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Physicochemical Characterization and Molecular Organization of the Collagen A and B Chains†

R. Kent Rhodes and Edward J. Miller*

ABSTRACT: Collagen containing the A and B chains has been isolated and purified from pepsin digests of human placenta, bone, and cartilage using convenient procedures which allow maximum recovery of the collagen in highly purified form. In addition, phosphocellulose chromatography has been employed to achieve complete resolution of the A and B chains following denaturation of the collagen. Utilizing these procedures, it has been observed that the stoichiometry of the A and B chains in collagen prepared from the different tissues is quite variable. The chain ratios for A and B chains in the collagen derived from placenta and bone are 1:1 and 1:1.6, respectively. On the other hand, the collagen derived from cartilage lacks the A chain but contains significant quantities of B chain. Molecular weight estimates for the chains, as determined by agarose molecular sieve chromatography and sodium dodecyl sulfate–polyacrylamide gel electrophoresis, indicate that the B chain is approximately 10% larger than the A chain. Thermal denaturation studies on collagen containing the A and B chains from placenta indicate that the melting transition is characterized by two distinct phases. Examination of the collagen denatured during the initial phase showed that it was comprised largely of A chains, whereas the collagen remaining in native conformation at this time was comprised largely of B chains. It is concluded from these data that the A and B chains exist in separate molecular species with the chain compositions $[A]_3$ and $[B]_3$ being the most likely and most prevalent forms.

Information on the genetic diversity of mammalian collagens has recently been extended by reports from this laboratory (Chung et al., 1976) and others (Burgeson et al., 1976) of new collagenous components. Preliminary characterization of the new chains, provisionally designated as the A and B chains, showed that each chain was approximately the size of α components derived from interstitial collagens. Nevertheless, the new chains differed markedly with respect to compositional features from the constituent chains of the known interstitial collagens. Moreover, though significant differences between the two chains exist, they both resemble chains derived from isolated basement membranes in their amino acid content. Such features include the presence of relatively large amounts

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of 3-hydroxyproline, relatively low levels of alanine, and elevated levels of hydroxylysine. The importance of the A and B chains and the collagen molecules in which they originate may be reflected in their apparent widespread distribution in a variety of connective tissues (Chung et al., 1976). Therefore, we have extended our earlier studies to further examine the physicochemical characteristics and molecular organization of these two collagen chains.

A major factor in the course of this work has been the improvement of techniques used for isolation and purification of the molecules containing these chains. These improved procedures have allowed the recovery of larger quantities of material. In addition, a new chromatographic system has been developed which achieves complete resolution of the two chains in any preparation and allows a more precise estimate of the chain stoichiometry. Use of these new procedures, then, has allowed us to resolve several questions concerning the A and B chains and the molecules from which they are derived. We report here that the A and B chains of Chung et al. (1976) are apparently identical to the αA and αB chains isolated by Burgeson et al. (1976). Moreover, more accurate estimates of the proportion of these chains occurring in several tissues as well as several other lines of evidence strongly suggest that the A and B chains originate in separate molecules.

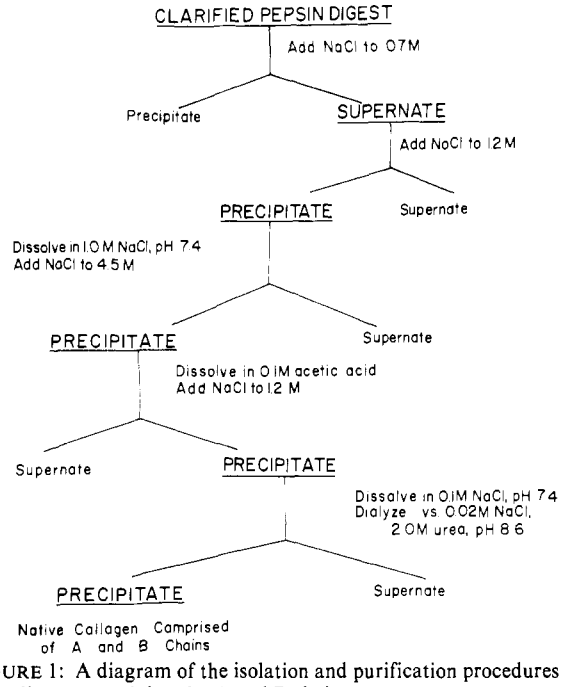

FIGURE 1: A diagram of the isolation and purification procedures used for collagen containing the A and B chains.

