CXCIX. THE FORMATION OF BACTERIAL PROTEASES, ESPECIALLY IN SYNTHETIC MEDIA.

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BRUNTON AND MACFADYEN [1889] showed that broth cultures of bacteria, moderately heated to kill the organisms, could bring about digestion of proteins. However, Fermi [1890] was apparently the first conclusively to demonstrate that an active proteolytic enzyme can be obtained from a sterile cell-free extract of the medium in which certain bacteria have been grown. Since that time difference of opinion has existed as to whether it is essential for the elaboration of such enzymes to have present in the medium proteins or protein derivatives. Some authors have reported the presence of enzymes after the growth of bacteria in synthetic media containing amino-acids only, or in some cases ammonium salts only, as the source of nitrogen. Others, on the other hand, have maintained that "organic" nitrogen in the form of peptone or some other complex substance, is required for protease formation. Much of the earlier work seems unreliable when viewed from a modern standpoint, since

(a) the concept of p_H , its application to bacteriology and its fundamental importance in enzymic activity had not been elaborated;

(b) the significance of vitamins in nutrition was unknown and discordant results with synthetic media may have been due to their presence in the amino-acids or other substances used;

(c) the organisms were not washed and consequently organic matter was probably carried over from the stock culture to the synthetic medium.

Bainbridge [1911] showed that pure native proteins are not attacked by bacteria and his results were confirmed and enlarged by Sperry and Rettger [1915]. Diehl [1919] stated that amino-acids are required in the medium for the elaboration of enzymes and he reported a curious specificity according to the amino-acid present. Merrill and Clark [1928], however, found that they obtained gelatinase from organisms growing in a synthetic medium of carefully purified salts, with ammonium chloride as the source of nitrogen, provided that salts of calcium and magnesium were present. No gelatinase was obtained in the absence of these salts, despite good growth. Wilson [1930] could not confirm Diehl's specificity. She criticised Merrill and Clark's results on the ground that poor growth only was obtained in the absence of salts of calcium

and magnesium and concluded that these salts are necessary for the elaboration of enzymes only in so far as they stimulate growth.

Numerous points remain to be settled. It is not evident from Bainbridge's work whether failure of the organism to grow was due to inability to utilise pure protein as a source of nitrogen or to lack of some other essential constituent such as the salts mentioned above. Non-decomposition of the protein may have been due as much to the inability of the enzyme to attack the protein, owing to high dilution or other non-optimal conditions, as to failure of the organism to produce enzyme. It is essential to guard against misinterpreting effects which may be due merely to dilution. For example, in the course of the present work it has been found that by diluting an enzyme preparation which formerly attacked two proteins, it may be made to attack only the one. If this latter dilution had been arbitrarily selected in the first instance a case of specificity might have been assumed. Again, if the proteolytic enzymes are concerned in the synthetic processes of the cell as well as in the degradation processes, it is to be expected that these enzymes will be formed whenever good growth occurs. In view of the conflicting views advanced, this latter point requires further investigation, and it is the main purpose of the present paper to attempt to settle it. It was necessary first of all to make a preliminary study of the formation of proteases in broth culture by the organisms selected (*i.e.* under optimum conditions) by way of control. The work was then extended, as far as possible with the same organisms, to synthetic media of known composition.

PROTEASE FORMATION IN BROTH.

Methods.

A single loopful of an overnight culture of the organism to be studied was inoculated into several hundred cc. of nutrient broth, p_H 7.4, spread in a thin layer in a large Fernbach flask. After incubation, during which it had been repeatedly shaken to ensure aeration of the culture, the broth was centrifuged at high speed for 30 minutes and sterilised by filtration through a Chamberland candle. As the result of preliminary tests 2–5 cc. lots of the filtrate were transferred to tubes containing 5–10 cc. of gelatin, caseinogen or egg-albumin solution. These were then incubated for several days and digestion estimated by the methods outlined below. The gelatin was made up according to the method of Wilson [1930] by dissolving 5 g. gold label gelatin in 100 cc. phosphate buffer on a water-bath at 60°, adding phenol to 0.5 % and adjusting the final p_H at 7.4. An approximately 1 % caseinogen solution was prepared by well stirring the required amount with phosphate buffer containing 0.9 % sodium chloride, filtering and adding thymol solution to 1 %, the final p_H again being 7.4. Egg-albumin was obtained from fresh eggs by the modified method of Hopkins used by Adair and Robinson [1930] and twice crystallised. It was dialysed in collodion sacs until a negative test for sulphates was obtained in the liquid outside the sac. Liquefaction of the gelatin was tested by immersing

