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# "γ-Glutamylase" as a Decapsulating Agent for *Bacillus anthracis*[*]

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## SUMMARY

γ-Glutamylase in a dog liver extract was extensively purified up to 780 fold. The homogeneous component was separated from the purified material by hydroxylapatite column chromatography. Monothioethylene glycol was found to be a stabilizer for γ-glutamylase.

A microscopical and a serological assay method, were devised for measuring the decapsulating activity. The specific decapsulating activity increased in parallel with that of the specific activity of the γ-glutamylase. Even after repeated chromatography the two specific activities increased in parallel. Thus γ-glutamylase is identical with the decapsulating agent.

Capsular glutamyl polypeptides of *B. anthracis* prepared from *in vitro* cultures or from body fluids containing the bacteria were shown to be susceptible to γ-glutamylase.

## INTRODUCTION

The decapsulation of *Bacillus anthracis* was first observed by Cromartie *et al.* (1947a, b) during histological studies on the infected foci of resistant animals (dog, rat, pig) and immunized rabbit. Therefore we studied the defence mechanisms of animals to *B. anthracis* and one of us (Torii, 1955) observed the decapsulation of *B. anthracis* and *B. megaterium* by a liver extract of dogs which are resistant to the former microorganism. The decapsulation of *B. megaterium* seemed to be due to hydrolysis of capsular GPP**. This concept was supported by recent work (Torii *et al.*, 1959). On the other hand, the decapsulation of *B. anthracis* was thought to be caused by another mechanism, because there was no evidence of hydrolysis of the capsular GPP of *B. anthracis*, except that the decapsulating activity for both microorganisms was always found in the same fraction during purification procedures.

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** The following abbreviations are used in this paper:
GPP: γ-glutamyl polypeptide; A-GPP: GPP of *B. anthracis*; M-GPP: GPP of *B. megaterium*; Glu: glutamic acid; DCA: decapsulating agent; AbN: antibody nitrogen; AgN: antigen nitrogen; HA: hydroxylapatite.

The nature of the decapsulating agent for *B. anthracis*, was studied from two aspects. In the first, the substrate specificity of highly purified $\gamma$-glutamylase ($\gamma$-glutamyl bond splitting enzyme) was studied, as already reported (Torii *et al.*, 1960). In the report, it was suggested that $\gamma$-glutamylase decapsulates *B. anthracis*. The other aspect was to investigate the fate of the decapsulating activity during extensive purification of $\gamma$-glutamylase. The present report describes studies which reveal that $\gamma$-glutamylase is the decapsulating agent.

## MATERIALS AND METHODS

### 1. *Bacteria*

*Bacillus anthracis* strain Vollum: This strain was given by Prof. J. Tomcsik of the University of Basel. The encapsulated cells were grown on a casamino acid-peptone-bicarbonate medium. The medium consisted of casein hydrolyzate (2.0 g), Polypeptone (Takeda Pharmaceutical Co.) (10.0 g), NaCl (2.0 g), $KH_2PO_4$ (3.0 g), yeast extract (Daigo Eiyo Co.) (2.0 g), agar (20.0 g), and water (1000 ml). The pH of the medium was adjusted to 6.0 with sodium hydroxide and after autoclaving and cooling to about 50°C, 130 ml of sterile 7 per cent sodium bicarbonate solution were added. The bacterial spores were inoculated onto slants, the cotton plugs were covered with Parafilm and the medium was incubated at 37°C for 16 hours.

*Bacillus megaterium* A5: This strain was also furnished through the courtesy of Prof. J. Tomcsik. It was named strain A5. The encapsulated cells were harvested from the medium at pH 7.0 above described, to which no sodium bicarbonate was added, after overnight incubation.

### 2. *Capsular glutamyl polypeptides (GPP)*

*A-GPP in vitro:* A-GPP *in vitro* was extracted from an encapsulated *B. anthracis* suspension by autoclaving and purified as the acid form according to the description of Hanby and Rydon (1946). There was 8.5 per cent L-Glu.

*In vivo A-GPP:* This was kindly given by Dr. D. W. Watson from the University of Minnesota. It had been prepared from body fluids of infected animals.

*M-GPP:* This was extracted by boiling an encapsulated *B. megaterium* suspension in water. It was purified as the sodium salt according to the method of Bovarnick (1942). The L- and D-Glu contents of the purified material were 50.7 and 49.3 per cent respectively.

### 3. *Antiserum to encapsulated B. megaterium*

The antiserum was prepared in rabbits as described previously (Utsumi *et al.*, 1959). This antiserum was used for the determination of capsular GPP liberated from *B. anthracis* by the action of the decapsulating agent. Homologous antisera could not be used, because other antigenic substances than capsular A-GPP also evoked precipitation. The antiserum contained 640 $\mu$g precipitable antibody N per ml with A-GPP.

### 4. *Determination of protein*

*U.V. absorption method*: The protein concentration was calculated according to the formula given by Kalckar (1947).

*Folin-Ciocalteau's method:* The microassay method described by Herriott (1941) was used. A purified bovine serum albumin preparation served as a standard for this method.

### 5. *Assay of $\gamma$-glutamylase*

The enzymic nature of $\gamma$-glutamylase and its assay have been described by Torii *et al.* (1960).

## RESULTS

### 1. *Purification of γ-glutamylase*

γ-Glutamylase was extensively purified to see whether it was identical with the decapsulating agent. Dog liver was chosen as the source of γ-glutamylase, because Torii *et al.* (1960) had studied the distribution of the enzyme and found dog liver the best source. The initial step of the purification procedures for γ-glutamylase in this report have already been described (Torii, 1959; Torii *et al.*, 1959; Torii *et al.*, 1960).

*Crude extract:* 2,180 g of liver from ten dogs were homogenized with 4,360 ml of saline containing 26.2 g of acetic acid. The homogenate was frozen and thawed twice, neutralized, and centrifuged at 2,800 rpm for 45 minutes. The chilled supernatant was again centrifuged at 34,800 g for 45 minutes, in a Servall refrigerated centrifuge. The supernatant of the crude extract, was further purified as follows.

*Step 1:* 1,480 g of ammonium sulfate were added to 4,175 ml of crude extract (50 per cent saturation) at 4°C and the precipitates were centrifuged off after 20 hours. A further 1,370 g of ammonium sulfate were added to 4,620 ml of the supernatant (100 per cent saturation) at 4°C and the precipitate centrifuged after 20 hours. It was dissolved in 250 ml of 0.01 M phosphate buffer at pH 6.60. The solution was dialyzed overnight against 5 l of 0.005 M phosphate buffer at pH 6.60 and then overnight against distilled water. The precipitates containing hemoglobinlike proteins were centrifuged and 830 ml of supernatant were obtained.

This preparation was heated at 56°C for 30 minutes at varying pH. Half the activity was retained at pH 4.0-5.0 and an abundant precipitate could be removed. Thus this treatment was used in the next step.

