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A Scanning Electron Microscopy study of human dentin and its interaction with third-generation dentin bonding systems

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ABSTRACT
This study describes regional variations of the organic contents of human dentin after acid-etching with two specific dentin conditioners, phosphoric acid and a 10% citric acid/3% ferric chloride solution. The relationship between the resinous components of two third-generation dentin bonding systems and the dentin substrate is also described. The clinical success of these dentin bonding systems is dependent upon their physical and chemical interation with dentin tissue. This investigation suggests two kinds of bonding mechanisms, either by mechanical interlocking of the methacrylates with microporosities created on dentin surfaces by specific etching agents, or by chemical reaction with the organic and inorganic components of dentin. In addition, backscattered electron imaging was performed to evaluate the relationship of the dentin organic components with the methacrylates included in the composition of the dentin bonding systems.

KEYWORDS
Collagen, dental bonding, dentin, electron microscopy, operative dentistry, scanning.

INTRODUCTION
Enamel is a highly mineralized and inert tissue containing 96% by weight of hydroxyapatite. Dentin is composed of 70% hydroxyapatite, 10% water, and 20% organic material which is primarily type I collagen [1]. Dentin contains numerous fluidfilled tubules with cellular extensions of the dental pulp called odontoblastic processes. The tubular dentinal fluid is normally under hydrostratic pressure which causes it to flow in an outward direction. In 1971, Van Hassel calculated an average of 25 mmHg hydrostatic pressure in normal pulp [2].

Odontoblastic processes are present only in tubules close to the pulp. The number of tubules is greatest close to the pulp with 45,000 per mm^2 having an average diameter of 2.5 µm, and least near the dentin-enamel junction havig 20,000 per mm^2 with an average diameter of 0.9 µm [3]. Peritubular dentin is collar formed around the inner wall of the tubule and is 40% more mineralized than intertubular dentin [1]. Intertubular dentin is a network of type I collagen fibers and ground substance in which hydroxyapatite is deposited [1].

Buonocore introduced a technique for etching enamel with phosphoric acid [4]. Strong bonds to enamel are readily created by mechanical interlocking of resin tags with etched enamel rods. Bonding to dentin represents a greater challenge than bonding to enamel due to the wetness of dentin surfaces , as well as its organic composition and the presence of a smear layer. The smear layer consists of debris left on tooth surfaces by the cutting instrument.

Dentin bonding systems (DBS) have been investigated for the last 35 years [5,6]. First - and second - generaton DBS were applied to dentin surfaces through the smear layer, to facilitate bonding of a tooth -colored restorative material called composite resin. Composite resin is a bispenol glycidyl methacrylate (Bis-GMA) resin filled with inorganic particles such as colloidal silica, quartz or glass [7,8]. Cohesive failures of the bonds were consistently observed within the

smear layer, and bond strengths were generally in the range of 1.7-6.8 MPa (1 MegaPascal=10 kg/cm^2) [9].

The third-generation dentin bonding systems either modify or remove the smear layer, also called "total-etch" dentin bonding systems, include an etching agent which is applied to enamel and dentin and exposes the tubule orifices. The etchant is followed by hydrophilic resinous primers (second step) that penetrate the tubules and copolymerize with a methacrylate-based unfilled bonding resin (third step). However, some third-generation DBS combine the etchant and the primer in one step. The primers serve as bifunctional coupling agents. They contain a hydrophilic group that bonds to dentin substrate and a hydrophobic group that bonds to the unfilled resin placed over it [10]. A resin-impregnated layer of dentin forms after application of these dentin bonding systems [11]. This has been described as a resin-infiltrated dentin layer located at the resin-dentin interface and it is believed to be the primary mechanism of bonding to dentin [12]. The *in vitro* dentin bond strengths associated with total-etch" dentin bonding systems have been reported to be as hight as 39.99 MPa [13].

The purpose of this investigation was to characterize the ultrastructure of the dentin substrate and to determine its interaction with the components of this newest generation of dentin bonding agents.

MATERIALS AND METHODS

Extracted human molars stored in a desinfectant solution of 0.2% thymol were used in this study. The teeth were sectioned with a Silverstone-Taylor hard-tissue microtome to obtain 300 µm thick dentin discs. To remove the smear layer the specimens were conditioned either with 10% and 32% phosphoric acid for 15 seconds or with a solution of 10% citric acid and 3% ferric chloride for 10 seconds, followed by vaccum-desiccation for 48 hours. The dentin discs were mounted on aluminum stubs with colloidal silver, sputter-coated with gold-palladium and observed in an Hiachi S-4000 Field Emission Scanning Electron Microscope (FESEM). FESEM was used because part of our work was routinely done with 15,000 to 30,000 times magnification. Dentin discs intented to be observed with the smear layer intact were fractured transversally and mounted to be observed in cross section. Amalgambond (Parkell, Farmingdale, NY) and All-Bond 2 (Bisco, Itasca, IL) dentin bonding systems were used to study the degree of resin penetration after smear layer removal. Cavities were prepared in extracted teeth and restored with the same techniques used in the *in vivo* conditions: a DBS ans a visible light-cured composite resin. After sectioning, the dentin discs were demineralized in 6N HCl for 30 seconds to dissolve the superficial dentin, followed by 1 week in 0.2% NaOCl in order to remove the organic component and expose the resin tags in the dentinal tubules [12].

