BIKEN'S JOURNAL 2, 165-176 (1959)

Immunochemical Studies of Bacterial Glutamyl Polypeptides

SAYAKA UTSUMI, MITSUO TORII, OSAMU KURIMURA, HIROSHI YAMAMURO AND TSUNEHISA AMANO

Department of Bacteriology, Osaka University Medical School, and Department of Immunology, Research Institute for Microbial Diseases, Osaka University.

(Received for publication, September 30, 1959)

SUMMARY

The antibodies homologous to glutamyl polypeptide (GPP) of *Bacillus megaterium* were produced in rabbits by long-term immunization with killed encapsulated cells.

The chemical structure of GPP of *Bacillus subtilis* was reconfirmed by immunochemical analysis to be a mixture of L-γ-GPP and D-γ-GPP, while that of *B. megaterium* was shown to be mainly a γ-copolymer of L- and D-glutamic acid.

The antibodies evoked by GPP of *B. megaterium* could be classified in three groups; the first was homologous to L-γ-GPP, the second to D-γ-GPP and the last was exclusively specific to the GPP of *B. megaterium*.

INTRODUCTION

Tomcsik *et al.* (1933) showed that the rabbit or horse antisera, prepared by immunization with a heat killed bacterial suspension of encapsulated *Bacillus anthracis*, contained specific antibodies capable of reacting with the purified capsular substance (*i. e.* γ-D-glutamyl polypeptide) of the organism. In addition, these antibodies were shown to cross-react with purified glutamyl polypeptides of *Bacillus subtilis* and of other nonpathogenic bacilli in the precipitin reaction (Ivánovics, 1937). It is well known that the glutamyl polypeptide of *B. subtilis* (S-GPP) consists of D- and L-glutamate (Watson *et. al.*, 1947, Thorne *et. al.*, 1954, Torii, 1956) and that of *B. anthracis* (A-GPP) is a D-glutamyl polypeptide (Ivánovics and Bruckner, 1937, Hanby and Rydon, 1946, Torii, 1956). There are two problems before it is possible to explain Tomcsik's finding that A-GPP antibodies could react with S-GPP. The first is to see whether A-GPP antibodies can react only with short γ-peptides of D-glutamate in S-GPP or secondly whether they can also react with those of L-glutamate as well as of D-glutamate. The question was solved by Bruckner *et. al.* (1958) and Ivánovics (1958) in their immunochemical studies on synthetic γ-glutamyl polypeptides. They demonstrated that A-GPP antibodies could precipitate synthetic γ-D-GPP and also alternative γ-copolymers of D- and L-glutamate but not γ-L-GPP. From these results S-GPP could be assumed to be either a mixture of γ-L-GPP and γ-D-GPP or γ-copeptides of

The following abbreviations are used: GPP, glutamyl polypeptide; S-GPP, glutamyl polypeptide of *B. subtilis*; M-GPP, glutamyl polypeptide of *B. megaterium*; A-GPP, glutamyl polypeptide of *B. anthracis*; AbN, antibody N; AgN, antigen N.

D- and L-glutamate. The chemical structure of S-GPP was further shown to be a mixture of γ-L-GPP and γ-D-GPP by Thorne and Leonard (1958).

For three years the authors have attempted to obtain antibodies in rabbits elicited by capsular glutamyl polypeptides of *Bacillus megatarium* (M-GPP). After continued immunizations, M-GPP antibodies became available. Immunological studies were made to clarify the immunochemical difference between S-GPP and M-GPP, which has almost the same nature as S-GPP as regards the optical isomers of glutamic acid and the peptide linkages (Torii, 1959), but is soluble at an acid pH unlike S-GPP. The acid-insolubility of S-GPP was the main subject of the studies of Thorne and Leonard (1958), in which S-GPP was shown to be a mixture of γ-L-GPP and γ-D-GPP.

MATERIALS AND METHODS

Strains of microorganisms.—Bacillus anthracis strain "Vollum": The strain was kindly sent to us by Dr. J. Tomcsik. It is highly virulent and produces capsule.

Bacillus megaterium A5: This was also given by Dr. J. Tomcsik. The strain number A5 was tentatively given in this laboratory.

Bacillus subtilis K: This strain was presented from Japan Antibiotics Research Association.

Antigens.—M-GPP: M-GPP was extracted by boiling the encapsulated *B. megaterium* suspension in water. It was purified as the sodium salt according to the method of Bovarnick (1942), who used it for the purification of S-GPP. The L- and D-glutamate contents of the purified material were 50.7 and 49.3 per cent respectively.

A-GPP: A-GPP was extracted by autoclaving from an encapsulated *B. anthracis* suspension and purified as the acid form according to the description of Hanby and Rydon (1946). The preparation used in this report was the same as that used in the previous report on the optical isomers of glutamic acid and was shown to contain D-glutamic acid exclusively (Torii, 1956).

S-GPP-1: This purified preparation (acid form) was kindly supplied by Dr. M. Bovarnick. The percentages of the L- and D-isomers of glutamic acid in the material were 51.2 and 48.8.

