The Nature of the Cross-linking of Proteins by Glutaraldehyde. Part I. Interaction of Glutaraldehyde with the Amino-groups of 6-Aminohexanoic Acid and of α-*N*-Acetyl-lysine

By **Paul M. Hardy, Alan C. Nicholls,** and **H. N. Rydon,*** Department of Chemistry, The University, Exeter EX4 4QD

The products of interaction of glutaraldehyde with 6-aminohexanoic acid and with α-*N*-acetyl-lysine have been separated and partially purified; studies of the most highly purified fraction are indicative of the formation of 1,3,4,5-tetrasubstituted pyridinium salts, such as (II), analogous to desmosine. It is suggested that similar structures are present in the cross-linked products formed by the action of glutaraldehyde on proteins.

ALTHOUGH the cross-linking action of glutaraldehyde on proteins is widely used, in such diverse fields as tanning,[1] enzymology,[2] electron-microscopy,[3] and *X*-ray crystallography,[4] its chemical nature remains unknown. All that is certain is that the reaction involves the ε-amino-groups of lysine side-chains and results in the appearance of an absorption maximum at *ca.* 265 nm. The stability of the cross-linkages to acid rules out simple Schiff's base formation. Up to the present only one attempt to explain the general mechanism of the cross-linking seems to have been made; Richards and Knowles [5] postulate a pathway involving a Michael-type addition of the side-chain amino-groups to αβ-unsaturated aldehydes formed by aldol condensation of the glutaraldehyde. We showed earlier [6] that, although commercial glutaraldehyde does contain unsaturated compounds, these are not present in aqueous solutions of purified glutaraldehyde; we now show that the tanning actions of aqueous solutions of both unpurified and purified glutaraldehyde are almost identical and it is clear that this cross-linking reaction at least is not dependent on the initial presence of unsaturated compounds. If the mechanism of Richards and Knowles is indeed operative, then the postulated aldol condensation must occur under the influence of the protein immediately prior to cross-linking. Furthermore, the suggested end-products seem unlikely to give rise to the acid-stable chromophore absorbing at 265 nm. At most this mechanism can only make a minor contribution to the overall cross-linking.

With the object of defining more closely the nature of its cross-linking action on proteins, we have investigated the reactions of glutaraldehyde with the model compounds, α-*N*-acetyl-lysine and 6-aminohexanoic acid, in unbuffered aqueous solution at room temperature. Most work was done with the latter compound since this gave products more amenable to chromatographic fractionation. There was a general similarity between the reactions with the two model compounds. In both cases a chromophore absorbing at 266 nm was generated; the optimal molar ratio of reactants was 2 : 1 (aldehyde to amino-acid) and reaction was complete in 7 days. The 266 nm chromophore was destroyed by reduction with sodium borohydride, being replaced by very weak chromophores absorbing at 235 and 300 nm. Alkaline hydrolysis also destroyed the chromophore. The reaction mixtures gave an intense orange colour with Dragendorff's reagent and fluoresced strongly in u.v. light.

Amino-acid analysis of the product from α-*N*-acetyl-lysine showed it to contain 0.126 μmol of lysine per mg (*i.e.* only 2% by weight); no other ninhydrin-positive material was eluted from the column in the normal buffer programme of the analyser, but regeneration of the column with 0.2M-sodium hydroxide removed considerable amounts of a dark-coloured material. This suggests the presence of strongly basic groups in the bulk of the product.

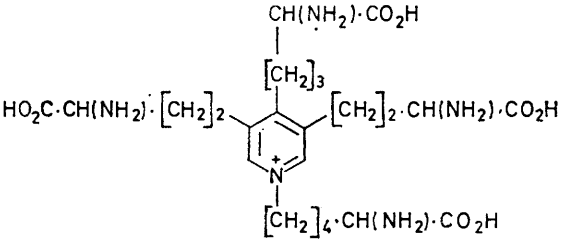
Evaporation of the reaction mixture from 6-aminohexanoic acid gave a brown oil which solidified in trituration with ethyl acetate; the weight of product was consistent with reaction of each mol of amino-acid with 1.8 mol of aldehyde. The absorbance of this product at 266 nm ($E_{1\ cm}^{1\%}$ 5.11) increased (to 8.65) on heating at 105 °C with 6M-hydrochloric acid; a similar increase was noted in other cases by Bowes and Cater [7] and ascribed to further condensation of the primary product. The crude solid product was a mixture of at least 12 components. Chromatography on SE-Sephadex G25 with phosphate–citrate buffer (pH 5.9) separated it into three approximately equal fractions. Further fractionation of one of these on DEAE-Sephadex, with 0.02M-acetic acid for elution, gave, in 10% overall yield, material which was eluted as a single symmetrical peak on gel-filtration on both G25 and G50 Sephadex.