Silver methenamine staining was performed in All-Bond 2 specimens to localize the collagen fibers with backscattered electron imaging. This staining has been reported to be specific for collagen in hypomineralized areas when used in optical [14] and transmission electron microscopy [15]. Specimens were demineralized in 6N acetic acid for 6 hours, immersed in 1% NaOCl for 24 hours and stained with silver methenamine [15].

RESULTS

After fracture, middle dentin contains a wide collar of peritubular dentin around the lumen of the tubule, as well as longitudinal collagen fibers (Fig. 1) described by other authors [16]. In the transition from middle to deep dentin the peritubular dentin is not so evident, but the longitudinal collagen fibers become more prominent (Fig. 2). Before conditioning, the instrumented dentin surface and entrance of the tubules is covered by debris called the smear layer which is crossed by fine grooves resulting from the cutting instrument (Fig. 3A and 3B). After etching with phosphoric acid from All-Bond 2 bonding system, dentin tubules are opened and in some specimens a fibrillar structure called the lamina limitans is observed (Fig. 4A). After fractures, it seems to be formed by collagen fibers (Fig. 4B). A cross-sectional view of dentin acid-etched either with 10% phosporic acid for 15 seconds or with the citric acid/ferric chloride solution for 10 seconds shows the entrance of the tubule with a funnel-shaped configuration (Fig.5).

High magnification examination of dentin etched with 32% phosphoric acid reveals the collagen around the tubule wall after dissolution of the peritubular dentin (Fig. 6A and 6B). This collagen is mostly embedded in a residual mineral structure. Collagen distribution in the superficial dentin has a different pattern

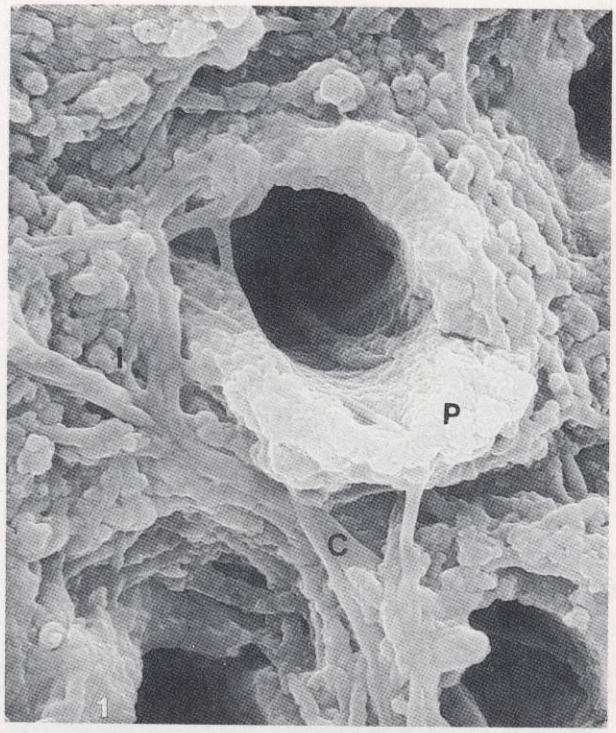


Fig. 1. Middle dentin after fracture showing peritubular(P) and intertubular dentin(I), as well as collagen fibers(C). X 13,200.

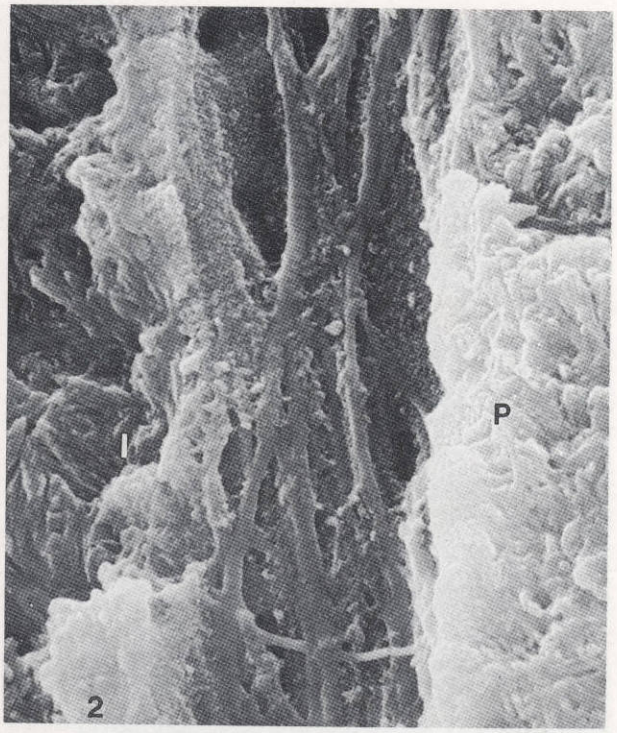


Fig. 2. Dentinal tubule in the superficial part of deep dentin. X 21,600.

