Fractionation of the Chymotryptic Precipitate of *Bombyx mori* Silk Fibroin

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1. A solution of *Bombyx mori* silk fibroin was digested with chymotrypsin. Amino acid analyses of the chymotryptic precipitate showed in addition to the main constituents Gly, Ala, Ser and Tyr, very small amounts of Lys, His, Arg, Asp, Thr, Glu, Pro, Cys, Val, Met, Ile, Leu, Phe and Trp. 2. A stable solution of the chymotryptic precipitate in 6M-urea was obtained by dialysing a solution in 50% (w/v) lithium thiocyanate against 6M-urea. 3. The dinitrophenylated chymotryptic precipitate in 6M-urea was fractionated by gel filtration and by ion-exchange chromatography. On Dowex 1 (X2), a main fraction I_D and three further fractions with different amino acid compositions and molecular weights were obtained. 4. Specific rearrangement and fission of the bonds involving the serine nitrogen atoms of fraction I_D and fractionation of the resulting mixture by gel filtration yielded five fractions. Two of these fractions had the compositions DNP-Ser-(Gly₆,Ala₄,Ser) and DNP-Ser-(Gly₄,Ala₂ or Ala₃,Ser) and are presumably double repeating units according to the proposed formula of Lucas, Shaw & Smith (1957), namely [Ser-Gly-(Ala-Gly)_n]₂, for *n* values of 2 and 1 respectively.

Drucker & Smith (1950) have shown that an aqueous solution of *Bombyx mori* silk fibroin can be separated into two fractions of different amino acid composition after being digested by a mixture of pancreatic enzymes. Subsequently Drucker, Hainsworth & Smith (1953) repeated their earlier investigations using the more specific hydrolytic enzymes, trypsin and chymotrypsin. Digestion with chymotrypsin yielded a precipitate (fraction Cp) with a molecular weight 4000 containing glycine, alanine and serine in the proportions 3:2:1 with a small amount of tyrosine. The soluble fraction consisted of a mixture of peptides with a mean chain length of five residues containing all the constituent amino acids of the fibroin.

Amino acid-sequence studies of fraction Cp were undertaken by Waldschmidt-Leitz & Zeiss (1955) and by Lucas *et al.* (1957). The results of these research groups were extremely divergent. According to Waldschmidt-Leitz & Zeiss (1955) the chymotryptic precipitate had a formula [Gly-Ala-Ser-Gly-Ala-Gly]₇-Tyr, whereas Smith and co-workers established the following composition: Gly-Ala-Gly-Ala-Gly-[Ser-Gly-(Ala-Gly)_n]₈-Ser-Gly-Ala-Ala-Gly-Tyr, in which *n* is usually 2 and always has a mean value of 2. The sequence of Gly-Gly derivable from the formula of Waldschmidt-Leitz & Zeiss (1955) was convincingly excluded.

A comparison of the hexapeptide Ser-Gly-Ala-Gly-Ala-Gly synthesized by Zahn & Schnabel (1957) and Schnabel & Zahn (1958) with the partially degraded chymotryptic precipitate favoured the formula proposed by Lucas *et al.* (1957). Therefore it seemed reasonable to prefer this formulation, although the value of *n* was not exactly defined. Despite numerous investigations, none of the above-mentioned authors was able to prove the homogeneity of the chymotryptic precipitate, as is seen in the statement of Lucas, Shaw & Smith (1958): 'It would, of course, be preferable before proceeding with sequence studies to separate the material into fractions which are terminally homogeneous, but the difficulties in doing this on a polypeptide of such size and insolubility are very great. Attempts in these laboratories to recrystallize it from the few solvents in which it will dissolve were unsuccessful.'

The purpose of the present paper is to describe attempts made to fractionate the chymotryptic precipitate of silk fibroin.

