The Formation of Collagen \(\alpha\) Chains in the Absence of Proline Hydroxylation*

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SUMMARY

The collagen synthesized by minced feet of 7- to 10-day-old chick embryos labeled with radioactive proline remained adsorbed to carboxymethyl cellulose columns under the usual conditions for eluting collagen \(\alpha\) and \(\beta\) components. In contrast, the collagen synthesized by 16- to 19-day-old lathyritic minces gave the expected \(\alpha1\) and \(\alpha2\) components under these chromatographic conditions. Such older, lathyritic minces were therefore used to examine the nature of the protocollagen that was synthesized when the hydroxylation of collagen prolyl residues was inhibited by \(\alpha,\alpha'\)-dipyridyl. Unlike the identically prepared collagen, such protocollagen samples failed to give appreciable \(\alpha\) components under the usual chromatographic conditions, but remained adsorbed to the column.

Inclusion of 4 M urea in the chromatographic buffers, however, caused appearance of an elution profile from protocollagen samples identical in position and number of peaks with that given by collagen. These collagen peaks could be shown to be \(\alpha\) components by disc electrophoresis. Two of the peaks from protocollagen were shown by gel filtration to be the same in size as the corresponding two peaks from collagen. These protocollagen species were, therefore, the size of \(\alpha\) chains. The elution position of these two peaks, as well as the effect of injecting \(\beta\)-aminopropionitrile or \(n\)-penicillamine into the embryos prior to preparing the minces, suggested that they represented, respectively, the \(\alpha1\) chain in which the \(\epsilon\)-amino group of a lysine residue had been deaminated to yield an aldehyde (\(\alpha1^{14}\)), and the \(\alpha1\) chain in which the lysine residue was still intact (\(\alpha1^{129}\)). Both peaks gave predominantly the original peak on rechromatography, except that much of the radioactivity remained adsorbed to the column, until eluted by a high concentration of salt. In the case of the \(\alpha1^{129}\) peak, addition of some of the original mince extract to the sample, prior to rechromatography, decreased this adsorption and enabled the original peak to reappear.

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After it became established that the hydroxylation of proline to form the hydroxyproline found in collagen occurred after proline hydroxylation had been incorporated into a polypeptide (2-4), considerable interest and discussion has focused on the nature of this polypeptide (5-7). In a previous report from this laboratory (8), the polypeptide substrate for collagen proline hydroxylase, referred to hereafter as protocollagen, that accumulates when minces of chick embryo are incubated with the hydroxylase inhibitor \(\alpha,\alpha'\)-dipyridyl, was examined by gel filtration measurements, and found to be approximately the size of the \(\alpha\) or \(\beta\) chains of tropocollagen. Subsequent attempts to make this identification more precise by more extensive gel filtration experiments, including recycling, were disappointing and gave an unsatisfactory resolution of \(\alpha\) and \(\beta\) chains. The elegant carboxymethyl cellulose chromatographic procedure developed by Piez, Eigner, and Lewis (9) for the separation of collagen \(\alpha\) and \(\beta\) chains was therefore applied to the study of this protocollagen.

Initially, extreme difficulty in obtaining chromatograms from protocollagen samples was experienced, as noted also by others (10). As detailed below, in order to chromatograph protocollagen, it was necessary to use the feet from older, lathyritic embryos as the source of the minces rather than the whole 8-day-old embryos worked with previously (8), and to modify the procedure of Piez et al. by inclusion of 4 M urea in the chromatographic buffers. This paper is concerned with the identity of the protocollagen peaks obtained from such chromatograms. It is concluded that polypeptide chains similar in size and charge to completed collagen \(\alpha\) chains continue to be synthesized when the hydroxylation of prolyl residues is prevented.

MATERIALS AND METHODS

Ichthyocol was a generous gift of Drs. Olga Blumenfeld and Paul Gallop. Chick embryo collagen was prepared from the tibiae of 19-day-old lathyritic embryos by the method of Lavenne and Gross (11). Gelatin was prepared from both collagens by adding water or buffer and warming for 2 to 3 min at 60°, followed by centrifugation. \(\beta\)-Aminopropionitrile fumarate was a generous gift from Abbott Laboratories or was purchased from Nutritional Biochemicals. \(n\)-Penicillamine-HCl was purchased from Pierce Chemical Company, Rockford, Illinois. Guanidine hydrochloride was Eastman grade from Distillation Products Industries and urea was Mallincrodt A.R.; both were used without purification although recent Cm-cellulose chromatograms eluted with urea that had been passed through the deionizing resin Amberlite MB-3 (Mallincrodt) gave elution profiles similar to those shown in this paper but with Peak 3 (see below) coming off somewhat later.

