SYNTHESIS OF GLUTAMIC ACID AND GLUTAMYL POLYPEPTIDE BY BACILLUS ANTHRACIS

III. Factors Affecting Peptide Production in Synthetic Liquid Media

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Received for publication October 10, 1952

A previous paper (Thorne *et al.*, 1952) described factors affecting production of glutamyl polypeptide (capsular material) by *Bacillus anthracis* on solid media. Large amounts of peptide were synthesized by three highly virulent strains, 99, 994, and M-36, only when $NaHCO_3$ was added to the medium or when the cultures were grown at a high pH in an atmosphere containing a high percentage of CO_2. It was shown further that $NaHCO_3$ had little or no effect on peptide synthesis by two avirulent strains, M and HM. Large quantities of peptide were produced by these strains in the absence of added CO_2.

In order to be able to study further factors affecting peptide synthesis and to investigate the biochemical mechanism involved, it was desirable to use a liquid medium for peptide production. The use of a liquid medium would facilitate the following of chemical changes such as the appearance or disappearance of various metabolites during growth of the organism.

It was soon learned that, although the mere addition of $NaHCO_3$ to liquid media resulted in production of capsulated organisms, the total amount of peptide produced was very small. Both stationary and shaken flasks plugged with cotton yielded poor results. Shaking the flasks of bicarbonate containing medium in an atmosphere containing CO_2 did not improve the peptide yield significantly. However, larger quantities of peptide were produced when the medium was aerated at a rapid rate with a mixture of CO_2 and air or when the cultures were grown in a bicarbonate containing medium in shaken flasks closed tightly with rubber stoppers. The results obtained with each of these methods and various factors affecting peptide production under these conditions are reported here.

EXPERIMENTAL METHODS

Cultures. The cultures of *B. anthracis* used, virulent strains, 994 and M-36, and the avirulent strain, M, were three of those studied previously (Thorne *et al.*, 1952). Spore suspensions were used for inocula.

Analytical methods. The methods for determining nitrogen, D- and L-glutamic acid, and glutamyl polypeptide were the same as those used previously (Housewright and Thorne, 1950; Thorne *et al.*, 1952). As will be shown later, autoclaving released most, but not all, of the bound D-glutamic acid from the cell. The method for quantitative measurement of glutamyl polypeptide involves the determination of glutamic acid before and after acid hydrolysis. In order to be certain that the bound glutamic acid, as measured in this manner, was actually glutamyl polypeptide, the peptide was isolated in a number of experiments. This was done by ion exchange procedures which will be described in a later publication. The isolated peptide gave only D-glutamic acid upon acid hydrolysis, and there was good agreement between the results obtained by the isolation procedure and by the direct hydrolysis method. Usually in routine analyses the whole culture rather than filtrate from the autoclaved culture was hydrolyzed. The amount of L-glutamic acid derived from the cells by this procedure, and thus assayed as peptide glutamic acid, was not large enough to affect the results significantly.

Glucose was determined by the method of Shaffer and Somogyi (1933).

Medium and conditions. The medium was a modification of the synthetic medium of Brewer

et al. (1946). It contained the following in grams per liter:

DL-alanine	0.45	L-tryptophan	0.26
L-arginine	0.52	DL-valine	0.29
DL-aspartic acid	0.33	K_2HPO_4	5.23
L-cystine	0.06	KH_2PO_4	4.08
L-glutamic acid	0.73	$MgSO_4 \cdot 7H_2O$	0.049
Glycine	0.15	$MnSO_4 \cdot H_2O$	0.015
L-histidine·HCl	0.48	$FeSO_4 \cdot 7H_2O$	0.014
DL-isoleucine	0.66	$CaCl_2 \cdot 2H_2O$	0.055
DL-leucine	0.66	$(NH_4)_2SO_4$	1.0
DL-lysine	0.15	Guanine	0.03
DL-methionine	0.15	D-ribose	0.12
DL-phenylalanine	0.33	Glucose	3.6
L-proline	0.58	Thiamin·HCl	0.02
DL-serine	0.11		mg per liter
DL-threonine	0.60	pH, 7.0–7.2 with NaOH	

Any changes made in the medium are given in the text.

