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

not at present much information concerning the effect of limited attack by alkali upon fibroin, or indeed upon proteins in general, and it seemed worthwhile to investigate the situation more fully.

MATERIALS AND METHODS

Bombyx mori silk fibroin. Japanese *B. mori* silk was purchased from H. T. Gaddum and Co. Ltd., Manchester, and was degummed as described by Drucker, Hainsworth & Smith (1953). Solutions were prepared by dissolving the fibroin in aq. 60% (w/v) LiSCN (1 g. in 5 ml.), diluting with water to 50 ml. and dialysing the solution in Visking $\frac{3}{8}$ in. cellophan dialysis tubing against a stream of water until free from SCN^- ions.

Chemicals. 6,9-Diamino-2-ethoxyacridine lactate was a commercial sample purporting to be of not less than 97% purity, purchased from the Aldrich Chemical Co. Inc., Milwaukee 10, Wis., U.S.A. 1-Fluoro-2,4-dinitrobenzene was also a commercial preparation, obtained from L. Light and Co. Ltd., Colnbrook, Bucks. Other chemicals were of analytical reagent grade.

Alkali-treatment. Solutions of fibroin containing about 2% (w/v) of protein were diluted with *N*-NaOH and with water so that they were 1% in protein and 0.1 *N* in NaOH. The pH of such solutions was found to be 12.8. In most of the experiments described in this paper the solutions were kept at 3° in a refrigerator, and were then brought to pH 6.2 by the addition of *N*-phosphoric acid. Such a preparation resulting from an alkali-treatment lasting 48 hr. is subsequently referred to as a solution of λ -fibroin (Gk. *λύειν*, to loose). Similar experiments were performed in which the solution at pH 12.8 was maintained at 25°.

Solutions at pH 11.0, 11.5, 12.0 and 12.5 were also prepared from 2% solutions of fibroin in water by the addition of equal volumes of an appropriate sodium borate buffer, 0.2 *M* in Na^+ ions: the pH values were finally adjusted by the addition of a few drops of *N*-NaOH or *m*-boric acid. The solution at pH 13.0 was obtained by the cautious addition of *N*-NaOH to the standard pH 12.8 solution described above.

Viscosity measurements. The flow times of solutions of fibroin, 1% (w/v) in protein, at a series of controlled values between pH 11.0 and 13.0, were measured in an Ostwald viscometer at 25°, at intervals between 0 and 24 hr. The results were plotted in the form:

$$\eta_{sp.} = \left(\frac{\text{flow time of solution}}{\text{flow time of solvent}} \right) - 1$$

against time. It was not possible to make accurate measurements of the viscosity of fibroin solutions at pH values below 11.0 because of surface denaturation.

Fractionation with rivanol. Solutions of fibroin and of alkali-treated fibroin, 1% in protein, were brought to pH 6.2 with phosphoric acid, and to about 3°. Aq. 1% rivanol was then added dropwise whilst the solution was maintained at $3 \pm 1^\circ$ in an ice-water bath. When further addition of rivanol produced no more precipitate, the mixture was allowed to stand for some minutes, and the precipitate, 'silk plastin', was collected by filtration through a sintered-glass crucible. It was washed with a little ice-water, with methanol and with ether, and then dried and weighed. Addition of too much rivanol resulted

in dissolution of some of the precipitate; this redissolved fraction could be recovered by dialysing the filtrate against water, when removal of the excess of rivanol resulted in its precipitation. The 'silk plastin' prepared in this way was granular in texture and pale yellow, owing to the presence of traces of strongly bound rivanol, the last of which could be removed by including methanol-0.1 *N*-HCl (9:1, v/v) in the washing sequence. Isolation of the dissolved 'silk fibrin' fractions in a dry state was not usually necessary, as analyses and end-group assays could be performed satisfactorily with the solutions remaining after removal of the excess of rivanol and the salts by dialysis; addition of ethanol or acetone to these solutions led to the production of very stable gels, from which it proved difficult to isolate the dry material. If a dry sample of the fraction was required, the best method was to shake the dialysed solution with a little activated charcoal to remove the remaining rivanol, and then to freeze-dry the resulting colourless solution: the preparation obtained in this way was only partially soluble in water.