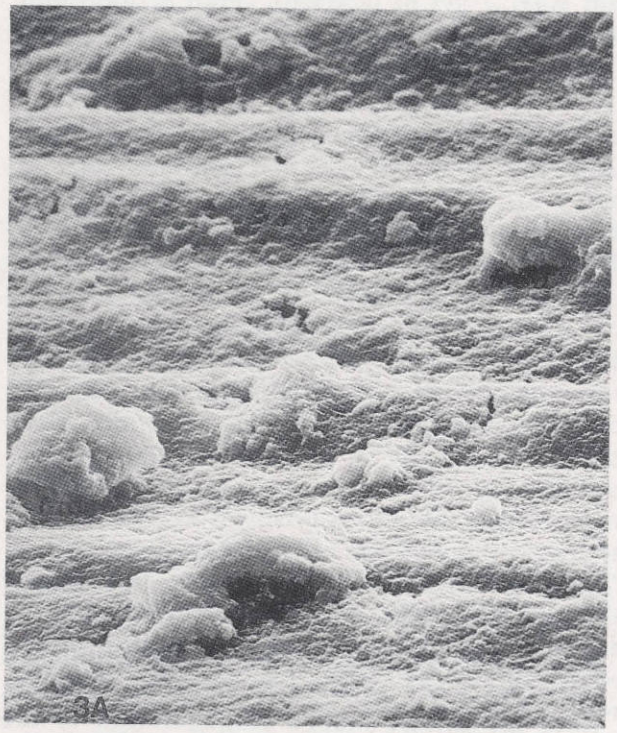


Fig. 3A. A smear layer created using a diamond disc with water coolant. Particle sizes range from 3 to 5 micron. X 6,000.

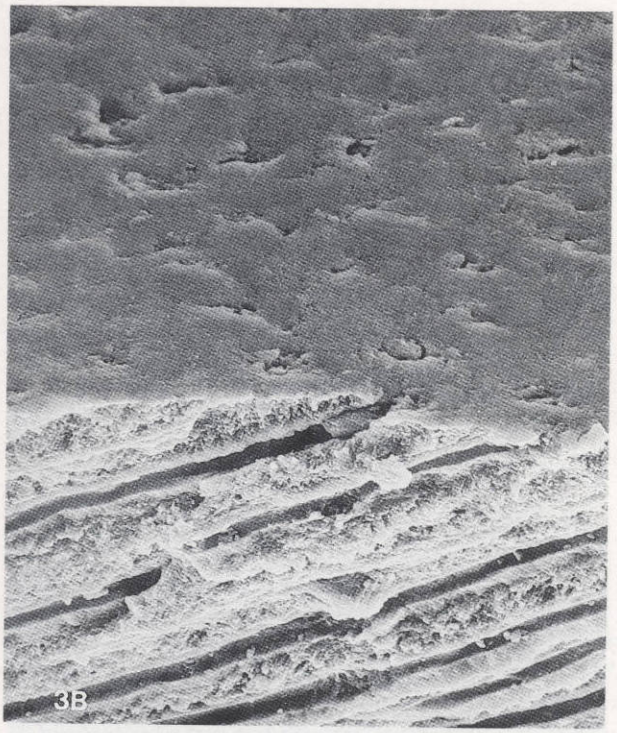


Fig. 3B. Another view of the smear layer which occludes the top of the dentin tubules. X 1,800.

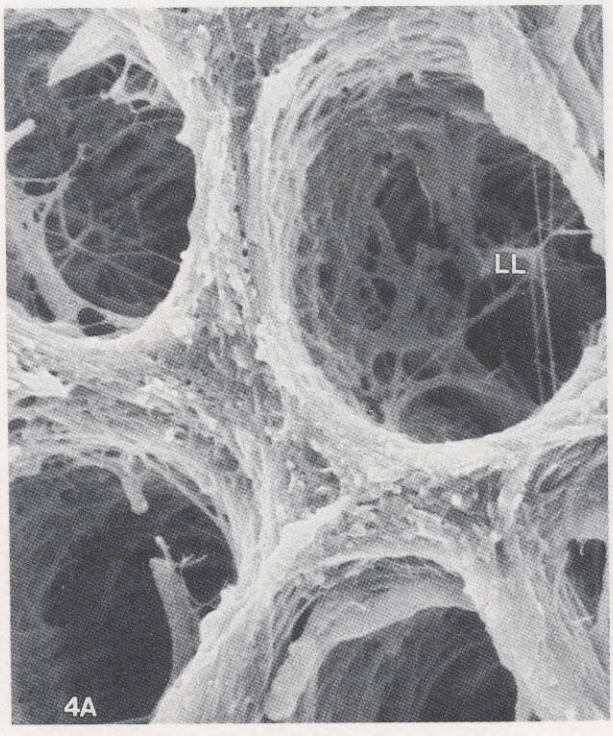


Fig. 4A. Deep dentin after acid etching with 10% phosphoric acid. The organic structure inside the tubules is called the "lamina limitans" (LL). X 12,000.

