4. Large quantities of ammonia but not methylamine were produced when soluble casein was added to the rumen. The maximum methylamine: ammonia ratio found was 1.53.

5. Methylamine was present in the contents of the digestive tract as far caudal as the ileum, but unlike ammonia did not appear to be produced in any region except the rumen.

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Fractionation of the Fibroin of *Bombyx mori* with Trypsin

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It is now well established that hydrolysis of dissolved *Bombyx mori* fibroin with chymotrypsin gives rise to an insoluble precipitate and a mixture of soluble peptides. The precipitate represents about 60 % of the protein by weight and is composed of sequences of amino acid residues in which glycine alternates with serine and alanine (Lucas, Shaw & Smith, 1957). The mixture of soluble peptides has also been shown to contain a substantial proportion of peptides in which glycine residues occupy alternate positions and are associated with residues of alanine, valine and tyrosine (Lucas, Shaw & Smith, 1962). Thus enzymic hydrolysis with chymotrypsin has proved to be a fruitful approach in studies of the structure of the fibroin molecule.

Less attention has been paid to the action of other proteolytic enzymes on fibroin. The first account of the hydrolysis of a solution of fibroin by

a purified trypsin preparation was given by Drucker, Hainsworth & Smith (1953). They obtained a 'tryptic precipitate', which they said contained 70 % of the total protein nitrogen, and a mixture of soluble peptides with a mean chain length of 5.0. Later, Waldschmidt-Leitz & Zeiss (1955) in a similar experiment found that the precipitated material represented 80 % (by weight) of the fibroin and that the soluble peptides had a mean chain length of about 7.

More recently, an account of a series of experiments by Zuber (1961) described the hydrolysis of a solution of fibroin by crystalline trypsin in the presence of hydrocinnamic acid, and recorded that 83 % of the protein nitrogen was precipitated in the form of a gel, while the peptides remaining in solution had a mean chain length of 31. Zuber (1961) investigated the gelatinous precipitate by hydrolysing it further with chymotrypsin, and

adduced some evidence that it was composed mainly of the sequences of glycine, alanine and serine residues that are precipitated when fibroin is hydrolysed with chymotrypsin, together with some other sequences which he did not precisely identify but which were composed largely of residues of glycine, alanine, valine and tyrosine.

In spite of the somewhat diverse findings of these workers, it is evident that the fraction that is precipitated by tryptic action constitutes a substantially larger proportion of the whole fibroin molecule than that precipitated by the action of chymotrypsin. It seemed that tryptic hydrolysis should prove to be a profitable line of attack in extending our knowledge of the molecular structure of fibroin, and it was the purpose of this work to clarify the mode of action of trypsin on fibroin, to investigate the composition of the 'tryptic precipitate' and to examine its relation to the structure of the fibroin molecule.

MATERIALS AND METHODS

Bombyx mori fibroin. The origin and preliminary treatment of the commercial raw silk from which the fibroin used in these experiments was prepared has been described previously (Drucker *et al.* 1953; Lucas *et al.* 1962).

Solutions of fibroin. Solutions were prepared by dissolving fibroin in aq. 60% (w/v) LiSCN (sp-gr. 1.20) at room temperature, diluting with water until the solution was 2.5% in protein, and dialysing against distilled water until the effluent gave a negative reaction for SCN^- ions with FeCl_3 . When a solution was prepared by the use of copper ethylenediamine, the method followed was that described by Drucker *et al.* (1953). Solutions prepared by either of these methods may contain a small amount of imperfectly dissolved fibroin that aggregates on dialysis: this was removed by centrifugation and disregarded.

Enzymes. Crystallized trypsin and chymotrypsin were purchased from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex; twice-crystallized trypsin was purchased from L. Light and Co. Ltd., Colnbrook, Bucks., and from Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Chemicals. Lithium thiocyanate was obtained from British Drug Houses Ltd., Poole, Dorset, and 1-fluoro-2,4-dinitrobenzene from L. Light and Co. Ltd. Other chemicals were of analytical reagent grade.

Hydrolysis of samples. Samples of peptides and proteins for amino acid analysis were hydrolysed in 6N-HCl at the boil for 24 hr. After evaporation of the hydrolysate, the residue was dissolved in water, filtered and made up to standard volume; the preferred concentration was approx. 0.5 g./l.

Amino acid analyses. These were performed by the method of Moore, Spackman & Stein (1958) with the minor variations described by Lucas *et al.* (1962). Some of the later analyses were performed on an automatic amino acid analyser based on the design of Spackman, Stein & Moore (1958) and constructed by the Phoenix Precision Instrument Co., Philadelphia, Pa., U.S.A. Tryptophan was estimated

by the colorimetric method of Spies & Chambers (1949), without prior hydrolysis of the samples.

Nitrogen estimations. The assays of total nitrogen, to which the amino acid analyses were related, were made by the micro-Kjeldahl method.

Mean chain lengths of peptide mixtures. These were determined by the FDNB* procedure (described below), or alternatively by treating a known amount of the peptide mixture, before and after hydrolysis, with ninhydrin under standard conditions and comparing the intensities of colour produced. This method is described in greater detail by Lucas *et al.* (1962), and is useful when the peptides concerned contain no basic amino acids; the presence of lysine, which gives a high colour yield with ninhydrin, reduces the accuracy of the method.