Although we do not claim this product to be a single chemical entity, we believe it to be a mixture of closely related compounds of similar molecular size about whose structure valid conclusions can be drawn. Electrometric titration showed the presence of a single titratable group (pK_a 4.7 ± 0.1), presumably a carboxy-group, and an equivalent of 300 ± 20. The u.v. spectrum of a sample in 0.05M-hydrochloric acid showed $\lambda_{max.}$ 268 nm, $E_{1\ cm}^{1\%}$ 89.0, corresponding to ε 2 670 per dissociating

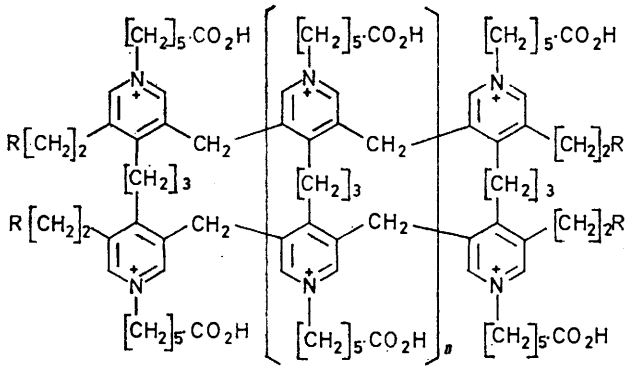
[1] L. Seligsberger and C. Sadlier, *J. Amer. Leather Chemists' Assoc.*, 1957, **52**, 8; M. L. Fein and E. M. Filachione, *ibid.*, p. 17.
[2] F. A. Quiocho and F. M. Richards, *Biochemtstry*, 1966, **5**, 4062.
[3] D. S. Sabatini, F. Miller, and R. J. Barnett, *J. Histochem. Cytochem.*, 1964, **12**, 57.
[4] F. A. Quiocho and F. M. Richards, *Proc. Nat. Acad. Sci. U.S.A.*, 1964, **52**, 853.
[5] F. M. Richards and J. R. Knowles, *J. Mol. Biol.*, 1968, **37**, 231.
[6] P. M. Hardy, A. C. Nicholls, and H. N. Rydon, *Chem. Comm.*, 1969, 565.
[7] J. H. Bowes and C. W. Cater, *Biochim. Biophys. Acta*, 1968, **168**, 341.

group on the basis of an equivalent weight of 300. The i.r. spectrum (KBr disc) showed $\nu_{max.}$ 3 440br (OH str.), 2 940 and 2 880 (CH_2 str.), 1 715 (carboxy C=O str.), and 1 640br cm^{-1} (C=C, C=N, and carbonyl C=O str.). The ^1H n.m.r. spectra of samples in trifluoroacetic acid and [$^2H_6$]dimethyl sulphoxide showed bands at τ 1.1, 5.5, 7.9, and 8.6; the weakness of the spectra and the overlap of the high-field bands precluded accurate integration. There was no obvious band in the position (τ *ca.* 0.2) expected for CHO, although the presence of some formyl groups was indicated by the development of absorption at 300 nm on treatment with *N*-methylbenzothiazolone hydrazone.[8]

On the basis of these properties, and by analogy with the structures of the quaternary pyridinium derivatives desmosine (I) and isodesmosine found in acid hydrolysates of elastin,[9] we postulate the polymeric quaternary pyridinium structure (II) for our purified product from glutaraldehyde and 6-aminohexanoic acid, which is



(I)



(II) R = CH_2·OH and/or CHO

probably a mixture of closely related compounds differing in the value of *n*.

