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REFERENCES

Baker, S. L., Butterworth, E. C. & Langley, F. A. (1946). *Biochem. J.* **40**, 291.
Bowes, J. H. & Kenten, R. H. (1949). *Biochem. J.* **45**, 281.
Dallemagne, M. J. (1943). *La Nature chimique de la substance osseuse*. Thesis. Liége.
Hammett, F. S. (1925). *J. biol. Chem.* **64**, 409.
Hess, A. F., Berliner, F. S. & Weinstock, M. (1931). *J. biol. Chem.* **94**, 9.
Lightfoot, G. H. & Coolidge, T. B. (1948). *J. biol. Chem.* **176**, 477.
Loofbourow, J. R., Gould, B. S. & Sizer, I. W. (1949). *Arch. Biochem.* **22**, 406.
Neumann, R. E. (1949). *Arch. Biochem.* **24**, 289.
Roche, J. & Garcia, J. (1936). *Bull. Soc. Chim. biol., Paris*, **18**, 1014.
Rogers, H. J. (1949). *Nature, Lond.*, **164**, 625.
Rogers, H. J. & Hall, D. A. (1951). Unpublished observations.
Stack, M. V. (1950*a*). *Biochem. J.* **46**, xi.
Stack, M. V. (1950*b*). *Nature, Lond.*, **166**, 1080.
Strobino, C. J. & Farr, L. E. (1949). *J. biol. Chem.* **178**, 599.
Vogt, J. H. (1949). *Acta med. scand.* **135**, 221.
Weidmann, S. M. & Rogers, H. J. (1950). *Biochem. J.* **47**, 493.

A Search for Specific Chemical Methods for Fission of Peptide Bonds

1. THE *N*-ACYL TO *O*-ACYL TRANSFORMATION IN THE DEGRADATION OF SILK FIBROIN

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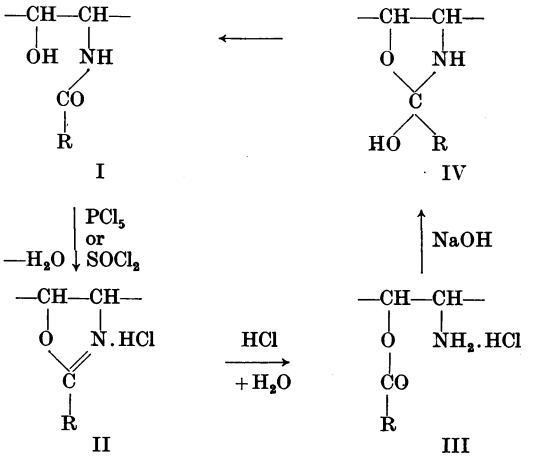
Partial degradation of proteins or polypeptides by chemical or enzymic hydrolysis is lacking in specificity and results obtained in this way have generally been very complex and difficult to interpret. Interest in the chemistry of the natural amino-acids has quickened considerably in recent years, the more complex amino-acids receiving particular attention. In the light of this recent knowledge it seems possible that the polypeptide chain could be broken in a predictable fashion by using a reagent showing selective reactivity with one amino-acid. It would be expected that only those amino-acids possessing functional groups in addition to the α-amino and carboxyl groups could be specifically attacked. The results described below show that serine displays distinctive chemical reactivity of the required type.

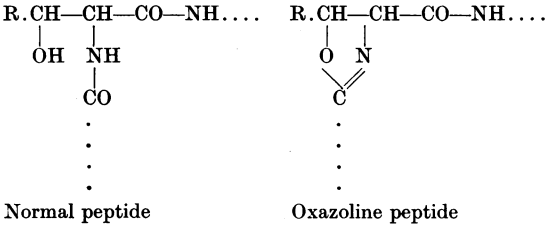
The migration of acyl groups in acyl derivatives of substances having a primary amino group and a hydroxyl group on adjacent carbon atoms was first observed in an aliphatic system by Bergmann, Brand & Dreyer (1921). Later, Bergmann & Miekeley (1924) demonstrated similar transformations in acyl derivatives of serine. The transformation of an *N*-acyl derivative (I) to an *O*-acyl derivative (III) and reconversion of the latter to the *N*-acyl derivative involved a number of steps which Bergmann, Brand & Weinmann (1923) showed to be as follows:



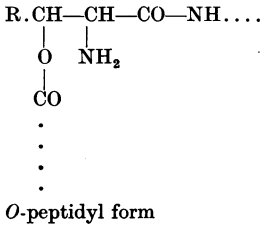
Compounds of types II and III were capable of independent existence and several examples were described by Bergmann and his collaborators, but compounds of type IV were assumed to have merely a transitory existence. Bergmann *et al.* (1923) were aware of the implications of their work in the protein field and put forward the suggestion that linkages such as those represented in II and IV might be found in proteins. Bergmann & Miekeley (1924) tried to substantiate this view by preparing an

oxazoline (type II) from glycylserine by treatment with thionyl chloride, but Bergmann, Miekeley & Kann (1925) showed that the compound obtained was 3-methylene-2:5-diketopiperazine.

In recent years a great many examples of the *O*, *N* migration have been discovered in compounds of varied type and have found important application in synthetic work, particularly where stereochemical problems were involved. Examples are to be found in the ephedrine field in which a careful study has been made by Welsh (1947) and in the synthesis of threonine (Elliott, 1948, 1949, 1950). As a result of methylation studies Blackburn, Middlebrook & Phillips (1942) suggested that oxazoline rings might be present in wool and silk. More recently Desnuelle & Casal (1948) have shown that in proteins the peptide bonds in which serine and threonine are linked through their nitrogen atoms are more labile than other peptide bonds to acid hydrolysis. This effect was more marked at low temperatures in the presence of very strong acid. These authors explained their results by assuming the intermediate formation of oxazoline rings in the peptide chain which were opened to give substances containing *O*-peptidyl bonds, the latter being more easily ruptured by acid hydrolysis than *N*-peptidyl bonds.