Paper electrophoresis and chromatography. Electrophoresis was carried out on Whatman no. 1 and no. 3MM paper strips, 15 cm. wide, with a sandwich type of apparatus. A potential gradient of about 50 v/cm. was commonly used. Electrolytes were 2.5% (v/v) formic acid and pyridine-acetic acid-water (10:1:89, by vol.) at approx. pH 6.1. Whatman no. 1 paper was employed for chromatography, which was carried out by downward development with butan-1-ol-acetic acid-water (4:1:5, by vol.). Peptides were demonstrated by spraying or dipping the dried papers in a solution of ninhydrin in ethanol (0.1%, w/v) and heating briefly at 80°.

Tryptic hydrolysis of fibroin

Solutions of fibroin in water. A solution of fibroin in water was diluted and buffered to bring the concentration of protein to approx. 5 g./l. and the pH to 8.0; the concentration of buffer salts was 11.0 g. of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ and 0.3 g. of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ /l. To this solution was added a solution of trypsin in a few millilitres of 0.01N-HCl; the ratio of enzyme to substrate was 1:100. At this stage samples of the solution were removed for nitrogen assay. The mixture was then incubated at 40°, whereupon the solution quickly became opaque and a substantial gelatinous precipitate appeared after 2–3 hr. At higher concentrations of substrate (1% or more) the whole solution set to a stiff gel which proved difficult to break down. After 24 hr. the gelatinous precipitate was centrifuged down, and samples of the supernatant were assayed for nitrogen; comparison of the two nitrogen estimations gives an accurate value for the percentage of fibroin nitrogen precipitated, provided that the volume change is negligible and that allowance is made for the contribution of the nitrogen in the enzyme. The gelatinous precipitate was collected by freezing the reaction mixture in a bath of solid CO_2 -acetone, thawing it, and washing the resulting comparatively compact solid product with water, dilute HCl, ethanol and ether. The dried product is referred to as fraction Tp. The supernatant from the enzymic digest was combined with the aqueous washings, brought to pH 7.0 by the addition of HCl and reduced to a small volume in a rotary evaporator. The resulting solution was centrifuged, if necessary, and freeze-dried. This peptide mixture is referred to as fraction Ts. When a salt-free product was required, the sodium phosphates and chloride were removed by desalting on a Zeo-Karb 225 column as described by Lucas *et al.* (1962).

* Abbreviation: FDNB, 1-fluoro-2,4-dinitrobenzene.

Variations of this procedure were studied to discover whether the percentage of protein nitrogen precipitated by tryptic action was dependent upon such factors as enzyme:substrate ratio, pH, concentration of substrate etc. Details of these experiments are given in the Results section.

Solutions of fibroin in aqueous LiSCN. Fibroin (1 g.) was dissolved in aq. 60% (w/v) LiSCN (5 ml.) at room temperature. Water (45 ml.), NaHCO_3 (0.5 g.) and trypsin (12 mg. dissolved in 2 ml. of 0.01 N-HCl) were added and the mixture (pH 8.3) was incubated at 40° for 16 hr. There was no precipitation under these conditions and the resulting hydrolysate was acidified by the addition of 1 ml. of acetic acid (care being taken to avoid excessive frothing) and was quantitatively transferred to a cellophan dialysis sac ($\frac{1}{8}$ in. inflated diameter). Water was used to complete the transfer. The solution was then dialysed against a stream of distilled water (flow rate about 1 l./hr.) for 24 hr., after which the effluent gave a negative reaction for SCN^- ions with FeCl_3 . The solution was removed from the sac, centrifuged if necessary to remove traces of solid, and assayed for nitrogen. Comparison of this nitrogen value with the known nitrogen content of the original weight of dry fibroin gave a measure of the proportion of non-diffusible peptide nitrogen. This non-diffusible material is referred to as fraction Tnd.

Secondary enzymic fractionation

Hydrolysis of fraction Tnd with chymotrypsin. To a solution of fraction Tnd in water (about 1 g. in 150 ml.) at 40° were added chymotrypsin (10 mg.) and NaHCO_3 (500 mg.). Samples of the solution were immediately removed for nitrogen estimation, and the remainder, whose pH was about 8.3, was incubated at 40° for 16 hr. After this time the fine granular precipitate, fraction Tnd-Cp, which had begun to separate after about 30 min., was centrifuged down, washed with water, dilute HCl, ethanol and ether, and dried. The supernatant, which contained a mixture of soluble peptides produced by the enzymic hydrolysis (fraction Tnd-Cs), was assayed for nitrogen, and the percentage of the nitrogen in fraction Tnd that had been precipitated by chymotryptic action was calculated. The supernatant was then combined with the aqueous washings from fraction Tnd-Cp, brought to pH 7 with HCl, evaporated to small bulk and freeze-dried. A similar experiment, in which aq. 0.05 N- NH_3 soln. was added periodically to the reaction mixture to maintain pH about 8.2 during the chymotryptic action, yielded approximately the same proportion of precipitate (Tnd-Cp), and evaporation and freeze-drying of the resulting peptide solution yielded a salt-free preparation of fraction Tnd-Cs. In this experiment the precipitate was collected and weighed to determine the proportion of fraction Tnd-Cp, since its estimation by nitrogen assay was not possible.