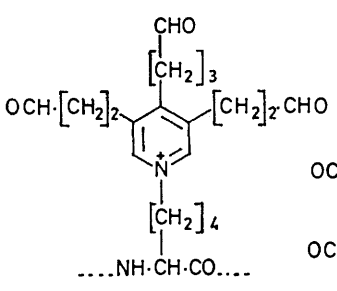
The presence of some sort of aromatic system is strongly supported by the u.v. and n.m.r. spectra, and the electrometric titration and ion-exchange chromatography (amino-acid analyser) results show that the amino-group has disappeared and has been replaced by a very strongly basic group. All this points to the presence of a quaternary pyridinium system, and this is supported by the sensitivity of the chromophore to the nucleophiles hydroxide and hydride (or borohydride) ions, in agreement with the known properties of pyridinium salts.[10] The reaction with Dragendorff's reagent and the fluorescence of the product are also typical of such compounds.

The u.v. and n.m.r. spectra are also indicative of the (desmosine) 1,3,4,5-substitution pattern postulated in structure (II), rather than the alternative (isodesmosine) 1,2,3,5-substitution pattern. Thus [9] desmosine (I) has $\lambda_{max.}$ 266 nm, close to $\lambda_{max.}$ (268 nm) for our product, whereas isodesmosine has $\lambda_{max.}$ 276 nm. The n.m.r. band at τ 1.1 is diagnostic for aromatic protons. It is a broad singlet, suggesting symmetrical substitution, and its position is that expected for 2- and 6-protons in pyridinium rings. Bedford and Katritzky [11] observed a single band at τ 1.45 for the 2- and 6-protons in desmosine, but two bands, at τ 1.43 and 1.70, respectively, for the 2- and 4-protons in isodesmosine; we observed separate bands at τ 1.05, 1.85, and 1.40, respectively, for the 2- and 6-, the 3- and 5-, and the 4-protons in *N*-(2-carboxyethyl)pyridinium chloride. The band at τ 5.5 is ascribed to the CH_2 groups adjacent to N^+, since the similar protons in desmosine and isodesmosine absorb at 5.5 and 5.45, respectively,[11] and those in *N*-(2-carboxyethyl)pyridinium chloride at τ 5.1; the area of this band is approximately equal to that of the band at τ 1.1, as expected for structure (II).

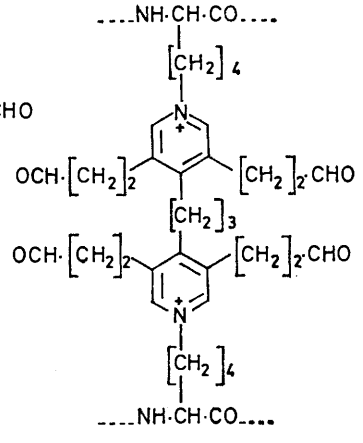
The literature indicates that, although the reactions of straight-chain aliphatic aldehydes with amines are seldom simple, there is a general major pathway leading ultimately to dihydropyridines; n-butyraldehyde, for example, initially forms a Schiff's base with butylamine, which undergoes further condensation with the aldehyde to give 1-n-butyl-3,5-diethyl-2-n-propyldihydropyridine.[12] Dihydropyridines are known to be readily oxidised to pyridinium salts,[12-15] and at least partial oxidation of this kind must be expected in the dilute aqueous solutions used in our work. The model experiments of Davis and Anwar [14] gave products with the isodesmosine (1,2,3,5-) rather than the desmosine (1,3,4,5-) substitution pattern we suggest for our purified product; however, in neither case are the yields high and the isolation of one particular product from the complex reaction mixture does not rule out the presence of other isomers. Both types of substitution undoubtedly occur in the cross-linkages in elastin, which are believed to arise from the interaction of the ε-amino-groups of lysine side-chains and the δ-formyl groups of allysine side-chains.[9,14,16]

