SOME CONDITIONS AFFECTING THE PRODUCTION OF GELATINASE BY PROTEUS BACTERIA[1]

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INTRODUCTION

It has been shown by previous investigators that the production of proteolytic enzymes by bacteria is affected by changes in the composition of the medium in which the organisms are grown, by variations in the acidity of the culture, and by the amount of oxygen available to the bacteria during growth.

Brunton and Macfadyen (1889) concluded that a stronger enzyme, capable of liquefying gelatin, was produced by bacterial cultures grown in meat broth media than in nutrient gelatin. Fermi (1892) showed that little or no gelatin-liquefying enzyme was obtained in cultures grown in his protein-free media while considerable was formed in nutrient gelatin.

Abbott and Gildersleeve (1903) stated that production of gelatinase was better in alkaline culture media than in acid culture media. At the 1919 meeting of the Society of American Bacteriologists, Clark (1920) reported an optimum production of gelatinase by Proteus bacteria in media near the pH of optimum activity of the enzyme and a decrease in production in media of original reaction of pH 6.5.

[1] Published by permission of the Surgeon General, U. S. Public Health Service.

Liborius (1886) showed a greater production of gelatin-liquefying enzyme in cultures exposed to oxygen than in cultures from which oxygen was excluded. Clark (1920) reported that anaerobiosis shuts off the production of gelatinase almost completely.

In the present paper, the investigations described include a description of the method of Palitzsch and Walbum (1912) as modified by Clark (1919) for the estimation of gelatinase; the quantitative data concerning the reports given at the meetings of the American Society of Bacteriologists by Clark (1919–1920)[3] and by Clark and Merrill (1925), and in addition, further studies on the effect of aerobic and anaerobic conditions on the production of gelatinase, and the development of a synthetic medium in which a good production of gelatinase by Proteus[4] is obtained and so controlled that uniform results can now be obtained.

EXPERIMENTAL

Cultures

For the study of the production of gelatinase by Proteus, strains of this group were obtained from L. A. Rogers, Bureau of Dairying, United States Department of Agriculture. Most of the work was carried on with the strain designated acx. The description determined by Rogers and co-workers for acx, is as follows: Isolated from spring water, it gives typical growth on agar, digests milk, liquefies gelatin, and with glucose gives off carbon dioxide and no hydrogen (ratio = ∞) gas, and forms acid. It does not ferment lactose. The gas obtained in fermentation with glucose was small and only recovered by the vacuum method. Proteus strains other than acx were used in the preliminary studies (Clark, 1920) as indicated in the charts.

[3] The preliminary studies (Clark, 1920) were made with the support of the Dairy Division, now the Bureau of Dairying, United States Department of Agriculture.

[4] Throughout this paper the term Proteus will be used to signify Proteus bacteria.

Estimation of gelatinase activity

The activity of gelatinase is usually determined by the rate of liquefaction of gelatin by the enzyme. The methods for determination of gelatin liquefaction are reviewed by Marras (1914). The procedure used by Fermi (1892), Palitzsch and Walbum (1912), Dernby (1918) and others was modified by Clark (1920) as follows. Melted gelatin and the medium containing the enzyme were mixed and after a period of digestion, a portion of the mixture was transferred to a test tube, and cooled in ice water. The time required for the mixture to set to a selected consistency was marked by a stop watch. This procedure is to be preferred to that of Palitzsch and Walbum, who plunged their mixture into ice water for ten minutes. To the consistencies observed, they gave arbitrary numbers which have no well defined meaning, while Clark's procedure calls for but one consistency.

Clark found empirically that the following equation could be used,

$$K = \frac{\log t_s - C}{T}$$

Here C is the logarithm of the constant time in minutes required for the gelatin control without enzyme to set to the selected state, t_s is the time in minutes required for the digested mixture to set in ice water, T is the time in minutes of incubation of the gelatin-enzyme mixture and K a constant, characterizing the enzyme strength. For this equation to be applicable, several conditions must be fulfilled as shown by Palitzsch and Walbum (1912). The temperature of incubation of the mixture must be constant. The concentration of gelatin must be constant. The pH of the gelatin mixture for the chilling test must be the same and such that the undigested gelatin sets well in ice water. Patten and Johnson (1919) have shown that the setting of gelatin is influenced by the pH of the medium and that within certain limits this hydrogen ion concentration effect is probably reversible. We chose pH 6.2 for the chilling test. The K values in comparative measurements must be determined with a single

batch of gelatin and if the mixtures of gelatin and enzyme are incubated at various pH values, each must be readjusted to the same value for the chilling test and brought to the same concentration of gelatin calculated as unattacked gelatin.

Accordingly, preliminary tests are made with indicators or electrode to determine the proportions of mixture and acid or alkali which must be used, in order that the mixture may be adjusted rapidly after the incubation period.

The stock gelatin contained 10 per cent Difco gelatin (washed free of calcium) 0.1 M H_3BO_3, and 0.1 M Na_2HPO_4 and was preserved with 0.5 per cent phenol. Before use 100 cc. of the melted stock gelatin was transferred to an Erlenmeyer flask, sufficient N sodium hydroxide solution added to bring to the desired pH, and distilled water then added to make the total volume 150 cc. This gelatin stock "solution" diluted from 100 cc. of the stock gelatin to 150 as just described we will designate as gelatin "solution" A.

An example of the procedure for estimation of gelatinase is as follows:

Proteus was grown in a medium made of 2.0 per cent Difco peptone, 1 per cent $Na_2HPO_4 \cdot 2H_2O$[5] and 2 per cent glucose adjusted to pH 7.0 with N hydrochloric acid solution. Fifty cubic centimeters of medium in a 1000-cc. Erlenmeyer flask, stoppered with a cotton plug to provide sufficient aeration, was inoculated with the bacteria and incubated at 30°. Within forty-eight hours considerable gelatinase was produced. To determine the gelatinase, a preliminary test was made to determine the number of cubic centimeters N sodium hydroxide solution required in the gelatin "solution" A so that on mixing with the culture solution, the final reaction of the mixture would be pH 8.4. In this case 5 cc. N sodium hydroxide solution was used in the gelatin "solution" A.

Twenty-five cubic centimeters of the bacterial culture solution were then well mixed with 100 cc. of gelatin "solution" A at 30° in a 300 cc. Erlenmeyer flask and the mixture incubated at 30°

[5] One per cent $Na_2HPO_4 \cdot 2H_2O$ was added to the culture medium to control the pH of the culture.

in a water bath. (The phenol prevented any further growth of the bacteria.)

Five cubic centimeters of the mixture were removed by means of a pipette at varying intervals and transferred to tests tubes. Five-tenths N hydrochloric acid solution was added to bring the reaction of the test mixture to pH 6.2 and the whole plunged into ice water. The test tube was gently shaken. A stop watch marked the time required to bring the mixture to a selected

TABLE 1

$$K = \frac{\log t_s - C}{T}$$

t_s	$\log t_s$	T	C ESTIMATED	K
minutes		*minutes*		
0.67	−0.17	8	−0.40	0.0288
0.75	−0.13	13		0.0208
1.08	+0.03	21		0.0205
2.00	+0.30	32		0.0219
3.83	+0.58	38		0.0258
Average				0.0236
0.67	−0.17	10	−0.40	0.0230
0.83	−0.08	15		0.0213
1.17	+0.07	22		0.0214
1.67	+0.22	28		0.0221
3.00	+0.48	36		0.0244
5.33	+0.73	42		0.0269
Average				0.0232

consistency of gelatin judged by partially inverting the test tube with its contents.