Fig. 4B. Deep dentin after fracture. Transverse septa similar to collagen fibers may be the same structures as in Fig. 4 A(arrow). X 12,000.

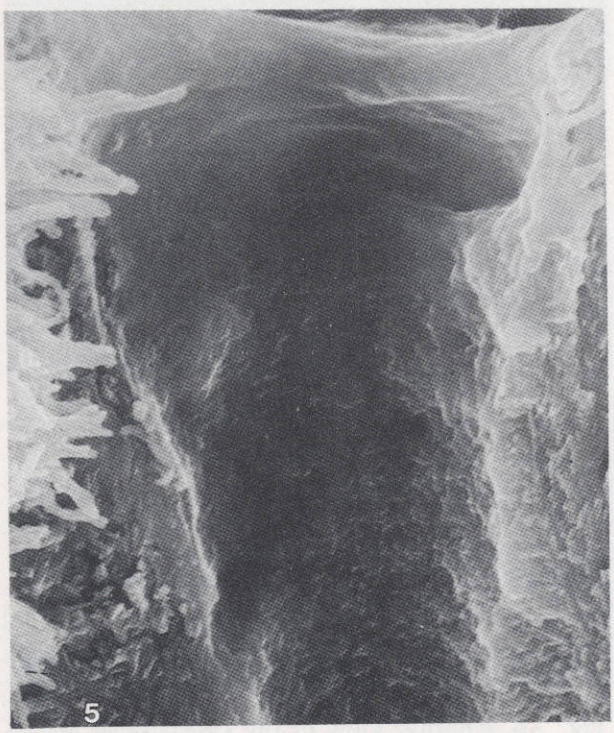


Fig. 5. Cross-sectional view of dentin etched with the citric acid/ferric chloride solution. Note the funnel-shaped configuration of the entrance of the tubule. X 18,000.

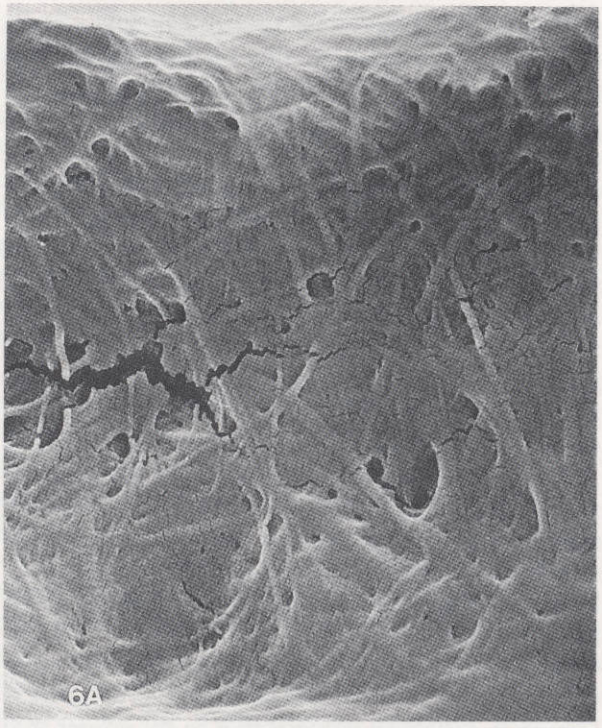


Fig. 6A. Middle dentin etched with 32% phosphric acid. The acid dissolved the peritubular dentin exposing the collagen fibers. These fibers are probably equivalent to the intertubular fibers observed in Fig 1. Note the characteristic banding pattern of collagen. X 18,000.

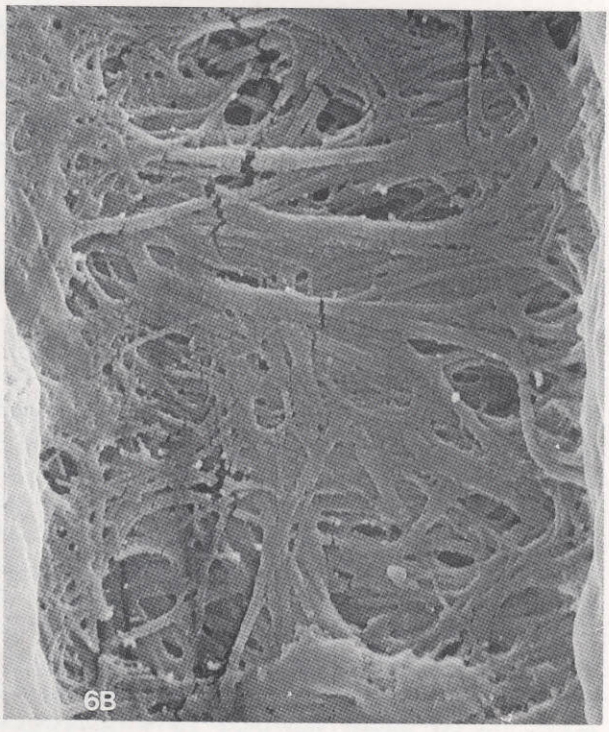


Fig. 6B. The equivalent image to Fig. 6A in deep dentin. The collagen fibers are thicker and more numerous. X 18,000.