[8] M. A. Paz, O. O. Blumenfeld, M. Rojkind, H. Hensen, C. Furfine, and P. M. Gallop, *Arch. Biochem. Biophys.*, 1965, **109**, 548.
[9] J. Thomas, D. F. Elsden, and S. M. Partridge, *Nature*, 1963, **200**, 652.
[10] E. M. Fry, *J. Org. Chem.*, 1964, **29**, 1647; P. S. Anderson and R. E. Lyle, *Tetrahedron Letters*, 1964, 153.
[11] G. R. Bedford and A. R. Katritzky, *Nature*, 1963, **200**, 652.
[12] T. M. Patrick, *J. Amer. Chem. Soc.*, 1952, **74**, 2984.
[13] D. Craig, L. Schaefger, and W. P. Tyler, *J. Amer. Chem. Soc.*, 1948, **76**, 1624.
[14] N. R. Davis and R. A. Anwar, *J. Amer. Chem. Soc.*, 1970, **92**, 3778.
[15] H. B. Charman and J. M. Rowe, *Chem. Comm.*, 1971, 476.
[16] R. A. Anwar and G. Oda, *Biochim. Biophys. Acta*, 1967, **133**, 151; S. R. Pinnell and G. R. Martin, *Proc. Nat. Acad. Sci. U.S.A.*, 1968, **61**, 708.

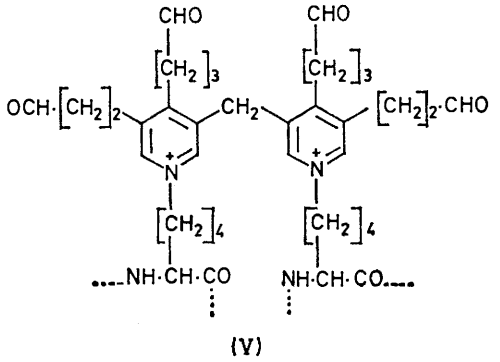
The mean values of three closely concordant elemental analyses of two specimens of the purified product from glutaraldehyde and 6-aminohexanoic acid were C, 58.59; H, 7.24; N, 4.66%. Owing to the presence of inorganic matter only the molar C : N ratio is significant. Structure (II; $n = 2$, $R = CH_2{\cdot}OH$) requires C : N 14.75 : 1, which is close to the found value (14.66 : 1). The calculated equivalent for (II; $n = 2$, $R = CH_2{\cdot}OH$) is 252 which, when corrected for inorganic matter on the basis of the carbon and nitrogen analyses, becomes 302, in good agreement with that found by electrometric titration. The stronger absorption of our products than of desmosine [9] on cation-exchange resins is explicable in terms of their polyelectrolyte nature, with several quaternary centres in each molecule. The absence of CHO signals in the ^1H n.m.r. spectra indicates considerable oxidation (CHO ⟶ CO_2H) or reduction (CHO ⟶ $CH_2{\cdot}OH$); the latter seems the more likely in view of the oxidation involved in the formation of the pyridinium nuclei.



(III)



(IV)



(V)

We suggest that cross-linkages of the same quaternary pyridinium type as those in (II) are also formed by the action of glutaraldehyde on proteins. Reaction of three molecules of glutaraldehyde with a single lysine side-chain would lead to (III), analogous to desmosine (I). Further reaction of (III) with another lysine side-chain and two more molecules of glutaraldehyde would give rise to cross-linkages of which (IV), involving the 4-side-chain of (III), and (V), involving the 3-side-chain, are the most symmetrical and the only ones containing the desmosine substitution pattern. We regard these as more likely than the corresponding isodesmosine analogues because the u.v. spectra of glutaraldehyde cross-linked proteins all show absorption maxima at *ca.* 265 nm, characteristic of the desmosine type of substitution. Further condensation of (IV) and (V) with glutaraldehyde and lysine side-chains can lead to increasingly complex types of cross-linkages, of which the lysine analogue of (II) is but one of the many possibilities. It is to be understood that the free formyl groups shown in structures (III)—(V) may be modified by oxidation or reduction. However, the persistance of some free formyl groups, with their potentialities for further cross-linking, would explain the observation that protein crystals treated with glutaraldehyde progressively darken,[17] as did our own glutaraldehyde–6-aminohexanoic acid condensation products.

Bowes and Cater [7] isolated a product from the acid hydrolysate of glutaraldehyde-treated collagen the elemental analysis of which agrees with that for the chloride from (III) with all the formyl groups oxidised to carboxy, if the not unlikely presence of a little sodium chloride is assumed (Found: * C, 52.0; H, 7.05; Cl, 9.7; N, 5.5. Calc. for $C_{27}H_{31}ClN_2O_8$ containing 3% NaCl: C, 51.5; H, 6.4; Cl, 9.1; N, 5.7%). We regard the isolation of this product as useful confirmatory evidence for our proposal.

