Microscopic Topography by Means of Surface Replicas[*]

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THE surfaces of solid substances have been studied directly with optical microscopes for many years. Biologists, metallographers, and petrographers have investigated the structures to be found on the natural surfaces of a great variety of objects. In addition, indications of underlying internal detail have been brought out on surfaces which have been created artificially by cutting or grinding through specimens. Almost all of the microscopy has been carried on with reflected light, from sources directed either obliquely or vertically toward the surfaces. Stereoscopic observation has been most popular for work at low power, but limitations in the depth of field afforded by glass lenses have made the use of magnifications over 150× impractical. Beyond this range, various types of microscopes with a single objective lens have ordinarily been employed.

The application of stereo-photographic techniques to optical microscopy has also been seriously restricted by the shallow depths of field, for only a very small area of the image remains in the focal plane when the specimen is tilted to the necessary angles. As a consequence, most recording of surface detail has been in the form of single photographs. It has been possible, however, to make tridimensional measurements, like those that could be made from stereoscopic photographs, directly from the specimens through the use of microscopes equipped with various types of micrometers.

For the past fifteen years the electron microscope has found widespread use in the observation of surface structure. In addition to providing tremendously increased resolving power and magnification, the electron microscope also exceeds the optical microscope in the depth of field afforded by its electromagnetic objective lens. Due to this latter advantage, an opportunity for greatly improved stereoscopic micrography is available, and a device for tilting of specimens has been included in the basic design of almost all types of electron microscopes. In actual practice, however, stereoscopic electron microscopy has not become especially popular. This has been due chiefly to the general features of the electron beam which serves as the illuminant, and to those of specimens which are suitable for examination. The standard instruments permit illumination of the specimen only by transmission of electrons. The electron beam itself has so little penetrating power that objects of more than a few tenths of a micron in thickness are opaque. Since direct examination of the surfaces of solids has thus been virtually precluded, a method has had to be developed whereby the structural detail can be studied indirectly. The basic technique consists in reproducing the surface structure in suitably thin micro-impressions, known as replicas, which can be examined under the electron microscope instead

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of the objects themselves. Almost as many procedures have been devised for preparing replicas as there have been problems at hand, and the applications have extended so far down the microscopic scale as to allow study of macromolecular arrays on the faces of crystals no larger than 2,000 angstrom units in their greatest dimension.(Ref. 1).

Under the electron microscope structural detail becomes visible largely as a result of scattering, rather than absorption, of electrons. Since replicas are extremely thin homogeneous films, their electron-scattering capacity is low, and, as a general rule, the microscopic images which are obtained from them are inherently lacking in contrast. It has become a standard procedure to enhance the contrast by means of a technique known as shadow-casting (Ref. 1). In this method a heavy metal is evaporated in high vacuum and is deposited obliquely upon the surface of the replica in a layer of only a few atoms in thickness. Those aspects of elevations on the replica which face the oncoming atoms directly are more heavily coated than those that are shielded from them. Different degrees of partial opacity are thus imparted to the preparation, and since the metallic atoms have a high electron-scattering capacity, contrast in the image is vastly improved. As a result of this treatment, elevations appear to cast long shadows, creating an effect much like that obtained in an aerial view of a landscape illuminated by early morning or late afternoon sunlight.

Photographs made from shadowed replicas are interesting in that they have a definite tridimensional appearance. It is this feature that has tended in large measure to minimize interest in stereoscopic micrography. Surface contours can readily be distinguished, and, if the geometric relationship between specimen and source of evaporated metal is known, quite accurate measurements of the heights of elevations can be made from the lengths of their shadows. Stereoscopic micrography is also made somewhat unrewarding by the fact that it has proved useful mainly at low power, due to the progressive decrease in depth of field which accompanies increasing magnification. Beyond the first few thousand diameters, the minute third dimension that can be brought out in stereomicrographs is as strikingly revealed in photographs more easily made from shadowed preparations.

