THE COMPOSITION AND MECHANISM OF FORMATION OF HUMAN DENTAL PLAQUE

A Thesis

Presented to

the Faculty of Graduate Studies

The University of Manitoba

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

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Department of Oral Biology

February 1967

ACKNOWLEDGEMENTS

For his guidance, encouragement and generous contribution of time, the author wishes to express his appreciation to Dr. I. Kleinberg who supervised these studies.

The author wishes to thank Dr. F. LaBella for carrying out the amino acid analysis, Dr. J. R. Trott for assistance in the staining of plaque specimens and Mr. D. Lane and Mr. A. Domokis for the art work.

Gratitude must also be expressed to the author's wife, Gail and Mrs. H. Strom for typing the thesis.

The author wishes to thank the Associate Committee on Dental Research, National Research Council of Canada for their award to the author of a Dental Research Fellowship.

This research was supported by grants to Dr. I. Kleinberg from the same committee and from the Colgate-Palmolive Company.

THE COMPOSITION AND MECHANISM OF FORMATION OF HUMAN DENTAL PLAQUE

Dental plaque, the bacterial films that readily form on tooth surfaces, was allowed to collect on the teeth of human subjects for a period of three days. The plaque was removed with a stainless steel spatula and transferred to test tubes containing 0.1 N NaOH for initial dispersal and solubilization of some of the plaque constituents. Repetitive exposure of the undispersed material to fresh NaOH resulted in a small percentage called the residue, remaining undispersed. The plaque cells suspended in the NaOH solutions were harvested by centrifugation at 12,800 x g and have been referred to in this study as the NaOH-suspended cells (NaOH-S cells). The supernatant was dialyzed against distilled water to remove the small molecular weight constituents, most of which probably originated from the cytoplasms of the plaque cells. The large molecular weight substances (called the NaOH-solubilized fraction or NaOH-S fraction) remained within the dialysis tubing and separated into two fractions, one a precipitate, the other the supernatant. The NaOH-S cells fraction comprised 28% of the total plaque N, the residue 8%, the NaOH solubilized fraction 32%, with the remaining 32% being dialyzable. The precipitate and supernatant components

of the NaOH-S fraction were 76% and 24% of this fraction respectively.

Carbohydrate, calcium and phosphate analyses performed on plaque and each of these fractions showed that all fractions contained some carbohydrate, calcium and phosphate. The carbohydrate was most in the NaOH-S-cells fraction, whereas the dialyzable fraction contained the largest percentage of the plaque calcium and phosphate.

The supernatant component of the NaOH-S fraction contained more carbohydrate, calcium and phosphate than the precipitate component. Amino acid analysis of the two sub-fractions showed that the supernatant component contained more acidic and less basic amino acids than did the precipitate component. Measurement of their solubilities as a function of pH, showed that the iso-electric point of the supernatant was lower than that of the precipitate (2.7 and 3.2 respectively), consistent with their difference in amino acid compositions.

Molecular sieving on gels of progressively higher porosities showed that the constituents of the supernatant and precipitate components had molecular weights greater than 100,000. Gel filtration on Bio-gel P200 resulted in the fractionation of the supernatant component into 5 sub-fractions and the precipitate component into 6. Varying amounts of carbohydrate were present in all sub-fractions and at least some calcium and phosphate in most. Comparison of the amino

acid composition of the total NaOH-S fraction to the composition of tooth pellicle, denture pellicle, salivary glyco-protein and salivary mucin, suggested that a large part of the NaOH-S fraction was of salivary origin. There was, however, some indication of the presence of nucleic acid, indicating that some of the NaOH-S fraction was of bacterial origin.

A study was also carried out to determine the factors affecting the aggregation of the cellular and acellular components of plaque, as a function of pH. Suspensions of plaque cells were titrated between pH 10 and 3 with 0.1 N HCL and the effect on aggregation over this pH range of the following factors; cell concentration, ionic strength, divalent cations and the various sub-fractions of the NaOH-S fraction, determined. Aggregation was measured by determining the O.D. of the suspension after centrifugation at 1780 x g for two minutes. As the pH was decreased, aggregation occurred. Increasing the cell concentration, the ionic strength, or the concentration of divalent cations, favoured the aggregation of the plaque cells at pH's higher than corresponding controls. All but one of the sub-fractions protected against aggregation. The one that did not (one of the sub-fractions of the supernatant component of the NaOH-S fraction) enhanced aggregation. These findings were consistent with the theory that lower pH levels on the surface of the teeth favour a more rapid rate of plaque formation.

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CHAPTER I

INTRODUCTION

In 1897, Williams first demonstrated by histological means, that bacterial films collected on those surfaces of teeth not readily accessible to cleansing. These films were shown by Williams and later others to consist of masses of bacteria within an organic matrix; these films are usually referred to as dental plaque. The importance of dental plaque in the pathogenesis of dental caries and periodontal disease has since been widely recognized but there is still a lack of information about the composition and mechanism of formation of plaque.

Dental plaque has been regarded in this thesis as a tissue, because it is composed of bacterial and some mammalian cells held together by an intercellular matrix of organic material, thought to be of salivary and bacterial origin. By applying to dental plaque some of the procedures used in the separation of other cellular systems (Ginzburg, 1961), plaque has been resolved into several acellular and cellular fractions. The chemical and physical properties of these fractions have been studied in relation to plaque composition and formation.

Purpose of the Study

The purpose of this study was to separate human dental plaque into as many components as possible, to chemically characterize each of these components and determine their role in plaque formation.

Organization of the Remainder of the Thesis

Chapter II will consider the contributions of previous investigators to (1) the relationship of dental plaque to caries and periodontal disease, (2) the microbial population of dental plaque, (3) the study of morphological changes during plaque formation and (4) theories of plaque formation.

Chapter III will report on the fractionation and characterization of the cellular and acellular components of dental plaque. In Chapter IV, studies on factors affecting the ability of the microorganisms in dental plaque to aggregate, are reported on and discussed with respect to plaque formation. A summary of the findings of the study along with the conclusions will be presented in Chapter V.

CHAPTER II

LITERATURE RELATED TO THE IMPORTANCE, COMPOSITION AND MECHANISM OF FORMATION OF DENTAL PLAQUE

The Relationship of Dental Plaque to Caries and Periodontal Disease

To explain the etiology of dental caries, Miller (1890) in his chemico-parasitic theory proposed that carbohydrate foodstuffs are decomposed by the oral bacteria to produce acids which subsequently decalcify the teeth. Williams (1897) and Black (1898) showed that these bacteria were localized on the teeth in films called dental plaque, permitting bacterial acids to accumulate and cause localized decalcification. More recently, Miller's postulate was expanded into a more generalized theory called the combined chemico-parasitic theory, to include both caries and periodontal disease (Kleinberg, 1958).

In the combined chemico-parasitic theory, the plaque microorganisms, based upon their metabolisms, were divided into two groups, those that produced acid from carbohydrates (the acid-producers) and those that produced base from nitrogenous substrates (the base-producers). Some organisms belong in both groups.

With respect to dental caries, because of the high concentration of cells in plaque and since materials can only

diffuse slowly through the plaque matrix, acids produced during the glycolytic breakdown of carbohydrate, readily accumulate. This accumulation of acid results in a rapid fall in pH which, if sufficiently large, can reach a certain critical level called the critical pH. At this critical pH, the calcium phosphate of tooth enamel underlying plaques in which acid has accumulated, will tend to go into solution to satisfy the solubility conditions within the plaque and adjacent saliva.

With respect to calculus formation, when nitrogenous substrates are metabolized, alkaline end-products accumulate in the plaque producing a rise in pH. Saliva subsequently contacting the plaque and diffusing into it would also rise in pH and become more supersaturated than previously with respect to calcium phosphate, favouring precipitation of calcium salts as calculus. In addition, calcium phosphate already present in the plaque matrix would be encouraged to dissolve.

Alteration of the plaque metabolism, would according to this theory affect plaque pH and in turn the solubility conditions existing within the plaque and between enamel and saliva. Lower plaque pH levels would favour caries, while higher pH levels would favour calculus formation.

The Microbial Population of Dental Plaque

Dental plaque contains a large variety of organisms including cocci (both gram positive and gram negative), rods, filamentous forms, epithelial cells and leucocytes. Twenty-seven types of microorganisms have been found in plaque by Hemmens et al, (1946) with micro-aerophilic organisms forming the largest share of the plaque flora (Winkler and Backer-Dirks, 1958).

To qualitatively assess the microbial composition of plaque, various methods including direct microscopic examination (Blayney, Kesel and Wach, 1936, and Bibby and Hine, 1938) and culturing on various types of media (Krasse, 1954, Hemmens, Blayney and Harrison, 1941 and Gibbons and Socransky, 1964) have been employed.

Mandel, Levy and Wasserman (1957) developed the technique for collecting plaque material on celluloid strips tied to the teeth, allowing them to compare day to day variations in plaque morphology. Histological examination of such strips showed that plaque, three to five days old, consists of both gram positive and gram negative cocci with occasional filamentous organisms. Mühlemann and Schneider (1959) using the same technique for collecting plaque found mostly cocci, rods and some leucocytes.

Turesky, Renstrup and Glickman (1961) showed that the percentage of filamentous and thread-shaped organisms in-

creased with plaque age and as calcification progressed, while the cocci and rods decreased.

The studies in the present investigation were with three day old plaque, sufficiently late to enable enough plaque to collect but sufficiently early so that true calculus had not yet formed. In addition, more is known of the metabolism of plaque at this age than at any other age (Kleinberg, 1961, Kleinberg and Jenkins, 1964),

Morphological Changes During Plaque Formation

McDougall (1963) histochemically examined the formation of an initial deposit, relatively free of microorganisms, on the surfaces of the teeth. Microorganisms then invaded this initial deposit in either of two ways. Firstly, by becoming attached to and embedded within this deposit and/or secondly by growing in from defects and lamellae in the enamel surface. Proliferation of the microorganisms then occurred and resulted in the appearance of numerous bacterial masses characteristic of older plaque. McDougall's observations have been confirmed by Bjorn and Carlsson (1964).

As a result of his studies, McDougall divided the process of plaque formation into two stages, an initial stage involving the formation of a non-bacterial deposit, and a second stage involving the attachment of bacteria to, invasion of and proliferation within this deposit. More

recently, Frank and Brendel (1966) showed in electron micrographs that bacteria can directly attach to enamel.

In this thesis, plaque formed during the initial stage has been referred to as primary or early plaque, while the term secondary or total plaque has been applied to plaque that is older.

Theories of Plaque Formation

Kirk (1910) suggested that the production of lactic acid by the oral bacteria, was responsible for the precipitation of mucin from saliva. Dobbs (1932), concluded from his *in vitro* studies that acid caused the precipitation of mucin onto the teeth particularly during sleep and that this precipitated mucin was then denatured by bacterial enzymes, presumably free in the saliva, to form a firm layer of plaque. Vallotton (1945) from his studies on the bacterial-free films that form on freshly cleaned teeth, suggested that in addition to bacterial enzymes, dehydration due to the passage of air over the teeth might have contributed to their denaturation.

Winkler and Backer-Dirks (1958) also concluded that salivary mucoid can be precipitated from saliva either by surface denaturation or by acid. These same authors pointed out that bacteria, which become attached to this layer of mucoid, could produce acid and precipitate further mucoid upon the teeth.

Leach (1963) suggested that a neuraminidase enzyme in saliva splits off sialic acid from sialomucins, resulting in an altered mucin with (because of the loss of the sialic acid carboxyl group) a raised iso-electric point. This altered mucin would precipitate under mildly acid or even neutral conditions and collect on the teeth as plaque. Lack of demonstrable amounts of sialic acid in plaque, he felt, supported his theory. Also in support of Leach's theory, was the observation of Knox (1953) that salivary mucinases liberate the carbohydrate moiety from salivary muco-protein and the observation of Gottschalk and Graham (1959) that a sialidase splits off sialic acid from bovine submaxillary mucoid.

McDougall (1963) precipitated salivary mucoid onto glass slides, incubated them in raw saliva for several hours and found that the staining reactions of the deposits were similar to the staining reactions of primary dental plaque. Considerable amounts of sialic acid were lost from the mucoid on the slides during the incubation and this led McDougall to conclude that removal of sialic acid from the mucoids occurred after the mucoids had precipitated onto the teeth, rather than before.

Trester and Kleinberg (1962) found that precipitation of nitrogenous material (presumably mucoprotein) from saliva occurred over a wide range, between pH 4 and pH 10 and that

the greatest amount of precipitation took place on the acid side of neutrality. They concluded that acid increased the rate of precipitation of this nitrogenous material.

Dawes (1964) questioned whether acid precipitation was important in plaque formation, since he failed to demonstrate the precipitation of protein in saliva above pH 3.5. However, since his results were based on visual observations of such changes in saliva, he might have missed the gradual changes in solubility measured spectrophotometrically by Trester and Kleinberg.

McGaughey and Stowell (1966b) showed that adsorption of salivary protein to hydroxyapatite increased, as the pH was decreased from 9.0 to 5.8, an observation that is consistent with the findings of Trester and Kleinberg (1962).

The finding in the present study, that the plaque cellular and acellular components decreased in solubility with decreasing pH, is also consistent with these findings.

CHAPTER III

FRACTIONATION AND CHARACTERIZATION OF THE CELLULAR AND ACELLULAR COMPONENTS OF DENTAL PLAQUE

INTRODUCTION

Prior to the present study attempts to fractionate plaque into its component parts have had very limited success. The majority of the investigators were primarily interested in separating plaque for the purpose of subsequently examining its bacterial composition. Stralfors (1950) developed an homogenization technique for this purpose while Green, Dodd and Inverso (1957) used ultra-sonic agitation. Earlier, Crowley and Rickert (1935) employed dilute NaOH to separate clumps of salivary bacteria in order to obtain a smear in which the distribution of organisms was uniform.

Fewer studies have attempted to examine the non-cellular components of plaque. Dobbs (1932) extracted mucin from plaque with 5% NaOH and showed that it constituted 27 to 52% of the total plaque. Another study that attempted to examine the acellular components of plaque was that of Ferguson (1964), who was able to separate seven components from samples of total plaque by starch electrophoresis. Examination of saliva by the same procedure and by the same

investigator suggested, from the location on the electrophoretic strips of the separated salivary and plaque components, that the plaque components might have originated from saliva. The disadvantage, however, of this analytical technique to study plaque composition is that much of the plaque sample remains at the origin.

In the present study, plaque has been successfully fractionated into cellular and acellular components. The total plaque and its fractionated components were analyzed for carbohydrate, calcium and phosphate because of the possible relationship of these chemical constituents, alone or in combination with other substances, to plaque formation. The nitrogen contents and dry weights of plaque and its fractionated components were also determined so that their individual carbohydrate, calcium and phosphate concentrations could be expressed in terms of either N or dry weight.

Previous analyses of plaque and calculus suggested that plaque carbohydrate is derived from both salivary and bacterial sources (Muhlemann and Schneider, 1959, Mandel, Hampar and Ellison, 1962 and McDougall, 1964). Much of this carbohydrate is present as mucoprotein, which has been implicated as an essential component in plaque formation (Kirk, 1910, Dobbs, 1932, Trester and Kleinberg, 1962 and Leach, 1963).

McGaughey and Stowell (1966a) suggested that calcium

and phosphate are necessary for salivary mucoids to adhere to the teeth. There is, however, no quantitative data available to support the role of these elements in plaque formation. The plaque analyses that have been done for calcium and for phosphorous have been only on total plaque (Allan and Moore, 1957, Dawes and Jenkins, 1962 and Kaminsky and Kleinberg, unpublished results), for purposes not directly concerned with plaque formation.

METHODS

Collection of Plaque

To obtain sufficient plaque for the various fractionations in the present study, plaque was removed from the teeth of several (five to ten) individuals who had not brushed their teeth for three successive days. On the morning of the fourth day, before any food or drink was ingested, plaque was removed with a stainless steel spatula from the various surfaces of the teeth upon which plaque had collected. As plaque was removed with the spatula, each portion was immediately transferred to 10 X 75 mm. test tubes containing 1.5 ml. of 0.1N NaOH and the plaque was dislodged by twirling the spatula.

Initial Separation of the Plaque into Cellular and Acellular Fractions

The plaque in the 10 X 75 mm. test tubes was mechanically dispersed in the NaOH by vibrating the tubes on a Vortex mixer. The tubes were allowed to stand for one hour at 4°C to permit further solubilization and suspension of plaque constituents. The tubes were then centrifuged at 1740 X g for 2-1/2 minutes to separate the suspended microorganisms and NaOH-soluble material in the supernatant from the remaining plaque. The supernatants (containing the suspended microorganisms and NaOH-soluble material) were pooled in a 50 ml. conical flask. The centrifugate was redispersed in 1.5 ml. of 0.1N NaOH and the whole procedure repeated. This was done five times. A residue still remained after these repeated exposures to NaOH and was composed mainly of clumps of mammalian and bacterial cells.