Materials and Methods

Isolation of Collagen. For the present study, collagen molecules containing the A and B chains were isolated chiefly from human placenta, infant calvaria, and infant epiphyseal cartilage. Due to the presence of varying proportions of other collagenous and noncollagenous substances, the tissues were processed in various ways prior to extraction of the collagen. All procedures were carried out at 4 °C. Whole placental tissue was minced and sequentially extracted with water, 1.0 M NaCl (0.05 M Tris,[1] pH 7.5), and 0.5 M acetic acid essentially as previously described for preparations of dermis (Epstein et al., 1971). Slices of cartilage were initially extracted with 4.0 M guanidinium hydrochloride (0.05 M Tris, pH 7.5), thoroughly washed with water, and then equilibrated with 0.5 M acetic acid (Miller et al., 1976). Pieces of calvaria were decalcified by dialysis vs. 0.5 M acetic acid (Miller et al., 1967). The tissue remaining following these preliminary extractions was then incubated with pepsin for 24 h. The usual incubation mixture was comprised of 20 g of tissue (wet weight) suspended in 100 mL of 0.5 M acetic acid containing 50 mg of pepsin (pepsin A, Worthington Corp.). Pepsin digests were centrifuged for 30 min at 48 000*g* to remove undigested debris and the supernatant solution was adjusted to 0.7 M NaCl by addition of crystalline NaCl. The solution was left standing for 24 h during which time a precipitate formed. This precipitate which contained largely the well-characterized interstitial collagens was removed by centrifugation. The NaCl concentration of the supernatant was increased to 1.2 M NaCl by further addition of crystalline NaCl. The solution again was allowed to stand for 24 h after which time the resulting precipitate was recovered by centrifugation. The latter precipitate was redissolved in 1.0 M NaCl (0.05 M Tris, pH 7.4). Undissolved material was removed by centrifugation and the collagen reprecipitated by increasing the NaCl concentration to 4.5 M. The precipitate was dissolved in 0.1 M acetic acid and reprecipitated at 1.2 M NaCl. Subsequently, the precipitate was redissolved in 0.1 M NaCl (0.05 M Tris, pH 7.4) and dialyzed against several changes of 0.02 M NaCl containing 2.0 M urea (0.01 M Tris, pH 8.6). The precipitate which formed on dialysis vs. the latter solvent was collected by centrifugation, redissolved in 0.5 M acetic acid, dialyzed extensively against 0.5 M acetic acid, and lyophilized. The lyophilized material was then used for all subsequent procedures. The purification procedures described above are summarized in Figure 1.

Phosphocellulose Chromatography. Dry phosphocellulose (Whatman P-11, Whatman, Inc.) was passed through a series of standard sieves, and particles falling in the 140–230 mesh range (U.S. series equivalent) were collected. The particles were washed in an excess of 0.5 N NaOH for 30 min, followed by rinsing with distilled water until the pH of the rinse solution reached 8.0. The particles were then collected by filtration in a Buchner funnel and resuspended in an excess of 1 N HCl for 30 min. Successive washings with water were performed until the pH of the rinse reached 4.0, at which time the particles were equilibrated with starting buffer, 0.03 M dibasic sodium phosphate adjusted to pH 6.3 with phosphoric acid. Lyophilized material containing native molecules comprised of the A and B chains was dissolved in starting buffer and denatured by warming to 43 °C for 30 min. Samples were then applied to a 1.5 × 9 cm column of phosphocellulose equilibrated with starting buffer and maintained at 43 °C by means of a circulating water bath. Elution of the sample from the column was achieved at a flow rate of 100 mL/h by employing a linear gradient from 0.0 to 0.2 M NaCl in starting buffer over a total volume of 400 mL. The column effluent was continuously monitored in the range of 222–230 nm by a Beckman DB-GT spectrophotometer and recorded by means of a Beckman 10-in. linear recorder. Fractions of approximately 5 mL were collected in an automatic fraction collector.

Molecular Sieve Chromatography. Molecular weight estimates on the chains resolved by phosphocellulose chromatography were obtained by rechromatography on a calibrated 1.5 × 150 cm column of agarose beads (Bio-Gel A-5m, 200–400 mesh, Bio-Rad Laboratories) as previously described (Chung et al., 1974). Samples containing the isolated chains were dissolved in 1.0 M $CaCl_2$ (0.05 M Tris, pH 7.5) and applied to the column which was eluted with the same solvent at a flow rate of 7.0 mL/h.

