XXXVII. THE INFLUENCE OF THE MEDIUM ON THE PRODUCTION OF BACTERIAL GELATINASE.

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IN a previous communication [Haines, 1931] it was shown that bacterial proteases are produced by organisms growing in simple synthetic media made up from carefully purified substances, and the influence of salts of calcium and magnesium on growth and protease formation was discussed. The methods used to estimate protease formation, namely, liquefaction of gelatin as evidenced by inability to set in ice-water and lack of coagulation of caseinogen on addition of acid, were however too crude to yield quantitative data as to the relative efficiency of the media in stimulating or inhibiting enzyme formation. In particular, it is to be expected that small amounts of gelatinase will be formed on certain media, insufficient in quantity to cause entire liquefaction, which will escape detection by such a method. More sensitive ways of measurement were therefore sought, and changes in viscosity of a mixture of gelatin and enzyme seemed to offer a ready means of following gelatinase action. Manning [1924] showed that the viscosity of 5 % gelatin could be measured in the ordinary way in an Ostwald viscometer provided that the determinations were carried out at 35° or above. Below 35° the gelatin is in gel form and possesses appreciable rigidity, but at 35° and above it is a sol. The shape of the viscosity-time curve also depends on the previous treatment of the gelatin, but is reproducible if the gelatin be made up under standard conditions.

EXPERIMENTAL.

The organisms used were freshly isolated from dung. In the previous work [1931] the strains of *Proteus* obtained would not grow in synthetic media. One strain has subsequently been isolated, however, which grew well, and the experiments described in this paper have been conducted with it and a strain of *Pseudomonas*. The characteristics of these organisms are listed in Appendix I. In general the organisms were grown on nutrient agar, p_H 7.4, overnight at 20°, the surface growth was scraped off, and washed and centrifuged in sterile saline three times. 1 cc. of the final suspension was used for inoculation purposes. The routine technique was the same as described in the previous paper, all substances being purified in a similar manner and carefully cleaned

pyrex glass-ware and ammonia-free re-distilled water used throughout. All media were made up by dilution from separate sterile solutions of their constituents. The gelatin was 5 % Gold Label in phosphate buffer of the required p_H , never more than half an hour's heating on the water-bath at not above 60° being employed. 0.5 % phenol was added and the filtered product stored in 12 cc. lots in tubes closed with corks coated with tin-foil. Cotton-wool had to be dispensed with owing to the trouble caused by small fibres finding their way into the viscometer. Blank viscosity determinations with a mixture of sterile medium and gelatin gave the type of curve shown in Fig. 1, constant for some hours and then decreasing slightly. To test gelatinase formation, the medium in which the organism had been grown was centrifuged at high speed for three quarters of an hour. By this means nearly all the organisms were removed, and the necessity of filtration through a Chamberland candle, experimentally tedious when a number of media are to be compared simultaneously, was avoided. Test experiments showed that the mixture of centrifuged medium and phenol-gelatin remained sterile. Comparison between filtered and unfiltered centrifugates also indicated that the few, if any, organisms remaining in the unfiltered preparations caused no increase in the digestion of the gelatin. In general, 5 cc. of the centrifugate were added to 10 cc. melted phenol-gelatin, both having been previously brought to 37° in the incubator, and 10 cc. of the well shaken mixture pipetted immediately into a clean dry Ostwald viscometer suspended in a water-bath at $37^\circ \pm 0.1^\circ$. A few minutes were allowed for the mixture to regain a temperature of 37° , because trial experiments showed that although both solutions were at 37° prior to mixing, and pipettes and viscometers had been stored at that temperature before use, a lowering of $2-3^\circ$ was inevitable during mixing. A first reading was then taken and others at half-hour intervals. Duplicate readings usually agreed to a fraction of a second, but the readings were taken to the nearest second. In the case of a very active broth centrifugate, however, the change at the beginning was so rapid that duplication of the initial reading was impossible with viscometers giving readings of the order of 90–150 seconds. Comparative experiments with several viscometers however gave identical results. All readings are expressed as percentages of the initial reading, thus cancelling variation from viscometer to viscometer. Relative rate of change of viscosity only has been measured and not absolute viscosity. Using four different viscometers and the same centrifugate, the four sets of figures nowhere differed by more than 2 %, and a change of viscosity of the order of 50 % is being measured with a broth culture. This is shown in Fig. 1, where the time for the rate of fall of the gelatin-enzyme mixture between the graduations on the viscometer, expressed as a percentage of the initial reading, is plotted against time of incubation. The control readings with gelatin and sterile broth are also shown.

