THE PROTEOLYTIC ENZYMES OF BACTERIA

II. The Peptidases of Some Common Bacteria[1]

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INTRODUCTION

A great deal of work has been done on the proteolytic enzymes of bacteria, especially those enzymes which are secreted into the medium by the living cell or as the result of its autolysis. The proteolytic enzymes of the cells themselves, however, have been less studied. Studies on bacterial peptidases in which substrates of known formulae (such as synthetic peptides) have been used, are few and fragmentary. In most cases, only a few data illustrating the fact that the peptides were hydrolyzed are given. A brief literature review is given in table 1.

In view of this lack of information, it was decided to investigate how the peptidase systems of bacteria of widely differing genera compared with one another and with the systems found in molds (Johnson and Peterson, 1935; Berger, Johnson and Peterson, 1937), yeast (Grassmann, 1930; Macrae 1933), and animal erepsin (Johnson, Johnson and Peterson, 1936; Johnson 1937). The first paper of this series (Berger, Johnson and Peterson, 1938) described in some detail the properties of the peptidases of *Leuconostoc mesenteroides*. The present paper will give a survey of the peptidases found in twelve organisms which were chosen so as to represent aerobic, anaerobic, facultative,

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proteolytic and non-proteolytic bacteria. The organisms were obtained from the stock cultures of the Bacteriology Department[2] and were as follows: *Escherichia coli*, *Bacillus megatherium*,

TABLE 1

Literature review of bacterial peptidases

ORGANISM	SUBSTRATE HYDROLYZED	pH OPTIMUM	AUTHOR
Staph. pyogenes-aureus.	Glycyl-*l*-tyrosine	(7)*	Otsuka, 1916
B. prodigiosus	Glycyl-*l*-tryptophane		
B. pyocyaneus†	Leucylglycine	8.4	Gorbach, 1930
	Leucyldiglycine	8.4	
B. casei	Leucylglycine	(7.5)	Tarnanen, 1930
	Glycylalanine	(7.5)	
Enterococcus, Gorini	Leucylglycine	(7.8)	Gorini, Grassmann, Schleich, 1932
	Leucyldiglycine	(7.0)	
Mammococcus, Gorini	Leucylglycine	4.8	Gorbach and Ulm, 1935
B. fluorescens	Diglycine	8.4	Gorbach and Pirch, 1936
	Leucylglycine	8.4	
	Leucyldiglycine	8.4	
Cl. histolyticum†	Leucyldiglycine	(7.8)	Weil and Kocholaty, 1937
Caseicoccus, Gorini	Leucylglycine	4.8, 7.0	Gorbach, 1937
Gastrococcus, Gorini	Leucyldiglycine	4.8	
Many aerobes, anaerobes†	Leucylglycine	(7.8)	Maschmann, (6 papers 1937-38)
	Leucyldiglycine	(7.2)	
Staph. aureus	Benzoyldiglycine	8.0	Imaizumi 1938a, 1938b
Staph. aureus, B. typhi	Leucyldiglycine	8.0	
Staph. aureus, B. typhi and *B. subtilis*	Chloroacetylphenylalanine	7.0	
14 organisms	Glycylphenylalanine	(8.0)	
14 organisms	Diglycine	8.0	
6 organisms	Benzoyldiglycine	(8.0)	

* Figures in parentheses represent the pH values at which the determination was made, a pH optimum not having been determined.

† Culture filtrates were used as enzyme source; in case of other organisms cell extracts were used.

Proteus vulgaris, *Pseudomonas fluorescens*, *Bacillus mesentericus*, *Bacillus subtilis*, *Clostridium butylicum* 21, *Clostridium sporogenes*,

[2] The authors with to thank Dr. Elizabeth McCoy and Miss Elizabeth Krauskopf for supplying transfers of these organisms.

Clostridium acetobutylicum (Pike strain), *Lactobacillus pentosus* 124-2, *Propionibacterium pentosaceum* P-11 and *Phytomonas tumefaciens.*

EXPERIMENTAL

Methods

When this survey was undertaken, it was recognized that if any one procedure were adopted, it could not represent the optimum conditions for the study of all the organisms. The extent to which different organisms would vary in the nature of their peptidases was, of course, unknown, but, as a first approximation, it was assumed that their properties would fall between the limits of those of the peptidases of molds, yeast and erepsin. For example, hydrolyses were carried out at pH 8 since known peptidase systems act optimally close to this value. Since a certain degree of uniformity was desirable, it was decided to keep constant as far as practicable, the composition of the medium, the growth period of the organisms and the method of enzyme extraction. Because of these limitations, the data reported in this paper are applicable only for the conditions and methods by which they were obtained.

The organisms were grown in 20-liter Pyrex bottles, each containing 14 liters of 0.5 per cent glucose, 0.5 per cent Bacto-peptone medium made with tap water. The pH of this medium was 6.8. Six hundred cubic centimeters of a 24-hour culture grown in the same medium were used as inoculum. All cultures were incubated at 37° for 24 hours; the cells were collected by centrifuging with a Sharples supercentrifuge and then placed in the freezing compartment of a refrigerator. These wet cells usually contained 80 to 85 per cent water. In the case of the anaerobes, no special precautions were taken to preserve anaerobiosis since the depth of the medium in the Pyrex bottles was about 30 cm. In the case of the aerobes (the first six organisms listed above), the medium was aerated throughout the whole period of incubation by bubbling through it filtered, sterile air at a rate which was limited by the amount of frothing. Unfortunately, this varied with the different organisms. In all cases the cells were

examined microscopically for freedom from contamination before harvesting.

