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THE FATE OF <u>IN-VITRO</u> CARIES-LIKE LESIONS
SEALED <u>WITHIN</u> TOOTH STRUCTURE

by
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THESIS

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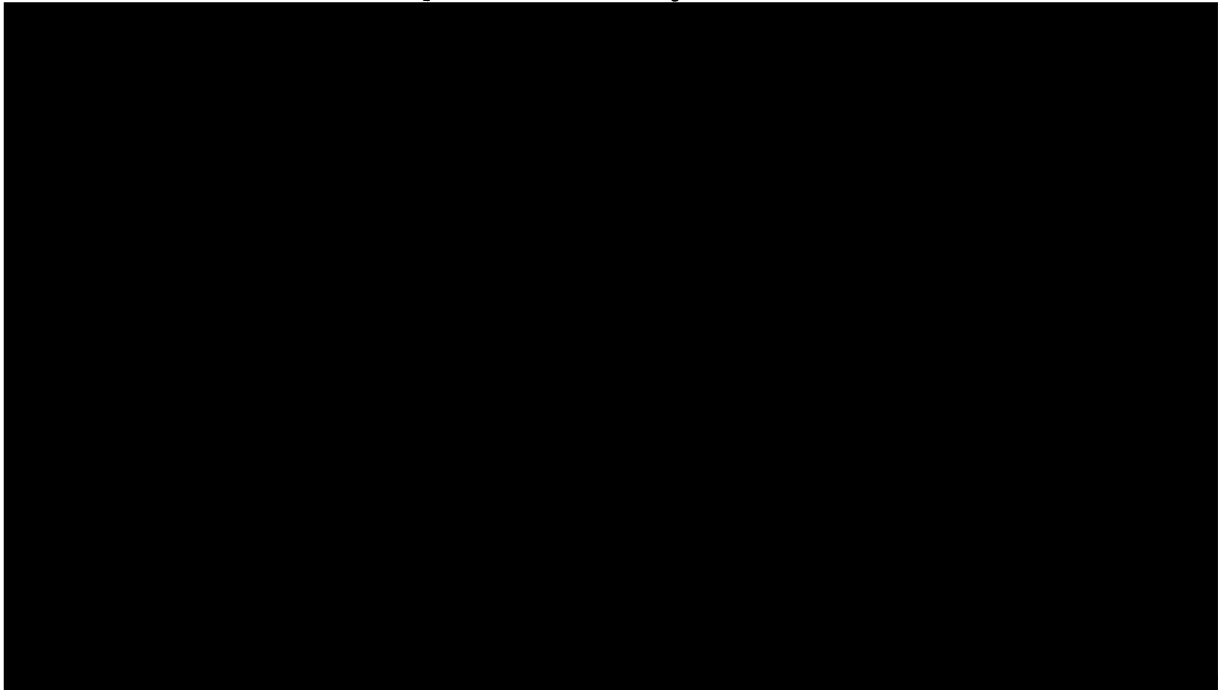
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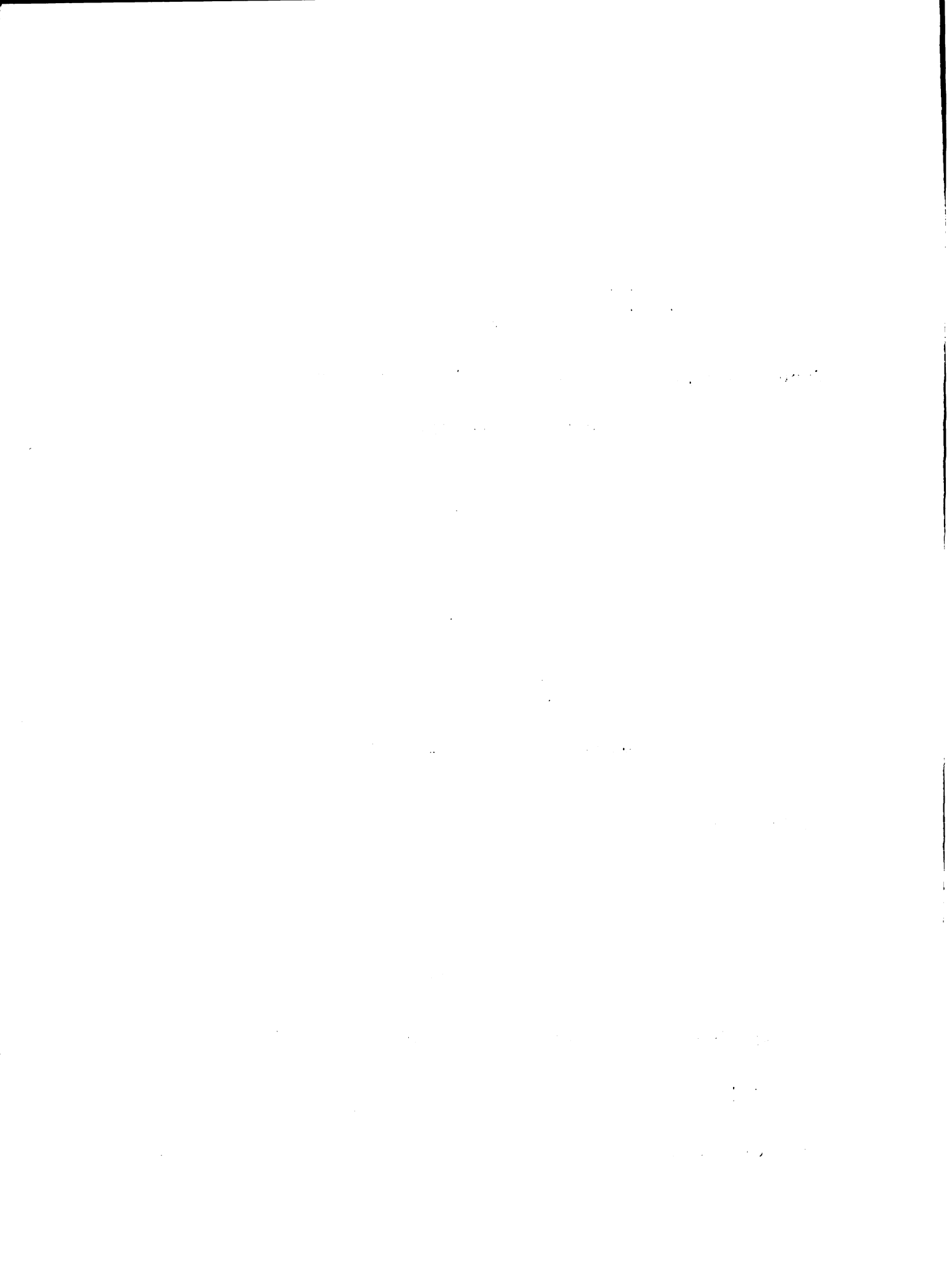
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I. INTRODUCTION

Dental caries is a localized progressive disease of the calcified tooth structure characterized by demineralization, cavitation, and bacterial invasion. Once a lesion has become established, the tissues involved cannot respond successfully unless factors responsible for the initiation and progress of the disease are removed or host resistance modified. Mechanical intervention and the substitution of prosthetic material for natural structure is almost always required. Restorative procedures, although not ideal in a biological sense, have generally provided a form of "cure" for diseased teeth in those persons who have had the opportunity to obtain individual professional attention.

The susceptibility of repaired teeth may decrease as certain more vulnerable areas are eliminated, but they are by no means immune to reinfection. The interface between tooth structure and the restoration for example, can become a new focus of carious activity. Furthermore, due to the professional time and the cost involved in mechanically treating a disease affecting almost the entire population, it can be anticipated that the condition runs its full course in a large number of cases. The ultimate erradication of dental caries therefore will not be provided by present or new restorative methods, but rather by preventive procedures capable of large scale application.

Currently, no single method for caries prevention (such as im-

munization) is anticipated, and so efforts are being made to develop and enhance procedures for partial control. Progress has been made in areas such as plaque inhibition by dietary regulation and oral hygiene methods, or by the use of chemical inhibitors. To date however, only fluoridation of water supplies or topical fluoride applications have proven to be effective public health procedures in the partial control of caries.

The recent introduction of "adhesive" resins for sealing occlusal pits and fissures has provided another potential method for caries prevention. Inherent in the concept of sealing the occlusal surfaces of erupted teeth, however, is the question of the fate of pre-existing bacterially infected demineralized areas at the base of sealed fissures. The presence of small occlusal lesions usually cannot be assertained with certainty by dentists using clinical diagnostic procedures. Furthermore, if the application of sealant is to be considered an effective preventive procedure it must be ameanable for use on a large scale basis by auxillary or para-medical personnel. Under such circumstances, many teeth that appear structurally sound but have existing carious lesions would be treated with sealant. If sealed lesions are not arrested, the usefullness of the material as a preventive substance is markedly reduced. The purpose of this investigation was to determine the activity of lesions sealed in tooth structure in an in vitro model, and assess the findings in relation to clinical conditions.

II. EXPERIMENTAL BACKGROUND AND PROBLEM UNDER INVESTIGATION

Structural and Biological Aspects of Occlusal Pits and Fissures

Carious dental areas are generally classified as smooth surface lesions or pit and fissure lesions according to the anatomical characteristics of the tooth location involved. On smooth surfaces, caries can be initiated by products of bacterial metabolism that are held in intimate contact with the enamel and are sequestered from the neutralizing effects of saliva by accumulations of adherent plaque. The plaque matrix consists mainly of insoluble high molecular weight glucans formed by the enzymatic action of certain oral streptococci on sucrose substrate. These streptococci are also acidogenic and have been shown to produce caries when inoculated into gnotobiotic animals.[1] In humans, the plaque is colonized by a variety of aerobic and anaerobic organisms which constitute the bulk of the attached mass. Concentration of destructive products on smooth tooth surfaces however can be limited mechanically and by other means which inhibit the retention and localization of plaque material.

Plaque cannot usually adhere to cusps and inclined planes of occluding teeth and therefore occlusal lesions almost always begin in pits and fissures. Saliva does not readily enter deep invaginated fissures to wash away or neutralize the products of the bacterial colonies, nor can these areas be mechanically cleaned.[2] Food and bacteria are impacted into pits and fissures during mastication and

plaque formation can continue undisturbed. Furthermore, since acidogenic microorganisms and substrate can be mechanically retained in the anatomical irregularities of pits and fissures, glucanogenic streptococci may not be specifically required for the production of this type of lesion. Fitzgerald for example, reports producing occlusal caries in gnotobiotic rats inoculated with a strain of Lactobacillus acidophilus.[3] In a later study,[4] Rosen used a strain of Lactobacillus isolated from human caries to produce lesions in the occlusal sulci of gnotobiotic rats. Plaque accumulation was not observed on the tooth smooth surfaces.

Occlusal surfaces with deep pits and fissures have repeatedly been noted to be the most susceptable locations for dental caries. Hyatt considered deep fissures to be defective enamel formations resulting from the lack of coalescence of developing lobes and reported the incidence of caries in molars to be four to twenty times greater in these areas than in other tooth surfaces.[5] In a sample of 1,160 first and second molars, Hyatt found only one per cent with caries-free fissures. Bodecker conducted extensive microscopic examinations of tooth sections and demonstrated numerous cases in which fissures, 60 μm or less in width, extended almost directly to the dentin.[6] He noted the problem of maintaining such areas free of food debris and plaque. He also noted the difficulty in diagnosing early lesions in deep fissures and demonstrated the presence of "hidden" caries in many teeth that appeared clinically sound. Bodecker and Applebaum,[7]

and Darling[8] utilized X-ray absorption techniques on ground sections

of teeth having no clinical evidence of caries and demonstrated hypo-

calcified zones identified as early lesions at the base of enamel

fissures. More recently, Granger and Reid,[9] and other investi-

gators[10, 11] have consistantly found the percentage of lesions on

the occlusal significantly higher than on other surfaces. Gillings

and Buonocore found deep invaginated pits and fissures in molars

and bicuspids in fifty-one out of fifty-two teeth investigated.[12] They

concluded that the pits and fissures should be considered normal

anatomical characteristics of posterior teeth rather than structural

malformations.

With the introduction of systemic and topical fluorides, the

differences in susceptibility to caries between occlusal pits and

fissures and other surfaces become even more apparent. Backer

Dirks observed caries reductions of approximately sixty to seventy

per cent on smooth surfaces of molars of twelve year old children

who began drinking fluoridated water at age four. The effect of

fluoridation on occlusal caries of the same group was minimal;

(approximately twelve per cent reduction).[13] This variation is not

well understood but has been attributed to differences in fluoride up-

take and to the thin and irregular nature of the enamel at the base of

the fissure. A depth of enamel of 0. 2 mm or less at the fissure base

is not uncommon, while the thickness on most other tooth locations

is approximately 1. 5 to 2. 0 mm (Figure 1). It is also known that
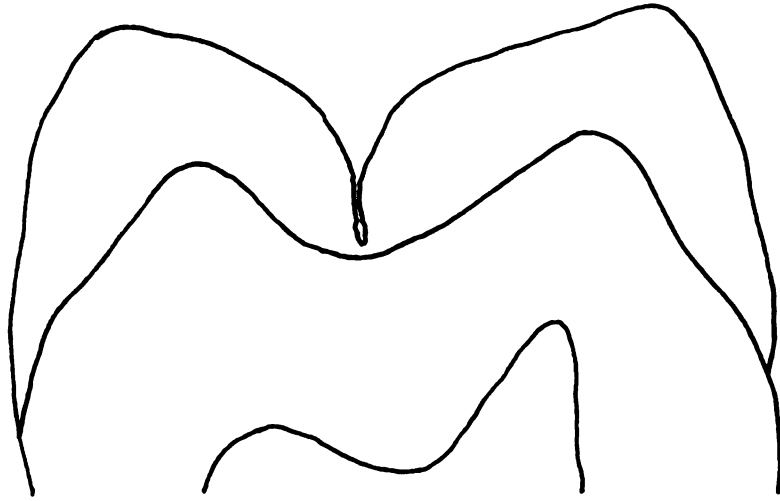
Figure 1 A. Diagram of a tooth slice showing a deep enamel fissure. The enamel at the fissure base has less depth than in most other coronal areas, and is inaccessible to cleaning procedures.

Enamel Depth
Approximately
1. 2 - 2. 0 mm

Enamel Depth
Approximately
0. 2 mm
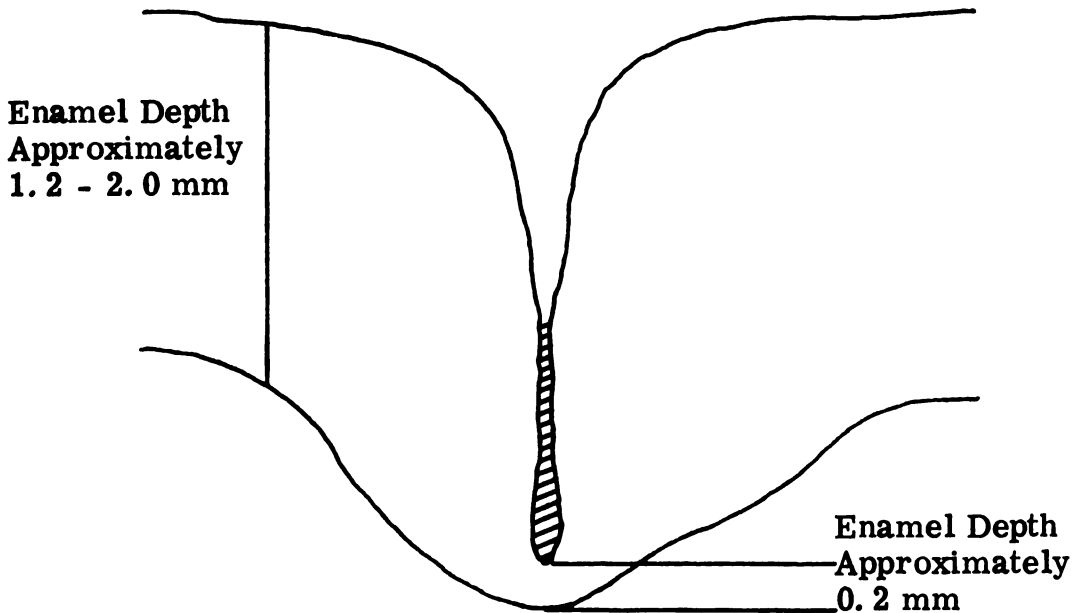
Figure 1 B. Detailed view of invaginated fissure area filled with plaque and debris. Width of fissure in this area is approximately 50 - 100µ .

fluoride is absorbed into the enamel surface after eruption and for the life span of the tooth from water, foods and saliva.[14] Backer Dirks also noted a significant topical effect of fluoridated water on the smooth surfaces of molars which had erupted shortly before fluoridation was initiated. The effect on pits and fissures of the same teeth was negligible.[13] Gedalia and Kalderon[15] report the mean fluoride content of a sample of 16-20 year old teeth at 380 ppm and that of 46-50 year old teeth at 672 ppm. The relative inaccessibility of the base of fissures to fluids in the mouth could lessen localized post-eruptive fluoride absorption as well as other enamel "maturation" effects.

The eventual development of effective methods for the control of cariogenic streptococci and plaque, may, combined with the effects of fluoride virtually eliminate the occurence of smooth surface caries. Such developments, however, might not significantly effect the incidence of occlusal lesions due to the structural characteristics of pits and fissures.

Factors affecting the Carious Process

The shape of an early pit and fissure lesion is largely determined by the anatomical features of enamel and the presence of developmental irregularities at the fissures base. Each enamel rod core is surrounded by a prism sheath or peripheral layer while an inorganic network of crystal fibers make up the interprismatic substance. The

rods are situated perpendicular to the concave dentino-enamel junction and therefore converge toward the occlusal surface (Figure 2). The caries process follows the general prism pattern but may also be influenced by the presence of enamel lamellae. These structures represent localized areas of hypocalcification or cracks extending from the surface to the dentino-enamel junction.[16] Developing occlusal enamel caries has the general shape of a cone with its apex at the tooth surface. When the lesion reaches the dentin, it spreads both along the dentino-enamel junction and pulpward. Using low power microscopic techniques with direct light, carious enamel is characterized by a change in color to brown or white due to the loss in translucency in the tissue. Under transmitted light, the lesion appears as a dark spot. Breaks in surface continuity may also be observed.

Although controversy still exists, the preponderance of experimental evidence indicates that the initial defect in coronal dental caries is produced by an acid attack on enamel.[17-20] A pH of approximately 5.0 - 5.4 is usually sufficient to initiate decalcification. The acids (mainly lactic) are formed by the enzymatic activities of certain bacteria on fermentable sugars in plaque or in other retentive areas on the teeth. Electronmicrographs of carious lesions demonstrate the penetration of bacteria through decalcified interprismatic areas in enamel, followed by a degradation of organic substances. The presence of a seemingly intact organic matrix in early lesions adds
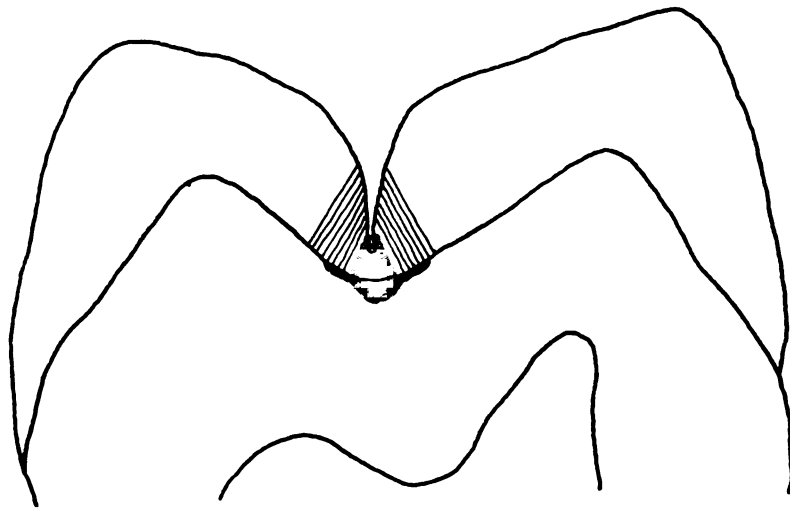
Figure 2. Diagram demonstrating the shape of an early penetrating fissure lesion in dentin and enamel.

credience to the concept that decalcification is the primary step in the caries process. A wave of demineralization also proceeds bacterial invasion into dentin. Bacteria enter the dentinal fibers, the tubules proper, and eventually the matrix, producing degenerative changes.[21, 22] MacDonald reports that bacteria have been shown to be able to attack organic components of demineralized teeth in vitro, but not intact tooth structure.[23] Burnett and Scherp also note that decalcification exposed dentin to bacterial invasion and eventual destruction or denaturation of organic components.[24] These and other studies on the morphology and development of carious lesions suggest that both the initiation and the progress of the pathology involves a cycle of activities perpetuated by the production of acids. It is conceivable that the continuation of the cycle depends directly on the availability of an exogenous supply of suitable carbohydrate substrate. Removal or limitation of this supply therefore could limit advancement of the carious process. Naturally occurring arrested lesions for example, are sometimes observed when carious surfaces become more cleanable and the available supply of substrate is decreased.[25] The chemical factors existing at the enamel intersurface with the oral fluids could then favor remineralization rather than degradation of mineral components.

Some workers contend, however, that once the pathological process has become established, the tooth itself can provide the required source of water, carbohydrate, and other nutrients either

directly from its organic matrix or by diffusion from the pulp chamber.[26, 27] Wachtel and Brown attribute the penetrating characteristics of natural caries to bacterial utilization of nutrients diffusing from within the tooth and suggest that this process favors the advancement of the lesion.[27] The limited "saucer" shaped morphology of caries in pulpless teeth and the unpenetrating nature of decalcified areas in certain in vitro caries systems could be explained by the absence of internal diffusion. It is also known, however, that vital teeth inhibit diffusion of fluids from the pulp chamber to carious areas by the formation of sclerotic or reparative dentin.[28] These secondary changes in dentinal structure may restrict bacterial growth if external sources of water and substrate are not available.

