NATIONAL BUREAU OF STANDARDS REPORT

NBS PROJECT		NBS REPORT
0708-11-07260	December 31, 1961	7407

Progress Report
on
Studies of Dental Calculus:
Organic Portion

by

John W. Stanford[1]
Alphonse F. Forziati[1]
George C. Paffenbarger[1]

[1] Research Associates, American Dental Association, National Bureau of Standards.

The data in this report were presented in a thesis by John W. Stanford in partial fulfillment of the requirements of the Graduate School, Georgetown University, Washington, D.C. for the degree of Doctor of Philosophy. Most of the work was conducted as a Guest Scientist in the Dental Division, Naval Medical Research Institute, National Naval Medical Center, Bethesda, Maryland.

This work is a part of the dental research program conducted at the National Bureau of Standards in cooperation with the Council on Dental Research of the American Dental Association; the Army Dental Corps; the Dental Sciences Division of the School of Aviation Medicine, USAF; the Navy Dental Corps, and the Veterans Administration.

U. S. DEPARTMENT OF COMMERCE
NATIONAL BUREAU OF STANDARDS

STUDIES OF DENTAL CALCULUS: Organic Portion*

Abstract

An investigation of the organic portion of dental calculus was undertaken with the view that knowledge so derived might help clarify the action, if any, of the organic components in the formation of salivary calculus.

Samples of composite and separate supragingival and subgingival calculi were decalcified by two methods: (1) 0.1 N HCl and (2) 15 per cent solution of ethylenediamine tetraacetate at a pH of 7.0 to 7.5. Chromatographic analyses of acid hydrolyzates indicate that the same amino acids make up the protein portion of the supragingival and subgingival calculi.

Samples of mucin precipitated from saliva were also hydrolyzed and examined by paper chromatography. The results obtained show that the same amino acids (qualitatively and quantitatively) are present in precipitated mucin and in salivary calculi. Paper chromatography was also used to study the carbohydrate fraction of the organic matrix of the calculi. Galactose, glucose, mannose, rhamnose and fucose were detected in resin hydrolyzates of the organic matrix. In addition there appears to be a hexosamine, a deoxy sugar, and a sialic acid.

*This investigation was supported in part by research grant D-902, Chemical Studies of Dental Calculus: Organic Portion, to the American Dental Association from the National Institute for Dental Research.

TABLE OF CONTENTS

1. INTRODUCTION

1.1 Statement of the Problem

Salivary calculus or tartar encompasses all calcified masses which are deposited on the surfaces of teeth or restorative appliances. Salivary calculus is a factor in periodontal diseases which affect the great majority of the adult population of the world. The majority of tooth losses in adults is caused by advanced periodontal disease (1). It is also generally accepted by the dental profession that dental calculus plays a very important role in the etiology of periodontal disease. Therefore, a better understanding of the mechanisms or mode of formation and a possible means of prevention of or reduction in formation become even more important in view of the average age of the American population.

The presence of calculus is seldom painful or annoying to most individuals, even in some of the more advanced stages. Occasionally, discomfort is experienced as a sequel to the presence of calculus, but because there is no apparent connection between the discomfort and the cause of periodontal disease in the lay mine, advice or treatment may not be sought until the damage is irreparable. An excellent example of this is shown in Figure 1. The lower central incisor shown before (Fig. 1, Top) and after the calculus deposit (Fig. 1, Bottom) was removed, was literally extracted by the presence of the calculus. As can be seen the tooth after removal of the calculus showed no signs of carious lesions.

If means could be found to prevent the formation of calculus, the ability of the dental profession to care for the dental needs of the public would be greatly enhanced. In attempting to assist in this problem, the analysis of the organic portion of dental calculus was undertaken with the view that knowledge so derived might help clarify the action, if any, of the organic components in the formation of salivary calculus. In addition preliminary investigations of the attachment of the calculus to tooth structure were also included.

The organic portion of the calculus was chosen for study since, as will be pointed out later, the inorganic or mineral part of dental calculus has been shown to be similar to the mineral part of human enamel. Therefore, any chemical agents which might reduce the formation of calculus by attacking the mineral part could possibly attack the mineral part of enamel also and thus would not be practicable.

2. HISTORICAL BACKGROUND

2.1 Structure of Salivary Calculus

In recent years, salivary calculus has frequently been divided into two classes: supragingival and subgingival. Supragingival calculus is believed to be deposited from saliva and occurs above the free gum margin. It is yellowish, soft and friable. Subgingival calculus is sometimes called serumal calculus. It consists of hard, dark deposits which are strongly adherent to the teeth. The foregoing is not meant to imply that the question of types of dental calculus has been settled. That two forms of calculus existed was the conception of Black (2) in 1886. The distinction was based upon the position and probable source of the deposit. This was also the conclusion of Wearn in 1935 (3). Burchard (4) believed, however, that there were five classes of calculus: (a) the yellowish white deposits upon the buccal surfaces of the upper molars, (b) the calculus found on the lower anterior teeth opposite the salivary glands, (c) the dark, flattened, hard, scalelike calculus found immediately beneath the gingival margins, (d) the small nodular calculus found deep in the pocket formations, and (e) material found on tooth roots in cases of gouty necrosis of the pericementum. Prinz (5) has gone to the opposite extreme. He has stated that there is only one type, that saliva is solely responsible for the constituents of tartar, and the location of deposits depended upon movement of the gingival margins. Thus, at one time the calculus could be classed as subgingival and at another, the same calculus could be classed as supragingival.

To ascertain the structure of calculus, Bibby (6) examined pieces of calculus which had been fixed in Zenker's solution and embedded in paraffin or celloidin for the preparation of the sections for microscopy. The sections were stained with hematoxylin and eosin and Gram's and Ljubinsky's stains. In those human specimens, gram-positive filaments appeared to be the only microorganisms present. These were arranged in irregular network in the central part of the specimen, but showed a regular palisade arrangement at the periphery. Between them there was an area which, with either the bacterial or the tissue stains, showed no structure.

Opinion has been divided as to whether lamination occurs in dental calculus as it does in some of the calculi in other parts of the body. Burchard (7) and Prinz (5)

found lamination, and Hulin (8) also found a stratified structure of calculus. Adamson (9) on the other hand was of the opinion that salivary calculus is pratically a homogenous material, and Smith (10) on examining the same material confirmed this view. Berke (11) stated as characteristic of the coronal type of calculus the regularity of the "fibers" arranged perpendicularly to the surface of the tooth. These were found to be fungi, gram-positive cocci, and to a lesser extent bacilli. He stated that the subgingival calculus is composed of the same elements arranged differently, being irregular and laminated. He also stated that the organic matrix precedes the deposit of the calcific salts; that is, the structural constituents are not entrapped by crystallization.

2.2 Origin and Formation of Salivary Calculus

Many theories have been proposed to explain the phenomena of how, when saliva is exposed to oral conditions, certain forces come into play which lead to the precipitation of dental calculus.

The many theories for the origin and formation of dental calculus may be summarized for convenience in three main groups, that is: (1) calcium salts are precipitated from the saliva by the action of bacteria; (2) physico-chemical processes cause precipitation of the calcium salts; and (3) enzymatic processes bring about the precipitation. An extensive review of these theories has been made by Hodge and Wah Leung (12). Since this report is concerned primarily with the chemical analysis of the organic portion of calculus and not directly with the theories of formation, the conclusion of Hodge and Wah Leung will be used to summarize the bacteriological and physico-chemical theories. They are of the opinion that the same mechanism of precipitation of the calcium salts has been postulated for the first two theories. An important difference lies in the specific factors held responsible for the decreased solubility of the calcium salts. The bacteriological theory requires the metabolic activity of bacteria to produce a local alkalinity and consequent decreased solubility. The physico-chemical theories, on the other hand, do not require the intervention of bacteria, but hold that the "normal" alterations in the physical and chemical properties of saliva, such as the loss of the colloidal protective action of the proteins or the escape of CO_2, are in themselves sufficient to lead to the precipitation of the calcium salts.

It would also appear, with the plausible source of phosphatase in the mouth being microorganisms (13, 14), that the enzymatic theory may be related to the bacteriological

theory and thus all three factors may be involved in the mechanism of calculus formation.

A recent report by Fitzgerald and McDaniel (15) extended the observations made by Fitzgerald in 1959 (16) and by Fitzgerald, Jordan and Stanley in 1960 (17) which suggested that dental calculus deposition could occur in the germ-free rat. Baer and Newton in 1959 (18) also reported the occurence on the maxillary molars of germ-free mice of hard, alizarin-staining deposits which they called calculus.

X-ray diffraction analyses by Fitzgerald and McDaniel (15) revealed the presence of hydroxyapatite in the pellicles removed from the maxillary molars of the germ-free rats. Periodic bacteriological checks on food and excreta of the animals revealed no viable microorganisms, and gram-stained smears of decalcified deposits confirmed their absence. Therefore, it seems apparent that dental calculus deposition can occur in the germ-free rat in the absence of a microbial flora. This observation does not rule out microbial participation in dental calculus formation in conventional animals where the oral microflora is ever present. However, findings in this study with germ-free animals do indicate that dental calculus deposition does not necessarily depend on the presence of microorganisms either living or dead.

2.3 Prevention of Calculus Formation

Burchard (7), because of his views on the cause of calculus, stated that the prevention of its formation is essentially the prevention of the formation of lactic acid. As a first means, however, a hygienic condition of the gingiva must prevail. Secondly, all fermentable material must be removed, and thirdly the microorganisms that produce the acid must be destroyed.

Black (19) in a study on himself, found that he could control the amount of calculus deposition by limiting his food intake and eating less rich foods. After eating heavy meals, he found that there would be a much greater deposition of calculus.

Head (20) described a method of preparing a solution of ammonium fluoride as a solvent for dental calculus. He stated that it must be used cautiously, as it has a caustic effect on the gingiva, but that it would not dissolve tooth structure.

Stewart (21) tested, on three patients, the effectiveness of mucinase in tooth powders. His basis for the use of mucinase was the finding of Pollard and Smith (cited

by Stewart) that algal polysaacharides adsorb salts of calcium, magnesium and iron. These are similar to the mucopolysaccharides of mucin; therefore, it was assumed that mucin could adsorb the precipitated salts of the saliva. Microorganisms could be enmeshed in the mucin, from which they can obtain nourishment as shown by Inouye (22) and Dreizen and Spies (23). The actinomycetes are the predominent organisms found in calculus, and Bartels (24) has shown that these organisms can and do produce calcium carbonate crystals in their growth medium. With initial crystal formation, further crystallization could be expected, as was reported by Leung (25). The submaxillary and sublingual glands produce the highest amount of mucin. The parotid gland produces albumins which are adhesive. Since the oral fluids are constantly moving about the mouth, the oral mucins along with the microorganisms and inorganic salts are probably carried to all corners of the mouth. These become adherent to the teeth and may initiate the cycle described. The mucins depolymerized with the mucinase would be less capable of holding the bacteria. From this very limited study, it has been proposed that a mucinase-containing dentifrice could prevent calculus formation.

Kerr and Field (26) used a sodium hexametaphosphate which has been used to dissolve phosphate stones with calcium metaphosphate being formed. If this reaction actually takes place, it should dissolve dental calculus. It has the ability to prevent the precipitation of complex calcium salts from colloidal solutions and dissolves salts already deposited. It appeared potentially useful in reducing calculus formation. Because of its wetting properties, it might also have a detergent action. Dilute solutions in vitro had very little effect, but in saturated solutions calculus was completely dissolved in 10 to 15 days. The stain was easily brushed from the teeth in a few days. The treatment had no effect on enamel but will dissolve silicate fillings. The use of the substance as a mouth wash prevented the formation of calculus up to six weeks or longer in patients having rapid calculus formation. This method has not met with general acceptance.

Leung (27) found that the inhibiting effect of silicones on synthetic calculus formation was negligible. Lactones were tested by adding daily to saliva in an experimental set up. They almost completely inhibited calculus formation but showed a whitening of the tooth that could be called decalcification. This was prevented by coating the teeth with

silicone before putting them in the glucone-lactone.

Aleece (28) tested three enzyme preparations on a total of 13 subjects. Three showed definite calculus reduction with Mylase L-1. These patients received a prophylaxis before the study was done. Grossman (29) attempted to determine: (1) whether some sequestrating or chelating agents are capable of dissolving a substitute for dental calculus, (2) whether these solutions will dissovle dental calculus itself, and (3) whether such agents affect the integrity of enamel. Of the agents tested, several effectively dissolved a calculus substitute and to a lesser extent calculus which had been removed from the teeth. Ten of the twenty-one solutions decalcified enamel. There was a direct proportion between the effectiveness of calculus removal and the decalcification of the enamel. In the following study (30) he tested one of the solutions that showed the greatest promise, EX 347. It was tried clinically with 31 patients having heavy calculus deposits. Of the 31, nineteen showed a marked reduction in calculus formation; in a number (not given) the teeth were calculus free. In eight cases the reduction was slight but definite. Three were uncooperative and one claimed no reduction. The solution did not etch the enamel when in contact with it for several weeks, in vitro.

Gunson (31) tested the substance EX 347 on 368 male prisoners of a state penitentiary in Pennsylvania. The EX 347 was supplied in a solution and a multi-tufted brush was given each subject. They were instructed in proper tooth brushing methods and asked to use the solution twice daily, after breakfast and before retiring, and told not to rinse the mouth after its use. The observation period extended over 12 weeks. All cases showed reduction of staining and calculus formation in 6 weeks and 95 per cent of subjects showed all the calculus and stain removed in the 12 week period. In the remaining 5 per cent the calculus was partially dissolved and new formations inhibited. None of the subjects showed any etching of the enamel surface.

2.4 Chemical Composition

The review of the literature on the chemical composition of calculus is divided into two parts; one, that which concerns the calculus formed in the mouth on the surfaces of teeth or restorative appliances and will be designated salivary, and two, that which concerns the calculus formed in

the salivary glands and will be designated duct.

Salivary Calculus

Dwinelle in 1844 (32) in a dissertation on salivary calculus reported his findings on repeated analyses of calculus as follows: "of 100 parts, there are of

Phosphate of lime ---------60.00
Carbonate of lime ---------14.00
Animal matter and mucus ---16.00
Water and loss ------------10.00"

Gorgas in 1892 (33) indicated the proportions of and composition of the inorganic and organic constituents of human salivary calculus as follows:

"It is composed of Phosphate and Carbonate of Lime, Magnesia, Fibrin or Cartilage, Mucus and Fat, 75 per cent of mineral matter, and the relative proportions of its constitutents vary according to its density."

The above quotation agrees with Dwinelle who further stated that the phosphate and carbonate of lime increased in quantity with the depth of its color and density, and vice versa; that is, the lighter colored calculus contained less lime and more animal matter.

The above two references are cited as early investigations which, despite the differences in analytical methods and in sources of samples, agree remarkably well with the results of later investigations (5, 34-38). Their results are summarized in Table 1. One of these, Prinz (5), referred to the organic matter contained in the calculus as mucus and food debris. The components of calculus based on an average of five analyses by Prinz are shown in Table 2.

Therefore, there has been reported about 15 to 25 per cent organic matter and 75 to 85 per cent inorganic matter in salivary calculus. The inorganic portion is comprised chiefly of calcium and phosphorous with smaller amounts of carbonate and magnesium, all in relatively constant proportions. This constancy might suggest the action of a uniform mechanism which precipitates calcium and phosphorous as a salt containing small but at least roughly proportionate amounts of magnesium and carbonate, with

organic matter in such a quantity that the ratio of inorganic to organic matter in the mass is equal to or slightly higher than that found in dentin. It may also be assumed that the precipitate does not represent mere sedimentation of the saliva, since the proportions of the constituents in the two differ widely as shown by Prinz (5).

X-ray crystallographic studies (38) have revealed a structure in calculus similar to apatite, but the lattice dimensions were so varied that it could not be defintely established as hydroxyapatite. Taylor and Sheard (39) found that the indices of refraction of three samples of salivary calculus (1.564, 1.563, 1.561) were similar to that of bone (1.562). They also reported that the x-ray diffraction pattern of calculus indicated an apatite structure. Phillip (40) has more recently obtained more definite evidence that dental calculus does contain hydroxyapatite.

Jensen and Dano (41), on the other hand, found Whitlockite often as a component of calculus and also that the deposition of apatite was often impeded.

King and others (42) compared the deposit from the teeth of hamsters and ferrets with that of human dental calculus. The samples were examined by both x-ray diffraction and microradiographic methods then heated to 900°C. to 1000°C. for one hour and then restudied by the same methods. In the unheated specimens the result showed that all had a hydroxyapatite pattern which was indistinguishable from that produced by bone and dentin. In the heated specimen some lines appeared in the diffraction pattern attributed to the presence of $\beta Ca_3(PO_4)_2$. Phillip (40) has suggested that the presence of $\beta Ca_3(PO_4)_2$ in heated human calculus was due to the formation of the calculus in the phosphate-rich medium of human saliva.

Salivary calculus then is mainly inorganic, the chief constituent (probably being) calcium phosphate either in the forms of $\beta Ca_3(PO_4)_2$ or hydroxyapatite or a mixture of both.

Prior to 1938 only a qualitative description of the organic content of dental calculus appeared in the literature. In 1938 Glock and Murray (38) reported the presence of 17.1 per cent organic material in subgingival calculus. According to these investigators, subgingival calculus consisted of ash (81.5%), CO_2 (1.39%), protein (8.34%), fat (2.71%) and water and undetermined organic material (6.06%).

They believed that protein fraction contained keratins, mucins, nucleoproteins or nucleic acids and suggested that the lipid fraction was made up of neutral fats, not phosphates. Cholesterol was not found by their method of analysis and nothing was said about the nature of a possible carbohydrate fraction.

Mandel, Levy and Wasserman (43) more recently conducted a histochemical study of calculus formation. They concluded that the structural and histochemical similarities between salivary calculus and other ectopic calcifications may indicate a common basic mechanism of calcification and that the carbohydrate-protein complexes present in the matrices of calcifying tissues may be the common denominator. Again in 1957 Mandel and Levy (44) reported on histochemical and chemical studies of suprigingival and subgingival calculus. Qualitative tests indicated the presence of cholesterol, cholesterol esters, phospholipids and fatty acids in the lipid fraction which constituted only 0.19 per cent of the organic material. They also detected the presence of reducing sugars in the carbohydrate fraction. The presence of a carbohydrate-protein complex in supragingival calculus was demonstrated by chemical and histochemical methods and in subgingival calculus by histochemical means. This varified their earlier report (43). The histologic appearance of subgingival calculus was found to be almost indistinguishable from that of supragingival calculus. Mandel and Levy also confirmed the observations of Naeslund (45) and others (6, 11, 46-48) of the predominantly filamentous bacterial nature of the calculus matrix. They observed that food debris, epithelial and inflammatory cells made up only a small fraction of the stroma. This appears to disprove that portion of the assumption made by Prinz (5) that desquamated epithelial cells and food in addition to bacteria act as the nucleus of a salivary deposit.

At the time the present study of the organic matrix of dental calculus was initiated, no evidence of the use of the "micro" techniques of paper and column chromatography, or infrared spectrophotometry was found in the literature. Recently, two reports were given at the Thirty-Ninth General Meeting of the International Association for Dental Research (49) concerning the carbohydrate components of supragingival calculus, and the organic matrix of dental calculus. The first report (50) showed that the carbohydrate fractions made up about 12 per cent of the organic matrix. Paper chromatograms indicated the presence of glucose, galactose, mannose, fucose, rhamnose, and either xylose or arabinose. The second report (51) showed that the carbohydrate, as assessed by Anthrone, accounted for 12 to 20 per cent of the organic

matrix and that nitrogenous components, calculated from micro-kjeldahl determinations, accounted for 36 to 40 per cent. Paper chromatography showed that 18 amino acids were consistently present in acid hydrolyzates of the matrix. Hydroxyproline, leucine and methionine appeared to be absent. Glactose, ribose and an unidentified non-mobile pentose were found in the carbohydrate fraction.

Duct Stones

A review of the analyses of calculus formed in the salivary ducts is included here, as it may be very similar to ordinary salivary calculus.

King and Boyce in 1957 (52) reported on the amino acid and carbohydrate composition of the mucroprotein matrix in various calculi. Acid hydrolyzates of residues, after decalcification of submaxillary gland calculi with ethylenediamine tetraacetic acid, were subjected to paper chromatography. The amino acids identified qualitatively are shown along with microbioligical assays for the detected amino acids in Table 3.

Portions of the residues were also subjected to resin hydrolysis and the resulting hydrolyzates subjected to chromatographic analysis for the carbohydrate components. The following carbohydrates were identified: glactose, glucose, mannose, rhamnose and fucose. Deoxypentose was found to be present by the diphenylamine test and hexosamine was also identified. Hexuronic acid, ketohexoses and sialic acid were not found to be present. No evidence of the presence of cholesterol or lipids was found.

A later report by Harrill, King and Boyce (53) confirmed the findings of King and Boyce (52) and included a histologic and histochemical study of calculi surgically removed from the salivary glands. The organic matrix stained poorly with hematoxylin.

The periodate Schiff leucofuchsin reaction was positive. Treatment of the matrix with diastase prior to the PAS reaction did not appear to diminish the reaction. Therefore, the authors concluded that glycogen was not a prominent component of salivary matrix. No epithelial or blood cells were seen in the matrices of the salivary calculi examined.

The authors suggested that calculi may form as a result of some abnormality of salivary or urinary mucoid. The molecules of mucoid might tend to coalesce into a gel and eventually into varying degrees of fibrillar or laminar

structures. The organic framework could therefore be thought of as forming an architectomic function in the deposition of calcigerous crystals.

3. METHODS AND RESULTS - PART I

3.1 Sampling and Separation of Calculus

Pooled salivary calculi, collected at random from adults,[1] were desiccated over anhydrous calcium sulfate for several days before reducing in a mullite mortar to a particle size which passed a U. S. Standard Sieve No. 150. The powdered samples were then frozen until used. For the first series of analyses no attempt was made to separate subgingival from supragingival calculus (such samples will be designated as composite calculus hereafter). However, such separations were effected for later analyses. The basis for separations of subgingival and supragingival calculi was by visual observation of the location at the time of removal.[1] In addition, samples of calculus which were removed from artificial dentures[2] were analyzed for comparison with the samples of calculus formed on natural teeth.

3.2 Sampling of Mucin

In addition a limited group of patients[3] was selected for salivary mucin analysis. The mouth of each donor was rinsed well with water before the collection of saliva was initiated. Approximately 50 ml. of stimulated saliva was then collected in a graduate. Three drops of 1N acetic acid for every 15 ml. of saliva were added to the graduate and then the contents of it were slowly transferred to an Erlenmeyer Flask which contained 200 ml. of acetone. The Erlenmeyer Flask was shaken during the addition of the saliva. The flask was then stoppered and set aside for 30 minutes. As much as possible of the supernatant liquid was decanted off the precipitate before centrifuging. After centrifugation the precipitate of mucin was washed four times using three milliliter portions of acetone for each wash. The precipitate was then placed on filter paper and allowed to air dry. This procedure was carried out in duplicate for each donor.

[1]Source: United States Public Health Service and Dental Hygienist's Association, Washington, D. C.

[2]Source: Rothstein Dental Laboratories, Inc., Washington, D. C.

[3]Source: Laboratory personnel located at Dental Research Section, National Bureau of Standards. Age range was 25 to 35 years.

3.3 Ashing of Samples of Calculus

Fifty to seventy-five milligram samples of the salivary calculi were ashed in small platinum foil cones. The samples were placed in an open furnace, the temperature of which was brought to 200°C. over a one-hour period. This temperature was maintained for one hour and then raised to 950 to 1000°C. with the furnace door closed. The samples were held within this temperature range for two hours and then cooled to 100°C. The cones were removed from the furnace and cooled to room temperature in a desiccator before weighing. The amount of ash determined by these tests ranged from 80 to 85 per cent of the weight of the original samples.