Hydrolysis of fraction Ts with chymotrypsin. To a solution of the fraction Ts adjusted to pH 8.0 was added sufficient chymotrypsin to give enzyme:substrate ratio 1:100. After incubation for 16 hr. at 40°, the solution of peptides was evaporated to small volume, adjusted to pH 7 with HCl and freeze-dried. The presence of inorganic salts in this fraction was not normally troublesome.

The fractionation of the fibroin molecule by enzymes, and the nomenclature of the resulting fractions, is shown diagrammatically in Fig. 1.

End-residue analysis

Fraction Tp. The freshly precipitated material obtained by hydrolysis of aqueous fibroin with trypsin was centrifuged down and washed several times with water and dilute HCl; it was treated with FDNB without drying, as it was thought that under these conditions the peptide material would be more accessible to the reagent, and the reaction therefore more nearly complete. For this purpose the gel was suspended in aq. 2% (w/v) NaHCO_3 (150 ml./g. of Tp), and to the suspension was added a solution of FDNB (1.2 g.) in ethanol (250 ml.). The mixture was shaken overnight at room temperature, and the yellow insoluble product was centrifuged down and washed with water, ethanol, dilute HCl, water, ethanol and ether in that order. The product, a friable yellow powder, was dried *in vacuo* over P_2O_5 to constant weight (DNP-Tp).

Fractions Ts and Tnd-Cs. Solutions of these materials containing about 0.1 g. of peptides in 100 ml. were assayed for total nitrogen, and to measured volumes of each was added NaHCO_3 (0.5 g./100 ml.) and a solution of FDNB in ethanol (0.5%, w/v). The ratio ethanol:water in the reaction mixtures was approximately 4:3 (v/v). The clear solutions were allowed to stand for 4 hr., after which time the ethanol was distilled off under reduced pressure, and the remaining solutions were extracted with ether to remove excess of FDNB; they were then made acid with HCl, and evaporated to dryness *in vacuo*. The residues were mixtures of DNP-Ts or of DNP-Tnd-Cs with dinitrophenol and inorganic salts.

Fig. 1. Diagram showing the enzymic fractionation of fibroin and the source of the fractions in the fibroin molecule. Phases I, II and III are defined in the text.

Fraction Tnd. To the solution of this fraction, containing about 1 g. of peptide in 100 ml., was added NaHCO_3 (2 g.) and ethanolic FDNB (175 ml. of ethanol containing 2 g. of FDNB). The reaction mixture, from which the insoluble DNP-peptide quickly separated, was shaken overnight at room temperature, and the yellow product was centrifuged down, and washed and dried in the same way as DNP-Tp.

Estimation of N-terminal residues. Weighed samples of the DNP-Tp and DNP-Tnd preparations were hydrolysed at 40° in 11.7N-HCl for 21 days; a ratio of about 50 ml. of HCl/g. of DNP-peptide was used. The crude DNP-Ts and DNP-Tnd-Cs preparations were similarly hydrolysed with 11.7N-HCl at 40° . It has been found (F. Lucas & J. T. B. Shaw, unpublished work) that under these conditions hydrolysis of peptide bonds is substantially complete, whilst decomposition of most DNP-amino acids is small (less than 10%). The hydrolysates were diluted with water, and the DNP-amino acids were isolated and estimated by the techniques described by Lucas, Shaw & Smith (1963). The yield of recovered DNP-amino acids, when related to the weights of DNP-Tp and DNP-Tnd, and to the nitrogen contents of the Ts and Tnd-Cs samples from which the DNP derivatives were prepared, gives a measure of the mean chain length of each fraction. For the calculation of these mean chain lengths it is necessary to relate the weight of DNP-peptide to the weight of peptide from which it is derived; this was done by making use of the known amino acid compositions of the fractions and by assuming that, besides the *N*-terminal amino groups, lysyl, tyrosyl and histidyl residues each react with 1 mole of FDNB. For the calculation of the chain length of the fractions Ts and Tnd-Cs, allowance was made for the contribution to the total nitrogen made by the side chains of residues of lysine, arginine, histidine and tryptophan.

Sedimentation analysis

The sedimentation properties of fraction Tnd were examined in a Spinco model E analytical ultracentrifuge with a schlieren optical system; the temperature was controlled at 20° . The sedimentation results were plotted as $S_{20,w}$ against concentration, and extrapolated to zero concentration. The relative viscosity of the solvents at 20° were determined in an Ostwald viscometer.

