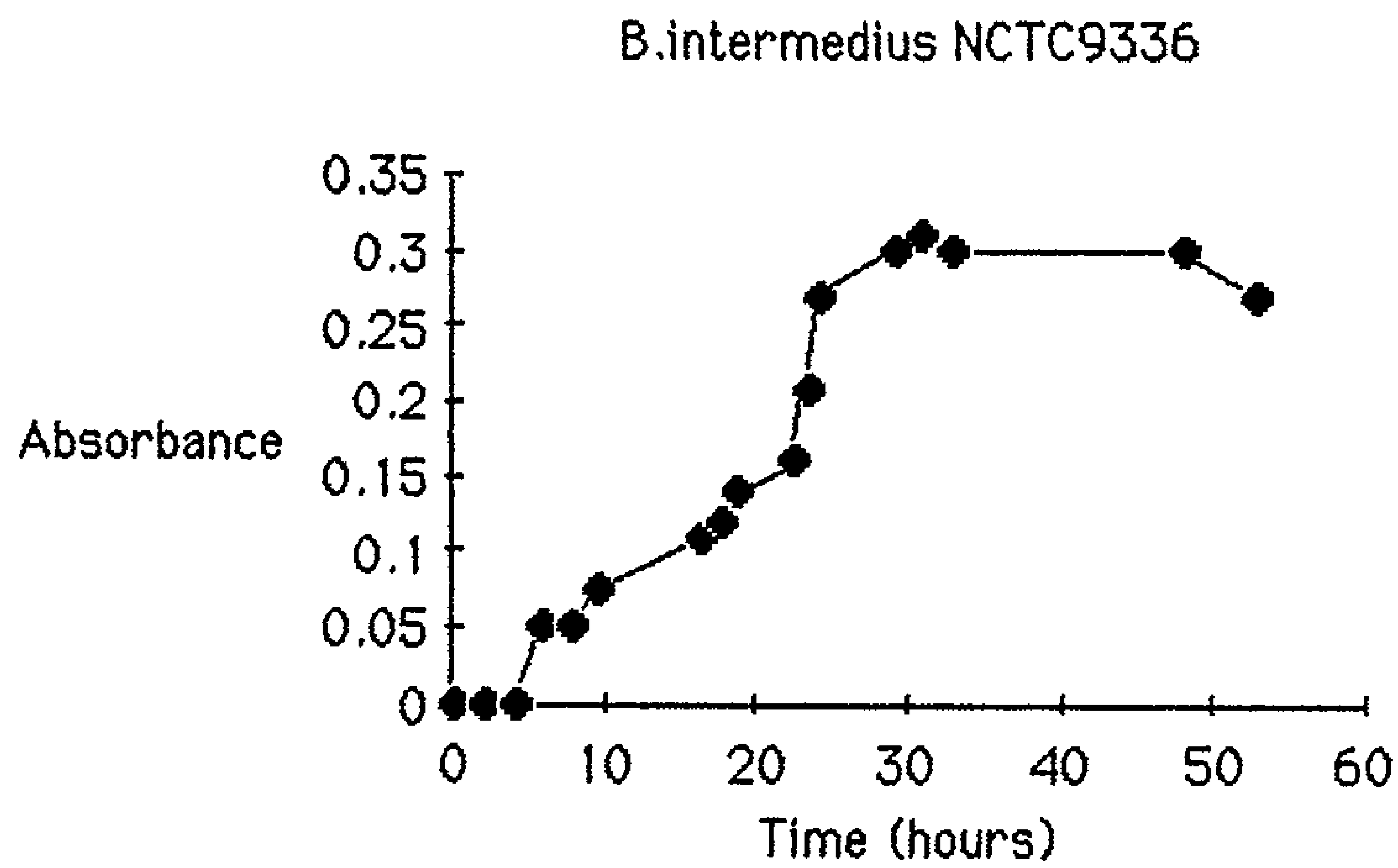

Figure 2.5. Growth of <u>B.intermedius</u> NCTC 9336 over 55 hours. An absorbance reading of 0.12 was gained after approximately 18 hours of growth.

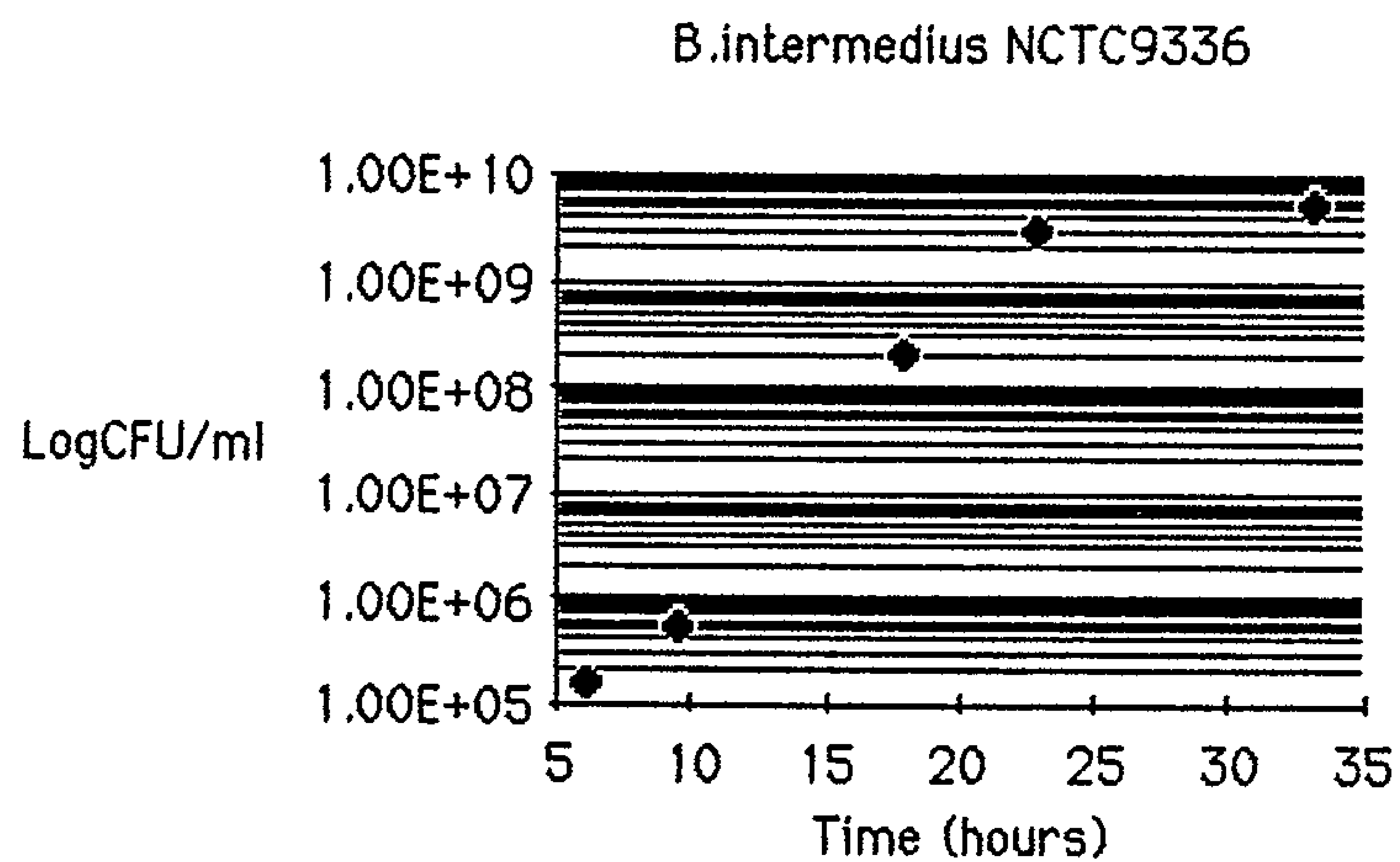

Figure 2.6. Growth curve of the Log of CFU/ml over a 33 hour period. Eighteen hours growth gives 10^8 CFU/ml. This occurs in the early portion of the log growth phase.

The three selected fluoride compounds were

1. Sodium fluoride- NaF (Sigma, St Louis, Lot no. 75F-0388)

2. Stannous fluoride- SnF_2 (Sigma, St Louis, Lot no. 35F-0667)

3. Silver fluoride- AgF (Creighton pharmaceuticals, Sydney, PP5245, WP167, D3107).

Sodium fluoride was obtained as a white powder and has a molecular weight of 42.00. This compound consists of 45.24% fluoride and 54.75% sodium. It is soluble in water, 4.3g/100mls at 25°C and is insoluble in alcohol (The Merck Index, 1983).

Stannous fluoride was obtained as a white powder and has a molecular weight of 156.7. The compound contains 24.24% fluoride and 75.75% tin. It is poorly soluble in water at 3g/100mls (The Merck Index, 1983).

Silver fluoride was obtained in fluid form, this was snap frozen and lyophilized to provide a yellow powder and the compound has a molecular weight of 126.88. It consists of 14.97% fluoride and 85.03% silver. It is very hygroscopic, darkens on exposure to light and is soluble in water when freshly prepared, 182g/100ml at 15.5°C (The Merck Index, 1983).

All fluorides that were used in the experiments were in a powder form and the fluoride concentration was determined on a weight per volume basis.

2.4 Test solution.

In order to compare equally the three fluorides, a test solution that did not bind the F^- or tin or silver ion was used. In the initial experiments deionized filtered water was used.

In the second set of experiments a test solution containing a protein solution of an equivalent concentration to that of gingival crevicular fluid was selected. Cimasoni (1983) reported that the total protein content of gingival crevicular fluid collected from inflamed gingivae varied from 5.5% to 9%. It was decided to use 6% bovine serum albumin (Sigma, St Louis. Lot no.92F-0630) as a test solution to represent gingival crevicular fluid from the gingival crevice.

The fluoride concentration of the deionized filtered water was determined, as this may provide fluoride in addition to that added. The procedure was to compare samples of known F^- concentration to the deionized water. A fluoride electrode (Orion Research) was standardized with glass distiled deionized filtered water, dried and then placed in the sample solution. Voltage readings were recorded at 3, 3.5, 4, 4.5 and 5 minutes . The electrode was washed and standardized between samples. The results were plotted on time response graph paper and the result at time infinity obtained (see Table 2.1). These results were plotted against the log parts per million (ppm) F^-. The deionized filtered water had 0.03ppmF^- content (see Figure 2.7).

SAMPLE	TIME (minutes)					
	3	3.5	4	4.5	5	α
$1000ppmF^-$	-197.2	-197.5	-197.6	-197.8	-197.9	-199
$500ppmF^-$	-182.2	-182.2	-182.2	-182.2	-182.2	-182.2
$100ppmF^-$	-139.4	-139.6	-139.6	-139.6	-140.9	-140.5
$10ppmF^-$	-79.5	-79.5	-79.6	-79.7	-79.7	-80
Water ($0.03ppmF^-$)	81.0	79.3	77.8	77.0	76.9	72

Table 2.1 Millivolt (mV) readings of the fluoride electrode (Orion Research) at time periods. Time at α calculated by plotting mV readings on time response graph paper. Fluoride concentration for deionized filtered water obtained from Figure 2.7.

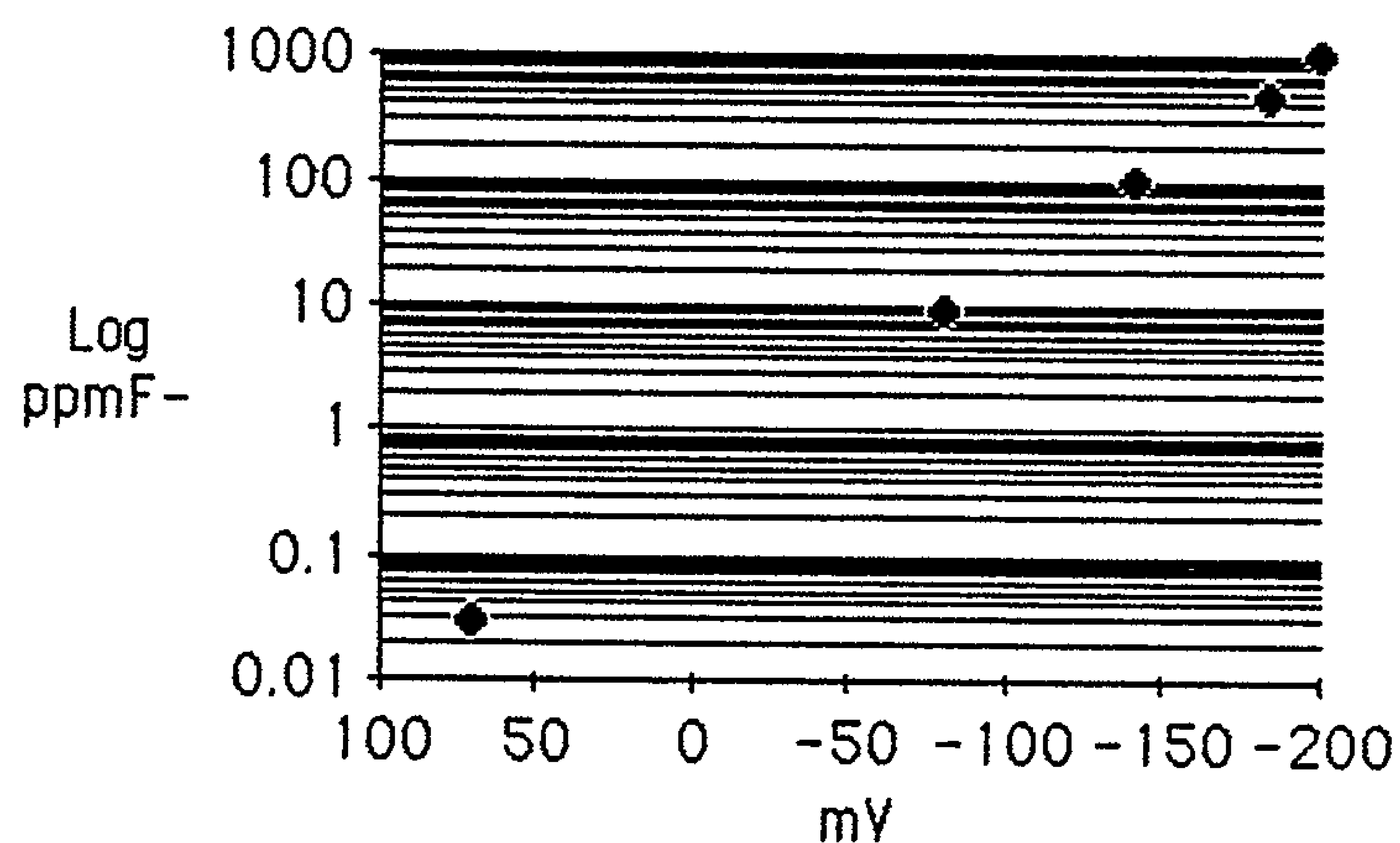

Figure 2.7 Fluoride determination. The log of fluoride concentration versus millivolts (mV). The graph was used to determine the fluoride concentration of filtered deionized water. Filtered deionized water has a fluoride concentration of $0.03ppmF^-$.

2.41 Preparation of solutions.

Only plastic disposable ware was used with the fluoride solutions. All solutions were freshly prepared and used within 30 minutes of preparation.

The required amount of prepared solutions were filter sterilized through a Millex-GS 0.22um filter unit (Millipore, Bedford) into sterile plastic disposable cultures tubes (Edwards Instruments, Narellen). Control tubes of filtered water and 6% albumin were similarly filter sterilized. All tubes were maintained at 37°C in a water bath before inoculation with the microorganisms and during the experiment.