The morphological aspects of clinical caries, and observations from in vitro studies convey the impression that both an external supply of fermentable carbohydrates and a favorable intradental environment are requirements for the general pattern of caries pathology. If removal of external sources of nutrients is a limiting factor in caries, then arrestment of the process is theoretically possible by the placement of an impervious sealant material on the overriding tooth structure.

Early Efforts at Controlling Pit and Fissure Caries

Since the consistantly high incidence of occlusal caries has long been recognized by clinicians and by dentists involved in research

activities, it is not surprising that numerous and varied procedures
for decreasing pit and fissure susceptibility have been proposed.
The principles of "extention for prevention" presented by Black
nearly three quarters of a century ago, are still widely accepted
and practiced. Black advocated that preparations on teeth under
treatment for occlusal caries be extended to eliminate non-carious
fissures thus rendering the surfaces more resistant to future
attack.[29] Hyatt, in a 1923 report, advanced the preventive aspects of
Black's concept by recommending operative procedures and the place-
ment of amalgam restorations in pits and fissures of newly erupted
posterior teeth before the appearance of clinical signs of decay.[30]
Cross referred to Hyatt's "prophylactic odontotomy" as "early treat-
ment" rather than true preventive dentistry, but advocated its wide-
spread use in clinics and private operatories.[31] Bodecker agreed
with Hyatt on the need to eliminate occlusal retentive areas prior to
clinical evidence of caries but recommended shaping pits and fissures
into wide nonretentive grooves rather than replacing them with rest-
orations.[32] He contended that the dentin exposed by the grinding
procedures would undergo secondary changes and become caries
resistant. Thirty-five years after his first report on the subject,
Bodecker continued to profess the need for "fissure eradication" and
proposed large scale evaluations of the technique.[33, 34] He also sug-
gested that the procedure would allow better utilization of the dentist's
clinical time especially since improved instrumentation permitted

more rapid grinding of fissures. Operative manipulations on teeth with no apparent lesions, however, did not become commonly accepted or employed techniques. Considering the professional time and the costs involved in performing fissure erradications or prophylactic odontotomies, it is apparent that they could not be conducted as public health prevention procedures.

Increasing the resistance of pits and fissures by the application of chemical solutions or dental materials was also considered by early workers. Zinc phosphate or copper cement was placed directly into fissures[35, 36] but these substances had little potential value due to their high solubility and poor retention to tooth structure. Silver nitrate was long considered to arrest caries when applied to tooth surfaces. The material, especially in ammoniated form, was reported to diffuse into enamel and dentin and increase the resistance of the area by forming complexes with protein components and by deposition of reduced silver.[37] Klein and Knutson, however, applied silver nitrate to pits and fissures and smooth surfaces of first molars in a controlled study, and found no. significant degree of caries prevention or arrestment.[38] A new rash of enthusiasm developed for impregnating solutions between 1940 and 1950, based on reports by Gottlieb and others that the primary route for the initiation of caries was by proteolytic action of organisms on the organic structures of enamel.[39] Dramatic caries reductions were reported by Younger using silver nitrate precipitated with saturated calcium chloride.[40]

Another impregnating solution consisting of zinc chloride and potas-

sium ferrocyanide was also reported to have a positive treatment

effect.[41] Ast, Bushel, and Chase[42] however, were critical of the

experimental procedures used in the studies evaluating these solu-

tions, and initiated a controlled investigation of the efficiency of the

zinc chloride and potassium ferrocyanide technique. They placed the

solution on the occlusal and other surfaces of the teeth on one side

of the mouth of 205 children and compared the one year caries in-

crement for the treated and untreated teeth. No significant differences

were observed. Obstruction of organic components of enamel did

not prove to be a successful approach to caries prevention.

Development of Occlusal Sealants

In recent years, interest in non-operative methods for increas-

ing the resistance of occlusal pits and fissures has focused on efforts

to develop polymeric materials capable of adhering to tooth structure.

It was long recognized by dental investigators that if adhesive dental

materials were available, they could be applied as a thin film to

enamel surfaces to effectively seal caries susceptible areas from the

external environment. The problems of forming durable bonds between

synthetic materials and tooth structure, however, are many. Enamel

and dentin are extremely incompatible substrates for adhesives as

their hydrophilic nature tends to favor the immediate or eventual

displacement of the absorbed molecules by water.[43] In addition,

dental sealants must fulfill the requirements of adhesives, and yet be biologically compatible with the oral environment.

In general, two substances will adhere together if one, in the form of a fluid, can closely penetrate the minute surface irregularities of the other, a solid substrate, and then solidify with a minimum of contraction. Physical bonding occurs as a result of interacting electromagnetic energies developed by electron movements within adjacent molecules of the two substances. An adhesive can also form stronger covalent or ionic bonds if it can chemically react with the substrate.[44, 45]

Close approximation at a molecular level, a requirement for adhesion, can only be achieved if the surface of the substrate is clean and thoroughly wetted by the adhesive liquid. Wetting or molecular dispersion of the liquid on the surface of the substrate will occur only if molecules at the solid - liquid interface are attracted more strongly to the solid. Inert and contaminated surfaces therefore provide poor areas for adhesion. Utilizing principles employed in industry, Buonocore etched the outer surface of samples of tooth structure with a 50 per cent phosphoric acid solution and observed enhanced retention of an acrylic filling material.[46] He concluded that the etching procedure produced an outer surface more representative of the underlying enamel, increased surface roughness for mechanical retention, increased the wetability of the surface by removing inert and nonadhesive substances, and increased the surface area for

adhesion. He also suggested that the etching procedure might increase the reactivity of the surface due to the absorption of highly polar phosphate groups.

In a later in vitro study, Buonocore, Matsui, and Gwinett placed samples of methyl-2-cyanoacrylate adhesive and a self curing methyl methacrylate resin on etched and unetched enamel surfaces.[47] The tooth samples were stored in physiological saline for periods of six to twelve months, and the adherance of the materials to the enamel was evaluated. Tests for microleakage at the polymer-enamel interface were also conducted using basic fuchsin dye and radioactive sulphate ($SO_4{}^{35}$). Tracer penetration and dislodgment of the test materials was observed with unetched samples, whereas etched samples displayed enhanced adhesion and little or no tracer penetration. It was suggested that chemical bonding may have occurred between the enamel and the cyanoacrylate and methyl methacrylate resins. Considering the composition and properties of the material, however, it is not likely that chemical bonds were formed. The investigators also decalcified and sectioned etched and unetched tooth samples covered with various resin filling materials. "Prism-like tags" up to 23μ in length were seen projecting from the resin samples placed over the etched enamel surfaces. They concluded that the resin material polymerized around the organic matrix on the prism surface, and penetrated into interprismatic spaces. The penetration of "tags" into micropores of enamel was thought to provide mechanical

retention for the resin. A persistance of "tags" and resistance of the enamel surface to decalcification was observed even after the bulk of the material was removed. Later reports by Gwinnett[48] and by Sharp and Grenoble[49] indicated that preferential removal of prism cores rather than of interprismatic spaces was the most common effect of etching with phosphoric acid. In another study, Lee and Swartz reported enhanced retention of a polyurethane material to enamel after etching the surface with a 50 per cent aqueous solution of citric acid.[50]

The phosphoric etching solution was considered safe for clinical trials since a similar concentration of acid was in general use in zinc phosphate cements.[51] The activity of the solution is also partially neutralized by the addition of seven per cent zinc oxide. The acid application was proposed to remove only acquired pellicle and enamel to a depth of approximately 5 μ , similar to concentrations removed during the application of acidulated topical fluoride compositions.[52] It is likely, however, that the total effect of the acid etch is greater considering the depth of penetration reported for "tags" of resin material. It should also be noted, that the outer layer of "mature" teeth is hypermineralized and more caries resistant than less superficial crystals. Gwinnett and Matsui for example, observed that teeth with longer exposures to the oral environment had an increased resistance to etching.[48] Wheras etching associated with topical fluoride application enhances enamel resistance, surfaces etched for

sealant application (and not covered) could possibly have an increased susceptibility. Although no such increases have been reported, this condition should continue to be assessed in clinical studies.

Although the conditioning procedures with acid solutions enhanced the possibilities for effective sealing of enamel surfaces, the characteristics of the polymer applied to the tooth were also very critical. In order to form strong durable bonds, it was essential that the material have an affinity for dental structure, polymerize with a minimum of shrinkage and no gas emission, experience minimum dimensional change on water absorption, and form physical or chemical bonds with enamel at the molecular level. In addition, the material was required to be non-toxic to oral tissues, to polymerize rapidly, and to be resistant to oral fluids. As mentioned above, substances bound to tooth structure by physical forces of adhesion or by micro-retention points in the etched enamel will tend to be displaced with water. The water molecules can diffuse from within the tooth, or at the tooth-sealant margin. True chemical bonds that alter the surface characteristics of the tissue are probably necessary for prolonged adhesion. Materials that form chemical bonds with enamel or dentin are not presently available although experimental models have been described and tested in vitro. One such system developed by Bowen[53] employs a coupling agent or "surface active copolymer". Bowen hypothesized that the coupling agent binds to the mineral component of the tooth with one molecular portion (by an

amphoteric 5 atom chelate ring with calcium atoms), while the other can polymerize with a monomer system. The coupling agent invest-igated is the addition reduction product of N-phenylglycine and glycidyl methacylate (NPG-GMA). A significant increase in bonding was ob-served when this agent is used on dentin or enamel. Lee reported a ten fold increase in adhesion of a resin material to bovine dentin when an organofunctional silane coupling agent was applied. He suggested that the silane formed a chemical bond between the hydroxyapatite of tooth structure and resin material.[43]

Numerous existing commercial adhesives and acrylic resins were screened for bonding efficiency to enamel by Ross, Lal, Williams, Falcetti[54] and other workers,[55] but none of the materials tested main-tained adhesion after prolonged immersion in water. In vitro tests with methyl-2-cyanoacrylate (Eastman 910 adhesive) conducted on etched teeth by Buonocore,[47] and by Gwinnett and Matsui [48] were promising, but clinical trials with the materials as occlusal sealants or cements produced mixed results. Swanson and Beck for example, cemented orthodontic bands to etched teeth in 24 patients with adhesive and observed loss of retention in all cases between 24 hours and four weeks.[56] Cueto and Buonocore,[57] however, applied a mixture con-taining methyl-2-cyanoacrylate, and a powdered filler as a sealant to the occlusal surfaces of posterior teeth in 201 subjects and reported an eighty-six per cent reduction in caries (after one year) in treated teeth as compared with untreated teeth in the same mouth. The

material was applied at six month intervals and was observed on seventy-one per cent of the treated surfaces after one year. Reduced caries levels were reported even in teeth from which the sealant was lost. Ripa and Cole[58] observed an eighty-four per cent reduction in new carious lesions over a one year period using a similar material on the teeth of ninety-one mentally handicapped children. In a later study, however, Parkhouse and Winter[59] observed no significant reduction in caries after six months in teeth in 108 children sealed with methyl-2-cyanoacrylate. The material was lost from almost all the study teeth and contrary to the observations of Cueto and Buonocore, the teeth had no residual protection.

A sealant material containing iosbutyl cyanoacrylate is currently being clinically evaluated in Maracaibo, Venezuela,[60] and in Millbrae, California.[61] One year results from the studies in Venezuela indicate that the material produces a statistically significant treatment effect.

Lee, Cupples, Schubert, and Swartz reported high adhesive strengths in vitro for a fluoride containing polyurethane resin applied to teeth etched with citric acid and treated with a silane coupling agent.[62] The material, however, displayed poor retention and solubility properties in vivo[63] and is currently considered primarily a vehicle for the application of topical fluoride.

A cross-linking thermosetting dimethacrylate monomer recently developed by Bowen[64] and now employed as the resin component in

most composite restorative materials was adapted for use as an

occlusal sealant. The dimethacrylate monomer is the addition re-

duction product of bis (4 hydroxyphenyl) dimethylmethane, (a di-

hydroxyl phenol), and glycidyl methacrylate, (an epoxy acrylic mon-

omer).[65]

BISPHENOL A   GLYCIDYL METHACRYLATE
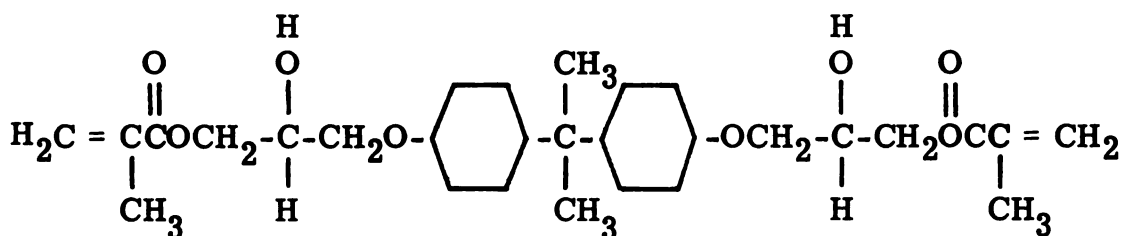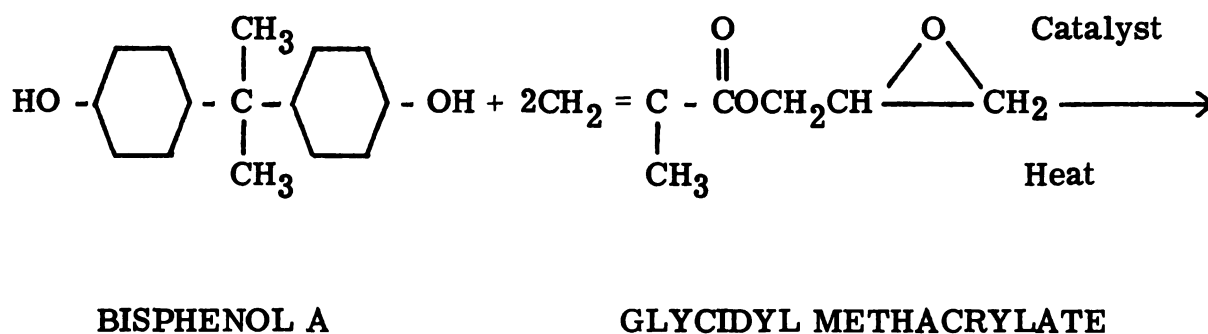
"BIS-GMA"

The reaction product, given the simplified name "BIS-GMA",

consists of phenoxy and methacrylate groups linked by hydroxy

glyceryl groups. The epoxy groups are no longer present, but the

molecule contains a polymerizable (unsaturated) group at the end of

the long connecting segment. The product is highly viscous and necessitates the addition of methyl methacrylate monomer, (1 part methyl methacrylate to 3 parts BIS-GMA by weight) to improve flow characteristics. The diluent copolymerizes with the dimethacrylate monomer during the curing process.

Addition polymerization of the sealant is initiated by a chemical catalyst which decomposes to form free radicals. The free radicals activate the monomer molecules by opening the double bonds, and the process continues by energy transfer from molecule to molecule.

$$-(CH_2 - \underset{\underset{O}{\overset{\|}{C}}}{\overset{\overset{CH_3}{|}}{C}} -)_n \quad -OCH_2- \underset{\underset{H}{|}}{\overset{\overset{OH}{|}}{C}} - CH_2O -\langle\ \rangle- \underset{\underset{CH_3}{}}{\overset{\overset{CH_3}{}}{C}} -\langle\ \rangle-$$
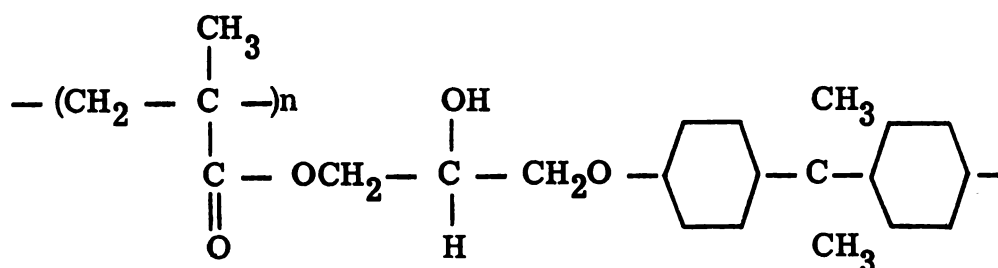
POLY (BIS-GMA)

Polymerization is initiated by conventional chemical methods (peroxide-amine catalyst system) or by long wave ultraviolet radiation (3,600Å) applied to the composition after it has been made ultra-violet sensitive by the addition of 2.0% benzoin methyl ether.

The material probably does not form chemical bonds with tooth structure, but was shown to have good physical properties in tests conducted in vitro.[66,67] The monomer mixture displays low shrinkage during polymerization, and has a relatively low coefficient of thermal

expansion. As a result of these characteristics, physical bonds and areas of mechanical retention between the material and an etched tooth surface are more likely to be formed and maintained.

Early results of clinical trials demonstrated that the sealant could inhibit the formation of new pit and fissure lesions. Roydhouse evaluated the effectiveness of the material (using a peroxide-amine polymerization system) in controlling occlusal caries among 130 children residing in a fluoridated community.[68] Three years after treatment, the material was observed to be 29 per cent effective in reducing the number of new carious surfaces compared with control teeth. Buonocore reported one hundred per cent protection from occlusal caries in selected permanent teeth one year after the application of a similar sealant,[69] and ninety-nine per cent effectiveness after two years.[70] Retention of the material was reported in eighty-seven per cent of the teeth after two years. The sealant employed by Buonocore utilized the benzoin methyl ether and ultraviolet radiation catalyst initiator system.

One year results of a study being conducted in Kalispell, Montana[60] utilizing the bisphenol A and Glycidyl methacrylate type polymer indicate approximately eighty-four per cent fewer carious lesions on the occlusal surfaces of treated than on untreated teeth. Approximately eighty-five per cent of the treated teeth were considered to have lost no sealant.

Although the results of the clinical trials of some sealant for-

mulations are promising, further in vitro and in vivo investigations

are required to assess the properties and limitations of this class of

materials. New formulations with improved bonding characteristics,

easier application methods, positive therapeutic effects, and other

refinements will undoubtedly be developed in the future. The infor-

mation gained from the studies on the presently available sealants

can assist in the development of the new materials and hasten their

clinical application.

Occlusal Sealants in Public Health Programs

Although strict maintenance of careful technique is required

during the application of occlusal sealants, the procedure does poten-

tially lend itself to use as a public health measure. The costs

associated with applying new preventive or treatment procedures,

however, must be considered in relation to anticipated benefits. In

the clinical trials for sealants conducted in Montana, for example,

approximately eighty-four percent fewer occlusal lesions were

observed in treated than in control teeth over a one year period.[60]

It was also noted that out of 1,160 surfaces treated, 294 (or one out

of four) were "prevented" from becoming carious. The occlusal sur-

faces of the other treated teeth either developed lesions or would

probably not have become carious during the one year period, even

if not treated. Assuming a child has eight teeth with susceptible

fissures, two surfaces could be considered to be "saved" from carious

lesions by the application of sealant. In other clinical studies,[71] it was observed that twenty-four minutes were required of the dentist's and assistant's time to examine the posterior teeth on each patient and to apply sealant ("BIS GMA" type) and approximately the same amount of time was required to restore two tooth surfaces. Consequently, there was little apparent saving in manpower and cost in performing sealant applications, especially if sealed teeth required yearly examinations and retreatments. In addition, if treated teeth developed proximal lesions requiring restorations, occlusal extensions of the cavity preparation would represent duplication of the protective effort of the sealant.

As noted by Cvar,[71] there are several defects in such forms of cost analysis, and the use of sealants in dental procedures could be indicated even if no significant savings in manpower are effected. Sound protected teeth, for example, have numerous advantages over repaired teeth. The time evaluations do suggest, however, that more rapid and less costly methods of sealant application should be investigated. The cost to benefit relationship of sealant applications could be favorably adjusted, for example, if highly trained dental personnel were not required to perform the treatments. One factor limiting the large scale utilization of the material by para-medical workers is the limited information available on the effect of sealing small carious lesions. If arrestment of the decay process is not assured, the teeth must be carefully examined prior to sealant application and

carious areas must be restored with a conventional filling material.
If sealant is placed on existing lesions, the progress of the caries
could be masked. If however, small sealed carious areas become
arrested, the prospects for utilizing the material on a mass applica-
tion basis are greatly enhanced.

Although the fate of bacteria remaining beneath conventional
dental restorations has for yearsbeen one of the most intriguing
aspects of the microbiology of dental caries, definitive information
on the subject is lacking. Reports of a number of in vivo and in vitro
studies indicate that bacteria remain viable under dental fillings,[72-78]
but the role of these microorganisms in the extention of the carious
process has not been clearly defined. On the basis of clinical obser-
cation, several authors have suggested that small areas of carious
dentin do not progress when isolated from the oral cavity.[74, 77, 78]
The accumulation of experimental data on the subject, however, has
been restricted by technical factors such as the properties of
conventional restorative materials and difficulties in accurately diag-
nosing the progress of carious lesions in vivo. Dental materials such
as amalgam, gold, silicates, and direct filling and composite resins
repeatedly have been shown to permit marginal leakage of substrate,[79-81]
and in some cases microorganisms,[82-84] into cavity preparations.
Carious lesions remaining under these restorations therefore, cannot
be considered isolated from substances in the mouth. In addition,
the extention of caries beneath a restoration could be attributed to the

invasion of additional microorganisms through the marginal areas,

rather than to the activities of bacteria in residual carious material.