Cotton-plugged 500 ml Erlenmeyer flasks containing 100 ml of medium were used when cultures were grown aerobically on the shaker. When cultures were grown in rubber stoppered flasks, 500 ml suction flasks were used. The side arm of each flask was equipped with a short piece of rubber tubing and a screw clamp. After the medium was sterilized by autoclaving at 15 pounds steam pressure for 20 minutes, cooled, and inoculated, a solution of $NaHCO_3$, which was sterilized by filtration, was added to give the desired concentration. The flasks then were stoppered tightly and allowed to incubate at 37 C on a reciprocating shaker (3 inch strokes, 90 to 100 per minute). After the desired incubation time, usually 72 hours, any accumulated pressure was released from the side arm through a tube passing into a disinfectant to prevent the scattering of infective aerosols. Samples then were taken for analysis.

For growing cultures under forced aeration 2 liter bottles were fitted each with a rubber stopper equipped with a sintered glass sparger of medium porosity, a sampling tube, and two cotton filters to sterilize the incoming and exhaust air. The sampling tube led to the bottom of a 50 ml side arm test tube. Usually 1,000 ml of medium were used, and it was sterilized along with a small amount of DC Antifoam A[1] in the assembled apparatus by autoclaving 20 minutes at 15 pounds steam pressure. The rubber stopper holding the sparger and tubes was secured by wire, and all openings were sealed with Fisher Pyseal Cement to prevent the escape of aerosols. When $NaHCO_3$ was used, it was sterilized by filtration and added with the inoculum through the sampling tube by applying air pressure to the side arm test tube. Incubation was at 37 C and samples were taken at various intervals.

[1] Product of Dow Corning Corporation, Midland, Michigan.

TABLE 1

*Effect of $NaHCO_3$ on peptide production by Bacillus anthracis, strain 994, in tightly stoppered shaken flasks**

$NaHCO_3$	RESIDUAL FREE GLUTAMIC ACID	BOUND GLUTAMIC ACID (PEPTIDE)
per cent	*mg per ml*	*mg per ml*
0	0.37	0.22
	0.39	0.28
0.2	0.37	0.70
	0.40	0.72
0.5	0.19	0.77
	0.18	0.74
0.8	0.29	0.49
	0.42	0.28
1.0	0.43	0.23
	no growth	—

* Incubation time, 40 hours. Results are for duplicate flasks.

RESULTS

Effect of $NaHCO_3$ and CO_2. Table 1 shows the effect of $NaHCO_3$ on peptide production by strain 994 in tightly stoppered shaken flasks. Very little peptide was produced when no $NaHCO_3$ was added to the medium. Similar results were obtained with strain M-36. For most of the later experiments 0.5 per cent $NaHCO_3$ was chosen as the level to be used. Data from experiments not reported here show that in the absence of added $NaHCO_3$ more peptide was produced in tightly stoppered flasks than in cotton-plugged flasks. Apparently the CO_2 produced by the organisms was sufficient to effect some peptide synthesis. The avirulent strain, M, produced high yields when grown in cotton-plugged flasks with no addition of

$NaHCO_3$. When cultures of strains 994, M-36, or M were incubated statically, growth was rather sparse and very little peptide was produced. No growth occurred when conditions were made completely anaerobic by flushing out the flasks with nitrogen.

In general, higher and more consistent yields of peptide were produced when the organisms were grown in aerated bottles as shown in table 2. The yields of peptide produced by strain M-36 with and without added CO_2 are compared.