S-GPP-2: GPP of *B. substilis* lacking in D-glutamic acid was prepared. *B. substilis* K was grown in the medium "E" containing 1.54×10^{-7} M of Mn^{++} described by Leonard *et al.* (1958). The GPP released into the medium was purified as an acid soluble fraction according to the description of Thorne and Leonard (1958). The percentage of the L-isomer in the total glutamic acid was approximately 90.

Polysaccharide: polysaccharide of *B. megaterium* A5 was prepared according to the method of Tomcsik and Guex-Holzer (1951).

Antisera.—A-GPP antisera: The encapsulated cells of *B. anthracis* "Vollum" were killed by heating at 100°C. for 30 min. Rabbits were immunized with this suspension according to the description of Tomcsik and Ivánovics (1938). The batches of antisera used in this report were No. 16 and 19.

M-GPP antisera: A cell suspension of the encapsulated *B. megaterium* A5 was killed by heating at 100°C. for 15 min. The optical density was adjusted to 0.3 when the sample of the suspension was diluted 1:5 as estimated by a Coleman junior spectrophotometer using a 6–310B cuvette. Each animal received intravenous injections of 0.5 ml., 1.5 ml., 1.5 ml. and 1.5 ml. of the antigen at 3 or 4 days intervals. After 7 to 10 days, a second course of injections was given. 4 doses of 1.5 ml. of the antigen were administered at 3 or 4 days intervals. 7 to 10 days later, a third course of injections was given. After an interval of 7 to 10 days injections were continued. The batch numbers of the antisera used in this study were 101, 102, 103 and 107. No. 102 and 107 were used separately, and No. 101 and 103 were pooled together (No. 101–103).

Quantitative precipitin reaction.—Precipitable antibody nitrogen was determined by the method of McDuffie and Kabat (1956) using a Beckman spectrophotometer. GPP has no absorption at 287 mμ in alkaline solution. The precipitated antibody nitrogen (AbN) of rabbit antibodies was calculated from the optical density (O.D.) by the following formula.

$$\text{AbN } \mu\text{g. per ml.} = \frac{\text{O.D. (at 287 m}\mu \text{ in 0.1 N NaOH)}}{0.0103}$$

Qualitative precipitin reaction.—Precipitin reactions were performed as follows. Serial 1:2 dilutions of antisera in 1 per cent saline solution of *gummi arabicum* were made. 0.05 ml. of these diluted antisera was placed in a 2 mm. diameter tube. On to the surfaces of these diluted antisera were placed 0.05 ml. of antigen solution diluted to 1:10000. The tubes were kept at 15°C. for from 2 to 4 hours.

Quantitative determination.—Total glutamic acid: The samples containing GPP were hydrolyzed in sealed tubes with 6 N HCl at 100°C. for 6 hours. This hydrolysis was enough to completely hydrolyze γ-glutamyl polypeptides. Total glutamic acids were determined by Sanger's DNP-method or the ninhydrin method according to the description of Troll and Cannan (1953). In the latter method the part of paper corresponding to the location of glutamic acid on the paper electro-chromatograms was cut off and the glutamic acid was extracted, and determined with ninhydrin.

L-Glutamic acid: L-glutamic acid decarboxylase of *E. coli* was used and the carbon dioxide liberated was estimated manometrically in a Warburg apparatus.

D-Glutamic acid: D-glutamic acid was calculated by subtracting the L-glutamic acid from the total glutamic acid, or it was directly determined manometrically with D-glutamic acid oxydase prepared from *Aspergilus ustus* according to the method of Mizushima *et al.* (1956).

RESULTS

Detection of M-GPP antibodies in antisera immunized with encapsulated B. megaterium: Ivánovics (1937) reported that A-GPP antibodies could cross-react with S-GPP. However, following problems have not yet been clarified, whether the antibodies evoked by D-glutamyl-γ-polypeptide of *B. anthracis* could react with short γ-peptides of L-glutamic acid as well as with those of D-glutamic acid of S-GPP, or whether they could only react with short γ-peptides of D-glutamic acid of S-GPP. The problem was made clear by Bruckner *et. al.* (1958) and Ivánovics (1958). They used synthetic γ-glutamyl polypeptides for the immunochemical studies of A-GPP antibodies. A-GPP antibodies could react with synthetic D-glutamyl-γ-polypeptide and also with alternative γ-copolymers of D- and L-glutamic acid. However, synthetic L-glutamyl-γ-polypeptide was not precipitated by these antibodies.

As the GPP antibodies so far studied were only homologous to D-glutamyl-γ-polypeptide, an antiserum containing antibodies exclusively homologous to L-glutamyl-γ-polypeptide was required. It was impossible to obtain because an organism producing a capsule exclusively consisting of the L-glutamyl-γ-polypeptide had not yet been found. The present studies were begun to obtain an antiserum containing both kinds of antibodies; the one homologous to the L-glutamyl-γ-polypeptide and the other to the D-glutamyl-γ-polypeptide. Encapsulated cells of *B. megaterium* A5 were tested as an immunizing antigen. The antigencity of the GPP of *B. megaterium* was demonstrated by us (Utsumi *et al.*, 1958) and also by Vennes and Gerhardt (1959).