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

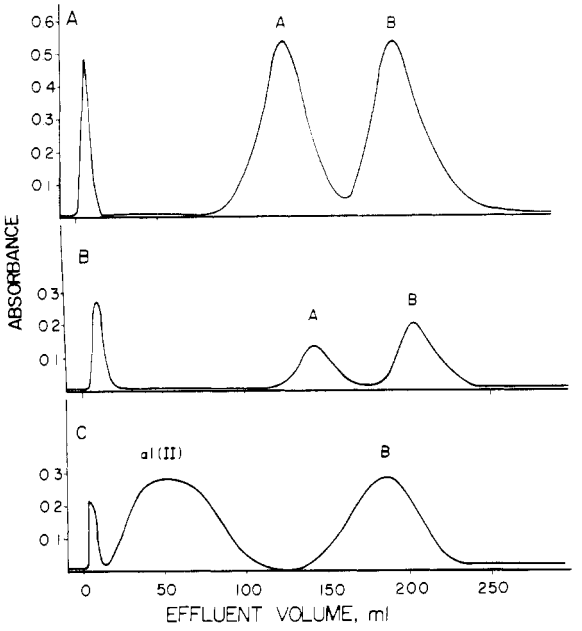

FIGURE 2: Phosphocellulose chromatograms illustrating the chromatographic properties of collagens derived from various tissues and purified as outlined in Figure 1. Samples were prepared for chromatography by denaturing at 43 °C, and elution was achieved as described in the text: (A) 20 mg of collagen prepared from placenta (column effluent monitored at 224 nm); (B) 8 mg of collagen prepared from calvaria (column effluent monitored at 230 nm); (C) 15 mg of collagen prepared from cartilage (column effluent monitored at 228 nm). The peak eluting at the front in each chromatogram represents highly absorbant noncollagenous material.

Amino Acid Analyses. Amino acid analyses were performed on acid hydrolysates of the isolated chains by means of a Beckman Model 119 automatic amino acid analyzer as described previously (Miller, 1972). Calculations of the amino acid analyses were adjusted for loss of threonine, serine, and tyrosine and incomplete release of valine using previously determined correction factors for collagen (Piez et al., 1960).

Polyacrylamide Gel Electrophoresis. $NaDodSO_4$ gel electrophoresis was performed using a previously described procedure (Furthmayr and Timpl, 1971). Samples of lyophilized native collagen or isolated chains recovered from phosphocellulose chromatography were dissolved in 0.01 M sodium phosphate, pH 7.2, containing 5.0 M urea and 0.2% $NaDodSO_4$ and denatured at 43 °C for 30 min. Electrophoresis of 10–30-μg samples was performed on 5% acrylamide gels (0.5 × 60 mm) in 0.1 M sodium phosphate containing 0.1% $NaDodSO_4$, pH 7.2, at 6 mA/gel. The samples were run until the Bromophenol Blue tracking dye rached the end of the gel at approximately 4 h. Gels were removed and stained for 1 h in a solution of 50% methanol and 5% acetic acid containing 0.25% Coomassie blue. Destaining was done by diffusion in 7% acetic acid.

Polarimetry. Optical-rotation measurements were performed using a Perkin-Elmer Model 241 polarimeter. Native collagen containing the A and B chains as well as standard preparations of pepsin-solubilized human dermal type I collagen (Chung and Miller, 1974) were dissolved in 0.5 M acetic acid at a concentration of approximately 1.0 mg/mL. Optical rotation was measured at 310 nm using a 1-dm jacketed cell with a mercury arc lamp as the light source. Thermal-denaturation curves were generated by stepwise temperature increases from 20 to 45 °C, allowing 30-min equilibration time at each temperature.

Denaturation Studies. In an attempt to more clearly define the significance of results obtained in the polarimetry studies, samples containing the A and B chains in native conformation were incubated in 0.5 M acetic acid for 1 h at various temperatures within the range at which loss of helicity occurs. Immediately upon removal from the water bath, the samples were rapidly cooled to 4 °C and the NaCl concentration was brought to 1.2 M by the addition of crystalline NaCl. The samples were allowed to stand overnight at 4 °C after which any precipitate which formed was collected by centrifugation and redissolved in 0.5 M acetic acid. The solution of precipitated material as well as the supernatant fluid were dialyzed vs. several changes of 0.5 M acetic acid and then lyophilized. The lyophilized material was then chromatographed on phosphocellulose and the proportions of the chains present in the precipitated material as well as the supernatant were estimated by planimetry of the peaks.