*Step 2:* 770 ml of distilled water were added to 830 ml of the supernatant obtained at step 1 to adjust the protein concentration to approximately 2 per cent. The pH was adjusted to exactly 4.0 with 0.1 M citrate. The temperature was maintained at 56°C for 4 minutes. The solution was chilled and the pH was adjusted to 6.7 with 0.4 M disodium phosphate. The precipitate formed was centrifuged off.

*Step 3:* 810 g of ammonium sulfate were added to 1,840 ml of the supernatant obtained at step 2 (62.5 per cent saturation) and the solution was chilled in an ice bath and stored for 24-48 hours in the cold. After centrifugation, 220 g of ammonium sulfate (to about 75 per cent saturation) were added to the supernatant (2,380 ml) and the solution was kept overnight in the cold. The precipitate was collected on a Buchner funnel using Hyflo Supercel. The residue was suspended in 0.01 M phosphate buffer at pH 6.0 and centrifuged. The supernatant was dialysed against distilled water. After dialysis, the solution was centrifuged and 125 ml of supernatant were obtained.

This preparation still contained lysozyme. Lysozyme was completely removed by bentonite adsorption, as described in our previous report (Torii *et al.*, 1959).

*Step 4:* 120 ml of supernatant (total protein content=600 mg) obtained at step 3 were concentrated to about 30 ml by dialysis against 40 per cent solution of Carbowax 6000. To the concentrated solution was added sodium phosphate buffer to adjust the total volume to 40 ml, the pH to 7.2 and the final phosphate concentration to 0.1 M. 300 mg of bentonite (Wako Chemicals, Inc.), washed twice with 0.1 M sodium phosphate buffer at pH 7.2 and suspended in a small amount of the buffer, were added to the concentrated solution (bentonite mg/protein mg=0.5). The mixture was shaken

for 15 minutes at room temperature and centrifuged. The precipitate was washed once with 5 ml of the buffer and the washings were added to the supernatant.

*Step 5:* To 50 ml (total protein content=365 mg) of the combined supernatant obtained at step 4 were added 50 ml of distilled water. The pH was adjusted to 6.2 with 0.05 M sodium dihydrogen phosphate. To this solution were added 18 ml of calcium phosphate gel containing 350 mg of dried matrial and the mixture was stirred for 30 minutes at room temperature (calcium phosphate mg/protein mg=0.96). After centrifugation at 2,000 rpm for 3 minutes, the precipitate was treated successively with the following buffer solutions: 1) 100 ml of 0.05 M sodium phosphate buffer at pH 6.5 for 20-30 minutes, 2) 100 ml of 0.05 M sodium phosphate buffer at pH 7.0 for 20-30 minutes, 3) twice with 100 ml of 0.05 M sodium phosphate buffer at pH 7.5 for 20-30 minutes, 4) 100 ml of 0.075 M sodium phosphate buffer at pH 7.2 for 5 minutes and 5 ) 100 ml of 0.1 M sodium phosphate buffer at pH 7.2 for 5 minutes. This washed calcium phosphate gel was added to 100 ml of 0.6 M ammonium dihydrogen phosphate-sodium hydroxide buffer at pH 7.2 and the mixture was stirred for 60 minutes. Thus the $\gamma$-glutamylase was eluted. The eluate was dialyzed against distilled water. 132 ml of purified $\gamma$-glutamylase were obtained. One half of it was stored at $-20°C$ and the other was lyophilyzed.

The $\gamma$-glutamylase activity during purification is shown in Table 1. As can be seen from the table, the specific activity was increased 780 fold. The most efficient step was step 5. However, a repetition of this step gave no further purification.

Table 1. $\gamma$-Glutamylase Activity during Purification

| Step | Volume | Protein | | $\gamma$-Glutamylase | | | | |
|---|---|---|---|---|---|---|---|---|
| | | mg/ml | Total | G-U/ml | G-U*/mg | Purification index | Total G-U | Recovery |
| | ml | | mg | | | | | % |
| Crude Extract | 4175 | 28.8 | 120450 | 15.3 | 0.5 | 1 | 64,000 | 100 |
| 1 | 830 | 46.4 | 38500 | 61.0 | 1.3 | 2.6 | 52,630 | 82 |
| 2 | 1840 | 4.4 | 8100 | 23.0 | 5.3 | 10.6 | 42,300 | 66 |
| 3 | 125 | 5.0 | 625 | 230 | 46 | 92 | 28,750 | 45 |
| 4 | 50 | 7.3 | 365 | 510 | 70 | 140 | 25,520 | 40 |
| 5 | 132 | 0.20 | 26 | 78.0 | 390 | 780 | 10,300 | 16 |

* G-U: $\gamma$-Glutamylase units.

2. *Assay of decapsulating activity*

For purification of the decapsulating agent for *B. anthracis* a method of assay of the activity had to be developed. Two methods were employed. One was microscopical observation using a negative stain. The other was by the quantitative precipitin reaction, in which the amount of capsular A-GPP liberated could be calculated by estimating the antibody precipitated by the A-GPP liberated from the capsules.

i) *Microscopical assay method*

a) *Procedure:* 0.1 ml each of serial dilutions of the preparation to be tested were put into a series of tubes containing 0.1 ml of the bacterial suspension described below, 0.1 ml of 10 per cent gelatin solution and 0.2 ml of 0.1 M phosphate

buffer at pH 5.6. As a control, 0.1 ml of saline was added to a tube in place of the preparation. The tubes were incubated at 37°C for 30 minutes and the pH of the mixture was raised to 8-9. A loopful of the contents of each tube was mixed with a droplet of India ink on a slide and examined under a microscope. The lowest activity in 0.1 ml of sample, which decapsulated the whole population, was taken as one unit. Thus the microscopical decapsulating units (MDC-U) per ml could be calculated as follows:

$$\text{MDC-U} = (\text{The dilution factor}) \times 10$$

b) *Bacterial suspension:* As capsules of the living, as well as heat-killed bacteria were susceptible to the decapsulating agent and the lysate of living *B. anthracis* contained a heat-labile substance which modified the capsules (Nordberg and Thorsell, 1955), the bacterial cells harvested from the medium were heated at 100°C for 30 minutes, thoroughly washed with saline and suspended in saline containing 0.5 per cent gelatin. The optical density of the suspension was adjusted to $OD_{550m\mu} = 1.20$ and the bacterial population contained $3.4 \times 10^9$ chains per ml. The suspension was stored in a refrigerator and could be used for about two months.

