# Structure of the Polyglutamic Acid from *Bacillus subtilis*

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The structure of the polyglutamic acid produced by certain strains of the genus *Bacillus* has been studied by a number of methods (literature reviews: Bricas & Fromageot, 1953; Waley, 1955). Bruckner & Ivanovics (1935) isolated and characterized the capsular substance of *B. anthracis* and showed that it consisted largely of units of D-glutamic acid in peptide linkage. More recent work on the soluble peptide from *B. subtilis* by Bovarnick (1942), the *B. anthracis* capsule by Hanby & Rydon (1946) and the soluble peptide from *B. mesentericus* by Pongor (1950) has shown that, to the extent of at least 99%, glutamic acid is the only nitrogen-containing substance in the peptide hydrolysates. The optical configuration of the glutamic acid appears to vary from one preparation to another although it is largely of the D form.

The molecular weight of the peptide depends on the method of preparation and values ranging from 5000 to 70 000 have been reported. The workers concerned, nevertheless, have not found the determination of molecular weight to be very straightforward. Physical measurements such as sedimentation and diffusion require much care as the results are markedly dependent on pH and salt concentration, owing to the poly-electrolyte nature of the material (Bovarnick, Eisenberg, O'Connell, Victor & Owades, 1954). Amino end-group analysis by the Sanger technique (Waley, 1955) is not very precise, for it is known that the reaction between a terminal glutamic acid residue and dinitrofluorobenzene can be taken to completion only with difficulty. Amino nitrogen determination with nitrous acid (Hanby & Rydon, 1946) is subject to the uncertainty of the behaviour of $\gamma$-peptide linkages with the reagent (Chibnall & Westall, 1932; Sachs & Brand, 1954).

The nature of the peptide linkage between the glutamic acid residues calls for more extended comment. Bovarnick (1942) concluded that the peptide from *B. subtilis* must be largely $\gamma$-linked on the basis of a negative biuret test and the absence of racemization on standing in alkaline solution. From titration data Hanby & Rydon (1946) concluded that both $\alpha$- and $\gamma$-linkages were present in the capsular substances from *B. anthracis*. Kovacs & Bruckner (1952) applied the Curtius degradation and Bruckner, Kovacs & Nagy (1953) the Hofmann degradation to the peptide from *B. subtilis*. In both cases the presence of $\gamma$-linkages was confirmed by the isolation of crystalline derivatives of $\beta$-formylpropionic acid. No evidence for $\alpha\gamma$-diaminobutyric acid, indicating an $\alpha$-linkage, was obtained, but the complete absence of this substance was not demonstrated even though it could be easily prepared by both degradation techniques from samples of synthetic poly-$\alpha$-glutamic acid (Kovacs, Bruckner & Kovacs, 1953; Bruckner, Kovacs & Kovacs, 1953). Waley (1955) compared the natural polyglutamic acid with synthetic $\alpha$-linked and $\gamma$-linked glutamyl peptides in respect to solubility in water, reaction with ninhydrin, titration constants, rate of acid hydrolysis and infrared spectra, and has concluded that the natural material most closely resembles the synthetic $\gamma$-linked material. The evidence thus suggests that the polypeptide is largely, if not wholly, $\gamma$-linked; nevertheless, the presence of a small percentage of $\alpha$-linkages has not been completely ruled out and the possibility that branch-peptide chains might occur has not been the subject of any specific inquiry.

The work reported in the present paper has been undertaken in the hope that the procedure of reduction of the ester with lithium borohydride would provide a final answer to these queries. The problem is really no more than a special, if somewhat simplified, example of the more general one of amide distribution dealt with successfully in the preceding paper (Chibnall, Haselbach, Mangan & Rees, 1958*a*). On the assumption that the polypeptide molecule consists of one peptide chain built up of glutamic acid residues, the ester should give $\delta$-hydroxy-$\gamma$-aminovaleric acid ($\delta$-$\gamma$-HAV) if these are $\gamma$-linked, $\alpha$-hydroxy-$\gamma$-aminovaleric acid ($\alpha$-$\gamma$-HAV) if they are $\alpha$-linked, and in both glutamidiol from the *C*-terminal residue. If, on the contrary, a reductive fission (Crawhall & Elliott, 1955) of the polypeptide itself should occur then any hydroxy-amino acid produced would have the reverse significance; $\delta$-$\gamma$-HAV would denote an $\alpha$-linkage

