

# EFFECTS OF SOME METALLIC IONS ON GLUTAMYL POLYPEPTIDE SYNTHESIS BY *BACILLUS SUBTILIS*

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Some factors influencing peptide production by *Bacillus subtilis* were reported in an earlier publication (Thorne *et al.*, 1954). Yields of peptide in excess of 15 mg per ml were obtained in a medium composed of glycerol, citric acid, L-glutamic acid,  $\text{NH}_4\text{Cl}$ ,  $\text{MgSO}_4$ ,  $\text{FeCl}_3$ , and  $\text{K}_2\text{HPO}_4$  (medium C). Maximum growth and peptide production were obtained only when tap water and a specific lot of  $\text{FeCl}_3$  were used in preparing the medium. This suggested that unidentified metallic ions in tap water and in the lot of  $\text{FeCl}_3$  were necessary. The present paper deals with the identification of these ions and presents a chemically defined medium (medium E) for optimum growth and peptide production. It also discusses the effects of various metallic ions on configuration of the glutamic acid comprising the polypeptide synthesized in this medium.

## MATERIALS AND METHODS

*Culture and medium.* *Bacillus subtilis*, ATCC 9945A, designated previously by us as strain CDII, was used. The basal medium (designated here as medium C) and methods for producing peptide in shaken flasks were the same as those described previously (Thorne *et al.*, 1954).

*Inorganic salts.* The inorganic salts used were the purest that were available. They were examined with a flame photometer or an emission spectrograph and, with the exception of one lot of  $\text{FeCl}_3$  which was contaminated with manganese, all were found to be of high purity.

*Analytical methods.* The methods for total glutamic acid, L-glutamic acid, precipitation and acid hydrolysis of peptide, and total nitrogen were the same as those used previously (Thorne *et al.*, 1954). D-Glutamic acid was determined directly with D-glutamic acid oxidase from *Aspergillus ustus* (Mizushima *et al.*, 1956) or estimated indirectly by subtracting the amount of the L-isomer from the total.

## RESULTS

*Metallic ion requirements for growth.* When medium C was prepared with distilled water, the organism did not grow except when a particular lot of  $\text{FeCl}_3$  was used. Analysis with a flame photometer revealed that this lot of  $\text{FeCl}_3$  was contaminated with a trace of  $\text{Mn}^{++}$ . When  $\text{Mn}^{++}$  was added to media prepared with distilled water and  $\text{FeCl}_3$  that did not contain detectable amounts of  $\text{Mn}^{++}$ , growth was obtained which was equivalent to that produced in the original medium C prepared with contaminated  $\text{FeCl}_3$ . The concentration of  $\text{Mn}^{++}$  required for maximum growth ( $>1.0 \times 10^9$  cells per ml in 24 hr) was  $1.5 \times 10^{-7}$  M. In this medium *B. subtilis* also required  $\text{K}^+$ ,  $\text{Fe}^{+++}$ , and  $\text{Mg}^{++}$  for growth and these ions were added at the concentrations found to be optimum in medium C.

*Metallic ion requirements for peptide synthesis.* Although by adding  $1.5 \times 10^{-7}$  M  $\text{Mn}^{++}$  to medium C made with distilled water a chemically defined medium was obtained which supported maximum growth, the amounts of peptide produced were small (8 mg per ml). However, when the residue obtained upon evaporating tap water to dryness was added, the usual high yields of peptide (15 mg per ml) were produced. Examination of the tap water with a flame photometer showed that the major metallic ions present were  $\text{Mg}^{++}$  and  $\text{Ca}^{++}$ . Since  $2.03 \times 10^{-3}$  M  $\text{Mg}^{++}$  was already present in the medium, the effects of  $\text{Ca}^{++}$  as well as concentrations of  $\text{Mn}^{++}$  higher than that required for maximum growth were investigated.