Fractionation with ammonium sulphate. λ -Fibroin solutions, about 1% (w/v) in protein and 0.1 *M* in Na^+ ions, were brought to 20° and pH 6.2, and were treated with a saturated solution of $(\text{NH}_4)_2\text{SO}_4$, with stirring. The $(\text{NH}_4)_2\text{SO}_4$ soln. was run in dropwise until 12 ml. had been added/100 ml. of protein solution. The mixture was allowed to stand for 10 min. and was then centrifuged. The precipitate ($\lambda\text{F-Ap}$) was washed once with $(\text{NH}_4)_2\text{SO}_4$ soln. (0.1 saturated) and was then dissolved in 5 *M*-urea and dialysed against very dilute sodium phosphate buffer, pH 7–8; the dialysed solution was later assayed for nitrogen. The supernatant from the centrifugation plus the washings were dialysed against water to remove $(\text{NH}_4)_2\text{SO}_4$ and buffer salts. When necessary, the product ($\lambda\text{F-As}$) was finally isolated by freeze-drying.

Nomenclature. It is proposed to adopt Narita's (1954) terminology in a general form and to call fractions of alkali-treated fibroin that can be precipitated with rivanol 'silk plastin' and those that cannot be so precipitated, 'silk fibrin'. However, more precise terms are needed to define the preparations of silk plastin and silk fibrin that we have investigated most fully, and the polypeptides obtained from standard λ -fibroin solutions by rivanol precipitation are subsequently called $\lambda\text{F-Rp}$ (plastin) and $\lambda\text{F-Rs}$ (fibrin), whereas those obtained by $(\text{NH}_4)_2\text{SO}_4$ fractionation are called $\lambda\text{F-Ap}$ (precipitate) and $\lambda\text{F-As}$ (soluble fraction).

Amino acid analyses. These were performed by the chromatographic methods of Moore, Spackman & Stein (1958) with the minor variations described by Lucas, Shaw & Smith (1962). Some of the analyses were subsequently confirmed on an automatic amino acid analyser. The samples were hydrolysed with boiling HCl (6 *N*) for 24 hr. and corrections were made for losses of serine and threonine by hydrolytic decomposition. The results were related to the total nitrogen content of the solutions, assayed by the micro-Kjeldahl technique. Tryptophan assays were performed on separate samples of unhydrolysed material by the colorimetric method of Spies & Chambers (1949).

End-residue analysis. *N*-Terminal residues of the fractions, and of fibroin, were identified and assayed by the FDNB* method. The techniques are essentially those of

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

Sanger (1945) but certain modifications were introduced. Treatment of the protein and peptide fractions with FDNB was carried out in ethanol-water (8:5, v/v) in the presence of an excess of NaHCO_3 and FDNB, and the reaction was allowed to continue overnight (16 hr.) at room temperature with shaking, to ensure completion. The fibroin samples were solutions in water (1–2%, w/v) that had been prepared by the use of LiSCN; the fraction $\lambda\text{F-Rs}$ and the alkali-treated fibroins were also available as solutions, and to each of these the appropriate amount of NaHCO_3 , dissolved in a little water, was added, followed by ethanolic FDNB. The $\lambda\text{F-Rp}$ fraction, which was not water-soluble, was reduced to a fine powder, suspended in water and then submitted to the same procedure. The products were centrifuged, thoroughly washed with dilute HCl, with water and with ethanol, and dried. Hydrolysis of weighed samples of the DNP derivatives was carried out in conc. (11.7N) HCl at 40° , and was considered to be substantially complete after 21 days. Under these conditions losses of DNP-amino acids, especially of DNP-glycine, are much reduced and are normally less than 10%. The liberated DNP-amino acids were isolated by column chromatography on buffered Celite and estimated spectrophotometrically as described by Lucas, Shaw & Smith (1963).

RESULTS

Viscosity changes. The change in specific viscosity of 1% (w/v) solutions of fibroin with time at values between pH 11 and 13 is shown in Fig. 1. These changes were found to be irreversible when the pH was returned to 6. Fig. 2 illustrates the fall in specific viscosity shown by 1% (w/v) solutions of fibroin in 0.1N-NaOH at 25° and at 3° : the pH of such solutions was between 12.7 and 12.8 and did not change perceptibly during the experiments.

Fig. 1. Action of alkaline buffers on solutions of fibroin: change of specific viscosity with time of treatment at 25° . ●, pH 11.0; △, pH 11.5; +, pH 12.0; ○, pH 12.5; ▲, pH 13.0.