1.5 ml. of 0.01M EDTA was added to the remaining residue for two successive periods of two hours each, to remove calcium and perhaps permit more cells to be liberated. This procedure was then followed by five successive additions of 0.1N HCl, again to liberate calcium and to attempt to release more cells from the residue. The number of cells freed from the residue as a result of both treatments (EDTA and HCl), with or without subsequent exposure to NaOH was insignificant.

The supernatants from the repeated NaOH extractions were centrifuged at 12,800 X g at $4^{o}C$ for ten minutes. This separated the cells from the NaOH-soluble material.

The supernatant from this centrifugation was decanted into a 50 ml. conical flask and the cells in the centrifugate were washed with 5 ml. of 0.1N NaOH to remove residual NaOH-soluble material. The washing procedure was repeated twice.

The NaOH-soluble material is referred to below as the _NaOH-soluble fraction_ (NaOH-S fraction for brevity), while the cells in the centrifugate are referred to as the _NaOH-suspended cells_ (NaOH-S cells).

Initial Fractionation of the NaOH-S Fraction

The NaOH-S fraction was dialysed in Visking quarter inch tubing against distilled water (100 X volume) for 96 hours (water changed six times), to remove small molecular weight substances such as free sugars, amino acids and minerals. A precipitate formed towards the end of the dialysis period, after which the contents of the dialysis bags were removed and centrifuged at 10,000 X g at $4^{o}C$. The supernatant was decanted, concentrated by freeze-drying and stored at $4^{o}C$, while the precipitate was washed three times with 5 ml. of distilled water to remove residual supernatant. Addition of 0.003N NaOH resolubilized the precipitate.

Sub-fractionation of the Precipitate and Supernatant Components of the NaOH-S Fraction

The technique of gel-filtration was employed to sub-fractionate the supernatant and precipitate components of the NaOH-S fraction and to obtain some idea of the size of the molecules in the resulting sub-fractions.

Because of the difficulty of obtaining enough of the precipitate and supernatant components of the NaOH-S fraction for the sub-fractionations, it was necessary to collect plaque from six individuals once each week for four weeks. After each collection the plaque was processed to the precipitate and supernatant stage, freeze-dried and stored at $4^{o}C$, until sufficient material for the sub-fractionations had been collected.

Gel-filtration was done on columns containing Bio-Gel (co-polymer of acrylamide and methylenebisacrylamide) of different sieve sizes and which had been equilibrated with 0.003N NaOH. 0.003N NaOH was used to solubilize the freeze-dried samples and as the eluant. Filtrations were carried out in a cold room at $4^{o}C$ and the effluents from the various columns collected in a micro-fraction collector and monitored at both 280 and 230 mμ in a Beckman DU spectrophotometer, adapted to read micro-samples.

Procedures Used to Characterize Plaque and Its Various Constituents

(a) Microscopic

Aliquots from suspensions of plaque, the NaOH-S cells and the residue after the NaOH, EDTA and HCl extractions, were transferred onto glass slides, air dried, fixed with 95% ethyl alcohol and then stained with hematoxylin-eosin. The slides were examined under oil immersion and photographed.

(b) Analytical

(i) Dry Weight Determinations

Dry weight determinations were done on plaque NaOH-S cells, residue and the precipitate and supernatant components of the NaOH-S fraction. Samples of each were placed on platinum boats, dried to constant weight by heating the boats in an oven at 100°C for one hour. Boats without samples added served as controls. Boats and samples were then dried in the oven at 100°C for one hour and then transferred to a desiccator containing concentrated H_2SO_4 as the desiccant. The samples were kept in the desiccator until they reached room temperature and were then weighed on an ultra-micro balance (Mettler UM-7).

N analyses (see below) were carried out on the weighed samples to determine their N/dry weight ratios.

(ii) Analyses of Plaque, NaOH-S Cells, Residue and the Various Fractions and Sub-fractions of the Acellular Portion of Plaque for Carbohydrate, Nitrogen, Calcium and Phosphate

Plaque and its various constituents were analyzed for carbohydrate, nitrogen, calcium and phosphate by micro-adaptations (developed in this laboratory) of the following procedures. Carbohydrate was determined by the method of Morris (1948). After samples had been digested with H_2SO_4, phosphate was determined on aliquots of the digest by the method of Kuttner and Cohen (1923) and nitrogen on other aliquots by Nesslerization (Hawk, Oser and Summerson, 1954). Calcium, was determined by the method of Williams and Wilson (1961) after digestion with aqua regia.

(iii) <u>Amino Acid Analyses of the Supernatant and Precipitate Components of the NaOH-S Fractions</u>

Samples of the precipitate and supernatant were hydrolyzed under vacuum in 6N HCl for 22 hours at 115°C. Nitrogen analyses were performed on the hydrolysates and samples from the hydrolysates, containing 100 µg of N, were analyzed for amino acids on an amino acid analyzer (Technicon).

(iv) <u>Determination of the Solubilities of the Precipitate and Supernatant Components of the NaOH-S Fraction as a Function of pH</u>

Samples of the precipitate and supernatant components of the NaOH-S fraction, each containing approximately 140 µg N, were solubilized in 200 µl of 0.003N NaOH. The resulting solutions were then titrated with 0.1N HCl between pH 10 and

pH 2. Changes in O.D. at 700 mμ were measured with a Beckman DU spectrophotometer, a decrease in solubility being shown by an increase in O.D.

RESULTS

A. Microscopic Appearance of the Total Plaque, Its NaOH-S Cells and Its Residue Fraction

The NaOH suspension of total plaque contained many individual microorganisms and large and small aggregates of bacterial and mammalian cells (Figure 1a). The NaOH-S cells fraction was free of mammalian cells and large bacterial clumps. It consisted only of individual bacteria and small aggregates of bacterial cells (Figure 1b).

The residue fraction contained virtually no individual microorganisms. It was composed mainly of epithelial cells and aggregates of bacteria. Large branched organisms similar to leptotrichia and actinomycetes were readily seen (Figure 1c).

On a dry weight basis there was four times as much NaOH-S cells as residue fraction (see Figure 2 below).

B. Fractionation of the Total Plaque into NaOH-S Cells, Residue, and Precipitate and Supernatant Components of the NaOH-S Acellular Fraction

(i) Percentage Composition in Terms of Nitrogen and Dry Weight

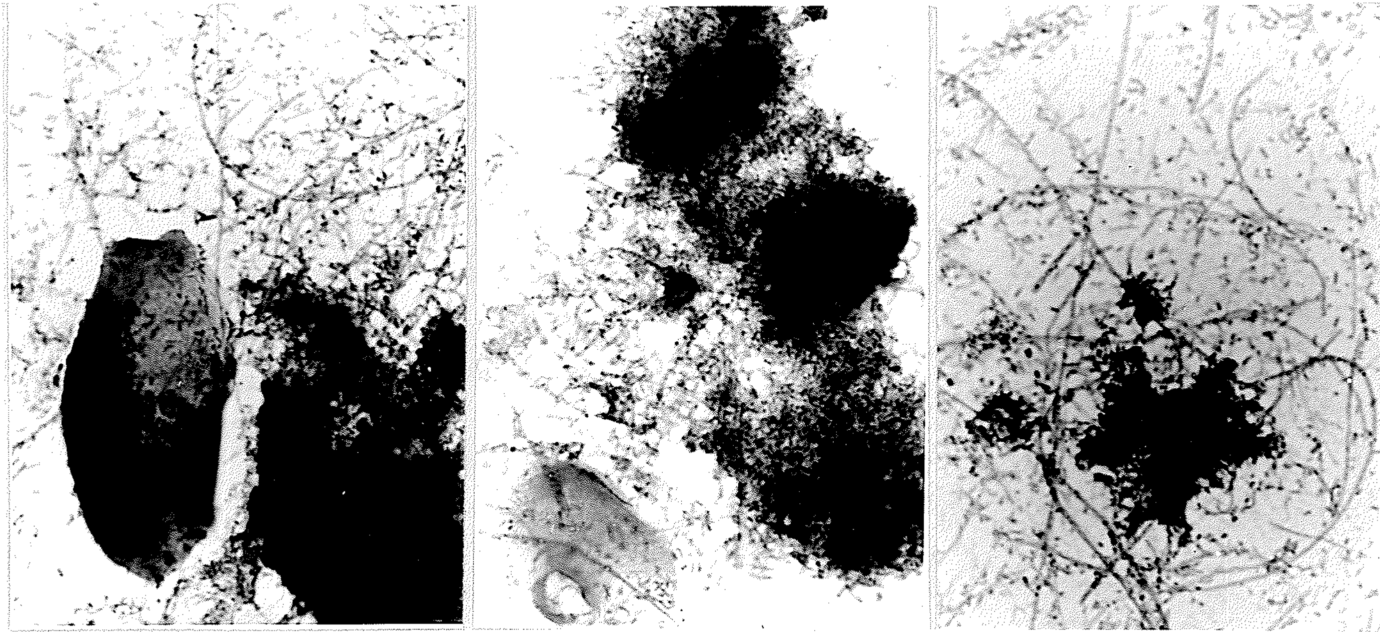

Figure 1a. Total plaque dispersed in 0.1N NaOH and stained with hematoxylin and eosin. Magnification x 1000.

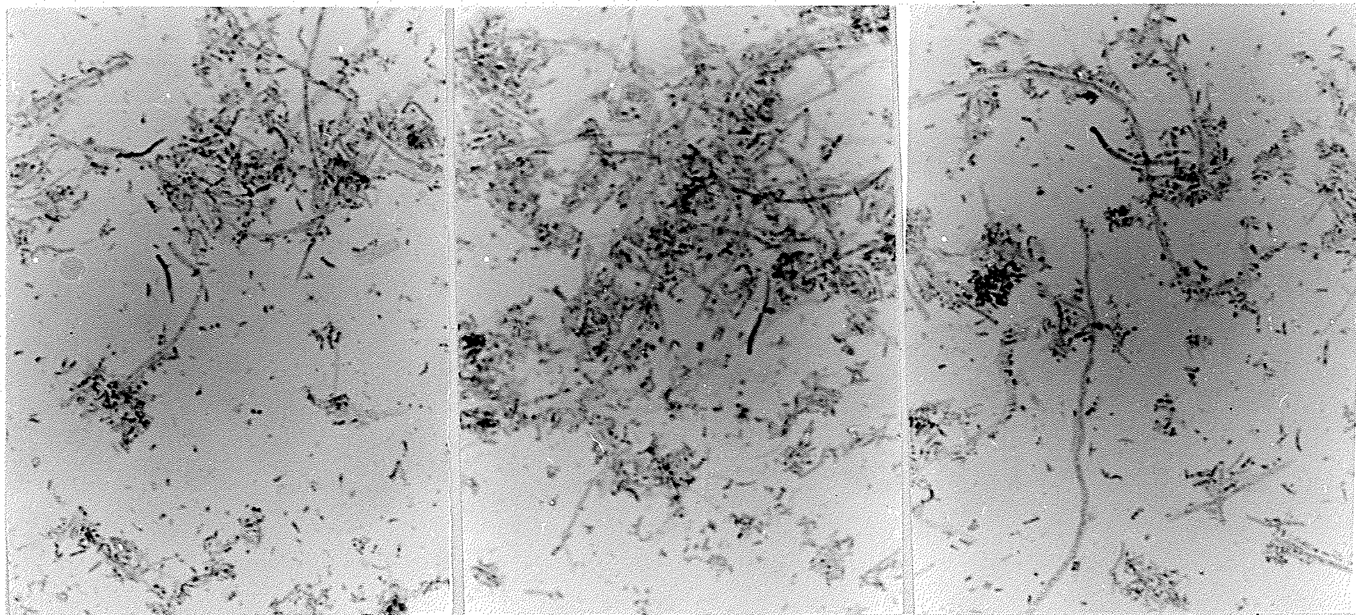

Figure 1b. NaOH-S cells suspended in 0.1N NaOH and stained with hematoxylin and eosin. Magnification x 1000.

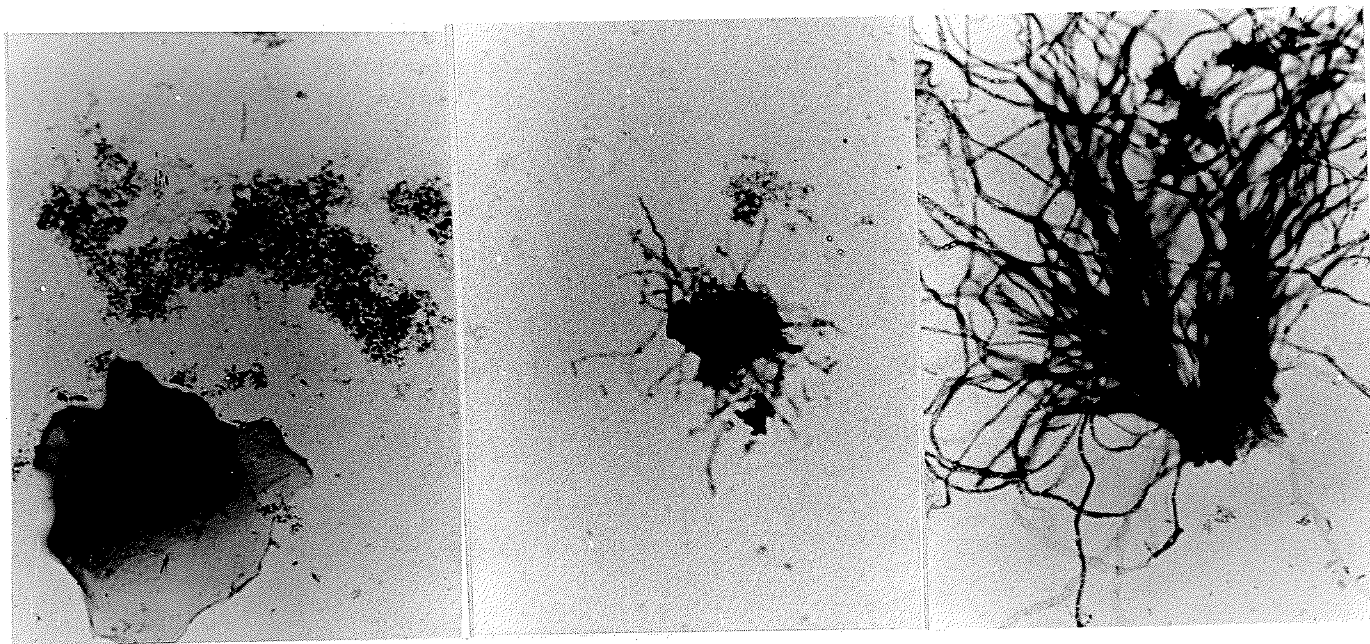

Figure 1c. Residue dispersed in 0.1N HCl and stained with hematoxylin and eosin. Magnification x 1000.