Extraction of cells. With *Bacillus megatherium*, a number of extraction methods were studied. The results of these experiments can be stated briefly as follows: Peptidases were extracted from fresh cells with difficulty (in keeping with the experience of many other investigators), but repeated freezing and thawing of the cells made the enzymes readily available for extraction by a number of procedures. These included autolysis in 40 per cent aqueous glycerol, autolysis under toluene of cells previously dried by acetone-ether treatment, and autolysis of wet cells with ethyl acetate and ether. Preliminary studies on the extraction of peptidases from *Escherichia coli* gave results very similar to those obtained above. On the basis of experiments with *Bacillus megatherium* and *Leuconostoc mesenteriodes* it was concluded that keeping bacteria in a frozen condition as long as three months did not result in a decrease in peptidase activity. It was therefore decided to use the following method in general for all the organisms:

Cells were frozen and thawed at least 5 times over a period of not less than a week, and then ground with sand in a mortar. Toluene was added and the pH was maintained at 6.8 to 7.0 by the careful addition of 0.1 N NaOH. The mass was then diluted to a volume which was roughly 5 to 10 times that of the cells and the suspension was allowed to extract at room temperature, usually for 24 hours. The solution was centrifuged in an angle head centrifuge at 4000 R.P.M. until it was clear. This crude enzyme solution was then analyzed for its ability to hydrolyze various substrates. In some cases, where the cells were poor in peptidases or the efficiency of the extraction was low, it was necessary to concentrate the crude enzyme solution *in vacuo* below 40°, in order to obtain a solution of sufficient activity to make determinations within reasonable lengths of time. This concentrate was then dialyzed against distilled water, usually for about 15 hours, to remove some of the impurities. The enzyme solutions were stored in a refrigerator at 2° to 5° in the

presence of toluene. Whenever an enzyme-substrate mixture was incubated for a long period where bacterial growth might occur, toluene was always added.

The extent of hydrolysis of the peptide substrates used was determined by the Linderstrøm-Lang titration (Linderstrøm-Lang, 1927). In all cases the substrate concentration was M/30 (M/15 for racemic peptides). Unless otherwise mentioned, the substrates were used at pH 7.8 to 8, being half neutralized with NaOH. They contained no added buffers. For all determinations of pH optima for hydrolysis of peptides, the regular substrates were adjusted to the desired pH by the addition of predetermined amounts of 1 N acetic acid or 1 N NaOH solution. Thus, there was no buffering other than that provided by the peptides themselves in the neighborhood of pH 8 and by the small amount of acetate at the lower pH values. The pH values of the enzyme-substrate mixtures were determined after the incubation period by means of the glass electrode. One cubic centimeter samples were titrated from 3.0 cc. of reaction mixture, made by diluting 2.0 cc. of peptide solution to a total of 3.0 cc. with enzyme and water. The maximum variation between duplicate determinations was equivalent to 2 to 5 per cent hydrolysis of the substrate, depending upon the amount of dissolved materials in the enzyme solution, since these sometimes interfered with the accurate reading of the end-point. In all cases, hydrolysis values have been reported as per cent hydrolysis of one optical component of racemic peptides.

The peptidases of aerobes

Proteus vulgaris. The rate of aeration of the medium in the growth of this organism was very much limited by the excessive frothing which occurred. From 14 liters of medium, 5.1 grams wet cells were obtained. The enzyme solution prepared from these cells (10 hour autolysis) was concentrated *in vacuo* and dialyzed against distilled water for 16 hours. The analysis of the final solution is given in table 2.

Johnson, Johnson and Peterson (1936) found that leucyl-

peptidase of hog erepsin was activated by Mg ions and that it hydrolyzed LG[3] and LGG at equal rates. Tests for Mg^{++} activation were therefore made with several bacterial enzyme solutions to see if any of these contained enzymes resembling animal leucylpeptidase. The solutions before testing were reduced in Mg content by acetone precipitation. The data of

TABLE 2

Peptidases of Proteus vulgaris

SUBSTRATE	CRUDE ENZYME*			HYDROLYSIS BY ACETONE-PRECIPITATED ENZYME†		RECOVERY OF ENZYME AFTER PRECIPITATION
	Time of incubation	Hydrolysis‡	Yield per 10 grams wet cells	No Mg	0.003 M Mg	
	hours	*per cent*	*cc.§*	*per cent*	*per cent*	*per cent*
dl-AG	1	20	13	25	27	6
dl-LG	3	20	4.3	14	76	48
GG	13	34	1.6	8	5	8
dl-AGG	15	29	1.3	6	7	13
dl-LGG	3	23	4.9	18	73	40
GGG	15	8	0.3	2	2	16

* 0.50 cc. (representing 77 mgm. of wet cells) of crude enzyme was used per determination.

† For demonstration of Mg^{++} activation, 8.0 cc. of crude solution at pH 6.3 were mixed with 16 cc. acetone, let stand 1 minute, and centrifuged. The precipitate was suspended in 8 cc. water, and recentrifuged. 0.50 cc. of the resulting clear solution was used in all cases; incubation was for 24 hours.

‡ Per cent hydrolysis of one optical component of racemic peptides. 0.003 M $MgCl_2$ was present in the substrates.

§ The basis for calculating yields has been described in a previous paper (Berger, Johnson and Peterson (1938)). Briefly stated, the values represent the hydrolysis which would be obtained by an extract from 10 grams wet cells in one hour at 40°, expressed as cc. of 0.2 N HCl.

table 2 show that the hydrolysis of LG and LGG by the leucylpeptidase of *Proteus vulgaris* was activated by Mg^{++}. Furthermore, the two peptides were hydrolyzed at approximately the same rate by the crude enzyme solution as well as by the

[3] In this paper the following abbreviations will be used: LG = leucylglycine, LGG = leucyldiglycine, AG = alanylglycine, AGG = alanyldiglycine, GG = diglycine, GGG = triglycine.

acetone-precipitated preparation, both in the absence and presence of Mg ions.

Pseudomonas fluorescens. 6.8 grams wet cells were obtained from 14 liters of medium. Here too, the rate of aeration was greatly reduced on account of excessive frothing of the medium. The enzyme solution prepared from these cells (10 hour autolysis) was concentrated *in vacuo* and dialyzed 16 hours. The analysis is given in table 3. It may be seen that the hydrolysis of LG,

TABLE 3

Peptidases of Pseudomonas fluorescens

SUBSTRATE	CRUDE ENZYME*			HYDROLYSIS BY ACETONE-PRECIPITATED ENZYME†			RECOVERY OF ENZYME AFTER PRECIPITATION
	Time of incubation	Hydrolysis	Yield per 10 grams wet cells	Time of incubation	No Mg	0.003 M Mg	
	hours	*per cent*	*cc.*	*hours*	*per cent*	*per cent*	*per cent*
dl-AG	2	36	8.7	2	20	20	56
dl-LG	2	62	15	8	14	57	23
GG	15	52	1.2	9	28	31	100
dl-AGG	2	30	7.3	8	36	41	34
dl-LGG	2	56	13	8	26	50	22
GGG	15	78	2.9	8	26	40	96

* 0.50 cc. (corresponding to 103 mgm. wet cells) of crude enzyme solution was used per determination. Substrates contained 0.003 M $MgCl_2$.