2.42 The pH of solutions.

The pH of the prepared solutions was measured with Merck pH strips (Merck, Germany). The pH readings were,

Solution.	F-concentration.	pH
Filtered water	0ppm	5.3
NaF	100ppm	5.3
	50ppm	5.3
	10ppm	5.3
SnF_2	100ppm	3.2
	50ppm	3.5
	10ppm	3.8
AgF	100ppm	5.3

	50ppm	5.3
	10ppm	5.3
6% Albumin	0ppm	6.5
NaF in Albumin	100ppm	6.5
SnF_2 in Albumin	100ppm	5.7
AgF in Albumin	100ppm	5.9

The marked change in pH observed with the SnF_2 in filtered water is due to the hydroxlylation of water by tin allowing an increase in hydrogen ions. If this solution is left to stand for several hours a white precipitate of stannous hydroxide forms (Tinanoff 1985). This effect was buffered by the albumin solution.

Both NaF and AgF caused no change of pH in water. The AgF with albumin showed no signs of precipitation. All solutions remained transparent during the experiments.

2.5 Experiments.

The experiments were performed in aerobic conditions.

2.51 Procedure 1. (see Figure 2.8)

S.sanguis G9B.

S.sanguis G9B was grown to an absorbance of 0.1. This provided approximately 10^9 CFU/ml. To ensure no chemicals from the TSB would bind to the fluoride solutions the microorganisms were centrifuged at 400rpm for ten minutes at 4^oC (Minor, Thomas

Optical and Scientific Company). The supernatant was removed and the pellet resuspended in 10ml sterile deionized filtered water. The above was repeated. The final suspension of microorganisms was brought to 37°C before the next step.

A 1 in 10 dilution was performed and then a 0.1ml aliquot was inoculated into duplicate tubes of 10ml of the test solutions. Four runs were performed, consisting of tubes containing,

Run 1. control- deionized filtered water

100ppm F^- NaF

50ppm F^- NaF

10ppm F^- NaF

Run 2. control

100ppm F^- SnF_2

50ppm F^- SnF_2

10ppm F^- SnF_2

Run 3. control

100ppm F^- AgF

50ppm F^- AgF

10ppm F^- AgF

The fourth run was included to investigate the effect of lowering the pH of the deionized water. The pH was adjusted to 3.5 (being a similar pH obtained with SnF_2 solutions) using 0.1M hydrochloric acid. This allowed a comparison of the effect of pH, SnF_2 and NaF.

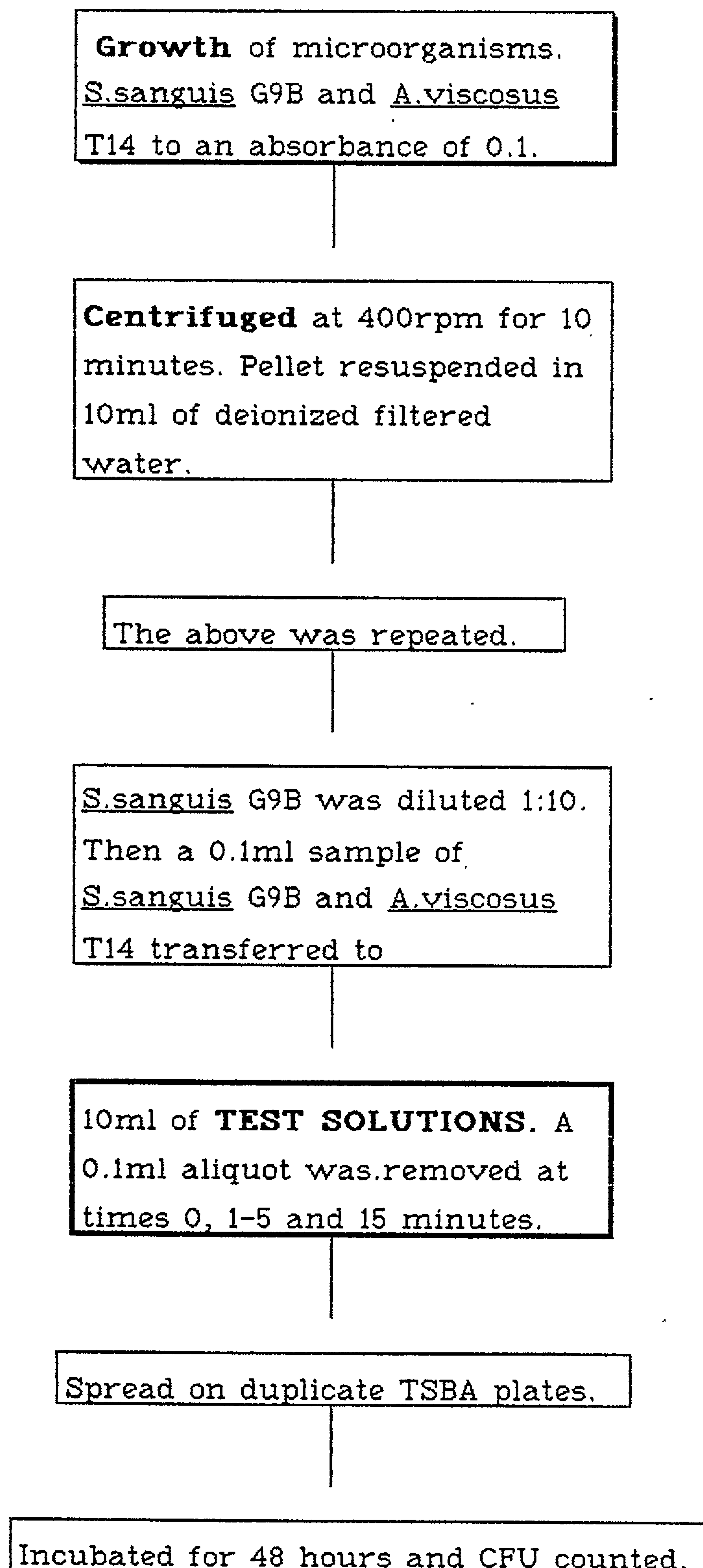

Figure. 2.8. Flow diagram of Procedure 1. Using the microorganisms. S.sanguis G9B and A.viscosus T14.

Run 4. control- 3.5pH

100ppm F^- NaF - 3.5pH

50ppm F^- NaF - 3.5pH

10ppm F^- NaF - 3.5pH

In each run a 0.1ml aliquot was removed from the control tube at time intervals 0, 1-5 and 15 minutes. For the fluoride containing tubes a 0.1ml aliquot was removed at 1-5 and 15 minutes. The aliquot was spread in the usual manner on duplicate TSBA plates. The plates were incubated aerobically at 37°C for 48 hours. The number of CFU were obtained with the use of a magnifying loop.

A.viscosus T14.

A.viscosus T14 was grown as previously described to an absorbance of 0.1. This provided approximately 10^8 CFU/ml. The microorganisms were centrifuged twice and resuspended in 10ml sterile deionized filtered water in the same manner as for S.sanguis G9B. This tube was brought to 37°C before continuing.

A 0.1ml aliquot of the A.viscosus T14 suspension was placed into duplicate tubes containing 10ml of test solution. Three run were performed with tubes containing,

Run 1. control- deionized filtered water

100ppm F^- NaF

50ppm F^- NaF

10ppm F^- NaF

Run 2. control

100ppm F^- SnF_2

50ppm F^- SnF_2

10ppm F^- SnF_2

Run 3. control

100ppm F^- AgF

50ppm F^- AgF

10ppm F^- AgF

A 0.1ml aliquot was obtained at similar times as above and spread onto duplicate TSBA plates. These were incubated anaerobically and counted as previously described.

B.intermedius NCTC 9336 was not tested by this procedure, see Procedure two.

2.52 Procedure 2.(see Figure 2.9)

In Procedure 1 the aliquot was plated out directly from the treatment tubes. It was reasoned that this may allow killing of the microorganisms after the testing time, as the fluoride solution remains in contact with the microorganisms on the agar plates.

Therefore, in procedure 2

(a) a larger initial inoculum was used and

(b) before plating out the microorganisms the sample was serially diluted through TSB.

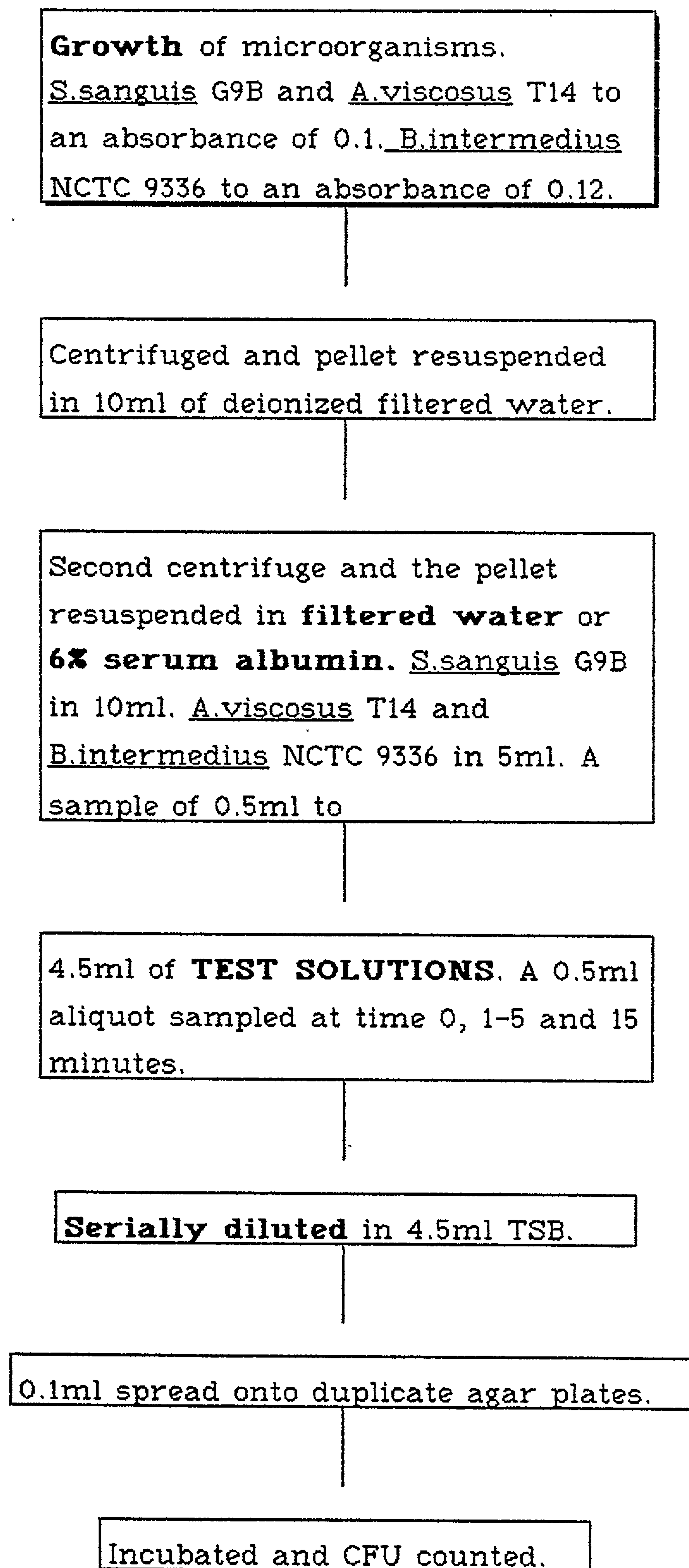

Figure. 2.9. Flow diagram of Procedures 2 and 3. Using the microorganisms S.sanguis G9B, A.viscosus T14 and B.intermedius NCTC 9336.

The concentration of fluoride in TSB that would allow survival of the microorganism was determined. S.sanguis G9B was grown and washed as per procedure 1. An inoculum of 0.5ml was placed into 4.5ml TSB with the following F^- concentration using the stannous fluoride powder, 100, 10, 1, 0.1ppm F^- and filtered water. At times 1 minute and 1 hour a 0.1ml aliquot was spread onto duplicate TSBA plates. At 24 hours the broth tubes were tested for presence of turbidity. Only the 100ppm F^- plates and tube showed no growth (see Table 2.2). Serial dilutions in TSB that were equal to or less than 10ppm F^- were only used for spreading on agar plates.

S.sanguis G9B

This microorganism was grown and washed as in procedure 1. After centrifuging the second time the pellet was resuspended in 10ml deionized filtered water. This tube was brought to 37°C. A 0.5ml aliquot was removed and placed into each test solution of 4.5ml. Two runs were done, consisting of a single tube of,

Run 1. Control - deionized filtered water

100ppm F^- of NaF

100ppm F^- SnF_2

50ppm F^- SnF_2

10ppm F^- SnF_2

TREATMENT	TIME		
	1-5 minutes	1 hour	24 hours
Control	339.5	531	yes
100ppmF	4.5	0	no
10ppmF	336	410.5	yes
1ppmF	366	413	yes
0.1ppmF	349	436	yes

Table 2.2. Viability of S.sanguis G9B grown in TSB with different fluoride concentrations of SnF_2. Mean number of CFU at times 1-5 minutes and 1 hour, and the presence of turbidity at 24 hours.

Run 2. Control - deionized filtered water

100ppm F^- of NaF

100ppm F^- AgF

50ppm F^- AgF

10ppm F^- AgF

At times 0, 1-5 and 15 minutes 0.5ml was removed from the control tube. At times 1-5 and 15 minutes 0.5ml was removed from the other tubes. This was serially diluted 1 in 10, three times in TSB. A 0.1ml aliquot was removed from each TSB tube and spread onto duplicate TSBA plates. These were incubated aerobically and counted after 48 hours.

A.viscosus T14.

A.viscosus T14 was grown and washed as previously described. The pellet from the final wash was resuspended in 5ml of deionized filtered water. A 0.5ml aliquot was removed and placed into each treatment tube. Two runs were performed as with S.sanguis G9B. Samples were taken, serially diluted and plated out as above. The plates were incubated anaerobically and counted after 48 hours.

B.intermedius NCTC 9336.

B.intermedius NCTC 9336 was grown to an absorbance of 0.12 in BM. This provided approximately 10^8 CFU/ml. The tube was centrifuged, the supernatant removed and the pellet resuspended in 10ml of sterile deionized filtered water. This was repeated and the pellet was resuspended in 5ml of filtered water. This was

brought to 37°C. A 0.5ml aliquot was removed and placed into each 4.5ml treatment tube. One run was carried out consisting of,

Run. Control

100ppm F^- NaF

10ppm F^- SnF_2

10ppm F^- AgF

Samples were removed at the times as described for S.sanguis G9B. They were serially diluted in TSB and a 0.1ml aliquot was spread onto duplicate ETSA plates. The plates were incubated anaerobically and the CFU counted after 7 to 10 days.

2.53 Procedure 3.(see Figure 2.9)

To simulate the protein content of gingival crevicular fluid a 6% bovine serum albumin was prepared (see Test solution.). The procedure design was the same as in procedure 2.

The three microorganisms were grown as previously described and centrifuged in the same manner. The pellet obtained was resuspended in 6% serum albumin solution. This tube was brought to 37°C. A 0.5ml sample was placed into the 4.5ml treatment tubes. A single run for each organism was performed consisting of,

Run. 6% Albumin Control

100ppm F^- NaF in albumin

100ppm F^- SnF_2 in albumin

100ppm F^- AgF in albumin

At the time periods 0, 1-5 and 15 minutes a 0.5ml aliquot was removed, serially diluted through TSB and a 0.1ml aliquot from these tubes spread onto duplicate agar plates as previously described for each microorganism.

The plates were incubated as previously described and the CFU counted.

The sequence for sampling and spreading onto agar plates was fluoride solutions first, highest ppm concentration to lowest and then the control. Where different fluorides were used in the same run the sequence was AgF, SnF_2 and finally NaF.

2.6 Statistical Analysis.

Statistical analysis was performed using a computer statistical program, Stat View 512+ (Brainpower Inc, 1986) on an Apple Macintosh Plus computer.

The control results for each microorganism and each procedure were combined.

Procedure 1.

S.sanguis G9B and *A.viscosus* T14.