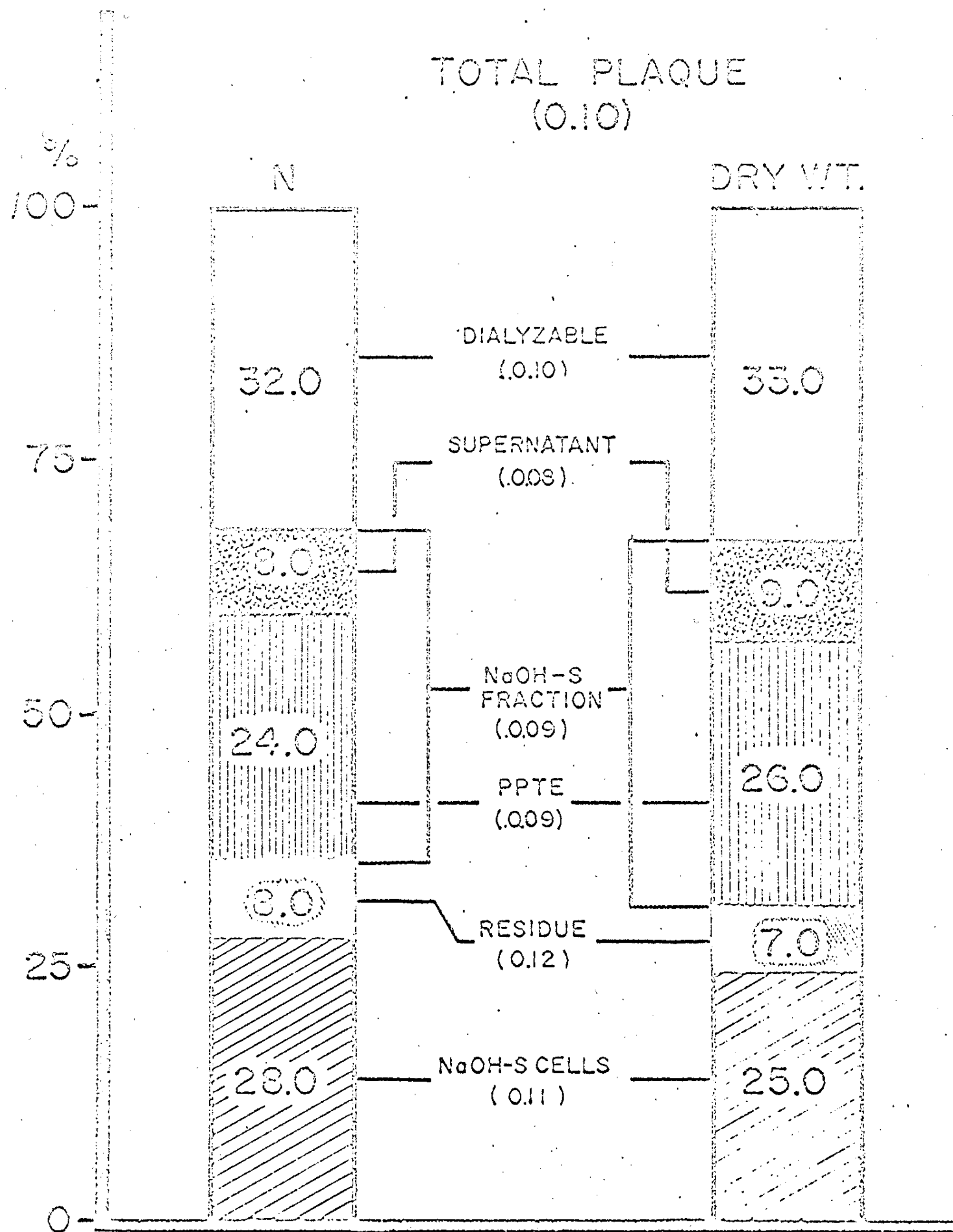

Figure 2. Per cent composition of total plaque in terms of nitrogen (N) and dry weight. The figures in the brackets are the N/dry weight ratios of plaque and its constituents.

Seven separate fractionations of total plaque into NaOH-S cells, residue and NaOH-S fraction were carried out and the N in the total plaque and each of these three determined. The mean values for each, expressed as a percentage of the total plaque N, are shown in Figure 2.

Nine fractionations of the NaOH-S fraction into precipitate and supernatant components were done separately and the N contents of the total NaOH-S fraction and of the two components determined. The proportion of the NaOH-S fraction represented by each of the two components, is also shown in Figure 2.

In separate experiments, the N/dry weight ratios of the individual plaque constituents were determined (5 or 6 values for each) and these are shown in brackets in Figure 2. From these ratios and the percentage N data, the percentage dry weights of the various fractions were calculated (Figure 2).

Whether expressed as unit nitrogen or unit dry weight, the percentage composition of total plaque was approximately the same.

Examination of the individual N/dry weight ratios showed certain characteristic differences. The cellular fractions of plaque (i.e. NaOH-S cells and the residue) had slightly higher N/dry weight ratios than the total plaque, while the acellular plaque constituents had lower ratios.

Of the two components of the acellular portion of the plaque (i.e. NaOH-S fraction), the supernatant component had a lower N/dry weight ratio than the precipitate component.

In the total plaque, the cellular fractions compose 36%, the acellular NaOH-S fraction 32% and the remaining dialyzable fraction 32%. Of the cellular portion of the plaque, there was four times as much NaOH-S cells as residue. 76% of the acellular NaOH-S fraction was made up of the precipitate component while the supernatant component accounted for 24%.

(ii) Carbohydrate, Calcium and Phosphate Content

The values for the total carbohydrate, calcium and phosphate contents of dental plaque per 100 µg of plaque N are shown in Figure 3 (insert).

The values for the carbohydrate, calcium and phosphate contents of the various plaque constituents, expressed as a percentage of the total plaque carbohydrate, calcium and phosphate values are shown in Figure 3. Their concentrations in terms of N are shown in Figure 4.

The fraction that contained the largest portion of the total plaque carbohydrate was the NaOH-S cells, while the dialyzable portion contained the most calcium and phosphate.

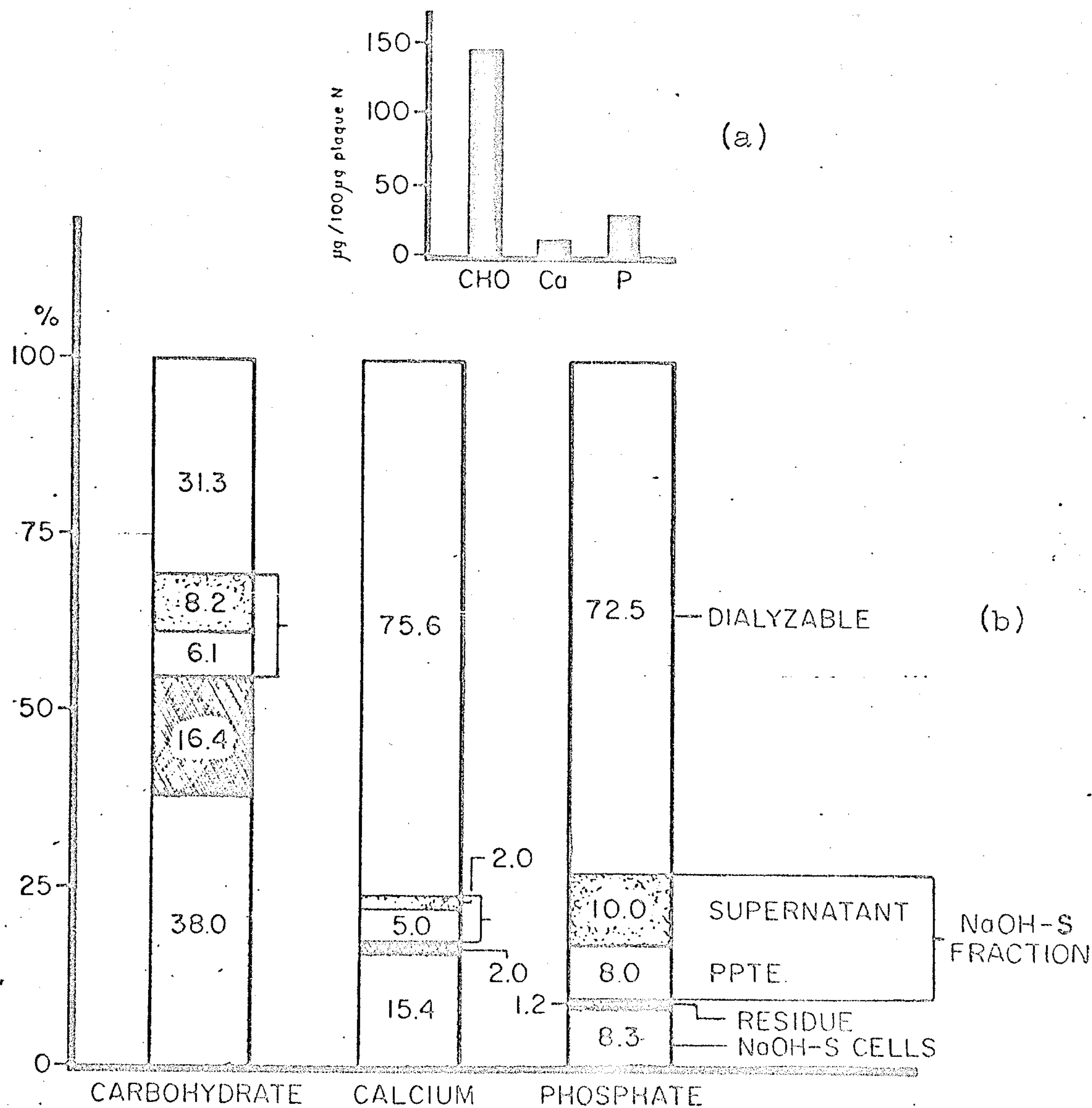

Figure 3. (a) Carbohydrate, calcium and phosphate contents of the various plaque constituents expressed as a percentage of the total plaque carbohydrate, calcium and phosphate. (b) The insert shows the amount of plaque carbohydrate, calcium and phosphate per 100 µg plaque N.

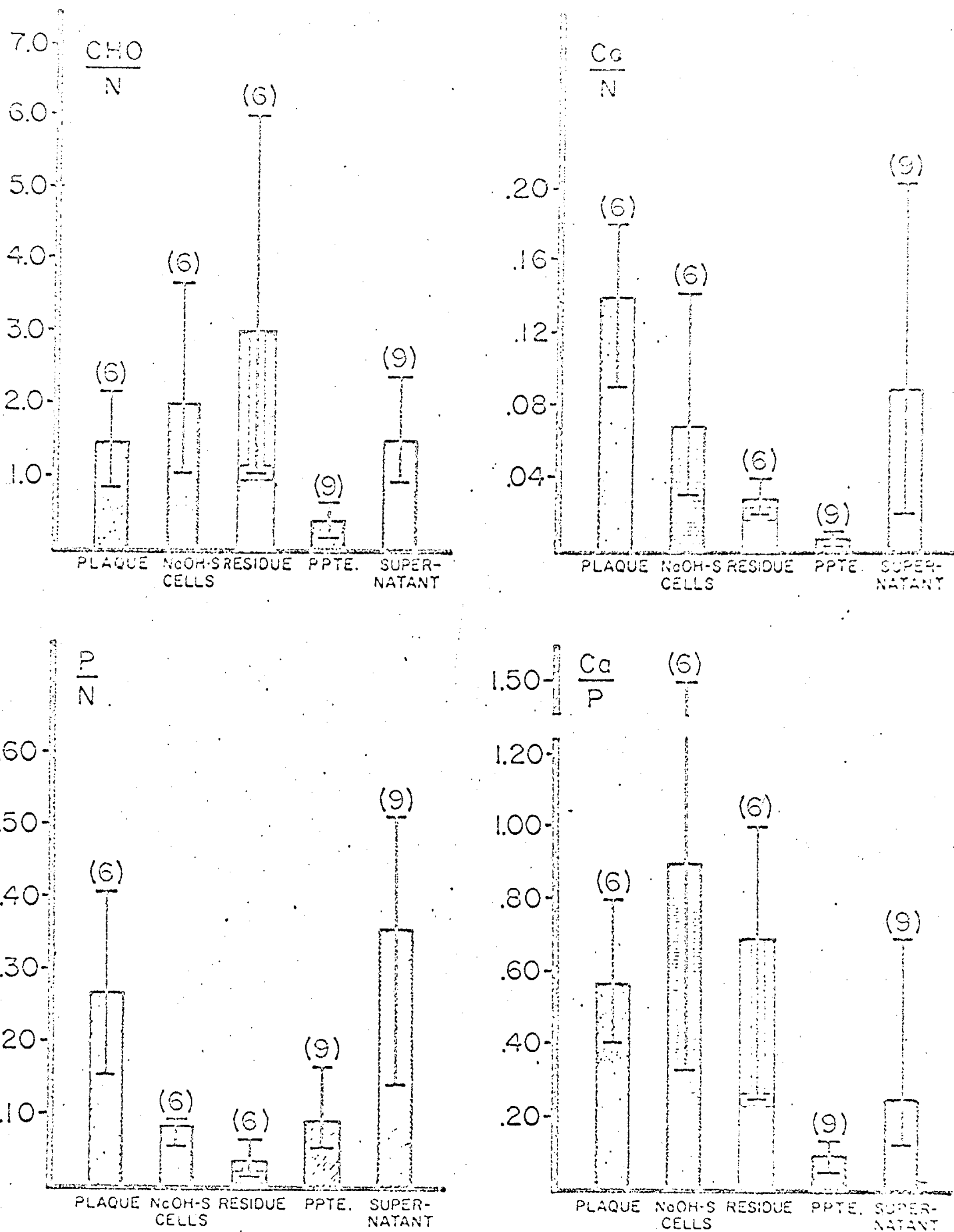

Figure 4. CHO/N, Ca/N, P/N and Ca/P ratios of plaque and its constituents. Figures in brackets represent the number of samples, while the vertical lines represent the range. The differences between CHO/N ratios of NaOH-S cells and residue and between Ca/P ratios of plaque and its constituents were not statistically significant at the p = .05 level.

Although the precipitate component of the NaOH-S fraction was three times the amount of the supernatant component, the supernatant contained more carbohydrate, calcium and phosphate. With respect to calcium, the supernatant component contained more than three times the amount of calcium present in the precipitate component.

(iii) <u>Amino Acid Analyses of the Precipitate and Supernatant Components of the NaOH-S Fraction</u>

The supernatant component of the NaOH-S fraction showed four times the ammonia content than that of the precipitate component (Table I). The supernatant component also had more acidic (glutamic and aspartic) and less basic (lysine, arginine, histidine) amino acids in its structure.

By an x - y plot of corresponding amino acids in the precipitate and supernatant component, glutamic acid, glycine and alanine were clearly higher in the former while the precipitate possessed more arginine, leucine and threonine. The unknown areas on the amino acid tracings were expressed as leucine equivalents.

(iv) <u>Solubilities of the Precipitate and Supernatant Components of the NaOH-S Fraction as a Function of pH</u>

The precipitate and supernatant components of the NaOH-S fraction were most soluble at the extreme ends of the pH range studied (Figure 5). The supernatant and precipitate

TABLE I

AMINO ACID ANALYSES OF THE PRECIPITATE AND SUPERNATANT COMPONENTS OF THE NaOH-S FRACTION OF PLAQUE*

Amino Acids	Precipitate	Supernatant
Ammonia	117	514
Hydroxyproline	0	0
Aspartic acid	97	94
Threonine	71	57
Serine	54	51
Glutamic acid	116	130
Proline	44	45
Glycine	98	139
Alanine	89	99
Cystine	0	0
Valine	90	84
Methionine	15	17
Isoleucine	47	44
Leucine	90	66
Tyrosine	32	22
Phenylalanine	37	30
Lysine	63	59
Histidine	18	16
Arginine	45	32
Unknown areas	6	15

* The results are expressed as moles per thousand moles of amino acid.

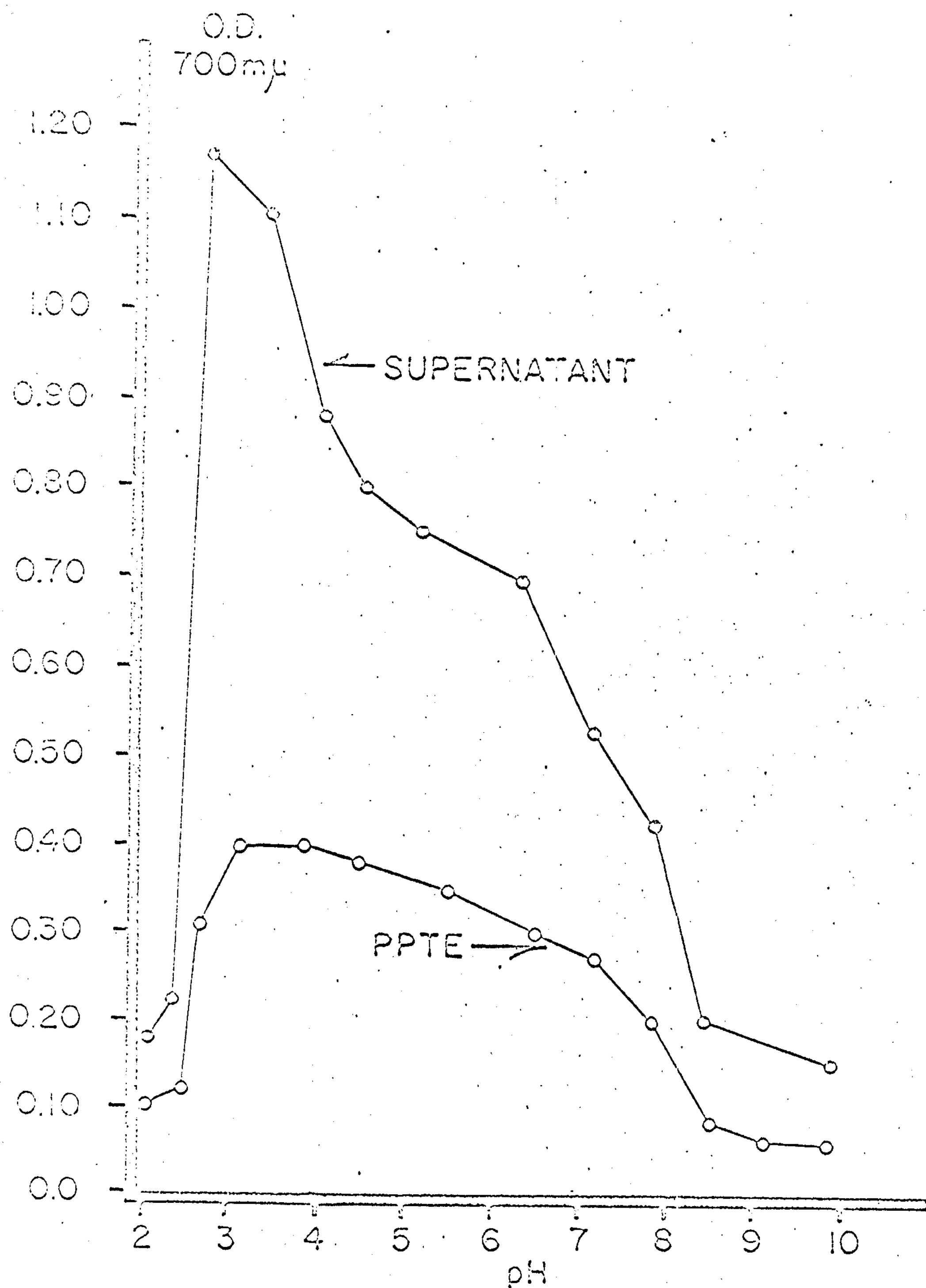

Figure 5: Solubility of the precipitate and supernatant components of the NaOH-S fraction as a function of pH, indicated by changes in O.D.

components were progressively less soluble as the pH was lowered, reaching their minimum solubilities at approximately pH 2.7 and pH 3.2 respectively.