TABLE 1. GPP Antibody Formation in Rabbits Injected with *B. megaterium*

Antigen \ Rabbit No.	101	102	103	107
	after 4 courses	4 courses	4 courses	4 courses
Polysac.	+++	+++	+++	+++
M-GPP	+	−	−	−
S-GPP	+	−	−	−
A-GPP	−	−	−	−
	after 0.5 course	0.5 course	1.0 course	0.5 course
Polysac.	+++	+++	+++	+++
M-GPP	+±	+	+±	+±
S-GPP	+±	+	+±	+±
A-GPP	+	−	−	+±
	after 1.0 course	0.5 course	0.5 course	1.0 course
Polysac.	++++	++++	+++	+++
M-GPP	++	+±	++	++
S-GPP	++	+±	++	++
A-GPP	+	+±	+	++
	after 1.0 course	1.0 course	1.5 course	1.5 course
Polysac.	++++	++++	++++	++++
M-GPP	++++	++++	+++	++++
S-GPP	+++	++++	+++	+++
A-GPP	++++	+++	+++	+++
Total term	8 months	8 months	8 months	6 months

Each antigen was diluted 1 : 10000 with saline (pH=7.0).

− : no detectable precipitation by the ring test;

± : positive precipitin reaction detectable with non-diluted serum;

\+ : positive precipitin reaction detectable with 1 : 2–1 : 4 diluted serum;

+± : positive precipitin reaction detectable with 1 : 4–1 : 8 diluted serum;

++ : positive precipitin reaction detectable with 1 : 8–1 : 16 diluted serum;

+++ : positive precipitin reaction detectable with 1 : 16–1 : 32 diluted serum;

++++ : positive precipitin reaction detectable with more than 1 : 32 dilution.

M-GPP antibodies were produced in a significant amount only after long-term immunization with killed encapsulated organisms as shown in Table 1. It can also be seen from the table that polysaccharide(s) antibodies were produced even at an early stage of immunization and that antibodies capable of precipitating M-GPP as well as S-GPP appeared earlier than those precipitating A-GPP in three of four rabbits listed. The potency of the latter kind of antibodies increased in the latter stage of long-term immunization to the same titer as that of the former. Therefore the antibodies evoked by M-GPP in these rabbits were heterogeneous in specificity.

M-GPP antisera absorbed with encapsulated B. anthracis:

To ascertain the heterogeneity in specificity of M-GPP antisera described above, attempts were made to remove the antibodies capable of precipitating A-GPP. M-GPP antisera were absorbed with heat killed encapsulated cells of *B. anthracis*.

A thick bacterial suspension of encapsulated *B. anthracis* was heated at 100°C. for 30 min., washed and lyophilized. 10 mg. of lyophilized bacterial cells were added to 1.0 ml. of M-GPP antiserum 102 and to antiserum 101–103. The mixtures were shaken at 37°C. for 30 min. and the supernatant after centrifugation was again mixed with 5 mg. of the cells. The suspension was kept at 4°C. overnight and centrifuged. The precipitin reactions of the absorbed supernatants were compared with those of unabsorbed antisera by the ring test. The results are presented in Table 2.

TABLE 2. Serological Activity of Immune Sera Absorbed by Capsulated *B. anthracis*

Serum	Serum / Antigen	Absorbed serum						Nonabsorbed serum			
		1 : 1	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 4	1 : 8	1 : 16	1 : 32
Serum 102	M-GPP	++++	+++	++	++	+	−	++	++	+	+
	S-GPP	++++	++	+	+	−	−	++	+	+	+
	A-GPP	−	−	−	−	−	−	++	+	+	±
Serum 101–103	M-GPP	++	++	+	+	−		++	+	+	±
	S-GPP	+	−	−	−	−		++	+	±	−
	A-GPP	—	−	−	—	−		++	++	+	±

As can be seen from the table, antibodies capable of precipitating A-GPP could not be detected in the absorbed antisera, although unabsorbed antisera possessed almost the same potency towards A-GPP as towards M-GPP or S-GPP.

To see if the absorbed antisera still contain antibodies capable of precipitating M-GPP or S-GPP, absorbed antiserum 102 was mixed with M-GPP solution and the precipitates were analyzed for GPP.

2.0 mg. of M-GPP, dissolved in 2.0 ml. of saline, were added to 25 ml. of absorbed antiserum 102, and incubated at 4°C. overnight. The resulting precipitates were centrifuged, washed three times with 25 ml. of saline, dissolved in 25 ml. of 0.1 N NaOH and diluted to 100 ml. with water. As GPP cannot be precipitated by 10 per cent trichloroacetic acid, 25 ml. of 50 per cent trichloroacetic acid solution were added to the solution.