Surface replicas have been used extensively throughout the world for the electron microscopic observation of the calcified body tissues (Ref. 2). They have provided the only means for studying the topographical configurations on the external surfaces of tooth enamel. Considerable interest has been centered upon the structural changes in surfaces which occur with advancing age, and the effects of treatment with chemical agents, such as fluorides, which are applied clinically for the control of dental caries. Replica methods have also proved invaluable in the study of the internal structure of fully calcified teeth and bones, for thus far it has not been possible to cut thin enough sections of these tissues for direct examination without subjecting them to preliminary softening through some form of demineralization. For these investigations of inner detail, replicas have been made from surfaces created artificially by grinding and polishing. The underlying structure is brought out on such highly polished surfaces by etching them lightly with acid or treating them with calcium-binding reagents. Most of the stereoscopic electron micrographs which have been published have been made from replicas of etched ground sections of teeth (Ref. 3 and 4).

The studies in this laboratory have been directed for the most part toward determining the fine structure of normal mature dental tissues, the alterations which occur as a result of malformation or disease, and the effects of various chemical agents on the structure and properties of tooth enamel. At the same time, in the course of developing replica techniques, possible applications of the methods in the examination of other solids have been explored. The illustrations which follow have been selected from the records of these investigations to demonstrate the fine detail that can be reproduced in surface replicas and the pronounced tridimensional effect that is characteristic of shadowed preparations.

Two general types of replicas have been made from external tooth surfaces or from the surfaces of ground sections. Negative, or single stage, replicas have been prepared by applying a dilute solution of collodion to the surface and allowing it to dry. The film which forms is suitably thin for electron microscopy, but it is too fragile to be removed by manual stripping. In order to recover the replica the specimen must be dissolved away by immersion in acid (Ref. 5). Positive replicas have been made by a two-stage process (Ref. 6). A preliminary collodion replica, much too thick for electron microscopy, is stripped from the surface. The detail on this replica is then reproduced in a thin film of carbon, deposited vertically on the collodion through the evaporation of graphite in high vacuum. The resulting positive carbon replica is easily recovered by removing the collodion with suitable solvents. Positive replicas have the advantages that the specimens from which they are made are preserved, that much more irregular surfaces can be reproduced than with negative replicas, and that their thickness can be more rigidly controlled. As implied by the nomenclature, the elevations and depressions are inverted in negative replicas, whereas in positive replicas they are restored to their original perspective.

Under certain conditions, the uppermost layer of a solid may become so firmly entrapped in a replica that it is actually removed with the film. When such an adherent layer is thin enough to be penetrated by the electron beam, it can often be examined profitably as part of the replica. Preparations of this type have come to be known as pseudo replicas (Ref. 1). They have had quite extensive application in studies of enamel and dentin.

Some examples of the structural detail revealed in replicas of the etched surfaces of ground sections of teeth are presented in Figure 1. Much that is new has been learned from such preparations about both the inorganic and organic components of enamel and dentin, many of which are so small that they can be seen only under the electron microscope. Pseudo-replicas of enamel, like the one shown in Figure 1A, have been particularly instructive, for the crystallites actually present can be observed and measured directly under the electron microscope. In addition, they can be identified as apatite (tricalcium phosphate) by subjecting the pseudoreplicas to electron diffraction. The marked tridimensional appearance of micrographs made from replicas of dentin is illustrated in Figure 1B–D. The varying heights of the elevations in these pictures are indicated clearly by the differences in the lengths of the shadows. A few of the other types of surfaces which may be studied by means of replicas are depicted in Figure 2.