C. Sub-fractionation of the Precipitate and Supernatant Components of the NaOH-S Fraction

(i) Separation by Gel-filtration

Fractionation of either the precipitate or supernatant components of the NaOH-S fraction by gel-filtration did not occur with gels that excluded molecules of molecular weight greater than 100,000. Separation did occur on Bio-gel P200, which has an exclusion limit of approximately 200,000.

Three sub-fractionations were carried out showing the same general characteristics. The most successful of the three, i.e., the one that gave the most sub-fractions (because the largest amount of starting material was used) is presented in Figures 6 to 11.

Precipitate separated into P_1, P_2, P_3 and P_4 sub-fractions (Figure 6). P_1, P_2 and P_3 were rechromatographed to effect further separation. Upon rechromatography of P_2, two sub-fractions were identified (Figure 7) and these have been labelled P_2A and P_2B. Rechromatography of P_3 gave rise to two more sub-fractions (Figure 8) and these are referred to as P_3A and P_3B. The net result was that the precipitate

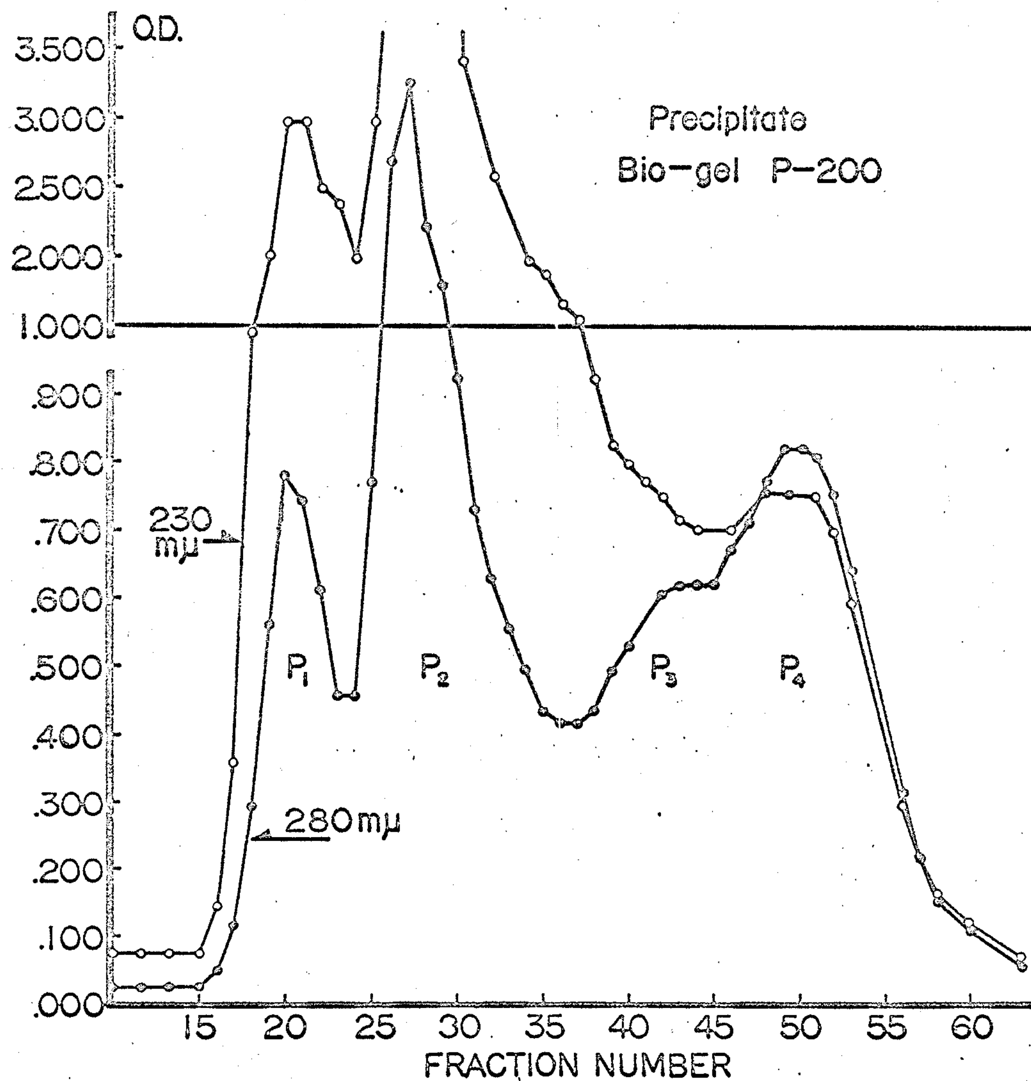

Figure 6. Sub-fractionation of the precipitate component of the NaOH-S fraction on Bio-gel P200.

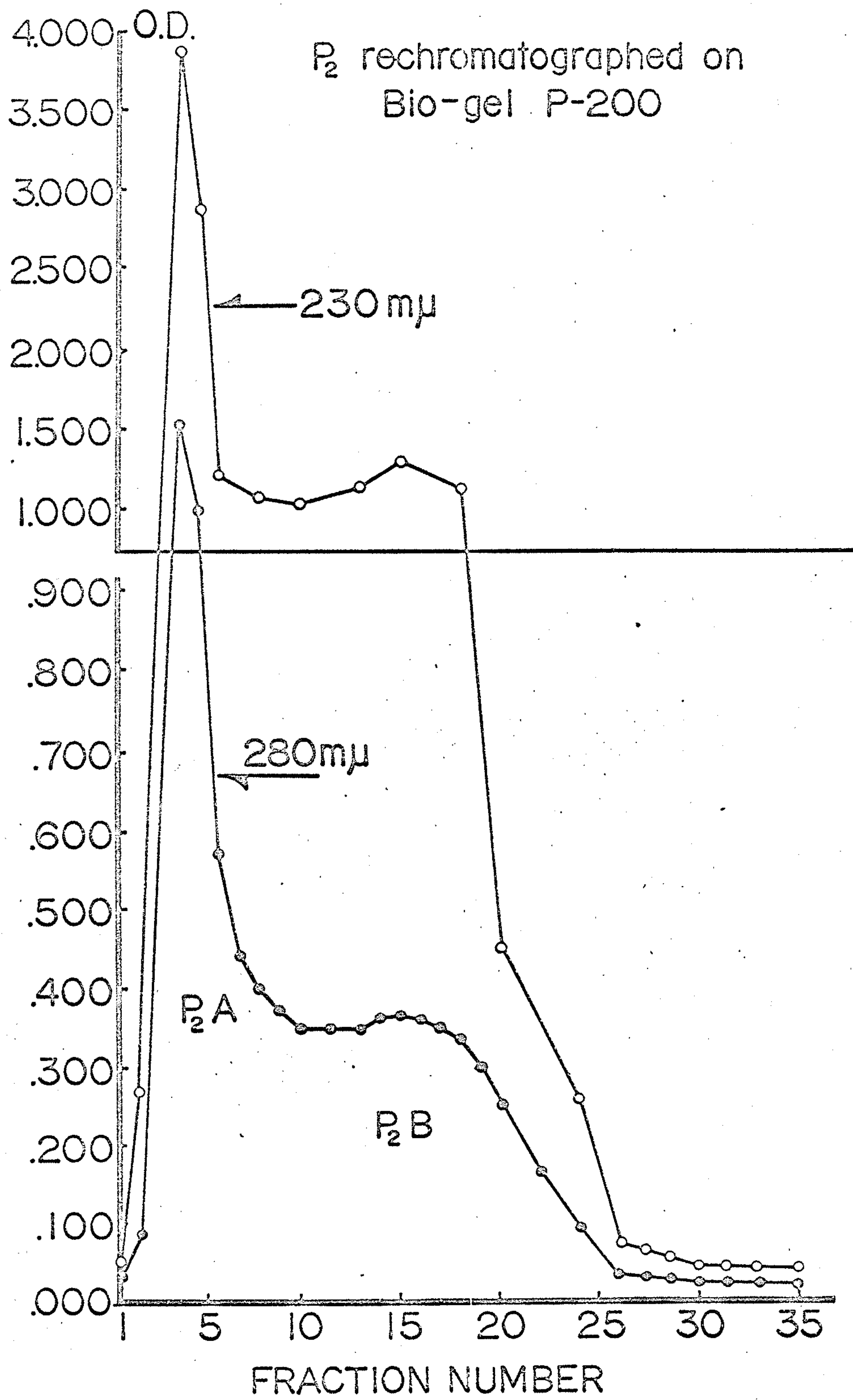

Figure 7. Rechromatography of P_2 to yield sub-fractions P_2A and P_2B.

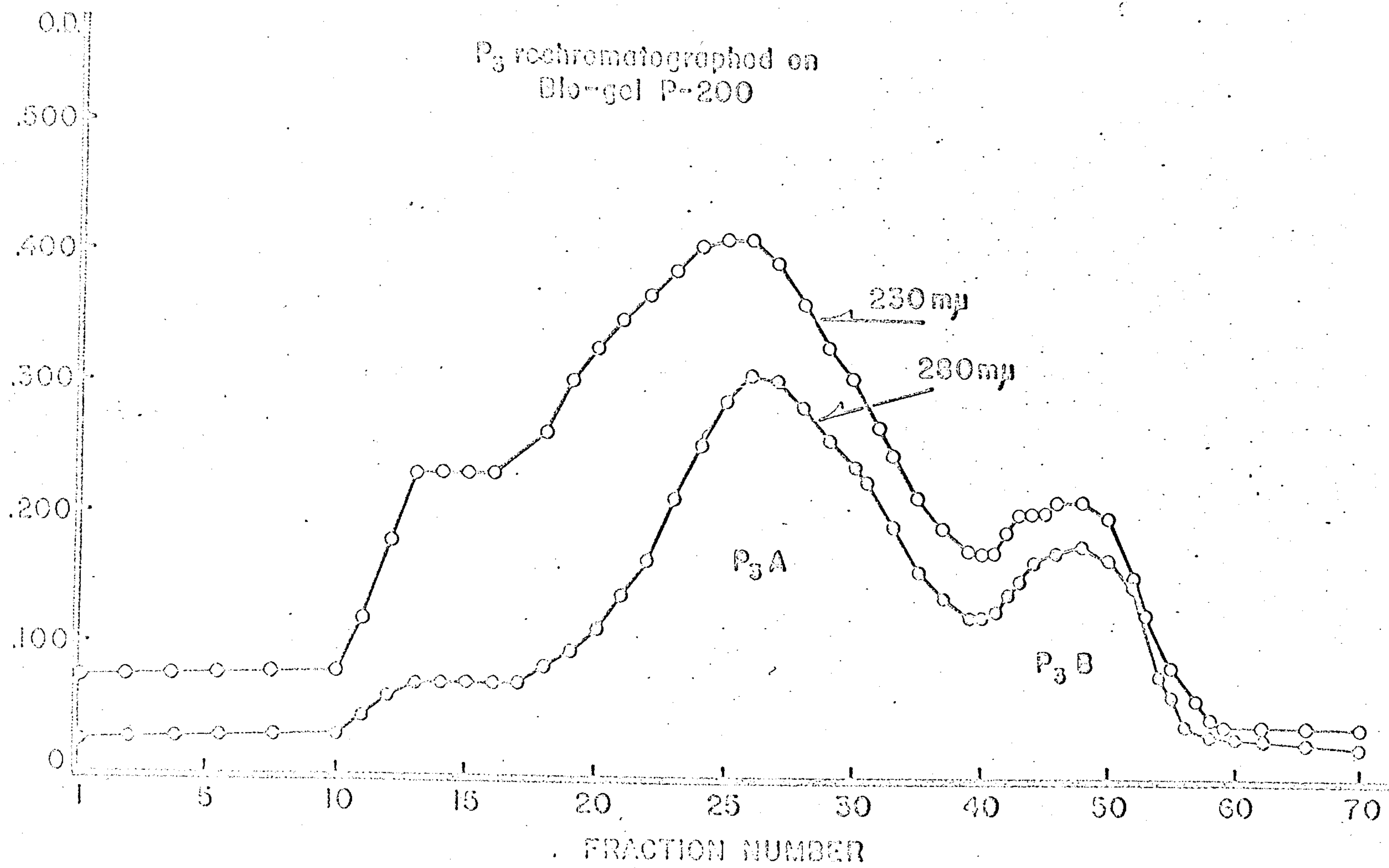

Figure 8. Rechromatography of P_3 to yield sub-fractions P_3A and P_3B.

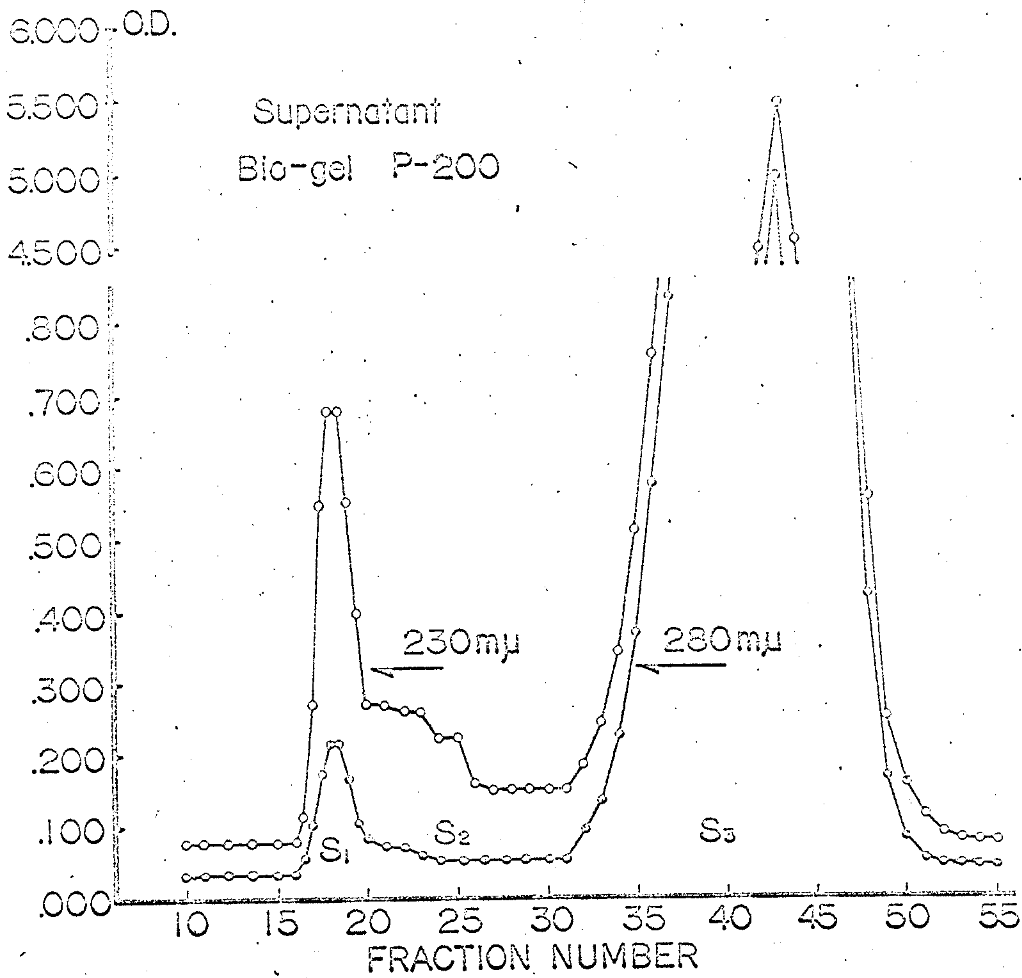

Figure 9. Sub-fractionation of the supernatant component of the NaOH-S fraction on Bio-gel P200.