† For demonstration of Mg^{++} activation, 8 cc. crude solution were mixed with 16 cc. acetone and centrifuged after 3 minutes. The resulting precipitate was suspended in 8 cc. H_2O, recentrifuged and 0.50 cc. of clear solution used per determination.

Basis for calculating yields same as in table 2.

LGG, GGG and possibly AGG was activated by Mg^{++}. It is very interesting to note that the GG- and GGG-splitting enzymes were recovered completely after acetone precipitation, whereas the enzymes of animal erepsin and mold which split these peptides are almost completely destroyed by acetone precipitation at room temperature.

Bacillus subtilis. 15.1 grams wet cells from 14 liters of medium were frozen 6 weeks with intermittent thawing, then autolyzed with toluene for 24 hours. The clear solution obtained after cen-

trifuging was tested for the presence of Mg-activated enzymes. As shown in table 4, the hydrolysis of LG was activated but the lack of activation of LGG hydrolysis was rather surprising since with *Proteus*, *Pseudomonas* and animal leucylpeptidase both LG and LGG hydrolyses were activated. The stability of the GGG-splitting enzymes to acetone precipitation was markedly different from the behavior of the GGG-splitting enzyme in erepsin towards this treatment and resembled that found in *Pseudomonas fluorescens* (table 3). LGG was found to be hydrolyzed by the

TABLE 4

Peptidases of B. subtilis

SUBSTRATE	CRUDE ENZYME*		HYDROLYSIS BY ACETONE-PRECIPITATED ENZYME†			RECOVERY OF ENZYME AFTER PRECIPITATION
	Time of incubation	Hydrolysis	Time of incubation	No Mg	0.003 M Mg	
	hours	*per cent*	*hours*	*per cent*	*per cent*	*per cent*
dl-AG	6	22				
dl-LG	24	70	24	46	70	100
GG	24	12				
dl-AGG	1	60	1	71	71	118
dl-LGG	1	60	1	59	59	100
GGG	24	70	24	61	49	87

* 1.0 cc. crude enzmye solution (corresponding to 125 mgm. wet cells) was used per determination. The substrates contained 0.003 M $MgCl_2$.

† To test for Mg^{++} activation, 10 cc. crude enzyme solution at pH 6.84 were mixed with 10 cc. of acetone; the precipitate obtained was centrifuged after 3 minutes, then suspended in 10 cc. H_2O, and recentrifuged. 1.0 cc. of the clear solution was used per determination.

acetone-precipitated enzyme preparation at a pH optimum of 7.7 as shown in figure 1.

Escherichia coli. During the course of this work 100 grams of wet cells were grown aerobically. The average yield was 25 grams from 14 liters of medium (strongly aerated). It was found that with *E. coli* just as with *B. megatherium* the peptidases were not readily extracted from fresh cells by a 17 hour autolysis in 40 per cent glycerol at 37°. (Only 12 units of LG-splitting enzyme were obtained per 10 grams wet cells.) Table 5 gives

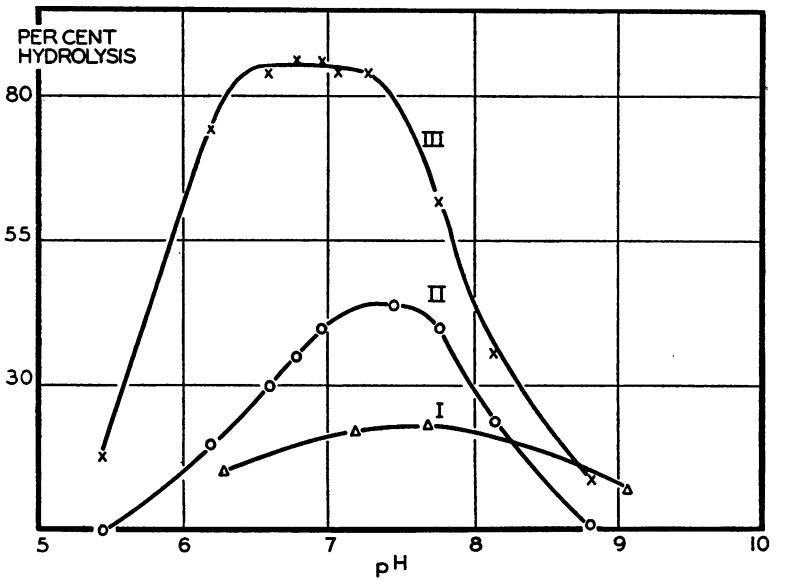

FIG. 1. pH optima for hydrolysis of LGG by *B. subtilis* (*I*, acetone-precipitated enzyme solution, incubation period 1 hour), and *C. sporogenes* (*II*, after 1 hour incubation, *III*, after 4 hours incubation at 40°).

TABLE 5

Peptidases of E. coli

SUBSTRATE	AEROBIC*		ANAEROBIC†	
	Hydrolysis	Yield per 10 grams wet cells	Hydrolysis	Yield per 10 grams wet cells
	per cent	*cc.*	*per cent*	*cc.*
dl-AG	94	127	24	7.0
dl-LG	41	56	18	5.2
GG	50	68	16	4.7
dl-AGG	67	91	52	15
dl-LGG	34	46	68	39
GGG	40	54	11	2.9

* 24 grams of wet cells were frozen 2 days with intermittent thawing, then autolyzed for 4 hours. 0.4 cc. crude enzyme solution, corresponding to 36.9 mgm. wet cells, was used per determination. Incubation time was one hour.

† 1.0 cc. crude enzyme solution, corresponding to 171 mgm. wet cells, was used per determination. Incubation time was 1 hour. Substrates contained 0.003 M $MgCl_2$.

Basis for calculating yields same as in table 2.

the results of the analyses on a representative enzyme preparation. The yield of peptidases may be seen to be higher than for any organism yet studied by us with the exception of *Leuconostoc mesenteroides*.