When the pH of the suspension was lower than 5.8, the bacterial cells agglutinated. Therefore a stabilizer was necessary at low pH. 2.0 per cent gelatin was found to be the most effective for this purpose. After addition of gelatin, the suspension was stable when the pH was kept above 5.4. From this reason the microscopical assay method could not be applied below pH 5.4, especially at pH 4.2 where $\gamma$-glutamylase was most active and the decapsulating agent for *B. megaterium* was also most active (Torii *et al.*, 1959).

ii) *Serological assay method**

As the former method was inapplicable at the optimal pH (4.2) of $\gamma$-glutamylase and serial dilutions caused unavoidable experimental errors, a more accurate assay method was necessary. While studying the cross reaction of A-GPP with antisera to encapsulated *B. megaterium* using the quantitative precipitin reaction it was found that M-GPP antibodies could cross react with A-GPP (Utsumi *et al.*, 1959). This cross reaction was used to assay the decapsulating activity by determining the antibodies precipitated. In this cross reaction, no other antigens of *B. anthracis* reacted with antisera to encapsulated *B. megaterium*.

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* Author's Note: Leonard and Thorne stated in the Journal of Immunology (vol. 87, no.1, p.175, 1961), which has arrived at this laboratory on the Sep. 28th. 1961, that the precipitins for A-GPP contained in the antisera to the encapsulated *B. anthracis* were not the authentic antibodies but the association products of basic proteins including lysozyme with $\gamma$-globulin. Even if their statement is true, we are sure that the serological assay method, based on the precipitation of A-GPP with the antiserum to the encapsulated *B. anthracis,* does not lose its value in the estimation of caspular A-GPP liberated by $\gamma$-glutamylase.

a) *Standard curve for the estimation of A-GPP:* The experimental procedures used were the same as described previously (Utsumi *et al.*, 1959).

Varying amounts (5 to 100 $\mu$g) of A-GPP dissolved in 0.5 ml of saline were added to a series of tubes containing 0.4 ml of the antiserum (No.120) to encapsulated *B. megaterium*. The tubes were incubated at 4°C for 48 hours and after washing twice with saline, the precipitates were dissolved in 3.0 ml of 0.1 N NaOH. The optical density was read at 287 m$\mu$. A duplicate series of experiments were performed. As a control, a tube was included to which 0.5 ml of saline was added in place of A-GPP solution. As A-GPP had no appreciable absorption at 287 m$\mu$, the amount of precipitated antibody N could easily be calculated according to the description of McDuffie and Kabat (1956). The results are shown in Table 2 and Fig. 1.

Table 2. Quantitative Precipitin Reaction of A-GPP by Antiserum to Encapsulated *B. megaterium*

| A-GPP added | $OD_{287}$ of dissolved precipitates | Ab N precipitated | Ab N/Ag N | Test on supernatant |
|---|---|---|---|---|
| 5$\mu$g(0.5 N $\mu$g) | 0.090 | 26 $\mu$g | 52 | Ab excess |
| 10 (1.1) | 0.215 | 62.5 | 63 | Ab excess |
| 20 (2.2) | 0.398 | 116 | 58 | Ab excess |
| 25 (2.7) | 0.486 | 142 | 57 | Ab excess |
| 30 (3.3) | 0.586* | 170 | 57 | Ab excess |
| 40 (4.3) | 0.644* | 188 | 47 | Ab excess |
| 50 (5.4) | 0.770* | 225 | 45 | Ag excess |
| 60 (6.5) | 0.870* | 253 | 42 | Ag excess |
| 80 (8.7) | 0.876* | 255 | | |
| 100 (10.9) | 0.788* | 230 | | |

* : In the estimation of $OD_{287}$ the sample was diluted 1 : 2 and the observed value was doubled.

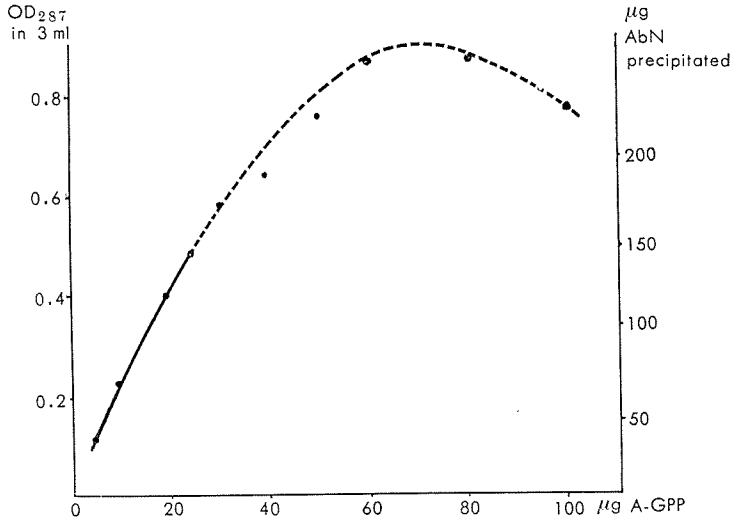


Fig. 1. Standard Curve for the Estimation of A-GPP

Thus the following formula was given for the standard curve in the range of 0.5-5.0 $\mu$g of A-GPP added,

$$(\text{Ab N}) = 67 \times (\text{A-GPP N}) - 4 \times (\text{A-GPP N})^2$$

and the second term could be neglected when the amount of added A-GPP was less than 2.3 $\mu$g N (21 $\mu$g) or the amount of the precipitated antibody was less than $OD_{287m\mu} = 0.45$.

b) *Bacterial suspension:* The stock bacterial suspension was the same as that used for the microscopical assay. The cells were washed before the experiment to remove spontaneously dissolved capsular A-GPP completely. This was done by repeated centrifugation and resuspension in saline containing 2 per cent gelatin.

c) *Recovery of A-GPP added to the bacterial suspension:* To prove the applicability of the quantitative precipitin reaction for the estimation of capsular A-GPP liberated, the recovery was tested. Aliquots of an inactivated $\gamma$-glutamylase preparation at varying pH below 6.0 were added to tubes containing A-GPP and the bacterial suspension, and the mixtures were centrifuged after 30 minutes incubation at 37°C. The amount of A-GPP was estimated in the supernatants. The least A-GPP was recovered in the supernatant of a preparation at pH 4.2 and more was obtained when the pH of the mixture was higher. To avoid this loss of added A-GPP, the pH of the mixture after incubation was raised to 9-10 and after 30 minutes the pH was readjusted to 7.2-7.6 and then the bacterial cells were centrifuged. In this way no loss of added A-GPP was observed, as shown in the following experiment.

