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CHARACTERIZATION OF A NEW TYPE OF CROSS-LINKAGE IN RESILIN, A RUBBER-LIKE PROTEIN

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SUMMARY

Native resilin as found in elastic ligaments in insects shows a bright blue fluorescence due to two compounds which are firmly connected to the protein, but which can be liberated by prolonged hydrolysis with acid or alkali. The two compounds have characteristic ultraviolet-absorption spectra with maximum absorption in acid solution at 286 and 283 mμ, respectively, and at 322 and 317 mμ in alkaline solution. This shift in the absorption maxima occurs for both compounds near neutrality and it is accompanied by an increased fluorescence. It is assumed that the group responsible for this shift is of phenolic nature.

Spot tests indicate that both compounds are aromatic α-amino acids containing a phenolic group; tests for diphenols and for an imidic nucleus are negative. One of the compounds is shown to be a diaminodicarboxylic acid and the other a triaminotricarboxylic acid. In the native protein the amino groups of the compounds do not react with dinitrofluorobenzene, indicating that they are built into the peptide structure, in this way linking the chains in a 3-dimensional network. The degree of cross-linking calculated from the amounts of these compounds present in resilin agrees well with the estimates from physical measurements.

INTRODUCTION

In 1960 WEIS-FOGH[1] described a new type of structural protein, resilin, which occurs in a pure state in some cuticular structures in insects. This protein has some unusual properties in that it shows long-range rubber-like elasticity and no lasting deformation even after considerable stretching for long periods of time. It is optically isotropic in the unstrained state but becomes birefringent on deformation. Although it is highly hygroscopic it is completely insoluble in water even at 140°, and agents such as 6 M urea with or without thioglycolic acid, alkaline thioglycolate, performic acid, and alkaline cupric ethylenediamine do not cause any solubilization of the material. This indicates that the insolubility is due neither to hydrogen bonds nor to disulphide bridges. Further studies by WEIS-FOGH[2,3] have shown that all the properties of resilin are consistent with the view that it is built from randomly coiled peptide chains, linked together in a stable three-dimensional network by a few covalent links of an unknown nature. Amino acid analysis showed that resilin has

Abbreviation: FDNB, dinitrofluorobenzene.

an unique composition[4], but failed to give evidence for the presence of any unusual compounds which could function as cross-linkages between the peptide chains.

In view of the fact that very few types of cross-linkages are known in proteins, we thought it rewarding to study the chemical nature of the cross-linking groups. In this paper two new amino acids will be described which have been isolated in small amounts from hydrolysates of resilin. They are built into the peptide chains of the protein, they have a structure well suited for linking the chains together, and the amounts are consistent with physical estimates of the degree of cross-linking.

MATERIALS AND METHODS

The main source of resilin for this investigation were the two elastic ligaments[1] found in the desert locust *Schistocerca gregaria* (Forskål), namely the prealar arm, which consists only of resilin and chitin, and the wing-hinge ligament part of which contains a second, fibrous protein. The latter part was cut off and discarded. Ligaments from deep-frozen locusts were dissected out under 70 % ethanol, carefully separated from all adhering tissue and stored in 96 % ethanol at 4° until required. The isolated ligaments were hydrolysed in a sealed tube with 0.1 N HCl at 100° for 6 h to dissolve the resilin without affecting the chitin present. The supernatant was then fully hydrolysed with 6 N HCl at 120° for 20 h and thereafter taken to dryness over solid NaOH at room temperature *in vacuo*.

Descending paper chromatography was performed on Whatman No. 1 paper after 1 h equilibration, using *n*-butanol–acetic acid–water (4:1:1, v/v/v) or isopropanol–concentrated ammonia–water (8:1:1, v/v/v) as solvents. A 0.2 % solution of ninhydrin in acetone was used as spray reagent to locate the amino acids, alone or after dipping the chromatogram in methanolic cupric nitrate solution whereby α-amino acids can be distinguished from other amines[5].

Folin's phosphomolybdic acid reagent was used after diluting 1:20 with water, whereafter the wet chromatogram was hung in an ammonia atmosphere for development of the colour. Other reagents tried were: Millon's reagent, the α-nitroso-β-naphthol reagent for tyrosine, diazotized sulphanilic acid, Ehrlich's reagent for indoles, ferric chloride, and ammoniacal silver nitrate, all prepared according to standard methods[6].

The ultraviolet absorption spectra were measured with a "Zeiss"-spectrophotometer (type PMQ II) using a hydrogen lamp as light source. The fluorescence activation spectra were measured with the same apparatus after placing a glass filter (Chance OY 10) behind the cuvette to cut off the activating light while transmitting the fluorescent light. Distilled water was used as blank. The emission spectra were determined by placing the cuvette containing the sample between the monochromator and the light source, which in this case was a low-pressure mercury lamp with maximum emission at 253.7 mμ. A solution of 19.2 g $CoSO_4 \cdot 7\,H_2O$ and 49.2 g $NiSO_4 \cdot 7\,H_2O$ in 100 ml water in a quartz cuvette of 1-cm light path was placed between the lamp and the sample to cut off the visible light. The fluorescence of pieces of native resilin was measured in the same manner except that the pieces were placed in a thin layer of water between foils of polyethylene.