Space will not be taken here to present the experimental data on the kinetics of the peptidases, since they were determined by the use of crude solutions. It was found that at pH 8, the hydrolysis of AG, LG, AGG, LGG and GGG proceeded at a

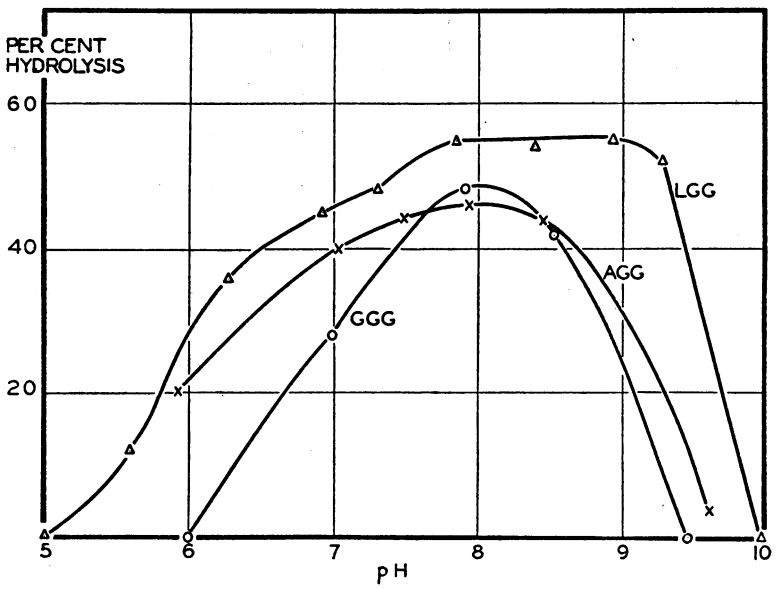

FIG. 2. pH optima for hydrolysis of LGG, AGG and GGG by peptidases of *E. coli*. Incubation time was 1 hour at 40°.

constant rate while the hydrolysis of GG was a first order reaction. That *E. coli* contains a leucylpeptidase was shown by the fact that in the presence of 0.003 M Mg^{++}, the hydrolysis of LGG by an acetone-precipitated enzyme preparation was activated fourfold, namely from 10 to 44 per cent.

Figures 2 and 3 summarize the data obtained for the optimum pH values for the hydrolysis of peptides by *E. coli* peptidases. From the shape of the curves obtained for LG and LGG hy-

drolysis, it appears that in both cases a second enzyme with pH optimum at 6 to 7 is involved. The very rapid decrease in the rate of hydrolysis of these two peptides after pH 9.3 and 9.6 is possibly due to the precipitation of the metal activator as $Mg(OH)_2$.

When *E. coli* was grown anaerobically, 8.6 grams wet cells (from 14 liters of medium) were obtained. The frozen cells were autolyzed 24 hours, and the resulting enzyme preparation, after

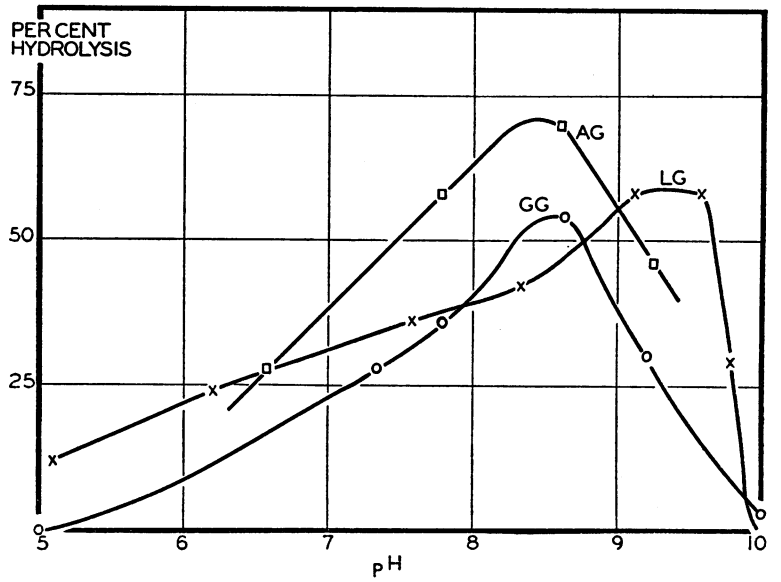

FIG. 3. pH optima for hydrolysis of AG, LG and GG by peptidases of *E. coli*. Incubation time was 1 hour at 40°.

clarification, was analyzed on 6 peptides. The results are given in table 5. When grown anaerobically, the cells appeared to contain less enzyme than when grown aerobically. Furthermore, the leucyl peptides were hydrolyzed more rapidly than the glycyl peptides, a reversal of the picture presented by the aerobically grown cells. A test for Mg-activatable peptidases gave negative results.

Bacillus megatherium. During the course of this study, 650

grams of wet cells were grown. The average yield was 40 grams wet cells per 14 liters of medium. Table 6 gives the data obtained for analyses on 6 peptides with a representative enzyme preparation. When a crude enzyme solution was used, it was found that the hydrolyses of LG, AG, GG and LGG were first order reactions while GGG and AGG were linear.

TABLE 6

Peptidases of B. megatherium

SUBSTRATE	ENZYME SOLUTION PER DETERMINATION	PREPARATION VIII*			
		Dialyzed enzyme solution†		Acetone-precipitated enzyme‡	Recovery of enzyme after precipitation
		Hydrolysis	Yield per 10 grams wet cells	Hydrolysis	
	cc.	*per cent*	*cc.*	*per cent*	*per cent*
dl-AG	0.1	68	71	30	44
dl-LG	0.3	48	17	20	42
GG	0.3	40	14	14	35
dl-AGG	0.3	54	19	14	26
dl-LGG	0.1	46	48	24	52
GGG	1.0	14	1.5	7	25

* Preparation VIII—62 grams wet cells were frozen and thawed 6 times during 2 days, then autolyzed with toluene for 12 hours. After clarification, the solution was concentrated in vacuo and dialyzed 10 hours.

† 1 cc. dialyzed solution corresponds to 480 mgm. wet cells. Incubation time was 1 hour.

‡ Acetone-precipitation—5 cc. dialyzed enzyme concentrate (pH 6.55) were mixed with 10 cc. acetone. The resulting precipitate was immediately centrifuged off, then suspended in 10 cc. H_2O. All substrates were incubated 2 hours, except GGG (4 hours).

Basis for calculating yields same as in table 2.

Acetone, ethyl alcohol, methyl alcohol, dioxan and combinations of some of these were used as precipitating agents. On the average, 40 to 50 per cent of the enzyme was recovered with acetone (table 6) or dioxan but less than 10 per cent with ethyl or methyl alcohol. When acetone was used to precipitate from a dilute enzyme solution, practically complete recovery of LG-, GG- and LGG-splitting enzymes was obtained. Preliminary

experiments on adsorption of enzymes on β-$Fe_2O_3 \cdot H_2O$ in aqueous solution showed that at pH 6.04, from dilute solution, the LGG-splitting enzyme was preferentially adsorbed, leaving behind in the filtrate a purer LG- and GG-splitting solution. $Al(OH)_3$-C_γ also adsorbed the LGG-splitting enzyme preferentially but did not give as sharp a separation.