The effects of  $\text{Mn}^{++}$  on peptide yields are shown in table 1. By increasing the concentration of  $\text{Mn}^{++}$  from  $1.5 \times 10^{-7}$  M to  $6.15 \times 10^{-4}$  M, the yields of peptide increased from 8 to 19 mg per ml with no significant change in total growth. Of particular interest was the effect of  $\text{Mn}^{++}$  on the proportions of the two isomers of

glutamic acid in the peptide. The proportion of the D-isomer increased gradually from 38 to 86 per cent of the total as the concentration of  $Mn^{++}$  was varied over a range from  $1.5 \times 10^{-7}$  M to  $2.46 \times 10^{-3}$  M. A concentration of  $4.92 \times 10^{-3}$  M was inhibitory to growth and peptide production.

An additional effect of  $Mn^{++}$  is shown also in table 1; although maximum growth was obtained with  $1.5 \times 10^{-7}$  M  $Mn^{++}$ , higher concentrations had a marked effect in prolonging cell viability. This effect could not be attributed to the occurrence of spores since the organisms did not sporulate in this medium.

As shown in table 2, the effect of  $Mn^{++}$  in prolonging cell viability was influenced by the concentration of  $K_2HPO_4$ . With  $1.54 \times 10^{-6}$  M  $Mn^{++}$  the number of viable cells per ml decreased

from  $10^9$  to  $10^4$  in 4 days regardless of the  $K_2HPO_4$  concentration. When the concentration of  $Mn^{++}$  was increased to  $6.15 \times 10^{-4}$  M and the  $K_2HPO_4$  was  $2.88 \times 10^{-3}$  M, the cell count remained at  $10^9$  per ml on the 4th day and was reduced to  $10^4$  per ml on the 6th day. However, with this concentration of  $Mn^{++}$  the number of viable cells remained at  $10^9$  per ml for 9 days when the concentration of  $K_2HPO_4$  was increased to  $5.76 \times 10^{-2}$  M. As pointed out above this effect was not a result of sporulation.

The effect of  $Ca^{++}$  on peptide production is shown in table 3. With  $1.54 \times 10^{-6}$  M  $Mn^{++}$ , which was below the optimum level for peptide production in the absence of  $Ca^{++}$ , maximum yields of peptide were produced when  $Ca^{++}$  was added at a concentration of  $1.02 \times 10^{-3}$  M. Although  $Ca^{++}$  stimulated peptide production it

TABLE 1  
*Effects of  $Mn^{++}$  on peptide synthesis and cell viability of *Bacillus subtilis*\**

MnSO <sub>4</sub>	No. of Viable Bacteria/ml, after Day:					Peptide as Glutamic Acid after Day:			
						2		4	
	1	2	3	4	5	Total	D-Isomer	Total	D-Isomer
$m \times 10^6$						mg/ml	%	mg/ml	%
0	$1.0 \times 10^6$	$1.0 \times 10^5$	$1.0 \times 10^4$			0		0	
0.07	$8.0 \times 10^8$	$1.0 \times 10^5$	$1.0 \times 10^4$			0.9		0.9	
0.15	$1.8 \times 10^9$	$1.7 \times 10^9$	$4.7 \times 10^7$	$1.0 \times 10^4$		7.7	38	7.9	39
1.54	$1.7 \times 10^9$	$1.8 \times 10^9$	$1.0 \times 10^7$	$1.0 \times 10^4$		7.3	49	8.0	46
30.8						11.2	75	13.8	68
154						12.7	80	16.7	74
308						13.3	86	18.2	78
615	$1.7 \times 10^9$	$1.8 \times 10^9$	$1.7 \times 10^9$	$1.5 \times 10^9$	$1.0 \times 10^8$	14.2	84	19.6	81
1230						12.6	84	18.8	84
2460						12.5	85	19.6	86
4920						6.3		8.9	86

\* Medium E with concentrations of  $MnSO_4$  as given in the table. No sporulation occurred.

TABLE 2  
*Effect of  $Mn^{++}$  and  $K_2HPO_4$  on viability of *Bacillus subtilis**

MnSO <sub>4</sub>	K <sub>2</sub> HPO <sub>4</sub>	No. of Viable Bacteria/ml,* after Day:				
		1	4	6	9	10
$m \times 10^6$	$m \times 10^2$					
1.54	2.88	$1.3 \times 10^9$	$1.0 \times 10^4$			
1.54	57.6	$1.0 \times 10^9$	$1.0 \times 10^4$			
615	2.88	$1.4 \times 10^9$	$1.3 \times 10^9$	$1.0 \times 10^4$		
615	57.6	$1.0 \times 10^9$	$1.0 \times 10^9$	$1.3 \times 10^9$	$1.1 \times 10^9$	$4.7 \times 10^7$

