FACTORS INFLUENCING THE EXTRACTION OF PROTEIN FROM ORAL BACTERIA AND DENTAL PLAQUE

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ABSTRACT

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In the past, attempts have been made to separate dental plaque into its bacterial and matrix components. The main uncertainty with these previous studies is the fact that the solvents used to extract the matrix may also have extracted some constituents from the bacterial cells and cell walls. An attempt has therefore been made to find the optimal conditions for separation of dental plaque into its bacterial and matrix components such that the bacteria would lose a minimum of intracellular or cell wall constituents into the extracting solvent.

Some of the factors influencing the extraction of protein from pure cultures of the predominant microorganisms found in dental plaque have been investigated and the findings have been related to the extraction of protein from dental plaque which was collected from subjects who had fasted for 10 hours. The following factors were investigated:

1) pH - 1.3, 4.0, 5.0, 7.0, 8.0, 9.0, 10.6, 12.7.
2) Temperature 0 - 23 - 37 - 60°C.
3) Presence of 2 mM calcium.
   Presence of 1 mM magnesium.

4) Osmotic shock

5) Ionic strengths of 0.05, 0.16 and 0.64.

6) Time of exposure.

7) Growth phase: - log, stationary, death.

8) Concentration of bacteria.

Various combinations of the above factors were used in each experiment.

Pure cultures of log phase streptococci, AHT, BHT, CHT, HHT, a Corynebacterium sp., a Veillonella sp., a Diphtheroid sp., and L. casei were harvested by centrifugation and washed twice with 0.4% saline to remove the growth medium. The cells were resuspended in 0.4% saline and 50 µl aliquots were added to tubes containing 0.95 ml of 0.05 ionic strength buffers over the pH range of 1.3 to 12.7. A time zero aliquot was taken and after 30 minutes the cells were centrifuged and supernatants collected. A protein analysis using the Lowry method, was done on the supernatant and time zero aliquots and the percent protein extracted was calculated.

The experiments were designed as complete randomized blocks with the treatments replicated on different days.

Preliminary experiments showed that logarithmic and stationary phase cells had similar amounts of protein extracted from them while death phase cells had significantly less protein

extracted from them. Hence, logarithmic phase cells were used throughout these experiments so that the maximal amount of protein would be extracted. The percent protein extracted was found to be independent of bacterial concentration over the range of 0 - 4 mg of total bacterial protein per ml.

Variations in ionic strength over the range tested had no significant effect on the percent protein extracted from the bacteria.

Osmotic shock caused a significant increase in the percent protein extracted from Veillonella sp. and BHT.

The mean percent protein extracted at each temperature was obtained from the eight microorganisms tested at eight pH levels with a total of 31 replications. The mean percent protein extracted at the four temperatures increased with an increase in temperature from $0^{o}C$.

The mean percent protein extracted from the eight microorganisms at the eight pH levels over the four temperatures showed that the minimum percent protein was extracted at pH 4.0 and 5.0 and that significantly more protein was extracted at pH 12.7 than at any other pH.

The effect of pH may perhaps be explained as follows. At pH 5.0 the charge on the permeability mechanism may be zero and therefore result in minimal leakage due to close proximity of the membrane components. As the pH is raised from 5.0 to 12.7 the permeability mechanism may become negatively charged and, due to repulsion of the membrane components, cause an increased leakage. The same would apply as the pH is lowered from 5.0 to 1.3, an increase

in positively charged ions would result in membrane repulsion and thereby cause an increase in leakage.

The microorganisms exhibited statistically significant variations in the amount of protein extracted at each of the eight pH levels tested. The largest variation in the mean percent protein extracted from the microorganisms occurred at pH 12.7

Calcium caused a significant reduction in the percent protein extracted from four of the eight microorganisms at pH 9.0, 10.6 and 12.7. No significant reduction in the percent protein extracted occurred at the lower pH levels. Magnesium also caused a significant reduction in the percent protein extracted from four of the eight microorganisms at pH 9.0, 10.6 and 12.7. No significant reduction in the percent protein extracted occurred at the lower pH levels. Divalent cations such as calcium or magnesium would favor a decrease in membrane repulsion at high pH by neutralizing the effective negative charge on the cell surface. These divalent ions could also bridge adjacent negatively charged groups on the cell surface, thereby facilitating decreased leakage.

At pH 4.0, there was no significant difference in the percent protein extracted from dental plaque and from a combined sample of microorganisms but at the other seven pH levels, significantly more protein was extracted from the dental plaque. Although the ratio of protein extracted from dental plaque to that extracted from the combined microorganisms was relatively

constant at approximately 4 : 1 over the pH range 8.0 - 12.7, the most protein was extracted at pH 12.7 and hence this was considered the optimum pH.

The increased extraction from dental plaque cannot be attributed to the fact that some of the cells of plaque might have been in the stationary phase of growth because, with the individual microorganisms, similar amounts of protein were extracted from log phase cells and from stationary phase cells. In addition, significantly less protein leaked from death phase cells than from log phase cells.

The increase in the percent protein extracted from dental plaque has been attributed to the matrix proteins. Since, at pH 12.7, approximately 77% of the protein extracted from dental plaque originated from the matrix and since, on average 37 ± 8% (S.D.) of the plaque protein was extracted, the matrix components must account from about 25 ± 10% (S.D.) of the total plaque protein.

In summary then, an attempt has been made to find the optimal conditions for separation of dental plaque into its bacterial and matrix components such that the bacteria would lose a minimum of intracellular or cell wall constituents into the extracting solvent.

The optimum conditions for extraction of the matrix from dental plaque involve the use of 0.05 N NaOH at $0^{o}C$ for about 1 hour. However, 20% of the protein obtained is probably extracted from the bacteria themselves. The matrix component of dental plaque appears to comprise about 25% of the total plaque protein.

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CHAPTER I

PURPOSE OF THE STUDY

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Dental plaque is a soft, concentrated mass consisting mainly of a large variety of microorganisms embedded in an intercellular matrix. It collects on the teeth in the absence of oral hygiene procedures and is associated with the two main dental disease processes: caries and periodontal disease. The formation of plaque is not fully understood but it is generally believed that the matrix is derived from salivary and/or bacterial constituents. The relative contribution of the bacterial and matrix components of plaque to the two dental disease processes is not understood. In the past, attempts have been made to separate the two components but the main uncertainty with these studies is the possibility that the solvents used to extract the matrix constituents have, in addition, extracted some constituents from the bacteria or their cell walls.

The purposes of the present investigation were to determine the proportion of protein matrix in dental plaque and to find the optimal conditions for separation of the plaque into its bacterial and matrix components.

Experiments were carried out initially to determine the effects of such factors as pH, temperature, cations, various solvents,

concentration of cells, and ionic strength on protein leakage from pure cultures of the predominant microorganisms in dental plaque. With this preliminary information it was hoped to obtain the optimum conditions for extraction of plaque that would have a minimal effect on the bacteria but would give adequate separation of the microorganisms from the matrix.

CHAPTER II

LITERATURE REVIEW

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LITERATURE REVIEW

The Dental Plaque

Dental plaque has been defined as the soft, concentrated mass, consisting mainly of a large variety of microorganisms embedded in an intercellular matrix, which attaches to the teeth surfaces when oral hygiene procedures are not carried out (Dawes et al, 1963). It is associated with the two main dental disease processes of caries and periodontal disease.

Miller (1890) proposed the chemico-parasitic theory to explain the etiology of dental caries. He proposed that carbohydrate foodstuffs are transformed by the oral bacteria to organic acids which may cause decalcification of the enamel. Kleinberg (1958) has expanded the chemico-parasitic theory to include both caries and periodontal disease. This combined chemico-parasitic theory, which is based on the metabolism of the organisms, divides the bacteria found in dental plaque into two groups: acid producers and base producers. The acid-producers break down carbohydrate to form acid, which increases the solubility of hydroxyapatite and results in caries. The base-producers break down nitrogenous substrates to produce base which causes a pH rise and a concomitant precipitation of calcium phosphate salts as calculus. The calculus may then irritate

the tissues and lead to periodontal disease.

Dental plaque has been known to contain bacteria since 1683 when Leeuwenhoek described the presence of "animalcules" in teeth scrapings and in saliva. It was not until 1897, however, that Williams demonstrated, by histological means, that a felt-like mass of acid-forming microorganisms was always seen lining the surface where decay had commenced.

Histological evidence for the existence of a matrix in dental plaque has been produced by McDougall (1963a) and Frank and Brendel (1966). Baer and Newton (1958) have shown that calculus may form in germ-free animals and this is evidence that a matrix may be deposited in the absence of bacteria.

Proportions and Proportional Changes with Time of the Various Types of Microorganisms Found in Dental Plaque

Microbial ecology is a study of the interactions of microorganisms with one another and with their environment. The environment has a selective action since it is responsible for the supply of nutrients. The nutrients man ingests are complex in nature and therefore favor the support of mixed microbial populations. In fact, the oral cavity supports one of the most dense and varied microflora indigenous to man (Rosebury et al, 1954).

The qualitative and quantitative relationships between oral microorganisms must be considered in a study of the dental plaque. The oral cavity has a temperature of 35 - 37°C (Jenkins, 1966) and a continual supply of intrinsic nutrients consisting mainly of salivary components, gingival crevice fluid and epithelial and white blood cells undergoing degeneration. Food is an additional intermittent source of extrinsic nutrients. Nutrients are necessary for synthesis of cell protoplasm, and for cell multiplication of the oral microbiota.

The environment controls the types of microorganisms that exist in a particular area of the oral cavity. Studies on the cultivable microbiota of the gingival crevice (Gibbons et al, 1963) dental plaque (Gibbons et al, 1964, Ritz, 1967) developing and mature dental calculus (Howell et al, 1965) and the human tongue (Krasse, 1953, Gordon and Gibbons, 1966) have shown how variable are the porportions of the different microorganisms in each ecological area.

Gibbons et al, (1964) reported a study of the predominant cultivable microbiota of human dental plaque. Five samples of dental plaque (location and age of plaque not stated) were collected from individuals (number not stated) who were 18 - 25 years old and had low to moderate caries experience. The samples were streaked on heart infusion blood agar plates supplemented with 5 µg/ml of menadione. The predominant cultivable organisms included facultative streptococci, 28%; facultative diphtheroids, 24%; anaerobic diphtheroids, 18%; peptostreptococci, 13% Veillonella, 6%; Bacteroides, 4%; fusobacteria, 4%; Neisseria, 0.04%; and vibrios, 2%. The predominant cultivable microbiota are listed in Table I - 1 according to their oxygen requirements and gram reaction.

Howell et al (1965) reported a study on the cultivable bacteria in developing and mature dental calculus. Six subjects (age 28 - 45) consisting of four males and two females with at least all their natural lower anterior teeth were utilized in the study. In each patient one lower incisor tooth was selected if the mesial, distal, and lingual surfaces were easily accessible. A thorough dental prophylaxis was completed prior to each plaque collection period. The results showed that streptococci were the predominant organisms in all samples up to and including four weeks. Actinomyces israeli and Actinomyces naeslundi comprised the largest percentage of plaque samples taken after 90 days. The results of the proportions of the organisms for two and four day plaque are listed in Table I - 1.

Table II - 1 Proportions and proportional changes of organisms in dental plaque.

Microorganism		Gram Reaction	Gibbons et al 1964	Howell et al 1965		Ritz 1967	
				2 day	4 day	1 day	3 day
Cocci	Facultative	+ve	28	50.6	39.7	46.0	69.0
		-ve	0.04				
	Anaerobic	+ve	13	-	-	1.5	3.8
		-ve	6	12.4	16.6	9.1	7.8
Total Cocci			47	63.0	56.3	56.6	80.6
Rods	Facultative	+ve	24	7.5	25.7	6.2	0.47
		-ve		2.8	1.3	0.3	2.0
	Anaerobic	+ve	18	1.3	1.0	3.2	0.11
		-ve	10	16.1	12.8		
Total Rods			52	27.7	41.5	9.7	2.6

Ritz in 1967 reported a study of microbial population shifts of selected organisms in developing human dental plaque. Plaque samples of 1, 3, 5, 7, and 9 days of age were obtained from the labial surfaces of maxillary and mandibular incisors and canines. Each subject had a dental prophylaxis on these surfaces prior to the start of each plaque collection period. The total count of microorganisms consisted of the highest total facultative - microaerophilic count added to the total number of the selected aerobes and anaerobes. Using these methods, 80 - 90% of the total flora could be accounted for in the 3 to 9 day plaque samples, while only 66% were accounted for in the one day plaque samples. The organisms unaccounted for were presumed to be lost because of the inhibitory effects of selective media. During the early stage of plaque development the streptococci, neisseria, and the nocardia predominated. Three day old plaque was predominated by streptococci (80%). As the plaque age and growth increased the proportion of aerobic organisms decreased and that of the anaerobic organisms increased. The results of the proportions of the organisms for one and three day plaque are listed in Table I - 1.

Several studies of the bacteriological and also histological composition of dental plaque have been reported. Generally, the bacteriological studies have been limited to selected organisms isolated from dental plaque (Hemmens et al, 1941, Appleman et al, 1955, Davis et al, 1959, Frisbie and Nuckolls, 1947, McDougall, 1963a).

Mandel et al (1957) attempted to correlate morphological and cultural examinations of early developing calculus on Mylar strips. On microscopic examination, three to five day plaque contained masses of gram positive and negative coccoid forms plus occasional filaments. By the seventh day coccoid and masses of filamentous forms were present; by the twelfth day the plaque was almost entirely filamentous. Several studies have been concerned with the formation of calculus (Mandel et al, 1957, Schroeder, 1963, Muhlemann and Schneider, 1959, Turesky et al, 1961).

The results from several of these investigations have shown that coccal forms are first established in early dental plaque (Slack and Bowden, 1964; Turesky et al, 1961; Mandel et al, 1957; and Appleman et al, 1955; Ritz, 1967; Howell et al, 1965). In the later stages of plaque formation filamentous forms increase in number (Howell et al, 1965; Mandel et al, 1957; Muhlemann and Schneider, 1959, Turesky et al, 1961, McDougall, 1963a).

The proportions of the different cultivable microbiota found by Gibbons et al, 1964, correlate fairly well with those found for four day plaque by Howell et al, (1965). One of the important differences in the work of Gibbons et al (1964), Ritz (1967), and Howell et al (1965) is that the patients for the latter two investigators had a thorough dental prophylaxis on the tooth surfaces which were to be used for plaque collection and secondly the sample age and sample location were given. The work of Ritz (1967) shows that the proportion of aerobic organisms decreases with the progression of plaque

development whereas the proportion of anaerobes increases. This may be caused by changes in the oxidation-reduction potential occurring in the plaque during development.

The studies of Ritz (1967) and Howell et al (1965) support the results of other investigators in that the proportions of filamentous organisms such as actinomyces, corynebacterium and fusobacterium increase as growth of plaque progresses.

It might seem surprising that anaerobic organisms are able to grow in the oral cavity which is continually exposed to atmospheric oxygen. In a very recent paper, Eskow and Loesche (1969), report that many of the anaerobic organisms indigenous to dental plaque will tolerate an oxygen concentration of 2 - 8%.

The facts which emerge from a review of the bacteriology of dental plaque will now be listed.

1. There are a large variety of different microorganisms in dental plaque.
2. The proportion of the various microorganisms varies with the age of the plaque. Early plaque (1 to 5 days) seems to be dominated by streptococci whereas later plaque seems to be dominated by filamentous microorganisms.
3. Methods of culturing all the different plaque microorganisms have not yet been developed.

4. The procedures used in these bacterial investigations are very complex and time consuming, and therefore the number of subjects that has been studied is small.

Plaque Formation

Jenkins (1968), Kleinberg (1968), and Dawes (1968) have recently reviewed the current theories of dental plaque formation. These are that salivary proteins may precipitate on the tooth surface by: Acid Precipitation, Surface Denaturation or Formation of Insoluble Calcium Proteinate.

Acid Precipitation

Kirk (1910) reported that addition to saliva of a drop or two of lactic acid caused precipitation of the mucin. He therefore concluded that the acid formed by the metabolism of oral bacteria could cause plaque formation by precipitation of salivary proteins.

Dawes (1964) confirmed that addition of unbuffered mineral or organic acids to saliva caused protein precipitation. However, this precipitation was found due to a localized denaturation of protein from exposure to the acid at a pH of less than 3.5. Addition of buffered organic acids to freshly secreted duct saliva did not cause precipitation of protein until a pH of 3.5 was reached. Since this pH is below the physiological range, he concluded that acid precipitation was not a primary factor in plaque formation.

Proteins are normally most insoluble at their isoelectric point and the isoelectric point of salivary, acid-insoluble proteins is about pH 2.6. (Inouye, 1930; Leach, 1967). However, removal of the sialic acid from salivary glycoproteins by the

action of the bacterial enzyme neuraminidase will increase the isoelectric point from pH 2.6 to approximately 7.0 (Leach, 1967). Hence, acid precipitation after sialic acid removal could be an important factor in plaque formation. Kleinberg (personal communication) has shown that when salivary sediment is incubated with glucose the pH falls and salivary protein precipitation occurs. Bacterial neuraminidase could be an important factor in the precipitation of salivary proteins under these conditions since the removal of sialic acid would raise the isoelectric point of the proteins over the time period studied.

Surface Denaturation or Formation of Insoluble Calcium Proteinate

Dawes (1964) reported that spontaneous precipitation of proteins occurred on shaking submandibular saliva. Slight agitation of submandibular saliva intraorally due to movement of the tongue might cause spontaneous precipitation of salivary glycoproteins. He also reported that some parotid and submandibular salivary glycoproteins were selectively precipitated by addition of calcium ions. The proteins precipitated by addition of calcium ions to saliva were similar to those precipitated by shaking submandibular saliva. The precipitation could have been caused by formation of an insoluble calcium proteinate or by the precipitation of hydroxyapatite if the addition of calcium, or the rise in pH due to loss of CO_2

on agitation of the saliva, caused the solubility product for hydroxyapatite to be exceeded. Salivary proteins could then adsorb on to these hydroxyapatite crystals.

Eleven high molecular weight protein fractions have been extracted from dental plaque with 0.1N NaOH by Silverman and Kleinberg (1967a). When these eleven fractions and the NaOH-soluble cells were titrated with dilute HCl from pH 10.0 one of the fractions precipitated at pH 8.0 and the others at pH 4.0 - 5.0. From this evidence they have concluded that acid precipitation of protein is a major factor in plaque formation. It should be noted that the plaque extracts would have lost most of their sugar moieties and the isoelectric point of the proteins would have been increased. This would cause the proteins to precipitate at a higher pH level than that for the freshly secreted proteins.

Other evidence contrary to the theory that acid precipitation causes the initial deposition of plaque matrix is that: matrix is deposited before the bacteria (McDougall, 1963a), matrix deposition occurs in germ-free animals (Baer and Newton, 1958), plaque formation can occur in patients whose only food intake is given by stomach tube and whose plaques do not form acid (Littleton et al, 1967), and that freshly secreted unstimulated saliva shows no evidence of precipitation at a pH as low as 5.5 (Dawes 1968).

Possible Source of Plaque Matrix

The most likely source of plaque matrix is salivary

glycoproteins but bacterial intracellular proteins, cell wall constituents, gingival crevice fluid, desquamated epithelial cells, white blood cells, and extracellular polysaccharides could be important.

McDougall (1963b) Hay (1966), and Silverman and Kleinberg (1967a) have given evidence to show that the source of plaque matrix is salivary glycoproteins. Ferguson (1964) separated seven proteins from dental plaque by starch gel electrophoresis. He claimed that these proteins had the same mobility patterns as certain parotid saliva glycoproteins. This finding is very surprising since the plaque proteins would have had most of their carbohydrate moieties detached. Hay (1966) showed that at least five of the glycoproteins from saliva could adsorb onto powdered hydroxyapatite and suggested that this may be important in acquired pellicle formation. Silverman and Kleinberg (1967a) separated eleven protein fractions from dental plaque and showed that the proportions of amino acids of the combined protein fractions corresponded quite well with the proportions of amino acids reported for salivary mucin by Armstrong (1966).

Kramer and Ramanathan (1966) showed that fluorescein labelled anti-human serum adsorbed to acquired pellicle indicating that the latter might be derived from serum proteins. They suggested that this might be an important method to study acquired pellicle and plaque formation. Brill (1958) showed that in dogs, fluid flows from the gingival crevice into the oral cavity. The serum proteins

present in this gingival crevice fluid could be incorporated into the acquired pellicle and dental plaque. Salkind et al (1963) have reported similar findings for the gingival crevices of humans.

Leucocytes are known to enter the oral cavity by way of the gingival crevice (Sharry and Krasse, 1960). The leucocytes rapidly disintegrate on exposure to the hypotonic saliva and components could conceivably be incorporated into the dental plaque.

Extracellular polysaccharides formed from sucrose have been implicated in plaque formation. Since plaque can form in patients nourished by stomach tube for a period of up to 52 months (Littleton et al, 1967) extracellular polysaccharides produced by metabolism of sucrose cannot be essential to plaque formation. They may, however, be important in giving bulk to the dental plaque in patients on a high sucrose diet.

In summary then, proteins from saliva, bacteria, gingival crevice fluid, desquamated epithelial and white blood cells are possible sources of plaque matrix. However, the most likely source of plaque matrix is salivary proteins.

The Separation of Dental Plaque into Its Bacterial and Matrix Components

In 1932, Dobbs suggested that the removal of dental plaque from the teeth by a chemical solvent might be useful from the standpoint of caries control. He therefore collected plaques and tried to solubilize them in a wide variety of solutions kept at 37°C. The amount dissolved was determined by the xanthoproteic test for protein. Although slight solubility of the plaques occurred, none of the solutions were of any value for the removal of dental plaque *in vivo*.

Dobbs extracted plaque with 5% NaOH and then filtered, dialyzed and precipitated the NaOH soluble proteins with HCl. The protein precipitated comprised 28-52% of the total plaque protein and Dobbs claimed that this proportion represented the "mucin" content of plaque.

Dawes (1962) found that when plaque was homogenized in 0.1N NaOH the matrix was solubilized and about 33% (range 15-51%) of the plaque nitrogen went into solution. Histologically the bacteria appeared to remain intact. Only 5% of the plaque nitrogen was solubilized in water.

Ferguson (1964) attempted to correlate the proteins of dental plaque with those of saliva by a starch gel electrophoretic technique. He reported, in an abstract, the separation of seven proteins from dental plaque which corresponded in mobility with seven proteins previously studied in parotid saliva from one subject

(d'Silva and Ferguson, 1962). The proteins took up carbohydrate stains demonstrating that they were glycoproteins. This finding seems to support the view that salivary proteins contribute to dental plaque, but it seems unlikely that only the parotid salivary proteins are found in dental plaque and that they have the same electrophoretic mobility as glycoproteins which have lost some of their carbohydrate residues (particularly sialic acid). The sialic acid components of salivary glycoproteins are rapidly metabolized by the oral bacteria (Dawes, 1962; Leach 1967).

Silverman and Kleinberg (1967a) used 0.1N NaOH to separate dental plaque into three major components; NaOH-soluble, NaOH-suspended cells, and NaOH-insoluble residue. Plaque was collected from all sufaces of the teeth from subjects who had not brushed for 3 days. The plaque was solubilized for one hour in tubes containing 0.1N NaOH at $0^{o}C$. The tubes were centrifuged at 1740 X g for 2.5 minutes and the NaOH-suspended cells and the NaOH-soluble material was collected. This solubilization was repeated a total of five times. The NaOH-soluble material plus NaOH-suspended cells was centrifuged at 12,800 X g at $4^{o}C$ for 10 minutes to separate the cells from the NaOH-soluble material. The NaOH-soluble protein was dialyzed and fractionated by column chromatography. Eleven high molecular weight protein fractions were collected.

Whether the eleven NaOH-soluble fractions were of bacterial or

salivary origin is unknown.

In summary,

1. Previous workers have attempted to solubilize the matrix of dental plaque with various solvents but particularly with 0.1N NaOH.
2. Eleven protein components have been fractionated after NaOH-solubilization of plaque matrix.
3. It has not been determined whether these protein fractions were derived only from plaque matrix or whether they may have been extracted from the bacteria.

Factors Affecting Viability and Leakage of Pure Bacterial Cultures

A. VIABILITY

Part of the present work involved a study of the effects of pH and temperature on protein leakage from pure bacteria. It was thought necessary to review not only the literature on bacterial leakage, but also that dealing with the effect of pH, temperature and several other factors on the viability or death rate of various microorganisms. The factors affecting viability and leakage have been separated into two sections but at times have been presented in parallel. Many previous workers have studied the viability of a bacterial population after it has been subjected to mild or severe stress situations. These studies were primarily applied to problems of sterilization and disinfection.

The following factors have been found to affect bacterial survival or death rate: Washing Procedure, Growth Phase, pH, Population Density, Cryptic Growth, Nutritional Status, Protective Substances, Osmotic Environment.

Removal of Growth Media

Most investigators have used saline, phosphate-saline buffer, phosphate buffer or distilled water to remove the growth medium from bacterial cultures. Postgate and Hunter (1962) and Strange and Shon (1964) have shown that the diluents used for

removal of growth medium may affect the subsequent viability of cultures. Postgate and Hunter (1962) reported that when A. aerogenes was washed once, twice, or three times with 0.85 g/l of saline and stored at 0^{o} or $22^{o}C$, the wash procedure did not affect the viability. After washing in saline some of the microorganisms were suspended in distilled water for 3 - 5 minutes. This caused the death rate to be faster than in those retained in the saline-tris buffer. If the microorganisms were stored in unbuffered saline for two hours viability decreased to 15%. This decreased viability was probably due to the fact that the pH of the unbuffered saline fell from 6.0 to 4.9 as a result of leakage into the medium of the products of endogenous metabolism.

In contrast, Strange and Shon (1964) showed that A. aerogenes washed in distilled water exhibited greater thermal resistance and therefore greater viability than similar microorganisms washed in aqueous solutions of saline, KCl, or phosphate-saline buffer. Salt solutions desorbed magnesium from the bacteria and this finding may explain the faster death rate of A. aerogenes in phosphate-saline than in distilled water.

Thus, the length of exposure and composition of the diluent used for removal of the media are important in bacterial survival. The temperature and pH of the subsequent experimental solutions are also important.

Since in the oral cavity, bacteria grow in saliva which is approximately isotonic with 0.4% saline, the microorganisms used in the present study were washed with 0.4% saline to remove growth media prior to being tested for protein leakage in various buffers.