3.4 Preparation for Analyses

Portions of the powdered samples of the various calculi were prepared for analyses in the following manner:

(1) demineralization in a 15 per cent solution of ethylenediamine tetraacetic acid (EDTA) at a pH of 7.0 to 7.5,

(2) demineralization with 0.1N HCl at 4°C.,

(3) extraction with boiling water, and

(4) extraction with ether and alcohol.

Demineralizations

EDTA.--The 15 per cent solution of EDTA was prepared by dissolving appropriate amounts of the disodium salt of ethylenediamine tetraacetic acid in distilled water. The pH of the resulting solution was adjusted to 7.0 to 7.5 by the addition of 5N NaOH. Samples of calculi weighing from 100 to 600 milligrams were introduced into pieces of cellophane tubing which had been tied at one end with dental floss. In general, 50 milliliters of EDTA solution was added to the samples as the inner fluid for the dialyzing process. The cellophane bags so formed were tied at the top with dental floss and suspended in a 400 milliliter beaker containing 250 milliliters of the EDTA solution. The bags containing the samples and the inner fluids were then lowered until the levels of the outer and inner fluids were the same. Agitation was maintained throughout the demineralization by magnetic stirring in the outer fluids. The outer fluids were renewed with 250 milliliters of fresh EDTA solution every 24 hours until demineralization was complete. The demineralizations by EDTA were carried out at 20 to 25°C. and

were considered complete when constant EDTA values were obtained by titration of excess EDTA with standardized $CaCl_2$ solution.

After demineralization, the residual calculi samples were washed thoroughly with distilled water by centrifugation and decantation. The residues were then dried to constant weights at 100°C. All residues thus obtained were stored in a desiccator at room temperature until examined. The residues recovered amounted to 10 to 14 per cent of the original weights of calculi.

0.1N HCl.--The dialyzing apparatus utilized was identical to that used in the demineralization procedure involving EDTA. Fifty milliliters of 0.1N HCl was added to the samples in the cellophane bags. The tops of the bags were tied off with dental floss as in the case of EDTA demineraliztion procedure. Each bag was lowered into a 400-milliliter beaker containing 250 milliliters of 0.1N HCl and each dialyzing apparatus was then transferred to a constant temperature room maintained at 4°C. Agitation was provided in the outer fluid by magnetic stirring. The outer fluid (250 milliliters) was changed every 24 hours. Demineralization was carried out for four days. The bags were then removed from the beakers. The inner fluids were transferred along with the residual calculi samples to centrifuge tubes. After centrifugation the supernatant liquids were decanted. The residues were washed with 0.1N HCl, followed by distilled water, dried to constant weight at 100°C., and stored in a desiccator until analyzed. The residues recovered amounted to 8 to 12 per cent of the original weights of calculi.

Extraction with Boiling Water

Two samples of powdered composite calculi were refluxed in distilled water for 24 hours. The resulting solutions were filtered and concentrated to about 1/4 volume under reduced pressure. The proteins were then precipitated by the addition of 10 volumes of absolute ethyl alcohol at 4°C. The precipitates were removed by centrifugation and washed with cold 50 per cent ethyl alcohol. They were then dried to constant weight at 100°C. and stored in a desiccator until analyzed. The precipitated residues amounted to about 1 per cent of the original samples of calculi.

Extraction with Alcohol and Ether

Two samples of composite calculi were placed in thimbles in Soxhlet Extractors. The samples were extracted with

absolute ethyl alcohol for 16 hours and then with anhydrous ethyl ether for an additional 16 hours. The alcohol and ether extracts were combined, filtered, and evaporated to dryness in tared flasks in a hood at room temperature. The flasks and contents were placed in a vacuum desiccator for drying at room temperature. The dry material was weighed after 3 days in the desiccator. The dry material contains the total lipids except cholesterol which is not extracted by these solvents.[1] The average of the two determinations on composite calculi samples weighing 1 to 1.5 gm., was 0.19 per cent.

Summary of Samples Prepared

A summary of samples prepared of the various salivary calculi is shown in Table 4. The demineralized calculi and extracted residues were subdivided for ash content determinations, nitrogen analyses, qualitative examinations of protein and carbohydrate, infrared spectroscopy, and chromatographic examinations of protein and carbohydrate.

3.5 Ashing of Residues

Five to ten milligram samples of the residues obtained upon demineralization and samples of mucin were ashed in small platinum foil cones. The samples were placed in an open furnace, the temperature of which was brought up to 200°C. in an hour. The furnace door was closed and this temperature was maintained for one hour; next the temperature was raised to 950 to 1000°C. The samples were held within this temperature range for two hours and then cooled to 100°C. The cones were removed from the furnace and cooled to room temperature in a desiccator before weighing. The amount of ash determined by these tests ranged from 7.8 to 8.8 per cent of the original weight of the EDTA-demineralized residues and from 4.2 to 4.6 per cent of the original weight of the HCl-demineralized residues. The ash content of mucin was 2.3 per cent.

3.6 Nitrogen Analyses

Procedure

Nitrogen was determined by a means of a Coleman

[1] Hess, W. C.; Lee, C. Y. and Peckham, S. C., The lipide content of enamel and dentin. J. Dent. Res. 35:273, 1956

Nitrogen Analyzer.[1] A sample weighing from 1 to 5 mg. of each residue after demineralization was weighed directly into a disposable aluminum boat. The boat containing the sample was then placed into the sample zone of the combustion tube and mixed with finely ground Cuprox.[2] The combustion tube was then installed into the combustion train where the sample was pyrolyzed at 850-900°C. During combustion, the sample was broken down into its elemental constituents and the gaseous combustion products were carried by CO_2 gas over oxidizing and reducing materials. The remaining nitrogen and CO_2 carrier gas were then passed through a 50 per cent aqueous solution of KOH to absorb the CO_2 carrier gas. The nitrogen was then collected and measured in a 5.000 cc. stainless steel syringe which is linked to a digital counter by a micrometer screw. A blank was determined for each series of determinations.

Calculation of Nitrogen Content

The volume of nitrogen collected is converted to per cent nitrogen in the sample by the following equation:[3]

$$\%N_2 = \frac{P_c}{T} \times \frac{V_c}{W} \times 44.90$$

where $P_c = P_o - (P_b + P_u)$

P_o = observed barometric pressure (mm. Hg)

P_b = barometric temperature correction

P_u = pressure correction for vapor pressure of KOH

T = temperature in degrees Kelvin

$V_c = V_o - (V_b + V_t)$

[1]Model 29, Coleman Nitrogen Analyzer, Coleman Instruments, Inc.

[2]Cuprox - Coleman Cat. No. 29-130 Cuprox Reagent (CuO)

[3]Operating directions for the Model 29 Coleman Nitrogen Analyzer, Coleman Instruments, Inc., page 21.

V_o = observed N_2 volume

V_b = volume of blank (µl)

V_t = volume correction for temperature (µl)

$= C_f(t_2 - t_1)$

W = sample weight in milligrams.

A sample calculation follows:

P_o= 750 mm. Hg.

W = 5.030 mg.

	Start	Finish
Counter readings, Blank	300 µl	303 µl
Counter readings, Sample	303 µl	819 µl

t_1= 27.5°C. t_2= 27.7°C.

V_o= 819 - 303 = 516 µl

V_c= 516 - [003 + $C_f(t_2 - t_1)$]

= 516 - [003 + 014(0.2)]

= 510 µl

P_c= 750 - 11 = 739

$$\%N_2 = \frac{739}{300.7} \times \frac{0.510}{5.030} \times 44.90$$

= 2.46 x 0.10 x 44.90

$\%N_2$= 11.05

The determined blank is the volume of unabsorbed gas which appears with nitrogen in the observed volume as a result of a completed combustion cycle but originates from sources other than the weighed sample. The blank must be small and reproducible under conditions identical to those that exist during an actual analysis, except for the presence of the sample. Blanks determined during the present analyses averaged 5 microliters with a reproducibility of ±1 microliter.

Results

The total nitrogen contents of the EDTA-demineralized calculi ranged from 7.62 to 8.61 per cent and those of the HCl-demineralized calculi ranged from 9.67 to 10.52 per cent. The average total nitrogen content of mucin was 7.82 per cent.

3.7 Qualitative Examinations

Protein

General Composition.--A small amount of powdered organic residue obtained after demineralization of dental calculi was heated in a dry test tube in which was suspended a strip of moistened red litmus paper. A piece of filter paper moistened with lead acetate solution was placed across the top of the tube. As the sample was heated it evolved fumes which turned the red litmus paper blue. This indicated the formation of ammonia from the nitrogen and hydrogen present. The lead acetate paper showed slight blackening which indicated the presence of sulfur.

Color Tests.[1]--(1). The Xanthoproteic Reaction which is due to the presence in the protein molecule of the phenyl group was positive. The particular complexes of the protein molecule which are of especial importance in this connection are those of tyrosine and tryptophan.

(2). Millon's Reaction due to the presence of the hydroxyphenyl group in the protein molecule was positive and indicated the presence of tyrosine.

(3). The Glyoxylic Acid Reaction (Hopkins-Cole) was positive. The positive reaction indicated the presence of the tryptophan group in the organic residues of dental calculi.

The above tests then indicated the presence of protein material within the organic residue. In addition the presence of both tyrosine and tryptophan were indicated.

Carbohydrate

The small amount of the organic residues of the dental calculi was placed in a test tube containing 2N HCl which

[1]Hawk, P. H., Osen, B. L., and Summerson, W. H., Practical Physiological Chemistry, p. 169, 1954.

was heated for 2 hours in a boiling water bath. The hydrolyzate was evaporated and the residue taken up in distilled water for color tests.

Color Tests.[1]--(1). The α-Naphthol Reaction (Molisch) was carried out on a portion of the solution obtained above. The test was positive and indicated the presence of furfural-yielding substances such as carbohydrates. The furfural or furfural derivatives are formed due to the action of the acid in the reaction upon the carbohydrate.

(2). The Phenylhydrazine Reaction was carried out on a small portion of the solution. The crystals obtained were examined microscopically. They were of a yellow crystalline nature and resembled the osazone formed by glucose, fructose and mannose. Because of the similarities in the molecular structure of these three sugars, they all give rise to the same osazone; therefore, the reducing sugar responsible for the osazone can not be distinguished on the basis of this test.

(3). Benedicts' Test was conducted on a portion of the solution and was positive indicating the presence of reducing sugars. This test depends upon the presence of a substance which reduces the cupric hydroxide to insoluble yellow or red cuprous oxide.

4. METHODS AND RESULTS - PART II

4.1 Examination by Infrared Spectroscopy

Samples Examined

Infrared spectra were made on calculus samples as they were collected, on the mineral portion after extraction by alkaline ethylene glycol, the residue after demineralization by 15 per cent ethylenediamine tetraacetic acid (EDTA) at a pH of 7.0 to 7.5, the residue after demineralization by 0.1N HCl, the residue precipitated by absolute ethanol from boiling water extracts of calculus, a sample of synthetic hydroxyapatite, and on samples of mucin precipitated from saliva.

Results

The spectra from the mineral portion of calculus (Fig. 2a) and the sample of synthetic hydroxyapatite (Fig. 2b) are quite similar with the exception of the 7 micron

[1]Hawk, P. H., Osen, B. L., and Summerson, W. H., Practical Physiological Chemistry, p. 63, 1954.

region. Qualitatively the inorganic fraction of the samples examined probably resembles largely hydroxyapatite and calcium phosphate. The absorption in the 7 micron region may be due to carbonate. The spectrum of the calculus as collected (Fig. 3) appears to be a composite of the spectra of the mineral portion (Fig. 2a) and the organic fraction after demineralization by EDTA (Fig. 4a) or after demineralization by 0.1N HCl(Fig. 4b). The spectra for the organic residues (Fig. 4a and 4b) indicate the presence of protein like substance (absorption at 6.0 and 6.5 microns presumably due to protein). The spectrum of the residue of the hot water extracts (Fig. 5) resembles the spectra for the organic residues obtained by demineralization (Fig. 4a and 4b). The absorption at 9.5 microns is more intense in relation to that of 6.0 and 6.5 microns (presumably due to protein) in the spectrum of the water extracts than in the spectra of the material obtained by demineralization. The absorption at 9.5 microns may be due to either organic or inorganic material. A spectrum for mucin precipitated from saliva (Fig. 6a) is compared to the spectrum for the organic residue obtained by EDTA decalcification (Fig. 6b). As can be seen the spectra are similar indicating the presence of protein in the organic residue (Fig. 6b). Mucin was chosen for comparison since the teeth and artifical applicances in the mouth are constantly bathed in saliva which contains mucin as its principal protein.

4.2 Examination of Structure and Attachment of Salivary Calculus

Methods and Apparatus

The lamp found to be most satisfactory in previous studies of the fluorescence of human dentition for irradiating the specimens was the General Electric AH6 (54). This is a 1,000 watt, water-cooled, mercury vapor lamp with a quartz jacket. It operates at 1,000 volts and at a pressure of approximately 2,000 pounds per square inch. Glass filters (Corning No. 9863 and No. 5860) were used to absorb the visible light and to transmit radiation in the region 3,200 to 3,900 A.U. A quartz condensing lens and a front surface mirror used with the lamp concentrated the ultraviolet radiation on the specimen. The lamp is shown in Figure 7. Longitudinal sections of tooth tissue and calculus approximately 0.060 inch thick were cut by means of a water-washed, rubber-bonded carborundum disk 3 inches in diameter and 0.015 inch thick. These sections were not ground or polished further. Initial attempts to prepare thin, polished sections resulted in destruction of the salivary calculus.

To make fluorophotomicrographs of the sections, a photomicrographic camera was used with either camera lens (Fig. 7) or a microscope according to the magnification desired. Exposure times varied from a few minutes for the low magnification picutres to about one hour for those made at high magnification.

Results

The tooth sections and adhering calculus deposits are shown in Figures 8 through 11. Four sections, each from different teeth, were utilized. The sections whether photographed by visible illumination or by flourescence all show lamination to a certain degree in the calculus deposits (Figs. 8-11). The sections photographed by visible illumination show varying degrees of opacity which can be seen as different layers or bands in the sections photographed by fluorescence. This can especially be seen in the layering of the deposit in Figures 9 and 10.

Examination of Figures 8, 9 and 10 all show in varying degrees a dark colored junction of the calculus and tooth tissue when the sections were photographed by visible illumination. When the same sections were photographed by fluorescence this band of colored material disappeared; that is, the area showed no distinct fluorescence which would suggest a band of material which was either all organic or inorganic in nature. The junction of calculus deposit and tooth tissue shown in Section 1(Fig. 8) was rephotographed at a greater magnification of approximately 100x. The photographs both by visible and fluorescence illumination are shown in Figure 12. Examination of these photographs indicates little if any inorganic material present in this band of material and it appears to be a film like substance between the enamel and the calculus.

4.3 Analyses of Protein

Hydrolytic Technics

Samples of the residues from the two demineralizing procedures of the various salivary calculi weighing between two and five milligrams were placed in small pyrex test tubes. The hydrolyzing medium used was 6N HCl (0.04 of a milliliter for every one milligram of sample). When samples were utilized to determine the presence of tryptophan, the hydrolyses were carried out in 5N NaOH for 6 hours at 105°C. The tubes containing the samples and hydrolyzing media were then sealed by an oxygen-air blowpipe. The sealed tubes

were transferred to an oven maintained at 105°C. Hydrolyses were carried out for 6 hours and 16 hours at 105°C. The tubes were then removed from the oven and allowed to cool to room temperature over a 45 minute period. After cooling the tubes were fractured about six centimeters above the level of the hydrolyzates and placed in a vacuum desiccator for evaporation of the hydrolyzates under reduced pressure. After evaporation the residues were dissolved in one tenth of one milliliter of distilled water and evaporated to dryness once more. This procedure was repeated three times to eliminate residual hydrochloric acid. The residues were then taken up in distilled water so that one microliter represented nominally 10 micrograms of residue.

Three hydrolyses were conducted in duplicate for each sample of organic residue examined. These were 6 hours and 16 hours at 105°C. using 6N HCl as the hydrolyzing medium and 6 hours at 105°C. using 5N NaOH as the hydrolyzing medium. The 6 hour and 16 hour periods of hydrolyzation were shown by Battistone and Burnett (55), in modifying the method of Wellington (56), in determining the amino acid content of dentin and enamel to be the maximum and minimum periods of hydrolysis for maximum recovery of the various amino acids detected. They found that the concentrations of cystine, methionine, tyrosine and histidine present were barely detectable if the hydrolyses were conducted for 16 hours. Most of the remaining amino acids could not be recovered completely from hydrolyzates run less than 16 hours. The results showed maximum recovery for cystine, methionine, tyrosine and histidine from the 6 hour hydrolyzates.

The 5N NaOH hydrolyses were conducted for the recovery of tryptophan because acid hydrolyses completely destroy this amino acid especially when carbohydrate is present in the protein. The prolonged heating necessary to effect hydrolysis by strong alkalies does not effect trytophan but does result in the partial or complete destruction of cysteine, cystine and arginine.

The weight of 6N HCl used in the hydrolyses was approximately 44 times the weight of the organic residues hydrolyzed. This dilution is necessary to minimize interaction of the amino acids and subsequent non-quantitative recovery.

Chromatographic Technics

Separation of Amino Acids.--The methods used were essentially those of Wellington (56) as modified by Battistone and Burnett (55). Whatman No. 1 filter paper was used. A line was drawn nine centimeters from the top edge of the paper.

A second line was drawn nine centimeters in from one side of the paper at a right angle to the line running parallel to the top edge. The point of intersection of these two lines was the spot of application of the hydrolyzate. Aliquots of the hydrolyzates ranging from 150 to 500 micrograms were applied to papers. To obtain maximum separation and uniform spots of the amino acids, the application of the samples was limited to a spot not more than four millimeters in diameter. A hair dryer was used to dry the spot as the aliquots were applied. After the hydrolyzates had been applied to the papers, the papers were placed in a large chromatographic cabinet. Two dimensional chromatography was then carried out. The solvent for the first dimension was absolute ethanol / tertiary butanol / NH_4OH/H_2O (60/20/5/15, v/v/v/v). This solvent was added to the papers for descending technics instead of ascending technics as given by Wellington. This was done as a matter of convenience. The time and length of solvent run was about 20 hours and 35 centimeters past the point of application of the hydrolyzate, respectively. The papers were dried at room temperature in a hood for six hours, trimmed, turned 90 degrees, then replaced in a chromatographic cabinet for the second dimensional run in water saturated phenol in an atomosphere of cyanide and ammonia. The time and length of solvent run were about 18 hours and 30 centimeters past the point of application of the hydrolyzate, respectively. The papers were then transferred to a hood at room temperature and dried for 30 hours before they were developed with two per cent ninhydrin in absolute ethyl alcohol. The color development was carried out at room temperature for 24 hours.

For quantitative estimates of the various amino acids, the spots were cut from the papers, cut into strips and placed into test tubes appropriately marked.

Blanks.--Three areas were cut from each paper to serve as blanks as follows: (1) a spot cut near the phenol front and equal to the size of the proline spot for proline and hydroxyproline, (2) a spot cut just below the lysine spot of a size equal to the largest spot for lysine, arginine, valine and the group leucine, isoleucine, and phenylalanine, and (3) a spot cut above glutamic acid of a size equal to the largest spot obtained for all the remaining amino acids. The three blanks for each chromatogram were placed in appropriately marked tubes.

Determination of Optical Densities.--Elutions of the developed colors were carried out using 50 per cent aqueous n-propanol at room temperature. Five milliliters of the aqueous n-propanol was added to each tube containing the colored spots and the tubes were shaken for 10 minutes to complete the elutions. The tubes were allowed to

stand for 15 minutes to allow lint from the papers to settle. Optical densities were measured, using a one-centimeter cell in an Optica Spectrophotometer, with a tungsten lamp as the radiation source, at 570 millimicrons for all amino acids except proline and hydroxyproline. For these two amino acids and their blank, a hydrogen lamp was used as the radiation source and measurements were made at 350 millimicrons.

Standard Curves.--Standard curves were prepared for each amino acid detected. These curves were prepared from data obtained from two-dimensional chromatograms handled in a manner identical with chromatograms prepared from hydrolyzates of the organic residues of salivary calculi. The standard curves were established on the basis of four concentrations of each acid. Each point for each concentration was the average of duplicate determinations making a total of eight chromatograms for each amino acid detected. These curves shown in Figures 13, 14, 15, and 16 served as standards for determining the unknown concentrations of the amino acids in the varioussalivary calculi samples and mucin.

Determination of Tryptophan

Aliquots of the hydrolyzates obtained using 5N NaOH for the detection of tryptophan were applied to Whatman No. 1 filter paper and the two dimensional solvent runs conducted in the same manner as described previously. Aliquots of standard solutions of tryptophan were also chromatographed. The papers which contained the NaOH hydrolyzates did not show good separation of the amino acids. A streaking occurred down from the spot of application of the hydrolyzate and no spot for tryptophan was detected on any of the papers examined.

Since color tests on samples of the organic matrix indicated the presence of tryptophan but the chromatograms did not, an additional test for clarification was conducted.

The procedure used was essentially that of Bates (57) as modified by Sullivan and Hess (58). The procedure was modified, in this investigation, to adjust it to a micro technique to be used in examining the organic residues of dental calculi.

To 5 milligrams of the organic residues in 0.2 milliliter of 0.1N NaOH were added 0.05 milliliters of the p-dimethylaminobenzaldehyde reagent, 0.02 milliliter (0.2 milligrams) of $NaNO_3$, and 0.25 milliliters of concentrated hydrochloric acid. The color was allowed to develop for 15 to 20 minutes. The solution was then diluted to 5 milli-

liters with 17.5 per cent hydrochloric acid. The calculi solutions were transferred to a Klett-Summerson Photoelectric Colorimeter using Filter 54 and read against tryptophan similarly tested.

The Bates method has the advantage of not requiring hydrolysis and therefore is a simple method of equal accuracy shown by methods involving hydrolytic procedures (58).

Comments on Procedures,

Reagents and Materials

Phenol.--Merck reagent grade phenol was distilled before use. Even with distillation this phenol left yellow streaks and dark solvent fronts on the paper. However, liquid phenol[1] (about 88 per cent carbolic acid) could be used satisfactorily without distilling. No streaking of the papers was observed.

Ninhydrin.--To eliminate possible differences in one lot to another of this reagent, a sufficient quantity of one lot[2] was obtained for this study.

Quality and size of Paper.--Whatman No. 1 and No. 4 papers are suitable. Schleicher and Schiill paper No. 507 is not suitable since the colored ninhydrin product cannot be completely eluted with the propanol-water solution. The Whatman No. 1 paper gave better separation of the amino acids; therefore, it was used throughout the protein analyses.

Temperature.--The chromatograms must be dried at room temperature after each run since heat applied to the wet chromatogram will cause loss of amino acids (56).

Time of Development.--According to Wellington (56) the miximum color develops for all amino acids 30 hours after reaction with the ninhydrin reagent and remains constant for at least another 24 hours. The papers should be dried in a well-ventilated darkened room free from HCl fumes.

[1]Fisher Certified Reagent-Phenol, Lot No. 793749, Fisher Scientific Company.

[2]Fisher Certified Reagent - Triketohyrindene Hydrate, Lot Number 792446, Fisher Scientific Company.

Size of Spot Applied.--It was necessary to keep the size of the spot of application of the protein as small as possible to obtain maximum separation and uniform spots of amino acids. To obtain spots of sufficient density to estimate the concentrations of cystine, histidine, methionine, and tyrosine, 500 µgm. aliquots of residue were required. All of the other amino acids could be determined from papers to which 150 µgm. aliquots of residue had been added. The spots for glycine and serine, for alanine and threonine, and for methionine and alanine were quite close; therefore, it was necessary to closely control the application of the hydrolyzates to the paper. A diagram of a typical chromatogram obtained is shown in Figure 17.

Separation of Amino Acid Spots.--The separation by cutting spots apart had to be done exactly at the same point in the same manner each time. Excellent reproducibility can be obtained according to Battistone and Burnett (55) if this procedure is rigidly adhered to for every paper. When running chromatograms routinely at same concentrations of total protein, the spots will be identical in size and shape and can be readily separated by cutting.