Replica techniques have proved very useful in studies of the physical and chemical properties of tooth enamel. Many of the experiments have been directed toward evaluating the effectiveness of various chemical agents in increasing the resistance of the outer enamel surface to acid action. Although the exact mechanisms through which enamel is destroyed in dental caries are still not clearly understood, there is considerable evidence that acid action may be a major factor. The ability to increase acid resistance of enamel is thus one of

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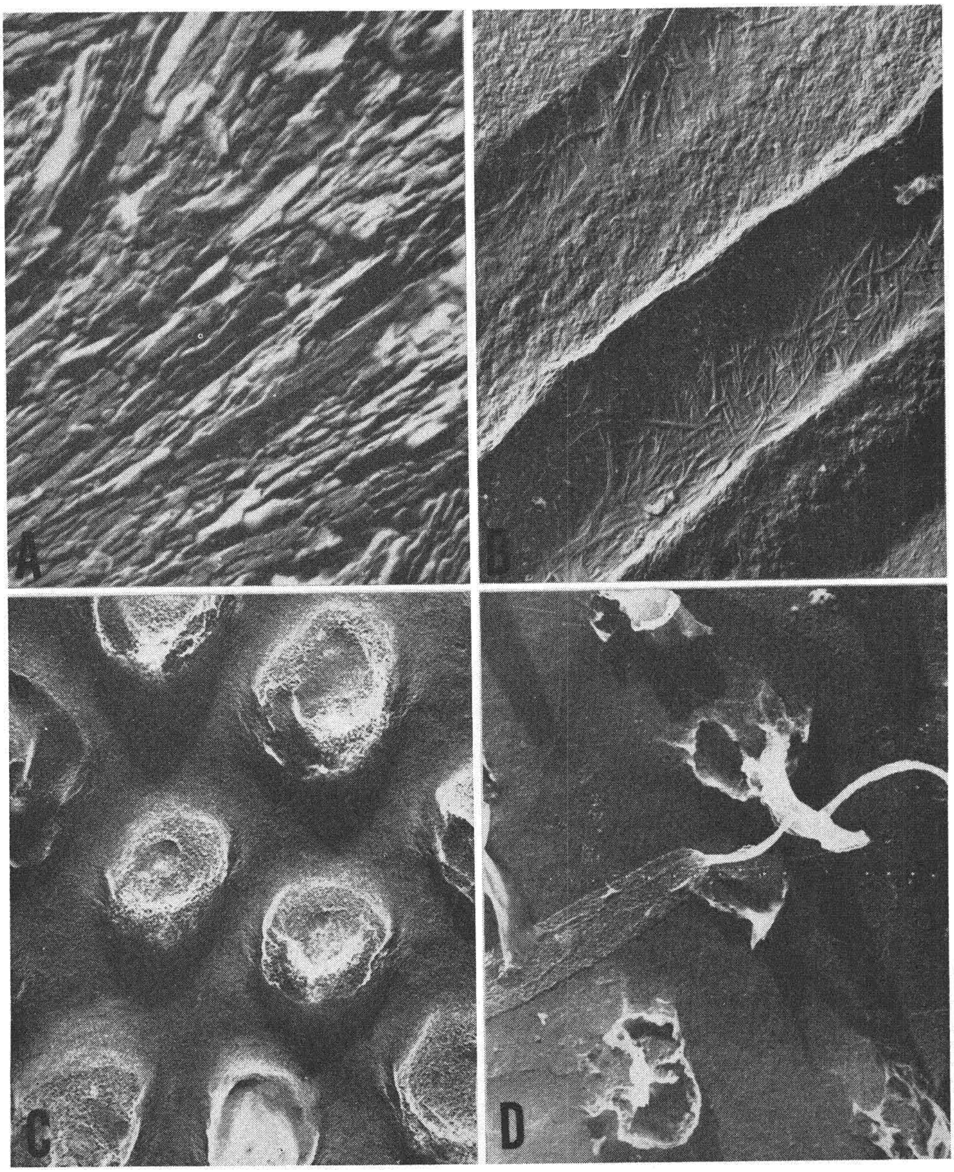

FIG. 1. Electron micrographs of shadowed replicas of etched ground sections of teeth.

