BACTERIAL DIGESTION OF COLLAGEN [1]

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(Submitted for publication February 20, 1953; accepted August 18, 1953)

That some species of Clostridia elaborate proteolytic enzymes has been known since the earliest days of bacteriology (1–3). Indeed, it was soon realized that such organisms were unusually active in this respect and played a major part in the natural processes of putrefaction (4–8). Yet neither these studies nor the much more detailed chemical investigations undertaken during and shortly after World War I (9–13) revealed the occurrence of any specific collagenolytic activity. It is true that Henry (14) reported that culture filtrates from *Cl. perfringens* had the property of disintegrating fresh muscle-tissue, but he does not seem to have appreciated the nature of this reaction which we now know to be due to the presence of a collagenase (15). Subsequently, however, in 1931 and 1932 Weinberg and Randin (16, 17) announced that filtrates from cultures of the anaerobe *Cl. histolyticum* and the aerobe *B. anthracoides* would slowly digest small pieces of fresh Tendo Achillis. But these important observations were ignored or forgotten until interest was once more aroused by the work of Maschmann on the protein metabolism of certain anaerobic organisms. In 1937, this worker (18, 19) isolated an enzyme from *Cl. perfringens* that would digest gelatin but had no effect on several other protein substrates with the solitary exception of collagen. He suggested the name "collagenase" for this agent but subsequently (20), for some undisclosed reason, withdrew the term. Several years later, MacFarlane and MacLennan (15) reported that in filtrates of *Cl. perfringens* Type A cultures, a substance was present which would dissolve pieces of fresh Tendo Achillis and destroy the collagen framework of muscle-tissue. They produced evidence that this activity was not due to any of the known enzymes elaborated by *Cl. perfringens,* a finding shortly afterwards confirmed by Oakley, Warrack, and Van Heyningen (21) by immunological methods. This new enzyme was later definitely identified as a collagenase by Bidwell and Van Heyningen (22). Subsequent work at first seemed to indicate that essentially similar enzymes were produced by other organisms (23–27). However, it has been clearly established that other bacterial proteases exist, unable to attack "native" collagen, but more or less readily hydrolyzing collagen which has been "denatured" by various physical and chemical means (28–31, 22).

[1] This work was supported in part by a grant from the Medical Research and Development Board, Office of the Surgeon General, Department of the Army, Contract No. DA 49-007-MD-14.

MATERIAL AND METHODS

Organisms

The majority of the strains used in the present study were obtained from the routine diagnostic service of the Surgical Bacteriology Laboratory. The three strains of Myxobacteria were kindly furnished by Dr. E. J. Ordal of the University of Washington. As for the Clostridia, many of these had been isolated by Dr. Ivan Hall during the bacteriological studies made on traumatic wounds by Dr. Frank Meleney and his co-workers during World War II. Others came from our own collected material (J. D. MacL.) and some were obtained from other sources; in particular Dr. C. L. Oakley of the University of Leeds and Dr. L. S. McClung of Indiana University.

Each organism was cultivated in a suitable fluid medium (usually nutrient broth with 1 per cent proteose peptone) for from 12 to 18 hours. A sample of the culture was then removed, clarified by centrifugation and examined for the presence of collagenolytic enzymes by the following methods.

a. "Natural" collagen: In these experiments the usual substrate was a small (approximately 5 by 3 by 2 mm.) piece of fresh rabbit Tendo Achillis. The culture sample (in various dilutions) was allowed to act at 37° C. for periods of up to 18 hours. Only complete digestion of the fragment of tendon was accepted as evidence for the presence of a collagenase. We subsequently used dried collagen prepared by the method of Bergmann as modified by Einbinder and Schubert (32), and with this material it was possible to get accurate quantitative estimations of collagenolytic activity; for the preliminary survey we were interested primarily in the mere occurrence of col-

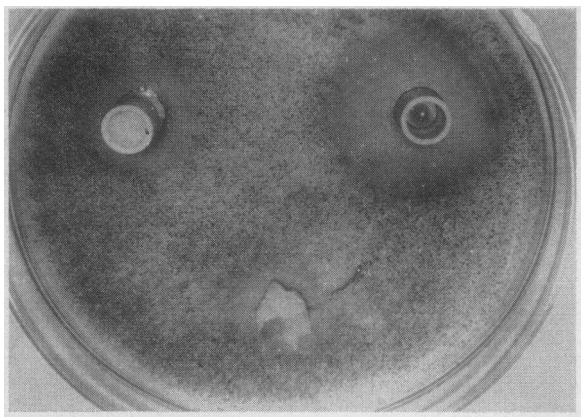

FIG. 1. AZOCOLL AGAR PLATE SHOWING DIGESTION OF THE AZOCOLL

lagenases, our only quantitative studies being obtained from the highest dilution of culture which gave complete digestion of the tendon.