The findings of Battistone and Burnett were confirmed in regard to the determinations of methionine, histidine and hydroxyproline. The methionine and histidine spots were close to hydroxyproline. However, when chromatograms were run with 150 µgm aliquots of residue, histidine did not appear while the methionine spot was very faint and did not touch the hydroxyproline spot which was yellow-orange. While the higher concentration of protein was run in order to estimate histidine and methionine, the hydroxyproline spot which was yellow-orange did touch the histidine and methionine spots when the papers were first developed, but both the spots for histidine and for methionine could be separated easily from the hydroxyproline and estimated at 570 mµ without interference of the hydroxyproline color which had faded almost completely after 24 hours.

The solvent systems used did not separate the spots for phenylalanine, leucine and isoleucine. To confirm qualitatively that all three were present, unidimensional chromatograms were made using a mixture of equal volumes of normal butanol and benzyl alcohol as the solvent system in an atmosphere of HCN (59). However, all quantitative estimates in the data presented for these three acids were based on the unseparated spot obtained by the two dimensional solvent systems.

The descending technic was used (as was by Battistone and Burnett) as a matter of convenience based on the equipment at hand.

Time of Drying.--The six hours drying off of the first solvent as recommended by Wellington (56) was not sufficient to get rid of the ammonia. The ammonia was subsequently seen as background color in the development of the papers with ninhydrin. When the drying off period was extended to ten hours, there was much less discernible background color on the papers after development.

Results

The results obtained on the insoluble residues after demineralization of samples of composite calculus, supragingival calculus, subgingival calculus and calculus removed from artificial dentures are given in Tables 5, 6, 7, and 8. Each table contains the recovered amino acids determined in terms of grams of amino acid per 100 grams of residue after demineralization. The column on the left in each table contains the results obtained from residues after demineralization by EDTA and the column on the right contains the results obtained from residues after demineralization by hydrochloric acid. The calculated nitrogen values for each amino acid estimated are included in each table along with the determined nitrogen content of the demineralized calculi. In addition the ash contents and total per cent recovery of amino acids by the methods used on the samples examined are shown.

Examination of the data in Tables 5, 6, 7, and 8 shows that, in general, the concentrations of alanine; glutamic acid; the amino acid group; phenylalanine, leucine, and isoleucine were lower and arginine were greater in the EDTA decalcified calculi while the concentrations of serine, tyrosine and methionine appear to be decreased, in general, by this method of demineralization. On the other hand, the concentrations of cystine, valine, glycine, threonine, lysine, proline, and histidine did not appear to be affected as much by the method of demineralization.

The concentrations of the majority of the recovered amino acids appear fairly uniform among the insoluble residues after demineralization of the various salivary calculi with the exception of aspartic acid, lysine and arginine. The aspartic acid content appears to be higher in residues obtained by both methods of demineralization of composite calculi and of calculus forming on artificial dentures than its concentration in supragingival and subgingival calculi. The concentrations of lysine in demineralized supragingival and subgingival calculi appear uniform and about twice that in the demineralized composite calculus and the demineralized calculus formed on artificial dentures. The values for arginine in the HCl-demineralized calculi appear to be more uniform than in the EDTA-demineralized calculi; however, the concentration of arginine

in the EDTA-demineralized composite calculus is more than twice that in the HCl-demineralized composite calculus.

Each table contains the estimated concentration of tryptophan detected in the various demineralized salivary calculi. In addition the concentration of hydroxy proline was estimated as about 0.1 per cent. Although hydroxyproline was detected in all residues, the orange color formed in the reaction with ninhydrin faded before elutions were made.

The recovered amino acids in grams per 100 grams of precipitated mucin are listed in Table 9. The concentrations of alanine; valine; the amino acid group; phenylalanine, leucine, and isoleucine; methionine; and histidine are somewhat lower than the estimated recovered concentrations for these amino acids in the insoluble residues of the various demineralized salivary calculi. On the other hand, the concentrations of glycine and proline are higher than the estimated concentrations for these amino acids in the salivary calculi. The concentrations of aspartic acid, serine, lysine, and arginine detected in mucin agree with the concentrations of these acids in the demineralized composite calculus and the demineralized calculus formed on artificial dentures. The estimated concentrations of the other detected amino acids in mucin compare favorably with the concentrations of these acids in the various demineralized salivary calculi.

4.4 Analyses of Carbohydrate

Amount Present by Anthrone Method

Procedure.--The amount of carbohydrate present in the various samples of calculi and precipitated mucin was determined by a modified anthrone colormetric technic as published recently by Beck and Bibby (60). Beck and Bibby modified the technic as recommended by Loewus in 1952 (61) in his improvement in the use of the anthrone method for the determination of carbohydrates.

Samples of the organic residues and mucin weighing from five to ten mg. were added to 30 ml. portions of dilute alkali (0.5N NaOH) in glass stoppered flasks. Each flask was shaken for thirty minutes on a mechanical shaker. Three aliquots (2 ml. each) from each flask were transferred to test tubes (18 x 150 mm.). To each tube 0.5 ml. of the anthrone reagent (2 per cent anthrone-ethyl acetate reagent) was added. Five milliliters of H_2SO_4 (96.7 per cent reagent grade) was added to each tube by carefully layering. The tubes were gently swirled until the ethyl acetate had hydrolyzed, as indicated by the appearance of a floc of an-

throne in the tubes. The contents of each tube were then throughly mixed by more rapid swirling to redissolve the anthrone in the sulfuric acid. As soon as all the tubes were mixed, they were transferred to a bath of boiling water, and each one was stirred with an individual glass stirring rod to insure complete mixing. The tubes were then covered with small watch glasses to prevent condensation of moisture in the upper part of the tubes and left in the boiling water for a total of 10 minutes. They were then transferred to an ice bath for five minutes and, following this, to a 20°C. bath for five minutes. Blanks were prepared in the same manner using two milliliters of distilled water and all reagents as described with the exception of the sample.

After the five minutes in the 20°C. bath, the contents of the tubes were transferred to 1 cm. cells and the optical densities read in a Beckman Spectrophotometer (Model DU), with a tungsten light source, at 630 mμ. The optical density of each sample was determined three times. The mean of the three readings was averaged with the means of the other two aliquots. The 100 per cent transmittance was adjusted with the distilled water-reagent-sulfuric acid blank when necessary before and between readings.

A standard curve was prepared using known concentrations of glucose handled in an identical manner as described for the calculi residues. Concentrations of 10, 20, 40, and 60 μgm. per ml. were used to establish the curve. All determinations were done in duplicate. The standard curve is shown in Figure 18.

The use of anthrone for the analysis of carbohydrate was first suggested by Dreywood (62) in 1946 and was a qualitative test based upon the production of a blue-green color when anthrone reagent was added to an aqueous solution or suspension of mono-, di-, or polysaccharides. Morse (63) and Morris (64) adapted Dreywood's qualitative test to the quantitative analysis of carbohydrate material. Their method has formed the basis for all subsequent technics. One of the more recent technics was described by Loewus (61). One of the major difficulites in the use of the anthrone method had been the darkening of the anthrone-sulfuric acid reagent with time. To overcome this, it was necessary to prepare fresh reagent each day and make a new standardization curve for each series of determinations. To avoid this Loewus suggested using a solution of 2 per cent anthrone (recrystallized from bensene and light petroleum ether) in reagent grade ethyl acetate, which Loewus claimed was stable for several weeks. Beck and Bibby (60) in 1961 reported that the anthrone reagent prepared in this manner was stable for 90

days. However, they found that a further modification in the technic was necessary to obtain results that were reliable and reproducible. The main difficulty appeared to be as has been pointed out by other investigators (65-67) that the amount of green coloration produced in the reaction is greatly influenced by the amount of heat of reaction of the sulfuric acid and water. Beck and Bibby (60) therefore studied the effect of controlling the temperature conditions under which the reaction took place. They found that by controlling the heating and cooling of the samples the accuracy as well as precision improved considerably in the determination of carbohydrate. They established the range of maximum accuracy of the anthrone technic at 20 to 60 µgm. per ml.

In the present investigation it was found necessary to crystallize the anthrone as received, from benzene and light petroleum ether, as was suggested by Loewus. This was necessary because it was impossible to dissolve a sufficient amount of the material as received to produce a 2 per cent solution in ethyl acetate.

Results.--The results obtained for the analyses of carbohydrates in the various demineralized calculi are presented in Table 10. The data in the column on the left are for the EDTA-demineralized calculi and the data in the column at the right are for the HCl-demineralized calculi. The value reported is the average determined upon two samples of each of the various calculi and mucin. The percentage carbohydrate contents obtained from EDTA-demineralized calculi ranged from 13 per cent for the subgingival calculus to 18 per cent for the composite calculus with the average for the various calculi being 16 per cent. There was no significance attached to the differences observed in the carbohydrate contents of the various calculi. However, in the case of the HCl-demineralized calculi the data show a lower recovery of carbohydrate for each of the various calculi. The amounts determined ranged from 8 per cent for the subgingival calculus to 12 per cent for the composite calculus with the average for the various calculi being approximately 10 per cent. It would appear therefore that there was a significant loss of carbohydrate during HCl demineralization; therefore, only the EDTA-demineralized calculi were utilized for further analyses.

Hydrolytic Technics

Procedures.--Portions of the residues from the EDTA demineralization procedures carried out on samples of the various calculi were utilized for these experiments.

The procedures of Glegg and Eidinger (68) were used for the hydrolyses of the residues for chromatographic analysis. The procedure involved a resin hydrolysis using a cation exchange resin, Permutit Q. This is a polystyrene sulfonic acid type resin. It was shipped in the sodium form and was acid -- regenerated by shaking with 4.4N HCl (900 milliliters/liter of resin) in a separatory funnel. The resin was washed repeatedly with distilled water to remove as much of the hydrochloric acid as possible. The final pH of the washings was 6. The resin was then allowed to air-dry.

Samples of the residues weighing between 9.5 and 10.5 milligrams were placed in small pyrex test tubes. The weight of the resin used was twelve times that of the samples. One milliliter of distilled water was added to each tube; the tubes sealed, and placed into an oven at 105°C. for 48 hours. At the end of the hydrolytic period the tubes were removed from the oven and cooled to room temperature. They were then opened and the hydrolyzates transferred to small tubes by decantation. The resin was then eluted three times with small quantities of 0.5N hydrochloric acid. The eluates were added to the decanted liquids, evaporated to dryness in a vacuum desiccator at room temperature and retained until paper chromatographic analyses were conducted.

Comments on Procedures.--Glegg and Eidinger (68) hydrolyzed carbohydrate-protein complexes using cation exchange resins prior to identifying monosaccharide components by paper chromatography. They determined that pentoses, methylpentoses and aldohexoses were easily identified on paper chromatograms after their solutions were heated with the cation exchange resin for 48 or even 96 hours. Ketohexoses were not detectable after heating with the resin for 48 hours. However, mineral acid hydrolysis also completely destroys ketohexoses.

Hexuronic acids were destroyed by heating at 100°C. with or without resin for 96 hours, but enough was preserved for identification when the hydrolysis was limited to 48 hours. Under these conditions Glegg and Eidinger found that decomposition was accompanied by development of dark brown solutions but that the brown solution did not interfere on subsequent chromatograms.

Glucosamine and N-acetyl glucosamine were destroyed by heating without resin at 100°C. However, glucosamine was protected from destruction by the presence of resin even after heating for 96 hours. This effect may be explained

by the absorption of glucosamine on the resin. It was found possible to elute the hexosamine from the resin with 0.5N HCl and then to identify it on chromatograms as the hydrochloride.

These findings by Glegg and Eidinger therefore indicated that, with the exception of ketoses, the monosaccharides were detectable after heating with the resin for 48 hours.

Chromatographic Technics

Separation of Carbohydrates.--The residues from the evaporated decanted liquids and eluates were taken up in one-tenth of one milliliter of distilled water. Twenty to fifty-microliter aliquots of each solution were used for chromatographic analysis. The papers used were Whatman No. 1 and Whatman No. 4, following essentially the procedures of Partridge (69). The aliquots were placed at points of origin along a horizontal line 9 centimeters from the top of paper strips 18 centimeters wide and 59 centimeters long. To each sheet 5 microliters of one per cent aqueous solution of the following sugars were added: glucose, galactose, rhamnose, mannose, fucose and glucosamine. Other sheets also contained 5 microliter aliquots of a one per cent aqueous solution of galacturonic acid. All chromatograms were run using a triple descending rather than a triple ascending technic of Partridge (69) or Glegg and Eidinger (68). This was done as a matter of convenience. Unidimensional chromatograms were prepared using the following solvent system:

1. n-butanol/glacial acetic acid/water (40/10/50, v/v/v).
2. Water saturated phenol in an atomosphere of cyanide and ammonia.

The time and length of solvent runs were from 20 to 22 hours and 30 to 35 centimeters, respectively. When the n-butanol/glacial acetic acid/water system was used, the aqueous phase was separated from the solvent phase which was placed in the tray. The aqueous phase was placed in the bottom of the chromatographic tank. In the case of the phenol system a 0.1 per cent aqueous solution of potassium cyanide was placed in the bottom of the tank to give a cyanide atmosphere during the runs.

After completion of the runs, the solvent fronts were marked and the solvents dried off in an oven at 100 to 105°C.

Identification Procedures

Reducing Sugars--After the papers were dried they were developed for identification of reducing sugars using two different reagent systems. The first reagent used was an ammonical silver nitrate solution recommended by Partridge (69) and the second reagent was a benzidine solution recommended by Horocks (70).

Silver Nitrate Reagent

The reagent was prepared by mixing equal parts of 0.1N silver nitrate and of 5N ammonium hydroxide. The dryed papers were sprayed evenly with the reagent and placed in an oven at 105°C. for five to ten minutes. The reducing sugars appeared as dark brown spots. While the ammonical silver nitrate solution will detect easily 5 to 40 µgm. of reducing sugar, the reagent is not stable to heat and light. Furthermore, urates interfer with the detection of many reducing sugars with ammonical silver nitrate since they produce a spot which "tails" and tends to mask the spots which would be produced by glucose, sorbose, arabinose, and fructose. This "tailing" was evident in the development of chromatograms containing hydrolyzates from residues of the calculi and possibly was caused by the presence of urates.

Benzidine Reagent

This reagent was prepared by dissolving 0.5 gm. of benzidine in 20 ml. of glacial acetic acid and 80 ml. of absolute ethyl alcohol. The dryed papers were sprayed with the reagent and then heated in an oven at 105°C. for 15 minutes. The benzidine reagent is only slightly affected by heat and light and the use of this reagent eliminates to a large degree the interference of the urates.

Ketoses and Hexuronic Acids.--The naphthoresorcinol reagent as suggested by Partridge was used to detect the presence of, if any, ketoses and hexuronic acids. The reagent was prepared fresh on day of use and consisted of equal volumes of two solutions: (1) 0.2 per cent of napthoresorcinol in ethanol (w/v) and (2) a 2.0 per cent solution of trichloracetic acid (w/v). Equal volumes of the two solutions were mixed immediately before use. The dryed papers were sprayed evenly with the mixture and dryed partially at room temperature. They were then placed in an oven at 105°C. for five to ten minutes. Fructose, sorbose, sucrose and raffinose if present will give very strong red spots, the color of which is stable for at least 12 hours (70). Separate series of

papers were also heated at 70-80°C. in a humid atmosphere. Pentoses and uronic acids if present give strong blue coloration in 10 to 15 minutes. The spots for ketoses under these conditions are orange-brown.

Hexosamines.--The Morgan and Elson (71) colorimetric estimation as modified by Partridge (69) for use with filter-paper techniques was used for the detection of hexosamines.

Two separate reagents were utilized. The first reagent was the acetylacetone reagent and consisted of the two following solutions:

(1) Solution 1 was prepared by dissolving 0.5 ml. of acetylacetone in 50 ml. of butanol and

(2) Solution 2 was prepared by mixing 5 ml. of 50 per cent aqueous KOH (w/v) with 20 ml. of absolute ethyl alcohol. One-half of one milliliter of solution 2 was added to 10 ml. of solution 1 immediately before the reagent was required. The second reagent was the p-dimethylaminobenzaldehyde reagent. One gram of p-dimethylaminobenzaldehyde, recrystallized from aqueous ethanol, was dissolved in 30 ml. of absolute ethyl alcohol. To this 30 ml. of concentrated HCl were added. The solution was then diluted with 180 ml. of redistilled butanol. The dryed papers were sprayed with the first reagent and heated in an oven for five minutes at 105°C. The dry paper strips were then sprayed with the second reagent and returned to the oven for an additional five minutes at 90°C. Under these conditions the hexosamines will give cherry-red colored spots.

Deoxy Sugars.--The thiobarbituric acid spray reagent by Warren (72) was used to detect the possible presence of deoxy sugars.

After the solvent was removed from the papers, they were sprayed with a 0.02M aqueous solution of sodium periodate and hung in air at room temperature to dry. After 15 minutes the papers were then sprayed with a solution consisting of ethylene glycol, acetone and concentrated sulfuric acid [50/50/0.3 (v/v/v)]. The papers were dried for 10 minutes and then sprayed with a 6 per cent aqueous solution of sodium 2-thiobarbiturate. The papers were then placed in an oven at 100°C. Deoxy sugars will appear as red spots on the papers after 5 minutes at 100°C. Under ultraviolet light the spots give a red fluorescence.

Sialic Acid.--The diphenylamine test as described by Winzler (73) was used to test for sialic acid and to detect if possible deoxy-sugar in demineralized calculi.

The procedure follows:

1. 4.8 ml. of 5 per cent trichloroacetic acid (TCA) were added slowly with shaking to 0.2 ml. of resin hydrolyzates of demineralized calculi, to 0.2 ml. of the sialic acid standard[1] (200 µgm. crystalline sialic acid/ml.) and to 0.2 ml. of 2-deoxyribose-5-phosphate standard[1] (400 µgm./ml.) in 15 x 150 mm. test tubes.

2. The tubes were then placed in a boiling water bath for exactly 15 minutes. The tubes were covered with small watch glasses to prevent evaporation. After heating the tubes were cooled by immersion in a water bath at 20-25°C. and the solutions were then filtered.

3. Aliquots of 2 ml. of each of the filtrates were pipetted into 15 x 150 mm. test tubes.

4. Then 4 ml. of the diphenylamine reagent[2] (DPA) were placed into each tube. A reagent blank containing 2 ml. of 5 per cent TCA and 4 ml. of the DPA reagent was also prepared.

5. The tubes were then mixed with separate small stirring rods which were left in each tube. The tubes were then covered with small watch glasses and immersed in a boiling water bath for exactly 30 minutes.

6. The tubes were then cooled in water at room temperature and the absorption curves determined with the reagent blank set at 100 per cent transmission using an Optica Spectrophotometer.

Comments on Identification Procedures.--The paper chromatographic technics provided clear separation of many carbohydrates. The clarity of the separations is a result of the combination of several factors -- the solvent used, the techniques of triple development and the fact that the solvent was allowed to move in the machine direction of the paper.

[1]Source: Dr. L. Warren, National Institutes of Health, Bethesda, Maryland.

[2]The diphenylamine reagent was prepared by recrystallizing 1 gm. of diphenylamine from ethanol and then dissolving it in a mixture of 90 ml. of glacial acetic acid and 10 ml. of concentrated sulfuric acid.

The n-butanol/glacial acetic acid/water system gave better separation of many of the carbohydrates than the phenol system. Even with prolonged drying to get rid of the phenol, there often was excessive background color development. Even with the atmosphere of cyanide a darkening of the papers near the solvent front took place on many of the chromatograms. Therefore, all results for the carbohydrates with the exception of hexosamine and the deoxypentose were determined from chromatograms developed with the n-butanol/glacial acetic acid/water system. The hexosamine hydrochlorides were determined from papers using phenol as the solvent because less tailing was seen with this solvent; therefore, clearly defined circular spots could be obtained for detection. The phenol solvent was used to separate the deoxypentose since this material has no detectable mobility in the other solvent used.

Results

The results obtained chromatographically are shown in Table 11. The following are arranged in the approximate order of decreasing color intensity after treatment with the benzidine reagent: galactose, glucose, mannose, rhamnose and fucose. These carbohydrates migrated identically with the standards in the solvent system used as seen by the R_f values for these five carbohydrates. The R_f values were corrected to 20°C. by the method of Partridge (69). All five carbohydrates were detected in all of the various decalcified calculi.

Deoxypentose and sialic acid were detected chromatographically in the decalcified calculi as shown in Table 11. The presence of the deoxypentose was detected by the method of Warren (72) and sialic acid was indicated by the appearance of a blue color with the diphenylamine test for sialic acid (73). The absorption curves determined on the diphenylamine reaction products are shown in Figure 19.

The R_f value for the hexosamine detected in the various demineralized calculi is shown also in Table 11, along with the R_f values for glucosamine and galactosamine. The R_f values determined indicated that the hexosamine detected in the various demineralized calculi was galactosamine.

The results of the test for hexuronic acid on the various decalcified calculi were not reproducible. In some instances a faint positive naphthoresorcinol reaction was obtained and in others negative results were obtained.

Hexosamine Content

Procedure.--The method of Hess and Lee (74) was used in the hydrolyses of residues for the quantitative determination of hexosamine content. Between 40 and 60 mg. of the demineralized calculi were sealed in tubes containing 2 ml. of 4N HCl and heated in a bath at 100°C. for ten hours. The hydrolyzates were diluted with water to a volume of 50 ml., shaken with about 0.5 gm. of decolorizing carbon and then filtered. The color was then developed with the Einbinder and Schubert technique (75).

After the acid hydrolyzates were diluted and filtered, 5.5 ml. of acetylacetone reagent were added to 2 ml. aliquots of the filtrates in 25 ml. volumetric flasks. The mixtures were heated in a boiling water bath for exactly 20 minutes. They were then quickly cooled and about 10 ml. of absolute ethyl alcohol was added with mixing. Then 2.5 ml. of Ehrlick's reagent were added and the volume made up to 25 ml. with absolute ethyl alcohol. After 24 hours, the optical densities of the solutions were read at 530 mμ on a Beckman Spectrophotometer (Model DU). The results were compared to a standard curve prepared from known concentrations of glucosamine shown in Figure 20.

Comments on Procedure.--The acetylacetone reagent was prepared from 4.76 gm. of anhydrous Na_2CO_3, 0.89 ml. of freshly distilled acetylacetone and 7.81 ml. of 1.00 M HCl, and made up of 100 ml. with distilled water. The Ehrlick reagent was made by dissolving 3.2 gm. of recrystallized p-dimethylaminobenzaldehyde in a mixture of 120 ml. absolute ethyl alcohol and of 120 ml. concentrated hydrochloric acid.

The colorimetric method for the determination of hexosamine was first developed by Elson and Morgan (76) and then modified by Einbinder and Schubert (75). Hess and Lee (74) found the latter modification to be suitable for their determinations of hexosamine in chondroitin sulfuric acid from dentin. However, they did find that the acidity of the test solution prior to color development had to be carefully controlled. From their experiments they concluded that the desired pink color could be obtained if the test solution had an acidity of 0.2N HCl.

Results.--The results for the quantititive determinations of the hexosamine are shown in Table 12. The hexosamine contents of the various demineralized calculi ranged from 3 to 5 per cent.

5. REVIEW AND DISCUSSION

5.1 Methods and Results - Part I

Sampling and Separation of Calculus

Two pooled samples of each type of salivary calculus were collected for physical and chemical studies. Each sample was composed of calculus from 40 to 60 patients and was subdivided for determination of ash content, infra-red spectroscopy and demineralization and extraction procedures. No attempt was made to determine the effect of storage on the composition of the samples as there was considerable delay in obtaining sizable samples necessary for the study.

Sampling of Mucin

Two samples of mucin were precipitated from saliva for comparison with the organic content of salivary calculus. It appeared logical to examine mucin since the presence of a mucoprotein has been suggested in salivary calculus and since the teeth are constantly bathed in saliva containing mucin.

Ashing of Samples of Calculus

Samples of the various salivary calculi were ashed in order to determine approximate amounts of organic residues which could be expected upon demineralization or extraction of samples.