\* Medium E with concentrations of  $MnSO_4$  and  $K_2HPO_4$  as given in the table. No sporulation occurred.

did not affect the proportions of the D- and L-isomers of glutamic acid in the peptide. With  $1.54 \times 10^{-6}$  M  $Mn^{++}$  the proportion of D-glutamic acid in the peptide was about 50 per cent regardless of the  $Ca^{++}$  concentration or the amount of peptide produced. The amount of total growth was not affected by the concentrations of  $Ca^{++}$  tested, and in contrast to  $Mn^{++}$ ,  $Ca^{++}$  had no effect on the length of time the cells remained viable.

The effect of increasing the concentrations of  $Mn^{++}$  in the presence of the optimum amount of  $Ca^{++}$  is shown in table 4. Under these conditions increasing the concentration of  $Mn^{++}$  beyond that required for maximum growth did not affect the total peptide yields significantly. However, the proportion of D-glutamic acid in the peptide increased from 39 to 84 per cent with increasing concentrations of  $Mn^{++}$  over a range from  $1.5 \times 10^{-7}$  M to  $2.46 \times 10^{-3}$  M.

The effects of  $Zn^{++}$  and  $Co^{++}$  on peptide synthesis are shown in tables 5 and 6, respectively.  $Co^{++}$ , but not  $Zn^{++}$ , replaced  $Mn^{++}$  for growth. In the presence of concentrations of  $Mn^{++}$  optimum for growth and of  $Ca^{++}$  optimum for peptide production,  $Co^{++}$  and  $Zn^{++}$  each had the same effect as  $Mn^{++}$  on the proportion of D-glutamic acid in the peptide. With increasing concentrations of either ion the proportion of D-glutamic acid increased from about 50 to about 85 per cent of the total. This effect of  $Zn^{++}$  and  $Co^{++}$  and that of  $Mn^{++}$  were not additive, i. e., high concentrations of  $Mn^{++}$  and  $Zn^{++}$  or of  $Mn^{++}$  and  $Co^{++}$  did not result in a proportion of D-glutamic acid higher than 85 per cent of the total. High concentrations of  $Zn^{++}$  or  $Co^{++}$ , unlike  $Mn^{++}$ , did not prolong cell viability.

TABLE 3

*Effect of  $Ca^{++}$  on peptide synthesis by *Bacillus subtilis*\**

CaCl <sub>2</sub>	Peptide as Glutamic Acid at 65 Hr	
	Total	D-Isomer
<i>M</i> × 10 <sup>6</sup>	mg/ml	%
0	7.9	46
0.34	13.6	52
1.02	17.0	51
3.40	17.3	49
34.0	17.9	50

\* Medium E with  $1.54 \times 10^{-6}$  M  $MnSO_4$ .

TABLE 4

*Effect of  $Mn^{++}$  in the presence of  $Ca^{++}$  on peptide synthesis by *Bacillus subtilis*\**

MnSO <sub>4</sub>	Peptide as Glutamic Acid at 90 Hr	
	Total	D-Isomer
<i>M</i> × 10 <sup>6</sup>	mg/ml	%
0.15	14.1	39
1.54	13.2	49
15.4	14.7	60
154	15.5	74
615	17.0	81
2460	15.4	84

\* Medium E with  $1.02 \times 10^{-3}$  M  $CaCl_2$ .

TABLE 5

*Effect of  $Zn^{++}$  on peptide synthesis by *Bacillus subtilis*\**

MnSO <sub>4</sub>	ZnSO <sub>4</sub>	Peptide as Glutamic Acid at 65 Hr	
		Total	D-Isomer
<i>M</i> × 10 <sup>6</sup>	<i>M</i> × 10 <sup>6</sup>	mg/ml	%
1.54	0	13.2	52
1.54	3.47	13.7	58
1.54	17.4	13.0	66
1.54	86.8	14.2	76
1.54	174	16.3	81
1.54	347	14.5	82
615	0	17.1	82
615	174	14.5	83

\* Medium E with  $1.02 \times 10^{-3}$  M  $CaCl_2$ .