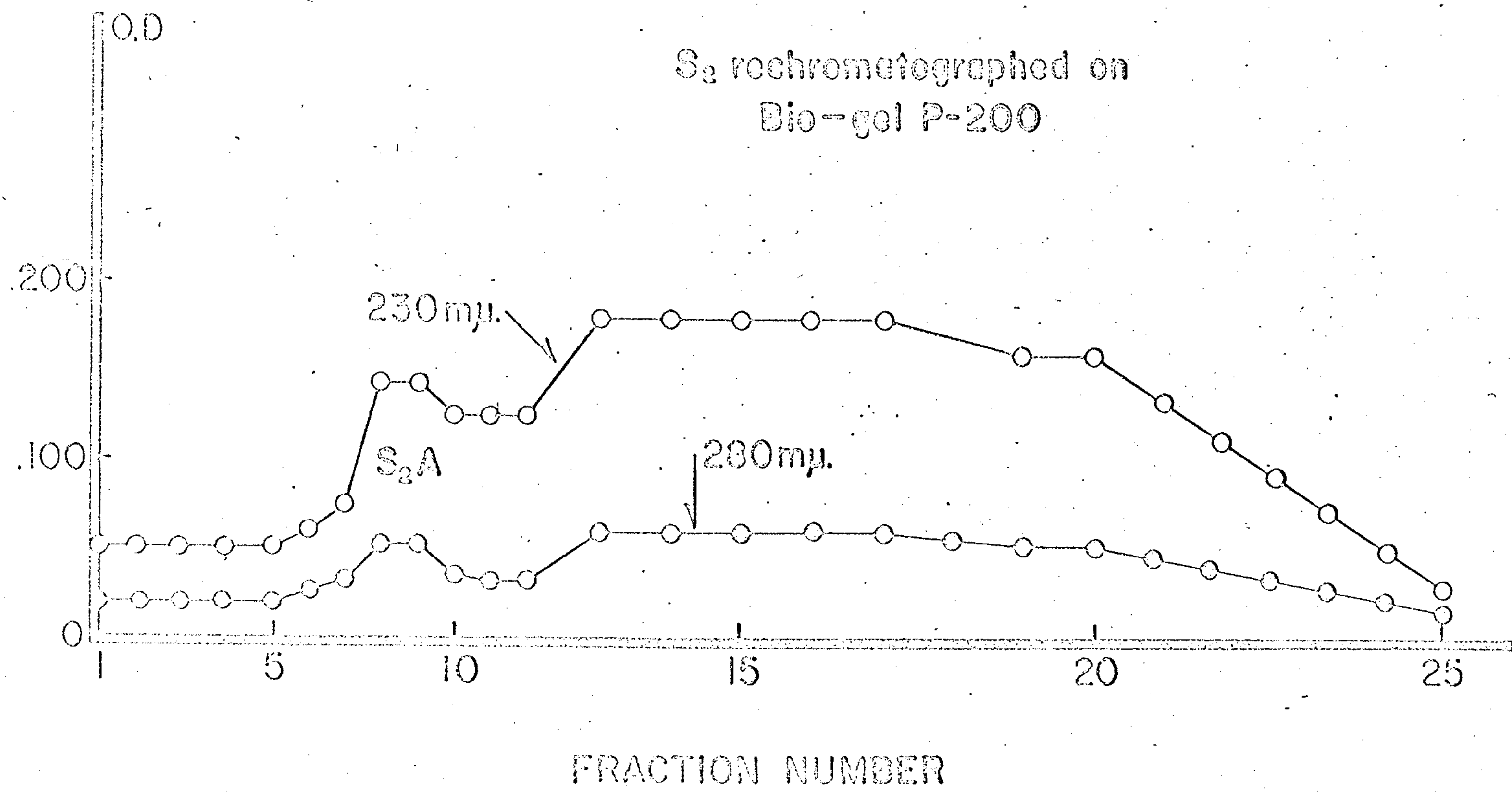

Figure 10a. Rechromatography of S_2 to yield S_2A.

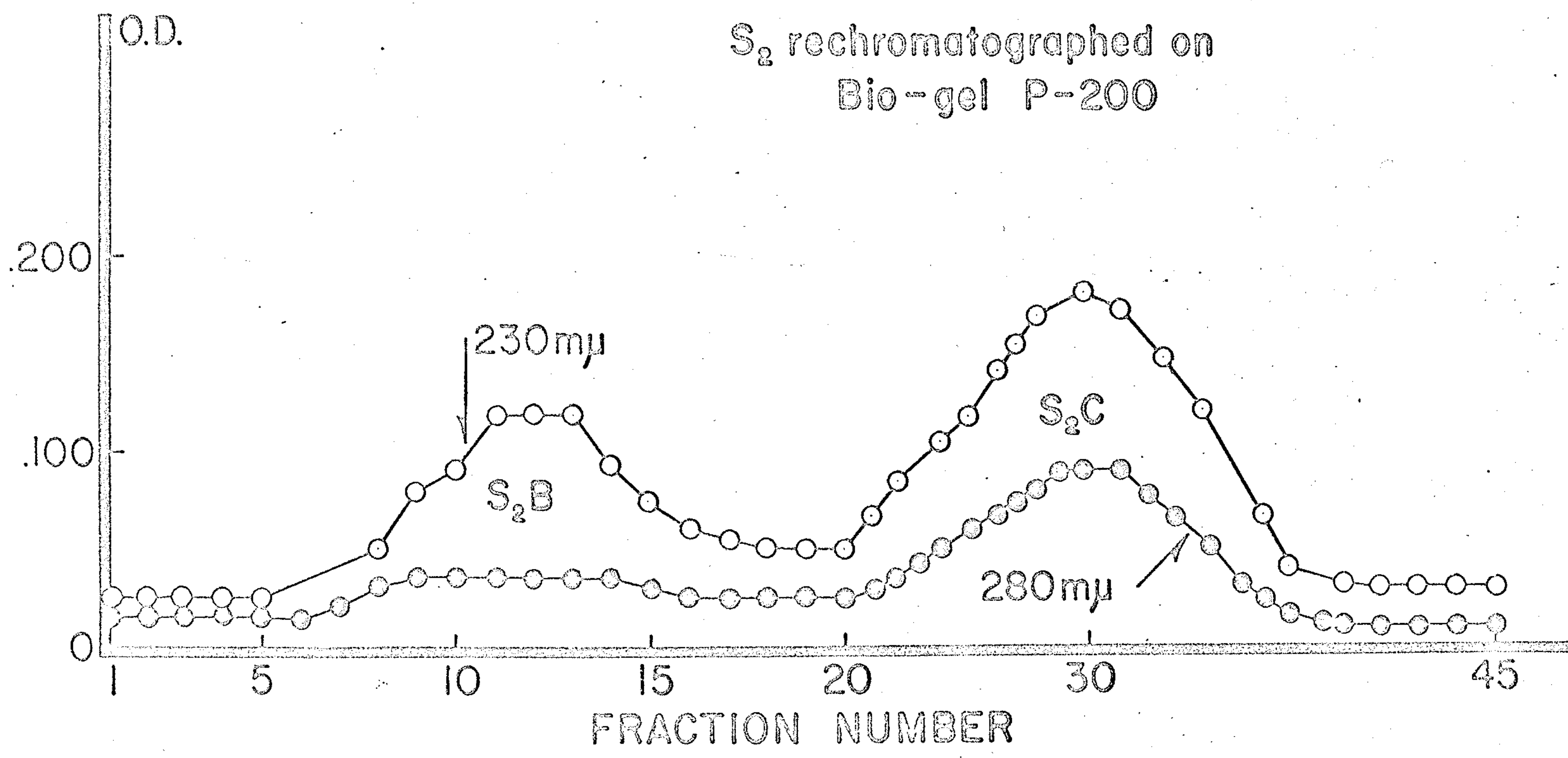

Figure 10b. Rechromatography of second peak in Figure 10a to yield S_2B and S_2C.

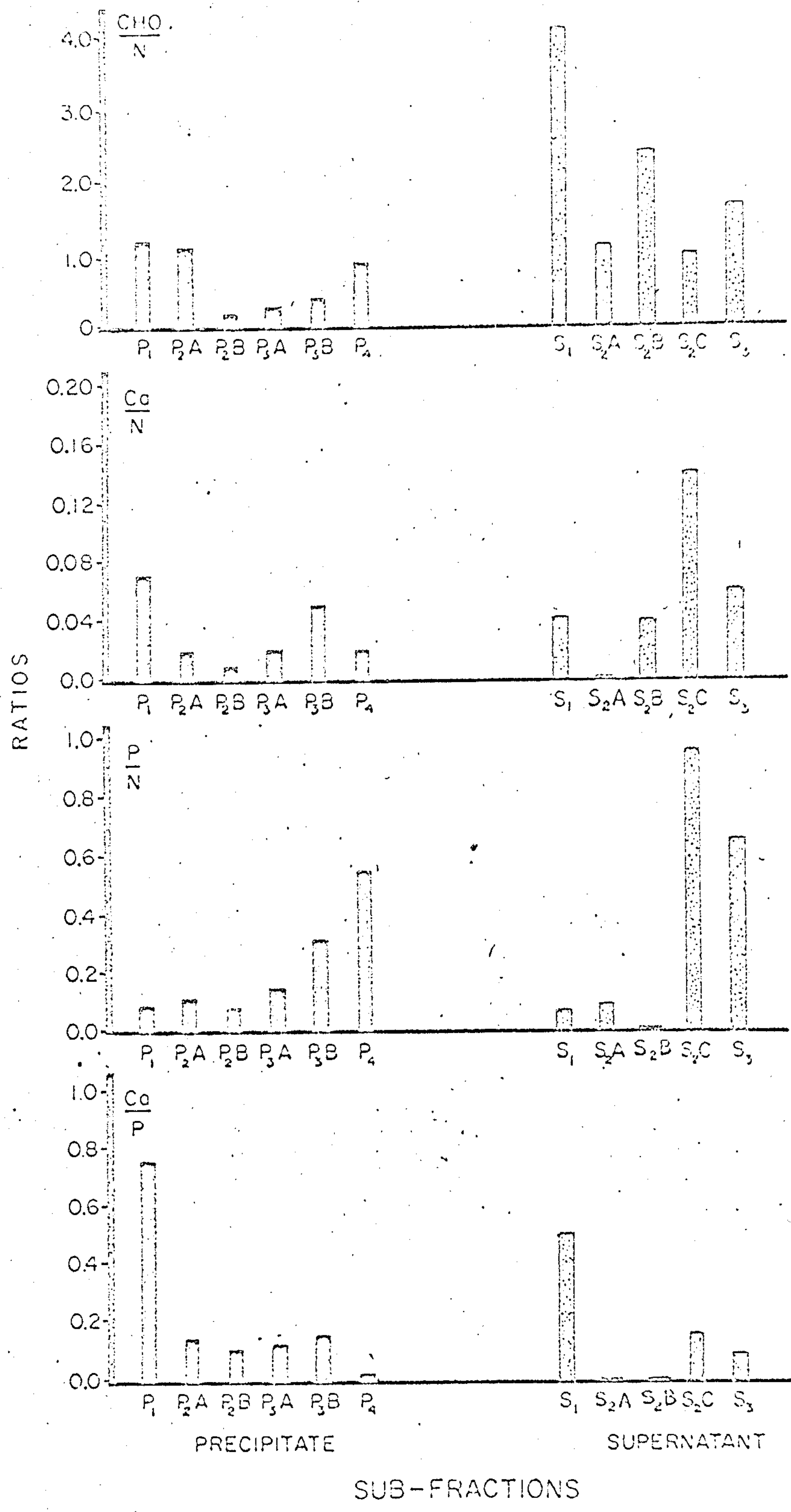

Figure 11. CHO/N, Ca/N, P/N and Ca/P ratios of the sub-fractions of the precipitate and supernatant components of the NaOH-S fraction.

component separated into six sub-fractions.

Supernatant separated into two major peaks (S_1 and S_3; Figure 9) with an elevated region between (S_2). Upon rechromatography of S_2, two sub-fractions S_2A and a second area which subsequently separated upon rechromatography into S_2B and S_2C were obtained (Figures 10a and 10b). A total of five supernatant sub-fractions resulted.

(ii) Carbohydrate, Calcium and Phosphate Analyses

All sub-fractions contained varying amounts of carbohydrate and all but S_2A contained varying amounts of calcium. The only sub-fraction that did not show phosphate was S_2B. S_2C had by far the highest Ca/N and P/N ratios.

The CHO/N, Ca/N, P/N and Ca/P ratios of all sub-fractions are shown in Figure 11.

DISCUSSION

The present study showed that dental plaque could be separated into several component parts by using dilute NaOH, a procedure used previously by Dobbs (1932) to extract mucin from plaque and by Crowley and Rickert (1935), to separate clumped salivary bacteria. This dispersion can be attributed to the plaque cells and matrix proteins becoming negatively charged at the high pH (caused by the addition of NaOH) and resulting in the repulsion of these plaque constituents.

The factors that determine the magnitude of the charges on the plaque cells and the effects on the aggregation of the plaque constituents, is dealt with in the next chapter.

The acellular portion of the dental plaque (the NaOH-S fraction) was 35% of the total plaque (on a dry weight basis), which compares well with the findings of Dobbs (1932) that 27 to 52% of the total plaque consisted of mucin. It is likely that most of this fraction consists of the matrix and extracellular proteins although there is some indication that nucleic acid is also present (see below).

The dialyzable fraction, comprising approximately 33% of the total plaque (allowing for the small amount of material lost during the various washing procedures) probably contained the small molecular weight substances that would be extracted from the cell cytoplasms.

<u>Comparison of the Plaque Calcium, Phosphate and Carbohydrate Contents Observed to Those Observed in Other Studies</u>

The mean Ca/N value of 0.13 (μg/μg or 13 μg/mg dry weight) for total plaque was higher than the figures reported by Dawes and Jenkins (1962) but is in close agreement with the figure of 14.4 μg Ca/mg dry weight reported by Allen and Moore (1957). The mean P/N value for the total plaque was 0.27 ($\frac{\mu g}{\mu g}$ or 27 μg/mg dry weight) and is higher than the figure of 16.5 μg/mg dry weight reported by Dawes and Jenkins (1962). Recent studies by Kaminsky and Kleinberg (unpublished results)

indicate that these differences can be attributed to differences in the location and age of the plaque studied.

The CHO/N values reported here for plaque are comparable to those found by Biswas, Vanry and Kleinberg (1965).

Comparison and Characterization of the Two Components of the NaOH-S Fraction

Separation of the acellular portion of dental plaque (NaOH-S fraction) into two components by dialysis against distilled water, was possible because of differences in solubility and slight differences in the iso-electric points of the two components. Removal of NaOH by dialysis served to lower the pH, so that the iso-electric point of the precipitate was reached before that of the supernatant component. The amino acid analyses of the supernatant and precipitate components showed more acidic and less basic amino acids in both their structures but the acidic/basic ratio was higher for the supernatant component.

Titrating these components with HCl and measuring their solubilities by O.D. measurement, also showed that the supernatant component had a lower iso-electric point than the precipitate component. The iso-electric point of the precipitate component was approximately 3.5, which can be accounted for by calculation from its amino acid composition.

The iso-electric point for the supernatant was 2.7, which was lower than the iso-electric point calculated from the amino acid analyses. The higher carbohydrate (if it contains COOH groups) and phosphate in the supernatant might be the source of the acidic groups necessary to account for this discrepancy.