TABLE I: Recovery of Collagen at Various Stages of Purification.

fraction	% of solubilized collagen		
	placenta	calvaria	cartilage
initial pepsin digest	100[a]	100[a]	100[a]
0.7 M NaCl ppt	84	96	96
1.2 M NaCl ppt (1st)	14	4	4
4.5 M NaCl ppt	14	4	4
1.2 M NaCl ppt (2nd)	12	3	3
0.02 M NaCl ppt, pH 8.6	6	3	3

[a] The proportion of total tissue collagen present in the initial pepsin digest is 60% for placenta, 70% for calvaria, and 75% for cartilage.

Results

Recovery of Collagens. Utilizing the purification procedures outlined in Figure 1, it was noted that approximately 6, 3, and 3% of the total collagen solubilized by limited pepsin proteolysis from placenta, bone, and cartilage, respectively, over a 24-h interval could be recovered in the fraction containing collagen comprised of the A and B chains (Table I). These estimates of recovery in various fractions are based on hydroxyproline determinations in acid hydrolysates of aliquots of the initial pepsin digest, the precipitates and supernates obtained on raising the NaCl concentration of the initial digest to 0.7 M, as well as the precipitates obtained during each step of the purification procedures. It is noteworthy that recovery of collagen containing the A and B chains is considerably facilitated by performing the initial precipitation of collagen from the pepsin digest at 0.7 M NaCl as opposed to higher NaCl concentrations as used in previous studies (Chung et al., 1976; Burgeson et al., 1976). At 0.7 M NaCl, essentially complete separation of type I, II, and III collagens from collagen containing the A and B chains is achieved. However, at 0.9 M NaCl, a significant proportion of collagens containing the A and B chains is precipitated along with the other collagens. Optimum recovery of collagen is therefore achieved following precipitation of the type I, II, and III collagens at the lower ionic strength. In addition, we observed no alterations in the chromatographic or electrophoretic behavior of the A and B chains derived from collagen obtained at the various stages of purification, indicating that the molecules were not substantially altered by nonspecific proteolysis during the lengthy purification procedures.

Phosphocellulose Chromatography. When chromatographed in denatured form on phosphocellulose, collagen prepared from placenta in the manner outlined in Figure 1 is resolved into two components, designated A and B chain (Figure 2A). On a routine basis, about 85% of the material applied to the column is recovered in these peaks, and the

TABLE II: Amino Acid Composition of the Components Recovered after Phosphocellulose Chromatography.[a]

amino acid	A chain[b]		B chain[b]			$\alpha 1$(II)[b]
	placenta	calvaria	placenta	calvaria	cartilage	cartilage
3-Hyp	3	2	5	5	5	1
4-Hyp	106	107	110	104	106	93
Asp	50	51	49	50	50	43
Thr	29	28	21	22	22	21
Ser	34	34	23	22	23	27
Glu	89	87	100	95	101	92
Pro	107	106	130	135	127	122
Gly	331	335	332	335	334	335
Ala	54	54	39	40	39	102
Val	27	29	17	19	16	17
Met	11	11	9	7	9	9
Ile	15	13	17	17	16	10
Leu	37	36	36	37	38	25
Tyr	2	2	4	3	4	1
Phe	11	11	12	11	12	13
Hyl	23	21	36	34	36	16
Lys	13	14	14	15	15	20
His	10	9	6	7	6	2
Arg	48	50	40	42	41	51
total	1000	1000	1000	1000	1000	1000

[a] The values presented represent averages of values obtained in at least four separate preparations of each component under conditions in which the coefficient of variation for each amino acid was 3.0%. [b] Residues/1000 total residues.

elution pattern indicates that the components are present in approximately equal proportions. This was confirmed not only by planimetry but by determinations of the amounts of material recovered after desalting and lyophilization of appropriate fractions containing the individual peaks. In some preparations the amount of A chain present actually exceeded the amount of B chain by as much as 10%, as indicated by peak area and amount of material recovered from the column. In contrast, material prepared from calvarial bone consistently chromatographs on phosphocellulose as indicated in Figure 2B. In this case, the amount of A chain present is always lower than the amount of B chain. The two chains are present in a ratio of about 1:1.6, as judged from planimetry measurements as well as determination of the amount of material recovered from each peak. The material prepared from cartilage chromatographs on phosphocellulose in yet another fashion (Figure 2C). One major component is apparently more acidic than the first peak found in extracts of placenta or bone and elutes significantly earlier in the gradient. This has been identified as $\alpha 1$(II) (see below). In addition, there is an apparent absence of A chain, whereas approximately one-half the collagen is eluted in the position corresponding to B chain. Slight variations in the actual elution position of the chains in the various preparations are due to differences in column packing and minor fluctuations in flow rate during elution.