1 The abbreviation used is: Cm-, carboxymethyl-.
The tissues used in these studies were the feet of 16-day-old Silver Cross chick embryos obtained by cutting the legs just above the ankle joint. Unless otherwise noted, the eggs were incubated on the chorioallantoic membrane on the 14th day with 24 mg of \( \beta \)-aminopropionitrile in 0.1 ml of H\(_2\)O. The extreme fragility of the embryos at Day 16 indicated the effectiveness of this treatment in inducing a lathyritic condition. Penicillamine-treated embryos were injected in the yolk sac on the 12th day with 100 mg of neutralized \( \beta \)-penicillamine-HCl in 0.9\% NaCl; on the 16th day the embryos were not as fragile as the lathyritic embryos, but were markedly more fragile than 0.9\% NaCl-injected controls. The preparations of radioactive protocollagen or collagen were modifications of those described previously (8).

The total incubation mixture for preparing radioactive protocollagen contained a single foot, 6 ml of Krebs-Ringer-phosphate (12) 1.56 mg of \( \alpha,\alpha' \)-dipyridyl, and either 100 \( \mu \)Ci of 3,4-\( ^3 \)H-proline or 15 \( \mu \)Ci of uniformly labeled \( ^{14} \)C-proline (250 \( \mu \)Ci per \( \mu \)mole). The tissue was first minced in a Dounce homogenizer after which it was added to the incubation flask which contained the dipyridyl. After 15 min of incubation at 37\( ^\circ \), the appropriate radioactive proline was added and the incubation was continued for 2 hours. The mince was separated from the medium by centrifuging for 2 min at approximately 5,000 \( \times \) \( g \), and was then extracted by homogenizing in a Dounce homogenizer in 6.5 ml of 0.5\% acetic acid at 3\( ^\circ \). The homogenate was shaken at 3\( ^\circ \) for 1 hour and was then centrifuged for 5 min at 15,000 \( \times \) \( g \). The supernatant was either dialyzed against distilled water or on standing at 3\( ^\circ \) (the material lost from solution could be shown to be present on the walls of the dialysis tubing or glass container). The percentage of the total

| Sample | Amount assayed | \( ^{3} \)H produced | Total counts per min released as \( ^{3} \)H |
|--------|----------------|----------------------|---------------------------------------------|
| Peak 1 | 450            | 4                    | 0.4 (average)                               |
| Peak 2 | 557            | 89                   | 16                                          |
| Peak 3; zero time | 5,560 | 4 | 0.1 |
| Peak 3; incubated | 5,560 | 901 | 15 |
| Peak 3; incubated | 11,120 | 1,662 | 16 |
| Peak 4 | 672            | 120                  | 18                                          |
| Peak 5 | 1,160          | 160                  | 14                                          |
| 5 \( \mu \) guanidine eluate | 6,800 | 1,090 | 10 |

Radioactivity was measured in an ambient temperature liquid scintillation system with a dioxane-based scintillation fluid. Quenching and efficiency were determined by adding \( ^{3} \)H or \( ^{14} \)C standards containing a known number of disintegrations per min to the sample vials. In double label experiments corrections were made for the spillover of \( ^{14} \)C counts into the \( ^{3} \)H channel. With protein samples it was found useful to add sodium dodecyl sulfate to the vials at a final concentration of 1 mg per ml to stabilize the counts which tended to decrease with time. The measurement of the radioactivity in hydroxyproline was done as described previously (4) except that liquid scintillation counting was used.