After being kept at 0°C. for 2 hours, the mixture was centrifuged in a refrigerated centrifuge. Trichloroacetic acid was removed by ether. After evaporation of the remaining ether, an equal volume of 12 N HCl was added and the mixture hydrolyzed in a sealed ampule at 100°C. for 6 hours. After hydrolysis the solution was concentrated and dried *in vacuo*. The dried residue was dissolved in water and neutralyzed. Ninhydrin positive substances other than glutamate were not detected by paper chromatography. Total glutamic acid was estimated by the DNP-glutamic acid method (Sanger, 1945).

Recovery from the precipitates was 0.98 mg. (49 per cent). Therefore the absorbed M-GPP antisera still contain GPP antibodies and hence M-GPP antibodies are heterogeneous in specificity.

As these antisera were prepared by immunizing rabbits with encapsulated *B. megaterium*, they contain polysaccharide(s) antibodies in addition to M-GPP antibodies as shown in Table 1. After antisera had been absorbed repeatedly with heat killed encapsulated cells of *B. megatrium*, the absorbed antisera were subsequently tested with three kinds of GPP and polysaccharide(s) of *B. megaterium* A5. A protocol of this experiment need not be presented here. Let it suffice to say that absorbed antisera did not contain GPP antibodies and that polysaccharide(s) antibodies were still retained almost unchanged in potency.

Acid solubility of M-GPP and S-GPP:

It can be seen in Table 2 that absorbed antiserum 101-103 could precipitate very little S-GPP but still had marked potency towards homologous M-GPP. Therefore there seems to be a structural difference between M-GPP and S-GPP, while they are almost equal in L- and D-glutamate content and both are solely constituted by γ-peptide linkages (Torii, 1959).

To verify this assumption, the solubilities of M-GPP and S-GPP at an acid pH were compared according to the description of Thorne and Leonard (1958).

100 mg. of S-GPP (D-glutamic acid 48.8 per cent, L-glutamic acid 51.2 per cent) were suspended in 2.0 ml. of water. The pH was adjusted to 7.0 with dilute NaOH, and S-GPP was dissolved. 2.0 ml. of 2 N HCl were added and the solution was kept at 0°C. for 20 hours. The resulting precipitates were centrifuged, washed twice with a small amount of water and dried in a desiccator. The dried material weighed 81 mg. (81 per cent). 100 mg. of M-GPP (D-glutamic acid 49.6 per cent, L-glutamic acid 50.4 per cent) were dissolved in 1.0 ml. of water and to this solution 1.0 ml. of 2 N HCl was added. It was kept at 0°C. for 20 hours. Almost no precipitate was found after centrifugation. The supernatant was neutralyzed and dialyzed against distilled water until Cl^- could not be detected. The dialyzed solution was lyophilized. The yield was 79 mg. (79 per cent). The contents of the optical isomers of glutamic acid were 49.3 per cent (D) and 50.7 per cent (L) respectively. The analytical values are shown in Table 3.

TABLE 3. GPP Used as Antigens

GPP	Form of GPP	L-Glutamic acid residue	D-Glutamic acid residue	Solubility
M-GPP	Na salt	50.7%	49.3%	Acid soluble
S-GPP	Free acid	51.2%	48.8%	Acid insoluble
A-GPP	Free acid	2.1%*	97.9%	Acid soluble

* This amount of L-glutamic acid estimated may be formed from D-glutamate during hydrolysis.

From the difference in acid solubility shown in this experiment, the chemical structure of M-GPP must differ from that of S-GPP, which is a mixture of L-GPP and D-GPP. Hence, M-GPP can be assumed to be copolymers of L- and D-glutamate.

Further purification of L-γ-GPP:

As L-γ-GPP was required for tests on supernatants in the quantitative precipitin reactions, the crude L-γ-GPP (S-GPP-2) was further purified by acid precipitation and by precipitation with A-GPP antiserum.

100 mg. of S-GPP-2, containing 90 per cent L-glutamic acid as described above, were dissolved in 2.0 ml. of water and 2.0 ml. of 2N HCl were added. It was kept at −10°C. for 30 min. The precipitates were removed by centrifugation. The supernatant was again kept at −10°C. for 30 min. The same procedure was repeated four times. The final supernatant, which gave no more precipitate at −10°C., was neutralized to pH 7.0, dialyzed against water and lyophilized. It contained approximately 100 per cent L-glutamic acid. A concentrated solution of this material still gave a slight precipitate with A-GPP antiserum 16. 5 mg. of the material were dissolved in 5 ml. of water. To this solution were added 10 ml. of A-GPP antiserum 16. The mixture was kept at 0°C. for 24 hours and subsequently centrifuged. The supernatant contained excess A-GPP antibodies. The supernatant was diluted to 150 ml. with water. 25 ml. of trichloroacetic acid solution were added and the mixture was

then centrifuged. The supernatant thus obtained was treated with ether to remove trichloroacetic acid and dialyzed after neutralization.

