cells spoken of as hemocytoblasts in a highly fatal disease of fowls. In many instances a retardation of mitotic activity both in the marrow and in the peripheral blood was demonstrable 24 hrs after treatment. Some evidence to indicate that these mustards have a lethal effect upon the causative virus in addition to causing death of the proliferating cells was indicated by the failure of blood drawn from treated birds to transmit the disease.

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In Vitro Studies of Caries of the Enamel in the Syrian Hamster

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In previous pathological studies of enamel caries in the human (*1*, *2*) and hamster (*4*) it was observed that two morphological types of microorganisms were constantly associated with the microscopic plaque and the early enamel lesion. Thread forms predominate in the more superficial areas of the plaque, whereas spheroidal forms were always present in the affected enamel beneath the plaque. As a follow-up of this observation, it seemed advisable to attempt to isolate and culture these organisms.

Scrapings were taken from the plaque overlying the early carious area, in which the first evidence of enamel caries is the appearance of a brown-stained spot in an apparently intact enamel surface. The scrapings were cultured, and a growth of filamentous forms was obtained which resembled those found predominating in the plaque of the earlier histological sections. These organisms developed on blood agar after incubation at 37° C for 4–5 days. An atmosphere of about 10% carbon dioxide seemed to be favorable for primary isolation. Subsequently the organisms will grow on a variety of media. Fermentation of the carbohydrates tested in the growth of the organisms was slow with the different strains tested. Gelatin was not liquefied, but the colonies developed a dark brown pigment in 1–2 weeks in this medium.

These organisms are similar to the actinomycetes, a group which has been called simple molds or pleomorphic bacteria. They are easily identified with some of the *Leptothrix* and *Cladothrix* described in the earlier literature under terms no longer used. Except for their more aerobic habits and the ease with which they are cultivated, they correspond closely with some strains isolated by several workers from normal mouths and throats, from carious detritus, gingivitis, and cervicofacial actinomycosis, and classified as *Actinomyces bovis* or *A. israeli*.

A high degree of pleomorphism is characteristic of these isolated filamentous organisms. Various small forms resembling diphtheroids, short bacilli, and irregular cocci, as well, were frequently observed to be associated with the filamentous cells in a single colony. After repeated subculture, some of these associated forms almost completely replaced the filaments. The change in the appearance of the filamentous types to other morphological forms may be accompanied by a change in the appearance of the colony, but this is not always the case. The filamentous colony is rough, whereas when transition to other forms takes place, the colony may have a smooth, glazed appearance. Neither the age of the culture nor the type of media has thus far been shown to be associated with the change in the morphological appearance of the cells. While the various cell types of the colonies most likely represent a single highly pleomorphic species, the possibility of a symbiotic relationship between two or more species which are difficult to separate in culture could not, at this point, be entirely ruled out.

Isolated filamentous cells growing on laked blood agar were selected for study and their multiplication was recorded with photomicrographs at definite intervals of time (Fig. 1). These observations were made at 2-hr

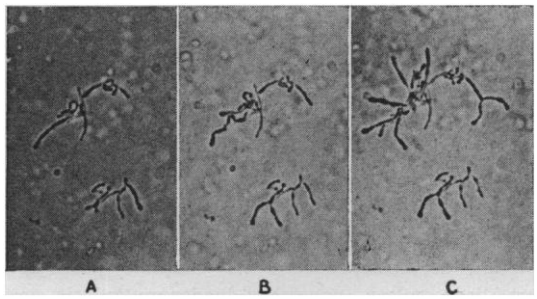

Fig. 1. Illustration of the filamentous organisms selected for microscopic study at incubation of: A, 7 hrs; B, 9 hrs; C, 13 hrs.

intervals for periods up to 33 hrs. In order to prevent this preliminary study from becoming too prolix, the experiment was limited to a relatively stable strain of the isolated organisms.

In the cultures of a single organism, the filaments were observed to divide into short bacillary bodies. Some of these in time elongated into filaments. Terminal or lateral branches were frequently found on the lengthening filaments. Occasionally a whole segment of a filament appeared to undergo autolysis, while the adjacent portion continued to proliferate. As the colony continued

to grow, through multiplication, the filamentous cells at the periphery assumed a palisade arrangement and appeared somewhat like the parallel alignment of organisms found on the tooth surface in the previously studied histological sections. We presume this characteristic arrangement may be the result of crowding in the colony. Further observations are being made by this method on other more pleomorphic strains.

In addition to the studies already described, an attempt is being made to determine whether or not the colonies composed of coccoid and diphtheroid cells have originally developed from filamentous organisms during the period of primary isolation from the tooth scrapings. If a change from the filamentous types to other forms does occur in primary culture, it would be exceedingly difficult, by routine bacteriological methods, to distinguish these colonies from those of other oral bacteria.