Table 3. Recovery of A-GPP Added

| | 1 | 2 | 3 | 4 | 5 | 6 | Control |
|---|---|---|---|---|---|---|---|
| A-GPP | 0.3 ml (60 $\mu$g) | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | — |
| Bact. suspension | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| DCA inactivated | 0.2 | 0.2 | 0.2 | — | — | — | — |
| $H_2O$ | — | — | — | 0.2 | 0.2 | 0.2 | 0.2 |
| Buffer | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| (pH) | (5.0) | (4.2) | (3.6) | (5.0) | (4.2) | (3.6) | (7.0) |
| Quantitative Preciptin Reaction after the Treatment Described in Text | | | | | | | |
| $OD_{287}$ of dissolved precipitates | 0.320 | 0.323 | 0.255 | 0.294 | 0.320 | 0.305 | 0.025 |
| $OD_{obs} - OD_{control}$ | 0.295 | 0.298 | 0.230 | 0.269 | 0.295 | 0.280 | — |

The contents of the experimental tubes are shown in Table 3. 30 minutes after incubation, the pH of the tubes was adjusted to 9-10 by adding 0.1 ml of 0.5 N NaOH. After 30 minutes the pH of the mixture was readjusted to 7.2-7.6 by adding 0.9 ml of 0.1 M phosphate buffer at pH 6.8. The contents were then centrifuged. 0.5 ml of each supernatant was added to a tube containing

0.4 ml of *B. megaterium* antiserum No. 120 and the tubes were treated as described above. The optical densities of the dissolved precipitates are also shown in Table 3.

As can be seen from the table, there was no loss of added A-GPP except at pH 3.6 and no appreciable quantity of capsular A-GPP was extracted from the encapsulated cells. As the optimal pH of $\gamma$-glutamylase was 4.2, the procedures were satisfactory for assay of the decapsulating activity. However, when a cruder $\gamma$-glutamylase preparation was used, recovery of A-GPP was not complete. Therefore this assay method could not be applied before step 2 in the preparation, as described below.

d) *The relationship between the amount of added decapsulating agent and the capsular A-GPP liberated:* To find the range of the amount of decapsulating agent which gave an approximately linear relationship with the capsular A-GPP liberated, the following experiment was performed.

**Table 4. A-GPP Liberated by Varying Amounts of DCA**

| Dilution of DCA | $OD_{287}$ | $OD_{obs} - OD_{control}$ | A-GPP liberated | Microscopical observation |
|---|---|---|---|---|
| 1 : 4 | 0.675 | 0.640 | 144 $\mu$g | complete decapsulation |
| 1 : 5 | 0.650 | 0.615 | 136 | 〃 |
| 1 : 10 | 0.690 | 0.655 | 148 | 〃 |
| 1 : 20 | 0.506 | 0.471 | 92 | decapsulation ++ |
| 1 : 25 | 0.424 | 0.389 | 72 | + |
| 1 : 30 | 0.325 | 0.290 | 52 | ± |
| 1 : 40 | 0.178 | 0.143 | 24 | − |
| Control | 0.035 | — | — | − |

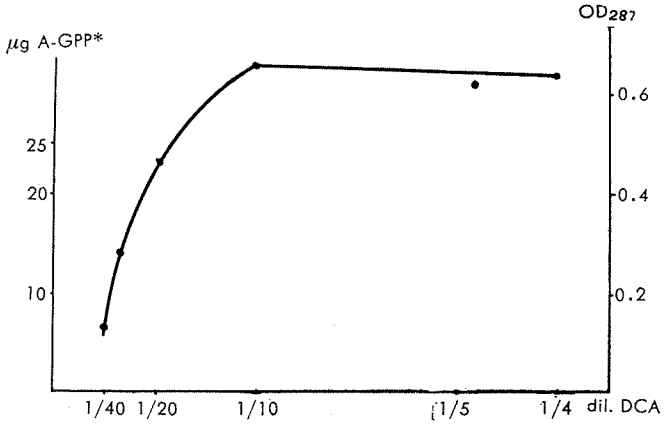


**Fig. 2. Relationship between A-GPP Liberated and DCA Added**

* This value is 1/4 of the total A-GPP liberated from the bacterial suspension.

0.2 ml aliquots of varying dilutions (1:4 to 1:40) of a partially purified preparation containing about 500 MDC-U per ml were put into graduated centrifuge tubes containing 0.5 ml of the bacterial suspension and 0.3 ml of 0.02 M citrate buffer at pH 4.5. In the control tubes, the decapsulating agent preparation was replaced by the same amount of distilled water. The tubes were incubated at 37°C for 30 minutes and the pH of the mixtures were brought to 9 to 10 by adding 0.1 ml of 0.5 N NaOH. The tubes were thoroughly shaken and left for 30 minutes. The pH was again adjusted to 7.2-7.6 by adding 0.9 ml of 0.1 M phosphate buffer at pH 6.8. The tubes were centrifuged and 0.5 ml of each supernatant was added to 0.4 ml of *B. megaterium* antiserum. The amount of precipitated antibody was estimated, and the difference between the experimental and corresponding control tube was calculated.

As can be seen in Table 4 and Fig. 2, an approximately linear relationship was observed when the OD at 287 mμ of the precipitated antibody was less than 0.45. The $OD_{287m\mu}$ and A-GPP added also increased approximately in parallel, as can be seen in Fig. 1. Therefore in later estimations of precipitated antibodies the $OD_{287m\mu}$ was always less than 0.45.

e) *The relationship between the amount of A-GPP liberated and the reaction time:* To find the reaction time, giving an approximately linear relationship with the amount of A-GPP liberated, the following experiment was carried out.

0.2 ml of 1:30 diluted preparation containing the decapsulating agent was put into a series of tubes, the contents of which were the same as described in the previous experiment. These were incubated at 37°C for 60 minutes and a tube was taken out every 10 minutes. The reaction was stopped by adjusting the pH to 9-10. Subsequent procedures were the same as those in the previous experiment.

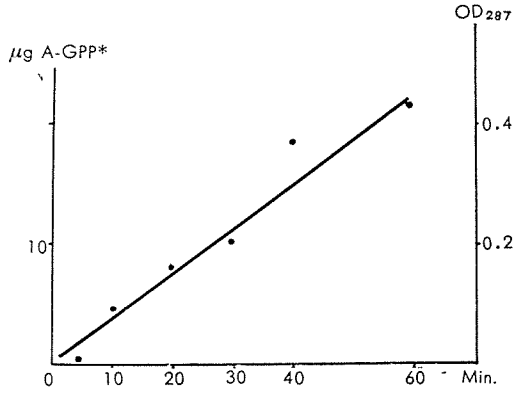


**Fig. 3. Relationship between A-GPP Liberated and the Reaction Time**

* This value is 1/4 of the total A-GPP liberated from the bacterial suspension.

As shown in Fig. 3 an approximately linear relationship was found for 60 minutes when the $OD_{287m\mu}$ was plotted against the reaction time. Therefore, a 60 minutes reaction time was chosen as suitable for assay of the decapsulating agent. Thus, the decapsulating activity could be assayed by the rate of liberation of A-GPP from the capsules.

f) *pH-activity curve:* Using the serological assay method the optimal pH was studied. The buffer used was 0.05 M citric acid-0.1 M disodium phosphate. The experimental conditions were as described above.

As can be seen in Fig. 4, the optimal pH was found to be 4.0, therefore pH 4.2 was chosen for the serological assay of the decapsulating activity. One unit of activity (SDC-U) was defined as the amount liberating 10 $\mu$g equivalent of GPP from the capsules of a standard bacterial suspension in 60 minutes at pH 4.2 at 37°C.