Normal peptide Oxazoline peptide



O-peptidyl form

Synge (1939) showed, however, that acid hydrolysis of DL-*N*-acetyl-*O*-benzoylserine yielded DL-*O*-benzoylserine, thus demonstrating the stabilizing effect of a charged nitrogen atom attached to a carbon atom adjacent to an ester group. On first consideration, the explanation offered by Desnuelle & Casal (1948) might appear to be incompatible with the observation of Synge (1939), but it should be pointed out that the yield of DL-*O*-benzoylserine obtained by Synge (1939) was small and it was not known whether any DL-*N*-acetylserine was produced simultaneously. A satisfactory comparison of relative rates of hydrolysis of *N*- and *O*-acyl bonds could only be made in a compound such as *ON*-diacetylserine in which both acyl groups are the same. The analytical data presented by Desnuelle & Casal supported the view that a shift of the peptide chain from nitrogen to oxygen in the linkages involving serine and threonine had occurred; the question whether or not an *O*-peptidyl bond is less stable than an *N*-peptidyl bond to acid hydrolysis cannot be decided on the evidence at present available. As a result of the work of Synge (1939) it seems probable that fission of an *O*-peptidyl bond by acid hydrolysis would require conditions at least vigorous enough to hydrolyse the more labile of the *N*-peptidyl bonds also. An attempt made by Desnuelle & Casal (1948) to bring about a rearrangement in egg albumin by the use of thionyl chloride was unsuccessful.

The purpose of the present investigation was to find a method of treating the protein molecule so that rearrangement of *N*-peptidyl linkages to *O*-peptidyl linkages occurred at the serine and threonine residues without at the same time causing hydrolysis of peptide bonds. It should then be possible to bring about a specific cleavage of the molecule. It seemed probable that the methods used by Bergmann and his co-workers in simple organic model substances would not be useful for proteins because these methods involved the use of organic solvents and vigorous reagents such as thionyl chloride and phosphorus pentachloride. In recent years ethanolic hydrogen chloride has been used for *N*, *O* acyl rearrangements (see, for example, Phillips & Baltzly, 1947), but it seemed unlikely that this reagent would be useful in the protein field. Reitz, Ferrel, Olcott & Fraenkel-Conrat (1946) found that when proteins were treated at low temperature with anhydrous sulphuric acid no peptide-bond fission took place unless the reaction was prolonged. The aliphatic hydroxyl groups were converted into mono-esters of sulphuric acid and some ring sulphonation occurred. Evidence supporting the view that no peptide-bond fission had occurred was obtained by Glendenning, Greensberg & Fraenkel-Conrat (1947) who found that insulin retained its biological activity after sulphuric acid treatment. Reitz *et al.* (1946) observed, however, that prolonged reaction of proteins with sulphuric acid caused a deep-seated change. This was indicated by the loss of sulphate bound by aliphatic hydroxyl groups, by the high amino-nitrogen values and by the low recoveries obtained when the crude reaction mixtures were poured on to crushed ice, neutralized and dialysed. This isolation procedure gave high recoveries with proteins given a short treatment with sulphuric acid. When the reaction with sulphuric acid was prolonged, breakdown of the molecule could be avoided by isolating the product in such a way as to avoid contact with

aqueous acid. These results could be interpreted on the assumption that the protein molecule had undergone rearrangement in a fashion analogous to the Bergmann transformations described above. As a result of the work of Reitz *et al.* (1946) it seemed probable that sulphuric acid would prove to be a very valuable reagent for the purpose in hand.

The experiments described below were concerned entirely with silk fibroin. This protein was chosen for study because of its availability, its relatively simple amino-acid composition and its high serine content. The degradation process consisted of three stages: (1) reaction of the protein with concentrated sulphuric acid at 21°, resulting in 'stage I protein'; (2) acetylation at pH 5, resulting in 'stage II protein'; (3) hydrolysis with dilute alkali, resulting in 'stage III acetylpeptide mixture'.

Rearrangement of silk fibroin with concentrated sulphuric acid

Treatment of silk fibroin with 97·5 % sulphuric acid at 21° for 3 days resulted in 62 % of the peptide bonds involving serine amino groups being converted into the *O*-peptidyl form. The extent of this conversion was not increased on further standing of the reaction mixture or under various other reaction conditions. It is shown in Table 1 that the amino nitrogen in the resulting stage I protein was considerably diminished after the material had been kept in a buffer solution at pH 9 for several hours. This behaviour would be expected from a substance having an *O*-peptidyl structure, because it would have been reconverted to the normal *N*-peptidyl form by the action of the alkali. It has been known for many years that acyloxyamines are extremely labile above pH 7; this transformation to *N*-acylaminoalcohol is generally instantaneous and complete. It is believed, therefore, that the difference between the two amino N values obtained before and after keeping stage I protein in alkaline buffer is a quantitative measure of the number of serine residues involved in the rearrangement. Rees (1946) found that the serine N in silk fibroin formed 11·6 % of the total N; on the basis of this figure the number of serine residues involved in the rearrangement was calculated to be 62 % of the total number present.