the tubes direct from the incubator in ice-water, and comparing the time required for setting with that of a control tube. Caseinogen digestion was indicated by absence of precipitate or turbidity on adding 0.4 cc. of alcoholic acetic acid (Diehl). Controls were here again carried out. Digestion of egg-albumin was estimated by adjusting the p_H of the solution approximately to the isoelectric point, coagulating by immersing for five minutes in boiling water, filtering either through Jena sintered glass filters or washed and tared filter-papers, drying at 100° for 4 hours and weighing.

Results.

Table I is an example of the type of result obtained with broth cultures of the organisms listed.

Table I.

Organism	Gelatin		Caseinogen		Egg-albumin	
	Blank	Exp.	Blank	Exp.	Blank g.	Exp. g.
<i>Proteus 3</i>	set 3 mins.	not set 24 hrs.	0	++	0.100	0.097
<i>B. mesentericus</i>	„ 3 „	„ „	0	++	0.098	0.084
<i>Pseudomonas 1</i>	„ 4 „	„ „	0	++	0.098	0.100
<i>Pseudomonas 2</i>	„ 3 „	„ „	0	++	0.088	0.077

In the first gelatin column is given the time of setting of the same mixture of enzyme and gelatin as in the second column, but heated to 100° for 10 min. In the egg-albumin column the mean weights of the respective coagula are given. In this and following tables these conventions are used for digestion of caseinogen: 0 = no difference from blank; * = doubtful; + = partial digestion; ++ = complete digestion.

The organism *Proteus 3* was a stock culture from the London School of Hygiene¹ and thought to be Felix and Weil's x 19 strain. *Bacillus mesentericus* was a stock laboratory strain from the Pathological Laboratory, Cambridge². *Pseudomonas 1* was isolated by the author from an abattoir but had been kept in culture for some months, and *Pseudomonas 2* was freshly isolated from cow-dung.

It will be seen that although sterile filtrates from all the above organisms liquefied gelatin and digested caseinogen beyond the acid-coagulable stage, filtrates from *Proteus 3* and *Pseudomonas 1* were without action on the native egg-albumin. Slight digestion beyond the heat-coagulable stage was apparently obtained with *B. mesentericus* and *Pseudomonas 2*. The latter organism was freshly isolated and sown immediately into broth, because it was thought that non-digestion of the albumin might be due to the fact that the stock strains had lost much of their vigour on repeated subculture. That this was not the case is evident from Table II in which it is seen that *Pseudomonas 2* produced a gelatinase so active that 0.01 cc. of filtrate liquefied 5 cc. of gelatin, while equal volumes of filtrate and albumin solution resulted in only the slight decomposition of albumin shown in Table I. Gelatin is apparently more easily attacked than caseinogen, since the higher dilutions which still liquefied gelatin were without action on the caseinogen.

¹ Supplied by courtesy of Dr Anderson.

² Supplied by courtesy of Dr Graham-Smith.

Table II. *Effect of dilution.**Pseudomonas 2* grown for 8 days at 20°, filtrate incubated for 3 days at 37°.

Volume of filtrate added to 5 cc. gelatin or caseinogen cc.	Gelatin		Caseinogen	
	Blank	Exp.	Blank	Exp.
	set 3 mins.	not set 24 hrs.		
0.01			0	0
0.05	" "	" "	0	*
0.10	" "	" "	0	+
1.00	" "	" "	0	++
2.00	" "	" "	0	++

Apparently, therefore, little or no decomposition of pure native albumin beyond the heat-coagulable stage occurred after incubation with filtrates containing a very active gelatinase and a moderately active casease. Failure to decompose the albumin can hardly in this instance be explained on the basis of specificity of amino-acids in the food supply, for there are presumably present in meat-extract peptone broth all the amino-acids required. Several possibilities present themselves, but further experimental evidence with better methods is desirable. Since the main object of this paper was the study of protease formation in synthetic media, the point has not at present been pursued further.