Fig. 2. Effect of p_H .

Fig. 1. Comparison of viscometers and broth control.

Gelatinase formation in broth.

Preliminary experiments were first carried out with the organism growing in nutrient broth, p_H 7.4, in order to follow the influence on gelatinase production of such factors as the age of the culture and the p_H of the digestion mixture.

The optimum p_H for gelatinase action. Batches of phenol-gelatin were made up in suitable buffers of p_H 5, 7.5, 8 and 9 approximately, checked by the colorimetric method. The centrifugate from a 94-hour culture of the *Proteus* organism was then added to each and the change in viscosity determined. The results are shown graphically in Fig. 2. It will be seen that the greatest change took place at p_H 8 and the least at p_H 5. The optimum p_H for the action of the gelatinase is thus 8, and all subsequent digestions were carried out at that p_H . This finding is in agreement with that of Merrill and Clark [1928] who also worked with *Proteus* bacteria and estimated the gelatinase activity by the time required for setting under standard conditions.

The age of the culture. 500 cc. of nutrient broth p_H 7.4 were inoculated with a single loopful of the *Proteus* organism in a litre pyrex flask and incubated at 20° with continued shaking. From time to time samples were withdrawn and gelatinase activity estimated, during the period 2–38 days. Plate counts of the rate of multiplication of the organisms were also carried out. The curves representing gelatinase activity are shown in Fig. 3. In order to obtain a numerical basis for comparison, the time for a 25 % change in viscosity has been found from each curve, and the reciprocal of this multiplied by ten, together with the corresponding plate counts, is shown in Table I. A similar experiment was carried out with the *Pseudomonas*, except that in this case 1 cc. of the suspension of the washed organism was inoculated into the broth.

Table I. *Growth and gelatinase formation in broth.*

t = time for 25 % change in viscosity.

<i>Proteus</i>				<i>Pseudomonas</i>			
Time in hrs.	Plate count Orgs./cc.	Time in hrs.	Gelatinase $1/t \times 10$	Time in hrs.	Plate count Orgs./cc.	Time in hrs.	Gelatinase $1/t \times 10$
0	95,000	46	3	0	1.7×10^6	20	<0.5
22	230×10^6	70	7	24	60×10^6	42	8
42	1060×10^6	94	14	49	1300×10^6	90	30
66	1400×10^6	118	14	73	4700×10^6	113	50
115	4200×10^6	142	20	97	7600×10^6	136	50
167	4600×10^6	214	25	119	5200×10^6	184	58
		360	33				
		574	33				
		912	33				

It will be seen that with the *Proteus* the gelatinase activity of the centrifugate increased rapidly up to about 4 days, after which it increased slowly until a limit was reached at about 14 days. The numbers of organisms, on the other hand, did not increase greatly after about 5 days. From 14 to 38 days

Fig. 4. Effect of age of culture. Clark's medium. *Pseudomonas*. Fig. 3. Effect of age of culture. Broth. *Proteus*.

the gelatinase activity remained approximately constant, after which no further measurements were made. The rate of multiplication of the *Pseudomonas* was of a similar order, but a more active gelatinase was obtained than from the former organism, and it was apparently formed somewhat more rapidly.

Gelatinase formation in synthetic media.

The media of Merrill and Clark and a slight modification of that of Fränkel, as listed in the previous paper, have been used, except that sodium lactate was substituted for ammonium lactate in Fränkel's medium. A modified Fränkel's medium in which glycerol was substituted for sodium lactate was found to yield as good growth as the original medium and was considerably less troublesome to prepare. This medium is referred to as Fränkel A, and it had the following composition:

10 cc. 4 % glycerol.
10 cc. 1 % solution of pure amino-acid.
2 cc. 2 % KH_2PO_4 .
2 cc. 5 % NaCl .

p_{H} adjusted to 7.4 with NaOH , and 1 cc. of 0.1 % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 1 cc. of a suspension of 0.1 % CaCO_3 added where required.