RESULTS

Hydrolysis of fibroin solutions in aqueous buffers with trypsin. The percentage of protein nitrogen precipitated during tryptic hydrolysis, after correcting for the contribution of the enzyme, was close to 90%; this corresponds to about 85% of the fibroin by weight. The proportion was only marginally affected by changes in any of the variables that were investigated. In particular, no effect was observed when catalytic amounts of Mg^{2+} ions were added to the hydrolysate, and the amount of precipitate was not dependent on the solvent used to dissolve the fibroin: solutions prepared by means of copper ethylenediamine gave the same result as those prepared with lithium thiocyanate. When higher concentrations of buffer salts and pH values about 7.5 were employed, the

precipitates were less gelatinous and more compact but there was again little change in the distribution of nitrogen. The variable that had a slight but perceptible effect on the extent of hydrolysis was the source of trypsin: the older sample (Armour Pharmaceutical Co. Ltd.) yielded slightly less, and that from L. Light and Co. Ltd. slightly more, precipitate than the sample from Worthington Biochemical Corp., but the differences were not very great. The addition of chymotrypsin to trypsin (1:8, w/w) reduced the percentage of fibroin nitrogen precipitated to 86.2, and the addition of a small amount of pancreatic elastase to the trypsin reduced the percentage to 66.0.

Fractions Tp and Ts. Amino acid analyses of the standard preparations of fractions Tp and Ts are given in Table 1. The serine values are corrected by 10% to allow for hydrolytic decomposition (Rees, 1946) and the ammonia estimates are correspondingly reduced. A set of values for whole fibroin, taken from Lucas, Shaw & Smith (1958), is included for comparison. Evidence for the existence of methionine in fibroin, first mentioned by Stokes, Gunness, Dwyer & Caswell (1945), receives support from its appearance in increased concentration in fraction Ts. The attribution of a small chromatographic peak amongst the basic amino acids to hydroxylysine must be regarded as tentative.

The results of the *N*-terminal estimations on the fractions Tp and Ts, carried out on the DNP-preparations, are presented in Table 2. The results are uncorrected for decomposition during hydrolysis or losses during chromatography: such losses are believed to be small, and will result in slight over-estimation of the mean chain length of the fractions. Measurement of the ninhydrin colour yield of fraction Ts, before and after hydrolysis, gave rather scattered values with an average of 11.2 for the mean chain length of the sample.

Hydrolysis of solutions of fibroin in aqueous lithium thiocyanate with trypsin. Hydrolysis under these conditions gives rise to a solution of peptides of which the smaller components may be removed by dialysis along with the lithium thiocyanate. If the dialysis is carried out while the solution is initially alkaline or neutral, much of the dissolved material quickly comes out of solution. If the solution is acidified before dialysis, however, all of the dissolved peptide remains in solution, with the exception of a small variable amount of precipitate representing less than 2% of the total, which has been disregarded. Under these conditions the resulting solution of non-diffusible peptides (fraction Tnd) is stable for some days. A series of determinations has shown that approx. 90% of the fibroin nitrogen is contained in fraction Tnd, and that the proportion is not further reduced on longer dialysis. Analysis of this fraction (Table 1) shows

Table 1. *Amino acid composition of Bombyx mori fibroin and of fractions Tp, Tnd, Tnd-Cp, Tnd-Cs and Ts*

Results are given in two forms: (1) amino acid nitrogen as a percentage of total nitrogen; (2) residues/100 residues. Values for fibroin are from Lucas *et al.* (1958).

	Fibroin		Tp		Tnd		Tnd-Cp		Tnd-Cs		Ts	
	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)
Glycine	43.7	44.5	46.5	46.4	44.3	45.8	47.2	49.0	39.3	41.0	12.6	16.4
Alanine	28.8	29.3	31.4	31.5	29.9	30.9	32.2	33.5	24.6	25.7	9.9	12.9
Valine	2.16	2.20	1.78	1.78	1.77	1.83	—	—	5.1	5.3	4.9	6.3
Leucine	0.52	0.53	0.15	0.15	0.41	0.42	—	—	0.66	0.69	2.6	3.4
Isoleucine	0.65	0.66	0.21	0.21	0.37	0.38	—	—	0.99	1.03	3.8	4.9
Serine	11.9	12.1	12.6	12.6	11.0	11.4	14.6	15.2	5.7	5.9	8.5	11.1
Threonine	0.89	0.91	0.67	0.67	0.59	0.61	0.43	0.45	1.34	1.40	2.8	3.6
Aspartic acid	1.28	1.30	0.66	0.66	0.83	0.86	—	—	2.46	2.57	9.8	12.8
Glutamic acid	1.00	1.02	0.74	0.74	0.72	0.74	—	—	1.75	1.82	5.6	7.3
Proline	0.35	0.36	—	—	0.24	0.25	—	—	0.79	0.82	1.6	2.1
Tyrosine	5.1	5.2	5.1	5.1	5.0	5.2	1.48	1.54	10.5	11.0	4.0	5.2
Phenylalanine	0.62	0.63	0.28	0.28	0.83	0.86	—	—	1.61	1.67	1.95	2.5
Lysine	0.63	0.32	0.19	0.09	1.00	0.52	0.69	0.36	0.95	0.50	6.4	4.2
Histidine	0.53	0.18	0.32	0.11	0.33	0.11	—	—	0.76	0.26	2.6	1.1
Arginine	1.83	0.47	0.40	0.10	0.54	0.14	—	—	1.30	0.34	14.2	4.6
Hydroxylysine	—	—	—	—	—	—	—	—	—	—	(0.70)	(0.45)
Tryptophan	0.33	0.11	—	—	—	—	—	—	—	—	0.80	0.35
Methionine	0.07	0.08	—	—	—	—	—	—	—	—	(0.55)	(0.72)
Ammonia	—	—	0.40	—	1.03	—	1.46	—	2.52	—	7.5	—

Table 2. *N-Terminal residues of fractions Tp, Ts, Tnd and Tnd-Cs*

Results are given in two forms: (1) m-moles/mole of main-chain peptide nitrogen; (2) moles/10⁵ g. of peptide.