From the amino acid analyses of both the precipitate and supernatant components of the NaOH-S fraction, these components appear to be predominately protein, of which (from the CHO/N ratios) an appreciable amount is muco-protein. The supernatant, which has a CHO/N ratio approximately four times that of the precipitate, also has four times the ammonia. Most of the ammonia observed in the amino acid analyses is probably derived from hexosamine, a sugar found by Leach (1963) in plaque muco-protein, although some may be derived from nucleic acid that might be present (see below).

Since calcium and phosphate were solubilized upon addition of NaOH to plaque (calcium phosphate is sparingly soluble at high pH), plus the fact that calcium and phosphate were found in both the precipitate and supernatant components after dialysis, strongly indicates that these mineral elements are bound. If the carbohydrate present in both components contains acidic groups, then these groups might be the sites for binding calcium, which in turn could

bind phosphate. This possibility is supported by the finding that the supernatant component, which has more carbohydrate than the precipitate component, also contains more calcium and phosphate. The fact that the supernatant is higher in calcium and is yet more soluble than the precipitate, could be explained by the greater negative charge possessed by the supernatant at a given pH.

The higher N/dry weight value for the precipitate than for the supernatant component, can be attributed to the lower amounts of carbohydrate, calcium and phosphate in the supernatant. Some of the phosphate in both components is probably part of phospho-protein and of nucleic acid.

Sub-fractions of the Precipitate and Supernatant Components of the NaOH-S Fraction

All the sub-fractions contained varying amounts of carbohydrate, indicating that they are or contain carbohydrate-protein. Ferguson (1964) separating plaque components by electrophoresis rather than by NaOH and gel filtration as in the present study, found that all of his fractions also contained carbohydrate. Most of the sub-fractions in the present study also contained calcium and phosphate. The S_2C sub-fraction contained the highest concentration of calcium and phosphate, while the S_1 sub-fraction contained the highest carbohydrate concentration. The S_2C sub-fraction favours

plaque cell aggregation most while S_1 favours it least (see next chapter).

Because both the precipitate and the supernatant components of the NaOH-S fraction separated on Bio-gel P200, and not on P100, this indicates that all the sub-fractions contain large molecules which have molecular weights of at least 100,000.

<u>Source of the NaOH-S Fraction</u>

The NaOH-S fraction consisted of a number of sub-fractions which could have originated from saliva, bacteria and/or gingival crevice fluid. Comparison of the amino acid analyses of the NaOH-S fraction to a similar analysis of precipitated human salivary mucin (Armstrong, 1966), showed a much closer similarity between these two materials (Figure 12) than a comparison between the same NaOH-S fraction and a number of others (Table II) suggesting that a large part of the NaOH-S fraction was of salivary origin. The similarity in changes in solubility between some of the salivary proteins (Trester and Kleinberg, 1962) and the NaOH-S fraction, supports this conclusion.

Most of the sub-fractions of the supernatant and precipitate components of the NaOH-S fraction contained varying amounts of bound calcium and phosphate. These minerals have been shown to be present in <u>initial</u> layers of plaque (Kaminsky

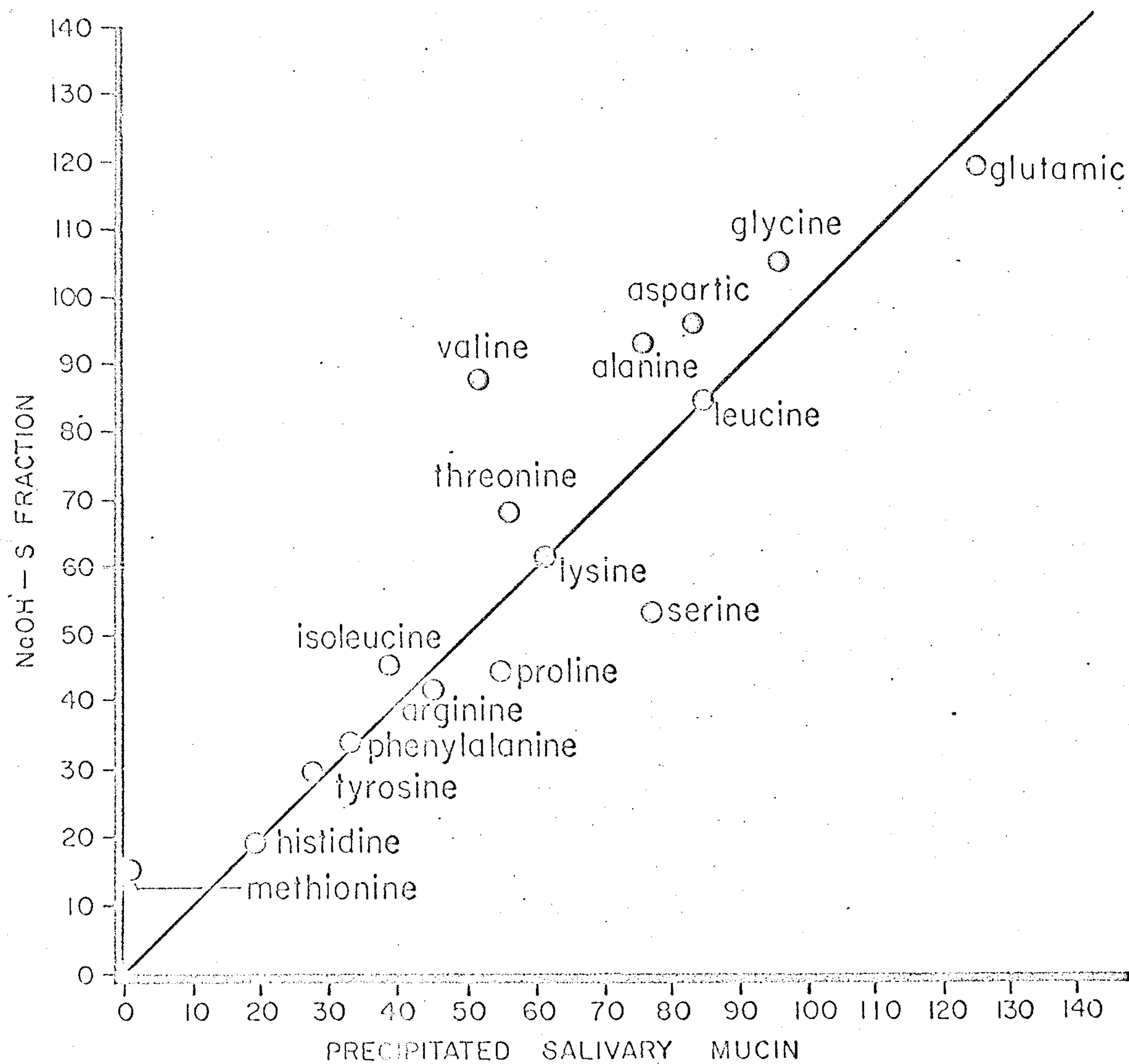

Figure 12. Plot of the corresponding amino acids in the NaOH-S fraction of plaque and precipitated human salivary mucin (Armstrong, 1966).

TABLE II

AVERAGE DEVIATION IN THE AMINO ACID COMPOSITIONS BETWEEN A NUMBER OF PLAQUE AND SALIVARY CONSTITUENTS

Moles per 1000 Moles Amino Acid

	NaOH-S Fraction	Precipitate	Supernatant	* Denture Pellicle	* Glycoprotein	** Tooth Pellicle	** Mucin
NaOH-S Fraction	-	6	12	66	55	27	11
Precipitate	-	-	17	64	54	28	18
Supernatant	-	-	-	70	58	28	22
Denture Pellicle	-	-	-	-	44	65	61
Glycoprotein	-	-	-	-	-	54	47
Tooth Pellicle	-	-	-	-	-	-	25

The figures in this table were calculated by use of the formula $D = \sqrt{\frac{\Sigma x^2}{N}}$, where D = Deviation, X = Number Moles that each amino acid differed from perfect correlation (as in Figure 12), N = Number Amino Acids.

* Smith (1964)

**Armstrong (1966)

and Kleinberg, unpublished results) and appear to be necessary for the adherence of salivary muco-protein to teeth (McGaughey and Stowell, 1966a).

Sub-fractions S_3 and P_4 may have originated from the bacteria, since they showed 230/280 ratios of 1:1 and P/N ratios of 0.7 and 0.6 respectively, suggestive of nucleic acid. Analysis of yeast nucleic acid by the anthrone procedure (Bailey, 1958), showed colour equivalent to a D-ribose content of 27% rather than the theoretical value of 46%. This would mean that nucleic acid would have a CHO/N ratio of 1.8, rather than 3.0 if the carbohydrate were determined by the anthrone procedure as in the present study. The figure of 1.8 is almost identical to the value for the CHO/N ratio for the S_3 sub-fraction (Figure 11). The P_4 sub-fraction, however, was slightly less than 1.0. This would indicate that the S_3 sub-fraction is probably nucleic acid, whereas the P_4 sub-fraction may contain nucleic acid along with protein.

The present study provides no information as to whether the NaOH-S fraction contains proteins originating from gingival crevice fluid.

CHAPTER IV

STUDIES ON FACTORS AFFECTING THE ABILITY OF THE MICROORGANISMS IN DENTAL PLAQUE TO AGGREGATE

Studies on many biological systems have shown that calcium plays an important part in the cohesion and adhesion of cells and in the holding together of cellular and intercellular components of tissues. Anderson (1953) found that to separate mouse liver cells from associated intercellular material, calcium must be removed from the intercellular cementing substance. Calcium has been shown to promote the adhesion of bacteria to phagocytes and facilitate the clumping of leucocytes (Mudd, McCutcheon and Lucke, 1934). The decreased cohesiveness of cancer cells has been attributed to a deficiency of calcium in their cell membrances (DeLong, Coman and Zeidman, 1950) and is consistent with cancerous cells having less calcium than normal tissue (Lansing, Rosenthal and Kamen, 1948). Jansen and Mendlik (1951) reported that deflocculated suspensions of yeast cells require calcium before reflocculation can take place, an observation confirmed by Chester (1965).

In studies on the nature of the intercellular substances holding the cells of pea root tips together, Ginsburg (1961) showed that aggregation of the cells and intercellular

material in this plant was affected not only by calcium, but also by other divalent cations, pH and ionic strength.

Because of the resemblance of plaque to some of these systems (e.g. it has a high calcium content and deaggregation occurs when the pH is raised) the effect of pH, cell concentration, ionic strength and divalent cations on plaque aggregation were examined. The effect of the acellular constituents of plaque (previously fractionated in Chapter III) on the same process, was also studied.

The purpose of the present study was to determine the role of the above factors on the integrity of the plaque system and on plaque formation.

METHOD

Cells, separated from total plaque with dilute NaOH, as described in the previous chapter, were harvested by centrifugation at 12, 800 X g for 10 minutes in a Lourdes centrifuge at $4^{o}C$. The cells were then washed three times with 0.1N NaOH and suspended in 0.1N NaOH at a cell concentration of 5% ($\frac{V}{V}$).

For each of the experiments in the present study, aliquots were removed from the 5% stock suspension, centrifuged at 12,800 X g for 10 minutes and the supernatants removed with careful suctioning to avoid disturbing the pellets. The small amount of NaOH that remained with

each pellet was intentionally left so that the cells in the pellet would resuspend upon subsequent addition of any one of a number of solutions. In each case, the volume of added solution was 1 ml.

Each cell suspension was titrated with 0.1N HCl from an Agla syringe (Burroughs Wellcome & Co.) between approximately pH 10 and pH 3. The pH after each addition of acid was read on a Beckman model G pH-meter, using glass and calomel electrodes. A salt bridge consisting of fine polyethylene tubing containing saturated KCl, lead from the calomel electrode to the measuring end of the electrode assembly.

After each addition of acid was made to each cell suspension, a 100 µl aliquot was removed and transferred to a 6 X 50 mm. Kimax test-tube and centrifuged at 1740 X g for one minute. Centrifuging at 1740 X g only removes aggregates of cells while cells that are not aggregated require a much higher centrifugal force (12,800 X g). To measure the cells remaining in suspension after centrifuging at 1740 X g, a 50 µl aliquot was removed from the supernatant in each tube and the optical density (O.D.) of the aliquot read at 500 mµ in a Beckman DU spectrophotometer adapted to read microsamples.

Solutions in Which the Cells Were Suspended

(1) Distilled water. This was used in experiments to determine the effect of cell concentration on cellular aggregation as a function of pH. It was also used in the controls in other experiments in this study.

(2) Potassium chloride. This was used to vary the ionic strength to determine its effect on cellular aggregation as a function of pH. The concentration range of KCl was 0 to 0.1M.

(3) Calcium and magnesium chloride. These salts were used to determine the effect of their divalent cations on cellular aggregation as a function of pH. Their concentrations were between 0 and 0.004M.

(4) Acellular plaque components. The total precipitate and total supernatant components of the NaOH-S fraction of plaque and their sub-fractions (Chapter III) were each combined with 0.001M $CaCl_2$ and their effects on cellular aggregation as a function of pH tested.

RESULTS

A. Effect of Varying Cell Concentration on Cell Aggregation as a Function of pH.

The cells from 25, 50, 100 and 150 µl of the stock 5%

($\frac{v}{v}$)cell suspension were resuspended in 1 ml. of distilled water, titrated with HCl and the pH measured as above. The results are shown in Figure 13.

With all four concentrations of cells (0.13, 0.25, 0.50, 0.75%), as the pH was lowered, the major drop in O.D. took place in the acid range. The 0.75% and 0.50% cell concentrations, both showed a slight decrease in O.D. which was not observed with the other two cell concentrations tested (pH 6.8 to 5.6). The precipitous drop in O.D. with the 0.25, 0.50 and 0.75% cell concentrations occurred at about the same pH, but that of the 0.13% concentration took place at a distinctly lower pH.

B. Effect of Ionic Strength

The cells from 40 µl of the stock 5% ($\frac{v}{v}$ cell suspension were resuspended in 1 ml. solutions of KCl ranging in concentration from 0 to 0.1M. These were each titrated with HCl and the pH measured. The results are shown in Figure 14.

With increasing concentrations of KCl and therefore increasing ionic strength, the O.D. curves shifted progressively downward and to the right. Consequently, increasing ionic strength increased cellular aggregation.

C. Effect of Divalent Cations

The cells from 50 µl aliquots of the stock 5% ($\frac{v}{v}$) cell suspension were resuspended in 1 ml. solutions of $CaCl_2$ and

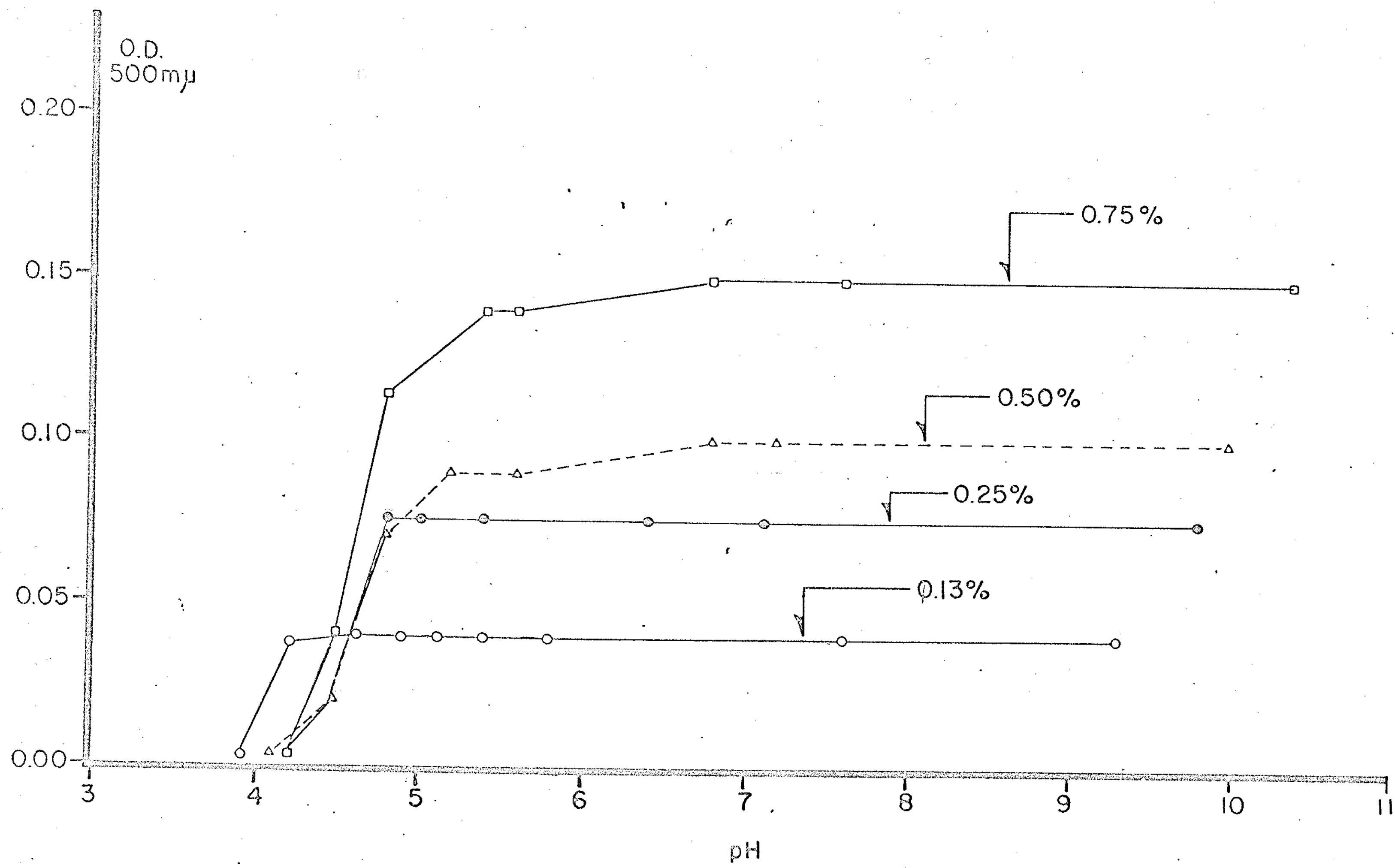

Figure 13. Effect of varying cell concentration on cellular aggregation as a function of pH.