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and δ-α-HAV a γ-linkage. In the ester, both types of linkage would lead to an increase in amount of glutamidiol. The results show that the glutamic acid residues are exclusively γ-linked. The presence or absence of branched chains and rings has been investigated by titration. If the product is a simple peptide chain of glutamic acid residues, one of them being *C*-terminal, the total number of titratable groups will be equal to the number of residues plus one. If branched chains should be present the number of *C*-terminal residues/mole would be correspondingly increased, but the total number of titratable groups/mole would remain unchanged. On the other hand, intramolecular folding to form closed rings, of the type that Ivanovics & Horvath (1953) suggest may be present in the parent capsular material of *B. anthracis*, would lead to a corresponding reduction in the total titratable groups/mole. The results suggest that branched chains and rings are not present.

## EXPERIMENTAL AND RESULTS

### *Esterification*

*Esterification.* The methods of esterification and of estimation of methoxyl are those given by Chibnall, Mangan & Rees (1958*c*).

*Polypeptide from* B. subtilis. Sample *A* (reputed mol.wt. 13 000) and sample *B* (reputed mol.wt. 39 000) were kindly provided by Dr M. Bovernick.

*Synthetic poly-α-glutamic acid.* This was kindly supplied by Dr J. Watson (Hanby, Waley & Watson, 1950), who informed us that the sample had been prepared from the ester by saponification with ethanolic KOH.

*Estimation of δ-hydroxy-γ-aminovaleric acid.* As the hydrolysate of the reduced bacterial peptide ester contained only glutamic acid and products derived from it, we assumed in the early stages of this work that a direct determination of the formaldehyde given on periodate oxidation (Rees, 1958) would be a valid measure of the total amount of δ-γ-HAV and glutamidiol present. Misleading deductions were indeed drawn as to the extent of reduction achieved before it was realized that the colour intensity on which the estimation was based could be lowered by the presence in the hydrolysate of boric acid derived from the reducing agent. The magnitude of the effect is illustrated by the following experiment. A suitable hydrolysate, prepared from a sample of peptide ester that had been reduced with a tenfold molar excess of $LiBH_4$, was taken and the formaldehyde formed on treatment with periodate was estimated on a sample. δ-γ-HAV equivalent to the formaldehyde found was then added to another sample and formaldehyde was again determined. The recovery of the hydroxyamino acid was only 83·3%. In another experiment, with an ester reduced with a 15-fold molar excess of $LiBH_4$, the recovery was 87%. To overcome such irregular interference an attempt was made to remove the boric acid as volatile methyl borate. In a trial experiment three 1 ml. samples of a solution of serine (13·7 mg. in 50 ml.) were treated in the following way. The first was evaporated to dryness *in vacuo* at room temperature. To the second was added 6·8 mg. of $LiBH_4$ in tetrahydrofuran (1 ml.). Excess of HCl was then added and the mixture evaporated to dryness *in vacuo* at room temperature. A similar amount of $LiBH_4$ in tetrahydrofuran was added to the third. The mixture was then heated with excess of methanolic HCl on the water bath for 3–4 min. and then evaporated to dryness *in vacuo*. The treatment with methanolic HCl was thrice repeated. Periodate–formaldehyde estimations on the three samples gave recoveries of 100, 44 and 99·8% serine respectively, showing that the estimation of serine, like that of δ-γ-HAV, is unreliable in the presence of boric acid. The latter can, however, be effectively removed as volatile methyl borate. Unfortunately, the modified procedure cannot be applied to the estimation of δ-γ-HAV as ring formation, to give pyrrolidonyl alcohol occurs to a small extent during the treatment with methanolic HCl. In the present work δ-γ-HAV has been estimated by column analysis.

*Column analysis of glutamic acid, δ-hydroxy-γ-aminovaleric acid, δ-hydroxy-α-aminovaleric acid and glutamidiol.* The method of Chibnall *et al.* (1958*a*) was used. In the absence of aspartic acid and its derivatives no difficulty was experienced in obtaining a good separation between the two hydroxyamino acids. As glutamidiol was the only amino alcohol concerned it could be determined by prolonging the elution until the fourth day.