Table 1. *Amino* N *values found on stage I and stage II proteins compared with the amino* N *of soluble silk*

	Percentage of total N		
	Stage I protein	Silk fibroin	Stage II protein
Amino N	9·2	0·55*	0·53
Amino N after keeping at pH 9	2·0	—	—
Difference (amino N due to *O*-peptidyl bond formation)	7·2	—	—

* The value given for silk fibroin is that found on the soluble form prepared as described by Coleman & Howitt (1947).

The results presented in Table 1 also show that the amino N of stage I protein after keeping in buffer solution at pH 9 was higher than the amino N of soluble silk. It is possible, therefore, that some non-specific fission of peptide bonds may have occurred in the reaction with sulphuric acid. The results obtained by the 1-fluoro-2:4-dinitrobenzene (FDNB) technique, on the other hand, indicate that only serine was involved in the reaction.

Experiments with fluorodinitrobenzene

It was not possible to react stage I protein under the usual conditions with FDNB because the reverse transformation from *O*-peptidyl to *N*-peptidyl form took place so rapidly, as is shown by comparison of Exp. 1 with Exp. 9 (Table 2). FDNB was found to be sufficiently reactive to combine with amino groups at pH 5, at which the *O*-peptidyl form was stable. The 2:4-dinitrophenyl (DNP) derivative obtained was then hydrolysed under three different sets of conditions as described by Porter (1950), and the resulting DNP-amino-acids were separated on silicagel columns. The band due to DNP-serine was very much stronger than other bands; no band due to DNP-threonine was observed. The only other substance identified with certainty was DNP-glycine, the remaining bands being so weak that they were not considered to be significant. Glycine was also found as an end group in silk fibroin itself. The yield of DNP-serine obtained from DNP-stage I protein was much greater than the yield of any of the DNP-amino-acids obtained from DNP-silk fibroin, but in spite of many attempts (see Table 2) the number of DNP residues introduced into stage I protein was less than expected from the amino N results given in

Table 2. *Amount of serine* N *in stage I protein which reacted with* FDNB *under various conditions of* pH, *time and temperature*

(Results expressed as a percentage of the serine N available for reaction: see Table 1.)

Exp. no.	Temp. (°)	pH	Time (hr.)	Serine N reacted (%)
1	20	5	4	23·4
2	20	5	8	26
3	20	5	19	19·2
4	20	5	19*	18·1
5	1·5	5	22	6·3
6	37	5	4·25	15·2
7	20	6	24	3·1
8	20	6	72	11·8
9	20	8·5†	2·5	4·5

* Experiment carried out in darkness.
† Using $NaHCO_3$ as buffer.

Table 1. It does not follow that the presence of glycine as an end group in stage I protein was an indication of hydrolysis, because this amino-acid was also present as an end group in silk fibroin. Further experiments with FDNB showed that a number of other end groups besides glycine were present in silk fibroin; owing to this complexity and also to the uncertainty of achieving quantitative reaction of the FDNB with silk fibroin or with stage I protein, it was not considered practicable or useful to make a quantitative comparison of the results obtained on the two substances.

It was hoped that specific fission of DNP-stage I protein could be brought about by mild alkaline hydrolysis, yielding a mixture of DNP-seryl-peptides by fission of the *O*-peptidyl bonds. Some preliminary experiments on the partial hydrolysis of DNP-stage I protein were carried out. Mild alkaline hydrolysis was not successful, but partial acid hydrolysis gave promising results. These experiments were abandoned, however, when it was realized that there was very little hope of improving the reaction of FDNB with stage I protein.

The results of dialysis experiments presented in Table 3 show that some breakdown occurred when silk fibroin was treated with concentrated sulphuric acid. It is not known whether the loss of nitrogen was due to destruction of some specific part of the fibroin molecule or whether it was the result of general breakdown of a small fraction of the total number of molecules. This question could not have been decided without a full amino-acid analysis of stage I protein. The value found for serine N on the stage III acetylpeptide mixture and recorded in Table 6 is not very different from the value of 11·6 % of the total N found for serine N in silk fibroin by Rees (1946). It is possible, therefore, that the soluble non-diffusible material recovered from the reaction with sulphuric acid had not suffered any change of amino-acid composition. The insoluble material remaining after dialysis was not examined.

Table 3. *Indiffusible* N *present at each stage in the degradation of silk fibroin*

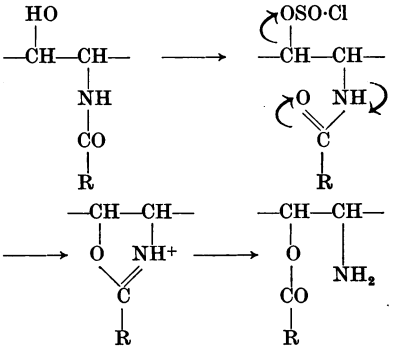
(Results expressed as a percentage of the total N in the system immediately before dialysis; for conditions see Experimental section.)

	Indiffusible N (%)
State I protein	65 (soluble) 22 (insoluble)
State II protein	97
Stage III acetylpeptide mixture	14

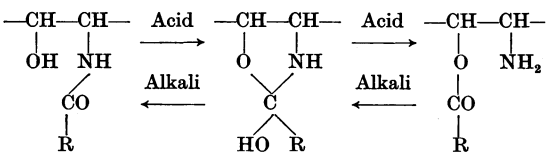
Gordon, Martin & Synge (1943) found that only about 50 % of the serine hydroxyl groups present in silk fibroin could be methylated by the action of dimethyl sulphate and alkali, in spite of continued treatment of the protein with the reagents. This finding could be correlated with the present observation that only 62 % of the bonds involving serine amino groups were rearranged by sulphuric acid; the reason for this lack of reactivity, however, is not understood.