PROTEASE FORMATION IN SYNTHETIC MEDIA.

Methods.

In the main Merrill and Clark's simple medium [1928], the media of Diehl [1919] and Fränkel [1894], or slight modifications of them, have been used. The exact composition of these media as used is listed in the Appendix. Pyrex glassware was used throughout. It was cleaned carefully by standing for 24 hours under strong chromic acid, 24 hours under strong alkali, several days under distilled water repeatedly changed and was finally autoclaved at two atmospheres pressure for one hour. Ammonia-free redistilled water was used exclusively. All salts, being of A.R. standard to begin with, were recrystallised two or three times. Glycerol and lactic acid were distilled once *in vacuo*. Glucose was crystallised three times from a mixture of alcohol and water. Good samples of crystalline asparagine and glycine were recrystallised from water. In the case of Merrill and Clark's and Fränkel's media, stock solutions of each of the constituents were made up separately in sterile water and finally sterilised by steaming once for 15 minutes. The sterilisation of the mixture in the final medium, which invariably resulted in decomposition of the glucose, was thus avoided, the mixture being made up by dilution from stock solutions with aseptic precautions. In all cases the final p_H of the medium was adjusted prior to inoculation to 7.4 approximately by the colorimetric method, using $N/5$ NaOH stored in a waxed bottle fitted with delivery burette. The organisms were grown on the surface of agar plates at the appropriate temperature overnight. The surface growth was then carefully scraped off with a sterile platinum needle, taking care not to break into the agar, and suspended in saline. After

Table III.

Organism	Medium																																	
	Clark with calcium and magnesium							Clark without calcium or magnesium							Clark with calcium only						Clark with magnesium only						Diehl							
	1	2	3	4	5	6	10	1	2	3	4	5	6	10	1	2	3	4	6	10	1	2	3	4	5	6	10	1	2	3	4	5	6	10
<i>Proteus 1</i>	+	*	0	.	.	.	.	+	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	0	.	.	.	.	.
<i>Proteus 2</i>	++	*	0	.	.	.	.	++	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	*	0	.	.	.	.	.
<i>Proteus 3</i>	+	*	0	.	.	.	.	+	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	*	0	.	.	.	.	.
" <i>Proteus 4</i> "	+++	+++	+++	+++	+++	+++	+++	++	+	+	+	*	.	.	.	.	.	.	.	.	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
<i>B. mesentericus</i>	20°	+++	+++	+++	+++	+++	+++	+	0	.	.	.	.	.	.	.	.	.	.	.	+++	+++	+++	+++	+++	+++	+++	+	.	.	.	.	.	.
	30°	+++	+++	+++	+++	+++	+++	+	+	.	.	.	.	.	+	+	+	*	.	.	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	37°	+++	+++	+++	+++	+++	+++	++	+	*	0	.	.	.	.	.	.	.	.	.	+++	+++	+++	+++	+++	+++	+++	.	.	.	.	.	.	.
<i>Pseudomonas 1</i>	+++	+++	+++	+++	+++	+++	+++	++	+	++	+	+	*	.	++	++	+	+	*	.	+++	+++	+++	+++	+++	+++	++	+	++	+++	++	+++	+++	+++
<i>Pseudomonas 2</i>	+++	+++	+++	+++	+++	+++	+++	++	++	+	+	+	*	.	++	++	++	++	+	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Fränkel																																		
Fränkel with calcium and magnesium																																		
Fränkel with calcium																																		
Fränkel with magnesium																																		
<i>Pseudomonas 1</i>	+++	+++	+++	+++	+++	+++	+++	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
<i>Pseudomonas 2</i>	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	++	++	+++	+++	+++	+++	+++	+++	+++	+++	.	.	.	.	.	.	.	.
<i>B. mesentericus</i>	20°	+++	+++	+++	+++	+++	+++	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	30°	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	.	.	.	.	.	.	+++	+++	+++	+++	+++	+++	+	.	.	.	.	.	.	.
	37°	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	++	+++	++	++	+++	+++	+++	+++	+++	+++	+++	+++	.	.	.	.	.	.	.	.