*Stability of δ-hydroxy-γ-aminovaleric acid and δ-hydroxy-α-aminovaleric acid on acid hydrolysis.* As unexpectedly large amounts of dissolved $NH_3$ were found in the hydrolysates of the reduced bacterial polypeptide it was necessary to investigate the stability of the two hydroxyamino acids under similar conditions of hydrolysis. The recovery of δ-γ-HAV, ascertained by estimation of periodate–formaldehyde, was 85% before column analysis (Table 1) and 72·5% after it (Chibnall *et al.* 1958*a*). Likewise the recoveries of δ-hydroxy-α-aminovaleric acid (δ-α-HAV),

Table 1. *Stability of δ-hydroxy-γ-aminovaleric acid and δ-hydroxy-α-aminovaleric acid on acid hydrolysis*

Acid used: 10N-HCl–formic acid (1:1, v/v).

| | δ-γ-HAV | | δ-α-HAV | | |
|---|---|---|---|---|---|
| Period of hydrolysis (hr.) | Equiv. periodate–formaldehyde (as % equiv. total N) | Ammonia N (as % of total N) | δ-α-HAV N (as % of total N) | Proline N (as % of total N) | Ammonia N (as % of total N) |
| 3 | 91·3 | 1·78 | — | — | — |
| 6 | 87·0 | 2·32 | — | — | — |
| 24 | 85·0 | 3·33 | 65·7 | 2·30 | 2·21 |

estimated with ninhydrin, were 65·7% before column analysis and 60% after it. Proline, which might be formed from δ-α-HAV by dehydration (Plieninger, 1950) was estimated by the column procedure of Moore & Stein (1954). Ammonia was estimated with ninhydrin by the Conway technique (Chibnall, Mangan & Rees, 1958*b*). As only limited supplies of the two synthetic hydroxyamino acids were available and the amount of $NH_3$ likely to be produced was small, particular attention was given to the purity of the reagents used. Redistilled constant-boiling HCl was brought to 10N with gaseous HCl; formic acid was redistilled. The saturated $K_2CO_3$ solution was aerated for some time to remove $NH_3$. The hydrolysis of the samples was carried out in a flask fitted with a long narrow air condenser to minimize adsorption of atmospheric $NH_3$. In the Conway estimation the hydrolysis blank was 0·15 μg., the apparatus blank was 0·46 μg. and the $NH_3$ measured was not less than 3·4 μg.

*Comment.* The data given in Table 1 show that both hydroxyamino acids undergo a slow change on hydrolysis. In each case ammonia is produced, but in amount much less than the equivalent of parent acid lost, or than might have been expected from the analysis of a reduced bacterial polypeptide hydrolysate. δ-γ-HAV must undergo ring formation to give pyrrolidonyl alcohol, whereas δ-α-HAV, which gives but very little proline, must go over to the δ-lactone. Under the conditions of hydrolysis and column analysis employed in the present work these changes, though of relatively large magnitude, were readily reproducible, and we have confidence in the correction factors used in the present as well as in previous papers (Chibnall & Rees, 1958; Chibnall *et al.* 1958*a*).

*Titratable carboxyl groups of the bacterial peptide*

On the assumption that sample *A*, of reputed mol.wt. 13 000, is a simple peptide chain bearing a *C*-terminal glutamic acid residue, it will contain 99 residues (or N atoms) and 100 titratable groups/mole or, expressed in another way, the titration value will be equivalent to 101% of total N. Likewise, sample *B* of reputed mol.wt. 39 000 would have a titration value equivalent to 100·3% of total N.

In a typical experiment sample *A* (35 mg.) was suspended in water (2 ml.) and brought to pH 7 (bromothymol blue) with 0·0143N-NaOH. The material dissolved during the operation and the end point was judged by colour comparison with a similar volume of buffer solution, pH 7, contained in a flask of like dimensions. The solution was then made to standard volume and total N determined. The titration value was equivalent to 94·5% total N and was easily reproducible to within 0·5%. Sample *B* gave a titration equivalent to 97·0% of total N. Under comparable conditions glutamic acid was fully titrated.

