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# FURTHER STUDIES ON THE CHROMATOGRAPHIC SEPARATION OF THE COLLAGEN MOLECULAR SUBUNITS

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SUMMARY

A study has been made of three chromatographic methods used for the separation of collagen molecular components. Two of the methods use a salt gradient, while the third uses a salt and pH gradient to elute the protein from the column. Optical absorption at 220, 230 and 278 mμ was used for analysis of the column effluent in one of the methods, in order to follow protein concentration and the distribution of the aromatic amino acids. These methods yield minor components which could correspond to acidic and basic telopeptides which are essential to the structure of collagen.

## INTRODUCTION

The chromatographic separation of the collagen molecular subunits has been based mainly on the simple linear gradient technique of PIEZ and co-workers[1,2], which uses CM-cellulose and acetate buffers. In general, the column effluent from these separations has been analysed spectrophotometrically at 230 mμ using flow cells of relatively large volume (± 1 ml), or by chemical means using bulk fractions (5 to 10 ml). While the method of PIEZ may resolve all the components present in a monomeric extract, it has been shown by several authors (see for example TRISTRAM, WORRALL AND STEER[3], COOPER AND DAVIDSON[4], and DAVIDSON AND COOPER[5]) that this simple gradient does not give complete resolution of all components present in a polymeric extract. Therefore some authors report using strong alkali and even 6 *M* urea to recover all the protein (see for example VEIS AND ANESEY[6]), while the present authors have developed a combined pH and salt gradient using phosphate buffers[4]. It is therefore of interest to study the effect of pH on the stability and solubility of soluble collagen.

In addition, the chromatographic resolution of soluble collagen has been improved with the aid of the Beckman Spectrochrom automatic chromatography analyser, which employs a micro cuvette for optical analysis of the column effluent. This micro cuvette has volumes of about 0.05 and 0.01 ml in its dual path length compartment of 10 and 2.5 mm respectively, compared with about 1 ml in most flow cells of 10 mm path length. Further the Spectrochrom is capable of continuously analysing the column effluent at any three fixed wavelengths. The distribution of the important aromatic amino acids in relation to the collagen subunits has therefore

been studied by optical absorption at 278 mμ. These aromatic amino acids play an important role in both intra- and intermolecular bonding[7].

## EXPERIMENTAL

### *Preparation of collagen*

The preparation and purification of the neutral-salt-soluble and acid-soluble collagen has been described previously[8].

### *Column chromatography*

The separation of heat-denatured soluble collagen was carried out using the following three methods:

(*1*) *Modified* PIEZ *method.* In this method a column kept at 40° was packed with CM-cellulose (Bio Rad Laboratories, Lot No. 4146) to give a bed 2.5 × 34 cm. The stock acetate buffer of PIEZ *et al.* (pH 4.8 and I = 0.5) was diluted to give their starting buffer, I = 0.06, and limiting buffer, I = 0.16. The linear gradient was prepared from 500 ml of each of these two buffers in separate flasks[1]. This was pumped onto the column (Beckman Accuflo pump) at 100 ml/h. After 900 ml of this gradient had been used, acetate buffer of a higher salt content (I = 0.26), prepared from the starting buffer by the appropriate addition of sodium chloride, was pumped through the column. The column effluent passed through the flow cell (1 cm path length, 1 ml volume) of a recording spectrophotometer (Beckman Model DB) set at 230 mμ, and was collected in 10 ml aliquots in a refrigerated fraction collector (Beckman Model 132). The collagen was dissolved in the starting buffer, to give solutions containing about 50 mg/25 ml, heat-denatured at 45° for 30 min, and run into the column with nitrogen at 10 p.s.i.

(*2*) *Spectrochrom method.* This method used the same column, column temperature, flow rate, gradient and sample preparation as above. The Spectrochrom was set to analyse the column effluent at 220, 230 and 278 mμ. At the same time continuous recordings of the conductivity and pH of the column effluent were obtained, which acted as a control on the gradient. The column effluent was collected in 10 ml aliquots by the drop counting method.

(*3*) *The pH and salt gradient method.* This method has been described in detail elsewhere[4].