The lyophilized material gave no precipitate with A-GPP antisera and almost 100 per cent L-glutamic acid was found after hydrolysis. D-Glutamic acid could not be detected by D-glutamic acid oxidase.

Quantitative precipitin reactions:

To substantiate the structural difference of M-GPP from S-GPP, quantitative precipitin reactions were made.

GPP used as antigens in these experiments were the same preparations as shown in Table 3.

First, the homologous system of M-GPP and its antisera was studied.

Varying quantities of M-GPP (0.5 to 46.5 μgN), dissolved in 0.5 ml. of saline, were pipetted into 10 ml. calibrated centrifuge tubes. 0.5 ml. of M-GPP antiserum 102 were then added to each tube. A serum control containing 0.5 ml. of the serum and 0.5 ml. of saline was included. The tubes were stoppered and incubated at 37°C. for 30 min. and then kept at 0°C. for 2 weeks.

The tubes were agitated daily and were finally centrifuged in the cold. The tubes containing precipitates were washed three times with 1 ml. aliquots of chilled saline and recentrifuged. Two drops of 0.5 N NaOH were added and the volume was brought up to 3.0 ml. with 0.1 N NaOH. The mixture was then incubated at 37°C. for 30 min. and then kept at 0°C. overnight. After the precipitates had been completely dissolved, the precipitated antibody N was estimated according to the method of McDuffie and Kabat (1956).

The supernatants were tested for antibody excess with homologous M-GPP as well as S-GPP and A-GPP. The antigen excess test was also conducted with the same antiserum 102 and A-GPP antiserum 16. The results are presented in Table 4.

Similar experiments were carried out with the same antiserum 102 and A-GPP or S-GPP The results are presented in Table 5 and 6. The results of these experiments are also summarized in Fig. 1.

TABLE 4. Quantitative Precipitin Reaction Between M-GPP and Serum 102

Tube No.	M-GPP added N μg.	Ppt. AbN from 0.5 ml. of serum μg.	AbN/AgN	Test on supernatant with			
				Serum 102	M-GPP	S-GPP & A-GPP	Serum 16
3	0.5	83.9	168	−	+++	+++	−
4	1.9	122.3	65	−	++	++	−
6	3.7	207.9	56	−	++	++	−
8	5.6	297.0	51	−	+	+	−
9	7.4	364.1	49	−	+	+	−
10	9.3	433.9	47	−	+	+	−
11	11.6	550.5	47	−	−	−	−
12	14.0	559.2	40	++	−	−	++
13	18.6	503.7	27	++	−	−	++
14	23.4	470.0	20	+++	−	−	+++
15	46.5	372.8	8	+++	−	−	+++

TABLE 5. Quantitative Precipitin Reaction Between A-GPP and Serum 102

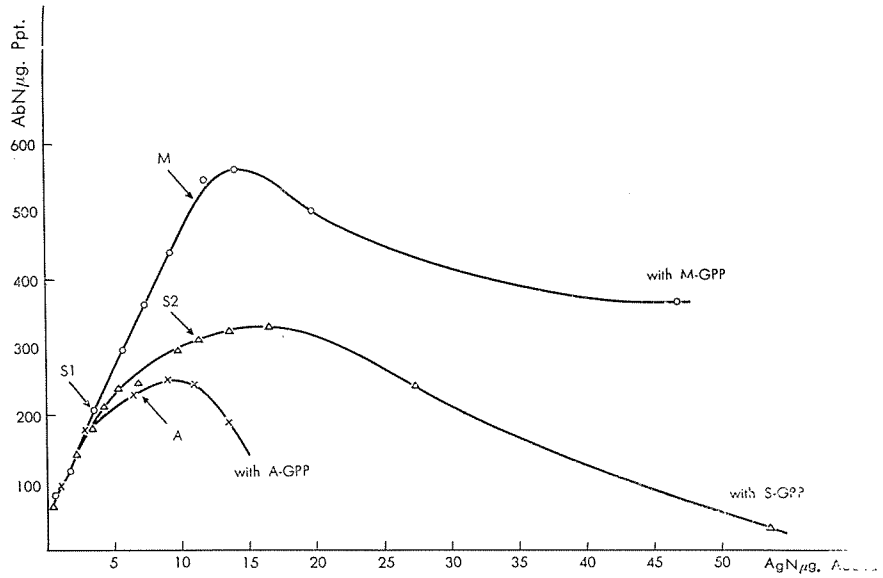
Tube No.	A-GPP added N μg.	Ppt. AbN from 0.5 ml. of serum μg.	AbN/AgN	Test on supernatant with				
				Serum 102	A-GPP	M-GPP	S-GPP	Serum 16
1	0.2	18.6	93	−	+++	+++	+++	−
2	1.1	93.4	85	−	++	+++	+++	−
3	2.7	174.2	65	−	+	+++	++	−
4	5.5	207.4	38	++	−	+++	++	++
5	6.5	227.2	35	++	−	+++	+	++
6	8.7	251.4	29	+++	−	+++	+	++
7	10.9	245.8	22	+++	−	+++	+	++
8	13.6	187.0	14	+++	−	+++	+	+++
9	16.4	(loss)	—	+++	−	+++	+	+++