Convention used: +++ = Very heavily turbid. Luxuriant growth. ++ = Heavily turbid. + = Definitely turbid. * = Doubtful. 0 = Negative, no growth.

Numbers at the head of the columns refer to the number of the subculture.

centrifuging at high speed for 30 minutes the deposit was ground up in a fresh quantity of sterile saline and the centrifuging repeated. In all, at least 3 washings were carried out. 1–2 cc. of the final suspension of about 50 cc. were inoculated into the various media. From 20 to 50 cc. of culture fluid were used in a thin layer on the bottom of 250–500 cc. Erlenmeyer flasks in order to ensure adequate aeration. Incubation was in general carried on from 2–5 days, for although the final growth was in many cases as luxuriant as in broth culture, the rate of growth was noticeably slower at the beginning. Doubtful cases were incubated for a week or more. At least ten subcultures were made to show that the organism was capable of continued growth in the synthetic medium. As a rule those organisms which could not flourish in the medium chosen died out at about the third subculture. Protease formation was measured in a similar manner to that used for broth cultures. Egg-albumin was dispensed with since the broth controls of the organism had given so little breakdown.

Results of experiments on growth in synthetic media.

The results of the experiments to determine the viability of the organisms in the respective media are summarised in Table III. *Proteus 1* was a strain of *P. vulgaris* obtained from the National Collection of Type Cultures, *Proteus 2* was isolated by the author from putrid ox-liver, *Proteus 3* was obtained as mentioned previously from the London School of Hygiene and was probably Felix and Weil's *x 19* strain. "*Proteus 4*" was stated by one authority to be a true *Proteus*, by another to be a *Zopfius*.

It will be seen that the *Proteus* strains 1, 2 and 3 all consistently failed to grow in any of the synthetic media used. Slightly better growth was obtained in a medium containing the following unpurified amino-acids: glycine, alanine, asparagine, tryptophan, histidine, cysteine, cystine and tyrosine. Even here, however, growth failed after 4–5 subcultures. The passage of oxygen through the culture also had a stimulating influence but failed to maintain continued growth. "*Proteus 4*" (*Zopfius*?) on the other hand grew well in Clark's and Diehl's media, provided salts of magnesium were present. In this connection it is interesting to note that Merrill and Clark [1928] reported good growth of "*Proteus bacteria*" in their synthetic media, whilst Wilson [1930] and Koser and Rettger [1919] obtained negative results in a variety of media. It is possible, but not yet proven, that certain strains of true *Proteus* organisms belong to the group which Hosoya and Kuroya [1923] showed would not grow without the presence of vitamin B. A broth control experiment indicated that protease formation with "*Proteus 4*" was feeble. Attempts to work with *Proteus* organisms had therefore to be abandoned. *B. mesentericus* and organisms of the *Pseudomonas* group proved suitable. Luxuriant growth was obtained in Merrill and Clark's simple medium with these bacteria provided salts of calcium and magnesium were present. Whilst requiring about 3 days to reach maximum turbidity as compared with 24 hours in broth, the final growth as

judged by turbidity was equally good in both cases. Growth in the absence of both calcium and magnesium was either feeble or definitely negative. In the presence of calcium but the absence of magnesium growth was little if any better than in the absence of calcium. In the presence of magnesium, on the other hand, luxuriant growth was again obtained. Good growth was also obtained in Diehl's medium which contained magnesium but no calcium. Apparently salts of magnesium are essential for the continued growth of bacteria in these simple synthetic media with an inorganic source of nitrogen. Such a salt is not universally necessary for growth, because continued multiplication of the bacteria took place in Fränkel's medium which contained no salt of calcium or magnesium. This medium, however, contains both ammonium lactate and asparagine. Even here these salts exerted a stimulating influence on growth, because, although good growth was obtained in their absence, more rapid and somewhat more luxuriant growth took place in their presence.