These methods are not ideal for fluorescence studies, and the activation and emission spectra of the isolated compounds in solution were therefore checked in

a Farrand spectrofluorometer equipped with a 150-W Hannovia xenon lamp. These control measurements confirmed the results obtained in the spectrophotometer which were found to be adequate for qualitative purposes.

Ion-exchange chromatography was performed with water-jacketed columns, 10 mm in inner diameter and 50 cm in length. Fractions of 1.5 ml were collected at a rate of 8–10 ml/h, and the ultraviolet-absorption of the fractions was measured in microcuvettes containing 0.5 ml, light path 2 cm.

Titrations were performed with a titrator, type TIIIb, from Radiometer, Copenhagen, the instrument being equipped with a semi-micro glass electrode, type G 222 C. The titration vessel was placed in a thermo-regulated bath with magnetic stirring. In some of the experiments CO_2 was excluded by bubbling CO_2-free air through the solution during the titration, but the exclusion of CO_2 had no effect and hence the absorption of CO_2 from the atmosphere must be negligible during the titration.

Before titration the isolated compounds were desalted as described later and the resulting solutions were freed from NH_3 and CO_2 by evaporation over concentrated H_2SO_4 after treatment with a small amount of dilute KOH, followed by acidification with dilute HCl and evaporation over solid NaOH. The residue was then dissolved in 0.5 ml 0.1 M KCl (CO_2-free), transferred to the titration vessel and titrated with 0.4 M KOH delivered from a microburette with a total volume of 0.2 ml. For comparison 0.5 ml of the KCl solution made slightly acid with HCl was titrated in a similar manner, and the results of this titration were used for correcting the titration value of the compound in question. From the corrected titration curve the amounts of the different groups titrated, and their apparent pK-values, were estimated, and from these values a theoretical titration curve was calculated and compared with the experimental curve.

After a titration a known fraction of the solution was withdrawn, diluted to 3 ml, and its ultraviolet-absorption was measured.

Paper electrophoresis was performed with a LKB-apparatus, type 3276. A voltage of 220 V was used and in the experiments with the unsubstituted compounds the ionic strength of the buffer solutions was chosen to give a current of 5 mA, except at extreme pH-values where larger currents were inevitable. The buffers used were: dilute HCl, citric acid–sodium phosphate mixtures, and sodium bicarbonate–sodium carbonate mixtures. Caffein was used as marker to indicate the electro-osmotic flow.

Partial dinitrophenylation was performed according to SILAEV *et al.*[7]. A spot of of a desalted solution of the compound in question was placed on a strip of electrophoresis paper, and treated, when dry, with 25 μl of a solution of FDNB in acetone (25 μl FDNB/ml acetone). The paper was incubated at 37° for 25 min. The reaction products were separated by electrophoresis in a mixture of 85 % formic acid – acetic acid – water (25 : 20 : 52) at 220 V for 6–7 h. The DNP-derivatives and the unmodified fluorescent compounds were localized by scanning the strips in the chromatography attachment of the spectrophotometer.

Dinitrophenylated resilin was prepared by treating whole ligaments with 70 % ethanol saturated with FDNB and $NaHCO_3$ for 6 h in the dark. The orange-coloured ligaments were washed repeatedly with 70 % ethanol and with distilled water until no more coloured material could be eluted. The DNP-resilin was hydrolysed in the same manner as untreated resilin, and after 6-fold dilution with water the hydrolysate

was extracted with ethyl acetate and the extracted material chromatographed two-dimensionally as described by LEVY[8]. The water phase was chromatographed in one direction with butanol–acetic acid as solvent.

The methoxycarbonyl derivatives were prepared according to the method of BAILEY[9].

RESULTS

Compounds I and II

Resilin *in situ*, isolated resilin both before and after boiling in water, as well as hydrolysates of resilin, all show a bright blue fluorescence in ultraviolet-light. When a hydrolysate is subjected to descending paper-chromatography in the solvent system butanol–acetic acid–water (4:1:1) the fluorescence is apparent in two spots with R_F-values of 0.05 (Compound I) and 0.18 (Compound II). Complete separation from all other amino acids can be obtained by first developing the chromatogram in isopropanol–concentrated ammonia–water (8:1:1), in which solvent neither fluorescent compound migrates, and then redeveloping in the same direction in the butanol–acetic acid solvent.

The fluorescence is rather weak in the presence of acetic acid but it becomes brilliant after treatment of the paper with ammonia vapour. Both compounds give a purple colour with ninhydrin but the reaction is negative if the paper is treated with cupric nitrate before spraying with ninhydrin, indicating that they are α-amino acids. Both compounds give a brownish-red colour with diazotized sulphanilic acid and with α-nitroso-β-naphthol. With Millon's reagent they give a red colour and with Folin's reagent for phenols they give a blue colour. No colour-formation could be observed after treatment with ammoniacal silver nitrate, ferric chloride, and *p*-dimethylaminobenzaldehyde. It is therefore concluded that both compounds react as mono-phenols and that they do not contain an indole nucleus.