### *Two dimensional high voltage electrophoresis and chromatography*

A Shandon water-cooled HVE apparatus and Whatman No. 4 paper was used[9]. The electrophoresis was carried out in a formic and acetic acid buffer (pH 1.6) at 6,000 V for 25 min. The chromatography was done with equal volumes of 2:6-lutidine, collidine and water for 24 h at 20°. The method was standardised with a known mixture of pure amino acids. The amino acids were detected by spraying the dried paper with ninhydrin.

### *Optical rotation and mutarotation*

These methods have been described previously[8,10]. A Perkin Elmer Model 141 digital-readout polarimeter was used at wavelengths of 589 and 365 mμ.

*Ultracentrifugation*

The same procedure was used as described previously[8].

*Amino acid analysis*

Amino acid composition was determined with a Beckman Unichrom amino acid analyser after hydrolysis with 6 *N* HCl under vacuum in sealed tubes at 100° for 24 h.

## RESULTS AND DISCUSSION

*Chromatographic separation of collagen components by the pH gradient method*

A typical separation of acid-soluble collagen by the pH gradient method into $\alpha$- and $\beta$-subunits plus the additional components a, b and c is shown in Fig. 1. From this it will be seen that the $\alpha$- and $\beta$-subunits elute at about pH 5.0, while component a elutes at pH 8.0, and components b and c at pH 11.0. The fact that these components elute at such relatively high pH values leads to interesting speculation as to their nature and relation to the $\alpha$- and $\beta$-subunits and tropocollagen. It was therefore considered important to follow the action of pH and temperature changes on the stability and solubility of acid-soluble collagen.

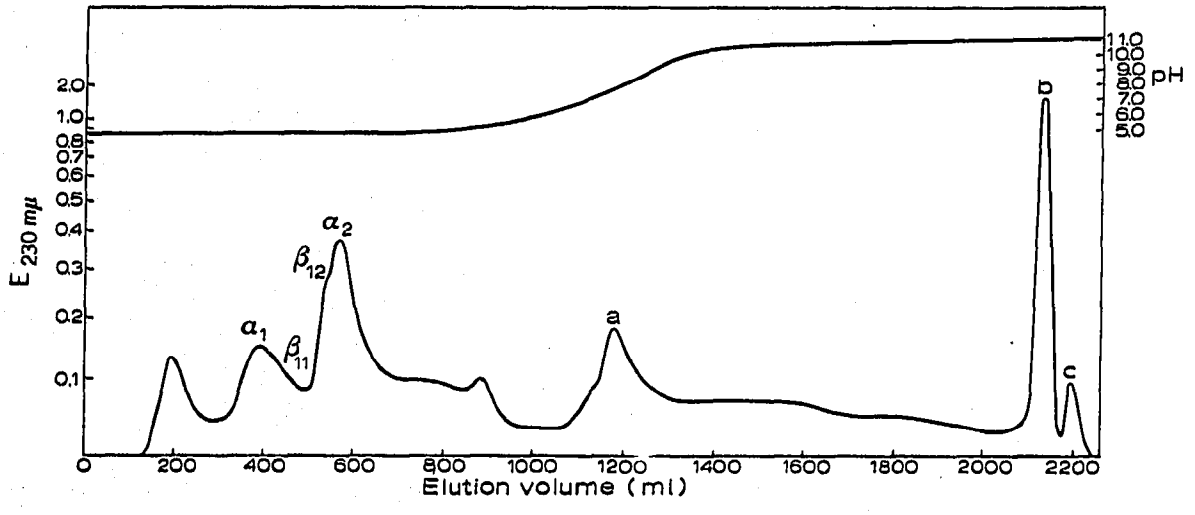


Fig. 1. Chromatography of 98 mg of acid-soluble collagen on CM-cellulose at 40° using a phosphate buffer gradient (see text). The upper curve shows the pH gradient.

In a series of experiments samples of acid-soluble collagen, as prepared and characterised in previous publications[5,8] were added to 0.15 *M* potassium acetate buffer (pH 4.8), which is a good solvent for mutarotation studies, to 0.1 *M* potassium phosphate buffer (pH 8.0), and to an alkaline phosphate buffer (pH 11.0). The latter corresponds to the solution used to elute components b and c from CM-cellulose[4]. The acid-soluble collagen was not readily soluble in any of these solvents at 4°, but on heating at 45° for 15 min the protein dissolved readily in all three instances. It is of interest to note that with the phosphate solution of pH 8 heating at 40° for 2 h did not result in the protein dissolving, but on raising the temperature to 45° the collagen dissolved readily. The melting point ($T_m$) of this acid-soluble collagen in 0.15 *M* acetic acid, as measured by optical rotation[5], was 38.1°.

