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GELATINASES PRODUCED BY GRAM-POSITIVE
AND GRAM-NEGATIVE BACTERIA

by

Eldon Creighton Couey
B.A. 1932, State University of Montana

Presented in partial fulfillment of the
requirement for the degree of
Master of Arts

State University of Montana

1936

Approved:

Galvin Watson

Chairman of Board
of Examiners

W. S. Bateman

Chairman of Committee
on Graduate Study

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E.C.

The Selective Action of Certain Deleterious Substances Upon Gelatinases of Gram-Positive and Gram-Negative Bacteria.

Part I

Introduction

The study of the enzymes of microorganisms dates back to about the middle of the last century. At that time, according to Buchanan and Fulmer,² there was some confusion with reference to the terms "ferment" and "enzyme". Pasteur (1861) used the former term to include living cells which produced fermentation. Later it was found necessary to expand the definition, for it developed that living cells might excrete or secrete substances which could produce chemical changes, and the term "organized ferment" was applied to living cells producing fermentation, while the term "unorganized ferment" was applied to excretions and secretions of cells which could cause fermentation. The subsequent discovery by Buchner (1897) that zymase could be extracted from the yeast cell led to the present concept of enzymes as unorganized ferments, or organic catalysts.

²

Buchanan and Fulmer define an enzyme as a "catalyst produced by a living cell (and showing a considerable degree of lability)," and list among the characteristics of enzymes such properties as their chemical composition, the specificity of their action, the reversibility of their action, and the effects of chemical and physical environment upon their action.

It has been known for some time that the proteolytic enzymes produced by bacteria differ widely in strength and quality. Thus Fowler⁹ (1911) demonstrated that on an ordinary gelatin plate culture prepared from sew-

age, the liquefying action around some of the colonies was not infrequently so rapid and intense that a few liquefying organisms caused the whole plate to become liquid before the remaining colonies had developed. At about the same time, Clark and Gage,⁴ of the Massachusetts State Board of Health, measured quantitatively the strengths of several proteolytic enzymes by observing the depth of liquefaction in tubes of gelatin of uniform bore, demonstrating that different organisms vary greatly in the strength of the gelatinases which they produce. Further, in a more recent investigation, Wells and Sherwood (1934)²⁵ suggest that "enzymes from gram-negative organisms liquefy a larger quantity of gelatin and are more active in alkaline medium than are enzymes produced by gram-positive organisms."

In addition to the strength and concentration of enzymes as varying factors, we find evidence which indicates the importance of such environmental factors as hydrogen ion concentration and temperature. Waksman and Davison (1926)²¹ note that "most enzymes are greatly influenced by the reaction of the medium in which they act...(and) there is an optimal hydrogen ion concentration for the activity of each enzyme, and there are also upper and lower limits of reactions, above or below which the enzyme is inactive or may even be rapidly destroyed." These authors cite the work of Sorenson (1908)¹⁸ as being among the most important contributions to the subject, his investigations being concerned with the influence of reaction upon the inversion of cane sugar by invertase. Cohnheim (1912)⁵ concludes that most enzymes are exactly adapted to the reaction of the solution in which they occur in nature, and that in

general "enzymes exhibit a remarkable adaptation to the conditions of their environment if these vary in nature."

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Relative to the temperature factor, Waksman and Davison observe that the velocity of enzyme reactions is accelerated as the temperature is increased until a certain optimum is reached. On further increasing the temperature the reaction velocity begins to diminish until it ceases completely. It is believed by these workers that each enzyme has an optimum temperature characteristic of that enzyme. These observations coincide with those of Cohnheim (1912) who concluded that the enzymes of the upper duodenum exhibit an optimum reaction temperature.

The importance of the study of the effects of activating and inactivating substances upon enzymes was voiced as early as 1912 by Euler in this statement; "concerning the action of paralyzers (inhibiting agents) we are almost completely in the dark, but these bodies are of considerable interest in enzymology since they are indispensable as sterilizing agents in all protracted experiments".

The factor of chemical environment is touched upon by Waksman and Davison who further suggest that the optimum of enzyme activity (see above) is greatly influenced, not only by the type of enzyme, but also by its concentration, the nature and concentration of the substrate upon which the enzyme acts, the reaction of the medium, and the presence of activating or paralyzing substances. Among the latter substances these authors list the salts of the heavy metals, notably mercuric chloride, the quantity of which necessary to stop completely the action of ptyalin have been accurately determined. Waksman and Davison further sug-

gest that certain salts and ions greatly stimulate the activity of enzymes, while other salts retard enzyme action. It is observed in this connection that much higher concentrations of the salts of alkalis and alkali earths are required to activate or produce injurious effects than of the salts of the heavy metals. Among the organic substances listed by these men which exert a deleterious effect upon enzyme action are hydrocyanic acid (which reduces the activity of catalase 50% in solutions of .000001 normality), formaldehyde, and alcohol.

The extreme sensitiveness of certain enzymes to their environment is shown by Doris and Pickford ¹⁵ who report that extract of the pancreas of amblystome embryos, which normally gives a strong amylase reaction, gives negative results if the extract is ground and kept in a pyrex mortar for several hours. This was not noticed when ordinary soft glass or quartz glass were used.

⁷ Euler (1912) states that the "injurious actions of 'poisons' (inhibiting agents) on enzyme solutions increases as the concentration of the enzyme diminishes", pointing out that the concentration of the enzyme and the purity of the solution condition the degree of inactivation by inhibiting agents.

The effects of dyes upon certain enzymes has been determined in several investigations. Quastel and Wheatley ¹⁷ (1931) demonstrated that many basic dyes at low concentrations inhibit dehydrogenase activities of bacteria and mammalian tissue, the effects being reversible. As a result of their investigations, Basu and Chakravartz ¹ (1935) concluded that acid dyes at concentrations of 1 in 2000 and 1 in 5000 would in-

hibit digestion by trypsin by as much as 36%. It was demonstrated by Quastel (1931)¹⁶ that impurities affect the inhibition of enzyme action by dyes, probably because the impurities were themselves absorbed by the enzymes.

In a recent investigation of the selective action of dyes and other disinfectants on bacteriophages, Wells and Sherwood (1933)²³ arrived at the conclusion that, in general, the phages which lysed gram-negative bacteria were more resistant to deleterious substances than were those which lysed gram-positive bacteria. A more recent investigation has been conducted by Wells and Sherwood (1934)²⁵ upon the effects of chemicals upon enzymic activity, with particular interest in the selective activity of dyes upon gelatinases of gram-positive and gram-negative bacteria. It was reported that the enzymes of gram-negative bacteria were, in general, more resistant to the inhibitory effect of gentian violet than were those of gram-positive organisms.