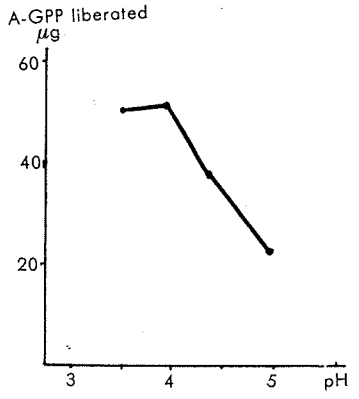


**Fig. 4. pH-activity Curve Assayed by the Serological Method**

As described above the optimal pH of the microscopical estimation method could not be examined, because of the agglutination of the bacterial cells in acid. However, after the completion of all the experiments described in this paper, a method to avoid agglutination in acid was found. The bacteria were grown at 30°C for 20 hours in the medium described above. Although growth was scanty, all the cells grown under such conditions were fully encapsulated and the suspension was quite stable down to pH 3.5 in the presence of 2 per cent gelatin. The experimental procedures used were the same as those described above. After in-

**Table 5. Optimum pH for Microscopical Assay of DCA**

| pH \ Time | 10′ | 20′ | 30′ | 60′ | 90′ | 120′ | control 120′ |
|---|---|---|---|---|---|---|---|
| 3.5 | ++ | ++ | 100 | 100 | 100 | 100 | no change |
| 4.0 | ++ | 100 | 100 | 100 | 100 | 100 | no change |
| 4.4 | ++ | ++ | ++ | 100 | 100 | 100 | no change |
| 4.8 | ± | + | ++ | 75 | 100 | 100 | no change |
| 5.2 | − | + | ++ | ++ | 100 | 100 | no change |
| 5.8 | − | − | + | ++ | ++ | 75 | no change |

−, +, ++ : Morphological change of capsule
++ : about 1/2 thickness
100, 75 : % of decapsulated bacilli

cubation, the pH of the medium was raised to 8-9 and the degree of decapsulation was studied microscopically by the India ink method. As can be seen in Table 5, the optimal pH was found to be 4.0. Thus the optimal pH was the same for both assay methods and coincided with that of the decapsulating activity for encapsulated *B. megaterium* (Torii *et al.*, 1959).

3. *The decapsulating activity during purification of γ-glutamylase*

As the γ-glutamylase preparation at step 5 still retained a high decapsulating activity for *B. anthracis*, this was estimated at each step in the purification of γ-glutamylase and the ratio of the decapsulating activity to γ-glutamylase activity was compared, as shown in Table 6. The serological estimation method for the decapsulating activity could only be applied after step 2, because GPP liberated from the bacterial capsules formed complexes with proteins in the crude γ-glutamylase preparation at pH 4.2 and some of these complexes did not dissociate on addition of sodium hydroxide. The decapsulating activity was parallel with that of the γ-glutamylase from step 1 to 4, when assayed microscopically, and from step 2 to 5 when assayed serologically.

Table 6. DCA during γ-Glutamylase Purification

| Step | G-U/ml | MDC-U/ml | MDC-U/G-U | SDC-U/ml | SDC-U/G-U |
|---|---|---|---|---|---|
| 1 | 61 | 200 | 3.3 | — | — |
| 2 | 23 | 64 | 2.8 | 146 | 6.3 |
| 3 | 230 | 640 | 2.8 | 1970 | 8.6 |
| 4 | 510 | 1200 | 2.4 | — | — |
| 5 | 78 | 120 | 1.5 | 670 | 8.6 |

G-U : γ-Glutamylase units
MDC-U : Microscopical units of DCA
SDC-U : Serological units of DCA

As the γ-glutamylase preparation contained 0.2 mg protein per ml at step 5, as shown in Table 1, the specific activity per mg protein of the decapsulating agent assayed microscopically was 600. When assayed serologically it was 3,350. Therefore 1.6 μg of protein was sufficient to decapsulate $3.4 \times 10^8$ chains of *B. anthracis* completely in 30 minutes at pH 5.6. Moreover 0.3 μg protein was sufficient to liberate 10 μg GPP from the capsules of $1.7 \times 10^9$ chains of the microorganism in 60 minutes at pH 4.2.

The purified preparation obtained at the last step was also very active on encapsulated cells of *B. megaterium*, and moreover, the activity was somewhat stronger for *B. megaterium* than for *B. anthracis*. In addition, the preparation was very active on living encapsulated cells of *B. anthracis* and *B. megaterium*. Thus the decapsulation of both microorganisms by dog liver extracts was displayed by the same agent.

Since the ratio of DCA to γ-glutamylase activity is essentially constant during the course of the 780-fold purification of γ-glutamylase it was assumed that a single agent is responsible for both activities.

4. *A stabilizer for γ-glutamylase*

As the highly purified preparation of γ-glutamylase was fairly unstable on storage in a refrigerator, some stabilizer was necessary. The stabilizing effects of monothioethylene glycol, gelatin and cystein were compared.

0.8 ml aliquots of the highly purified preparation of γ-glutamylase at step 5 containing 123 units, were put into tubes containing, 0.1 ml of 10 per cent gelatin, 0.1 ml of 0.2 M cystein, 0.1 ml of 0.2 M monothioethylene glycol and 0.1 ml of distilled water. To each of these tubes 0.1 ml of 0.1 M Cetavrone was added as a preservative. The tubes were kept at room temperature (10-15°C) for 6 days. 0.2 ml samples were taken out every two days and their γ-glutamylase activity measured. The decapsulating activity could not be estimated because Cetavrone lysed the bacterial cells. Cetavrone had no harmful effect on γ-glutamylase (Torii *et al.*, 1960). An additional experiment was carried out, in which a tube containing 0.5 ml of γ-glutamylase preparation was placed in a deep freeze at −20°C and assyed after 6 days.

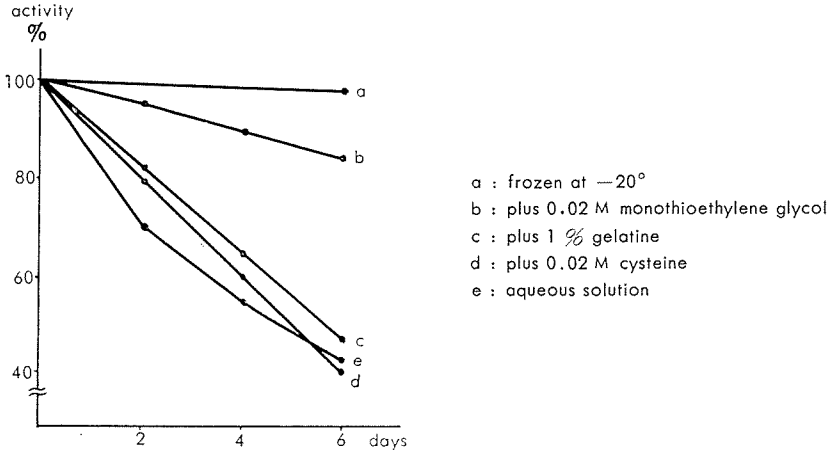


**Fig. 5. Stability of γ-Glutamylase with Various Reagents**

As can be seen, monothioethylene glycol in 0.02 M final concentration gave the best stabilization. Preparations remained most active in the deep freeze at −20°C.