Gelatinase formation has been measured in these media using varying sources of nitrogen. As a rule the first subculture and some subsequent one such as the third or sixth in any given medium have been tested, for there seems to be a general tendency for slightly more rapid growth and rather better gelatinase formation with repeated subculture. It is unlikely, therefore, that despite the washing with saline, anything has been carried over from the original agar culture, because in that case gelatinase activity would be expected to be greater at first and then to decrease on subculture. While it was not possible to carry out plate counts in every case, care has been taken to avoid misinterpretations of gelatinase activity owing to poor growth, the final growth having been estimated by turbidity measurements against BaSO_4 standards, made up in the manner described by Peskett [1927].

Gelatinase formation in Merrill and Clark's medium.

Gelatinase formation in Merrill and Clark's simple medium with NH_4Cl as the source of nitrogen and with calcium and magnesium salts present was first followed. 1 cc. of a three times washed suspension of the *Pseudomonas* organism in saline was added to 250 cc. medium in a 500 cc. Erlenmeyer pyrex flask, and approximately 10 cc. lots were withdrawn at intervals and gelatinase activity was estimated. Control experiments were carried out with gelatin *plus* sterile medium containing Ca and Mg salts. The curves so obtained were similar to that for gelatin *plus* sterile broth shown in Fig. 1, and are shown in Figs. 5 and 6. Plate counts were also carried out. These counts are shown in Table II together with the turbidity measurements and the figure obtained for 25 % change in viscosity. The complete curves are shown in Fig. 4.

Fig. 6. Gelatinase production in Merrill and Clark's medium.
Third subculture 9 days. *Proteus*.

Fig. 5. Gelatinase production in Merrill and Clark's medium.
First subculture 6 days. *Proteus*.

Table II. *Gelatinase formation in Merrill and Clark's medium at 20° with Pseudomonas, in the presence of Ca and Mg salts.*

Time in hrs.	Plate count Orgs./cc.	Turbidity	Time in hrs.	Gelatinase 1/t × 10
0	2.5×10^6	< <i>M</i> /1000	20	< 0.5
24	8×10^6	< <i>M</i> /1000	42	< 0.5
49	71×10^6	<i>M</i> /400	66	36
73	520×10^6	<i>M</i> /320	90	36
97	870×10^6	<i>M</i> /200	113	40
119	1700×10^6	<i>M</i> /80	136	44
			184	52
			208	52

Turbidity was measured in terms of molar K_2SO_4 used in making up the standards. Thus *M*/80 signifies that one-eightieth molar K_2SO_4 solution was added to excess $BaCl_2$ solution. 10 cc. lots were used in sealed comparator tubes [see Peskett, 1927]. The method is not accurate with these bacteria owing to the difficulty of comparison between the fine bacterial suspension and the more coarsely granular $BaSO_4$. It served, however, as a useful basis of comparison, more accurate than the loose terms "good" or "poor" growth. Approximate calibration is provided for *Pseudomonas* by the figures of Table II; results with *Proteus* were of a similar order.

It will be seen that both growth and production of gelatinase, though slower in the synthetic medium, were eventually of a similar order to those obtained in broth culture. Preliminary experiments with both *Proteus* and *Pseudomonas* organisms in the presence and absence of salts of calcium and magnesium indicated that although greater production of gelatinase took place in the appropriate medium with *Pseudomonas*, yet this organism was more rigid in its requirements than *Proteus*. The formation of gelatinase in the absence of calcium and magnesium salts with the former organism, using NH_4Cl or certain amino-acids, was very much less than with *Proteus*, growth also being very poor. *Proteus* gave measurable amounts of gelatinase and fair growth with a wide range of sources of nitrogen, while the *Pseudomonas* seemed definitely to yield better results with the simplest medium containing NH_4Cl . For that reason *Proteus* was chosen as the more interesting organism for the majority of the experiments, and in the following examples it is to be understood that that organism is referred to unless it is expressly stated otherwise.