Moreover, these investigators point out that the selective inhibition of activity of gelatinases by gentian violet does not separate the gram-positive from the gram-negative bacteria as distinctly as was noted with bacteriophage, altho there seems to be some similarity. Wells and Sherwood (1934)²⁵ concluded that the results obtained "suggest a close relationship between the bacteria and their respective enzymes and bacteriophages."²¹ Moreover, it has been suggested by Wakaman and Davison that the lytic principle in the Twort d'Herelle phenomenon is an enzyme rather than an ultra-microscopic phage.

Wells and Sherwood (1934) used twelve strains of bacteria that were

rapid liquifiers, equal numbers of which were gram-positive and gram-negative. Enzymes were prepared by passing the products of liquefaction thru Berkefeld filters. The inhibiting agent used was gentian violet in dilution of 1 in 200 and 1 in 400 (of a saturated alcoholic solution) and tests were made in both acid and alkaline media (pH 6 and pH 7.8). Solutions of dye and enzyme were prepared by mixing equal quantities of dye solution of desired concentration and enzyme filtrates of desired pH. After a period of incubation for inhibition, tests were conducted to determine the degree of inactivation by transferring one wire loopful of each mixture to a gelatin plate. A similar inoculation was made, using uninhibited enzyme (control); and after four days incubation at 20 degrees Centigrade, the relative sizes of the concave depressions produced by liquefaction were observed and the percentage of inhibition approximated. As a result of this investigation it was shown that five of the enzymes of gram-positive organisms were inhibited 100%, while the sixth varied from none to 25% inhibition. Of the six enzymes from gram-negative organisms, two showed complete (100%) inhibition, while four showed degrees of activity varying from no inhibition to 50% inhibition. To quote Wells and Sherwood: "Not enough difference in results was obtained between the acid and alkaline mediums to permit a conclusion to be drawn regarding the more favorable reaction for inhibition of the enzyme."

In this work we are particularly interested in the effects of chemical environment on the action of enzymes, and in particular, the selective action of deleterious substances upon gelatinases produced by gram-positive and gram-negative bacteria. It was decided to attempt to repeat the work of Wells and Sherwood (1934), using the same technique

but extending the investigation by the use of more types of bacterial enzymes and the addition of sodium hydroxide as an inhibiting agent. As an additional modification the use of three different dilutions of each inhibiting agent was introduced in an attempt to make the determinations more quantitative.

Part I

Experimental Procedure

Twenty gelatin-liquefying organisms were isolated, half of which were gram-positive and the rest gram-negative, and no two of which showed the same cultural characteristics. (Table 7) Twentyfour hour cultures were prepared from these and inoculated so that the growth would cover the slant. One cubic centimeter of sterile saline solution was added to each tube, the growth scraped loose with a sterile glass rod, and a uniform suspension made by agitating the tube. This suspension was transferred to the surface of gelatin in Petri dishes, which had already been prepared. Deep dishes were used (about $1\frac{1}{2}$ cm.), filled about half full of 25% gelatin, and covered with clay tops. Over the surface of the gelatin in each of the twenty plates the suspension of an organism was spread, using sterile glass rods bent for the purpose. Care was taken to prevent contamination. The plates thus prepared were left at room temperature for 72 hours, the liquefied gelatin^{was} poured off, and filtered through sterile Berkefeld filters, using a suction flask to hasten the filtration. After adjusting the reaction of these enzyme filtrates to pH 7.6 they were incubated for 24 hours to insure against contamination and were then ready for use.

In preparation for setting up the experiment a supply of clean, cotton-plugged culture tubes and two or three dozen clean 5cc pipettes were sterilized. A saturated solution of sodium hydroxide was prepared (109 g./100 cc water), and from this were made dilutions of 1 in 100, 1 in 400 and 1 in 1000 (by volume). Similarly, dilutions of 1 in 100, 1 in 400 and 1 in 1000 of gentian violet were prepared from a saturated alcoholic solution (15.21 g./100 cc 95% alcohol).

Four tubes were prepared for each enzyme as follows, care being exercised to keep all liquids sterile at all times: In tube #1 were placed 1 cc of sterile saline and 1 cc of the sterile enzyme filtrate. In tube #2 were placed 1 cc of sterile enzyme filtrate and 1 cc of the 1 in 100 dilution of gentian violet. Tubes #3 and #4 contained 1 cc of sterile enzyme filtrate and 1 cc of the 1 in 400 and 1 in 1000 dilutions of dye respectively.

Three tubes were prepared for each enzyme similar to tubes #2, #3 and #4 using dilutions of 1-100, 1-400 and 1-1000 of the sodium hydroxide instead of gentian violet.

These seven tubes for each enzyme (140 in all) were prepared with great care to prevent contamination, and were placed in the 37 degree incubator (Centigrade) along with the following controls:

- 1 cc of each enzyme, each diluted with 1 cc of sterile saline
- 1 cc completely inactivated enzyme filtrate (autoclaved) and 1 cc of sterile saline
- 1 cc completely inactivated enzyme filtrate and 1 cc of 2% alcohol (to see if the alcohol would liquefy gelatin)
- 1 cc of 1-100 dilutions each of gentian violet and sodium hydroxide.

At the end of a twenty-four hour incubation at 37 deg. C, the 144 tubes were removed from the incubator and tested for enzyme activity. Eleven Petri dishes were poured half full of 25% gelatin and chilled to solidification. The bottom of each dish was marked in quarters and a space in each quartile designated for each mixture of dye and enzyme (3 dilutions) and for the mixture of enzyme and saline (control). Another quartile was similarly marked for each enzyme for the three dilutions of sodium hydroxide and the control. In this manner a space was provided for an inoculation from each of the 140 tubes and the controls containing un-inactivated enzyme and saline (for comparison with the inhibited enzyme). The bottom of the eleventh plate was marked to designate regions for inoculation with the rest of the controls.

Inoculations were made with a standard loop prepared by bending the wire of an ordinary inoculating needle around an eight-penny nail. Using sterile technique, the loop was dipped into each incubated mixture and the adherent drop transferred to its designated place on the surface of the gelatin in the Petri dish. Care was taken to get as nearly as possible the same amount of inoculum on the loop at each transfer. This could be varied by the speed and direction of withdrawal from the solution. Care was also exercised when placing the drop of inoculum on the gelatin that the material spread over the same area of gelatin each time, that the shape of the droplets was uniform, and that approximately the same amount of liquid was removed from the loop at each inoculation. It was found convenient to arrange the inoculations as shown in the accompanying photograph (p. 18b), with the control "spot" toward

the center of the plate and the mixtures of the three dilutions of inhibiting agent and enzymes around the edge. The latter were placed in a manner to allow a maximum spread of the liquefied areas without running together. The four other controls were similarly inoculated onto the eleventh plate.