The resins systems utilized as occlusal sealants have been reported

to form tight margins with tooth structure, and therefore may permit

an accurate assessment of the fate of sealed carious areas.[64, 65]

In Vitro Methods for Studies on Caries

One of the main problems in assessing the effect of sealants on

caries progress in vivo is the absence of accurate methods of measur-

ing the size of carious lesions over various time periods, or in deter-

mining when a lesion has been arrested. As indicated above, the

mere presence of viable organisms in enamel or dentin is not a clear

indication that the organisms are actively engaged in producing acids

or other destructive products. Bacteria are known to remain quies-

cent for long periods of time when deprived of adequate supplies of

water or substrate. Present radiographic techniques are not quanti-

tative, nor at times even diagnostic for occlusal lesions. Histological

methods have also not been effective in determining the state of acti-

vity of existing lesions. Massler and co-workers[85, 86] have reported

the presence of microscopic differences between active and arrested

caries in dentin, but only in chronic areas where recalcification was

occurring. Arrested incipient lesions or lesions in earlier states of

inhibition could probably not be diagnosed by their methods. Other

problems also arise when doing in vivo caries studies on humans.

Existing lesions cannot be permitted to progress for the periods of time that might be required to determine differences between test and control teeth. In addition, normal permanent teeth cannot be removed for sectioning at the conclusion of a study. Investigations on animal subjects are costly and are limited by many of the same factors mentioned for human studies. Furthermore, of the animals available for caries research, only primates have teeth that provide adequate study models for treatment with sealant.

In spite of the limitations mentioned above, various forms of in vivo studies are vital to understanding the effect of sealing carious lesions, and should be conducted. Complimentary information, however, obtained from controlled in vitro studies, can be valuable in determining the extent and direction for in vivo studies and can aid in the interpretation of results.

The in vitro production of areas of decalcification in enamel and dentin resembling natural caries has been reported by numerous investigators including such early workers as Tomes and W. D. Miller.[87] The results of many of these studies were consequently used to support the concept that the carious process is primarily the result of an external attack on the tooth surface by acids produced locally by bacteria. Microscopically, for example, both in vivo and in vitro lesions in enamel were observed to progress along the interprismatic substance in advance of changes in the organic matrix.

The basic methodology for the formation of in vitro caries has

been similar in most reported cases. A variety of solutions that in-
duce demineralization, however, have been employed. Enright,
Friessell and Trescher[88] exposed teeth to inorganic acids and report-
ed the production of natural appearing lesions. Weisenberger[89] in-
cubated teeth in a medium containing glucose and inoculated with
a mixed oral flora, whereas Hughill and Box[90] observed lesions
with "microscopic exactness to the true pathological lesion" when
teeth were placed in solutions containing acids and proteolytic
enzymes but not when acids and enzymes were used alone. Darling[91]
observed subsurface lesions after exposing enamel to 0.075% lactic
acid or to lactose broth inoculated with acidogenic microorganisms.
Other workers placed substances such as acidified gelatin,[92, 93]
lactate[94] or acetate buffers,[95] or inoculated glucose-tomato juice
agar[96] on tooth surfaces and reported the production of caries-like
changes. Besic,[97] and Coolidge, Besic and Jacobs[98] noted that
when calcium and phosphate ions were added to an acid buffer solu-
tion in contact with a tooth surface decalcification was inhibited.
According to the investigators, high concentrations of calcium and
phosphate completely retarded decalcification, while moderate con-
centrations permitted the formation of lesions with the characteristics
of true caries.

Although these and other workers report producing natural appear-
ing lesions in teeth in vitro, acceptance of these lesions as true anal-
ogues of in vivo caries has not been universal. Some researchers, for

example, have reported the production of microscopic penetrating caries like areas in enamel at a neutral pH.[99, 100] It is also noted that localization of attack does not occur with the decalcification methods unless the tooth is covered with paraffin or some other inert material and a small window is placed in the area where decalcification is desired. Dietz,[101] and Burnett and Scherp[102] do not agree that lesions so formed are identical to true caries. Dietz[101] claimed to have produced localized lesions by exposing tooth to saliva at pH 6. 0 and placing a hydrolyzable carbohydrate substrate on a specific enamel area. He attributed this condition to plaque formation and a localized acidity in the area covered by the substrate. The lesions produced by this system, however, did not penetrate more than half the thickness of the enamel nor did they have the characteristic flame-like morphology of natural caries.

Pigman, Elliott, and Laffare[103] developed an apparatus for the _in_ _vitro_ production of caries in which culture medium was allowed to drop slowly on the coronal portion of a tooth. A plaque-like material developed on the enamel and pH values of 4. 8 - 6. 2 were measured on the tooth surface. The formation of penetrating pits was observed in some cases. The investigators reported that a localization of the process could be obtained if the teeth were brushed twice daily. Presumably, the cleaning procedure permitted the maintenance of a relatively high pH over most of the occlusal surface while the acidity increased in pits and fissures and other protected areas. The lesions

produced by this method, however, were also observed not to have

the penetrating flame-like characteristics of true pit and fissure caries.

An in vitro technique for the formation of caries that utilizes

the permeability characteristics of dentin and enamel was described

by Brown, Wachtel, and Wheatcroft.[104] This method was reported to

produce penetrating lesions with bacterial invasion that were typical

of natural pit and fissure caries. Studies in vivo, have shown that

teeth function as modified semipermeable membranes between the

fluids of the mouth and the blood and interstitial fluid of the pulp

chamber. Water, ions, and small molecules have been reported to

pass through the membrane dependant on osmolarity conditions and

possible chemical reactions with the tooth structure.[105-107] In vivo

and in vitro studies have demonstrated that substances such as isotopes,

dyes, and various biological growth factors diffuse rapidly through den-

tin, the rate being a function of the molecular weight of the diffusant.[108]

The permeability properties of the tooth, however, are determined by

the enamel which is considerably more resistant to fluid flow than

dentin. Diffusion through enamel occurs most readily in areas of

minimal thickness such as in deep pits and fissures or where there

are natural or artificial defects in the calcified structure (e.g. lamellae,

cracks). As noted above, characteristics of the lesion within the tooth

may be influenced by water and nutrient availability through the dentin

and enamel. Bodecker[109] noted that an artifact in in vitro caries

systems was the absence of fluid ("dental lymph") in the extracted

tooth.

In one of their investigations, Brown, Wachtel, and Wheat-
croft[104] diffused a niacin solution or a complete medium from the

pulp chamber of a tooth and placed a niacin-free medium and a

bacteria requiring this growth factor, on the occlusal aspect. Local-

ized lesions resembling natural caries were produced by this method,

but only generalized decalcification was observed when complete

growth medium was used occlusally. The investigators concluded

that the lesions were due to rapid bacterial growth and acid produc-

tion in areas where niacin diffusion through the enamel was most

rapid. These areas (deep fissures, pits, lamellae) or artificially

prepared holes became locations of optimum nutriment whereas a

deficiency in the medium existed on the main tooth surface. It was

felt that the bacteria were stimulated to proliferate inward toward

the direction of the growth factor. Stained sections verified the

presence of bacteria in the enamel and dentin to the depth of the de-

calcified lesion. On the other hand, no bacteria were observed in

dentin when water was substituted for the niacin solution. It was

concluded that microbial growth was dependant on diffusion of nut-

rient and was not supported by the structural components of the

tooth.

In order to determine the appearance of susceptible areas prior

to the initiation of caries, and to be able to observe the developing

carious area, Wachtel and Brown[27] also applied the procedures des-

cribed above to 250μ tooth slices. With this method, the appearance of

susceptible areas could be determined prior to the initiation of caries and the lesion could be observed through various stages of development.

The organism utilized in the diffusion technique for in vitro caries was Lactobacillus plantarum. This bacteria has several exacting nutritional requirements, in addition to compounds utilized essentially as energy sources. An external supply of niacin is needed to form the pyridine nucleotide coenzymes involved in carbohydrate fermentation.[110]

Niacin (as nicotinomide) is the active constituent of the coenzymes nicotin-amide-adenine dinucleotide (NAD) and nicotinamide-adenine dicleotide phosphate (NADP).
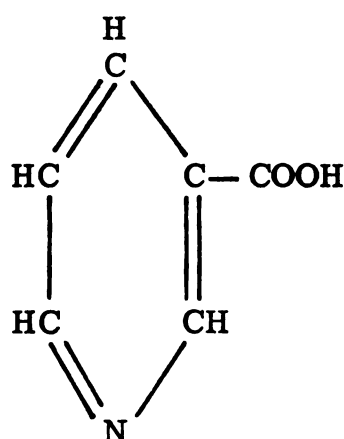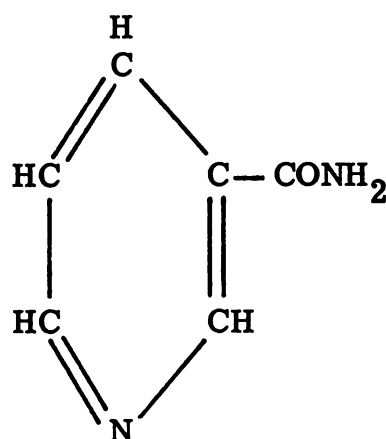
NICOTINIC ACID   NICOTINAMIDE

NAD and NADP are essential for hydrogen and electron transport during biological oxidation-reduction reactions catalyzed by dehydrogenases. During the oxidation of a substrate, a pair of electrons and

and a hydrogen atom are transferred to the pyridine ring. The reduced coenzyme can then be reoxidized to the original state and undergo another cycle.[111]

OXIDIZED FORM   REDUCED FORM
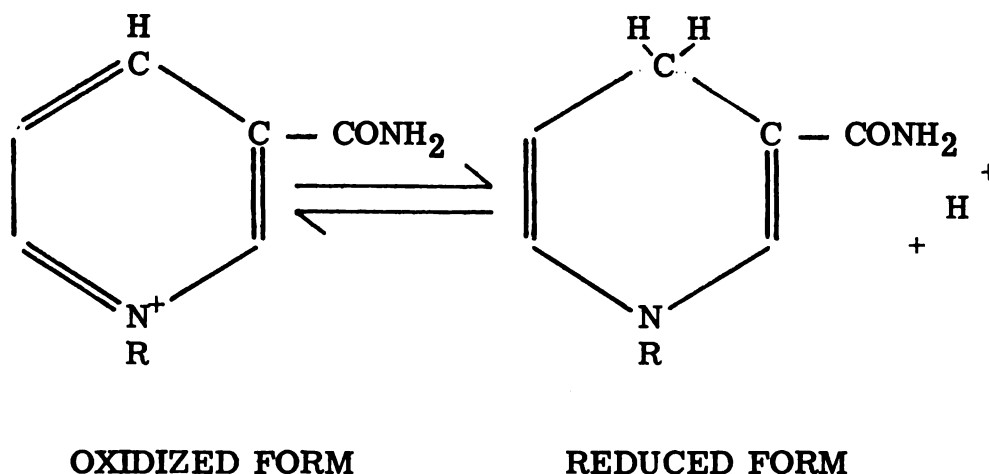
Although a metabolic pathway other than the conventional Embden-Meyerhof-Parnas scheme is reported to be used by L. plantarum, all the enzymes of the EMP pathway are present. At least two pyridine nucleotide dehydrogenases are required: glyceraldhyde phosphate dehydrogenase and lactic dehydrogenase. They are used in the breakdown of glyceraldehyde 3-phosphate in the formation of ATP and lactic acid.[112]

Glyceraldehyde 3-phosphate ⟶ Lactate
ATP   ATP

The amount of NAD in the organism is limited and new quantities must be generated for the continuation of metabolism. An external source of niacin is required by L. plantarum for NAD production

as the organism does not biosynthize this substance.

L. plantarum can utilize nicitonic acid and nicitinamide with equal efficiency.[113] Nicitonic acid, a pyridine-3-carboxylic acid, has a molecular weight of 123.11, is water soluble, and is stable in an aqueous solution. It withstands autoclaving at 15 pounds pressure for 5 hours, and incubation conditions (37°C) for several months without loss of potency. It is weak acid in solution, a 1% solution having a pH of 4.0. No apparent loss in potency is observed when nicitonic acid is mixed with phosphate buffer and the pH maintained at 6.0. Nicotinamide has a molecular weight of 122.12 and has similar solubility and potency properties as nicotinic acid. A freshly mixed 1% solution has a pH of 6.0. The pH gradually drops during incubation, however, probably due to the hydrolysis of the nicotinamide to nicotinic acid.

Snell and Wright[114] developed a standard microbiological assay for niacin by preparing a chemically defined, niacin-free growth medium for L. plantarum. Unknown samples incubated with the inoculated medium could be assayed for niacin content by quantitating pH changes and increases in turbidity. This assay procedure was adopted by Wachtel and Brown to determine niacin diffusion rates through tooth structure, and L. plantarum and the assay medium were utilized for the production of in vitro caries.

The limitations of studying the effect of sealant on caries in an in vitro system are numerous. None of the methods described above

are impeccable. Teeth studied in vitro are not readily subjected to typical occlusal stresses, thermal changes, and variable environmental conditions found in vivo. All these factors can effect the physical properties of the sealant material. Teeth cannot be stored, sterilized, and incubated without producing structural changes that could effect conditions such as susceptibility to demineralization. Perhaps the most important artifact of an in vitro caries study is the absence of dynamic conditions associated with a vital pulp. The diffusion of substances from the blood stream and responsive changes seen in vital teeth do, as mentioned above, influence caries development.

On the other hand, in vitro systems provide certain investigative advantages. Lesions can be induced in caries-free teeth at a specific time period and be monitored under relatively controlled conditions. Extensive caries can be permitted to progress and teeth can be sectioned at the conclusion of the study. A method has also been described that allows the visualization of developing or arrested lesions during the course of the study. In vitro caries studies are also far less costly than in vivo studies in humans or animals and are more rapid.

III. STUDY OBJECTIVE

The primary purpose of this investigation was to obtain information that will assist in determining whether carious lesions in teeth can be arrested from further development when isolated from external sources of substrate. The specific experimental objective was to study the effects of the application of occlusal sealant on teeth with enamel or dentinal caries in an in vitro system.

IV. METHODOLOGY

Study Design

The in vitro caries method employed in this study utilizes the principle that localized lesions could be produced by bacterial activity in enamel and dentin when a growth factor required by the bacteria is diffused from the pulp chamber. This technique was selected because it was reported to induce penetrating lesions resembling natural pit and fissure caries[104] and because it could best be adapted for work with occlusal sealants.

Two variations of the basic methodology, each with specific advantages, were incorporated in this investigation. One technique utilized whole teeth and the other used 750μ tooth slices. Whole teeth were considered more representative of natural conditions, whereas tooth slices had the advantage of providing direct visualization into the depth of the fissures. Niacin-free growth medium was placed on the occlusal surfaces of sterilized teeth or tooth slices while the root and pulpal areas were isolated and exposed to a niacin solution. The medium was inoculated with a culture of an acidogenic organism that required niacin for sustained growth. As the niacin diffused through the tooth structure, bacterial metabolism progressed and decalcified areas were formed. Previous reports discussed above[27, 104] indicate that bacterial activity occurs predominately in deep enamel fissures or pits or in natural or artificial defects where niacin can enter most

readily. The lesions that were produced were then isolated from the inoculated medium by the placement of an occlusal sealant or were left as controls. After an additional period in the in vitro system, the samples were removed and assessed for the effect of the sealant application on the continuation of the carious process.

In studies with whole teeth, lesions were induced on the mesial and distal aspects of the occlusal surface of each tooth. The pits and fissures on one side were then treated with sealant while those on the other side were left as controls. Some of the whole teeth were also prepared with a small hole drilled through the mesial and distal pits in the occlusal enamel to the depth of the dentino-enamel junction. The holes were made in order to accelerate the formation of caries-like areas in dentin, and to mechanically provide the teeth with two equally susceptible locations. After primary lesions were formed, one hole and the surrounding fissures were sealed and the other left unsealed. Studies with 750 µ sections relied on the production of carious areas in the fissures of two slices from each tooth. One slice was then treated with sealant and the other remained as an untreated control.

A rough estimation of the extent of the lesions in whole teeth prior to treatment with sealant was made on the basis of radiographs and by physical examination. Actual measurements were made at the conclusion of the study when the teeth were sectioned and stained. The lesions in tooth slices were observed and measured during their devel-

opment, at the time of treatment, and after the final incubation period.

Experimental Procedures

The teeth selected for samples were selected from human non-carious unerupted or partially erupted third molars extracted from eighteen to twenty-five year old persons. Only teeth with sound enamel and no apparent developmental defects were chosen. The teeth were obtained at the U.S. Public Health Service Hospital, San Francisco, and at the Department of Oral Surgery, Letterman General Hospital, San Francisco, California. All periodontal and pulpal soft tissues were removed, and the teeth were then prepared as whole tooth units or as tooth slice units.

Whole Tooth Units

Twenty teeth having deep pits and fissures in both mesial and distal aspects of the occlusal surface were selected from the study population, radiographed, and photographed. Each tooth was mounted in a diffusion apparatus consisting of two pieces of silicone rubber tubing* (7/16 inch i.d.). A twelve inch length of tubing was sealed to the root portion of the tooth (with silicone adhesive[+]) and a shorter length to the crown, effectively isolating the two segments (Figure 3). The unit was mounted on an eight by eight inch sheet of fiberboard with the longer

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* Silastic Tubing, Down Corning Corporation, Midland, Michigan.

[+] Medical Adhesive Silicone Type A, Dow Corning Corporation, Midland, Michigan.

Figure 4. Diffusion apparatus with tooth mounted between two sections of silicone rubber tubing.

Figure 3. Detailed view of tooth-tube connections.

tube bent into a "U" shaped configuration (Figure 4). A small amount
of water was placed into the tubes to prevent dehydration of the tooth
structure during the setting of the silicon adhesive. After twelve
hours, the system was tested for leakage by determining whether a
vacuum could be maintained within each tube segment. The tubes
were rinsed with distilled water and the open ends were covered
with stainless steel culture tube covers. The units were sterilized
in 100% ethylene oxide gas at $30^{o}C$ for three hours in a large capacity
gas sterilizer[*] and then aired for twelve hours to permit the dissipa-
tion of residual gas. Unaired units produced bactericidal effects on
the test organisms. Five milliliters of sterile niacin-free growth
medium[+] was then placed over the coronal portion of each tooth and
a sterile solution containing 100µg of niacin[#] per milliliter was plac-
ed into the lower tubes. The volume of the niacin solution was brought
to the same level as the medium in order to equalize hydrostatic
forces. If leakage was not observed at the tube-tooth interfaces, the
upper tube on each unit was inoculated with three drops of a dilute
suspension of Lactobacillus plantarum (ATCC 8014), and units were
incubated at $37^{o}C$.

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[*] Steri-vac, Minnesota Mining and Manufacturing Company, St. Paul
Minnesota.

[+] Niacin Assay Medium, Difco Laboratories, Inc., Detroit, Michigan.

[#] Calbiochem, Los Angeles, California.
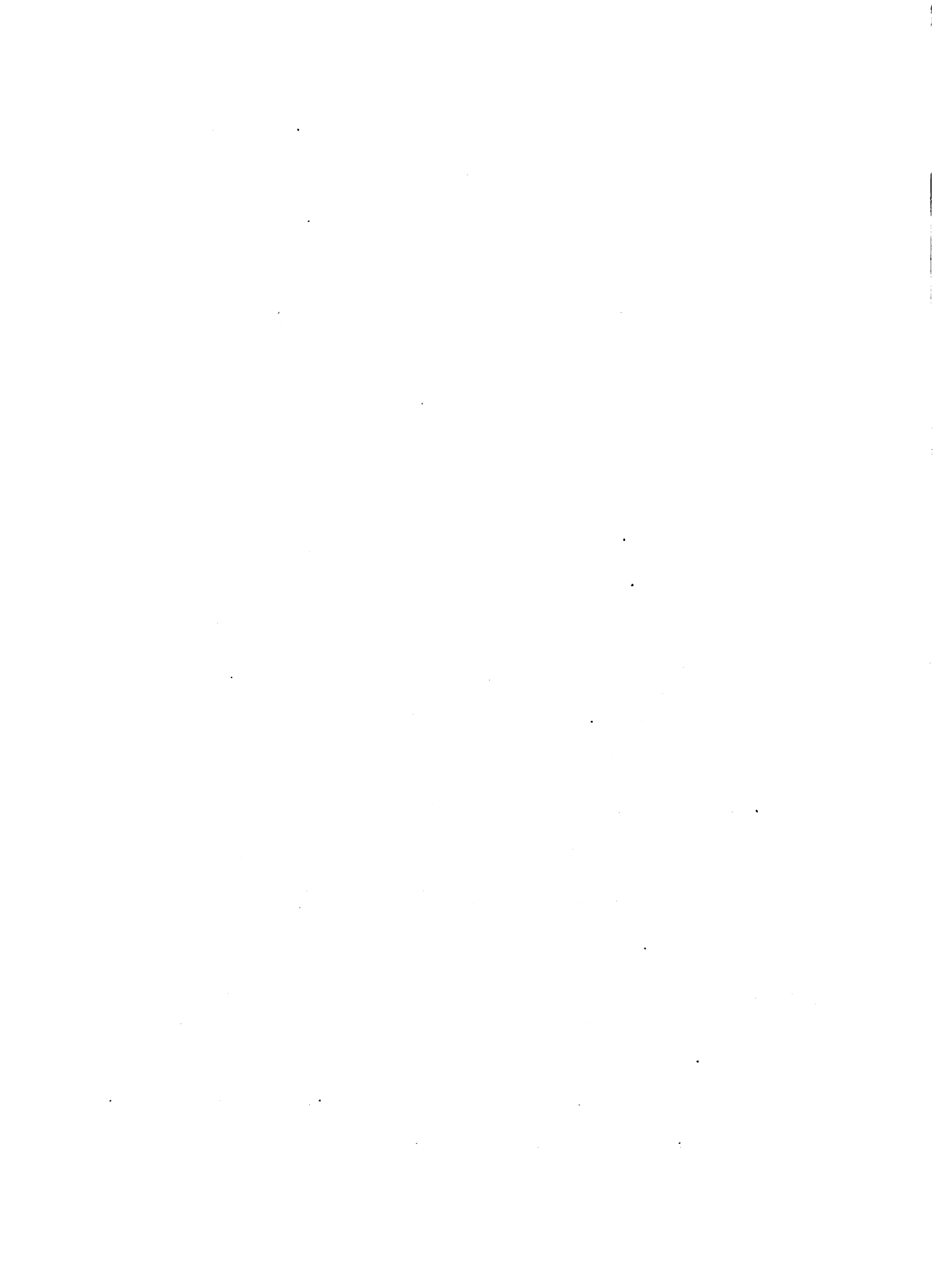
L. plantarum is a bacterial species classified in the family

Lactobacillaceae, the tribe Lactobacilleae, the genus Lactobacillus.