Fractionation with rivanol. Solutions of fibroin were kept at pH 12.8 at 3° and at 25° and portions were removed at intervals and tested for precipitability with rivanol. The results are shown in Fig. 3. It is clear that a steady state is reached after about 2 days at 3° and after about 10 hr. at 25° . After these times of treatment, 10% by weight of the fibroin is precipitable with rivanol. A solution of fibroin that had been treated with pH 11 buffer for 3 hr. at 25° was completely precipitated with rivanol at pH 6.2; complete precipitation also occurred when a solution of fibroin prepared with copper ethylenediamine as described by Drucker *et al.* (1953), instead of with aq. lithium thiocyanate, was tested without pretreatment.

Fig. 2. Action of 0.1N-NaOH on solutions of fibroin (pH 12.8): changes in specific viscosity with time of treatment at (a) 3° and (b) 25° .

Fractionation with ammonium sulphate. Treatment of a solution of λ -fibroin with ammonium sulphate under the conditions described in the Materials and Methods section resulted in the production of a precipitate (λ F-Ap) representing about 10% by weight of the total protein. The remaining dissolved polypeptide (λ F-As) could be dialysed against water without significant loss.

Solubility properties. The λ F-Rs and λ F-As fractions were soluble in water and in aqueous buffers at all pH values. The solutions were stable and did not gel even on prolonged standing. The dissolved peptides could be fractionally precipitated by the addition of trichloroacetic acid or of ethanol; this suggested the presence of a mixture of peptides, probably heterogeneous with respect to chain length. The λ F-Ap fraction could be dissolved in aqueous urea, and the resulting solution survived dialysis. The fraction remained soluble in aqueous buffers at values above pH 5.8 and below 3.5, but was insoluble between these values; between values about pH 5.8 and 7.0 the solution was opalescent. The λ F-Rp fraction showed similar properties after the absorbed rivanol had been removed.

Amino acid composition. The amino acid analyses of *B. mori* fibroin, and of the four principal fragments here described, λ F-Rp, λ F-Rs, λ F-Ap and λ F-As, are given in Table 1 together with Narita's (1954) analyses of 'silk fibrin' and 'silk plastin'. The values for serine are corrected by a factor of 10% for losses due to hydrolytic decomposition and those for threonine by 5%. The tentative

Fig. 3. Action of 0.1N-NaOH on solutions of fibroin (pH 12.8): precipitability with rivanol after treatment at (a) 3° and (b) 25°.

Table 1. *Amino acid composition of fibroin and of some fractions of fibroin*

Results are given in two forms: (1) as amino acid nitrogen as a percentage of total nitrogen; (2) as amino acid residues/100 residues.

	Fibroin*		λ F-Rp		λ F-Ap		λ F-Rs		λ F-As		'Silk plastin'†	'Silk fibrin'†
	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(1)
Glycine	43.7	44.6	14.0	18.3	16.3	21.2	44.7	46.4	44.5	45.7	36.1	47.8
Alanine	28.8	29.4	12.6	16.5	14.0	18.2	29.3	30.4	30.0	30.8	26.4	29.3
Valine	2.16	2.20	4.12	5.40	3.84	5.00	1.87	1.94	2.13	2.19	1.84	2.41
Leucine	0.52	0.53	4.23	5.54	3.99	5.19	0.15	0.16	0.1	0.1	2.75	0.48
Isoleucine	0.65	0.66	3.98	5.21	3.86	5.02	0.31	0.32	0.35	0.36		
Serine	11.9	12.1	7.92	10.4	8.3	10.8	10.6	11.0	11.7	12.0	11.5	11.5
Threonine	0.89	0.91	2.00	2.62	1.77	2.30	0.78	0.81	0.75	0.77	1.38	0.44
Aspartic acid	1.28	1.30	8.22	10.8	7.5	9.8	1.02	1.06	1.09	1.12	4.10	0.12
Glutamic acid	1.00	1.02	4.98	6.52	4.39	5.71	0.79	0.82	0.74	0.76	2.12	0.56
Proline	0.35	0.36	2.34	3.1	2.0	2.6	0.4	0.4	—	—	0.70	—
Tyrosine	5.07	5.17	3.63	4.75	3.66	4.78	4.77	4.95	4.70	4.83	5.12	4.05
Phenylalanine	0.62	0.63	1.90	2.49	2.02	2.62	0.53	0.55	0.6	0.6	1.25	—
Lysine	0.63	0.32	2.40	1.57	2.21	1.43	0.44	0.22	0.48	0.25	1.68	—
Histidine	0.53	0.14	3.15	1.38	2.53	1.08	0.34	0.11	0.39	0.13	—	—
Arginine	1.83	0.47	12.6	4.12	11.9	3.88	0.56	0.14	0.79	0.20	10.7	1.29
Tryptophan	0.33	0.11	1.0	0.6	1.05	0.69	0.30	0.10	0.35	0.12	—	—
Hydroxylysine	—	—	0.56	0.36	0.43	0.28	—	—	—	—	—	—
Methionine	—	—	0.26	0.34	0.26	0.30	—	—	—	—	—	—
Ammonia	—	—	6.5	—	7.14	—	1.0	—	1.4	—	—	—