from middle and deep dentin in which collagen bundles are coarser and more numerous. The 640 Å periodicity of collagen is visible in Fig. 6A. After acid-etching with 10% phosphoric acid and application of a mixture of acetone - based primers (All-Bond 2 Primers A and B), the collagen is enclosed by resin particles (Fig. 7). The penetration and relationship of the unfilled bonding resin with dentin after demineralization and removal of the superficial organic components is illustrated in Fig. 8. The surface roughness of the resin is an impression of the rough walls of the dentinal tubules suggesting a good impregnation when the bonding system is applied to a moist substrate. Resin tag "necks" [12] are visible in Fig. 8. When the resin is applied to dry dentin substrates, the resin tags appear smooth and lack the characteristic funnel-shaped or neck appeareance (Fig. 9). After acid-etching with a solution of 10% citric acid with 3% ferric chloride from Amalgambond bonding system, the collagen fibers are not so evidently exposed as they were after etching with phosphoric acid (Fig. 10). However, following the application of Amalgambond primer, which is a solution of 2-hydroxyethyl methacrylate (HEMA), resinous particles appear to be in close association with dentinal fibrous structures that were not evident immediately after acid-etching (Fig.11).

The backscattered electron images of All-Bond 2 specimens processed with silver methenamine suggest that collagen is not present at the resin-dentin interface, but around the resin tags (Fig. 12 and 13).

DISCUSSION AND CONCLUSION

Dentin tubules are lined by a membrane-like structure called the lamina limitans which can be confused with an odontoblastic process [17, 18]. Transverse septa originating from this organic membrane divide the tubules into a system of chambers [14]. However, the evidence of these chambers inside the tubules has not been fully demonstrated. In our study the rganic sheets

that compose the septa were only visible in the deep dentin near the pulp chamber (Fig. 4A and 4B).

The role of the longitudinal fibers within tubular walls remains unexplained. These collagen fibers were not observed in superficial dentin which is in agreement with other studies [16]. However, our observations suggest that in middle dentin these longitudinal fibers are partially mineralized or embedded in peritubular dentin and not "lying freely within the tubule" [16]. These discrepancies may be related to the different preparation of the specimens. At an average distance of 50 µm from the pulp these fibers seem to coalesce and to form a wide collagen fiber not surrounded by peritubular dentin (Fig. 14). The periodicity of these fibers avoids any confusion with the odontoblastic process.

Instrumentation of dentin with rotary or hand instruments forms a smear layer of debris on its surface [19] which has been described to be made up of small particles of mineralized collagen matrix spread over the dentin surface [20]. Microprobe analysis has identified the particles as containing sulfur, nitrogen, and carbon [21]. The smear layer has further been characterized as being composed of globular appearing particles between 0.5-15 µm in diameter [21]. In our study, smear layers were created using a diamond disc with water coolant (Fig. 3 A). The particle size seems to be more consistent: 3 to 5 µm.

Acid-etching of dentin removes the smear layer and peritubular dentin from the top 5-10 µm of the tubules yielding a funnel-shaped orifice [22]. After acid-etching with 32% phosphoric acid, collagen fibrils are evident in dentin walls. Their distribution varies according to different depths. This may be an adaptation of the dentin substrates to withstand the loads constantly applied by masticatory forces on enamel. Acid-etching with the citric acid/ferric chloride solution did not produce the same superficial etching pattern as did phosphoric acid; however, the tubules displayed the same funnel-shaped profile (Fig.4.B) . It has been reported that acid-etching with 37% phosphoric acid for 30 seconds exposes 3-5 µm of the superficial collagen, whereas acid-etching with the citric acid/ferric chloride solution exposed 1-2 µm of collagen [23]. If the smear layer is totally removed, tubules are open and available for increased retention and surface collagen is exposed for possible linkage with resins. Removing the smear layer from dentin surfaces seems to be a logical approach to obtaining higher dentin bond strengths. It permits the penetration of resins into the dentinal tubules thereby providing either mechanical retention or entanglement of the resinous primers within the collagen fibrils [10].