	Tp		Ts	Tnd		Tnd-Cs
	(1)	(2)	(1)	(1)	(2)	(1)
Glycine	0.91	1.23	9.1	1.03	1.39	80.2
Alanine	0.17	0.23	11.7	0.38	0.51	6.2
Valine	0.082	0.11	3.7	0.089	0.12	—
Leucine/isoleucine	0.035	0.048		0.050	0.067	—
Serine	0.37	0.51	7.4	0.50	0.68	7.9
Threonine	0.097	0.13	3.6	0.12	0.15	1.7
Aspartic acid	0.16	0.22	8.9	0.093	0.12	2.9
Glutamic acid	0.11	0.14	4.9	0.17	0.23	
Tyrosine	0.21	0.28	11.1	0.28	0.38	—
Phenylalanine	0.019	0.026	1.6	0.018	0.024	—
Lysine	0.029	0.040		0.066	0.089	—
Others*	0.28	0.56	14.0	0.53	0.71	10.0
Total	2.47	3.52	76.0	3.33	4.48	108.9
Mean chain length (residues)	405		13.1	300		9.2

* Includes unidentified DNP-amino acids and some incompletely hydrolysed DNP-peptides.

that it is closely similar in composition to fraction Tp, and end-residue assay gives a value of 300 for its mean chain length. If the pH of a solution of fraction Tnd is raised to 8.5 by the addition of sodium hydrogen carbonate, the dissolved peptides are quickly precipitated from solution.

Sedimentation properties. Fraction Tnd sedimented in aq. 10 % (w/v) lithium thiocyanate and in 2M-urea as a single approximately symmetrical peak with a low rate of diffusion. The sedimentation coefficients, $S_{20,w}^0$, were 2.09s in lithium thiocyanate and 2.06s in urea.

Hydrolysis of fraction Tnd with chymotrypsin.

The precipitate that results when fraction Tnd is further hydrolysed with chymotrypsin is closely similar to the precipitate (Cp) obtained by direct chymotryptic action on a solution of fibroin and resembles it in composition and in the proportion of the whole fibroin that it represents. Estimations of the percentage of Tnd nitrogen precipitated on chymotryptic hydrolysis varied between 62 and 66 %, with a mean value 65 %. Thus this fraction (Tnd-Cp) represents 59 % of the total fibroin nitrogen (after allowance has been made for the

contribution of the nitrogen in the enzymes), or slightly less than the 62 % precipitated by direct chymotryptic action. An analysis of fraction Tnd-Cp is included in Table 1, and it is seen that, like fraction Cp, it is composed almost entirely of residues of glycine, alanine, serine and tyrosine, with traces of lysine and threonine. An analysis of the soluble peptide mixture, fraction Tnd-Cs, is also given in Table 1. An approximate chain-length determination of this fraction by the ninhydrin method gave a value of 6.9, and end-group assay by the FDNB method showed that the *N*-terminal residues were predominantly glycine.

Hydrolysis of fraction Ts with chymotrypsin. Further hydrolysis of fraction Ts with chymotrypsin reduced the mean chain length determined by

means of the ninhydrin colour ratio (after hydrolysis/before hydrolysis) from 11.2 to 6.5.

Examination of fractions Tnd-Cs and Ts-Cs. Examination of fraction Tnd-Cs by paper electrophoresis and by paper chromatography showed that it was composed mainly of neutral peptides (Fig. 2). Comparison with peptides previously isolated from fraction Cs (Lucas *et al.* 1962), whose structure had been established, justified the identification of the four predominant components of the mixture as Gly-Ala-Gly-Tyr, Gly-Val-Gly-Tyr, and the two octapeptides with alternating glycy residues, Gly₄Ala₃Tyr and Gly₄Ala₂ValTyr. The octapeptides were present in higher concentration than the tetrapeptides and the amount of Gly-Val-Gly-Tyr was small, but the molar proportions were not determined. Electrophoresis of fraction Ts-Cs

Fig. 2. Paper electrophoresis and chromatography: (a) chromatography in butan-1-ol-acetic acid; (b) electrophoresis in 2.5 % (w/v) formic acid, 3000 v, 2 hr.; (c) electrophoresis in pyridine-acetic acid-water (10:1:89, by vol.), pH 6.1, 2000 v, 3 hr. Samples: 1, Gly-Ala-Gly-Tyr; 2, Gly-Val-Gly-Tyr; 3, Gly-Ala-Gly-Ala-Gly-Ala-Gly-Tyr; 4, Gly-(Gly₃Ala₂Val)-Tyr; A, Tnd-Cs; B, Ts-Cs; C, Ts.

showed that it was composed mainly of basic and acidic peptides. The small neutral component of the fraction did not contain appreciable amounts of the two tetrapeptides and the two octapeptides mentioned above.