The influence of salts of calcium and magnesium on gelatinase production in Merrill and Clark's medium.

The results obtained from centrifugates of Merrill and Clark's medium with both calcium and magnesium salts present, and with each salt separately and neither salt, in the first and third subcultures of the *Proteus* are shown graphically in Figs. 5 and 6. The numerical figures in terms of 25 % change in viscosity, and the growth as estimated by turbidity, are given in Table IV for first, third and sixth subcultures. It will be seen that good growth and the

best gelatinase production occurred in the presence of both salts. Nearly as good gelatinase production was obtained with calcium salts alone, although the growth was much less. With magnesium salts alone growth was as good as with both calcium and magnesium and much better than with calcium salts alone or with neither, and yet gelatinase production was much less than with the former and appreciably less than with the latter. In order to clinch this point, counts were carried out in a similar manner to those of Table II, with *Pseudomonas* instead of *Proteus* as used above, growing in Merrill and Clark's medium with magnesium salts only, so as to make the figures comparable. The results are shown in Table III.

Table III. *Growth and gelatinase formation in Merrill and Clark's medium at 20° with Pseudomonas, in the presence of Mg salts only.*

Time in hrs.	Plate count Orgs./cc.	Time in hrs.	Gelatinase
0	10×10^6	22	<0.5
19	57×10^6	172	<0.5
72	1020×10^6	264	<0.5

It will be seen that the rate of multiplication is essentially similar in the two media, but gelatinase formation very much less in the latter one. Magnesium salts therefore have a stimulating influence on growth but an inhibitory action

Fig. 7. Gelatinase production in Fränkel's medium. *Proteus*.

on gelatinase formation. Calcium salts have no stimulating action on growth but stimulate gelatinase production. The two salts together stimulate both growth and gelatinase production. It appears that the salt content of the medium is of much greater importance in determining the gelatinase production than the extent of growth of the organisms. While slight variation occurred in the results from subculture to subculture they are essentially similar in every case.

Gelatinase production in Fränkel's medium.

Fig. 7 represents the results of an experiment in Fränkel's medium with and without added salts. In this case salts of magnesium again stimulate growth (see Table IV) but although multiplication of the organisms is much

Table IV. *Gelatinase activity of the centrifugate, obtained from viscosity curves, = $1/t \times 10$ where t = time for 25 % change.*

<i>Proteus</i> organism.						
Medium	Source of nitrogen	Added salts	No. of subculture	Age of subculture days	Growth	Gelatinase $1/t \times 10$
Clark	NH ₄ Cl	None	1	6	M/400	4
"	"	Mg	1	6	M/80	2
"	"	Ca	1	6	M/800	12
"	"	Ca + Mg	1	6	M/80	17
"	"	None	3	9	M/800	5
"	"	Mg	3	9	M/80	1
"	"	Ca + Mg	3	9	M/80	20
"	"	Ca	3	6	M/800	14
"	"	None	6	11	M/800	3
"	"	Mg	6	11	> M/80	1
"	"	Ca	6	10	M/800	14
"	"	Ca + Mg	6	11	> M/80	13
Fränkel	Asparagine	None	1	7	M/400	4
"	"	Mg	1	7	M/80	4
"	"	Ca	1	7	M/400	12
"	"	Ca + Mg	1	7	M/80	22
"	"	None	4	9	M/200	5
"	"	Mg	4	7	M/80	6
"	"	Ca	5	9	M/80	17
"	"	Ca + Mg	5	9	M/80	18
Fränkel A	Glycine	None	1	8	M/400	3
"	"	Mg	1	7	M/80	3
"	"	Ca	1	5	M/200	2
"	"	Ca + Mg	3	7	M/80	14
"	"	None	3	7	M/400	6
"	"	Mg	3	6	M/320	1
"	"	Ca	3	6	M/400	2
"	"	Mg	2	5	M/80	3
"	"	Ca + Mg	4	10	M/80	17
"	Alanine	None	1	8	M/400	1
"	"	"	3	7	M/400	5
"	"	Ca + Mg	1	4	M/80	17
"	"	Ca + Mg	3	17	M/80	25
"	Arginine	None	1	6	M/400	< 0.5
"	"	Ca + Mg	1	4	M/200	6
"	"	Ca + Mg	3	13	M/80	13
"	Glutamic acid	None	1	6	M/200	3
"	"	Ca + Mg	1	4	M/80	14
"	Tyrosine	None	1	8	??	< 0.5
"	"	Ca + Mg	1	7	?	2

* It was impossible to estimate the turbidity in the presence of tyrosine owing to undissolved substance, but growth was slight.