Protocollagen was assayed by an unpublished method devised in this laboratory, but since the details of a similar method have subsequently been published (13), only a minimal description will be given here. The assay depends on the release of an atom of tritium to water when 3,4-\( ^3 \)H-proline, previously incorporated into the polypeptide backbone of protocollagen, is hydroxylated to yield 4-hydroxyproline. Under the conditions of this assay (excess hydroxylase and limiting protocollagen substrate), the formation of tritiated water is directly proportional to the amount of protocollagen present, as shown in Table I for Peak 3. The source of the hydroxylase was an extract of feet from 16-day-old embryos prepared by blending the tissue in an equal volume of 0.1 M Tris-HCl buffer (pH 7.5), 12 mM KCl, 3.3 mM MgCl\(_2\), 1.3 mM \( \alpha \)-ketoglutaric acid, 2.7 \( \mu \)M ascorbic acid, and 0.1 \( \mu \)M ferrous sulfate. After 2 hours of incubation at 37\( ^\circ \) in 25 ml parafilm capped flasks, the reaction mixtures were transferred to "soldiers" suitable for lyophilizing (type 1 distilling tubes, Burket Glass, Inc., Millville, New Jersey) and 0.06 ml of 70\% HClO\(_4\) was added to stop any further reaction. After the reaction mixture had been lyophilized to dryness, 1 ml of the collected water was added to 10 ml of scintillation fluid and counted. If a sample of protocollagen required dialysis prior to being assayed it was essential to determine the total radioactivity recovered before assay, since with certain Cm-cellulose peaks almost quantitative loss of material could occur on dialysis against distilled water or on standing at 3\( ^\circ \) (the material lost from solution could be shown to be present on the walls of the dialysis tubing or glass container). The percentage of the total
tritium released to water was arbitrarily calculated from the counts per min in the 1 ml counted, rather than from the 1.5 ml of original incubation volume, since the total water recoverable by lyophilization showed the final incubation volume was closer to 1 than 1.5 ml.

Disc electrophoresis was according to the procedure of Sakai and Gross (14) except that 8 M urea was included in all solutions and samples (15). Urea tended to thicken the width of the bands, but was added to raise the recovery of radioactivity from the gels. Samples from the gradient portion of Cm-cellulose chromatograms could be run without dialysis, samples eluted by high concentrations of NaCl or by 5 M guanidine hydrochloride required prior dialysis against 4 M urea starting buffer. Concentration when necessary was effected by putting the sample in dialysis tubing which was then coated with dry Sephadex G-200. To determine the radioactivity of sliced gels, the slices were put in counting vials and covered with 0.5 ml of 3% H2O2. The vials were capped and kept at 55-65° until the gel had dissolved. One milliliter of H2O and 10 ml of scintillation fluid were then added and the vials were counted. Repeated counts were necessary since in some cases the H2O2 caused spurious counts which declined on standing to a stable value.

RESULTS

Breakdown of Collagen and Protocollagen by Mincing of Whole Embryos—Embryonic feet were used in these studies, rather than whole embryos as previously (8), since two lines of evidence indicated that the collagen species extracted from minces of whole embryos were subject to considerable breakdown. First, gel filtration through Sephadex G-200 showed the presence of newly synthesized collagen that was smaller in size than α chains from incubations containing whole 8-day-old embryos, than from incubations of legs and feet. Second, protocollagen extracted from incubations with whole 8-day-old embryos proved to be unstable under the conditions of Piez et al. (9) for Cm-cellulose chromatography (pH 4.8, 42°) as judged by the loss of ability to be enzymatically hydroxylated, whereas protocollagen extracted from incubations with feet was stable under the same conditions. Some protection of the protocollagen synthesized by whole 8-day-old embryos could be obtained by heating the extract to 100° for 5 min, a fact which further indicated that proteolytic activity was probably responsible for the apparent instability.

Anomalous Behavior of Protocollagen during Cm-cellulose Chromatography under Conditions of Piez et al.—The first attempts to chromatograph radioactive collagen on Cm-cellulose under the conditions of Piez et al. (9) were unsuccessful. Extracts from incubations of either whole 8-day-old embryos or of their feet failed to give the expected α or β peaks and almost all of the added collagen remained adsorbed to the columns. Among a variety of prior treatments of these extracts, only incubation with the aqueous extract from whole 8-day-old embryos (originally prepared as a source of proline hydroxylase as described above in connection with the assay of protocollagen) caused the appearance of a radioactive peak from Cm-cellulose columns in the position expected for α1 chains. This procedure, however, was not adopted because incubation with such preparations caused some decrease in size of the collagen species, as judged by gel filtration experiments. The possibility that the collagen from older embryos would show these difficulties less severely was then examined. Comparison of the collagen chromatograms from embryos of various ages from 8 to 20 days produced the generalization that the older the embryo, the higher the probability of getting a normal collagen elution profile on Cm-cellulose chromatography. Better recovery of collagen from such columns was also obtained if the embryos were made lathyritic by prior injection of β-amino-propionitrile with the result that typical lathyritic elution profiles could be obtained from radioactive collagen synthesized by minces of 16- to 19-day-old lathyritic feet, as shown in Fig. 1. The initial effluent, which contained both material which did not adsorb at all to the column, as well as free proline added as a marker, contained little radioactive hydroxyproline. Subsequently, a large α1 peak and a smaller α2 peak (one-third or less of α1) were eluted and, finally, material that stuck still more tightly, was eluted by 5 M guanidine. Disc electrophoresis confirmed the identity of the α peaks. Since repeatedly each of these peaks after the initial eluate (α1, α2, and material eluted by 5 M guanidine) gave approximately the same radioactivity in hydroxyproline to total radioactivity (37% when labeled from 14C proline), they were apparently radiochemically pure collagen. However, when protocollagen, prepared identically except for the presence of dipryridyl in the incubation medium, was chromatographed under these conditions, very low yields of α peaks were obtained, with most of the radioactivity sticking to the column. The difference in behavior between protocollagen and collagen was most clearly seen when they were co-chromatographed, as in Fig. 1. From the ratio (0.09) of radioactive protocollagen to collagen in the sample, the protocollagen peaks (Δ) should be 0.9 the height of the collagen peaks (●), as plotted, whereas it can be seen that the protocollagen present in the α peaks is much less than this (it is barely detectable) and much more of the protocollagen sample is in the 5 M guanidine eluate, relative to the collagen sample.