Amino Acid Analyses. Amino acid analyses were performed on each of the peaks recovered from phosphocellulose. These data are presented in Table II. The analyses for A chain prepared from placenta and calvaria agree quite well and are likewise very similar to previously published analyses for A chain derived from dermis (Chung et al., 1976). Similarly, the analyses for B chain from placenta, calvaria, as well as cartilage indicate that the chain derived from these sources is indeed the same. Moreover, these analyses confirm the general compositional features previously established for the B chain derived from dermis (Chung et al., 1976). It should be noted that neither chain contains cysteine. In addition, analyses of the more acidic component recovered from preparations of cartilage collagen allowed its identification as $\alpha 1$(II) based on comparison with previous studies on $\alpha 1$(II) chains derived from human tissues (Miller and Lunde, 1973).

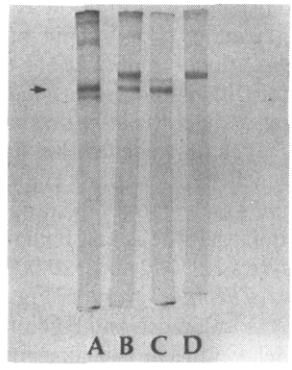

FIGURE 3: $NaDodSO_4$–polyacrylamide gel electrophoresis of: (A) a standard preparation of pepsin-solubilized type I collagen, (B) a preparation of collagen containing the A and B chains from placenta, (C) a sample of A chain from placenta following chromatography on phosphocellulose, (D) a sample of B chain from placenta following chromatography on phosphocellulose. The arrow indicates the position of $\alpha 1$(I) in gel A.

$NaDodSO_4$ Gel Electrophoresis. In order to more carefully assess the value of the isolation and chromatographic procedures, samples containing either the A and/or B chains derived from placenta as well as a standard preparation of type I collagen were electrophoresed on $NaDodSO_4$–polyacrylamide disc gels. In Figure 3, gel A presents the electrophoretic pattern of denatured pepsin-solubilized type I collagen, which is shown to be comprised largely of $\alpha 1$(I) and $\alpha 2$ chains along with smaller amounts of β and γ components. Gel B is an electrophoretic pattern of denatured collagen which contains the A and B chains prior to phosphocellulose chromatography. Gels C and D, then, present electrophoretic patterns of the A and B chains, respectively, following resolution of these components by phosphocellulose chromatography. These data clearly indicate that the A and B chains are recovered after phosphocellulose chromatography in essentially pure form. A minor component present in the mixture of A and B chains (gel B) chromatographs in the A chain fraction during phosphocellulose chromatography (gel C). This component appeared in

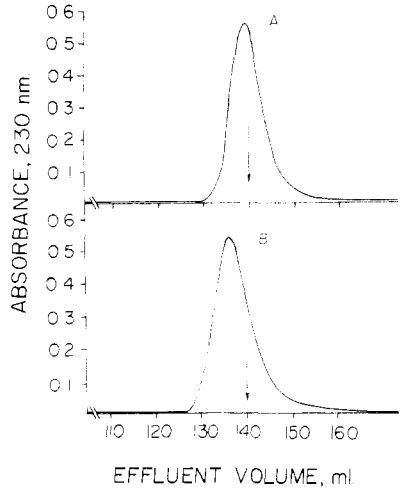

FIGURE 4: Agarose molecular-sieve chromatography (Bio-Gel A-5m) of A chain (upper panel) and B chain (lower panel) recovered following phosphocellulose chromatography of placenta collagen. The arrow indicates the elution position of collagen $\alpha 1$(I), $\alpha 2$, $\alpha 1$(II), or $\alpha 1$(III) chains.

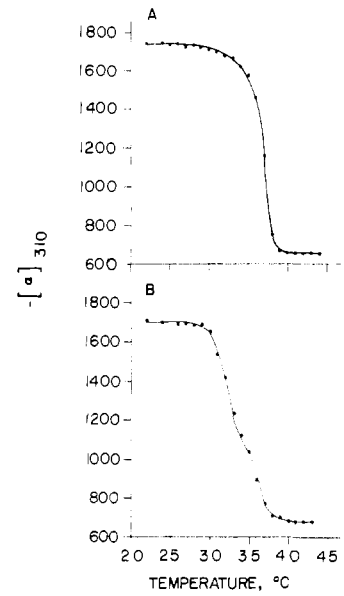

FIGURE 5: Thermal-denaturation curve of pepsin-solubilized human type I collagen (A) and collagen containing the A and B chains derived from placenta (B). The collagens were dissolved initially in 0.5 M acetic acid at a concentration of 1 mg/mL, and the curves were generated during stepwise increases in temperature over the indicated range.