MATERIALS AND METHODS

Preparation of fraction Cp and its dinitrophenyl derivative. Fibroin of *Bombyx mori* was prepared from 26/30 denier Japanese raw silk dissolved in cupri-ethylenediamine according to the method of Drucker *et al.* (1953). The fibroin

solution was dialysed in Visking 36/32 cellophan dialysis tubing. Enzymic digestion was with α -chymotrypsin I (3-times crystallized, CDI 6114-5; Worthington Biochemical Corp., Freehold, N.J., U.S.A.). 1-Fluoro-2,4-dinitrobenzene was prepared as described by Zahn & Würz (1951), and fraction Cp was dinitrophenylated according to the method of Lucas *et al.* (1957). Lithium thiocyanate (pure) was purchased from Fluka A.-G., Buchs, Switzerland, and further purified by twofold recrystallization from water. A concentrated solution was prepared and adjusted to a 50% (w/v) content by titration according to the method of Volhard. In the same way we prepared a 76.0% (w/v) solution of LiBr (ultrapure; E. Merck A.-G., Darmstadt, Germany).

Analytical methods. High-voltage paper electrophoresis was carried out at 50 v/cm. in buffers of pH 1.9 and pH 3.5 described by Kückhöfen & Westphal (1952). For thin-layer chromatography 0.5 mm. layers of Kieselgel (HF 254; E. Merck A.-G.) were prepared on glass plates. The chromatograms were developed with the following solvents: DNP-peptides, butan-2-ol-formic acid-water (150:27:23, by vol.); amino acids, phenol-water (4:1, w/v); DNP-amino acids, chloroform-methanol-acetic acid (14:6:1, by vol.); chloroform-benzyl alcohol-acetic acid (70:30:3, by vol.). For all quantitative determinations the products were dried over P_2O_5 *in vacuo* at 75°.

Amino acid analyses were done with a Beckman-Spinco automatic amino acid analyser (Spackman, Stein & Moore, 1958). The conditions for hydrolysis and the corrections for destruction during hydrolysis of serine, threonine and tyrosine were those described by Moore & Stein (1963). To determine the content of amino acids present in fraction Cp in very low percentages we applied a suitable amount of hydrolysate to the columns and stopped the flow of ninhydrin at the breakthrough of glycine and alanine to avoid blockage of the Teflon coil. The following amounts of fraction Cp were, after hydrolysis, applied to the columns for determination of the respective amino acids: 20 mg. for proline and the basic amino acids, 0.25 mg. for glycine and alanine, 40 mg. for cysteic acid, 2 mg. for the other neutral and acidic amino acids. Tryptophan was determined as described by Spies & Chambers (1948) and cystine after oxidation to cysteic acid by the technique of Moore (1963). Quantitative determination of DNP-amino acids was carried out by chromatography on nylon-powder columns according to the method of Siepmann & Zahn (1965). DNP-peptides were hydrolysed with 6N-HCl at 95° for 16 hr. in a sealed tube. Corrections for destruction during hydrolysis were made as follows: DNP-glycine according to Weigmann (1963); the other *N*-DNP-amino acids according to Middlebrook (1951); *O*-DNP-serine 23% loss, *O*-DNP-tyrosine 6% loss according to Beyer (1966).

Sedimentation-equilibrium measurements were performed in a Spinco model E ultracentrifuge at 35600 rev./min., phase-plate angle 80° and a temperature of 20° in a double-sector capillary cell. Samples of DNP-Cp (52.2 mg.) and fraction I_D (52.5 mg.) were dissolved at 30–40° in 3.29 ml. of 76% (w/v) LiBr and made up to 10 ml. with distilled water. Then 5 ml. of each solution was filtered and dialysed 24 hr. in Visking 18/32 tubing, against 25% (w/v) LiBr. Before dialysis, the tubing was prepared as follows: 1 hr. boiling in 0.1M-NaHCO₃, three times in distilled water, three times washed with ethanol and finally washed with distilled water.

From the schlieren patterns we calculated the molecular-weight distribution according to the method of O'Donnell & Woods (1961).

Fractionation of the DNP-Cp. For fractionation we used the DNP-Cp because in the product all peptides carry DNP residues at the amino end groups and at the phenolic hydroxyl groups of tyrosine. The fractionation was easily observed by measuring the extinction at 254 m μ . By dialysing a solution of DNP-Cp in LiSCN against 6M-urea we obtained a solution of DNP-Cp in 6M-urea which was suitable for fractionation by gel filtration and ion-exchange chromatography.