At the conclusion of the inoculation the 144 tubes were returned to the incubator for another twenty-four hours, at the end of which time the procedure of inoculation was repeated on eleven more plates. After a final incubation of twenty-four hours (seventy-two hours in all), a third set of plates was prepared and the 144 tubes cleaned preparatory to another determination.

The three sets of plates thus prepared were "read" at 24, 48, and 72 hour intervals, being kept at room temperature (about 20 deg. C.) At the end of the 72-hour reading on the plates prepared from the material which had incubated for 72 hours, all plates were cleaned and the entire procedure repeated.

Reading the Plates

It was observed that the gelatin beneath the places inoculated was liquefied to varying degrees, producing concave depressions (see photograph, p. 18 b). These varied in diameter and depth and were nearly always circular in shape. The depressions resulting from the activity of uninhibited enzymes (controls) were nearly always larger than the depressions produced by those which had been inhibited. These controls also varied in size for the twenty enzymes, the larger ones being produced by the more rapid liquefiers. No depressions were ever observed to have

reached the bottom of a plate. The four control inoculations were always negative (i.e. showed no liquefaction), indicating that liquefaction was not due to the dye, the sodium hydroxide, the alcohol present in the dye, or any substance in the enzyme filtrate not destroyed by heating sufficiently to destroy the enzyme.

The degree of inhibition of enzyme activity was approximated by comparison of the relative sizes of the depressions produced by the uninhibited enzymes (controls) and those produced by mixtures of enzyme filtrates and an inhibiting agent. Readings were recorded as "percentage inhibition". For example, if the depression produced by the activity of enzyme "A" with a 1-100 dilution of gentian violet was judged to be one-fourth as large as that produced by the same enzyme uninhibited, the reading would be "75% inhibition". Since readings were made from the same plates at 24, 48, and 72 hour intervals, sterile precautions were observed in examining them as well as in inoculating them.

An attempt was made to secure more quantitative readings by actual measurement of the depressions, but the results were unsatisfactory. The depth of the liquefied areas could not be measured and the diameters of the depressions were not a good index of the amount of gelatin liquefied.

In consideration of the inaccuracy and more or less subjective nature of this method of reading, an attempt was made to check on the validity of the observations. A good plate was selected, sodium hydroxide having been used as the inhibiting agent, and a private reading made and recorded. Ten disinterested persons were then selected at random and individually, and each requested to make a reading on the plate. At the

conclusions of these observations the various readings were compared with the reading which had been previously recorded. It was noted (Table I) that in a few places the opinions of the individuals differed considerably with reference to the degree of inhibition. In one particular place the readings of two individuals varied from 0 to 42% inhibition. On the other hand, taken as a whole, the observations of the individuals correspond quite favorably with each other and with the recorded reading. The readings of the ten observers were averaged for each dilution of inhibiting agent and for each of the four enzymes used. (Table I). The percentages of deviation of these averages from the recorded reading were computed and, as will be noted, these vary from a maximum positive error (reading higher than the recorded reading) of 33% to a maximum negative error (reading too low) of 16.1%. The average deviation tending to be too high was 7.72%, while the average deviation below the recorded reading 11.35%.

In spite of the occasional great variety of opinions, it will be noted that the readings of each individual were quite consistent, in demonstrating that three of the enzymes were inhibited to a great degree (over 75%) and the other one was inhibited in the neighborhood of 40%.

Table I

Results of Experiment to Check Accuracy of Plate Readings

| Enzyme and dilution of NaOH used | Readings of Observers in Percentage Inhibition | | | | | | | | | | Recorded |
|--|--|-----|----|-----|----|----|----|----|----|----|----------|
| | LJF | AYW | RW | AH | FD | FS | RR | EJ | MB | EO | |
| P. vulgaris | | | | | | | | | | | |
| 1-200 | 95 | 98 | 95 | 98 | 95 | 98 | 95 | 97 | 98 | 99 | 99 |
| 1-400 | 80 | 90 | 90 | 80 | 85 | 95 | 75 | 75 | 90 | 95 | 95 |
| 1-600 | 80 | 75 | 85 | 80 | 85 | 85 | 60 | 60 | 85 | 90 | 90 |
| 1-800 | 78 | 75 | 75 | 80 | 80 | 95 | 50 | 40 | 80 | 88 | 90 |
| B. subtilis | | | | | | | | | | | |
| 1-200 | 35 | 25 | 15 | 30 | 50 | 25 | 25 | 50 | 24 | 50 | 30 |
| 1-400 | 40 | 25 | 15 | 25 | 50 | 20 | 20 | 30 | 30 | 40 | 25 |
| 1-600 | 40 | 25 | 15 | 35 | 60 | 15 | 20 | 45 | 35 | 50 | 25 |
| 1-800 | 42 | 0 | 0 | 20 | 15 | 5 | 0 | 10 | 15 | 20 | 10 |
| Wells 3S | | | | | | | | | | | |
| 1-200 | 85 | 95 | 75 | 80 | 97 | 80 | 90 | 85 | 90 | 90 | 90 |
| 1-400 | 90 | 90 | 70 | 75 | 92 | 70 | 75 | 75 | 80 | 85 | 85 |
| 1-600 | 90 | 80 | 70 | 80 | 85 | 60 | 70 | 70 | 70 | 80 | 80 |
| 1-800 | 90 | 80 | 70 | 75 | 80 | 60 | 65 | 60 | 60 | 80 | 80 |
| ECC #10 | | | | | | | | | | | |
| 1-200 | 99 | 100 | 98 | 100 | 97 | 95 | 99 | 97 | 95 | 99 | 98 |
| 1-400 | 95 | 98 | 98 | 90 | 97 | 90 | 95 | 80 | 90 | 95 | 95 |
| 1-600 | 92 | 98 | 98 | 90 | 95 | 90 | 90 | 85 | 87 | 89 | 90 |
| 1-800 | 90 | 95 | 85 | 85 | 92 | 90 | 85 | 80 | 85 | 85 | 85 |