The results obtained with mixtures of gelatin with the same culture solution, in two separate determinations, are shown in table 1 and figure 1.

C was estimated as follows: T was plotted as ordinate and $\log t_s$ as abscissa as in figure 1. If K is a constant and T is zero, $\log t_s = C$. Hence project the "best" straight line through the experimental points to $T = 0$. Ideally, C should be the logarithm

of the time of setting of the gelatin mixture without enzyme. While such direct control furnishes values of C of the right order of magnitude, there is found an appreciable difference between C as determined graphically and as determined in the mixture. However, the graphically estimated C values were selected arbitrarily in the estimation of K.

Table 1 and figure 1 show that if the time of setting is very short, near that of the control, the error in judging the time of attainment of the selected consistency makes a larger proportional error in the true value of t_s.

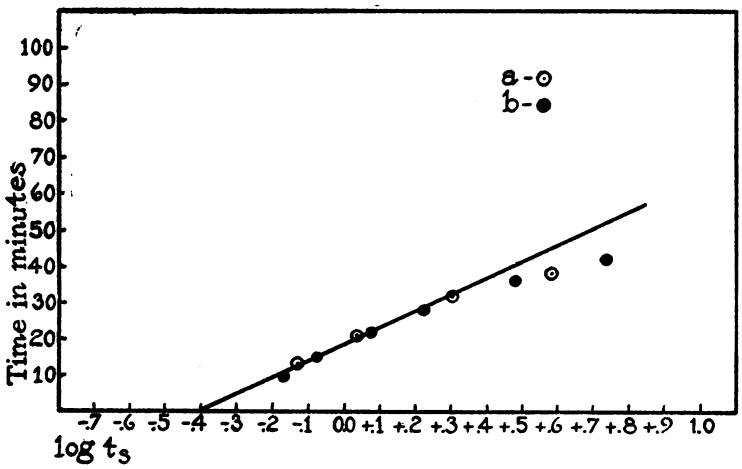

FIG. 1

On the other hand, if the time of setting is very large it means that extensive alteration of the gelatin has taken place and the equation found empirically to fit a short range of conditions would not be expected to hold. Hence the intermediate values of t_s in a series of measurements with different incubation time T were chosen as the more probable determinations of K. See table 1 and figure 1.

Effect of dilution on gelatinase activity

In order to determine the effect of dilution on gelatinase activity, mixtures of gelatin were made as previously described in

which varying amounts of phenolized bacterial culture solution were added to gelatin "solution" A, the final volumes of the

TABLE 2

K as a function of enzyme strength

Cubic centimeters phenolized enzyme solution mixed with 100 cc. phenolized gelatin "solution" A diluted to 125 cc.

CULTURE SOLUTION	K	$\frac{K}{s}$
cc.		
25	0.037	0.00148
20	0.029	0.00145
15	0.023	0.00153
10	0.015	0.00150
5	0.008	0.00160
2.7	0.004	0.00159
Average		0.00153

mixtures being kept constant by the addition of water and the final pH of the mixture for digestion with the enzyme controlled to pH 8.4 by previous additions of the proper amount of N

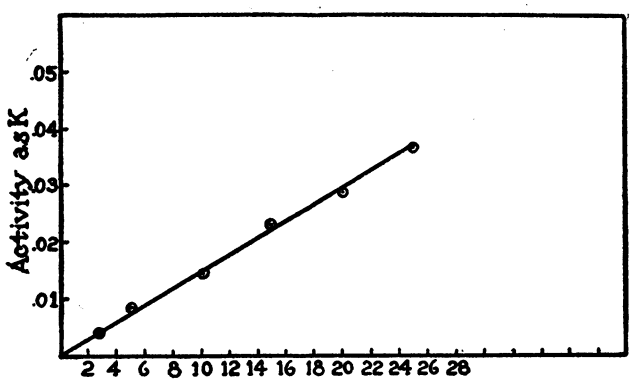

FIG. 2

sodium hydroxide solution. The test portions were all adjusted to pH 6.2 before cooling.

The effect of dilution of the gelatinase on the activity K is shown in table 2 and figure 2.

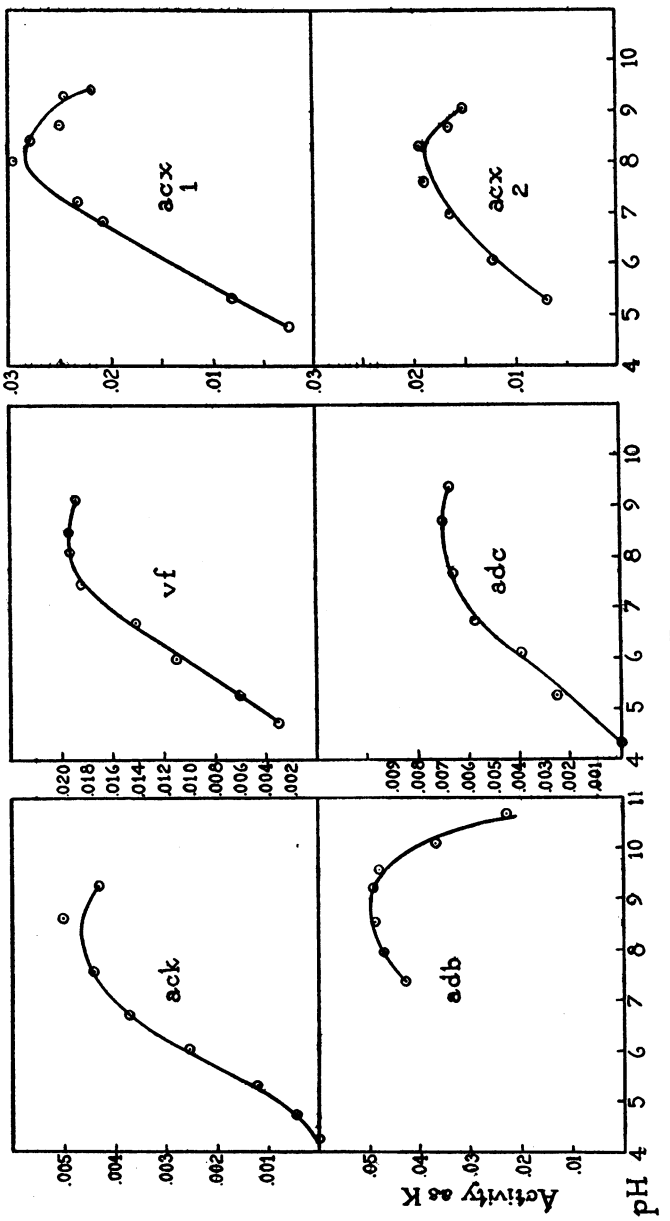

Fig. 3

From a study of table 2 and figure 2, it is seen that at the dilutions studied the activity K varies with the concentration of the gelatinase solutions. We therefore conclude that the activity K determined in cultures of the same strain of Proteus indicates the amount of gelatinase present in the culture solution.