A 5 g. sample of DNP-Cp was dissolved in 150 ml. of 51.3% (w/v) LiSCN at 20° and the solution was dialysed in Visking 18/32 tubing against 6M-urea (urea pure, E. Merck A.-G.) until the dialysis fluid gave a negative test for thiocyanate with FeCl₃. At that time the solution inside the tubing was also free of thiocyanate. The solution was filtered to remove traces of precipitate. The content of DNP-Cp in this solution was determined as follows: 3 ml. of the solution was diluted with 30 ml. of distilled water and saturated with (NH₄)₂SO₄. After standing overnight the whole of the DNP-Cp had precipitated, the supernatant liquor being colourless. The precipitate was collected on a sintered-glass suction filter, washed with distilled water and weighed, after drying over P₂O₅ in a vacuum desiccator. The solution contained 19.5 mg. of DNP-Cp in 100 ml.

Gel filtration on Sephadex G-50 and G-75 was carried out in 175 cm. $\times$ 1.7 cm. columns. Sephadex, suspended in 6M-urea, was poured into the column according to the method of Pharmacia, Uppsala, Sweden. Then 1 ml. of the DNP-Cp solution in 6M-urea was applied to the column and the elution commenced with 6M-urea. The extinction of the eluate at 254 m μ was measured and recorded by a Uvicord 4701A spectrophotometer (LKB Produkter AB, Stockholm, Sweden) and 5 ml. fractions were collected automatically.

Dowex 1 (X2; 200–400 mesh; Serva, Heidelberg, Germany) was purified and converted into the acetate form (Rudloff & Braunitzer, 1961). Stepwise elution was carried out with buffers made 0.1M in ammonium acetate and 6M in urea with increasing concentration of acetic acid as follows: A, 0.1N; B, 1N; C, 5N. The fractions I_D and II_D were eluted with A, fraction III_D with B and IV_D with C (see Fig. 1). Further fractions were obtained by elution with a 10N-acetic acid buffer, but, as their total yield was only about 1% by wt. of the applied starting material, these were discarded. The fractions were dialysed in Visking 36/32 tubing against distilled water. After a short time the fractions I_D and II_D precipitated in the dialysis tubing and were centrifuged off and successively washed twice with distilled water, 0.02N-HCl, distilled water, methanol and ether.

The fractions I_{G-75}, III_D and IV_D remained in solution. These were dialysed for 3 days and evaporated to dryness in a rotatory evaporator at 45°. After being dried and weighed they were suspended in a small volume of distilled water; the suspension was then dialysed against distilled water for 1 day. After this procedure had been repeated the weight of the fractions remained constant.

Specific fission of fraction I_D and investigation of the resulting products. Specific fission of the bonds involving serine nitrogen atoms was carried out by the method of Desnuelle & Bonjour (1951) and Elliott (1952). A 1 g.

sample of fraction I_D was cooled to -30° and 20 ml. of 98% H₂SO₄, cooled to -10°, was added. The solution was kept in a sealed flask for 3 days in the dark, then cooled to 0° and poured into 600 ml. of dry ether at 0°. The yellow precipitate was collected by centrifuging and washed five times with 300 ml. of dry ether. After drying over P₂O₅ *in vacuo* the yield was 1.16 g. The ester linkages in this product were hydrolysed by a method similar to that of Zahn & Schnabel (1957): the rearranged product was dissolved in 20 ml. of 6N-HCl, the solution kept for 5 hr. at 20° in the dark and evaporated to dryness in a rotatory evaporator at 45°. The residue was dissolved in 20 ml. of distilled water and neutralized with NaHCO₃. With stirring, 2 g. of NaHCO₃ and 0.3 g. of 1-fluoro-2,4-dinitrobenzene in 40 ml. of ethanol were added and stirring was continued for 6 hr. at 40°. Then a further 0.3 g. of 1-fluoro-2,4-dinitrobenzene was added and the mixture stirred again for 6 hr. The mixture was neutralized with dilute HCl and extracted five times with ether to remove unchanged 1-fluoro-2,4-dinitrobenzene, dinitrophenol and dinitroaniline.

The final ether extract was colourless; all ether extracts were discarded. To determine the content of DNP-peptides and inorganic salts, 4 ml. of the solution was evaporated to dryness in a weighed crucible on a steam bath, the residue dried over P₂O₅ in a vacuum desiccator and weighed: the yield was 0.2352 g. The residue was ashed and weighed after cooling: the yield was 0.1089 g. The solution thus contained 32 mg. of DNP-peptides/ml. and 27 mg. of inorganic salts/ml.