Mechanism of the rearrangement

As a result of recent work it is evident that more than one mechanism can operate in the rearrangement (cf. Welsh, 1949; Phillips & Baltzly, 1947). Thionyl chloride forms oxazolines from acylaminoalcohols and to obtain the acyloxyamine it is necessary to treat the oxazoline with dilute acid. Elliott (1949) has shown that when the carbon atom carrying the hydroxyl group is asymmetric then an inversion of configuration may occur on that carbon atom on treatment of the substance with thionyl chloride:



The use of this reagent involves a two-stage process from acylaminoalcohol to acyloxyamine. Phillips & Baltzly (1947) have shown, however, that ethanolic hydrogen chloride causes rearrangement of acylaminoalcohols with production of relatively small amounts of oxazoline. These authors are probably correct in assuming that a hydroxyoxazolidine is the intermediate in this case, the oxazoline being formed by a side reaction involving dehydration of the hydroxyoxazolidine. In support of the conclusions of Phillips & Baltzly (1947) it has been found that DL-*N*-benzoyl*allo*threonine ethyl ester is converted into the corresponding *O*-benzoyl derivative without change of configuration by the action of ethanolic hydrogen chloride. The hydroxyoxazolidine structure, which was postulated many years ago by Bergmann *et al.* (1923) as an intermediate in the reverse transformation under the influence of alkali seems now to be well established as an intermediate when proceeding in either direction:



It cannot be decided at present which mechanism operates in the rearrangement of silk fibroin with concentrated sulphuric acid. In some of the preliminary experiments on the reaction of silk fibroin with sulphuric acid the protein was isolated in the dry state by precipitation and washing with dry ether. No evidence was obtained for the presence of oxazoline rings in this material: the amino N value remained unchanged after standing for several hours at pH 1, but the sulphuric acid used was not completely anhydrous and it is possible that oxazoline rings were produced and immediately opened again by the water present. It would be possible to establish the mechanism of the rearrangement using sulphuric acid, if threonine could be induced to take part in the reaction, by examining the configuration of the threonine obtained on acid hydrolysis of the stage I protein, but at present there is no evidence suggesting that threonine behaves in the same fashion as serine. In order to decide this question it would be better to use a protein containing a higher proportion of threonine than does silk fibroin.

Acetylation of stage I protein at pH 5

It has already been pointed out that a charged nitrogen atom attached to a carbon atom adjacent to an ester group renders this group more stable to acid hydrolysis than an ordinary ester group. It seemed probable, therefore, that it would not be very easy to find conditions for the preferential fission of the *O*-peptidyl linkages in stage I protein by acid hydrolysis, because of this stabilization phenomenon. Alkaline hydrolysis was impossible owing to the intervention of the reverse transformation from *O*-peptidyl to *N*-peptidyl form. These difficulties were overcome by blocking the nitrogen atom with an acetyl group which prevented the reverse transformation taking place and rendered the stage II protein susceptible to mild alkaline hydrolysis. It was necessary to find a method of acetylating stage I protein below pH 7, the pH at which the reverse transformation to *N*-peptidyl form occurred. Fortunately reaction of stage I protein with acetic anhydride in acetate buffer at pH 5 caused rapid acetylation of the amino groups; a small fraction remained unacetylated under these conditions. It is shown in Table 1 that the amount of amino N in stage II protein was almost equal to the amount present in soluble silk. It is known that the ϵ-amino group of lysine will react with nitrous acid under the conditions normally used for proteins (Chibnall, 1942) and using the value quoted by Traill (1950) for the lysine content of silk fibroin it can be calculated that about 75 % of the amino N of soluble silk is attributable to the ϵ-amino group of lysine. A possible explanation of the small but definite amino N value found in stage II protein is that the ϵ-amino groups of lysine may have failed to react with acetic anhydride at pH 5. It was found that even after several treatments with acetic anhydride the amino N of stage II protein, although diminished, did not become zero. The ease with which a base is acetylated probably depends on the amount of free amine which is present during the reaction. At a low pH a strong base would be largely in the $-NH_3^+$ form, thus making acetylation more difficult than in the case of a weak base. The ϵ-amino group of lysine would be more basic than the α-amino group of serine having its carboxyl group in peptide combination, because in the latter case the electrophilic carbonyl group is attached to the same carbon atom. This question of the reactivity of the ϵ-amino group of lysine was not considered to be very important at this stage of the investigation and has not been further studied. It seemed desirable, however, to show that a simple model substance was acetylated under the conditions used for stage I protein and it was found that *O*-benzoylserine was converted into *N*-acetyl-*O*-benzoylserine by acetic anhydride at pH 5.

Alkaline hydrolysis of the stage II protein

Titration of the aqueous solution of stage II protein with 0·01N-potassium hydroxide revealed that a small number of acidic groups was present; some of these may have been carboxyl groups and some may have been sulphonic acid groups introduced into aromatic rings (see below). The results of this direct titration were not particularly significant.

Table 4. *Bound* —COOH *groups found by titration in three different preparations of stage II protein, compared with the bound* —COOH *groups calculated from the amino* N *in stage I protein*

(Results expressed as number of —COOH groups per 100 N atoms.)

Preparation no.	No. of —COOH groups: Calc.	No. of —COOH groups: Found by titration
1	7·2	8·1
2	7·2	8·7
3	7·2	7·4

When an excess of potassium hydroxide was added, however, a number of additional acid groups were liberated. This was demonstrated by back titration after 1·5 hr. It was concluded that saponification of the ester bonds had occurred. Two additional observations supported this conclusion: the amount of alkali consumed in the saponification process was in good agreement with the theoretical amount calculated from the amino N results on stage I protein (see Tables 1 and 4) and the alkali treatment had converted an indiffusible substance into a product of which 86 % of the nitrogen was diffusible.