A two factor analysis of variance (ANOVA) was calculated to compare CFU/ml versus Time and Treatment groups for each microorganism. A one factor ANOVA was then calculated for time intervals 1-5 and 15 minutes comparing CFU/ml versus Treatment groups for each organism. Multiple comparison tests using Fisher's Protected Least Significant Difference (PLSD) were

calculated to indicate where the differences between treatments occurred, a 99% confidence level was used (Winer 1971).

Procedure 2 and Procedure 3.

S.sanguis G9B, A.viscosus T14 and B.intermedius NCTC 9336.

A three factor ANOVA was calculated for each procedure to compare CFU/ml versus Microorganisms, Time and Treatment groups. Then a two factor ANOVA was calculated for each procedure to compare CFU/ml versus Time and Treatments. Multiple comparison tests (Fisher PLSD) were used to indicate where the differences occurred between treatments at times 1-5 and 15 minutes.

RESULTS

3.1 Procedure 1.

Streptococcus sanguis G9B

The control for *S.sanguis* G9B showed a gradual decline in the number of colony forming units (CFU) over the 15 minutes period. Both silver fluoride and stannous fluoride treatments had the most profound effect on the microorganism at the 1-5 and 15 minutes, with no CFU counted at these times. This effect was achieved at the lowest fluoride level used, 10ppmF^-. Sodium fluoride treatment had minimal effect at 10ppmF^- and 50ppmF^-, however at 100ppmF^- and after 15 minutes a difference from the the control was significant. Altering the pH to 3.5 produced a difference between control treatment groups and after fifteen minutes the acidulated NaF produced a similar effect to SnF_2 and AgF treatment. The two factor ANOVA indicated a significant difference occurred within treatments and times, and between treatment and times ($p \leq 0.0001$, See Table 3.1, Figures 3.1, 3.2, 3.3 and 3.4).

At the 1-5 minute mark multiple comparison tests (Fisher Protected Least Significant Difference (PLSD)) indicated differences occurred between the control and all other treatments ($p \leq 0.01$), except 10ppmF^- and 50ppmF^- NaF. The AgF, SnF_2 and NaF pH3.5 (50ppmF^- and 100ppmF^-) treatment groups produced the most reduction. The lowest fluoride concentration which provided the

TREATMENT	TIME		
	0.minutes	1-5 minute	15 minute
Control SD Number	12030 3067 12	11715 2509 12	9727 2448 12
Control pH 3.5 SD No.	9505 2938 4	7350 1545 4	2990 780 4
10ppmF⁻ NaF SD No.	10900 288 4	9265 1643 4	5750 1807 4
50ppmF⁻ NaF SD No.	10900 788 4	9573 660 4	4940 1071 4
100ppmF⁻ NaF SD No.	10900 788 4	8145 1026 4	485 117 4
10ppmF⁻ NaF pH3.5 SD No.	9505 2938 4	4420 2587 4	143 66 4
50ppmF⁻ NaF pH3.5 SD No.	9505 2938 4	325 260 4	13 5 4
100ppmF⁻ NaF pH3.5 SD No.	9505 2938 4	155 73 4	3 5 4
10ppmF⁻ SnF_2 SD No.	9715 1470 4	10 12 4	0.3 0.5 4
50ppmF⁻ SnF_2 SD No.	9715 1470 4	0 0 4	0 0 4
100ppmF⁻ SnF_2 SD No.	9715 1470 4	0 0 4	0 0 4
10ppmF⁻ AgF SD No.	15475 2660 4	0 0 4	0 0 4
50ppmF⁻ AgF SD No.	15475 2660 4	0 0 4	0 0 4
100ppmF⁻ AgF SD No.	15475 2660 4	0 0 4	0 0 4

Table. 3.1. Procedure 1. CFU/ml.of the microorganism <u>S.sanguis</u> G9B compared to different treatments with time. The two factor ANOVA: Time (A) F=467.457, P≤0.0001. Treatment (B) F=38.269, P≤0.0001. AB F=13.653, P≤0.0001.

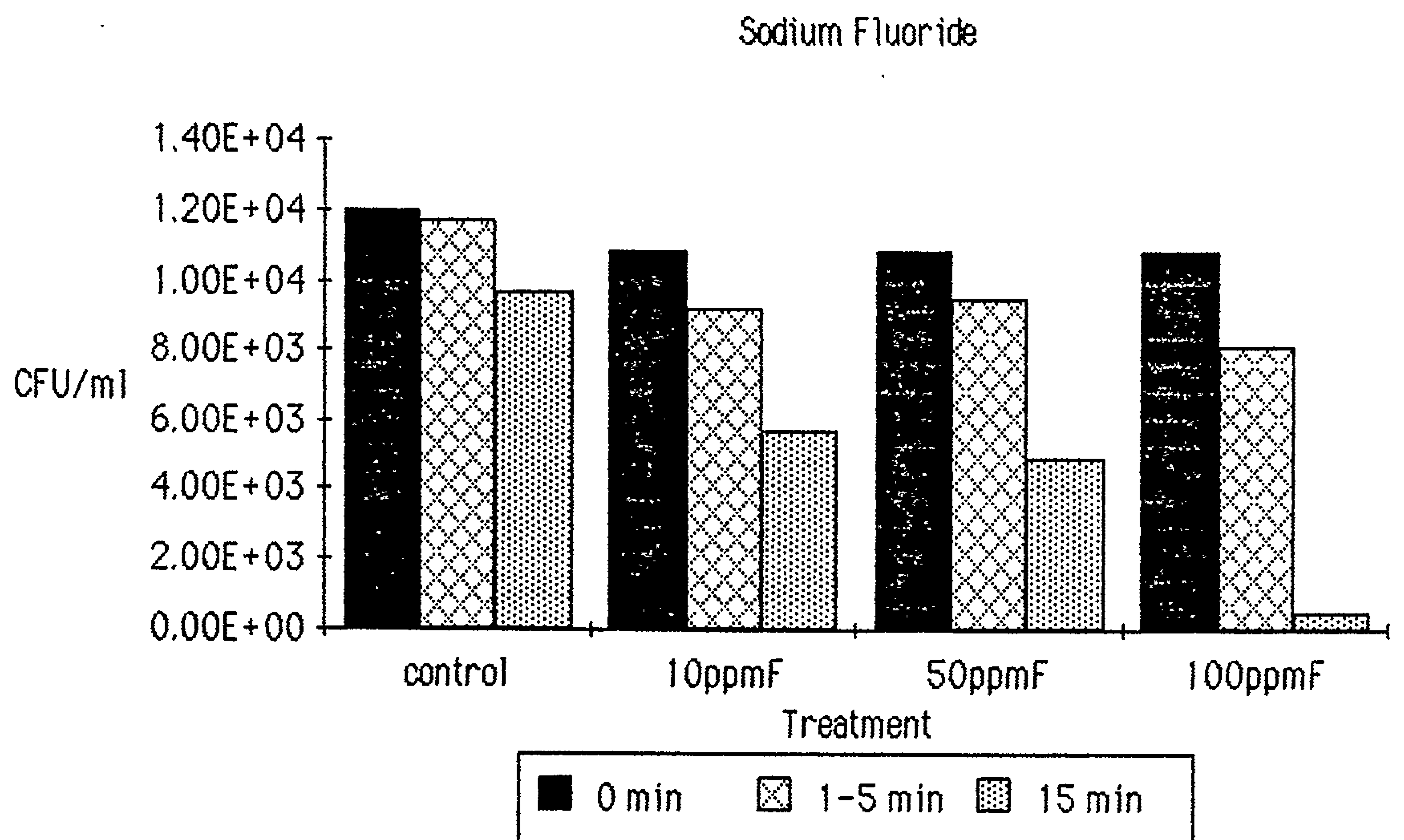

Figure 3.1 Procedure 1. S.sanguis G9B treated with sodium fluoride.

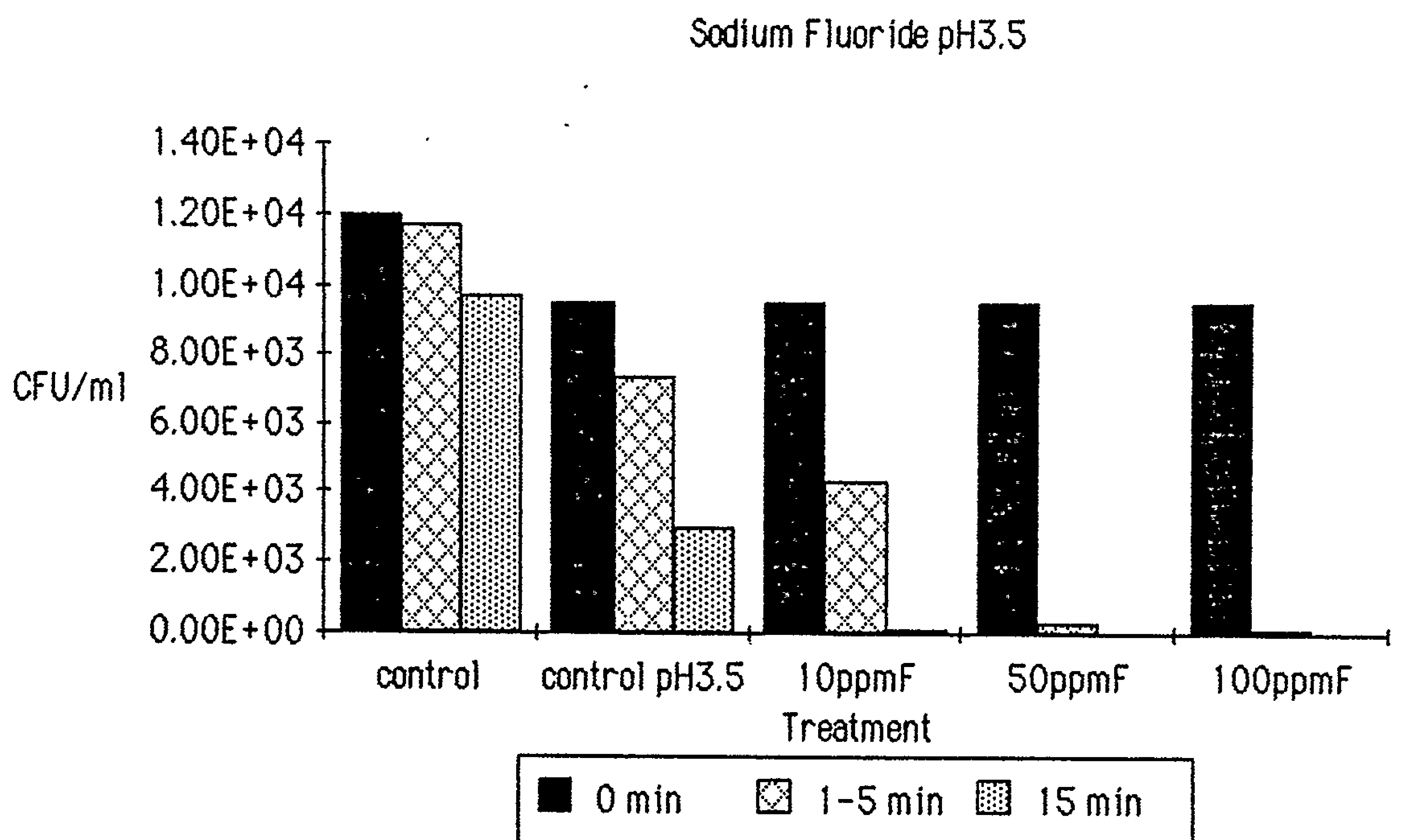

Figure 3.2. Procedure 1. S.sanguis G9B treated with sodium fluoride adjusted to pH 3.5.

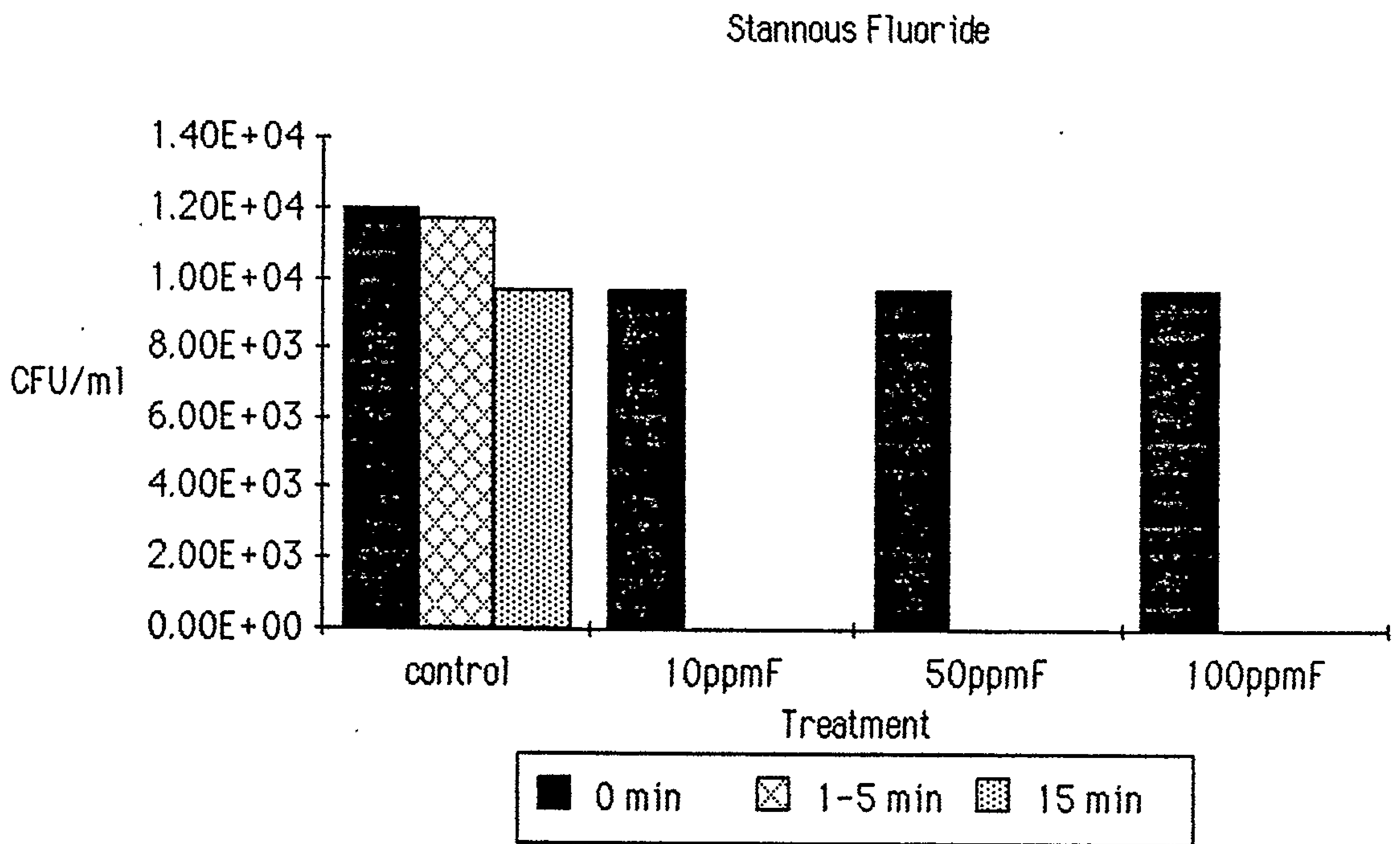

Figure 3.3. Procedure 1. S.sanguis G9B treated with stannous fluoride.