Table IV (*cont.*).

Medium	Source of nitrogen	<i>Pseudomonas</i> organism.		Age of subculture days	Growth	Gelatinase 1/t × 10
		Added salts	No. of subculture			
Clark	NH ₄ Cl	None	1	4	M/1000	<0.5
"	"	Mg	1	4	M/80	<0.5
"	"	Ca	1	4	M/1000	<0.5
"	"	Ca + Mg	1	4	M/80	33
"	"	None	2	19	M/800	<0.5
"	"	Ca + Mg	2	19	> M/80	33
"	"	None	3	7	M/1000	<0.5
"	"	Mg	3	7	M/80	<0.5
"	"	Ca	3	7	M/1000	<0.5
"	"	Ca + Mg	4	8	> M/80	35
Fränkel A	Asparagine	None	1	3	M/800	<0.5
"	"	Ca	1	3	M/800	<0.5
"	"	Mg	1	3	M/80	<0.5
"	"	Ca + Mg	1	3	M/200	4
"	"	None	3	7	M/1000	<0.5
"	"	Ca	3	7	M/1000	<0.5
"	"	Mg	3	7	M/80	<0.5
"	"	Ca + Mg	3	7	M/80	10
"	Glycine	None	1	4	M/1000	<0.5
"	"	Ca	1	4	M/1000	<0.5
"	"	Mg	1	4	M/200	<0.5
"	"	Ca + Mg	1	4	M/200	10
"	"	None	3	6	M/1000	<0.5
"	"	Ca	3	6	M/1000	<0.5
"	"	Mg	3	6	> M/80	<0.5
"	"	Ca + Mg	3	6	M/80	28

greater in Fränkel's medium *plus* magnesium salts than in this medium alone, gelatinase formation is almost identical in the two cases. With calcium salts growth was much less than with either magnesium, or calcium *plus* magnesium salts, yet gelatinase production was greater than in the unaltered medium or medium *plus* magnesium salts, and nearly the same as in the medium with both salts. Similar results were obtained from subsequent subcultures, and these are given in Table IV.

In Fränkel's medium, therefore, magnesium salts stimulate growth but have no action, either stimulative or inhibitive, on gelatinase production, but salts of calcium, while not affecting growth, stimulate the formation of gelatinase. As in other media, the greatest amount of gelatinase is yielded in the presence of both salts.

The production of gelatinase in other media.

Other sources of nitrogen have been substituted for asparagine in the medium Fränkel A and complete curves obtained representing a measure of the quantity of gelatinase produced. The figures found from these curves for 25 % change in viscosity of the gelatin-enzyme mixture are grouped together in Table IV.

DISCUSSION.