Effect of pH of digestion mixture on gelatinase activity

The effect of hydrogen ion concentration on the rate of digestion of the gelatin by the enzyme produced in Proteus cultures, was determined by the method described above. One-half of 1 per cent phenol was added to a culture to prevent further bacterial action and digestion of gelatin determined at varying hydrogen ion concentrations. Before a test was made in ice water, the portion of gelatin-enzyme mixture removed was brought in these experiments, to pH 6.2.

In figure 3, the velocity constant is seen to be greatest in gelatin mixtures at a range near pH 8 to 9, and decreases on either side. At pH 4.7 the enzyme effect is small, a result which is in accord with those of Northrop (1922) who showed that trypsin digestion is at a minimum at the isoelectric point of gelatin.

Our results indicate the tryptase type of protease which Dernby (1921) found in his work on bacterial cultures. Within the limits of the proximate method we have found the optimum pH of activity of gelatinase in gelatin digestion to be about 8.4. Accordingly in subsequent experiments having to do with factors controlling gelatinase production, gelatin digestion was carried on near pH 8.4. Controls were run with gelatin at pH 8.4 without addition of gelatinase. No liquefaction was noted in these controls when chilled at pH 6.2.

Effect of pH of culture on production of gelatinase

Figures 4 and 5 illustrate experiments performed to determine the effect of the pH values during culture upon production of gelatinase.

The medium used was made as follows: 2 per cent Difco peptone, 0.5 per cent $Na_2HPO_4 \cdot 2H_2O$, 0.5 per cent KH_2PO_4, and 0.1 per cent NH_4Cl. Adjustments to the different initial pH

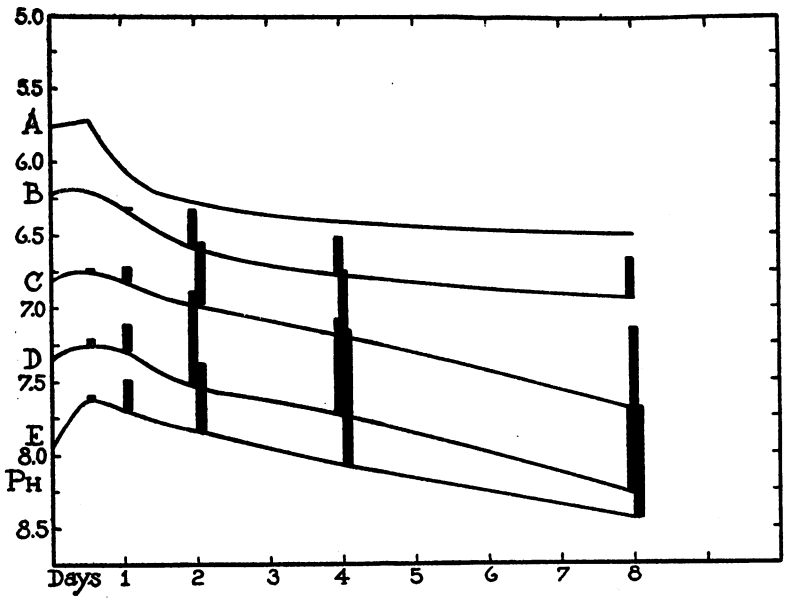
5.0
5.5
A
6.0
B
6.5
C
7.0
D
7.5
E
8.0
PH
8.5
Days 1 2 3 4 5 6 7 8

FIG. 4

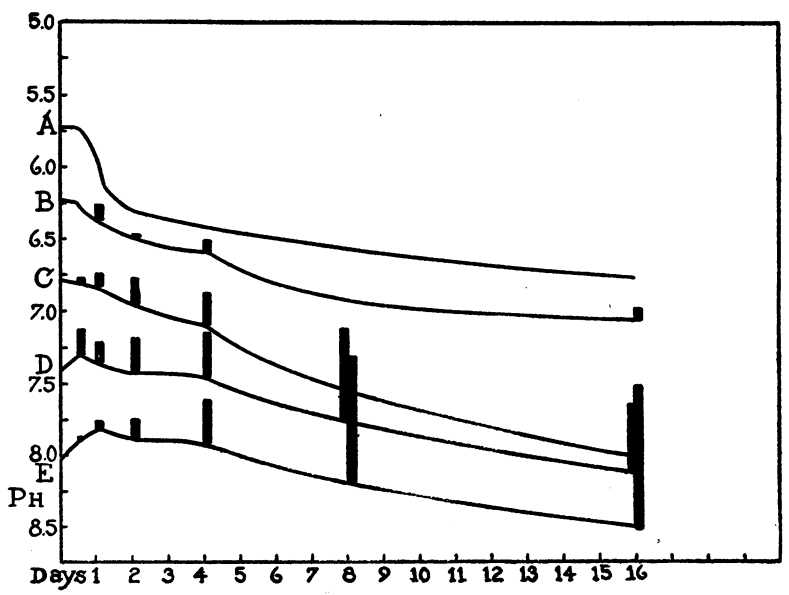
5.0
5.5
A
6.0
B
6.5
C
7.0
D
7.5
8.0
E
PH
8.5
Days 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

FIG. 5

values were made with hydrochloric acid or sodium hydroxide solution. The initial pH values were those measured after sterilization. An aliquot of each medium was placed in a 1000-cc. flask, inoculated with Proteus and incubated at 30°. Portions were withdrawn with sterile pipettes for the several tests.

In figures 4 and 5 the heights of the blacked rectangles represent the relative strengths of the enzyme produced as measured by the method outlined above. Each experiment was run in duplicate and the average value of each pair recorded.

The best production of gelatinase by Proteus in these experiments was found to be in cultures of reactions between pH 7 and 8. However, the data must be interpreted as applying to the particular conditions used. In cultures grown later in glucose media of original pH 7, we often obtained good production of gelatinase although the final culture reactions were quite acid.

Effect of aeration of culture on production of gelatinase

Liborius (1886) and others have noted the favorable effect of aeration on the production of proteolytic enzyme. The following experiments were made to study the effect of aeration on the production of gelatinase.

Proteus adc was grown in a medium of 2 per cent Difco peptone, 0.5 per cent $Na_2HPO_4 \cdot 2H_2O$ and 0.5 per cent KH_2PO_4 at a temperature of 30°. The conditions of aeration were as follows:

A_1 and A_2	Culture highly evacuated and sealed
B_1 and B_2	Culture in test tube with marble to delay diffusion
C_1 and C_2	Culture in open test tube
D_1 and D_2	50 cc. culture in 200 cc. Erlenmeyer
E_1 and E_2	50 cc. culture in 1 liter Erlenmeyer
F	Culture in tube with intermittent bubbling of O_2.

The production of gelatinase under these conditions is shown in table 3. The production of gelatinase as shown in this table evidently increases with increased aeration.