Quantitative isolation

For further studies of the fluorescent compounds larger amounts of the pure substances were required for which purpose ion-exchange chromatography was used. Dowex resins were found to be unsuited due to strong adsorption of the two compounds: compound I could not be eluted from Dowex-50, even with 0.2 N NaOH.

Complete separation of the two compounds from each other and from other amino acids was obtained by means of DEAE-cellulose. The resilin hydrolysate was brought to pH 8.8 and run onto a column of DEAE-cellulose equilibrated with 0.02 M Na_2HPO_4. The salt- and pH-gradient for elution was established by mixing 0.06 M NaH_2PO_4 with 0.02 M Na_2HPO_4 contained in a reservoir (300 ml). Fig. 1 shows that compound II is eluted soon after the tyrosine peak but before there has been any change in the pH of the eluate, and that compound I accompanies a sharp drop in pH, when the buffer capacity of the DEAE-cellulose has been exhausted. Paper-chromatography of samples of the two fluorescent peaks shows that neither compound is contaminated with other amino acids.

Solutions of the two compounds can be desalted by adsorbing them from acid solution (pH 2) onto a small column of cellulose phosphate (ammonium form). After washing the column with distilled water the compounds are eluted with dilute ammonia. The fluorescent portion of the eluate is located in ultraviolet light and evaporated over concentrated H_2SO_4.

Optical properties

The ultraviolet absorption spectra of the compounds are shown in Figs. 2 and 3. The spectra are rather similar to that of tyrosine but small yet conspicuous differences are present. The absorption maximum in acid solution is found to be at 286 mμ and 283 mμ for compounds I and II respectively, compared with 275 mμ for tyrosine. In alkaline solution the maxima are shifted to 322 mμ, 317 mμ and 293 mμ respectively. With all three compounds the absorption at the maximum is increased in going from acid to alkaline reaction, for tyrosine the increase is by a factor of 1.8, for compound I by 1.1, and for compound II by 1.4.

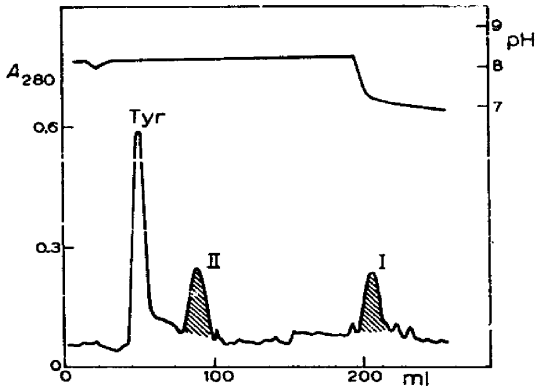

Fig. 1. Fractionation of resilin hydrolysate on DEAE-cellulose in equilibrium with 0.02 M Na_2HPO_4. The elution was performed at room temperature and the gradient for elution was established by running 0.06 M NaH_2PO_4 into 300 ml 0.02 M Na_2HPO_4. The fluorescence is indicated by cross-hatching. The upper curve shows the pH in the effluent.

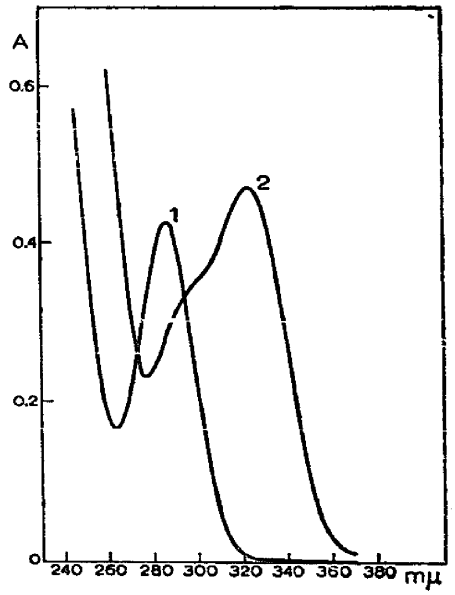

Fig. 2. Ultraviolet absorption spectra for compound I in (1) acid solution and (2) alkaline solution.

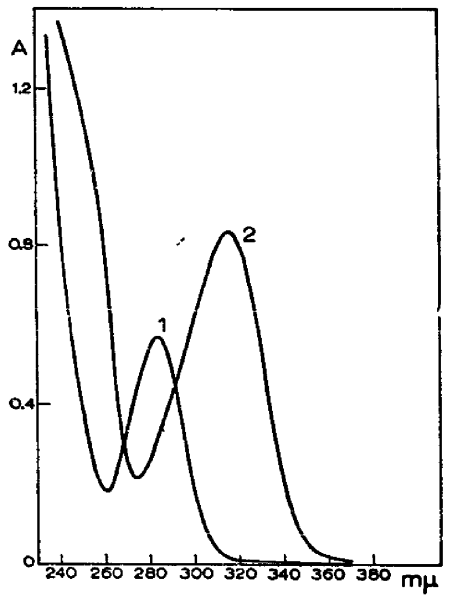

Fig. 3. Ultraviolet absorption spectra for compound II in (1) acid solution and (2) alkaline solution.