A. Positive carbon pseudo-replica of enamel. The actual mineral crystallites present were entrapped in the original collodion replica and transferred in situ to the final carbon film. 13,500×.

B. Positive carbon replica of dentin. The deep trough represents one of the tubules (which course through the calcified matrix) that has been longitudinally bisected and emptied of its contents. 7,000×.

C. Negative collodion replica of dentin. The elevated structures represent cross-sectioned tubules that have been etched to a greater depth than the surrounding matrix. 5,000×.

D. Negative collodion pseudo-replica of dentin. The branched process emanating from the cross-sectioned tubule is an actual organic dentinal fiber that remained attached to the replica after the tooth substance was dissolved away. 5,000×.

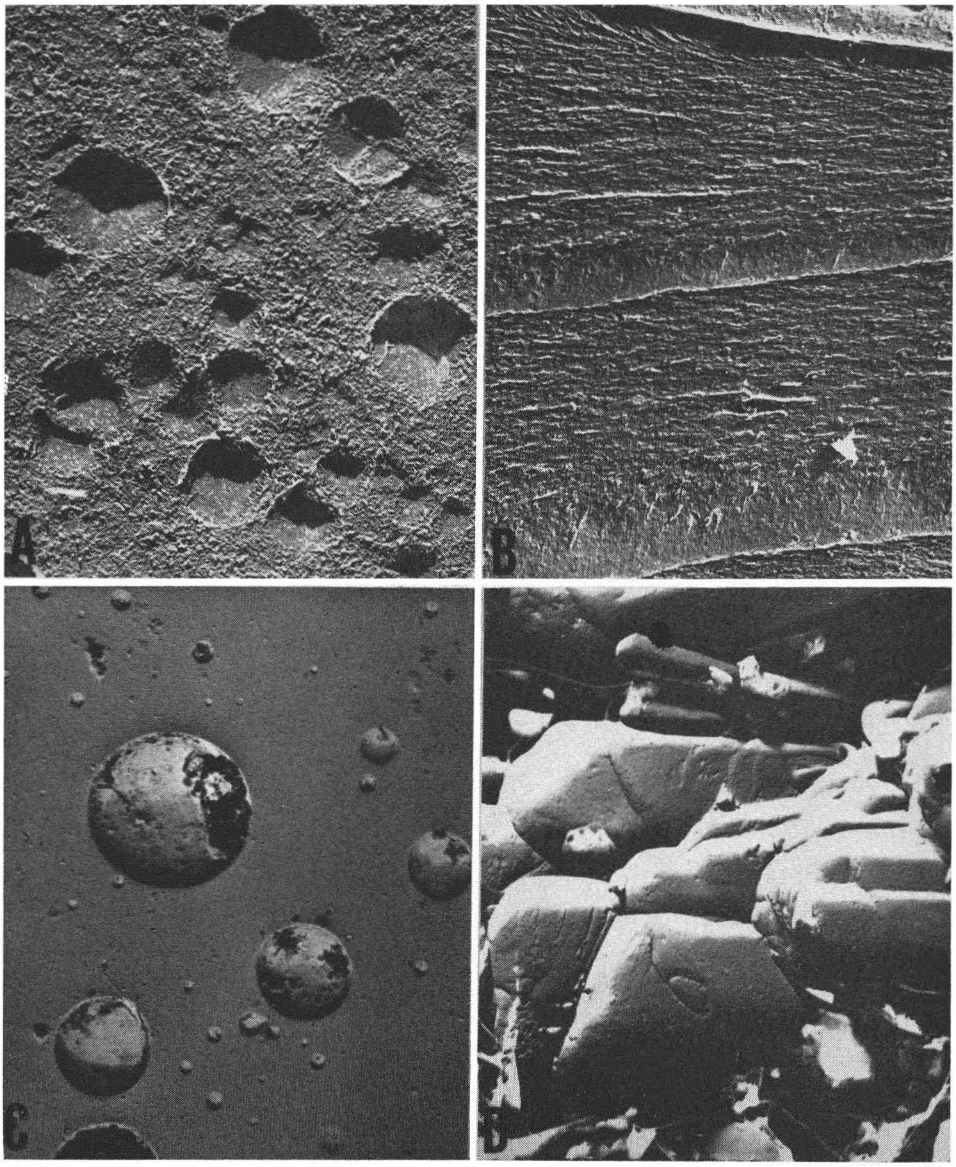

FIG. 2. Further examples of typical electron micrographs made from shadowed replicas.

A. Negative collodion replica showing the calcium fluoride cyrstals which formed on the outer enamel surface of a tooth immersed for fifteen days in a sodium fluoride solution. Micrographs of this type must be carefully oriented for examination, so that elevations and depressions are seen in their proper perspective. If this picture is rotated through 180 degrees the detail appears to be inverted. 11,000×.

B. Positive carbon replica of the surface of a planed piece of walnut wood, showing the parallel arrangement of the constituent fibers. 5,000×.

C. Positive carbon replica of a glazed ceramic building tile, showing the small pits which characterize the surface. 5,500×.

D. Positive carbon replica of the surface of a block of plaster of paris, showing the irregularly overlapped arrangement of the component crystals. 8,000×.

the principal criteria in laboratory screening tests, through which specific compounds, such as certain fluoride salts, are selected for clinical trial as possible agents for the control of caries. In testing such compounds, similar areas on treated and untreated surfaces are subjected to the same acid treatment, and the depths of etching are compared by means of shadowed replicas. Because of variations in the response to acid between various regions on the same tooth, and between different teeth, actual measurement of etch-depth has not seemed feasible. It has proved more practical to classify the etch-patterns on the basis of a previously established pictorial scale showing progressively increased de-