The amount of ash determined by these tests ranged from 80 to 85 per cent of the weight of the original samples. This indicated that one could expect approximately 15 to 20 per cent residue after demineralization neglecting solubilization of organic components and moisture content.

Preparation for Analyses

Demineralizations.--Portions of the same samples of calculus were demineralized in solutions of ethylenediamine tetraacetic acid (EDTA) and hydrochloric acid. These procedures were chosen because:

1. EDTA is a powerful chelating agent which has been used to decalcify tooth tissue at a neutral or alkaline pH as shown by Battistone and Burnett (55) with a minimal effect on the protein of the tooth tissues.

2. The 0.1N HCl represents the inorganic acids and has been used in previous studies on the amino acid composition of tooth structure by Hess and co-workers (77) and Battistone and Burnett (55).

Extractions.--Portions of samples of composite calculus were extracted with boiling water and with alcohol and ether.

1. Calculus was extracted with boiling water in the same manner as had been reported by Battistone and Burnett. In working with dentin, they found that the protein extracted by refluxing samples in boiling water was identical to that obtained by decalcification of dentin with 15 per cent EDTA; therefore, this had implied a simple technique which possibly could be used to extract the protein of the calculus. Only about 1 per cent of the weight of the original sample could be extracted; therefore, this procedure was discontinued and no analytical results, with the exception of the infrared spectrum, are reported for this method of separation of mineral and organic residues of salivary calculus.

2. Extractions with absolute ethyl alcohol and anhydrous ethyl ether were conducted to determine the lipid content of salivary calculus. The average of two determinations showed that only approximately 0.2 per cent of the weight of the original sample was extracted. Although theoretically lipid may play a part in calcification of salivary calculus, the presence of larger amounts of a carbohydrate-protein complex, subsequently determined, would appear to be of more significance. Therefore, for the present investigation, no further tests were conducted on the lipid fraction.

Ashing of Residues

The precentage residue obtained upon ashing samples of the various HCl-demineralized calculi was only about one-half that obtained in ashing the EDTA-demineralized calculi indicating that a more complete demineralization was obtained in HCl than in the EDTA medium.

Nitrogen Analyses

The nitrogen determinations were made with the Coleman Nitrogen Analyzer, which is an automatic version of the micro-Dumas method, as a matter of convenience to the investigator. This method was chosen instead of the micro-Kjeldahl method only because of the availability of the nitrogen analyzer to the investigator and the

short time (about 8 minutes) required per determination. The instrument permits measurements of nitrogen values from 5 to 50 per cent in samples in the 1 to 10 milligram range.

The nigrogen content of samples of ammonium sulfate determined in order to verify the accuracy of the instrument were within ±0.30 per cent of the theoretical value. In terms of weight per cent nitrogen, ±0.30 per cent of the theoretical nitrogen content of ammonium sulfate would be ±0.04 per cent nitrogen. Therefore, this accuracy was assumed in subsequent analyses of nitrogen content of samples under study.

The average total nitrogen content of the HCl-demineralized salivary calculi was 9.90 per cent and that for the EDTA-demineralized salivary calculi was 8.13 per cent. The greater ash content of the EDTA-demineralized calculi might account for a part of this difference observed since the actual weight of organic material would be smaller, if corrected for ash content, than that used to calculate nitrogen content.

Qualitative Examinations

General composition and color tests indicated the presence of nitrogen, sulfur, protein-like material (tyrosine and tryptophan) and reducing sugars in the demineralized salivary calculi.

5.2 Methods and Results - Part II

Examination by Infrared Spectroscopy

The infrared spectra obtained on the mineral portion of calculus and on synthetic hydroxyapatite were quite similar indicating the presence of either hydroxyapatite or calcium phosphate or both in the mineral portion of calculus. The spectra of the demineralized calculus and the residue extracted by boiling water were similar and revealed proteinaceous material as seen when compared to the spectrum for mucin.

Examination of Structure and Attachment of Salivary Calculus

Microscopic examination by white light and by ultraviolet radiation of sections of teeth having calculus deposits adhering to them were conducted as a possible means of investigating the structure of salivary calculi

and of its attachment to tooth tissues.

In other studies human teeth have been investigated by irradiating with ultraviolet light (54). When irradiated with ultraviolet light, the teeth emitted visible light or fluoresced. Photomicrographs of this fluorescence have been shown to reveal details of structure not revealed by other methods in examining human teeth.

Photographs prepared by visible illumination and by fluorescence excited by ultraviolet illumination revealed laminations in the calculus and also suggested a band of organic material between the enamel and the calculus.

The observance of laminations was also reported by Mandel and others (43). In a histochemical study of calculus formation, they found that the calcifying areas of calculus were frequently laminated by alternating dark and light staining bands which produced a concentric ring-like appearance similar to that of urinary calculi. This band of material was termed by Mandel as an amorphous, nonstaining cuticularlike material present at the junction of the plaque and the celluloid strip fixed to teeth in their histological study of calculus forming in vivo. This cuticular material was also occasionally present at different levels within the deposit itself. Optically the material resembled the cuticle which is seen over the surface of enamel and cementum. More recently, Meckel (78), in an oral presentation on the formation and properties of organic films on teeth, reported the deposition of an organic film on cleaned enamel surfaces. This renewable film or pellicle appeared to be identical to the secondary enamel cuticle. This pellicle withstood average toothbrushing and was found to be practically free of bacteria. Histochemical staining reactions were very similar for pellicles and salivary films of a variety of known origins including mucin from saliva.

The reported tenacity of the organic film to tooth structure may be a key to the attachment of the calculus deposits to the tooth structure and it would seem logical that additional layers of this organic film could be deposited on the layers of calculus as the deposit forms over a long period of time.

The presence of an organic layer at the junction of the calculus deposit and the tooth tissue and throughout the deposit would tend to support the possible role of an organic complex in the calcification of salivary calculus and substantiates the need for further knowledge of the

chemical nature of the organic matrix.

Analyses of Protein

The results for the recovered amino acids of the various salivary calculi indicate a general similarity between the supragingival calculus, the subgingival calculus and the calculus removed from artificial dentures. The composite calculus, in general, represents a mixture of supragingival and subgingival calculi. Considering the number of patients involved, the results for the amino acid compositions determined indicate fair uniformity in the recovered residues after demineralization. Moreover, the estimated recovered amino acid composition of precipitated mucin appears similar to that of the various demineralized salivary calculi. Small but detectable amounts of methionine, leucine and hydroxyproline were found in all demineralized calculi. Little (51) did not detect these three amino acids in her study of the organic matrix of dental calculi. King and Boyce (52) however did find methionine and leucine in duct stone matrices. No mention was made by them concerning the presence or absence of hydroxyproline in their study.

The percentage ash was much less for the residues obtained by HCl demineralization than was determined on the residues obtained by EDTA demineralization. This would indicate that a more complete demineralization was obtained in HCl than in the EDTA medium. The average total recovery of amino acids from the various EDTA demineralized calculi, not corrected for ash, was 49.27 per cent while the average total recovery from the various HCl demineralized calculi, not corrected for ash, was 50.52 per cent. On this basis therefore, the recovered amino acids would account for about 50 per cent of the demineralized calculi. This agrees with the total amino acids recovered from demineralized duct stones by King and Boyce (52) but is higher than that estimated by Little (51) in salivary calculi (36 to 40 per cent) based on total nitrogen content.

The average result for amino acid nitrogen calculated on the basis of recovered amino acids from the various calculi accounts for approximately 75 per cent of the average total nitrogen determined on the demineralized calculi indicating loss or the presence of nitrogenous constitutents other than amino acids. The more labile acids especially in the presence of carbohydrates would be partially or totally destroyed. Either amide nitrogen or ammonia or both might account for a part of the nitrogen nonbalance. Ammonia has been reported present in duct calculi by Harril, King and Boyce (53).

Little (51) does not agree with the findings of King and Boyce (52). Little if any deoxypentose was indicated by the absorption curve determined on the diphenylamine reaction products of calculus. This may be due to the amount of deoxypentose being so small as to be masked by the absorption characteristics of the sialic acid reaction with diphenylamine.

Hexosamine appears to be present in salivary calculi as well as in salivary mucin. No significance can be attributed at this time to the differences in hexosamine content observed in the various salivary calculi. A browning of the hydrolyzate took place during heating with some possible decomposition of hexosamine. At least 20 per cent decomposition of hexosamine has been shown by Eastoe and Eastoe (80) in attempts to quantitatively recover known added amounts of hexosamine to gelatin. Therefore, the actual amount of hexosamine in salivary calculi is probably higher than the amounts determined. However, there was little if any hexuronic acid recovered from the salivary calculi. One cannot, however, conclude definitely that hexuronic acid is not present in the salivary calculi. Hexuronic acid is in part dializable and may have been lost to some extend during this procedure on the various salivary calculi. In addition there is destruction of hexuronic acid also in the resin hydrolysis procedure.

5.3 Recommendations for Future Studies

The preliminary studies on the structure and attachment of calculus to tooth tissue revealed a band of material, thought to be organic in nature, between the calculus and tooth tissue. The importance of this layer in future studies should not be overlooked as a possible key to the adhesion of calculus to the tooth as has been suggested by Leung (81). Moreover, since the results of this study have shown the presence of a carbohydrate-protein complex in the organic portion of calculus, it is suggested that the best avenue for investigating the prevention of or reduction of calculus lies in a possible attach of the organic portion in preference to an attack of the mineral portion.

To advance further, it might be advisable to investigate the organic portion of calculus formed *in vitro* to compare it with the formed *in vivo*. If they are similar then one could work *in vitro* in future studies with reagents to reduce the formation of salivary calculus before attempting to conduct similar studies *in vivo* without harm to the patient.

It would also seem advisable to study calculus as it forms; that is, determine the organic content of calculus formed *in vivo* at definite intervals during calcification. Also, the effectiveness of reagents in reduction of calculus could be studied at the same time intervals in calcification.

The above recommendations are based on the indicated regularity of the presence of a mucoprotein in all the calculi examined which might account for the reduction seen in calculus formation in the mucinase studies of Stewart (21) and Aleece and Forscher (28). This regularity would suggest that the mucoprotein plays a role in calcification and deposit of the calculus on the teeth or artificial dentures. The indicated consistency of the recovered amino acids and carbohydrates, in general, in both salivary and duct calculi would suggest also that the organic matrix is not a chance inclusion but that it is a prerequisite to calculus formation, and that crystal deposition is a secondary phenomenon. The fact also that salivary calculus tends to deposit in greatest concentrations opposite the orifices of the submaxillary and parotid ducts suggests a similar mechanism for formation of salivary and duct calculi.

6. Summary and Conclusions

1. The present investigation was concerned primarily with the insoluble organic portion of various dental calculi. The samples examined were composite calculus (a mixture of supragingival and subgingival calculi), supragingival calculus, subgingival calculus, and calculus from artificial dentures. In addition samples of mucin precipitated from saliva were also examined. A brief preliminary study was also conducted on the structure and attachment of salivary calculi to natural teeth.

2. Three methods were used to separate the organic matrix from the mineralized portion of the various salivary calculi. They were: (1) demineralization with 15 per cent EDTA at a pH of 7.0 to 7.5, (2) demineralization with 0.1N HCl at 4°C. and (3) extraction with boiling water. The extraction with boiling water did not prove practicable since the amounts of recovered protein upon precipitation by ethanol were very small.

3. Gross examination by infrared spectroscopy revealed proteinaceous material in the demineralized calculi while spectra for the mineral portion of calculi and synthetic hydroxyapatite proved similar.

4. Microscopic examination of sections of calculus adhering to tooth structure revealed laminations in the calculus and also indicated a band of organic material between the enamel and the calculus.

5. The amino acids recovered from acid hydrolyzates of the various demineralized calculi were identified by paper chromatography. Estimations of quantities of amino acids, with the exception of tryptophan, recovered were conducted by elution of the ninhydrin reaction products, determination of optical densities of these elutions and subsequent estimations of concentrations for each amino acid using prepared standard curves. The percentage carbohydrate in the various demineralized calculi was estimated by the Anthrone Method. With the Anthrone Tests less carbohydrate was detected in HCl-demineralized calculus than in EDTA-demineralized calculus. Therefore, only the EDTA-demineralized calculi were utilized for subsequent examinations. Separation and identification of the recovered carbohydrates from resin hydrolyzates were conducted by paper chromatography and absorption spectroscopy. In addition, estimations of hexosamine contents were performed on acid hydrolyzates. The residue obtained upon extraction of calculi with ethyl alcohol and ethyl ether was recorded as the lipid content. A summary of the percentage recovery of protein, carbohydrate and lipid from EDTA-demineralized calculi is shown in Table 13. The total recovery of amino acids, carbohydrates, lipid and ash were about 80 per cent of the insoluble EDTA-demineralized composite calculi. Further studies are necessary to account for the remainder of the insoluble matrix.

6. The results obtained for the recovered amino acids from the various EDTA-demineralized salivary calculi, duct calculi and mucin are summarized for comparison in Table 14. The detected carbohydrates are summarized in Table 15. The results for the recovered material indicate that supragingival calculus and subgingival calculus are similar. The recovered organic substances from calculus formed on artifical dentures apparently is comparable to those recovered from the other salivary calculi.

7. While it is realized that there could possibly be other proteins such as nucleoproteins from bacteria present in calculi, the recovered amino acids and carbohydrates at least indicate that a glyco- or mucoprotein may be present. This would also be substantiated by the similarity of the recovered amino acids from the glyco-protein, mucin, with those recovered from the various salivary and duct calculi.

8. It is proposed that salivary calculus formation may therefore begin with the deposition upon the tooth surface of a film of salivary mucoid in which possibly bacteria and other sediments are entrapped. This film would serve at least two important functions. It would provide a matrix for holding, together the mineral precipitates and it would furnish the means for the firm attachment of the calcified deposit to the tooth surface.

7. REFERENCES

1. Allen, E. F. Statistical study of the primary causes of extraction. J. Dent. Res. 23:453, 1944.

2. Black, G. V. Diseases of the Periodontal Membrane; The American System of Dentistry, Vol. 1, Lea Brothers and Co., 1886.

3. Wearn, E. E. Salivary and serumal calculus. Aust. J. Dent. 7:293, 1935.

4. Burchard, H. H. Varieties of dental calculi. Dental Cosmos 40:1, 1898.

5. Prinz, H. Origin of salivary calculus. Dental Cosmos 63:231, 1921.

6. Bibby, B. G. Formation of salivary calculus. Dental Cosmos 77:668, 1935.

7. Burchard, H. H. Origin of salivary calculus. Dental Cosmos 37:821, 1895.

8. Hulin, C. H. Nature et mode de formation des calculus salivairs. L'Odontologie 66:201, 472, 1928.

9. Adamson, K. T. Calculi, with special reference to those found in the mouth. Austral. J. Dent. 33:235, 1929.

10. Smith, G. H. Factors affecting the deposition of dental calculus. Austral. J. Dent. 34:305, 352, 1930.

11. Berke, J. Etiology and histology of dental tartars. Dental Cosmos 77:134, 1935.

12. Hodge, H., and Leung, S. W. Calculus formation. Jour. of Periodont. 21:211, Oct. 1950.

13. Citron, S. R. Role of actinomyces israeli in salivary calculus formation. J. Dent. Res. 24:87, 1945.

14. Ennever, J., and Warner, B. Phosphatase and oral actinomyces. J. Dent. Res. 31:25, 1952.

15. Fitzgerald, R. J., and McDaniel, E. G. Dental calculus in the germfree rat. Arch. Oral Biol. 2:339, 1960.

16. Fitzgerald, R. J. Discussion of "A review of dental research using germfree animals" by F. J. Orland. Ann. N. Y. Acad. Sc. 78:288, 1959.

17. Fitzgerald, R. J., Jordan, H. V., and Stanley, H. R. Experimental caries and gingival pathology in the gnotobiotic rat. J. Dent. Res. 39:923, 1960.

18. Baer, P. M., and Newton, W. L. The occurrence of periodontal disease in germfree mice. J. Dent. Res. 38:1238, 1959.

19. Black, G. V. Deposit of Salivary Calculus. Dental Rev. 26:337, 1912.

20. Head, J. A tartar solvent especially useful in work. Dental Cosmos 51:41, 1909.

21. Stewart, G. Mucinase, A possible means of reducing calculus formation. J. Periodontology 23:85, 1952.

22. Inouye, J. M. Biochemical studies of salivary mucin. J. Dent. Res. 26:472, 1947.

23. Dreizen, S., and Spies, T. D. Further studies on the association between products of protein putrefaction and dental caries activity. J. Dent. Res. 27:305, 1948.

24. Bartels, H. A. Bacterial growth and crystal formation: a possible factor in calculus formation. J. Dent. Res. 29:436, 1950.

25. Leung, S. W. Uneven distribution of calculus in the mouth. J. Periodontology 22:7, 1951.

26. Kerr, C. A., and field, H. Sodium hexometaphosphate as an aid in the treatment of periodontal disease. J. Dent. Res. 23:313, 1944.

27. Leung, S. W. The effect of silicones and lactones on synthetic calculus formation. J. Dent. Res. 33:670, 1954.

28. Aleece, A. A., and Forscher, B. K. Calculus reduction with a mucinase dentifrice. J. Periodontology 25:122, 1952.

29. Grossman, L. I. Clinical evaluation of a salivary calculus preventitive agent. Oral Surg., Oral Med., and Oral Pathol. 7:607, 1954.

30. Grossman, L. I. Effect of chemical agents on a calculus substitute. Oral Surg., Oral Med., and Oral Pathol. 7:484, 1954.

31. Gunson, J. J. Clinical studies on a new calculus dissolving agent. Dent. Survey 31:1248, 1955.

32. Dwinelle, W. H. Dissertation on salivary calculus. Amer. J. Dent. Sc. 5:32, 1844.

33. Gorgas, F. J. S. Questions and Answers, Snowden and Cowman, Baltimore, 1892.

34. Schehevetskey, cited by Talbot (35).

35. Talbot, E. S. Interstitial gingivitis and pyorrhea alveolaris. Ranson and Randolph, Toledo, 1913.

36. Kranz, P., Cited by Karshan and Schroff (37).

37. Karshan, M., and Schroff, J. Composition of some salivary calculi. J. Dent. Res. 8:454, 1928.

38. Glock, G. E., and Murray, M. Chemical investigation of salivary calculus. J. Dent. Res. 17:257, 1938.

39. Taylor, N. W., and Sheard, C. Microscopic and x-ray investigation on the calcification of tissue. J. Biol. Chem. 81:479, 1929.

40. Phillip, A. Uber die strucktur von zahnstein, calculus dentalis supragingivalis, Hoppe-Seylev's Z. physiol. chem. 233:209, 1935.

41. Jensen, A. T., and Dano, M. X-ray crystallographic examination of calculi from salivary glands. J. Dent. Res. 31:620, 1952.

42. King, T. O., Rowles, S. L., Little, K., and Thewlis, J. Chemical and x-ray examination of deposits removed from the teeth of golden hamsters and ferrets. J. Dent. Res. 34:650, 1955.

43. Mandel, I. D., Levy, B. M., and Wasserman, B. H. Histo-chemistry of calculus formation. J. Periodontology 28:132, 1957.

44. Mandel, I. D., and Levy, B. M. Studies on salivary calculus. Oral Surg., Oral Med., and Oral Pathol. 10:874, 1957.

45. Naeslund, C. Studies of tartar formation. Acta path. et microbiol. Scandinav. 3:637, 1926.

46. Taylor, W. E. Association of bacteria with dental calculus. J. Dent. Res. 14:237, 1934.

47. Grynthe, O. The role of actinomyces and leptothrix as an etiological factor in the formation of tartar and salivary calculi. Norske Tandl. Tidende. 48:433, 1938.

48. Siegel, E. Aktinomyzeter als zahnstein-bildner. Arch. Hyg. 113:223, 1935.

49. Thirty-Ninth General Meeting, International Association for Dental Research, Boston, Mass. March 1961.

50. Hampar, B., Mandel, I. D., and Ellison, S. A. The carbohydrate components of supragingival calculus. Oral presentation, Boston, Mass. March 1961.

51. Little, M. F., Casciani, C., and Lensky, S. The organic matrix of dental calculus. Oral presentation, Boston, Mass. March 1961.

52. King, J. S., Jr., and Boyce, W. H. Amino acid and carbohydrate composition of the mucoprotein matrix in various calculi. Proc. Soc. Exp. Biol. and Med. 95:183, 1957.

53. Harrill, J. A., King, J. S., Jr., and Boyce, W. H. Structure and composition of salivary calculi. The Laryngoscope. 69:481, 1959.

54. Dickson, G., Forziati, A. F., Lawson, M. E., Jr., and Schoonover, I. C. Fluorescence of teeth: a means of investigating their structure. J. A. D. A. 45:661, 1952.

55. Battistone, G. C., and Burnett, G. W. III. The amino acid composition of human dentin protein. J. Dent. Res. 35:255, 1956.

56. Wellington, E. F. An ultramicro method for quantitative determination of amino acids. Canad. J. Chem. 30:581, 1952.

57. Bates, R. W. A rapid method for quantitative determination of tryptophan. Proc. Am. Soc. Biol. Chem., J. Biol. Chem. 119:7, 1937.

58. Sullivan, M. X., and Hess, W. C. A study of the various procedures for the estimation of tryptophan. J. Biol. Chem. 155:441, 1944.

59. Consden, R., Gordon, A. H., and Martin, A. J. P. Qualitative analysis of proteins: a partition chromatographic method using paper. Biochem. J. 38:224, 1944.

60. Beck, D. J., and Bibby, B. G. A modified anthrone colorimetric technique for use in investigations related to the cariogenicity of foodstuffs. J. Dent. Res. 40:161, 1961.

61. Loewus, F. A. Improvement in anthrone method for determination of carbohydrates. Anal. Chem. 24:219, 1952.

62. Dreywood, R. Qualitiative test for carbohydrate material. Ind. and Eng. Chem., Anal. Ed., 18:499, 1946.

63. Morse, E. E. Anthrone in estimating low concentrations of sucrose. Ind. and Eng Chem., Anal. Ed. 19:1012, 1947.

64. Morris, D. L. Quantitative determination of carbohydrates with Dreywood's anthrone reagent. Science 107:254, 1948.

65. Seifter, S., Dayton, S., Novic, B., and Muntwyler, E. The estimation of glycogen with the anthrone reagent. Arch. Biochem. 25:191, 1950.

66. Scott, T. A., Jr., and Melvin, E. H. Determination of dextran with anthrone. Anal. Chem. 25:1656, 1953.

67. Viles, F. J., Jr., and Silverman, L. Determination of starch and cellulose with anthrone. Anal. Chem. 21:950, 1949.

68. Glegg, R. E., and Eidinger, D. Hydrolysis of polysaccharides by a cation exchange resin and identification of monosaccharide components by paper chromatography. Anal. Chem. 26:1365, 1954.

69. Partridge, S. M. Filter-paper partition chromatography of sugars. Biochem. J. 42:238, 1948.

70. Horocks, R. H. Paper partition chromatography of reducing sugars. Nature 164:444, 1949.

71. Morgan, W. T. J., and Elson, L. A. A colorimetric method for the determination of N-acetylglucosamine and N-acetylchondrosamine. Biochem. J. 28:988, 1934.

