HYDROLYSIS OF PROTEIN IN CARTILAGE AND BONE

BY

P. PINCUS, D.D.Sc.Melb., Ph.D.Lond.

(From the Medical School, Westminster Hospital)

The purpose of the work described below was to test the effect on cartilage and on bone of bacteria associated with dental caries.

Dental caries may be defined as loss of tooth structure apart from fracture or abrasion. Harrow (1946) describes dentine as 23% organic matter, mainly collagen, and 77% inorganic matter. Dental caries, then, constitutes loss of both organic and inorganic matter. On the aetiology of caries two schools of thought exist: (1) the inorganic matter, mainly calcium salt (Harrow, 1946), is first removed by acid formed by bacterial breakdown of ingested carbohydrate material, and proteolysis follows; (2) proteolysis constitutes the initial attack and the calcium salts are lost later.

The fact that in carious teeth the protein collagen is hydrolysed suggested that strains of bacteria capable of hydrolysing collagen should be found in such teeth.

Effect of Caries Bacteria on Cartilage and Proteins

Carious material was obtained from a human tooth, immediately after extraction, by scraping the carious lesion with a sterile, slowly revolving, large-size rose-head dental bur (No. 10), allowing the scrapings to drop into 2 ml. of ordinary nutrient broth. For each experiment carious material was prepared in this way. A sterile container was taken to the dental surgery, the extracted tooth placed in it direct from the mouth, and the carious material prepared at once. The period between dental extraction and the inoculation of media with carious material was generally less than 30 minutes; inoculation was done with a sterile glass tube 7 mm. in diameter and about 5 cm. long, with a rubber teat to allow the scrapings to be sucked up and then used as inoculum.

As a source of collagen, cartilage was used. It was obtained from the abattoirs, cleaned, cut into pieces 1 cm. at their largest, and covered with 0.9% NaCl solution in test-tubes. The cartilage was then sterilized without delay, by autoclaving, by steaming, or by heating to 57° C. for one hour on each of eight successive days. In other experiments cartilage was cleaned, cut up, and as before placed in test-tubes covered with saline (0.9% NaCl), no attempt being made to attain or preserve sterility. Serum was often seen to exude from the cartilage.

Carious material was inoculated into broth, on to nutrient-agar and blood-agar plates, on more than 150 consecutive occasions. On incubation at 37° C. bacterial growth was observed in all but about half a dozen; failure to obtain growth on these occasions may have been due to bactericidal substances associated with the practice of dental surgery, such as phenol or creosote. The presence of living bacteria in carious material was thus confirmed.

Cartilage in test-tubes, either sterilized as described or freshly obtained but unsterilized, was inoculated with carious material and incubated at 37° C. Whether the cartilage was sterilized or not, it appeared from Gram-stained smears and aerobic cultures made at frequent intervals, sometimes each day, that the same types of bacteria were present in the cartilage-saline preparations on incubation. At first mixed bacteria grew, but in from two to four days Gram-positive cocci predominated. Following this, Gram-

negative bacilli grew and outnumbered all other forms seen; at times they were the only organisms found in Gram-stained smears.

After about seven to ten days' incubation cartilage was seen to be lysed, the separate white pieces darkening to a yellowish powdery mass. The familiar odour resembled that noticed when proteolytic organisms grow in cooked-meat media and, without actual test in the present work, ascribed to such products of protein degradation as indole and skatole.

Fig. 1 shows this change, with the light wooden sticks, identical in weight and length and carrying paper markers, gradually slipping down the tubes as the cartilage loses its springiness. Twenty-three experiments of the kind described have been carried out on cartilage; in all but one the result was as recorded.

It has been said above that Gram-positive cocci and Gram-negative bacilli were seen in cartilage-saline preparations inoculated with carious material and incubated at 37° C. Both these organisms have been grown in pure culture, readily on some occasions; the use of penicillin media has assisted isolation. At times difficulty has been met with in obtaining free growth of the Gram-negative bacillus. Cultural characteristics of the organism are set out below. Isolation of the Gram-negative bacillus was carried out on 17 occasions, pure cultures being obtained. Sterile cartilage-saline preparations were then made, inoculated with the Gram-negative bacillus, and incubated at 37° C. Gross lysis of cartilage occurred, showing that this organism was capable of proteolysis.

Media containing other proteins were then exposed to cultures of the Gram-negative bacillus at 37° C. and observations of free amino-groups made by the use of Sorenson's formol titration method; broth, cooked meat.

FIG. 1.—Cartilage undergoing bacterial hydrolysis, at the same time losing its characteristic springiness and allowing the light wooden sticks to slip down; on the extreme right the stick rests on the bottom of the tube.

serum, and sericin (from silk) were tested in this way. Copious growth was most readily obtained with sericin media.