Effect of Growth Phase

Growth phase has a marked effect on bacterial survival. Since the time of Boer (1890) suspensions of log phase cells in buffers have been known to exhibit a faster death rate than that of stationary phase cells.

Several investigators (Sherman and Albus, 1923; Ellicker and Frazier, 1938; White, 1951, 1953; Lemcke and White, 1959) have indicated that the heat resistance of bacteria varies with the growth phase of the culture. Early logarithmic phase cells have been found to be the least resistant.

Strange et al (1961) studied in detail the survival of suspensions of A. aerogenes as a function of growth phase. The death rate of log phase cells was faster than that of early stationary phase cells; in turn, death rates of early stationary phase cells were faster than those of late stationary phase cells. These differences in viability were not apparent until the bacteria had been suspended in buffer for at least 24 hours.

Strange and Shon (1964), however, have shown that when A. aerogenes was grown in a carbon-limited defined medium, the influence

of growth phase on thermal resistance depended on the washing procedure used for removal of growth medium. Under certain conditions the log phase cells were more resistant than stationary phase cells. When A. aerogenes was washed with distilled water and resuspended in phosphate-saline, log phase cells were more heat resistant than were stationary phase cells; however, after washing in phosphate-saline, the stationary phase cells were more resistant to heat than were log phase cells.

Sato and Takahashi (1968) have shown that the death rate of cold shocked E. coli cells is faster for logarithmic phase cells than for early or late stationary cells.

In general, log phase cells have been shown to be the most sensitive to stress conditions.

Effect of Temperature

Elevated temperatures have been shown to accelerate the death rate of bacteria (Cohen, 1922; Watkins and Winslow, 1932; Postage and Hunter, 1962; Allwood and Russell, 1967). The latter two authors reported a direct relationship between death rate and temperature over the temperature range of 3°C to 50°C. This effect of temperature may have been due to denaturation of bacterial enzymes or more probably to breakdown of RNA at the higher temperatures (Strange and Shon, 1964).

Effect of pH

The optiumum pH for storage of bacteria has often been shown

to be different from the optimum pH for growth (Cohen, 1922, Winslow and Falk, 1923, Strange et al, 1961; Postgate and Hunter, 1962). For instance, Postgate and Hunter (1962) found that A. aerogenes showed maximum viability when stored in aqueous suspensions between 6.0 and 6.5 whereas the optimum pH for growth was 7.0 - 7.5.

pH may be an important factor in the denaturation of protein and also may be a very important factor affecting membrane structural orientation. Exactly why survival is enhanced at a pH less than that optimal for growth is unknown.

Effect of Population Density

It has been shown, or implied, since the time of Geppart (1889 a & b) that viability is dependent upon the concentration of microorganisms. More recently, Harrison (1960), Postgate and Hunter, (1962), in studies with A. aerogenes showed that 10^8 microorganisms/ml was the optimum for a minimal death rate. At higher or lower concentrations the death rate was accelerated.

An increase in the concentration of cells may decrease viability due to an accumulation of toxic metabolites, a decrease in the concentration of cells may result in the insufficent excretion of protective substances for cryptic metabolism.

Cryptic Growth

Cryptic growth is defined as growth of microorganisms from the leakage and lysis products of dead microorganisms. Lange (1922)

reported that dead cells had a protective effect on live cells. Postgate and Hunter (1962) reported that with stored suspensions of A. aerogenes cryptic growth started after seventy-two hours. The magnitude of this cryptic growth was one division for every forty-seven dead microorganisms. This factor would not be important in our studies but could conceivably take place in the inner layers of the dental plaque.

Strange et al (1961) showed that cryptic growth could be substantially reduced when A. aerogenes was grown in cellophane sacs and dialyzed against buffered saline. This decreased growth was probably caused by loss of small molecular weight compounds that are readily metabolized.

Effect of Nutritional Status

Strange et al (1961) reported that A. aerogenes grown in tryptone glucose medium exhibited 95% survival for longer periods of time than the same microorganisms grown in tryptic meat broth or defined medium.

The bacteria grown in the glucose-containing medium were able to form intracellular polysaccharides which could subsequently be metabolized in the absence of exogenous nutrients.

The work of Strange et al (1961) suggests that those microorganisms which can synthetize intracellular polysaccharides are subsequently better able to survive under starvation conditions.

In our study, seven microorganisms were grown in media

containing glucose and the Veillonella sp. was grown in a medium containing lactate. These organisms were thus presumed to be in a n optimal nutritional state.

Effect of Osmotic Environment

Certain bacterial cells have been shown to withstand osmotic pressures of 3 to 20 atmospheres as well as a very low osmotic pressure such as in distilled water (Mitchell and Moyle, 1956). The optimum osmotic pressure for survival depends on the type of bacteria. Gorrill and McNeil (1960) showed that the lethal effect of distilled water on P. pyocyanea was greater than that of Ringer's solution. Strange et al (1962), however, reported that the death rate of A. aerogenes was less in distilled water than in buffered saline or buffer at pH 6.5. The latter two authors have shown that the lethal effect depended on the diluent and the particular type of bacteria.

Neu and Heppel (1965) have used a procedure called osmotic shock to release proteins from E. coli. Suspensions of bacterial cells are exposed to a 20% sucrose solution for 10 minutes and then centrifuged. After the sucrose solution is removed, a dilute solution of $MgCl_2$ is added, the microoganisms expand and release protein, enzymes, and 260 mμ-absorbing material into the medium.

B. LEAKAGE

A limited number of studies have been done on the factors affecting leakage from pure bacterial cultures. Of the studies completed most have investigated the effect of temperature on leakage over a given period of time. In very few studies has a statistical analysis been carried out. The present study was to investigate the effects of pH, temperature, the presence of divalent cations and several other factors on leakage of protein from pure bacterial cultures. Similar experiments were then carried out on dental plaque.

The following materials are amongst those that have been found to leak out of bacteria; amino acids, peptides of low molecular weight, potassium ions, ATP, protein and ammonia, RNA and RNA degradation products such as hypoxanthine, inorganic phosphate, ribose.

Leakage of the above materials from bacteria has been shown to depend on the following factors: Temperature of the Medium, Growth Phase, Length of Exposure to the Solutions, Type of Bacteria, pH, Type of Diluent.

Effect of Temperature

The effect of temperature on leakage from bacterial cultures has been studied previously in two ways: 1) organisms grown at 37°C

were harvested, washed and resuspended in diluent at 22°C or 37°C and then rapidly chilled to 0°C to study the effect of cold shock. 2) microorganisms grown at 37°C were slowly chilled to 0°C, centrifuged, washed and resuspended at 0°C. Aliquots were then added to other diluents to study the effect on leakage of an increased temperature.

Using the first method, Strange et al (1961) showed that the initial rate of leakage of amino acids and ATP from A. aerogenes chilled at 0°C was much greater than at 20°C. However, the total amount of leakage at 20°C over several hours was greater than the leakage from this microorganism at 0°C. This finding was believed to be caused by greater degradation of RNA at the higher temperature. Neither acid-insoluble protein nor RNA were detected as leakage products from the chilled bacteria but an absorption maximum occurred at 260 mμ.

With the second method, Allwood and Russell (1967) using S. aureus reported that an increase in temperature from 3°C to 55°C caused an increase in leakage material. At 60°C coagulation occurred causing a decrease in the total amount of leakage relative to 55°C.

In general, increased leakage occurs with an increase in temperature up to 60°C where coagulation probably occurs.

Effect of Growth Phase

Allwood and Russell (1968) studied the effects of temperature (50° and 60°C) on the leakage of free amino acids, protein and

260 mμ-absorbing material from logarithmic and stationary phase cells of S. aureus. At 50°C, leakage of 260 mμ-absorbing material was greater from log phase cells than from stationary phase cells, but no differences in the amino acid or protein leakage were detected. At 60°C, RNA and amino acid leakage occured more rapidly from log phase cells, than from stationary phase cells. At 60°C, they reported that a small amount of protein leakage occurred and concluded that this decrease in leakage resulted from protein coagulation.

With respect to both leakage and viability, log phase cells appear to be more sensitive than stationary phase cells.

Effect of Time

Allwood and Russell (1967) showed that the amount of leakage from S. aureus, as monitored by 260 mμ-absorption, increased with time. They reported that minimal leakage occurred at 3°C. At 37°C, the amount of leakage increased for the entire eight hours time period. At 60°C, leakage increased to a maximum in one hour after which it was constant for the remaining three hour time period.

Califano (1952) reported that the temperature at which leakage of RNA material into the medium occurred depended on the bacterial species. For example, with Staphylococci it began at 60°C and for pneumococci it began at 40°C.

In general then, studies have shown that leakage increases with time. In no other studies than the present one have the leakage patterns of several microorganisms been compared.

pH

Leakage from suspensions of pure bacteria has usually been studied at or near neutral pH levels (6.5 - 7.0). The present study appears to be the only one in which protein leakage was investigated on a quantitative basis from several bacteria and over a wide range of pH.

Effect of Calcium and Magnesium on Leakage

The presence of calcium and magnesium ions in suspensions of bacteria has been shown to protect bacteria against loss of viability.

Strange and Dark (1962) showed that bacteria chilled in the presence of calcium, magnesium, or manganese were protected against loss of viability. They suggested that calcium and magnesium decrease the permeability of the cytoplasmic membrane in the intact cell during chilling.

Sato and Takahashi (1968) have recently shown that for suspensions of E. coli the loss of viability caused by cold shock is substantially reduced in the presence of 0.1 M calcium chloride and magnesium sulphate.

The Physiological Variables in the Oral Cavity

The range of the physiological variables which appear to be tolerated by the plaque microorganisms in vivo will now be outlined.

Plaque pH can vary from as low as 4.5 with glucose utilization

to as high as 9.0 with urea utilization (Kleinberg, 1967 a & b). These pH changes may last for only a few minutes or for several hours depending on the concentration and clearance rate of substrate present. Liquids commonly consummed such as orange juice and Coca-cola, which have pH's as low as 3.5 and 2.5 respectively, could probably lower plaque pH considerably despite the buffering capacity of plaque.

The temperature of dental plaque can vary from 0°C during consumption of ice cream or a glass of ice water to 55 - 60°C for a cup of coffee.

Dental plaque is known to concentrate calcium above the level in saliva (Dawes and Jenkins, 1962). Since calcim and magnesium are known to stabilize bacterial protoplasts it was postulated that these two cations might decrease the leakage of proteins from dental plaque microorganisms.

The ionic strength of the fluids in the oral cavity is normally about 0.05 but may range from zero to 0.6 depending on whether distilled water is being consumed or whether a strong salt mouthwash is being used.

CHAPTER III

METHODS AND MATERIALS

CHAPTER III

METHODS AND MATERIALS

Introduction

A series of preliminary experiments (Chapter IV) were completed prior to the initiation of a more thorough investigation (Chapter V) of the leakage pattern of eight microorganisms over the pH range 1.3 to 12.7. The purpose of the preliminary work was to establish the effect of several factors on leakage from pure cultures of bacteria. A set of conditions was then selected to study the effect of pH, temperature, calcium, magnesium and osmotic shock on the percent protein extracted from eight dental plaque microorganisms and from dental plaque.

Microorganisms

The following organisms were used in this study:

1. Four Streptococcal strains
   AHT, BHT, CHT, HHT
2. Two lactobacilli
   Lactobacillus casei, *Lactobacillus acidophilus*
3. One *Veillonella* species
4. One *Diphtheroid* species
5. One *Corynebacterium* species

These organisms were isolated from human oral flora. For simplicity the streptococcal strains, AHT, BHT, CHT, HHT are designated by the first letter (i.e. Streptococcus A). HT signifies that the source is human teeth. The streptococcal strains were originally isolated by Zinner (1966) and were obtained from Dr. H.J. Sandham, Department of Microbiology, University of Manitoba. Of the four steptococci AHT, BHT and HHT are cariogenic whilst CHT is non-cariogenic. It was suspected that differences in leakage patterns between these microorganisms might occur since they are known to show differences in growth rates, in fluorescent antibody reaction, and in their ability to produce caries in hamster. (Zinner et al 1965; Jablon and Zinner, 1966). Mr. Steve Ng, University of Manitoba, provided the strain of Veillonella. The Department of Medical Microbiology provided the Corynebacterium and Lactobacilli strains. The author isolated the Diphtheroid using the procedure outlined by Rasmussen and Gibbons (1966).

Preparation of Buffers

Two methods were employed for the preparation of buffers. The buffers used in the experiment described in Chapter IV were prepared on the basis of molarity and all were made to 0.05M, except for the HCl and NaOH, which were made to 0.1N to give a final pH of 1.0 and 13.0 respectively. The 0.1N NaOH was used to provide a comparison with the work of Silverman and Kleinberg (1967 a and b). When the different buffers are made to a constant molarity the ionic strength of the buffers are not the same and vice versa. For the experiments

described in Chapter V the buffers were made to the same ionic strength and therefore the molarity varied. The buffers are shown in Table III - 1.

(a) For the experiments described in Chapter IV, buffers over the pH range of 1.0 - 13.0 were prepared using the Henderson-Hasselbalch equation:

$$pH = pKa + (salt) / (acid)$$

to get the correct proportion of salt to acid. The molarity of the buffers was chosen as 0.05 and the correct amounts of salt form and acid form were weighed and made to a specific volume to give the correct pH. The pH was verified on a Radiometer #26 pH meter and adjusted if necessary.

Hydrochloric acid and sodium hydroxide were used at the extremes of the pH scale and were made to 0.1N to give pH's of 1.0 and 13.0 respectively.

These buffers were used for the following sections of the results reported in Chapter IV: Effect of Growth Phase, Effect of Number of Extracts, Correlation of Nitrogen and Protein Values.

(b) For the experiments described in Chapter V, buffers over the pH range of 1.3 (0.05N HCl) to 12.7 (0.05N NaOH) were used in all of the experiments. The buffers were also used for the experiments described in the following sections of Chapter IV: Effects of Time of Exposure to Buffers, Effects of Buffer Type, Ionic Strength and Bacterial Concentration. Buffers were chosen such that the pH values would be as close as possible to the buffer pK values, in order

Table III - 1 Buffers used in the experiments to be described in Chapter IV and V.

pH	Chapter IV Buffers	Chapter V Buffers
1.0	0.1N HCl	-------
1.3	-----	0.05N HCl
4.0	Acetate	Lactate
4.5	Acetate	----
5.0	Acetate	Acetate
5.5	Acetate	-----
6.0	Phosphate	-----
7.0	Phosphate	Phosphate
8.0	Phosphate	Bicarbonate
9.0	Borate	Borate
10.6	Carbonate	Carbonate
12.7	-----	0.05N NaOH
13.0	0.1N NaOH	-----

that maximum buffering capacity would be exerted. Using the Lewis - Randall formula:

$$\mu = \frac{1}{2} \Sigma cz^2$$

where c equals concentration and z equals valence, buffers were made for three different ionic strengths. These ionic strengths, 0.05, 0.16 and 0.64 were chosen because:

(1) unstimulated saliva has approximately the same ionic strength as the 0.05 μ buffers

(2) plasma and probably gingival crevice fluid have approximately the same ionic strength as the 0.16 μ buffer

(3) the most concentrated salt mouthwash might contain 10% NaCl and have an ionic strength of 0.64.

Experiments were carried out after the addition of magnesium or calcium to some of the buffers to give final concentrations of 1 mM and 2 mM respectively. The addition of these two cations changed the ionic strength of the buffers only slightly.

Composition and Preparation of Media

a) Veillonella

Veillonella sp. was grown in a Lactate medium similar to that proposed by Rogosa in 1956. It contained tryptone 10 gm, yeast extract 5 gm, sodium monooleate(Tween 80) 1 ml, sodium lactate (42.5%) 24 ml, KH_2PO_4 6.9 gm and sodium thioglycollate 0.75 gm per litre of deionized water. The pH of the medium was adjusted to 6.5 with Na_2CO_3.

b) The Streptococcus sp. and the Diphtheroid sp. were grown on Trypticase Soy Broth (Baltimore Bacteriological Laboratories, BBL) containing trypticase 17.0 gm, phytone 3 gm, sodium chloride 5 gm, dipotassium hydrogen phosphate 2.5 gm and dextrose 2.5 gm per litre of deionized water. The final pH was adjusted to 7.1.

c) The Lactobacilli were grown in a selective medium proposed by Rogosa in 1951. It consisted of Trypticase (BBL) 10 gm, yeast extract (Difco) 5 gm, KH_2PO_4 6 gm, ammonium citrate $[(NH_4)_2HC_8H_5O_7]$ 2 gm, salt solution 5 ml*, glucose 20 gm, Tween 80 1 ml, sodium acetate 25 gm, glacial acetic acid (99.5%) 1.32 ml per litre of distilled water. The final pH was adjusted to 5.4.

*) Salt solution contained $MgSO_4.7H_2O$ 11.5 gm; $MnSO_4.2H_2O$ 2.4 gm; $FeSO_4.7H_2O$ 0.68 gm; distilled water to 100 ml.

Growth and Temperature Conditions

For all experiments and all growth conditions the organisms were grown in their respective media at 37°C.

Organisms were maintained for 48 hours in 5 ml of their respective media then transferred to fresh media, placed in an anaerobic jar and degassed four times, the air being replaced with 95% N_2 and 5% CO_2 each time.

Prior to the start of an experiment or growth curve, the organisms under study were transferred every 24 hours from their daily culture tubes. For each experiment 5 ml of broth was added aseptically to 195 ml of sterile medium in a 500 ml Erlenmeyer flask which was immediately sealed. The flask was degassed four times, the air being replaced after each degassing with 95% N_2 and 5% CO_2. After 8 hours at 37°C ±0.1°C in a Precision Scientific Model #2 Incubator the organisms were transferred to 800 ml of sterile media in a 2 litre flask sealed with a rubber stopper containing a degassing tube and an inverted Klett tube. The flask was degassed four times and after each degassing the air was replaced with 95% N_2 and 5% CO_2. The growth of the organisms was monitored by inverting the flask and inserting the Klett tube into a Klett colorimeter set at a wavelength of 640-700 mμ. This was repeated every 15 minutes until the organisms reached the stationary phase of growth (Figure III - 1).

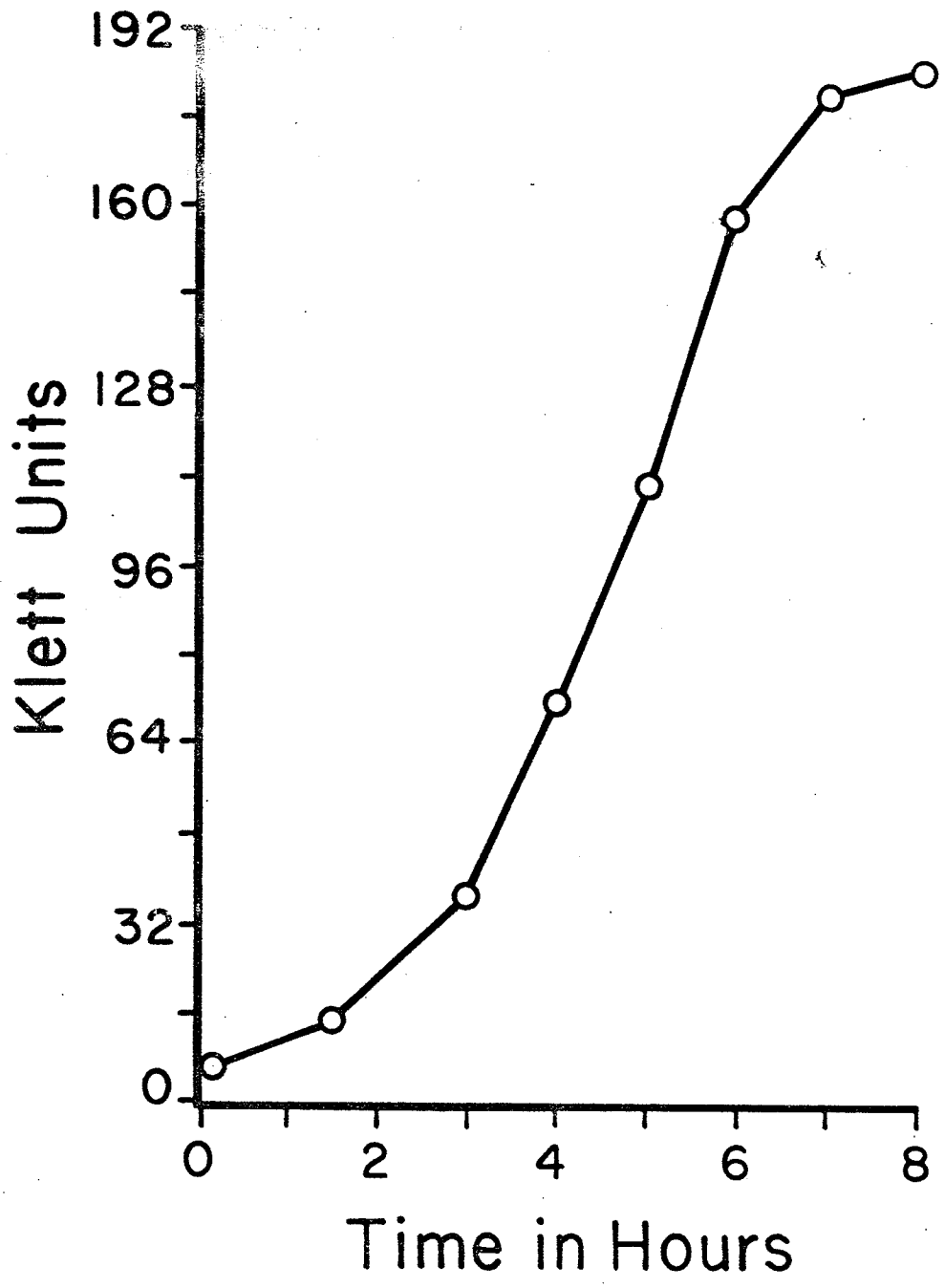


Figure III - 1 Growth curve of Veillonella sp.

For experimental purposes the cells were grown 1/2 to 2/3 (Figure III - 1) of the way into the logarithmic phase of growth, (unless stated otherwise), at which point the cells were harvested as described below.

Methods of Harvesting the Cells

Two methods were used for harvesting the cells and therefore each will be described separately. The method used for the experimental work in Chapter V was found more efficient than the method adopted initially for the work described in Chapter IV.

a) Chapter IV

After 1/2 to 2/3 of the logarithmic phase of growth was reached, 10 ml of the media plus cells was pipetted into 12 ml Beckman polyallomer centrifuge tubes and centrifuged at 10,000 x g at 0°C for 10 minutes in a Lourdes centrifuge. The tubes were removed from the centrifuge, and the medium was suctioned off. 1.5 ml of 0.4% saline was added to each tube using a Mini-pet. The organisms were redispersed by agitation on a vortex mixer and centrifuged at 10,000 x g for 5 minutes and the supernatant wash material was removed by suctioning. This washing procedure was repeated two more times. After the final wash the supernatant was removed by suction and 1.5 ml of the desired buffer was added to the tubes. After addition of the buffer and vortexing, a 40 µl time zero aliquot was taken from each tube. These tubes,

with the bacteria dispersed in the buffers, were left at 0°C or 23°C for thirty minutes. After centrifugation (10,000 x g for 10 minutes), the supernatants were collected and stored in sealed tubes. The remaining pellet was redispersed and aliquots were taken for nitrogen and protein analyses; nitrogen and protein analyses were also done on the supernatant. The percentage of nitrogen and protein release could then be calculated. The time zero aliquot was used for a nitrogen analysis to check recovery (which was 95 to 105%).

b) Chapter V

One litre of organisms was grown to 1/2 to 2/3 of the logarithmic phase of growth, poured into 250 ml bottles, and centrifuged at 12,000 x g for 10 minutes in a Sorval RB2 centrifuge at 0°C. The organisms were washed twice with 40 ml of hypotonic saline (0.4%) and resuspended in 5 ml of the hypotonic saline after the final wash. Hypotonic saline was used because it has about the same ionic strength as saliva and because it was shown to extract the least amount of protein and nitrogen from the bacterial cells. One 50 µl aliquot of the bacterial suspension was added into each 15 ml polypropylene tube containing 0.95 ml of buffer over the pH range of 1.3 to 12.7. The organisms were in the buffer for 30 minutes and a 25 µl time zero aliquot was taken from each tube. After 30 minutes the suspension was centrifuged at 12,000 x g for 10 minutes and the supernatants collected. The time zero aliquots and supernatants were analyzed by the protein method of Lowry et al (1951) and the percentage of protein extracted was calculated. The effects of pH, temperature,

calcium and magnesium concentrations, and ionic strength on the eight microorganisms were investigated by this procedure.

Investigation of the effects of osmotic shock required modification of the above procedure. After washing, the microorganisms were resuspended in 5 ml of hypotonic saline. Two ml of the bacterial suspension was removed and used as unshocked cells.

Three ml of 40% sucrose was added to the remaining organisms in the 3 ml of hypotonic saline and the mixture was gently shaken at 23°C. 100 µl aliquots were pipetted into 24 labelled tubes which were allowed to stand for 10 minutes from the time of addition of sucrose and then centrifuged at 12,000 x g for 5 minutes. The sucrose was removed by suction from the tubes containing the shocked cells. One ml of buffer was added to each tube and a 25 µl time zero aliquots was taken. During a 30 minute period 16 of the tubes were placed in a water bath at 37°C and 8 were left at room temperature. All tubes were centrifuged for 10 minutes at 12,000 x g and the supernatants collected. The supernatants and the time zero aliquots were analyzed by the method of Lowry et al (1951) and the percentage of protein extracted was calculated.

Plaque Collection

Subjects, mainly first and second year dental students,were instructed to brush their teeth thoroughly after breakfast on a given day and then not to brush again for two to three days to allow fresh

plaque to form. Prior to breakfast, on the morning of the third day, the plaque was gently removed with a Gracey # 10 curette, weighed immediately on a Torsion balance to 0.1 mg and placed in 5 ml of 0.4% saline. For each experiment, the plaque was placed into 0.4% saline to allow mixing of the plaque obtained from several individuals. Initial attempts to store the plaque in 100% humidity resulted in some drying and therefore this method was not adopted. A combined total of 10-80 mg of plaque per person was obtained from all areas of the teeth except the lingual surfaces of the lower anteriors. The plaque and saline were vortexed and aliquots of the suspension were added to the buffers. Time zero aliquots were taken for analysis. After thirty minutes the tubes were centrifuged and the supernatants collected. Protein and nitrogen analyses on the time zero aliquots and supernatant samples were completed and the percentage of protein and/or nitrogen leakage was calculated.