b. *"Denatured" collagen:* For "denatured" collagen Azocoll was employed as a substrate. "Azocoll" is the name given by Oakley, Warrack, and Van Heyningen (21) to a preparation of hide powder coupled with a red azo-dye. Hydrolysis of the hide powder results in the liberation of the dye, permitting an estimation of enzymatic activity by colorimetric means (21, 22).

In the present study Azocoll has been used in several different ways.

A standard amount of culture filtrate has been added to a weighed sample of Azocoll, allowed to react under standard conditions, and the amount of color released estimated. This technique, which is essentially that of Oakley *et al.* (21), is peculiarly adapted for quantitative estimations and is described in detail elsewhere (33).

A simpler method for qualitative studies has been to disperse 70 to 100 mg. in 10 ml. of nutrient agar in a petri dish. The resulting agar plate appears diffusely red, not unlike a blood agar plate. When test organisms are streaked across such a plate and incubated for 12 to 18 hours at 37° C., the presence of an enzyme capable of digesting the Azocoll is indicated by the appearance of a clear zone similar to that seen around a hemolytic organism on a Blood Agar Plate (Figure 1).

With organisms such as *Proteus vulgaris* that give a spreading type of growth, another technique may be employed. The organisms are grown in a fluid medium and then the clarified culture medium is inoculated into small porcelain cups, placed on an Azocoll plate as in antibiotic testing. The plate is then incubated at 37° C. and examined periodically for signs of clarification of the Azocoll around the porcelain cups (Figure 1).

It may again be noted here that Azocoll is not a specific substrate for collagenases. Other proteolytic enzymes which have little or no action on collagen will attack it. On the other hand, the use of fresh unaltered collagen is a true indicator of collagenase activity.

RESULTS

In all, 162 strains of bacteria were examined by the methods detailed above. The results of the study are set out in the following tables (I and II).

These studies show that, apart from two species of anaerobic spore-bearing bacilli, *Cl. perfringens* and *Cl. histolyticum,* no organism examined was capable of digesting native collagen. The ability to produce enzymes active against Azocoll was rather commoner, being found in almost all clostridia in the series and in certain strains of pyococci, of aerobic spore-bearers (Bacilli) and of *P. vulgaris.* The enzymes, both collagenases and proteinases elaborated by *Cl. histolyticum* were by far the most potent of any studied.

With regard to the other organisms, the significance of our findings may be summarized as follows.

1. *Cocci:* Although none of the pyococci investigated produced a true collagenase, one out of six strains of *Staph. aureus* was capable of attacking Azocoll, although only weakly. The fact that even one strain of staphylococcus could hydrolyze Azocoll is of interest, not only because the proteolytic activities of this organism have been considered negligible, but also because Gillespie and Adler (34) in a recent study report that coagulase-positive staphylococci are without action on Azocoll. As for the hemolytic streptococci, our findings agree with those of Todd (28) and Elliott and Dole (35, 36). It is probable that the positive reactions obtained against Azocoll are due to the proteinase described by Elliott as active against streptococcal M-protein.

2. *Aerobic spore-bearers:* The ability of four out of the nine strains of *Bacillus* examined to pro-

TABLE I

Miscellaneous organisms (50 strains)

Organism	No. of strains	Collagen positive	Azocoll positive
Staph. aureus	6	0	1 (±)
Staph. albus	6	0	0
Strept. pyogenes	5	0	2 (±)
B. mesentericus	3	0	2
B. cereus	4	0	2
B. anthracis	2	0	0
E. coli	6	0	0
P. vulgaris	6	0	2
Myxobacteria	3	0	0

duce enzymes capable of degrading hide-powder was unexpected. While most members of this genus can liquefy gelatin, few can attack the higher proteins. It would probably be of interest to compare the activities of these proteinases against a wide variety of substrates. No strain produced a collagenase, not even *B. anthracis,* which gives rise to an extremely necrotic type of lesion in the animal body.