Following a personal communication from Dr. M. Stephenson, basal medium No. 7 (Stephenson, 1939) was prepared. It contains KH_2PO_4 1 g., MgSO_4 0.7 g., NaCl 1 g., FeSO_4 0.03 g., water 1,000 ml. Sericin from silk was used as the source of nitrogen. The method adopted was to place 1 g. of cleaned raw silk in a phial, cover with 15 ml. of Stephenson's medium, autoclave 30 minutes at 15 lb. pressure (121° C.), and adjust to pH 7 with sterile N/100 NaOH. The sterile supernatant liquid gave positive biuret, Millon, and xanthoproteic reactions. On inoculating sericin media with the Gram-negative bacillus and

incubating at 37° C., a maximum titration with formol was reached in seven to ten days; later a fall in free amino-groups was noted. Results of two such experiments were as follows.

Table showing titration of free amino-groups against N/10 NaOH; Gram-negative bacillus growing in sericin + Stephenson's basal salt media at 37° C.

Days	ml. of N/10 NaOH		
0	0.05	0.05	0.05
2	0.06	0.06	0.06
8	0.14	0.31	0.31
16	0.25	0.51	0.51
28	0.11	0.45	0.45

Demonstration of the presence of protein-lysing bacteria in carious material rests on these points: the naked-eye appearance of cartilage undergoing lysis (Fig. 1); its microscopical appearance; the typical odour assumed to be due to the presence of indole and skatole, possibly associated with the breakdown of serum present in cartilage; and the quantitative estimations of free amino-groups (see Table).

Effect of Caries Bacteria on Bone

A femur freshly removed from a cow aged about 5 years was stripped of skin and muscle and sawn into blocks about 1 by 0.5 by 0.5 cm. These blocks, two or three to a tube or screw-top phial, were covered with a mixture of one part broth to 20 parts saline, some sterilized by heating to 57° C. for one hour on each of eight successive days, others autoclaved. These bone preparations were inoculated with the Gram-negative bacillus isolated from carious teeth, and incubated at 37° C. for several weeks, some being opened and examined from time to time. A good growth was difficult to get; the addition of serum was of doubtful value. Possibly the best method was the use of a heavy inoculum.

FIG. 2.—Slice of femur from 5-year-old cow. Markings show type of block sawn out.

Fig. 2 shows a slice of mature cow femur; a piece typical of those used for experiments is outlined. Fig. 3 shows mature bone after lysis, the periosteum being indicated. An enlarged view of mature bone after lysis is shown in Fig. 4.

Observations were made on lysed mature bone at two stages: when lysis was half complete and when the bone was reduced to a fragmentary condition. During a routine

FIG. 3.—Microtome preparation of lysed mature bone; the periosteum is indicated by an arrow. (× ca. 32.)

examination it was noticed that some bone was soft and sponge-like after about 15 days' exposure to the lysing preparation. The soft condition enabled microtome preparations to be made without the usual preliminary acid decalcification. In places structure typical of mature bone may

still be recognized. Bone undergoing lysis is not readily examined unless the phial or tube is emptied, because of the considerable deposit that forms in the lower part of the container. This yellow or yellowish-brown material is in a very finely divided state, and if shaken up may take hours to settle. Eventually bone-saline preparations were prepared and sterilized as set out above, inoculated with the

FIG. 4.—Enlarged view of lysed mature bone. (× ca. 100.)

culture of the Gram-negative bacillus, and allowed to remain for several weeks at 37° C.

Frequently, sometimes each day, colorimetric estimations of the pH value have been made. Variations between 6.5 and 7.5 have been found, with end-results of prolonged incubation near 8 or even 8.5. Lysis of bone occurred more readily in screw-top phials than in test-tubes plugged with cotton-wool. The nutrient broth did not reduce Fehling's solution and gave a negative Molisch reaction.

Cultural Characteristics of the Two Organisms

Coccus.—Gram-positive; pairs or very short chains; able to grow in the presence of bile; final pH in glucose broth 4.5; resists heating to 60° C. for 30 minutes; growth is easily emulsified; mannite, lactose, raffinose, saccharose, all give acid and gas; gelatin not liquefied; litmus milk clotted, turned acid, and was decolorized; readily isolated by routine methods. The organism closely resembles an enterococcus.

Bacillus.—Gram-negative, some motility but not a "spreader"; haemolysis variable from none to clearing about 3 mm. wide; litmus milk, either no acid or doubtful; urea attack doubtful; glucose acid but no gas; salicin, mannite, lactose, maltose, saccharose, dulcitol, all negative for acid and for gas (all 48 hours' incubation); gelatin liquefaction either absent or slight unless prolonged incubation is given; after isolation the organisms died out on several occasions. The organism resembles a strain of *Proteus morgani*.