After heating at 45° for 15 min to dissolve the collagen, these solutions were stored at 4° for one week. Samples of the solutions of known concentration were then heated at 45° for 15 min and immediately placed in a polarimeter tube thermostated at 15°. The mutarotation of these solutions was then followed for 48 h at 15° and 589, 578, 546, 436 and 365 mμ. The results as measured at 365 mμ are shown in Fig. 2. In Table I the specific rotation values are given for the original collagen solutions and for the solutions after heating and keeping at 15° for 48 h. From these results it is evident that at pH values of 4.8 and 8.0, and presumably at pH values in between these, the mutarotation and final specific rotation are largely unaffected, whereas at pH 11 the specific rotation is significantly reduced. The specific rotation at this pH ($[\alpha]_D^{15} = -198°$ and $[\alpha]_{365}^{15} = -649°$) is, however, still greater than the value for the rotation of α-helix-forming proteins and collagen in the denatured form ($[\alpha]_D^{15} = -90°$ to $-120°$, $[\alpha]_{365}^{15} = -450°$; HARRINGTON AND VON HIPPEL[11]), showing that even after heating at 45° and pH 11, acid-soluble collagen on cooling still retains a degree of helical content. At this pH the mutarotation after 48 h at 15° gave a 90 % recovery (Table I).

Ultracentrifugation at 35° of acid-soluble collagen after heating at 45° in the phosphate solution of pH 11 showed the presence of low molecular weight degradation products (Fig. 3), but a large proportion of material with a relatively high sedimen-

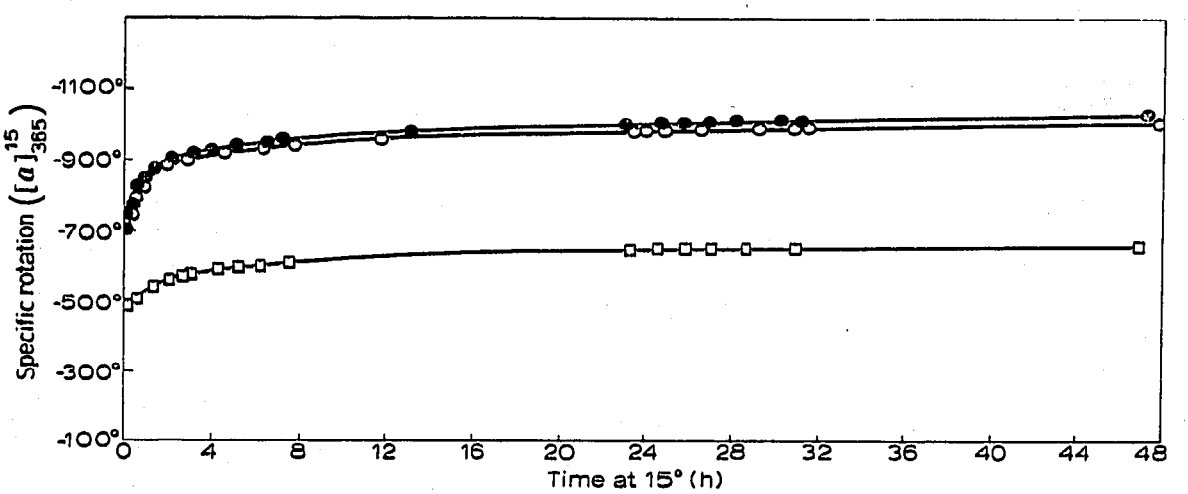


Fig. 2. Specific rotation ($[\alpha]_{365}^{15}$) as a function of time for solutions of acid-soluble collagen (1.40 mg/ml) at various pH values, heat-denatured at 45° for 15 min and then kept at 15°. (●) pH 4.8; (○) pH 8.0; (□) pH 11.0.