approximately one-half the preparations of collagen from placenta and was never present when the collagens were prepared from bone. Since its presence did not alter the amino acid composition of A chain preparations (Table II), we have concluded that it represents a form of the A chain which migrates slower than the majority of A chain material due either to variable posttranslational modifications or to incomplete proteolysis with pepsin. In addition, it was noted that the major A chain band migrates under the indicated conditions slightly slower than the $\alpha 1$(I) chain. On the other hand, the B chain is retarded significantly relative to $\alpha 1$(I) or A chains. Using known collagen chains and peptides as standards, it was estimated from these data that the A and B chains exhibit a molecular weight of about 105 000 and 120 000, respectively.

Molecular Sieve Chromatography. Figure 4 shows the elution patterns of the isolated A and B chains when chromatographed on Bio-Gel A-5m. The A chain (Figure 4, top) elutes at the same position as an $\alpha 1$(I), $\alpha 2$, or $\alpha 1$(II) chain (arrow), indicating an apparent molecular weight of 95 000 as determined by molecular-sieve chromatography. However, B chain is consistently eluted somewhat earlier in a position corresponding to a molecular weight of approximately 105 000 (Figure 4, bottom). Thus, although the molecular weights determined by agarose chromatography were approximately 10% lower for each chain, an apparent difference in molecular weight was still obvious.

Polarimetry. Melting curves for both a pepsin-solubilized type I collagen sample and collagen containing the A and B chains from placenta were generated by measuring optical rotation during stepwise increases in temperature over the range of 20–45 °C. The preparation of type I collagen exhibits a characteristic sharp transition from helical to denatured form with a melting temperature of about 37 °C (Figure 5A). Although collagen containing the A and B chains exhibits virtually identical initial and final specific optical rotations when compared to preparations of type I collagen, such samples do not exhibit a sharp transition in optical rotation as a function of temperature. Indeed, in five different preparations of this collagen, a definite deflection in the melting curve was observed in the 33–35 °C range (Figure 5B), suggesting that at least two separate molecules with somewhat different melting points are present.

Denaturation Studies. The apparent presence of at least two molecular species with different melting temperatures in the

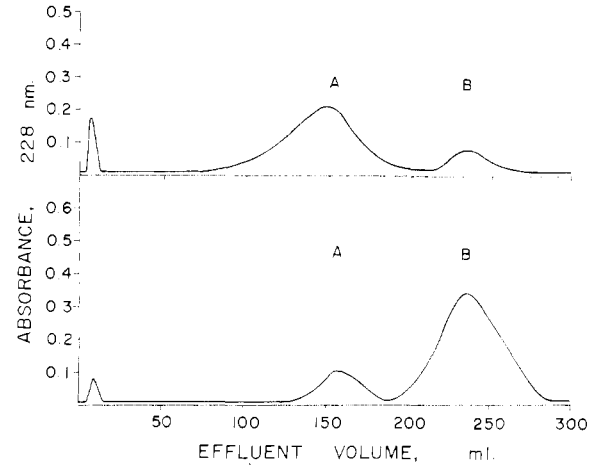

FIGURE 6: Phosphocellulose elution pattern of the collagen denatured (upper panel) and remaining native (lower panel) on incubation of collagen containing the A and B chains for 1 h at 33 °C.

placental collagen samples containing the A and B chains suggested that partial denaturation of the material could provide a means of separating the molecular forms. This was based on preliminary observations, indicating that when such samples were completely denatured by incubation at 44 °C for 1 h in 0.5 M acetic acid the completely denatured collagen could no longer be precipitated on cooling to 4 °C by the addition of NaCl to a concentration of 1.2 M. Therefore, samples were incubated for 1 h in the range of 33–35 °C, cooled rapidly to 4 °C, and immediately adjusted to 1.2 M NaCl. The precipitate that formed over the subsequent 24-h interval was collected by centrifugation, redissolved in 0.5 M acetic acid, and dialyzed extensively vs. 0.5 M acetic acid and lyophilized. The supernatant solutions collected following centrifugation were likewise extensively dialyzed vs. 0.5 M acetic acid and lyophilized.