72. Warren, L. Thiobarbituric acid spray reagent for deoxy sugars and sialic acids. Nature 186:237, 1960.

73. Winzler, R. J. Determination of serum glycoproteins. Methods of Biochemical Analysis 2:279, 1955.

74. Hess, W. C., and Lee, C. The isolation of chondroitin sulfuric acid from dentin. J. Dent. Res. 31:793, 1952.

75. Einbinder, J., and Schubert, M. Separation of chondroitin sulfate from cartilage. J. Biol. Chem. 185:725, 1950.

76. Elson, L. A., and Morgan, W. T. J. Determination of glucosamine and chondrosamine. Biochem. J. 27:1824, 1933.

77. Hess, W. C., Lee, C. Y., and Neidig, B. A. Dentinal protein: amino acid composition. J. Dent. Res. 31:791, 1952.

78. Meckel, A. H. Formation and properties of organic films on teeth. Oral presentation, Boston, Mass., March 1961.

79. Mandel, I. D. Personal communication.

80. Eastoe, J. E., and Eastoe, B. The organic constituents of mammalian compact bone. Biochem. J. 57:453, 1954.

81. Leung, S. W. Calcification in Biological Systems. AAAS, Pub. No. 64, p. 307, 1960.

TABLE 1

PERCENTAGE COMPOSITION OF DENTAL CALCULUS

Source* of Calculus	Calcium Phosphate	Calcium Carbonate	Calcium Fluoride	Magnesium Phosphate
Supra	67.18	8.13	1.55	1.07
Sub	63.08	3.70	--	0.98
Sub	72.73	--	--	4.91
Sub	67.47	3.44	--	0.79
Sub	62.80	11.66	--	0.20
Supra	63.17	5.03	--	1.74
Supra	63.42	9.14	--	1.31
Duct	72.6	4.33	--	1.54
Supra	75.97	3.17	--	3.77

*Supra - supragingival
Sub - subgingival

TABLE 1--Continued

Organic Matter	H_2O	H_2O Soluble Substances	Water Insoluble		Reference
			Organic	Inorganic	
	22.07				34
	32.24				35
27.00	4.48				35
--	5.38	3.66	19.21	71.75	36
--	5.68	5.36	14.22	70.36	36
--	11.02	4.95	13.67	70.36	36
15.16	5.68	5.24			5
20.0					37
	17.1				38

TABLE 2

COMPONENTS OF CALCULUS*

Component	Per Cent
Water	5.69
Water soluble substances	5.24
Organic constituents (mucus and food debris)	15.16
Magnesium phosphate	1.3
Calcium carbonate	9.14
Calcium phosphate	63.42
Total	99.95%

*Source: Prinz, H., Origin of salivary calculus. Dental Cosmos 63:231, 1921.

TABLE 3

RESULTS OF MICROBIOLOGICAL ASSAY OF CALCIGEROUS STONE MATRIX

Amino Acid	Duct Stone Range	
	Range	Mean
	Wt. %	Wt. %
Alanine	2.4 - 4.90	3.65
Glycine	2.1 - 3.26	2.82
Valine	1.8 - 2.12	2.11
Leucine	3.41- 8.47	5.39
Isoleucine	2.23- 4.00	2.88
Proline	1.10- 2.11	1.53
Phenylalanine	2.11- 2.46	2.29
Tyrosine	1.60- 1.88	1.75
Tryptophan	0.43- 0.74	0.53
Serine	2.70- 5.36	4.07
Threonine	4.21- 4.75	4.40
Methionine	0.91- 0.98	0.95
Arginine	3.05- 4.08	3.60
Histidine	1.19- 1.52	1.30
Lysine	2.60- 5.28	4.05
Aspartic	3.76- 5.40	4.08
Glutamic	5.87- 6.45	6.10
Total	41.47-63.76	51.50

TABLE 4

SUMMARY OF SAMPLES PREPARED
SALIVARY CALCULI*

Method	Composite	Supragingival	Subgingival	From Artificial Dentures
Demineralization				
15 per cent EDTA	2	2	2	2
0.1N HCl	2	2	2	2
Extraction				
Boiling water	2	--	--	--
Ether and Alcohol	2	--	--	--

*The samples of the various dental calculi were subdivided for the demineralization and extraction procedures. Each sample included calculus from 40 to 60 patients each.

TABLE 5

RECOVERED AMINO ACIDS FROM DEMINERALIZED COMPOSITE CALCULUS

(Expressed as Grams Per Cent)[a]

Amino Acid	Demineralizing Media			
	EDTA[b]	N_2	HCl	N_2
Alanine	5.5 ± 0.3	0.86	5.9 ± 0.2	0.94
Arginine	3.5 ± 0.2	1.12	1.4 ± 0.3	0.45
Aspartic	6.5 ± 0.2	0.68	5.3 ± 1.6	0.56
Cystine	0.13± 0.03	0.02	0.10± 0.02	0.01
Glutamic	9.5 ± 0.3	0.90	11.4 ± 0.4	1.08
Glycine	1.8 ± 0.4	0.34	2.1 ± 0.3	0.39
Histidine	1.3 ± 0.1	0.35	1.3 ± 0.5	0.35
Hydroxyproline	0.1	0.01	0.1	0.01
Lysine	2.2 ± 0.1	0.42	2.3 ± 0.3	0.44
Methionine	0.3 ± 0.1	0.03	0.7 ± 0.1	0.06
PLI[c]	9.9 ± 0.6	0.99	10.8 ± 1.1	1.07
Proline	2.0 ± 0.2	0.24	2.2 ± 0.2	0.27
Serine	2.3 ± 0.2	0.31	2.4 ± 0.1	0.32
Threonine	2.2 ± 0.3	0.26	1.8 ± 0.3	0.21
Tryptophan	0.6 ± 0.2	0.08	0.5 ± 0.2	0.07
Tyrosine	0.4 ± 0.2	0.03	0.7 ± 0.3	0.06
Valine	2.7 ± 0.3	0.32	2.6 ± 0.3	0.31
Total residues	50.93		51.60	
Ash	8.8		4.6	
N calculated		6.96		6.60
N determined		7.62		9.67

[a]Averages of duplicate determinations on two samples.
[b]Ethylene diamine tetraacetic acid
[c]Phenylalanine, leucine and isoleucine

TABLE 6

RECOVERED AMINO ACIDS FROM DEMINERALIZED SUPRAGINGIVAL CALCULUS

(Expressed as Grams Per Cent)[a]

Amino Acid	Demineralizing Media			
	EDTA[b]	N_2	HCl	N_2
Alanine	5.3 ± 0.2	0.83	5.9 ± 0.1	0.94
Arginine	2.6 ± 0.2	0.84	1.9 ± 0.2	0.61
Aspartic	1.3 ± 0.2	0.14	1.1 ± 0.2	0.12
Cystine	0.06± 0.01	0.07	0.10± 0.03	0.01
Glutamic	8.6 ± 0.3	0.82	9.6 ± 0.4	0.91
Glycine	2.5 ± 0.1	0.47	1.8 ± 0.1	0.34
Histidine	1.9 ± 0.1	0.51	1.7 ± 0.1	0.46
Hydroxyproline	0.1	0.01	0.1	0.01
Lysine	4.2 ± 0.4	0.80	4.5 ± 0.2	0.86
Methionine	0.6 ± 0.2	0.06	0.6 ± 0.1	0.06
PLI[c]	9.2 ± 0.2	0.92	9.6 ± 0.2	0.96
Proline	2.0 ± 0.1	0.24	2.3 ± 0.1	0.28
Serine	1.1 ± 0.2	0.15	2.2 ± 0.2	0.29
Threonine	1.6 ± 0.2	0.19	1.2 ± 0.2	0.14
Tryptophan	0.6 ± 0.2	0.08	0.4 ± 0.1	0.05
Tyrosine	0.9 ± 0.1	0.07	1.0 ± 0.1	0.08
Valine	2.5 ± 0.2	0.03	3.1 ± 0.2	0.37
Total residues	45.06		47.10	
Ash	8.3		4.3	
N calculated		6.50		6.49
N determined		8.61		10.52

[a]Averages of duplicate determinations on two samples.
[b]Ethylene diamine tetraacetic acid
[c]Phenylalanine, leucine and isoleucine

TABLE 7

RECOVERED AMINO ACIDS FROM DEMINERALIZED SUBGINGIVAL CALCULUS

(Expressed as Grams Per Cent)[a]

Amino Acid	Demineralizing Media			
	EDTA[b]	N_2	HCl	N_2
Alanine	5.5 ± 0.2	0.86	5.8 ± 0.2	0.91
Arginine	2.4 ± 0.1	0.77	1.3 ± 0.1	0.42
Aspartic	3.1 ± 0.2	0.33	1.1 ± 0.2	0.12
Cystine	0.10± 0.02	0.01	0.10± 0.02	0.10
Glutamic	11.0 ± 0.3	1.05	10.9 ± 0.2	1.04
Glycine	2.4 ± 0.2	0.45	2.0 ± 0.1	0.37
Histidine	1.3 ± 0.1	0.35	1.5 ± 0.1	0.41
Hydroxyproline	0.1	0.01	0.1	0.01
Lysine	4.8 ± 0.2	0.92	4.8 ± 0.2	0.92
Methionine	0.7 ± 0.0	0.06	0.6 ± 0.2	0.06
PLI[c]	9.9 ± 0.4	0.99	11.9 ± 0.2	1.18
Proline	1.6 ± 0.1	0.19	2.2 ± 0.1	0.27
Serine	1.8 ± 0.2	0.24	2.4 ± 0.1	0.32
Threonine	1.9 ± 0.1	0.22	2.0 ± 0.2	0.24
Tryptophan	0.5 ± 0.2	0.07	0.6 ± 0.2	0.08
Tyrosine	0.7 ± 0.1	0.06	0.4 ± 0.1	0.03
Valine	2.2 ± 0.2	0.26	3.5 ± 0.2	0.42
Total residues	50.00		51.20	
Ash	8.1		4.4	
N calculated		6.84		6.81
N determined		7.97		9.71

[a]Averages of duplicate determinations on two samples.
[b]Ethylene diamine tetraacetic acid
[c]Phenylalanine, leucine and isoleucine

TABLE 8

RECOVERED AMINO ACIDS FROM DEMINERALIZED CALCULUS FROM ARTIFICIAL DENTURES

(Expressed as Grams Per Cent)[a]

Amino Acid	Demineralizing Media			
	EDTA[b]	N_2	HCl	N_2
Alanine	5.7 ± 0.2	0.89	5.9 ± 0.1	0.94
Arginine	2.8 ± 0.1	0.90	2.4 ± 0.2	0.77
Aspartic	4.6 ± 0.3	0.48	4.8 ± 0.2	0.50
Cystine	0.10± 0.02	0.01	0.08± 0.03	0.09
Glutamic	9.3 ± 0.6	0.88	9.7 ± 0.4	0.92
Glycine	2.6 ± 0.1	0.48	2.3 ± 0.2	0.43
Histidine	1.1 ± 0.2	0.30	1.3 ± 0.1	0.35
Hydroxyproline	0.1	0.01	0.1	0.01
Lysine	2.2 ± 0.1	0.42	2.3 ± 0.2	0.44
Methionine	0.4 ± 0.05	0.04	0.6 ± 0.1	0.06
PLI[c]	10.9 ± 0.8	1.08	11.4 ± 0.6	1.13
Proline	2.2 ± 0.1	0.27	2.3 ± 0.2	0.28
Serine	2.3 ± 0.2	0.31	2.3 ± 0.2	0.31
Threonine	2.4 ± 0.1	0.28	2.5 ± 0.2	0.29
Tryptophan	0.5 ± 0.1	0.07	0.6 ± 0.2	0.08
Tyrosine	1.3 ± 0.3	0.10	1.1 ± 0.2	0.08
Valine	2.6 ± 0.1	0.31	2.5 ± 0.2	0.30
Total residues	51.10		52.18	
Ash	7.8		4.2	
N calculated		6.83		6.98
N determined		8.34		9.72

[a] Averages of duplicate determinations on two samples.
[b] Ethylene diamine tetraacetic acid
[c] Phenylalanine, leucine and isoleucine

TABLE 9

RECOVERED AMINO ACIDS FROM PRECIPITATED MUCIN

(Expressed as Grams Per Cent)[a]

Amino Acid	Residue	N_2
Alanine	2.2 ± 0.1	0.34
Arginine	4.9 ± 0.3	1.58
Aspartic	5.8 ± 0.2	0.61
Cystine	0.10± 0.03	0.01
Glutamic	9.7 ± 0.5	0.92
Glycine	4.0 ± 0.3	0.75
Histidine	0.9 ± 0.1	0.24
Hydroxyproline	--	--
Lysine	3.3 ± 0.1	0.63
Methionine	0.1 ± 0.02	0.01
PLI[b]	7.7 ± 0.3	0.77
Proline	7.4 ± 0.2	0.90
Serine	3.3 ± 0.1	0.44
Threonine	1.6 ± 0.2	0.19
Tryptophan	0.4 ± 0.2	0.05
Tyrosine	0.9 ± 0.2	0.07
Valine	1.1 ± 0.2	0.13
Total residues	53.40	
Ash	2.3	
N calculated		6.64
N determined		7.82

[a]Average of duplicate determinations on two samples.
[b]Phenylalanine, leucine and isoleucine

TABLE 10

CARBOHYDRATE CONTENT[a]

(Anthrone Method)

Sample	Residues after demineralization by	
	EDTA[b]	HCl
	Percent	Percent
Composite	18	12
Supragingival	16	10
Subgingival	13	8
Calculus from artificial dentures	17	11
Average	16	10
Mucin	28	

[a]Reported to nearest whole per cent.
[b]Ethylene diamine tetraacetic acid

TABLE 11

CARBOHYDRATE - R_f VALUES*

Carbohydrate	Standards		EDTA-Demineralized Calculi	
	Solvent		Solvent	
	n-butanol/acetic acid/H_2O [40/10/50 (v/v/v)]	Phenol 1% NH_3 KCN	n-butanol/acetic acid/H_2O [40/10/50 (v/v/v)]	Phenol 1% NH_3 KCN
Galactose	0.16		0.16	
Glucose	0.18		0.18	0.38
Mannose	0.20		0.21	
Fucose	0.27		0.28	
Rhamnose	0.37		0.37	
Deoxyribose		0.73		0.72
Glucos-amine-HCl		0.62		
Galactos-amine-HCl		0.65		0.66

*R_f values determined were corrected to 20°C. by the following (75):

$$a_t = \frac{R_f \text{ value of glucose at } 20°C.}{R_f \text{ value of glucose at } t°C.}$$

Corrected $R_{f20°C.} = R_{ft°C.} \times a_t$.

TABLE 12

HEXOSAMINE CONTENT

EDTA-Demineralized Calculi

Sample	Percent*
Composite	4
Supragingival	3
Subgingival	5
Calculus from Artificial Dentures	3

*Hexosamine content reported to nearest whole per cent.

TABLE 13

PERCENTAGE RECOVERY
EDTA-DEMINERALIZED COMPOSITE CALCULUS

Recovered	Amount*
Amino acids	6.8
Carbohydrates	2.4
Lipid	0.2
Ash	1.2
Total recovered	10.6
Insoluble residue after demineralization	13.3
Loss and/or other undetermined organic constituents	2.7

*Expressed as grams per 100 grams of calculus.

TABLE 14

SUMMARY OF RESULTS - RECOVERED AMINO ACIDS[a]

Salivary Calculi, Duct Calculi and Mucin

Amino Acid	Composite	Supragingival	Subgingival	Artificial Denture	Duct[b]	Mucin
Alanine	5.5	5.3	5.5	5.7	3.65	2.2
Arginine	3.5	2.6	2.4	2.8	3.60	4.9
Aspartic	6.5	1.3	3.1	4.6	4.08	5.8
Cystine	0.13	0.06	0.10	0.10	--	0.10
Glutamic	9.5	8.6	11.0	9.3	6.10	9.7
Glycine	1.8	2.5	2.4	2.6	2.82	4.0
Histidine	1.3	1.9	1.3	1.1	1.30	0.9
Hydroxyproline	0.1	0.1	0.1	0.1	--	--
Lysine	2.2	4.2	4.8	2.2	4.05	3.3
Methionine	0.3	0.6	0.7	0.4	0.95	0.1
PLI[c]	9.9	9.2	9.9	10.9	10.56	7.7
Proline	2.0	2.0	1.6	2.2	1.53	7.4
Serine	2.3	1.1	1.8	2.3	4.07	3.3
Threonine	2.2	1.6	1.9	2.4	4.40	1.6
Tryptophan	0.6	0.6	0.5	0.5	0.53	0.4
Tyrosine	0.4	0.9	0.7	1.3	1.75	0.9
Valine	2.7	2.5	2.2	2.6	2.11	1.1
Total recovered acids	50.93	45.06	50.00	51.10	51.50	53.40

[a]Results for salivary calculi are based on the EDTA-demineralized calculi.

[b]King, J. S., Jr. and Boyce, W. H. Amino acid and carbohydrate composition of the mucoprotein matrix in various calculi. Proc. Soc. Exp. Biol. and Med. 95:183, 1957.

[c]Phenylalanine, leucine and isoleucine

TABLE 15

SUMMARY OF RESULTS - DETECTED CARBOHYDRATES[a]

Carbohydrate	
Galactose	
Glucose	
Mannose	
Fucose	
Rhamnose	
Deoxyribose	
Galactosamine	
(Hexuronic Acid)[b]	
Sialic acid	
Carbohydrate content (Anthrone):	13 to 18 per cent
Hexosamine content :	3 to 5 per cent

[a]Results are based on the EDTA-demineralized samples.

[b]Faint spot inconsistently seen.

USCOMM-NBS-DC

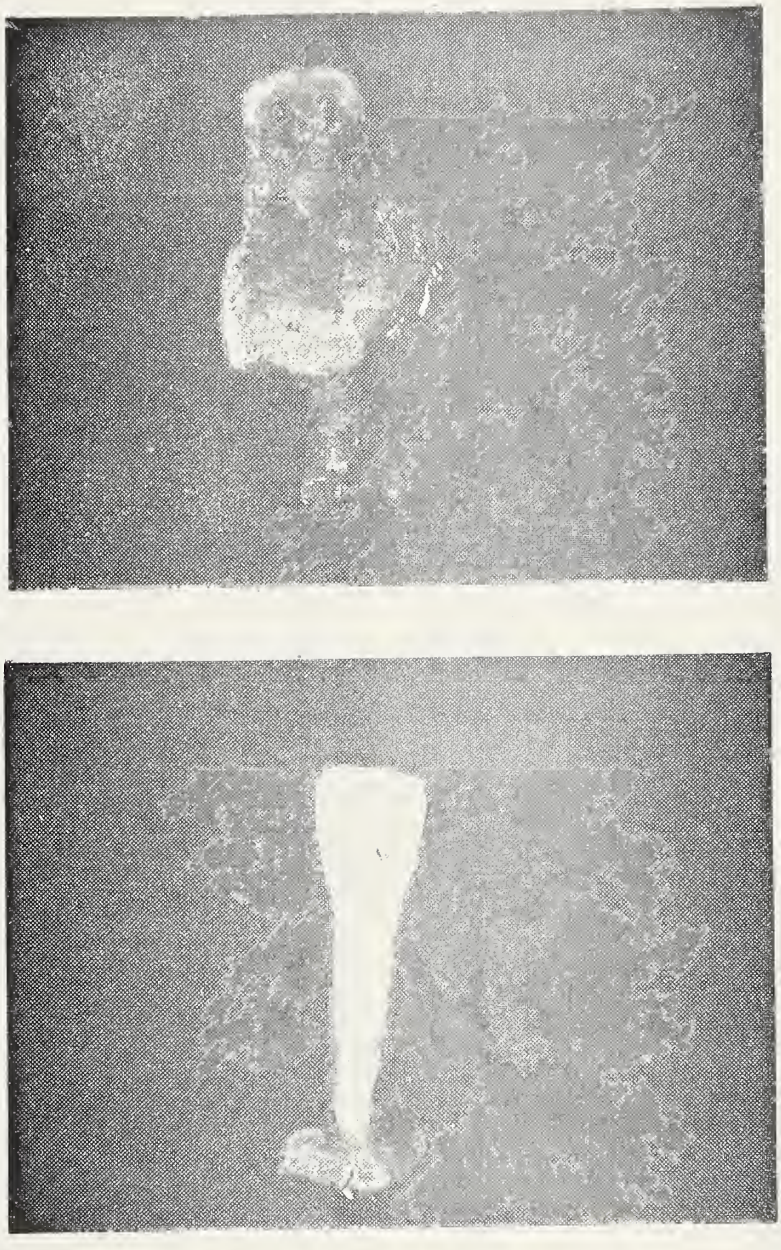


Fig. 1.--A lower central incisor: Top - tooth with calculus deposit, Bottom - tooth after calculus deposit removed.