As can be seen from the foregoing discussion, the preponderance of filamentous organisms in the surface plaque on enamel, together with their pleomorphism when cultured, invites speculation that the round forms seen in the initial phase of early enamel caries might result from the metamorphosis of the filamentous forms which are characteristically seen on the surface.

An attempt was made to produce caries *in vitro* with the strains of actinomycetes which were isolated from the teeth scrapings. Since it is possible to determine whether erupted human teeth are noncarious only by histological examination, they are not suitable for *in vitro* work. Likewise, the chance of contamination with other mouth organisms when removing unerupted teeth from the oral cavity, together with the scarcity of human material, causes these procedures to be highly unsatisfactory.

The method used in earlier histological studies in which rats' teeth were dissected from their crypts just prior to eruption offered what seemed to be the most ideal method for this kind of study. The Syrian hamster was selected for this phase of the experiment, since its molars are peculiarly susceptible to dental caries which more closely resemble that observed in human teeth (*3*). It was possible to obtain the molars from the maxilla in a sterile condition just prior to eruption by the following procedure:

Unerupted first and second molars of the hamster were dissected from crypts of the maxilla. The oral mucosa was first stripped off, exposing the teeth in their crypts. Following the removal of the teeth, under aseptic conditions, they were placed in Difco Proteose Peptone No. 3 broth and incubated for 10 days to establish their sterility. The cotton plugs of the tubes were replaced with sterile rubber stoppers to prevent evaporation of the broth. Following the 10-day incubation period, the broth containing the teeth was inoculated with a pure culture of the filamentous organisms. Strains obtained from the human as well as from the hamster were used for the inoculations. The tubes were then incubated for a period of 3–5 months. At 3- and 4-week intervals the broth was pipetted off and fresh broth added. The culture was examined for contamination, and the pH was measured at these intervals. It was noted that at no time did the pH of the cultures drop below 6.5.

At the termination of the incubation period the teeth were removed and placed in neutral formol in preparation for histological examination. Following fixation and decalcification, they were imbedded in nitrocellulose and sectioned serially on the microtome to a thickness of 4 μ. The sections were then stained after several bacteriological methods. To date, the histological preparations of only two of these infected teeth have been completed because of the process involved.

Molar I was inoculated with strain #80, which was obtained from scrapings of hamster teeth. This strain is highly pleomorphic. The viscid sediment which develops in the broth completely enveloped the tooth and seemed to adhere loosely to it. The pH of this culture was 6.5 on the occasions when it was tested. After two months incubation, the tooth began to undergo a slight brownish discoloration. The discoloration was in contrast to the control teeth, which were not inoculated. This tooth was removed from the culture on the 74th day and placed in the fixative agent. The histological examination showed a dense intertwining mat of filamentous cells on the surface of the tooth and around the remnants of the ameloblastic layer which is adherent to the enamel surface (Fig. 2, A). Many filaments were observed to project down into the enamel rods and interrod intervals for considerable depths. In some positions the organisms involved rods far ahead of the area of general breakdown. The irregular round bodies were especially numerous in these zones (Fig. 2, E). The rod matrix adjacent to the organisms exhibited an intense acidophilic staining reaction. In general, the picture was identical in every way to that observed in human caries and caries

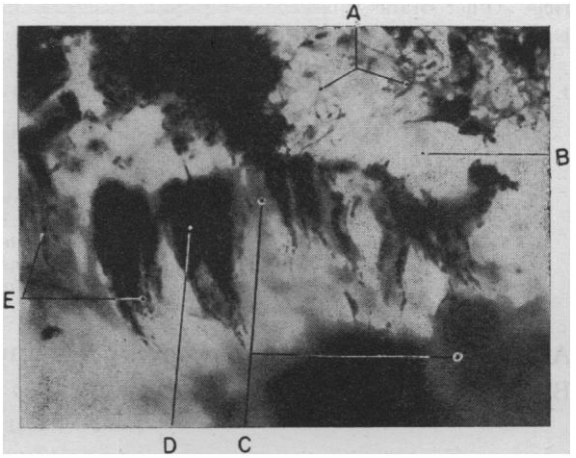

FIG. 2. Photomicrograph of a serial section taken from *in vitro* studies in which a hamster molar was infected from a pure strain of filamentous organisms. 5 μ neutral formol fixation MacCallum's Gram × 1,000. A—Filamentous organisms in the ameloblastic layer. B—Artifact due to separation. C—Uninvolved enamel matrix. D—Infected enamel undergoing degradation. E—Organisms penetrating deeply into the enamel. Note the spheroidal-appearing bodies occupying a position well in advance of areas of general breakdown.

in the Syrian hamster *in situ*. The organisms penetrate along selected channels and appear to invade and utilize the organic matrix in satisfying their metabolic requirement.