3. *Gram negative bacilli:* Among the strains of *Enterobacteriaceae* tested, the results were negative both with strains of *E. Coli* and *Proteus vulgaris* even though the latter species may cause massive destruction of tissue in some wound infection. Dr. Wm. Altemeier has stated in personal communications that strains of *Proteus vulgaris* exist which produce an active collagenase. However, these collagenases, unlike those elaborated by the Clostridia, appear to be adaptive enzymes, and our methods of examination were not well suited to the detection of such enzymes. Indeed, it is worth suggesting that further surveys of this group of bacteria be made in which collagen or gelatin is incorporated in the test medium.

4. *Myxobacteria:* The three strains of *Chondrococcus columnaris* were included because they can cause in fish a highly fatal infection associated with much destruction of tissue (37). None of the strains produced enzymes capable of attacking hide powder, although Ordal and Rucker (37) state that gelatin is rapidly liquefied.

5. *Clostridia:* Apart from the *Cl. perfringens* and *Cl. histolyticum,* no species of clostridium examined could liquefy collagen. The ability of *Cl. sporogenes* to attack Azocoll may be significant since it is an actively proteolytic organism which has been used in the past for the enzymic debridement of foul necrotic wounds (38, 39).

It has been known for some time that *Cl. perfringens* could produce a collagenase. The distribution of this enzyme amongst the various toxigenic types has been set out in Table III. This table is in general agreement with the findings of Oakley *et al.* (21) in that all Type A strains can digest collagen. However, Oakley and his collaborators (21) report that Types B, C, and D also elaborate collagenase, although only in small amounts. This we did not encounter. However, the number of strains we studied was relatively small. Furthermore all four Type B strains and one out of three Type C strains gave positive reactions against Azocoll. Since Type B strains form at least two proteinases (29), one capable of attacking "native" and "denatured" collagen, and the other only "denatured" collagen, while Type C strains form only the true collagenase, some of the positive Azocoll results may be due to traces of collagenase, too weak to digest the relatively large amounts of fresh tendon, but capable of releasing color from Azocoll powder.

TABLE II

Clostridia

Organism	No. of strains	Collagen positive	Azocoll positive
Cl. perfringens	30	20	25
Cl. histolyticum	82	82	82
Cl. novyi	2	0	0
Cl. septicum	2	0	1
Cl. bifermentans	2	0	2
Cl. sporogenes	3	0	3

TABLE III

Cl. perfringens

Organism	No. of strains	Collagen positive	Azocoll positive
Type A	20	20	20
B	4	0	4
C	3	0	1
D	3	0	0

Indeed, even with Type A strains the amounts of collagenase produced are small—the highest dilution of culture giving positive results being $1/16$, as compared with frequent readings of $1/256$ and occasionally of $1/512$ in the case of *Cl. histolyticum.*

Cl. histolyticum

In view of the relatively high activity of the proteolytic enzymes produced by *Cl. histolyticum,* we decided to limit our future investigations to this organism. Hence we re-examined our collection of 82 strains and selected two—No. H.4 for its ability to produce proteinase and No. 230–2 for its ability to produce collagenase. Strain No. H.4 was isolated in the United States during World War II in the course of the Contaminated Wounds Project, while Strain No. 230–2 came from the collection of Dr. L. S. McClung of Indiana University. Further details concerning these organisms are not available.

Development of optimal medium to produce enzymes

Since this study was undertaken in an effort to discover bacterial enzymes of therapeutic value, the development of a fluid medium which would consistently favor the production of large amounts of a highly active proteinase by *Cl. histolyticum* has involved an extensive series of trials. It soon became clear that one containing high concentrations of peptone was superior to all others. The effect of peptone concentration, vitamins, minerals, pH, ionic strength, and Fe^{++} was then studied, and the following formula devised.

To each liter of distilled water are added:

15	Gm.	Trypticase Soy Base
50	Gm.	Proteose Peptone
9	Gm.	$Na_2 HPO_4$
1.92	Gm.	$KH_2 PO_4$
0.08	Gm.	$MgSO_4 7H_2O$
5	ml.	Vitamin Solution containing 200 mg. each of Ca Pantothenate, Nicotinic Acid, Pyridoxine and Thiamine, 20 mg. of Riboflavin per liter
6	ml.	$FeSO_4 7H_2O$/100 ml. N/10 HCl NaOH to adjust pH to 7.4

The concentration of the Vitamin Solution does not appear to be of critical value; that of the ferrous salt and the peptone does. Moreover, certain other metallic salts—in particular calcium, magnesium and cobalt—may have a beneficial effect, either in place of or in addition to the ferrous iron.