An experiment to determine the range of the percent protein extracted from the dental plaque was as follows: Thirteen fasted subjects contributed 16 samples of 3 day old plaque which was placed in 0.05 N NaOH at $0^{o}C$ for one hour. A time zero aliquot was taken for determination of total protein and, after centrifugation, aliquots of the supernatant were removed for analysis. The percent protein extracted was then calculated.

Analytical Methods

a) Nitrogen

A micro-adaptation of the Nessler's reaction (Hawk et al 1954) was used for nitrogen determination. Ammonium sulphate was used as the nitrogen standard and the method was sensitive for 0-4 µg of nitrogen.

Bacterial and dental plaque samples were digested overnight in 10 by 75 mm pyrex tubes containing 80 µl of 50% sulphuric acid. The black residue which developed was oxidized by addition of 50 µl of 30% hydrogen peroxide to each tube and a further three hours of digestion was allowed to permit oxidation with evaporation of the water and CO_2 release in all tubes. A second addition of hydrogen peroxide was made if necessary. To the 40 µl of concentrated acid remaining after digestion and evaporation, 280 µl of deionized water was added to make the volume in each tube up to 320 µl and the solution approximately 4.5N with respect to H_2SO_4. Duplicate 100 µl aliquots were removed and placed into tubes in an ice bath. 400 µl of cold Nessler's reagent was then added to each tube which was vortexed immediately. Four tubes at a time were then removed and allowed to warm to room temperature before the optical density was read in a Unicam SP 500 spectrophotometer at 465 mµ.

The Nessler's solution, was prepared by dilution of 17.5 ml of stock Nessler's solution (Koch & McMeekin, 1924) to a 100 ml with

fresh 8.5% NaOH. Digested, and undigested (not heated) $(NH_4)_2SO_4$ standards had exactly the same nitrogen values; urea nitrogen recovery following digestion was 100%. The coefficient of variation with 19 aliquots containing 1.00 µg of nitrogen was 0.9%.

b) Protein

A micro-adaptation of the method of Lowry et al (1951) was used for protein determination. The reagents were:

1) 0.5% $CuSO_4 \cdot 5H_2O$ in 1% Na tartrate

2) 4% Na_2CO_3 in 0.2 N NaOH

3) Folin Ciacolteau Phenol Reagent

Hammersten Casein was used as the standard protein. The method is sensitive to 0-50 µg of protein. Standards and samples were pipetted into 10 x 75 mm pyrex tubes, made up to 700 µl volume with deionized water and vortexed. Fresh alkaline copper solution was then prepared by adding 4 ml of reagent No. (1) to a 100 ml volumetric flask and making to volume with reagent No. (2). Also at this time, fresh phenol reagent was made by adding one part pure phenol reagent to two parts water. At timed intervals (15 seconds), 500 µl of alkaline copper solution was added to a tube which was vortexed immediately. At least 12 minutes were allowed to pass from the addition of alkaline copper solution before 100 µl of phenol reagent was blown directly into the liquid in each tube. Each tube was vortexed immediately and after a further 35 minutes the optical

density was read at 600 mμ in a Unicam SP 500 spectrophotometer. The coefficient of variation in 14 aliquots of 32 μg of protein from the same sample was 0.8%.

When phosphate buffer in the concentration of 0.1M or greater was present, a white precipitate occurred when the phenol reagent was added but the precipitate could be centrifuged down without affecting the optical density due to the presence of protein. When the concentration of NaCl was greater than 10% it interfered with the protein method.

The bacterial time zero samples were solubilized in 10N NaOH and neutralized with HCl to between pH 7.0 and 9.0 prior to the protein analysis.

Experimental Design and Statistical Analysis

After completion of the preliminary studies, nine experiments outlined in Table 111 - 2 were conducted to investigate the effects of several factors on the percent protein extracted from microorganisms and dental plaque. The material included eight microorganisms (AHT, BHT, CHT, HHT, L. casei, Diphtheroid sp., Veillonella sp., Corynebacterium sp.), dental plaque (DP) and a mixture of the microorganisms. The mixture of the microorganisms is called the combined microorganisms (CM). The combined microorganisms consisted of 50% gram positive and gram negative facultative and anaerobic cocci plus 50% gram positive and negative facultative and anaerobic rods. These

Table III - 2 Summary of materials and factors used in the Experiments of Chapter V.

Experiment Number	Material	Factors & Number of Levels	Number of Treatment Combinations	Number of Replications per organism	Total Number of Replications	Combined Analysis of Variance
1	8 Microorganisms	pH - 8 Temp.-4	32	2-6	31	Yes
2	8 Microorganisms	pH-8 Calcium-2	16	2-9	37	Yes
3	8 Microorganisms	pH-8 Magnesium-2	16	2-9	37	Yes
4	BHT *Veillonella sp.*	pH-8 Temp.-2 Osm. Shock-2	32	1-3	4	No
5	BHT	pH-8 Temp.-2 Growth Phase-2	48	2	2	No
6	Dental Plaque (DP)	pH-8 Temp.-3	24	2	2	No
7	Combined (CM) Microorganisns	pH-8 Temp.-3	24	2	2	No
8	DP & CM	pH-8 Temp.-3	24	2	2	Yes
9	DP & CM	Time-7 Temp.-3	21	2	2	Yes

microorganisms were used: CHT 44%; Veillonella sp. 6%; Diphtheroid sp. 25%; Corynbacterium sp. 25%. A description of the factors involved in these nine experiments is as follows:

a) pH

Eight pH levels (1.3, 4.0, 5.0, 7.0, 8.0, 9.0, 10.6, 12.7) were used throughout the experiments. These pH levels were selected to provide a comparison between the percent protein extracted from dental plaque with that extracted from microorganisms isolated from dental plaque, as well as to provide a comparison between each of the microorganisms. These buffers had a constant ionic strength approximately equal to that of saliva. In Experiment 9 where pH was not a factor, a pH of 12.7 was used to make this experiment comparable to the work of Silverman and Kleinberg (1967a).

b) Temperature

In Experiment 1, four temperatures (0-23-37-60^{o}C) were used. In other experiments involving this factor the temperature of 60^{o}C was not used. In Experiment 4 only 23^{o}C and 37^{o}C were used. In Experiments 2 and 3, where temperature was not a factor, it was held constant at 37^{o}C.

c) Calcium

The effect of calcium on the percent protein extracted was studied in Experiment 2 at levels; 0 and 2 mM of $CaCl_2$. The concentration of calcium in whole saliva is about 2 mM (Jenkins, 1966)

d) Magnesium

The effect of magnesium on the percent protein extracted was studied in Experiment 3 at two levels; 0 and 1 mM of $MgCl_2$. The concentration of magnesium in whole saliva is somewhat less than 1 mM (Lear and Gron, 1968).

e) Osmotic Shock

The effect of osmotic shock on the percent protein extracted was studied in Experiment 4 at two levels; with and without osmotic shock. This procedure was described by Neu and Heppel (1965).

f) Time

The effect of time on the mean percent protein extracted from the material was studied in Experiment 9 at seven levels: 0.25, 0.50, 1, 2, 3, 4 and 5 hours. In all other experiments this factor was constant at 30 minutes except for the tubes at 60°C which were held at that temperature for only ten minutes.

g) Growth Phase

The effect of growth phase on the mean protein extracted was studied in Experiment 5 at two levels; logarithmic and early death phase.

All experiments were designed as complete randomized blocks with the treatments replicated on different days.

A detailed description of Experiment 1 is presented below as an example of the experimental method and statistical analysis used for all experiments.

Experiment 1 Temperature and pH

The thirty-two treatment combinations (8 pH and 4 temp.) for each organism were replicated on different days. The days were considered to be the blocks in the analysis of variance. The number of replications (r) ranged from two for the Corynebacterium sp. to six for AHT. An analysis of variance for each organism was performed according to the following distribution of the degrees of freedom.

SOURCE OF VARIATION	DF
Blocks (days)	r-1
pH	7
Temp.	3
pH X Temp.	21
Experimental Error	31(r-1)
TOTAL	32r-1

In addition, a combined analysis of variance which included the eight organisms was performed according to the following distribution of the degrees of freedom.

SOURCE OF VARIATION	DF
Blocks within Microoganisms	23
Microorganisms	7
pH	7
Temperature	3
Temp. X pH	21
Micro. X pH	49
Micro. X Temp.	21
Micro. X Temp. X pH	149
Experimental Error	713
TOTAL	991

One of the merits of the factorial analysis of variance as applied to Experiment 1 is that it enables an examination of the main effects (Temperature, pH, and Microorganisms) as well as all the possible interactions between the factors (pH X Microorganisms, Microorganisms X Temperature, pH X Temperature, pH X Microorganisms X Temperature).

CHAPTER IV

PRELIMINARY RESULTS

CHAPTER IV

PRELIMINARY RESULTS

Removal of Media from the Microorganisms

Figure IV - 1 shows the effect of five consecutive washes with hypotonic saline (0.4%) on *Veillonella sp.* at 0°C. The amount of nitrogen present in 1.5 ml of hypotonic saline after centrifugation decreased from 27 µg for the first wash to 7 µg for the second and the third washes. Since nitrogen containing compounds were probably extracted from the bacteria during each wash the amount of nitrogen remaining after any wash would never decrease to zero. The nitrogen from the medium could be adequately removed after two or three washes with hypotonic saline.

Absorption Spectrum of Leakage Products

Leakage products extracted with 0.1N NaOH from *Veillonella sp.* exhibited an absorption maximum at 260 mµ as did the supernatants left after the wash procedure. This is probably due to nucleic acids which absorb very strongly at this wave length.

Effect of Growth Phase

The effect of growth phase on the percent protein extracted from *Veillonella sp.* was studied. Similar amounts of protein were

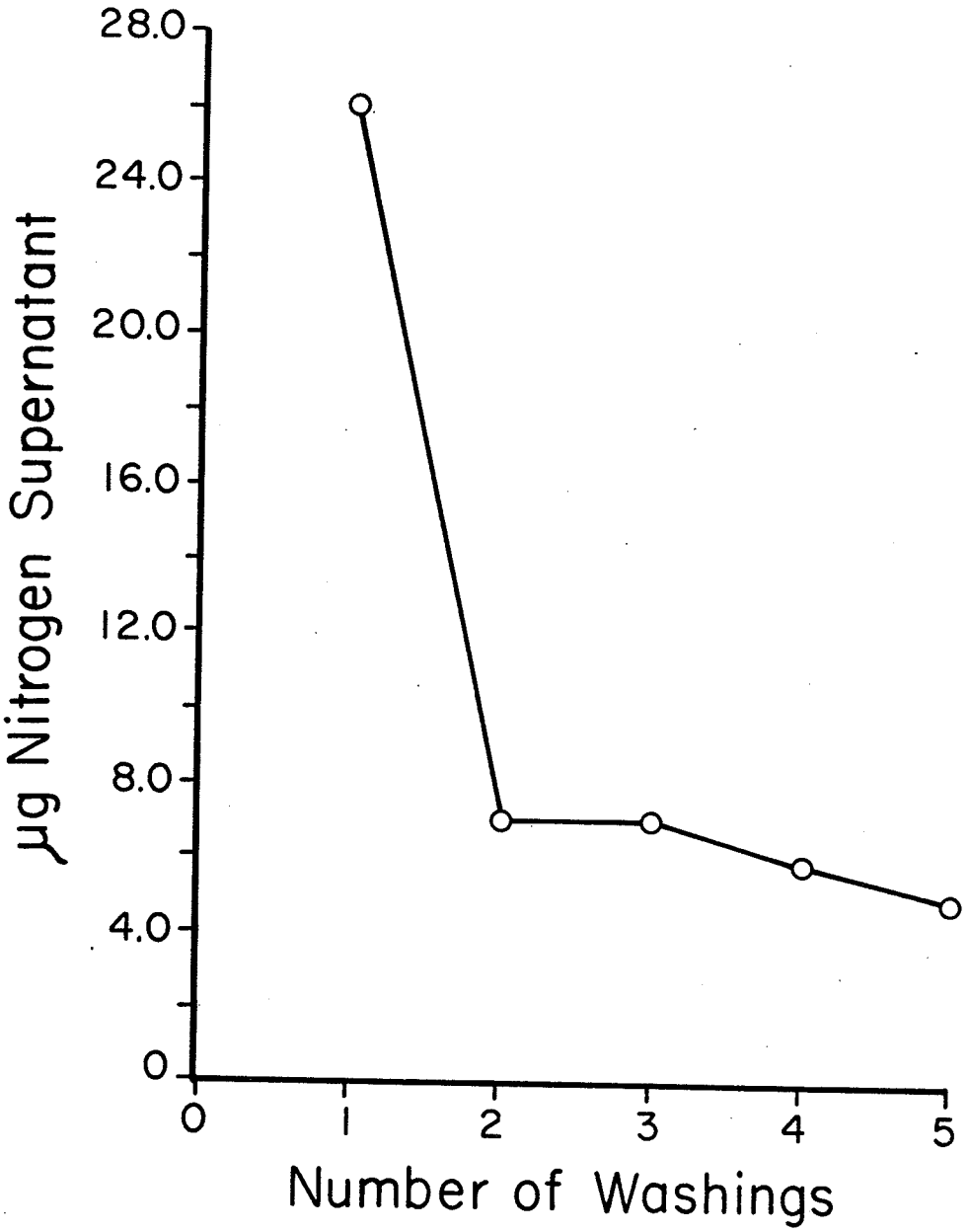


Figure IV - 1 Nitrogen release into supernatant during successive washing of bacteria.

extracted from the cells in the logarithmic phase of growth and from cells in the stationary phase of growth. Since other investigators (Allwood and Russell 1967) have studied the effect of growth phase on cell leakage and obtained similar results, this problem was not pursued in detail.

Effect of Time of Exposure to Buffers

Figures IV - 2, IV - 3, IV - 4, show the effect of time on the the leakage of 260 mμ-absorbing material. The experiments were carried out at three different temperatures (0°C, 37°C, 60°C) and six different pH levels. Since the effect of pH and temperature will be dealt with in detail in Chapter V, only the effect of time will be presented here.

Figure IV - 2 represents the effect of six pH levels on the leakage of HHT at 0°C over a three and one quarter hour time period. At this temperature, the amount of leakage almost reached a maximum level in fifteen minutes.

At 37°C (Figure IV - 3), leakage of material which absorbed at 260 mμ almost reached a constant level after forty-five minutes. The accumulation of leakage products was less between fifteen and forty-five minutes than betweeen zero and fifteen minutes. The accumulation of leakage products between forty-five minutes and three and one-quarter hours depended on the pH of the extracting solution.

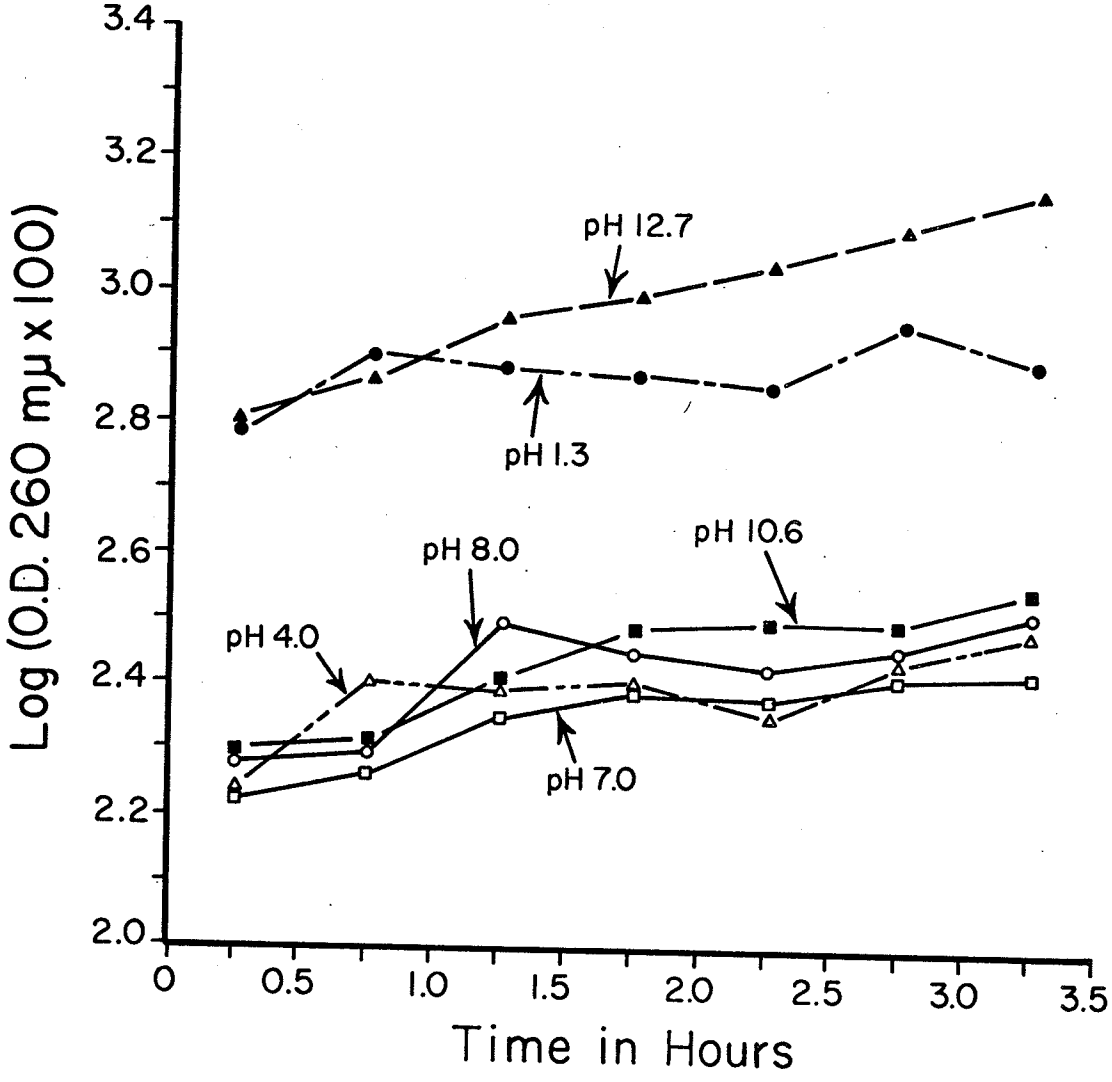


Figure IV - 2 Effect of time and pH on the leakage of 260 mμ-absorbing material from HHT at 0°C.

Figure IV-2

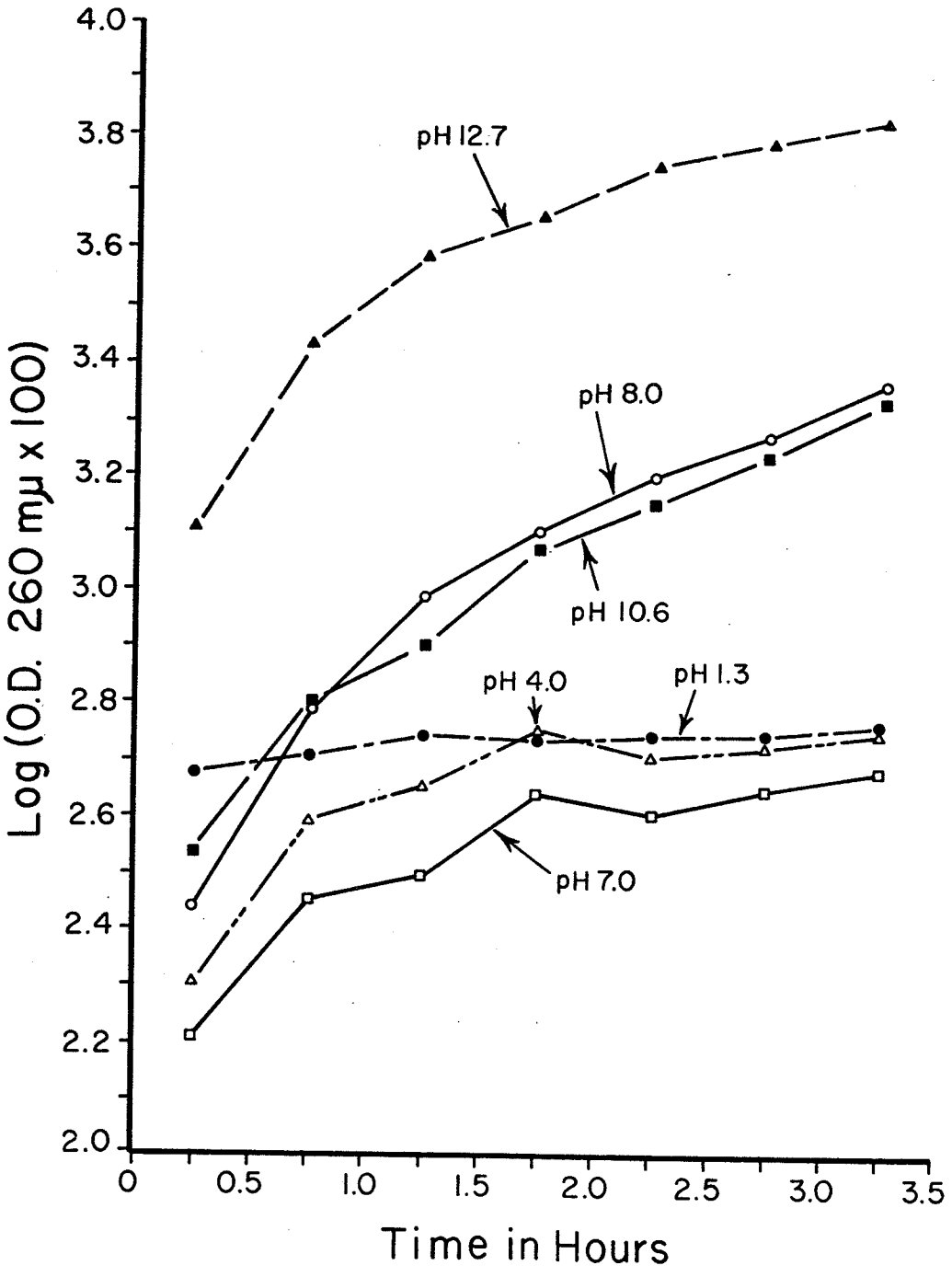


Figure IV - 3 Effect of time and pH on the leakage 260 mμ-absorbing material from HHT at 37°C.

At 60°C, (Figure IV - 4) leakage at pH 1.3 and at pH 4.0 reached a maximum after fifteen minutes; whereas at pH 8.0, 10.6 and 12.7 it required forty-five minutes. Leakage at pH 7.0 reached constant level after one and three quarter hours.

Effect of the Number of Extracts

The effect of the number of extracts on Veillonella sp. was studied. Table IV - 1 shows the percent protein and/or nitrogen extracted by two consecutive extracts with 0.1N NaOH, bicarbonate buffer and hypotonic saline (0.4%). This table clearly shows that a second extract increased the amount of protein and/or nitrogen extracted.

Table IV - 1 Effect of the number of extracts on the mean percent protein and nitrogen extracted from Veillonella sp.

Buffer	Extract	Length of time in hours	% Protein Extd.	% N Extd.
0.1N NaOH, pH 13.0	1st	1	35.5	44.1
	2nd	1	53.1	58.4
0.01M Bicarbonate pH 9.35	1st	1	3.25	
	2nd	1	8.59	
Hypotonic saline (0.4%)	1st	1	1.14	
	2nd	1	3.17	

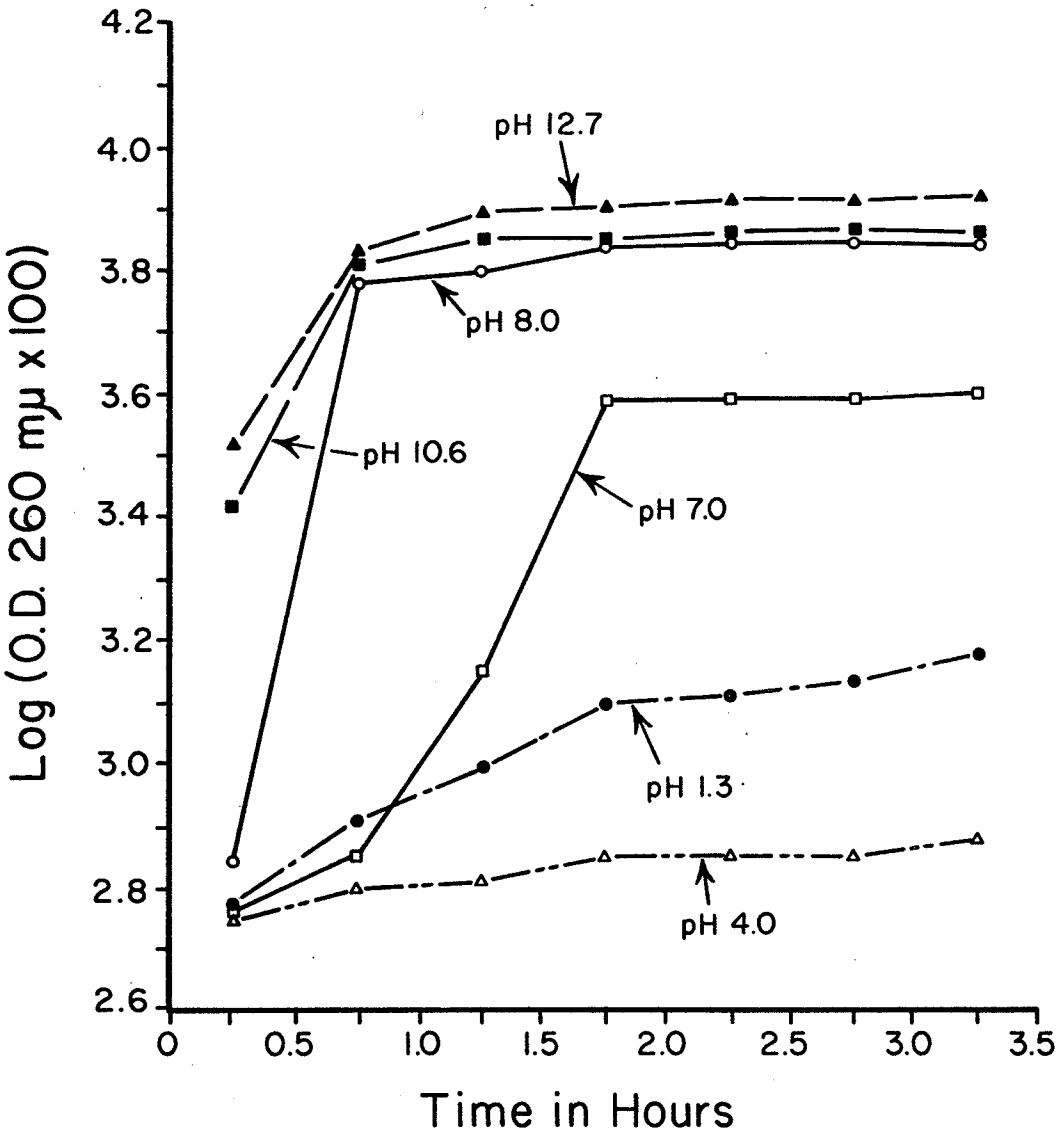


Figure IV - 4 Effect of time and pH on the leakage of 260 mμ-absorbing material from HHT at 60°C.