DISCUSSION

Values of 70, 80 and 90 % have been reported for the proportion of dissolved fibroin nitrogen precipitated during tryptic hydrolysis, but in the experiments described here consistent values close to 90 % were obtained. This value agrees with that of Kirimura (1962), who has reported the precipitation of 90 % of fibroin when a solution taken from the silk gland was hydrolysed with trypsin. Possible reasons for the disparity between these and earlier results were tested, and the most probable explanation was concluded to be the variable purity of the enzymes used. Recent commercial preparations of pure trypsin that were used in these experiments gave consistent results, but an older sample produced smaller and less reproducible amounts of precipitate (65–82 %). This sample, and those used by earlier workers, may have been contaminated by proteolytic enzymes other than trypsin. It has been found that pancreatic elastase attacks fibroin solutions by hydrolysing bonds linking alanine and serine residues with those of glycine (J. T. B. Shaw, unpublished work), and treatment of a fibroin solution with a mixture of trypsin and elastase resulted in the precipitation of only 66 % of the protein nitrogen. On the other hand, the addition of 12 % of chymotrypsin to a sample of trypsin led to only a small reduction in the proportion of fibroin nitrogen precipitated.

Drucker & Smith (1950) showed that it is possible to divide the fibroin molecule into two parts, quite different in composition, by chymotryptic hydrolysis and the results in Table 1 show that the disproportionation is even more marked with trypsin. Fractions Tp and Tnd, which are obtained in approximately the same yield (about 85 % by wt. of fibroin), have closely similar amino acid compositions. They derive from regions of the fibroin molecules that are composed of long sequences of the amino acids glycine, alanine, serine, valine and tyrosine, of which glycine represents almost half. Though it is not known how disperse in chain length are fractions Tnd and Tp, the Tnd molecules are too large to pass through cellophan dialysis tubing and the diversity of *N*-terminal residues in both fractions shows that they have no true molecular homogeneity. The evidence suggests that fraction Tnd is composed of molecules of similar composition, perhaps with a measure of dispersity in size, rather than of a mixture of physically or chemically distinct species, and the sedimentation properties of the fraction accord with this view.

The discovery that tryptic hydrolysis of fibroin proceeds normally in a solvent containing 6 % of lithium thiocyanate, and that the products remain in solution, provided a convenient method for investigating the larger fragments produced by the hydrolysis. For this purpose the concentration of lithium thiocyanate is critical: at higher concentrations, e.g. 9 %, hydrolysis is incomplete, and at lower concentrations the product (fraction Tp) comes out of solution. Harris (1956), investigating the effect of urea upon the rate of hydrolysis of a synthetic substrate by trypsin, concluded that the enzyme does not retain its activity in an unfolded form, and it is likely that the concentration of lithium thiocyanate used in our experiments was just sufficient to prevent the chains of fraction Tnd from aggregating, but not sufficient to disrupt the configuration of the active trypsin molecule.

The solubility of fraction Tnd is surprising, since it is composed of long sequences of amino acid residues with few polar side chains. It is significant that this fraction remains in solution only if acid is added before the removal of lithium thiocyanate by dialysis. The presence of basic residues in exposed positions at the *C*-terminal ends of the peptide chains are probably relevant to the solubility of the fraction, and the occurrence of some 5 % of the bulky and hydrophilic residues of tyrosine in fractions Tnd and Tp may contribute to the solubility of the first and the gelatinous character of the second by preventing or restricting close juxtaposition of the peptide chains.

Two explanations can be suggested to account for the apparent difference in chain length between fractions Tp (405 residues) and Tnd (300 residues). The first concerns the reaction between FDNB and the terminal residues of the peptides, which is more likely to be complete when both reactants are originally in solution, as with fraction Tnd, than when one is insoluble. Incomplete reaction between FDNB and fraction Tp would result in low recovery of end residues and a consequent over-estimation of mean chain length, although the relative proportions of the *N*-terminal residues would be expected to be correct. Another factor is the different circumstances of the tryptic hydrolysis. During the hydrolysis of fibroin in aqueous lithium thiocyanate to produce fraction Tnd the products remain in solution, and there is no obvious reason why any potentially labile bonds should not be broken. On the other hand, the precipitation of fraction Tp occurs at a comparatively early stage in the absence of lithium thiocyanate, and any subsequent action of the trypsin (within the gel) would be very restricted; consequently fraction Tp may contain a proportion of peptides containing bonds that are theoretically labile to tryptic action, but which have not been split. Such peptides would be

longer than the corresponding peptides in fraction Tnd, and would explain the higher mean chain length of fraction Tp. For these reasons the end-residue results obtained with fraction Tnd are likely to be the more significant.

The regions of the fibroin molecules that yield fraction Ts are very distinctive. The glycine content is only 12.6 residues/100 residues compared with 44 % in fibroin and 46 % in fraction Tp; thus there is little opportunity for alternation of glycyl residues in these regions. The alanine and serine contents are similarly reduced and the content of amino acids with basic and acidic side chains is correspondingly increased; the accumulation of lysine and arginine in these regions (4.2 and 4.6 residues/100 residues respectively) explains their sensitivity to tryptic hydrolysis; if each basic residue were at the *C*-terminal end of a peptide, that is if tryptic action were complete, the mean chain length of the peptide mixture would be 11.5, in good agreement with the experimental result.