Since the aeration may affect the production of gelatinase by its effect on growth alone, an attempt was made to determine

the effect of aeration by contrasting cultures grown under apparently slight differences of air exposure.

For this experiment, a peptone-broth medium containing 1.0 per cent peptone, and 1.0 per cent $Na_2HPO_4 \cdot 2H_2O$, was adjusted to pH 7.2. Fifty cubic centimeters of the medium were placed in a 1000-cc. Erlenmeyer flask[6] and 500 cc. of the medium placed in another 1000-cc. Erlenmeyer flask and sterilized. The 50 cc. medium had a depth of 1.0 cm. and the 500 cc. medium had a depth of 4.4 cm., so that the aeration of the first was notably greater than that of the second. Both were inoculated with Proteus, strain acx, so that about 400 bacteria were added per

TABLE 3

Effect of aeration on production of gelatinase by Proteus

EXPERIMENT	*K* VALUES
A_1	0.0005
A_2	0.0004
B_1	0.0007
B_2	0.0009
C_1	0.008
C_2	0.009
D_1	0.027
D_2	0.027
E_1	0.064
E_2	0.043
F	0.050

cubic centimeter of media. The cultures were incubated at 30° and at intervals the number of bacteria per cubic centimeter was determined in each culture in order to determine the growth.

In table 4, the bacterial counts during growth are tabulated.

Table 4 shows that the growth of the bacteria is about the same in both cultures after sixteen hours. After that period there are more bacteria found per cubic centimeter in the shallow culture

[6] In all reference in this paper to cultures in a 1000 cc. Erlenmeyer flask, an Erlenmeyer flask of this volume is specified with a maximum diameter of approximately 13 cm. and a height of approximately 23 cm. with a cotton plug inserted in the neck, to allow aeration of the contents.

than in the deep culture. After fifty hours growth the activity of the gelatinase produced in the cultures was $K = 0.0207$ in the shallow culture and $K = 0.0015$ in the deep culture. In this experiment the greater aeration of the shallow culture resulted in a somewhat better growth of the bacteria, and production of a stronger gelatinase than was found in the culture of more depth and less aeration.

TABLE 4

Effect of aeration due to depth of culture on growth of Proteus

TIME OF GROWTH	NUMBER OF BACTERIA PER CUBIC CENTIMETER IN CULTURE OF DEPTH 1.0 CM.	NUMBER OF BACTERIA PER CUBIC CENTIMETER IN CULTURE OF DEPTH 4.4 CM.
hours		
1		
2		
3	$10^{2.8}$	$10^{2.8}$
4	$10^{3.5}$	$10^{3.5}$
5	$10^{4.0}$	$10^{4.3}$
6	$10^{4.6}$	$10^{4.6}$
7	$10^{5.0}$	$10^{4.9}$
8	$10^{5.9}$	$10^{6.3}$
9	$10^{6.6}$	$10^{6.8}$
10	$10^{7.3}$	$10^{7.3}$
11	$10^{7.7}$	$10^{7.9}$
12	$10^{8.3}$	$10^{8.0}$
13	$10^{8.3}$	$10^{8.8}$
14	$10^{8.3}$	$10^{8.3}$
15	$10^{8.6}$	$10^{8.5}$
16	$10^{8.7}$	$10^{8.6}$
17	$10^{8.5}$	$10^{8.3}$
19	$10^{9.0}$	$10^{8.3}$
21	$10^{8.9}$	$10^{8.3}$
23	$10^{9.3}$	$10^{8.6}$
42	$10^{10.0}$	$10^{9.3}$
52	$10^{10.0}$	$10^{9.0}$

The number of bacteria and gelatinase production was determined in two cultures of Proteus, strain acx, grown in a medium made of 2 per cent peptone, 2 per cent glucose, and 1 per cent $Na_2HPO_4 \cdot 2H_2O$, at a temperature of 30°, for seventy-two hours. The cultures were grown in 1000-cc. Erlenmeyer flasks, one in a 50 cc. portion of depth approximately 1 cm., the other in a 300 cc. portion with a depth of 3 cm.

In this experiment the small difference in numbers of bacteria in the cultures of different depth, does not account for the greater production of gelatinase in the more shallow culture which has the greater aeration, as shown in table 5.

In order to study the effect of aeration still further, another experiment was carried out in which the aeration was more definitely controlled. Two cultures of Proteus strain acx were grown in peptone-broth containing 1.0 per cent $Na_2HPO_4 \cdot 2H_2O$. One culture was grown in 50 cc. of medium in a 1000-cc. Erlenmeyer flask providing good aeration. The other was grown in

TABLE 5

Effect of varying depth of culture on growth and production of gelatinase

DEPTH	FINAL NUMBER OF BACTERIA PER CUBIC CENTIMETER	K
cm.		
1	$10^{9.6}$	0.0168
3	$10^{9.4}$	0.0037

TABLE 6

Effect of aeration on growth and production of gelatinase by Proteus

CULTURE IN	FINAL NUMBER OF BACTERIA PER CUBIC CENTIMETER	GELATINASE ACTIVITY AS K	FINAL pH OF CULTURE
50 cc. open to aeration	$10^{8.8}$	0.0316	8.4
1000 cc. closed to aeration	$10^{8.6}$	0.0002	6.9

1000 cc. of medium held in a 1000-cc. Florence flask from which air was excluded by means of a rubber stopper with an outlet tube dipping into mercury to allow escape of evolved gases. The number of bacteria per cubic centimeter at the beginning of growth was about 300, and the original pH of the medium was pH = 7.2.

In table 6 are given the number of bacteria per cubic centimeter and the activity of the gelatinase determined in the culture after ninety-six hours incubation at 30°. From the results in this table, it appears that the small difference in growth of the bacteria

in the two cultures does not account for the much greater production of gelatinase in the culture which is well aerated. The difference in pH of the culture is not the reason for the difference in production of gelatinase, as, in many cultures previously studied, a good production of gelatinase was found in aerated cultures at pH 6.9.

From the experiments on the effects of aeration on the production of gelatinase by Proteus, it appears that access of air to the culture results in stimulation of production of gelatinase due to aerobic effects, in themselves not associated with numbers of bacteria or with hydrogen ion concentration, since the number of bacteria under anaerobic conditions may be near that of aerobic cultures, and the difference in hydrogen ion concentration does not account for so large a difference in production of gelatinase.

A further discussion of the effect of aeration on production of gelatinase by Proteus will be given in the section on synthetic media.

REPRODUCIBILITY OF RESULTS

While very fair duplications of data were obtained in parallel experiments made at the same time, with the same materials and with the same culture, it was difficult to reproduce the data with experiments made at other times and with different samples of peptone in the medium.

Under apparently the same conditions a variation of the brand of peptone used causes a wide variation in gelatinase production and it was realized that other factors beside pH and aeration had still to be considered. We therefore began the study and development of synthetic media of well controlled composition which might take the place of the peptone-broth in the growth of Proteus with production of gelatinase.