This mixture of DNP-peptides was separated into the fractions I, II, III and IV on a 175 cm. × 1.7 cm. column of Sephadex G-25 (fine grade) in water (see Fig. 2). Further fractionation of fraction IV on a 175 cm. × 1.7 cm. column of Sephadex G-10 yielded the components IVa and IVb.

RESULTS

Amino acid composition of the chymotryptic precipitate. The investigations of the qualitative composition of the chymotryptic precipitate by high-voltage electrophoresis on paper with buffers at pH 1.9 and 3.5 showed in addition to Gly, Ala, Ser and Tyr small amounts of Thr, Asp, Glu, Lys and Arg.

In Table 1 the quantitative composition of silk fibroin and of its chymotryptic precipitate is recorded.

Fractionation of the dinitrophenylated chymotryptic precipitate. As mentioned above it was possible to prepare a stable solution of the DNP-Cp in 6M-urea by dissolving the material in 50% (w/v) lithium thiocyanate and subsequent dialysis against 6M-urea.

Column chromatography of the DNP-Cp on Sephadex G-50 resulted in a separation into about six components, which overlapped. When Sephadex G-75 was used a high-molecular-weight fraction (I_{G-75}) was clearly separated having the amino acid composition given in Table 2.

Four fractions were obtained by separation on ion-exchanger Dowex 1 (X2; acetate form) (see

Table 1. *Amino acid composition of silk fibroin (Bombyx mori) and of its chymotryptic precipitate (fraction Cp)*

Values are expressed as amino acid N, as % of total N.

	Fibroin	Fraction Cp
Lys	0.61	0.09
His	0.50	0.05
Arg	1.96	0.18
Asp	1.61	0.26
Thr†	0.95	0.39
Ser†	11.57	16.39
Glu	1.24	0.22
Pro	0.42	0.10
Gly	44.60	50.95
Ala	28.61	34.63
Cys	0.13	0.04
Val	2.02	0.38
Met	0.08	*
Ile	0.59	0.10
Leu	0.45	0.06
Tyr†	5.37	1.75
Phe	0.62	0.22
Trp	0.43	0.06

* Obtained peak not quantitatively evaluated.

† Corrected for decomposition during hydrolysis (Moore & Stein, 1963).

Fig. 1). A survey of the total weight of all fractions is shown in Table 3. From these values it can be deduced that the fractions I_D, II_D, III_D and IV_D comprised about 85% in total of the DNP-Cp compound. The remaining 15% was lost during fractionation. Table 2 indicates the amino acid composition of the four fractions as compared with the unfractionated DNP-Cp.

Ultracentrifugation of fraction I_D, dissolved in 25% (w/v) lithium bromide, by sedimentation equilibrium at 35 600 rev./min. gave the molecular-weight distribution shown in Table 4. From these values the weight-average molecular weight of the dissolved DNP-Cp was derived as 7600, whereas the value of fraction I_D was about 6000 (calculated according to van Holde & Baldwin, 1958). Based on N-terminal group estimations for DNP-Cp and the fractions obtained after chromatography on Dowex 1 (X2) the following molecular weights were calculated: DNP-Cp, 8000; I_D, 6800; II_D, 25 300; III_D, 17 400; IV_D, 10 100.

Specific fission of fraction I_D and investigation of its products. The mixture of DNP-peptides obtained after rearrangement of fraction I_D with concentrated sulphuric acid, followed by hydrolytic fission of ester bonds and subsequent dinitrophenylation, was separated by thin-layer chromatography. This mixture comprised five components having *R_F* values 0.05, 0.15, 0.22, 0.32 and 0.44 [in butan-2-ol-formic acid-water (150:27:23, by vol.)].

Table 2. *Amino acid and DNP-amino acid composition of DNP-Cp and its fractions obtained by gel filtration on Sephadex G-75 (I_{G-75}) and by ion-exchange chromatography on Dowex 1 (X2) (fractions I_D , II_D , III_D , IV_D)*

Values are expressed as amino acid N, as % of total N. Corrections for losses during hydrolysis: Thr, Ser, Tyr according to Moore & Stein (1963); DNP-Gly according to Weigmann (1963); other *N*-DNP-amino acids according to Middlebrook (1951); *O*-DNP-Ser loss 23%, *O*-DNP-Tyr loss 6% according to Beyer (1966). N.D., not determined.