The pH at which this bathochromic shift occurs is different for all three compounds. For tyrosine it occurs at about pH 10 and for compounds I and II at pH 6.3 and 7.2 respectively, as illustrated in Fig. 4.

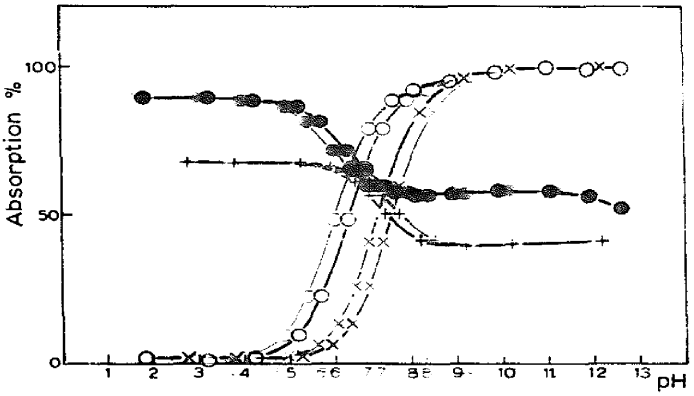

Fig. 4. The pH-dependence of the ultraviolet absorption of compounds I and II at two wavelengths. ●—●, compound I at 285 mμ; ○—○, compound I at 325 mμ; +—+, compound II at 285 mμ; ×—×, compound II at 315 mμ.

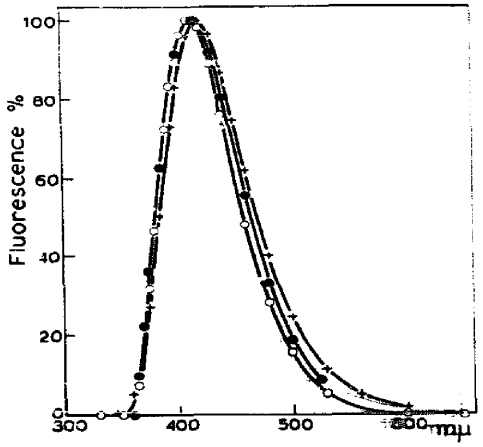

Fig. 5. The spectra of the fluorescent light from compounds I and II and from native resilin, all at alkaline reaction. Activation at 253.7 mμ. ●—●, compound I; ○—○, compound II; +—+, native resilin.

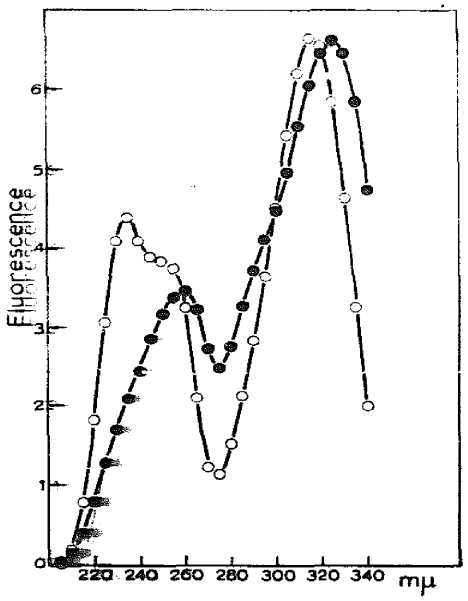

Fig. 6. The activation spectra for compounds I and II. ●—●, compound I at pH 8; ○—○, compound II at pH 11. The spectra are not corrected with respect to the different light intensity of the hydrogen lamp at different wavelengths.

The spectra of the fluorescent light emitted from alkaline solutions of compounds I and II and from native resilin are shown in Fig. 5. The maximum is near 415 mμ, and the differences between the three curves are so small that they cannot be considered significant. In acid solution the fluorescence is much weaker but the spectra of the fluorescent light are identical with those in alkaline solution.

The activation spectra in alkaline solution for the two compounds are shown in Fig. 6. As the measurements have not been corrected with respect to the changes in light intensity with wavelength, the values found at the shorter wavelengths are too small compared with those at the longer wavelengths. The activation maxima are identical with the absorption maxima both in acid and alkaline solution. Figs. 7 and 8 show the pH-dependence of the fluorescence intensity of compounds I and II together with that of the methoxycarbonyl derivatives. Both unmodified compounds,

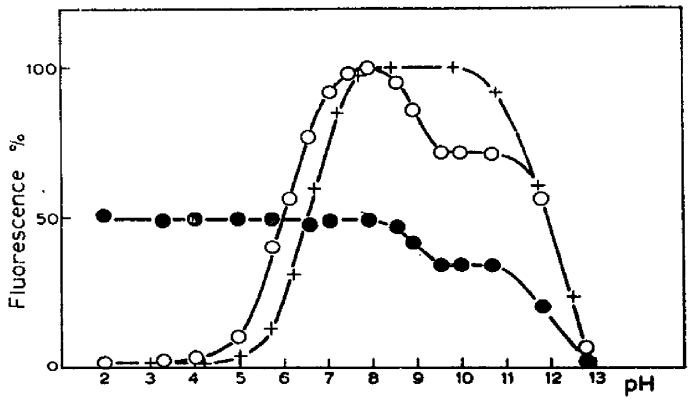

Fig. 7. The pH-dependence of the fluorescence of compound I activated at 285 mμ (● — ●) and at 325 mμ (O — O) and of the methoxycarbonyl derivative of compound I activated at 325 mμ (+ — +).