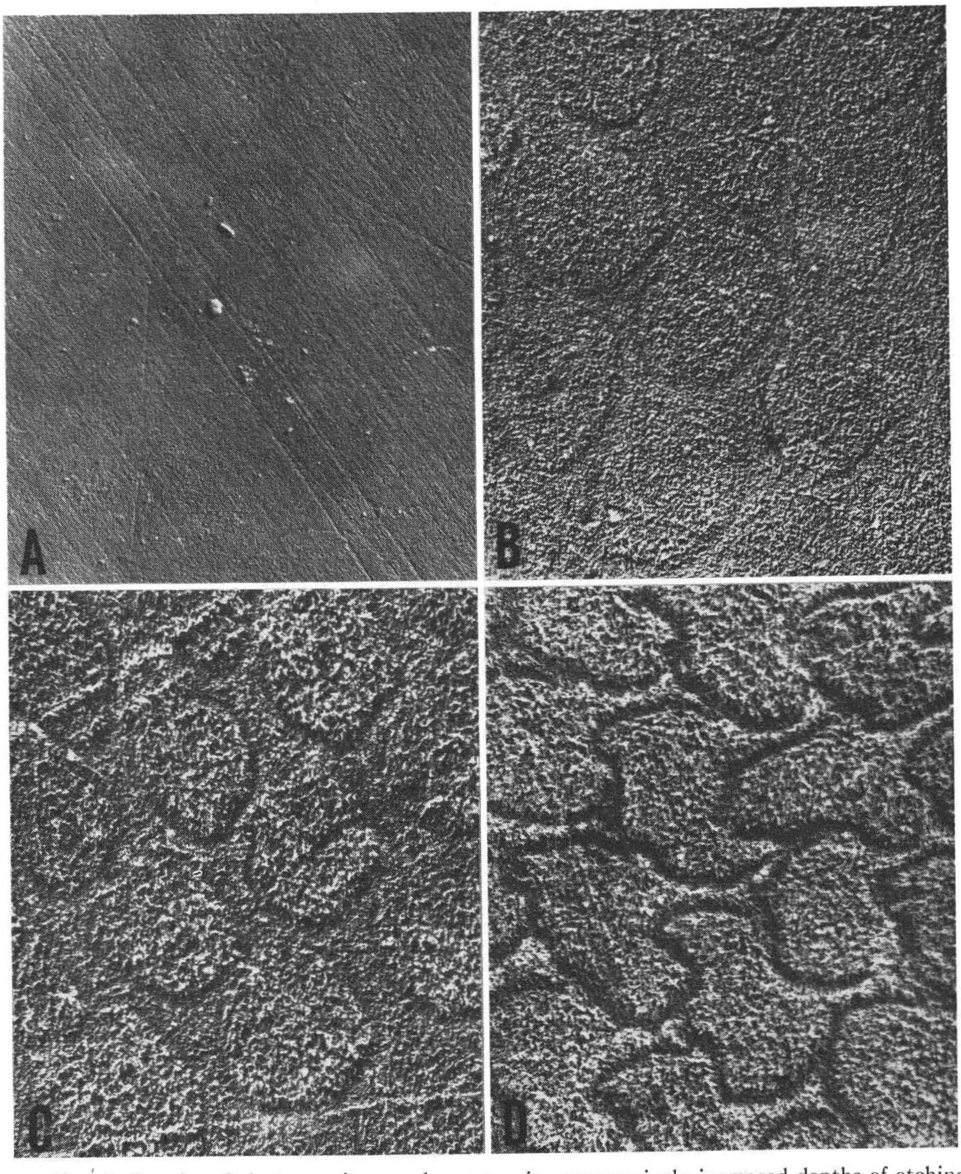

FIG. 3. A series of electron micrographs portraying progressively increased depths of etching (A through D) on acid-treated outer enamel surfaces which can be visualized by means of positive carbon replicas. 4,000×.