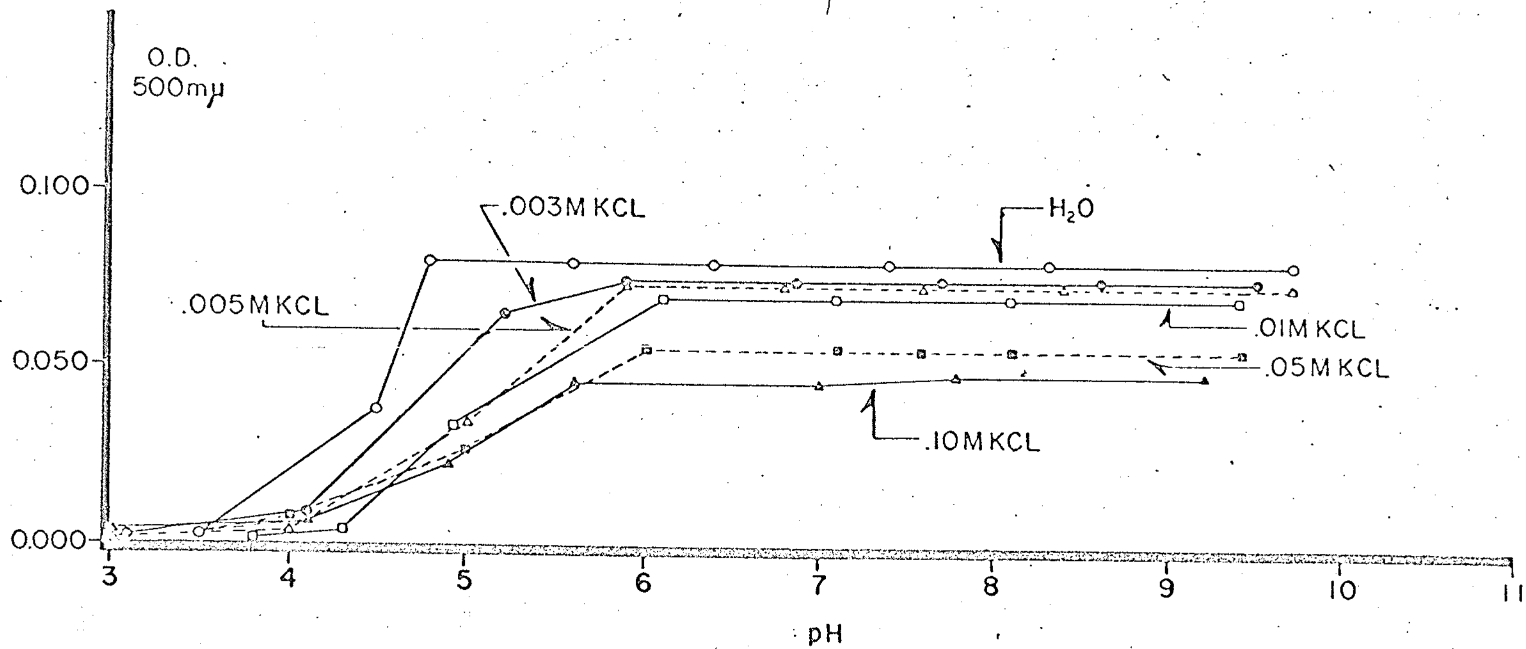

Figure 14. Effect of increasing ionic strength on cellular aggregation as a function of pH.

$MgCl_2$. These were titrated with HCl and the pH measured. One experiment comparing the effects on cell aggregation of the Mg and Ca chlorides is shown in Figure 15a, while another showing the effects of calcium alone is shown in Figure 15b.

Both experiments showed that increasing concentrations of calcium and magnesium caused the O.D. curves to shift progressively downwards and to the right, indicating increased aggregation. The extent of the effect was the same with either of the divalent cations.

D. Effect of the Acellular Components of Plaque on Cellular Aggregation in the Presence of 0.001M $CaCl_2$

(i) Precipitate and Supernatant Components of the NaOH-S Fraction of Plaque

The cells from 50 µl aliquots of the stock 5% ($\frac{v}{v}$) cell suspension were resuspended in 1 ml. of 0.001M $CaCl_2$ containing either the precipitate or supernatant component of the NaOH-S fraction of plaque; the concentrations of precipitate and supernatant component were both 5.8 µgN/ml. The mixtures with these components, along with distilled water and 0.001M $CaCl_2$ controls, were titrated with HCl and the pH measured.

The O.D. readings in the mixtures containing the precipitate and supernatant components, were higher over the whole pH range examined than in the $CaCl_2$ control and lower

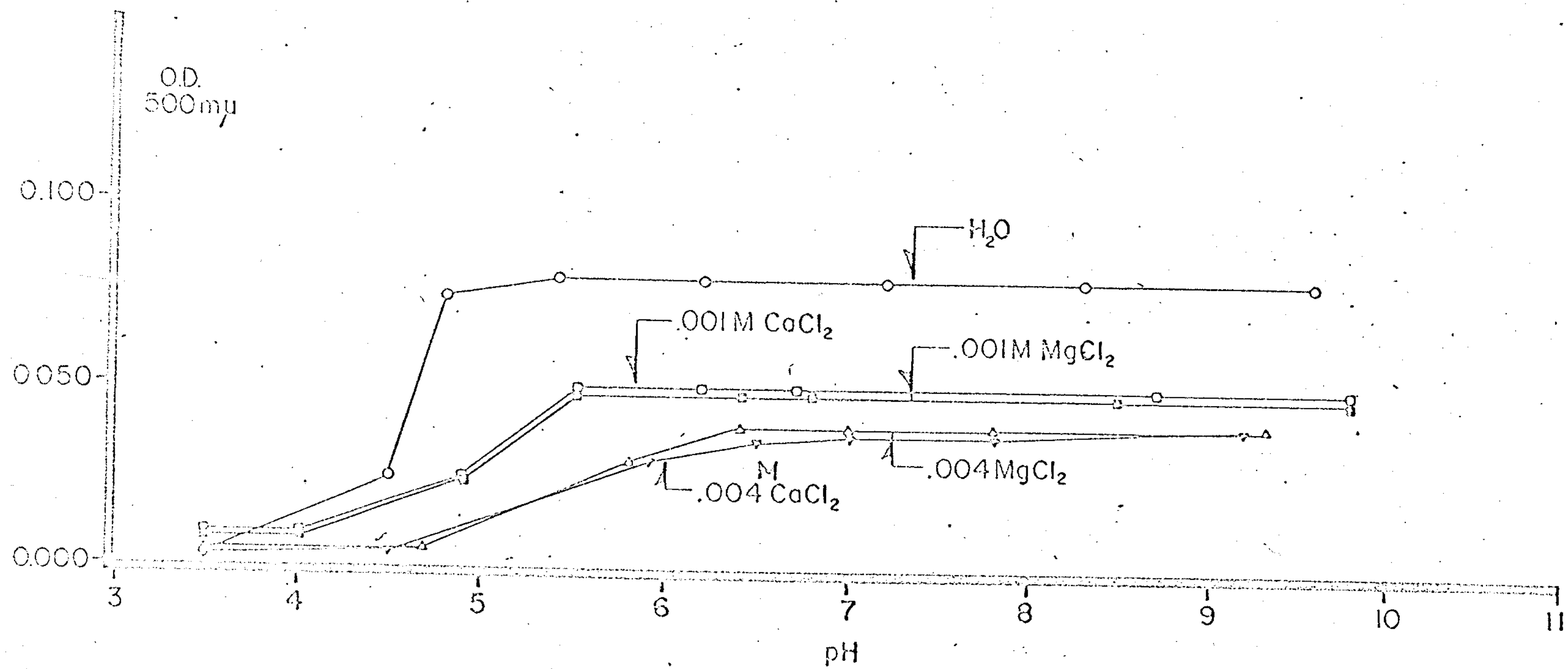

Figure 15a. Effect of $CaCl_2$ and $MgCl_2$ on cellular aggregation as a function of pH.

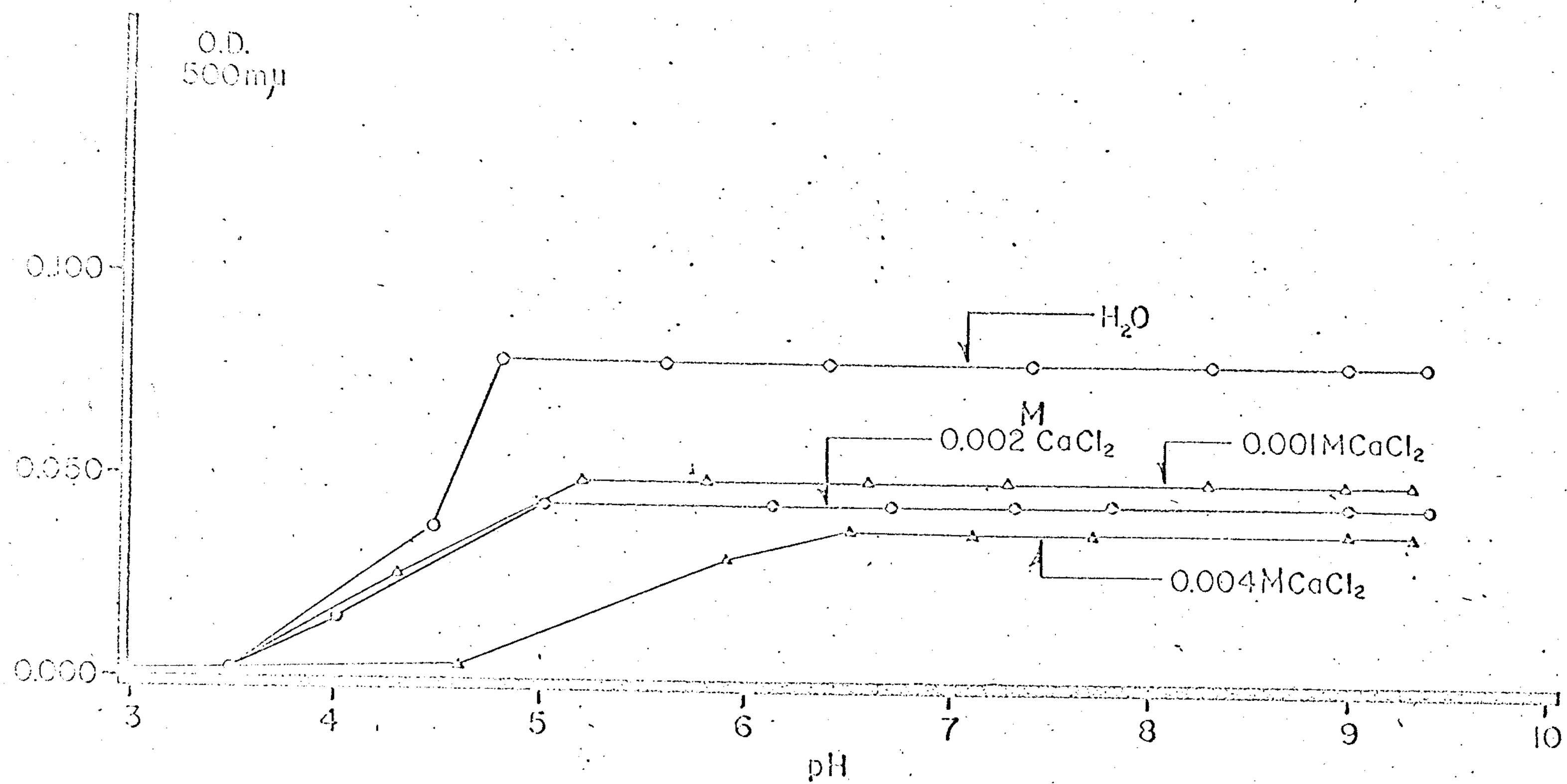

Figure 15b. Effect of increasing concentration of $CaCl_2$ on cellular aggregation as a function of pH.

than in the water control (Figure 16). This indicates that the two plaque components protect the cells from the tendency for calcium to cause their aggregation.

(ii) <u>Sub-fractions of the Precipitate and Supernatant Components of the NaOH-S Fraction of Plaque</u>

The cells from 50 ul aliquots of the stock 5% ($\frac{v}{v}$) cell suspensions were resuspended in 1 ml. 0.001M $CaCl_2$ solutions containing the individual sub-fractions of the precipitate and supernatant components of the NaOH-S fraction. The concentrations of the precipitate and supernatant sub-fractions used were approximately 5.0 ug N/ml. Each solution along with distilled water and 0.001M $CaCl_2$ controls were titrated with HCl and the pH measured.

The effects with the majority of the sub-fractions were the same as those observed in (i) above, with the precipitate and supernatant components (Figure 17). Sub-fractions S_1 and S_2C, however, showed some differences (Figure 18).

The O.D. in the mixture containing the S_1 sub-fraction was much higher over the pH range tested than the O.D. in the $CaCl_2$ controls. However, at pH 3.5, when the cells in both the $CaCl_2$ and water controls were completely aggregated, some of the cells in the mixture with the S_1 sub-fraction were still not aggregated.

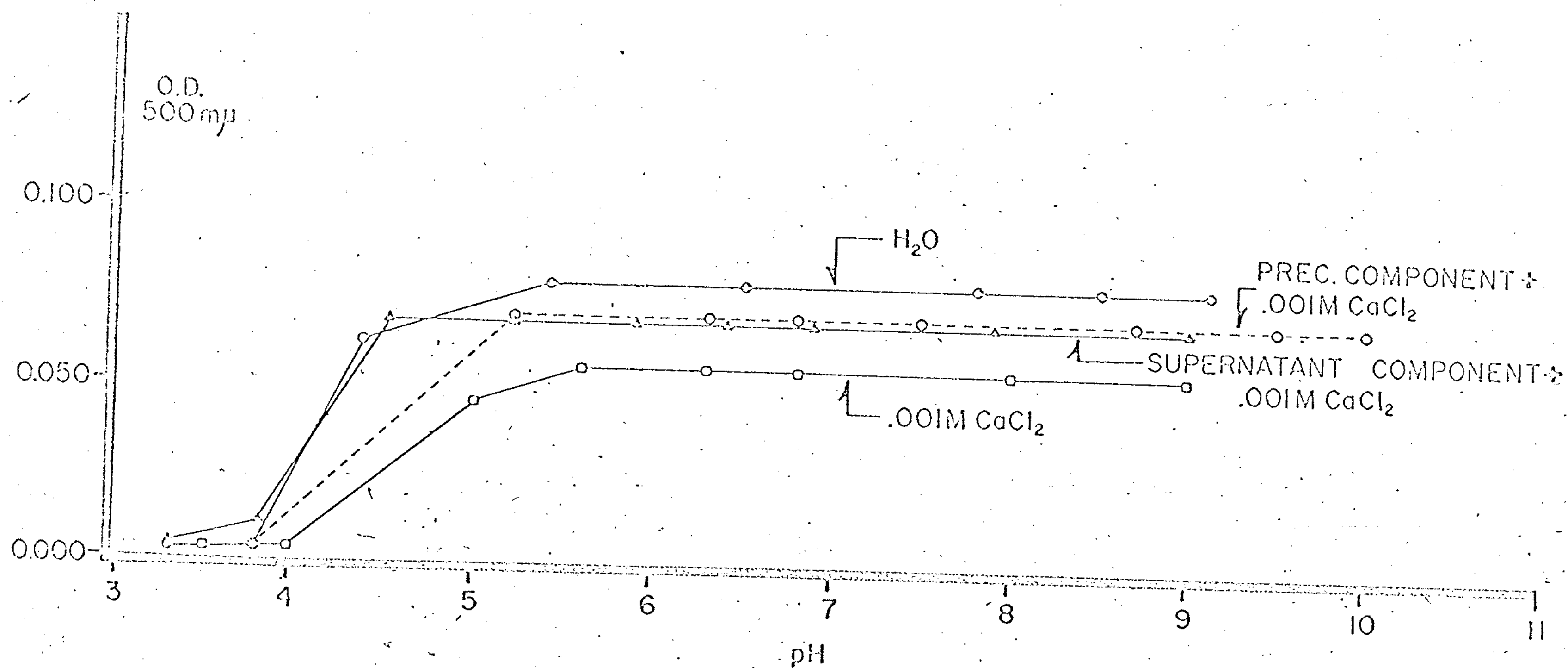

Figure 16. Effect of the precipitate and supernatant components of the NaOH-S fraction on cellular aggregation as a function of pH and in the presence of 0.001M $CaCl_2$.