TABLE I

MUTAROTATION OF HEAT-DENATURED ACID-SOLUBLE COLLAGEN AT 15° AND VARIOUS pH VALUES

| *pH* | *Specific rotation* | | | |
|---|---|---|---|---|
| | *Initial* | | *After 48 h at 15°* | |
| | $[\alpha]_D^{15}$ | $[\alpha]_{365}^{15}$ | $[\alpha]_D^{15}$ | $[\alpha]_{365}^{15}$ |
| 4.8 | −318° | −1025° | −318° | −1025° |
| 8.0 | −292° | − 998° | −292° | − 998° |
| 11.0 | −206° | − 721° | −198° | − 649° |

tation constant ($S_{20,w} = 2.2$) was still present. This may be compared (Fig. 3) with the sedimentation at 35° of acid-soluble collagen in formate buffer (pH 3.75) after denaturing at 45°. Here the $\alpha$- and $\beta$-subunits are clearly shown ($S_{20,w} = 2.6$ and 3.4, respectively). It is evident from Fig. 3 that the alkaline treatment decomposes the $\beta$-subunit. DOTY AND NISHIHARA[12] and ASTRUP *et al.*[13] report a similar decrease in the $\beta$-subunit at pH values above 9.

While the above results show the effect of high pH on acid-soluble collagen as a whole, in the actual chromatography procedure used (Method 3) the pH gradient only reached high values after the bulk of the acid-soluble collagen had been eluted from the column. Therefore component b was isolated by this chromatography procedure

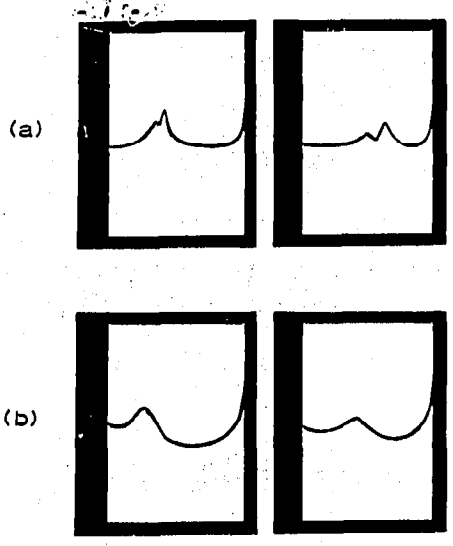


Fig. 3. Sedimentation patterns of (a) heat-denatured acid-soluble collagen in sodium formate buffer, pH 3.75, and (b) heat-denatured acid-soluble collagen in phosphate solution, pH 11.0. The photographs were taken after 80 and 120 min at 56100 rev/min and 35°. The solutions contained 0.4% of protein. Sedimentation is from left to right.

(Method 3), dialysed against several changes of distilled water and freeze-dried. This was then dissolved in potassium acetate buffer (pH 4.8) and its optical rotation measured. The specific rotation of component b ($[\alpha]_D^{15} = -159°$, $[\alpha]_{365}^{15} = -527°$) is only slightly greater than that of collagen in the denatured form ($[\alpha]_D^{15} = -90°$ to $-120°$, $[\alpha]_{365}^{15} = -450°$), showing that component b under the conditions of its preparation has a low helical content in comparison with tropocollagen.

*Chromatographic separation of collagen components by the Spectrochrom method*

A typical separation obtained by the method of PIEZ *et al.*[1] is shown in Fig. 4, where the optical absorption was measured at 230 m$\mu$. The improved resolution using the micro cuvette of the Spectrochrom method is illustrated in Fig. 5. This shows the optical absorption at 220 m$\mu$, due to peptide bonds, and at 278 m$\mu$ from the presence of aromatic amino acids. These results confirm previous findings[4,5] that the method of PIEZ *et al.*[1] does not give a complete picture of the chromatographic components. The components obtained using the Spectrochrom method (Fig. 5) are in many respects similar to those found by the more complicated and longer method of step-wise gradients[5], and by the pH-gradient method (Fig. 1). The distribution of the components, particularly the $\alpha$- and $\beta$-subunits, was studied in detail for the separation