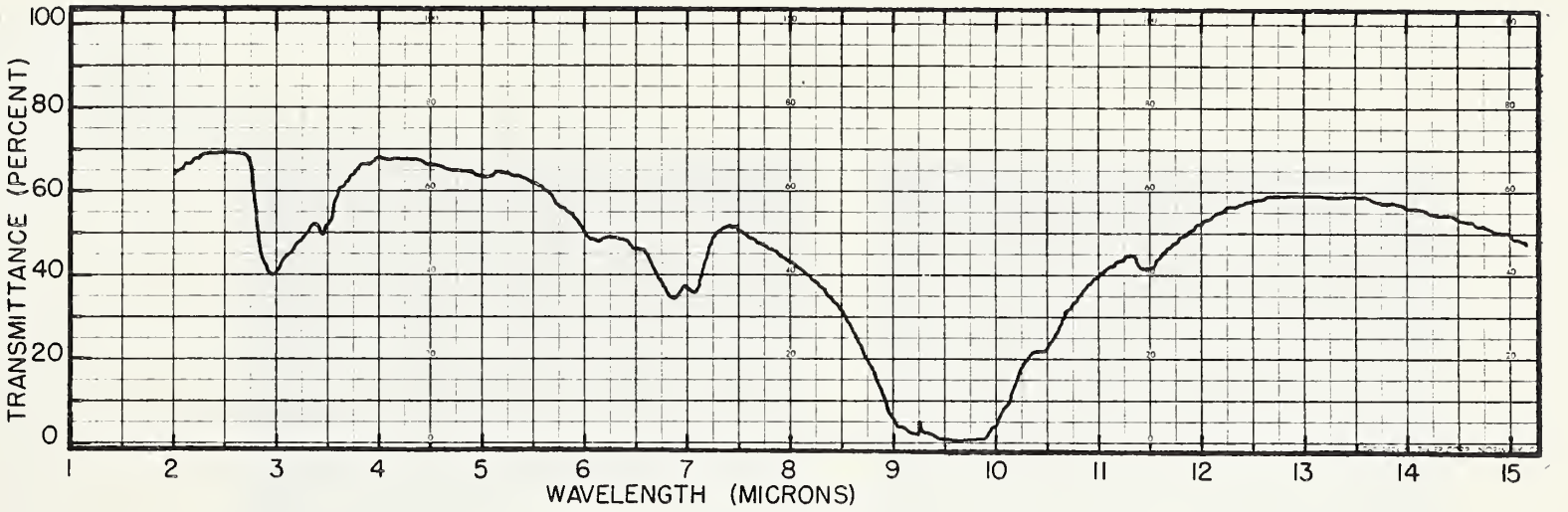


Fig. 2a.--Infrared spectrum of mineral fraction of calculus

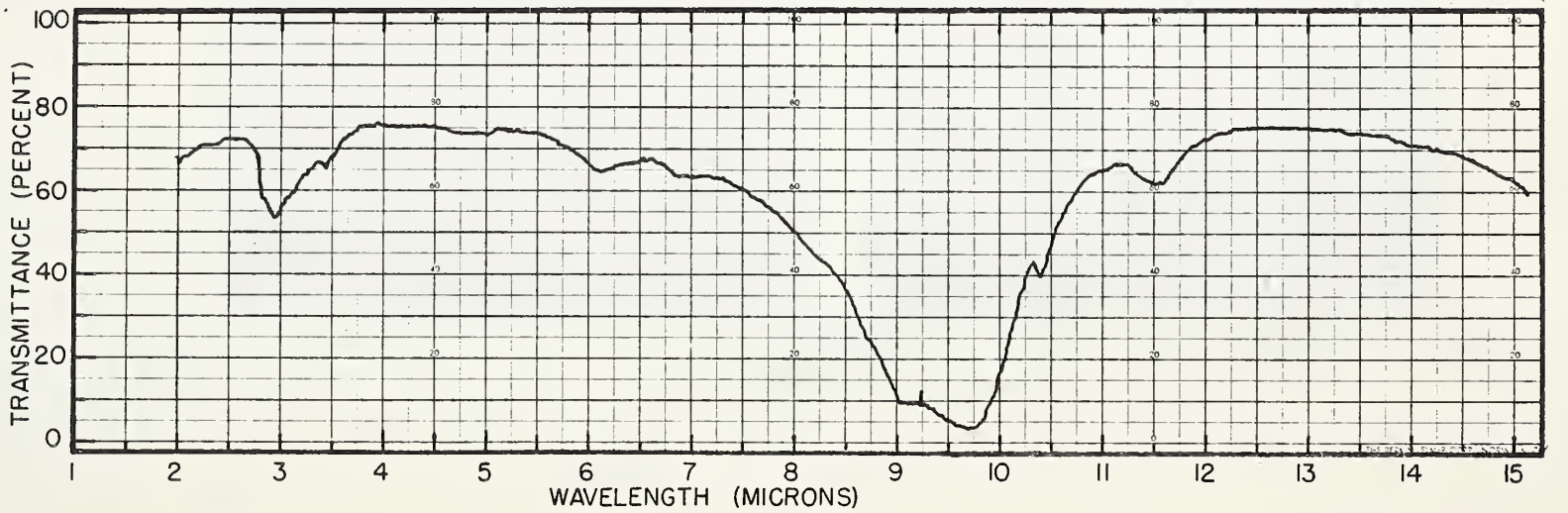


Fig. 2b.--Infrared spectrum of synthetic hydroxyapatite

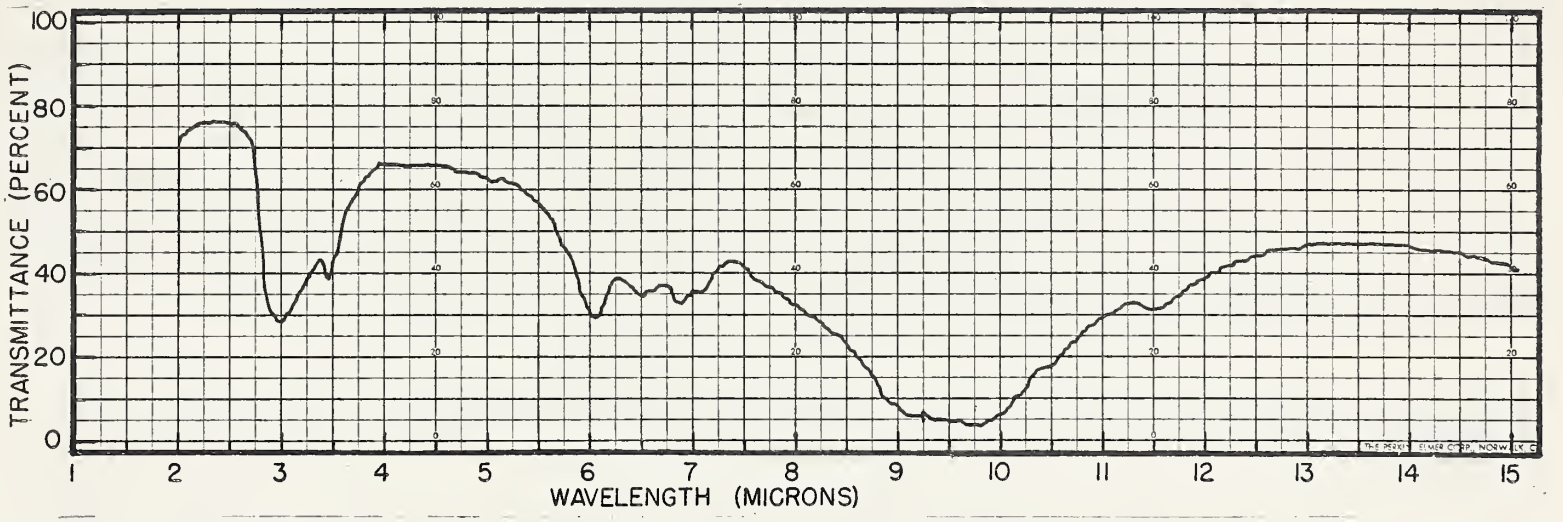


Fig. 3.--Infrared spectrum of calculus as collected

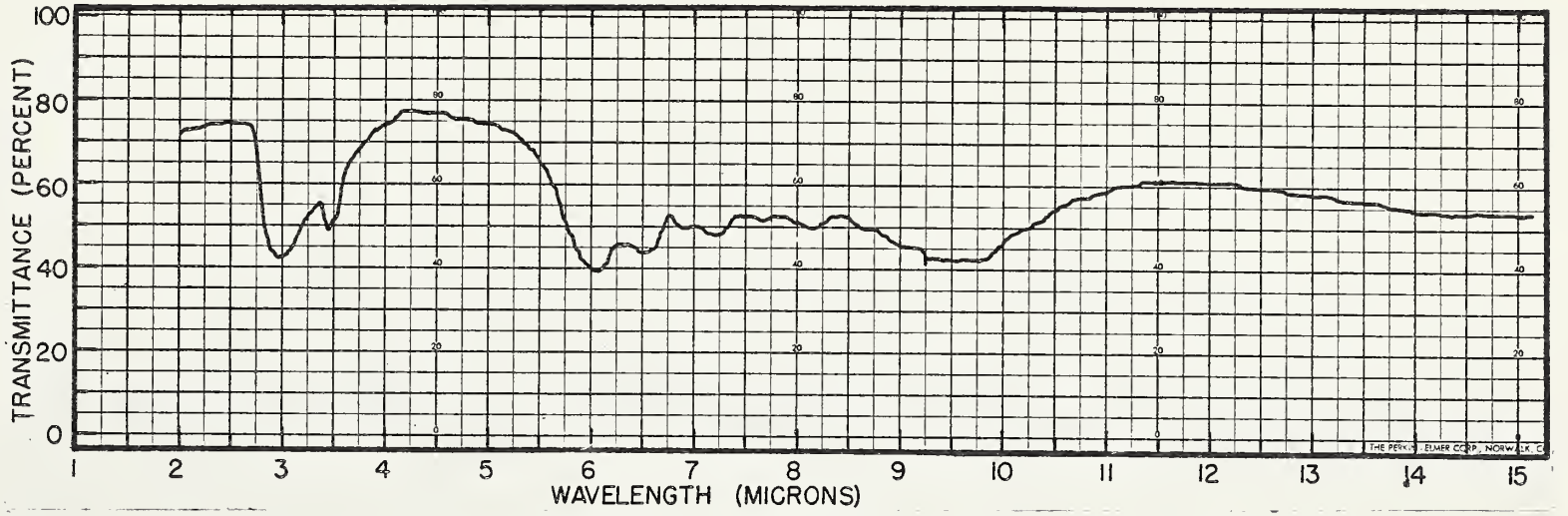


Fig. 4a.--Infrared spectrum of EDTA - demineralized calculi

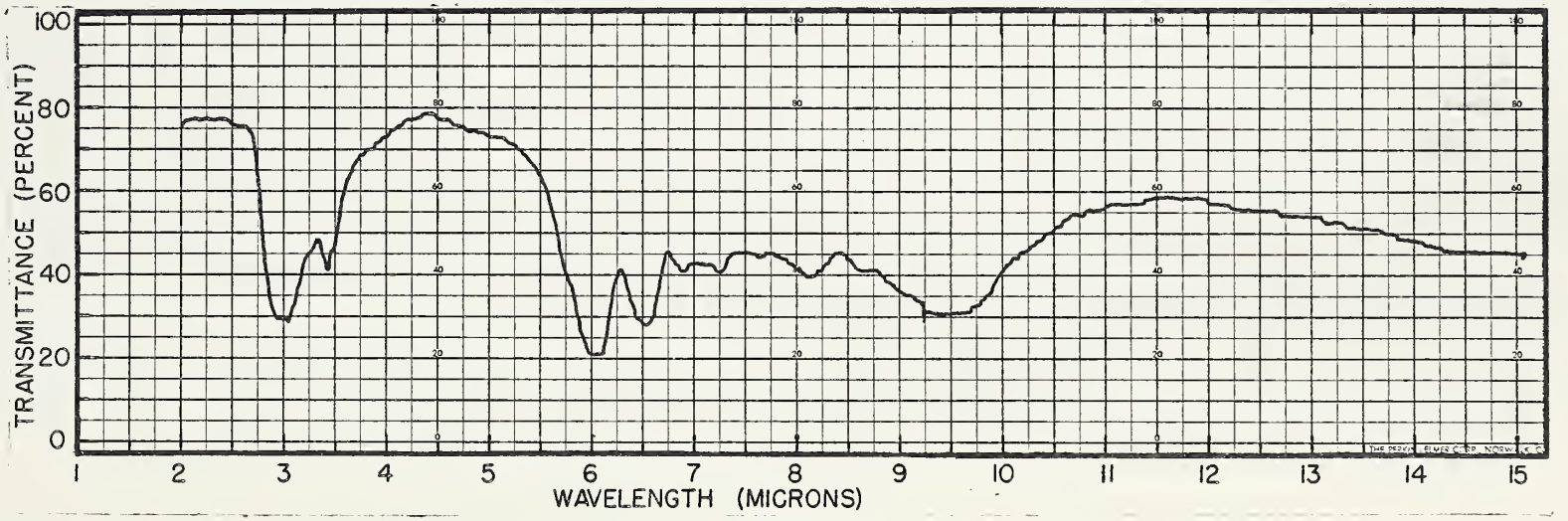


Fig. 4b.--Infrared spectrum of HCl - demineralized calculi

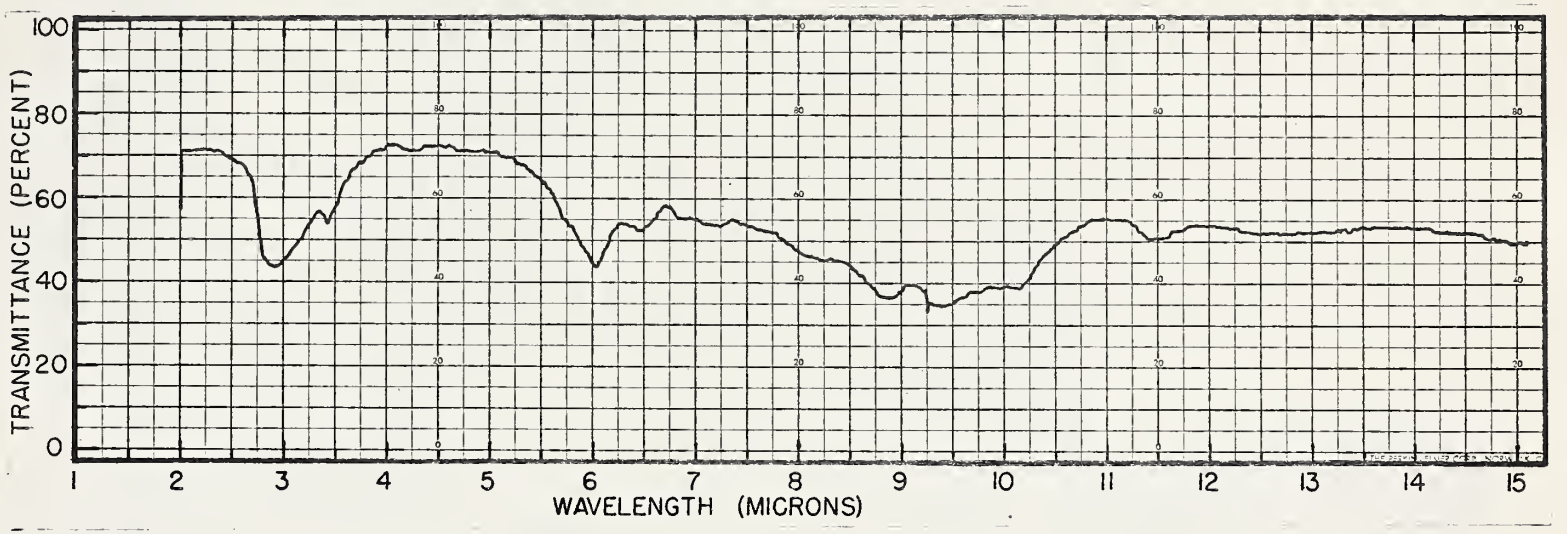


Fig. 5.--Infrared spectrum of hot water extract of calculus

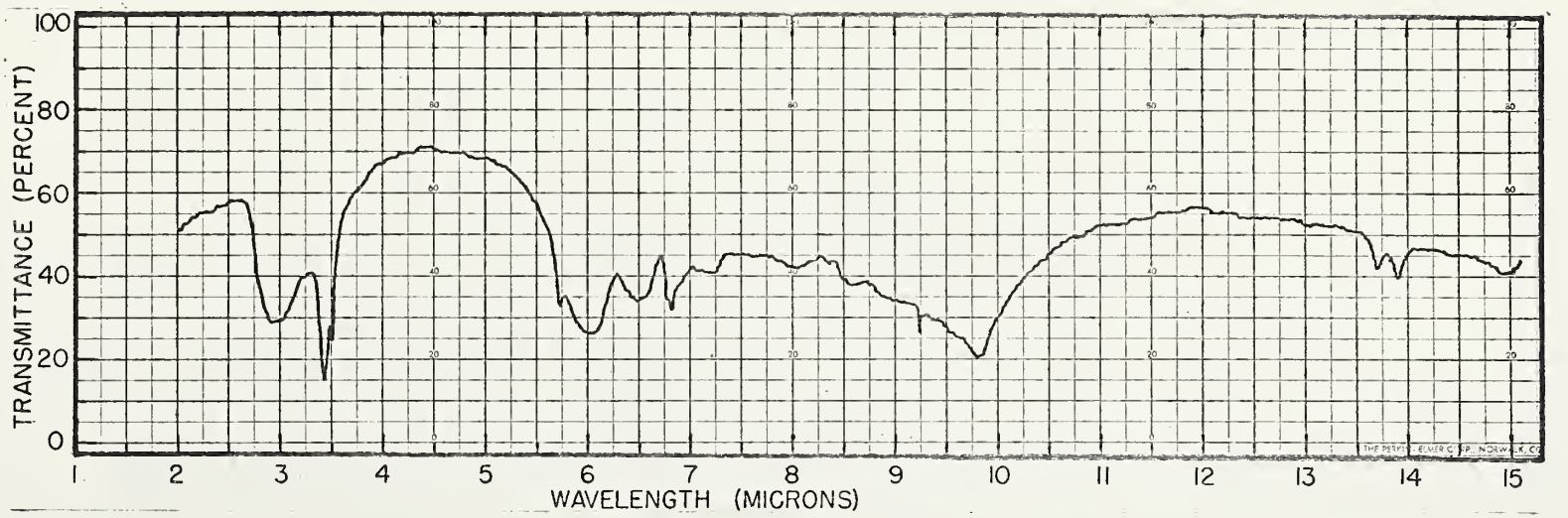


Fig. 6a.--Infrared spectrum of mucin precipitated from saliva

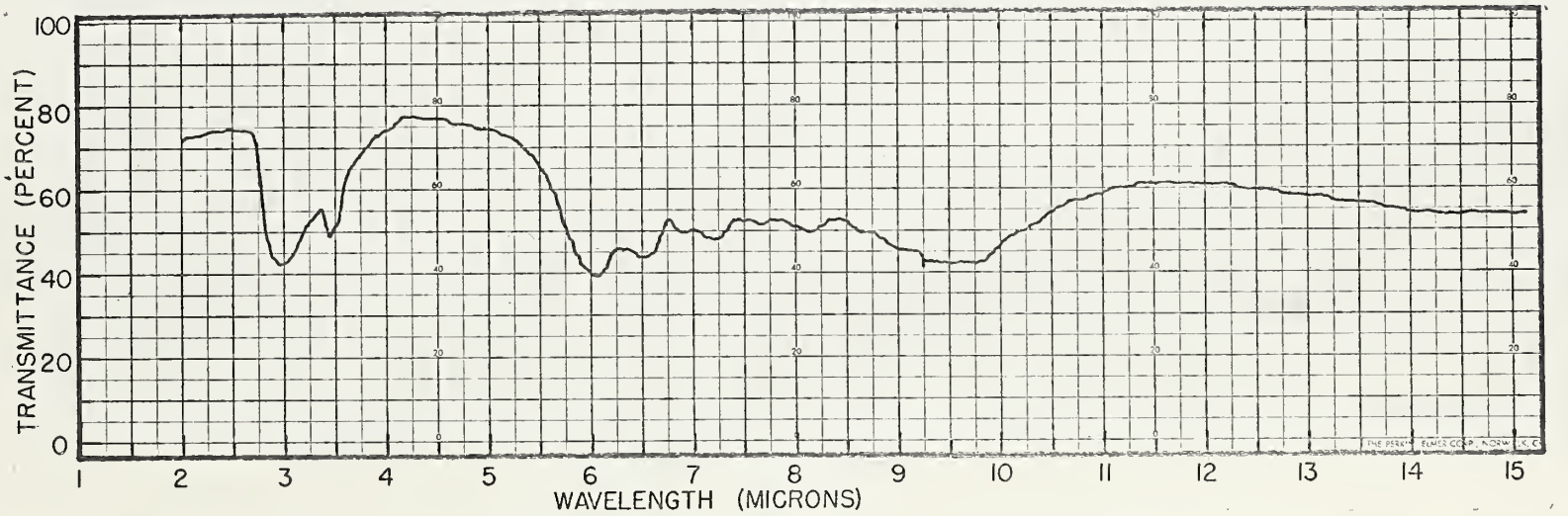


Fig. 6b.--Infrared spectrum of EDTA - demineralized calculi

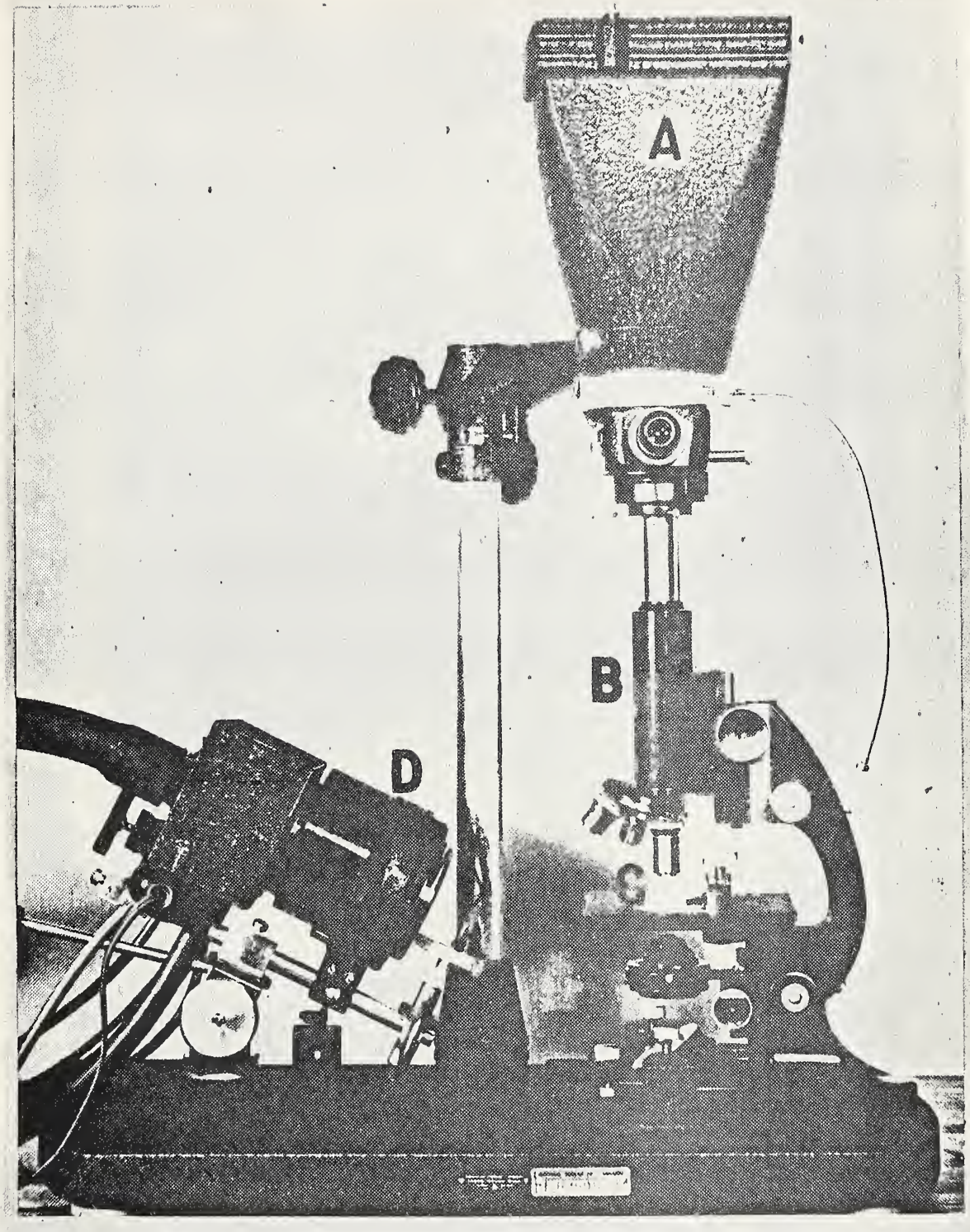


Fig. 7.--Apparatus used to produce fluorophotomicrographs. A. Camera B. Microscope C. Section of tooth tissue and calculus D. Mercury vapor ultraviolet lamp.

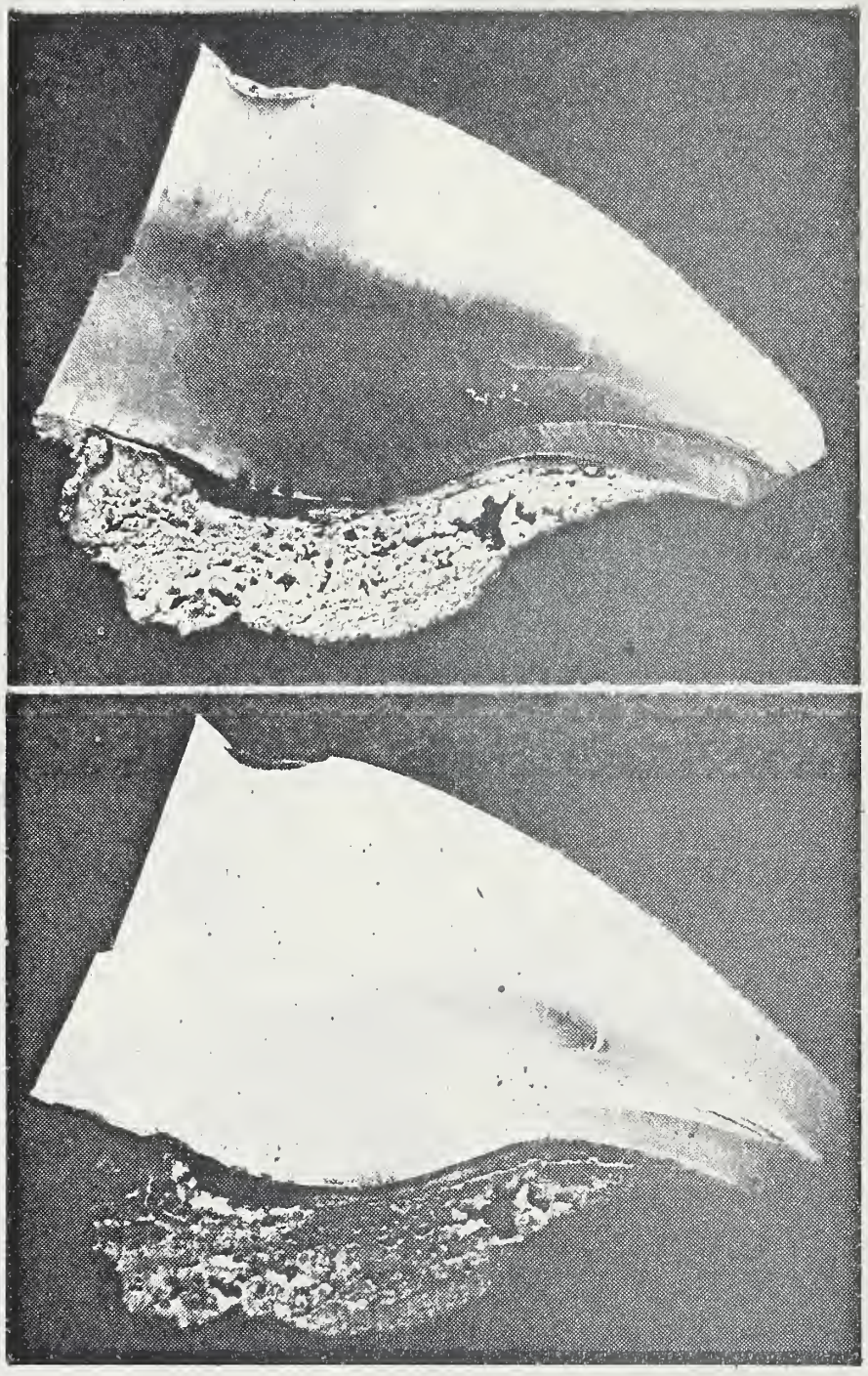


Fig. 8.--Section 1. Tooth section and adhering calculus photographed by visible illumination, top, and by fluorescence excited by ultraviolet illumination, bottom. Magnification approximately 10x.