SYNTHETIC MEDIUM DEVELOPED FOR CULTURE OF PROTEUS WITH PRODUCTION OF GELATINASE

It has been shown by Brunton and Macfadyen (1889), Fermi (1892), Jordan (1906), Diehl (1919) and others that the same bacteria which produce proteolytic enzyme in one kind of a

medium will not produce it in another medium, although the growth may be luxuriant in both.

Fermi, who found good enzyme production in gelatin cultures of certain species of bacteria, also grew a number of bacteria in synthetic media in which ammonium salts were the only source of nitrogen. A trace of proteolytic enzyme was found in the cultures of but three species of bacteria out of the number studied and he concluded that little or no proteolytic enzyme could be produced by bacteria in protein-free nutrient media.

Jordan used a synthetic medium in which asparagin was the source of nitrogen. He obtained enzyme but his tests were made on cultures many weeks old.

Diehl obtained good enzyme in synthetic media with three species of bacteria when asparagin was present as a source of nitrogen but none when ammonium salts were the only form of nitrogen. He concluded that organic nitrogen was necessary for the production of proteolytic enzyme.

Auerbach (1897) obtained a better production of proteolytic enzyme in bacterial cultures when he added calcium or magnesium carbonate to the peptone broth media. He ascribed this effect to the neutralizing action of these salts. In our work we have shown that the effect of calcium or magnesium salts is due to some other factor than the pH of the culture.

Soppeland and Levine (1924) found that calcium and sodium chloride together accelerate the proteolysis of milk by certain strains of bacteria. They did not show whether this was due to an accelerating effect on the action of the enzyme on its substrate, or to a greater production of proteolytic enzyme. Levine and Soppeland (1926) studied the effect of sodium chloride and calcium chloride on proteolysis of gelatin by bacteria but here again, the bacteria were grown in the gelatin medium, and one cannot separate the effects of the salts on the production of enzyme by the bacteria from the effects on the liquefaction of the gelatin by the enzyme. Delezenne (1905) and Delezenne and Zunz (1906) have shown that calcium salts may accelerate proteolysis by pancreatic extract.

Falk (1923), in a review of synthetic media, has shown that Ca

is often left out of the composition of synthetic media while Mg is frequently retained.

In our work we have found that calcium and magnesium salts have considerable influence on the production of proteolytic enzyme by bacteria and that this enzyme may be produced by Proteus when ammonium salts are the only source of nitrogen if calcium or magnesium salts be present.

The first synthetic medium was made as follows:

Synthetic medium no. 1

$Na_2HPO_4 \cdot 2H_2O$*	8.8 grams
K H phthalate	2.0 grams
Asparagine	1.0 gram
Glucose	1.0 gram
Glycerol	1.0 gram
Distilled H_2O	to 1000 cc.

Adjusted to pH 7.0 with N/1 HCl.

* The $Na_2HPO_4 \cdot 2H_2O$ was added as usual in the synthetic media in part for its action as a buffer salt in the culture.

Fifty-cubic-centimeter portions of synthetic medium no. 1 were placed in 1000-cc. Erlenmeyer flasks under aseptic conditions. Inoculation was made with Proteus strain acx so that 10^3 bacteria were added per cubic centimeter medium. After five days incubation at 30° the bacteria had increased to $10^{9.0}$ per cubic centimeter. No gelatinase was found in this medium. Transfers were made of this culture to fresh synthetic medium several times with the same result. Growth was slow but the same number of bacteria were finally obtained as in peptone media.

Next a more concentrated synthetic medium was made of different composition.

Synthetic medium no. 2

Na_2HPO_4	10 grams
KH_2PO_4	2 grams
Asparagine	10 grams
Glucose	10 grams
Distilled H_2O	to 1000 cc.

Adjusted to pH 7.0 with N/1 HCl.

When 0.2 per cent cystine or 0.2 per cent sodium thiosulfate was added to this medium, growth was obtained but no gelatinase.

A synthetic medium no. 3 was made up as follows with ammonium salts as sole source of nitrogen.

Synthetic medium no. 3

$Na_2HPO_4 \cdot 2H_2O$	10 grams
KH_2PO_4	2 grams
NH_4Cl	10 grams
Glucose	10 grams
Distilled H_2O	to 1000 cc.

Adjusted to pH 7.0.

When Proteus strain acx, was transferred to 50 cc. of synthetic medium no. 3 in a 1000-cc. Erlenmeyer flask, fair growth of the bacteria was obtained but no production of gelatinase. However, when 0.05 per cent calcium carbonate and 0.05 per cent magnesium sulfate were added to 50 cc. medium no. 3 and other cultural conditions repeated, Proteus strain acx grew to the same maximum numbers as in peptone broth and a good production of gelatinase was obtained.

Preparation of synthetic media

Precautions were taken never to sterilize the ammonium chloride and sugar solutions together with heat. The ammonium chloride solution and the phosphate solution were each sterilized separately at 15-pound pressure in the autoclave. When heated together free ammonia was given off. The sugar solution was passed through a Berkefeld filter to make it sterile. Care was taken not to pass ammonium or phosphate solutions through the Berkefeld as calcium and magnesium salt impurities might well be extracted from the filter under these conditions. The Berkefeld filter was well washed with distilled water before use to remove soluble salts developed when sterilized by heat. The solutions were mixed in the proper proportions after sterilizing as above.

All work with bacteria was done under aseptic conditions.

Salts and sugar were recrystallized twice except for the calcium and magnesium salts which were high grade analyzed salts used

in small quantities. All glassware was made specially clean before use. Flasks used for cultures were of Pyrex glass.

Proteus acx[7] was adapted to the synthetic medium used, and after growth diluted to 10^7 and 1 cc. added to fresh synthetic medium so that from 100 to 1000 bacteria were added per cubic centimeter of medium. Transfers were made several times so that growth effects were known to be due to the synthetic medium without traces of any peptone. Fifty cubic centimeters of the synthetic medium were added to 1000-cc. Erlenmeyer flasks to insure surface aeration of shallow cultures. Calcium and magnesium salts in weighed amounts were sterilized in the flasks previous to addition of the solutions.

The strength of the gelatinase produced in synthetic medium cultures was not as great as that produced in peptone broth cultures. Nevertheless on mixing the cultures with gelatin in the same proportion as used in previous determinations of gelatinase, it was found that the best gelatinase production in synthetic media resulted in liquefaction of the gelatin in from two and one-half to three hours digestion time. This production of gelatinase is called "good" in the tables on results obtained in synthetic medium.

When the production of gelatinase was such that it took twenty-four hours digestion with the gelatin to obtain liquefaction, the gelatinase is called "fair." When only a slight liquefaction took place after three days digestion, the enzyme is designated "small."

Effect of calcium and magnesium salts

Since a "good" production of gelatinase was obtained when Proteus was grown in synthetic medium no. 3 to which both calcium carbonate and magnesium sulfate had been added, the effect of these salts was studied in a series of experiments conducted, in the main, as already described. In those cultures in which no magnesium sulfate was present 0.025 gram sodium

[7] In all work with the synthetic media Proteus strain acx was used. The results recorded apply to strain acx. The effects of the synthetic media on other strains of Proteus have not yet been investigated.

sulfate was added so that the sulfate ion might be present in the control. Twenty-five-thousandths gram sodium carbonate was added to those cultures in which no calcium carbonate was present to insure the carbonate ion. The initial pH of all cultures was 7.0 to 7.1.