As can be seen in Table 4, the system of M-GPP and antiserum 102 was a homologous one, and the equivalence point was located in tube No. 11. The results of tests on supernatants shown in Table 5 clearly demonstrate that the equivalence point of A-GPP and D-γ-GPP antibodies in M-GPP antiserum was located between tube No. 3 and 4. Thus, excess antibody was found in tubes 1 to 3 with A-GPP and excess antigen (A-GPP) was found in tubes 4 to 9 being quite parallel with the M-GPP antiserum 102 as well as with the A-GPP antiserum 16. However, excess antibody could be detected in all these supernatants tested with M-GPP as well as S-GPP. From these results, it was confirmed that M-GPP antibodies are heterogeneous in specificity.

TABLE 6. Quantitative Precipitin Reaction Between S-GPP and Serum 102

Tube No.	S-GPP added N μg.	Ppt. AbN from 0.5 ml. of serum N μg.	AbN/AgN	Test on supernatant with					
				Serum 102	L-γ-GPP	S-GPP	M-GPP	A-GPP	Serum 16
1	0.05	53.9		−	++	+++	+++	+++	−
2	0.2	66.4	332	−	+	+++	+++	+++	−
3	0.5	71.9	144	−	+	+++	+++	+++	−
4	2.2	140.4	64	−	+	++	+++	+++	−
5	3.3	177.7	54	−	−	++	+++	+++	−
6	4.4	212.5	48	+	−	++	+++	++	−
7	5.5	241.5	44	+	−	++	++	++	−
8	6.5	249.0	38	+	−	+	++	+	−
9	8.7	294.2	34	+	−	+	++	+	−
10	10.9	311.7	28	++	−	−	++	−	+
11	13.6	320.4	24	++	−	−	+	−	+
12	16.4	337.9	21	++	−	−	+	−	++
14	27.3	249.0		+++	−	−	+	−	++
15	54.4	35.5		+++	−	−	+	−	+++

Fig. 1. Quantitative Precipitin Reaction Between M-GPP Antiserum and Bacterial GPP



M, S_1, S_2 and A indicate the equivalence points of M-GPP, L-GPP in S-GPP, D-GPP in S-GPP, and A-GPP respectively.

The results of the tests on supernatants of the system of S-GPP and M-GPP antibodies shown in Table 6, indicate that M-GPP and S-GPP differ in their chemical structure since M-GPP and antiserum 102 was a homogeneous system. The table shows that excess antibody could be detected over all the range tested with M-GPP. However, excess antibody was only found in tubes 1 to 9 with S-GPP quite parallel with A-GPP. As excess D-γ-GPP could be detected with A-GPP antiserum in tubes 10 to 15, the equivalence point of D-γ-GPP in S-GPP and D-γ-GPP antibodies in M-GPP antiserum must be located between tubes 9 and 10. On the other hand, the excess antibody for L-γ-GPP could be found only in tubes 1 to 4. The positive antigen excess tests in tubes 6 to 9 detected with antiserum 102 can be interpreted as a precipitin reaction evoked by excess L-γ-GPP of S-GPP and L-γ-GPP antibodies in M-GPP antiserum 102, since the D-γ-GPP of S-GPP could only be detected in tubes 10 to 15. Therefore, the equivalence point of L-γ-GPP in S-GPP and L-γ-GPP antibodies in M-GPP antiserum must be in tube 5. Therefore S-GPP has two equivalence points corresponding to its L-γ-GPP and D-γ-GPP. These experimental results indicate also that M-GPP antibodies are heterogeneous in specificity and must be divided into three groups: the first is D-γ-GPP antibodies, the second is L-γ-GPP antibodies and the third is exclusively homologous to M-GPP. Probably the third would be elicited by fragments of M-GPP containing γ-copolymers of D- and L-glutamate or by some fixed steric structure which is not present in S-GPP and A-GPP.

Analysis of S-GPP and M-GPP in precipitates:

To show, by appropriate antiserum, the independent presence of both L- and D-γ-GPP in S-GPP the following experiments were performed.

1.0 ml. of S-GPP solution containing 0.9 mg. was added to 7 ml. of M-GPP antiserum 101–103 and incubated at 0°C. for 48 hours. The precipitates formed were thoroughly washed with chilled saline, dissolved in 20 ml. of 0.1 N NaOH and deproteinized with 10 per cent trichloroacetic acid. They were analysed for glutamic acid after hydrolysis. A similar experiment was also made with 1.0 mg. of M-GPP and 10 ml. of M-GPP antiserum. The same experiments were also performed with A-GPP antisera using S-GPP and M-GPP as antigen. The results are presented in Table 7 and 8.