* Results for untreated fibroin are from Lucas, Shaw & Smith (1958).

† Values for 'silk plastin' and 'silk fibrin' are from Narita (1954).

Table 2. *N*-Terminal residues of fibroin and of some fractions of fibroin

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

	Fibroin		λ -Fibroin		λ F-Rs		λ F-Rp		'Silk fibrin' [†]	'Silk plastin' [†]
	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)		
Glycine	0.19	0.15	3.80	2.96	4.28	3.17	1.46	1.41	0.93	0.92
Alanine	0.13	0.10	1.93	1.50	2.12	1.57	0.88	0.85	0.56	0.71
Serine	0.18	0.14	1.16	0.90	1.22	0.90	0.74	0.71	0.66	1.05
Aspartic acid	0.17	0.13	0.35	0.28	0.33	0.24	0.24	0.23	—	—
Others*	0.14	0.11					1.18	1.14	—	—
Total	0.81	0.63	7.24	5.64	7.95	5.88	4.50	4.34	2.15	2.68
Mean chain length	1587		178		170		230		—	

* This fraction includes small amounts of unidentified DNP-amino acid and some incompletely hydrolysed DNP-peptides.

[†] Results from Narita (1954) for 'silk-fibrin' and 'silk-plastin' fractions, in the form moles/10⁵ g. of DNP-peptide.

Fig. 4. Liberation of *N*-terminal residues during treatment of dissolved fibroin with 0.1 *N*-NaOH at 3°. O, Total; ●, glycine; △, alanine; ▲, serine.

identification of a minor component in some hydrolysates as hydroxylysine is based wholly on its chromatographic behaviour and needs confirmation. The evidence for the presence of methionine as a minor component of the fibroin molecule (Shaw, 1964) is further supported by the analysis of fractions λ F-Ap and λ F-Rp.

End-residue assay. The results of estimations of *N*-terminal residues with dissolved fibroin, with λ -fibroin and with the fractions λ F-Rp and λ F-Rs are presented in Table 2; the results of end-group analyses performed on fractions λ F-Ap and λ F-As were similar to those for the corresponding fractions obtained with rivanol, and are not recorded. Narita's (1954) estimates of the *N*-terminal residues in his 'fibrin' and 'plastin' preparations are also included for comparison. The present results are not corrected for losses during hydrolysis and

chromatography, but such losses are likely to be small. The rate of production of total *N*-terminal residues, and of the three principal *N*-terminal residues glycine, alanine and serine, when a fibroin solution is maintained at pH 12.8 at 3°, is illustrated in Fig. 4.

DISCUSSION

These results show Narita's (1954) original postulate that dissolved fibroin can be fractionated into two chemically distinct fragments by precipitation with rivanol to be incorrect, and Narita has subsequently withdrawn this opinion in a personal communication; he suggested instead that breakdown of the fibroin molecules may result from the dissolution of the protein in copper ethylenediamine. However, solutions of fibroin prepared with copper ethylenediamine are here shown to behave towards rivanol in exactly the same way as those prepared with lithium thiocyanate, and it follows that the fractionation must have been due to some other cause. This cause is the breakdown of the fibroin molecule from the use of boiling ammonia solution in the preparation of the fibre. Shimizu (1957) confirmed that, though dissolved fibroin from the silk gland was completely precipitable by rivanol, fibrous fibroin, boiled with aq. 0.5 % ammonia solution and dissolved in copper ethylenediamine, was not completely precipitable; the amount of precipitate decreased with increasing duration of the ammonia treatment.