Chromatography of Protocollagen and Collagen on Cm-cellulose in 4 M Urea—In an attempt to answer whether some kind of aggregate was responsible for protocollagen sticking to these Cm-cellulose columns, pilot runs on Sephadex columns (both G-25 and G-200) were made, with the unexpected finding that under the same conditions much of the protocollagen also stuck to the Sephadex columns and failed to appear in the effluent. When, however, gel filtration was carried out in buffer containing 4 to 8 M urea or 5 M guanidine, the protocollagen could in large part be recovered. Therefore, Cm-cellulose chromatography was tested in the presence of 4 M urea. Under these conditions, typically a 5-peaked pattern, as seen in Fig. 2 for protocollagen (Δ), and collagen (●), was obtained, followed by a final peak when 5 M guanidine was added at the end of the gradient. The first peak is prior to the addition of guanidine, and are referred to throughout this paper as Peaks 1 through 5, respectively, in the order in which they were eluted (in Fig. 2, Peak 4 is barely detectable in the collagen sample and very small, possibly insignificant protocollagen peak just before the guanidine eluate is ignored). The higher radioactive seen in the initial eluate (Peak 1) of the protocollagen sample of Fig. 2, relative to the collagen sample, was not found consistently; since these initial peaks were low in protocollagen, as judged by hydroxylase assay, as well as in hydroxyproline in the case of collagen samples, they apparently represented mostly noncollagenous material. Every peak beyond Peak 1, however, including the guanidine eluate, consistently contained quite radiochemically pure collagen or protocollagen (see below). Columns calibrated with ichthyocal gelatin or lathyritic gelatin from chick embryo tibiae showed that α1 chains were eluted in the region of Peak 3, and α2

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The fraction size was approximately 2 ml, of which 0.2 ml was counted. The protocol collagen sample contained 181,000 cpm and the collagen sample 1,999,000 cpm. An additional 75 ml of 5 m unlabeled proline (5.8 mg) was added as an internal marker. Against starting buffer, heated for 3 min at 60°C, and centrifuged. Two standard incubation mixtures (each incubation was equivalent to 4 ml-incubations) were mixed after dialysis for protocollagen and collagen. The relative heights of the peaks also varied between collagen and protocollagen samples labeled from 3,4-3H-proline were incubated with proline hydroxylase. The direct proportionality between the 3HOH produced in this assay and the amount of protocollagen present is illustrated by Peak 3 in Table I. As also shown in this table, the initial peak from 4 m urea chromatograms was very low in protocollagen. Every subsequent peak, however, including the material that required more drastic conditions to be eluted consisted largely of protocollagen. A figure of approximately 15% for the fraction of counts per min released as water was given so consistently by a variety of protocollagen samples as to suggest that it represents the ratio for radiochemically pure protocollagen under the conditions of this assay. Since this ratio is calculated from the counts per min in the 1-ml sample of water that was counted, whereas the original incubation volume was 1.5 ml, the true yield of tritiated water is closer to 1.5 × 15% or 22% of the total radioactivity, a figure in excellent agreement with the theoretical maximum yield from protocollagen that is totally unhydroxylated pure collagen, on the assumption that the susceptible prolines represent about 0.44 of the total prolines (16) and that they release 50% of their tritium on hydroxylation (13).