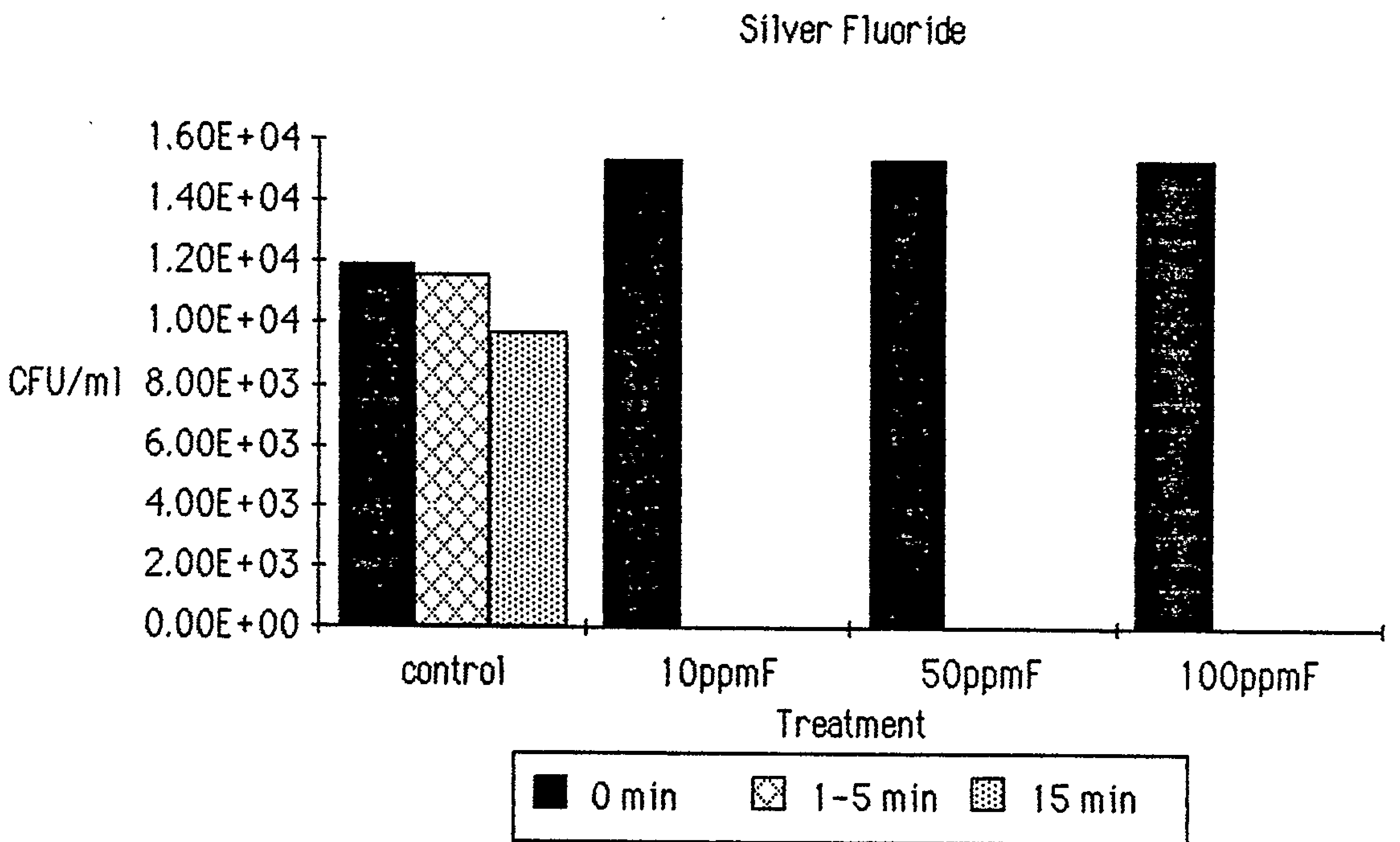

Figure 3.4. Procedure 1. S.sanguis G9B treated with silver fluoride.

most significant growth reduction was 50ppmF^- NaF at pH3.5, 10ppmF^- SnF_2 and 10ppmF^- AgF.

At 15 minutes all treatments were significantly different ($p \leq 0.01$) from the control treatment. The NaF at 10ppm$F^-$and 50ppmF^- were the least effective treatments. There was no statistically significant difference at the $p=0.01$ level for the other groups. Although an apparent trend would indicate 10ppmF^- SnF_2, 10ppmF^- AgF , 100ppmF^- NaF and 10ppmF^- NaF at pH3.5 are the lowest concentrations that produce significant reduction at 15 minutes.

<u>Actinomyces viscosus</u> T14

The control treatment for <u>A.viscosus</u> T14 produced a gradual decline in the number of CFU counted over the 15 minutes. Both SnF_2 and AgF treatments produced inhibition of growth, with no CFU counted at the two time intervals. NaF produced a gradual inhibiting effect over the time period. This effect was most profound with the 100ppmF^- NaF treatment. The two factor ANOVA indicated a statistically significant difference within the time periods and within treatments and between times and treatments ($p \leq 0.0001$, see Table 3.2, Figures 3.5, 3.6 and 3.7).

At the 1-5 minute interval only the SnF_2 and AgF treatments were significantly different from the control treatment ($p \leq 0.01$). There was no difference between SnF_2 and AgF treatments. NaF 100ppmF^- was more effective than the other two NaF concentrations.

TREATMENT	TIME		
	0 minutes	1-5 minute	15 minute
Control	15522	13895	12243
SD	4680	3943	3061
Number	12	12	12
10ppmF^- NaF	19675	18850	13575
SD	4480	1201	492
No.	4	4	4
50ppmF^- NaF	19675	16175	6290
SD	4480	3133	2132
No.	4	4	4
100ppmF^- NaF	19675	10055	2088
SD	4480	693	1438
No.	4	4	4
10ppmF^- SnF_2	11665	0	0
SD	4010	0	0
No.	4	4	4
50ppmF^- SnF_2	11665	0	0
SD	4010	0	0
No.	4	4	4
100ppmF^- SnF_2	11665	0	0
SD	1130	0	0
No.	4	4	4
10ppmF^- AgF	15225	0	0
SD	1130	0	0
No.	4	4	4
50ppmF^- AgF	15225	0	0
SD	1130	0	0
No.	4	4	4
100ppmF^- AgF	15225	0	0
SD	1130	0	0
No.	4	4	4

Table. 3.2. Procedure 1. CFU/ml of the microorganism _A.viscosus_ T14 compared to different treatments with time. The two factor ANOVA: Time (A) $F=220.203$, $P \leq 0.0001$. Treatment (B) $F=49.237$, $P \leq 0.0001$. AB $F=7.718$, $P \leq 0.0001$.

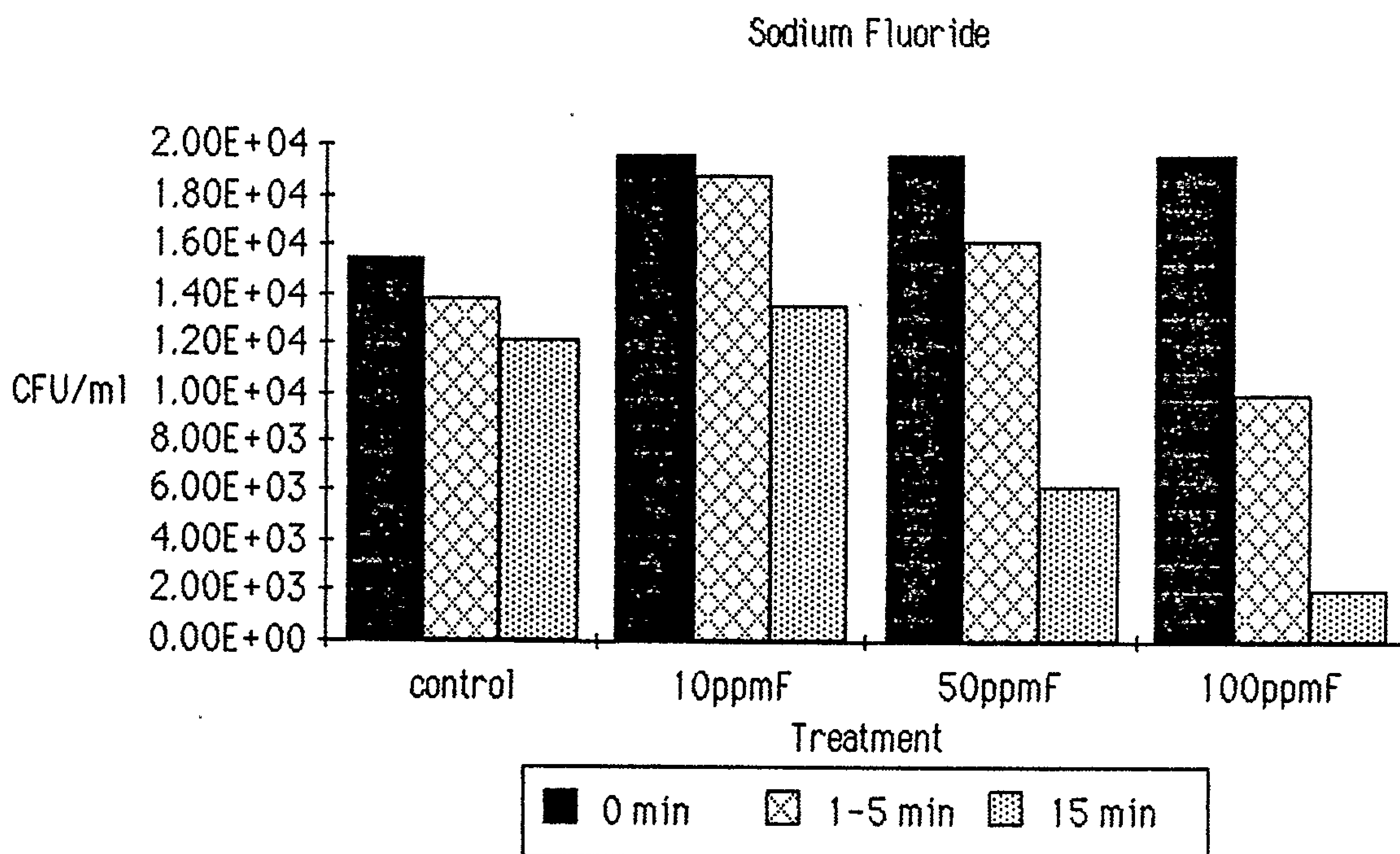

Figure 3.5. Procedure 1. A.viscosus T14 treated with sodium fluoride.

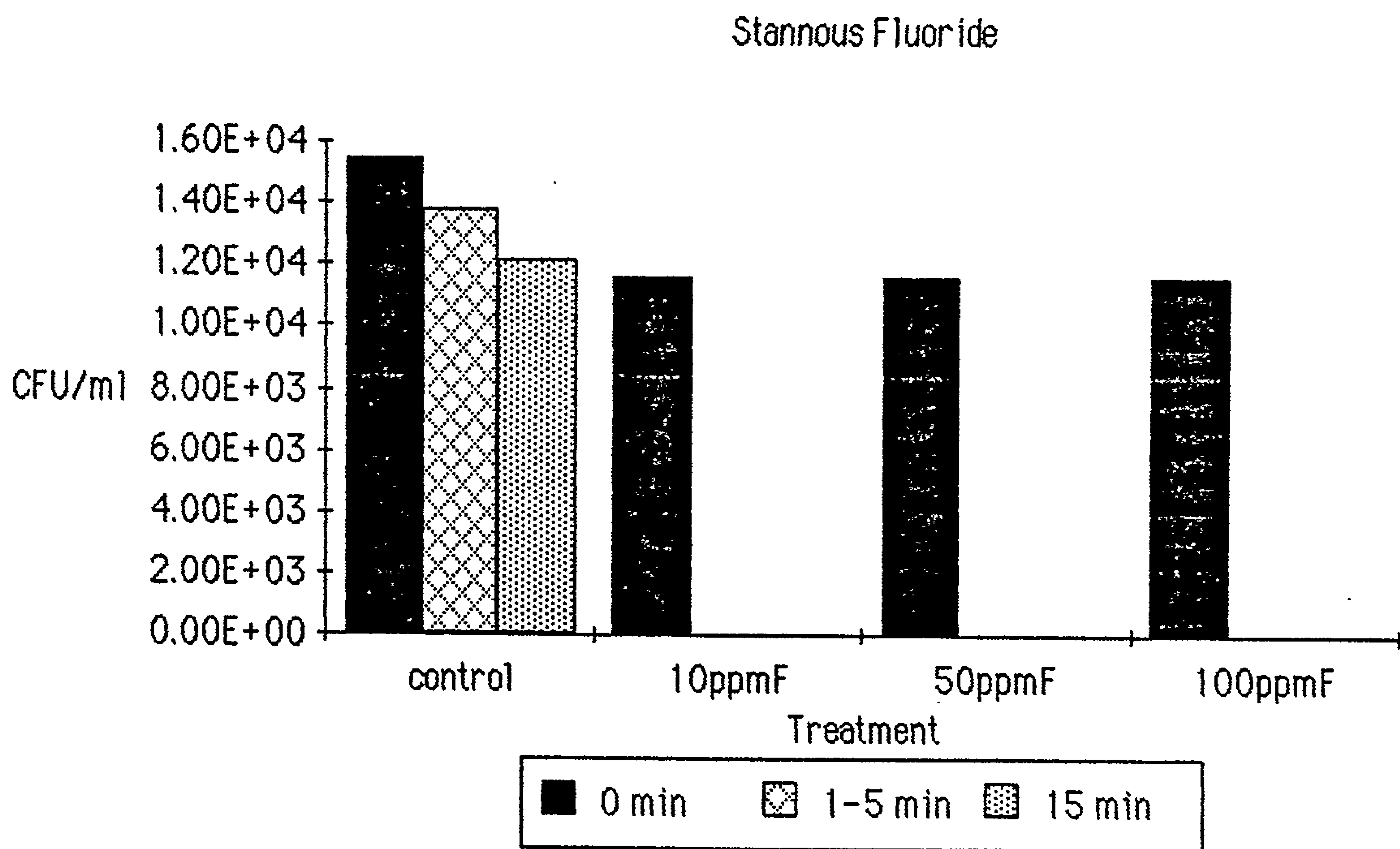

Figure 3.6. Procedure 1. A.viscosus T14 treated with stannous fluoride.

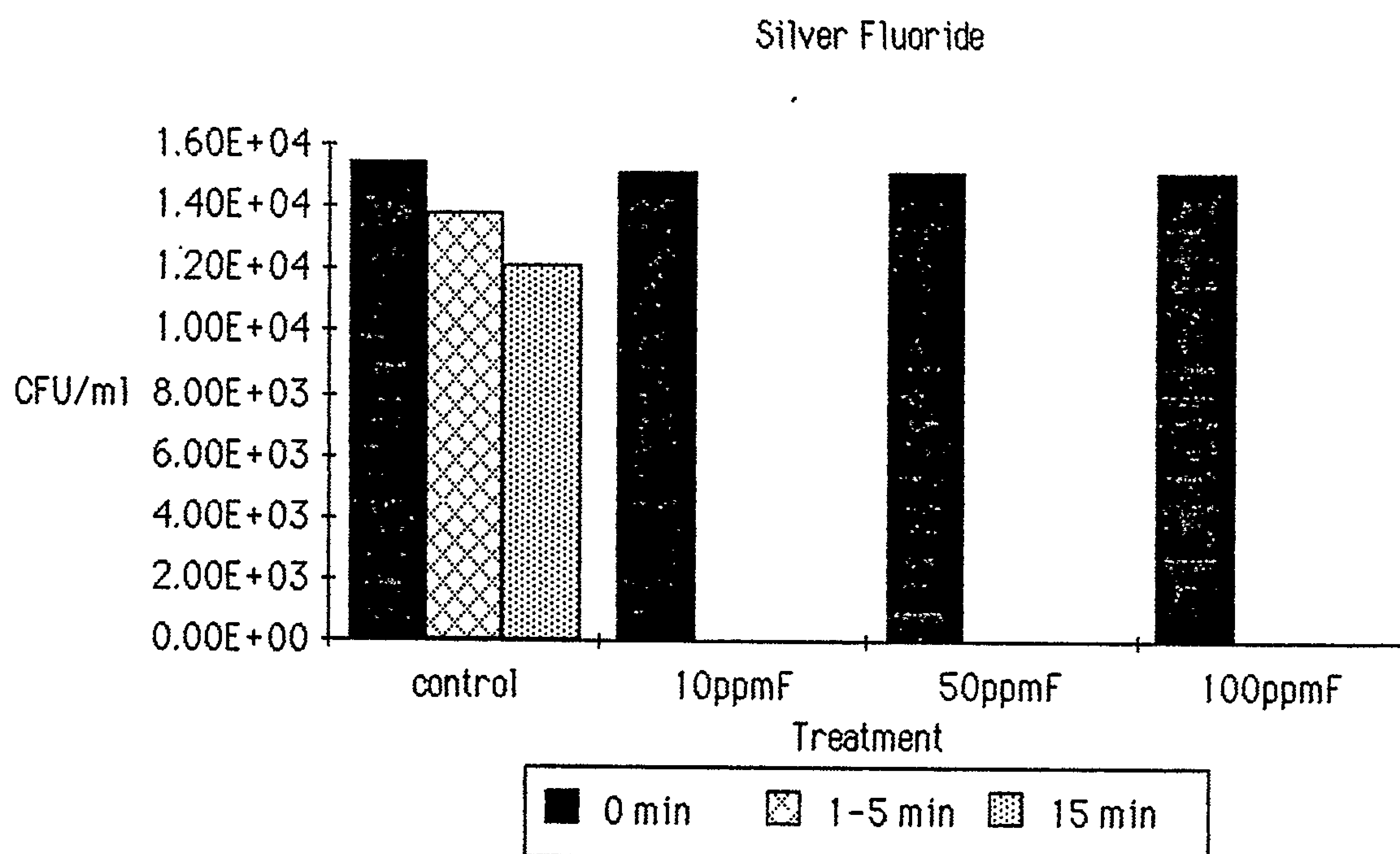

Figure 3.7. Procedure 1. A.viscosus T14 treated with silver fluoride.