Protease formation.

The results of a number of experiments are summarised in Table IV. It will be seen that good protease formation took place in Merrill and Clark's medium in the presence of salts of calcium and magnesium, gelatin being liquefied and caseinogen digested. Little or no protease could be detected in the presence of calcium without magnesium in this medium, the growth, however, being relatively poor. Nevertheless counts showed that fair multiplication had occurred. In the same medium together with magnesium salts only luxuriant growth occurred in every way as good as in the presence of both salts. Yet in the latter case protease formation was negative or at the best very doubtful. Hence the position taken up by Wilson [1930] that these salts influence protease formation only in so far as they stimulate growth is not tenable. Either calcium salts alone, or a mixture of calcium and magnesium salts, have a specific action on the liberation of proteases in this medium. Magnesium salts alone have not this ability, or possess it only to a very slight extent, although they stimulate growth. The action of calcium salts only, or a mixture of calcium and magnesium salts, apart from their growth-stimulating properties, could not be tested in Merrill and Clark's medium, because so little growth occurred in their absence. A medium was required which would give good growth in the absence of these salts as well as in their presence. After some preliminary tests a slight modification of Fränkel's medium [1894] was adopted, as mentioned above. Continued growth of both *Pseudomonas* and *B. mesentericus* was obtained in this solution. All attempts to demonstrate the presence of protease in the medium without any addition of salts of calcium and magnesium have consistently failed, despite good growth with both organisms. A little enzyme was detectable in the presence of calcium salts, but good protease formation occurred on the addition of both calcium and magnesium salts. That the action of these salts was not due to activation of the enzyme during the incubation of a filtrate containing them and protein was shown by the fact that no difference

Table IV.

Temperature of incubation 37°.

Organism	Medium	Growth	Temp. of incubation of culture °C.	No. of sub-culture	Age of sub-culture days	Time of incubation of filtrate hrs.	Protease formation		
							Blank	Gelatin	Caseinogen
<i>Pseudomonas 2</i>	Clark with Ca and Mg salts	++	20	3	8	48	Set 3 mins.	>10 hrs.	++
	Clark without Ca and Mg salts	+	20	3	8	48	" 3 "	3 mins.	0
	Clark with Ca salts only	++	20	3	7	48	" 3 "	7 mins.	*
	"	++	20	4	8	48	" 3 "	6 mins.	*
	"	++	20	5	4	48	" 3 "	15 mins.	*
	Clark with Mg salts only	++	20	3	7	48	" 4 "	4 mins.	*
	"	++	20	4	8	48	" 3 "	3 mins.	0
	"	++	20	5	4	48	" 3 "	4 mins.	0
	Diehl	++	20	4	7	48	" 3 "	5 mins.	0
	Fränkel alone	++	20	5	9	48	" 3 "	3 mins.	0
	"	++	20	8	4	48	" 3 "	3 mins.	0
	Fränkel with Ca and Mg salts	++	20	2	2	48	" 3 "	>10 hrs.	++
	"	++	20	3	5	48	" 3 "	>10 hrs.	++
	Fränkel with Ca salts only	++	20	5	4	48	" 4 "	>10 hrs.	++
	Fränkel with Mg salts only	++	20	2	2	48	" 3 "	3 mins.	*
<i>B. mesentericus</i>	Fränkel with Mg salts only	++	20	5	5	48	" 3 "	4 mins.	0
	"	++	20	5	4	48	" 3 "	4 mins.	*
	Clark with Ca and Mg salts	++	37	1	5	48	" 4 "	>10 hrs.	+
	"	++	37	2	6	48	" 3 "	>10 hrs.	+
	Clark with Ca salts only	++	30	8	11	48	" 3 "	>10 hrs.	+
	Clark with Mg salts only	++	30	3	11	48	" 3 "	3 mins.	*
	Fränkel alone	++	37	1	5	48	" 3 "	5 mins.	0
	"	++	30	6	11	48	" 3 "	3 mins.	0
	Fränkel with Ca and Mg salts	++	37	1	5	48	" 3 "	4 mins.	*
	Fränkel with Mg only	++	37	3	6	48	" 3 "	>10 hrs.	+
	"	++	37	3	6	48	" 3 "	3 mins.	+
	"	++	37	3	6	48	" 3 "	3 mins.	0