Discussion

The experiments described above show that the organic framework of bone can be destroyed by the proteolytic enzymes of bacteria. It is therefore considered that the main purpose of the experiments has been met. The concept underlying the present experiments is that of hydrolysis of the organic framework of bone. Knowledge of the process of either physiological or pathological resorption of bone appears far from complete. In this respect Brash (1934) states that "the fundamental problem is . . . the control of resorption and not of accretion of bone." Lovatt

Evans (1939), quoting Brash, adds: "This general conclusion, however qualified, is inescapable. It raises resorption to a position of great importance and significance." In 1943 McLean stated: "It thus appears that the decalcification of bone by resorption, involving simultaneous dissolution of both its organic and its inorganic constituents, must be substituted for that of decalcification."

The present work was limited to the isolation in pure culture of one particular organism constantly found in carious material and capable of attack on collagen in cartilage and in bone. Lysis of bone seemed to occur more readily in screw-top phials than in test-tubes plugged with cotton-wool. Lowering of O_2 tension and accumulation of CO_2 occur in cultures in sealed containers.

In 1896 Mall tested the effect of enzymes and of heat (72° and 100° C.) on proteins, finding that exposure to heat followed by exposure to a proteolytic enzyme (e.g., trypsin) produced a greater degree of hydrolysis than either heat alone or the enzyme alone. In the present work there seemed little difference when cartilage was exposed to either 57° C. or 100° C., or autoclaved at about 121° C., though quantitative estimations of hydrolysis were not carried out on cartilage, gross observation appearing adequate. Maximow and Bloom (1942) consider the organic framework of bone to be apparently identical with the collagen of ordinary connective tissue. It is called bone collagen, or ossein. Gelatin is formed from collagen by moist heat. Before they were exposed to proteolytic bacteria, moist heat was applied to both cartilage and bone to sterilize them. The results seen, then, followed exposure to two sets of hydrolytic agents—heat and bacteria. Since Bhagvat and Sreeramamurthy (1944) showed that gelatin (with acid added) was hydrolysed at 120° C. in 45 minutes, it will be clear that sterilization may have caused some degree of hydrolysis.

Some of the above points have been paralleled by other workers. Kowaleski (1940) found collagen to be attacked by mixed cultures from leather works and that Gram-negative bacilli were partly responsible for this attack. Gram-negative bacilli have been shown in the present work to be capable of proteolytic action. The fact that gelatin-peptone bouillon may slowly darken to brown on incubation with bacteria at 37° C. was noted by Drummond (1914). I observed similar appearances with cartilage lysis. Kowaleski (1940) found no bacterial hydrolysis of collagen at pH 5 or 6.5, but maximum action at 7.5. I found the pH value of cultures in which lysis of bone has occurred to vary between 6.5 and 7.5.

These various points bear on relevant aspects of the work, and it will in fact appear that the underlying concept—i.e., that Gram-negative bacilli might attack both the collagen of cartilage and that of bone—existed in full before the present work was started, though it provides evidence bearing out these findings and joining them in one conclusion.

Summary

Tests of the effect on cartilage and bone of bacteria associated with dental caries are described.

Cartilage and bone sterilized by heat are shown to be broken down when incubated at 37° C. in the presence of bacteria taken from carious teeth.

Pure cultures of a bacillus repeatedly isolated from carious teeth are shown to be capable of attacking some proteins.

Following bacterial hydrolysis at 37° C., quantitative estimations of free amino-groups have been made by the use of Sorenson's formol titration method. Bacterial hydrolysis of bone collagen is shown to have occurred in media with pH values close to 7.

Two stages of attack on bone are presented, one more advanced than the other.

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The research, which is primarily on dental caries, is now, through the kindness of Prof. Samson Wright, being carried on in the Department of Physiology, Middlesex Hospital Medical School.

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THE YEARLY RE-EXAMINATION OF A FACTORY GROUP BY MASS MINIATURE RADIOGRAPHY

BY

W. POINTON DICK, M.R.C.S., L.R.C.P.

Physician in Charge, M.C.C. Mass Radiography Unit

The importance of the periodic re-examination of factory and similar groups by mass miniature radiography after an initial survey is generally recognized, and only the shortage of sets and personnel has prevented its routine application. There has not, however, been the same agreement upon the interval which should elapse between surveys. For instance, a three-monthly check has been advocated in some quarters as necessary if the early case of tuberculosis is not to be missed, while Reid (1940, 1941), in America, has formed the conclusion, after considerable experience of the radiological re-examination of personnel of the Metropolitan Life Insurance Company, New York, that a yearly interval is adequate.

The present paper gives the results of the yearly re-examination for four years of personnel of Factory A of the Medical Research Council's Report on Mass Miniature Radiography of Civilians (1945). The findings of the initial re-examination have been published previously as a supplement to the above report.

The surveys under review differ from those reported by Reid in two important particulars—namely, no pre-employment radiography was undertaken, and the annual fluorographic examinations were entirely voluntary and in no way part of a yearly medical check-up. Nevertheless, as is shown below, there has been a marked diminution in the number of significant lesions found in the factory.