The action of alkalis on proteins has not been very widely studied, largely because the hydrolytic processes are complicated by secondary effects including the racemization and breakdown of amino acids, especially serine, threonine, cystine and arginine (Sanger, 1952). However, the pattern of the hydrolysis of a protein with alkali differs from that with acid owing to the reversal of the charge effects and to the comparative resistance of certain bonds, for example those involving the

amino groups of the hydroxyamino acids. Consequently, hydrolysis with alkali may provide useful results if extreme conditions are avoided.

There is a marked contrast between the sensitivity of dissolved and fibrous fibroin towards attack by alkali, and also in the nature of this attack. The action of 2*N*-sodium hydroxide on fibrous fibroin at 40° has been described by Shaw & Smith (1961); the fibre was quite rapidly attacked, and after 24 hr., when 73 % of the material had been dissolved, no evidence was found either from amino acid analysis or by X-ray diffraction for any pronounced differentiation in composition or structure between the dissolved and the undissolved fragments. Treatment with cold 0.1*N*-sodium hydroxide gives rise to considerable disruption of the dissolved fibroin molecule, but has no perceptible effect on fibrous fibroin; this difference is explicable in terms of accessibility. Fibroin molecules probably exist in solution as structureless 'random-coils'; the rate and pattern of attack by alkali is therefore governed by the relative labilities of the peptide bonds (or other covalent linkages), since all are equally accessible. In these circumstances the principal factor governing the rate of fission of a particular bond is the distribution of charge in its environment, and this in turn is determined by the sequence of the amino acids adjacent to it.

Figs. 1-3 show that the dissolved fibroin molecules are attacked by alkali even at low temperatures and under comparatively mild conditions of pH. The resulting molecular disruption leads to a substantial and irreversible drop in the viscosity of the solutions. Figs. 1 and 2 show that the disruptive effect increases markedly at pH values above 12, and that in 0.1*N*-sodium hydroxide at 3° the specific viscosity of a 1 % solution of fibroin is reduced to 50 % of its original value in about 6 hr., and to about 15 % in 48 hr. These changes in viscosity are accompanied by changes in precipitability with rivanol; a comparison of the graphs of this property (Fig. 3) with those illustrating the viscosity change (Fig. 2) demonstrates the resemblance very clearly.

The results in Table 1 show that fractionation of dissolved fibroin by protein precipitants after a limited and controlled attack by alkali effects the separation of the molecule into two grossly dissimilar parts. The fractionation may be compared with that encountered in tryptic hydrolysis (see the preceding paper). The amino acid composition of the Tp (trypsin-precipitated) fraction is strikingly similar to that of fractions λ F-As and λ F-Rs, and all three are recovered in yields corresponding to about 90 % of the whole protein. This, the major building block of the fibroin molecules, has a glycine content approaching 50 %, and is composed

to a very large extent of sequences in which glycyl residues alternate with those of alanine, serine, valine and tyrosine. The remaining 10 % of the fibroin molecule is totally dissimilar, and it is the isolation of this fragment that these procedures have made possible; it is, of course, also present in the tryptic hydrolysate of fibroin, but as the mixture of short soluble peptides that constitute fraction Ts. This minor component can be isolated, and therefore presumably occurs in the original protein, in long sequences of about 230 residues.

Narita's (1954) analysis and end-residue estimations of 'silk fibrin' and 'silk plastin' (Tables 1 and 2) confirm that these fractions were derived from fibroin that had been attacked by alkali, but less extensively than the λ -fibroin preparation described here. Narita's 'silk plastin' was composed of longer peptide chains than fraction λ F-Rp, and its composition shows that it may be considered as a preparation of λ F-Rp with some sequences of λ F-Rs. From the rivanol-precipitability values, it appears that Narita's fibroin had been degraded to an extent corresponding to a fibroin solution after treatment for 20-30 hr. with 0.1*N*-sodium hydroxide at 3°.