Correlation of Nitrogen and Protein Values

Table IV - 2 and Figure IV - 5 show the percent protein and/or nitrogen extracted from L. acidophilus at 0°C and 23°C over the pH range of 1.0 to 13.0. The percent nitrogen extracted is usually higher than the percent protein extracted at a given pH and temperature.

Effect of Buffer Type

In order to eliminate the possibility of a specific buffer anion effect on the percent protein extracted, phosphate and bicarbonate buffers were made (Chapter V method) to pH 7.0 and an ionic strength of 0.05. At pH 7.0, the bicarbonate and phosphate buffers respectively extracted 1.69 and 1.71 percent protein from Veillonella sp. at 37°C. These results demonstrate that the type of anion had little or no effect at the pH level studied.

Ionic Strength

The effect on the mean percent protein extracted from AHT and BHT of buffers at three different ionic strengths over the pH range of 1.3 to 12.7 was studied. Ionic strength had no statistically significant effect under these conditions.

Bacterial Concentration

The effect of differences in the concentration of microorganisms was studied in separate tubes containing one ml of pH 7.0 phosphate buffer at 37°C and is presented in Figure IV - 6. After centrifugation, the concentration of protein in the supernatant was determined

as was the total present in each tube at zero time. The percent protein extracted was independent of the bacterial concentration in the range 0 - 4 mg/ml.

Table IV - 2 Mean percent protein and nitrogen extracted from L. acidophilus at eleven pH levels.

pH	PERCENT PROTEIN EXTRACTED		PERCENT NITROGEN EXTRACTED	
	0°C	23°C	0°C	23°C
1.0	4.40	7.80	16.03	16.57
4.0	0.36	0.78	1.54	1.67
4.5	0.25	0.52	0.88	1.32
5.0	1.28	0.77	1.16	1.84
5.5	0.35	0.88	1.41	2.98
6.0	0.42	0.88	1.30	2.89
7.0	0.83	1.31	1.44	3.36
8.0	1.64	1.06	1.54	2.65
9.0	0.90	0.90	1.50	3.67
10.8	4.99	13.27	9.57	11.90
13.0	34.67	34.23	26.90	26.90

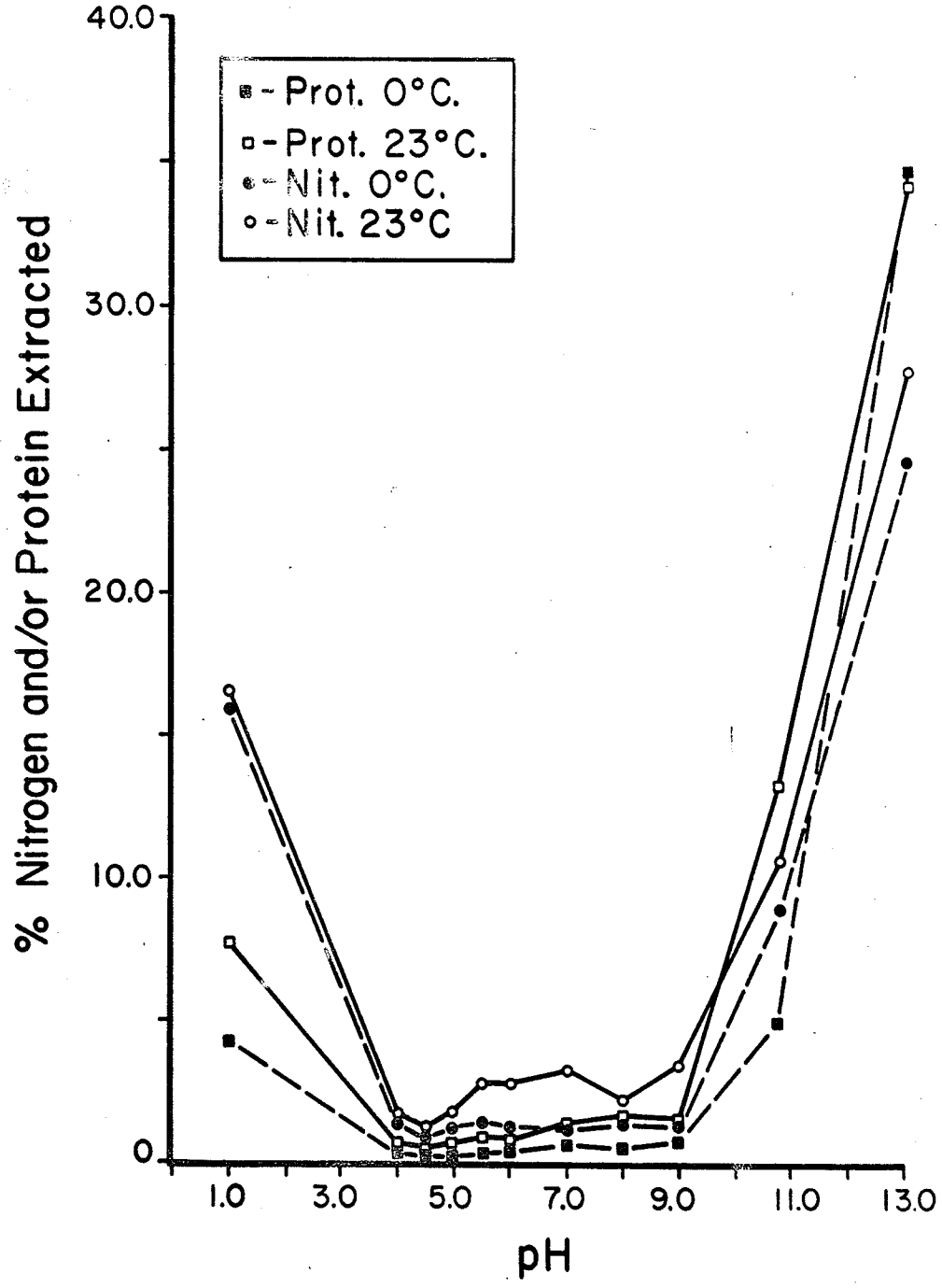


Figure IV - 5 Mean percent protein and nitrogen extracted from L. acidophilus at eleven pH levels.

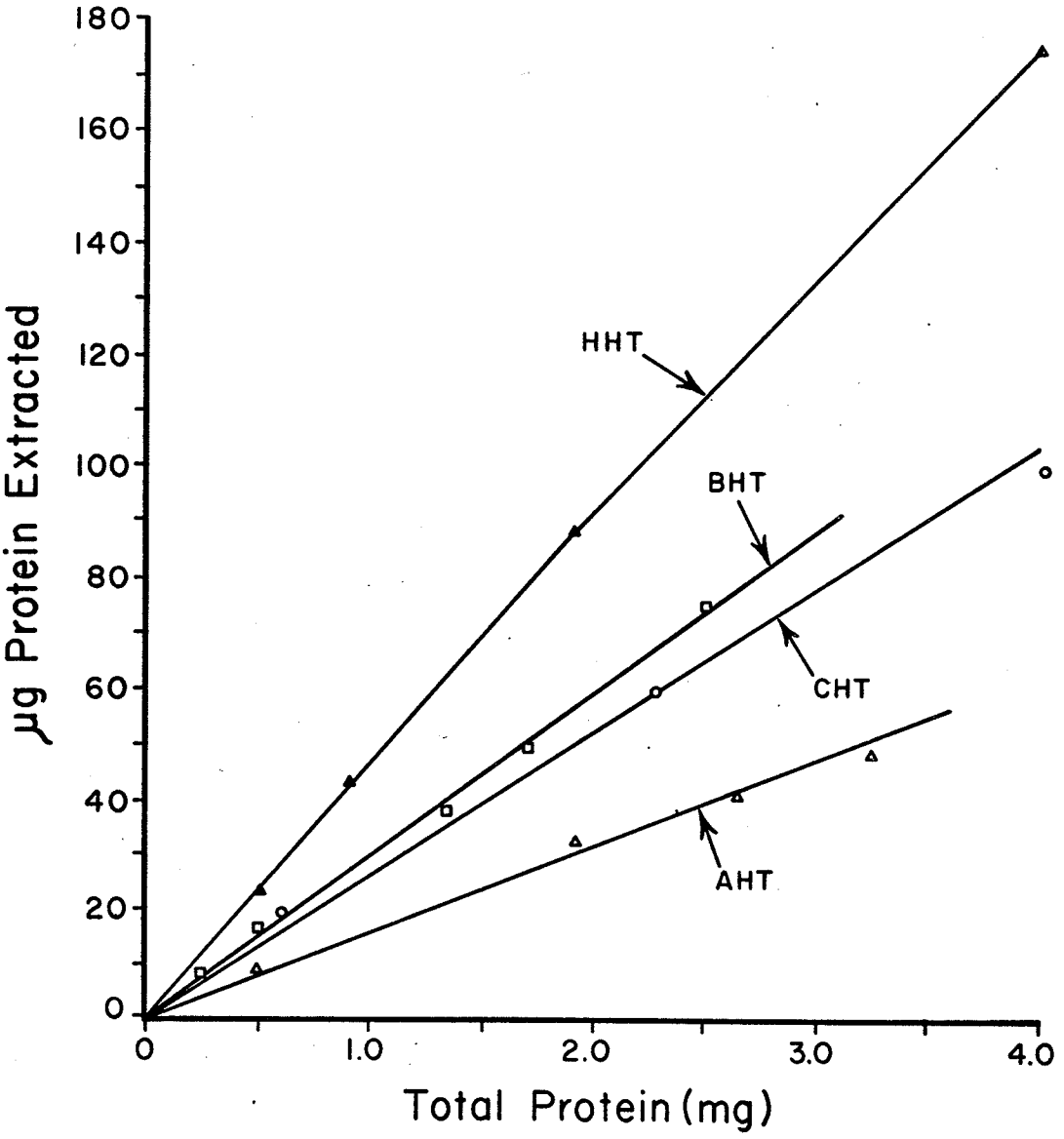


Figure IV.- 6 Relationship of percent protein extracted to the total protein of HHT, BHT, CHT, and AHT.

CHAPTER V

RESULTS

CHAPTER V

RESULTS

EFFECTS OF TEMPERATURE AND pH ON THE EXTRACTION OF PROTEIN FROM EIGHT DENTAL PLAQUE MICROORGANISMS

The combined analysis of variance for the mean percent protein extracted from the eight oral microorganisms studied in Experiment 1 is shown in Table V - 1.

All main effects and interactions were significant at the 1% level. The means and standard error are presented in Tables V - 2 to V - 8 and in Appendix 1.

Main Effects

1. Temperature

Table V - 2 and Figure V - 1 show the effect of temperature on the mean percent protein extracted. The mean percent protein extracted at each temperature was obtained from eight microorganisms tested at eight pH levels with a total of thirty-one replications. The means for the percent protein extracted at the four temperatures are significantly different ($p < 0.01$) from each other. The mean percent protein extracted increased with an increase in temperature from $0^{o}C$.

Table V - 1 Combined analysis of variance for the effect of temperature and pH on the percent protein extracted from the oral microorganisms.

SOURCE OF VARIATION	DF	MEAN SQUARE	SIGNIF. LEVEL
Replications	23	18.22	1%
Temperature	3	1022.38	1%
pH	7	1588.65	1%
Microorganism	7	281.47	1%
Micro. X Temp.	21	13.33	1%
Micro. X pH	49	143.77	1%
Temp. X pH	21	21.05	1%
Micro. X pH X Temp.	147	6.54	1%
Experimental Error	713	2.23	
Total	991		

Table V - 2 Mean percent protein extracted at the four temperatures over the eight microorganisms and eight pH levels.

TEMPERATURE	MEAN PERCENT PROTEIN EXTRACTED	STANDARD ERROR
0°C	4.24	±0.09
23°C	4.66	±0.09
37°C	6.38	±0.09
60°C	8.71	±0.09

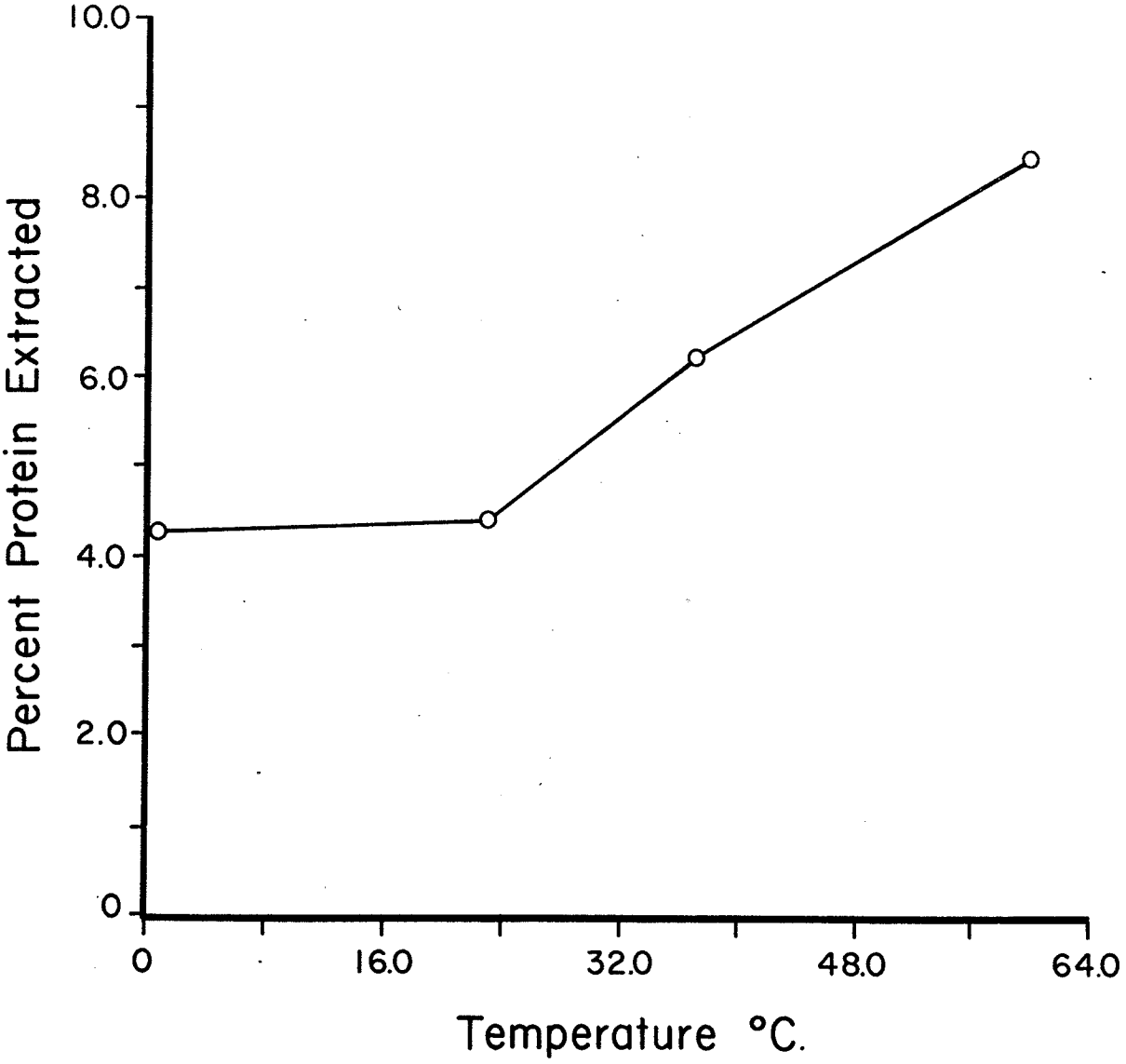


Figure V - 1 Mean percent protein extracted at the four temperatures over the eight microorganisms and eight pH levels.

2. pH

Table V - 3 and Figure V - 2 show the effect of pH on the mean percent protein extracted. The mean percent protein extracted at each pH was obtained from the eight microorganisms tested at four temperatures with a total of thirty - one replications. No significant difference in the means for the percent protein extracted were found between pH 8.0 and at pH 9.0. The means for the percent protein extracted at all other pH values were significantly different ($p < 0.01$) from each other. The minimum percent protein extracted was at pH 5.0 and the maximum was at pH 12.7.

3. Type of Microorganism

Table V - 4 and Figure V - 3 show the mean percent protein extracted from each microorganism. The percent protein extracted from each microorganism is obtained from thirty-two treatment combinations (eight pH X four temperature). The number of replications varied from 2 - 6 for the individual microorganisms resulting in different standard errors. Significantly more protein was extracted from the L. casei than from the other microorganisms ($p < 0.01$). In addition, more protein was extracted from the Veillonella sp. than from the Diphtheroid sp. ($p < 0.01$). There were no significant differences in the mean percent protein extracted from the other five microorganisms and these values were significantly less than those from L. casei, the Veillonella sp. and the Diphtheroid sp. ($p < 0.001$).

Table V - 3 Mean percent protein extracted at the eight pH levels over the eight microorganisms at the four temperatures.

pH	MEAN PERCENT PROTEIN EXTRACTED	STANDARD ERROR
1.3	7.20	±0.13
4.0	3.37	±0.13
5.0	2.83	±0.13
7.0	4.16	±0.13
8.0	5.30	±0.13
9.0	4.97	±0.13
10.6	6.00	±0.13
12.7	14.14	±0.13

Table V - 4 Mean percent protein extracted from each microorganism over the 32 treatment combinations.

MICROORGANISM	MEAN PERCENT PROTEIN EXTRACTED	STANDARD ERROR
HHT	4.92	±0.12
AHT	4.97	±0.11
BHT	5.13	±0.15
CHT	5.23	±0.12
Corynebacterium sp.	5.24	±0.18
Diphtheroid sp.	7.25	±0.13
Veillonella sp.	7.84	±0.15
L. casei	8.98	±0.15

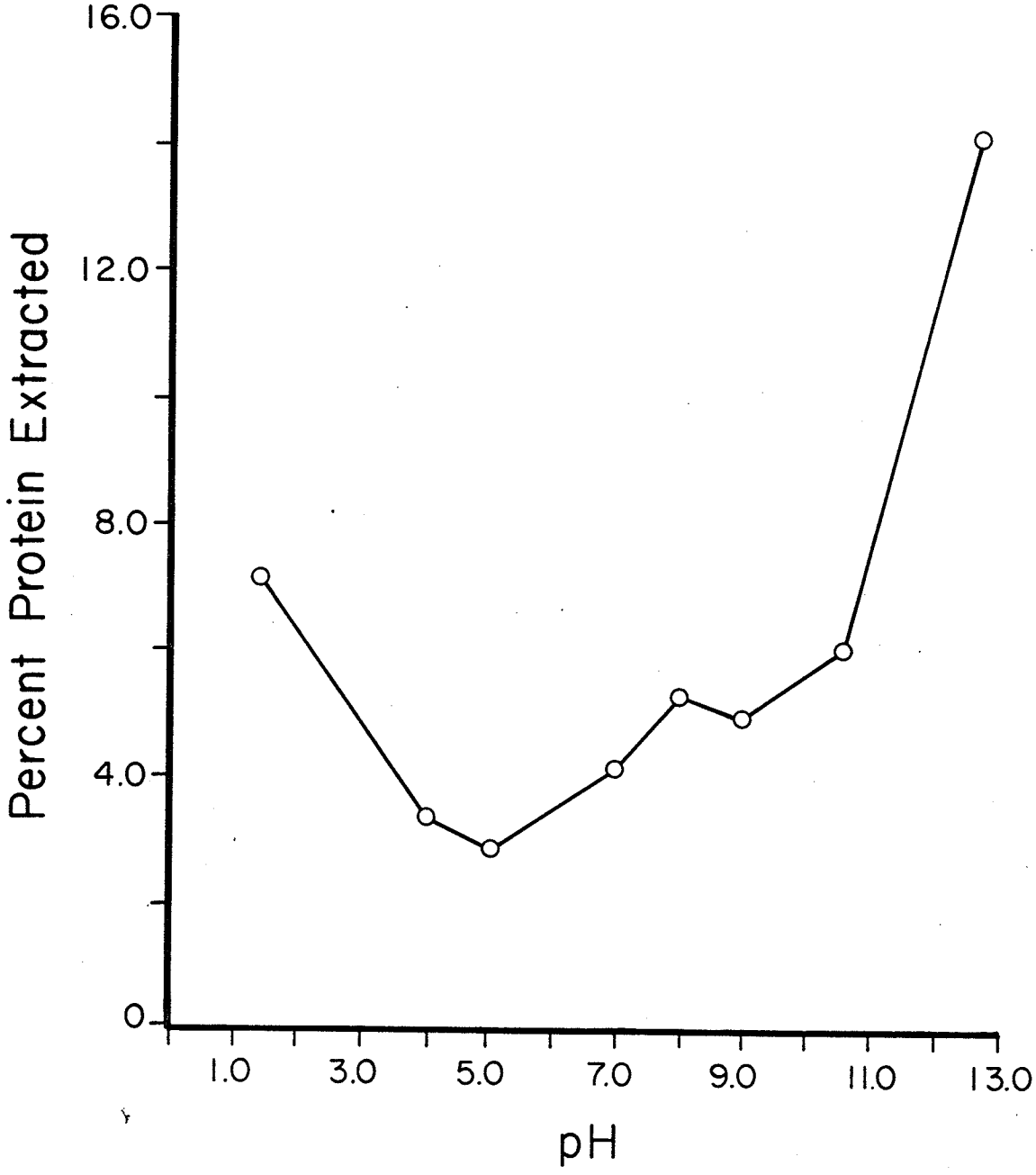


Figure V - 2 Mean percent protein extracted at the eight pH levels over the eight microorganisms at at the four temperatures.

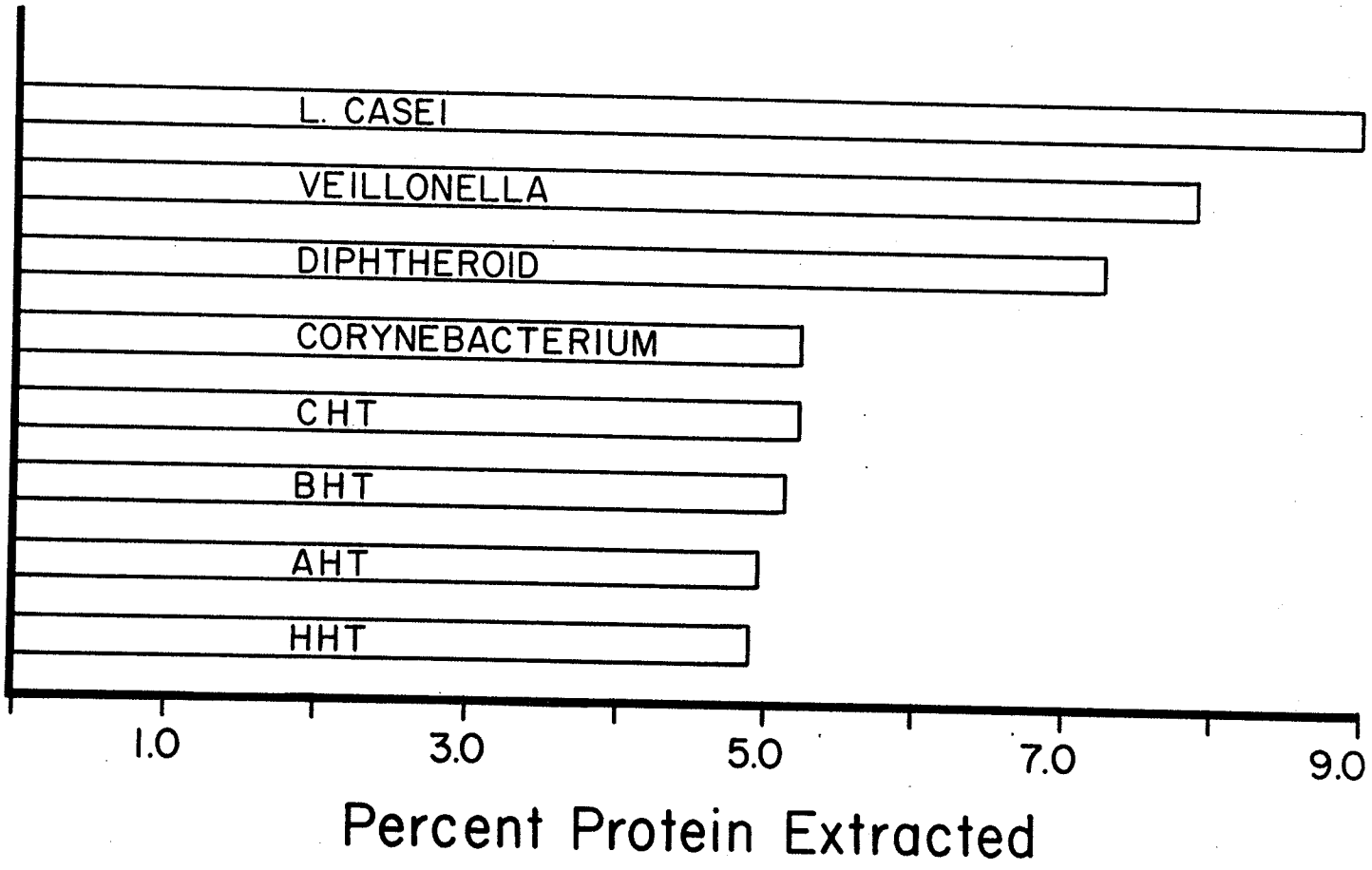


Figure V - 3 Mean percent protein extracted from each micro-organism over the thirty-two treatment combinations.