Although our suggestion satisfactorily explains many features of the cross-linking action of glutaraldehyde on proteins, the postulated conversion of lysine side-chain amino-groups into quaternary pyridinium groups accounts only partially for the observed changes in the proton-binding properties of the side-chains.[18] As Bishop and Richards [18] point out 'the well-known plethora of formaldehyde–protein reactions serves as a warning of possible complexities' and it would be unwise to assume that the type of cross-linking we suggest is the only one present in glutaraldehyde-treated proteins.

EXPERIMENTAL

N.m.r. spectra were recorded with a Perkin-Elmer R10 60 MHz or a JEOL JMH-100 100 MHz spectrometer, u.v. spectra with a Pye-Unicam SP 800 recording spectrometer, and i.r. spectra with a Hilger–Watts Infrascan recording spectrometer. Chromatographic column effluents were monitored with a continuous flow LKB Uvicord U.V. Absorptiometer, type 8301A. Acidic hydrolyses were

* Cater has informed us that the oxygen analysis was obtained by difference.

[17] Personal communication from Professor D. C. Hodgkin.

[18] W. H. Bishop and F. M. Richards, *J. Mol. Biol.*, 1968, **33**, 415; E. F. Jansen, Y. Tomimatsu, and A. C. Olson, *Arch. Biochem. Biophys.*, 1971, **144**, 394; Y. Tomimatsu, E. F. Jansen, W. Gaffield, and A. C. Olson, *J. Colloid Interface Sci.*, 1971, **36**, 51.

carried out with redistilled 6M-hydrochloric acid in evacuated sealed tubes at 110 °C for 24 h.

Glutaraldehyde.—Aqueous solutions were prepared from the freshly distilled aldehyde.[6] The tanning action of such solutions was compared with that of a commercial solution (Koch–Light), showing considerable absorption at 235 nm, as follows.

Acetone-dehydrated sheepskin (1 g) was swollen in water and then shaken at room temperature for 6 h with aqueous 5% glutaraldehyde (1.5 ml) diluted to 10 ml with water (experiments at pH 4.0) or 5% sodium hydrogen carbonate (experiments at pH 8.1). The glutaraldehyde content of the tanning solution was determined by the method of Frigerio and Shaw.[19] After tanning the skins were removed from the tanning solution and washed with water; the aldehyde content of the supernatant and washings was determined. The washed skins were acetone-dried, a portion was hydrolysed with acid, and the hydrolysate was subjected to amino-acid analysis. The shrinkage temperature of the tanned skins was also measured. The results are given in the Table.

Glutaraldehyde	pH	Loss of lysine + hydroxylysine (residues per 10^5 g)	Glutaraldehyde uptake (mmol g^{-1})	Shrinkage temp. (°C)
None (control)	4.0			57.2
Commercial	4.0	15.4	0.36	84.8
Purified	4.0	14.6	0.43	83.1
None (control)	8.1			58.4
Commercial	8.1	20.6	0.55	86.1
Purified	8.1	20.2	0.63	84.0

α-N-*Acetyl-*L*-lysine.*—A modification of the procedure of Neuberger and Sanger,[20] involving decomposition of the intermediate copper complex with disodium ethylenediaminetetra-acetate (EDTA) instead of hydrogen sulphide was used.

The finely ground copper complex of ε-*N*-benzyloxycarbonyl-L-lysine [20] (4.8 g), suspended in water (30 ml) at 60 °C, was treated with EDTA (5.5 g), added in portions over 30 min, while the temperature was maintained at 60—70 °C. A final portion of EDTA (4.3 g) in acetone (20 ml) was added to complete decomposition of the complex. The mixture was then cooled and the precipitate filtered off and washed with water. Recrystallisation from water yielded ε-*N*-benzyloxycarbonyl-L-lysine (3.5 g, 94%), m.p. 245—246° (lit.,[20] yield 80%; m.p. 242—243°).

Acetylation and subsequent hydrogenolysis by the method of Neuberger and Sanger [20] gave α-*N*-acetyl-L-lysine (80%), m.p. 250—252°, $[\alpha]_D^{20}$ +5.9° (*c* 1.9 in H_2O) (lit.,[20] yield 62%; m.p. 250°; $[\alpha]_D^{20}$ +4.7°) (Found: C, 51.2; H, 8.8; N, 14.9. Calc. for $C_{18}H_{16}N_2O_3$: C, 51.0; H, 8.5; N, 14.9%).