The bonding mechanism of the primers with the dentin substrate remains controversial and undefined. The depth of resin penetration into the tubules has been reported as being 10-20 µm in vital teeth, versus several hundred microns in old extracted teeth [24]. Chemical bonding to collagen has been hypothesized [25, 26]. Alternatively, other authors have proposed a micromechanical bonding [27, 28]. The All-Bond 2 system utilizes phosphoric acid to remove the smear layer and etch the dentin surface. All-Bond 2 primers (second step) are composed of 2% N-tolyglycine glycidyl methacrylate (NTG-GMA) and 16% biphenyl dimethacrylate (BPDM) in an acetone solution [29]. NTG-GMA is known to act as a surface-active co-monomer that triggers the polymerization of one of the hydrophilic components used in the composition of some primers, PMDM, which is the reaction product of pyromellitic dianhydride and hydroxyethyl methacrylate [30]. Because Bis-GMA resins are hydrophobic, surface-active comonomers are needed to compete with water on dentin surfaces [8]. BPDM is similar to PMDM, but has two benzene rings in the center of the molecule, whereas PMDM just has one [13]. The aromatic ring of NTG-GMA is electron-rich while that of PMDM/BPDM is electron-poor. Thus, there may be an affinity between the surface active co-monomer NTG-GMA and the coupling agent PMDM [31]. PMDM contains two carboxyl groups in the aromatic ring [30,32] and BPDM contains four free carboxyl groups, two in each ring (B.I. Suh, personnal communication). This may explain its affinity for dentin. The four polymerizable groups present in the BPDM molecule may be responsible for the copolymerization with the unfilled resin (third step). The acetone-based primers have a water-chasing action making it possible to penetrate and impregnate moist dentin walls [27]. The unfilled bonding resin (third step) contains a hydrophobic part, 2-hydroxyethyl methacrylate (HEMA), and hydrophobic components, urethane dimethacrylate and Bis-GMA, that bond to the composite resin restoration placed over the bonding agent.

The amalgambond system removes the smear layer with a solution of 10% citric acid and 3% ferric chloride, before a hydrophilic primer (HEMA) is applied to the dentin. The catalyst tri-n-butyl borane (TBB) is mixed with 4-methacryloxyethyl trimellitate anhydride (4-META) resin and is said to be activated by the presence of water and oxygen to trigger the polymerization of the resin [25]. The 4-META resin is then hydrolyzed into 4-MET which has been reported to spontaneously penetrate 6 μm into dentin substrate. Raman spectroscopy analysis demonstrated that the concentration of 4-MET in the resin-dentin hybrid layer was more than four times the concentration of its original solution, which illustrates its affinity for dentin [33].

Backscattered electron microscopy using silver methenamine staining suggests that collagen is present around the All-Bond 2 resin tags, but is not in close contact with them. The hybrid layer that has been described as being located at the resin-dentin interface may be formed of resin and hydroxyapatite (without any organic components). Our observations suggest that the bonding mechanism may be primarily micromechanical due to the following reasons: 1) the surface roughness of the superficial part of the resin tags when All-Bond 2 is applied to moist substrates; 2) the superficial region of these tags is a detailed impression of the morphology of etched dentin; and 3) the absence of organic structures at the resin-dentin interface.

However, there does seem to be some evidence that a chemical bonding to collagen occurs. In Figure 7, the All-Bond 2 primers enclose the collagen fibrils, suggesting some kind of chemical attachment. In Figure 15 the Amalgambond resin tags are bonded to a fibrous structure. The bonding to collagen amino groups has been suggested [26,34]. The significance of the chemical component of this bonding mechanism has not been determined. In order to clarify the ultrastructure of the resin-impregnated layer and the mechanism of bonding to dentin, a combined field emission scanning and transmission electron microscopy study is currently underway.

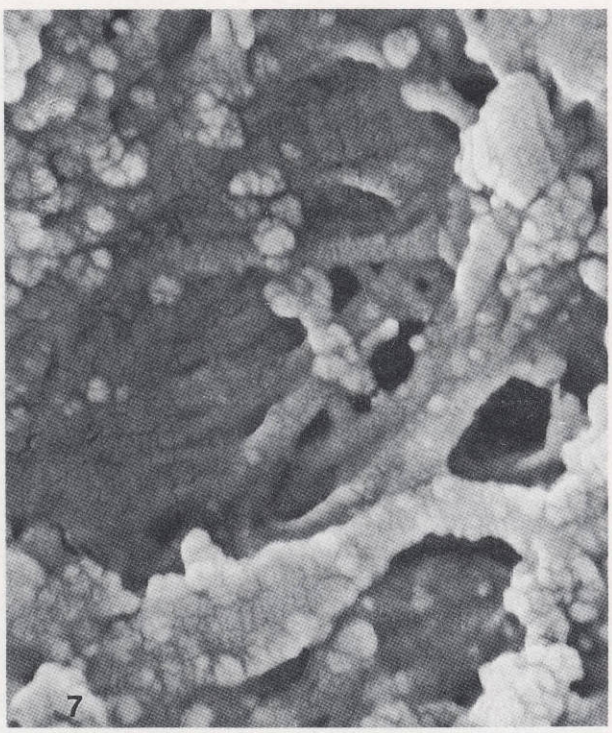


Fig. 7. Collagen fibers after acid-etching with 10% phosphoric acid and application of All-Bond 2 primers. X 60,000.