Average of Readings and Comparison with Recorded Reading

| | 1-200 | | 1-400 | | 1-600 | | 1-800 | | | | | |
|--|----------|-------|----------|-------|----------|-------|----------|-------|-------|------|----|-------|
| | Av. Rec. | %Dev. | Av. Rec. | %Dev. | Av. Rec. | %Dev. | Av. Rec. | %Dev. | | | | |
| Vulg. | 96.9 | 98 | 1.1% | 86.4 | 95 | 9.1% | 80.5 | 90 | 10.5% | 75.5 | 90 | 16.1% |
| Sub. | 31.0 | 30 | 3.3% | 29.5 | 25 | 18.0% | 33.2 | 25 | 33.0% | 12.5 | 10 | 25.0% |
| 3S | 87.0 | 90 | 3.3% | 80.6 | 85 | 5.2% | 76.8 | 80 | 4.0% | 72.7 | 80 | 9.1% |
| 10 | 97.9 | 98 | .1% | 93.0 | 95 | 2.0% | 91.4 | 90 | 1.5% | 88.0 | 85 | 3.5% |
| Maximum deviation higher than recorded | | | | | | | 33.00% | | | | | |
| Maximum deviation lower than recorded | | | | | | | 16.10% | | | | | |
| Average deviation higher than recorded | | | | | | | 7.72% | | | | | |
| Average deviation lower than recorded | | | | | | | 11.35% | | | | | |

Part I

Experimental Results

The twenty enzymes were tested in the manner described six consecutive times. It was discovered that the best readings were ^{obt} from plates prepared from materials which had been incubated 72 hours, and that the 48 and 72 hour readings of these plates were little different from the 24 hour reading. Although the depressions were larger, the relative sizes were very nearly the same.

In simplifying the readings it was found most convenient to average the results as follows: The percentages of inhibition of enzyme activity with each dilution of dye and sodium hydroxide were averaged for each organism, using the readings from the best plates, uncontaminated and having uniformly shaped areas of liquefaction. These averages were then averaged for all gram-positive organisms, and similarly for all gram-negative organisms. (See Tables II and III - pp. 17 and 18)

It was discovered that, in general, the activities of the enzymes from gram-positive organisms were inhibited to a greater degree than were the enzymes from gram-negative organisms when 1-100 dilutions of gentian violet and sodium hydroxide were used as inhibiting agents. The average inhibition of activity of the ten enzymes from gram-positive organisms by a 1-100 dilution of sodium hydroxide was 81.8%, as compared to an inhibition of 75.8% of the activity of the enzymes of gram-negative bacteria, using the same inhibiting agent. Similarly, using a 1-100 dilution of gentian violet as an inhibiting agent, the enzymes of gram-

positive organisms were inhibited 91.6% as compared to a 79.3% inhibition of those from gram-negative organisms.

On examination of Table II it will be noted that there were four gram-negative organisms, the activities of the enzymes of which appear to be decided exceptions to the general rule of inhibition when gentian violet was used as an inhibiting agent. These exceptional qualities show up in all three dilutions of the dye and three of these enzymes show these same characteristics when a 1-100 dilution of sodium hydroxide was used as an inhibiting agent. The exception is this: although these four organisms were exceptionally rapid liquefiers, the activity of their enzymes was retarded to a greater degree than were those of less rapid liquefiers and the rest of the enzymes of gram-negative organisms. One exception will also be noticed among the enzymes of gram-positive organisms (Table IIb) when gentian violet was used as an inhibiting agent. (Enzyme E). The seeming ready inhibition of the action of this enzyme persists throughout all three dilutions of the dye, but did not reappear when sodium hydroxide was used as inhibitor. Instead, the different organisms show this same property throughout the three dilutions of the sodium hydroxide (Enzymes D and J).

These so-called exceptions may perhaps be due to individual differences in the strains of bacteria. Waksman and Davison (22) suggest that, since bacteria vary in the nature of the nutrients which they can utilize, and in the conditions under which these nutrients are utilized, the nature of the enzymes produced by the different organisms will vary.

Excluding these "exceptions" from the averages of inhibition for

the six determinations and reaveraging the averages for enzymes of gram-positive and gram-negative organisms with both gentian violet and sodium hydroxide as inhibiting agents, we find that the results are even more conclusive, as may readily be observed in Table IV, p. 19.

Table II
Percentage Inhibition of Enzyme Activity by Gentian Violet

(a) Inhibition using enzymes of gram-negative organisms.

| Enzyme | Percentage inhibition | | |
|---|-----------------------|-------------------|--------------------|
| | 1-100 dilution | 1-400 dilution | 1-1000 dilution |
| III | 52% | 48% | 37% |
| IV | 57 | 46 | 43 |
| 3S | 98 ^a | 95 ^a | 89 ^a |
| A | 75 | 61 | 47 |
| B | 65 | 58 | 42 |
| H | 97 ^a | 91 ^a | 76 ^a |
| M | 75 | 67 | 53 |
| HB | 94 ^a | 76 ^a | 86 ^a |
| SP | 83 | 71 | 64 |
| Vulg | 97 ^a | 96 ^a | 91 ^a |
| Average Inhibition--- | 79.3% | 70.9% | 62.8% |
| Av. Inhib. ex- clusive of exceptions ^b --- | 68.0% | 58.0% | 48.0% |

(b) Inhibition using enzymes of gram-positive organisms.

| Enzyme | Percentage inhibition | | |
|---|-----------------------|-------------------|--------------------|
| | 1-100 dilution | 1-400 dilution | 1-1000 dilution |
| I | 94% | 79% | 60% |
| D | 95 | 58 | 54 |
| E | 79 ^a | 36 ^a | 18 ^a |
| F | 82 | 72 | 56 |
| G | 93 | 63 | 41 |
| J | 93 | 84 | 62 |
| K | 98 | 83 | 40 |
| R | 95 | 73 | 31 |
| Sub | 95 | 66 | 35 |
| 10 | 92 | 77 | 69 |
| Average Inhibition--- | 91.6% | 69.2% | 46.6% |
| Av. inhibition exclusive of exceptions ^b ----- | 93.0% | 73.0% | 48.0% |

(c) Summary

| Dilution of gen. violet | Average Percentage Inhibition | |
|-------------------------|-------------------------------|---------------|
| | Gram-positive | Gram-negative |
| 1-100 | 83% | 68% |
| 1-400 | 73% | 58% |
| 1-1000 | 49% | 48% |

Table III
Percentage Inhibition of Enzyme Activity by Sodium Hydroxide

(a) Inhibition using enzymes of gram-negative organisms.