There remained the possibility that acetylation of the hydroxyl group of tyrosine had occurred during the acetic anhydride treatment of stage I protein. These *O*-acetyl groups would have been readily removed on treatment with excess of alkali, and the titration results would have been invalid. Herriott (1935) has shown that when the Folin colorimetric estimation of phenolic groups is carried out at pH 8 instead of the usual pH 11, *O*-acetyltyrosine does not react. This method was used to compare the apparent tyrosine content of stages I and II proteins. The reaction was found to be dependent on concentration, and so comparison of the two proteins was made at approximately the same nitrogen concentration. It was not possible to interpret these results on a quantitative basis, because it has been shown by Reitz *et al.* (1946) that sulphuric acid causes sulphonation of tyrosine residues in proteins and that tyrosinesulphonic acid has a lower chromogenic power than tyrosine itself in the Folin estimation. It was not known how many of the tyrosine residues in stage I protein had been sulphonated. The results given in Table 5 do show, however, that very few, if any, of the tyrosine hydroxyl groups had been acetylated by the acetic anhydride treatment.

Table 5. *Estimation of tyrosine* N *in stage I and stage II proteins by the Folin colorimetric method at* pH 8

(For conditions see Experimental section.)

Substance	Total protein N taken for analysis (mg.)	Tyrosine N (% of total protein N)
Stage I protein	0·445	2·72
	0·99	2·26
	1·335	2·05
Stage II protein	0·42	2·55
	0·84	2·24
	1·26	1·97

Serine and threonine analyses carried out on stage III acetyl peptide mixture showed that almost all of the serine and threonine were in the diffusible portion (see Table 6).

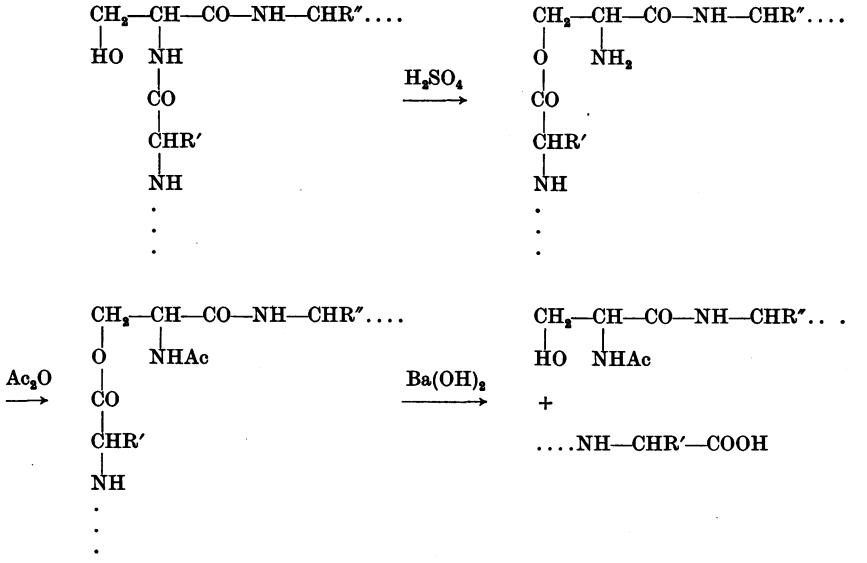
Table 6. *Distribution of serine* N *and threonine* N *in the diffusible and indiffusible fractions of stage III acetylpeptide mixture*

(Results expressed as percentages of the total N in the mixture taken for dialysis.)

	Serine N	Threonine N
Acetylpeptide mixture before dialysis	12·3	0·68
Indiffusible fraction	0·62	0·098
Diffusible fraction	11·7*	0·58*

* Value calculated by difference.

It is concluded that the degradation steps described in this paper can be formulated as shown below:



EXPERIMENTAL

Methods of analysis

Total nitrogen. This was determined by the Kjeldahl method.

Amino nitrogen. The Van Slyke method (reaction time 15 min.) was used for the manometric measurement of the nitrogen evolved on reacting the substance with HNO_2 in the Van Slyke-Neill apparatus. Several determinations were carried out on each sample. No increase in the reproducibility of the results occurred when the modified procedure of Kendrick & Hanke (1937) was used.

Alkali titrations. These were carried out in a N_2 atmosphere, using 0·01 N-KOH in methanol and bromothymol blue as indicator. To determine bound —COOH groups in stage II protein, excess of alkali was added to the protein solution and the solution back titrated after 1·5 hr.

Colorimetric estimation of phenolic groups at pH 8. This was carried out by the method of Herriott (1935) except that the colours were allowed to develop for 1 hr. at 40° before reading in the Hilger 'Spekker' photoelectric absorptiometer, using a visible light source and Ilford filter no. 608. These conditions gave more reproducible results than the 15 min. at 37° recommended by Herriott (1935). A standard reference curve was prepared for tyrosine under the same conditions.

Estimation of serine and threonine. These amino-acids were determined by the method of Rees (1946). Hydrolyses of stage III acetylpeptide mixture were carried out for 24 hr. at 115° in a sealed tube with 20 % (w/v) HCl. The hydrolysate was evaporated to dryness under reduced pressure, the residue was dissolved in water, brought to pH 6 with NaOH and made up to a suitable volume for Kjeldahl and periodate estimations.