In tables 7 and 8 the effects of 0.05 per cent calcium and magnesium salts are shown on the growth and production of gelatinase by Proteus grown in 50 cc. synthetic medium no. 3 in 1000 cc. Erlenmeyer flasks under aerobic conditions. Inoculations were

TABLE 7

The effect of calcium and magnesium salts on the growth and production of gelatinase by Proteus in synthetic medium no. 3

ADDITION OF Ca AND Mg	NUMBER OF BACTERIA PER CUBIC CENTIMETER AFTER						PRODUCTION OF ENZYME AFTER 4 TO 10 DAYS GROWTH
	24 hours	48 hours	72 hours	96 hours	120 hours	144 hours	
grams							
0.025 $CaCO_3$ 0.025 $MgSO_4$	10^3	$10^{5.5}$	$10^{8.6}$	$10^{9.6}$	$10^{9.6}$	$10^{9.7}$	"Good"
0.000 $CaCO_3$ 0.000 $MgSO_4$ 0.025 Na_2SO_4	$10^{2.3}$	$10^{4.3}$	$10^{7.9}$	$10^{8.8}$	$10^{8.5}$	$10^{8.6}$	"None"
0.025 $CaCO_3$ 0.000 $MgSO_4$ 0.025 Na_2SO_4	$10^{1.0}$	$10^{4.1}$	$10^{7.3}$	$10^{8.7}$	$10^{8.8}$	$10^{8.7}$	"Fair"

made so that about 100 bacteria were added per cubic centimeter medium.

For the determination of gelatinase 2.5 cc. of culture was added to 10 cc. of gelatin "solution" A, the pH of which was such that on mixing with the culture the pH of the mixture would be 8.4. The mixtures were kept in test tubes in a water bath at 30° and portions removed as usual for tests.

The pH of these cultures, after several days' growth, was 6.8 to 7.0 so that the differences in the results were not due to variation of the hydrogen ion concentration of the culture.

Gelatinase was found in the culture containing both calcium

and magnesium on the fourth day of growth when the growth had attained the maximum value. This enzyme increased in strength up to the seventh day of growth.

In the culture containing calcium but no magnesium, a weak gelatinase was found on the fourth day of growth. This enzyme increased in strength at a later period but did not attain the strength of the gelatinase in the first culture containing both calcium and magnesium.

TABLE 8

The effect of calcium and magnesium salts on the growth and production of gelatinase by Proteus in synthetic medium no. 3

ADDITION OF Ca AND Mg	NUMBER OF BACTERIA PER CUBIC CENTIMETER AFTER						PRODUCTION OF ENZYME AFTER 4 TO 10 DAYS' GROWTH
	24 hours	48 hours	72 hours	96 hours	120 hours	144 hours	
grams							
0.025 $CaCO_3$ 0.025 $MgSO_4$	$10^{2.6}$	$10^{5.1}$	$10^{8.5}$	$10^{9.5}$	$10^{9.8}$	$10^{9.7}$	"Good"
0.000 $CaCO_3$ 0.000 $MgSO_4$ 0.000 Na_2SO_4*	$10^{2.7}$	$10^{5.5}$	$10^{8.3}$	$10^{8.8}$	$10^{8.8}$	$10^{8.7}$	"Trace"
0.000 $CaCO_3$ 0.025 $MgSO_4$ 0.025 Na_2CO_3	$10^{2.7}$	$10^{5.7}$	$10^{8.5}$	$10^{9.4}$	$10^{9.7}$	$10^{9.8}$	"Small"
0.025 $CaCO_3$ 0.025 $MgSO_4$ 0.025 Na_2CO_3	$10^{2.6}$	$10^{4.8}$	$10^{8.3}$	$10^{9.3}$	$10^{9.6}$	$10^{9.7}$	"Good"

*Sodium sulfate was added to the control in the previous series without apparent effect.

No enzyme was found at all in the culture in which there was no calcium or magnesium.

In this series (table 8) gelatinase was found in the culture containing calcium and magnesium after four days when the number of bacteria had reached maximum values. In the culture containing magnesium and no calcium only a slight production of gelatinase was found after eight days' growth. The number of bacteria determined was practically the same in both

these cultures and the difference in production of gelatinase is not due to any significant difference in growth of these two cultures.

Only a trace of gelatinase was found in the culture containing no calcium and no magnesium. Although the original pH of the cultures was 7.0 to 7.1 the pH of the cultures in the second series, after eight days incubation, was 6.5 to 6.4. This hydrogen ion concentration was not the cause of the difference in enzyme production as both comparatively good and poor production of gelatinase occurred at this pH of the series. It is apparent that the addition of calcium and magnesium salts results in production of gelatinase, which is best when both salts were added, although with calcium alone a fair production of gelatinase is obtained. The difference in production of enzyme was not due to the effect of the anions of the salts, as they were added in the controls as sodium sulfate or sodium carbonate.

The smaller numbers of bacteria do not seem to be the cause of the apparent *lack* of enzyme in the cultures without calcium or magnesium salts, as digestion with gelatin was prolonged three days within which time a relatively small enzyme would have given liquefaction had it been present.

When calcium sulfate was substituted for the carbonate a good production of gelatinase was obtained in these cultures.

An experiment was made which showed that the differences found in the gelatinase activity of cultures were due to calcium and magnesium salt effects in the cultures and not to salt effects on the digestion of the gelatin by the enzyme. A series of cultures was made in synthetic medium as in the previous experiments, with and without the addition of calcium and magnesium salts. After several days growth the gelatinase was tested as usual. Two-and-five-tenths cubic centimeters of each culture solution was added to 10 cc. portions of gelatin "solution" A in test tubes. At the same time 2.5 cc. of each culture solution was added to 10 cc. portions of gelatin "solution" A to which had been added a solution of calcium or magnesium salts, so that each gelatin mixture contained the calcium or magnesium salt lacking in the culture added to it. Care was taken to keep the volumes

and pH of these mixtures alike. It was of interest to find that in this experiment the velocity of digestion of the gelatin by the gelatinase in the cultures was unaffected by the absence or presence of the calcium or magnesium salts in the gelatin digestion mixture.

The culture solution lacking both calcium and magnesium salts did not liquefy the gelatin mixture to which calcium and magnesium salts were added. The culture solution containing magnesium salt but no calcium salt showed the same "small" gelatinase activity in the gelatin mixture with and without addition of calcium salt; and the culture solutions containing calcium and no magnesium, showed the same "fair" gelatinase activity in the gelatin mixture to which magnesium salt had been added as in its control. It may be concluded then that the salt effects found in the cultures grown in our synthetic medium were due to effects on production of gelatinase in the cultures themselves.

It was thought that possibly the salt effects might be due to autolysis of the bacterial cells. Phenol was added to cultures containing no calcium or magnesium during growth and these killed bacterial mixtures were added to calcium and magnesium salts and allowed to stand several weeks. No indication of enzyme due to autolysis was found.