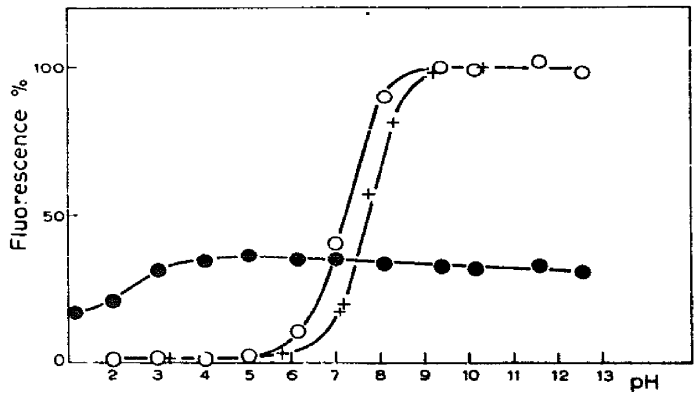

Fig. 8. The pH-dependence of the fluorescence of compound II activated at 285 mμ (● — ●) and at 315 mμ (O — O) and of the methoxycarbonyl derivative of compound II activated at 315 mμ (+ — +).

when activated at about 320 mμ, show an increase in fluorescence at the same pH-values where the change in ultraviolet absorption is found, but only compound I shows decreasing fluorescence at higher pH-values. The decrease found near pH 9 must be due to the amino groups as it can be abolished by reacting the compound with methoxycarbonyl chloride. Another difference in the fluorescent behaviour of the compounds is that compound II shows a decrease in fluorescence between pH 2 and 3 when activated at 285 mμ. This decrease may be connected with the dissociation of a carboxylic group.

Electrophoresis

Fig. 9 shows how the electrophoretic migration of the fluorescent compounds depends on pH. The isoelectric point of both compounds is between pH 4.5 and 5.0, and in both cases the dissociation occurs in three steps. As spot tests indicate that the compounds are α-amino acids, the dissociation occurring about pH 2.5 should be due to carboxylic groups and the dissociation occurring near pH 9 should be due to α-amino groups. The dissociation seen near neutrality must be due to the groups which are responsible for the changes both in ultraviolet absorption and in fluorescence.

A significant fact emerging from Fig. 9 is that the changes in the speed of migration are appreciably larger at the first and third steps in the dissociations than at the step near neutrality. This indicates that in these compounds there are present more carboxylic and amino groups than groups dissociating near neutrality, a view which is confirmed by the titration experiments.

Titration

Compound II which is the more abundant of the two compounds was titrated at three different temperatures. The results were basically alike but since the second and the third step in the dissociation were separated best at low temperatures, only the results of the titration at 0° are shown by the circles in Fig. 10. The fully drawn curve is the calculated titration curve, it being assumed that for each group dissociating at pH 7.3 the compound contains two groups dissociating at pH 2.5 and two groups dissociating at pH 9.9. The titrations at the higher temperatures gave the same ratio between the three groups, and further purification of the compound did not influence the results. The conclusion can be drawn that compound II contains twice as many carboxylic groups and amino groups as groups with dissociation at pH 7.3.

The determination of the number of carboxylic groups cannot be considered very precise due to less accurate pH-determinations below pH 2. In order to check the

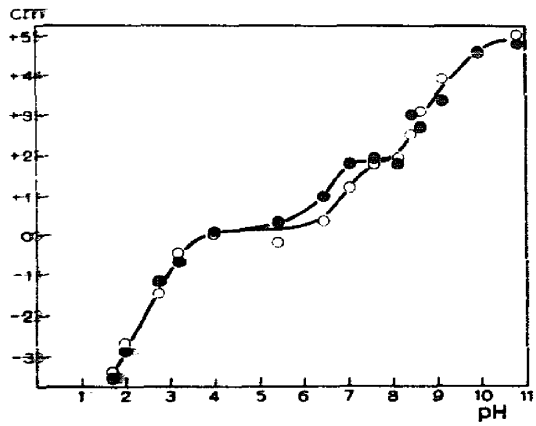

Fig. 9. Electrophoretic migration on paper of compound I (●—●) and of compound II (○—○). The vertical axis shows the distance travelled in two hours after correction for electro-osmosis.