TREATMENT	TIME		
	0 minutes	1-5 minute	15 minute
Control SD Number	2750000 373720 4	2737500 411856 4	2670000 556166 4
100ppmF^- NaF SD No.	2750000 373720 4	2562500 99457 4	2080000 733394 4
10ppmF^- SnF_2 SD No.	2840000 594000 2	1450000 155563 2	0 0 2
50ppmF^- SnF_2 SD No.	2840000 594000 2	83000 28284 2	0 0 2
100ppmF^- SnF_2 SD No.	2840000 594000 2	0 0 2	0 0 2
10ppmF^- AgF SD No.	2660000 184000 2	0 0 2	0 0 2
50ppmF^- AgF SD No.	2660000 184000 2	0 0 2	0 0 2
100ppmF^- AgF SD No.	2660000 184000 2	0 0 2	0 0 2

Table. 3.3. Procedure 2. CFU/ml of the microorganism S.sanguis G9B compared to different treatments with time. The two factor ANOVA: Time (A) $F=178.35$, $P \leq 0.0001$. Treatment (B) $F=38.372$, $P \leq 0.0001$. AB $F=10.43$, $P \leq 0.0001$.

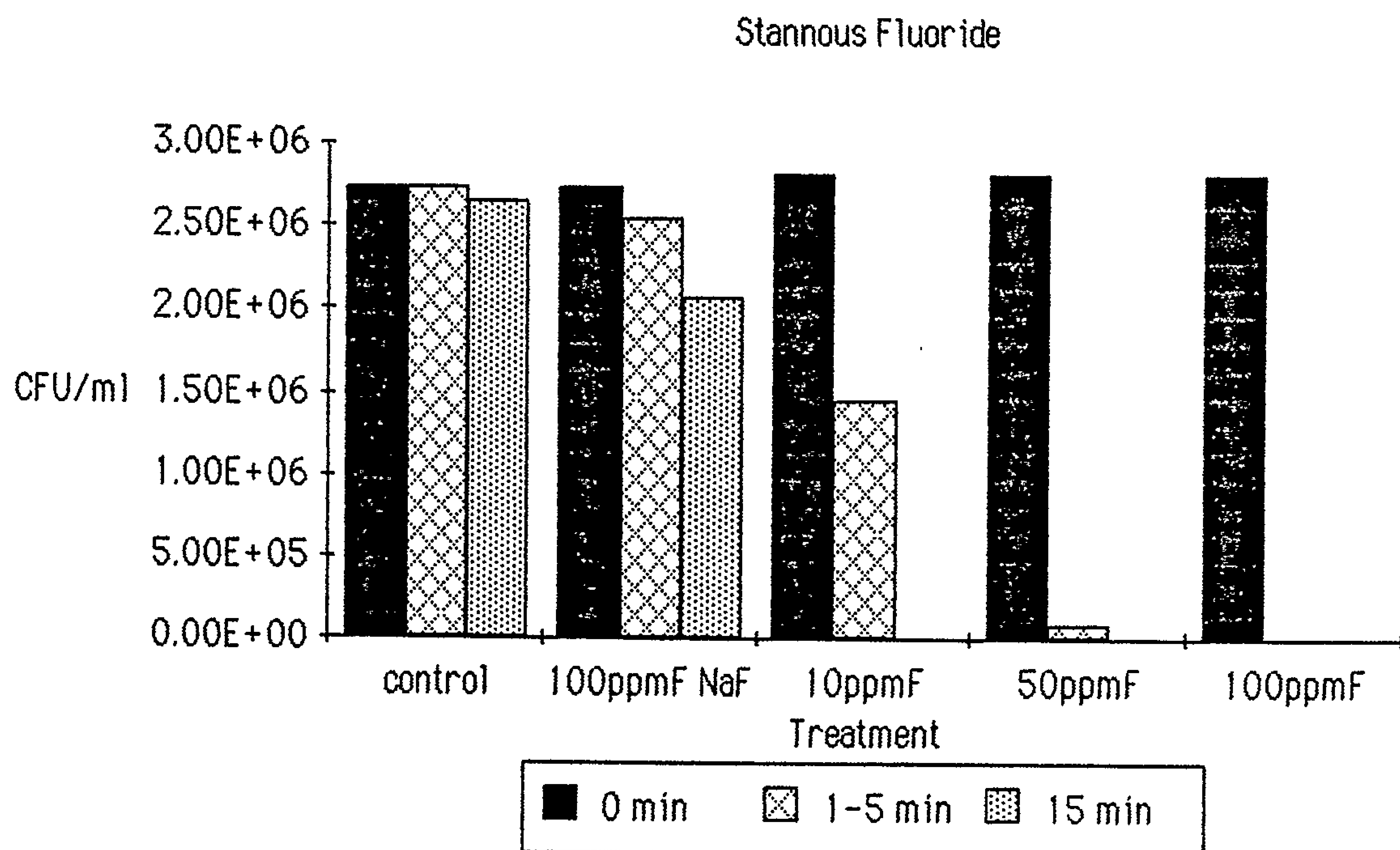

Figure 3.8. Procedure 2. S.sanguis G9B treated with stannous fluoride.

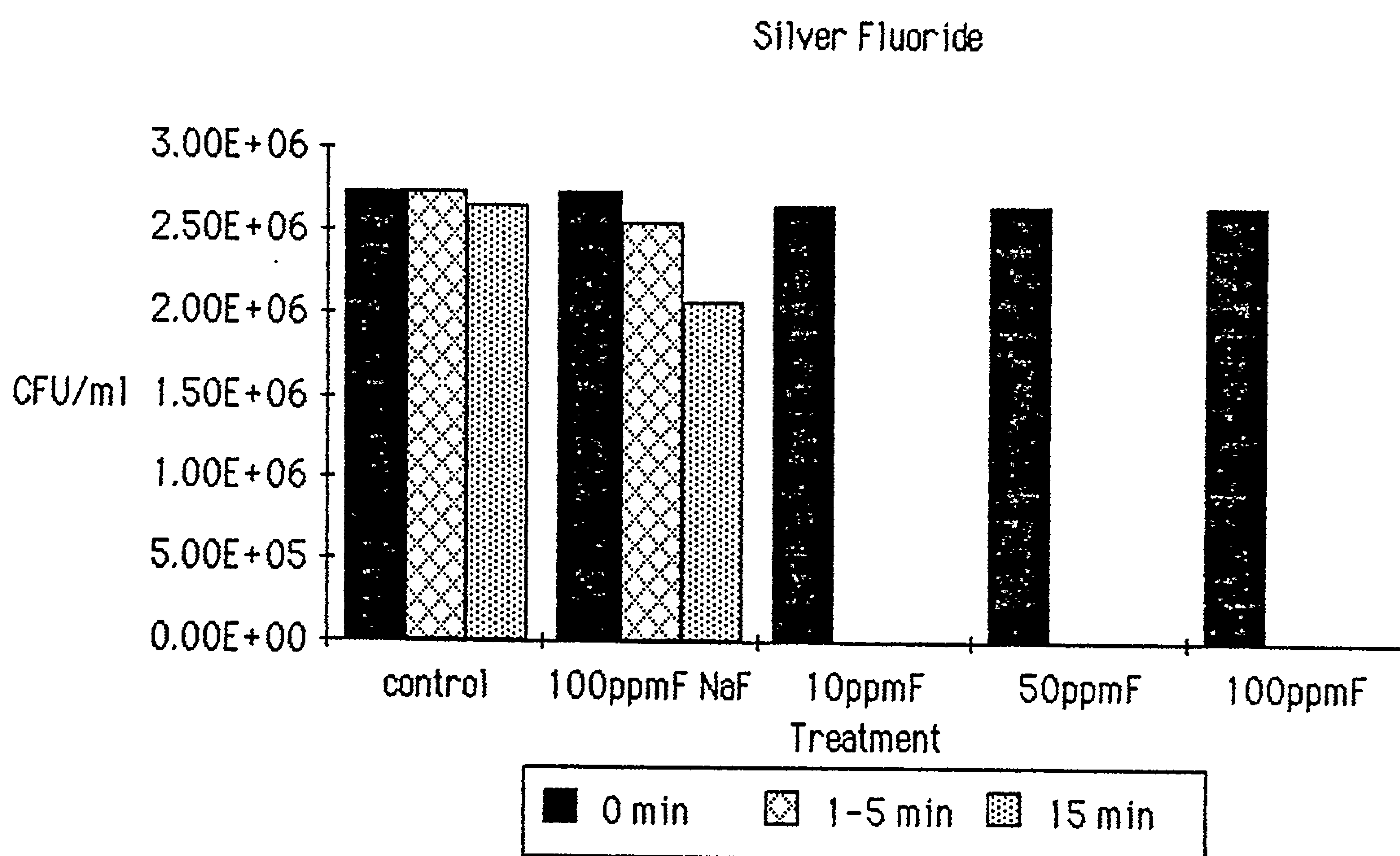

Figure 3.9. Procedure 2. S.sanguis G9B treated with silver fluoride.

After 15 minutes there was no difference between the control and NaF 10ppmF$^-$. Both 50ppmF$^-$ and 100ppmF$^-$ NaF produced a statistically significant inhibition of the microorganism compared to the control ($p=\leq 0.01$). Both AgF and SnF_2 treatments produced the most profound effect, with no CFU counted. The lowest fluoride concentration that produced the most inhibition of A.viscosus T14 at 1-5 and 15 minutes was 10ppmF$^-$ of SnF_2 and AgF.

3.2 Procedure 2.

S.sanguis G9B

A gradual decrease in the number of CFU occurred with the control treatment. The was no noticeable difference between the effect of the control and the NaF 100ppmF$^-$ treatments. Silver fluoride consistently totally inhibited growth over the fifteen minutes. Stannous fluoride totally inhibited growth at 1-5 minutes with 100ppmF$^-$ and at 15 minutes with all three concentrations. The two factor ANOVA confirmed a significant difference occurred within the treatments and times and between the treatments and times ($p \leq 0.0001$, see Table 3.3, Figures 3.8 and 3.9).

At the 1-5 minute interval 100ppmF$^-$ SnF_2 and 10ppmF$^-$ AgF were the lowest concentrations of fluoride to produce no growth. A statistically significant difference (Fisher PLSD, $p \leq 0.05$) exists between 10ppmF$^-$ AgF and 10ppmF$^-$ SnF_2 exists at 1-5 minutes. This indicated 10ppmF$^-$ AgF is the lowest concentration of fluoride to inhibit growth.

TREATMENT	TIME		
	0.minutes	1-5 minute	15 minute
Control	3407500	2830000	1665000
SD	283240	428486	807642
Number	4	4	4
100ppmF^- NaF	3407500	2982500	1645000
SD	283240	629464	439811
No.	4	4	4
10ppmF^- SnF_2	3315000	10650	0
SD	163000	1485	0
No.	2	2	2
50ppmF^- SnF_2	3315000	4750	0
SD	163000	919	0
No.	2	2	2
100ppmF^- SnF_2	3315000	0	0
SD	163000	0	0
No.	2	2	2
10ppmF^- AgF	3500000	0	0
SD	424000	0	0
No.	2	2	2
50ppmF^- AgF	3500000	0	0
SD	424000	0	0
No.	2	2	2
100ppmF^- AgF	3500000	0	0
SD	424000	0	0
No.	2	2	2

Table. 3.4. Procedure 2. CFU/ml of the microorganism A.viscosus T14 compared to different treatments with time. The two factor ANOVA: Time (A) $F=331.589$, $P \le 0.0001$. Treatment (B) $F=31.869$, $P \le 0.0001$. AB $F=9.798$, $P \le 0.0001$.

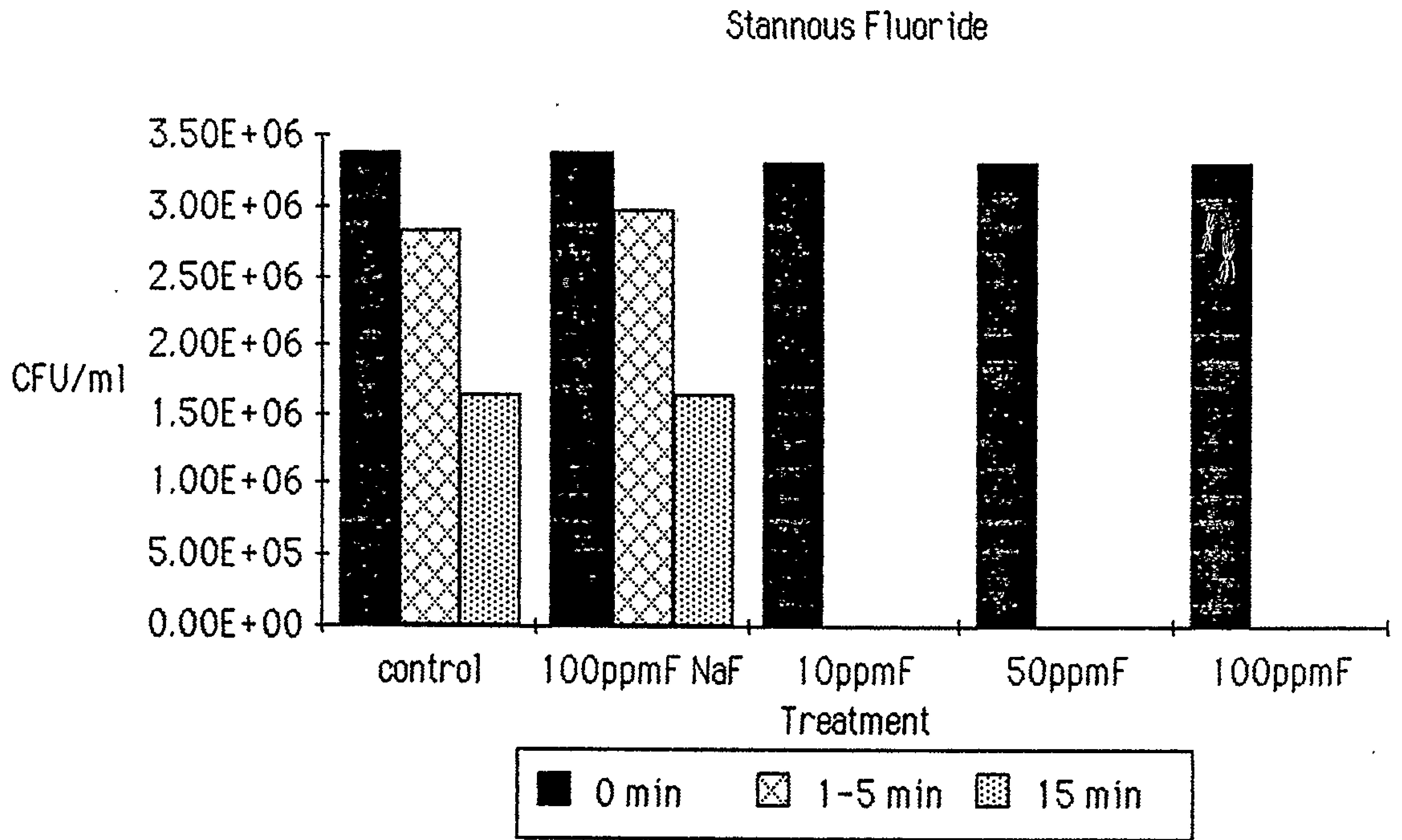

Figure 3.10. Procedure 2. A.viscosus T14 treated with stannous fluoride.