5. *Chromatography of γ-glutamylase*

To demonstrate the identity of γ-glutamylase with the decapsulating agent, γ-glutamylase preparations were studied chromatographically. The ion exchange resin, IRC-50, gave a low yield. DEAE-cellulose and CM-cellulose gave some-

what better yields, but they were unsatisfactory to prove the identity of the two agents. Hydroxylapatite column chromatography, according to the method of Tiselius *et al.* (1956) gave the best separation of proteins and the highest γ-glutamylase yield.

To a 2×18 cm column of hydroxylapatite saturated with 0.001 M sodium phosphate buffer at pH 6.80, 25 mg protein of a preparation at step 2 were applied. Then sodium phosphate buffer of stepwise increasing concentration at pH 6.80 was added. The volumes and concentrations used were: 180 ml of 0.01 M, 120 ml of 0.025 M, 120 ml of 0.05 M, 150 ml of 0.1 M, 120 ml of 0.2 M, 120 ml of 0.4 M and 120 ml of 0.6 M.

Fig. 6 shows a chromatogram of the γ-glutamylase preparation obtained at step 2. Both γ-glutamylase and the decapsulating agent were found in the 0.4 M fraction. After this fraction had been dialyzed against 40 per cent Carbowax 6000 solution to remove phosphate, it was rechromatographed.

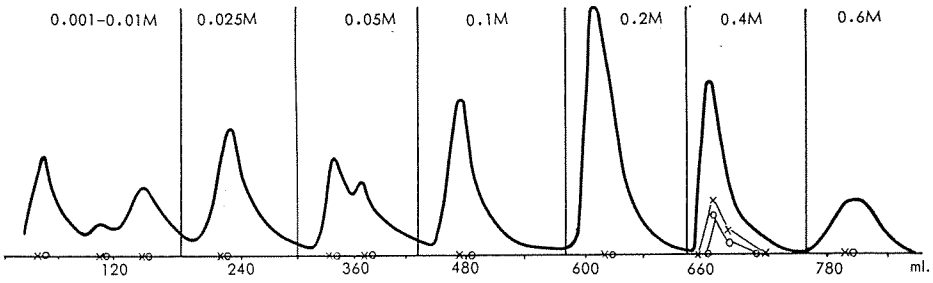


**Fig. 6. HA Column Chromatogram of γ-Glutamylase at Step 2**

– × –, – ○ –: activity-curves of γ-glutamylase and DCA

For rechromatography, the same sized column was used and elution was made successively with 240 ml of 0.025 M, 120 ml of 0.05 M, 120 ml of 0.1 M, 90 ml of 0.2 M, 90 ml of 0.4 M and 120 ml of 0.6 M buffer at pH 6.80.

Fig. 7 shows the rechromatography. Both activities were again in the 0.4 M fraction.

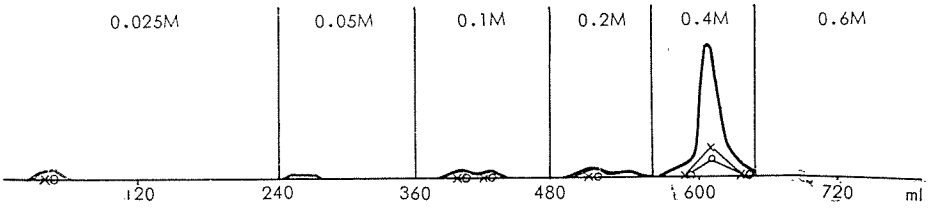


**Fig. 7. Rechromatography of 0.4 M Fraction shown in Fig. 6**

After the first run, 60 per cent of both activities were lost and after the second 85 per cent of original activities were lost. Figs. 8 and 9, respectively shows the chromatography of γ-glutamylase preparations at steps 3 and 5.

In the experiment shown in Fig. 8, a 2×18 cm column was used and 13.2 mg of protein of the preparation at step 3 were applied. Stepwise elution was made with 120 ml each of 0.01 M, 0.02 M, 0.05 M, 0.1 M, 0.2 M and 0.4 M buffer and finally with 180 ml of 0.6 M buffer at pH 6.80. For Fig. 9, a 1×10 cm column was used and 2.0 mg of protein of the preparation at step 5 were applied and then eluted successively with 240 ml of 0.01 M, 120 m of 0.05 M, 120 ml of 0.1 M, 90 ml of 0.2 M, 90 ml of 0.4 M and lastly with 120 ml of 0.6 M buffer at pH 6.80.

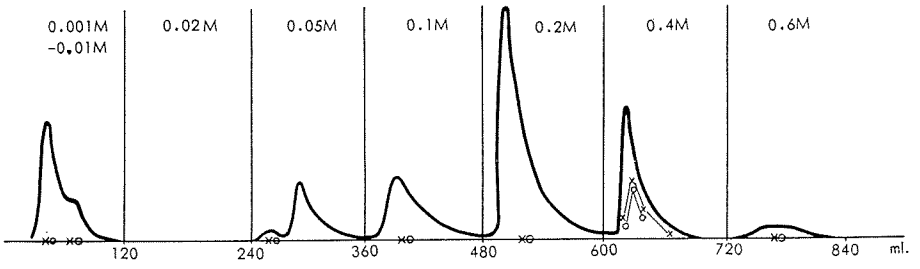


Fig. 8. HA Column Chromatography of γ-Glutamylase at Step 3

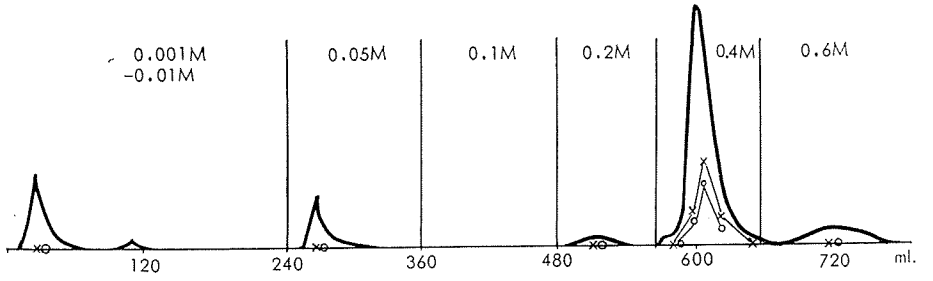


Fig. 9. HA Column Chromatagraphy of γ-Glutamylase at Step 5

In these experiments also both activities were found in the 0.4 M fraction. After chromatography as shown in Fig. 9, 43.2 per cent of the γ-glutamylase activity was recovered and the pattern of the 0.4 M fraction indicated that this fraction still contained from 20 to 25 per cent impurities.