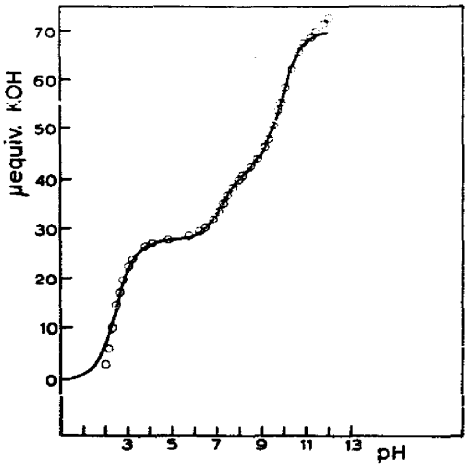

Fig. 10. Compound II titrated at 0°. The circles are the measured values corrected for the titration of water. The fully drawn curve is calculated on the assumption that there are present 28, 14, and 28 μequiv. of groups with pK'-values of 2.5, 7.3, and 9.9, respectively.

determination of these groups a sample of methoxycarbonyl compound III was prepared and titrated. The pK'-value of the carboxylic groups of this derivative was found to be 3.7, a shift of this order of magnitude being expected when a neighbouring amino group becomes blocked. In two independent determinations the ratio of the number of carboxylic groups to that of groups titrated near neutrality was 1.8 and 2.0, in accordance with the above results.

Only a single titration of compound I has been performed so far because the amount of material was too small to make accurate determinations possible. The result is seen in Fig. 11 which indicates that in this compound three amino groups and three carboxylic groups are present for each group with dissociation at pH 6.2. This interpretation was confirmed by experiments with partial dinitrophenylation.

In the titrations of both compounds deviations can be seen above pH 10 between the experimental and the calculated values, but it has not been investigated whether this is due to experimental inaccuracy or to some unknown group.

Partial dinitrophenylation

The principle of the method is that the amino compound in its alkaline form is treated on paper with FDNB in the absence of buffer[7]. The hydrogen fluoride evolved will stop the reaction before it is completed and the resulting mixture of substitution products can be separated by paper electrophoresis in a strongly acid solvent. Fig. 12 shows that the reaction mixture from the treatment of compound III with FDNB can be separated into two yellow and one fluorescent band, whereas compound II gives three yellow and one fluorescent band. The slowest moving DNP-derivative

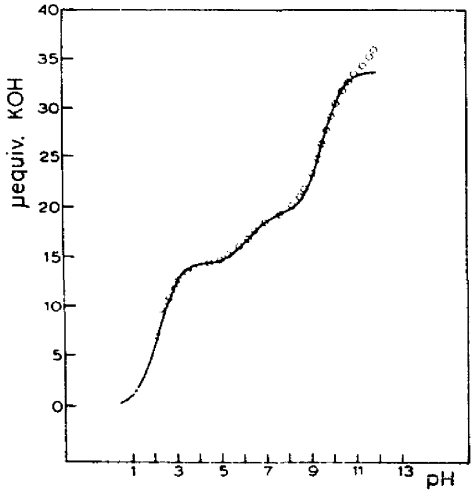

Fig. 11. Compound I titrated at 23°. The circles are the measured values corrected for the titration of water. The fully drawn curve is calculated on the assumption that there are present 14.4, 4.8, and 14.4 μequiv. of groups with pK'-values of 2.2, 6.2, and 9.6, respectively.

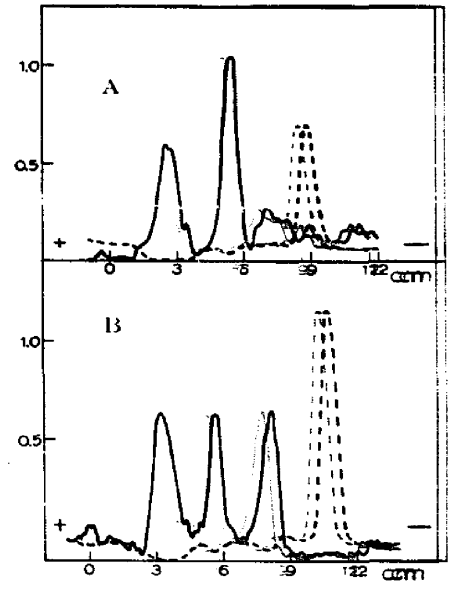

Fig. 12. Paper electrophoretic separation of the reaction mixtures from partial dinitrophenylation of compound II (A) and of compound I (B). The fully drawn lines show the light absorption at 420 mμ, and the broken lines show the fluorescence when activation is performed at 285 mμ.

was not separated from dinitrophenol but could, nevertheless, be distinguished from this substance by its yellow colour at acid reaction and by its positive reaction with Folin's phenol reagent. In contrast to the other yellow bands this one gave no reaction with ninhydrin, indicating that all its amino groups had reacted with FDNB.

The number of reaction products found in the two cases shows that compound I contains three amino groups per molecule and that compound II contains two. The number of carboxylic groups cannot be determined by this method but since the isoelectric point is near pH 5 for both compounds the number of carboxylic groups must equal the number of amino groups.

Quantitative determinations

The results of the titrations together with the partial dinitrophenylations show that both fluorescent compounds contain only one group per molecule dissociating near neutrality, and therefore the molar absorption at the wavelength with maximum absorption can be found. The value found for compound I is 8000, for compound II it is 5400, and for the methoxycarbonyl derivative of compound II it is 5300.

The molar absorption coefficients make it possible to determine the number of residues of the two compounds in resilin and to calculate the average chain length between two cross-linkages. This calculation is based on the assumption that these compounds are the cross-linking agents and that compound I links three peptide chains together and that compound II links two chains together. The results of three determinations are given in Table I.