TABLE 2

*Peptide production by Bacillus anthracis, strain M-36, in aerated liquid medium**

HOURS	AERATION GAS	RESIDUAL FREE GLUTAMIC ACID	BOUND GLUTAMIC ACID (PEPTIDE)
		mg per ml	*mg per ml*
0		0.74	
20	air	0.14	0.20
	30% CO_2	0.62	0.05
27	air	0.19	0.16
	30% CO_2	0.16	0.62
33	air	0.08	—
	30% CO_2	0.12	0.93
53	air	0.18	0.13
	30% CO_2	0.10	0.97
75	air	0.14	0.13
	30% CO_2	0.10	1.25

* Aeration rate, 500 ml per minute. Medium which was aerated with 30 per cent CO_2 had 0.75 per cent $NaHCO_3$ added at time of inoculation.

In this experiment the aeration gases were air and air with 30 per cent CO_2. Very little bound glutamic acid was produced when air was the aeration gas, but a high yield resulted when CO_2 was used. Other experiments showed little differences in peptide yields when the amount of CO_2 in the air-CO_2 mixture was varied from 25 to 50 per cent. The medium in bottles which were aerated with air-CO_2 contained 0.5 to 0.75 per cent $NaHCO_3$. When the bicarbonate containing medium was aerated with air, the pH rose very high as the bicarbonate decomposed, and there was poor growth and little peptide production. High yields of peptide were obtained also under air-CO_2 aeration when $NaHCO_3$ was omitted, and the medium was adjusted after autoclaving to pH 8.5 to 9.0 with NaOH. Under these conditions or when $NaHCO_3$ was added, the pH during growth was 7.0 to 7.5. When $NaHCO_3$ was omitted and no pH adjustment was made, the organisms grew well under air-CO_2 aeration, but little or no peptide was produced. Under these conditions the pH during growth was approximately 6.0. Strains 994 and M-36 produced little or no peptide in the absence of added CO_2, regardless of the pH of the medium. As will be shown later, the avirulent strain, M, produced high yields of peptide under aeration with air.

Effect of various amino acids. Only three of the amino acids in the synthetic medium are absolutely essential for growth of strains 994, M-36, and M. They are leucine, valine, and methionine. The organisms did not grow when any one of these was omitted from the medium. When each of the other amino acids was omitted, one at a time, there was not much effect on growth. It was only when various pairs and combinations of these amino acids were omitted that growth was affected significantly. When each of the amino acids which were not required for growth was tested for effect on peptide synthesis, it was found that DL-isoleucine, DL-phenylalanine, and L-glutamic acid were the only ones which affected the peptide yield significantly. When any of the other amino acids was omitted, with the exception of those required for growth, there was little or no effect on peptide yield.

Table 3 shows the effect of L-glutamic acid, DL-phenylalanine, and DL-isoleucine on peptide production by strain 994 in tightly stoppered shaken flasks. Results obtained with strain M-36 were similar. There was poor utilization of glutamic acid, and much less peptide was synthesized when isoleucine was omitted. When phenylalanine was omitted, free glutamic acid accumulated and the peptide yield was low. This free glutamic acid was the L-isomer. Although the data in table 3 show no effect of omitting glutamic acid, the results of other experiments were variable. Usually the initiation of growth was much slower in the absence of added glutamic acid and peptide yields varied greatly even among replicates in a single experiment. In experiments using $C^{14}O_2$ with strains

994 and M-36, which will be reported in a later publication, it was desirable to use very little, if any, glutamic acid since it was learned that added glutamic acid resulted in the incorporation of less $C^{14}O_2$ in the peptide and a lower specific activity of the peptide glutamic acid. Therefore, experiments were done to determine whether very small amounts of L-glutamic acid were adequate. Amounts as low as 50 to 100 μg per ml resulted in as consistently good initiation of growth and peptide yields as higher amounts. The addition of D-glutamic acid to the medium had no effect on peptide yield.