The values given by both samples of polypeptide were less than the theoretical minimum for a single or branched-chain molecule, and for reasons discussed below it was considered that this might be due to slight esterification brought about by the use of alcohol during the preparation of the samples from the bacterial capsule. Methoxyl determinations were accordingly made and these were found to be equivalent to 5·0 and 2·95% of total N for samples *A* and *B* respectively; each value remained unchanged if the material was twice dissolved in water and dried from the frozen state to remove any excess of methanol. Adding these values to the titration values, both products show a close agreement between the number of carboxyl groups and the number of residues/mole, indicating the probable absence of ring structures.

*Esterification of the bacterial polypeptide*

According to our experience, proteins, when suspended in methanol and treated with methanolic HCl of the requisite strength, will dissolve readily as the hydrochloride and full esterification can be achieved under suitable conditions (Chibnall *et al.* 1958*a*). The bacterial polypeptide and also synthetic poly-α-glutamic acid, however, will not disperse when treated in this way, presumably owing to the large excess of carboxyl groups (cf. Hanby *et al.* 1950). The following procedure was found to be effective for the purpose.

The bacterial polypeptide or the poly-α-glutamic acid (125 mg.) was suspended in water (1 ml.) and 0·96N-LiOH added with stirring until the product had dissolved (0·825 ml.). The clear solution was then freeze-dried. The residue, a hard mass, was ground up with methanolic HCl equivalent to the LiOH added, when solution was effected in about 3 min. By the appropriate addition of methanolic 0·94N-HCl and methanol the volume was then brought to 25 ml. and the HCl to 0·1N or 0·05N as the case may be. After the solution was brought to 25° a sample (2 ml.) was withdrawn and run into acetone–ether (1:1; 12 vols.). The precipitated material was centrifuged off, washed once with the same volume of acetone–ether and then dissolved in water (8 ml.). The extent of esterification was ascertained by titration, as described in the preceding section, or by Zeisel analysis. The main solution was kept at 25°, and further samples were withdrawn at intervals for treatment in the same way.

*Comment.* The data given in Table 2 show that the γ-carboxyl groups of the poly-α-glutamic acid are more reactive than the carboxyl groups of the bacterial polypeptide; indeed, the former product is more than 90% esterified in only 10 min. That γ-glutamyl groups should esterify with such great rapidity was unexpected, because in a more comprehensive investigation of β-lactoglobulin, with a similar esterifying medium, only 69·1% of such residues were esterified in 30 min. and 86·6% in 1 hr. (Chibnall *et al.* 1958*c*), a reaction rate which is impressive and greater than that for the bacterial polypeptide, but much lower than that for the poly-α-glutamic acid.

One other observation needs mention. About 93–94% esterification of the bacterial polypeptide occurs in about 3 hr. with methanolic 0·1N-HCl and the product remains soluble in the medium. When precipitated by acetone–ether, as described above, the centrifuged and washed material dissolved readily in water. If, on the contrary, the period of esterification was extended beyond about 3 hr. there was a slow separation of the fully esterified material, and this, when dried as before, was no longer soluble in water. The significance of the difference in behaviour will appear later.

### *Reduction of the bacterial polypeptide and its methyl ester*

The results are given in Table 3. Experience has shown that a tenfold molar excess of $LiBH_4$ was the most suitable for present purposes, but under certain conditions discussed below it was found difficult to effect complete reduction of the peptide ester, even with a much greater excess of reductant than this. The degree of dispersion of the polypeptide and ester was one of the factors concerned, and this was controlled in the following way.

The polypeptide (25 mg.) suspended in water (0·5 ml.) was treated with sufficient aq. LiOH soln. to effect solution. It was then precipitated by adding methanol (20 ml.) containing 10% (w/v) of LiCl. The product was washed with acetone (20 ml.), then three times with dry ether (20 ml.) and finally three times with tetrahydrofuran (12 ml.). During treatment the polypeptide was in contact with methanol for 3–5 min. The ester hydrochloride (0·1265 g.), prepared as described earlier, was washed first with ether and then three times successively with tetrahydrofuran (7 ml.) and finally brought to a fine suspension in that solvent. The polypeptide, as a fine suspension in

Table 2. *Esterification of polyglutamic acids in methanolic hydrochloric acid at 25°*

The bacterial polypeptide was sample *A*, of reputed mol.wt. 13 000 (see text). Progress of esterification was measured by titration or, where marked *, by Zeisel determination of methoxyl. Results are expressed as percentage total esterification.

