Characterization of the Collagen Chains Synthesized by Cultured Smooth Muscle Cells Derived from Rhesus Monkey Thoracic Aorta†

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ABSTRACT: Five different collagen chains and one smaller collagenous fragment have been isolated from the collagens found in the combined cell layer and medium of rhesus monkey aortic smooth muscle cell cultures. The collagen chains which can be identified are α1(III), α1(I), α2, A and B. The smaller collagenous peptide exhibits an apparent molecular weight of 45 000 and has been designated CP45 (Mayne, R., et al. (1977), *Arch. Biochem. Biophys. 181*, 462). Smooth muscle cells continue to synthesize the collagens from which these components are derived for at least eight passages in culture. At each passage the α1(III) chain consistently represents about one-half of the total collagen which is recovered after initial fractionation by agarose gel chromatography. The results show that smooth muscle cells derived from rhesus monkey thoracic aorta are phenotypically stable for many generations in vitro.

The synthesis of collagen by cultures of aortic smooth muscle cells has been demonstrated both morphologically (Jarmolych et al., 1968; Ross, 1973) and biochemically (Faris et al., 1976). Several analyses of smooth muscle cell cultures specifically for collagen types have shown the synthesis of both type I and type III collagens, with the synthesis of type I collagen usually exceeding that of type III collagen (Layman and Titus, 1975; Layman et al., 1977; Leung et al., 1976; Barnes et al., 1976; Rauterberg et al., 1977). Recently, Burke et al. (1977) and Scott et al. (1977) have fractionated the procollagens isolated from the medium of monkey and porcine aortic smooth muscle cell cultures, respectively, and reported the isolation of relatively more type III procollagen than type I procollagen. Burke et al. (1977) showed that the isolation of collagen after incubation with pepsin at 15 °C to convert procollagen species to collagen resulted in an underestimation of the relative amount of type III collagen when compared with type I collagen. Mayne et al. (1977) isolated collagens from guinea pig aortic smooth muscle cell cultures after incubation with pepsin at a lower temperature (4–7 °C) and reported the isolation of approximately equal proportions of type I and type III collagens. In addition, a collagenous peptide with an apparent molecular weight of 45 000 (designated CP45[1]) was also isolated, which appeared to be a specific marker for the smooth muscle phenotype, since it could not be isolated from cultures of dermal or striated muscle fibroblasts. The latter results were obtained only on examination of cells in the first passage from the initial outgrowth. Later passages showed a profile of collagen biosynthesis more typical of a fibroblast population with low type III relative to type I collagen, and little or no synthesis of CP45.

In the present paper, we present a detailed analysis of the collagen chains which can be isolated from cultured smooth muscle cells derived from rhesus monkey thoracic aorta. The results demonstrate the continued synthesis for many passages both of an excess of type III collagen relative to type I collagen, together with the continued synthesis of CP45. In addition, the synthesis of two more collagen chains has been demonstrated, which are similar, if not identical, to the A and B chains described by Chung et al. (1976) or the αA and αB chains isolated from human fetal placental membranes by Burgeson et al. (1976). Recent experiments using the intact human placenta and comparing the isolation procedures for A and B with those described for αA and αB suggest that these chains are the same (Rhodes, Gay, and Miller, manuscript in preparation), and throughout this manuscript these chains will be referred to as A and B.

Materials and Methods

Materials. Minimum essential medium (Earle's salts), fetal calf serum, glutamine, and antibiotic–antimycotic mixture were obtained from Grand Island Biological Co. The radioactive precursor [2-^{3}H]glycine (11.7 Ci/mmol) was purchased from New England Nuclear Corp. Collagenase (form III) was purchased from Advance Biofactures Corp., Lynbrook, N.Y. Carrier human type I and type III collagens were prepared either from fetal skin or placenta by previously published procedures (Chung and Miller, 1974). Carrier type II collagen was prepared from pepsin-digested chicken sterna as described previously (Mayne et al., 1975).