TABLE 3

*Effect of glutamic acid, phenylalanine, and isoleucine on peptide production by Bacillus anthracis, strain 994, in shaken flasks**

AMINO ACID OMITTED	RESIDUAL FREE GLUTAMIC ACID	BOUND GLUTAMIC ACID (PEPTIDE)
	mg per ml	*mg per ml*
None	0.38	0.67
DL-Isoleucine	0.77	0.27
DL-Phenylalanine	1.30	0.28
L-Glutamic acid	0.08	0.62

* Tightly stoppered shaken flasks, 0.8% $NaHCO_3$. Incubation time, 70 hours. Results are averages of 4 experiments.

The effect of isoleucine on strains 994 and M-36 in aerated bottles was the same as in tightly stoppered shaken flasks. However, the omission of phenylalanine under these more aerobic conditions had little or no effect on peptide yield. Table 4 shows the effect of glutamic acid, phenylalanine, and isoleucine on peptide production by strain M-36 in aerated bottles. The yield of peptide in the absence of isoleucine was less than half that in the complete medium, but yields in the absence of phenylalanine or glutamic acid were practically the same as in the control. Growth was retarded in the absence of glutamic acid as is evidenced by the data on both cell nitrogen and disappearance of glucose. However, the yield of peptide at the end of 83 hours was as high as that when glutamic acid was included in the medium. The final amount of cell nitrogen was practically the same in the samples which had isoleucine, phenylalanine, or glutamic acid omitted, but the amount of peptide produced in the absence of isoleucine was much less than in the other samples. The cell nitrogen was highest in the complete medium, although the peptide yield in this medium was about the same as that obtained when phenylalanine or glutamic acid was omitted. Thus, it is difficult, if not impossible, to establish a correlation between peptide yield and growth as measured by cell nitrogen. Capsulated cells contain more nitrogen per cell than noncapsulated cells, and the percentage of peptide free in the medium varies with the age of the culture.

TABLE 4

*Effect of glutamic acid, phenylalanine, and isoleucine on peptide production by Bacillus anthracis, strain M-36, in aerated bottles**

AMINO ACID OMITTED	HOURS	RESIDUAL FREE GLUTAMIC ACID	BOUND GLUTAMIC ACID (PEPTIDE)	GLUCOSE	CELL NITROGEN
		mg per ml	*mg per ml*	*mg per ml*	*mg per ml*
None	0	0.74	0	3.50	0
	19	0.58	0.04	2.85	0.01
	27	0.10	0.56	1.14	0.13
	35	0.05	0.89	0.26	0.19
	51	0.05	1.20	—	0.14
	83	0.05	1.23	—	0.18
DL-Isoleucine	0	0.74	0	3.50	0
	19	0.52	0.01	1.84	0.03
	27	0.45	0.35	1.78	0.08
	35	0.53	0.47	0.28	0.08
	51	0.70	0.49	—	0.10
	83	0.72	0.54	—	0.12
DL-Phenylalanine	0	0.74	0	3.50	0
	19	0.50	0.14	1.75	0.01
	27	0.47	0.33	1.40	0.07
	35	0.47	0.53	0.26	0.09
	51	0.68	0.95	—	0.13
	83	0.65	1.17	—	0.13
DL-Glutamic acid	0	0	0	3.50	0
	19	0	0	3.15	0.01
	27	0	0	—	0.01
	35	0	0.35	1.46	0.09
	43	0.10	0.75	0.33	0.13
	51	0.07	1.03	—	0.13
	83	0.09	1.25	—	0.13

* 0.75 per cent $NaHCO_3$. Aerated with 30 per cent CO_2. Cells were washed 3 times with distilled H_2O before nitrogen analyses were done.