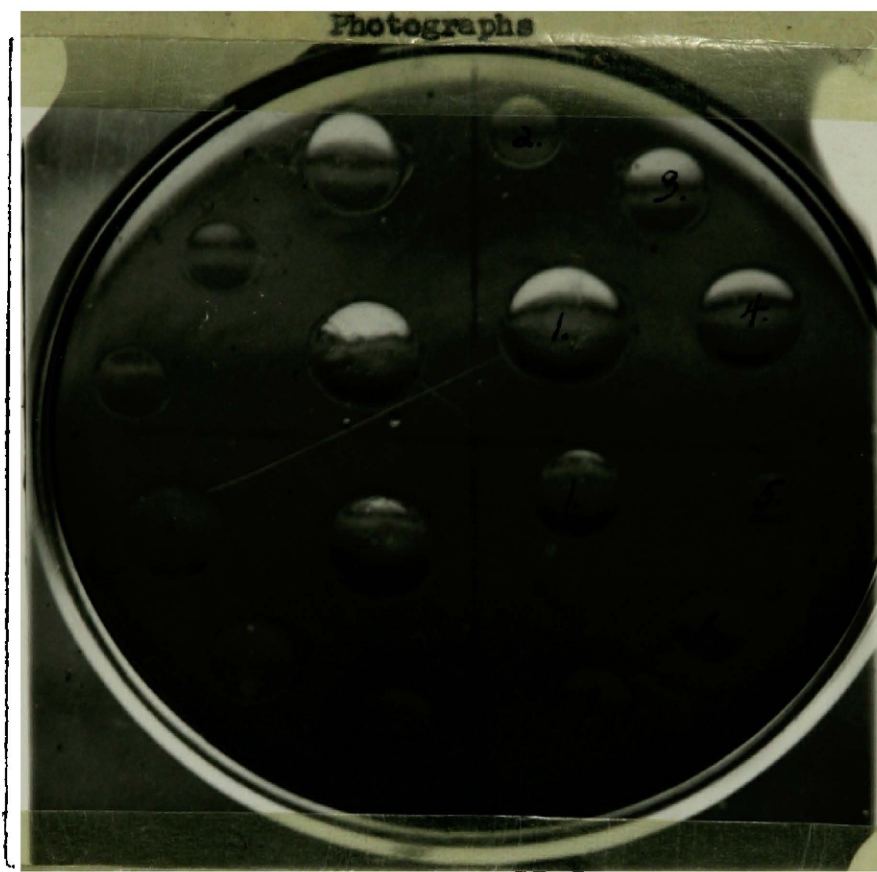
| Enzyme | Percentage inhibition | | |
|--|-----------------------|-------------------|--------------------|
| | 1-100 dilution | 1-400 dilution | 1-1000 dilution |
| III | 65% | 45% | 34% |
| IV | 87 | 49 | 43 |
| 3S | 95° | 34 | 33 |
| A | 33 | 29 | 33 |
| B | 87 | 36 | 37 |
| H | 88 | 43 | 34 |
| M | 54 | 43 | 41 |
| HB | 97° | 44 | 40 |
| SP | 55 | 44 | 42 |
| Vulg | 97° | 40 | 39 |
| Average inhibition ----- | 75.8% | 40.8% | 37.6% |
| Average inhibition exclusive of exceptions ----- | 67.0% | 40.8% | 37.6% |

(b) Inhibition using enzymes of gram-positive organisms.

| Enzyme | Percentage inhibition | | |
|--|-----------------------|-------------------|--------------------|
| | 1-100 dilution | 1-400 dilution | 1-1000 dilution |
| I | 86% | 42% | 44% |
| D | 45° | 31° | 33° |
| E | 94 | 34° | 36 |
| F | 98 | 38 | 38 |
| G | 85 | 46 | 41 |
| J | 44° | 27° | 32° |
| K | 93 | 45 | 39 |
| R | 81 | 34 | 31 |
| Sub | 99 | 40 | 42 |
| 10 | 93 | 37 | 35 |
| Average inhibition - | 81.8% | 37.6% | 37.1% |
| Average inhibition exclusive of exceptions ----- | 91.1% | 39.5% | 38.3% |

(c) Summary

| Dilution of Sod. Hyd. | Average Percentage Inhibition | |
|-----------------------|-------------------------------|---------------|
| | Gram-positive | Gram-negative |
| 1-100 | 91.0% | 67.0% |
| 1-400 | 39.5% | 40.8% |
| 1-1000 | 38.3% | 36.6% |



Photograph of Gelatin Plate Showing Areas of Liquefaction

(Enzyme mixtures incubated 48 hours at 37 deg. C. Plate incubated at 20 deg. C. for 48 hours. Liquefied areas are concave depressions)

Legend

1. The Control (uninhibited enzyme)
2. Mixture of enzyme and 1-100 gentian violet
3. " " " " 1-400 " "
4. " " " " 1-1000 " "
5. Mixture of enzyme and 1-100 sodium hydroxide
6. " " " " 1-400 " "
7. " " " " 1-1000 " "

Table IV

(Summary)

Average Percentage Inhibition of Enzymes of Gram-Positive and Gram-Negative Organisms Using Gentian Violet and Sodium Hydroxide.

| Dilutions | Gram-Positive | | Gram-Negative | |
|-----------|----------------|------------------|----------------|------------------|
| | Gentian Violet | Sodium Hydroxide | Gentian Violet | Sodium Hydroxide |
| 1-100 | 93.0% | 91.0% | 68.0% | 67.0% |
| 1-400 | 73.0% | 39.5% | 58.0% | 40.8% |
| 1-1000 | 48.0% | 38.3% | 48.0% | 37.6% |

It is interesting to note that of the organisms used by Wells and Sherwood, ²⁵ 25% were exceptions, of which the greater number were gram-negative. Of the twenty organisms used in this work, five, or twenty per cent were exceptions to the general rule, and four of these were gram-negative.

An interesting experiment was performed relative to the effect of age upon the enzyme filtrates. Five cubic centimeter portions of each of the twenty enzyme filtrates were placed in sterile tubes, stoppered with cotton, corked, and sealed with paraffin. These were kept for a period of two and one-half years in a cool dark place and then tested in identically the same manner as heretofore described, using the same concentrations of sodium hydroxide and gentian violet as inhibiting agents. Very little difference was noticed in the behaviour of these filtrates, as compared to the behavior of the same filtrates tested two and one-half years previous.

Part I

Summary and Conclusions

1. In general, the activities of the gelatinases of gram-positive organisms are more readily inhibited by a 1-100 dilution of gentian violet or sodium hydroxide than are those gelatinases of gram-negative organisms.
2. An enzyme may be a very rapid gelatin liquefier and at the same time be inhibited to a greater extent by a 1-100 dilution of gentian violet or sodium hydroxide than other less rapid liquefiers.
3. Gelatinases present in enzyme filtrates change little in liquefying power and relative resistance to the inhibitory effects of gentian violet and sodium hydroxide over long periods of time.

Part II

Introduction

Because of the inaccuracy and somewhat subjective nature of the foregoing determinations, it was decided to develop a suitable method by which the strengths of the enzyme filtrates could be quantitatively determined. Attempts were made to determine the amount of change produced in the substrate, thus getting an index of the activity of the enzyme. According to Waksman and Davison¹⁷ the velocity of an enzyme reaction is an index of concentration, and the rate of change of the substrate is directly proportional to the concentration of the enzyme.