It will be seen that a certain amount of gelatinase is detectable whatever the medium used, when sufficient growth has occurred. The setting-power of the gelatin is not a reliable criterion of gelatinase production, because unless sufficient enzyme has been formed to cause a change of 40–50 % in the viscosity the gelatin will still set when immersed in ice-water. It has been found that within this range the ability to set is somewhat indefinite. For example, a sample whose viscosity was 46 % of the original value set, while one with viscosity of 48 % of initial value did not. Possibly the added salts exerted an influence here. In a medium containing no added salts it makes little difference whether the source of nitrogen be ammonium chloride or any of the amino-acids used, except in so far as rather better growth is obtained with some amino-acids than others. The figures obtained for gelatinase production are not quite so good with tyrosine, arginine and alanine as with asparagine, glutamic acid, glycine and ammonium chloride, but a more accurate measure of growth than the turbidity tests would probably account for these relatively small differences. In every case, however, in a synthetic medium salts of calcium and magnesium together greatly stimulate formation of gelatinase, whatever the source of nitrogen. With ammonium chloride as the source of nitrogen, salts of magnesium alone seem partially to inhibit enzyme production, although at the same time stimulating growth, while salts of calcium alone, having no influence on growth, stimulate gelatinase formation. This effect is very marked, excellent growth with little gelatinase being obtained with the magnesium salts, and only medium or poor growth and good gelatinase with the calcium salts. Moreover, the production of enzyme in the medium containing the magnesium salt and giving excellent growth was less than that in the medium containing no added salts and giving moderate growth. While the factor of the amount of growth taking place must not be lost sight of, emphasis must be laid on the salt content of the medium as of fundamental importance in determining the amount of gelatinase occurring in it. The concentration of the salts causing these marked effects is of the order of 0.01–0.004 % as so far investigated; it remains to be seen what effect increasing this concentration would have. Also, while no marked diminution in the ability to produce enzyme in the synthetic medium with subculture has yet been noted, it must be remembered that the washed organism has so far been carried through only about six subcultures. Freshly isolated cultures seem to yield more active gelatinase preparations than those which have been kept on ordinary nutrient agar for some time, so that further experiment may show a similar effect in synthetic media. With asparagine as source of nitrogen magnesium salts stimulate growth but not enzyme production, while calcium salts again affect the latter without influencing the former. With glycine, however, neither calcium nor magnesium singly affects the gelatinase although magnesium salts here again stimulate growth, but the two salts combined exhibit their

usual effect. The reasons for the varying influence of the single salts and yet the almost constant effect of their combination, with different sources of nitrogen, are not at present apparent. Explanation must be sought, however, in some action on the bacterial cell during growth. An experiment was tried in which 0.1 cc. of the suspension of calcium carbonate was added to a centrifugate from a culture of Clark's medium *plus* magnesium salts. The curves obtained both with and without addition of calcium salts were identical and similar to the top curve of Fig. 6. That is, salts of calcium added subsequently had no activating influence on the gelatinase produced previously in the medium containing magnesium salts.

SUMMARY.

1. By following the change in viscosity of a mixture of gelatin and sterile centrifugate from the medium in which the organism had been grown, a certain amount of gelatinase was demonstrated whatever the medium used, providing adequate growth occurred.

2. In a synthetic medium containing no magnesium or calcium salts, no single amino-acid was markedly superior to any other or to ammonium chloride in its effect on gelatinase production, except in so far as rather better growth was obtained with some than others.

3. In all cases salts of calcium and magnesium together greatly stimulate the production of gelatinase.

4. In some cases salts of magnesium alone, while stimulating growth, partly inhibit gelatinase formation. Salts of calcium alone have little or no growth-stimulating action but in most cases greatly stimulate gelatinase production.

5. In a medium containing ammonium chloride, glucose and phosphates, together with salts of calcium and magnesium, gelatinase is produced in amount comparable with that obtained in broth culture.

6. The explanation of the function of these salts must be sought in some action on the cells apart from stimulation of growth or activation of the enzyme.

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REFERENCES.

- Haines (1931). *Biochem. J.* **25**, 1149.
Manning (1924). *Biochem. J.* **18**, 1085.
Merrill and Clark (1928). *J. Bact.* **15**, 267.
Peskett (1927). *Biochem. J.* **21**, 460.

APPENDIX. *Cultural characteristics of the organisms used.*

<i>Proteus. Motile Gram-negative rod</i>	<i>Pseudomonas. Motile Gram-negative rod</i>
Agar—Raised, smooth, white to cream	Moist, raised, cream growth, bright green fluorescence
Gelatin—Crater liquefaction, becoming stratiform	Crater liquefaction
Potato—Moist, raised, cream to brown	Spreading, dirty cream to brown
Litmus milk—Alkaline, peptonised	Alkaline, peptonised
Broth—Pellicle and sediment	Heavy turbidity
Nitrates—Reduced	Reduced
Indole—Doubtful	Not formed
Lactose—Not fermented	Not acid
Maltose—Acid and gas	Not acid
Sucrose—Acid and gas	Acid, no gas
Dextrose—Acid and gas	Acid, no gas
Mannitol—Acid and gas	Not acid
Galactose—Acid, no gas	Acid