occurred on adding 0.2 cc. of their solutions to inactive filtrates together with protein, prior to incubation.

The original observation of Merrill and Clark is therefore confirmed. Salts of calcium and magnesium are apparently of fundamental importance to the bacterial cell for the liberation of proteases into the surrounding medium, in the media so far tested. The best effect is obtained by the combination of the two salts: magnesium salts alone have a stimulating influence on growth but affect protease production but little if at all. Calcium salts alone have no apparent stimulating influence on growth, but a trace of protease seems to be detectable in their presence. The action of these salts cannot be explained as a process of stimulation of growth only. So far, only ammonium chloride, ammonium phosphate, ammonium lactate and asparagine have been used as possible sources of nitrogen for the organism. All these can readily be obtained in a state of purity. It remains to be seen whether the action of these salts is of a fundamental nature and common to all media containing other and more complex sources of nitrogen. While there is as yet no experimental evidence, it is interesting to speculate that the action of salts of calcium and magnesium may in some way be linked up with their well-known characteristic effects at certain interfaces and their influence on the reversal of certain emulsions. It is possible that proteolytic enzymes are already present in the cell and assisting in the processes of metabolism, but that they are only secreted into the surrounding medium with a change in the cell surface caused by magnesium and calcium ions. In that case the calcium salts of serum may play an important rôle in the phenomena of immunity.

SUMMARY.

1. Pure native egg-albumin is apparently more resistant to bacterial enzymes than commercial caseinogen or gelatin. Sterile filtrates of broth cultures of *B. mesentericus* and a *Pseudomonas* which readily attacked the latter had but little action on the albumin.

2. Mixtures of salts of calcium and magnesium, or magnesium salts alone, exert a stimulating influence on the growth of micro-organisms in simple synthetic media. Calcium salts alone have but little action in this respect.

3. Proteases are formed in simple synthetic media with ammonium chloride as the source of nitrogen provided that salts of calcium and magnesium are present. Little or no protease formation occurs in the presence of magnesium salts alone, despite good growth. In a medium containing ammonium lactate and asparagine protease formation is stimulated by these salts.

4. The influence of salts of calcium and magnesium on protease formation cannot be explained on the basis of stimulation of growth only.

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APPENDIX. *Composition of media used.**Merrill and Clark's medium.*

NH ₄ Cl	...	...	10 g.
Na ₂ HPO ₄ , 2H ₂ O	...	...	10 g.
KH ₂ PO ₄	...	...	2 g.
Glucose	...	...	10 g.
Water	...	...	1 litre

5 cc. of 0.1 % MgSO₄, 7H₂O and 2 cc. well shaken suspension of 0.1 % CaCO₃ in 50 cc. of medium where required. Final *p_H* 7.4.

Diehl's medium.

(NH ₄) ₂ HPO ₄	...	...	1.75 g.	Final <i>p_H</i> adjusted to 7.4
K ₂ HPO ₄	...	...	0.25 g.	
Na ₂ HPO ₄ , 2H ₂ O	...	...	0.5 g.	
MgSO ₄ , 7H ₂ O	...	...	0.5 g.	
Glycerol	...	...	20.0 cc.	
Water	...	...	1 litre	

Fränkel's medium.

NaCl	...	...	5.0 g.	Final <i>p_H</i> adjusted to 7.4
KH ₂ PO ₄	...	...	2.0 g.	
Ammonium lactate	...	...	6.0 g.	
Asparagine	...	...	4.0 g.	
Water	...	...	1 litre	

Calcium and magnesium salts added as above when required.