Fig. 8. All-Bond 2 DBS applied on moist dentin. The dentin has been dissolved leaving only the resin(R). Note the funnel or "neck" configuration of the resin tags. X 6000.

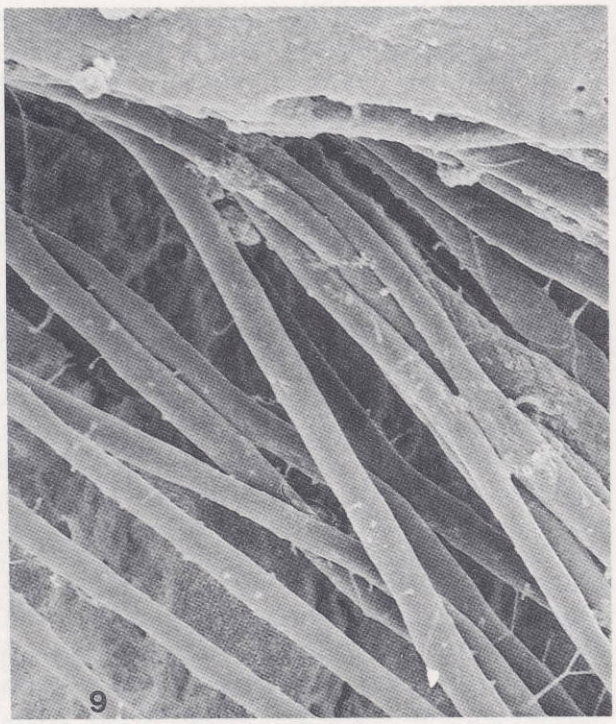


Fig. 9. All-Bond DBS applied on dry dentin. Note the absence of the "neck" configuration and the consistent smoothness of the resin tags. X 3,600.

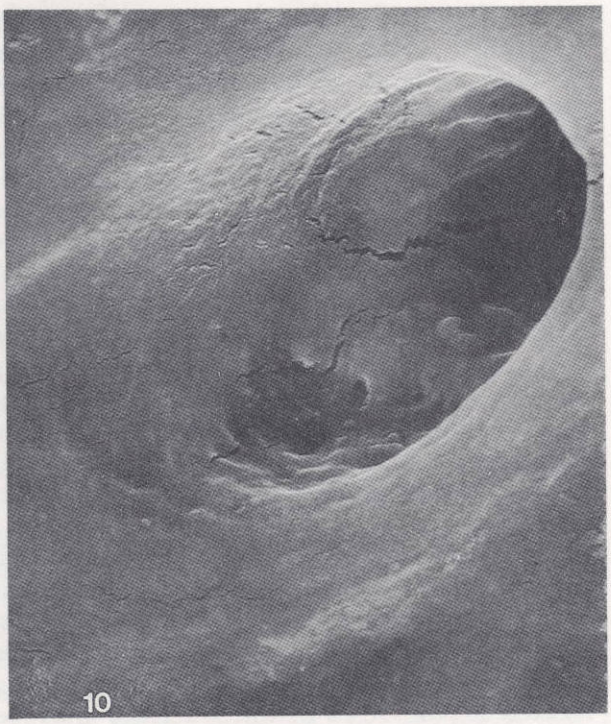


Fig. 10. Dentinal tubule after acid-etching with a solution of 10% citric acid/3% ferric chloride for 10 seconds. X 18,000.