Table 5 shows the effect of various levels of isoleucine and phenylalanine on peptide production by the avirulent strain, M. The peptide yield was very low when either of these was omitted from the medium. The optimum level of isoleucine appeared to be around 0.11 to 0.33 mg per ml. When amounts below or above this level were used, the peptide yield was reduced. After this was learned, the amount of isoleucine used routinely in all media was reduced from 0.66 to 0.33 mg per ml. The results show no significant difference in peptide yields when the amount of phenylalanine was varied over a range

TABLE 5

*Effect of isoleucine and phenylalanine on peptide production by Bacillus anthracis, strain M, in shaken flasks**

AMINO ACID ADDED		RESIDUAL FREE GLUTAMIC ACID	BOUND GLUTAMIC ACID (PEPTIDE)
DL-Phenylalanine	DL-Isoleucine		
mg per ml	*mg per ml*	*mg per ml*	*mg per ml*
0.33	0	1.96	0.27
0.33	0.06	0.68	0.64
0.33	0.11	0.24	1.69
0.33	0.33	0.38	1.55
0.33	0.66	0.39	1.01
0.33	1.32	0.22	0.79
0	0.66	1.82	0
0.05	0.66	0.33	0.96
0.10	0.66	0.37	1.11
0.20	0.66	0.28	1.12
0.33	0.66	0.39	1.01

* 100 ml medium in 500 ml cotton-plugged Erlenmeyer flasks. Incubation time, 70 hours. Results are averages of duplicate flasks.

of 0.05 to 0.33 mg per ml. However, it should be pointed out that the various amounts of phenylalanine were tested with isoleucine at a level above the optimum. When both D- and L-isoleucine were tested, only the L-form was active. D-Phenylalanine was not tested, but the L-form was active.

The effect of D- and L-glutamic acid on peptide production by strain M is shown in table 6. D-Glutamic acid did not replace the L-form, and increasing the level of glutamic acid above the regular level (0.73 mg per ml) in the medium resulted in increased peptide yield. This was shown to be true in many experiments.

Effect of autoclaving glucose with the medium. Glucose was required for maximum peptide production by all three strains. The amount of glucose in the regular medium was 3.6 mg per ml. Higher amounts did not improve the peptide yield although the organisms were able to use up to 10 mg per ml during the usual incubation period. When glucose was omitted, initiation of growth was erratic and retarded.

Table 7 shows that strains 994 and M-36 produced much higher yields of peptide when glucose was autoclaved in the medium than when it was sterilized separately, either by filtration or autoclaving. The opposite was true for strain M.

TABLE 6

*Effect of D- and L-glutamic acid on peptide production by Bacillus anthracis, strain M, in shaken flasks**

GLUTAMIC ACID ADDED	RESIDUAL FREE GLUTAMIC ACID	BOUND GLUTAMIC ACID (PEPTIDE)
mg per ml	*mg per ml*	*mg per ml*
L-Glutamic acid		
0	0.36	0.23
0.73	0.26	0.59
1.46	0.22	0.96
2.19	0.39	0.94
2.92	0.61	0.92
D-Glutamic acid		
1.46	1.48	0.37

* 100 ml medium in 500 ml cotton-plugged Erlenmeyer flasks. Incubation time, 70 hours. Results are averages of duplicate flasks.

In order to obtain high yields of peptide with this strain it was necessary to sterilize the glucose separately. With all of the strains the amount of growth did not appear to be affected by the manner of sterilization of glucose.

Distribution of peptide between the cells and culture filtrate. We have confirmed the observation of others (Bovarnick, 1942; Ivánovics and Bruckner, 1937) that the glutamyl peptide produced by *Bacillus subtilis* occurs in the medium free from the cells. In our experiments most of the peptide produced by *B. anthracis* occurred bound to or inside the cell, and the percentage of total peptide free in the culture filtrate increased as the culture aged. Data are shown in table 8. Bound glutamic acid was determined on samples of culture filtrate, filtrate from cultures that had

been autoclaved, and whole cultures. Most of the peptide which was bound to the cells was released upon autoclaving. However, the data shown here (experiment 3 in table 8) and those obtained in many other experiments show that cells contained bound D-glutamic acid even after autoclaving at 15 pounds steam pressure for 45 minutes.