Further modification of the synthetic medium

It was found that when Proteus was grown in synthetic media in which the amount of glucose and ammonium chloride was reduced to 0.5 per cent, a final pH of 6.8 resulted when calcium and magnesium were present; while in a medium which contained larger amounts of glucose and ammonium chloride a tendency was noted for the final pH of the culture to be lower. A somewhat stronger enzyme was obtained in the cultures grown in the modified synthetic media in which the final reaction was pH 6.8. In this experiment 50 cc. of synthetic media in 1000-cc. Erlenmeyer flasks were inoculated as usual and the results are shown in table 9.

A further change was then made. Lactic acid, neutralized with N sodium hydroxide solution[7] was substituted for glucose in

the synthetic media; with calcium and magnesium salts added this modified medium gave good growth of Proteus and "good" production of gelatinase. The culture became alkaline as the organic acid was decomposed by the bacteria. The effect of

TABLE 9

Effect of decreasing ammonium chloride and glucose in the synthetic medium on bacterial numbers, pH and enzyme activity K

AMMONIUM CHLORIDE	GLUCOSE	FINAL NUMBER OF BACTERIA PER CUBIC CENTIMETER	FINAL pH OF CULTURE	$100 \times K$
per cent	*per cent*			
0.5	0.5	$10^{9.8}$	6.8	0.393
1.0	0.5	10^{10}	6.8	0.333
1.0	1.0	$10^{9.8}$	6.2	0.192

TABLE 10

The effect of calcium and magnesium salts on the final number of bacteria, pH and production of gelatinase by Proteus in lactate synthetic medium

NUMBER	Ca AND Mg	FINAL NUMBER OF BACTERIA PER CUBIC CENTIMETER	FINAL pH OF CULTURE	PRODUCTION OF ENZYME
	grams			
1	0.043 $CaSO_4 \cdot 2H_2O$ 0.025 $MgSO_4$	$10^{9.0}$	7.6	"Good"
2	0.043 $CaSO_4 \cdot 2H_2O$ 0.000 $MgSO_4$	$10^{9.3}$	7.2	"Fair"
3	0.000 $CaSO_4 \cdot 2H_2O$ 0.025 $MgSO_4$	$10^{9.3}$	7.7	"Trace"
4	0.000 $CaSO_4 \cdot 2H_2O$ 0.000 $MgSO_4$ 0.025 Na_2SO_4	$10^{8.5}$	7.0	"None"

calcium and magnesium salts was studied in a series in which 0.5 per cent lactic acid was substituted for glucose in the synthetic medium and 0.5 per cent ammonium chloride was used instead of 1 per cent. Calcium was added as calcium sulfate and in amount equivalent to that used in the first and second series.

The cultures were grown in 50 cc. shallow layers in 1000-cc. Erlenmeyer flasks as in the previous series and the initial pH was 7.0 to 7.1.

Table 10 records the findings at the end of four days' growth. In this series, the growth is the same for the first three cultures and somewhat less in the culture in which no calcium or magnesium is present.[8] The lack of enzyme in no. 3 in which magnesium is present but no calcium is not due to any decrease in numbers of bacteria since the growth is as good as in 1 and 2.

In no. 4, in which no calcium or magnesium is present, the number of bacteria, although somewhat less than in the other cultures, cannot account for the fact that no enzyme was produced as digestion with gelatine was prolonged sufficiently long to give indication of any enzyme present.

The final hydrogen ion concentration of the cultures is not the cause, as at pH 7.6 and 7.7, a good enzyme is produced with calcium and magnesium together and a poor one with magnesium alone. At pH 7.2 and 7.0, a fair enzyme is produced with calcium alone and no enzyme is produced in the culture without either calcium or magnesium.

It appears again that calcium and magnesium together give the strongest enzyme; that calcium alone gives a fair enzyme and that magnesium alone gives a trace of enzyme, while in cultures with neither calcium nor magnesium, no determinable enzyme is produced during the period studied.

After ten days' growth, the alkalinity increased in cultures nos. 1, 2 and 3 to a pH of 8.1 to 8.4. The increasing alkalinity had resulted in a slight decrease in numbers of bacteria from $10^{9.3}$ to $10^{8.5}$ and the enzyme produced had been partially destroyed.

No enzyme was found in the lactate culture without calcium or magnesium, even *after twenty-eight days'* growth.

[8] The error in determination of bacterial numbers is not small, but from consideration of the numbers of bacteria found in cultures grown in the synthetic medium referred to in this paper, one may conclude that the production of gelatinase may be small in spite of large numbers of bacteria. In cultures containing magnesium and no calcium, the number of bacteria was as great as in cultures containing calcium, but in the former case, very little gelatinase was produced compared to the latter.

The amounts of calcium and magnesium salts used in the above experiments were more or less arbitrary and usually resulted in the presence of a solid phase.

A series of Proteus cultures was grown in 50 cc. portions of media containing decreasing amounts of calcium and magnesium salts in the lactate synthetic medium. The cultures were grown in 1000 cc. flasks as before. It was found that "fair" production of gelatinase was obtained in those cultures containing calcium chloride equivalent to as little as 0.0025 per cent $CaCO_3$, and no magnesium salt. No solid phase occurred when 0.005 per cent Ca salt was added alone.

The fact that so little as 0.0025 per cent calcium salts in cultures grown in synthetic medium resulted in a "fair" production of gelatinase, while without calcium salt in the control culture no gelatinase was found, impresses the investigator with the importance of careful cleansing of glassware used in work of this character.

Only a "small" production of gelatinase took place in those cultures containing magnesium sulfate alone, although the strength of the enzyme was the same in those cultures containing 0.0024 per cent magnesium sulfate as in those of 0.05 per cent magnesium salt content. When 0.012 per cent calcium and 0.012 per cent magnesium salts were both present a "good" production of gelatinase was obtained.

With neither calcium nor magnesium salts, no gelatinase was produced in the synthetic medium cultures during the period of observation.

EXPERIMENTS ON ANAEROBIC GROWTH AND PRODUCTION OF GELATINASE BY PROTEUS IN SYNTHETIC MEDIA

Anaerobic cultures of Proteus were grown in 1-liter Florence flasks, filled to the neck with synthetic media. A rubber stopper was inserted, through which two glass tubes allowed nitrogen to bubble until little or no air was left in the flask. This nitrogen had been deoxygenated by passage over reduced copper at 700°. The inlet tube was then closed, the bent end of the outlet passing into mercury which allowed any evolved gases to escape while no air could enter.

In the first series 0.5 per cent lactic acid (neutralized with sodium hydroxide) was substituted for glucose in the synthetic media. Calcium and magnesium salts were present 0.025 per cent each.

In one flask, sodium nitrate was added to make a concentration of 0.25 per cent.

After ten days' incubation, it was found that the bacterial numbers had increased from $10^{2.8}$ to $10^{7.2}$ per cubic centimeter in the culture to which nitrate had been added, while in the cultures containing no nitrate, there was an increase to only $10^{4.0}$ bacteria per cubic centimeter. Also, a fairly strong gelatinase was found in the culture containing nitrate but none in the culture without it. The final pH of both was about 7.0.