| Time after addition of methanolic HCl (min.) | Esterification of bacterial polypeptide in Methanolic 0·1N-HCl | Methanolic 0·05N-HCl | Esterification of synthetic poly-α-glutamic acid in methanolic 0·1N-HCl |
|---|---|---|---|
| 0 | 10·2 | 10·2 | 27·0 |
| 10 | — | — | 91·6 |
| 20 | — | — | 96·7 |
| 30 | 49·4 | 44·5 | 98·0* |
| 60 | 69·4 | 65·1 | — |
| 90 | 78·9 | — | — |
| 120 | 86·6 | 80·0 | — |
| 165 | 92·5 | — | — |
| 240 | — | 92·0 | — |
| 1080 | 98·0, 97·0* | — | — |

Table 3. *Analytical data for polyglutamic acids and their methyl esters, with details of reduction with lithium borohydride*

The reputed molecular weight of sample *A* of the bacterial peptide was 13 000, and that of sample *B* 39 000 (see text).

| Expt. no. | Bacterial peptide (sample *B*) 1 | 2 | 3 | Bacterial peptide methyl ester (sample *B*) 4 | 5 | 6 | 7 | 8 | Bacterial peptide methy ester (sample *A*) 9 | 10 | Synthetic poly-α-glutamic acid methyl ester 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Methoxyl (% by wt.) | 2·95 | 2·95 | 2·95 | 93·8 | 98·0 | — | — | — | — | 97·0 | — |
| Titratable carboxyls (% total carboxyl groups) | 97·0 | 97·0 | 97·0 | — | — | — | — | — | 7·3 | — | — |
| $LiBH_4$ (mol./mol. of peptide N) | — | — | 10 | 15 | 10 | 8 | 9 | 19 | 10 | 10 | 8 |
| Molarity of $LiBH_4$ | — | — | 0·33 | 0·5 | 0·33 | 0·5 | 0·6 | 1·2 | 0·33 | 0·33 | 0·5 |
| Reduction time (hr.) | — | — | 6 | 6 | 6 | 5 | 18 | 94 | 6 | 6 | 18 |
| Hydrolysis time (hr.) | 2 | 24 | 3 | 16 | 6 | 2 | 2 | 2 | 7 | 6 | 6 |

tetrahydrofuran (1 ml.), or a sample of the ester suspension (1 ml. = 25·3 mg.), was then reduced under reflux for 6 hr. with $LiBH_4$ (33 mg.) in a total volume of 3 ml. of tetrahydrofuran. At the end of the reaction the cooled mixture was treated with excess of methanolic HCl and allowed to stand overnight. The time interval is advantageous in that borate complexes are thereby decomposed and most of the borate passes into solution. The reduced product was then centrifuged off, washed with ether and dried *in vacuo*. It was hydrolysed with 10N-HCl–formic acid (1:1, v/v; 5 ml.) for the required time.

## DISCUSSION

The bacterial polypeptide itself (Table 4, no. 3) exhibited but little change on reduction. The data given in Table 4 show that in all the experiments the amount of glutamidiol given by the polypeptide ester is in excess of that which could arise from the presence of 1 *C*-terminal residue/mole (0·33 % for sample *A*, 1 % for sample *B*). As was mentioned earlier, the finding could be interpreted as evidence for branch-peptide chains, each of which would carry a *C*-terminal residue. Under such conditions one would expect to find that the amount of glutamidiol produced was more or less independent of the reductive conditions employed and this is definitely not the case. The diol production is clearly a function of both reductant concentration and reaction time, and was due, almost certainly, to reductive cleavage.

In none of the experiments with bacterial material quoted in Tables 3 and 4 was δ-α-HAV encountered, and an amount representing less than 0·05 % could have been readily detected. As this particular hydroxyamino acid can undergo dehydration on acid hydrolysis to give proline a sample of a reduced ester from sample *B* was analysed for this amino acid on a standard ion-exchange column (Moore & Stein, 1954); none was found. It may be concluded therefore that α-glutamyl peptide linkages do not occur in either sample of bacterial polypeptide, and the possible small excess of δ-γ-HAV produced on reduction of the polypeptide itself must be due to slight esterification during the initial treatment to disperse the product or, less likely, to a direct reduction of the α-carboxyl group.