A number of times enzyme preparations of *B. megatherium* were acetone-precipitated and the resulting preparations tested

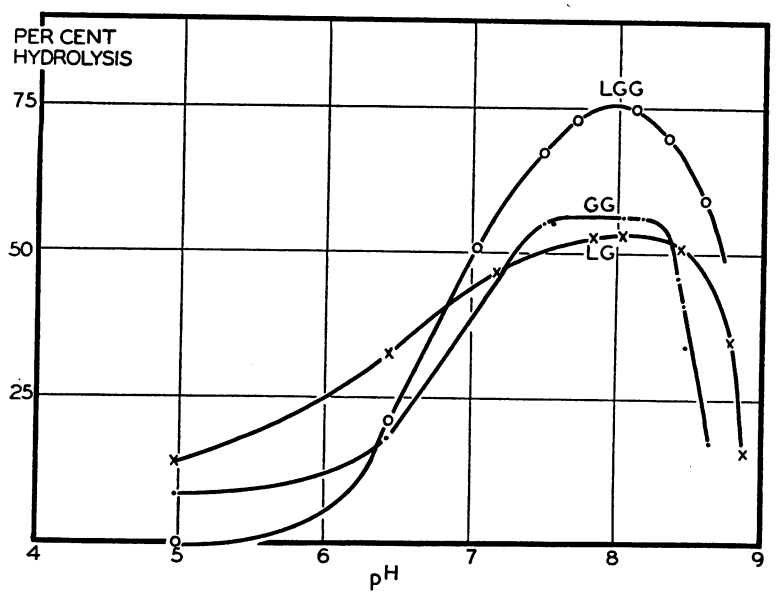

FIG. 4. pH optima for hydrolysis of LG, GG and LGG by peptidases of *B. megatherium*. Incubation time was 1 hour.

for the hydrolysis of the usual 6 peptides in the presence and absence of 0.003 M Mg^{++}. No activation was observed at any time. Another procedure to test for Mg^{++} activation was also tried. An enzyme solution was dialyzed until it had decreased 50 per cent in activity and then was tested on LG and LGG in the presence and absence of Mg^{++}. No activation was observed.

From figure 4 it may be seen that the pH optimum for hydrolysis of GG was 7.6 to 8.0 and of LG and LGG, 8.0.

Phytomonas tumefaciens. This organism was grown on a synthetic medium of the following composition:[4]

Sucrose	10.0 grams
$(NH_4)_2SO_4$	7.5 grams
Glutamic acid	2.5 grams
$MgSO_4 \cdot 7H_2O$	0.2 gram
NaCl	0.2 gram
K_2HPO_4	10.0 grams
$CaCl_2$	0.1 gram
Distilled water	1000 cc.

pH was adjusted to 6.8

The culture was grown in 6-liter Pyrex bottles with continuous aeration by sterile, filtered air for 88 hours at 26°. The cells

TABLE 7

Peptidases of P. tumefaciens and B. mesentericus

SUBSTRATE	P. TUMEFACIENS*		B. MESENTERICUS†	
	Hydrolysis	Yield per 10 grams wet cells	Hydrolysis	Yield per 10 grams wet cells
	per cent	*cc.*	*per cent*	*cc.*
dl-AG	64	3.1	14	1.9
dl-LG	78	3.7	10	1.4
GG	50	2.4	2	0.3
dl-AGG	26	1.2	34	4.7
dl-LGG	48	2.3	42	5.8
GGG	10	0.5	6	0.8

* 1.0 cc. crude enzyme solution corresponding to 210 mgm. wet cells was used per determination. Incubation time was 5 hours.

† 1.0 cc. dialyzed enzyme solution, corresponding to 180 mgm. wet cells was used per determination. Incubation time was 2 hours.

Basis for calculating yields same as in table 2.

were centrifuged off, frozen two weeks and thawed intermittently at least 5 times. They were then suspended in water, the pH adjusted to 7 and the mixture allowed to autolyze with toluene at room temperature. Samples were analyzed daily for LG- and LGG-splitting enzymes until no further increase appeared

[4] This medium was devised by J. M. Van Lanen, A. J. Riker and I. L. Baldwin at the University of Wisconsin. It gives a yield of 70 grams wet cells (considerable of which appears to be gum) from 14 liters of medium.

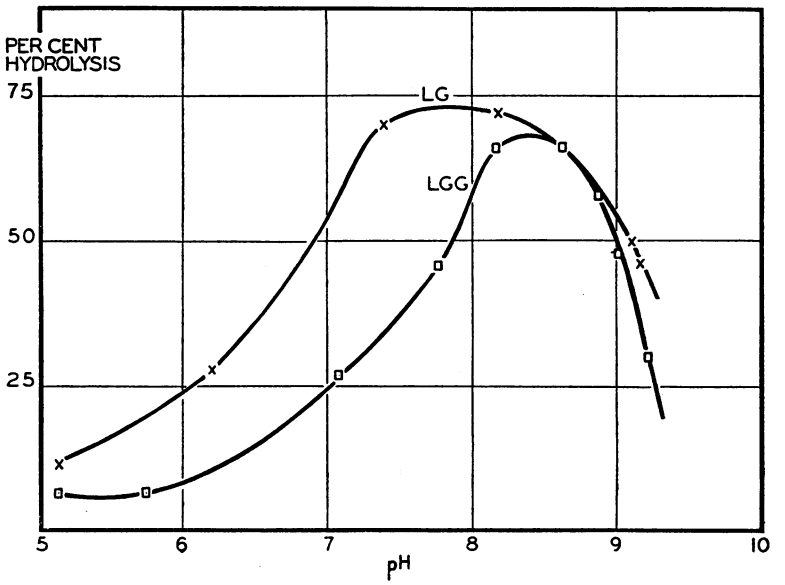

FIG. 5. pH optima for hydrolysis of LG and LGG by peptidases of *P. tumefaciens*. Incubation time was 6 hours at 40°.