In the case of collagen also, Peak 1 contained little collagen, as judged by its low content of radioactive hydroxyproline, whereas all peaks after Peak 1 contained radiochemically pure collagen, as judged by the ratio of the counts per min in hydroxyproline to the total, a result completely analogous to that obtained from chro-
Identification of Collagen Peaks 2, 3, 4, and 5 from Chromatography in 4 M Urea as α Chains by Disc Electrophoresis—Although this technique could not be applied to the protocollagen peaks directly (see below), they could be shown by gel filtration to be the same size as the corresponding collagen peaks. The identification of the latter was therefore important in characterizing these protocollagen species.

The belief, based on the behavior of these collagen samples on chromatograms developed under the conditions of Piez et al., that at least the major early and late radioactive peaks from collagen chromatograms in which 4 M urea was included in the buffers (Fig. 2, ■), were, respectively, α1 and α2 chains, was confirmed by disc electrophoresis (Fig. 3). In fact, both of the two radioactive peaks appearing in the α1 region (Peaks 2 and 3) and both of the peaks in the α2 region (Peaks 4 and 5) were α chains, as shown by the presence of the major part of the recovered radioactivity in the α area of the gel. A sample of lathyritic chick embryo gelatin is included at the top of the figure to show where cr (the larger band) and ot2 moved in this system; the smaller amount of the faster moving ot2 component, this preparation. The stained bands in the gels containing

FIG. 2. Co-chromatography of 14C-protocollagen (●) and H-collagen (●) on Cm-cellulose in 4 M urea. The samples were from the same extracts used in Fig. 1, and similar amounts were taken. After dialysis against the starting buffer of Piez et al. (9), and heating for 3 min at 60°, solid urea was added to a calculated molarity of 4 (since an increase in volume occurred as a result of this addition, the actual molarity was somewhat less). The samples were combined and centrifuged to remove any insoluble material. Further procedures were as given in the legend to Fig. 1. The graph shows the counts per min in the volume of sample that was counted, which was 0.1 ml through Fraction 146, after which 0.05 ml- aliquots were counted. The protocollagen sample contained 540,000 dpm and the collagen sample 6,993,000 dpm. The recoveries were 67 and 89%, respectively.

Matography of collagen under the conditions of Piez et al., as described above.

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FIG. 3. Disc electrophoresis of collagen fractions obtained by chromatography on Cm-cellulose in 4 M urea. The bands that appeared after staining with Amido black are shown, with the fainter ones being stippled. The gels were sliced as indicated after staining and the radioactivity of each slice was determined. The percentage of the total eluted radioactivity present in each slice is indicated. The total recoveries, expressed as counts per min and as percentage of original sample, for Peaks 2 through 5 were, respectively, 815 cpm, 52%: 344 cpm, 47%; 415 cpm, 62%; and 292 cpm, 76%. The lathyritic chick embryo collagen shown in the top gel and the Peak 2 sample were subjected to electrophoresis in the same run for 1 hour at room temperature, as were Peaks 4 and 5 after the addition of 1 μg of ichthyocol to each sample. The Peak 3 sample was subjected to electrophoresis in a separate run for 2 hours at 3°, so significance should not be attached to the difference in migration compared to the top two gels. In fact, the stained bands in the gels containing Peak 2 and Peak 3 represent endogenous α chains in both cases, since numerous experiments have shown that the only major stained bands seen in collagen samples from these lathyritic embryos are α components (compare top gel). The Peak 2 sample (0.1 ml) represented approximately 0.01 of the Peak 2 from chromatography of 1.6 X a standard 14C-protocollagen and 0.3 X a standard 1H-collagen incubation mixture; Peak 3 (0.15 ml) was from a single 2-ml fraction (Fraction 25) from the Peak 3 region of the same chromatogram. In both gels the recoveries of counts per min in tritium were too low to be significant. Peaks 4 and 5 contained the equivalent of 0.3 ml (concentrated to 0.1 ml) of Fractions 64 and 68, the approximate peak fractions of Peaks 4 and 5 from a chromatogram in which 5-ml fractions were collected. The sample put on this latter column consisted of the extract from two-thirds of a standard 1H-collagen incubation mixture, mixed with 20 mg of ichthyocol gelatin; the α2 peak of the latter was eluted slightly ahead of the radioactive Peak 4 from the chick embryo collagen.