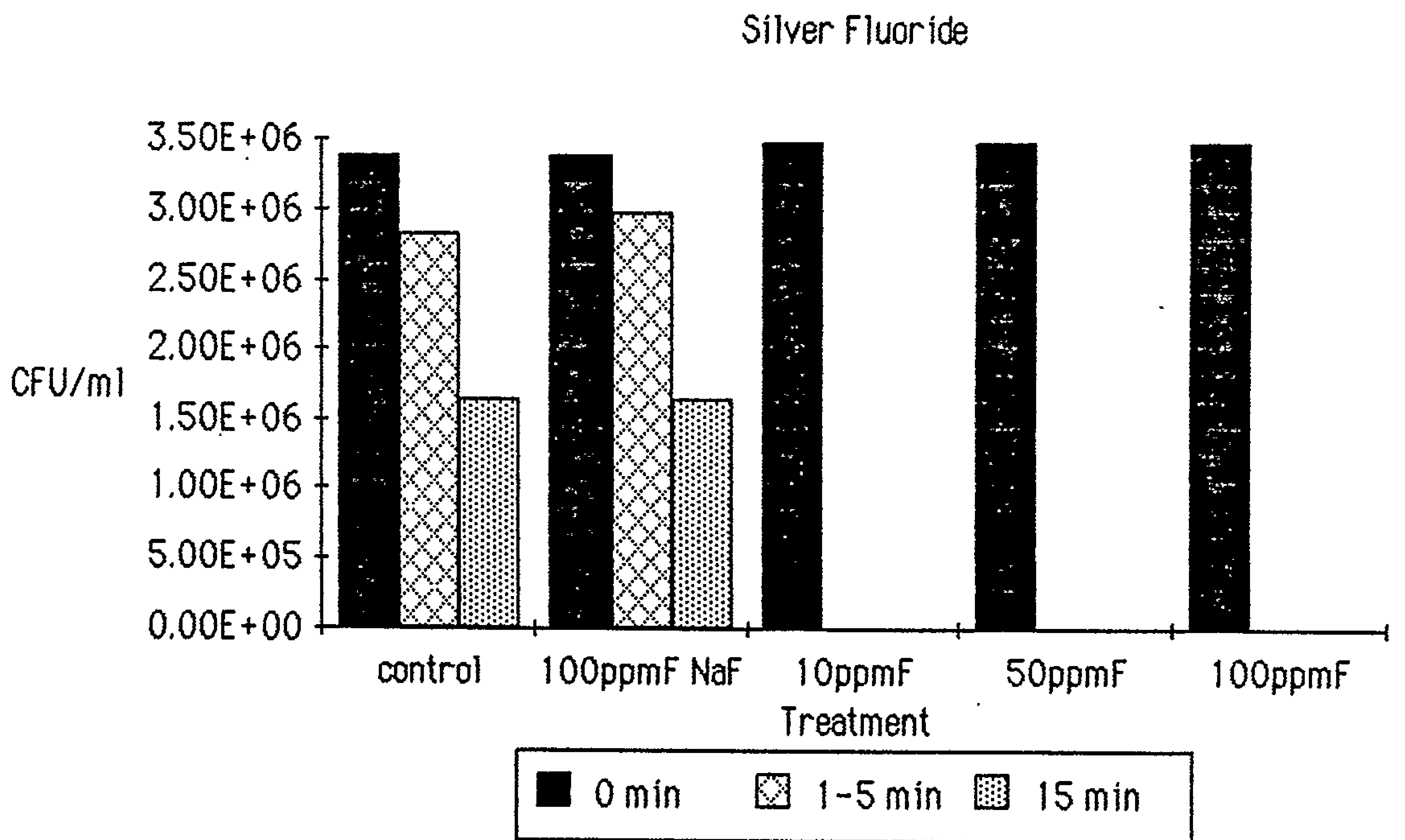

Figure 3.11. Procedure 2. A.viscosus T14 treated with silver fluoride.

TREATMENT	TIME		
	0.minutes	1-5 minute	15 minute
Control SD Number	605000 35355 2	665000 247487 2	475000 77782 2
100ppmF^- NaF SD No.	605000 35355 2	260000 113137 2	400000 113137 2
10ppmF^- SnF_2 SD No.	605000 35355 2	0 0 2	0 0 2
50ppmF^- SnF_2 SD No.	605000 35355 2	0 0 2	0 0 2
100ppmF^- SnF_2 SD No.	605000 35355 2	0 0 2	0 0 2
10ppmF^- AgF SD No.	605000 35355 2	0 0 2	0 0 2
50ppmF^- AgF SD No.	605000 35355 2	0 0 2	0 0 2
100ppmF^- AgF SD No.	605000 35355 2	0 0 2	0 0 2

Table. 3.5. Procedure 2. CFU/ml of the microorganism <u>B.intermedius</u> NCTC 9336 compared to different treatments with time. The two factor ANOVA: Time (A) $F=301.759$, $P\leq0.0001$. Treatment (B) $F=29.548$, $P\leq0.0001$. AB $F=8.31$, $P\leq0.0001$.

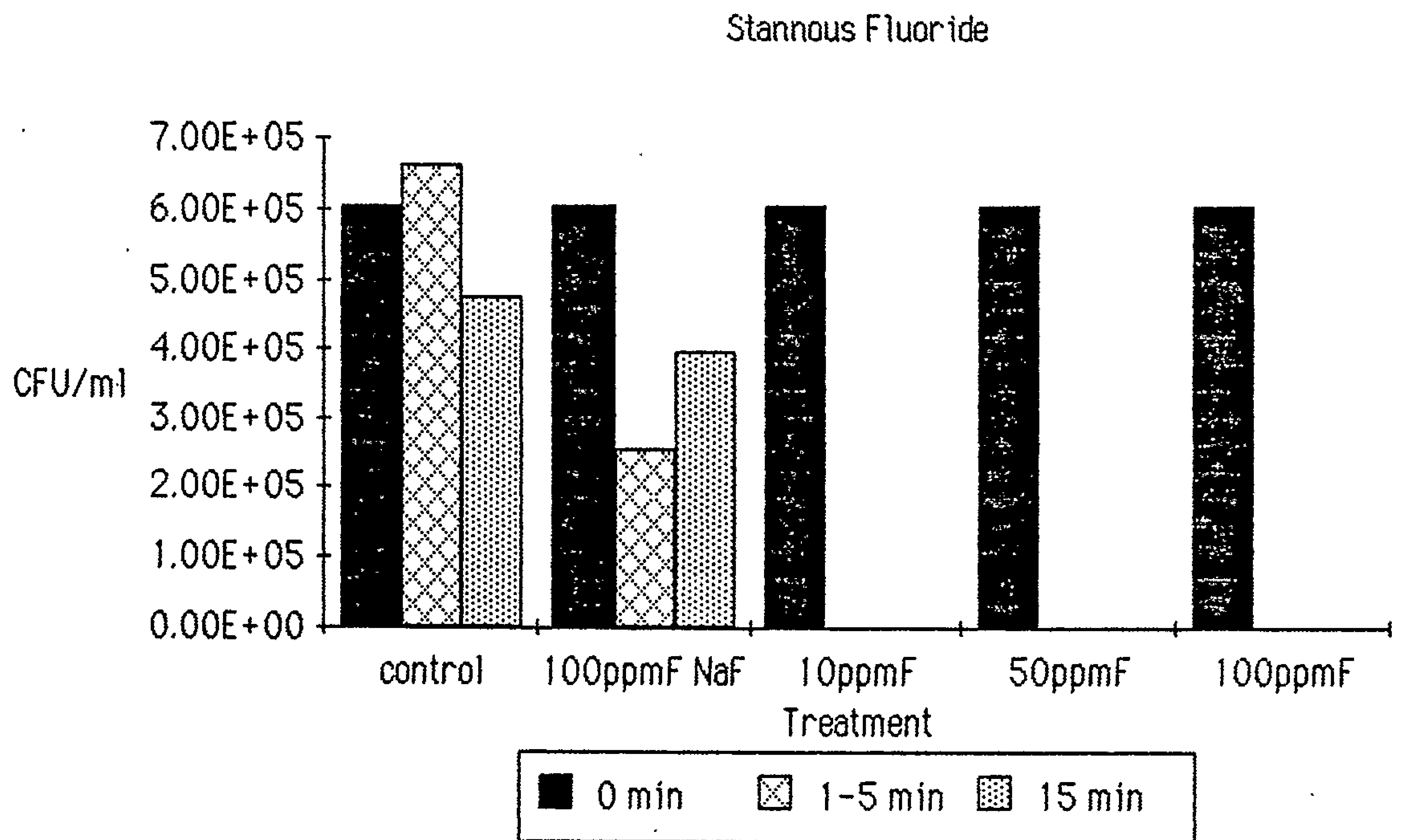

Figure 3.12 Procedure 2. B.intermedius NCTC 9336 treated with stannous fluoride.

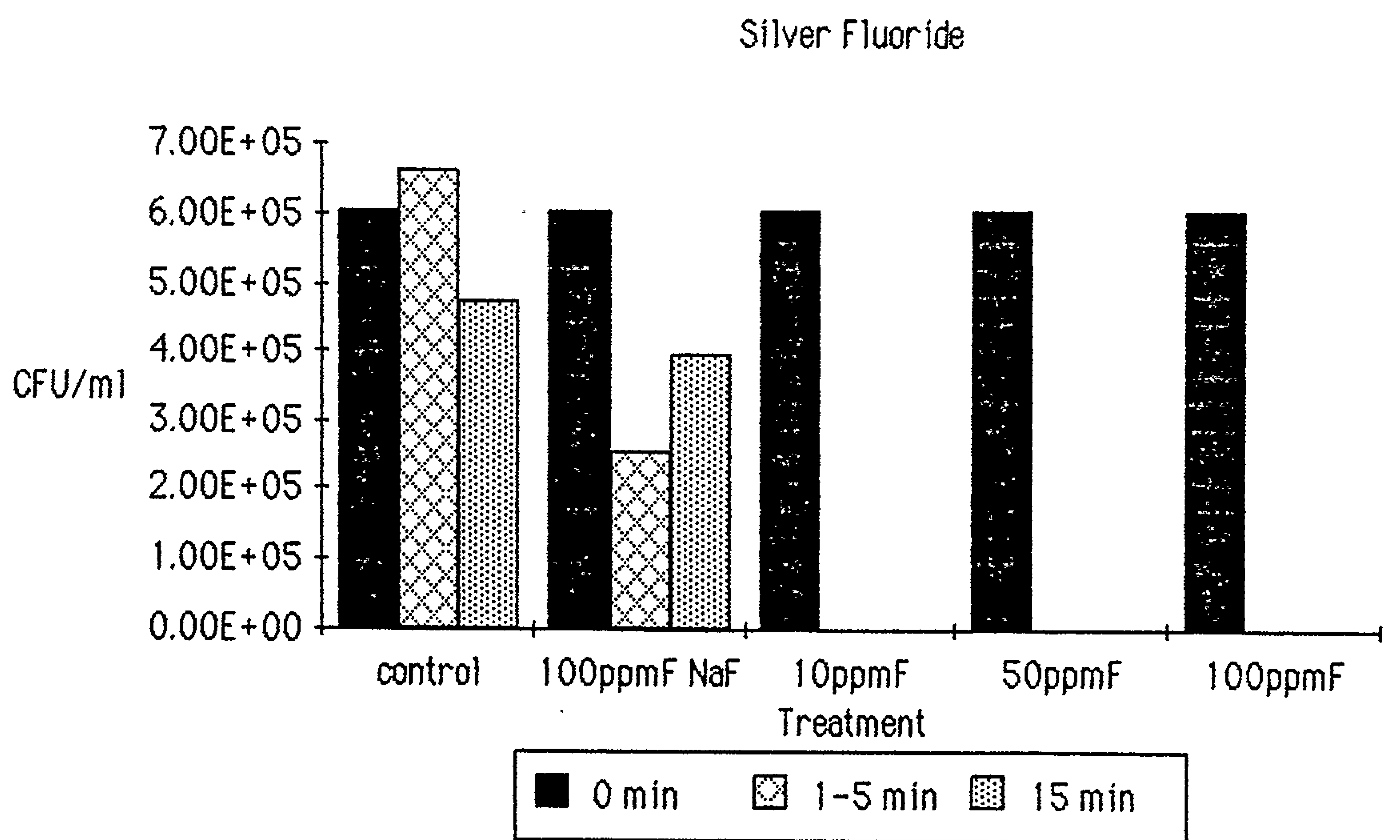

Figure 3.13. Procedure 2. B.intermedius NCTC 9336 treated with silver fluoride.

After 15 minutes the was no difference between the AgF or SnF_2 treatments. Both treatments were significantly different to the control and 100ppmF⁻ NaF ($p \leq 0.01$).

<u>A.viscosus</u> T14

Both the control treatment and 100ppmF⁻ NaF had a similar effect on the number of CFU counted. The two way ANOVA confirmed a difference within and between treatments and times ($p=0.0001$, see Table 3.4, Figures 3.10 and 3.11).

At 1-5 minutes there was no CFU recorded for the AgF treatments and 100ppmF⁻ SnF_2 treatment. This would imply 10ppmF⁻ AgF provided the most effective means of inhibiting survival of the microorganism. Multiple comparison tests indicated no significant differences between the AgF and SnF_2 treatments at the 95% level.

After 15 minutes both SnF_2 and AgF treatments produced no growth of <u>A.viscosus</u> T14. Hence, after 15 minutes 10ppmF⁻ AgF or 10ppmF⁻ SnF_2 are equally effective at inhibiting growth.

<u>Bacteroides intermedius</u> NCTC 9336

A decline in the number of microorganisms was produced with time in the control treatment. Sodium fluoride 100ppmF⁻ produced a similar decline. Both AgF and SnF_2 treatments produced no growth at 1-5 or 15 minutes. The two factor ANOVA confirmed the within and between differences of treatments and times ($p \leq 0.0001$, see Table 3.5, Figures 3.12 and 3.13).

At both the 1-5 and 15 minute intervals AgF and SnF_2 were equally effective at producing no growth with a fluoride

concentration of 10ppm. The 100ppmF^- NaF produced a significant decline in numbers at the 1-5 minutes, while at 15 minutes the was no statistical difference to the control. The effects of the fluoride appears more profound on B.intermedius NCTC 9336 than the other microorganisms. The aerobic conditions under which the procedure was performed are not ideal for this microorganism.

A three factor ANOVA was calculated for procedure 2. A statistically significant difference occurred within and between the groups, treatments, time and microorganisms ($p \leq 0.0001$).