Cell Culture. Smooth muscle cell cultures were initially prepared as outgrowths from small strips of the inner media of the thoracic aorta of young rhesus monkeys (*Macaca mullata*) essentially as described by Ross (1971). Cells were grown in the presence of minimum essential medium, 10% fetal calf serum, 1% glutamine, and 1% antibiotic–antimycotic mixture, and after 4–5 weeks were passaged using Ca^{2+}- and Mg^{2+}-free saline and trypsin (0.125%) for 30–45 min. Further passages of the cells were carried out every 2–3 weeks by the same procedure with a dilution factor at each passage of 1:10. At the sixth passage some cells were placed in liquid nitrogen at a concentration of 2×10^6 cells/mL of complete medium which contained additionally 10% glycerol. Collagen analyses were performed between the 2nd and 8th passages. Human skin fibroblasts were obtained as outgrowths from a biopsy of a young adult male and were grown for five passages before being stored in liquid nitrogen. The cells were grown in conditions identical with the monkey smooth muscle cells, and

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[1] Abbreviations used: CP45, a collagenous peptide of apparent molecular weight 45 000; Tris, tris(hydroxymethyl)aminomethane; CNBr, cyanogen bromide; CM-cellulose, carboxymethylcellulose.

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collagen analyses were performed between the 6th and 9th passages. In all experiments radioactive labeling procedures were carried out at the high densities of cells achieved after growth for 2–3 weeks. All cultures were labeled for 24 h with complete medium containing [2-^{3}H]glycine (50 μCi/mL) in the presence of β-aminopropionitrile (100 μg/mL) and ascorbic acid (50 μg/mL).

Isolation of Collagen from Cell Cultures. In most experiments collagen was isolated from the combined cell layer, medium, and wash as described in previous publications (Mayne et al., 1975, 1977), with the exception that pepsin was present at concentrations of either 100 μg/mL or 1 mg/mL. Occasionally, the medium and the cell layer were analyzed separately in which case human type I collagen (0.1 mg/mL) was dissolved in the medium before precipitation with ammonium sulfate (25% saturation). In some experiments radioactively labeled collagen was isolated in the presence of both type I and type II carrier collagens (1 mg/mL each), and the collagen was subsequently fractionated by differential salt precipitation (Trelstad et al., 1972). After precipitation of both collagens with ammonium sulfate, the precipitate was redissolved in 10 mL of 1.0 M NaCl, 0.05 M Tris-HCl, pH 7.5. Type I collagen was then selectively precipitated by dialysis at 4 °C against 2.6 M NaCl, and type II collagen was subsequently precipitated by dialysis against 4.4 M NaCl.

Molecular Sieve Chromatography. Collagen chains were isolated by chromatography on a column of agarose beads as previously described (Mayne et al., 1977), with the exception that the column was eluted with 1 M $CaCl_2$, 0.05 M Tris-HCl, pH 6.5 (Piez, 1968). Molecular weights of CNBr peptides derived from isolated collagen chains were determined on a standard column of agarose beads as described previously (Mayne et al., 1977).

Peaks obtained after molecular sieve chromatography were desalted either by elution with 0.1 M acetic acid from a column of Bio-Gel P-2 (Bio-Rad Laboratories), or by dialysis against several changes of 0.5 M acetic acid, followed by lyophilization.

CM-Cellulose Chromatography. Further fractionation of peaks obtained after molecular sieve chromatography was carried out in the presence of carrier type I collagen (10 mg) using one or other of the two procedures previously described (Mayne et al., 1977).

CNBr Cleavage. Labeled and carrier collagen chains eluted from either molecular sieve or CM-cellulose columns were mixed with an additional 50 mg of type I or type III carrier collagens, and cleavage at methionyl residues was achieved as previously described (Mayne et al., 1977).

Reduction and Alkylation. Labeled α1(III) chains or CP45 obtained after agarose chromatography were reduced with 2-mercaptoethanol and subsequently alkylated with iodoacetic acid (Mayne et al., 1977). The samples were then applied directly to a calibrated agarose column (Bio-Gel A-5m) for a determination of molecular weights.