Further hydrolysis of fractions Tnd and Ts with chymotrypsin results primarily in the fission of bonds involving the carboxyl groups of tyrosine and phenylalanine residues. Hydrolysis of fraction Ts with chymotrypsin would be expected to free the octapeptides Gly₄,Ala₃,Tyr and Gly₄,Ala₂,Val,Tyr and the tetrapeptides Gly₂,Ala,Tyr and Gly₂,Val,Tyr from any longer runs of such sequences. The absence of these peptides from fraction Ts-Cs implies that such sequences are not present in fraction Ts. There is a reduction in mean chain length from 11.2 to 6.5 when fraction Ts is further hydrolysed with chymotrypsin. It can be calculated from the tyrosine and phenylalanine content that complete hydrolysis of the bonds involving these residues would result in a reduction of the mean chain length from 11.2 to 6.0 and

consequently it is clear that the action of the enzyme is substantially complete.

Fraction Tnd gives rise on chymotryptic hydrolysis to a precipitate, substantially identical with that (fraction Cp) obtained by direct action of chymotrypsin on fibroin. It is composed of sequences of glycyl residues alternating with those of alanine and serine; these sequences are about 60 residues long and end in a *C*-terminal tyrosine residue (Lucas *et al.* 1957). The soluble peptides derived by chymotryptic hydrolysis of fraction Tnd consist of two octapeptides (Gly₄,Ala₃,Tyr and Gly₄,Ala₂,Val,Tyr) and two tetrapeptides (Gly₂,Ala,Tyr and Gly₂,Val,Tyr) together with a very small amount of other peptide material. These four peptides had been previously isolated from the chymotryptic digest of fibroin and the structures of three of them have been reported (Lucas *et al.* 1962). The fourth peptide (Gly-Val-Gly-Tyr) has been characterized more recently (F. Lucas, unpublished work). The fact that fraction Tnd is composed almost entirely of these sequences of established composition proves that a very large proportion of the fibroin molecule is built up of long sequences in which glycine residues alternate with a limited range of other amino acid residues, principally those of alanine, serine, tyrosine and valine.

It is necessary to be cautious in giving a precise quantitative value to the proportion of the residues in fibroin that take part in sequences with alternating glycine. Table 3, which gives the distribution of amino acids in the different fractions, shows that about 30 residues/100 residues appear in fraction Tnd-Cs and about 61 in Tnd-Cp. Of these, all but the apparently small amount of unidentified material in fraction Tnd-Cs are known to occur in sequences with alternating glycine, and it follows

Table 3. *Approximate distribution of 1000 amino acid residues in Bombyx mori fibroin before and after fractionation with pancreatic enzymes*

	Fibroin	Ts	Tp and Tnd	Tnd-Cp	Tnd-Cs
Glycine	444	15	429	301	128
Alanine	293	12	281	201	80
Valine	22	6	16	0	16
Leucine	5	3	2	0	2
Isoleucine	7	4	3	0	3
Serine	121	10	111	93	18
Threonine	9	3	6	3	3
Aspartic acid	15	11	4	0	4
Glutamic acid	10	7	3	0	3
Proline	4	2	2	0	2
Tyrosine	52	5	47	9	38
Phenylalanine	6	2	4	0	4
Lysine	3	2	~1	<1	<1
Histidine	2	1	1	0	1
Arginine	5	4	1	0	1
Others	2	2	0	0	0
Total	1000	89	911	607	303

that the proportion of these sequences may be as high as 90 %. What proportion of fraction Tnd-Cs this unidentified material represents is difficult to estimate, for peptides of high chain length with low ninhydrin colour yields might not be revealed by the techniques that have been used. Analysis shows fraction Tnd-Cs to be composed of glycine, alanine, valine and tyrosine residues to the extent of 83 %, and some of the remaining 17 % of residues are derived from the trypsin and chymotrypsin with which the fraction is contaminated. Consequently it is very unlikely that less than 80 % of the residues of fraction Tnd-Cs are present in peptides other than the four that have been characterized, and it follows that between 85 and 90 % of the residues in fibroin take part in sequences of alternating glycine residues.

When the analytical results in Table 1 are considered, it should be remembered that no attempts were made to remove the enzymes from fractions Tnd, Tnd-Cs and Ts at the end of the experiments, and consequently the analytical values for these fractions include amino acids derived from the trypsin and chymotrypsin. Their proportion is small for fraction Tnd (1.0–1.5 %), but fraction Ts is contaminated with about 10 % of trypsin, and fraction Tnd-Cs with about 6 % of a mixture of trypsin and chymotrypsin. Nevertheless, the amount of methionine found in fraction Ts (0.72 residue/100 residues) is substantially greater than can be accounted for by the trypsin, which contains about 0.9 % of this amino acid; it is therefore evident that methionine is a component of fibroin.

In the intact fibroin molecule, it is convenient to designate as phase I the sequences of glycine, alanine and serine that give rise on chymotryptic action to fraction Cp, and to call the sequences of glycine, alanine, valine and tyrosine residues that occur with phase I in fractions Tp and Tnd, phase II. The remaining sequences, comprising mainly the polar and high-molecular-weight residues, may be called phase III. In fibrous fibroin it is likely that aggregates of phase I sequences constitute the highly crystalline β -structure that contributes most to the characteristic X-ray diffraction pattern of the fibre, whereas phase II sequences may pack in imperfectly ordered, but not entirely disoriented, arrays. Phase III probably constitutes the truly amorphous part of the fibroin structure. Of 100 amino acid residues in fibroin, approximately 61 occur in phase I, 30 in phase II and nine in phase III.