In the first attempt, the principle of Gates (1930)¹⁰ was followed, the principle of which was a determination of the change produced in the opacity of the emulsion on an exposed photographic film by the progressive proteolysis of the gelatin and consequent liberation of some of the silver salt. The density of the resulting film was compared to that of an unchanged film (control) by the use of a colorimeter. Results were obtained in percentage of gelatin unaffected by the enzyme. Gates showed that the ratio of the final to the control reading was proportional to the enzyme activity at the time and under the conditions of the test.

The method was not successful and was abandoned largely because of the difficulty of finding a suitable temperature at which the enzyme would work satisfactorily and still not be warm enough to soften the emulsion on the film. If this occurred, the particles of silver would

clump together and make it impossible to read accurately with the colorimeter. This same principle has been used by Burdon and Lafferty³ with success, however, in a rapid procedure for the quantitative estimation of antitrypsin, eliminating the use of the colorimeter.

A second attempt involved the determination of total nitrogen by the Kjeldahl method. Measured quantities of enzyme filtrate were added to standard amounts of gelatin in tubes of the same bore. A fixed time was allowed for liquefaction, after which the fluid resulting was washed off and diluted to a standard volume. Similar tubes were prepared, using inactivated enzyme and mixtures of enzymes and various dilutions of inhibiting agents. Kjeldahl determinations were run on all, and the percentage of nitrogen calculated. The increase in the amount of nitrogen in the tubes containing uninhibited enzyme was taken as an index of the amount of activity of the enzyme. In a like manner the degree of inhibition by the inhibiting agents used was determined. The Kjeldahl method was abandoned largely because of the small amount of nitrogen present; and, therefore, the corresponding magnitude of experimental error. It was also necessary to run a great many determinations, and the method was time-consuming.

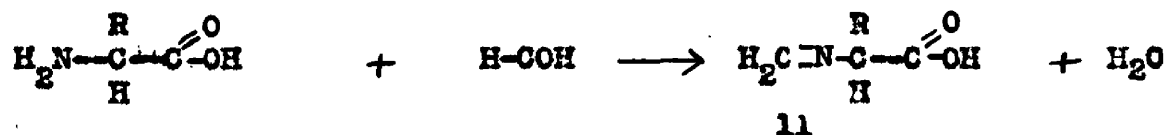
The method which was finally adopted was Sorensen's formal-titration of the amino acids formed as a result of the activity of the enzymes.

The principle of the method is briefly stated by Macleod¹³ as follows:

"A most important reaction consists in the condensation of aldehydes with the amino group. This occurs particularly readily with formaldehyde, water being eliminated in the reaction and the basic nature of

the amino acids being thus destroyed.....the titration is performed by rendering the solution of amino acids neutral, then adding formaldehyde and titrating with standard acid, using phenolphthalein as an indicator, and thus finding to what degree the acidity of the solution has been increased as a result of the formaldehyde. Since the increase in acidity must depend upon the number of amino groups, it furnishes us with an indirect estimate of the concentration of the amino acids."

The reaction takes place according to the following equation:



According to Hawk and Bergeim (1927) ammonia will likewise react with formaldehyde as well as polypeptides and still more complex protein derivatives. Thus the results do not strictly represent amino-acid nitrogen.

The amount of amino acids actually produced and present in the enzyme filtrates is debatable. Among the decomposition products of gelatin Hawk and Bergeim list 25.5% glycocoll, 9.5% proline, 8.2% arginine and 14.1% hydroxyproline. It is not likely that the amount of digestion produced by bacterial enzymes in the time allowed in this investigation would result in the formation of all of these in large amounts. However, Mesernitzky (1910) described a gelatinase from *Bacterium prodigiosum* which produced glycocoll among the hydrolytic products. Tirabaschi (1905) concluded that "all enzymes which liquefy gelatin hydrolyze it at least to peptones".

Whether the acidic constituents present with which the formaldehyde

reacts are amino acids or more complex products of protein digestion matters little in this work, since the experiment was conducted with the end in view of obtaining a quantitative estimate of the amount of substrate changed. The formaldehyde has been reported to react with decomposition products other than amino acids and ¹¹, according to Waksman and Davison ²¹ the amount of these products formed by the action of the enzymes is an index of the activity of the enzymes. Therefore, if a suitable technique could be developed for titrating the products of the reaction, the strengths of the enzymes could be quantitatively determined. Similarly, the degree to which an enzymic reaction had been inhibited by deleterious substances could be determined by comparing the amount of substrate which it could digest with the amount digested by an uninhibited enzyme.

Part II

Experimental

In the previous work (Part I) considerable variation was noticed in the strengths of enzymes which had to be refiltered because of contamination. Considerable error was also introduced by occasional contamination of plates or enzymic materials. For these reasons it was decided to add a preservative to the enzyme filtrates.

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Cohnheim suggests that all observations on enzymes, made without the addition of antiseptics, must be regarded with doubt and mistrust, and he recommends the use of glycerin in concentrations of 70% to 80% to check bacterial growth.

It was discovered that the addition of glycerin to 50% concentra-

tion was satisfactory. Gelatin was liquefied by eleven rapid liquefiers as in Part I, the products centrifuged, an equal volume of chemically pure glycerin added to each clear supernatant fluid, and this mixed thoroughly.

Three-cubic-centimeter portions of melted gelatin were pipetted into flat-bottomed glass tubes of uniform bore ($2\frac{1}{2}$ cm. in diameter and 10 cm. deep). These were plugged with cotton, sterilized in an Arnold sterilizer, and chilled to solidification in a refrigerator.

Four tubes were prepared for each enzyme filtrate. To the first tube were added 1 cc. of sterile saline and 1 cc. of the stock enzyme filtrate containing 50% glycerin. The second tube contained 1 cc. of enzyme stock solution and 1 cc. of a 1-100 dilution of gentian violet. To the third tube were added 1 cc. of enzyme solution and 1 cc. of a 1-100 dilution of sodium hydroxide. The final tube, the control, contained 1 cc. of sterile saline and 1 cc. of completely inactivated enzyme filtrate. This was prepared by autoclaving a few cubic centimeters of the stock material containing 50% glycerin. The contents of all tubes were well mixed by agitation and placed where the temperature was very nearly constant over long periods of time (about 18 deg. C.). The tubes were agitated occasionally to allow fresh enzymes to replace the reaction products in contact with the solid medium.