Figure 6 (top) depicts the phosphocellulose elution pattern of material which could not be precipitated from samples preincubated at 33 °C and was therefore presumably dena-

tured at the latter temperature. When compared to the starting material (depicted in Figure 2A), the elution pattern of this fraction, which represents approximately 40% of the incubated collagen, shows a considerable enrichment in A chain components. These results, then, strongly suggest the presence of molecules with the chain composition $[A]_3$ which exhibit a relatively low-melting temperature. In confirmation of this interpretation, Figure 6 (bottom) depicts the phosphocellulose elution pattern of material which was precipitable from a sample preincubated at 33 °C. This fraction accounted for the remainder of the collagen in the sample and clearly contains a comparable enrichment in B chains, suggesting the presence of molecules with the chain composition $[B]_3$ which have a higher melting temperature than the $[A]_3$ molecules.

Discussion

The present investigations have established that collagens containing the A and B chains comprise a significant and readily retrievable proportion of the pepsin-solubilized collagen from a variety of tissues. In this regard, these studies have led to a practical and somewhat simplified method for recovery and purification of these collagens by taking advantage of the fact that the initial selective precipitation at 0.7 M NaCl effectively removes the more abundant interstitial collagens (types I, II and III) from solution, thus facilitating purification of molecules containing the A and B chains. However, it should be noted that our data indicate (Figure 2C) that a small proportion of type II collagen from cartilaginous tissues is not removed by these procedures and apparently remains with molecules comprised of B chains in all subsequent steps.

Subsequent purification of molecules containing the A and B chains involves precipitation by increasing the NaCl concentration of the digest to 1.2 M and redissolving the precipitate in a neutral salt solvent (1.0 M NaCl, 0.05 M Tris, pH 7.4). These two steps respectively provided a method of separating the molecules containing the A and B chains from highly aggregated, largely noncollagenous substances and inactivated any remaining pepsin activity. The collagen dissolved in the neutral salt solvent may then be reprecipitated at relatively high ionic strength (4.5 M NaCl). This similarity in precipitation behavior between the molecules described here and the molecules comprised of what Burgeson et al. (1976) designated as αA and αB indicates that the A and B chains are identical to αA and αB. Following precipitation from a neutral salt solvent at high ionic strength, we found it advantageous to redissolve the collagen in an acidic solution (0.1 M acetic acid) and perform an additional precipitation from this type of solvent, adjusting the ionic strength to 1.2 M NaCl. Final purification of the collagen was achieved on redissolving the collagen in a neutral salt solvent (0.1 M NaCl, 0.05 M Tris, pH 7.4) and dialysis vs. a low ionic strength buffer at slightly alkaline pH (0.02 M NaCl containing 2.0 M urea, 0.01 M Tris, pH 8.6). Under these conditions, the collagen was precipitated and small quantities of residual noncollagenous glycoprotein remained in solution.

The present studies have also led to the development of a new chromatographic system employing phosphocellulose for the resolution of the A and B chains. The indicated procedures allow complete resolution of these chains and thus represent a considerable improvement over techniques employing carboxymethylcellulose as utilized in previous reports (Chung et al., 1976; Burgeson et al., 1976).

In addition to the advances in methodology described above, the present report provides considerable information regarding the molecular organization of the A and B chains. Thus, the relative proportions of these chains in collagen isolated from different tissues are quite variable. They occur in approximately a 1:1 ratio in extracts of placenta and in a 1:1.6 ratio in extracts of bone, whereas substantial quantities of B chain unaccompanied by detectable levels of A chain are recovered from extracts of cartilage. The absence of A chain in the collagen preparations derived from cartilage cannot be attributed to selective denaturation of A chain molecules during preliminary extraction of the cartilaginous tissues with denaturing solvents such as 4.0 M guanidinium hydrochloride at neutral pH (Miller et al., 1976), since identical preliminary extractions of placenta or bone tissue did not alter the recovery of A and B chains from the isolated collagens. Based on what is known concerning the molecular organization of collagen in molecules comprised of three individual chains, it is evident that the chain ratios observed in these studies are not compatible with the view that the A and B chains originate from a single molecular species of collagen as suggested in earlier studies (Burgeson et al., 1976). Indeed, the evidence presented here strongly favors the concept that these chains originate from two molecular species with the chain composition $[A]_3$ and $[B]_3$.

Support for this view may be derived from additional evidence presented here on the thermal denaturation of collagen containing the A and B chains. In this regard, chick type I and II collagens exhibit somewhat different melting profiles in 0.5 M acetic acid (Miller, 1971), and a biphasic thermal-denaturation curve is observed when equimolar mixtures of these collagens are studied under the same conditions used for collagen containing the A and B chains. In conjunction with the polarimetry studies, one must also consider the results obtained following partial denaturation of the samples. Although complete separation of two molecular species was not obtained, it was clear that the A chain is the predominant chain derived from the collagen denaturing during the initial portion of the melting transition and that the collagen which apparently remains native at this time is comprised predominantly of B chains. These data, then, are compatible with the notion that these chains occur in separate molecules and that the $[A]_3$ molecule exhibits a lower melting temperature than the $[B]_3$ molecule.