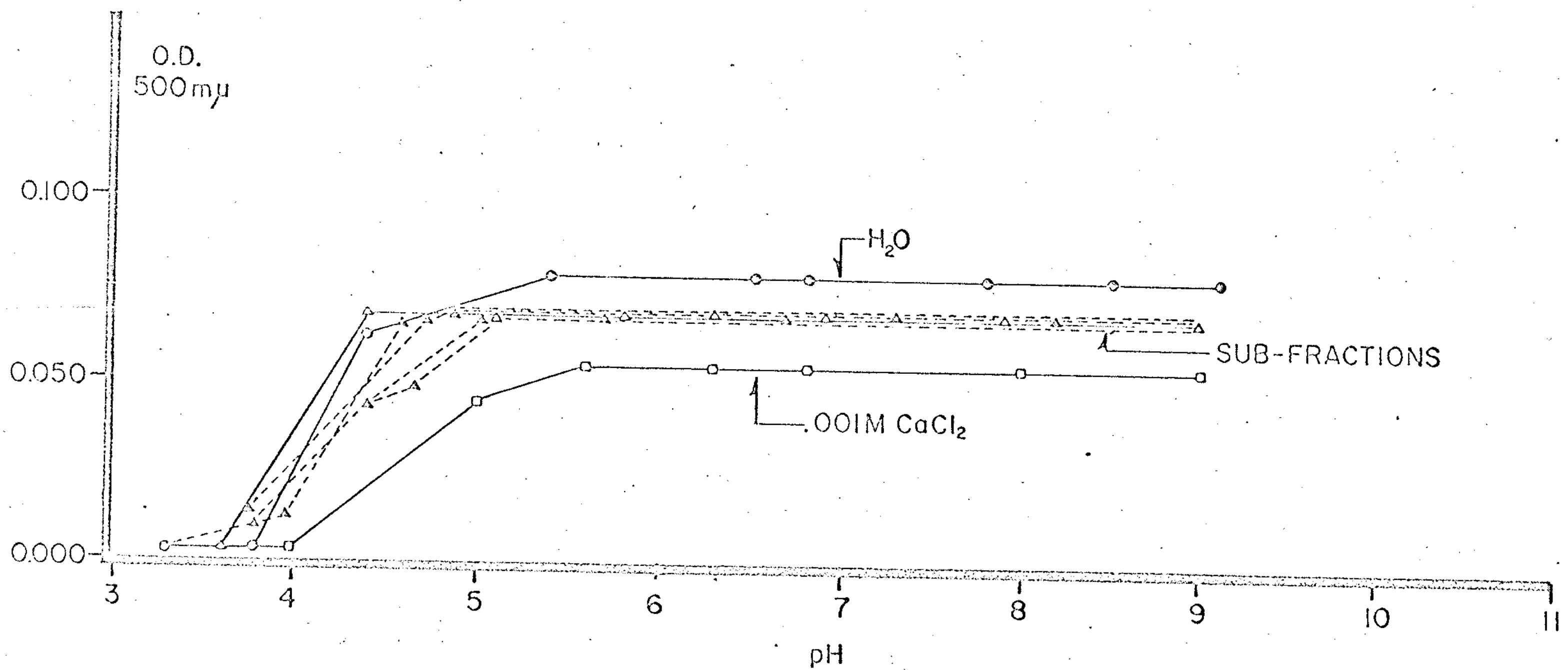

Figure 17. Effect of the precipitate and supernatant sub-fractions other than S_1 and S_2C, on cellular aggregation as a function of pH and in the presence of 0.001M $CaCl_2$.

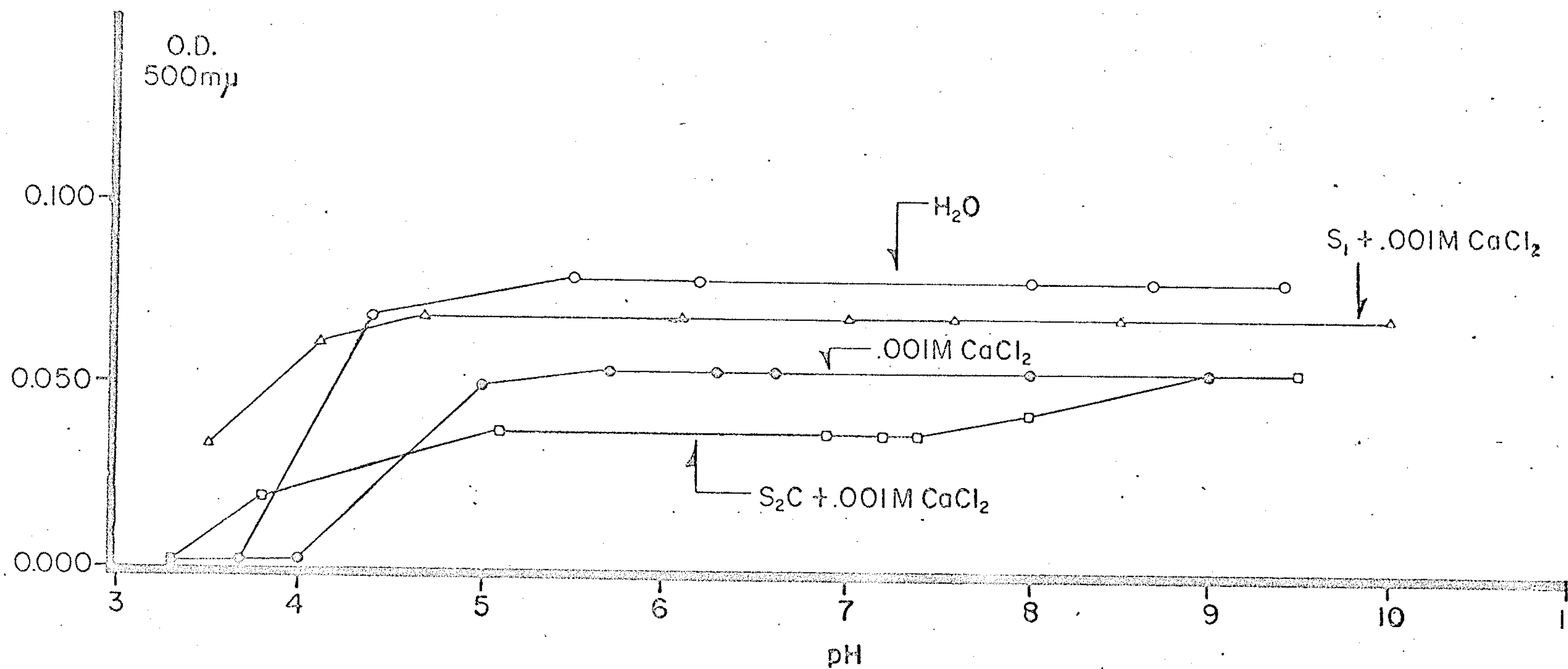

Figure 18. Effect of the S_1 and S_2C sub-fractions on cellular aggregation as a function of pH and in the presence of 0.001M $CaCl_2$.

The suspension with the S_2C sub-fraction showed the same O.D. as the $CaCl_2$ control between pH 9.5 and pH 9. Below this pH range and down to pH 4.6, the O.D. with this sub-fraction was below that in the $CaCl_2$ control. The relationship of their O.D. were reversed below pH 4.6 and was similar to that observed with the S_1 sub-fraction.

DISCUSSION

The effect of pH on the aggregation of the plaque cells can be explained as follows. At pH 10, the surfaces of the cells (probably surface proteins) will possess a net negative charge, causing the cells to repel each other and remain suspended. As the pH is lowered, the negative electrostatic charges on the cell membranes would decrease, allowing the cells to come closer together. When the charges on the cell surfaces reach zero, aggregation between the cells can occur. The favouring of aggregation with higher cellular concentrations can be attributed to the reduction in the mean free path between the cells in suspension, increasing their tendency to aggregate.

Increasing the ionic strength by increasing the concentration of KCl, would favour aggregation by decreasing the effective charges on the cell membrane sites, and thus reducing the repulsion between the cells.

Divalent cations such as calcium or magnesium would

favour aggregation by neutralizing the charge on the cell membranes more effectively than monovalent cations. Equations describing the binding of calcium to cell membranes have been reported by Gent, Trounce and Walser, (1964). Besides neutralizing the charge on the plaque cells, these divalent cations could also bridge these cells and facilitate their cohesion.

The precipitate and supernatant components of the NaOH-S fraction and all but one of their sub-fractions protected against the effect of calcium and this could have occurred in either of two ways; (1) by each component or sub-fraction binding calcium and preventing its action, and (2) by each component or sub-fraction interacting with the cells and lowering the iso-electric points of this cell-protein combination. Binding of macro-molecules to cell membranes is a general phenomenon that can occur in many cellular systems (Katchalsky, 1964).

The best protection was with the S_1 sub-fraction, which had the highest CHO/N ratio. The S_2C sub-fraction, instead of inhibiting aggregation, favoured this phenomenon between pH 9 and pH 4.6. Between pH 9.5 and pH 9, it had no effect. As the pH was lowered below pH 9 and down to pH 4.6 more aggregation occurred than in the controls, which can be attributed to the high calcium content of the S_2C sub-fraction (Ca/N = 0.14). The calcium in this sub-fraction might favour

the aggregation of the plaque cells by assisting in the formation of a floc. The S_2C sub-fraction would be the bridging molecule between the plaque cells, with the calcium bridging the negatively charged groups on the surfaces of the plaque cells and on the S_2C sub-fraction.

Below pH 4.6 the S_2C sub-fraction instead of favouring aggregation, actually showed a small protective effect. This protection might have resulted by a similar mechanism(s) to that proposed above for the other sub-fractions and components, after the bound calcium has been displaced by H ions. In other words, displacement of the calcium in this low pH range might enable the S_2C sub-fraction to form a protein-cell combination that is slightly more soluble than the cells alone.

The effects of pH, ionic strength and divalent cations on plaque cellular aggregation are similar to the effects observed on the pea root tip system studied by Ginzburg (1961) and may be characteristic of tissue systems in general. Ginzburg, showed that the integrity of his system was affected by changes in pH, ionic strength and concentration of divalent cations. Increases in pH and ionic strength and a decrease in the concentration of divalent cations favoured separation of the cells. Jansen and Mendlik (1951) in their experiments with yeast cells, also demonstrated that pH and the presence of electrolytes influenced the flocculation

velocities of the yeast cells and that flocculation was determined by the colloidal properties of the cell wall.

With respect to plaque formation, the present study suggests that the salivary bacteria have the capability of (1) becoming attached to and invading a primary layer of protein material, if this should form first on the surfaces of the teeth, (2) becoming attached to the teeth directly (Frank and Brendel, 1966), or (3) becoming bound to salivary protein or remnants of mammalian cells (Wildman, 1963) before precipitating onto the teeth to form the primary plaque. Whether any or all three processes occur, the various interactions would likely be favoured by the presence of calcium ions from the saliva.

Once aggregates of salivary microorganisms or protein-microorganism combinations settle onto the teeth, formation of acid by microbial breakdown of carbohydrate can take place. This would accelerate the rate of precipitation of further salivary muco-proteins and of secondary plaque, particularly as the cells increase in number.

Since a low pH favours both aggregation of the plaque cells and a decreased solubility of the precipitate and supernatant components of the NaOH-S fraction, plaque will be more soluble at neutral than at acid pH. These properties are consistent with the findings of Trester and Kleinberg (1962) and their postulate that plaque forms less readily at neutral

or alkaline pH's than at acid pH.

CHAPTER V

SUMMARY AND CONCLUSIONS

Raising plaque to a pH of approximately 12.0 by suspending it in dilute NaOH, resulted in its separation into cellular, acellular and residue fractions. The residue fraction was a portion of the plaque that could not be solubilized or suspended in the NaOH and consisted of clumps of bacteria, epithelial cells and large organisms resembling actinomycetes. The cellular fraction (NaOH-S cells) consisted of the plaque cells which were suspended in NaOH, while the acellular fraction (NaOH-S fraction) contained the material solubilized by the NaOH.

The acellular or NaOH-S fraction was separated from the NaOH-S cells by centrifugation and then fractionated by dialysis against water, into precipitate and supernatant components.

Microscopic examination showed that the NaOH-S cells consisted of individual bacteria and small aggregates of bacteria, while the residue was made up of epithelial cells, larger organisms and clumps of bacteria.

The percentage composition of total plaque was approximately the same, whether its constituents were expressed per unit nitrogen or per unit dry weight. In terms

of nitrogen, total plaque was composed of 28% NaOH-S cells, 8% residue, 32% NaOH-S fraction and 32% of dialyzable material. The NaOH-S fraction was composed of 76% precipitate component and 24% supernatant component.

The carbohydrate, calcium and phosphate analyses performed on total plaque and its constituents showed that the largest portion of carbohydrate was contained in the NaOH-S cells and that most of the calcium and phosphate were in the dialyzable portion. The supernatant component of the NaOH-S fraction contained more carbohydrate, calcium and phosphate than the precipitate component.

Amino acid analyses of the supernatant and precipitate components showed that the supernatant protein had more acidic and less basic amino acids in their structures than the proteins in the precipitate component. This finding was consistent with the results of the HCl titrations of the two components, which showed that the iso-electric point of the supernatant component was lower than that of the precipitate component. HCl titration of the two components also showed that although the largest amount of precipitation of these plaque components occurred in the low acid range, precipitation also occurred at neutral and alkaline pH's.

Separation of the precipitate and supernatant components into eleven sub-fractions was accomplished by gel-filtration on Bio-gel P200. The molecular weights of all the

sub-fractions were at least 100,000 or greater. Varying amounts of carbohydrate were present in all the sub-fractions, suggesting that they are, or contain muco-protein. The majority of the sub-fractions possessed a certain amount of bound calcium and phosphate.

Comparison of the NaOH-S fraction to other material indicated that a large part of the NaOH-S fraction is likely of salivary origin. Sub-fractions P_4 and S_3 are likely from bacteria in view of evidence that they are or contain nucleic acid.

In a separate series of experiments, the influence of several factors on the ability of plaque cells to aggregate as a function of pH was determined. NaOH-S cells were suspended at approximately pH 10 and titrated with 0.1N HCl between this pH and pH approximately 3. Aliquots removed during the titration were centrifuged at 1740 X g for one minute and aggregation of the cells was determined by measuring changes in the O.D. in the supernatant. It was found that the presence of calcium, increasing ionic strength, decreasing pH and increasing cell concentration, all favoured aggregation of the plaque cells. The precipitate and supernatant components of the NaOH-S fraction protected the cells from aggregating, either by binding the cells and protecting them from the aggregating effect of calcium or by binding calcium or both. Compared to either the precipitate or

supernatant components of the NaOH-S fraction, one of the supernatant sub-fractions (S_2C) increased cellular aggregation, while another supernatant sub-fraction decreased it (S_1).

With respect to plaque formation, the present study suggests that the salivary bacteria may (1) attach to the teeth directly, (2) attach to a layer of protein material that has formed first on the surfaces of the teeth, or (3) be bound to protein material from saliva (or remnants of mammalian cells) before this material precipitates onto the teeth.

The accumulation of acids due to the decomposition of carbohydrate by the attached bacteria, would increase the rate of precipitation of salivary mucoprotein and thus increase the rate of formation of secondary plaque.

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