Peak 2 and Peak 3 were from the endogenous α1 chains present in the lathyritic feet used in the incubation; as would be expected, these chains were eluted from Cm-cellulose columns in the area of Peaks 2 and 3. The stained bands of the lower two gels, which contained added ichthyocol gelatin as a marker, show the γ components at the start of the running gel, the β components (stippled), and the two α components, α1 and α2; the narrow, fastest moving band did not correspond to a peak of radioactivity and appeared to be a degradation product of the ichthyocol. Peaks 4 and 5, which had been judged to be α2
The reasons for this difference in behavior from collagen are not to be removed from solution on dialysis or on storage. Similarly identical conditions by electrophoresis, such poor recoveries of collagen peaks obtained from Cm-cellulose columns under grams and their content of radioactivity relative to Peaks 2 and 3, both from the elution position on Cm-cellulose chromatography than the CrL Peaks, 2 and 3.

When attempts were made to separate and estimate the procollagen peaks obtained from Cm-cellulose columns under identical conditions by electrophoresis, such poor recoveries of radioactivity were obtained that it was impossible to establish the size of the radioactive material in those peaks by this method. The reasons for this difference in behavior from collagen are not known, although they may be related to the greater tendency of procollagen to stick to Cm-cellulose or Sephadex columns and to be removed from solution on dialysis or on storage. Similarly it has not been possible to date to identify by this technique the size of the collagen or procollagen components eluted at the end of the gradient by high concentrations of NaCl in 4 M urea or by 5 M guanidine hydrochloride.

Comparison of Protocollagen and Collagen by Gel Filtration—Since the molecular size of the Cm-cellobiose procollagen peaks could not be estimated by acrylamide disc electrophoresis because of their poor recoveries, an estimate of the size of Peaks 2 and 3 was obtained by comparing their behavior on Sephadex G-200 with the corresponding collagen peaks (Fig. 4). In the experiments illustrated, 3H-protocollagen and 14C-collagen samples were co-chromatographed on Cm-cellobiose in 4 M urea and the indicated peaks were subjected to gel filtration. The single peak (Fractions 59 through 75) obtained from gel filtration of Peak 2 contained 85% and 98%, respectively, of the recovered procollagen and collagen. Fractions 90 through 172 were pooled and found to contain negligible radioactivity. The total recoveries from this column were 92 and 65%, respectively; the lower recovery of procollagen was not unexpected in view of its greater tendency to stick to Cm-cellobiose columns and to dialysis tubing or glass. When the Peak 3 from the same Cm-cellobiose chromatogram as this Peak 2 was subjected to gel filtration, 76 and 92%, respectively, of the recovered procollagen and collagen were present together in a peak as narrow as shown for Peak 2 (total recoveries were approximately 60 and 100%). The Peak 3 sample, from a different chromatogram, shown in Fig. 4, again indicated that Peak 3 consisted predominantly of a single size species of collagen and procollagen and that this procollagen species was the same size as the collagen species. In these samples, however, there was evidence for some breakdown, since only 48 and 69% of the recovered activity of protocollagen and collagen, respectively, were in the single peak; the rest was smeared throughout the later fractions. In all three gel filtration experiments described here the absorbance at 232 m\mu (not shown) was also found to give a single peak that coincided with the radioactive peak. This absorbance was presumably due in part to endogenous \( \alpha \) chains.

The finding by gel filtration that procollagen Peaks 2 and 3 were the same size as collagen Peaks 2 and 3, coupled with the evidence from disc electrophoresis that these collagen peaks were \( \alpha \) chains, showed that these procollagen peaks also consisted of \( \alpha \) chains.