3.3 Procedure 3.

S.sanguis G9B

Over the time interval of 15 minutes the microorganism grew in the 6% albumin solution. Growth was not completely inhibited by any of the treatments over the fifteen minutes. Both 100ppmF^- SnF_2 and 100ppmF^- AgF treatments produced a reduction in the number of CFU counted. NaF 100ppmF^- had a similar effect to the control treatment. There was a significant difference within and between result for treatments and times ($p \leq 0.0001$, see Table 3.6, Figure 3.14).

At 1-5 minutes there was no difference between the control and the 100ppmF^- NaF treatment. The 100ppmF^- SnF_2 treatment was significantly different to all the other treatments and had the most profound effect on the microorganism. The 100ppmF^- AgF treatment was significantly different to the 100ppmF^- SnF_2 ($p \leq 0.05$).

TREATMENT	TIME		
	0.minutes	1-5 minute	15 minute
Control	2880000	3225000	3710000
SD	56569	91924	98995
Number	2	2	2
100ppmF⁻ NaF	2880000	3105000	3405000
SD	56569	77782	219203
No.	2	2	2
100ppmF⁻ SnF_2	2880000	85500	1450
SD	56569	4950	71
No.	2	2	2
100ppmF⁻ AgF	2880000	2715000	24000
SD	56569	49497	0
No.	2	2	2

Table. 3.6. Procedure 3. CFU/ml of the microorganism S.sanguis G9B in 6% serum albumin compared to different treatments with time. The two factor ANOVA: Time (A) F=329.251, P≤0.0001. Treatment (B) F=967.707, P≤0.0001. AB F=392.197, P≤0.0001.

TREATMENT	TIME		
	0.minutes	1-5 minute	15 minute
Control	360000	925000	1070000
SD	84852	120208	113137
Number	2	2	2
100ppmF⁻ NaF	360000	800000	960000
SD	84852	56569	98995
No.	2	2	2
100ppmF⁻ SnF_2	360000	390000	303500
SD	84852	56569	23335
No.	2	2	2
100ppmF⁻ AgF	360000	59000	0
SD	84852	1414	0
No.	2	2	2

Table. 3.7. Procedure 3. CFU/ml of the microorganism A.viscosus T14 in 6% serum albumin compared to different treatments with time. The two factor ANOVA: Time (A) F=18.717, P≤0.0002. Treatment (B) F=90.944, P≤0.0001. AB F=24.144, P≤0.0001.

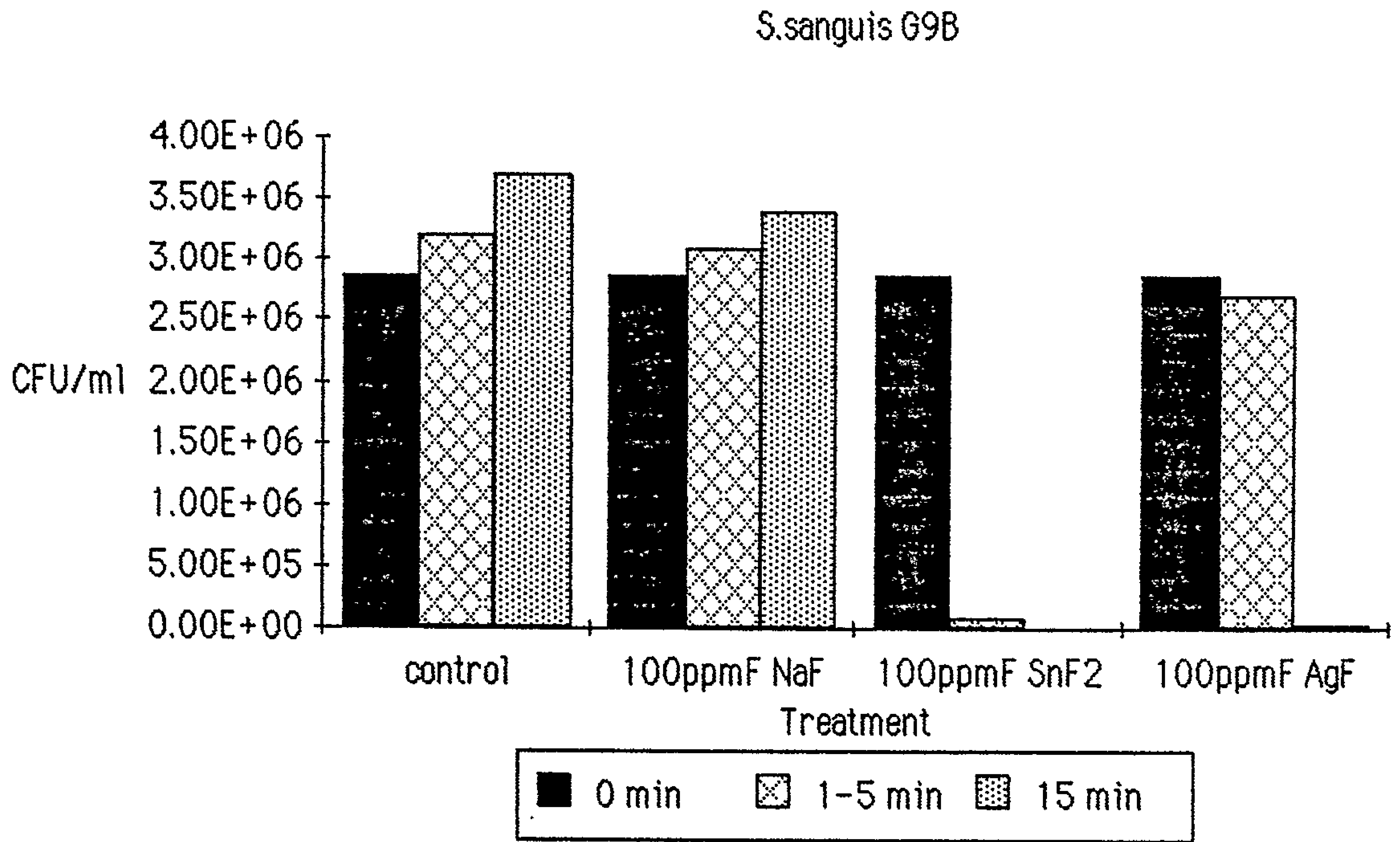

Figure 3.14. Procedure 3. S.sanguis G9B treated with 100ppmF in 6% bovine serum albumin.

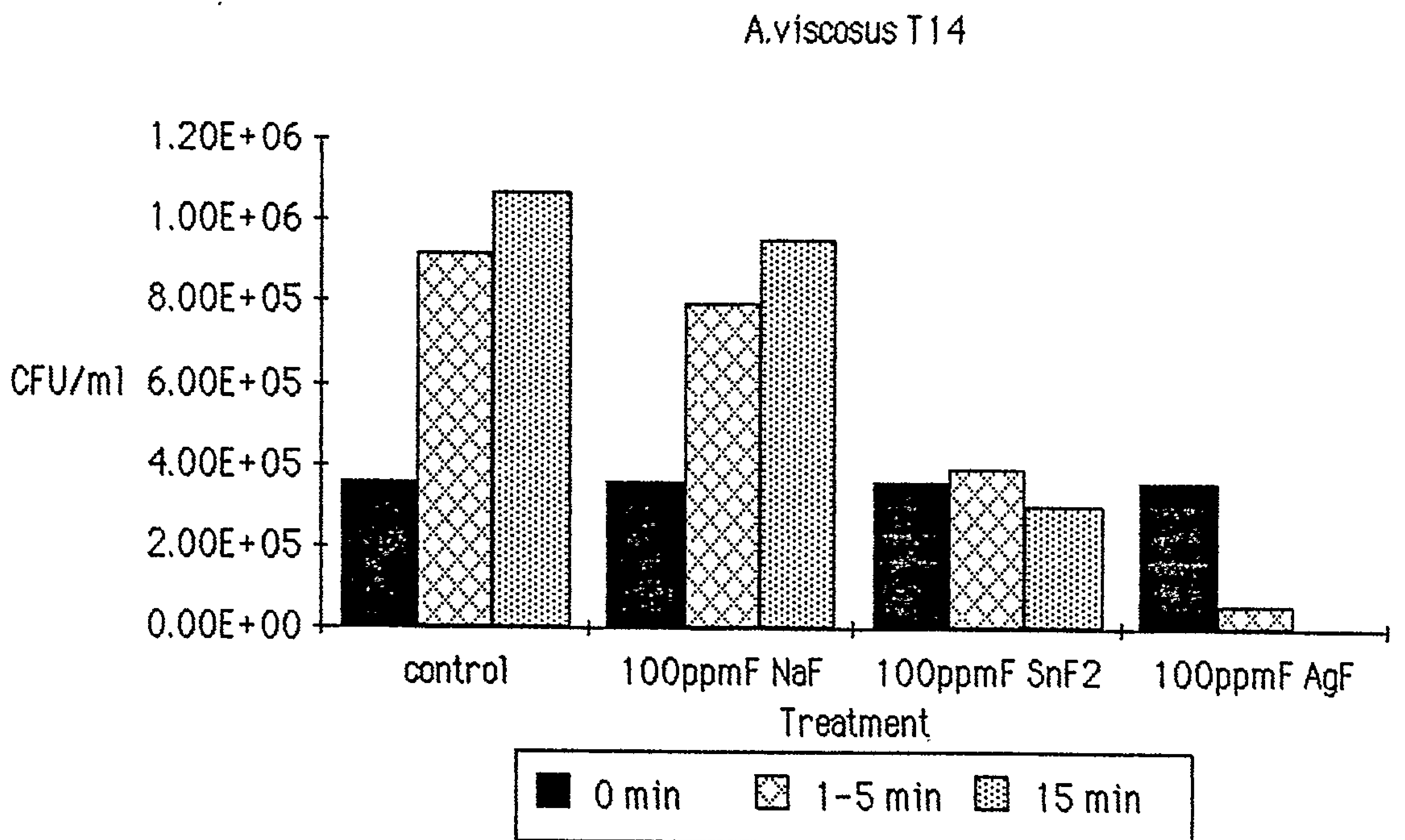

Figure 3.15. Procedure 3. A.viscosus T14 treated with 100ppmF in 6% bovine serum albumin.

After the 15 minutes all treatments did not totally inhibit growth. In fact 100ppmF$^-$ NaF had allowed growth to occur. There was no statistically significant difference between the 100ppmF$^-$ SnF_2 and 100ppmF$^-$ AgF treatments. Both these treatments produced the most significant reduction in the number of CFU.

<u>A.viscosus</u> T14

Both the control and 100ppmF$^-$ NaF allowed growth of the microorganism during the test period. The 100ppmF$^-$ SnF_2 treatment produced an equilibrium between growth and killing over the fifteen minutes. The number of CFU counted at 0 minutes for the control was similar to the number counted for SnF_2 100ppmF$^-$ at the 1-5 and 15 minutes. AgF 100ppmF$^-$ was the only treatment to decrease the number of CFU over the time period. The two factor ANOVA indicated a significant difference within times ($p \le 0.0002$), within treatments ($p \le 0.0001$) and between treatments and times ($p \le 0.0001$, see Table 3.7, Figure 3.15).

At the 1-5 minute interval there was no difference between the control and NaF 100ppmF$^-$ treatment. SnF_2 100ppmF$^-$ treatment was more effective compared to the control treatment. The 100ppmF$^-$ AgF treatment was different to all other treatments (significant at the $p \le 0.01$ level).

After 15 minutes similar comparisons were present as after 1-5 minutes. AgF 100ppmF$^-$ treatment was the most effective compound with no CFU counted

B.intermedius NCTC 9336

The number of CFU decrease over the time interval for control treatment. Both NaF 100ppmF$^-$ and SnF_2 100ppmF$^-$ treatments produced a similar decline in numbers grown. AgF 100ppmF$^-$ allowed no growth of microorganisms over the time period. The two factor ANOVA indicated a significant difference within times ($p \leq 0.0001$) and within treatments ($p \leq 0.0033$). The difference between times and treatments was not highly significant ($p \leq 0.0432$, see Table 3.8, Figure 3.16).

There was no significant difference between control, NaF 100ppmF$^-$ and SnF_2 100ppmF$^-$ treatments at the 1-5 minute interval. The AgF 100ppmF$^-$ treatment was significantly different from all the treatments ($p \leq 0.01$).

After 15 minutes SnF_2 100ppmF$^-$ and NaF 100ppmF$^-$ treatments caused a decrease in the CFU compared to the control ($p \leq 0.01$). AgF 100ppmF$^-$ treatment allowed no growth of the microorganism, although this was not statistically significant from the reduction caused by the SnF_2 100ppmF$^-$ treatment.

A comparison between the microorganisms, times and treatments in Procedure 3. using a three factor ANOVA indicated a highly significant difference between and within the three groups tested ($p \leq 0.0001$).

TREATMENT	TIME		
	0.minutes	1-5 minute	15 minute
Control	7825000	2810000	2605000
SD	954594	339411	601040
Number	2	2	2
100ppmF$^-$ NaF	7825000	2190000	980000
SD	954594	268701	155563
No.	2	2	2
100ppmF$^-$ SnF_2	7825000	2715000	600000
SD	954594	827315	240416
No.	2	2	2
100ppmF$^-$ AgF	7825000	0	0
SD	954594	0	0
No.	2	2	2

Table. 3.8. Procedure 3. CFU/ml of the microorganism B.intermedius NCTC 9336 in 6% serum albumin compared to different treatments with time. The two factor ANOVA: Time (A) F=262.885, P≤0.0001. Treatment (B) F=8.042, P≤0.0033. AB F=3.165, P≤0.0423.

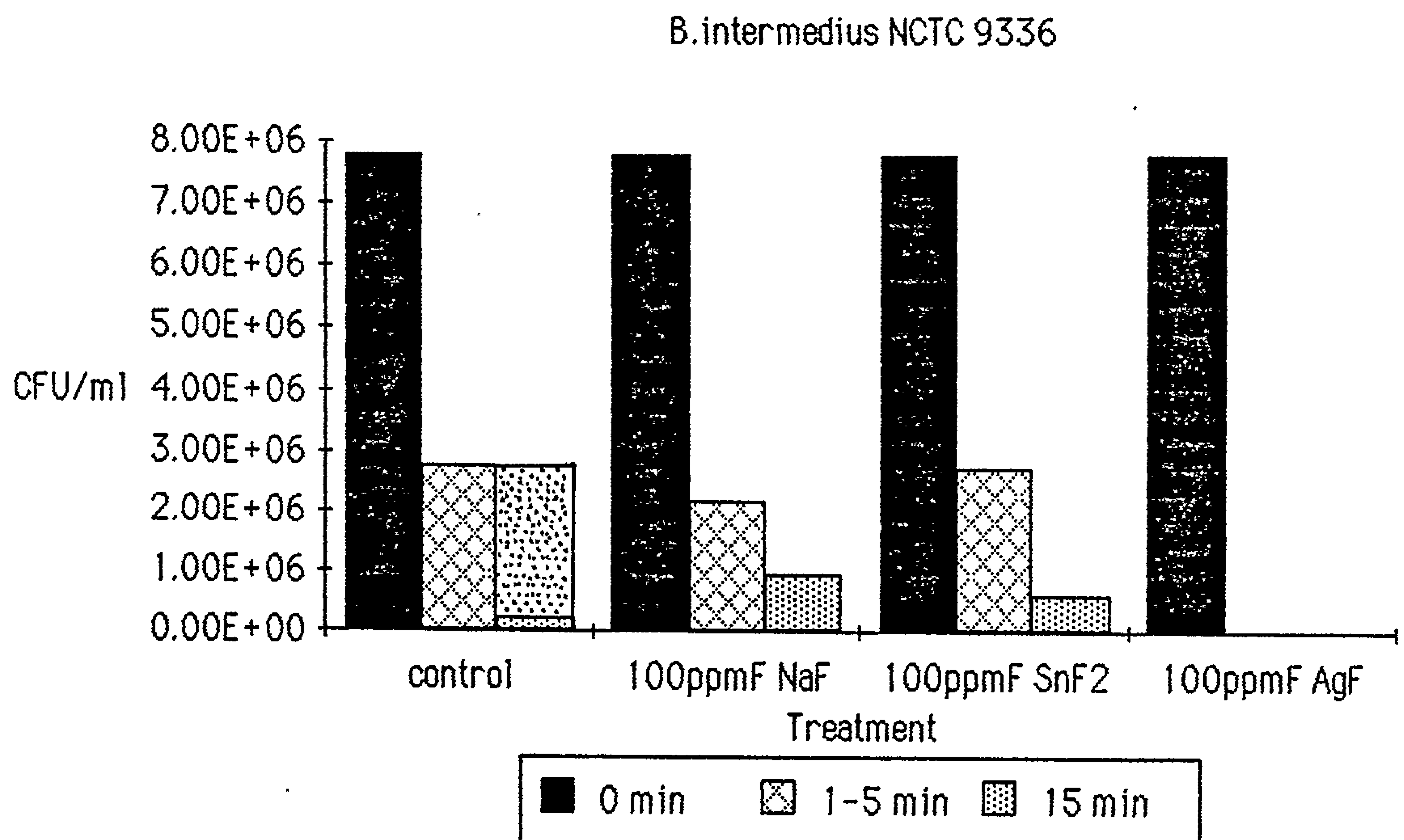

Figure 3.16. Procedure 3. B.intermedius NCTC 9336 treated with 100ppmF in 6% bovine serum albumin.