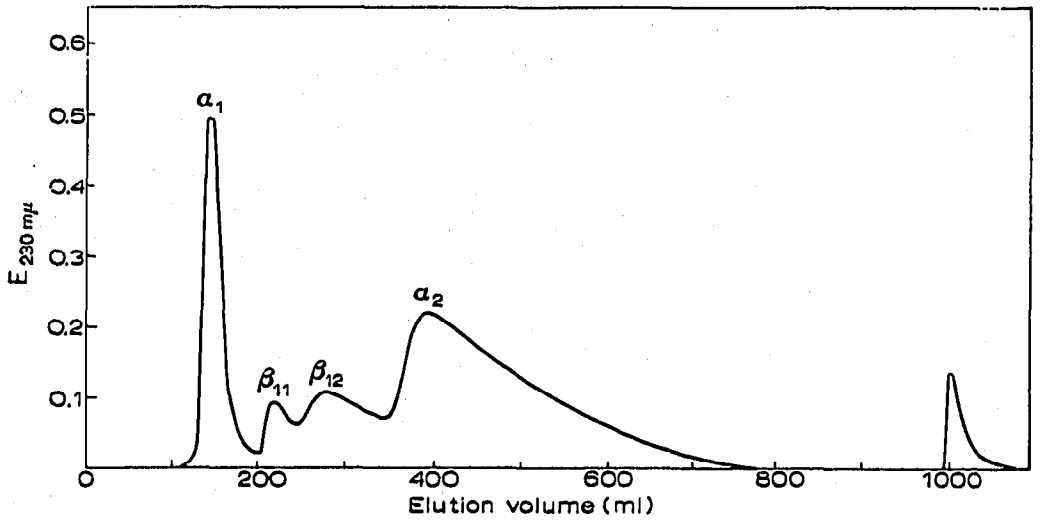


Fig. 4. Chromatography of 50 mg of acid-soluble collagen on CM-cellulose at 40° using a modified PIEZ gradient (see text).

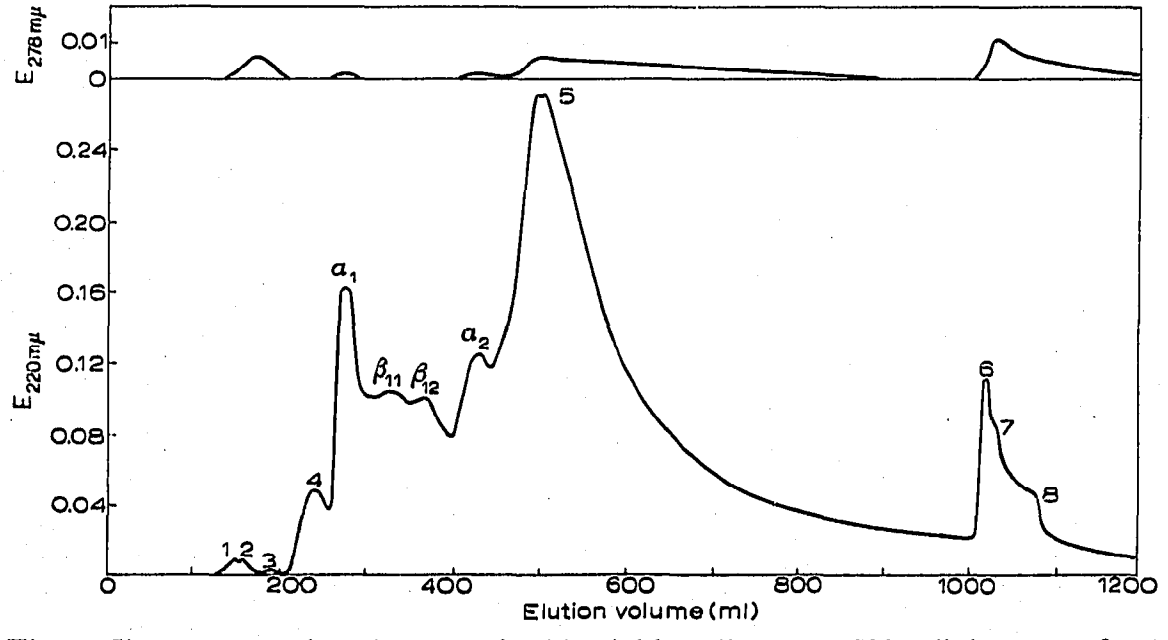


Fig. 5. Chromatography of 50 mg of acid-soluble collagen on CM-cellulose at 40° using the Spectrochrom method (see text). The lower curve represents the optical absorption at 220 m$\mu$ and the upper curve at 278 m$\mu$.

obtained by the method of step-wise gradients[5], and was used here to confirm the allocation of the $\alpha$- and $\beta$-subunits in the separation by the Spectrochrom method. Sedimentation studies have shown the absence of $\gamma$-components[5,8] in the collagen preparations used here. It is suggested that component 4 may correspond to the $\alpha_3$-subunit[14]. Both methods were reproducible and gave similar results for several preparations of neutral salt-soluble and acid-soluble collagen, before and after lyophilisation.