	DNP-Cp	I_{G-75}	I_D	II_D	III_D	IV_D
Lys	0.05	1.06	—	—	—	0.63
His	*	0.83	—	—	—	0.23
Arg	0.15	1.94	—	—	—	2.34
Asp	0.33	1.64	—	0.38	1.05	4.70
Thr	0.43	0.75	—	0.52	0.79	2.27
Ser	15.36	15.11	14.81	14.86	14.21	12.45
Glu	0.20	0.14	—	0.26	0.70	2.24
Pro	*	0.39	—	—	0.20	0.76
Gly	46.40	44.00	49.74	48.00	46.60	37.20
Ala	32.87	30.32	33.05	33.65	32.43	25.32
Val	0.40	0.84	—	0.36	0.80	1.76
Met	*	0.11	—	—	0.03	0.27
Ile	0.06	0.40	—	0.08	0.23	0.97
Leu	0.05	0.73	—	0.13	0.29	1.02
Tyr	0.09	—	—	0.17	0.19	0.39
Phe	0.21	0.20	—	0.19	0.44	1.49
<i>N</i> _{im} -DNP-His	*	N.D.	—	—	0.74	1.32
<i>O</i> -DNP-Ser	0.30	N.D.	0.11	0.11	0.20	0.42
<i>N</i> _ε -DNP-Lys	*	N.D.	—	—	—	1.03
<i>O</i> -DNP-Tyr	1.57	N.D.	1.25	0.87	1.30	1.45
DNP-Asp	0.01	N.D.	—	—	0.01	0.05
DNP-Glu	0.01	N.D.	—	—	0.02	0.07
<i>N</i> -DNP-Ser	0.07	N.D.	0.06	0.07	0.15	0.03
<i>N</i> -DNP-Thr	*	N.D.	—	—	—	—
DNP-Gly	0.75	N.D.	0.96	0.22	0.27	0.03
DNP-Ala	0.09	N.D.	0.03	0.01	0.05	0.02

* Obtained peak not quantitatively evaluated.

Fig. 1. Fractionation of DNP-Cp by ion-exchange chromatography on Dowex 1 (X2). A solution of 975 mg. of DNP-Cp in 50 ml. of 6*M*-urea was applied to a 52 cm. $\times$ 5 cm. column with Dowex 1 (X2; 200–400 mesh, acetate form). Stepwise elution was carried out with buffers which were 0.1*M* and 6*M* with respect to ammonium acetate and urea and of increasing acetic acid concentration as follows: A, 0.1*N*; B, 1*N*; C, 5*N*. Fractions I_D and II_D were eluted with A, fraction III_D with B and fraction IV_D with C. Elution rate was 250 ml./hr. Ultraviolet absorption of the eluate was measured and recorded at 254 $m\mu$ by means of a Uvicord spectrophotometer (LKB Produkter AB).

Table 3. *Yield of fractions obtained by ion-exchange chromatography of DNP-Cp on Dowex 1 (X2)*

The amount of starting material was 975 mg.

Fraction	Weight (mg.)	Percentage of starting material
I_D	704	72.2
II_D	48	4.9
III_D	48	4.9
IV_D	30	3.1
Total	830	85.1

Separation of the mixture on a preparative scale was attempted on Sephadex G-25 columns, resulting in four fractions as shown in Fig. 2. Their homogeneity has been investigated by paper and thin-layer chromatography, including high-voltage paper electrophoresis. Fractions I and II did not seem to be as homogeneous as fraction III. Fraction IV contains two components, which could be separated on Sephadex G-10 into IVa and IVb. The corresponding R_F values on thin-layer chromatography were: fraction III, 0.22; IVa, 0.32; IVb, 0.44.

Table 4. *Molecular-weight distribution of DNP-Cp and fraction I_D at sedimentation equilibrium*

Ultracentrifugation was carried out in a Spinco model E ultracentrifuge. The rotor speed was 35 600 rev./min., temperature 20°, phase-plate angle 80°. The initial concentrations were 3.94 mg. of DNP-Cp/ml. and 4.61 mg. of fraction I_D/ml. respectively in 25% (w/v) LiBr. Schlieren photographs were taken after the system had reached equilibrium. Results are given in the form of weight averages of apparent molecular weight at every point of the cell.