Second Order Interactions

1. Microorganism x Temperature

The results are shown in Table V - 5 and Figure V - 4.

(a) For each individual microorganism a comparison was made between the percent protein extracted at each of the four temperatures.

The mean percent protein extracted from AHT, for example, was not significantly different at $0^{o}C$ and $23^{o}C$. At $23^{o}C$, $37^{o}C$, and $60^{o}C$, however, significant differences ($p < 0.01$) in the percent protein extracted were found, more protein being extracted the higher the temperature. Similarily, for the other individual microorganisms, there was no significant difference in the percent protein extracted at $0^{o}C$, $23^{o}C$ but the amounts extracted at $23^{o}C$, $37^{o}C$, and $60^{o}C$ were significantly different.

(b) For each of the four temperatures the mean percent protein extracted from the eight microorganisms was compared.

$0^{o}C$

At $0^{o}C$, significantly more protein ($p < 0.01$) was extracted from *L. casei* than from the other seven microorganisms. The amounts extracted from the *Diphtheroid sp.* and the *Veillonella sp.* were significantly greater ($p < 0.01$) than the amounts extracted from the other five microorganisms.

Table V - 5 Mean percent protein extracted from each of the eight microorganisms at the four temperatures over the eight pH levels.

MICROORGANISMS	TEMPERATURE (°C)				STANDARD ERROR
	0	23	37	60	
Corynebacterium sp.	3.02	3.52	5.20	9.23	±0.37
HHT	3.15	3.63	5.38	7.52	±0.24
CHT	3.35	4.08	5.94	7.52	±0.24
AHT	3.61	3.80	5.52	6.96	±0.22
BHT	4.00	4.23	5.39	6.90	±0.30
Diphtheroid sp.	4.95	5.46	8.19	10.40	±0.26
Veillonella sp.	5.43	5.81	7.52	12.47	±0.30
L. casei	7.60	8.02	8.75	11.55	±0.30

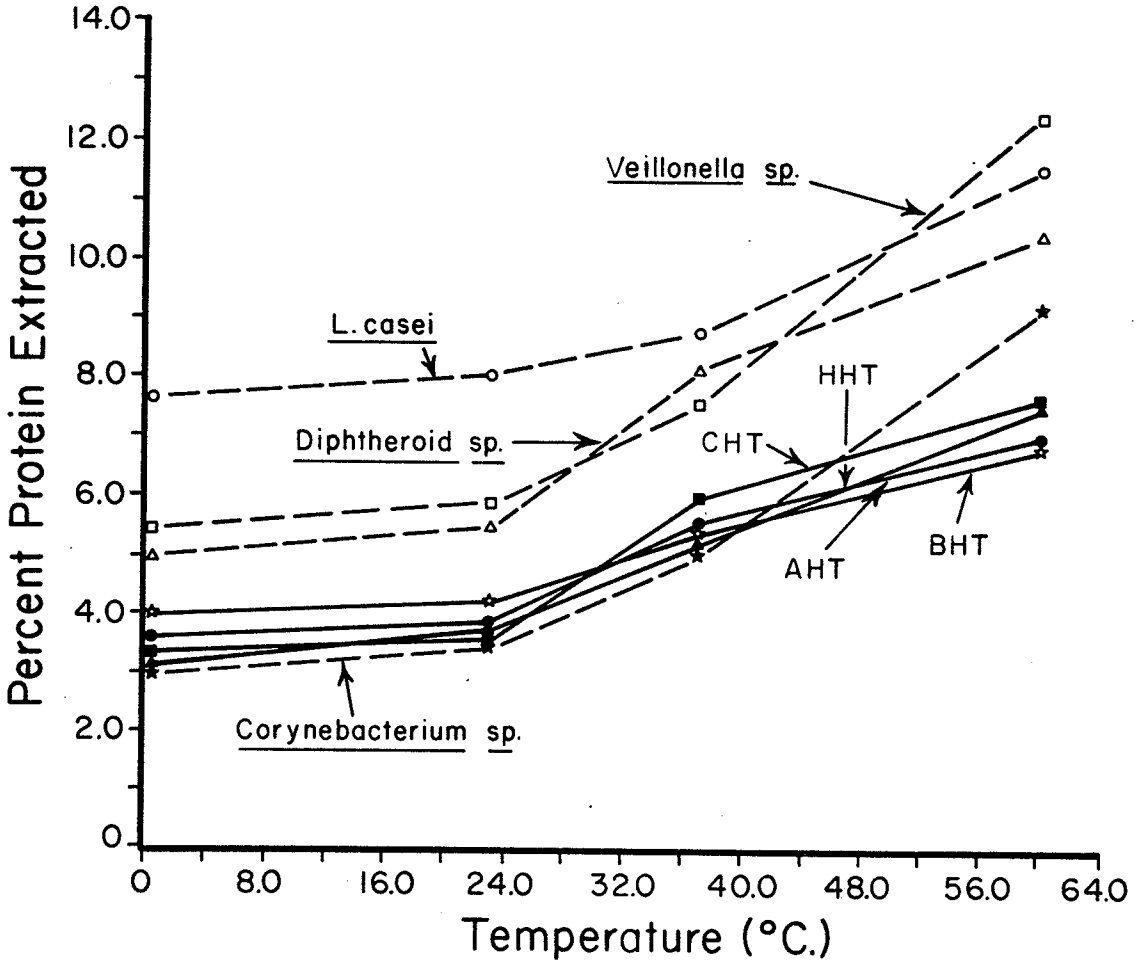


Figure V - 4 Mean percent protein extracted from each of the eight microorganisms at the four temperatures over the eight pH levels.

23°C

At 23°C, the amount of protein extracted from L. casei was significantly greater ($p < 0.001$) than that from AHT, BHT, CHT, HHT, and the Corynebacterium sp. The amounts of protein extracted from the Diphtheroid sp. and Veillonella sp. were significantly greater than those from the other five microorganisms (AHT, BHT, CHT, HHT, Corynebacterium sp.). No significant differences in the amounts of protein extracted from the four Streptococcus sp. and the Corynebacterium sp. were found.

37°C

At 37°C, more protein was extracted from the L. casei than from the Diphtheroid sp. ($p < 0.05$) and from the other six microorganisms ($p < 0.001$). The percent protein extracted from the Veillonella sp. and the Diphtheroid sp. was significantly greater ($p < 0.001$) than that from AHT, BHT, CHT, HHT, and the Corynebacterium sp.. No significant differences in the amount of protein extracted from AHT, BHT, CHT, HHT, and the Corynebacterium sp. were found,

60°C

At 60°C, the amount of protein extracted from L. casei was significantly greater ($p < 0.001$) than that extracted from AHT, BHT, CHT, HHT, the Corynebacterium sp. and the Diphtheroid sp.; however, at this one temperature, the amount extracted from the L. casei was significantly less ($p < 0.01$) than that extracted from the Veillonella sp. The amount of protein extracted from the

Corynebacterium sp. and Diphtheroid sp. was significantly greater ($p < 0.001$) than that from the AHT, BHT, CHT, HHT. As shown previously, at the other three temperatures, there were no significant differences in the amount of protein extracted from the different streptococci.

2. Microoganism x pH

The results are shown in Table V - 6 and Figure V - 5.

(a) For each of the eight microorganisms the mean percent protein extracted at the eight pH levels was compared.

AHT

With AHT, the amount of protein extracted at pH 5.0 was significantly less ($p < 0.01$) than the amount extracted by the other buffers except the pH 7.0 buffer. The difference in the percent protein extracted between pH 5.0 and pH 9.0 was only significant at the 5% level. The amount of protein extracted by the pH 12.7 buffer was significantly greater ($p < 0.01$) than that extracted by the other seven pH buffers.

BHT

For BHT, the results were similar to those obtained with AHT. The amount of protein extracted at pH 5.0 was significantly less than the amount extracted at pH 7.0, at pH 4.0 ($p < 0.05$), and at the other five pH values ($p < 0.01$). At pH 12.7 and pH 1.3 the amount

Table V - 6 Mean percent protein extracted from the eight microorganisms at the eight pH levels over the four temperatures.

MICROORGANISMS	pH 1.3	4.0	5.0	7.0	8.0	9.0	10.6	12.7	STANDARD ERROR
AHT	7.48	3.98	2.75	3.56	4.01	3.82	4.40	9.75	±0.30
BHT	7.28	4.35	2.97	4.21	4.64	4.55	4.97	8.07	±0.43
CHT	5.44	3.35	2.86	4.30	5.30	4.55	5.27	10.73	±0.40
HHT	5.72	3.30	2.91	4.47	4.49	4.48	4.66	9.34	±0.33
Diphtheroid sp.	8.32	3.68	3.80	5.55	6.57	6.42	8.01	15.65	±0.37
L. casei	12.68	4.11	3.54	5.83	8.69	8.00	10.51	18.48	±0.43
Veillonella sp.	7.35	1.38	1.28	2.01	4.64	4.13	6.62	35.33	±0.43
Corynebacterium sp.	3.66	1.54	1.84	2.81	5.47	5.10	5.83	15.70	±0.53

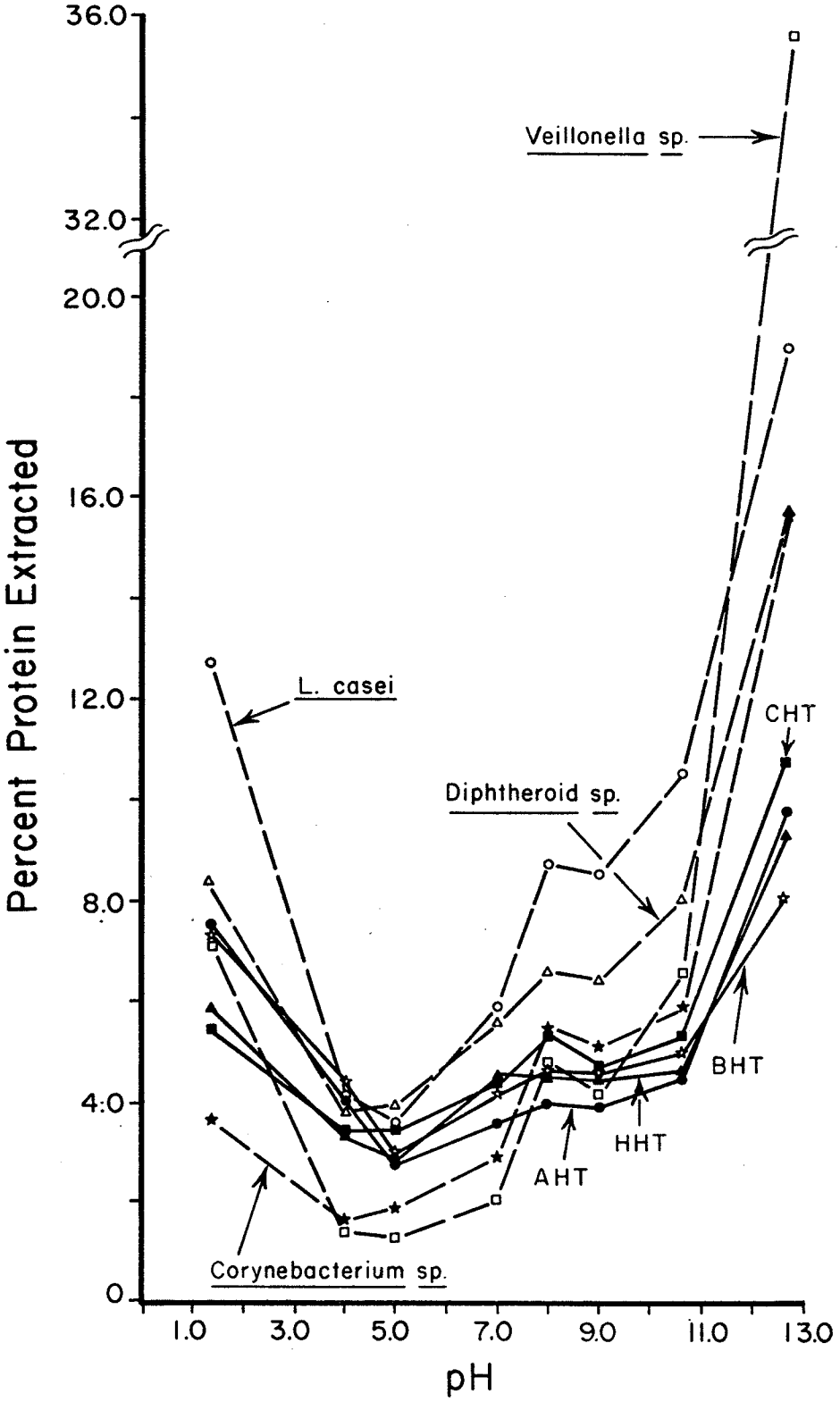


Figure V - 5 Mean percent protein extracted from the eight microorganisms at the eight pH levels over the four temperatures.

of protein extracted was significantly greater ($p < 0.01$) than at the other six pH values; however, no significant difference in the percent protein extracted at pH 12.7 and at pH 1.3 was found.

CHT

With CHT, the amount of protein extracted at pH 5.0 was significantly less ($p < 0.01$) than the amount extracted at the other seven pH levels except pH 4.0 The amount of protein extracted at pH 12.7 was significantly greater ($p < 0.001$) than that at the other seven pH levels.

HHT

With HHT, the amount of protein extracted at pH 5.0 was significantly less ($p < 0.01$) than the amount extracted at the other seven pH levels with the exception of pH 4.0. The amount of protein extracted at pH 12.7 was significantly greater ($p < 0.001$) than that extracted at the other seven pH levels.

Diphtheroid species

With the Diptheroid sp. the percent protein extracted was significantly less ($p < 0.001$) at pH 4.0 and at pH 5.0 than the amount extracted at each of the other six pH levels. The amount of protein extracted at pH 12.7 was significantly greater ($p < 0.001$) than that extracted at the other seven pH levels.

Lactobacillus casei

With L. casei the amounts of protein extracted at pH 4.0 and

at pH 5.0 were not significantly different from each other. Also, the amounts of protein extracted at pH 8.0 and at pH 9.0 were not significantly different from each other. The maximum percent protein extracted was at pH 12.7 and the minimum was at pH 4.0 and at pH 5.0.

Veillonella species

With Veillonella sp. the means for the percent protein extracted at pH 5.0, pH 4.0 and at pH 7.0 were not significantly different, but these buffers extracted significantly less protein ($p < 0.001$) than at each of the other five pH levels. No significant difference in the percent protein extracted occurred at pH 8.0 and at pH 9.0 The amount of protein extracted at pH 12.7 was significantly greater ($p < 0.001$) than the amount of protein extracted at the other seven pH levels.

Corynebacterium species

With Corynebacterium sp. the amounts of protein extracted at pH 4.0, at pH 5.0, and at pH 7.0 were not significantly different from each other; but all three levels were significantly less ($p < 0.01$) than the amounts extracted at the other five pH levels. The amount of protein extracted at pH 12.7 was significantly greater ($p < 0.001$) than the amount extracted at the other seven pH levels.

(b) For each pH the mean percent protein extracted from the eight individual microorganisms was compared.

pH 1.3

At pH 1.3, the amount of protein extracted from the Corynebacterium sp., was significantly less ($p < 0.01$) than the amount extracted from the other seven microorganisms. The amounts of protein extracted from CHT and from HHT were not significantly different; but, both had significantly less ($p < 0.01$) protein extracted from them than BHT, AHT, the Veillonella sp. and the Diphtheroid sp. Significantly more protein was extracted from L. casei than from each of the other seven microorganisms.

pH 4.0

At pH 4.0, significantly less ($p < 0.01$) protein was extracted from the Corynebacterium sp. and the Veillonella sp. than was extracted from the other six microorganisms. The amounts of protein extracted from the other six microorganisms were not significantly different from each other.

pH 5.0

At pH 5.0, the percent protein extracted from the Veillonella sp. was significantly less ($p < 0.01$) than the amount extracted from the other seven microorganisms. The amount of protein extracted from the Corynebacterium sp. was significantly less than the amount extracted from the Diphtheroid sp. Similar amounts of protein were extracted from AHT, BHT, CHT, HHT, L. casei and the Diphtheroid sp. .

pH 7.0

At pH 7.0, the percent protein extracted from the Veillonella sp. was not significantly different from the amount extracted from the Corynebacterium sp., but it was significantly less ($p < 0.01$) than the amounts extracted from the other six microorganisms. The percent protein extracted from L. casei was significantly greater ($p < 0.01$) than the amount extracted from BHT, CHT, HHT, the Veillonella sp. and the Corynebacterium sp. The means for the percent protein extracted from the four streptococci were not significantly different from each other.

pH 8.0

At pH 8.0, the amount of protein extracted from L. casei was significantly greater ($p < 0.001$) than the amount extracted from the other seven microorganisms. The percent protein extracted from the Diphtheroid sp. was significantly greater than the amount extracted from the AHT, BHT, HHT, and the Veillonella sp. No significant differences in the percent protein extracted were found with the other six microorganisms.

pH 9.0

At pH 9.0, the results were similar to those obtained for pH 8.0 except for the Diphtheroid sp.; the percent protein extracted from the Diphtheroid sp. was significantly greater ($p < 0.001$) than that from AHT, BHT, CHT, HHT, and the Veillonella sp..

pH 10.6

At pH 10.6, the means for the percent protein extracted from the L. casei were significantly greater than the means for the other seven microorganisms. There was no significant difference in the means for the percent protein extracted from the Veillonella sp. and Diphtheroid sp., but both were significantly greater ($p < 0.01$) than the means for the other five microorganisms, (AHT, BHT, CHT, HHT, and Corynebacterium sp.) There were no significant differences in the percent protein extracted from these five microoganisms.

pH 12.7

The largest variation in the percent protein extracted occurred at this pH. The following combinations of the percent protein extracted from the microorganisms were not significantly different: Corynebacterium sp. and Diphtheroid sp. BHT and HHT, AHT and HHT, and AHT and CHT. All other possible combinations of the means for the percent protein extracted were significantly different from each other.

3. pH x Temperature

For each individual pH and temperature the percent protein extracted is shown in Table V - 7 and Figures V - 6 and V -6a.

Table V - 7 Mean percent protein extracted at the four temperatures and eight pH levels over the eight microorganisms.

TEMPERATURE	pH 1.3	4.0	5.0	7.0	8.0	9.0	10.6	12.7	MEAN
0°C	5.93	2.70	1.20	2.47	3.16	2.80	3.64	11.22	4.24
23°C	6.37	2.59	1.92	2.61	3.42	2.96	4.29	13.10	4.66
37°C	6.66	3.59	2.79	4.11	6.27	5.63	7.32	14.67	6.38
60°C	9.83	4.60	4.59	7.48	8.34	8.47	8.76	17.59	8.71
Mean	7.20	3.37	2.83	4.16	5.30	4.97	6.00	14.14	

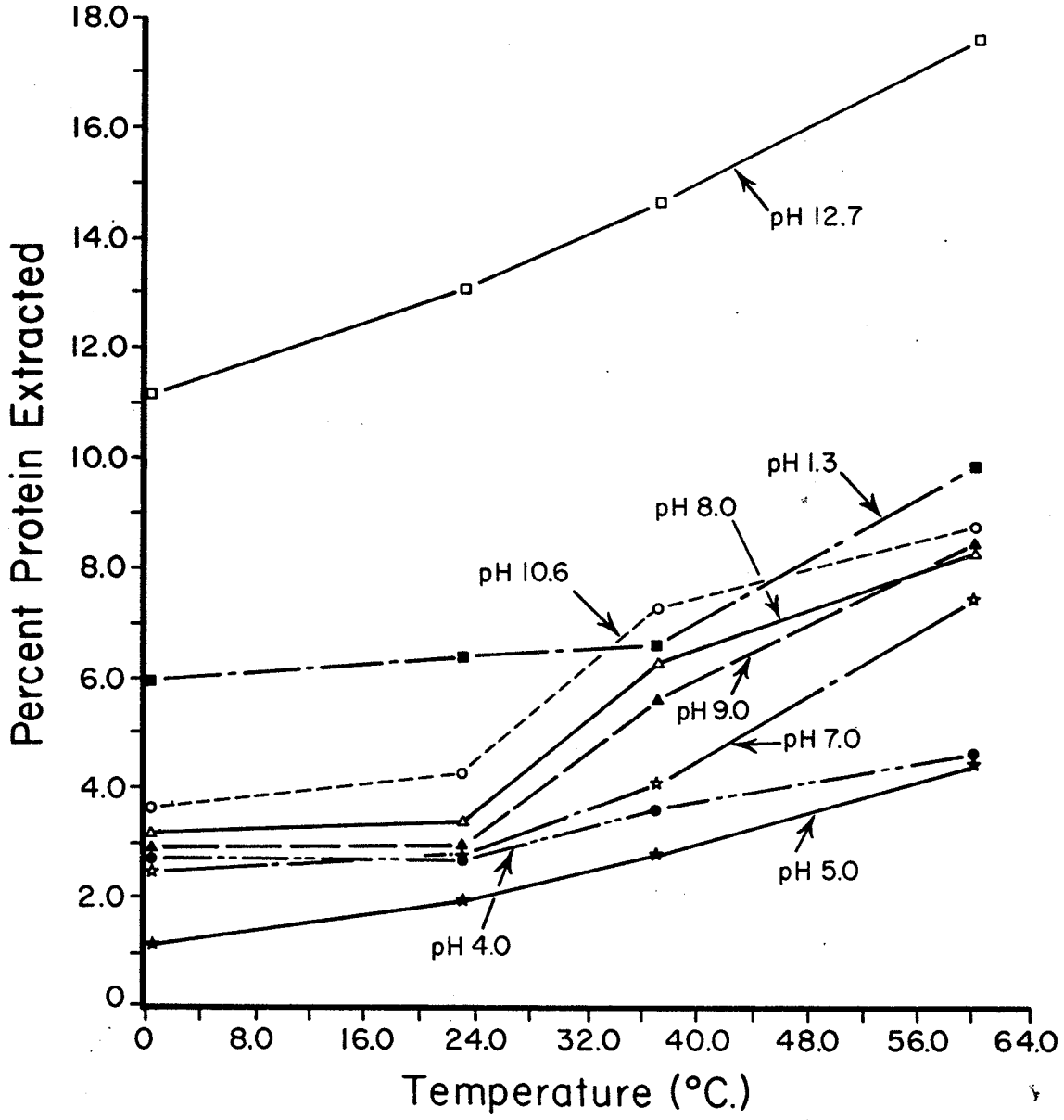


Figure V - 6 Mean percent protein extracted at the eight pH levels and four temperatures over the eight microorganisms.

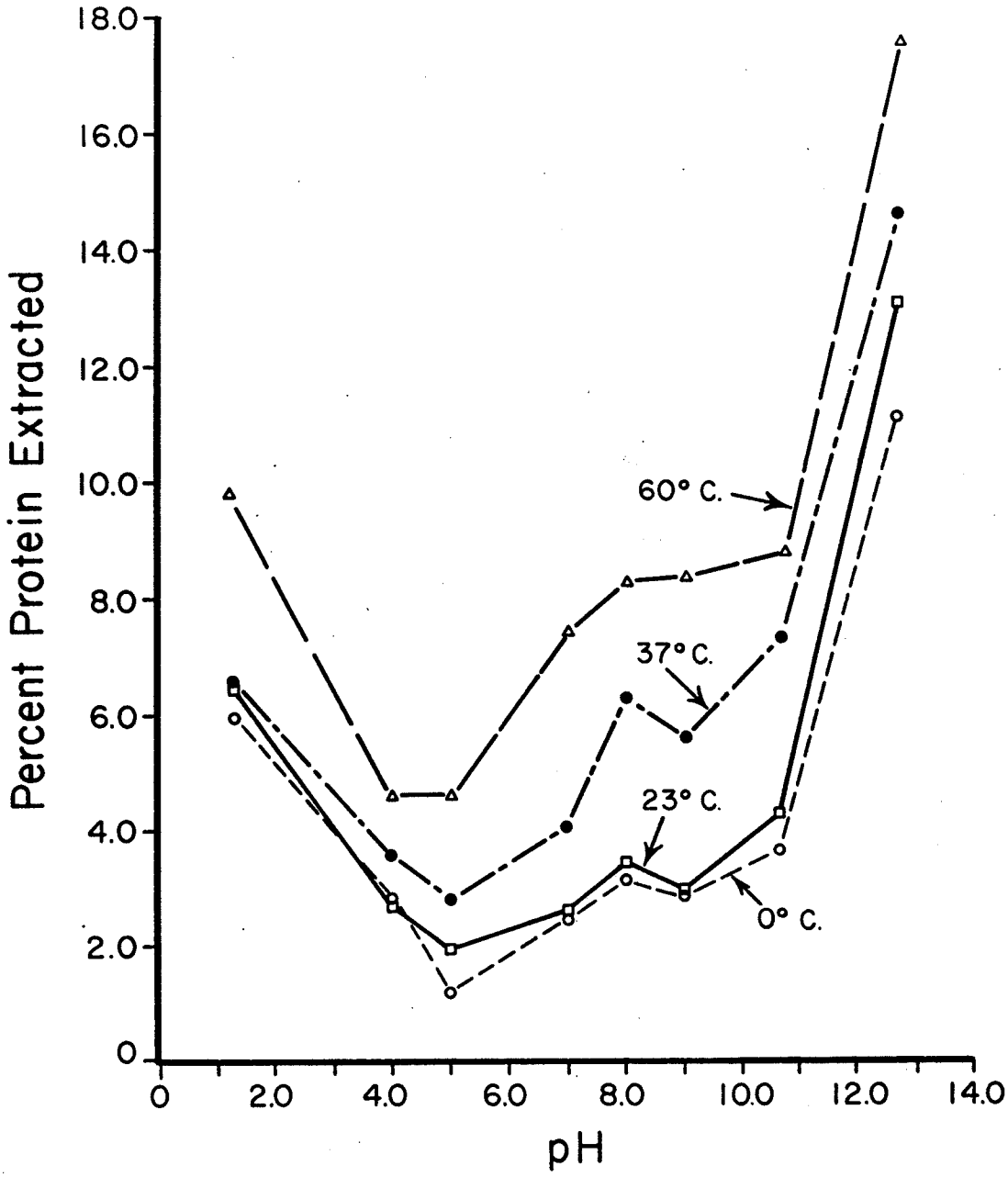


Figure V - 6a Mean percent protein extracted at the four temperatures and eight pH levels over the eight microorganisms.