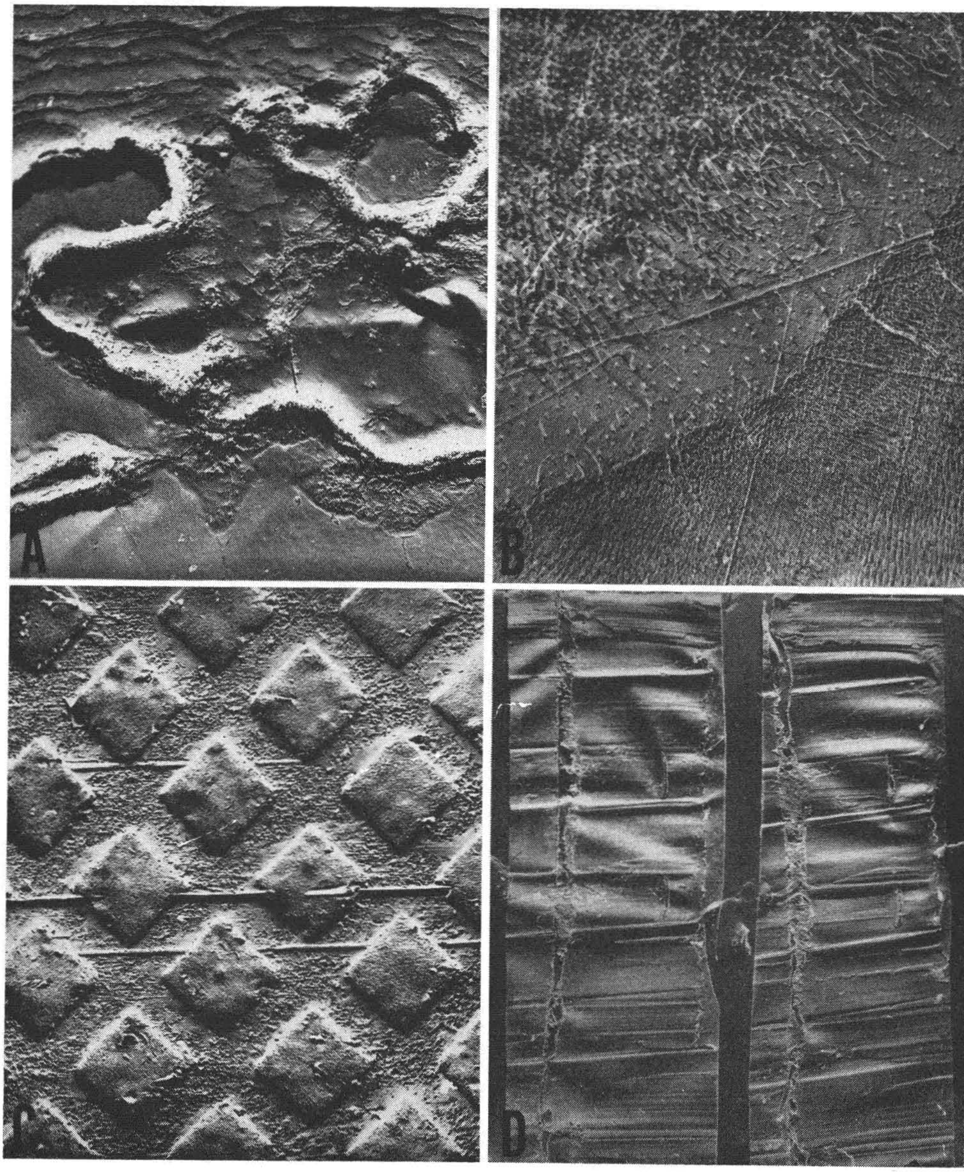

FIG. 4. Optical micrographs made from shadowed negative replicas.

A. Collodion replica of the external surface of a tooth, showing the irregular junction between the enamel of the crown (above) and the cementum of the root (below). 105×.

B. Collodion replica of the surface of a ground cross-section through a tooth, showing the region of the junction between dentin (above) and enamel (below). 105×.

C. Cellulose acetate replica of the etched surface of a copper rotogravure printing cylinder. The square elevations, representing depressions in the copper, are the minute wells in which ink is held. 105×.

D. Cellulose acetate replicas of the surfaces of two bullets fired from the same revolver. Many points of structural similarity can be noted. In order to show the entire replicas the magnification has been reduced photographically to 3×.

grees of etching. Four electron micrographs from such a standard series are shown in Figure 3.

Although the replica methods have had their greatest exploitation in electron microscopy, they have also been applied profitably in studies made with the optical microscope (Refs. 7, 8, 9, 10). Negative surface replicas adequate for optical examination are very easy to prepare. Most of the techniques that are used parallel those employed in the preparation of specimens for electron microscopy, except that, inasmuch as visible light is much more readily transmitted by them, the films can be made thick enough so that they can be stripped manually from the surfaces with little difficulty. The simplest procedure consists in applying a strip of plastic sheeting under finger pressure to the surface, which has previously been wetted with a solvent for the plastic. The structural detail of the surface is thus imprinted into the superficially softened plastic, which then sets as the solvent evaporates. If cellulose acetate and acetone are used, a replica can be stripped in less than one minute. The optical micrographs in Figure 4 illustrate the type of pictures that can be obtained from metal-shadowed collodion and cellulose acetate replicas.

The examples presented above demonstrate clearly that, even in the absence of stereoscopic pairs of photographs, much can be learned about the third dimension of surface structures from single micrographs of shadowed replicas. Experience has shown that the information thus derived is adequate for the purposes of most investigations. At the electron microscopic level, the procedure generally followed in surface studies has been to make single photographs from shadowed replicas routinely, and stereo pairs from unshadowed replicas in the event of confusion in interpretation or when the tridimensional effect is not sufficiently pronounced.

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