1-(2-*Carboxyethyl*)*pyridinium Chloride.*—Prepared by the procedure of Hamilton and Adams [21] in 37% yield, this had m.p. 158—159.5° (lit.,[22] 160°); τ (60 MHz; D_2O) 1.05br (2 H, d), 1.4br (1 H, t), 1.85br (2 H, d), 5.1br (2 H, d), and 6.8 (2 H, t, *J* 6 Hz); $\nu_{max.}$(KBr) 1 735, 1 635, 870, 828, 798, and 697 cm^{-1}; $\lambda_{max.}$ 259 nm (ε 4 550).

Interaction of Glutaraldehyde and α-N-*Acetyl-lysine.*—α-*N*-Acetyl-L-lysine (37.6 mg, 0.2 mmol) was dissolved in water (1 ml) and treated with aqueous glutaraldehyde (0.8 mmol in 2 ml). The mixture (pH 6) slowly became dark brown. After 7 days at 20 °C, t.l.c. showed only a trace of acetyl-lysine. Evaporation of the mixture gave an oil, which solidified on trituration with ethyl acetate. The product (65 mg) had τ (60 MHz; D_2O) 1.2 (1 H), 4.75 (2 H), 5.1 (1 H), 5.9 (6.2 H), 7.95 and 8.01 (*ca.* 13 H), and 8.4 (2.5 H); $\nu_{max.}$(KBr) 3 300, 2 950, 2 850, 1 730, 1 645, 1 587, 1 445, 1 405, 1 380, and 1 050 cm^{-1}; $\lambda_{max.}$ 265 nm ($E_{1\ cm}^{1\%}$ 46.5) (Found: C, 58.9; H, 7.9; N, 6.4%). Acidic hydrolysis of 2.1 mg was followed by amino-acid analysis; the hydrolysate contained 0.265 mol of lysine (*i.e.* 0.126 μmol mg^{-1}); enzymic hydrolysis of 1.0 mg with hog renal acylase at pH 7 gave 0.08 μmol mg^{-1}.

Solutions of α-*N*-acetyl-L-lysine (0.2 mmol) and various amounts of glutaraldehyde were kept at 20 °C in water (30 ml); the absorbance at 266 nm was measured, in suitably diluted solutions, after 7 days. The results are plotted in Figure 1. T.l.c. showed that reaction was

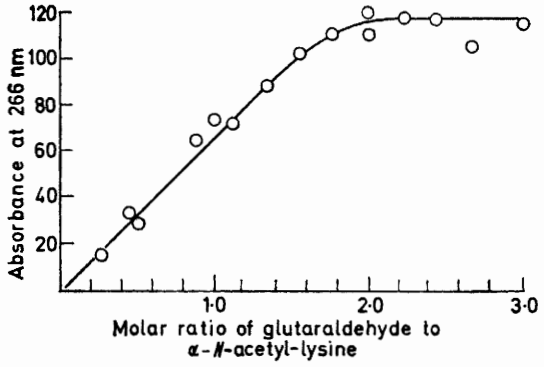


FIGURE 1

substantially complete after 24 h for mixtures containing more than 2 mol of aldehyde per mol of acetyl-lysine; with less aldehyde, unchanged acetyl-lysine was present and the amount of this, and also the absorbance at 266 nm, remained unchanged for up to 1 month.

α-*N*-Acetyl-L-lysine (37.6 mg, 0.2 mmol) in water (1.0 ml), aqueous 5% sodium hydrogen carbonate (5.0 ml), and glutaraldehyde (0.75 mmol) in water (1.0 ml) were mixed. The mixture (pH 9.3) was kept at 20 °C for 2 h. Unchanged glutaraldehyde was removed by extraction with ether. The residual aqueous layer was brown and had $\lambda_{max.}$ 266 and 300 nm. Treatment with sodium borohydride (67 mg, 1.77 mmol), added in small portions, followed by stirring overnight, led to a straw-coloured solution with very weak absorption at $\lambda_{max.}$ 230 and 300 nm; the maximum at 266 nm had completely disappeared.