Molar II was inoculated with strain #82, isolated from scrapings of a microscopic human carious lesion. This strain was also highly pleomorphic. The pH of the culture, when tested, was in the neighborhood of 7.1. On the 75th day of incubation the molar was removed from the culture and placed in the fixative. An examination of the culture at this time showed it to be contaminated. It is presumed that the contamination occurred when the broth was changed 5 days earlier, since it was uncontaminated at that time. In the histological preparation of this tooth a considerable amount of the matrix was lost. The fragments which were recovered, however, exhibited a penetration by filamentous forms similar to that seen in Molar I.

It is admitted that the *in vitro* experiments to date are too few to make a definite conclusion, but we feel that the studies advance a method by which further studies may be conducted. The observations indicate that there may be an etiological relationship between the oral actinomycetes and caries of the enamel. The *in vitro* work is being repeated, using the same strains of organisms. Other strains will also be employed and other oral bacteria will be tested by the above method.

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A Paradoxical Zone Phenomenon in the Bactericidal Action of Penicillin *in Vitro*

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If varying concentrations of penicillin are added to suspensions of bacteria *in vitro*, and if at intervals thereafter one determines the number of organisms surviving at each concentration, one usually finds that the susceptibility of the particular organisms can be defined in terms of three concentrations of penicillin: (1) the concentration which serves only to reduce the rate of multiplication, (2) a somewhat higher concentration at which the organisms die faster than they multiply, with a slow decrease in the number of viable organisms, and (3) a maximally effective concentration, which varies between 2 and 10 times the "sensitivity level" of the organism as ordinarily defined. At this optimal concentration of penicillin the organisms are killed at a maximal rate which is not further affected even by a 20,000-fold increase in the concentration of the drug.

These relationships are illustrated in Table 1 for the C-203 strain of *Streptococcus pyogenes*. Different species of bacteria, and different strains of the same species, vary with respect to both the effective concentrations of penicillin and the maximum rate at which the organisms can be killed. Qualitatively, however, with most strains so far studied, there is a maximally effective level as defined above.

Paradoxical and as yet unexplained results have been obtained with certain strains of streptococci and staphylococci. With these strains there were the same three critical concentrations of penicillin, including a concentration at which the organisms were killed at a maximal rate. With further increase in the concentration of penicillin, however, the rate of bactericidal action did not remain constant as it did with, for example, *Treponema pallidum*, *Diplococcus pneumoniae*, and the group A strains of *Streptococcus pyogenes* so far tested, but instead was strikingly reduced. This is illustrated by the experiment of Table 2, carried out with the Smith strain of *Staphylococcus aureus*. In that experiment, after, for example, 6 hrs exposure to penicillin at varying concentrations, there were 400 times as many viable organisms at a concentration of 2,048 μg/cc than there were at the optimum concentration of 0.096 μg/cc, and it required 27 hrs instead of 5 to kill 99.9% of the organisms.

To test the regularity of this phenomenon, 13 strains of β-hemolytic streptococci (Lancefield groups A, B, C), 7 strains of *Str. faecalis*, 4 other strains of α-hemolytic streptococci, 7 strains of *Staph. aureus*, and 2 strains of *Staph. albus* were tested by the same technic.

Five of the 7 strains of *Str. faecalis* were rapidly killed at an optimal concentration of 4 μg/cc, at which 99.9% of the organisms were rendered nonviable in 5–7 hrs. All 5 of these strains were killed much more slowly at higher concentrations of penicillin. Thus, at 512 μg/cc it required 36–46 hrs to kill 99.9% of the organisms, instead of 5–7 hrs. This zone phenomenon was equally apparent if the results were expressed in terms of the proportion surviving after, for example, 6 hrs. Three of the other 4 strains of α-hemolytic streptococci tested showed a similar zone.

Of the β-hemolytic streptococci, the 5 group A strains failed to show the zone phenomenon. However, all 4 of the group B strains, and 2 of the 4 group C strains tested, showed a zonal susceptibility to penicillin resembling that illustrated in Table 2. Three of the 7 strains of *Staph. aureus* tested, and 1 of the 2 strains of *Staph. albus* showed the zone phenomenon in varying degree.

The paradoxically reduced bactericidal activity of penicillin in high concentrations was not due to the appearance of resistant strains at the higher concentrations. With *Str. faecalis*, the last few organisms surviving from an initial inoculum of approximately 1,000,000/cc, when subcultured and tested by the same technic as the original strain, proved to have the same resistance as the original culture, judged either by the effective concentrations of

[1] With the technical assistance of Arlyne D. Musselman.