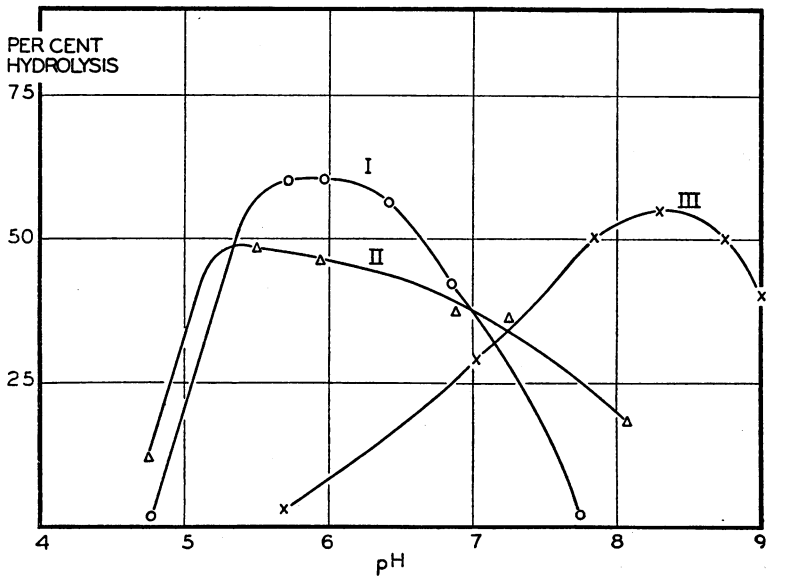

FIG. 6. pH optima for hydrolysis of AG by *P. pentosaceum* (*I*), and of LGG by *L. pentosus* (*II*) and *B. mesentericus* (*III*).

to occur (nine days). The solution was then filtered. Its analysis is summarized in table 7 (which includes also data for *Bacillus mesentericus*). *P. tumefaciens* also contains a Mg- activated leucylpeptidase since the hydrolysis of LGG by an acetone-precipitated enzyme preparation increased from 26 to 44 per cent when 0.003 M Mg^{++} was added. From figure 5 it may be seen that the crude enzyme solution hydrolyzed LG at an optimum pH of 7.6 to 8.2, while LGG was hydrolyzed most rapidly at pH 8.4.

Bacillus mesentericus. 10.6 grams wet cells from 14 liters of medium were frozen 3 months, autolyzed with toluene for 24 hours, and the final enzyme solution dialyzed 14 hours before analysis. The results are given in table 7. It may be seen that leucyl and alanyl peptides were hydrolyzed more rapidly than glycyl peptides, a characteristic common to all the aerobic organisms studied with the exception of *Escherichia coli.* The pH optimum for the hydrolysis of LGG was found to be 8.4, as shown in figure 6.

Specificity of bacterial peptidases

In table 8 are given the data for the hydrolysis of a number of peptides and their derivatives by peptidases of *Escherichia coli* and *Bacillus megatherium.* It may be seen that *E. coli* peptidases hydrolyze glycyl peptides more rapidly than leucyl peptides, while the reverse is true for *B. megatherium.* Substitution of a methyl group for a hydrogen atom of the free amino group such as in sarcosylglycine, N-methylalanyldiglycine and N-methylleucyldiglycine inhibited hydrolysis very markedly with both organisms. *B. megatherium* peptidases, however, hydrolyzed sarcosyldiglycine more rapidly than GGG. Acylated peptides were not split by either organism. For *B. megatherium,* separate determinations (not given in the table) showed that chloroacetylglycine was not hydrolyzed, while prolylglycine and prolyldiglycine were both very slowly hydrolyzed at about 1/50 the rate of LG. Prolylglycine was split 1.7 times as fast as prolyldiglycine. Decarboxylation of peptides such as in leucylmethyl-

amine and glycylmethylamine inhibited hydrolysis by peptidases of both organisms, but *E. coli* peptidases split both compounds very slowly. Yeast dipeptidase and presumably ereptic dipeptidase require a free carboxyl group on the dipeptide to be hydrolyzed (Bergmann *et al.*, 1935), but leucylpeptidase (Johnson

TABLE 8

Hydrolysis of synthetic peptides by E. coli and B. megatherium peptidases

Data are expressed as per cent hydrolysis of one optical component of racemic peptides. Thus 200 per cent splitting of AGG means that 2 linkages (presumably of the natural form of the peptide) have been completely hydrolyzed with the formation of 3 amino acid molecules.

SUBSTRATE	E. COLI (AEROBIC PREP. II)*			B. MEGATHERIUM (PREP. VII)†		
	Per cent hydrolysis of one linkage					
	1 hour	6 hours	24 hours	1 hour	6 hours	24 hours
dl-AG	94	100	107	41	88	94
dl-LG	41	100	101	13	47	83
dl-Leucylmethylamine	4	9	12	1	5	5
GG	50	84	90	9	31	49
Glycylmethylamine	2	4	12	0	0	0
Sarcosylglycine	0	5	23	0	0	3
Sarcosyl-*l*-tyrosine	0	2	4	0	1	10
dl-AGG	67	184	201	11	47	138
dl-N-Methylalanyldiglycine	4	20	58	4	5	65
dl-LGG	34	162	202	36	102	136
dl-N-Methylleucyldiglycine	5	13	83	1	1	7
GGG	40	140	183	0	1	18
Sarcosyldiglycine	6	29	169	1	3	35
Benzoyldiglycine	0	1	1	0	0	0
Tetraglycine	33	164	272	2	4	16
Chloroacetyl-*l*-tyrosine	0	0	1	1	1	6

* 0.40 cc. crude enzyme solution (corresponding to 36.9 mgm. wet cells) was used per 3 cc. of reaction mixture in all determinations.

† 0.30 crude enzyme solution (corresponding to 50 mgm. wet cells) was used per 3 cc. of reaction mixture in all determinations.

et al., 1936) is able to split decarboxylated dipeptides. Since *E. coli* contains an LGG-splitting enzyme whose activity is accelerated by Mg^{++}, and since decarboxylated dipeptides are hydrolyzed by the crude enzyme solution, the presence of an animal-leucylpeptidase-like enzyme is strongly suggested.

An activation appears to occur in the hydrolysis of N-methylleucyldiglycine and sarcosyldiglycine between 6 and 24 hours of incubation (*E. coli*) and in the hydrolysis of N-methylalanyldiglycine and sarcosyldiglycine by *B. megatherium* peptidases. The reason for this is not clear.