DNP-Cp	I _D
5914	5335
6017	5461
6101	5565
6161	5729
6201	5860
6224	5895
6233	5984
6493	6052
6452	6099
6643	6071
6659	6330
6787	6435
7005	6512
7057	6616
7270	6549
7610	6611
8208	6691
9767	6708
11701	6782
14117	6759
15949	6794
	6806
	6866
	6839
	6945
	7072
	7804
	8308

Since no migration of fractions III, IVa and IVb on high-voltage electrophoresis in buffer pH 1.9 was observed, and staining with ninhydrin gave no positive colour, it was concluded that all three fractions were free of unprotected peptides.

Small samples of all fractions after hydrolysis and ether extraction were further investigated by thin-layer chromatography to identify the amino acids and DNP-amino acids present. Fractions I, II, III and IVb all contained Gly, Ser and Ala in apparently different proportions, whereas only fraction I contained *O*-DNP-Tyr. Qualitative estimation of the ether-soluble DNP-amino acids by thin-layer chromatography were carried out on the fractions, yielding the following results: fraction I, DNP-Gly; II, DNP-Gly and DNP-Ala; III, DNP-Ser; IVb, DNP-Ser. Additionally, quantitative examination of fractions III and IVb gave the compositions

Fig. 2. Fractionation of fission product of fraction I_D by gel filtration on Sephadex G-25. A solution of 32 mg. of fission product in 1 ml. of water was applied to a 175 cm. × 1.7 cm. column of Sephadex G-25. Elution was performed with distilled water at a rate of 25 ml./hr. Ultraviolet absorption of the eluate was measured and recorded at 254 mμ by means of a Uvicord spectrophotometer (LKB Produkter AB).

Table 5. *Amino acid and DNP-amino acid composition of fractions III and IVb obtained by gel filtration on Sephadex G-25 and G-10 of fission product of fraction I_D*

Correction for losses during hydrolysis: Ser according to Moore & Stein (1963), DNP-Gly according to Weigmann (1963), DNP-Ala and *N*-DNP-Ser according to Middlebrook (1951).

	No. of amino acid residues/100 g. of DNP-peptide		Rounded-off numerical proportion of amino acid residues	
	III	IVb	III	IVb
Gly	0.66	0.54	6	4
Ala	0.43	0.34	4	2.6
Ser	0.11	0.13	1	1
DNP-Gly	0.005	0.01	—	—
DNP-Ala	0.01	0.01	—	—
<i>N</i> -DNP-Ser	0.08	0.12	0.7	0.9

shown in Table 5. From these values it can be deduced that the formulae are as follows: fraction III: DNP-Ser-(Gly₆,Ala₄,Ser); fraction IVb: DNP-Ser-(Gly₄,Ala_{2.6},Ser).

DISCUSSION

The conclusions drawn from the results of our determination of amino acid composition of fraction Cp are that this material cannot be homogeneous, because the amounts of those amino acids which fraction Cp contained in addition to Gly, Ala,

Ser and Tyr were far too small for every fraction Cp molecule of molecular weight about 8000, to include at least one residue of each of them. The question therefore arises as to the manner in which these 14 amino acid residues are combined with each other and with Gly, Ala, Ser and Tyr in the peptide chains of fraction Cp. Theoretically two extreme cases of possible structure can be discussed: (1) the total amount of those 14 amino acid residues constitutes one single polypeptide; (2) one or only a few residues of the 14 amino acids are distributed in each molecule of fraction Cp.

In the first case it can be calculated that about 3% by wt. of fraction Cp has an amino acid composition different from the abundant structure composed of Gly, Ala, Ser and Tyr. If we consider the second case, it can be calculated that each molecule of fraction Cp must have a different amino acid composition. Both possibilities are improbable and therefore the real structure should lie between the two extreme cases.