Up to the present, all studies of the end-residues of fibroin have revealed more than one *N*-terminal residue, and glycine, alanine, serine and aspartic acid are usually found (Zuber, 1961; Narita, 1954; J. T. B. Shaw, unpublished work); thus it seems

probable that fibroin is a complex of several peptide chains. Whether the chains are joined together in the fibre only by hydrogen bonds, or whether they are combined by covalent forces in a single molecular unit, is not certain. Attempts to characterize chemically distinct components in solutions of fibroin have not been fruitful and, if the fibroin unit is of the first type, it is highly likely that the peptide chains are similar in size and in chemical composition. On the present evidence it seems probable that the chains of the molecule are joined by interchain bonds that are not broken when fibroin is dissolved; possible cross-links are the S-S bonds of a severely limited number of cystine residues, or, as Zuber (1961) suggests, ester bonds between serine or tyrosine hydroxyl groups and the β - and γ -carboxyl groups of aspartic acid and glutamic acid residues; the presence of about 0.15 % of cystine in fibroin has been reported by Zuber, Ziegler & Zahn (1957) and by Schroeder & Kay (1955). The low yield of end-residues is compatible with a 'multi-chain' molecule composed of a small number of long peptide chains. Whether such chains contain mixtures of all three phases or whether the composition of each chain is restricted to a single phase or to a combination of two phases is not known.

As the fraction Tnd-Cp: fraction Tnd-Cs ratio is approximately 2:1 in terms of amino acid residues, and as the chain length of fraction Tnd is about 300 residues, it follows that, of these, about 200 are in phase I-type sequences and 100 in phase II-type sequences. As phase I occurs in units of 60 residues, it follows that fraction Tnd contains three of these units combined with some 90 residues derived from phase II. It seems more likely that the transition from the small, potentially crystalline, amino acid residues of phase I to the bulky and amorphous phase III is effected through the interposition of phase II sequences rather than by direct conjunction. In support of this view phase I sequences can be isolated in a very pure form (the analysis of Tnd-Cp shows a composition of over 99 % of glycine, alanine, serine and tyrosine residues), whereas the fraction derived from phase II (Tnd-Cs) is contaminated with peptides containing amino acids other than glycine, alanine, valine and tyrosine. The small amounts of extraneous peptides encountered in fraction Tnd-Cs may derive from phase III and constitute the junctions between this phase and phase II.

SUMMARY

1. Hydrolysis of a solution of fibroin with trypsin gives rise to a precipitate containing 90 % of the protein nitrogen and to a mixture of short soluble peptides.

2. If tryptic hydrolysis is carried out in a medium containing 6 % of lithium thiocyanate no precipitation occurs, and the hydrolysate may be acidified and dialysed to give a solution of peptides of high molecular weight.

3. Further hydrolysis of this fraction with chymotrypsin shows that it consists predominantly of long sequences of amino acid residues throughout which glycyl residues alternate with alanine, serine, valine and tyrosine residues. Such sequences make up almost 90 % of the fibroin molecule.

4. Amino acid analyses of the fractions obtained by enzymic hydrolysis are presented, and the distribution of the amino acids in fibroin is discussed.

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Fractionation of the Fibroin of *Bombyx mori* with Alkali

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In the course of a series of experiments on the silk fibroin of *Bombyx mori*, Narita (1954) described the use of rivanol (6,9-diamino-2-ethoxyacridine lactate) to fractionate the dissolved protein. He used samples of fibroin that had been 'degummed' with hot aqueous ammonia solution; these were dissolved in copper ethylenediamine and the resulting solutions were dialysed against water and brought to pH 6.2 with phosphate buffer. By adding aqueous rivanol under controlled conditions to such a solution Narita obtained two fractions: a precipitate which represented about 15 % of the total protein, and a soluble fraction which constituted the remainder. The precipitate, which Narita called 'silk plastin', was amorphous by X-ray-crystallographic standards, and in composition it was substantially poorer in glycine and alanine and richer in most other amino acids than whole fibroin. The soluble material he called 'silk fibrin'; this was 'crystalline' by the same standards

when isolated from solution by precipitation with acetone, and was composed almost entirely of glycine, alanine and serine, with a little tyrosine and valine.

When a solution of *B. mori* fibroin prepared in these laboratories was treated with rivanol under the conditions used by Narita, a different result was obtained: no fractionation was effected and the whole of the dissolved protein was precipitated. This fibroin preparation had been obtained from raw silk by degumming with hot soap solution, and the solution was prepared by the use of concentrated aqueous lithium thiocyanate instead of copper ethylenediamine; thus the two preparations differed in that Narita's had been exposed to more prolonged and vigorous attack by alkaline reagents. It appears that attack by alkali is capable of breaking down the fibroin molecule in such a way that subsequent fractionation gives rise to two or more grossly dissimilar fractions. There is