The evidence for the presence of γ-glutamyl peptide linkages on the other hand is unequivocal, as shown by the extensive production of δ-γ-HAV. Nevertheless, in the early stages of the work certain of the reduced ester hydrolysates gave results which were not readily interpretable in terms of a single-chain poly-γ-glutamyl molecule. Two unusual features of sporadic and irregular occurrence puzzled us, and until these had been clearly defined and their full import realized, much effort was wasted in trying to obtain concordant results with

Table 4. *Amino acid analysis of polyglutamic acids and their methyl esters after reduction with lithium borohydride*

| Expt. no. ... ... ... | N (% of total N) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| Amino N after hydrolysis* | 98·0 | 96·0 | 96·2 | 91·3 | 82·2 | — | — | — | — | — | 90·3 |
| Glutamic acid | — | — | 92·5 | 8·0 | 20·0 | 22·4 | 6·6 | 2·9 | 5·5 | 22·0 | 5·7 |
| δ-γ-HAV | — | — | 3·6 | 82·2 | 60·5 | 57·8 | 72·5 | 64·3 | 83·7 | 63·1 (0·85)† | 4·3 |
| δ-α-HAV | — | — | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 77·8 |
| Glutamidiol | — | — | 0 | 2·0 | 1·2 | — | 1·8 | 3·9 | 2·7 | 1·4 | 3·0 |
| $NH_3$ | 0 | 0·4 | 1·3 | 6·0 | 14·1 | — | — | — | 7·0 | 9·4 | 1·3 |
| Total accounted for | — | — | 97·4 | 98·2 | 95·8 | — | — | — | 98·9 | 95·9 | 92·1 |

* Corrected for $NH_3$ present.

† Representing *N*-terminal δ-γ-HAV in peptides, see text.

reduced products prepared under what were thought at the time to be comparable conditions. First, the extent of reduction appeared to be unpredictable and was not brought under control until its dependence on the degree of esterification had been elucidated. Paradoxically, both sample *A* and sample *B* under comparable conditions underwent less reduction when fully esterified than when esterified to only about 93% (Table 4, nos. 5 and 10, contrasted with nos. 4 and 9). Secondly, the ammonia produced on hydrolysis of the reduced esters varied in the same irregular way and was also shown to be subject to the degree of esterification. In this case, however, the fully esterified product gave more than that esterified by only 93%.

We believe that these anomalies are a reflexion of the state of aggregation of the products concerned. Where less than full esterification has been achieved ionizable polar groups are still present. Presumably the individual peptide chains retain their intrinsic property of free movement and in an aqueous acid medium $H^+$ ions will have ready access to the peptide bonds. It is thus not surprising that such a product should be soluble in water and readily hydrolysed by aqueous acid. Under the usual conditions the polypeptide itself is fully hydrolysed in 2 hr., whereas 3 hr. was sufficient if it had been esterified to 93% before reduction. In the fully esterified product, on the contrary, the *N*-terminal residue carries the only polar group. It is to be expected therefore that the long peptide chains will tend to aggregate under the influence of van der Waals forces and will be insoluble in water. The approach of $H^+$ ions to the peptide bonds may thus be hindered and hydrolysis in aqueous acid slowed. It has, indeed, been observed that under the usual conditions of hydrolysis peptides containing *N*-terminal δ-γ-HAV residues which will react with periodate are still present in small amounts after 6 hr. On column analysis they emerge immediately behind δ-γ-HAV itself (Table 4, no. 10). It is more difficult to argue about susceptibility to reduction in a non-aqueous medium, but the results given in Table 4 are consistent with the view that the approach of the reducing agent is the more difficult when the product is fully esterified.

All of these observations are in keeping with the suggestion that the fully esterified product, both before and after reduction, has a more closely knit configuration than the one that is esterified to a lesser degree, and accordingly may require more drastic treatment than usual with strong acid to fragment it. It is to be noted that during the hydrolysis of the reduced ester the derived δ-γ-HAV has undergone more extensive decomposition, with production of ammonia, than when present as the free hydroxyamino acid in the hydrolysis medium (Table 1), and this is especially marked in the fully esterified products. The evidence thus seems fairly conclusive that the samples of bacterial polypeptide, of high as well as of low molecular weight, are single-chain poly-γ-glutamyl peptides.