The organism is a gram positive, microaerophilic, homofermenta-

tive rod whose energy requirements are supplied mainly be the an-

erobic breakdown of carbohydrates.[110] Lactic acid is the principal

fermentative end product with only traces of acetic acid and earbon

dioxide being formed. Like other members of the genus, L. plan-

tarum is both acidogenic and aciduric. When the organism was

grown in a complete medium, active acid production and a terminal

pH of 3.8 to 4.0 was observed.

Inoculums were prepared for use in this study by subculturing

from a stock of L. plantarum into 10 ml of a complete growth medium[*]

and incubated for 24 hours at 37°C. The culture was then transferred

aseptically to a sterile centrifuge tube and centrifuged 30 min. at 7700

Xg. The supernatant culture fluid was decanted and the cell mass

washed with sterile isotonic sodium chloride and then centrifuged again.

After two washings, the cells were resuspended in 10 ml of sterile

isotonic saline and then diluted 1-1000. The faintly visible suspension

was used to inoculate the study samples. The bacteria were washed

in order to remove nutrients dissolved in the culture medium and those

adhering to the cells. Some essential nutrients remained within the

cells and therefore a diluted inoculum was used. A typical three drop

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[*] Micro Inoculum Broth, Difco Laboratories, Inc., Detroit, Michigan.

inoculation of the diluted cell suspension reduced the pH by less than

0. 25 units when incubated in a niacin-free medium.

Aseptic technique was strictly adhered to throughout the study

in order to avoid contamination of the medium with other microor-

ganisms. Extraneous bacteria could increase variations between sam-

ples and could lead to the failure of the in vitro caries system.

A solution containing 100µg of nicotinic acid was prepared for

use in this study. The solution was autoclaved for 15 minutes at

15 pounds per square inch pressure and stored under refrigeration

until needed.

The experimental growth medium (niacin assay medium) is

nutritionally complete for L. plantarum (ATCC 8014) except for the

absence of nicotinic acid and its analogue. It is a synthetic medium

containing a chemically defined composition based on the formula

described by Snell and Wright.[114] The freshly prepared medium

(pH 6.5) was autoclaved for 10 minutes at 15 pounds pressure (p.s.i.)

prior to use with the study samples.

All glassware, materials, and tooth samples were throughly

rinsed with distilled water prior to use with the medium to avoid

contamination with exogenous sources of niacin.

The pH of the medium in the whole tooth units was measured

electrometrically at daily intervals for the first 7 days and then at

7 day intervals for 20 weeks. The medium was changed and the teeth

rinsed with sterile distilled water, weekly. Viability of the organisms

was demonstrated by visible growth and by increases in the acidity of the medium. Niacin solution was added to the lower tube weekly to compensate for loss by evaporation. Biological assays of the potency of the niacin solution in the tubes were made periodically. This was accomplished by placing three drops of the niacin solution into a tube of inoculated niacin assay medium and observing the degree of growth. Little or no loss in niacin activity was observed. Checks of culture purity were made at monthly intervals by plating on heart infusion agar containing 5% citrated blood, and by observing stained smears of samples from each tube.

After approximately twelve weeks of incubation, the study teeth were radiographed and prepared for treatment with sealant. Previous reports[27, 115] indicated that lesions ranging from enamel etchings to dentinal involvement could be formed in twelve weeks in this type of in vitro system. The occlusal surface of each tooth was rinsed with sterile saline and wiped with a cotton applicator until all loose plaque and debris was removed. Direct access to the tooth was obtained by aseptically excising the rubber tubing approximately one-half inch above the occlusal surface (Figure 5). Care was taken to avoid contamination during the treatment procedures. All instruments used, including an explorer, spatula, mixing pad, and brushes were sterilized by autoclaving. The teeth were examined and the presence of softened decalcified areas in the pits and fissures or in other locations was noted. The occlusal surface of each tooth was divided visually

Figure 6. Study tooth remounted in silicon rubber tube.

Figure 5. Application of sealant to test area of a whole tooth.

into two roughly symmetrical segments (mesial and distal), one of

which was chosen (by the toss of a coin) to be the test area. The

segment to be sealed was etched by the one minute application of a

50% phosphoric acid mixture. The tooth was held at an angle to in-

hibit the flow of the etchant into the control area. The acid was

applied with a brush and rinsed off with copious quantities of sterile

saline. The sealant material* used was BIS-GMA type formula-

tion and consisted of a liquid monomer mixture and a catalyst. These

ingredients could not be subjected to sterilizing procedures and were

used directly as supplied. Due to their chemical composition, how-

ever, the monomer mixture and benzoin methyl ether catalyst are

highly resistant to microbial contamination and samples of the unpoly-

merized mixture produced no bacterial growth when incubated in a

complete medium. The sealant was applied with a sterile brush and

polymerized by the one minute application of high intensity ultraviolet

radiation. The treated tooth was examined to determine if polymeri-

zation was complete. If any sealant was observed in the untreated

(control) area it was removed with an explorer. The tooth was then

enclosed in a new silicone rubber tube (1/2 inch i.d.) that was fitted

over the stump of the previous segment and bonded with sterile

silicone adhesive (Figure 6). Niacin-free medium was inserted and

the units were incubated for an additional period of ten weeks (Table 1).

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* Nuva-Seal, L.D. Caulk Company, Milford, Delaware.

TABLE 1

Summary of the Whole Tooth Units Prepared and the Incubation Schedule

TYPE OF UNIT	NUMBER OF TEETH USED	INCUBATION SCHEDULE (37°C)
EXPERIMENTAL (Undrilled)	20	20 Weeks Incubation (Treatment period during 12th week)
CONTROL (Undrilled)		
a. Treated at start of study	2	20 Weeks Incubation
b. Saline Substituted for Niacin	2	20 Weeks Incubation
c. Complete Medium Used on Occlusal	2	20 Weeks Incubation
d. Medium not Inoculated	2	20 Weeks Incubation
EXPERIMENTAL (Drilled)	8	12 Weeks Incubation (Treatment period during 6th week)
CONTROL (Drilled)		
Treated at Start of Study	4	12 Weeks Incubation

At the completion of the final incubation period, the teeth were photographed, fixed in 10 percent phosphate buffered formalin (pH 6), imbedded in acrylic resin, and cut into approximately 300 - 500μ sections. The sections were studied under a dissecting microscope using reflected and transmitted light and photographed at X3 and X7 magnification. The most extensive lesions on the treated and untreated sides were classified according to the categories in Table 2. If caries-like areas were present in dentin, they were measured (depth and width) using a dissecting microscope with an eyepiece mounted filar micrometer gauge (Figure 7). The extent of dentinal caries in the treated and untreated areas was compared. Some of the sections were ground and stained with a pararosaline-potassium metabisulphite-mixture to further define the confines of the lesion.[116]

Eight additional teeth were mounted and sterilized as described above. Two of these teeth were treated with sealant prior to the first incubation period, and were then inoculated and incubated for twenty weeks. The other six teeth were not treated but were used as controls of the in vitro caries system. Two of the six teeth were not inoculated; distilled water was substituted for niacin solution in two others; and a complete growth medium was used in place of the niacin-free medium on the remaining two. These units were also incubated for a total of twenty weeks, sectioned, and examined.

In another phase of the study, two small holes were drilled into the enamel of eight whole teeth with a 1/4 round dental bur. The holes

TABLE 2

Classification of Lesions in Fissures on Treated and Untreated Sides
of Whole Tooth Units at the End of the Final Incubation Period

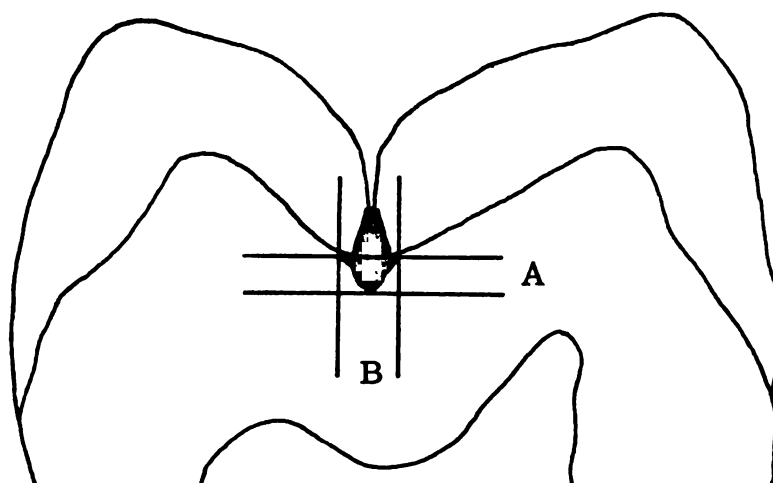| CLASS	DESCRIPTION OF LESION
O	No penetrating lesion or decalcification
E	Penetrating lesion in enamel only
D	Penetrating lesion in dentin
G	Generalized decalcification only

Figure 7. Measurements made on sections cut through treated and
untreated segments of whole tooth units.
A. Distance from dentino-enamel junction to base of decalcified
area in dentin.
B. Width of widest area of decalcification in dentin.

(approximately 0. 6 mm in diameter and 2 mm in depth) were placed

into pits on the mesial and distal aspects of the occlusal surface to

a depth just beyond the dentino-enamel junction (Figure 8). The

extent of the preparations were checked with radiographs and teeth

with holes extending more than slightly into dentin or teeth with holes

of unequal depth were eliminated from the study. The teeth were

placed into the diffusion apparatus, inoculated, and incubated for

six weeks, and radiographed. Six weeks was considered a sufficient

incubation time to produce lesions in drilled teeth. One hole and the

adjacent fissures on the occlusal of four teeth were treated with

occlusal sealant. Zinc phosphate cement was substituted for sealant

on the other samples. This material is known to permit marginal

leakage and was used for control purposes. The teeth were incubated

for an additional six weeks, sectioned, and examined. The lesions

beneath the treated and untreated areas were measured by the same

methods used for undrilled teeth.

Four other whole teeth were drilled and treated (two with seal-

ant and two with zinc phosphate cement) at the start of the study. They

were mounted, inoculated and incubated for twelve consecutive weeks,

and then sectioned, and examined.

The mean acidity of the media in the various groups of experi-

mental and control units was calculated for each time period by

averaging the antilogs of the pH units. The mean antilog values ($[H^+]$)

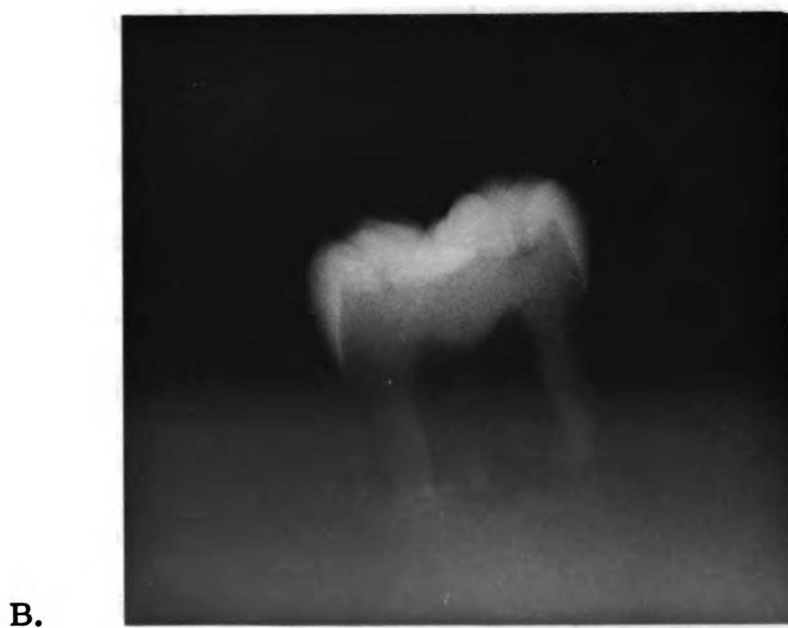were then converted to log functions and plotted on a graph.

A.

B.

Figure 8.
A. Study tooth with two holes drilled into occlusal surface.
B. Radiograph showing the extent of the preparations.

Tooth Slice Units

Twenty teeth with well developed fissures were cut into slices, approximately 750μ thick. The cuts were made buccal-lingually through the long axis of the tooth on a Gillings-Hamco hard tissue sectioning machine.[*] The device utilizes a 4 inch diameter diamond wheel (300μ thick) with a rotation speed of 5000 rev/min. Only teeth that provided a minimum of three slices containing moderate or deep non-carious fissures were used. The slices were examined under a dissecting microscope for areas of decalcification or discoloration (dark spots in transmitted light) in the fissures. The presence of these areas was noted, and only slices with slight or no fissure de-calcification or discoloration were used.

The cut sides of the slices were etched with a 50% phosphoric acid solution and treated with the same adhesive resin used in this investigation to seal pits and fissures. The sealant was applied care-fully in several increments under a dissecting microscope in order to insure that all cut surfaces were covered and that no material had spread into the occlusal fissures. Although the sealant is less reten-tive to dentin than to enamel, no diffusion of liquid was observed at the adhesive-dentin interface under the conditions of this study. The pulp chamber of each tooth slice was then enclosed between two 18 by 18 mm (250μ thick) plastic cover-slips. A water tight cell was formed by flow-

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[*] Hamco Machines, Inc., Rochester, New York.

ing dental sticky wax around the edges of the plastic segments and between the plastic and the tooth slices (Figure 9). A small opening was left at the base of the cell for the addition of solutions.

Two slice units from the same tooth were suspended (pulpal side up) within a 23 by 150 mm test tube. This was accomplished by attaching one end of a stainless steel wire to the wax at the base of each unit and bending the other end over the lip of the tube (Figure 10). Twenty tubes, each containing a pair of units were prepared. The tubes were covered with stainless steel culture tube covers. The tooth slice units were sterilized in 100% ethylene oxide gas at $30^{o}C$ for three hours and then aired for 12 hours. Approximately 2 ml of a sterile solution of niacin (100 µg/ml) was aseptically injected into the pulp area of each unit with a tuberculin syringe, and the cell was sealed (Figure 11).

The tubes were filled with sufficient niacin-free medium to completely cover the tooth slices (approximately 30 ml) and were incubated at $37^{o}C$. Samples taken from each tube and placed into complete medium were also incubated. If no growth was observed after 24 hours, the units were assumed to be sterile and were inoculated with three drops of a dilute suspension of L. plantarum.

A third slice unit from each study tooth was placed into a separate test tube. Twenty tubes containing these single slices were prepared according to the procedures outlined above but were not inoculated with the organism. These units were used in order to determine the

Figure 10. Test and control tooth slice units suspended in a test tube.

Figure 9. Tooth slice with pulp area enclosed in plastic cell.
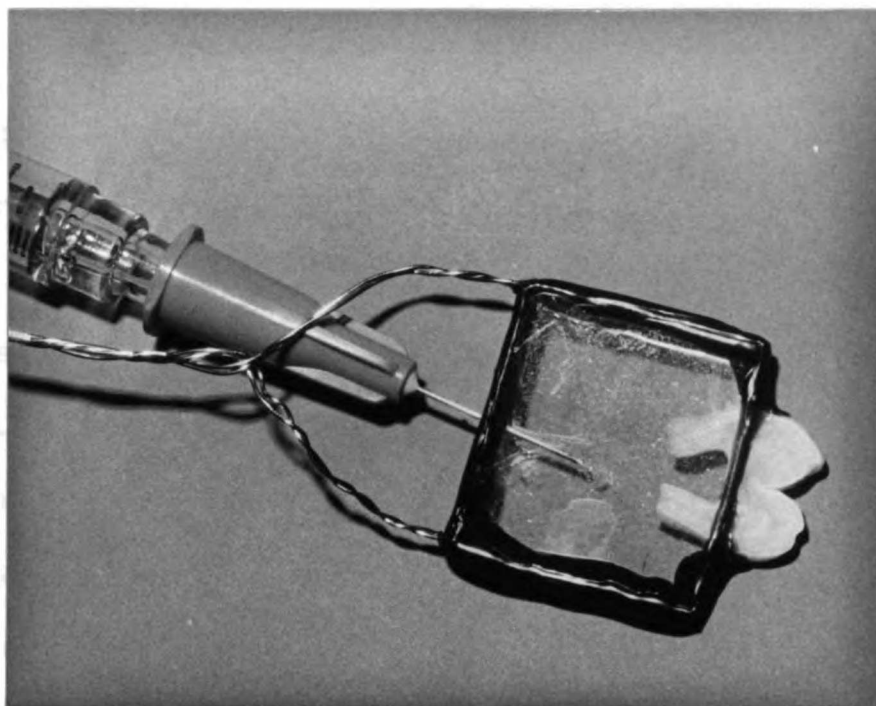
Figure 11. Placement of niacin solution into pulp chamber of a tooth slice.
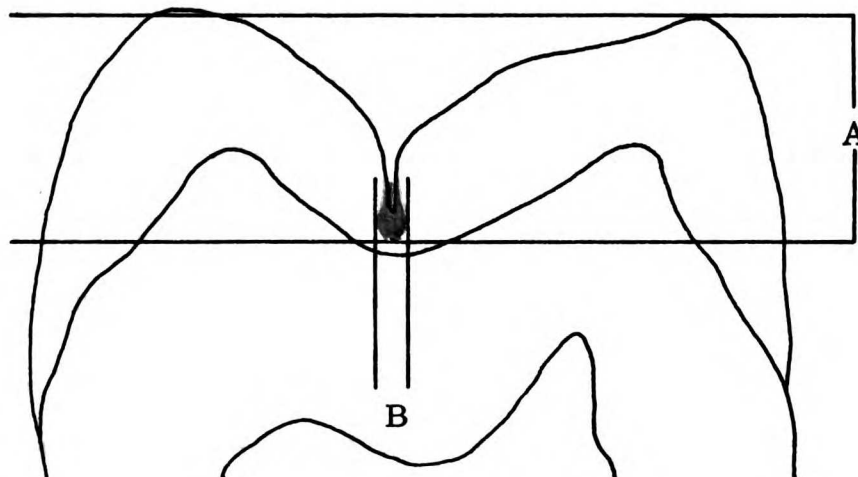
Figure 12. Measurements made on each tooth slice at the treatment period and at the end of the final incubation period.
A. Distance from a line connecting two cusp tips to the base of a lesion (if any) below the fissure.
B. Width of the widest area of decalcification associated with the fissure base.

effect of the solutions alone on the tooth slices. Four additional

tubes containing single units were prepared as controls for the in

vitro caries system. Saline was used in place of niacin solution in

the pulp chamber of two of the units while the other two units were

immersed in a complete growth medium in place of the niacin-free

medium.

The units were incubated for approximately eighteen weeks at

37°C (Table 3). An assay of the viability and purity of the organisms

in the medium were made every four weeks in a manner similar to

that used with the whole tooth units. The growth medium was changed

twice monthly and the niacin solution at monthly intervals. The pH

of the medium was recorded weekly. At two to three week periods

throughout the study, the tooth slices were studied under a dissecting

microscope for changes in appearance.

Ten weeks following the start of the incubation period, the

twenty pairs of tooth slices comprising the experimental group were

examined and photographed. If caries-like lesions were present in the

enamel fissures, they were measured according to the criteria in

Figure 12, and the area of involvement recorded (depth and width).

One tooth slice from each pair was randomly selected (by the toss of

a coin) for treatment with occlusal sealant. Aseptic techniques

similar to those used during the application of sealant to the whole

tooth units were used. The tooth slice to be treated was placed on a

sterile towel and washed several times with saline. The occlusal

TABLE 3

Summary of the Tooth Slice Units Prepared and the Incubation Schedule

TYPE OF UNIT	NUMBER OF TUBES USED	INCUBATION SCHEDULE (37°C)
EXPERIMENTAL (Two slices/Tube)	20	18 Weeks Incubation (Treatment Period During 10th Week)
CONTROLS (One slice/Tube)		
a. Medium not Inoculated	20	18 Weeks Incubation
b. Saline Substituted for Niacin in Pulp Chamber	2	18 Weeks Incubation
c. Complete Medium Placed in Tube	2	18 Weeks Incubation

surface was wiped thoroughly with a cotton pellet, examined with an explorer, and then etched with a 50% phosphoric acid solution. The surface was rewashed and dried. Sealant was applied to the occlusal pits and fissures and polymerized by ultraviolet radiation. The treated slices were returned to the tube and incubated for an additional eight weeks.