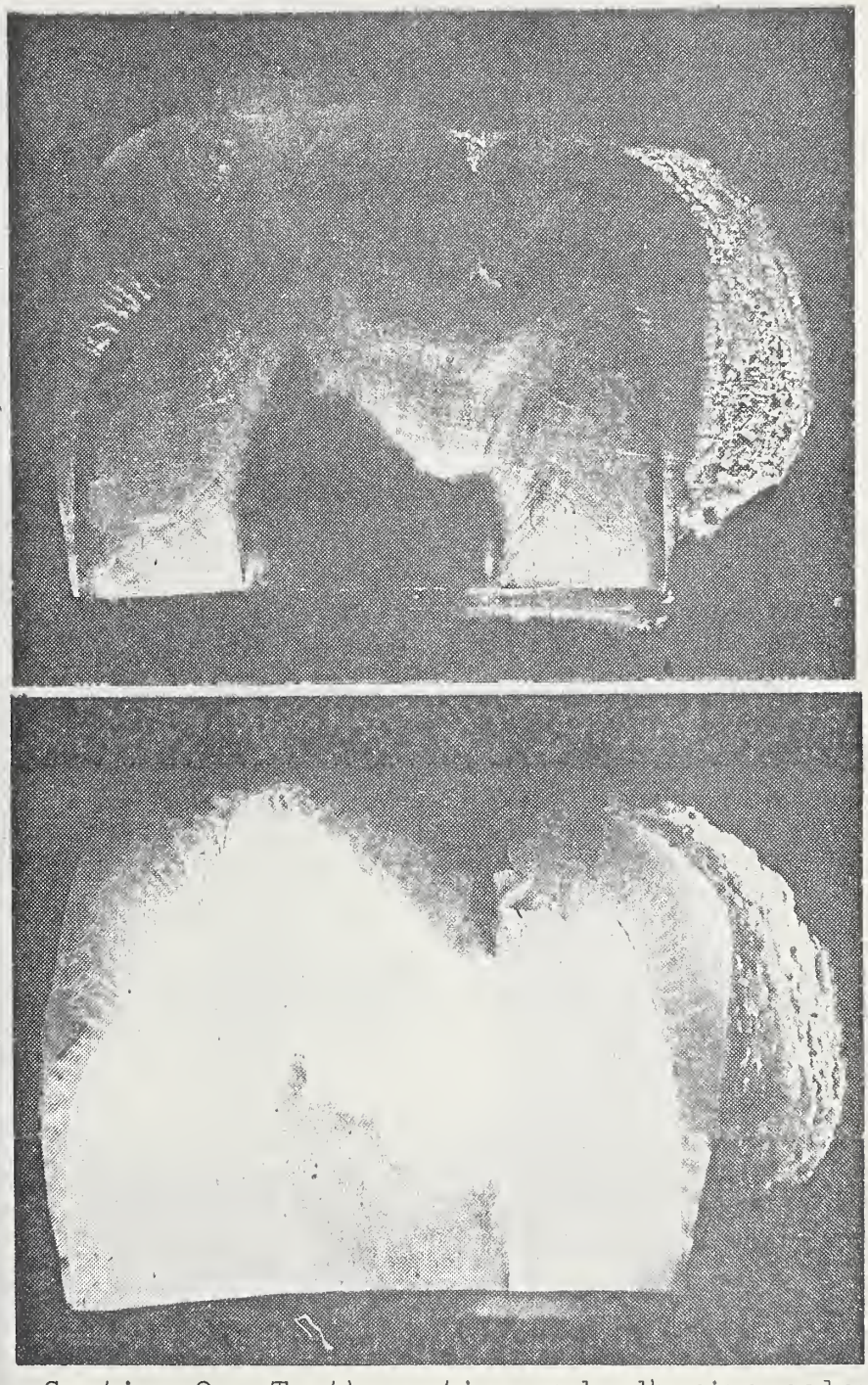


Fig. 9.--Section 2. Tooth section and adhering calculus photographed by visible illumination, top, and by fluorescence excited by ultraviolet illumination, bottom. Magnification approximately 10x.

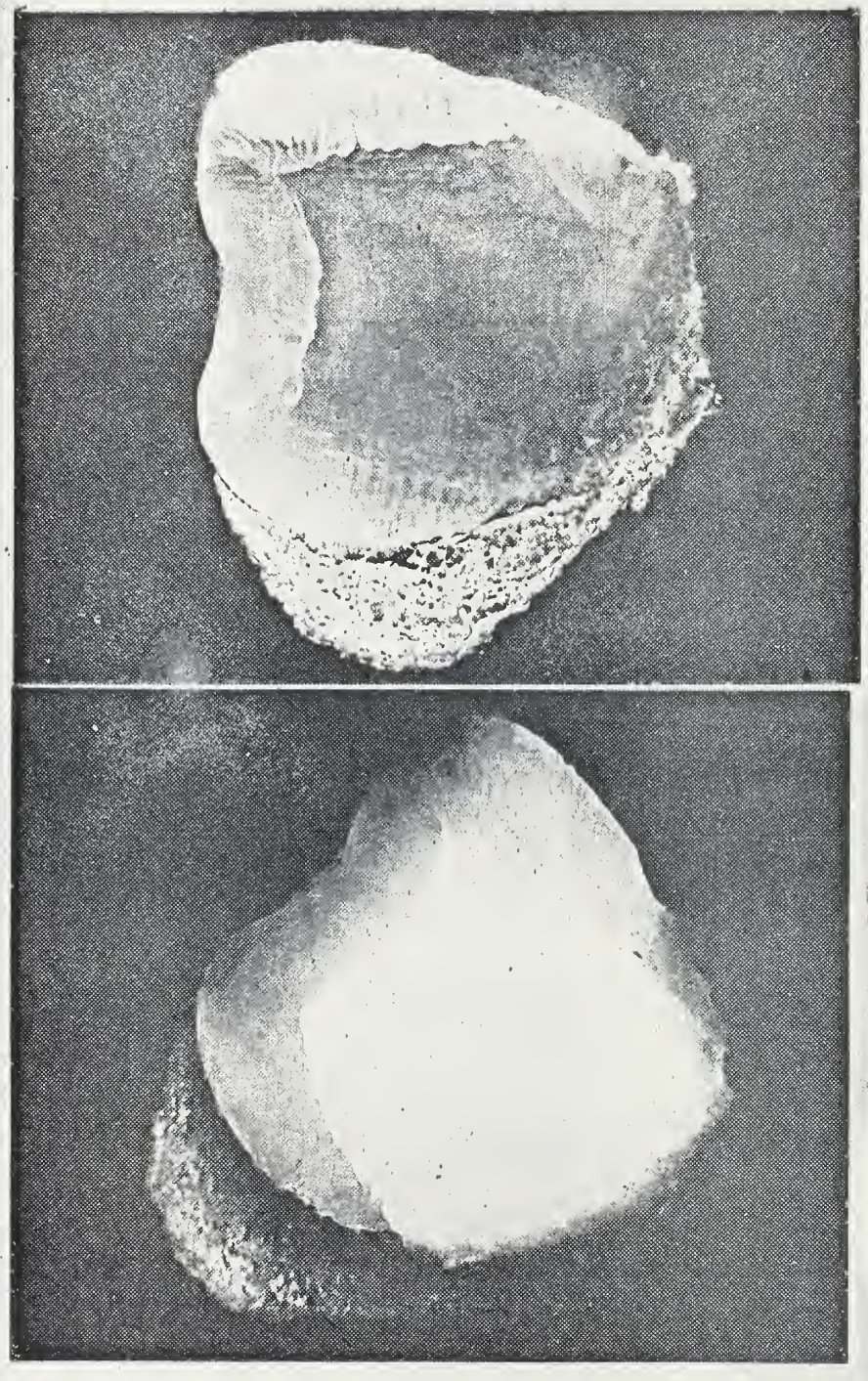


Fig. 10.--Section 3. Tooth section and adhering calculus photographed by visible illumination, top, and by fluorescence excited by ultraviolet illumination, bottom. Magnification approximately 10x.

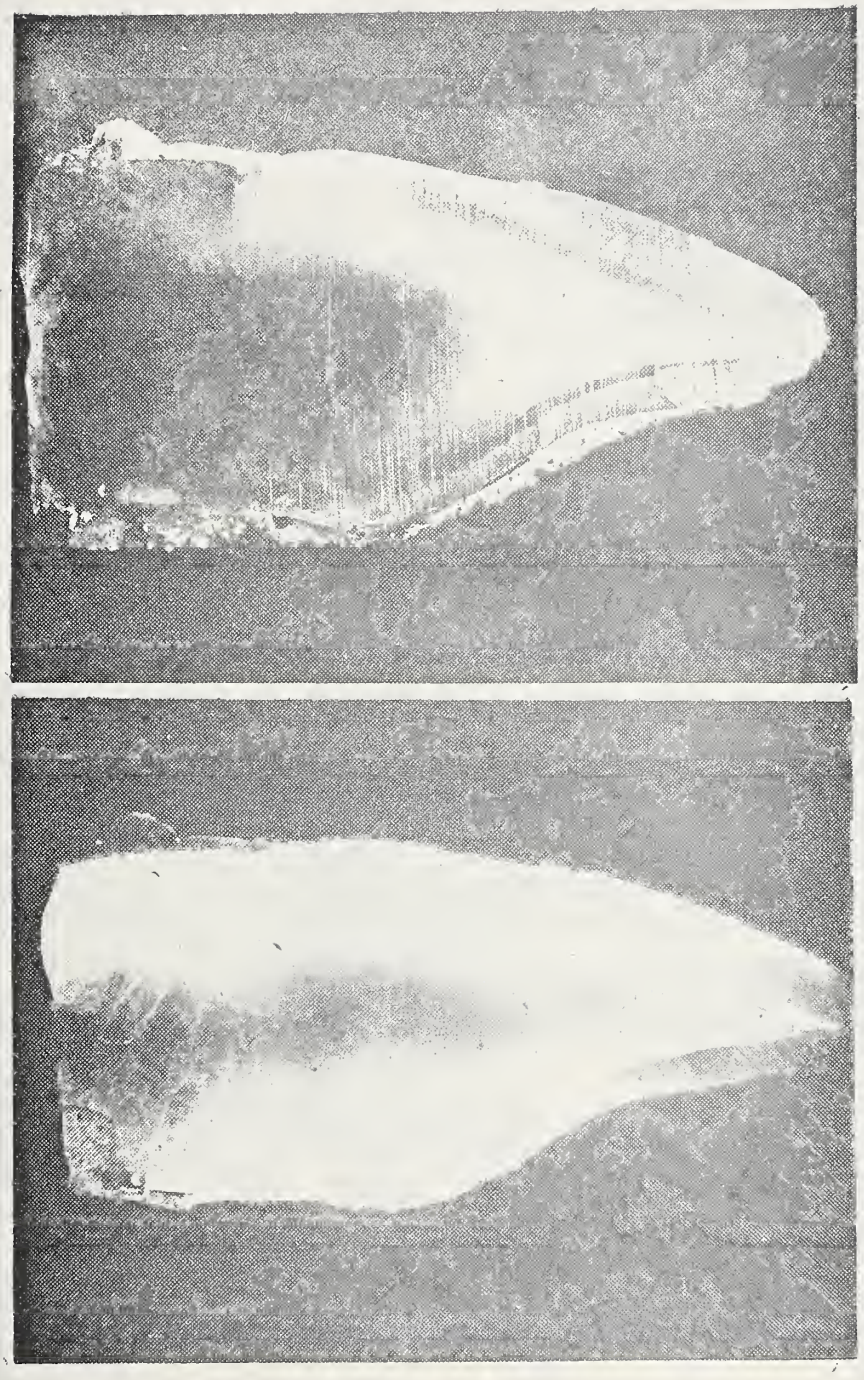


Fig. 11.--Section 4. Tooth section and adhering calculus photographed by visible illumination, top, and by fluorescence excited by ultraviolet illumination, bottom. Magnification approximately 10x.

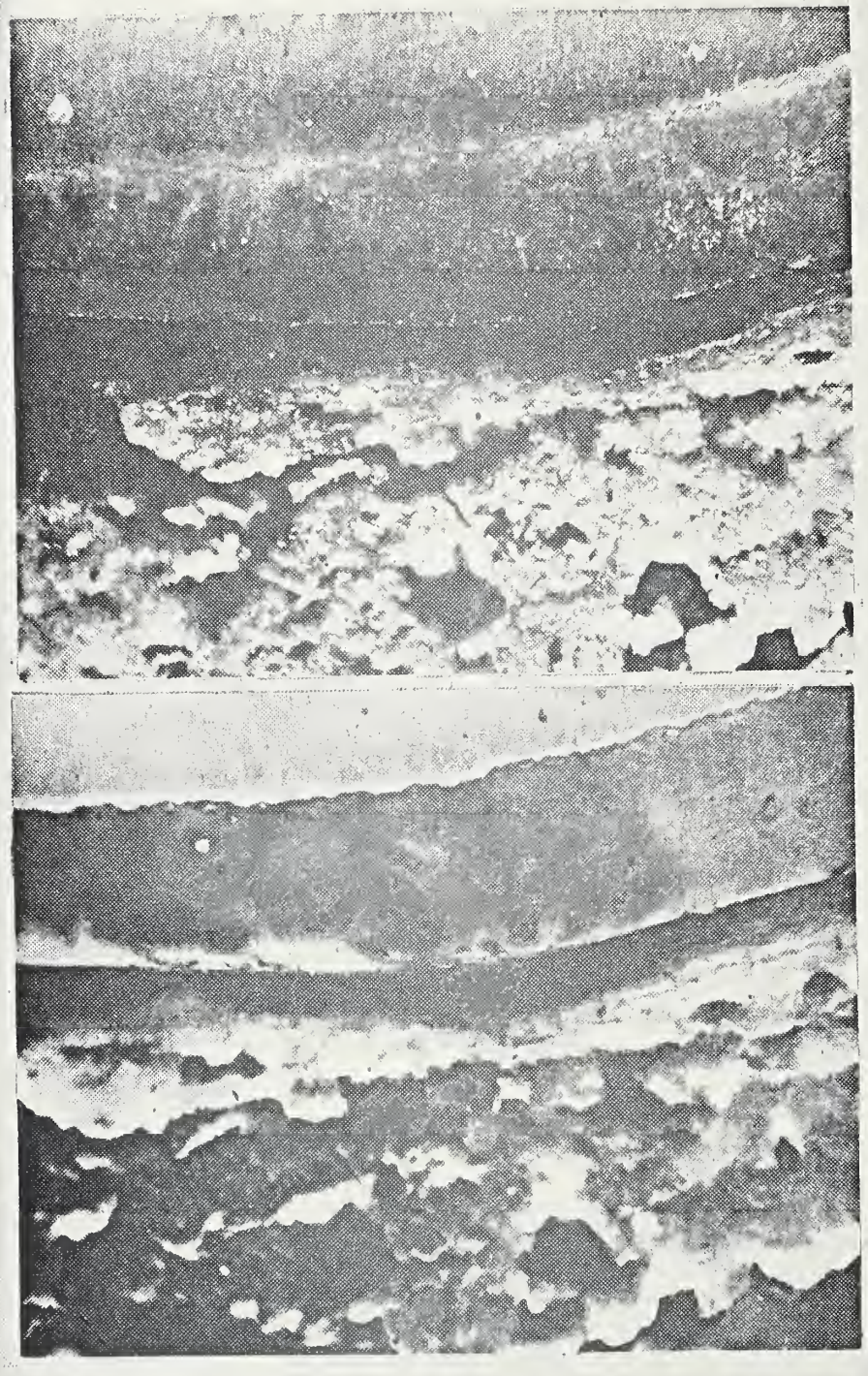


Fig. 12.--Section 1. Magnification of junction of tooth tissue and calculus (Fig. 8) photographed by visible illumination, top, and by fluorescence excited by ultraviolet illumination, bottom. Magnification approximately 100x.