TABLE 7. Analysis of Glutamic Acid Recovered from Specific Precipitates with M-GPP Antiserum 101-103 after Deproteinization

Antigen added	Serum 101–103	Precipitates			Test on supernatant with			
		Total glutamic acid μg.	L-Glutamic acid μg. (%)	D-Glutamic acid μg. (%)	M-GPP & S-GPP	A-GPP	Serum 101–103	Serum 16
M-GPP 1 mg.	10 ml.	497	238 (48)	259 (52)	−	−	+	++
S-GPP 0.9 mg.	7 ml.	394	80 (20)	314 (80)	±	+	+	−

TABLE 8. Analysis of Glutamic Acid Recovered from Specific Precipitates with A-GPP Antiserum 16 and 19.

Antigen added	Serum 16–19	Total glutamic acid μg.	L-Glutamic acid μg. (%)	D-Glutamic acid μg. (%)
S-GPP 2.8 mg.	18 ml.	995	32.0 (3%)	963 (97%)
M-GPP 2.8 mg.	18 ml.	1867	896 (48%)	971 (52%)

As shown in Table 7, the amount of M-GPP added corresponded to a slightly antigen-excess point, where the maximal amount of antibody N was precipitated, and the amount of S-GPP added approximately corresponded to the midpoint between the equivalence point of L-γ-GPP and that of D-γ-GPP in S-GPP. Thus, the midpoint was located in the antigen-excess zone of L-γ-GPP and in the antibody-excess zone of D-γ-GPP. Though the yields of total glutamic acid in both experiments were almost one half of the theoretical value, the contents of the optical isomers of glutamic acid were approximately equal in the case of M-GPP added, and 20 per cent of L-glutamate and 80 per cent of D-glutamate were found in S-GPP precipitate. Although the reason of the loss of 50 per cent of the GPP added is not yet known, there was probably loss because the precipitates would not be separated completely into antigen and antibody molecules at alkaline pH.

The recovery of the optical isomers of glutamic acid in the case of S-GPP reconfirmed the findings of Thorne and Leonard that S-GPP is a mixture of L-γ-GPP and D-γ-GPP. It also substantiated the conclusion obtained in the preceding experiment, that a system of S-GPP and M-GPP antiserum 102 was composed of two antigen-antibody systems, and that two independent equivalence points were indicated in the system. As the amount of S-GPP added was located in the

antigen-excess zone of L-γ-GPP and in the antibody-excess zone of D-γ-GPP, there must be far less L- than D-glutamic acid recoverable from precipitates.

The findings of Thorne and Leonard on the chemical structure of S-GPP were again reconfirmed from the result shown in Table 8. Though the yield of total glutamic acid was again low as compared to the theoretical value, the yield of L-glutamic acid was quite small as compared to that of D-glutamic acid. The value of the yield of L-glutamic acid was not corrected for the amount of L-glutamic acid formed from D-glutamic acid during hydrolysis. According to our experience, about 2 per cent of D-glutamic acid is converted to L-form during hydrolysis (Torii, 1956). Therefore, only D-γ-GPP of S-GPP was precipitated by A-GPP antiserum.

If M-GPP is a γ-copolymer of D- and L-glutamic acid, the recoveries of L- and D-glutamic acid from precipitates of M-GPP with A-GPP antiserum should be equal to the contents of the optical isomers of glutamic acid in the M-GPP used. As can be seen from Table 8, almost the same amounts of D- and L-glutamic acid were recovered, although the yield of total glutamic acid was not quantitative. Therefore M-GPP is mainly composed of the γ-copolymer of L- and D-glutamic acid.

DISCUSSION

In the experimental work which has been presented here evidence of a quite different nature has been brought to bear on the subject. This fully substantiates the conclusions on the chemical structure of S-GPP reported by Thorne and Leonard (1958). Antibody formation to M-GPP in rabbits has enabled the serological analysis of S-GPP, and it was shown that S-GPP was a mixture of L-γ-GPP and D-γ-GPP.

Physico-chemical evidence on the nature of M-GPP led us to the assumption that M-GPP is a γ-copolymer of L- and D-glutamic acid. A system of M-GPP and its antiserum was demonstrated to be a homogeneous one, differing from a system of S-GPP and M-GPP antiserum, in which two equivalence points were demonstrated corresponding to the L-γ-GPP and D-γ-GPP in S-GPP. Additional and more conclusive evidence was obtained from the analysis of the optical isomers of glutamic acid in a precipitate of M-GPP with A-GPP antiserum, which contained only D-γ-GPP antibodies. The result of the analysis showed that the ratio of L- to D-glutamic acid of precipitated M-GPP was almost the same as that of the original M-GPP. These results fully substantiate the chemical structure of M-GPP as mainly a γ-copolymer of L- and D-glutamic acids.

The sequence of L- and D-glutamic acid in the γ-copolymer of M-GPP cannot be assumed because M-GPP antibodies can be classified in three groups with regard to their specificity; the first is specific to L-γ-GPP, the second to D-γ-GPP and the last was exclusively specific to M-GPP itself. If the determinant group(s) homologous to the last were clarified, the sequence of D- and L-glutamic acid in M-GPP could be assumed.