3.4 Summary.

The results from Procedure 1 and 2 indicate that AgF and SnF_2 compounds at a low concentration of 10ppmF^- are effective in inhibiting growth of the three microorganisms. This effect being obtained at the 1-5 minute period. Decreasing the pH of NaF compounds markedly improves the effectiveness of this compound. However, it is apparent that acidic solutions (control pH3.5) are also effective at retarding growth in this experimental system. There is a trend to indicate that the susceptibility of microorganisms to fluorides differs between the microorganisms tested, S.sanguis G9B being the least susceptible and B.intermedius NCTC 9336 being the most susceptible. The effectiveness of the fluoride compound does not rely entirely on the fluoride concentration but also the anion associated with the fluoride.

Procedure 3 demonstrates that the pH of the solution does affect the results. A higher fluoride concentration of 100ppmF^- was required to produce similar results as in Procedure 1 and 2. Stannous fluoride shows less effect on A.viscosus T14 and B.intermedius NCTC 9336 compared to silver fluoride.at the 1-5 minute interval. For S.sanguis G9B, SnF_2 would appear to be more effective than AgF in the early time period. Silver fluoride is the most effective agent used to inhibit growth in the three procedures.

Discussion.

4.1 Comparison of fluorides.

Results from the present study indicate the effectiveness of fluorides against oral microorganisms. This is in agreement with previous in vitro studies (Yoon and Berry 1979, Yoon and Newman 1980). The effectiveness of different fluoride compounds is also shown in the present study.

Sodium fluoride at pH 5.3 and pH 6.5 had minimal effect at the concentrations used. Mandel (1983) found sodium fluoride to be effective at a minimum of 128ppmF (parts per million fluoride) over 24 hours exposure in a broth solution. By altering the pH of the test solution to pH 3.5 (see Table 3.1) there was a significant improvement in its effect on Streptococcus sanguis G9B. Other authors (Yoon and Berry 1979, Yoon and Newman 1980) have noted that acidulated phosphate fluoride (APF) is more effective than sodium fluoride. In the present experiment an acidic solution of 3.5 pH had more effect than the control, indicating an acidic solution can also affect the growth of microorganisms. This effect is not as profound as with fluoride ions present.

Stannous fluoride in procedures 1 and 2 is more effective than sodium fluoride in inhibiting growth (Tables 3.1, 3.2, 3.3 and 3.4). This effect has been shown in vitro (Yoon and Berry 1979, Yoon and Newman 1980, Mawhew and Brown 1980) and clinically by Andres et al. (1974). In procedure 3, stannous fluoride is more

effective than sodium fluoride at inhibiting growth of S.sanguis G9B and as effective on the other microorganisms (Tables 3.6, 3.7 and 3.8).

Silver fluoride is effective at inhibiting the growth of the microorganisms in all three procedures. The results confirm the work by Oppermann and Johansen (1980) that silver fluoride is more effective than stannous fluoride.

4.2 Role of pH.

The present study confirms that stannous fluoride in solution has an inherent low pH (Tinanoff 1985). However, in procedure 1 this low pH does not account entirely for the inhibition of growth. Sodium fluoride adjusted to pH 3.5 affects growth to a lesser degree than stannous fluoride at a similar fluoride concentration. In procedure 3, using serum albumin as the vehicle, stannous fluoride 100ppmF is buffered to pH 5.7. This alters stannous fluoride's effectiveness in inhibiting growth. This is similar to the findings of Mayhew and Brown (1980). From the present study it is not possible to confirm if the tin or the fluoride component accounted for the compound's effectiveness. It appears, as suggested by Yoon et al. (1980), that it is the tin and fluoride combination that contributes to the effect.

Silver fluoride has the advantage that it does not appear to depend on pH for its effectiveness. However, Waler and Rölla (1982) found that as a mouthrinse, silver fluoride may not be as effective as chlorhexidine due to the formation of insoluble silver chloride upon contact with saliva.

4.3 Effective fluoride concentration.

In Procedure 1 and 2 the minimum effective fluoride concentrations that killed the microorganisms were significantly less than other reports. The main reason is that the test solution (deionized filtered water) used did not allow maintenance or growth of the microorganisms. However, the solution did allow an equal comparison between the three fluoride solutions. Most in vitro studies have utilized broth solutions which will affect the amount of free ions available and the inherent pH of the compound (especially with SnF_2).

The washing of the microorganisms prior to inoculation in the test solution ensured no carry over of the broth solution. This would affect the free ion concentration available. This problem has been previously noted by Newman et al. (1979). To date no other study has used this precaution, as all in vitro studies have used broth solutions. Caufield and Wannemuehler (1983) approached the problem by growing the microorganisms on filter membranes. After 5 minutes contact with the fluoride solution the filter membranes were transferred to a fresh broth solution. They used a growth inhibition zone of ¼ inch to indicate susceptibility.

Most in vitro studies have used a prolonged contact time between the fluoride solution and the microorganism (24-48 hours). Caufield and Wannemuehler (1983) used an exposure time of 5 minutes. Clinical studies have utilized contact times of 1-3 minutes. Hence, the use of 1-5 and 15 minutes intervals of exposure to the fluoride in this study would seem more appropriate for extrapolation into a clinical situation.

In Procedure 2 and 3, after exposure in the fluoride solutions, the microorganisms were diluted through broth solutions. This was to ensure minimal carry over of fluoride onto the agar plates and hence, giving a definite cut off in exposure time.

In Procedure 3, 6% serum albumin was utilized as the test solution. This solution allowed growth and maintenance of S.sanguis G9B and Actinomyces viscosus T14. Bacteroides intermedius NCTC 9336 was not maintained in this solution, this was due to the aerobic conditions used to perform the experiment. Anaerobic conditions were not utilized as rapid serial dilution and plating out were required to maintain consistency of the exposure times used in this experiment. This procedure would have been hampered under anaerobic conditions. To obtain similar inhibition of microbial growth in the albumin solution a higher fluoride concentration was required (100ppmF). This concentration was similar to the results of studies using broth solutions. Silver fluoride was the most effective agent against A.viscosus T14 and B.intermedius NCTC 9336. Stannous fluoride was more effective against S.sanguis G9B at 1-5 minutes.

4.4 Susceptibility of microorganisms.

The difference in the susceptibility of the microorganisms to fluoride demonstrated here is the same as shown in other studies (Newman et al. 1979, Yoon and Berry 1979. Yoon and Newman 1980, Mandell 1983, Iida et al. 1986), Streptococcus sp. being the least susceptible, the Bacteroides sp. being the most susceptible and Actinomyces sp. in between. This may be due to the way the fluoride affects the enzyme systems of the different

microorganisms. The Streptococcus sp. being more robust microorganisms are better able to survive adverse conditions. The Bacteroides sp. needs specific growth requirements for survival, such that minor alterations in their environmental conditions will adversely affect their survival.

The greater effect of silver fluoride on A.viscosus G9B and B.intermedius NCTC 9336 may be used to affect the sequential nature of dental plaque development (Moore et al. 1984) and coaggregation between microorganisms (Cisar 1982).

4.5 Study design.

The significance of the study could be improved by repeating the procedures a number of times. The present results suffer from the lack of numbers, although the results gained are consistent in the three procedures. The study would also benefit if a positive control, such as chlorhexidine was used for comparison. The design and exposure times used in this study allow a more effective comparison of fluoride solutions and extrapolation to clinical situations than in vitro studies using a long exposure time.

Performing the experiment under anaerobic conditions may improve recoverable numbers of microorganisms and enhance the microorganisms' ability to recover from fluoride exposure. This would be especially true for B.intermedius NCTC 9336. However, the problem of performing the experimental tasks in a short time period necessitated the use of aerobic conditions.

4.6 Further studies.

Further studies of the clinical use of fluorides in periodontitis are warranted. These should be designed to compare the effectiveness of fluorides to other commonly used agents.

The combination of serum albumin and silver nitrate is known to reduce the caustic nature of the latter. In addition the effects of the silver may be slowly released from the protein solution and have an enhanced therapeutic effect (Russell et al. 1981). The potential of this property with silver fluoride should be investigated.

4.7 Summary.

The effectiveness of fluorides in inhibiting growth of oral microorganisms has been established in this and other studies. Their use in the clinical treatment of periodontal disease warrants further research.

Conclusion.

The results of the present study may be summarized.

-Fluorides are effective antimicrobial agents against oral microorganisms. Stannous fluoride and silver fluoride are the most effective agents and sodium fluoride the least effective.

-These agents are effective after a short exposure time, after 1-5 and 15 minutes.

-The effectiveness of fluoride solutions are determined by the pH of the solution, especially with stannous fluoride and sodium fluoride, and the availability of free ions.

-Different oral microorganisms are affected by different fluoride concentrations and different fluoride compounds.

-Silver fluoride is consistently the most effective agent tested.

-Further research is warranted for the clinical use of fluoride as an adjunctive aid in the treatment of periodontal disease.

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Appendix.

Procedure 1.

Streptococcus sanguis G9B.

2 factor Analysis of Variance. CFU/ml vs Time vs Treatment.

Source	df	Sum of Squares	Mean Squares	F-test	P value
Time(A)	2	3027934090.36	1513967045.18	467.457	.0001
Treatment(B)	13	1.611E9	123943472.431	38.269	.0001
AB	26	1.15E9	44217086.097	13.653	.0001

Actinomyces viscosus T14.

2 factor Analysis of Variance. CFU/ml vs Time vs Treatment.

Source	df	Sum of Squares	Mean Squares	F-test	P value
Time(A)	2	3.503E9	1.752E9	220.203	.0001
Treatment(B)	9	3.525E9	391655228.627	49.237	.0001
AB	18	1.105E9	61392093.904	7.718	.0001

Procedure 2.

3 factor Analysis of Variance. CFU/ml vs Time vs Treatment vs Microorganisms.

Source	df	Sum of Squares	Mean Squares	F-test	P value
Time(A)	2	1.122E14	5.610E13	508.653	.0001
Treatment(B)	7	4.709E13	6.72E12	60.986	.0001
AB	14	2.526E13	1.804E12	16.357	.0001
Organism(C)	2	4.697E13	2.349E13	212.925	.0001
AC	4	3.235E13	8.087E12	73.314	.0001
BC	14	1.211E13	8.653E11	7.845	.0001
ABC	28	9.441E12	3.372E11	3.057	.0001

S.sanguis G9B

2 factor Analysis of Variance. CFU/ml vs Time vs Treatment.

Source	df	Sum of Squares	Mean Squares	F-test	P value
Time(A)	2	5.066E13	2.533E13	178.35	.0001
Treatment(B)	7	3.815E13	5.450E12	38.372	.0001
AB	14	2.074E13	1.481E12	10.43	.0001

A.viscosus T14.

2 factor Analysis of Variance. CFU/ml vs Time vs Treatment.

Source	df	Sum of Squares	Mean Squares	F-test	P value
Time(A)	2	9.898E13	4.949E13	331.589	.0001
Treatment(B)	7	3.329E13	4.756E12	31.869	.0001
AB	14	2.047E13	1.462E12	9.798	.0001

Bacteroides intermedius NCTC 9336.

2 factor Analysis of Variance. CFU/ml vs Time vs Treatment.

Source	df	Sum of Squares	Mean Squares	F-test	P value
Time(A)	2	2.588E12	1.294E12	301.759	.0001
Treatment(B)	7	8.868E11	1.267E11	29.548	.0001
AB	14	4.988E11	3.563E10	8.31	.0001

Procedure 3.

3 factor Analysis of Variance. CFU/ml vs Time vs Treatment vs Microorganisms.

Source	df	Sum of Squares	Mean Squares	F-test	P value
Time(A)	2	8.901E13	4.451E13	312.906	.0001
Treatment(B)	3	2.095E13	6.984E12	49.101	.0001
AB	6	1.259E13	2.099E12	14.754	.0001
Organism(C)	2	1.168E14	5.840E13	410.568	.0001
AC	4	1.333E14	3.334E13	234.371	.0001
BC	6	1.187E13	1.979E12	13.913	.0001
ABC	12	1.332E13	1.110E12	7.804	.0001

S.sanguis G9B.

2 factor Analysis of Variance. CFU/ml vs Time vs Treatment.

Source	df	Sum of Squares	Mean Squares	F-test	P value
Time(A)	2	4.808E12	2.404E12	329.251	.0001
Treatment(B)	3	2.120E13	7.066E12	967.707	.0001
AB	6	1.718E13	2.864E12	392.197	.0001

A.viscosus T14.

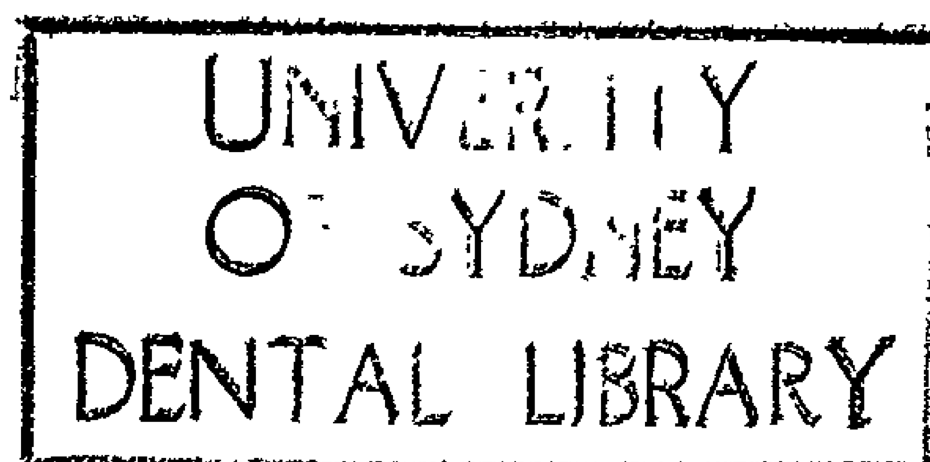
2 factor Analysis of Variance. CFU/ml vs Time vs Treatment.

Source	df	Sum of Squares	Mean Squares	F-test	P value
Time(A)	2	2.271E11	1.135E11	18.717	.0002
Treatment(B)	3	1.655E12	5.517E11	90.944	.0001
AB	6	8.788E11	1.465E11	24.144	.0001

B.intermedius NCTC 9336.

2 factor Analysis of Variance. CFU/ml vs Time vs Treatment.

Source	df	Sum of Squares	Mean Squares	F-test	P value
Time(A)	2	2.173E14	1.087E14	262.885	.0001
Treatment(B)	3	9.972E12	3.324E12	8.042	.0033
AB	6	7.850E12	1.308E12	3.165	.0423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