**Effect of \( \beta \)-Aminopropionitrile and of \( \beta \)-Penicillamine on Synthesis of Peaks 2 and 3—**Peaks 2 and 3 seemed likely to be the two forms of \( \alpha \) chains previously reported by Pfea et al. (17) to be detectable on Cm-cellobiose chromatograms, and which these workers showed to “differ in that a lysyl residue in one (\( \alpha 1^{\text{syn}} \)) is present as an aldehyde in the other (\( \alpha 1^{\text{Lys}} \)).” Since \( \alpha 1^{\text{Lys}} \) was found to elute later than \( \alpha 1^{\text{syn}} \), as expected from its possession of an extra positively charged \( \epsilon \)-amino group, Peaks 2 and 3 would correspond to the \( \alpha 1^{\text{Lys}} \) and \( \alpha 1^{\text{syn}} \), respectively. Although the embryos used in the present study had all been made lathyritic by injection of \( \beta \)-aminopropionitrile which inhibits the conversion of \( \alpha 1^{\text{syn}} \) to \( \alpha 1^{\text{Lys}} \) (18), appreciable Peak 2 material was frequently present. However, when \( \beta \)-aminopropionitrile (10 mm) was included in both the incubation medium and in the 0.5 M acetic acid used for extraction of procollagen, as well as being injected into the eggs, it was possible to obtain considerably increased yields of Peak 3 and decreased yields of Peak 2, as shown in Fig. 5. In this chromatogram the yield of Peak 3 was 28% of the input radioactivity and Peak 2 was present only as a
small shoulder on Peak 1. The apparent absence of α2 peaks may have been due to the addition of 5 M guanidine before they had been eluted; the significance, if any, of the second peak in the guanidine eluate has not been pursued, although such a peak was frequently seen. Unfortunately, for reasons that are still undiscovered, it was not possible to recover such an elevated Peak 3 from every β-aminopropionitrile-containing protocollagen incubation, some of which gave chromatograms with little peak 2 or Peak 3 and with almost all of the recovered radioactivity in the guanidine eluate; a number of times one sample of an extract gave a chromatogram such as shown in Fig. 5 and a sample treated identically except for its storage (either frozen or at 3°) stuck to the column and required guanidine for its elution. (Similar decreases in Peaks 2 and 3 were seen equally frequently in extracts from standard protocollagen incubation mixtures to which β-aminopropionitrile had not been added.) Since, however, protocollagen chromatograms with an exceptionally large Peak 3 and low Peak 2 have been repeatedly obtained from incubations containing β-aminopropionitrile and have not been obtained in its absence (compare Fig. 2), the evidence is suggestive that the presence of β-aminopropionitrile is responsible for the elevated Peak 3, by preventing its conversion to Peak 2 and to possibly more cross-linked material.

The effect of n-penicillamine on Peaks 2 and 3 is less well documented, but is described here, since it was of the type expected from the report (19) that it causes accumulation of an elastin rich in α-aminoadipic δ-semialdehyde, the product of deamination of the lysine ε-amino group. A recent report (20) indicates that the collagen extracted by neutral salt solutions from n-penicillamine-treated rats also has an elevated aldehyde content. If the assignment of Peak 2 as α141 is correct, one might expect this peak to be increased by penicillamine. When a 3H-collagen preparation from a penicillamine-injected embryo was co-chromatographed with a 14C-collagen preparation from a β-aminopropionitrile-injected embryo (the two incubations were otherwise identical (and standard) and were done together), the yield of Peak 2 from the lathyritic embryo was 3% of the input, compared to 4.5% in the penicillamine-treated mince; in Peak 3 the yields were, respectively, 21 and 18%; and in the guanidine eluate the yields were 25 and 32%, respectively. The increase in Peak 2 due to penicillamine was modest (1.5-fold), but significant in view of the increase in the ratio of Peak 2 to Peak 3. In addition, the isolated Peak 2 represented a minimum estimate of that originally present in the extract, since this peak tended to be unstable and to give rise to material that appeared in the 5 M guanidine eluate. Thus, another portion of the same lathyritic 14C-collagen preparation, but dialyzed for an additional 4 hours against 4 M urea starting buffer at room temperature, failed to give any Peak 2, although the yield of Peak 3 remained constant. Attempts to block the loss of Peak 2 by adding 10 mM penicillamine to the incubation mixture were complicated by the finding that this level of penicillamine also inhibited proline hydroxylase.

Rechromatography of Cm-cellulose Peaks—Both Peak 2 and Peak 3 derived from collagen samples ran true on rechromatography under the original conditions. (The further characterization of Peaks 4 and 5, including their behavior on rechromatography, remains to be done.) In these experiments the fact that free proline, added as a marker, happened to elute in the same position as Peak 2 proved very useful since Peak 2 material on rechromatography gave a major peak coinciding with proline, whereas the major peak from Peak 3 samples appeared later than proline. Recoveries were never very high (about 60% was the maximum), apparently due in part to breakdown of the original peak, as indicated by the presence of radioactivity in other fractions, and to irreversible adsorption to the column. The latter phenomenon may be seen in Fig. 6 (●), where only 17% of the original radioactive Peak 3 collagen sample was recovered in Peak 3 and 27% was eluted by 5 M guanidine; the missing radioactivity (56%) was still stuck to the column.