(a) For each pH the effect of the four temperatures on the percent protein extracted was compared.

The percent protein extracted by each of the eight buffers at 0°C was not significantly different from the amount extracted at 23°C except that the pH 12.7 buffer extracted significantly more protein ($p < 0.001$) at 23°C than at 0°C.

Except at pH 1.3, the amount of protein extracted by each of the eight buffers at 37°C was significantly greater ($p < 0.05$) than the amounts extracted at 0°C.

The amount of protein extracted by each buffer at 37°C, except the pH 1.3 buffer, was significantly greater ($p < 0.05$) than that extracted at 23°C.

The percent protein extracted by each buffer at 60°C was significantly greater ($p < 0.01$) than that extracted by the corresponding buffer at the other three temperatures.

(b) For each temperature the effect of the eight pH levels on the percent protein extracted was compared.

0°C

At 0°C, the percent protein extracted at pH 5.0 was significantly less ($p < 0.001$) than that extracted at the other seven pH levels. The percent protein extracted at pH 12.7 was significantly greater ($p < 0.01$) than that extracted at each of the other pH levels.

23°C

At 23°C, the means for the percent protein extracted at pH 4.0, at pH 5.0, and at pH 7.0 were not significantly different from each other. Significantly more protein ($p < 0.001$) was extracted at pH 12.7 than at any of the other pH levels.

37°C

At 37°C, the percent protein extracted at pH 5.0 was significantly less ($p < 0.05$) than that extracted at each of the other pH levels. Significantly more protein ($p < 0.001$) was extracted at pH 12.7 than at any of the other seven pH levels.

60°C

At 60°C, the means for the percent protein extracted at pH 4.0 and at pH 5.0 were significantly less than those from the other six pH levels. Again, the most protein was extracted at pH 12.7 ($p < 0.001$).

Third Order Interactions

pH X Temperature X Microorganisms

Although the third order interaction was significant it is not discussed here due to its complexity. The means for the percent protein extracted are presented in Appendix I.

EFFECTS OF CALCIUM AND pH ON THE EXTRACTION OF PROTEIN FROM EIGHT DENTAL PLAQUE MICROORGANISMS

The combined analysis of variance for the mean percent protein extracted from the eight oral microorganisms studied in Experiment 2 is shown in Table V - 8.

All main effects and interactions were significant. The means and standard errors are presented in Tables V - 9 to V - 14 and in Appendix II.

Main Effects

1. Calcium

The percent protein extracted with or without calcium (Table V - 9) was obtained from the eight microorganisms tested at eight pH levels with thirty-seven replications. Calcium, in the concentration of 2 mM, caused a significant decrease ($p < 0.001$) in the percent protein extracted from the microorganisms.

2. pH

The effect of pH was similar to that found in Experiment 1 in that minimum protein extraction occurred at pH 5.0 and maximum extraction at pH 12.7 (Table V - 10).

Table V - 8 Combined analysis of variance for the effect of calcium and pH on the percent protein extracted from the oral microorganisms.

SOURCE OF VARIATION	DF	MEAN SQUARE	SIGNIF. LEVEL
Replications	29	10.21	1%
Calcium	1	40.21	1%
pH	7	744.77	1%
Microorganisms	7	111.82	1%
Micro. X Calcium	7	6.32	1%
Calcium X pH	7	38.05	1%
Micro. X pH	49	70.94	1%
Micro. X pH X Ca	49	8.27	1%
Experimental Error	435	1.62	
Total	591		

Table V - 9 Mean percent protein extracted at the two calcium levels over the eight microorganisms at the eight pH levels.

	PERCENT PROTEIN EXTRACTED	STANDARD ERROR
Without Calcium	6.18	±0.06
With 2mM Calcium	5.66	±0.06

3. Microorganism

The results obtained were similar to those recorded in Experiment 1 in that the amount of protein extracted from the individual microorganisms decreased in the order L. casei, Diphtheroid sp., Veillonella sp., AHT, AHT, HHT, BHT and Corynebacterium sp. (Table V - 11).

Second Order Interactions

1. Calcium X pH

The effect of 2 mM calcium on the mean percent protein extracted at each of the eight pH levels is shown in Table V - 12 and Figure V - 7. Calcium caused a significant decrease ($p < 0.001$) in the percent protein extracted at pH 9.0, 10.6, and 12.7. At pH 4.0, 5.0, 7.0, and 8.0, calcium had no effect on the percent protein extracted. At pH 1.3, significantly more protein ($p < 0.01$) was extracted with calcium present.

2. Calcium X Microorganisms

The effect of 2 mM calcium on the mean percent protein extracted from each of the eight microorganisms is shown in Table V - 13. Calcium caused a significant decrease in the amount of protein extracted from BHT ($p < 0.05$), the Diphtheroid sp., the Veillonella sp. and the Corynebacterium sp. ($p < 0.01$) but not

Table V - 10 Mean percent protein extracted at the eight pH levels over the eight microorganisms at the two calcium levels.

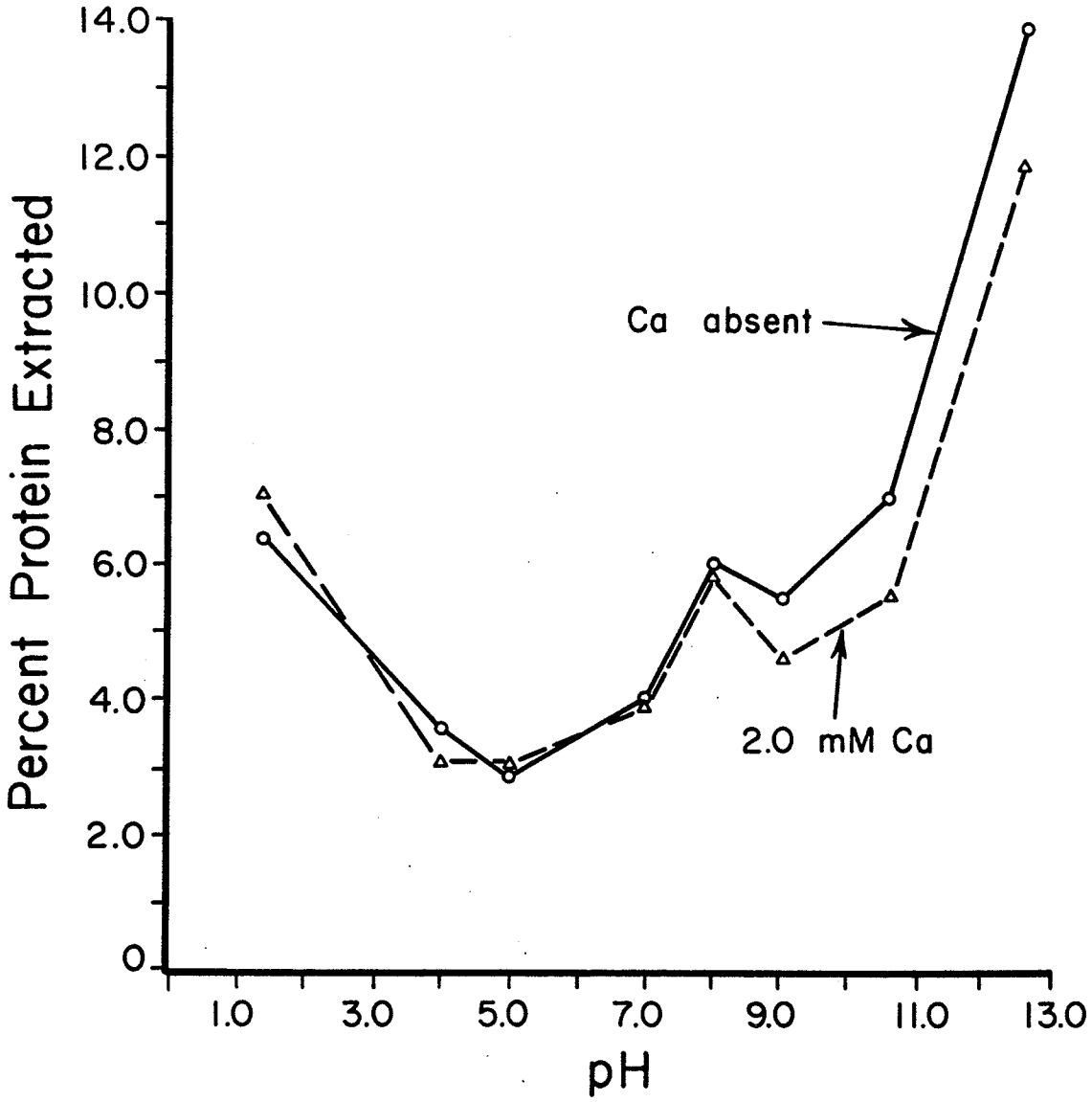
pH	MEAN PERCENT PROTEIN EXTRACTED	STANDARD ERROR
1.3	6.79	±0.12
4.0	3.38	±0.12
5.0	2.97	±0.12
7.0	3.99	±0.12
8.0	5.95	±0.12
9.0	5.07	±0.12
10.6	6.25	±0.12
12.7	12.94	±0.12

Table V - 11 Mean percent protein extracted from each microorganism over the 16 pH and calcium treatment combinations.

MICROORGANISM	MEAN PERCENT PROTEIN EXTRACTED	STANDARD ERROR
AHT	5.36	±0.11
BHT	5.19	±0.09
CHT	5.79	±0.12
HHT	5.23	±0.12
Diphtheroid sp.	7.32	±0.13
L. casei	8.95	±0.16
Veillonella sp.	6.78	±0.16
Corynebacterium sp.	4.25	±0.19

Table V - 12 Mean percent protein extracted at the eight pH and two calcium levels over the eight microorganisms.

	pH							
	1.3	4.0	5.0	7.0	8.0	9.0	10.6	12.7
Without calcium	6.45	3.60	2.87	4.03	6.02	5.52	6.96	13.97
With 2 mM calcium	7.14	3.15	3.07	3.96	5.88	4.62	5.53	11.91



Figures V - 7 Mean percent protein extracted at the eight pH and two calcium levels over the eight microorganisms.

Table V - 13 Mean percent protein extracted from each of the eight microorganisms at the two calcium levels over the eight pH levels.

MICROORGANISM	CALCIUM (mM) 0.0	2.0	STANDARD ERROR
AHT	5.52	5.20	0.16
BHT	5.32	5.06	0.13
CHT	5.94	5.65	0.17
HHT	5.38	5.07	0.17
Diphtheroid sp.	7.95	6.68	0.19
L. casei	8.74	9.16	0.22
Veillonella sp.	7.52	6.03	0.22
Corynebacterium sp.	5.19	3.32	0.27

from the AHT, CHT, HHT, or L. casei.

3. pH X Microorganisms

Although this interaction is significant it will not be discussed here. The means and standard errors of this interaction are presented in Table V - 14.

Third Order Interaction

pH X Microorganisms X Calcium

Although the third order interaction is significant it will not be discussed here due to its complexity. The means for the percent protein extracted are presented in Appendix II.

Table V - 14 Mean percent protein extracted from each microorganism at eight pH levels, over the two calcium levels.

MICROORGANISMS	pH								STANDARD ERROR
	1.3	4.0	5.0	7.0	8.0	9.0	10.6	12.7	
AHT	7.83	4.13	2.75	4.01	5.13	4.47	5.42	9.13	±0.31
BHT	6.33	3.69	2.72	4.07	5.09	5.21	5.45	8.99	±0.25
CHT	5.81	3.55	3.78	4.29	6.44	5.03	5.95	11.51	±0.34
HHT	6.04	3.34	3.43	4.58	5.39	4.75	5.12	9.17	±0.34
Diphtheroid sp.	7.11	3.86	4.36	4.55	8.38	6.15	5.56	14.59	±0.38
L. casei	10.52	3.27	2.98	5.51	11.30	9.57	10.60	17.87	±0.49
Veillonella sp.	6.76	1.14	1.01	1.28	3.21	1.86	5.35	33.60	±0.44
Corynebacterium sp.	3.84	1.99	1.75	2.17	3.67	3.05	4.05	13.50	±0.54

EFFECTS OF MAGNESIUM AND pH ON THE EXTRACTION OF PROTEIN FROM EIGHT DENTAL PLAQUE MICROORGANISMS

The combined analysis of variance for the mean percent protein extracted from the eight oral microorganisms studied in Experiment 3 is shown in Table V - 15.

All main effects and second order interactions were significant at the 1% level. The means and standard errors are presented in Tables V - 16 to V - 21 and in Appendix III.

Main Effects

1. Magnesium

The percent protein extracted with or without magnesium (Table V - 16) was obtained from the eight microorganisms tested at eight pH levels with thirty-seven replications. Magnesium, in the concentration of 1 mM, caused a significant decrease ($p < 0.001$) in the percent protein extracted from the microorganisms.

2. pH

The effect of pH was similar to that found in the Experiment 1 in that minimum protein extraction occurred at pH 5.0 and maximum protein extraction at pH 12.7 (Table V - 17).

3. Microorganism

The results obtained were similar to those recorded in the Experiment 1 in that the amount of protein extracted

Table V - 15 Combined analysis of variance for the effect of magnesium and pH on the percent protein extracted from the oral microorganisms

SOURCE OF VARIATION	DF	MEAN SQUARE	SIGNIF. LEVEL
Replications	29	11.75	1%
Magnesium	1	56.67	1%
pH	7	702.17	1%
Microorganism	7	118.69	1%
Micro. X Mg	7	8.05	1%
Magnesium x pH	7	9.48	1%
pH X Microorganisms	49	78.55	1%
Micro. X pH X Mg	49	1.35	-
Experimental Error	435	1.55	
Total	591		

Table V - 16 Mean percent protein extracted at two magnesium levels over the eight microorganisms

	MEAN PERCENT PROTEIN EXTRACTED	STANDARD ERROR
Without Magnesium	6.21	±0.07
With 1 mM Magnesium	5.56	±0.07

Table V - 17 Mean percent protein extracted at the eight pH levels from the eight oral microorganisms at the two magnesium levels.

pH	MEAN PERCENT PROTEIN EXTRACTED	STANDARD ERROR
1.3	6.78	±0.14
4.0	3.51	±0.10
5.0	2.88	±0.10
7.0	3.92	±0.10
8.0	5.87	±0.10
9.0	5.04	±0.10
10.6	6.36	±0.10
12.7	12.69	±0.10

Table V - 18 Mean percent protein extracted from each microorganism over the 16 pH and magnesium treatment combinations.

MICROORGANISM	MEAN PERCENT PROTEIN EXTRACTED	STANDARD ERROR
Corynebacterium sp.	4.23	±0.13
Bht	5.13	±0.11
AHT	5.20	±0.13
HHT	5.24	±0.14
CHT	5.75	±0.16
Veillonella sp.	6.65	±0.18
Diphtheroid sp.	7.67	±0.16
L. casei	8.71	±0.18

from the individual microorganisms decreased in the order L. casei, Diphtheroid sp., Veillonella sp., CHT, HHT, AHT, BHT, and the Corynebacterium sp. (Table V - 18).

Second Order Interactions

1) Magnesium X pH

The effect of 1 mM magnesium on the mean percent protein extracted at each of the eight pH levels is shown in Table V - 19 and Figure V - 8. Magnesium caused a significant decrease ($p < 0.01$) in the percent protein extracted at pH 9.0, 10.6 and 12.7. At the other five pH levels, however, magnesium had no effect on the mean percent protein extracted.

2) Magnesium X Microorganisms

The effect of 1 mM magnesium on the mean percent protein extracted from the eight microorganisms is shown in Table V - 20. Magnesium caused a significant decrease in the percent protein extracted from HHT ($p < 0.01$) AHT, BHT, the Veillonella sp., and the Corynebacterium sp. ($p < 0.01$) but not from the L. casei, BHT, CHT or Diphtheroid sp.

3) pH X Microorganisms

Although this interaction is significant it will not be discussed here. The mean and standard errors of this interaction are presented in Table V - 21.

Table V - 19 Mean percent protein extracted at the eight pH and two magnesium levels over the eight microorganisms.

	pH								MEAN
	1.3	4.0	5.0	7.0	8.0	9.0	10.6	12.7	
Without Magnesium	6.45	3.60	2.87	4.03	6.02	5.52	6.96	13.97	6.20
With 2 mM Magnesium	6.94	3.42	2.89	3.81	5.68	4.57	5.57	11.41	5.56
Mean	6.70	3.51	2.88	3.92	5.87	5.04	6.36		

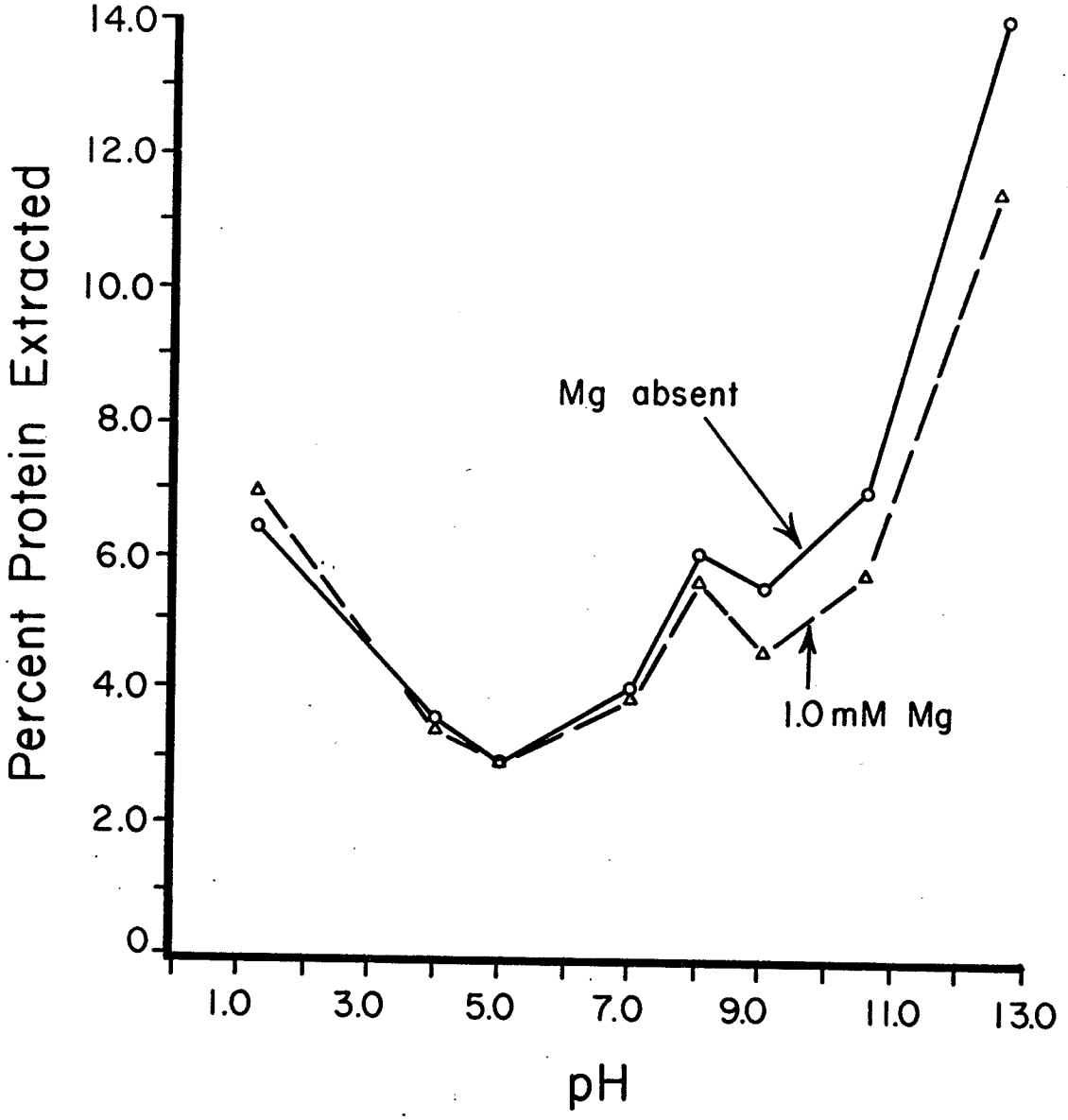


Figure V - 8 Mean percent protein extracted at the eight pH and two magnesium levels over the eight microorganisms.

Table V - 20 Mean percent protein extracted from each microorganism at two magnesium levels over the eight pH levels.

MICROORGANISM	MAGNESIUM		STANDARD ERROR
	0 mM	1 mM	
AHT	5.52	4.88	±0.18
BHT	5.32	4.93	±0.15
CHT	5.94	5.54	±0.20
HHT	5.38	5.09	±0.10
Diphtheroid sp.	7.95	7.39	±0.22
L. casei	8.74	8.37	±0.25
Veillonella sp.	7.52	5.85	±0.25
Corynebacterium sp.	5.19	3.27	±0.31

Table V - 21 Mean percent protein extracted from each microorganism at the eight pH levels over the two magnesium levels.

MICROORGANISM	pH								STANDARD ERROR
	1.3	4.0	5.0	7.0	8.0	9.0	10.6	12.7	
AHT	7.31	4.51	2.84	3.87	4.71	4.26	5.26	8.88	±0.36
BHT	6.25	3.93	2.82	3.84	5.18	5.15	6.40	10.87	±0.29
CHT	5.80	3.62	3.38	4.57	6.18	5.15	6.40	10.87	±0.39
HHT	6.01	3.59	3.22	4.52	5.35	4.67	5.12	9.14	±0.39
Diphtheroid sp.	7.30	4.03	4.23	4.44	8.59	6.42	11.57	14.80	±0.44
L. casei	11.65	2.75	2.74	5.09	11.33	9.32	10.73	16.12	±0.51
Veillonella sp.	6.46	1.03	0.91	1.29	3.18	1.76	5.39	33.50	±0.51
Corynebacterium sp.	4.03	1.99	1.65	2.46	3.47	3.18	3.45	13.61	±0.62

EFFECTS OF OSMOTIC SHOCK ON THE PERCENT PROTEIN EXTRACTION FROM BHT AND VEILLONELLA

The analysis of variance for the mean percent protein extracted from BHT and *Veillonella sp.* in Experiment 4 is shown in Table V - 22 and Table V - 23.

For BHT all the main effects are significant at the 1% level. For the *Veillonella sp.* only two of the main effects (pH and Osmotic Shock) are significant ($p < 0.01$).

The means and standard errors for osmotic shock are presented in Tables V - 24 and V - 25 and in Appendix IV. The effect of pH and temperature will not be discussed as they have been presented previously.

Main Effects

1. Osmotic Shock

BHT

Table V - 24 shows the effect of osmotic shock on the percent protein extracted from BHT. Osmotic shock caused a significant increase ($p < 0.01$) in the percent protein extracted from BHT.

Veillonella sp.

Table V - 25 shows the effect of osmotic shock on the percent protein extracted from *Veillonella sp.* Osmotic

Table V - 22 Analysis of variance for the effect of osmotic shock, pH, and temperature on the mean percent protein extracted from BHT.

SOURCE OF VARIATION	DF	MEAN SQUARE	SIGNIF. LEVEL
Replications	2	14.53	1%
Osmotic Shock	1	8.71	1%
pH	7	75.44	1%
Temperature	1	86.91	1%
pH X Osmotic Shock	7	1.59	
pH X Temperature	7	2.39	
Osmotic Shock X Temp.	7	2.93	
pH X Osmotic shock X Temp.	7	0.50	
ERROR	62	1.15	
TOTAL	95		

Table V - 23 Analysis of variance for the effect of osmotic shock, pH, and temperature on the mean percent protein extracted from Veillonella sp.

SOURCE OF VARIATION	DF	MEAN SQUARE	SIGNIF. LEVEL
Osmotic Shock	1	33.87	1%
pH	7	70.44	1%
Temperature	1	3.54	
pH X Osmotic Shock	7	1.77	
pH X Temperature	7	2.87	
Osmotic Shock X Temp.	1	0.23	
pH X Osmotic Shock X Temp.	1	2.30	
TOTAL	31		

Table V - 24 Mean percent protein extracted with and without osmotic shock from BHT.

BHT	PERCENT PROTEIN EXTRACTED	STANDARD ERROR
Unshocked	5.11	±0.15
Shocked	5.71	±0.15

Table V - 25 Mean percent protein extracted with and without osmotic shock from *Veillonella sp.*

Veillonella sp.	PERCENT PROTEIN EXTRACTED	STANDARD ERROR
Unshocked	6.37	±0.38
Shocked	8.43	±0.38

shock caused a significant increase ($p < 0.01$) in the percent protein extracted from *Veillonella sp.*

2. Temperature and pH

The results for the effect of temperature and pH on these two microorganisms have been presented previously and will not be recapitulated at this stage.

EFFECTS OF TEMPERATURE AND pH ON THE EXTRACTION OF PROTEIN FROM BHT IN THE LOGARITHMIC AND EARLY DEATH PHASE OF GROWTH

The analysis of variance for the mean percent protein extracted from the logarithmic and death phase cells of BHT in Experiment 5 is shown in Table V - 26.

All main effects were significant at the 1% level; pH X Logarithmic and Death Phase cells was the only significant second order interaction. The means and standard errors are presented in Tables V - 27 to V - 30 and in Appendix V.

Main Effects

1) Logarithmic Phase Cells and Death Phase Cells of BHT

Table V - 27 shows that significantly less protein ($p < 0.01$) was extracted from BHT in the death phase than from the cells in the logarithmic growth phase.

2). pH

The effects of pH on the mean percent protein extracted are similar to those in previous sections and therefore will not be recapitulated (Table V - 28).

Table V - 26 Combined analysis of variance for the effect of pH and temperature on the mean percent protein extracted from logarithmic and death phase BHT cells.

SOURCE OF VARIATION	DF	MEAN AQUARE	SIGNIF. LEVEL
Replication	1	3.12	1%
Log. Death Phase(LvD)	1	27.33	1%
pH	7	44.71	1%
Temperature	2	14.40	1%
pH X Temperature	14	0.79	—
pH X LvD	7	4.37	1%
pH X Temp. X LvD	14	0.74	
ERROR	47	0.52	
TOTAL	95		

Table V - 27 Mean percent protein extracted from BHT at two levels of growth phase over eight pH levels and three temperatures.