The peptidases of anaerobes

In the investigation of the 7 aerobes, the characteristics of the peptidases did not appear to differ markedly from the properties of known peptidases. The enzymes hydrolyzed their peptide substrates at pH optima close to pH 8, and were reasonably stable on incubation at this pH value and 40°. There was no particular reason to suspect that a procedure different from the one used would give better results. However, in the study of 5 anaerobes, differences, apparently in the nature of the peptidases, soon became obvious. With further investigation, the number of these differences increased until it was realized that anaerobes contained peptidases which could not be studied properly under the same conditions that were used for the aerobes. These differences in stability, pH optima and activation by reducing agents will be pointed out for the organisms where they appeared.

Clostridium sporogenes and *Closteridium butylicum.* 11.8 grams wet cells of *C. sporogenes* (from 14 liters of medium) were frozen 1 week, thawed 5 times and autolyzed 10 hours. 18.6 grams wet cells of *C. butylicum* grown on a 0.5 per cent tryptone, 0.5 per cent glucose medium, were frozen 3 months, then autolyzed for 79 hours. The analyses of these enzyme solutions are given in table 9 (which also includes data for *Clostridium acetobutylicum*). When cells of *C. butylicum* grown on the regular peptone-glucose medium were frozen 1 week and autolyzed 10 hours, 2.7 and 8.5 units of AGG- and LGG-hydrolyzing enzymes respectively per 10 grams wet cells were obtained. In an incubation period of 2 hours, no hydrolysis of the other 4 peptides tested was obtained.

We noticed in our work a marked instability of the peptidases of these two organisms and also of certain other anaerobes. In

a previous paper (Berger, Johnson and Peterson, 1938) it was shown that the unstable AGG-hydrolyzing enzyme of *Leuconostoc mesenteroides* was activated by a large number of heavy metals. It has subsequently been found that reducing agents such as thioglycolic acid, cysteine, H_2S, HCN and monomethyl-paramido phenol sulfate ("metol") also have a marked activating effect on this peptide hydrolysis. Data are given in table 10. The AGG-hydrolyzing peptidases of *C. butylicum* (and *Lactobacillus*

TABLE 9

Peptidases of C. sporogenes, C. butylicum and C. acetobutylicum

SUBSTRATE	C. SPOROGENES*		C. BUTYLICUM†		C. ACETOBUTYLICUM‡		
	Hydrolysis	Yield per 10 grams wet cells	Hydrolysis	Yield per 10 grams wet cells	Time of incubation	Hydrolysis	Yield per 10 grams wet cells
	per cent	*cc.*	*per cent*	*cc.*	*hours*	*per cent*	*cc.*
dl-AG	2	0	22	3.9	24	30	0.5
dl-LG	1	0	22	3.9	24	26	0.5
GG	1	0	6	1.1	24	6	0.1
dl-AGG	16	4.9	14	2.5	6	18	1.3
dl-LGG	50	15	44	7.8	6	14	1.0
GGG	2	0	10	1.8	24	6	0.1

* *C. sporogenes*—Substrates contained 0.003 M $MgCl_2$. 0.5 cc. crude enzyme solution (corresponding to 82 mgm. wet cells) was used per determination. Time of incubation was 2 hours, except for GGG (4 hours)

† *C. butylicum*—0.75 cc. crude enzyme solution (corresponding to 140 mgm. wet cells) was used per determination. Incubation time was 2 hours throughout.

‡ *C. acetobutylicum*—Substrates contained 0.003 M $MgCl_2$. 1.0 cc. of crude enzyme solution (corresponding to 115 mgm. wet cells) was used per determination.

Basis for calculating yields same as in table 2.

pentosus) were also activated by "metol." Investigations at present under way indicate that the peptidases of many anaerobic species are activated by reducing agents. Weil and Kocholaty (1937) and Maschmann (1938) have reported activation of bacterial proteinases by reducing agents, but did not mention activation of peptidases.

The pH optimum for the hydrolysis of LGG by *C. sporogenes* peptidases was found to be 7.4 as shown in figure 1. When determinations were made after a 4-hour incubation period

instead of 1 hour, the pH optimum appeared to be 6.5 to 7.2. This, however, is probably the pH stability range. These data suggest that only determinations in which relatively short periods of incubation are used can be relied upon to give true pH activity optima. In the literature review in table 1, 24 hours was the shortest incubation period used in the determination of any of the pH optima. We have found the pH optimum for the hydrolysis of AGG in the presence of 10^{-3} molar "metol" by *C. butylicum* peptidases to be 7.5 (1 hour incubation at 40°).

The hydrolysis of AGG and LGG by an acetone-precipitated enzyme preparation from *C. sporogenes* was not activated by

TABLE 10

Activation of peptidases by reducing agents (L. mesenteroides)

MOLAR CONCENTRATION OF ACTIVATOR	PER CENT HYDROLYSIS OF AGG				
	Cysteine	H_2S	HCN	Thioglycolic acid	"Metol"
None	27	27	27	27	34
10^{-2}	104	66	66	73	92
10^{-3}	29	44	54	106	94
10^{-4}	29	79	42	30	96
10^{-5}	29	88	36		71

The same enzyme solution gave 80 per cent hydrolysis of AGG in the presence of 10^{-3} M Zn^{++}.

Incubation was for one hour.

0.003 M Mg^{++} at pH 8. Thirty-one per cent of the LGG-splitting enzyme was recovered after the acetone precipitation.

Clostridium acetobutylicum. 13.8 grams wet cells from 14 liters of medium were frozen 6 weeks with intermittent thawing, then autolyzed with toluene for 24 hours. The data obtained on the analysis of the enzyme solution are contained in table 9. The low yields of enzyme are possibly due to the inefficiency of the extraction method as well as to the instability of the peptidases in the absence of suitable activating agents.

Lactobacillus pentosus and *Propionibacterium pentosaceum.* 26.7 grams wet cells of *Propionibacterium* and 10.9 grams wet cells of *Lactobacillus* were obtained per 18 liters of a medium which

contained in addition to the usual components, 0.5 per cent of sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$) and 100 cc. of yeast water[5] per liter of medium. The pH of this medium at the time of inoculation was about 5.8. Cells of both organisms were frozen 3 months with intermittent thawing, then autolyzed with toluene for 24 hours. The enzyme solutions were dialyzed 10 hours. The analyses of these solutions are given in table 11. Unfortunately, these data do not give the true picture of the distribution of the peptidases in these organisms since the pH optima for the hydrolysis of AG by *Propionibacterium* and of LGG by *Lactobacillus* were found to be 5.8 and 5.5, as shown in figure 6,

TABLE 11

Peptidases of Lactobacillus and Propionibacterium

SUBSTRATE	L. PENTOSUS	P. PENTOSACEUM
	Hydrolysis	
	per cent	*per cent*
dl-AG	6	20
dl-LG	10	14
GG	6	10
dl-AGG	6	6
dl-LGG	3	0
GGG	0	2

Time of incubation was 2 hours.