To remove these impurities by repeating chromatography, it was necessary to stabilize the activities. As a stabilizer monothioethylene glycol was added to the eluent buffer at a final concentration of 0.02 M. However monothioethylene glycol disturbed the estimation of protein by UV absorption or by Folin's reagent. Thus the first run was carried out in the presence of the stabilizer in the same manner as the experiment of Fig. 9. The presence of γ-glutamylase in the 0.4 M fraction was confirmed after dialysis. The recovery was 80 per cent. The dialyzed 0.4 M fraction was rechromatographed. The experimental conditions for chromatography were the same as those used in the first run but no stabilizer was added.

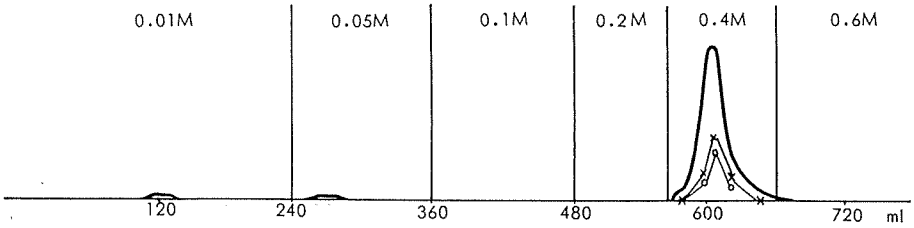


Fig. 10. Rechromatography of 0.4 M Fraction of $\gamma$-Glutamylase at Step 5 in the Presence of Monothioethylene Glycol

Fig. 10 shows the rechromatography. The recoveries of both activities in the 0.4 M fraction were equal and were approximately 40 per cent of the initial activity. Very little protein was found in the 0.01 M and 0.05 M fractions. However the pattern of the 0.4 M fraction indicated the presence of a minor component. To eliminate this, 1.0 mg of the preparation at step 5 was applied to a $1 \times 10$ cm column which was then eluted successively with 120 ml of 0.05 M and 0.1 M buffer, 45 ml of 0.25 M, 60 ml of 0.4 M and 90 ml of 0.6 M buffer at pH 6.8.

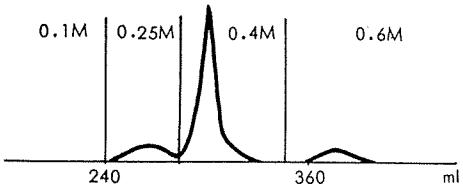


Fig. 11a. Elimination of Minor Contaminants from 0.4 M Fraction (First Chromatography)

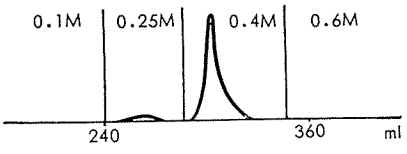


Fig. 11b. Rechromatography of 0.4 M Fraction of Fig. 11a

Fig. 11a shows the chromatogram. The pattern of the 0.4 M fraction became homogeneous.

The homogeneity of the 0.4 M fraction was confirmed by rechromatography, as shown in Fig. 11b. Though monothioethylene glycol was not added to the buffer, the homogeneous 0.4 M fraction contained both activities and, in addition, this fraction also decapsulated *B. megaterium*. From these results it can be stated that $\gamma$-glutamylase and the decapsulating agent for *B. anthracis* and *B. megaterium* are identical.

6. *The decomposition of capsular GPP of B. anthracis by a highly purified $\gamma$-glutamylase*

Since $\gamma$-glutamylase was found to be identical with the decapsulating agent for *B. anthracis*, it was necessary to investigate the effect of $\gamma$-glutamylase on the capsular GPP of *B. anthracis*. As the capsular GPP of *B. anthracis* contained a very

small amount of L-glutamate and γ-glutamylase can split γ-D-Glu-L-Glu, it was probable that the γ-peptide bond linking L-Glu to D-Glu could be hydrolyzed. The hydrolysis was estimated by the change in viscosity of the reaction mixture.

To three Ostwald viscosimeters containing varying amounts (1.0, 0.5 and 0.3 ml) of the purified γ-glutamylase preparation at step 5 preincubated at 37°C were added 1.0 ml aliquots of 0.1 M acetate buffer at pH 4.2 containing 5.0 mg of A-GPP, which had also been warmed to 37°C. The volume was adjusted by adding appropriate amounts of warm water. After experiments, the viscosimeters were thoroughly washed and control experiments were performed. In this case the γ-glutamylase preparation was replaced by a preparation inactivated by heating at 100°C for 3 minutes. From the values of the relative viscosity obtained in the experimental and control viscosimeters, the specific viscosity was calculated. There was an obvious change in viscosity, except in the control experiments, and no detectable precipitates were formed in either experimental or control vessels.

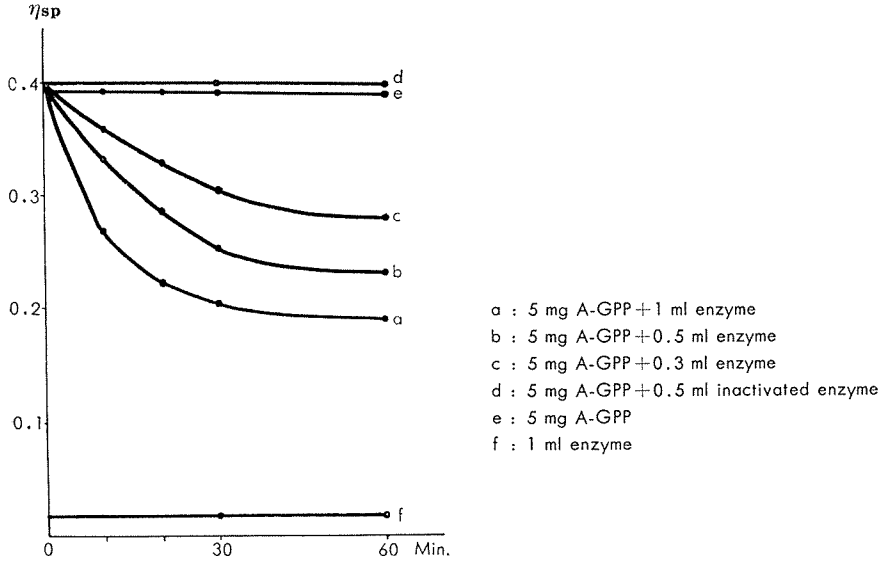


**Fig. 12. Break-down of *in vitro* A-GPP by γ-Glutamylase**

**Table 7. Break-down of *in vivo* A-GPP by γ-Glutamylase**

| Time | Experiment | Control a | Control b | Control c |
|---|---|---|---|---|
| 0 | 1′40″6 | 1′40″2 | 1′38″2 | 59″2 |
| 30 | 1′39″6 | 1′40″6 | 1′38″2 | 59″2 |
| 60 | 1′38″8 | 1′40″6 | 1′38″2 | 59″2 |
| 90 | 1′38″6 | 1′40″6 | 1′38″2 | — |
| 120 | 1′38″2 | 1′40″6 | 1′38″2 | 59″2 |

Experiment : 10 mg A-GPP+0.5 ml enzyme+buffer
Control a : 10 mg A-GPP+0.5 ml inactivated enzyme+buffer
Control b : 10 mg A-GPP+0.5 ml $H_2O$+buffer
Control c : 0.5 ml enzyme+0.5 ml $H_2O$+buffer

The results are shown in Fig. 12. The results indicate that GPP prepared from the capsules of *B. anthracis in vitro* was susceptible to $\gamma$-glutamylase.