GROWTH PHASE	PERCENT PROTEIN EXTRACTED	STANDARD ERROR
Logarithmic	4.32	±0.10
Death	3.25	±0.10

3). Temperature

The effects of temperature on the percent protein extracted were similar to those in previous sections and will not be recapitulated (Table V - 29).

Second Order Interactions

pH X Logarithmic and Death Phase Cells

Table V - 30 and Figure V - 9 show that significantly more protein ($p < 0.05$) was extracted from the logarithmic phase cells than from the early death phase cells at pH 4.0, 7.0, 8.0, 9.0, 10.6 and 12.7. Significantly more protein ($p < 0.01$) was extracted from the early death phase cells than from the logarithmic phase cells at pH 1.3. No significant difference in the mean percent protein extracted occurred at pH 5.0.

Table V - 28 Mean percent protein extracted from BHT at the eight pH levels over the two growth phases.

pH	PERCENT PROTEIN EXTRACTED	STANDARD ERROR
1.3	6.66	±0.21
4.0	2.88	±0.21
5.0	1.57	±0.21
7.0	2.74	±0.21
8.0	3.14	±0.21
9.0	2.91	±0.21
10.6	3.45	±0.21
12.7	6.90	±0.21

Table V - 29 Mean percent protein extracted from BHT at the three temperatures over eight pH levels and two growth phases.

TEMPERATURE ^{o}C	PERCENT PROTEIN EXTRACTED	STANDARD ERROR
0	3.31	±0.13
23	3.49	±0.13
37	4.55	±0.13

Table V - 30 Mean percent protein extracted at each pH from logarithmic and death phase BHT cells over the three temperatures.

pH	GROWTH PHASE		STANDARD ERROR
	LOG	DEATH	
1.3	5.88	7.45	±0.29
4.0	4.01	1.73	±0.29
5.0	1.73	1.41	±0.29
7.0	3.66	1.84	±0.29
8.0	3.90	2.39	±0.29
9.0	3.53	2.30	±0.29
10.6	4.29	2.62	±0.29
12.7	7.54	6.26	±0.29

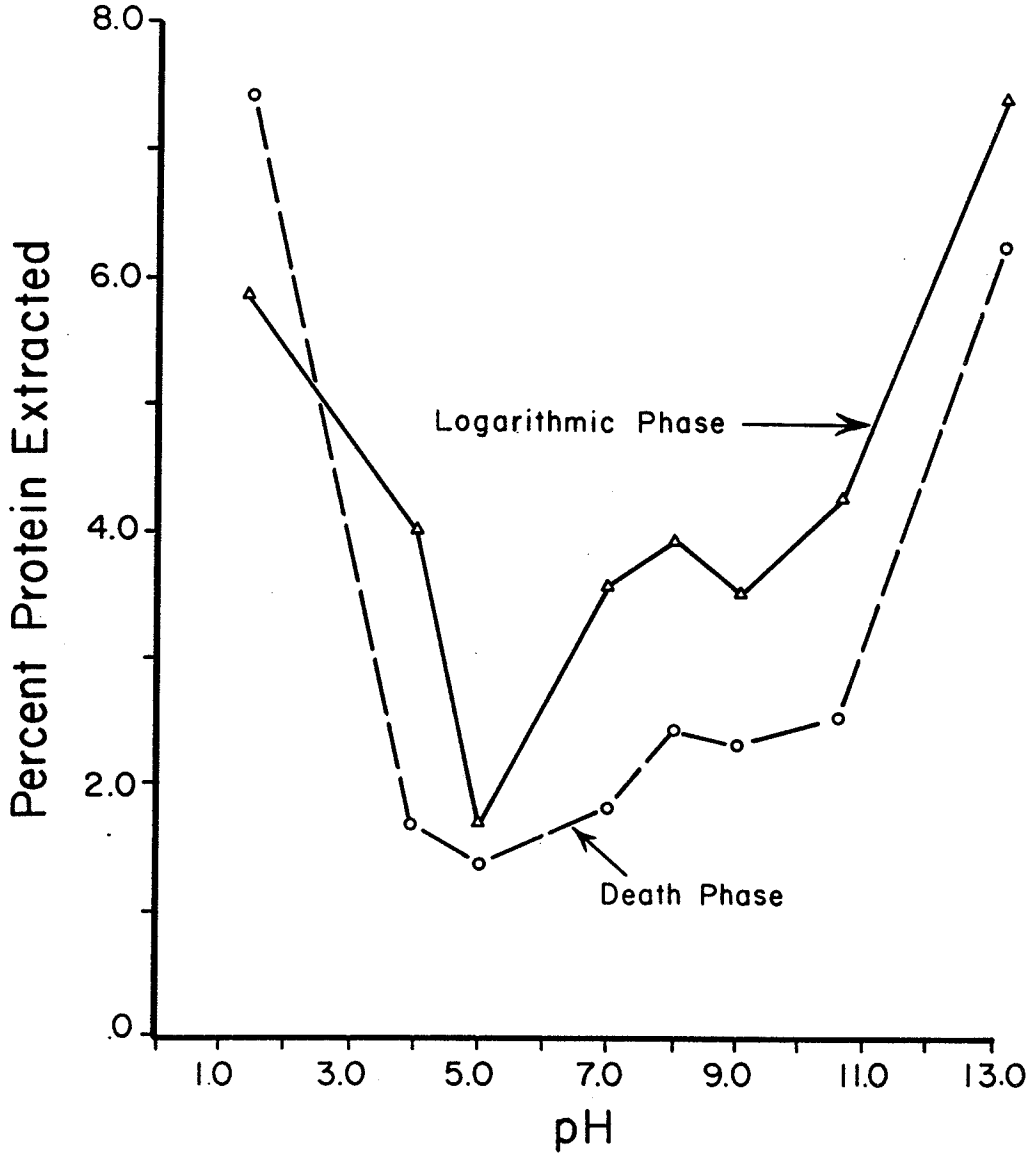


Figure V - 9 Mean percent protein extracted at each pH from logarithmic and death phase BHT cells at three temperatures.

EFFECTS OF TEMPERATURE AND pH ON THE EXTRACTION OF PROTEIN FROM THE COMBINED MICROORGANISMS

The analysis of variance for the mean percent protein extracted from the combined microorganisms in Experiment 6 is shown in Table V - 31.

The main effects (pH and temperature) are significant at the 1% level. The means and standard errors are presented in Table V - 32 and V - 33 and in Appendix VI.

Main Effects

1) Temperature

Table V - 32 shows the effect of temperature on the mean percent protein extracted. The percent protein extracted at each temperature was obtained from the combined microorganisms tested at eight pH levels and a total of two replications. Significantly more protein ($p < 0.01$) was extracted at $37^{\circ}C$ than was extracted at $0^{\circ}C$ and $23^{\circ}C$.

2) pH

Table V - 33 and Figure V - 10 shows the effect of pH on the mean percent protein extracted. The minimum percent protein extracted occurred at pH 5.0 and at pH 4.0 and the maximum was extracted at pH 12.7.

Table V - 31 Analysis of variance for the effect of pH and temperature on the mean percent protein extracted from the combined microorganisms.

SOURCE OF VARIATION	DF	MEAN SQUARE	SIGNIF. LEVEL
Replication	1	14.13	1%
pH	7	91.31	1%
Temperature	2	10.22	
pH X Temperature	14	0.47	
Experimental Error	23	0.61	
Total	47		

Table V - 32 Mean percent protein extracted at the three temperatures from the combined microorganisms at eight pH levels.

TEMPERATURE ^{o}C	PERCENT PROTEIN EXTRACTED	STANDARD ERROR
0	3.70	±0.20
23	3.72	±0.20
37	5.09	±0.20

Table V - 33 Mean percent protein extracted at the eight pH levels from the combined microorganisms at the three temperatures.

pH	PERCENT PROTEIN EXTRACTED	STANDARD ERROR
1.3	4.85	±0.32
4.0	1.75	±0.32
5.0	1.32	±0.32
7.0	3.57	±0.32
8.0	2.76	±0.32
9.0	2.56	±0.32
10.6	3.11	±0.32
12.7	13.45	±0.32

EFFECTS OF TEMPERATURE AND pH ON THE EXTRACTION OF PROTEIN FROM DENTAL PLAQUE

The analysis of variance for the mean percent protein extracted from dental plaque is shown in Table V - 34.

The main effect (pH) was significant at the 1% level. The means and standard errors are presented in Table V - 35 and in Appendix VII.

Main Effects

1) pH

Table V - 35 and Figure V - 1 show the effect of pH on the mean percent protein extracted. The minimum mean percent protein was extracted at pH 4.0 ($p < 0.01$); and the maximum was extracted at pH 12.7.

PERCENT PROTEIN EXTRACTED FROM DENTAL PLAQUE WITH 0.05 N NaOH AT $0^{o}C$ FOR ONE HOUR

The percent protein extracted from sixteen separate samples of dental plaque was 37% ± 8% (S.D.) with a range from 29-45%.

Table V - 34 Combined analysis of variance for the effect of pH and temperature on the mean percent protein extracted from dental plaque.

SOURCE OF VARIATION	DF	MEAN SQUARE	SIGNIF. LEVEL
Replications	1	46.57	-
pH	7	109.21	1%
Temperature	2	3.68	-
pH X Temperature	14	1.02	-
Error	23	10.72	
Total	47		

Table V - 35 Mean percent protein extracted at each of the eight pH levels from dental plaque at three temperatures.

pH	PERCENT PROTEIN EXTRACTED	STANDARD ERROR
1.3	23.77	±1.34
4.0	4.24	±1.34
5.0	6.77	±1.34
7.0	12.77	±1.34
8.0	12.44	±1.34
9.0	10.94	±1.34
10.6	16.02	±1.34
12.7	46.50	±1.34

EFFECTS OF TEMPERATURE AND pH ON THE EXTRACTION OF PROTEIN FROM DENTAL PLAQUE AND A COMBINED SAMPLE OF THE MICROORGANISMS

The combined analysis of variance for the mean percent protein extracted from dental plaque and the combined microorganisms in Experiment 8 is shown in Table V - 36.

The main effects are significant at the 1% level. The means and standard errors are presented in Tables V - 37 to V - 40 and in Appendix VI and VII.

Main Effects

1) Dental Plaque versus the Combined Microorganisms

Table V - 37 shows that significantly more protein ($p < 0.01$) was extracted from the dental plaque than from the combined microorganisms.

2) Temperature

Table V - 38 shows the effect of temperature on the mean percent protein extracted from the dental plaque and the combined microorganisms. The amount of protein extracted at $37^{o}C$ was significantly greater ($p < 0.01$) than that at $0^{o}C$ and $23^{o}C$. The amounts of protein extracted at $0^{o}C$ and $23^{o}C$ were not significantly

Table V - 36 Combined analysis of variance for the effect of pH and temperature on the mean percent protein extracted from dental plaque and the combined microorganisms.

SOURCE OF VARIATION	DF	MEAN SQUARE	SIGNIF. LEVEL
Replications	1	4.70	
Plaque and Organisms (DP&CM)	1	3219.47	1%
pH	7	944.51	1%
Temperature	2	44.82	1%
pH X Temperature	14	6.28	
pH X DP&CM	7	317.53	1%
Temperature X DP&CM	2	4.78	
pH X Temp. X DP&CM	14	5.09	
ERROR	47	6.74	
TOTAL	95		

Table V - 37 Mean percent protein extracted from dental plaque and the combined microorganisms over eight pH levels and three temperatures.

	PERCENT PROTEIN EXTRACTED	STANDARD ERROR
Dental Plaque	15.75	±0.37
Combined Micro.	4.17	±0.37

different from each other.

3) pH

Table V - 39 and Figure V - 10 show the effect of pH on the mean percent protein extracted from the dental plaque and the combined microorganisms.

Significantly less protein was extracted at pH 4.0 than that at pH 7.0 ($p < 0.05$) and at the other six pH levels ($p < 0.01$) except for the pH 5.0 buffers. The maximum amount of protein was extracted at pH 12.7 ($p < 0.01$).

Second Order Interactions

pH X Dental Plaque Versus Microorganisms

The results were shown in Table V - 40 and Figure V - 10.

a) For dental plaque and for the combined microorganisms, the percent protein extracted at the eight pH levels were compared.

Dental Plaque

With dental plaque, the amount of protein extracted at pH 4.0 at pH 5.0 and at pH 7.0 was significantly less than the amount extracted by the other five buffers. There was no significant difference in the amount of protein extracted at pH 4.0, pH 5.0

Table V - 38 Mean percent protein extracted at the three temperatures from dental plaque and the combined microorganisms at the eight pH levels.

TEMPERATURE ^{0}C	PERCENT PROTEIN EXTRACTED	STANDARD ERROR
0	9.31	±0.40
23	9.25	±0.46
37	11.33	±0.46

Table V - 39 Mean percent protein extracted at the eight pH levels from dental plaque and the combined microorganisms at the three temperatures.

pH	PERCENT PROTEIN EXTRACTED	STANDARD ERROR
1.3	14.31	±0.75
4.0	3.00	±0.75
5.0	3.33	±0.75
7.0	5.17	±0.75
8.0	7.60	±0.75
9.0	6.75	±0.75
10.6	9.57	±0.75
12.7	29.97	±0.75

Table V - 40 Mean percent protein extracted from the dental plaque and the combined microorganisms at the eight pH levels over the three temperatures

	pH								
	1.3	4.0	5.0	7.0	8.0	9.0	10.6	12.7	STANDARD ERROR
Dental Plaque	23.77	4.24	5.34	6.77	12.44	10.94	16.02	46.50	±1.05
Combined Microorganisms	4.85	1.75	1.32	3.57	2.76	2.56	3.11	13.45	±1.05

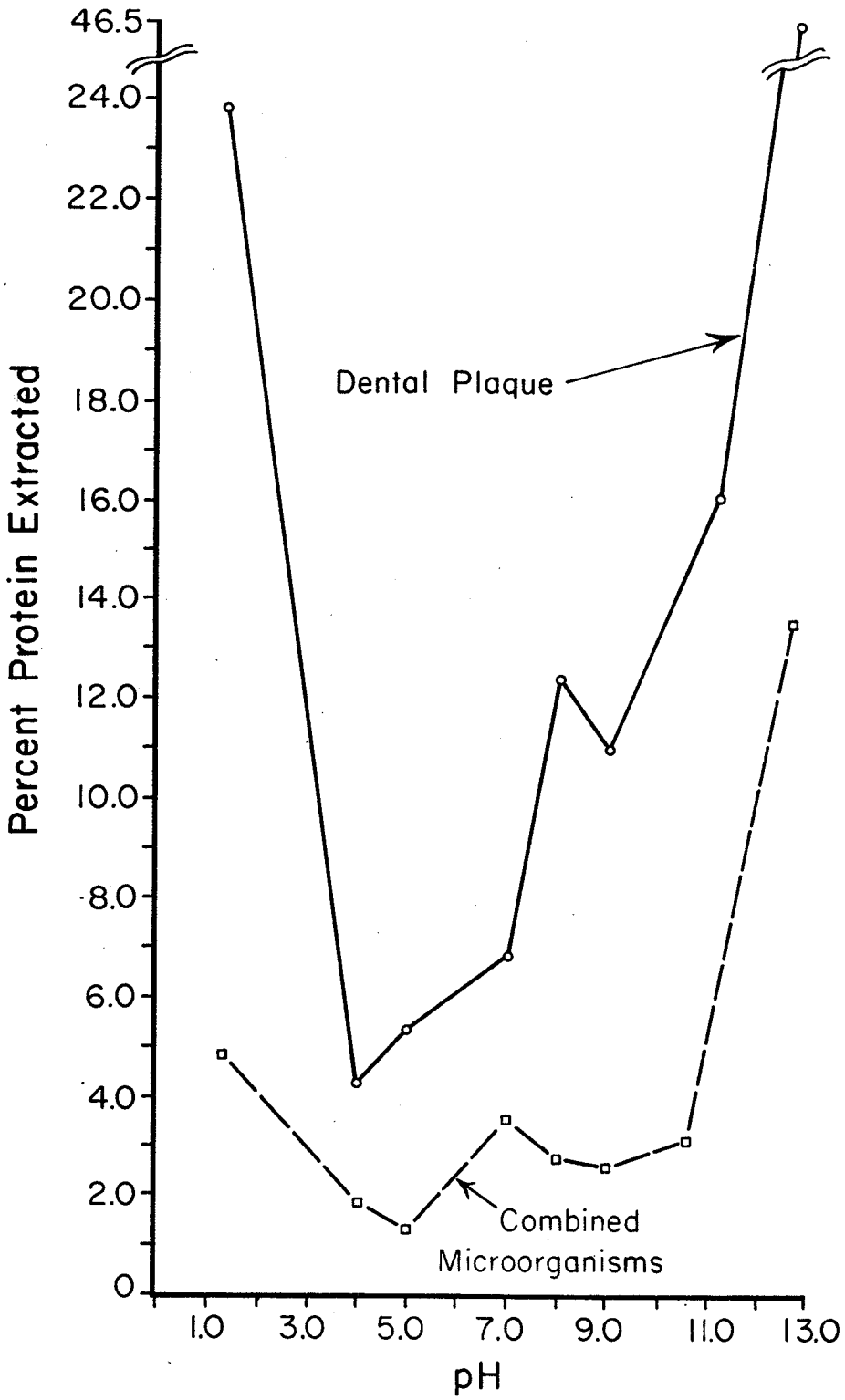


Figure V - 10 Mean percent protein extracted from the dental plaque and the combined microorganisms at the eight pH levels over the three temperatures.

and pH 7.0. This may have been caused by limited sampling. The amount of protein extracted at pH 12.7 was significantly greater ($p < 0.01$) than the amount extracted by the other seven pH buffers.

Combined Microorganisms

The maximum amount of protein was extracted at pH 12.7. The differences in the amount extracted at the other pH levels were not significant; this may have been caused by limited sampling.

b) For the eight pH levels the mean percent protein extracted from dental plaque and the combined microorganisms was compared.

Significantly more protein was extracted from dental plaque than from the combined microorganisms at pH 1.3, 5.0. 9.0, 10.6, 12.7 ($p < 0.01$) and at pH 7.0 ($p < 0.05$). No significant differences in the amount of protein extracted from the dental plaque and the combined microorganisms occurred at pH 4.0.

EFFECTS OF A FIVE HOUR CONTINUOUS EXTRACTION WITH 0.1N NaOH OF PROTEIN FROM DENTAL PLAQUE AND THE COMBINED MICROORGANISMS

The combined analysis of variance for the mean percent protein extracted from dental plaque and the combined microorganisms in Experiment 9 is shown in Table V - 41.

The main effects are significant at the 1% level. The means and standard errors are presented in Tables V - 42 to V - 45 and in Appendix VIII.

Main Effects

1) Dental Plaque versus Combined Microorganisms

Table V - 42 shows that significantly more protein ($p < 0.01$) was extracted from dental plaque than from the combined microorganisms.

2) Effect of Temperature

Table V - 43 show that significantly more protein was extracted at 23°C than at 0°C.

3) Effect of Time

Table V - 44 shows that the amount of protein

Table V - 41 Combined analysis of variance for the effect of time on the percent protein extracted from dental plaque and the combined microorganisms.

SOURCE OF VARIATION	DF	MEAN SQUARE	SIGNIF LEVEL
Replication	1	1.89	
Dental Plaque vs. Combined Microo. (DPvCM)	1	20225.51	1%
Temperature	1	268.74	1%
Time	6	90.36	1%
DPvCM X Temp.	1	118.38	1%
DPvCM X Time	6	16.09	
Temp. X Time	6	5.37	
DPvCM X Temp. X Time	6	3.45	
ERROR	27	11.56	
TOTAL	55		

Table V - 42 Mean percent protein extracted from dental plaque and the combined microorganisms over the seven time intervals.

	PERCENT PROTEIN EXTRACTED	STANDRAD ERROR
Dental Plaque	55.59	±0.64
Combined Microorganisms	17.58	±0.64

extracted after two hours was significantly greater ($p < 0.01$) than that extracted after 15 minutes. The amount of protein extracted after five hours was significantly greater ($p < 0.05$) than the amount extracted after two hours.

Second Order Interaction

Temperature X Dental Plaque vs Combined Microorganisms

Table V - 45 shows that significantly more protein ($p < 0.01$) was extracted from dental plaque than from the combined microorganisms at $0^{o}C$ and at $23^{o}C$. Also, significantly more protein ($p < 0.001$) was extracted at $23^{o}C$ than at $0^{o}C$ from the dental plaque.

Table V - 43 Mean percent protein extracted at the two temperatures from the dental plaque and the combined microorganisms at the seven time intervals.

TEMPERATURE °C	PERCENT PROTEIN EXTRACTED	STANDARD ERROR
0	34.39	±0.64
23	38.77	±0.64

Table V - 44 Mean percent protein extracted at the seven time intervals from the dental plaque and the combined microorganisms at two temperatures.

TIME IN HOURS	PERCENT PROTEIN EXTRACTED	STANDARD ERROR
0.25	31.88	±1.20
0.50	34.32	±1.20
1.0	33.88	±1.20
2.0	37.47	±1.20
3.0	38.41	±1.20
4.0	38.62	±1.20
5.0	41.53	±1.20

Table V - 45 Mean percent protein extracted from dental plaque and the combined microorganisms at 0°C and 23°C over the seven time intervals.

	TEMPERATURE		STANDARD ERROR
	0°C	23°C	
Dental Plaque	51.94	59.23	±0.91
Combined Microorganisms	16.84	18.32	±0.91

CHAPTER VI

DICUSSION

CHAPTER VI

DISCUSSION

STUDIES ON THE INDIVIDUAL MICROORGANISMS

Relationship of Growth Phase to Leakage

For *Veillonella sp.* the percent protein extracted from logarithmic phase cells was similar to that extracted from stationary phase cells. This finding is in agreement with that of Allwood and Russell (1968) on *S. aureus*.

However, for BHT the percent protein extracted from the logarithmic phase cells was significantly greater than that from the early death phase cells. Strange et al (1961) found that late stationary phase cells survive better than early stationary phase cells and logarithmic phase cells. Results from other studies (Sherman and Albus, 1923) show that logarithmic phase cells are more sensitive to minor stress conditions (e.g. cold shock) than are stationary phase cells.

The percent protein extracted from the logarithmic phase cells of BHT was significantly greater than that extracted from death phase cells. This could by explained by the fact that the osmotic barrier has been shown to be intact in approximately 50% of dead *A. aerogenes* (Postgate and Hunter, 1962). If the osmotic barrier were not intact in the other 50% of the dead cells, leakage of the

intracellular constituents could have occurred during death or during the washing procedure and therefore not have been detected under the conditions used in our experiments. On the other hand, Califano (1952) has reported that lag and logarithmic phase cells liberate more nucleic acids into the medium than do stationary phase cells. Hence the log phase cells may really be more leaky than death phase cells.

The effect of growth phase is important when the percent protein extracted from the pure cultures of organisms isolated from the oral cavity is compared with that extracted from dental plaque. The reason for this is that at any given time the growth phase of the microorganisms in dental plaque is uncertain. The growth phase of the plaque microorganisms probably depends on such factors as metabolic requirements, substrate availability, plaque thickness, and oxygen potential. Since the thickness of dental plaque increases markedly during the first few days of collection it seems unlikely that many of the bacteria of the dental plaque are in death phase.

Effects on the Organisms of Time of Exposure to Buffers

An increase in the amount of 260 mμ-absorbing materials was observed at all temperatures and pH levels as the length of time of exposure to the buffers increased. These results are similar to those of several previous workers (Allwood and Russell, 1967; Iandolo and Ordal, 1966).

This continual leakage of nutrients may be important in the

phenomenon of cryptic growth or cryptic metabolism in pure cultures or in mutually dependent metabolic systems as found in the oral cavity and in dental plaque in particular.

Correlation of Nitrogen and Protein Values

The percent nitrogen extracted from the various bacteria was greater than the percent protein extracted. This implies that non-protein nitrogenous substances leak from bacteria more readily than do proteins. Since many nitrogen-containing molecules such as urea and amino acids are of low molecular weight this would account for their greater ability to leak through the cell wall.

Effect of Buffer Type

To test the possiblity of a specific buffer anion effect two different buffers of the same pH were selected. The percent protein extracted by phosphate and bicarbonate buffers was exactly the same at pH 7.0 indicating that, at least at this pH, the buffer anion had no effect. A slight decrease in the percent protein extracted was observed at pH 9.0 in relation to pH 8.0. This may have been due to a specific effect of the borate buffer which can complex negatively charged groups at alkaline pH levels (Michaelis, 1931) thereby reducing the effective charge on the membrane and facilitating decreased leakage.

Ionic Strength

Variations in ionic strength over the range of 0.05 - 0.64 had no statistically significant effect on the percent protein extracted

from AHT and BHT. Some investigators (Gorrill and McNeil, 1960; Allwood and Russell, 1967) have shown that in very dilute ionic environments bacterial viability and leakage is affected.

Bacterial Concentration

Protein leakage as a percentage of total protein was independent of bacterial concentration over the range of 0 - 4 mg/ml. This important finding does not appear to have been reported previously in the literature. Hence, on these fairly dilute suspensions, bacterial aggregation must not have been of a sufficient magnitude to influence the degree of protein leakage.

Temperature

At the temperatures studied (0^{o}, 23^{o}, 37^{o} and $60^{o}C$) there was a significant increase in the percent protein extracted with each increase in temperature. It has been postulated that temperature controls the permeability mechanism by influencing the proximity of the various membrane components, thereby affecting the amount of leakage, (Meynell, 1958; Iandolo and Ordal, 1966).

A temperature of $23^{o}C$ appears to be a critical one because the amount of protein leakage at $0^{o}C$ was only slightly, although significantly less than the amount of leakage at $23^{o}C$. Huang et al, (1964) showed that model membrane formation was affected by temperature. At $20^{o}C$ membrane formation did not occur but at $36^{o}C$ it occurred very rapidly. At $20^{o}C$, certain lipid components of the model membrane underwent a liquid to solid phase transition.

Such a transition in a bacterial cell wall might increase the cell wall stability and account for the minimal change in the degree of protein leakage over the temperature range of 0 - 23°C.