TABLE 6

*Effect of  $Co^{++}$  on peptide synthesis by *Bacillus subtilis*\**

CaCl <sub>2</sub>	MnSO <sub>4</sub>	CoCl <sub>2</sub>	Peptide as Glutamic Acid at 90 Hr	
			Total	D-Isomer
<i>M</i> × 10 <sup>3</sup>	<i>M</i> × 10 <sup>6</sup>	<i>M</i> × 10 <sup>6</sup>	mg/ml	%
0	0	1.09	8.3	—
0	0	10.9	12.9	66
0	0	109	13.6	72
1.02	1.54	0	14.6	49
1.02	1.54	1.09	13.5	47
1.02	1.54	21.9	13.0	51
1.02	1.54	109	13.3	66
1.02	1.54	439	15.0	82
1.02	1.54	1760	15.0	85
1.02	615	439	17.0	85

\* Medium E.

TABLE 7  
 Medium E for production of peptide by  
*Bacillus subtilis*

	g/L
L-Glutamic acid.....	20.0
Citric acid.....	12.0
Glycerol.....	80.0
NH <sub>4</sub> Cl.....	7.0
K <sub>2</sub> HPO <sub>4</sub> .....	0.5 (2.88 × 10 <sup>-3</sup> M)
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.5 (2.03 × 10 <sup>-3</sup> M)
FeCl <sub>3</sub> ·6H <sub>2</sub> O.....	0.04 (1.44 × 10 <sup>-4</sup> M)
CaCl <sub>2</sub> ·2H <sub>2</sub> O.....	0.15 (1.02 × 10 <sup>-3</sup> M)
MnSO <sub>4</sub> ·H <sub>2</sub> O*.....	0.000026 (1.54 × 10 <sup>-7</sup> M) to 0.42 (2.46 × 10 <sup>-3</sup> M)

Distilled water to 1 L  
 pH 7.4 with NaOH

\* The concentration of Mn<sup>++</sup> can be varied to give the desired proportion of D-glutamic acid in the peptide.

*Effects of other inorganic ions.* Various concentrations of K<sup>+</sup>, NH<sub>4</sub><sup>+</sup>, Fe<sup>+++</sup>, Mg<sup>++</sup>, and PO<sub>4</sub><sup>=</sup>, up to levels inhibitory to growth or peptide synthesis, were tested for their effects on the proportions of D- and L-glutamic acid in the peptide. Although each of these was essential for growth or peptide production, none had an effect on the proportions of the two isomers in the peptide. None of these ions or Ca<sup>++</sup>, when tested at the highest concentration permitting optimum growth and peptide synthesis, was antagonistic to Mn<sup>++</sup> in its effect on the percentage of D-glutamic acid in the peptide.

*Final medium for peptide production.* Table 7 defines synthetic medium E. It is the same as the original medium C except that Mn<sup>++</sup> and Ca<sup>++</sup> have been added and distilled water has been substituted for tap water. In this medium the concentration of Mn<sup>++</sup> can be varied from 1.5 × 10<sup>-7</sup> M to 2.46 × 10<sup>-3</sup> M to give the desired proportion of D-glutamic acid in the peptide. Ca<sup>++</sup> was added to insure high yields of peptide with any concentration of Mn<sup>++</sup> used.

#### DISCUSSION

The findings that Mn<sup>++</sup>, K<sup>+</sup>, Fe<sup>+++</sup>, and Mg<sup>++</sup> were required for growth of the strain of *B. subtilis* used here are in agreement with the results of Feeney *et al.* (1947) and Feeney and Garibaldi (1948) who found that these ions were required for growth of their subtilin-producing

strain. In addition these workers found that a trace of Zn<sup>++</sup> was required. A requirement for Zn<sup>++</sup> could not be confirmed for our organism, but the possibility was not ruled out that sufficient Zn<sup>++</sup> was being added as a contaminant in some other components in the medium.