1.0 cc. dialyzed enzyme solution was used in each determination, corresponding to 188 mgm. wet cells of *Lactobacillus*, or 534 mgm. for *Propionibacterium*.

and not pH 8 at which value the routine determinations were made. *Propionibacterium* and *Lactobacillus* thus appear to contain acidopeptidases similar to those reported in acidoproteolytes by Gorbach (1937).

Enzymes in the culture medium

Considerable controversy exists concerning the secretion of proteinases and peptidases into the medium by living bacterial cells. Gorbach and Pirch (1936) have claimed that the secretion of proteinase occurs only after partial cell autolysis while Virtanen

[5] The clear extract from 200 grams pressed yeast autoclaved in 1 liter of water.

and Suolahti (1937) insist that living cells secrete proteinase. Whatever the true situation may be, we have found peptidase activity in filtrates from 24- to 48-hour cultures of proteolytic as well as non-proteolytic organisms. This is shown in table 12 from which it may be seen that filtrates of *Escherichia coli* (non-proteolytic) and *Bacillus megatherium* (proteolytic) contained very considerable amounts of peptidases. Filtrates of cultures of *Clostridium sporogenes*, *Clostridium butylicum*, *Clostridium acetobutylicum*, *Proteus vulgaris*, *Bacillus subtilis* and *Bacillus mesentericus* did not contain sufficient LG- or LGG-splitting peptidases to give increases in titration in 10- to 24-hour incubation periods that were significantly greater than experimental error. Cells of these organisms, if analyzed for in the same proportional dilution, would also have given no significant titration, so it is impossible to conclude in these cases whether cells or medium contained more enzyme.

TABLE 12

Peptidases found in culture media

ORGANISM	PREPARATION	SUBSTRATE	TIME OF INCUBATION	HYDROLYSIS	APPROX. YIELD FROM 14 LITERS MEDIUM	
					Medium	Cells*
			hours	*per cent*	*cc.*	*cc.*
B. megatherium†	I	GG	48	26	38	56
	II	*dl*-LGG	6	24	280	192
		dl-LGG	4	27	472	192
	III	*dl*-LG	10	54	378	68
		dl-LGG	5	52	728	192
		GGG	10	8	56	6
E. coli‡		GG	24	26	23	170
		dl-LGG	18	56	65	115

* Values for enzyme yields from cells were taken from tables 5 and 6. 25 grams wet cells of *E. coli* and 40 grams wet cells of *B. megatherium* were taken as average cell yields per 14 l. medium.

† 1 cc. filtrate of 24 hour cultures was used per determination.

‡ 3 liters of filtrate from a 45 hour culture were concentrated in vacuo (below 40°) to 900 cc.: this was dialyzed 18 hours, then 1 cc. used per determination.

Basis for calculating yields same as in table 2.

DISCUSSION

In a wide survey of this kind, optimum conditions for analysis of all the organisms could not be maintained. For the aerobes, the general procedure adopted proved to be very satisfactory. When the anaerobes were subjected to this procedure, however, it was found that their peptidases had properties differing very considerably from those of any previously studied peptidases. The instability under the experimental conditions employed, the activation by reducing agents and heavy metals, and the acidic pH optima of some of the peptidases greatly increased the difficulties involved in their analysis. This study has outlined the general properties of a large number of bacterial peptidases and also suggested the peculiar properties possessed by some peptidases. These characteristics must be kept in mind in a detailed investigation of any one species of bacteria.

SUMMARY

1. A survey has been made of the peptidases in cell-free extracts of twelve bacterial species, including *Escherichia coli*, *Bacillus megatherium*, *Proteus vulgaris*, *Pseudomonas fluorescens*, *Bacillus mesentericus*, *Bacillus subtilis*, *Clostridium butylicum*, *Clostridium sporogenes*, *Clostridium acetobutylicum*, *Lactobacillus pentosus*, *Propionibacterium pentosaceum* and *Phytomonas tumefaciens*.

2. An extraction procedure has been found, involving repeated freezing and thawing of cells, followed by a toluene autolysis, which gives satisfactory enzyme preparations with a degree of activity which is measurable within short periods of time.

3. The pH optima have been determined for the hydrolysis of AG by 2 organisms, LG by 3 organisms, GG by 2, AGG by 2, LGG by 7 and GGG by 1. In most cases the optimum value for peptide hydrolysis was between pH 8 and 9. Two organisms, however, *Lactobacillus pentosus* and *Propionibacterium pentosaceum* were found to contain acidopeptidases which split their substrates at optimum pH values of 5.5 to 6.0.

4. In most cases the peptidases were quite stable at pH 8

and 40° but the LGG-splitting enzymes of the anaerobes (*Clostridium sporogenes, Clostridium butylicum*) were very unstable under these conditions. This instability was partly overcome by the addition of reducing agents, which for *Clostridium butylicum* activated the hydrolysis of AGG and LGG.

5. The specificity of the peptidase systems of *Bacillus megatherium* and *Escherichia coli* was studied in some detail. Dipeptides and tripeptides were readily hydrolyzed but acylated or decarboxylated peptides were hydrolyzed only very slowly or not at all. Substitution of a methyl group for a hydrogen atom of the free amino group on a peptide resulted in a very marked decrease in hydrolysis.

6. In four organisms, namely *Escherichia coli, Proteus vulgaris, Pseudomonas fluorescens* and *Phytomonas tumefaciens* a leucylpeptidase-like enzyme was found whose hydrolysis of LGG was activated by 0.003 M Mg^{++}.

7. Appreciable amounts of peptidases were found in culture filtrates of *Escherichia coli* and *Bacillus megatherium*. With *Escherichia coli*, more enzymes could be extracted from the cells than from the medium on which they were grown, as might be expected with a non-proteolytic organism. With the proteolytic organism *Bacillus megatherium*, more peptidases were consistently found in a given volume of medium than could be obtained from the cells grown on the same volume of medium.

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