Quastel and Stephenson (1925) have shown that Proteus is able to grow anaerobically in their synthetic media. Here, lactic acid was the organic carbon compound, and nitrate was present. Quastel, Stephenson and Whetham (1925) found that certain bacteria ceased to grow when 0.4 per cent nitrite was formed by reduction.

In our experiment fair growth was obtained when only 0.25 per cent nitrate was added. We have shown that a fairly strong gelatinase is produced under these anaerobic conditions with the number of bacteria somewhat below the maximum number, while without nitrate, no enzyme is found.

Another series was then run under anaerobic conditions in which the synthetic medium contained glucose instead of lactate, and calcium and magnesium were added. The bacterial numbers increased to $10^{6.6}$ per cubic centimeter in the culture to which no nitrate was added and a fairly strong gelatinase was found, which shows that glucose without nitrate not only supports anaerobic growth of Proteus but also allows a small production of gelatinase in this synthetic medium under anaerobic conditions.

In a culture grown as above, but with magnesium and no calcium present, no enzyme was found, showing again the more favorable influence of calcium as previously determined under aerobic conditions.

Dernby and Blanc (1921) obtained a proteolytic enzyme in cul-

tures of anaerobes grown in broth to which 1 per cent glucose had been added and a "minimal" dose of calcium sulfide. Our results differ in that a facultative anaerobe was used instead of the obligate spore-forming anaerobe used by Dernby and Blanc, and that our cultures were grown in synthetic media instead of in broth.

The importance of the composition of the medium in which bacteria are grown for the study of the effect of anaerobiosis on the production of gelatinase is shown in our results obtained with synthetic media.

DISCUSSION

The importance of using a synthetic medium in place of peptone-broth for growth of bacteria in investigations dealing with production of gelatinase by bacteria, has been shown in our present work. Synthetic media such as those described in this paper, can be easily duplicated while the composition of peptone-broth varies with the peptone used. We have been able to duplicate our results a number of times in synthetic media but results with peptone-broth are not uniform.

It is of considerable interest that the presence of calcium or magnesium salts in the synthetic media should have such an effect on the production of gelatinase by Proteus. This effect is not due to numbers of bacteria in the cultures as good growth was obtained in presence of magnesium salts with only "small" production of gelatinase, while "fair" or "good" production of gelatinase was obtained in presence of calcium.

Without either calcium or magnesium, the small decrease in growth of the bacteria which took place does not explain the apparent lack of gelatinase produced.

We have shown that the calcium and magnesium salt effects were not due to an effect on the liquefaction of gelatin by gelatinase but on the production of gelatinase in the culture solutions containing the salts.

The reason for the calcium and magnesium effects on production of gelatinase by Proteus is not apparent. It may be due to specific effects on the bacteria during growth. The antagonistic

action of salts is often discussed in regard to results with salts in bacteriological media, but we have no evidence on this point.

In regard to studies of the anaerobic effects on the production of gelatinase by bacteria, we have shown that the composition of the media is of great importance, since Proteus can produce gelatinase under anaerobic conditions, when glucose is present or when small amounts of nitrate are added to a lactate synthetic medium.

SUMMARY

The method for estimation of gelatinase as modified by Clark has been described in detail.

The effects of pH and aeration on production of gelatinase in cultures of Proteus have been studied.

The development of a synthetic medium in which Proteus gives good growth and production of gelatinase has been presented.

The effect of calcium and magnesium salts on the production of gelatinase has been studied and discussed.

The production of gelatinase by Proteus under anaerobic conditions has been successfully obtained in certain synthetic media.

REFERENCES

ABBOTT, A. C., AND GILDERSLEEVE, N. 1903 A study of the proteolytic enzymes and of the so-called hemolysins of some of the common saprophytic bacteria. Jour. Med. Res. **10**, 42.

AUERBACH, W. 1897 Über die ursache der hemmung der gelatine-verflüssigung durch bacterien durch zuckerzusatz. Arch. Hyg., **31**, 311.

BRUNTON, T. L., AND MACFADYEN, A. 1889 The ferment-action of bacteria. Proc. Roy. Soc., **46B**, 542.

CLARK, W. M. 1920 The production and activity of proteus gelatinase in relation to pH. Abst. Bact., **4**, 2.

CLARK, W. M., AND MERRILL, A. T. 1925 Production of gelatinase by proteus. Abst., Bact., **9**, 37.

DELEZENNE, C. 1905 Activation du suc pancreatique par les sels de calcium. Compt. rend. Soc. Biol., **59**, 476.

DELEZENNE, C., AND ZUNZ, E. 1906 Ibid., **60**, 1070.

DERNBY, K. G. 1918 A study on autolysis of animal tissues. Jour. Biol. Chem., **35**, 179.

DERNBY, K. G., AND BLANC, J. 1921 On the growth and the proteolytic enzymes of certain anaerobes. Jour. Bact., **6**, 419.

DIEHL, H. S. 1919 The specificity of bacterial proteolytic enzymes and their formation. Jour. Inf. Dis., **24**, 347.

FALK, I. S. 1923 The rôle of certain ions in bacterial physiology. Abst. Bact., **7**, 33.

FERMI, C. 1892 Weitere untersuchungen über die tryptischen enzyme der mikroörganismen. Arch. f. Hyg., **14**, 1.

JORDAN, E. O. 1906 Experiments with bacterial enzymes. Biol. Studies by the Pupils of W. T. Sedgwick, 124.

LEVINE, M., AND SOPPELAND, L. 1926 Proteolysis by bacteria from creamery wastes. Bull. 82, Engineering Experiment Station, Iowa State College.

LIBORIUS, P. 1886 Beitrage zur kenntnis des sauerstoffbedurfnisses der bacterien. Z. Hyg., **1**, 115.

MARRAS, F. M. 1914 Methoden zum nachweis und zur untersuchung der tryptoproteasen. Centrbl. f. Bakter., Orig., I, **74**, 505.

NORTHROP, J. H. 1922 The mechanism of the influence of acids and alkalies on the digestion of proteins by pepsin or trypsin. Jour. Gen. Physiol., **5**, 263.

PATTEN, H. E., AND JOHNSON, A. J. 1919 The effect of hydrogen ion concentration on the liquefaction of gelatin. Jour. Biol. Chem., **38**, 179.

PALITZSCH, S., AND WALBUM, L. E. 1912 Über die optimale wasserstoffionenkonzentration bei der tryptischen gelatineverflüssigung. Biochem. Z., **47**, 1.

QUASTEL, J. H., AND STEPHENSON, M. 1925 Further observations on the anaerobic growth of bacteria. Biochem. Jour., **19**, 660.

QUASTEL, J. H., STEPHENSON, M., AND WHETHAM, M. D. 1925 Some reactions of resting bacteria in relation to anaerobic growth. Biochem. Jour., **19**, 304.

SOPPELAND, L., AND LEVINE, M. 1924 Effect of salt concentration on bacterial proteolysis. Abst. Bact., **8**, 4.