Degradation of silk fibroin

Reaction with H_2SO_4. Commercially purified silk fibroin was washed successively with ether, ethanol, dilute acetic acid and water. It was dried for several weeks in a vacuum desiccator over NaOH, by which time the moisture content had fallen to about 5 %. The silk (2 g.) was quickly weighed and after teasing apart the fibres with a pair of tweezers it was added to 97·5 % (w/w) H_2SO_4 (40 ml.). The flask was securely stoppered to prevent entry of moisture and gently shaken by hand at 21° until the silk had dissolved. The solution was then allowed to stand at 21° for 72 hr. The solution, which was originally pale yellow, had become somewhat darker in colour, but the appearance did not suggest that appreciable charring had occurred. The solution was then cooled to − 30° and to it was added Na-dried ether (600 ml.) previously cooled to − 30° to − 40° and the mixture thoroughly shaken. The protein precipitate was centrifuged off and washed once by centrifugation with a second portion (600 ml.) of dry ether at room temperature. To the precipitate, which was still moist with ether, was then added about 150 g. of crushed ice and the mixture well stirred. A 10 % (w/v) solution of hydrated sodium acetate was then added very carefully, with stirring to avoid local excess, until the solution had pH 3–4; it was then warmed to room temperature. A considerable amount of the protein remained undissolved in the form of a sticky gum, which was dissolved by stirring in solid NaCl in small portions. The amount of NaCl added did not appear critical, no precipitation of protein being observed when excess was added. After a final adjustment of the pH if necessary, the solution was dialysed in cellophan against distilled water saturated with CO_2, until Cl^- and SO_4^{--} were removed. The volume of the solution in the dialysis bag was allowed to increase freely during the dialysis; this seemed to assist the separation of insoluble material. At the end of the dialysis the contents of the bag were removed and centrifuged at 2000 rev./min. for 5 min. The supernatant was a pale-yellow solution which foamed readily; the sticky brown insoluble material was rejected. The recovery of N after dialysis in this and the subsequent stages in the degradation process is given in Table 3; other analyses of this material are given in Tables 1 and 5. The soluble protein is referred to subsequently as stage I protein.

Acetylation at pH 5. An aqueous solution of stage I protein (385 ml.) prepared and purified as described above and containing 0·567 mg. N/ml. was mixed with 200 ml. 4 M-acetate buffer. The buffer was prepared by mixing 15 vol. of 4 M-acetic acid solution with 35 vol. of 4 M-sodium acetate solution; at this concentration the pH of the buffer was 5·2, but on dilution with the protein solution the pH fell to 5·0. Acetic anhydride (27 ml.) was then added and the mixture shaken for a few minutes until the solution was homogeneous; it was then allowed to stand for 1 hr. at room temperature. A further portion of acetic anhydride (27 ml.) was then added and the process repeated; the amino N had then fallen to a very low value (see Table 1). It was found that no loss of amino N occurred when the mixture of protein solution and buffer was allowed to stand without addition of acetic anhydride. The acetylated protein was then dialysed against distilled water until the acidity, as determined by titration with 0·01 N-KOH, had reached a constant value, which was generally equivalent to about 0·25 ml. of 0·01 N-KOH/mg. of protein N. This method of testing for the removal of the last traces of acetate was preferable to the lanthanum nitrate test which could only be applied to the much greater volume of liquid outside the dialysis membrane. The recovery of nitrogen in this dialysis experiment is given in Table 3. Analyses carried out on the dialysed protein solution are given in Tables 1, 4 and 5. This protein is subsequently called stage II protein.

Hydrolysis of stage II protein. A solution of the acetylated protein (935 ml.) containing 0·227 mg. N/ml. was mixed with 0·1 N-$Ba(OH)_2$ (25 ml.) in an atmosphere of N_2 and the solution allowed to stand 1·5 hr. at room temperature. An exact equivalent of 0·1 N-H_2SO_4 was then added and the $BaSO_4$ removed by centrifugation. The aqueous solution was concentrated *in vacuo* to 30 ml. A small amount of $BaSO_4$ which separated during the evaporation was removed by centrifugation. This contained only 0·1 mg. of N and was rejected. The amount of $Ba(OH)_2$ required for the hydrolysis was determined by titration with 0·01 N-KOH as described above; the amount actually used was a 50 % excess over that required for saponification of all the ester groups. Analyses given in Tables 3 and 6 were carried out on the concentrated solution of acetylpeptides. This mixture is referred to subsequently as stage III acetylpeptide mixture.

Experiments with fluoro-2:4-dinitrobenzene (FDNB)

Method of reacting stage I protein with FDNB at various pH *values.* In all experiments the stage I protein was in the form of solid material obtained by dry ether washing of the precipitate produced on addition of dry ether to the H_2SO_4 reaction mixture until the washings were free from SO_4^{--}. This material was therefore contaminated with the insoluble substance which separated during the dialysis procedure described above. Amino N determinations on protein prepared in this way gave rather erratic results. This may have been due to the presence of insoluble material. An average of many determinations gave approximately the same value as that found for the stage I protein after dialysis. About 25 mg. of the protein was mixed with 1 ml. of M-acetate buffer of the required pH. A solution of 0·2 ml. of FDNB in 1·8 ml. ethanol was then added and the mixture shaken vigorously for various times and at different temperatures. The contents of the reaction vessel were evaporated to dryness under reduced pressure at room temperature. The residue was hydrolysed for 24 hr. with 6 N-HCl and the 2:4-

dinitrophenyl (DNP)-amino-acids isolated as described by Porter (1950). The mixture of DNP-amino-acids was added to a silica-gel column and the DNP-serine band separated and estimated by the method described by Porter (1950). The remaining bands were very weak by comparison with the DNP-serine band and were ignored. The results of these experiments are given in Table 2. The amount of serine N available for reaction was calculated from the amino N results given in Table 1.