It seemed that this problem could have been solved by using ion-exchange chromatography for the fractionation of DNP-Cp, where all free amino groups plus the hydroxyl groups of tyrosine had reacted with 1-fluoro-2,4-dinitrobenzene. Thus the amino groups had lost their basic character and only the carboxyl groups were able to carry charges. A fractionation on anion-exchange columns (Dowex 1) should have taken place with the elution firstly of DNP-peptides containing only one C-terminal carboxyl group followed by those with the additional carboxyl groups of aspartic acid and glutamic acid residues. Such a proposed separation occurred as shown in Table 2. A fraction of about 72% by wt. had an amino acid composition with molar proportions glycine 40:alanine 26:serine 12:tyrosine 1. This result fits more closely to the first case than to the second one mentioned above. The residual fractions investigated, which amounted up to 13%, differed widely from each other. By summing all the fractions obtained, a loss of about 15% by wt. was observed, representing a part of DNP-peptides which was irreversibly adsorbed by the ion-exchanger. Presumably this fraction has a composition dissimilar to that of fraction I_D. If this is true then we would expect that almost 30% by wt. has an amino acid composition which is different from the main fraction (I_D). The heterogeneity in respect to the chain length, indicated by ultracentrifuge measurements, can now be compared with the behaviour during gel filtration. The distribution of the weight average of the apparent molecular weights under sedimentation equilibrium for DNP-Cp ranges between 6000 and 16000. When the same conditions are applied to fraction I_D a molecular-weight distribution of 5000 to 8000 is observed (see Table 4). The ultracentrifuge

measurements have been confirmed by the results of gel filtration. Though on Sephadex G-50 a separation into six components is indicated, we were able to separate a high-molecular-weight fraction I_{G-75} with Sephadex G-75, which showed, according to the limit of total exclusion, a molecular weight of at least 50000. These findings lead to the conclusion that fraction I_{G-75} is very similar to fraction II_D because of its similarity in molecular weight, derived from N-terminal groups and with respect to amino acid composition (see Table 2). The remarkable molecular-weight fractionation on Dowex 1 (X2) is due to the fact that fractions containing more carboxyl groups equally have higher molecular weights.

The main fraction (I_D) proved to be only slightly heterogeneous if we consider its chain length (see Table 4), the amount of N-terminal groups (92% of Gly) and amino acid composition. However, the question of its homogeneity with respect to the amino acid sequences is left open. In the formula for fraction Cp suggested by Lucas *et al.* (1957) a certain micro-heterogeneity within the repeating units was taken into account by providing a variable value for *n*. After specific fission of fraction I_D followed by the separation of the DNP-peptide mixture, five fractions were obtained. The first fraction probably represents unrearranged starting material, since the volume of elution on Sephadex G-25 is equal to limit of exclusion from which a molecular weight about 5000 could be concluded. Furthermore only DNP-Gly as N-terminal group was obtained.

Presumably fraction II represents unrearranged but partly unspecifically cleaved material, because of the absence of DNP-Ser as end group.

From the formulae of fraction III [DNP-Ser-(Gly₆,Ala₄,Ser)] and fraction IVb [DNP-Ser-(Gly₄,Ala₂₋₆,Ser)] the following conclusion can be drawn. Fraction III corresponds to the double repeating unit of [Ser-Gly-(Ala-Gly)_{*n*}]₂ with *n* 2, as postulated by Lucas *et al.* (1957). By considering the DNP-peptide of fraction IVb no final formula is acceptable since the value of Ala is about 2.6 residues. If the value Ala₂ is considered, then a repeating unit with *n* 1 could result, hence: [Ser-Gly-(Ala-Gly)₁]₂. On the other hand, the value Ala₃ leads to a sequence that would not be compatible with the formula suggested by Lucas *et al.* (1957).

It is peculiar that we have obtained only dimers of the repeating units after specific rearrangement, since this is contrary to the findings of Lucas *et al.* (1957) and Zahn & Schnabel (1957). Both authors were able to isolate only the single repeating unit of the hexapeptide. This could be due to our modification of the conditions of rearrangement. According to our procedure it would not be expected

that fractions III and IVb would be composed of both DNP-peptides together with free peptides. This supposition is confirmed by the results of high-voltage paper electrophoresis at pH 1.9.

Since we found repeating units with $n = 1$, it can be deduced from the value for serine that repeating units with $n > 2$ also must occur. However, such peptides have not yet been detected.

The aim of further investigations is to formulate a complete amino acid sequence of fraction I_D without the variable value of n . It is realised that this aim will only be achieved if the degree of sequential heterogeneity is not too great.

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