At the completion of the final incubation period, the slices were studied under the dissecting microscope and caries-like areas were measured as previously described. Changes in the extent of the decalcification process in the treated and untreated slices were compared. The tooth slice pairs were classified according to the criteria in Table 4. Lesions were considered to have advanced if the length or the width of the decalcified zone increased by 0.100 mm or more between the two measurement periods. This adjustment factor was used to compensate for measurement error using the micrometer eyepiece which was observed to range from zero to ± 0.040 mm.

Photographs were taken of the fissure areas of the 750µ sections at X3 and X7 magnification on a metallurgic microscope[*] using a high intensity fiber optic light source.[+] At the conclusion of the above procedures, ground sections were made of selected slices to allow better visualization and photography of the fissure areas.

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[*] Unitron Metallograph, Unitron Instrument Company, Newton Highlands, Massachusetts.

[+] American Optical Company, Southridge, Massachusetts.

TABLE 4

Classification of Tooth Slice Pairs According to the Status of Lesions
in Fissures at the End of the Final Incubation Period

| CLASS | STATUS OF LESIONS* | |
	TREATED SLICE	UNTREATED SLICE
I	Not Advanced	Advanced
II	Not Advanced	Not Advanced
III	Advanced	Advanced
IV	Advanced	Not Advanced

* A fissure lesion is considered "advanced" if there is an increase
of 0.100 mm or more in the length or width of the area of involvement
between the treatment period and the end of the final incubation period.

The mean acidity of the medium in the experimental units and of the medium in the various groups of control units was determined for each time period as described above. The values were plotted and compared.

V. OBSERVATIONS

Whole Tooth Units

The effect of the diffusion of niacin from the pulp chamber of whole teeth was reflected by increases in the mean acidity of the media in the upper segment of the diffusion apparatus. The pH changes for the drilled and undrilled experimental and control units during the first week of incubation are presented in Figure 13. When saline rather than niacin was diffused through the tooth, bacterial growth was minimal and decreases in pH were only slightly more than those observed in uninoculated medium. More rapid and extensive declines in pH were observed in the experimental units. The mean acidity of the medium in the eight units with drilled teeth was observed to be higher than the mean value for the medium in the twenty units with intact teeth. The steepest pH gradient was observed in control units which contained inoculated complete growth medium in the upper tube in place of niacin-free medium. Control units treated at the start of the study displayed pH changes similar to those observed in experimental units.

The pH of the medium in the undrilled experimental units fell to an increasingly lower level through six weekly test periods but then changed at a constant rate throughout the remainder of the study. The pH of the medium in drilled teeth changed at a constant rate after three weeks. The mean pH of the growth media in whole tooth units at the
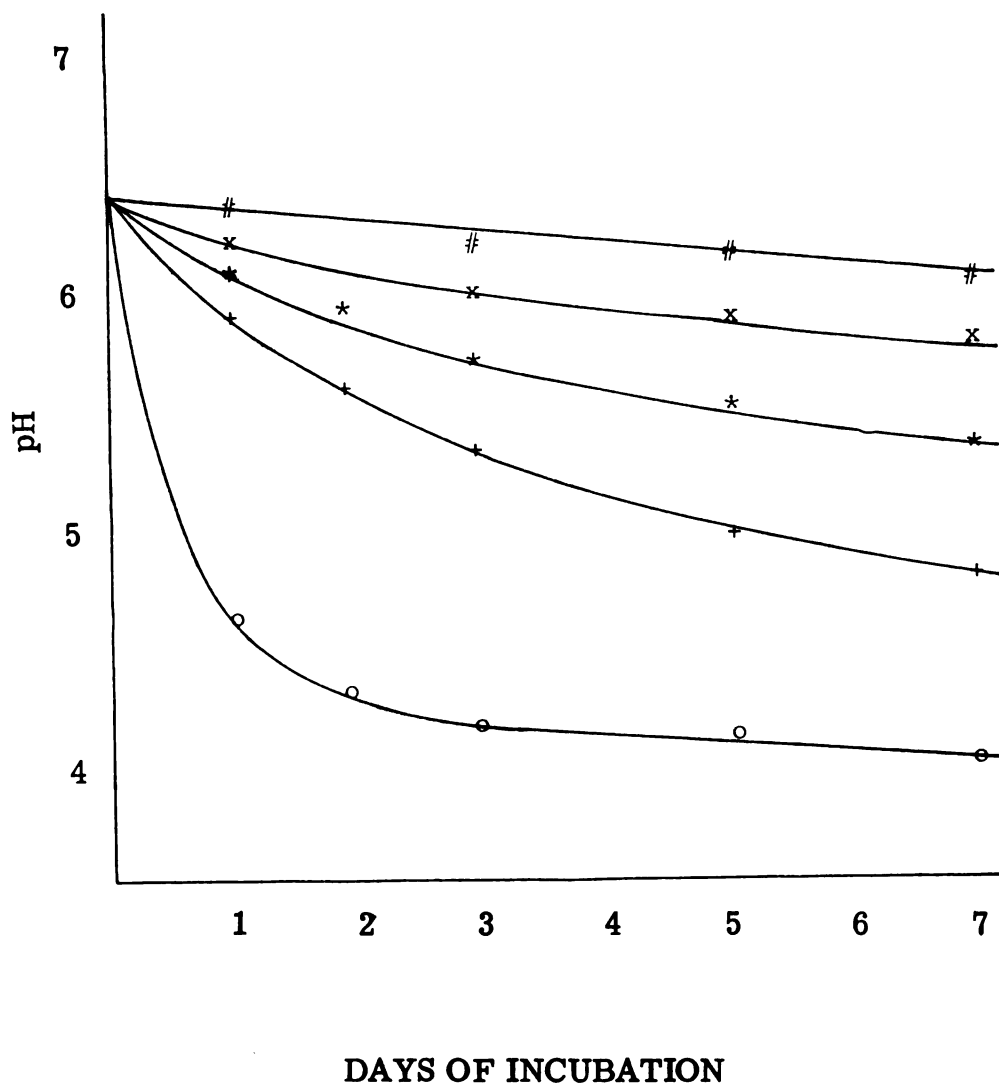
DAYS OF INCUBATION

Figure 13. The mean pH of the growth media of whole tooth units during the first week of incubation.
#: medium from uninoculated control units.
x: medium from units in which saline was substituted for niacin.
*: medium from experimental units (undrilled).
+: medium from experimental units (drilled).
o: sample from units containing complete inoculated growth medium.

end of weekly intervals during the twenty weeks of incubation is presented in Figure 14. The mean pH for the medium in experimental units with drilled teeth after the third week of incubation was approximately 4.5 with individual values ranging from 4.3 to 4.8. The mean pH for the medium in the twenty experimental units with intact teeth was slightly higher (4.7), with a range of values of 4.3 to 5.0. No differences were noted in the rate or extent of pH change in the media after the treatment period.

Radiographic evidence of lesions resembling caries in undrilled teeth was limited. Only four of the twenty experimental teeth (and none of the controls) had distinct radiolucent areas in dentin after twenty weeks of incubation. Gross radiolucencies, however, were observed radiating into dentin from the base of the holes in drilled teeth. The caries-like areas were present under both holes of all eight units scheduled for treatment after six weeks of incubation. At the end of the final incubation period, increases in the size of the existing radiolucent areas was observed. There was no apparent inhibitory effect by either the sealant or the zinc phosphate cement placed after the first incubation period. Radiolucent areas were also present beneath the filled and unfilled holes in the teeth treated at the start of the study with zinc phosphate cement. Teeth treated with sealant at the baseline period, however, showed radiolucencies only under the unfilled holes.

The occlusal surface characteristics of the drilled and intact

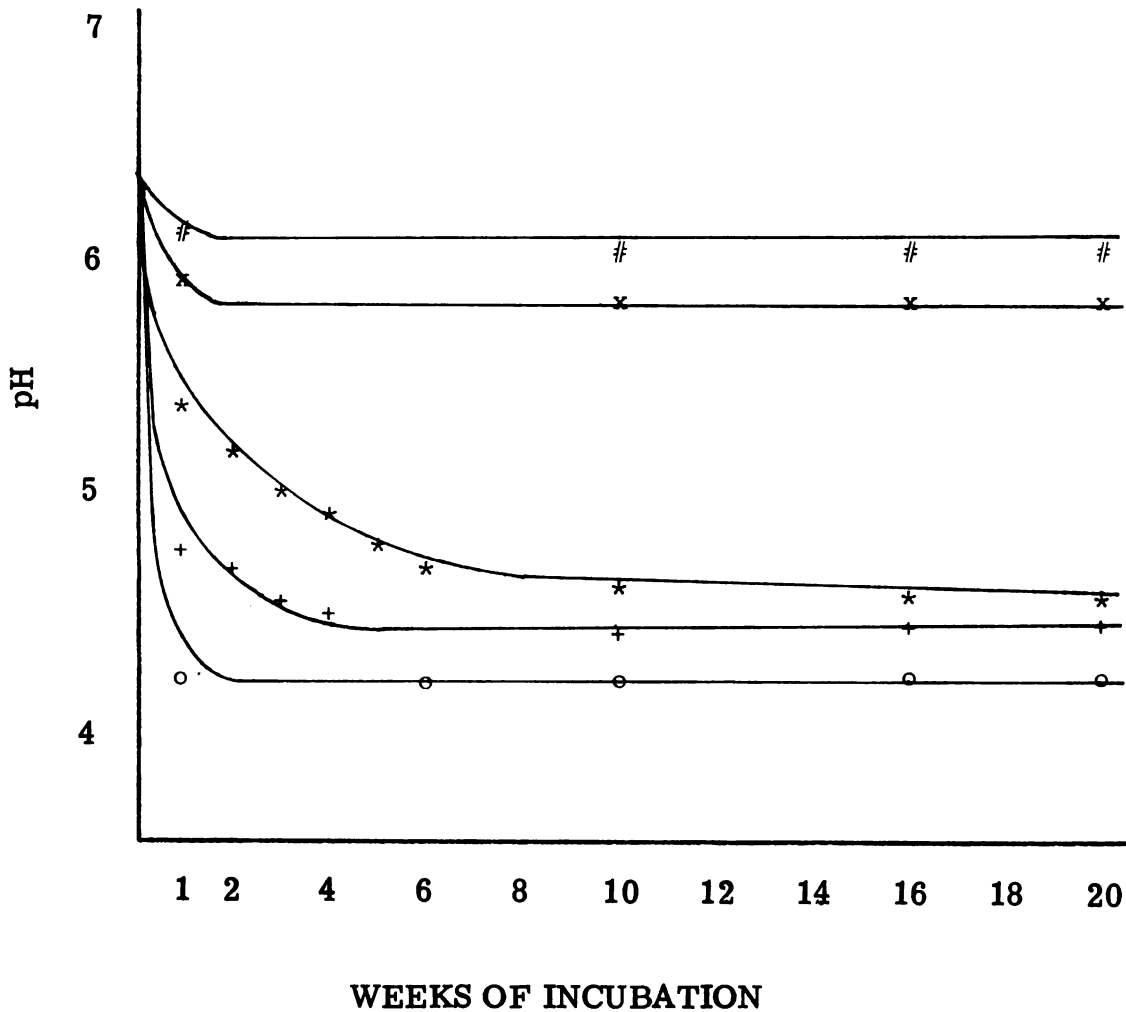Figure 14. The mean pH of the growth media of whole tooth units of the end of weekly periods during twenty weeks of incubation.

#: medium from uninoculated control units.

x: medium from units in which saline was substituted for niacin.

*: medium from experimental units (undrilled).

+: medium from experimental units (drilled).

o: samples from units containing complete inoculated growth medium.

teeth ranged in appearance from normal or slightly etched to generally demineralized when examined visually (with the aid of an explorer) at the end of the final incubation period. Microscopic examination (at X20) of the sections prepared from the whole teeth revealed the presence of penetrating areas of demineralization in the deep fissures of sixteen of the twenty intact study teeth and in the dentin beneath the holes in drilled teeth. The areas were also observed beneath the holes of drilled teeth treated with zinc phosphate cement at the start of the study. Only generalized demineralization was observed on four of the intact teeth. The enamel and dentin beneath sealant placed on intact or drilled teeth before the initiation of the first incubation period appeared normal.

Control teeth which were incubated in uninoculated medium, or those in which saline was used as a diffusant in place of niacin, displayed no surface demineralization on gross or microscopic examination. The enamel of control teeth incubated in complete medium, however, was almost completely destroyed and no specific areas of penetration were present. Examples of the enamel surface characteristics and lesions observed in the experimental and control teeth are presented in Figure 15.

The demineralized areas on the treated and untreated sides of the twenty experimental whole teeth were evaluated and classified according to the criteria described in Table 1. The results are presented in Table 5. In twelve of the samples, lesions on the treated

Section from experimental tooth.
Lesion in enamel only.
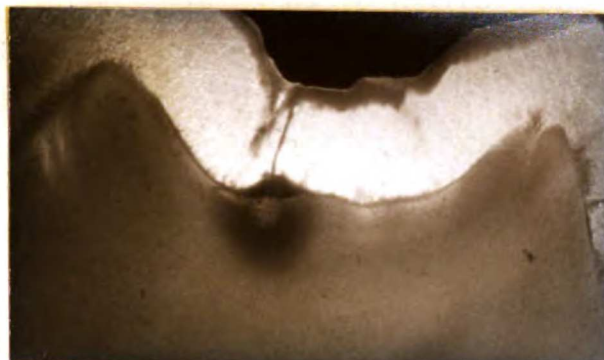
Section from experimental tooth.
Penetrating lesion in dentin.

Section from control tooth;
saline substituted for niacin.
No lesions or demineralization.

Section from control tooth;
complete medium used.
Generalized demineralization only.

Figure 15.   Examples of the effects produced in experimental and
control teeth in vitro.

TABLE 5

Classification of Lesions in Fissures on Treated and Untreated Side of Undrilled Whole Teeth at the End of the Final Incubation Period

WHOLE TOOTH NUMBER	TREATED SIDE CLASS				UNTREATED SIDE CLASS			
	O	E	D	G	O	E	D	G
1				X				X
2			X				X	
3			X				X	
4		X					X	
5			X				X	
6		X					X	
7				X				X
8		X					X	
9				X				X
10				X				X
11		X					X	
12		X					X	
13		X					X	
14		X					X	
15			X				X	
16		X					X	
17		X					X	
18		X					X	
19		X					X	
20		X					X	

side were relatively shallow and limited to the enamel, whereas the decalcified areas on the untreated side extended past the dentino-enamel junction (Figure 16). In four teeth, the lesions were restricted to enamel on both the treated and control side. These samples, however, were grossly decalcified and displayed no evidence of penetration of the decalcification process; they therefore did not contribute to data analysis. The lesions in four teeth extended into the dentin on both the treated and untreated sides (Figure 17). The extent of the lesions in these teeth was measured according to the method described in Figure 7. No appreciable difference between the extent of the dentinal lesions on the treated and untreated side was observed (Table 6). Of the sixteen teeth contributing to data analysis, arrestment of the caries-like process was considered to have occurred in twelve, while the other four were considered treatment "failures". This result is not statistically significant at the five percent point, as evaluated by the binomial test.

Measurements were also made of the caries-like areas in dentin in the twelve experimental and control teeth treated with sealant or zinc phosphate cement (Table 7, 8). Examples of the type of lesions observed are presented in Figure 18. No essential difference in the extent of the lesions beneath filled or unfilled holes was observed in the experimental teeth treated with either material. The extent of the caries process also appeared similar beneath both holes of the teeth treated with zinc phosphate cement at the start of the study. Teeth
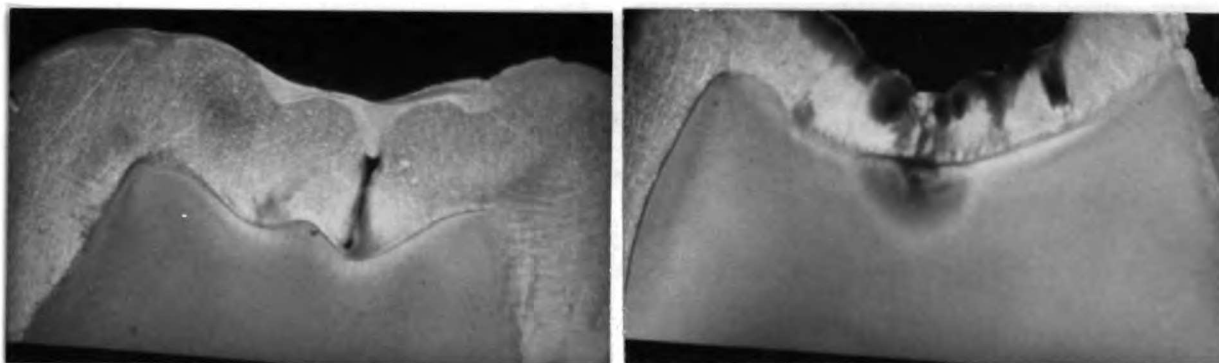
Section from treated side.   Section from untreated side.

Figure 16. Sections from an undrilled whole tooth. Lesion on treated side limited to enamel while lesion on untreated side has extended into dentin.
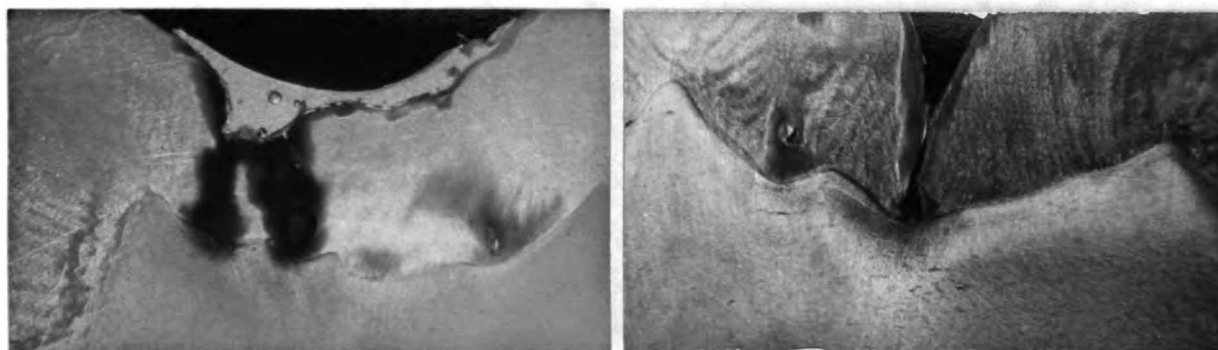
Section from treated side.   Section from untreated side.

Figure 17. Sections from an undrilled whole tooth. Lesions on both the treated side and untreated side have extended into dentin.

TABLE 6

Measurements (mm) of the Extent of Caries-like Lesions in Dentin
on Treated and Untreated Sides of Undrilled Whole Teeth

WHOLE TOOTH NUMBER	EXTENT OF CARIES-LIKE LESIONS IN DENTIN*			
	TREATED SIDE		UNTREATED SIDE	
	DEPTH	WIDTH	DEPTH	WIDTH
1	0.44	0.78	0.37	0.59
2	1.30	0.97	1.37	1.14
3	1.08	1.56	0.80	1.42
4	0.76	0.84	0.74	1.49
MEAN	0.88	1.06	0.84	1.16

* See Figure 7. for measurement methods.

TABLE 7

Measurements (mm) of the Extent of Caries-like Lesions in Dentin
on Treated and Untreated Sides of Drilled Whole Teeth
Treatment Material: Sealant

| WHOLE TOOTH NUMBER | EXTENT OF CARIES-LIKE LESION IN DENTIN[*] | | | |
| | TREATED SIDE | | UNTREATED SIDE | |
	DEPTH	WIDTH	DEPTH	WIDTH
D1	0.68	1.18	1.16	1.34
D2	0.36	0.93	0.57	0.99
D3	0.74	1.25	0.68	1.37
D4	1.25	1.44	1.03	1.39
MEAN	0.86	1.20	0.76	1.27
CONTROLS[+]				
1	0.00	0.00	1.16	1.05
2	0.00	0.00	1.01	1.37

* See Figure 7. for measurement methods.
+ Treated prior to the initiation of incubation.