Either Mn<sup>++</sup> alone or Ca<sup>++</sup> plus the minimum concentration of Mn<sup>++</sup> required for growth replaced tap water in fulfilling the metallic ion requirements for peptide synthesis. The increased yields of peptide in the presence of high concentrations of Mn<sup>++</sup> could be accounted for partially by the prolonged viability of the cells. However the rate of peptide synthesis, which apparently was increased by the presence of Ca<sup>++</sup>, also seemed to be influenced by the concentration of Mn<sup>++</sup> when Ca<sup>++</sup> was absent. The effect of Mn<sup>++</sup> in increasing the proportion of D-glutamic acid in the peptide was not a reflection of its effect on cell viability. With a given concentration of Mn<sup>++</sup> in the medium the proportion of D-glutamic acid in the peptide did not change significantly although the total yield of peptide increased as the time of incubation was extended.

Preparations of polypeptide from *B. subtilis* have been reported to contain various proportions of the D- and L-isomers of glutamic acid (Bovarnick, 1942; Watson *et al.*, 1947; Thorne *et al.*, 1954). The results reported here show that by varying the concentration of Mn<sup>++</sup> in the medium it was possible to control the proportions of the isomers in the peptide without affecting the total yield. Thus far we have been unable to show an effect of Mn<sup>++</sup> on any of the isolated enzyme systems that have been implicated in the synthesis of either D-glutamic acid or polypeptide. These enzyme systems include  $\gamma$ -glutamyl transferase (Williams and Thorne, 1954*a*, *b*), L- and D-amino acid transaminases (Thorne *et al.*, 1955) and alanine racemase (Wood and Gunsalus, 1951; Thorne *et al.*, 1955).

Although glutamyl peptides with chain lengths of 2 to 6 glutamic acid residues were synthesized by  $\gamma$ -glutamyl transferase in cell-free preparations from *B. subtilis* (Williams *et al.*, 1955), the possibility was not ruled out that another mechanism exists for synthesis of the polypeptide in growing cultures. Since the  $\gamma$ -glutamyl transferase preparations also were active in hydrolyzing the polypeptide to glutamic acid, it is possible that the primary function of this enzyme is that of hydrolysis rather than synthesis. In

another publication (Thorne and Leonard, 1958) we have described the isolation of an L-glutamyl polypeptide and a D-glutamyl polypeptide from culture filtrates of *B. subtilis* and have presented evidence that this organism does not synthesize a peptide with large proportions of both isomers in the same peptide chain. Perhaps two different enzyme systems for the synthesis of peptide are present in *B. subtilis*, one specific for the incorporation of L-glutamic acid and another specific for the incorporation of D-glutamic acid. If this is true, the latter enzyme system is probably activated by  $Mn^{++}$ ; another possibility is that  $Mn^{++}$  antagonizes the enzyme system incorporating L-glutamic acid into peptide.

A further possibility is that one enzyme system synthesizes both D- and L-peptides and that the specificity is controlled by metallic ions. If such a system included the  $\gamma$ -glutamyl transferase mentioned above,  $Mn^{++}$  might produce its effect by controlling the proportions of the D- and L-isomers of glutamine that are synthesized and made available as substrates for the transferase.

In recent experiments peptide synthesis has been obtained with protoplasts of *B. subtilis* and the effect of metallic ions on this system will be investigated in future studies.

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#### SUMMARY

The synthesis of glutamyl polypeptide by *Bacillus subtilis* during growth in a chemically defined medium was dependent upon the metallic ion composition of the medium. Although addition of  $1.5 \times 10^{-7}$  M  $Mn^{++}$  resulted in maximum growth, the yield of peptide was low. Increasing the concentration of  $Mn^{++}$  to  $6.15 \times 10^{-4}$  M resulted in maximum yields of peptide. Likewise, addition of  $1.02 \times 10^{-3}$  M  $Ca^{++}$  in the presence of the minimum concentration of  $Mn^{++}$  required for growth gave maximum yields of peptide. The proportion of D-glutamic acid in the peptide was independent of the  $Ca^{++}$  concentration but varied from 38 to 86 per cent with increasing concentrations of  $Mn^{++}$ ,  $Co^{++}$ , or  $Zn^{++}$ . Other inorganic ions in the medium did not affect the proportions of D- and L-isomers in the peptide.

$Mn^{++}$  was also effective in prolonging the

viability of cells and this effect was enhanced by increasing the concentration of  $K_2HPO_4$ .

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