At the end of the incubation period (72 hours) the liquid material on the gelatin remaining in each tube was removed by washing with distilled water, making the total volume up to 50 cubic centimeters. This material was then titrated as follows:

To the 50 cc. of solution was added 1 cc. of .5% phenolphthalein solution (.5 g. phenolphthalein in 50 cc. of alcohol and 50 cc. of water) and sufficient c.p. barium chloride crystals to produce saturation (about two grams). To this solution was added a sufficient amount of a saturated solution of barium hydroxide to produce a faint pink color, and then an excess of 5 cc. This red mixture was then filtered and the clear filtrate diluted to 100 cc. with freshly boiled water. Eighty cubic centimeters of this solution were made neutral to litmus by adding .2N hydrochloric acid and the volume again made up to 100 cc. To 50 cc. of this neutral solution was added enough .2N sodium hydroxide to produce a faint pink color, and then one drop more, producing a decided pink. This solution was placed in a large culture tube and reserved for later comparison. To the other 50 cc. of the neutral solution was added enough .2N sodium hydroxide to make its color identically the same as that just previously prepared. To this were added 25 cc. of freshly prepared formaldehyde solution * and the resulting mixture titrated back to the exact color of the control with a .2N sodium hydroxide.

Each of the four tubes for each enzyme was titrated in this manner. The use of the control for matching colors in titrating was found to be indispensable, especially when titrating mixtures containing dye. However, the color of the dye did not seriously interfere with the match-

* To 300 cc. of formaldehyde, add 6 cc. of .5% phenolphthalein sol. and 11 cc. of .2N sodium hydroxide, thus making the solution slightly basic to litmus.

ing, although some difficulty was experienced in the 1-25 dilution. It seemed that the darker the solutions the more difficult it is to get an exact match. The pink color of the end-point blended with the purple color of the dye to produce a light purple or lavender. By using a control tube, however, and preparing a new control tube every other titration, it was possible to read quite accurately.

Part II

Experimental Results

Six determinations were completed for each of the eleven enzymes using dilutions of 1-100 of gentian violet and sodium hydroxide as inhibiting agents, and three determinations were completed for each enzyme using dilutions of 1-50 and 1-25 of these reagents.

The amount of .2N sodium hydroxide needed to neutralize the acid material in the control tube (containing inactivated enzyme) was found to vary slightly for different enzymes but was fairly consistent for each enzyme. The amount of base used in the other three tubes varied from .45 cc. to 1.40 cc.

The true amount of acid formed due to the action of the enzyme was determined by deducting the amount of acid in the control from the amount found in the tube containing the uninhibited enzyme. Similarly, this same amount (control) was subtracted from the amount of acid discovered by titration to have been formed in the tubes containing enzyme inhibited by gentian violet or sodium hydroxide.

It is possible to determine the exact number of grams of nitrogen equivalent to each cubic centimeter of the .2N sodium hydroxide. Each cubic centimeter of .2N alkali or acid solution is equivalent to .0028 grams of nitrogen. The normality of the sodium hydroxide used was .1958, making each cubic centimeter of base equivalent to .00274 grams of nitrogen in the material tested.

However, since the relative amount of nitrogenous products liberated was all that was required in this investigation, it was found to be just as satisfactory and much more convenient to determine the degree of inhibition directly from the number of cubic centimeters of .2N sodium hydroxide used in the titration. For example, if a titration using enzyme "X" gave the following data:

| | |
|--|------------------|
| Tube #1 containing inactivated enzyme used | .35 cc. .2N base |
| Tube #2 " active " " | 1.08 cc. " " |
| Tube #3 " a 1-25 dilution gentian violet | .50 cc. " " |
| Tube #4 " " " sodium hydrox. | .45 cc. " " |

the calculations would be as follows:

| | |
|--|--------|
| Total cc. of .2N acid formed by enzyme action (#1 subtracted from #2) | .73 |
| Total cc. of .2N acid formed by mixture of enzyme and 1-25 sodium hydroxide; (#1 subtracted from #4) | .15 |
| Total cc. of .2N acid formed by mixture of enzyme and 1-25 dye; (#1 subtracted from #3) | .10 |
| % inhibition by 1-25 NaOH = $\frac{73-15}{73} \times 100 =$ | 79.40% |
| % inhibition by 1-25 dye = $\frac{73-10}{73} \times 100 =$ | 86.30% |

The percentages of inhibition of each enzyme by each dilution of each inhibiting agent were determined and averaged for the number of

determinations completed. These averages were then averaged for all enzymes of gram-positive organisms and for each dilution of the two inhibiting agents, and similarly for all enzymes of gram-negative organisms. (See Table V, which follows).

Table V

Average Percentage Inhibitions of Enzymes of Gram-positive and Gram-negative Organisms by Gentian Violet and Sodium Hydroxide, as Determined by Sorenson's Formol Titration.

| (a) Using Gentian Violet as Inhibiting Agent | | | | (b) Using Sodium Hydroxide as Inhibiting Agent | | | |
|--|-------|------|------|--|-------|------|------|
| Dilutions | | | | Dilutions | | | |
| | 1-100 | 1-50 | 1-25 | | 1-100 | 1-50 | 1-25 |
| Gram-Positive | 27% | 44% | 74% | Gram-Positive | 29% | 43% | 88% |
| Gram-Negative | 28% | 31% | 64% | Gram-Negative | 21% | 27% | 65% |

It will be noticed that in dilutions of 1-100 of both inhibiting agents (sodium hydroxide and gentian violet) there is little selectivity of inhibition with respect to gram staining reactions of the organisms; but in higher concentrations, especially in the 1-25 dilutions of sodium hydroxide, there is marked selectivity (i.e. the enzymes of gram-positive organisms are noticeably inhibited to a greater degree than are those of gram-negative organisms).

In this investigation (Part II), the organisms III, IV, 3S, Vulg., and Sub. were used which had also been used in Part I. It will be remembered that two of these organisms (3S and Vulg) were exceptional in their behavior in that they were rapid liquefiers, but that the activity of their enzymes was inhibited more readily than that of other enzymes. These results were duplicated in this portion of the investigation, the

enzyme 3S being inhibited an average of 90% by gentian violet and 83% by sodium hydroxide in 1-25 dilutions, as compared to inhibitions like 54%, 70%, 59%, 57%, 48% and 53% among the enzymes of the other gram-negative organisms.

Two new gram-negative organisms (Tr and Py) were added in Part II as well as one gram-positive (#VIII).

Summary and Conclusions

1. The results of this investigation confirm those reached in Part I with respect to selective inhibition; namely, that enzymes of gram-positive organisms are generally more susceptible to the deleterious action of gentian violet and sodium hydroxide than are those of gram-negative organisms.
2. The formol titration method for determining amino acid nitrogen is entirely satisfactory as a means of titrating the strengths of enzymes when used as herein described.

Cultural characteristics and description of organisms.