The proportions of the various components are given in Table II. One of the most important features of these data is the relative proportions of components with optical absorption at 230 m$\mu$, which is due to peptide bonds and is therefore a measure

of protein concentration, and the corresponding absorption at 278 mμ due to the presence of aromatic amino acids. Thus the minor components 1, 2, 3, 6, 7 and 8 which constitute only 6 % of the whole preparation contain about 43 % of the aromatic amino acids, while the α- and β-subunits comprise 29 % of the preparation and only contain 8 % of the aromatic amino acids. The major component 5 comprises 63 % of the preparation yet contains only 49 % of the aromatic amino acids. The presence of components similar to 1 to 8 in acid-soluble collagen was found by a completely different chromatography procedure on several preparations of soluble collagen[5].

TABLE II

COMPOSITION OF CHROMATOGRAPHIC COMPONENTS

| *Component* | *% Composition* | | |
|---|---|---|---|
| | PIEZ *method at 230 mμ* | *Spectrochrom method* | |
| | | *At 220 mμ* | *At 278 mμ* |
| 1, 2, 3 | — | 0.5 | 10.8 |
| 4 | — | 2.4 | 0 |
| $\alpha_1$ | 21 | 8.2 | 3.3 |
| $\beta_{11}$ | 18 | 6.5 | 5.0 |
| $\beta_{12}$ | | 5.9 | |
| $\alpha_2$ | 61 | 8.3 | |
| 5 | | 62.9 | 49.3 |
| 6, 7, 8 | — | 5.3 | 31.6 |

Components 1, 2 and 3 eluted close to the void volume of the column, and therefore have little affinity for the CM-cellulose, which is a cation exchanger. They are therefore acidic in nature. On the other hand, components 6, 7 and 8 required a high ionic strength or pH to elute them. These are therefore either polymeric forms[3,5], or are strongly basic in character. Components 1, 2 and 3 were separated by chromatography from the rest of the collagen, evaporated to dryness under vacuum, and then hydrolysed for 24 h at 100° in a sealed tube with 6 *N* HCl. These components were not dialysed against water to remove excess salt before hydrolysis, since there was some doubt as to the size of these polypeptides and therefore they could possibly have been lost on dialysis. Components 6, 7 and 8 were, however, dialysed against several changes of distilled water before being freeze-dried, and hydrolysed for amino acid analysis.

Both preparations were then analysed by high voltage electrophoresis followed by chromatography. Repeat runs showed that components 1, 2 and 3 contained no amino acids detectable by the method used, but that ninhydrin-positive material remained at the origin after electrophoresis and migrated in a long streak after chromatography. The inability to detect amino acids in this preparation was due to the low concentration, arising from the fact that only a few milligrams of this preparation were available (see below). Components 6, 7 and 8 were shown to contain lysine (+ + +), arginine (+ +), histidine (+ + +), alanine (+ + +), glycine (+ +), serine (+), valine (+), leucine (+), glutamic acid (+ +), and aspartic acid (+ +), where the plus signs indicate the intensity of the spots as assessed visually. In addition

TABLE III

AMINO ACID COMPOSITION (RESIDUES/1000 RESIDUES)

| *Amino acid* | *Components 1, 2, 3* | *Components 6, 7, 8* | *Component 9 —corresponding to components 6, 7, 8 corrected for collagen* |
|---|---|---|---|
| Ala | 44.0 | 103.2 | 64.8 |
| Arg | Trace | 47.8 | 66.1 |
| Asp | 122.1 | 47.6 | 51.8 |
| Glu | 191.7 | 95.4 | 136.7 |
| Gly | 263.7 | 307.7 | 237.0 |
| His | Trace | 10.8 | 25.1 |
| Hyl | — | 4.9 | 4.1 |
| Hyp | — | 62.5 | 0.0 |
| Ile | — | 13.0 | 17.4 |
| Leu | — | 24.1 | 26.5 |
| Lys | Trace | 34.5 | 52.9 |
| Met | — | 0.6 | 0.0 |
| Orn | Trace | 16.9 | 46.6 |
| Phe | 72.0 | 12.1 | 14.3 |
| Pro | — | 99.0 | 49.3 |
| Ser | 175.8 | 77.9 | 157.1 |
| Thr | — | 22.5 | 35.3 |
| Tyr | 130.6 | 5.6 | 10.5 |
| Val | — | 13.4 | 4.7 |

similar ninhydrin-positive material as that found in components 1, 2 and 3, but not corresponding to amino acids, was also present.