The mechanism of the fragmentation of the fibroin molecule by alkali is obscure. There is now considerable evidence for the existence of more than one species of peptide chain in the fibroin molecule; certainly end-residue assays invariably show three or four different *N*-terminal residues. The chains may be joined together either by a severely limited number of cystine cross-links or by some other type of covalent bond, perhaps ester links involving the plentiful seryl residues. Either of these types of cross-link might be labile under alkaline conditions and release of the individual peptide chains may well be an important feature of the attack. However, it is not possible to establish this unequivocally, for end-group assay of the treated fibroins demonstrates progressive release of *N*-terminal residues that can only be attributed to concomitant main-chain cleavage.

Fig. 3 shows that prolongation of the alkali treatment at 3° beyond 48 hr. does not result in a further reduction of material precipitable with rivanol; from this it follows that most of the peptide bonds in this fraction, derived from phase III (for definition of terminology see Shaw, 1964) of the fibroin molecule, are resistant to cleavage by alkali. It is the sequences of alternating glycyl residues (phases I and II) that are most sensitive to alkaline hydrolysis, as is shown by the progressive release of *N*-terminal glycyl residues and, less rapidly, those of alanine and serine, which continued throughout the 25-day period for which it was studied. Comparison of the chain lengths shows that fractions λ F-Rs and λ F-As (mean chain

length 170 residues) are somewhat degraded relative to the corresponding Tp and Tnd fractions (mean chain length about 350 residues). Consequently, though alkali treatment is at present the only method available for isolating phase III of the fibroin molecules, enzymic hydrolysis, being more specific, is to be preferred for isolating phases I and II.

The use of rivanol as a protein precipitant was, until recently, largely confined to the separation of the proteins of blood plasma and in particular to the isolation of γ -globulin (Horejsi & Smetena, 1956). Subsequently, Steinbuch & Quentin (1959) isolated ceruloplasmin by stepwise precipitation of plasma with rivanol under different conditions of pH, and Kaldor, Saifer & Vecsler (1961) have studied the interaction between rivanol and bovine serum albumin under different conditions of pH, ionic strength and concentration. They concluded that, in general, the interaction of serum albumin with rivanol resembled that with simple metal cations rather than with cationic detergents. However, Narita's (1954) observations that silk plastin is redissolved by the addition of an excess of rivanol, corroborated by our experience with fraction λ F-Rp, suggests that this interpretation may not be valid in all circumstances.

It appears that rivanol is capable of forming complexes with proteins that exhibit appropriate arrangements of accessible polar side chains, and that the consequent obstruction of these polar groups renders the rivanol-protein complex insoluble. Rivanol is a strongly cationic dye (pK_a 11.04) and it is not surprising that, of the plasma proteins, γ -globulin, which is not precipitated, is the most basic. The fact that the precipitable fraction of alkali-treated fibroin (λ F-Rp) has a large excess of acidic (carboxyl) over basic side chains, and is thus a predominantly anionic molecule, corroborates the view that rivanol functions largely by combining with the acidic side chains of electronegative proteins. It follows that the fractional precipitation of proteins with rivanol is likely to be critically dependent upon pH, and Narita (1954) observed that the precipitation of his 'silk-plastin' fraction was most complete at values between pH 6.0 and 6.4, whereas at values below pH 5.0 and above 8.0 the amount of precipitate was much reduced. In the soluble fraction λ F-Rs, which is very deficient in polar side chains, the few acidic and basic groups are present in approximately equivalent amounts and precipitation at pH 6.2 would not therefore be expected. Rivanol is not strongly bound to either fraction: it can be removed from the dissolved λ F-Rs by shaking with charcoal and from the precipitated fraction λ F-Rp by washing with methanol containing a little dilute hydrochloric acid.

The relatively high chain length of the phase III fragments that have now been isolated (about 230 residues), considered with the fact that this phase represents only about nine amino acid residues/100 residues in fibroin, suggests that if the fibroin molecule is a unique entity it contains more than 2000 residues and thus must have a molecular weight in excess of 170 000. Recent evidence accords with the view that the molecular weight of fibroin is very high; ultracentrifuge studies by Prati, Moruzzi & Centola (1958), with a solution of fibroin in aqueous lithium bromide, afforded a value 270 000, and light-scattering experiments gave values around 300 000 (Hyde & Wippler, 1962). Moreover the end-group estimations quoted in Table 2 of this paper imply a mean chain weight of about 120 000 (taking 78 as the mean residue weight); consequently, if a four-chain molecule with terminal alanine, aspartic acid, glycine and serine residues is postulated, a molecular weight of at least 400 000 must be envisaged for fibroin. Clearly some of the most vital questions about the fibroin molecule that remain unanswered concern the existence and nature of interchain linkages.