TABLE 8

Measurements (mm) of the Extent of Caries-like Lesions in Dentin
on Treated and Untreated Sides of Drilled Whole Teeth
Treatment Material: Zinc Phosphate Cement

WHOLE TOOTH NUMBER	EXTENT OF CARIES-LIKE LESIONS IN DENTIN[*]			
	TREATED SIDE ($ZnPO_4$)		UNTREATED SIDE	
	DEPTH	WIDTH	DEPTH	WIDTH
D 5	0.36	1.08	0.59	1.03
D 6	1.14	1.22	1.10	1.56
D 7	0.61	1.20	0.86	1.08
D 8	1.41	1.08	0.89	1.22
MEAN	0.88	1.15	0.86	1.22
CONTROLS[+]				
1	0.27	0.86	0.53	0.99
2	0.86	1.08	0.93	1.14

[*] See Figure 7. for measurement methods
[+] Treated prior to the initiation of incubation.
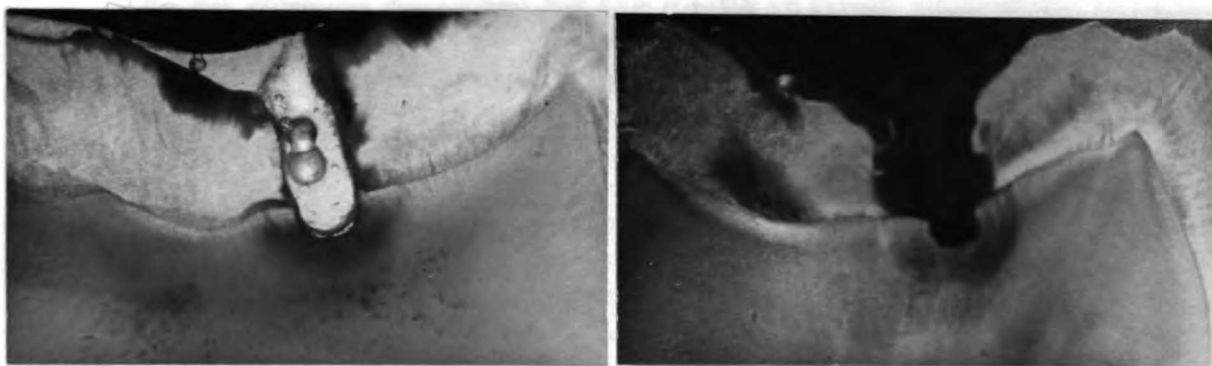
Section from treated side.   Section from untreated side.
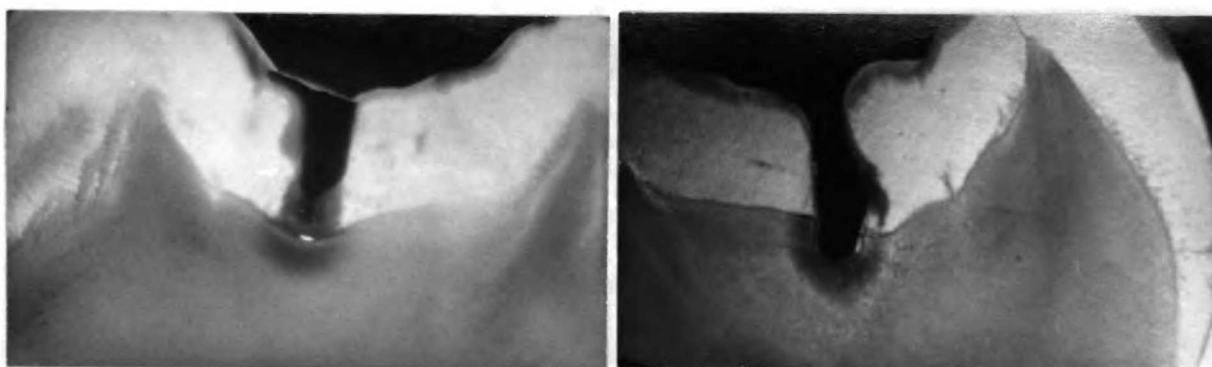
Treatment Material: Sealant

Section from treated side.   Section from untreated side.

Treatment Material: Zinc Phosphate Cement

Figure 18. Sections from experimental drilled whole teeth. Lesions on both the treated and untreated side have extended approximately the same distance into dentin.

treated with sealant at the start of the study, however, demonstrated demineralizations only beneath the unfilled hole.

Tooth Slice Units

The pH changes recorded for the media in tubes containing tooth slices followed the same general pattern as was observed with whole teeth (Figure 19). The actual pH values for the tubes with control slices were essentially the same as those observed in the corresponding whole tooth controls. The mean acidity, however, was lower in the medium surrounding the experimental tooth slices than in the medium in the experimental whole tooth units. The medium in the tubes with experimental samples had a mean pH of approximately 5.4 while individual values among the twenty tubes ranged from 5.3 to 5.8.

None of the tooth slices comprising the twenty experimental pairs demonstrated signs of gross generalized demineralization at the time of treatment with the sealant or at the end of the final incubation period. The enamel on the occlusal cusps and the inclined planes of the samples in seven tubes, however, was slightly etched and had an opaque or brown stained appearance. The tooth slices placed into uninoculated medium and those whose pulp chamber contained saline rather than niacin, showed essentially no changes in surface or internal characteristics over the entire study period. The tooth slices placed into complete medium, on the other hand, were extensively demineralized with only small traces of translucent enamel remaining.
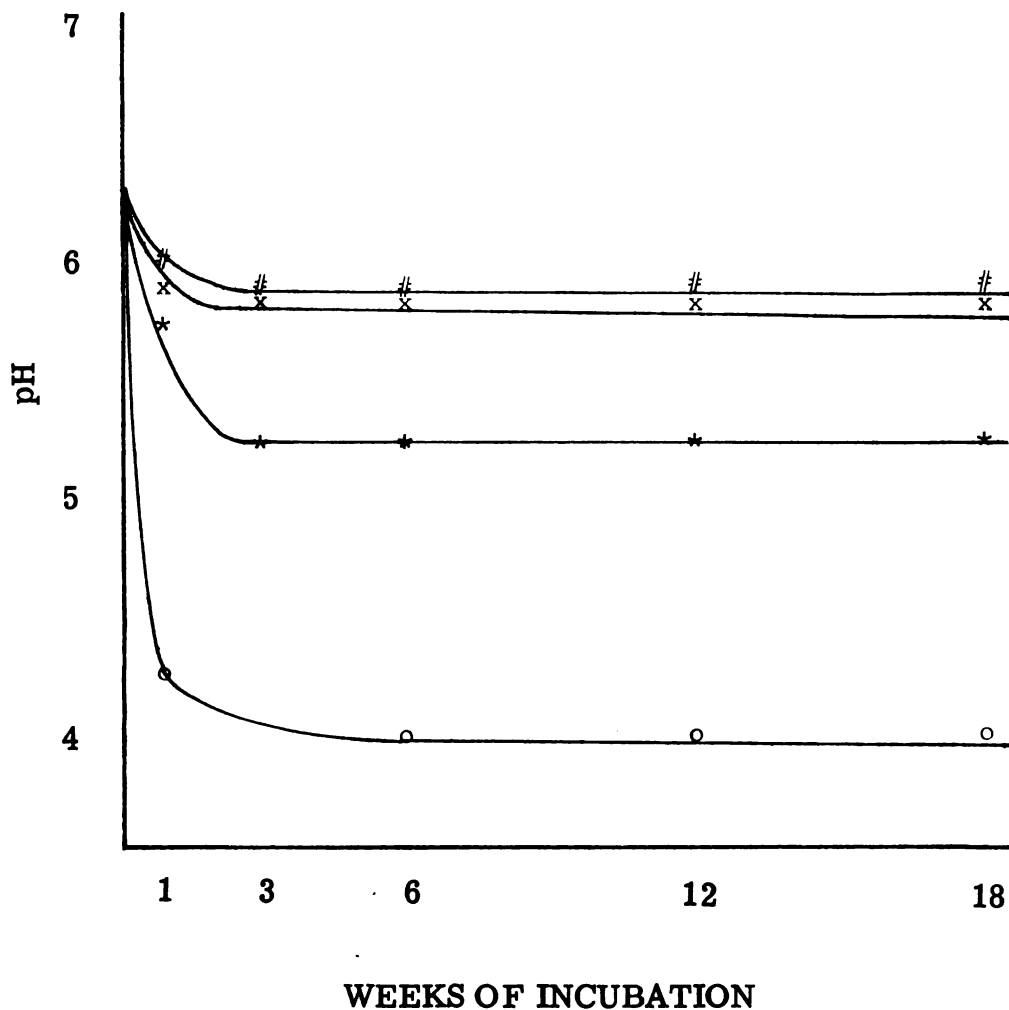
Figure 19. The mean pH of the growth media in tubes with tooth
slices at the end of weekly periods during eighteen weeks of incubation.
#: medium from uninoculated control tubes.
x: medium from tubes with saline control units.
*: medium from tubes with experimental units.
o: medium from control tubes with complete medium.

There was no evidence of selective penetration in these samples, and the lesions had no resemblance to true caries.

Localized areas of demineralization within deep fissures were observed in all twenty pairs of experimental tooth slices after the first ten weeks of incubation. The demineralized areas observed ranged from 0. 02 mm to 1. 65 mm in width and extended from the base or sides of the fissures. The lesions were limited to the enamel or to the immediate area of the dentino-enamel junction. Although changes in the size of the decalcified areas during the final incubation period were small, four cases of dentinal penetration were observed in untreated slices at the end of the study. Photographs of the 750 μ sections demonstrating the variety of effects observed in test and control samples are presented in Figures 20-22.

The results of measurements made of lesions appearing in the tooth slices at the treatment period and at the end of the final incubation period are presented in Table 9. The measurements were made according to the method described in Figure 12. The mean change in the depth of the fissure decalcification in the treated tooth slices was +0. 04 mm and the mean change in width was +0. 02 mm. In un-treated slices, the mean changes were +0. 24 mm and +0. 19 mm respectively.

The tooth slices were classified as presented in Table 10. Sixteen of the twenty tooth slice pairs were placed in classification I. In these cases, advancement of the decalcified fissure area was
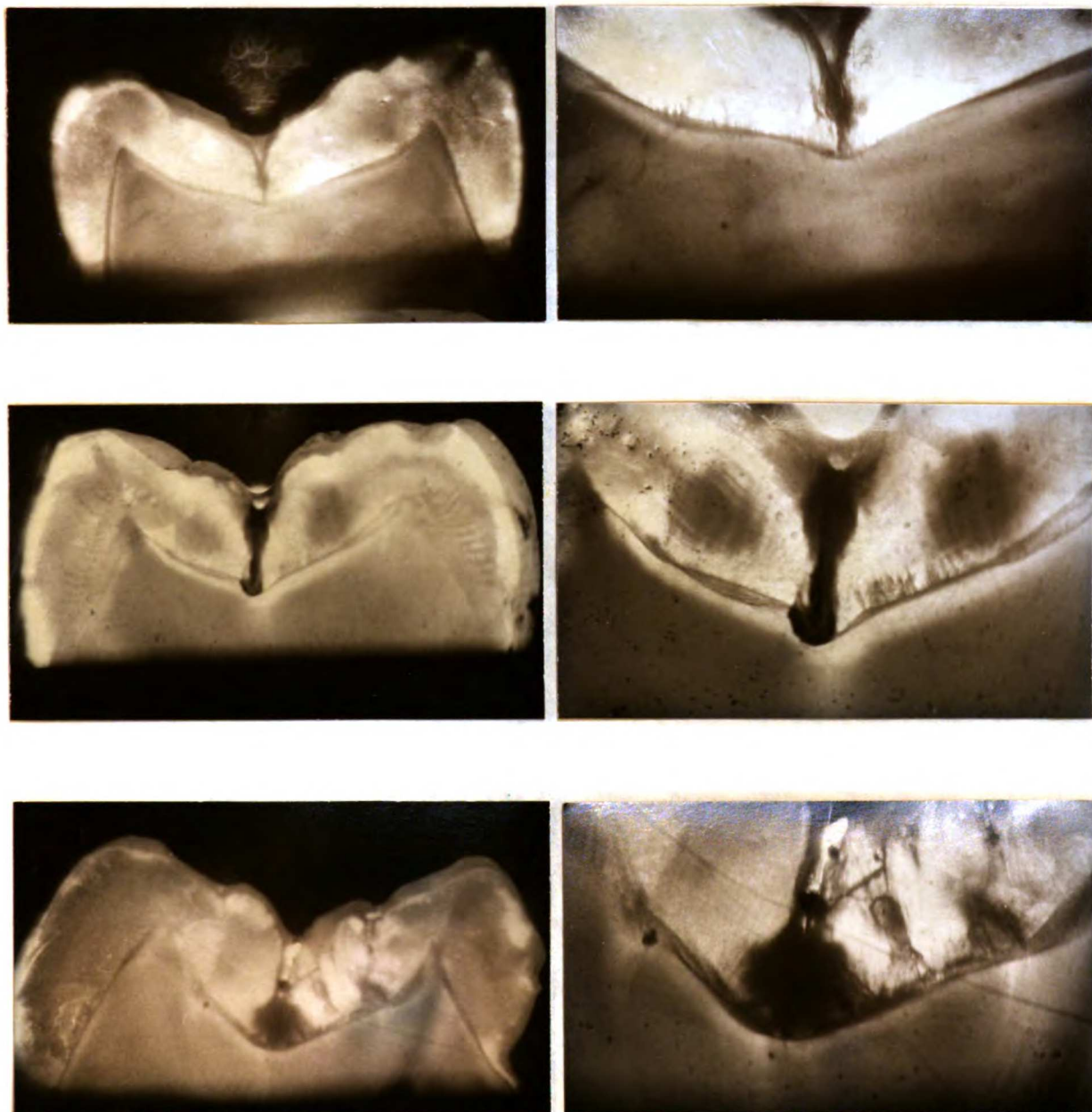
Column A   Column B

Figure 20.   Examples of caries-like areas formed in enamel in 750_μ tooth slices.   Photographs taken through test tube.   (Final magnifications: Column A approx. X10, Column B approx. X30).
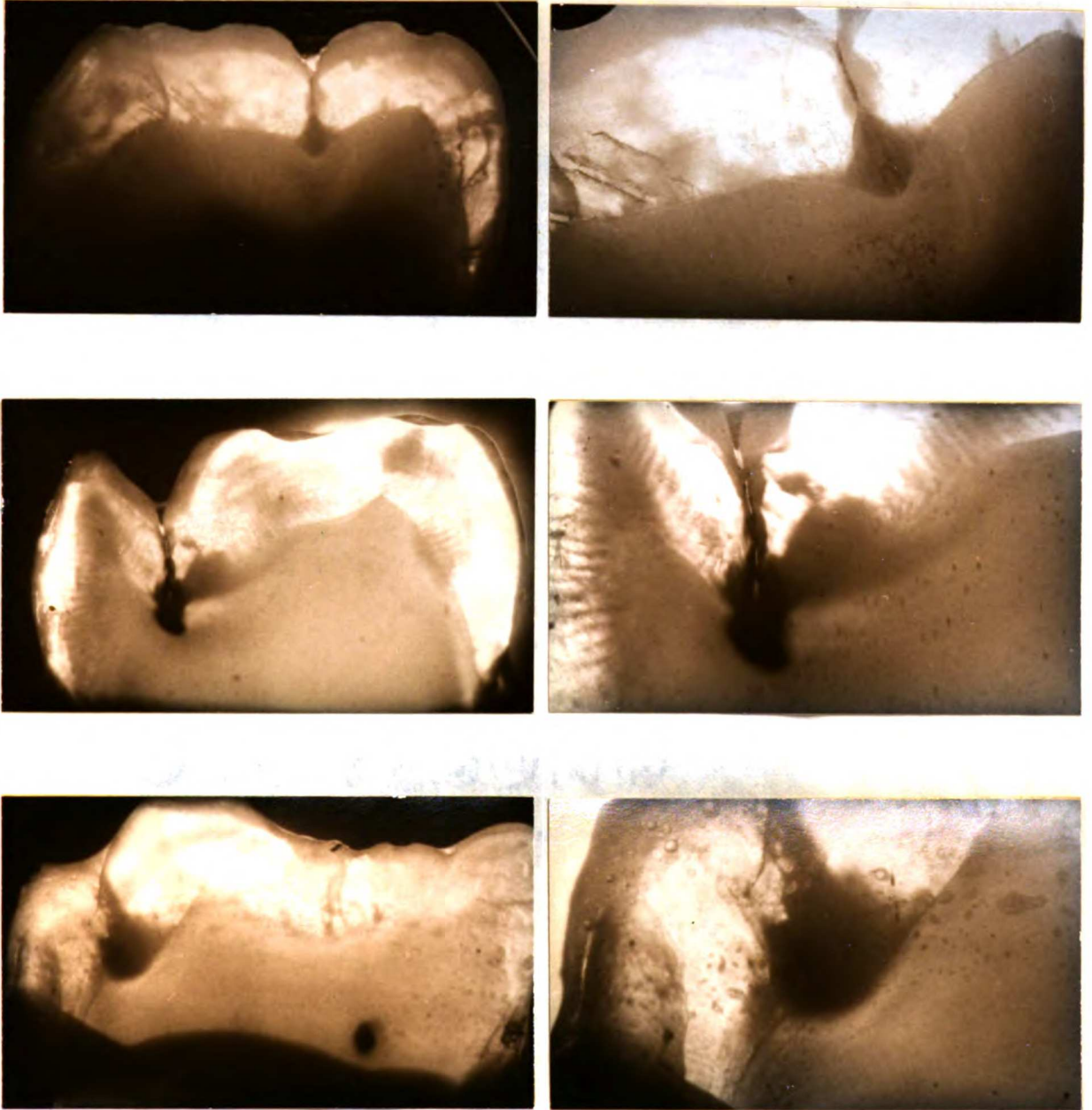
Column A   Column B

Figure 21.   Examples of caries-like areas formed in dentin in 750μ tooth slices.   Photographs taken through test tube.   (Final magnifications: Column A approx X10, Column B approx. X30).

A.

B.

Figure 22A.   Control tooth slice unit incubated in complete medium.
Figure 22B.   Control tooth slice unit incubated in uninoculated
medium.

TABLE 9

Increase in the Extent (Length and Width) of Caries-like Lesions in Treated and Untreated Tooth Slices Between the "Treatment Period" and the End of the "Final Incubation Period"

| TOOTH NUMBER | INCREASE IN THE EXTENT OF CARIES-LIKE LESIONS (mm)[*] | | | |
| | TREATED SLICE | | UNTREATED SLICE | |
	DEPTH	WIDTH	DEPTH	WIDTH
1	0.02	0.02	0.20	0.13
2	0.00	0.04	0.08	0.04
3	0.00	0.00	0.15	0.13
4	0.00	0.06	0.43	0.23
5	0.06	0.00	0.14	0.16
6	0.02	0.02	0.38	0.10
7	0.04	0.00	0.23	0.15
8	0.00	0.04	0.17	0.42
9	0.02	0.00	0.17	0.21
10	0.15	0.00	0.68	0.13
11	0.06	0.02	0.48	0.15
12	0.04	0.02	0.27	0.21
13	0.00	0.02	0.28	0.16
14	0.02	0.00	0.27	0.13
15	0.23	0.02	0.00	0.76
16	0.00	0.00	0.27	0.17
17	0.04	0.00	0.04	0.02
18	0.04	0.00	0.13	0.15
19	0.04	0.00	0.48	0.25
20	0.00	0.02	0.00	0.02
MEAN	0.04	0.02	0.24	0.19

* See Figure 12. for measurement methods

TABLE 10

Summary of the Characteristics of In Vitro Lesions in Twenty Pairs
of Tooth Slices at the End of the Final Incubation Period

CLASS*	NO. OF PAIRS	EXTENT OF LESIONS			
		TREATED SLICE		UNTREATED SLICE	
		ENAMEL ONLY	DENTIN	ENAMEL ONLY	DENTIN
I	16	16	0	12	4
II	3	3	0	3	0
III	1	1	—	1	—
IV	0	—	—	—	—
TOTAL:	20	20	0	16	4

*   See Table 3 for classification system.

observed in the untreated samples but no corresponding increase was seen in the slices treated with sealant. Both the treated and untreated slices in three tubes demonstrated no changes in the size of the fissure lesions for the period surveyed. In one tube, both the treated and untreated tooth slices from one tooth had lesions that appeared "advanced". The results were analyzed by the binomial test and were statistically significant beyond the 99 percent level of confidence ($p < 0.01$). The sealant appeared to be well retained in all cases. Ground sections of treated slices indicated that the material was well adapted to the fissure walls and had penetrated to the fissure base (Figure 23).
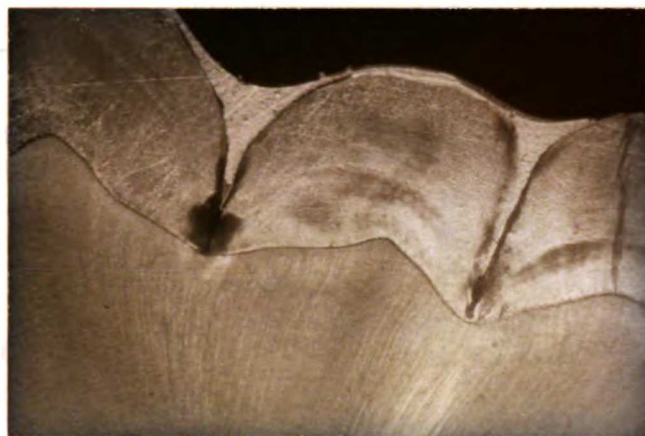
Figure 23. Ground sections of treated tooth slices. (Final magnification approx. X30).

VI. DISCUSSION AND INTERPRETATION OF THE RESULTS

Dynamics of the In Vitro Caries System

The pH values observed for the inoculated media in the exper-
imental and control whole tooth units were a function of the variable
concentrations of available niacin. The terminal acidity level (pH
4. 0) was rapidly reached when a complete medium was used. The
pH values measured in the niacin-free medium reflect the levels of
diffusion of the growth factor from the pulp chamber of the mounted
teeth. With drilled teeth, the niacin diffusion rate appeared constant
after the first two to three weeks of the study. The diffusion rate in
units with intact teeth increased gradually over a six week period
and then became constant. Presumably, the surface decalcifications
that occurred during the first weeks of incubation enhanced the per-
meability of the enamel. At the end of weekly incubation periods
throughout the study, however, the pH of the medium in the experiment-
al units was still considerably higher than in control units containing
complete medium.

The minimal growth capabilities of L. plantarum without regular
sources of niacin, and the inability of tooth structure to provide the
nutritional requirements for the organism, were indicated by the
small increases in acidity noted when saline was used as the diffusant.

Higher pH values were observed in the medium in the tubes with
experimental tooth slices than in the corresponding whole tooth units.

This difference can be attributed to the small surface area available for niacin diffusion in the tooth slices, and the higher concentration of niacin-free medium in the tubes (30 ml vs. 5 ml). The maximum acidity level was also reached, however, when complete medium was utilized in place of niacin-free medium.