Protocollagen Peaks 2 and 3 were also found to run true on rechromatography, but they seemed to show even more severely the problems of breakdown and, particularly, adsorption, described above for collagen, so that sometimes on rechromatography no radioactivity was recovered except in the 5 M guanidine eluate. The only difference between the original chromatography and the rechromatography appeared to be that the other components of the original extract were present in the sample put on the first column but were absent from the isolated Peak 2 or 3 sample put on the second column. Therefore, the effect of

Fig. 5. Cm-cellulose chromatography of 3H-protocollagen from an incubation containing β-aminopropionitrile. This compound was included in a standard incubation mixture and in the 0.5 M acetic acid used for extraction of the incubated mince, at a concentration of 10 mM. The column was loaded with the entire extract from the standard incubation, which contained 5.11 X 10⁴ dpm, in 8.3 ml (this volume resulted from rinsing the dialysis tubing in which the extract was dialyzed with 2 to 3 ml of warm starting buffer). The recoveries in Peaks 1 and 2 together (Fractions 8 to 15), in Peak 3 (Fractions 29 to 34) and in the guanidine-eluted peaks (Fractions 132 to 169) were, respectively, 11.5%, 26% and 32%. An additional 3% was found on either side of Peak 3 (Fractions 16 to 28, 35 to 43), bringing the total recovery to 72%. The slight difference in elution position of this Peak 3 and of those in Fig. 6 was most probably due to the presence of contaminating salts in the urea which caused slight variations in the final pH and ionic strength of the starting buffer; if the urea was deionized first (see "Materials and Methods"), the Peak 3 was eluted later. Peak 3 was frequently eluted in a slightly different place on rechromatography and, similarly the elution position of the Peak 3 from the same batch of unbleached chick embryo gelatin showed this much variation on different chromatograms. These observations indicated that the variation in elution position was due to the chromatographic conditions and not to differences between the various samples of Peak 3.
adding back the original amount of extract, but from a nonradioactive incubation, to a Peak 3 sample prior to rechromatography was tested, with the result that such additions were found to increase considerably the recovery of the original peak. The Peak 3 protocollagen sample shown in Fig. 6 (△) was first rechromatographed in the absence of unlabeled extract (not shown) and gave no recovery in the Peak 3 area; in fact the radioactivity eluted from the column was recovered in the 5 M guanidine fractions, and amounted to a total recovery of 14%. The same sample in the presence of unlabeled extract (Fig. 6, △) gave 39% recovery in the original Peak 3 position (with an additional 46% recovery in the guanidine eluate). Experiments with small columns (4 ml; 1.3 cm, internal diameter) confirmed the greater tendency of Peak 3 protocollagen samples to stick to Cm-cellulose compared to Peak 3 collagen samples and showed that sufficient unlabeled extract (2 ml) either mixed with the sample or added just ahead allowed quantitative recovery of Peak 3 material from these small columns under the original elution conditions.

**DISCUSSION**

Although the conclusion had been reached earlier, both in this laboratory (8) and according to Kivirikko and Prockop (10), that material approximately as large as α chains was made when hydroxylation of proline was inhibited, the present studies provide a considerably more precise identification of at least two of these protocollagen species. In view of the evidence presented above, it is clear that hydroxylation may be severely inhibited without preventing the formation of α chains. The extent of hydroxylation of the protocollagen after subsequent incubation with hydroxylase, indicates that hydroxylation was effectively inhibited under these conditions, a conclusion also reached earlier by measuring the content of radioactive hydroxyproline before and after incubation with hydroxylase (8). Although further work is needed to identify with certainty Peak 2 as α(L), by measurement, for example, of its content of α-aminoadipic δ-semialdehyde, the circumstantial evidence presented here makes this assignment appear very plausible. It seems, therefore, that the oxidation of at least some lysine residues to form aldehydes, can also proceed in the absence of hydroxylation.

It is tempting to suppose that the radioactive material that remains adsorbed to the Cm-cellulose columns after α and β components have been eluted, consists in part of more highly cross-linked species. Isolated Peak 3 samples on rechromatography may, however, adsorb tightly to these columns in a similar manner, and simply mixing such samples with some of the original unfractionated mince extract can decrease this adsorption. Similarly, although not reported above, addition of such extracts to material that originally required 5 M guanidine hydrochloride or 2 M NaCl in 4 M urea to be eluted causes most of the radioactivity of these samples to elute earlier in the area of α and β components.

These facts indicate the need for further work to clarify these observations, and they suggest caution in concluding that the material which sticks tightly to these columns is necessarily covalently cross-linked. The recent identification of hydroxylysine as a participant in two types of covalent links between collagen chains (21) suggests the possibility that inhibition of hydroxylation would interfere with cross-linking.

The reasons for the greater tendency of protocollagen relative to collagen to adsorb tightly to Cm-cellulose, and the means by which urea lessens this tendency, are unknown. This tendency appears, however, to be an intrinsic property of the protocollagen, since it is exhibited by isolated Peak 3 samples as well as by the original extracts before chromatography.

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