TABLE I

NUMBER OF RESIDUES OF COMPOUNDS I AND II IN LOCUST RESILIN AND THE CALCULATED AVERAGE MOLECULAR WEIGHT OF PEPTIDE CHAINS BETWEEN TWO NEIGHBOURING CROSS-LINKAGES

Material	*Amount of resilin*	*Residues of compound I in 10^5 g resilin*	*Residues of compound II in 10^5 g resilin*	*Chain weight*
Prealar arm	6.98 mg*	4.2	9.2	3200
Wing hinge	2.78 mg	6.1	8.8	2800
Wing hinge	7.71 mg	5.1	9.3	3000

* In this determination the amount of resilin was calculated on the assumption that the prealar arms contain 76% resilin[12].

Treatment of whole ligaments with FDNB

The structures of the two compounds are ideally suited to serve as cross-linkages between the peptide chains in the native protein, and the amino groups should then be involved in peptide linkages and unable to react with FDNB. 16 mg of resilin was therefore treated with FDNB, hydrolysed with HCl, and the hydrolysate was repeatedly extracted with ethyl acetate whereafter both the organic phase and the water phase were subjected to paper chromatography. The chromatograms of the organic phase showed, besides the spots corresponding to dinitrophenol and dinitroaniline, two distinct spots of equal size corresponding to DNP-serine and DNP-glutamic acid (or DNP-aspartic acid) and a barely visible spot corresponding to DNP-alanine. Furthermore, very close to the origin a small yellow spot was present,

the identity of which could not be established. If it represents some reaction product of one of the fluorescent amino acids, it can only correspond to an insignificant fraction of the total amount.

The chromatograms of the water phase showed only one visible spot, corresponding to ε-DNP-lysine. When the chromatograms had hung for a day or more two brown spots became visible between the origin and the ε-DNP-lysine. Measurements of these regions of the paper before the brown colour had developed showed absorption spectra resembling that of *O*-DNP-tyrosine with absorption maximum near 310 mμ. When the water phase was subjected to paper electrophoresis in the formic acid–acetic acid mixture the compounds giving rise to the brown colour migrated faster towards the cathode than ε-DNP-lysine, *i.e.* they appear to carry more positive charges. This makes it probable that they are *O*-DNP-derivatives of the fluorescent compounds, but this possibility was not further investigated, the main point being that no spots were found which could correspond to any significant amount of *N*-DNP-derivatives of these compounds.

Enzymic digestion

A few experiments have been performed in which resilin was hydrolysed by means of subtilisin B, a bacterial proteinase obtained from the Novo Pharmaceutical Company, Copenhagen. Rather small peptides should be expected as end products due to the broad specificity of this enzyme. At intervals samples were drawn from the digestion mixture and the ultraviolet absorption measured (Fig. 13). As the digestion was performed at pH 8 the fluorescent compounds are mostly present in their alkaline form with absorption maximum near 320 mμ, and it is evident that the solubilization of these compounds goes parallel with that of tyrosine. Paper chromatography of

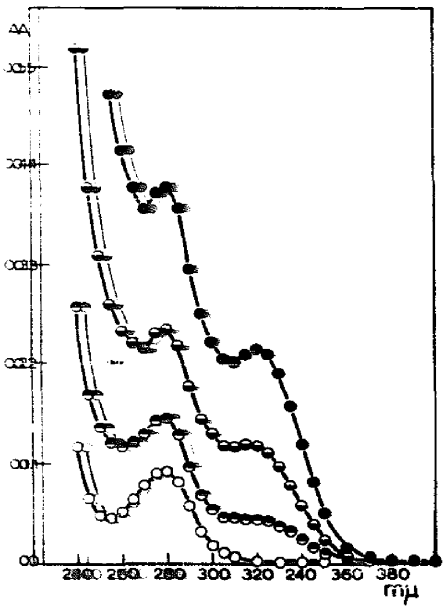

Fig. 13. Solubilization of resilin when treated with subtilisin B at room temperature. Ultraviolet absorption spectra of the supernatant after 0 h, (○ — ○), after 1 h (◓ — ◓), after 3 h (◒ — ◒) and after 19 h (● — ●).

such digests shows that the fluorescent compounds are not free but combined with peptide material. The isolation of such fluorescent peptides in a pure state has not been achieved as yet, but work in this direction is in progress.

DISCUSSION

The possibility that the two fluorescent compounds are artefacts can be ruled out for a number of reasons. (a) Whole untreated ligaments show the same fluorescence as the isolated compounds and (b) the pH-dependence of the fluorescence is the same in the two cases. It has not been possible to determine the absorption spectrum of native resilin, but (c) an enzymic digest of resilin has an absorption spectrum which can be considered the summation of the spectra of the fluorescent compounds and that of tyrosine. (d) Alkaline hydrolysis of resilin (1 N KOH at 100° for 4 h) liberates two fluorescent compounds which in all respects seem to be identical with the two compounds liberated by acid hydrolysis. It is therefore reasonable to conclude that these compounds exist as such in the native protein.