Interaction of Glutaraldehyde and 6-*Aminohexanoic Acid.*—Experiments similar to, but less extensive than, those summarised in Figure 1 showed that the optimal ratio of aldehyde to amino-acid in this reaction was likewise 2 : 1. Accordingly, glutaraldehyde (400 mg, 4 mmol) and 6-aminohexanoic acid (262.5 mg, 2 mmol) were kept at 20 °C in water (30 ml) for 7 days; t.l.c. then showed that there was very little unchanged amino-acid. The solution was evaporated under reduced pressure and the residual brown oil triturated with ethyl acetate to give the crude product (618 mg), $\lambda_{max.}$ 266 nm ($E_{1\ cm}^{1\%}$ 51) in water; heating at 100 °C with 6M-HCl intensified the absorption (to $E_{1\ cm}^{1\%}$

[19] N. A. Frigerio and M. J. Shaw, *J. Histochem. Cytochem.*, 1969, **17**, 176.

[20] A. Neuberger and F. Sanger, *Biochem. J.*, 1943, **37**, 515.

[21] T. S. Hamilton and R. Adams, *J. Amer. Chem. Soc.*, 1928, **50**, 2210.

[22] A. Kirpal and B. Wojnar, *Ber.*, 1938, **71B**, 1261.

75); heating with 2M-NaOH caused the disappearance of the band, which was replaced by a weak broad band (λ 230—290 nm) superimposed on general absorption.

A column of SE-Sephadex (38 × 2.5 cm) was equilibrated with citrate–phosphate buffer [pH 5.9; from citric acid (19.7 ml) and 0.2M-Na_2HPO_4 (30.3 ml), made up to 100 ml with water]. The crude interaction product (60.4

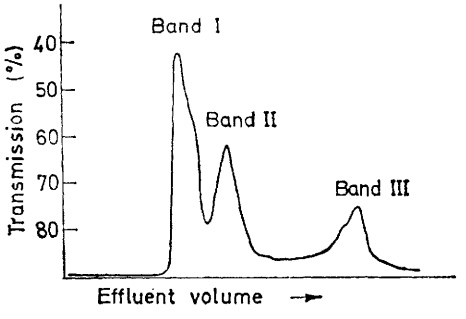


FIGURE 2

mg), in this buffer (3 ml), was applied to the column. Elution was conducted at 14 ml h^{-1}, and was monitored by light absorption at 266 nm, revealing three main peaks (Figure 2). The fractions corresponding to each peak were pooled and concentrated. The products were desalted on columns of Dowex AG 50 × 4 (H^+ form). After washing the resin well with water, the products were eluted with aqueous 3% ammonia. On evaporation, Band I gave 22.5 mg of material, Band II 22.7 mg, and Band III 23.1 mg (86.5% total recovery). This separation was repeated successfully several times with loadings of up to 100 mg of crude product. With the heavier loadings, separation of Bands I and II was improved if the crude mixture was loaded at pH 5.8.

A column of DEAE-Sephadex (25 × 1 cm) was converted into the acetate form and thoroughly washed with water. A sample of Band I (5 mg) was applied to the column in 0.05M-ammonium acetate and the column washed with water until all unabsorbed material had been eluted. Elution with increasingly concentrated acetic acid up to 0.01M did not remove any product. Elution with 0.02M-acetic acid desorbed a major component as a symmetrical peak. Stronger acetic acid displaced material with the solvent front as unsymmetrical peaks; some product remained firmly bound and was not desorbed even by 2M-acetic acid. On the preparative scale (50 mg) the product eluted with 0.02M-acetic acid was lyophilised to give a brown powder (15 mg; 11% overall) the properties of which are described in the Discussion section. It was eluted as a single symmetrical peak on gel filtration on G25 and G50 Sephadex in 0.1M-ammonium formate (V_e/V_o = 1.015 and 2.32, respectively).

We thank the S.R.C. and the British Leather Manufacturers' Research Association for a C.A.P.S. Studentship (to A. C. N.) and Dr. J. H. Bowes and Mr. C. W. Cater for discussions. The tanning experiments were carried out in the B.L.M.R.A. laboratories at Egham.

[5/1775 *Received*, 15*th September*, 1975]