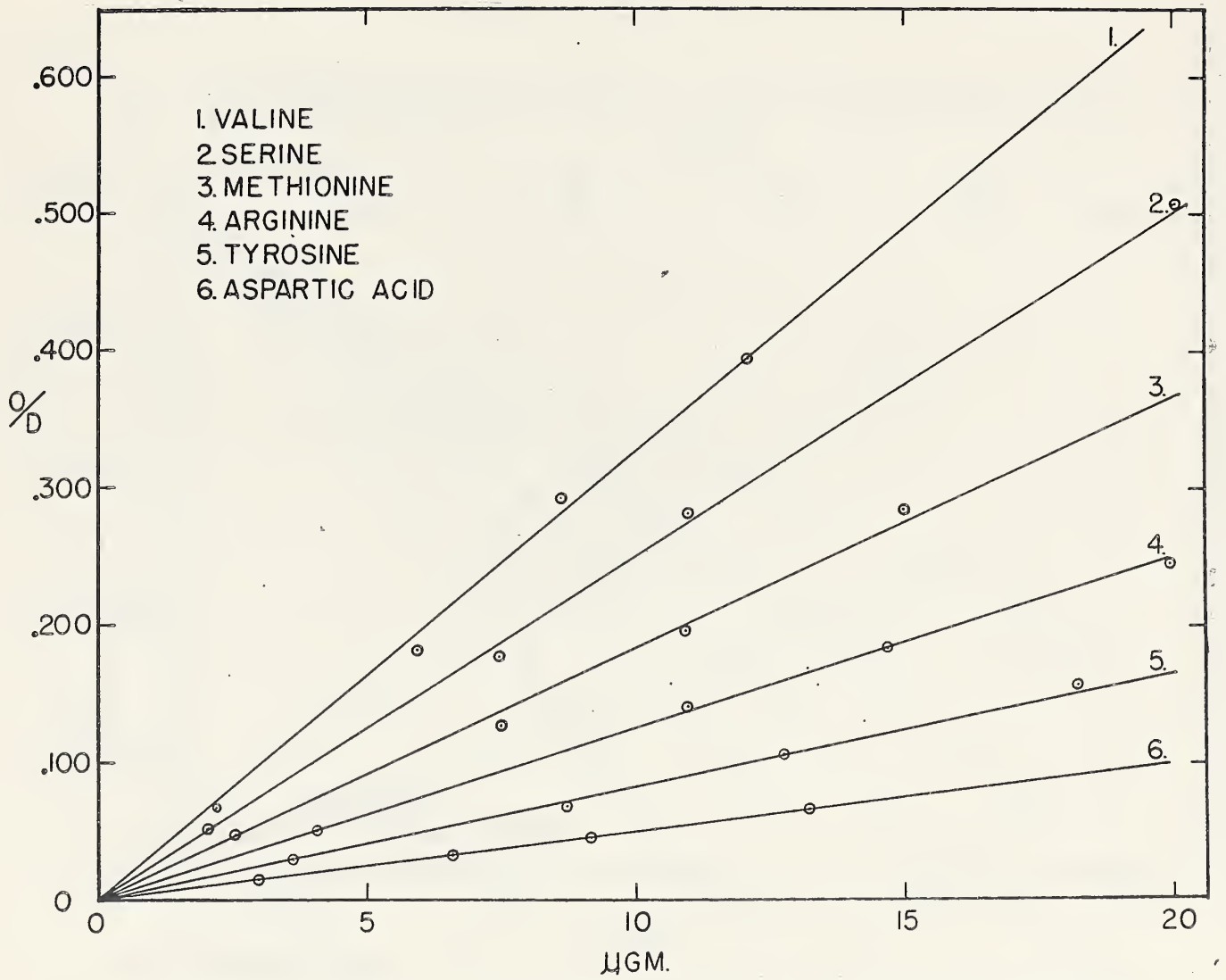


Fig. 13.--Standard curves for six amino acids read at 570 mµ

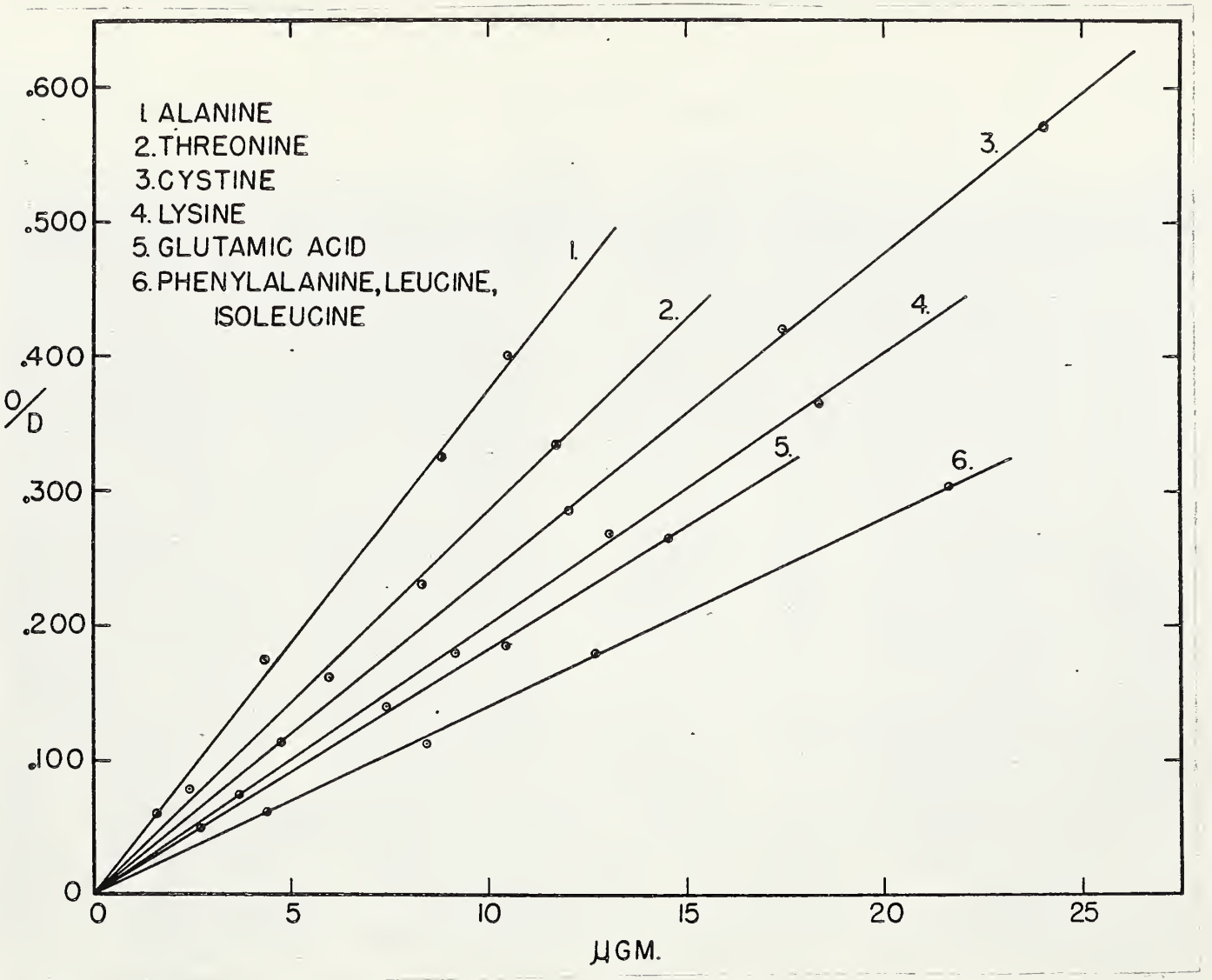


Fig. 14.--Standard curves for six amino acids read at 570 mµ

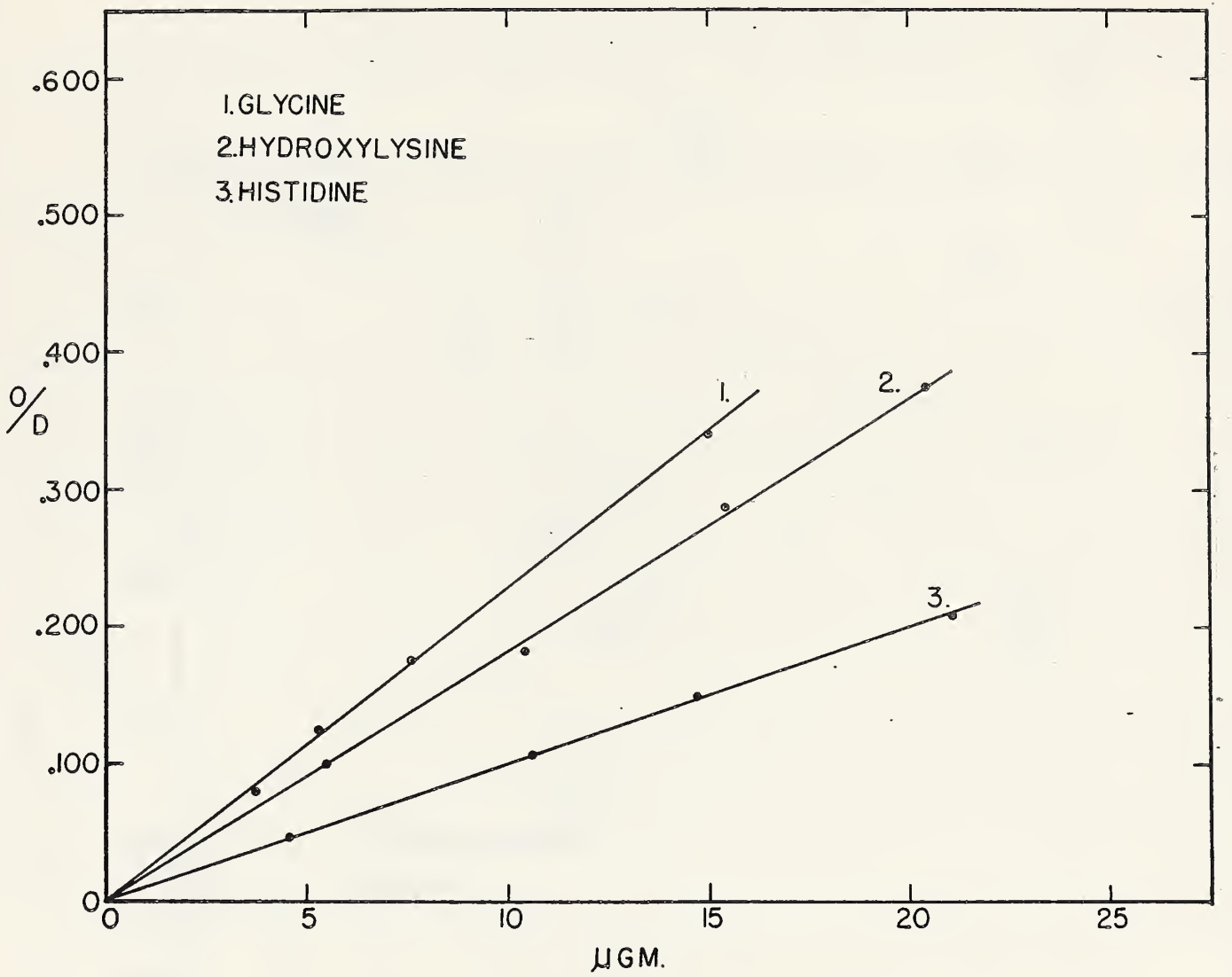


Fig. 15.--Standard curves for three amino acids read at 570 mµ

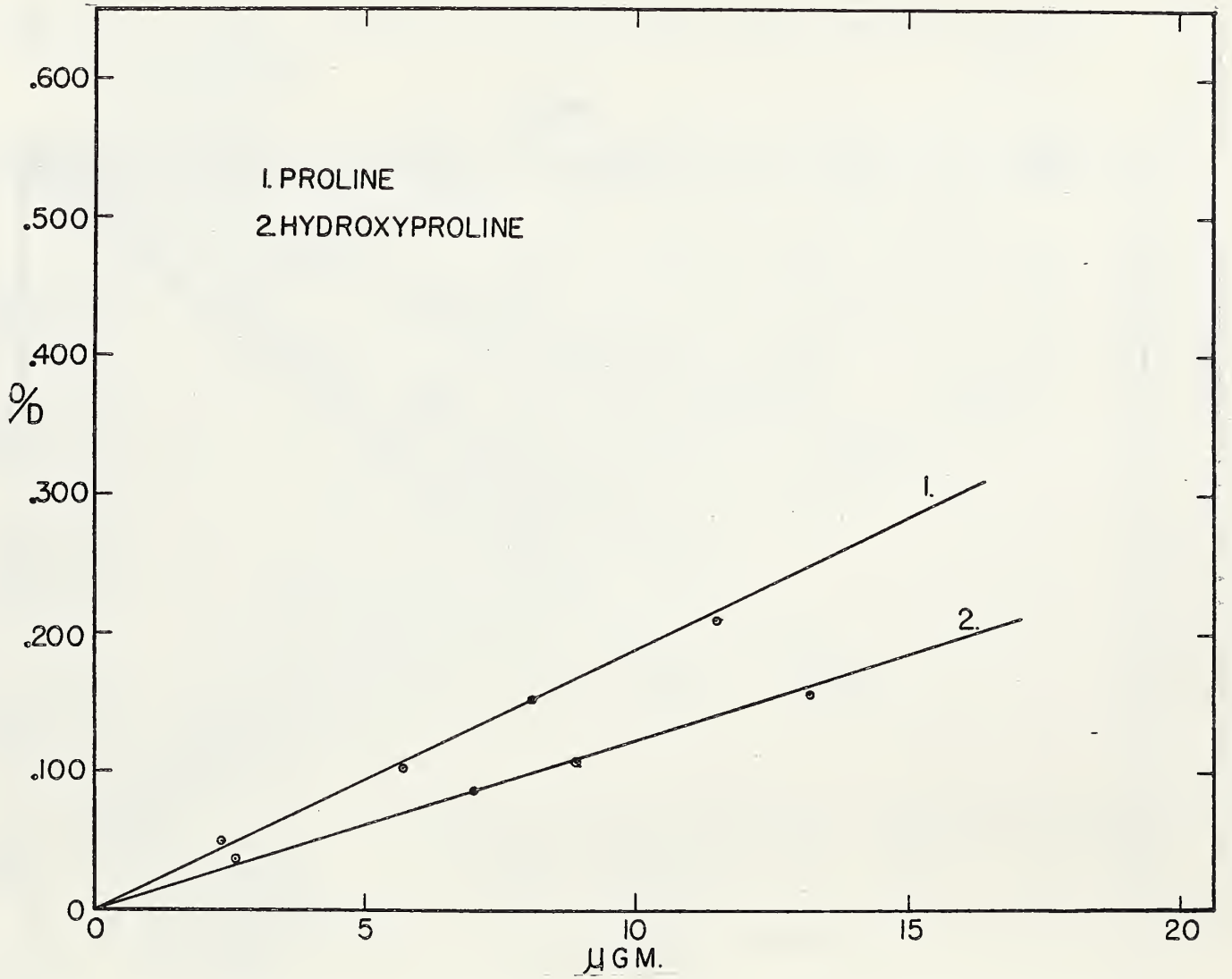
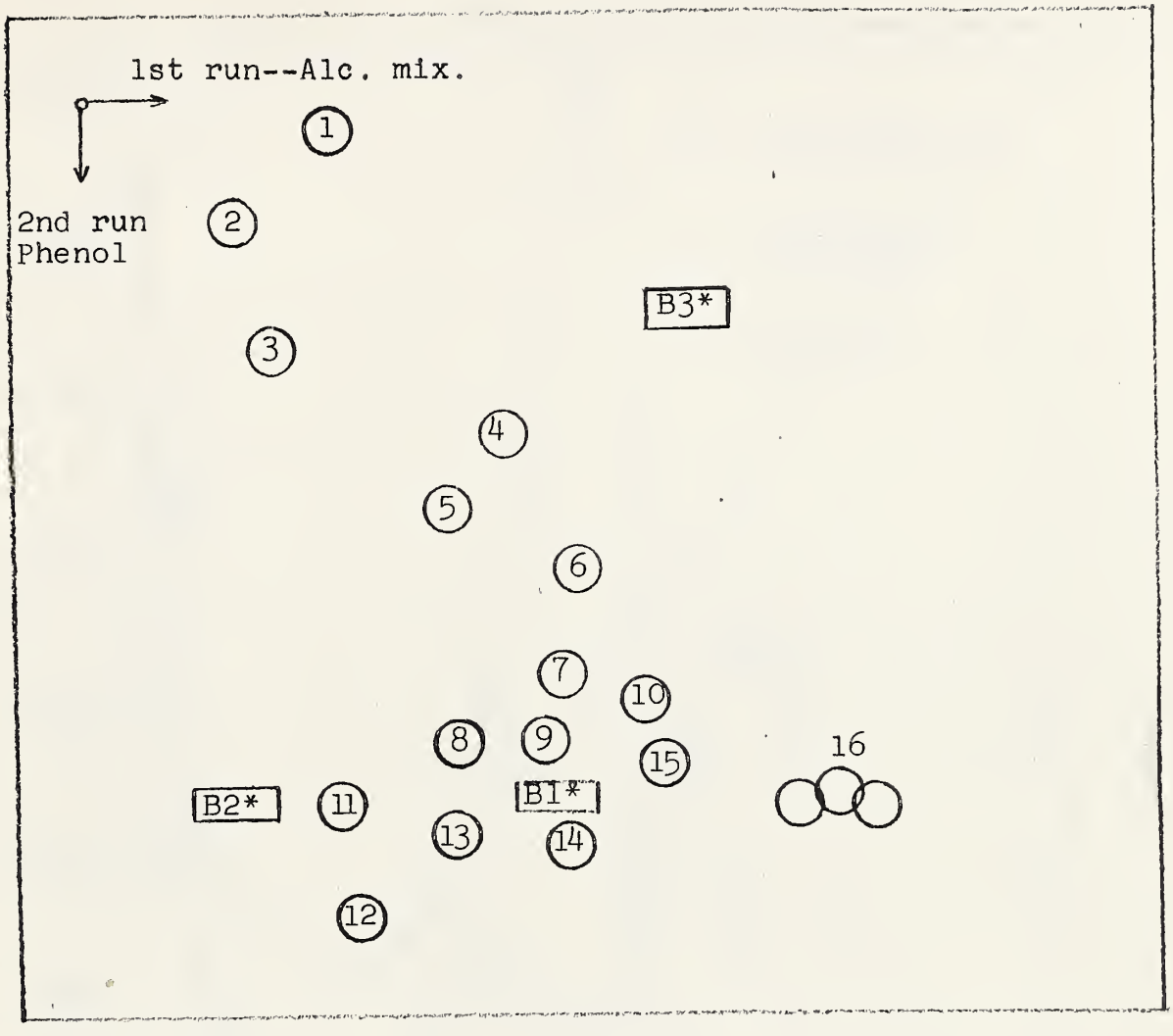


Fig. 16.--Standard curves for two amino acids read at 350 mμ



*Blanks

1) Cystine
2) Aspartic
3) Glutamic
4) Serine
5) Glycine
6) Threonine
7) Alanine
8) Hydroxyproline
9) Methionine
10) Tyrosine
11) Lysine
12) Arginine
13) Histidine
14) Proline
15) Valine
16) Phenylalanine
Leucine
Isoleucine

Fig. 17.--Chromatogram of acid hydrolyzates

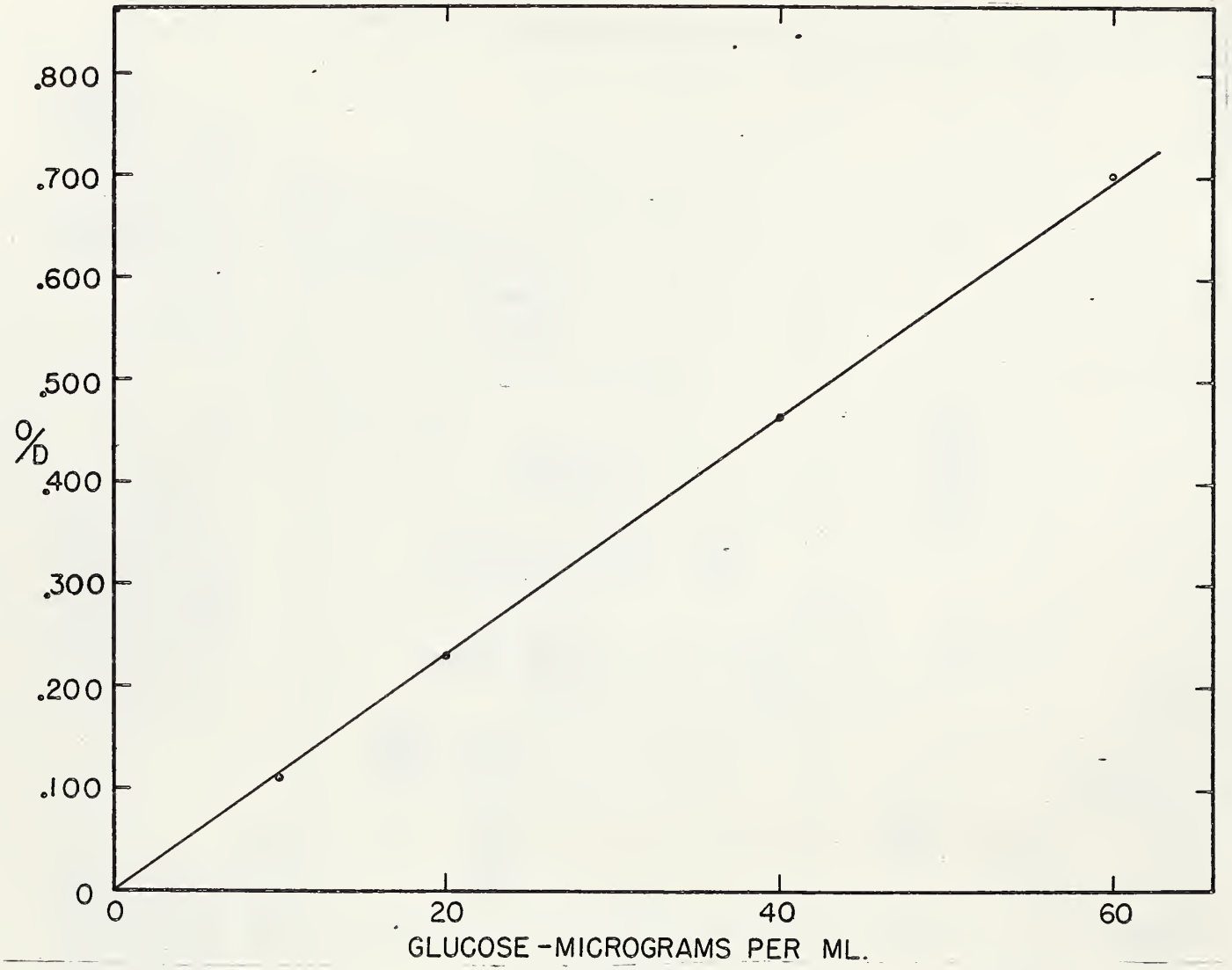


Fig. 18.--Standard curve for carbohydrate determinations by anthrone method

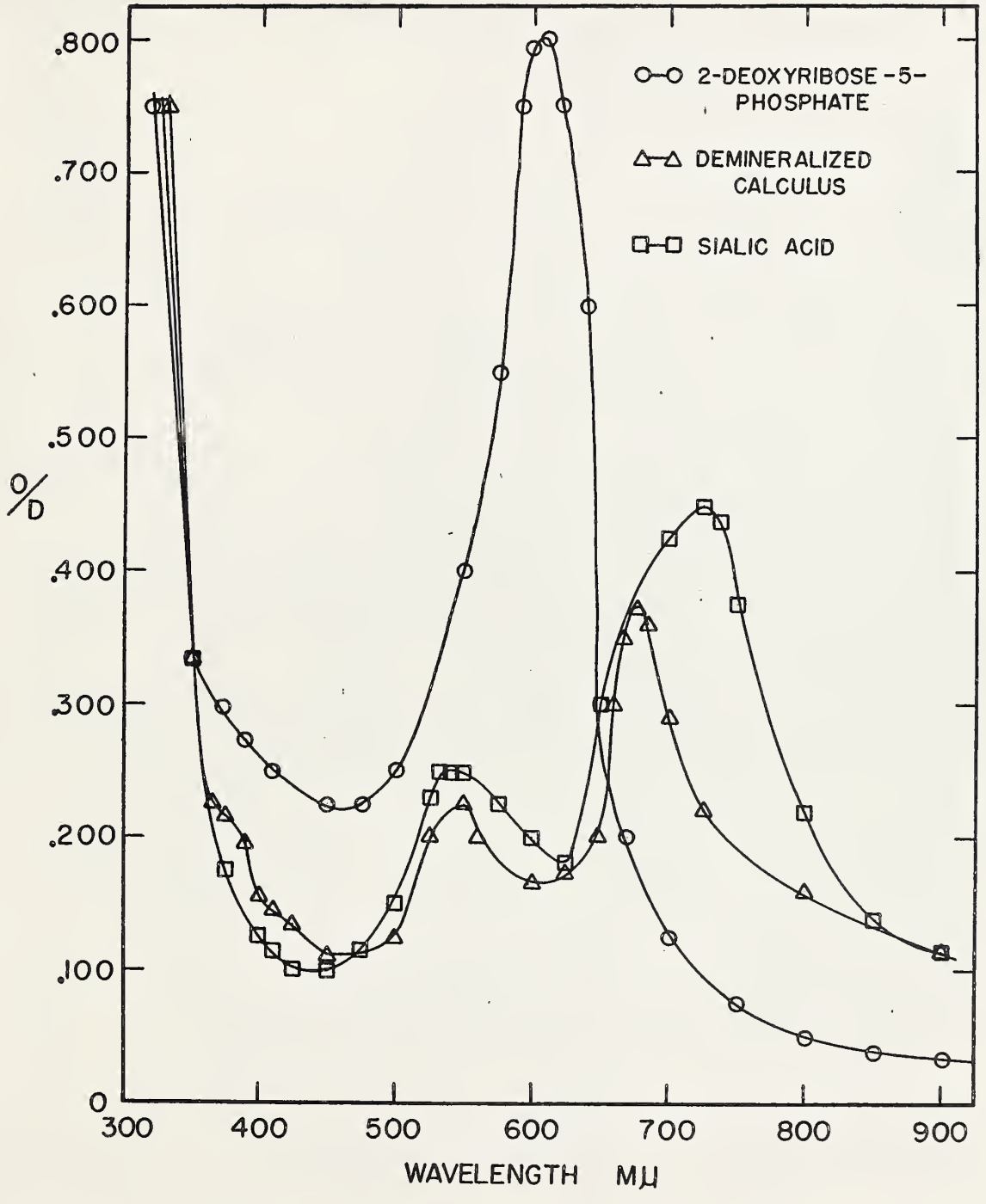


Fig. 19.--Absorption curves of diphenylamine reactions

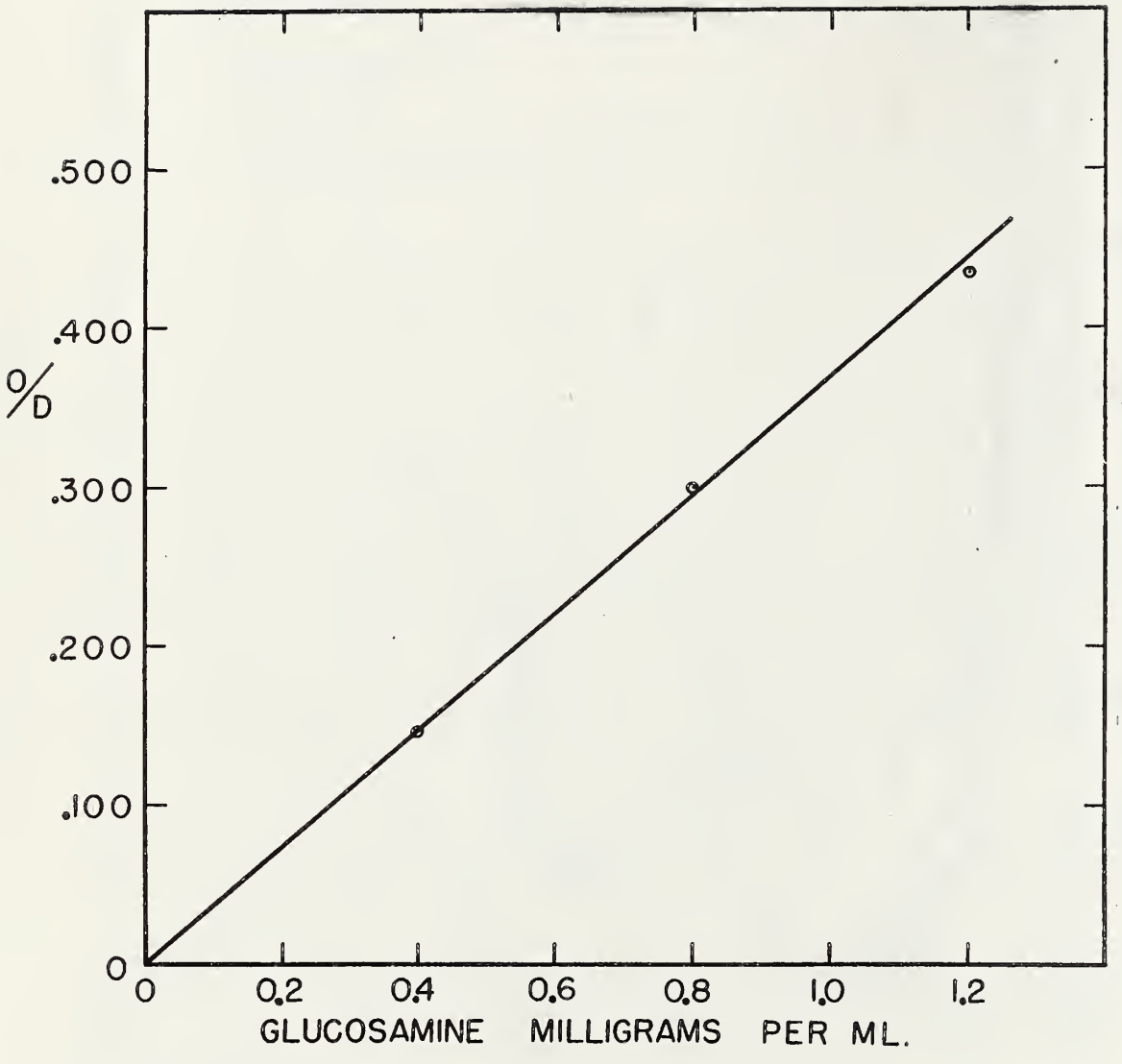


Fig. 20.--Standard curve for hexosamine read at 530 mμ.