It is noteworthy that the studies presented here strongly suggest that the B chain is significantly larger than the A chain. This conclusion is based on molecular-weight estimates derived from $NaDodSO_4$–polyacrylamide gel electrophoresis as well as agarose molecular sieve chromatography. Although collagenous components may show anomalous migration behavior in either of these systems, the molecular-weight estimates discerned by agarose molecular-sieve chromatography have been substantiated in studies on the cyanogen bromide peptides derived from each chain, which indicate that the B chain contains approximately 100 more amino acid residues in a typical collagen-like sequence than the A chain (R. K. Rhodes and E. J. Miller, manuscript in preparation).

Initial reports of the occurrence of these two collagenous components have already stimulated investigation into their distribution and possible biological roles. We have confirmed in this report the earlier observations that the distribution and relative proportions of the A and B chains present in tissue extracts are a function of the tissue under investigation (Chung et al., 1976). In addition, recent results have indicated that smooth-muscle cells in culture synthesize A and B chains (Mayne et al., 1978). Moreover, recent studies indicating that molecules comprised of A and B chains are localized in muscle endomysium (Duance et al., 1977) agree with our previous suggestion (Chung et al., 1976) that the molecules arise from certain basement-membrane-like structures. Definitive information concerning the prevalence, distribution, and phys-

iological roles played by these collagens will be the subject of future investigations.

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Chemical and Physical Properties of Aequorin and the Green Fluorescent Protein Isolated from *Aequorea forskålea*†

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ABSTRACT: The calcium-sensitive photoprotein aequorin and the green fluorescent protein were isolated and purified from *Aequorea forskålea*. Purified aequorin shows electrophoretic microheterogeneity but appears as a single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Amino-terminal sequence determination by the automated Edman technique revealed a single NH_2-terminal sequence: Val-Lys-Leu-(Thr)-Pro-Asp-Phe-Asn-Asn-Pro-(−)-Trp-Ile-Gly-Arg-His-aequorin exists as a single polypeptide chain. Apparent molecular weights were determined by sedimentation equilibrium, urea–sodium dodecyl sulfate/polyacrylamide gel electrophoresis, gel filtration of native protein, and gel filtration of denatured protein in 6 M guanidine hydrochloride; all methods suggest an apparent molecular weight of 19 500 ± 2000 for aequorin. Under some conditions the protein dimerizes by disulfide bond formation. Aequorin has a sedimentation coefficient of 2.31 S and a Stokes radius of ~19 Å; the extinction coefficient ($E_{1cm}^{1\%}$) was calculated to be 27.1. Amino acid analysis revealed a slight preponderance of acidic residues; no carbohydrate moieties were found. Aequorin contains at least one free sulfhydryl group, chemical modification of which results in irreversible loss of luminescent activity. The apparent molecular weight of the green fluorescent protein is 30 000 when determined by urea–sodium dodecyl sulfate/polyacrylamide gel electrophoresis and gel filtration of denatured protein in 6 M guanidine hydrochloride. Amino acid analysis revealed an absence of tryptophan.

The jellyfish *Aequorea forskålea* exhibits a bright green bioluminescence when stimulated chemically or physically. The biochemical system responsible for the bioluminescence comprises two proteins which occur in close association in vivo, aequorin and a green fluorescent protein. Aequorin has the property of emitting light when exposed to calcium ions (Shimomura et al., 1962, 1963a,b; Shimomura & Johnson, 1969) and requires other exogenous cofactors. The light emitted by aequorin is distinctly blue, however, and the green light seen in vivo is probably a consequence of transfer of energy from aequorin to the green fluorescent protein.

The intriguing properties of this system have attracted the interest of two types of investigators, those interested in the phenomenon of bioluminescence per se and those wishing to utilize the protein as a biological calcium indicator (Blinks et al., 1976). Irrespective of specific interests, however, the physicochemical properties of the protein must clearly be known. Of these probably the most fundamental is molecular weight. Yet, despite a number of studies from several laboratories (Shimomura et al., 1962; Shimomura & Johnson, 1969; Blinks et al., 1969; Hastings & Morin, 1969; Kohama et al., 1971), there has been no agreement on the molecular weight of aequorin.

Published estimates of the molecular weight of aequorin fall into two groups: those near 20 000 and those near 30 000. Thus

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