These two preparations were then analysed quantitatively for their amino acid content. The results are presented in Table III. In agreement with the findings from chromatography components 1, 2 and 3 contain relatively large amounts of aspartic and glutamic acids (313.8 residues/1000 residues) and small amounts of the basic amino acids, thus making these components acidic in nature. The amount of material available made it possible only to detect traces of the basic amino acids. It was also found in the analysis that relatively large amounts of ammonia were present which indicated that the acidic amino acids were present to some extent as amides. Therefore components 1, 2 and 3 could correspond to an acidic telopeptide. Also of significance are the relatively large amounts of glycine, phenylalanine and tyrosine, the latter two accounting for the optical absorption at 278 mμ found in the chromatography.

The analysis for components 6, 7 and 8 shows that these contain hydroxyproline, and therefore collagen. If all the hydroxyproline is assumed to be contained in the collagen, and at the elution position of components 6, 7 and 8 this would correspond to polymeric forms of collagen[5], then the amino acid analysis of components 6, 7 and 8 minus that of acid-soluble collagen corresponds to that shown in Table III. For the sake of clarity this is referred to as component 9. This analysis shows that component 9 contains 194.8 residues/1000 residues of basic amino acids and 188.5 residues/1000 residues of acidic amino acids. The analysis also showed the presence of relatively large amounts of ammonia (1.04 %, w/w amide nitrogen) which would indicate that the acidic amino acids are largely present as amides, thus making component 9 basic in nature. This confirms the deduction made from the chromato-

graphy data. Component 9 contains lower amounts of phenylalanine and tyrosine than components 1, 2 and 3, which is also confirmed by the chromatographic data at 278 mμ (Table II). Therefore components 6, 7 and 8 appear to contain collagen and polypeptides which could correspond to basic telopeptides.

The presence of large amounts of material other than amino acids in component 9, as found by high voltage electrophoresis followed by chromatography, was confirmed by the fact that the amino acid analysis only gave a recovery of 35.2 % of amino acids. The presence of ornithine in these components probably arises from the decomposition of arginine[15], although the hydrolysis conditions used had not previously produced ornithine in studies on collagen and the non-collagenous proteins of skin[8]. This would also account for the relatively large amount of ammonia found in these amino acid analyses.

In the amino acid analysis two unidentified peaks were obtained for both components 1, 2, 3 and 6, 7, 8. These eluted in the positions of glycerophosphoethanolamine and phosphoethanolamine, but still have to be identified. These could account for a portion of the large amount of non-amino acid material shown to be present.

In some respects, notably in the high contents of acidic amino acids, glycine, tyrosine and phenylalanine, the peptide in components 1, 2, 3 resembles the telopeptides obtained from calfskin tropocollagen by proteolytic enzymes[7] and the two cyanogen bromide peptides obtained from ratskin collagen[16]. The latter authors found that the $NH_2$-terminal region of ratskin collagen is unusual in being more acidic than collagen, in lacking hydroxyproline, and in not containing glycine in every third position. By present concepts this region cannot assume the helical configuration characteristic of collagen.

These acidic and basic peptides were found in repeat runs on four different preparations of acid-soluble collagen, and also in the re-chromatography of collagen molecular subunits[5]. As these peptides were present in very small quantities, the possibility that they are impurities in the collagen must be considered. The acid-soluble collagen preparations used in these studies, however, were extracted from skin after the neutral-salt-soluble and non-collagenous impurities had been removed, and the acid-soluble collagen was re-purified by different methods[5,8].

The first component obtained by the phosphate gradient chromatography method (Fig. 1) would appear to correspond to components 1, 2, 3 from the Spectrochrom method (Fig. 5), while components b and c from the phosphate method could correspond to components 6, 7, 8 of the Spectrochrom method. In the latter case the basic nature attributed to these components would agree with this conclusion. Optical rotation studies show that component b still retains some helical nature, which is in accord with the fact that amino acid analysis showed the presence of collagen in components 6, 7, 8.

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