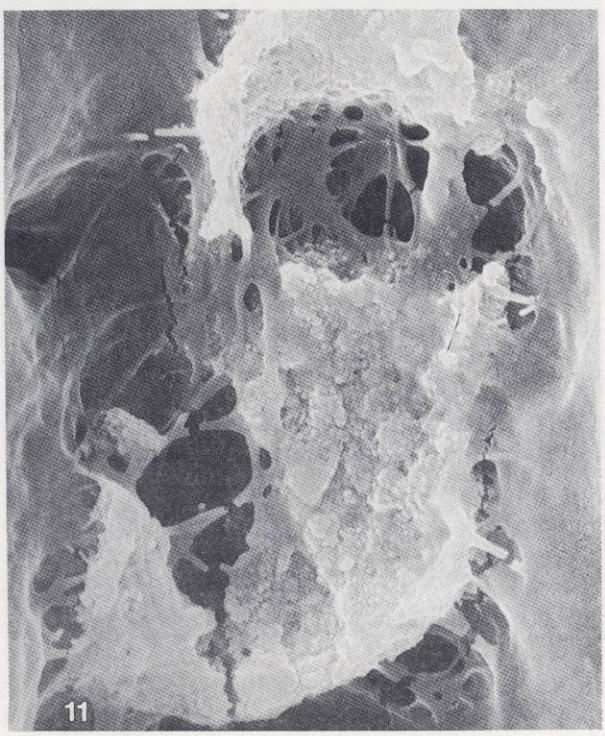
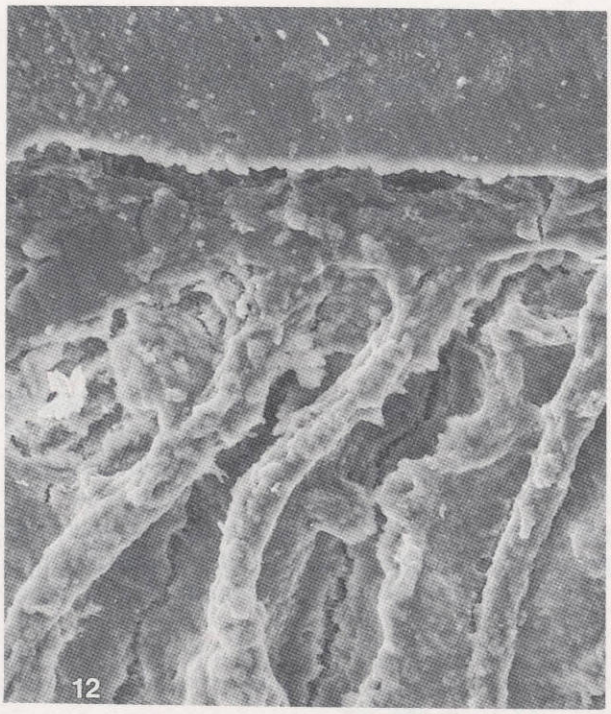
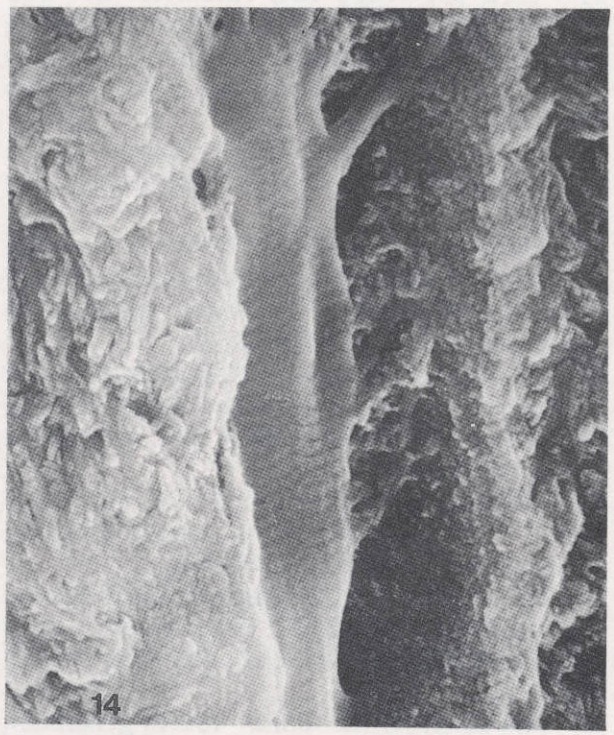


Fig. 11. The same specimen as in Fig. 10 but with the primer applied immediately after treatment with the etching agent. X 18,000.



Figs. 12 and 13. Secondary versus Backscattered electron images of an All-Bond 2 specimen stained with silver methenamine. Note the staining around the tags which indicates that collagen is present in that area(arrows). X 3,600.



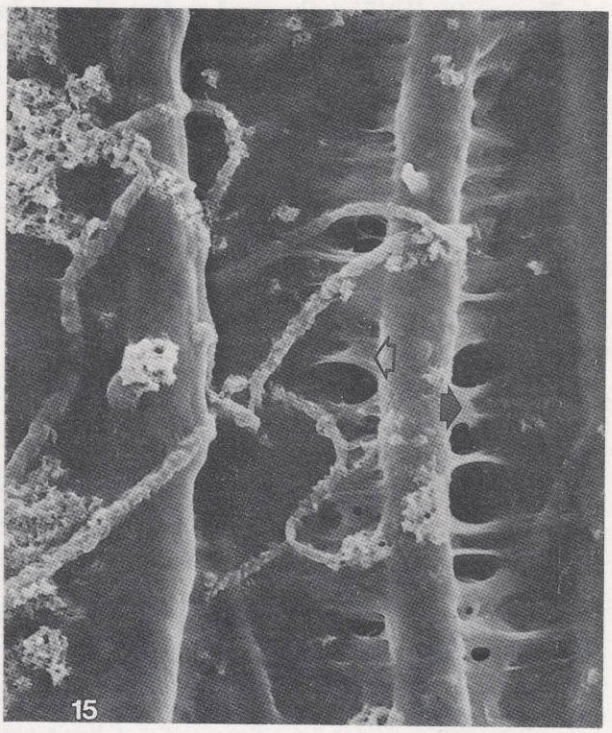


Fig. 14 In deep dentin the intertubular collagen fibers coalesce into wider fibers that penetrate the pulp spaces. Note the characteristic collagen banding pattern. X 30,000.

Fig. 15. Amalgambond specimen displaying a resin tag attached to fibrous structures(arrows). X 7,200.

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