Of the amino acids usually present in proteins only phenylalanine, tyrosine, and tryptophan fluoresce in ultraviolet light, and in contrast to compounds I and II they have their emission maximum in the ultraviolet range[10]. Compounds I and II can thus be distinguished easily from all the other amino acids found in protein hydrolysates. They appear to be α-amino acids because of their positive reaction with ninhydrin and their ability to form copper complexes. The behaviour on ion-exchange material is further evidence for their amphoteric nature. They are adsorbed on to cellulose cation-exchangers from acid solution and to cellulose anion-exchangers from alkaline solution and they can be eluted from both at neutral reaction.

As aromatic compounds tend to be more or less strongly adsorbed on to Dowex resins[11], it is not surprising that compounds I and II are adsorbed too, but it was rather unexpected that the adsorption of compound I was so strong that all attempts to elute it were unsuccessful. This strong adsorption of the compounds on to the resin also explains why they have been overlooked so far.

The presence in both compounds of a group with dissociation near neutrality is demonstrated by the spectral measurements as well as by titration and electrophoresis. The pH-dependence of both the ultraviolet absorption and of the fluorescence indicates that the group is phenolic. The presence of a phenolic group is indicated independently by the positive reactions with Millon's reagent and with α-nitroso-β-naphthol, and also by the ability of the compounds to couple with diazotized sulphanilic acid. The tests for diphenols were all negative, and as the compounds are completely stable to alkaline hydrolysis it is unlikely that they contain more than one phenolic group unless more than one aromatic ring is present. It is remarkable that the phenolic groups present are much more acid than the group in tyrosine; presumably this is due to some substituent in the aromatic ring system the nature of which is unknown.

The most important feature of the compounds is the presence of more than one amino group and more than one carboxylic group per molecule. Since there is good evidence that the amino groups are all unable to react with FDNB in the native protein, and since fluorescent peptides can be liberated by means of enzymes, the compounds are presumably built into the peptide chains in the same manner as the other

amino acids but in such a way that they form cross-linkages between the ordinary linear peptide chains, giving rise to a large three-dimensional network.

Although this is the most plausible explanation of the results, a more thorough investigation is needed in order to clear up exactly how these amino acids are built into the protein chains. One should remember that the absence of *N*-dinitrophenylated products of these compounds after treatment of resilin with FDNB could mean that the amino groups in some way are unavailable for reaction without being bound covalently to the peptide structure. Also, we have no information concerning the state of the carboxylic groups of the compounds in native resilin.

Although more conclusive evidence is needed, all present results strongly indicate that both fluorescent compounds function as cross-linkages between peptide chains, compound II linking two chains together and compound I linking three chains together at one junction point.

Chemical tests and physical measurements predicted the presence of cross-linkages in resilin[1,2] and, in the case of dragonfly resilin[3], the average molecular weight of the chains between two neighbouring junction points was estimated to be 5000. This calculation is based on the assumption that four half-chains meet in all the junction points. The presence of junction points where six half-chains meet will make the calculations more uncertain but will have no influence on the general physical properties of the protein. Only some of the junction points need be chemical cross-linkages since others may represent physical entanglements, making an average molecular weight of chains between two neighbouring chemical cross-linkages of between 5000 and 10000 most probable for dragonfly tendon resilin.

The average chain weight between chemical junction points in locust resilin was found to be 3000 (Table I) based on the assumption that the fluorescent amino acids constitute the cross-linkages. This value is somewhat lower than the value based on physical measurements, but as resilins from the two sources are known to differ in the degree of cross-linking[13], locust resilin being more cross-linked than dragonfly resilin, the agreement between the two estimates is reasonably good.

Thus all the results we have got so far support the hypothesis that these fluorescent compounds form the cross-linkages in resilin. Other types of cross-linkages are apparently not present: we have been able to rule out disulphide linkages, phosphate groups, ester groups, carbohydrates, and amide linkages involving the ε-amino group in lysine.

If compounds I and II are involved in cross-linking one should expect to find them in resilin from different animal species. So far resilin from only three representatives of arthropods, a locust (*Schistocerca*, Insecta, Orthoptera), a dragonfly (*Aeshna*, Insecta, Odonata), and a crayfish (*Astacus*, Crustacea, Decapoda) have been investigated in this respect and the compounds were found to be present in all of them. On the other hand, it has not been possible to find even traces of them in other proteins from the same species, so compounds I and II can apparently be considered amino acids which are specific for resilin.

As resilin is a cuticular structure it is notable that its formation in some respects must deviate much from that of the ordinary cuticle, where proteins are thought to be laid down in bulk and then rendered insoluble by tanning with low-molecular quinoid substances derived from the metabolism of tyrosine[14]. Agents which dissolve such tanned proteins do not bring resilin into solution[1]. Also, in resilin the deposition

of the protein and of the fluorescent compounds must occur simultaneously, or nearly so, as there is proportionality between the amount of material laid down in the ligaments and the amounts of the fluorescent amino acids present during the whole period of deposition[12] (2–3 weeks). This means that if the fluorescent amino acids constitute the cross-linkages, the peptide chains must be cross-linked as soon as they have been secreted from the synthesising cells.

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