REFERENCES

Bovarnick, M. (1942). The formation of extracellular D(−)-glutamic acid polypeptide by *Bacillus subtilis*. *J. Biol. Chem.* **145,** 415–424.

Bruckner, V., Kajtár, M., Kovács, J., Nagy, H. und Wein, J. (1958). Synthese des immunospezifischen, polypeptidartigen Haptens der Anthrax-Subtilis Bazillen-Gruppe. Ein synthetischer Beweis der Konstitution der natürlichen Polyglutaminsäuren. *Tetrahedron* **2,** 211–240.

Hanby, W. E. and Rydon, H. N. (1946). The capsular structure of *B. anthracis*. *Biochem. J.* **40,** 297–307.

Ivánovics, G. und Bruckner, V. (1937). Chemische und immunologische Studien ueber den Mechanismus der Milzbrandinfektion und -Immunität. I. Die chemische Struktur der Kapselsubstanz des Milzbrandbazillus und der serologisch identischen spezifischen Substanz des *Bacillus mesentericus*. *Z. f. Immunitätsf.* **90,** 304–317.

Ivánovics, G. (1937). Das Vorkommen der spezifischen Kapselsubstanz der Milzbrandbazillen in verschiedenen aeroben, sporentragenden Saprophytenbazillen. *Zentr. Bakteriol. Parasitenk., Abt., I, Orig.* **138,** 211–220.

Ivánovics, G. (1958). Vergleichende serologische Untersuchungen synthetischer Polyglutaminsäuren verschiedner Konstitution. *Tetrahedron* **2,** 236–240.

Leonard, C. G., Houswright, R. D. and Thorne, C. B. (1958). Effects of some metalic ions on glutamyl polypeptide synthesis by *B. subtilis*. *J. Bacteriol.* **76,** 499–503.

McDuffie, F. C. and Kabat, E. A. (1956). Comparative study of methods used for analysis of specific precipitates in quantitative immunochemistry. *J. Immunol.* **77,** 193-197.

Mizushima, S., Izaki, K., Takahashi, H., and Sakaguchi, K. (1956). Studies on the metabolism of D-amino acid in microorganismus, II. *Bull. Agr. Chem. Soc. Japan* **20,** 36–41.

Sanger, F. (1945). The free amino groups of insulin. *Biochem. J.* **39,** 507–515.

Thorne, C. B., Gomez, C. G., Noyes, H. E. and Houswright, R. D. (1954). Production of glutamyl polypeptide by *Bacillus subtilis*. *J. Bacteriol.* **68,** 307–315.

Thorne, C. B. and Gomez Leonard, C. (1958) Isolation of D- and L-glutamyl polypeptides from culture filtrates of *B. subtilis*. *J. Biol. Chem.* **233,** 1109–1112.

Tomcsik, J. and Guex-Holzer, S. (1951). Anthrax-Polypeptid und andere spezies-spezifische Substanzen der Kapsel in der Bazillus-Gruppe. *Schweiz. Z. f. Allgem. Pathol. und Bakteriol.* **14,** 515–522.

Tomcsik, J. and Ivánovics, G. (1938). Ueber der Hellstellung des Antikapsel-Immunkörpers des Milzbrandbazillus. *Z. f. Immunitätsf.* **93,** 196–208.

Tomcsik, J. und Szongott, H. (1933). Ueber ein spezifisches Protein der Kapsel des Milzbrandbazillus. *Z. f. Immunitätsf.* **78,** 86–99.

Torii, M. (1956) Optical isomers of glutamic acid composing bacterial glutamyl polypeptides. *Med. J. Osaka Univ.* **6,** 1043–1046.

Torii, M. (1959). Studies on the chemical structure of bacterial glutamyl polypeptides by hydrazinolysis. *J. Biochem.* **46,** 189–200.

Troll, W. and Cannan, R. K. (1953). Modified photometric ninhydrin method for the analysis of amino acid and imino acid. *J. Biol. Chem.* **200,** 803–811.

Umbreit, W. W. and Gunsals, I. C. (1945). The function of pyridoxine derivatives. Arginine and glutamic acid decarboxylases. *J. Biol. Chem.* **159,** 333–341.

Utsumi, S., Torii, M., Kurimura, O., Yamamuro, H. and Amano, T. (1958). Heterogeneity of antibodies evoked by a glutamyl polypeptide of *Bacillus megaterium*. *Biken's J.* **1,** 201–202.

Vennes, J. W. and Gerhardt, P. (1959). Antigenic analysis of cell structures isolated from *Bacillus megaterium*. *J. Bacteriol.* **77,** 581–592.

Watson, D. W., Cromartie, W. J., Bloom, W. L., Heckley, R. J., McGhee, W. J. and Weissman, N. (1947). Studies on infection with *Bacillus anthracis*. V. The isolation of an inflammatory factor from crude extracts of lesions of *B. anthracis* infection and its biological and chemical relationship to glutamyl polypeptide. *J. Inf. Dis.* **80,** 121–136.