- #I Organism:** short gram-positive rod, rounded ends, singly and in pairs, non-motile, spore-bearer
Culture : acid in dextrose, sucrose, positive nitrate reduction of litmus pigment; milk completely digested in 72 hours, color reduced; gelatin fair, stratiform.
Source : sidewalk
Colony : rough, gray to white, fairly dry, crenated, raised.
- C. Organism:** gram-positive rod, rounded ends, very long chains and very pronounced spores; motile ---.
Culture : acid in dextrose and sucrose, reduction of pigment in lactose, negative indol, positive nitrate, gelatin fair and infundibulate. Litmus milk digested and half decolorized in 72 hours.
Source : genitalia
Colony : rather dry, gray, not distinct and do not stand out well. Growth of colony produces a fringe.
- D. Organism:** large gram-positive rod, fairly long and with round ends; spores toward one end and spore bearers not motile-others about - motility
Culture : acid in dextrose and sucrose with slight reduction of pigment; blue pellicle on lactose and mannose; positive nitrate and indol tests with heavy pellicle; litmus milk slightly digested in 48 hours, pigment of upper half of tube wine-colored in 72 hours.
Source : contamination in broth. (gel.3---cupped)
Colony : much like "C", spreading more and becoming slightly brown and drier when older.
- E. Organism:** fairly long gram-positive rod, some chains, which are very motile; prominent spores in 24-hour culture.
Culture : acid in dextrose, positive nitrate, negative indol, good stratiform gelatin liquifier, white pellicle and coagulation in peptone, stringy growth in broth and good growth in Treece medium; litmus milk completely decolorized with purple pellicle in 72 hours.
Source : contamination in gelatin.
Colony : much like "D", but darker.
- F. Organism:** short gram-positive rod, some slightly motile in young cultures; in pairs or chains with spores.
Culture : acid in dextrose, positive nitrate, slight indol, good pellicle in other sugars; acid and pept. litmus milk in 72 hours; gelatin, good infund.
Source : gelatin contamination.
Colony : like "C", "D", and "E" but more moist; follow streak on plate; lighter color than above.

- G. Organism:** medium-sized, non-motile, gram-positive rod, in chains and with spores.
Culture : acid in dextrose with slight gas; pellicle on other sugars and peptone with surface growth on Treese medium; litmus milk acid and peptonized in 48 hours with gray sediment in bottom; positive nitrate and negative indol. Fair craterform liquefaction.
Source : -- --
Colony : gray, rough raised, with slight depression on top.
- I. Organism:** non-motile, gram-positive rod.
Culture : acid and reduction dextrose, blue pellicle mannose and lactose, acid and reduction sucrose, positive nitrate, slight indol, litmus milk completely digested in 72 hours; gelatin stratiform liquefaction 2-
Source : sidewalk
Colony : round, rough, gray, and dry.
- J. Organism:** gram-positive, slightly motile rod, round ends, spores toward one end and not in chains, as a rule.
Culture : acid and slight gas in dextrose and mannose, acid and reduction in sucrose, negative in litmus milk in 24 hours but digested in 72 hours with flocculent material on top. Fair infundib. liquifier.
Source : horse manure.
Colony : white, rough and does not follow streaks on agar.
- K. Organism:** short, thick, non-motile rods; gram-positive; in chains, and usually with spores.
Culture : acid in dextrose, with slimy pellicle, and a pellicle in mannose and sucrose which sinks if shaken; positive in nitrate; gelatin fair stratiform liquefaction.
Source : street
Colony : indistinct, irregular and spreading. White and rough.
- R. Organism:** short, thick, gram-positive, non-motile rod, occurring in chains or singly.
Culture : acid and reduction in dextrose, with a pellicle; slight reduction in bottom of sucrose; thick growth and pellicle with positive nitrate; negative peptone; litmus milk negative in 48 hours; acid and pept. in 72 hours with blue ring left at the top; gelatin fair infund.
Source : tap water
Colony : spreading, white, and rather flocculent appearing; very irregular.
- Sub. Org. :** gram-positive rod, spores near the pole; long chains with spores; very motile in young cultures.
Culture: acid in dextrose and sucrose, positive nitrate; slight reac. in Treese medium; alk. and pep. of milk. good gelatin liquifier.

Colony : dry, corrugated pellicle; white to gray and granular; flat and slightly convex.

*10 Organism: short, thick, gram-positive rod with rounded ends and bearing spores; occur singly or in chains; non-motile.

Culture : acid in dextrose and sucrose.

Source : sewage

Colony : gray, white, glistening, wet, and flat.

III Organism: very small gram-negative rod; slightly motile.

Culture : acid and gas in all sugars, positive nitrate, acid in litmus milk in 24 hours. Gelatin --

Source : sewage.

Colony : very small in 24 hours, smooth, shiny and wet.

IV Organism: small, gram-negative, motile rod.

Culture : negative in all sugars; Gelatin --

Source : sewage

Colony : like III except lighter and green-gray in color.

38. Organism: very small, short, gram-negative rod; very motile.

Culture : acid and gas in dextrose; acid and slight gas in sucrose turning alk. in 72 hours; positive nitrate. Very good gelatin liquifier.

Source : A.Y. Wells

Colony : gray, smooth, wet and shiny; convex to pulvinate.

A. Organism: very short, gram-negative rod; extremely motile, some organisms resting for a moment in the drop and then resuming motion in a straight line.

Culture : acid and slight gas in dextrose, acid and reduction in mannose, acid in sucrose, positive nitrate, and negative indol; light pellicle on sugars and milk; latter digested and acid in 48 hours. Gives slight Treece reaction in several days. Gelatin, fair infundibulate liquifier.

Source : tap water

Colony : moist, translucent, grayish brown (color of mucilage); center of old colonies dark.

B. Organism: apparently a gram negative diplococcus; non-motile

Culture : negative in all media. Acid and pep. of milk in two weeks. Fair gelatin liquifier-napiform.

Source : contamination in gelatin shake.

Colony : white, opaque and isolated; slightly wrinkled edges in old colonies; moist and glistening.

H. Organism: small gram-negative rod; slightly motile.

Culture : acid and slight gas in dextrose in 48 hours; acid and gas in mannose and sucrose; positive nitrate; litmus milk negative in 48 hours, slightly acid in 72 hours. Infun. liquef. gelatin.

Source : urinal
Colony : smooth, wet, shiny

M. Organism: very small gram-negative rods; non-motile.
Culture : acid and slight gas in dextrose; acid and gas in mannose;
acid and slight gas in sucrose; acid and slight coag. in
in litmus milk in 24 hours; in 48 hours a red sediment
settles. Positive nitrate; very good stratiform to infund.
liquef. of gelatin.

Source : horse manure.
Colony : round, smooth, blue-green and opalescent.

Vulg. Organism: small, gram-negative rod; very motile.
Culture ; acid and gas in dextrose; acid sucrose; acid and reduction
in litmus milk; positive nitrate; positive Treece.
Colony ; smooth, thin.

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