TABLE 7

*Effect of glucose on peptide production by strains 994, M-36, and M in shaken flasks**

STRAIN	GLUCOSE	RESIDUAL FREE GLUTAMIC ACID	BOUND GLUTAMIC ACID (PEPTIDE)
		mg per ml	*mg per ml*
994	Added aseptically	0.19	0.50
	Autoclaved in medium	0.09	0.88
	None	0.56	0.37
M-36	Added aseptically	0.25	0.47
	Autoclaved in medium	0.15	0.79
M	Added aseptically	0.18	0.94
	Autoclaved in medium	0.12	0.21
	None	1.06	0.25

* Strains 994 and M-36 were grown in tightly stoppered shaken flasks with 0.5 per cent $NaHCO_3$ and 0.73 mg L-glutamic acid per ml. Strain M was grown in cotton-plugged shaken flasks with 1.46 mg L-glutamic acid per ml and no $NaHCO_3$.

TABLE 8

*Distribution of peptide between the cells and culture filtrate of strain M-36**

EXPERIMENT	HOURS	BOUND GLUTAMIC ACID						
		Culture filtrate		Filtrate from autoclaved culture			Whole culture	
		mg per ml	*per cent of total*	*mg per ml*	*per cent of total*	*per cent* D-	*mg per ml*	*per cent* D-
I	27	0.17	32	0.36	70		0.52	
	33	0.36	42	0.66	76		0.86	
	53	0.54	51	0.96	89		1.07	
II	27	0.15	26				0.56	
	43	0.69	57				1.21	
	83	0.87	70				1.23	
III	40			1.53	78	97	1.97	95

* Grown in bottles aerated with 30 per cent CO_2.

DISCUSSION

Small amounts of isoleucine and phenylalanine were required for maximum peptide production and glutamic acid utilization. However, free L-glutamic acid was synthesized in the absence of these amino acids, the accumulation of free glutamic acid being most marked when phenylalanine was omitted. This indicates that these two amino acids do not function directly in peptide synthesis, i.e., they do not serve as a carbon source for peptide, but that they may be essential for synthesis of one or more enzymes involved in peptide production.

The function of CO_2 or bicarbonate in peptide synthesis by the CO_2 requiring strains is not understood. Studies with $C^{14}O_2$, not yet reported, show that large amounts of CO_2 were incorporated into the glutamic acid molecule of the peptide. If CO_2 functioned only in the synthesis of glutamic acid, one might expect added glutamic acid to replace the CO_2 requirement. Although results with $C^{14}O_2$ show that added glutamic acid did reduce the amount of $C^{14}O_2$ incorporated into peptide, CO_2 or bicarbonate was still necessary for peptide synthesis.

SUMMARY

Conditions for the production of glutamyl polypeptide by *Bacillus anthracis* on a synthetic liquid medium and various factors affecting the yield of peptide are reported. Under the best conditions yields of 1.0 to 1.5 mg per ml were produced.

The following differences were observed in the physiology of the virulent strains, 994 and M-36, and the avirulent strain, M: (1) strains 994 and M-36 required added CO_2 for peptide synthesis while strain M did not; (2) strain M required high levels of L-glutamic acid (1 to 2 mg per ml) for maximum yields of peptide while the other strains did not, although small levels of L-glutamic acid (0.05 to 0.1 mg per ml) did stimulate initiation of growth of these strains and improve the peptide yield in many experiments; and (3) strains 994 and M-36 produced more peptide when glucose was autoclaved with the medium while for high yields with strain M it was necessary to sterilize glucose separately.

Only three amino acids, leucine, valine, and methionine, were essential for growth. Isoleucine and phenylalanine, while not essential for growth,

were essential for maximum peptide production under conditions reported here.

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