It is encouraging to see that, in fractions λ F-Ap and λ F-Rp, several amino acids occur in proportions that bear simple relations to one another. For example, leucine, isoleucine and valine occur in closely similar molar amounts, whereas aspartic acid occurs twice, and threonine about half, as frequently. Other relations, more or less precise, can be found. Although this fraction is not a single molecular species, further work may show that it is composed of a fairly simple and tractable mixture of components, capable of resolution into entities suitable for sequential studies. If this is so, it will be possible to determine whether the unique regularity of structure now evident throughout almost 90 % of the fibroin molecule has its counterpart in the remaining fraction.

SUMMARY

1. Dissolved *Bombyx mori* fibroin was treated with 0.1 N-sodium hydroxide at 3°, and the rate of peptide-bond cleavage was measured, together with the fall in viscosity.

2. Solutions of fibroin that had been treated with 0.1 N-sodium hydroxide at 3° for 48 hr. were fractionated by means of the cationic dye rivanol (6,9-diamino-2-ethoxyacridine lactate) and by the addition of saturated ammonium sulphate.

3. The precipitated fractions, which represented about 10 % of the total protein, were found to be substantially different in composition from the soluble fractions. They contained little glycine and had mean chain lengths of about 230 residues.

4. The fractionation of fibroin by alkali is compared with that obtained by hydrolysis with trypsin, and the relation of the fractions to the fibroin molecule is discussed.

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Acetoacetate Formation by Liver Slices from Adult and Infant Rats

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It has become evident in recent years that fat is the main energy source for most infant mammals, particularly the rat. In that animal milk represents in fact a high-fat diet (Glass, 1956), and fat is not only rapidly utilized but is also laid down during the first few days after birth (Hahn & Koldovský, 1960). Reflexions of this state are high lipid concentrations in the blood of fed infant rats (Grafnetter, Hahn, Koldovský & Viktora, 1960) and a rapid suppression of lipogenesis in the liver very soon after birth (Vilée, 1958).

Since the end product of fatty acid oxidation in the liver is acetoacetate (Lehninger, 1945; Quastel & Wheatley, 1933; Edson, 1935), it might be expected that more of this substance is formed in the liver during the suckling period than later in life, when the fat content of the diet and of the liver is low. Surprisingly, this has not previously been examined. Even concentrations of ketone bodies in the blood of young rats have been determined only after weaning (Wick & McKay, 1940).

In the present work an attempt has been made to follow developmental changes in acetoacetate formation by liver slices and to correlate these with changes in the ketone-body concentration in the blood. A preliminary report of part of this work has appeared (Drahota, Hahn, Kleinzeller & Kostolánská, 1963).

MATERIALS AND METHODS

Animals. Male white Wistar rats fed on a standard laboratory diet (Fábry, 1959) were used throughout. Rats younger than 23 days were employed regardless of sex. Litters were always reduced to eight on the second day after birth. When animals were starved, rats younger than 23 days were kept at 30° without the mother animal in an incubator. Adult animals were kept at room temperature (about 22°) and were deprived of both food and water.

Tissue preparations. Rats were decapitated and the livers were rapidly removed and chilled. Tissue slices were cut by hand. They were gently dried on filter paper and weighed on a torsion balance, and 50–80 mg. portions placed in chilled Warburg vessels containing 3 ml. of saline medium (see below). The slices were incubated at 37° in oxygen in a Warburg apparatus. The central well of each flask contained 0.2 ml. of 20% (w/v) KOH solution. Since high concentrations of phosphate inhibit acetoacetate formation (Quastel & Wheatley, 1933), the composition of the saline medium was adjusted to the following: NaCl, 134 mM; KCl, 5.6 mM; CaCl₂ 2.25 mM; potassium phosphate, 4.2 mM; MgSO₄, 1.4 mM; the final pH was 7.2. Other additions were made as indicated in the text. The Q_{O_2} and respiratory quotient (R.Q.) values were determined by the direct Warburg method (see Dixon, 1943).

Analytical methods. Acetoacetate was determined colorimetrically according to the method of Walker (1954). Sodium acetoacetate used for calibration was prepared according to the method of Ljunggren (1924) and standardized according to the method of Edson (1935).