Strains of L. plantarum with similar properties to the ATCC 8014 variety are widely distributed in nature and have been reported to account for approximately three per cent of the total lactobacilli concentrations in the human mouth. Lactobacilli comprise less than one per cent of the bacterial flora of saliva or plaque, but they are usually the predominant organisms in carious dentin. The precise role of lactobacilli in caries however remains unclear. Although other organisms may be more important in the initiation of the carious process, it is likely that lactobacilli contribute to caries extension and development due to their acidogenic and aciduric characteristics.[117]

Characteristics of the Lesions Produced

The lesions that developed in the majority of the undrilled whole teeth had certain similarities to naturally occurring caries. The demineralized areas in enamel fissures appeared to penetrate inward following the direction of the enamel rods, and then spread along the dentino-enamel junction. Penetrations into dentin were observed in many of the samples, especially on the untreated sides of experimental

teeth. Lesions in dentin in both undrilled and drilled whole teeth displayed discoloration, demineralization, and cavitation also resembling natural caries.

There were, however, certain aspects of the lesions produced in vitro in whole teeth that are generally not observed in true caries. A thin band of partially demineralized enamel for example, was observed along the entire exposed coronal surface in most of the experimental units. This condition was expected considering the mean acidity observed in the medium on the occlusal aspects of the teeth during the twenty weeks of incubation. The sections with demineralized enamel bands, however, were considerably different than sections from teeth incubated in complete medium. In the latter case, almost all the calcified enamel structure was lost, and no specific area of penetration was observed. The influence of the demineralized surface of the experimental teeth on the properties of the sealant was not determined although the retention of the material did not seem adversely affected.

The advancing lesion in dentin in some of the drilled and undrilled whole teeth also displayed certain features not common in natural caries. Although the areas resembled true caries radiographically, examination of sections from several teeth revealed cases of elongated penetration of the discoloration and demineralization process through the dentinal tubules. Whether this condition represented bacterial proliferation or merely downward diffusion of the acidic

medium was not determined. Using a similar methodology, however, Brown, Wachtel, and Wheatcroft[101] reported observing microorganisms at the depth of in vitro lesions in dentin. The rapid penetration seen in some of the advancing lesions in dentin could be attributed to the absence of secondary changes that are observed in natural teeth. While the diffusion of growth factor through the tooth enhanced the penetration of bacterial products, the inability of the dentin to respond to the invasion apparently influenced the shape of the resulting lesion.

The decalcified areas observed in tooth slices also resembled true caries, but due to the relatively short incubation periods used, most of the lesions produced were small and limited to enamel. Wachtel and Brown[27] reported utilizing incubation periods of six to eighteen months to produce relatively large lesions in dentin in tooth slices. The principal advantage of utilizing tooth slices in this study, however, was that they provided a mechanism for observing and measuring the extent of lesions at the time the sealant was applied. Even small advances in the extent of the caries-like process therefore, could be assessed. Furthermore, since the pH of the medium surrounding the tooth slices was usually maintained above 5.4, a band of demineralization on the surface enamel was not observed.

Properties of the Sealant Under Study Conditions

The retentive and adaptive properties of the "BIS-GMA" resin to tooth structure under the condition of this study were good. Al-

though leakage was observed under material "flash" that extended on to cuspal planes, a tight seal appeared to be formed and maintained in the fissure areas. When the sealant was applied to undrilled or drilled teeth prior to the initiation of incubation, no leakage of substrate or bacteria at the tooth-sealant interface was observed, whereas gross leakage was observed when a control material was used. No evidence of surface deterioration or solubility of the sealant was observed when the teeth were examined at the conclusion of the study.

Comments on the Assay Procedures

The measurements used in this study to assay the characteristics and the extent of the in vitro lesions were limited to microscopic examinations of the tooth slices and sections from whole teeth, and to measurements with an eyepiece filar micrometer. The micrometer gauge was mounted on a dissecting microscope and was calibrated for measurements at X20 magnification. Each unit on the micrometer dial was equivalent to 3.8_{μ}, and measuring variability generally ranged between 10 and 15 units. Both the depth and the width (breadth) of the decalcified areas were measured, since it was felt that an increase in either of these dimensions represented an advance in the lesion.

Although care was taken to minimize uncertainty during measurements, errors could have resulted from several factors. Arbitrary decisions had to be made for example, concerning the specific point that constituted the farthest extent of an area of demineralization. The three dimensional characteristics of the tooth slices added to this

problem. Furthermore, since the treated samples could be readily identified and since all measurements were made by one person, the possibility of examiner bias cannot be discounted. After several practice and calibration sessions, however, the measurement procedures produced repeatable results. In addition, sample measurements and observations made by other workers were similar to those reported here. Assay methods such as microradiography, polarized-light microscopy, electron microscopy, and micro-hardness analysis, could provide additional information and should be considered in future investigations.

In whole teeth, the extent of lesions could not be determined at the treatment period, and therefore only relatively large differences between treated and control areas noted at the end of the study were considered meaningful. A caries-like area was classified as "not advanced" if it was limited to enamel, while the paired location on the same tooth had a lesion that extended into dentin. These cases were ranked as treatment successes, and no measurements were made. If lesions in dentin associated with both treated and untreated sides of a tooth, were observed, measurements of the extent of the areas were made. The measurements included the width of the lesions, and the depth below the dentino-enamel junction. It was felt that caries arrestment effects, if present, could be detected after the final ten week incubation period since relatively rapid advances in process were expected once dentin was involved. An arbitrary system was
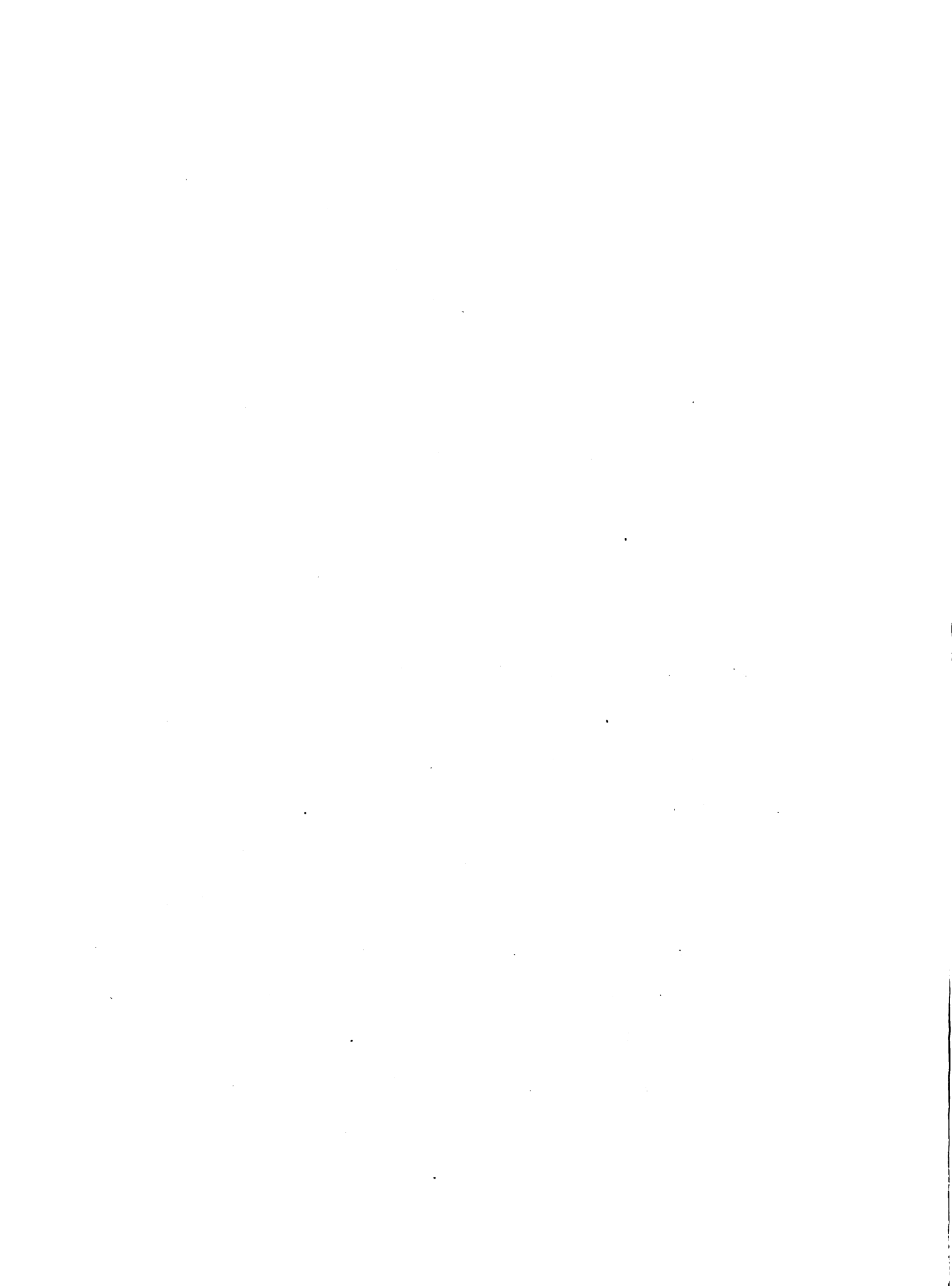
used in scoring the effect of sealant in teeth with two dentinal lesions. If the average of the measured length and width of the lesion on one side of the tooth did not exceed the average size of the lesion on the other side by more than 0.5 mm, the two sides were ranked equally. These samples were scored as treatment failures, or cases where arrestment did not occur. The results of the study indicate that differences much smaller than 0.5 mm were generally observed between the treated and untreated sides.

The drilled teeth were used to determine if equalizing the initial susceptibility of the experimental areas would produce different results than observed in undrilled teeth. The assay procedures for determining the extent of caries-like areas were the same as those used for dentinal lesions in undrilled teeth.

The caries-like process in tooth slices advanced more slowly than in whole teeth, and a majority of the lesions had not penetrated into dentin by the conclusion of the study. The methods for determining the status of lesions in tooth slices, however, were more precise than those used with whole teeth and the progress of caries-like areas in enamel could be measured. Two sets of measurements were made for each tooth slice, one at the treatment period and one at the end of the final incubation period. The differences between the measurements for the treated slice were compared with the differences between the measurements for the paired untreated slice. Considering the potential measuring error associated with this procedure, a "correction"

factor was applied to the results. Only decalcified areas that in-
creased 0.1 mm or more in length or width were considered
"advanced". In these procedures, the depth of the lesion was
measured using a line joining the cusp tips as a reference point
rather than the dentino-enamel junction. This method could be used
effectively with tooth slices since evaluations were based on differen-
ces between successive measurements and not on absolute values.
The width of the lesion was measured at the widest point associated
with the fissure base. The outlines on the cavities were drawn during
the first examination and the direction of the measurements was in-
dicated. These diagrams served as a guide for orientation of the
tooth slice during the final examination and measurement procedure.

Analysis of the Data

The results of the studies with whole undrilled teeth indicate
that apparent arrestment of the in vitro caries process was effected
in twelve of the twenty samples. In these instances, lesions on the
side treated with sealant were limited to enamel, while lesions on the
untreated side extended into dentin. Since caries-like areas did not
develop on either the treated or untreated sides of four whole teeth,
these teeth did not contribute to data analysis. The four samples
with dentinal lesions of approximately equal extent on treated and control
sides were considered cases in which arrestment did not occur. The
binomial test for related samples was appropriate for statistical
analysis of these data. With $n = 16$, the difference between treated

and untreated areas was not significant (0. 10>p>0. 05).

In drilled teeth, the progress of the caries-like process did not appear to be affected by sealant applied after twelve weeks of incubation. The lesions under treated holes extended to approximately the same degree as lesions in unfilled holed in the same tooth. In addition, no differences were observed in the progress of lesions treated with sealant and those treated with zinc phosphate cement. This result was particularly significant since obvious areas of leakage were noted at the tooth-phosphate cement margin, while the sealant appeared in tight contact with the tooth structure.

The data from the study with tooth slices was also analyzed according to the binomial test for related samples. In sixteen tubes, no changes were observed in the size of the caries-like area in treated slices, while the corresponding paired control slices had progressing lesions. In one tube, both treated and untreated slices had lesions that were judged "advanced" after the final incubation period. No progressing lesions were observed in either the treated or the control slices comprising the other three study pairs, and they were not included in the statistical analysis. With n = 17, a statistically significant difference (p < 0. 01) in the progress of enamel lesions was observed after ten weeks of incubation between tooth slices treated with sealant and untreated controls.

Further review of the data from the study using undrilled whole teeth indicates that the absence of caries arrestment in sealed lesions
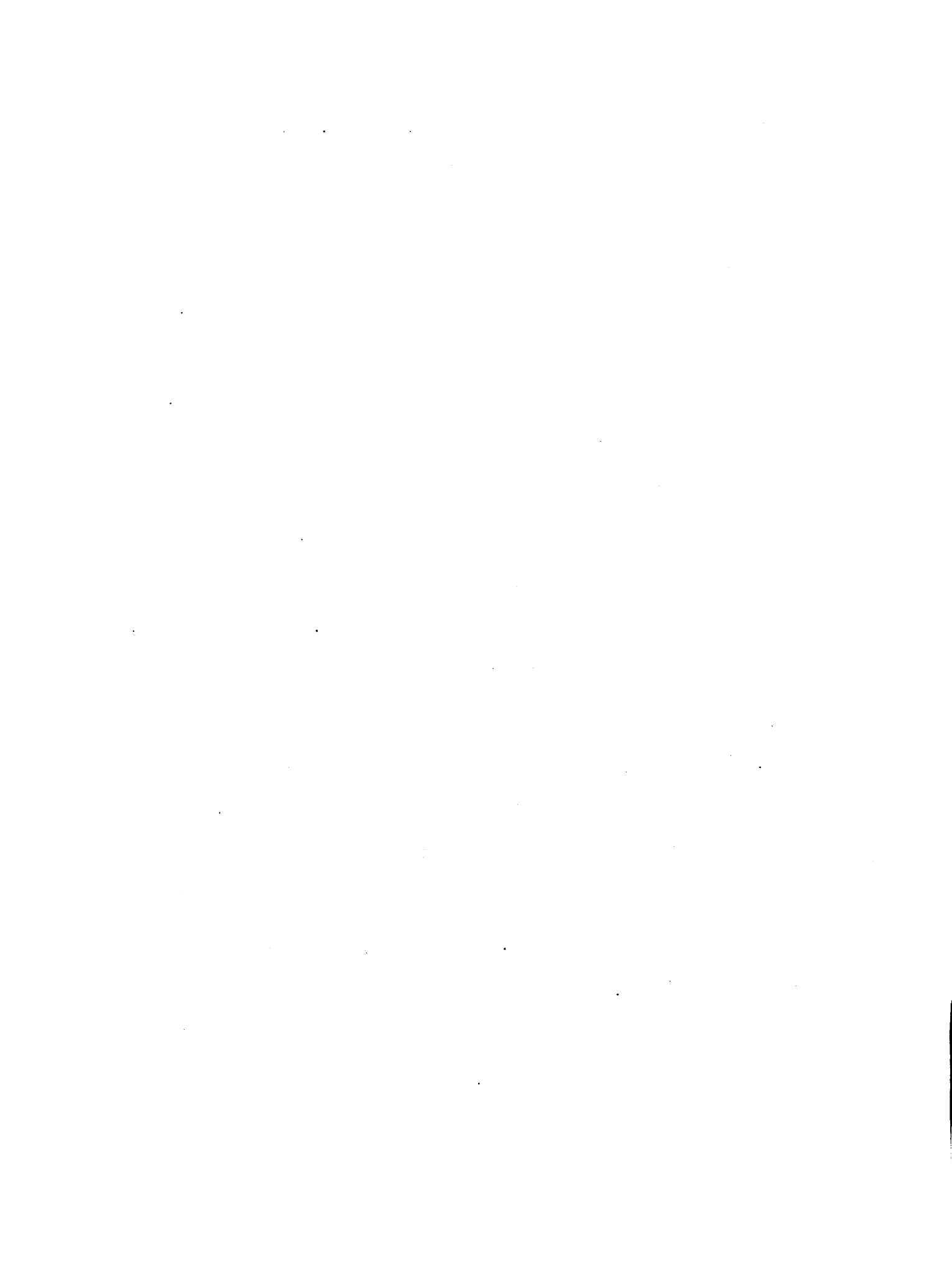
was observed only in samples in which both treated and untreated sides of the tooth were associated with lesions in dentin. Almost all sealed enamel lesions in whole teeth and in tooth slices appeared arrested.

The reasons for the inability of sealant to inhibit the progress of dentinal lesions under the conditions of this study is not completely understood, but can possibly be related to the diffusion of the growth medium through the dentin. Bacteria surviving in the caries-like lesion under sealant could have been nourished by substrate diffusing from the upper tube of the diffusion apparatus and niacin diffusing from the lower tube. Medium from the upper tube could have entered the dentin through the unsealed hole in drilled teeth or through carious areas on the control side of undrilled teeth, and then diffused to the sealed lesion. Dye solution placed into one hole of a drilled study tooth indicated that diffusion occurs rapidly downward through the dentinal tubules, and then secondarily across the dentino-enamel junction to other areas of dentin. In vitro studies by Buonocore[45] also indicated that substances entering restored teeth through areas of marginal leakage moved along the dentino-enamel junction and entered the dentin beyond the area of the cavity preparation. Since sclerotic dentin formation in vital teeth would tend to isolate carious areas, the conditions observed in this aspect of the study could be artifacts of the in vitro system.

VII. SIGNIFICANCE OF THE RESULTS AND CONCLUSIONS

Although factors such as the short periods of time available for incubation of samples, the limitations of the assay procedures used for determining the nature and extent of caries-like lesions, and the possibility of examiner bias during evaluations detracted from the potential value of this investigation, certain useful conclusions can be made. The results of the investigations with whole teeth and with tooth slices indicate that penetrating zones of demineralization with characteristics resembling natural caries can be produced in vitro. "flame-like" areas in enamel and extensions of the process into dentin were frequently noted in the study samples. The morphological features of the lesions suggested that they were initiated by localized bacterial metabolism and subsequent acid formation in deep enamel fissures.

The caries-like activity in enamel was restricted from further development by the application of occlusal sealant over the involved location. The viability of the bacteria beneath the sealant was not determined by this study. There was, however, an apparent inhibition of metabolic activity within the fissure due to the adequate isolation of the area from the source of substrate and additional microorganisms.

Microscopic examinations revealed a close adaptation and firm retention of the resin to the tooth surface. Further demonstrations of the sealing properties of the test material used in this investigation were noted in the drilled and undrilled tooth samples which were treat-

ed prior to being placed in contact with the inoculated medium. Leakage of bacteria or medium was not observed at the tooth-material interface when the sealant was employed, but was noted when a control material was used. The absence of pH changes in samples in which saline rather than niacin was diffused through the tooth structure indicated that the enamel did not provide adequate substrate for the growth of the study organism.

Lesions with dentinal penetration in whole teeth were not inhibited by the application of sealant under the conditions of this study. The method employed utilized one segment of the occlusal surface of a study tooth for the application of sealant while another occlusal segment of the same tooth served as an untreated control area. The development of the carious area under the sealant therefore, could have been related to the diffusion of substrate through the dentin from natural or artificial lesions in the enamel on the untreated side. Sealed enamel lesions did not appear to become reactivated by these conditions. This result can be attributed to the restricted permeability of the enamel to sufficient quantities of complete substrate, and to the possibility that the enamel fissure was sterilized by the application of the etching solution and the sealant material. Bacteria in deeper dentinal lesions on the other hand, were more likely to survive the treatment process.

The inability to arrest dentinal lesions by the application of sealant in this in vitro study can be viewed in the context of clinical

situations. If similar conditions to those observed in this investigation prevailed in vivo, caries that had become dormant after the application of sealant could be reactivated by diffusants entering the dentin through a carious area on another surface. Since small lesions cannot always be accurately assessed for dentinal penetration, placement of sealant over any lesion could present a potential hazard. Furthermore, the mere necessity for thorough examinations would limit the use of conventional sealants in public health procedures. Secondary changes occuring in vital teeth however, are probably sufficient to restrict the diffusion of adequate substrate through dentin.

The observed limitations in using non-vital teeth to study dynamic processess involving infectious agents and host defense mechanisms make it unlikely that questions concerning the effect of sealing dentinal lesions can be resolved by investigations conducted in vitro. The overwhelming need for additional information, and the important public health significance of understanding the fate of sealed lesions indicate that in vivo studies on this subject should be actively pursued.

The results of this study strongly suggest that dental caries limited to enamel will be arrested if occlusal sealant is placed over the involved area. The observations of the fate of sealed dentinal lesions, however, can probably not be directly related to clinical conditions. The experimental goal of this investigation therefore, has only been partially resolved, and perhaps more questions have been presented than answered. It is hoped, however, that the overriding

objective of providing additional background toward the understanding

of caries arrestment will be advanced.