Another series of experiments were carried out in which 25 mg. of protein were shaken with 1 ml. pH 5 M-acetate buffer, 0·2 ml. of FDNB and 1·8 ml. of ethanol for a certain period of time, when a second addition of buffer and FDNB, equal to the previous amounts, was made and the shaking continued for a further period. In some experiments a third portion of the reagents was added and shaking continued. The results of these experiments need not be quoted as no improvement in the yield of serine N blocked by FDNB resulted. Variations in the amount of ethanol used also brought about no improvement.

Stage I protein (25 mg.) was reacted with FDNB according to the conditions of Exp. 2 (Table 2). The DNP-protein was hydrolysed for 4 hr. with 6 N-HCl and the resulting mixture of DNP-amino-acids separated in silica-gel columns. Apart from the band due to DNP-serine there was a weaker band which was identified as DNP-glycine by comparison with authentic material in several different solvent systems. The intensity of the DNP-glycine band was about 20% of that of the DNP-serine band; other bands were very much weaker and no attempt was made to identify them. After hydrolysis under the conditions used by Porter (1950) to identify DNP-proline, no band attributable to this substance was found.

End groups in silk fibroin. Silk fibroin (0·5 g.) was cut into small pieces and shaken with a solution of $NaHCO_3$ (1 g.) in water (20 ml.), ethanol (40 ml.) and FDNB (0·33 ml.) for 24 hr. After addition of 0·33 ml. of FDNB, shaking was continued for another 48 hr. A third portion (0·33 ml.) of FDNB was then added and the shaking continued until the total reaction time was 6 days. The DNP-protein was hydrolysed for 24 hr. with 6 N-HCl and the DNP-amino-acids were examined. The mixture was very complex and no attempt was made to identify all the bands. The presence of DNP-serine and DNP-alanine was definitely established by comparing the *R* values of the bands in several solvents with the *R* values of authentic material. In a separate experiment, in which the DNP-protein was hydrolysed 4 hr. with 6 N-HCl, DNP-glycine was identified with certainty. No attempt was made to carry out these experiments on a quantitative basis. Similar results were obtained when soluble silk (Coleman & Howitt, 1947) was used.

Attempted partial hydrolysis of DNP-stage I protein. The DNP-protein was prepared under the conditions of Exp. 2 (Table 2). It was found to be completely decomposed on standing at room temperature with 0·1 N-NaOH. No DNP-peptides were produced, neither were any DNP-amino-acids found when the reaction mixture was subjected to HCl hydrolysis. When the DNP-protein was refluxed 20 min. with N-HCl and the solution extracted exhaustively with ethyl acetate a mixture of DNP-peptides was obtained. By means of silica-gel columns with $CHCl_3$ containing various amounts of butanol as the moving phase, the mixture was separated into at least eight DNP-peptides. These were separately hydrolysed. The DNP-amino-acid forming the end group was found to be DNP-serine in every case. Glycine and alanine were also found in every peptide; no other amino-acid was found. This is in agreement with recent data obtained by Drucker & Smith (1950) on the localization of serine, glycine and alanine in silk fibroin. Some preliminary experiments on methanolysis of the *O*-peptidyl bonds in the DNP-protein by treatment with methanolic HCl were not as successful as the partial acid hydrolysis.

Experiments with compounds of known structure

Rearrangement of DL-N-*benzoyl*allo*threonine ethyl ester with ethanolic hydrogen chloride.* 1·25 g. of the ester were refluxed 1·25 hr. with 5 ml. ethanol containing 10% (w/v) HCl. The mixture was then evaporated to dryness under reduced pressure, the residue dissolved in 10 ml. of M-acetate buffer (pH 5) and extracted with ether. The aqueous layer was made alkaline with $NaHCO_3$ and again extracted with ether. The first ether extract should have contained the weakly basic oxazoline, if present, and the second contained the *O*-benzoyl ester. Both extracts gave the same picrate on addition of ethereal picric acid; this proved to be the picrate of DL-O-*benzoyl*allo*threonine ethyl ester*, m.p. 170–171° (Found: N (Dumas), 11·6. $C_{19}H_{20}O_{11}N_4$ requires N, 11·7%) which was not depressed on admixture with an authentic sample of the substance (m.p. 169–170°: Found: N, 11·4%). This was prepared from DL-*cis*-4-carbethoxy-2-phenyl-5-methyl-Δ^2-oxazoline (Elliott, 1949) by treatment with excess of cold dil. HCl for several hours at room temperature followed by evaporation to remove excess HCl, addition of saturated sodium picrate solution and crystallization of the precipitate from ethanol. The picrate of the *cis*-ozaxoline, already mentioned, has m.p. 148–150°, that of the corresponding *trans*-oxazoline has m.p. 128–129° and that of DL-*O*-benzoylthreonine ethyl ester has m.p. 140·5° (Elliott, 1949). The structure assigned to the picrate having m.p. 170–171° is therefore probably correct.

Acetylation of DL-O-*benzoylserine at* pH 5. The amino-acid (50 mg.) was shaken with 8 ml. of M-acetate buffer (pH 5) and acetic anhydride (1 ml.). The solution became homogeneous in 1 min. The solution was allowed to stand 1 hr. at room temperature and N-HCl (5·6 ml.) was then added. The solution was evaporated to dryness and the residue extracted with ethanol. DL-*O*-Benzoyl-*N*-acetylserine (32 mg.) separated from the extracts on cooling. It had m.p. 191°. Synge (1939) gives m.p. 192–194°. (Found: C, 57·9; H, 6·0; N, 5·8. Calc. for $C_{12}H_{13}O_5N$: C, 57·4; H, 5·2; N, 5·6%.)