At 37°C, significantly more protein was extracted than at 23°C. At 60°C, more protein was extracted than at 37°C. At 60°C, protein coagulation probably occurs rapidly (Allwood and Russell, 1968; Califano, 1952) and this may be the reason why the percent protein extracted at this temperature reached a maximum level within 45 minutes and did not increase with further exposure of organisms to buffer (Figure IV - 1). This effect of temperature in increasing leakage confirms the finding of several previous investigators (Allwood and Russell, 1968; Califano, 1952; Postgate and Hunter, 1962).

pH

The minimum percent protein was extracted at pH 5.0 and the maximum was extracted at pH 12.7. Curtis (1967) has reported that the iso-electric point of many bacteria is in the range of 3.5 to 5.0. Hence, at pH 5.0 the net charge on the permeability mechanism could be zero and this might account for the minimal leakage due to the close proximity of the membrane components. As the pH is raised from 5.0 to 12.7 the permeability mechanism may become negatively charged and repulsion of the membrane components could cause an increased leakage. The same would apply as the pH is lowered from 5.0 to 1.3, an increase in positively charged ions would result in membrane repulsion and thereby cause increased leakage.

Temperature X pH

The effect of temperature and pH, independent of the microorganisms was such that the minimum percent protein was extracted at pH 5.0 and the maximum was extracted at pH 12.7. The increase in the percent protein extracted as the temperature increased from 0^{o} to 37^{o} to $60^{o}C$, was minimal at pH 5.0 and 4.0 but at the other pH values tested it was considerably greater. This finding has been interpreted to mean that at pH 5.0 the membrane has very little net charge, is therefore closely packed and this results in minimal leakage despite an increase in thermal vibrations at higher temperatures. At the higher pH levels the membrane is more negatively charged and therefore increased leakage occurs due to mutual repulsion of the membrane components. At pH 1.3 and 12.7 the effect of the charge on the membrane and the increase in thermal vibrations at higher temperatures complement each other and cause a markedly increased leakage of protein from the bacteria.

Type of Microorganisms

The mean percent protein extracted from each microorganism was obtained from thirty-two treatment combinations (8 pH X 4 temperatures). There were no significant differences in leakage patterns between the cariogenic and non-cariogenic streptococci. The reason for the variations in response to the various treatments by the other microorganisms is not certain but

is most likely due to differences in cell wall composition such that at a given pH the cell walls are charged to different extents.

Microorganisms X Temperature

L. casei, the Diphtheroid sp. and the Veillonella sp. exhibited a greater loss of protein at all temperatures studied than did the four streptococci and the Corynebacterium sp. The protein molecules of thermophilic bacteria have been shown to have a greater heat stability than those of mesophilic bacteria (Rose, 1967) and ribosomal RNA from a thermophilic bacillus has been shown to have a greater heat stability than the corresponding RNA from a mesophilic bacteria (Tecce and Toschi, 1960). Whether the differences in amount of leakage from the above microorganisms could be caused by differences in enzyme or RNA stability is uncertain. If certain cell wall proteins were particularly susceptible to heat denaturation, this may have resulted in structural changes in the membrane such that leakage of cell contents was facilitated.

Microorganism X pH

For the four streptococci the minimum percent protein extracted occurred at pH 5.0; for the other four microorganisms (L. casei, Diphtheroid sp., Veillonella sp., Corynebacterium sp.) the minimum percent protein was extracted at pH 4.0 and 5.0. The four streptococci were the least affected by changes in pH and showed the least variation in the percent protein extracted over the entire pH range of 1.3 to 12.7. The mean percent protein extracted from the four streptococci at pH 12.7 was approximately 10%.

The variation in the percent protein extracted from the different microorganisms at pH 12.7 was very large when compared with the range at the other seven pH levels; this is probably due to maximal disruption of the membranes at the extremes of the pH range. The large variation in the percent protein extracted from the microorganisms at pH 12.7 might account for the large variation in the percent protein extracted from different samples of dental plaque since the proportions of the various types of microorganisms found in plaque show considerable variation.

Effect of Calcium and Magnesium

Calcium and Magnesium caused a significant decrease in the percent protein extracted from four of the eight microorganisms at pH 9.0, 10.6 and 12.7.

Since the isoelectric point of many bacteria cells has been shown to be in the pH range of 3.5 - 5.0 (Curtis, 1967), the cells will be negatively charged at alkaline pH values. Calcium has been shown to be important in the aggregation of cells and Curtis (1967) discussed two possible calcium binding mechanisms:

a) calcium could bridge two negatively charged carboxyl groups from two separate cells.

b) A calcium-protein-calcium bridge could hold two cells together.

Calcium and magnesium probably reduced leakage from the bacterial cells by binding to two adjacent, negatively charged groups on the cell surface. This binding would act to neutralize

the excess negative charge and the repulsive forces on the membrane. There was no evidence of bacterial aggregation with the concentration of calcium and magnesium used.

Osmotic Shock

Osmotic shock (Neu and Heppell, 1965) caused a significant increase in the percent protein extracted from BHT and Veillonella sp. The procedure was reported by Neu and Heppell to be effective for removal of degradative enzymes from gram negative organisms which did not metabolize sucrose. The effects of osmotic shock cannot be specific for such organisms since BHT, an organism which can metabolize sucrose, also showed increased leakage of protein when subjected to this procedure. Bacteria within the oral cavity may often be exposed to high concentrations of sucrose or glucose and this may have some osmotic shock effects although high glucose concentrations do inhibit metabolism slightly (Kleinberg, 1961). As the glucose concentration decreases the bacteria begin to metabolize again and appear to suffer no deleterious effects. This fits in with the present results in that although osmotic shock caused a statistically significant increase in protein leakage, the absolute increase was relatively small.

STUDIES ON THE COMBINED MICROORGANISMS AND ON DENTAL PLAQUE

The effects of pH and temperature on the percent protein extracted from the combined microorganisms were very similar to

those expected from the results for the eight individual microorganisms. This would support the view that there occurred no aggregation or other interaction of the cocci and rod bacteria to alter the behaviour of the combined bacteria as compared to when they were tested as single strains.

Dental Plaque versus Combined Microorganisms

The minimum mean percent protein was extracted from dental plaque at pH 4.0 and the maximum was extracted at pH 12.7. For the eight pH levels the mean percent protein extracted from dental plaque and the combined microorganisms was compared. Significantly more protein was extracted from dental plaque than from the combined microorganisms at all of the eight pH levels studied except pH 4.0. In our attempts to find the optimal conditions for separation of dental plaque into its bacterial and matrix components, a buffer of a pH somewhat less that 12.7 was expected to cause minimal leakage from the microorganisms. However, over the pH range of 8.0 to 12.7, the ratio of the percent protein extracted from dental plaque to that extracted from the combined microorganisms was approximately 4 : 1 (Table VI - 1). That is, 80% of the protein extracted from dental plaque was probably matrix and 20% was probably of bacterial origin. Since this ratio was constant over the pH range of 8.0 - 12.7 it was concluded that the pH 12.7 buffer was the best one to separate the matrix from the bacteria since this buffer extracted the largest amount of protein from plaque.

Table VI - 1 Comparison of the percent protein extracted from dental plaque, the combined microorganisms, and the eight microorganisms at the eight pH levels over the three temperatures.

pH	Dental Plaque : Eight Micro-organisms	Dental Plaque : Combined Microorganisms
1.3	3.7 : 1	4.9 : 1
4.0	1.5 : 1	2.4 : 1
5.0	2.5 : 1	4.0 : 1
7.0	2.3 : 1	1.9 : 1
8.0	2.9 : 1	4.5 : 1
9.0	2.8 : 1	4.3 : 1
10.6	3.1 : 1	5.2 : 1
12.7	3.3 : 1	3.5 : 1

Effect of Time

An increase in the length of time of extraction caused an increase in the percent protein extracted from the dental plaque and the combined microorganisms. However, with extraction times longer than one hour, the absolute increase in protein extracted from dental plaque was equal to the increase in protein extracted from the combined microorganisms. Hence, the increased extraction with increased time after one hour was due only to increased extraction from the bacteria and the matrix was probably removed in one hour.

Effect of Temperature

Significantly more protein was extracted from dental plaque at 37°C than at 0°C or 23°C. No significant difference in the percent protein extracted from dental plaque occurred at 0° and 23°C. Since the increase in the percent protein extracted at 37°C as compared with 0°C or 23°C was similar for both dental plaque and the combined microorganisms, then the increased extraction from dental plaque at the higher temperature is probably from the microorganisms and not from the matrix. Therefore 0°C would seem to be the optimal temperature for dental plaque separation.

Effect of Calcium

Dawes and Jenkins (1962) reported that the calcium content of 2-day dental plaque was approximately 5 µg/mg dry weight. Since 20 mg wet weight of plaque was used in the various buffers in the

present study the calcium concentration could be 0.5 mM. From the results on individual bacteria using 2 mM calcium, the 0.5 mM calcium could only cause a very slight reduction in the percent protein extracted from dental plaque.

Detailed Calculation of Matrix Component of Dental Plaque

The exact calculation of the matrix contribution to the total protein of dental plaque is as follows:

a mg bacteria + b mg matrix = c mg plaque

Since at pH 12.7 and 0°C the mean percent protein extracted from the combined microorganisms after one hour was 16.0% and the mean percent protein extracted from dental plaque was 37.1 ± 8.1% (S.D.) with a range of 28.9 - 45.3% then:

$$a \times \frac{16.0}{100} + b = \frac{37.1}{100} c$$

or

$$16.0\ a + 100\ b = 37.1\ c$$

solving for b and a

$$b = 0.252\ c$$

$$a = 0.748c$$

Therefore, of the total plaque protein 25.2% is derived from the matrix and 74.8% from the bacteria.

When similar calculations are carried out for the range of the percent protein extracted, then 25.2 ± 9.8% (S.D.) of the plaque consists of matrix.

For these calculations the assumption has been made that

the microorganisms in plaque respond to the 0.05 N NaOH in exactly the same way as the cultures of microorganisms *in vitro*. If the microorganisms in plaque exhibited more or less protein leakage than the sample of combined microorganisms then the calculation for plaque matrix content would be in error. However, a variation of 20% in the extraction of protein from the bacteria would only change the mean value of 25.2% for the plaque matrix to 22.2 or 27.9%.

Some of the variation in the results obtained for plaque matrix content can be explained on the basis of different samples of plaque containing widely different proportions of the various bacteria. For instance, results show that *Veillonella sp.* is particularly susceptible to 0.05 N NaOH.

If some matrix is laid down before the bacteria (McDougall, 1963a) then the smaller the amount of plaque formed, the higher will be the matrix content. Hence variations in plaque thickness may account for some of the variation in plaque matrix content.

The range of percent protein extracted from dental plaque was 28.9 to 45.3. This range correlates extremely well with the range reported by Dobbs (1932) as he found that 28 - 52% of the total plaque protein was extracted with 5% NaOH.

Results from the present study support the work of Silverman and Kleinberg (1967a) in which they used 0.1 N NaOH at $0^{o}C$ for 5 hours for separation of matrix from the bacteria of

dental plaque. However, the time used for separation need be no longer than one hour since the increased protein extraction after one hour appears to be derived only from the bacteria.

The experiments described were not designed to give information about the source of the plaque matrix, whether it be of salivary, bacterial or other origin. The results merely show that in the process of matrix extraction some protein is released from the bacteria. The latter could conceivably contribute protein during the formation of matrix *in vivo*.

CHAPTER VII

SUMMARY AND CONCLUSIONS

CHAPTER VII

SUMMARY AND CONCLUSIONS

The purpose of this study was to find the optimal conditions for the separation of dental plaque into its bacterial and protein matrix components. This purpose was justified by the fact that the the solvents used previously are known to extract protein from pure cultures of bacterial cells.

The effects of pH, temperature, calcium and magnesium ions, osmotic shock, ionic strength, time of exposure and growth phase on the percent protein extracted from eight of the predominant microorganisms found in dental plaque were investigated.

With the eight individual microorganisms the minimum percent protein was extracted at pH 5.0 and the maximum was extracted at pH 12.7. The effect of pH may perhaps be explained as follows. At pH 5.0, the charge on the permeability mechanism could be about zero and therefore result in minimal leakage due to the close proximity of the membrane components. As the pH is raised from 5.0 to 12.7 the permeability mechanism may become negatively charged and repulsion of the membrane components could cause an increased leakage. The same would apply as the pH is lowered from 5.0 to 1.3, an increase in positively charged ions would result in membrane repulsion and thereby cause increased leakage.

At the four temperatures studied, 0^{o}, 23^{o}, 37^{o} and $60^{o}C$, the

percent protein extracted increased significantly with each increase in temperature.

The divalent cations, calcium and magnesium, caused a significant reduction in the percent protein extracted at the alkaline pH levels of 9.0, 10.6 and 12.7. However, only four of the eight microorganisms had significantly less protein extracted from them when calcium or magnesium were present in the buffers. These divalent cations would favour a decrease in membrane repulsion at high pH levels by neutralizing the effective negatively charged groups, facilitating decreased leakage.

Osmotic shock caused an increase in the percent protein extracted from BHT and Veillonella sp.

Over the pH range of 1.3 to 12.7 at 23°C buffers of various ionic strengths (0.05, 0.16 and 0.64) had no significantly different effects on the percent protein extracted from the bacteria.

The amount of 260 mμ-absorbing material extracted from HHT over six pH levels in the range of 1.3 - 12.7 and three temperatures (0°, 37°, 60°C) increased with an increase in the time of extraction from 0.25 to 3.25 hours.

Similar amounts of protein were extracted from lag, logarithmic, and stationary phase cells of Veillonella sp. Significantly more protein was extracted from logarithmic phase cells of BHT than was extracted from death phase cells of BHT.

The effects of pH and temperature were then studied on a combined sample of microorganisms and on dental plaque itself. The effects of pH and temperature (8 pH and 3 temperature) on the percent protein extracted from a combined sample of microorganisms were very similar to those expected from the results obatined from the eight individual microorganisms, suggesting that the microorganisms in combination did not behave differently from when present as single strains.

The effects of pH and temperature (8 pH and 3 temperature) on the percent protein extracted from dental plaque were then investigated. The minimum percent protein was extracted at approximately pH 4.0 - 5.0 and the maximum was extracted at pH 12.7. There was no significant difference in the percent protein extracted from dental plaque and the combined microorganisms at pH 4.0 suggesting that plaque matrix was not extracted at this pH. However, signifcantly more protein was extracted from dental plaque at the other seven pH levels and this difference was attributed to the matrix proteins found in dental plaque but not found in the pure cultures of microorganisms. Significantly more protein was extracted from both dental plaque and the combined microorganisms at 37°C than at 0° or 23°C.

Over the pH range of 8.0 to 12.7 the ratio of the percent protein extracted from dental plaque to that extracted from the combined microorganisms was relatively constant at approximately 4 : 1. It was therefore concluded that use of the pH 12.7 buffer at 0°C would be the most effective for extraction of the matrix from

the dental plaque since the most protein was extracted at this pH whilst the porportion of protein extracted from the bacteria was no greater than at lower pH values. With this solvent, the mean percent of protein extracted from dental plaque in one hour was 37 ± 8%(S.D.). Taking into account the protein leakage from the bacteria, the results suggest that matrix comprises about 25 ± 10% of the total plaque protein.

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APPENDIX

APPENDIX I

Mean percent protein extracted from the eight microorganisms over the four temperatures and eight pH levels.

MICRO-ORGANISM	Temp.	pH 1.3	4.0	5.0	7.0	8.0	9.0	10.6	12.7
	0°C	5.39	3.56	1.82	2.39	2.50	2.08	2.95	8.17
	23°C	6.42	3.27	1.59	2.36	2.60	2.48	2.95	8.69
AHT	37°C	7.33	4.35	2.51	4.17	5.38	4.69	5.89	9.83
	60°C	10.78	4.76	5.09	5.32	5.58	6.04	5.82	12.30
	0°C	5.91	4.32	2.61	3.00	2.76	2.58	3.36	7.47
	23°C	5.78	4.40	2.29	3.25	3.42	3.61	3.39	7.69
BHT	37°C	6.80	4.16	2.35	4.67	5.89	5.58	6.13	7.72
	60°C	10.63	4.50	4.61	5.93	6.49	6.64	6.99	9.39

APPENDIX I

(cont.)

MICRO-ORGANISM	Temp.	pH 1.3	4.0	5.0	7.0	8.0	9.0	10.6	12.7
	0°C	4.41	2.48	1.93	2.14	2.85	2.64	3.40	6.97
	23°C	4.47	2.71	1.97	2.74	3.61	2.69	4.00	10.21
CHT	37°C	5.62	3.85	3.45	4.69	6.57	5.37	6.24	11.74
	60°C	6.97	4.37	4.09	7.64	9.15	7.48	7.45	14.03
	0°C	4.77	2.24	1.95	1.86	2.26	2.67	2.94	6.55
	23°C	4.91	2.57	2.98	2.11	2.67	2.62	3.31	8.74
HHT	37°C	5.87	3.86	3.39	4.79	5.45	4.59	5.22	9.88
	60°C	7.33	4.53	4.22	9.11	7.56	8.05	7.18	12.20

APPENDIX I

(cont.)

MICRO-ORGANISM	Temp.	pH 1.3	4.0	5.0	7.0	8.0	9.0	10.6	12.7
Diphtheroid sp.	0°C	7.86	3.15	3.15	3.83	4.23	3.51	3.91	10.00
	23°C	6.90	2.63	2.87	3.96	4.30	4.28	5.83	12.85
	37°C	7.42	4.32	4.08	4.53	7.91	8.58	11.90	16.75
	60°C	11.09	4.62	5.09	9.86	9.85	9.32	10.45	22.90
L. casei	0°C	11.07	2.80	2.41	3.71	7.67	4.84	8.25	20.00
	23°C	14.35	2.14	2.51	3.53	7.31	4.48	10.64	18.97
	37°C	9.10	3.25	2.68	2.27	11.55	10.90	11.87	16.23
	60°C	16.14	8.27	6.53	11.60	8.29	11.55	11.29	18.73

APPENDIX I

(cont.)

MICRO-ORGANISM	TEMP.	pH 1.3	4.0	5.0	7.0	8.0	9.0	10.6	12.7
	0°c	5.54	1.12	.66	1.21	1.53	1.58	2.67	30.0
Veill-onella	23°C	5.38	0.75	0.66	1.04	1.53	1.24	2.72	33.07
sp.	37°C	6.56	1.09	0.86	1.38	3.55	1.98	7.98	36.78
	60°C	11.93	2.56	2.94	4.41	11.87	11.73	13.11	41.33
	0°C	3.35	0.74	1.06	1.50	2.20	3.45	2.56	9.30
Coryne-bacterium	23°C	3.25	0.92	0.91	1.50	2.33	2.42	2.61	14.13
sp.	37°C	4.06	1.93	1.70	2.66	3.78	3.78	4.36	19.29
	60°C	3.96	2.55	2.78	5.45	13.55	10.76	13.76	20.08

APPENDIX II

Mean percent protein extracted from the eight microorganisms over the eight pH and two calcium levels.

MICRO-ORGANISM		pH 1.3	4.0	5.0	7.0	8.0	9.0	10.6	12.7
	0 Ca	7.33	4.35	2.30	4.16	5.38	4.69	5.89	9.83
AHT	2mM Ca	8.33	3.91	3.00	3.84	4.88	4.24	4.95	8.42
	0 Ca	5.85	3.87	2.05	3.98	5.10	5.87	5.46	9.47
BHT	2mM Ca	6.82	3.50	2.48	4.15	5.07	4.54	5.44	8.50
	0 Ca	5.61	3.85	3.45	4.69	6.57	5.38	6.24	11.74
CHT	2mM Ca	6.01	3.25	4.11	3.89	6.30	4.68	5.66	11.28
	0 Ca	5.87	3.86	3.39	4.79	5.45	4.59	5.23	4.88
HHT	2mM Ca	6.21	2.82	2.46	4.37	5.32	4.90	6.01	8.45

APPENDIX II

(cont.)

MICRO-ORGANISM		pH 1.3	4.0	5.0	7.0	8.0	9.0	10.6	12.7
Diphtheroid sp.	0 Ca	7.42	4.32	4.08	4.53	7.91	6.82	11.90	16.75
	2mM Ca	6.80	3.39	4.63	4.58	8.66	5.48	7.22	12.43
L. casei	0 Ca	8.95	3.25	2.68	4.47	11.54	10.91	11.87	16.21
	2mM Ca	12.09	3.230	3.28	6.45	11.06	8.23	9.32	19.53
Veillonella sp.	0 Ca	6.65	1.09	.86	1.38	3.55	1.98	7.99	36.79
	2mM Ca	6.96	1.19	1.15	1.18	2.88	1.73	2.71	30.45
Corynebacterium sp.	0 Ca	4.06	1.93	1.69	2.66	3.78	3.74	4.3	19.29
	2mM Ca	3.62	2.04	1.81	1.68	3.56	2.36	3.74	7.71

APPENDIX III

Mean percent protein extracted from the eight microorganisms over the eight pH and two magnesium levels.

MICRO-ORGANISM		pH 1.3	4.0	5.0	7.0	8.0	9.0	10.6	12.7
AHT	0 Mg	7.35	4.35	2.50	4.16	5.38	4.69	5.89	9.84
	1mM Mg	7.30	4.67	3.17	3.57	4.04	3.82	4.57	7.91
BHT	0 Mg	5.85	3.87	2.93	3.98	5.10	5.38	6.24	11.74
	1mM Mg	6.65	3.98	2.69	3.70	5.26	4.91	6.56	10.00
CHT	0 Mg	5.66	3.85	3.45	4.69	6.57	5.38	6.24	11.74
	1mM Mg	5.95	3.38	3.30	4.45	5.79	4.91	6.56	10.00
HHT	0 Mg	5.87	3.86	3.39	4.79	5.45	4.59	5.23	9.88
	1mM Mg	6.15	3.32	3.04	4.25	5.24	4.74	5.00	8.96

APPENDIX III

(cont.)

MICRO-ORGANISM		pH 1.3	4.0	5.0	7.0	8.0	9.0	10.6	12.7
Diphtheroid sp.	0 Mg	7.42	4.22	4.08	4.53	7.91	6.82	11.90	16.75
	1mM Mg	7.18	3.84	4.37	4.36	9.28	6.03	11.24	12.06
L. casei	0 Mg	9.10	3.25	2.68	4.47	11.54	10.91	11.87	16.22
	1mm Mg	12.36	2.26	2.80	5.71	10.52	7.72	9.58	16.03
Corynebacterium sp.	0 Mg	4.06	1.93	1.69	2.66	3.78	3.74	4.36	19.29
	1mm Mg	3.99	2.04	1.60	2.25	3.15	2.61	2.54	7.93
Veillonella sp.	0 Mg	6.56	1.09	0.86	1.38	3.55	1.98	7.99	36.79
	1mm Mg	6.35	0.96	0.96	1.20	2.81	1.54	2.78	30.22

APPENDIX IV

Mean percent protein extracted from osmotic shocked and unshocked *Veillonella sp.* at eight pH levels and two temperatures.

	TEMP.	1.3	4.0	5.0	7.0	8.0	9.0	10.6	12.7
UNSHOCKED	23°C	4.47	0.72	0.59	0.83	0.96	1.11	1.37	37.60
	37°C	8.10	1.15	0.91	1.39	3.06	2.28	3.78	44.80
SHOCKED	23°C	5.32	0.99	0.83	1.18	1.88	1.79	3.64	38.70
	37°C	7.42	1.30	1.48	2.56	5.15	4.37	8.51	38.64

APPENDIX IV

(cont.)

Mean percent protein extracted from osmotic shocked and unshocked (control) BHT at eight pH levels and two temperatures.

		pH							
	TEMP.	1.3	4.0	5.0	7.0	8.0	9.0	10.6	12.7
UNSHOCKED	23°C	7.35	3.78	2.26	2.51	3.15	2.94	3.86	8.84
	37°C	10.1	4.05	3.11	3.72	5.52	5.04	6.09	9.49
SHOCKED	23°C	8.78	4.16	2.23	3.77	3.12	3.11	3.13	8.43
	37°c	12.01	5.31	4.69	5.40	6.56	4.13	6.64	9.99

APPENDIX V

Mean percent protein extracted from logarithmic and death phase cells of BHT at eight pH levels and three temperatures.

		pH							
	TEMP.	1.3	4.0	5.0	7.0	8.0	9.0	10.6	12.7
LOGARITHMIC PHASE	0°C	5.94	4.08	1.46	3.23	2.72	2.36	3.39	7.34
	23°C	5.57	4.10	1.51	3.37	3.28	3.04	3.33	7.79
	37°C	6.14	3.89	2.21	4.40	5.72	5.20	6.15	7.50
DEATH PHASE	0°C	6.23	1.09	1.68	1.50	2.16	1.80	2.18	5.84
	23°C	7.38	1.35	1.19	1.45	1.91	1.84	2.33	6.49
	37°C	8.75	2.78	1.38	2.57	3.11	3.26	3.36	6.46

APPENDIX VI

Mean percent protein extracted from the combined microorganisms at the eight pH levels and three temperatures.

		pH							
	TEMP.	1.3	4.0	5.0	7.0	8.0	9.0	10.6	12.7
COMBINED	0°C	4.24	1.44	0.96	3.61	1.97	2.24	2.37	12.76
MICROORGANISMS	23°C	4.63	1.51	1.42	3.23	2.02	1.90	2.23	12.86
	37°C	5.69	2.31	1.59	3.87	4.29	3.55	4.74	14.72

APPENDIX VII

Mean percent protein extracted from dental plaque at the eight pH levels and three temperatures.

		pH							
	TEMP.	1.3	4.0	5.0	7.0	8.0	9.0	10.6	12.7
DENTAL PLAQUE	0°C	18.43	4.05	8.15	7.15	10.65	10.10	15.80	48.06
	23°C	23.55	3.60	4.52	6.50	13.24	11.28	13.30	42.04
	37°C	29.34	5.08	6.37	6.65	13.24	11.43	18.98	49.42

APPENDIX VIII

Mean percent protein extracted from the dental plaque and the combined microorganisms at the seven time intervals and two temperatures.

		TIME (in hours)						
	TEMP.	0.25	0.50	1.0	2.0	3.0	4.0	5.0
DENTAL PLAQUE	0°C	49.34	49.38	47.57	52.66	52.36	54.03	58.27
	23°C	50.85	56.27	54.58	60.91	62.61	60.99	68.43
COMBINED	0°C	13.19	14.88	15.97	17.16	18.35	19.31	19.06
MICROORGANISMS	23°C	13.98	16.77	17.41	19.16	20.34	20.18	20.39