The reduction of methyl poly-α-glutamate (Tables 3 and 4, no. 11) was allowed to proceed for 18 hr. to emphasize any reductive cleavage and the presence of glutamidiol in excess of that derived from one *C*-terminal residue shows that this has indeed occurred to a small extent. The production of 4·3% of δ-γ-HAV suggests that the sample of γ-ethyl glutamate used in the synthesis of the poly-α-glutamic acid contained about 5% of the α-homologue. In this experiment proline was not determined, and the omission is in part responsible for the rather low recovery of nitrogen accounted for.

Finally, there are two observations arising out of the present study which need particular emphasis, for they undoubtedly have implications in general protein chemistry. The first is the ease with which γ-carboxyl groups in a long peptide chain will esterify. The second is the clear demonstration that the destruction of residues in the early stages of hydrolysis, when they are still in peptide linkage, may greatly exceed that of the free amino acid under the conditions used for hydrolysis (cf. Martin & Synge, 1945).

## SUMMARY

1. Titration of two different samples of poly-glutamic acid from *Bacillus subtilis* suggests that they are built up exclusively of open peptide chains, and that closed ring structures are not present.

2. When esterified and reduced with lithium borohydride, the products given on subsequent acid hydrolysis show that the glutamic acid residues in the polypeptide are exclusively γ-linked and that the presence of branch chains is unlikely.

3. The properties of the ester, the extent of reduction achieved and the composition of the hydrolysate of the reduced ester are dependent in an unusual way on whether the degree of esterification is less than 93% or exceeds this value.

4. It is suggested that this difference in ester behaviour can be correlated with the number of residual carboxyl groups present.

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# Determination of the *N*-Terminal Residues in Proteins with Methoxycarbonyl Chloride

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The well-known procedure of Sanger (1945), which employs 1-fluoro-2:4-dinitrobenzene (FDNB) for the determination of *N*-terminal residues in proteins, has been widely used, but in certain cases, e.g. tropomyosin, where the presence of a *C*-terminal residue can be demonstrated by means of carboxypeptidase, it has so far failed to reveal the presence of any *N*-terminal residue at all. This apparent conflict of evidence led Bailey (1953) and Desnuelle (1953) to suggest tentatively that the *N*-terminal amino group might be linked with an $\omega$-carboxyl group in the peptide chain to give a structure which can be schematically described as a 'figure 6'. Bailey recognized, however, that the failure to detect *N*-terminal residues with FDNB might be due either to steric factors or to the instability of the derived dinitrophenyl-(DNP)-amino acid given on subsequent acid hydrolysis. There is one other possibility. When a protein is treated with FDNB under the usual conditions the DNP-protein rapidly comes out of solution. Tropomyosin is rich in lysine residues, and it might well be that these react to give an insoluble product before the *N*-terminal residues have become fully substituted.

To probe these possible limitations to the use of FDNB an alternative reagent has been sought which would fulfil the following three conditions. First, it should be of low molecular weight so as to reduce the possibility of steric hindrance. Secondly, it should condense with proteins to give products which remain soluble in the reaction mixture, thereby favouring the attainment of full substitution. Thirdly, the substituted protein should be readily soluble in concentrated hydrochloric acid at a temperature around 37°. To effect solution of DNP proteins it is generally necessary to treat with boiling 6N-hydrochloric acid for some time. Such drastic conditions of hydrolysis, however, might not be necessary to split off the *N*-terminal residue, for the stability of the peptide bond concerned would be lowered by the elimination of the strongly charged terminal amino group. It was hoped that hydrolysis with concentrated hydrochloric acid at 37° over a period of several days (Gordon, Martin & Synge, 1941) would be suitable for the purpose, and thus permit the employment of a reagent giving an *N*-substituted amino acid which might not survive prolonged boiling in 6N-hydrochloric acid.

Methoxycarbonyl (MC) chloride (I), which readily condenses with an amino acid (II) to give the methoxycarbonylamino acid (III), was found to satisfy these conditions.

$$\underset{\text{(II)}}{HO_2C\cdot CH(R)\cdot NH_2} + \underset{\text{(I)}}{Cl\cdot CO\cdot OCH_3} \rightarrow \underset{\text{(III)}}{HO_2C\cdot CH(R)\cdot NH\cdot CO\cdot OCH_3} + HCl$$