The maximum yield of enzyme is obtained after from 17 to 18 hours' incubation at 37° C., the warm medium being heavily inoculated with a young culture of the selected strain of *Cl. histolyticum* (Figure 2).

Other bases and supplements tested, including various casein-digests, phytones and peptones, collagen and collagen derivatives such as hide powder and gelatin, glucose and other carbohydrates were not as satisfactory.

Although the above medium was best in our studies, it would be extremely expensive to use on a commercial scale. We have, accordingly, tested various digest preparations as an alternative source of protein. Through the courtesy of

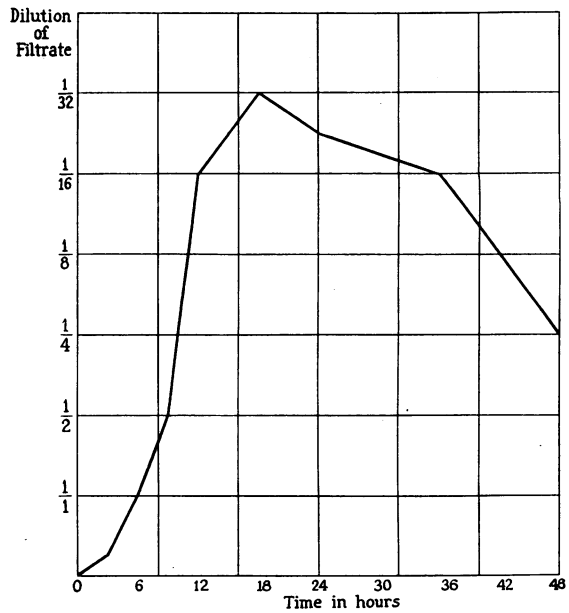

FIG. 2. COLLAGENASE PRODUCTION

Maximum collagenase activity is plotted in dilutions of the filtrate against time in hours.

Dr. Irwin S. Danielson of Lederle Laboratories seven preparations were obtained and tested in various ways. These preparations are listed in Table IV.

None of these media, in varying concentrations and supplemented by various salts, vitamins and growth factors, has given us results in any way comparable to those obtained with our own medium. The most satisfactory was the Pancreatic Liver Cake Digest; and yet even at its best this gave us yields of the proteinase at least 50 per cent lower.

The very high (5 per cent) concentration of proteose peptone required for optimum yields suggested to us that some "growth factor" might be present. The peptone preparations used in bacteriological work contain considerable amounts of Blood Group A substance, but neither the addition of purified Blood Group A substance nor of various other fractions obtained from peptone in

TABLE IV

1. Pancreatic Beef Heart Digest
2. Pancreatic Liver Cake Digest
3. Peptic Liver Cake Digest
4. Peptic Veal Muscle Digest
5. Peptic Placenta Digest
6. Papain Liver Cake Digest
7. Tryptic Beef Heart Digest

any way enhanced the value of a medium in which proteose peptone was absent or present in sub-optimal amounts. Other attempts to separate a "growth factor" proved no more successful.

A technical point of importance is that the strains of *Cl. histolyticum* used in these studies have a tendency to develop rough variants. Such variants give consistently low yields of proteolytic enzymes. It has, therefore, been our custom periodically to plate out the strain under study on Blood Agar and to select a smooth colony from which to grow the inoculum in ordinary nutrient broth. By selecting several such colonies from a single plate and testing out the resultant broth cultures both for collagenase and proteinase activity, we have had no difficulty in maintaining strains of high potency.

SUMMARY AND CONCLUSIONS

1. Methods for determining a) the collagenase and b) other proteinase activities of micro-organisms have been described.

2. One hundred and sixty-two strains of bacteria have been examined for such activities.

3. Few organisms other than the spore-bearing bacilli (aerobic and anaerobic) exhibited proteinase activity, none other than the clostridia possessed collagenase activity.

4. By far the most active organism, both with regard to proteolytic and collagenolytic powers, was *Cl. histolyticum*.

5. A collection of 82 strains of this bacterium was examined in detail and certain very potent strains selected for further study.

6. A medium which favors greatly increased production of this proteinase has been evolved.

7. Unsuccessful attempts to simplify this medium are described.

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