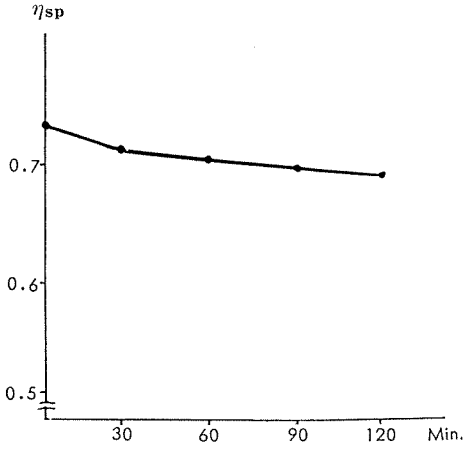


Fig. 13. Break-down *in vivo* A-GPP by $\gamma$-Glutamylase

An A-GPP preparation obtained from the body fluids of infected animals was kindly given to us by Dr. D. W. Watson. Ten mg of the *in vivo* A-GPP was tested with 0.5 ml of a $\gamma$-glutamylase preparation, in the same manner as described above. Table 7 shows the flow time recorded in the experimental and control vessels (10 mg of A-GPP plus 0.5 ml of the heat-inactivated $\gamma$-glutamylase preparation) in 120 minutes. Fig. 13 shows the change in specific viscosity. Though

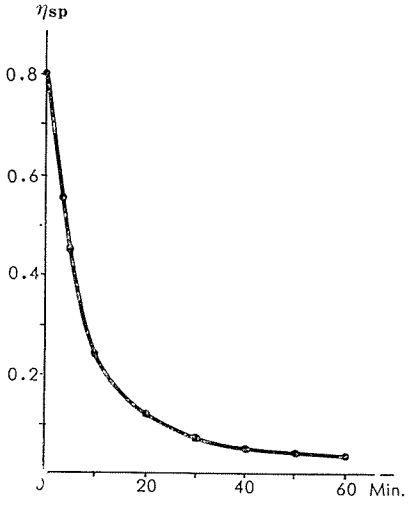


Fig. 14. Break-down of M-GPP by $\gamma$-Glutamylase

the viscosity change in 120 minutes was not great, it is still apparent that the *in*

*vivo* A-GPP was slightly susceptible to γ-glutamylase. The reason for its low susceptibility is discussed below.

Fig. 14 shows the high susceptibility of M-GPP, which consists of a γ-copolymer of D- and L-Glu (Utsumi *et al.*, 1959). The high susceptibility of M-GPP may explain the fact that the capsules of *B. megaterium* dissolve much quicker than those of *B. anthracis*.

## DISCUSSION

Chemical information on the nature of the decapsulating agent in dog organs and tissues led us to conclude that this immunologically important agent is γ-glutamylase, which hydrolyzes the γ-peptide bonds of glutamic acid such as γ-L-Glu-L-Glu and γ-D-Glu-L-Glu (Torii *et al.*, 1960). At first the decapsulating agent was thought to be an enzyme like γ-glutamylase. In the previous report Torii (1955) could not demonstrate the hydrolysis of A-GPP by a crude extract of dog liver but found that M-GPP could be hydrolyzed by the extract. Next this agent was suspected to be a polyase and the effect of the crude extract on a polysaccharide preparation of *B. anthracis* was tested, but without success. Thus we reinvestigated the M-GPP splitting enzyme and Torii found that it attacked L-glutathione (1959). The next year we found that γ-L-Glu-L-Glu could be hydrolyzed by the γ-glutamylase of a crude extract and it became possible to assay the enzyme. Using a partially purified preparation the substrate specificity of γ-glutamylase was studied and γ-D-Glu-L-Glu was found to be the substrate. In addition γ-glutamylase was shown to be an endopeptidase (Torii *et al.*, 1960). These facts encouraged us to purify γ-glutamylase further using γ-L-Glu-L-Glu as substrate. Extensive purification was achieved, as described in this paper.

We had previously studied the cross reactions of bacterial glutamyl polypeptides quantitatively (Utsumi *et al.*, 1959). These experiments enabled us to develop a serological assay method for the decapsulating activity without difficulty. In this method the rate of decapsulation is determined with almost the same degree of accuracy as the γ-glutamylase.

The extensive purification was most helpful in the identification of γ-glutamylase as the decapsulating agent. If γ-glutamylase were an entity distinct from the decapsulating agent, the ratio of the two activities would not be constant during purification of γ-glutamylase. As the parallelism of the two activities was still retained even after rechromatography on a hydroxylapatite column, it can be concluded that γ-glutamylase and the decapsulating agent are identical. As γ-glutamylase is present in high concentration in dog leucocytes (Torii *et al.*, 1960) and the decapsulation of bacteria in infected foci took place after foci had been infiltrated by leucocytes (Cromartie *et al.*, 1947a, b), it can be assumed that decapsulation in dog tissues is also mediated by the γ-glutamylase of the infiltrating leucocytes.

The mechanism of decapsulation can be understood as due to γ-glutamylase, because the hydrolysis of A-GPP could be demonstrated in viscosimetric experiments using the most highly purified preparation. If the crude preparation were used in place of the most highly purified one, no hydrolysis could be shown in viscosimetric experiments as in the results of Torii (1955). The fact, that *in vivo* A-GPP was slightly sensitive to γ-glutamylase, can be explained by the effect of pretreatment *in vivo* with γ-glutamylase, although γ-glutamylase in anthrax-sensitive animals is not very active. However the hydrolysis of capsular GPP may not be the whole of the decapsulation mechanism, because the chemical structure of the connection between the cell wall and the capsule is not yet known and an unknown chemical structure which is very susceptible to γ-glutamylase may be present there.

The significance of the presence of γ-glutamylase in dog tissues is partly on one hand, concerned in the metabolism of reduced L-glutathione and folic acid. Because of the DCA activity on encapsulated *Bacillus anthracis* demonstrated in this paper, γ-glutamylase may also have a protective action in dogs which are resistant to anthrax.

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