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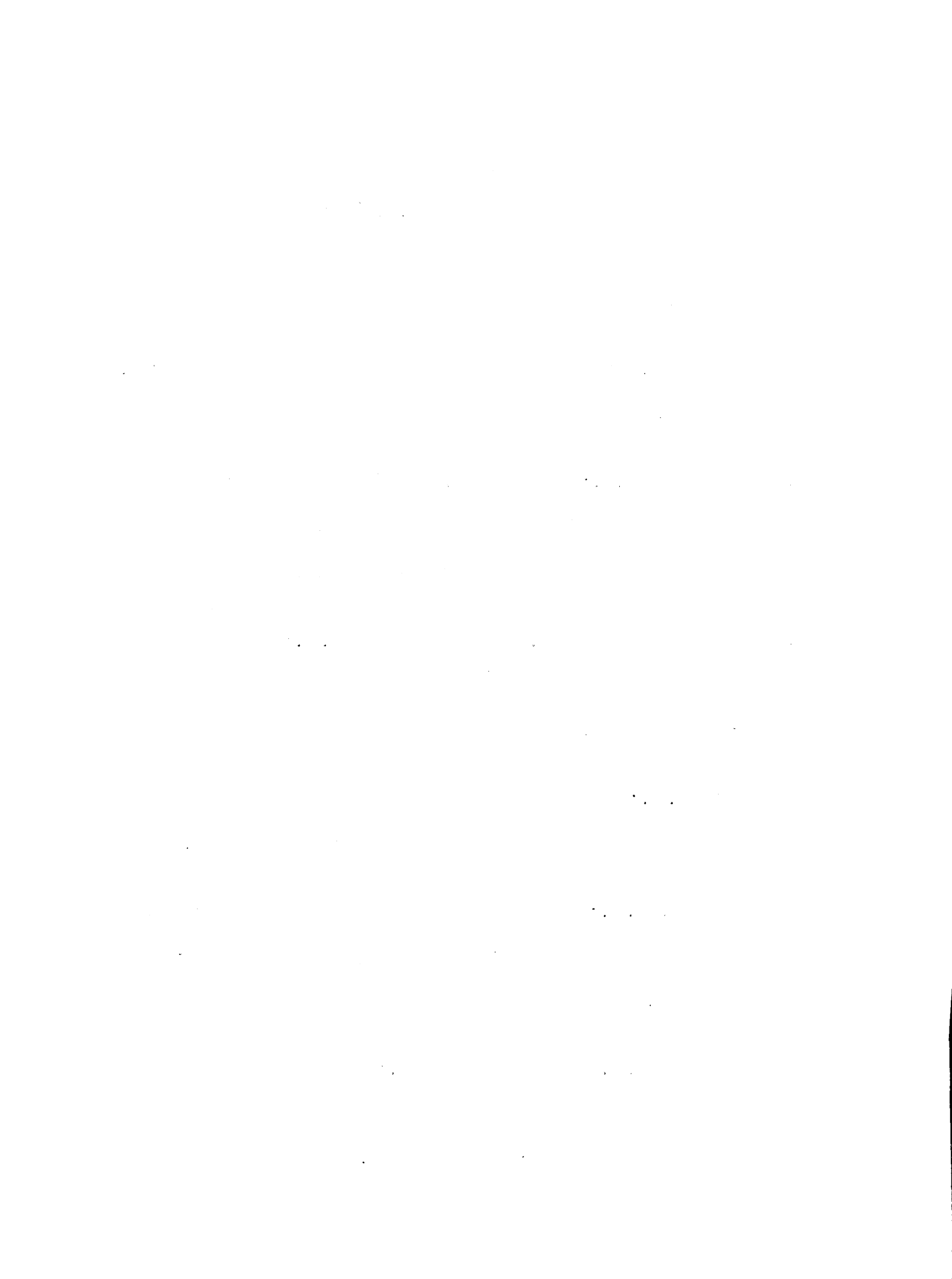
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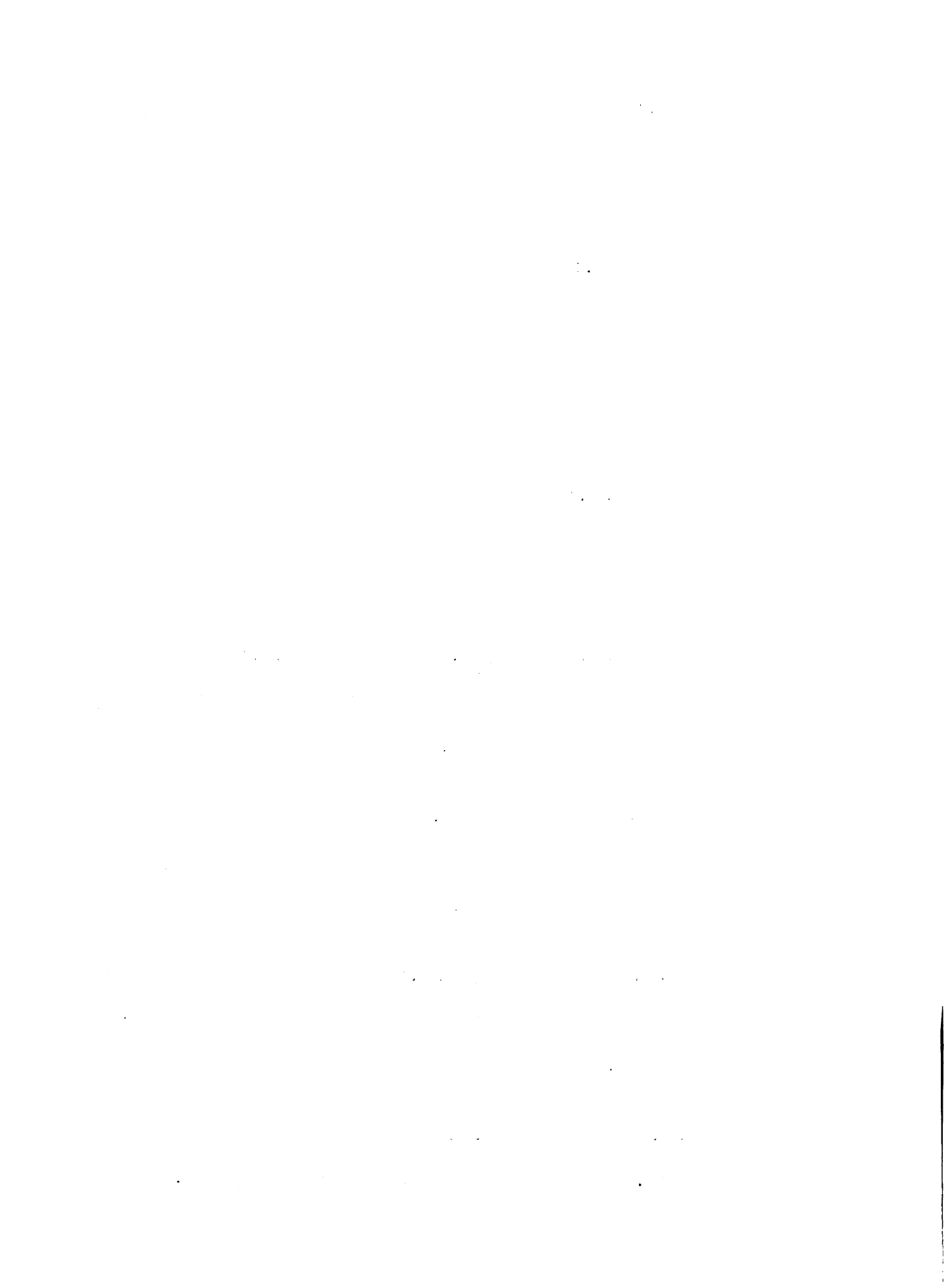
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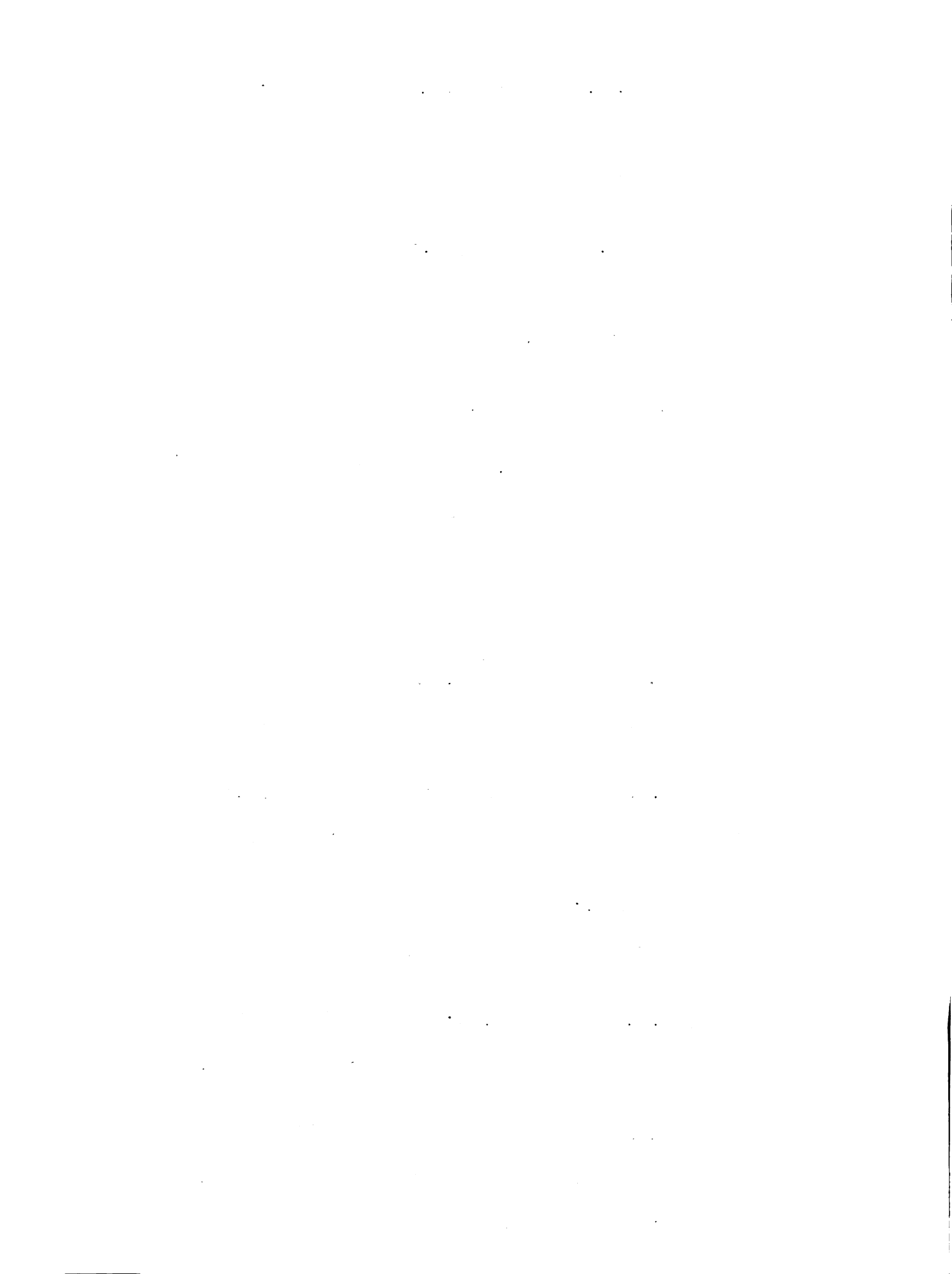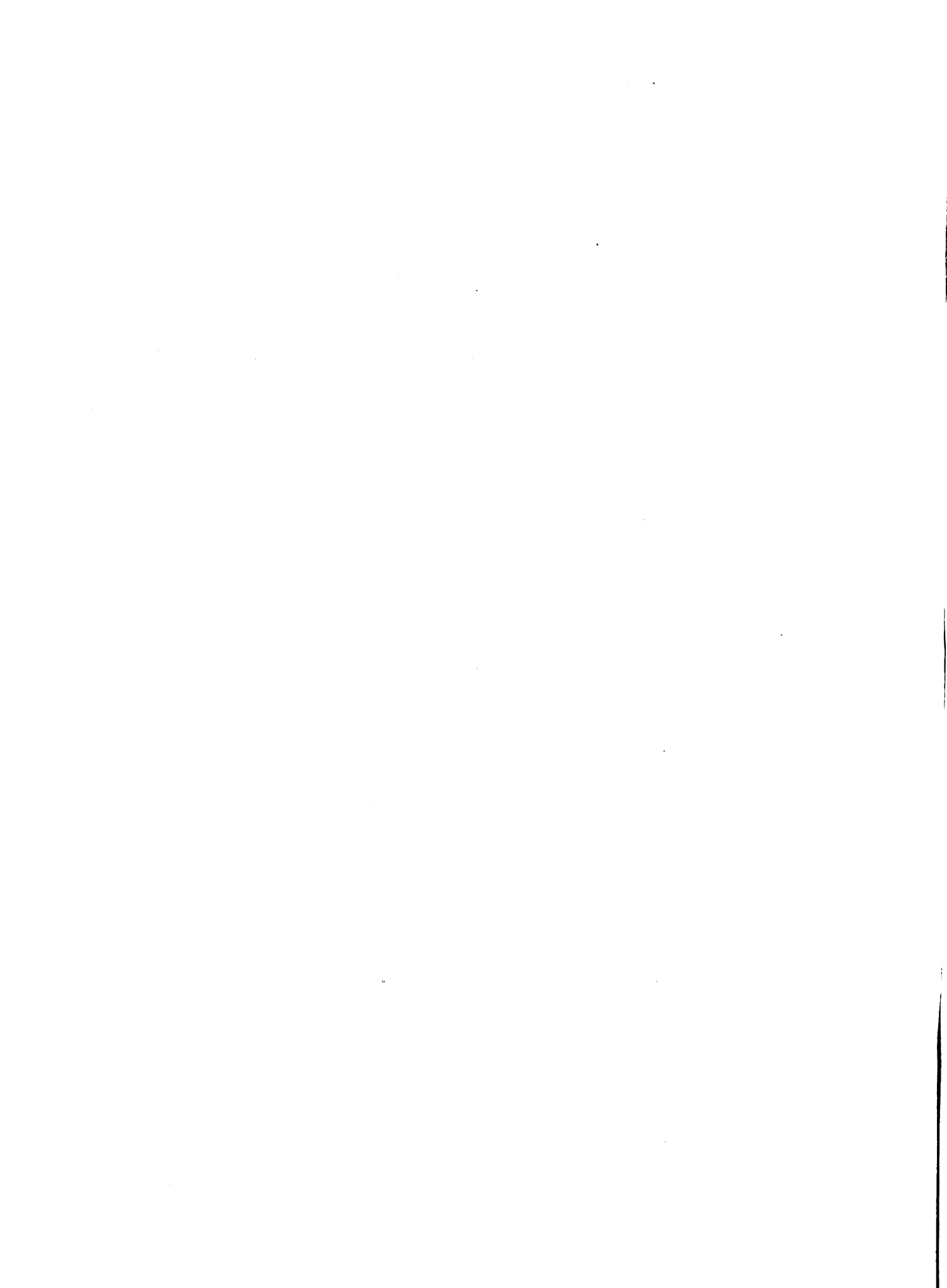
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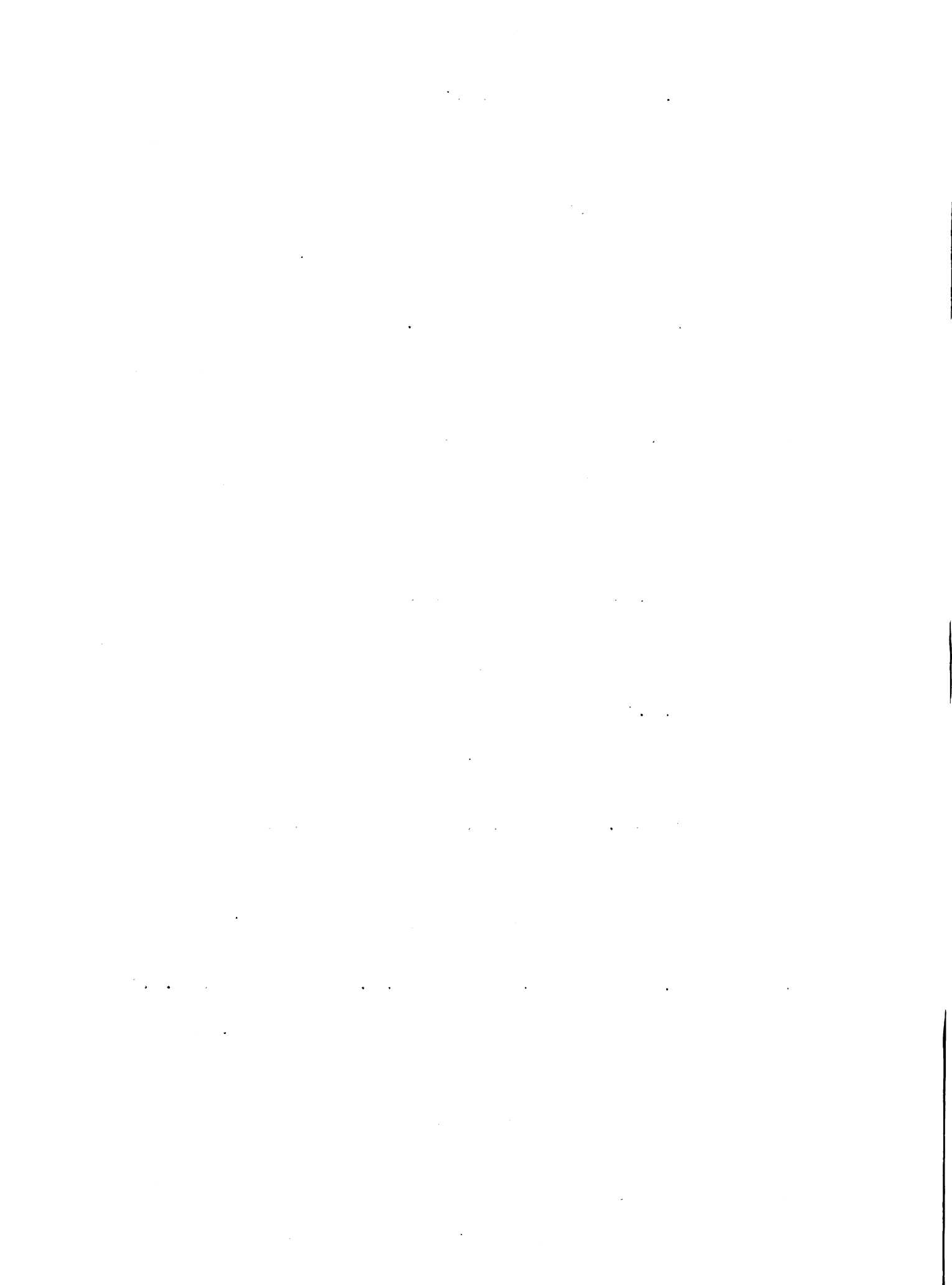
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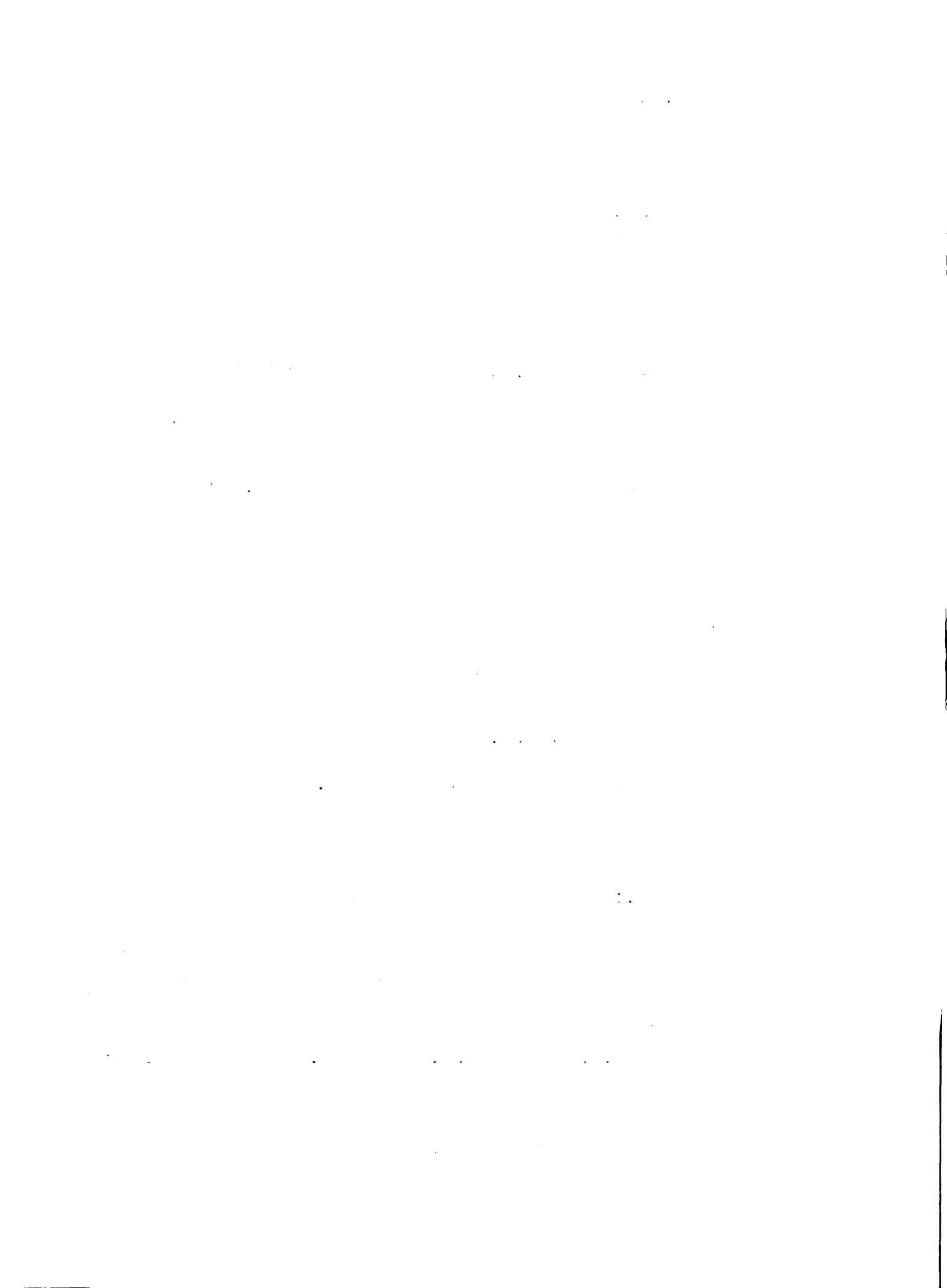
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