SUMMARY

1. Treatment of silk fibroin with concentrated sulphuric acid at 21° brought about a transfer of approximately 60% of the peptide chains linked to the nitrogen atoms of serine residues to the hydroxyl groups of these residues.

2. The resulting material was acetylated at pH 5 and then treated with excess of dilute alkali which caused hydrolysis of the *O*-peptidyl bonds and yielded a mixture of acetylseryl peptides.

3. Non-specific hydrolysis of peptide bonds may have occurred simultaneously with the rearrangement during the sulphuric acid treatment, but only to a relatively small extent.

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REFERENCES

Bergmann, M., Brand, E. & Dreyer, F. (1921). *Ber. dtsch. chem. Ges.* **54**, 936.
Bergmann, M., Brand, E. & Weinmann, F. (1923). *Hoppe-Seyl. Z.* **131**, 1.
Bergmann, M. & Miekeley, A. (1924). *Hoppe-Seyl. Z.* **140**, 128.
Bergmann, M., Miekeley, A. & Kann, E. (1925). *Hoppe-Seyl. Z.* **146**, 247.
Blackburn, S., Middlebrook, W. R. & Phillips, M. (1942). *Nature, Lond.*, **150**, 57.
Chibnall, A. C. (1942). *Proc. roy. Soc.* B, **131**, 136.
Coleman, D. & Howitt, F. O. (1947). *Proc. roy. Soc.* A, **190**, 145.
Desnuelle, P. & Casal, A. (1948). *Biochem. Biophys. Acta*, **2**, 64.
Drucker, B. & Smith, S. G. (1950). *Nature, Lond.*, **165**, 196.
Elliott, D. F. (1948). *J. chem. Soc.* p. 310.
Elliott, D. F. (1949). *J. chem. Soc.* p. 589.
Elliott, D. F. (1950). *J. chem Soc.* p. 62.
Glendenning, M. B., Greensberg, D. M. & Fraenkel-Conrat, H. (1947). *J. biol. Chem.* **167**, 125.
Gordon, A. H., Martin, A. J. P. & Synge, R. L. M. (1943). *Biochem. J.* **37**, 538.
Herriott, R. M. (1935). *J. gen. Physiol.* **19**, 283.
Kendrick, A. B. & Hanke, M. E. (1937). *J. biol. Chem.* **117**, 161.
Phillips, A. P. & Baltzly, R. (1947). *J. Amer. chem. Soc.* **69**, 200.
Porter, R. R. (1950). In *Methods in Medical Research*, ed. by R. W. Gerard, Vol. 3, p. 256. Chicago: The Year Book Publishers.
Rees, M. W. (1946). *Biochem. J.* **40**, 632.
Reitz, H. G., Ferrel, R. E., Olcott, H. S. & Fraenkel-Conrat, H. (1946). *J. Amer. chem. Soc.* **68**, 1024.
Synge, R. L. M. (1939). *Biochem. J.* **33**, 1924.
Traill, D. (1950). *Chem. & Industr.* p. 23.
Welsh, L. H. (1947). *J. Amer. chem. Soc.* **68**, 128.
Welsh, L. H. (1949). *J. Amer. chem. Soc.* **71**, 3500.

Studies in Carotenogenesis

3. IDENTIFICATION OF THE MINOR POLYENE COMPONENTS OF THE FUNGUS *PHYCOMYCES BLAKESLEEANUS* AND A STUDY OF THEIR SYNTHESIS UNDER VARIOUS CULTURAL CONDITIONS

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Zechmeister & Sandoval (1945, 1946) described phytofluene, a colourless polyene* showing a bluish-green fluorescence in ultraviolet light, which accompanies carotenoids* in the parts of higher plants, such as fruit and flower petals, which contain little, if any, chlorophyll. It has also been shown to occur in three classes of fungi: in the Schizomycete *Rhodotorula rubra* (Bonner, Sandoval, Tang & Zechmeister, 1946), in the Ascomycete *Neurospora crassa* (Haxo, 1949) and in the Basidiomycete *Cantharellus cinnabarinus* (Haxo, 1951). Phytofluene is more saturated than the carotenoids, containing only seven ethylenic bonds, five of which are conjugated. Porter & Lincoln (1950) consider that it is probably dodecahydrolycopene. If, as first suggested by Bonner *et al.* (1946), phytofluene is an intermediate in the biogenesis of carotenoids, it is of interest to see if it is produced by *Phycomyces blakesleeanus*, the fungus at present in use in this laboratory to study carotenogenesis. The possibility that polyenes, such as phytoene (Porter & Lincoln, 1950), which are even more saturated than phytofluene, also occur has been investigated.

In addition to the already well established occurrence in *Phycomyces* of large amounts of β-carotene and traces of α-carotene (Schopfer, 1935; Karrer & Krause-Voith, 1948; Bernhard & Albrecht, 1948; Garton, Goodwin & Lijinsky, 1951), the present investigation has shown that very small amounts of four other carotenes also occur; Bernhard & Albrecht (1948) had previously stated that traces of lycopene were present and whilst the work now reported was in progress Schopfer & Grob (1950) stated that five carotenoids exist in their strain of *Phycomyces*, but they did not discuss identification.

As colourless polyenes were found in *Phycomyces*, it was considered important to determine whether

* In this paper the term polyene refers to all the C_{40} compounds discussed, whilst the term carotenoid is used when reference is made to the coloured members of the series.