Collagenase Digestion. Samples of CP45 were incubated with bacterial collagenase, and the reaction mixture was subsequently applied directly to an agarose A-5m column as previously described (Mayne et al., 1977).

Polyacrylamide Gel Electrophoresis. The procedure of Furthmayr and Timpl (1971) was used, and sections (1 mm) of the gels were prepared using a Gilford Aliquogel fractionator. Each gel section was subsequently dissolved in hydrogen peroxide (30%, w/w, 55–60 °C, 18 h) and a radioactivity determination made of the solution.

Radioactivity Determination. These were performed as described in an earlier publication (Mayne et al., 1977).

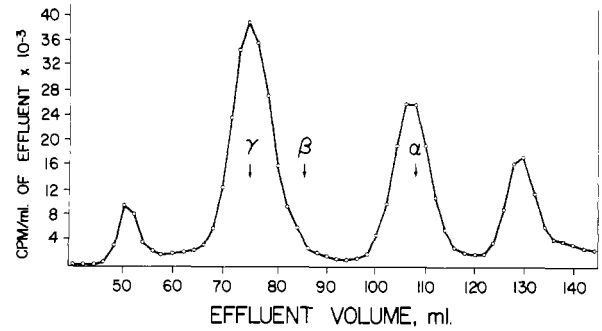

FIGURE 1: Agarose (Bio-Gel A-5m) molecular sieve elution pattern of radioactively labeled collagen isolated in the presence of pepsin (1 mg/mL) from the combined cell layer and medium of cultures of monkey smooth muscle cells at the 4th passage. The column (1.5 × 155 cm) was eluted with 1 M $CaCl_2$, 0.05 M Tris-HCl, pH 6.5, at a constant flow rate (7.6 mL/h). Arrows designate the elution positions of γ, β, and α components.

Results

Figure 1 depicts a representative elution pattern of [^{3}H]-glycine-labeled collagen isolated from smooth muscle cell cultures when chromatographed in denatured form on a column of agarose beads. As in earlier work with guinea pig smooth muscle cells (Mayne et al., 1977), the separation of collagen into three peaks was achieved by this procedure, the three peaks consisting of a γ component (mol wt 285 000), α chains (mol wt 95 000) and the collagenous peptide of apparent mol wt 45 000 designated CP45. Each of these peaks together with carrier collagen was subsequently desalted, lyophilized, and then subjected to further analysis.

Characterization of the γ Component. Previous studies in which cells or tissues were labeled in the presence of β-aminopropionitrile, and the collagen subsequently isolated by limited proteolysis with pepsin at low temperature, have shown that type III collagen is extracted entirely as a γ component consisting of three disulfide-bonded α1(III) chains (Chung et al., 1975; Mayne et al., 1977). The γ component isolated from monkey smooth muscle cell cultures also consisted entirely of type III collagen and this was shown in several ways. Reduction and alkylation of the γ component followed by rechromatography on agarose A-5m showed a quantitative conversion to material eluting as α chains (data not shown). Rechromatography of the γ component on CM-cellulose in the presence of type I carrier collagen showed a single peak of radioactivity eluting in the region of β12 components (data not shown). Previous studies have shown that type III collagen from human (Chung and Miller, 1974; Chung et al., 1974; Epstein, 1974; Seyer et al., 1976) or monkey (Burke et al., 1977) will elute at this same general position. Finally, the labeled γ component was cleaved with CNBr together with 50 mg of human type III carrier collagen, and the cleavage products were chromatographed on CM-cellulose (Figure 2). Good agreement was observed between the radioactivity and absorbance patterns, except that monkey α1(III)CB5 and α1(III)CB6 appear to coelute. This was confirmed by a molecular weight determination of the labeled peptides present in this region of the chromatogram. Two peptides were found of molecular weights 19 000 and 8000, which compare closely with published values of 20 000 for α1(III)CB5 and 8000 for α1(III)CB6 isolated from human Type III collagen (Chung et al., 1974).

Characterization of the α Chains. Initial attempts were made to fractionate the α chains by CM-cellulose chromatography in the presence of carrier human type I collagen. Four peaks of radioactivity were always present, two of which coe-

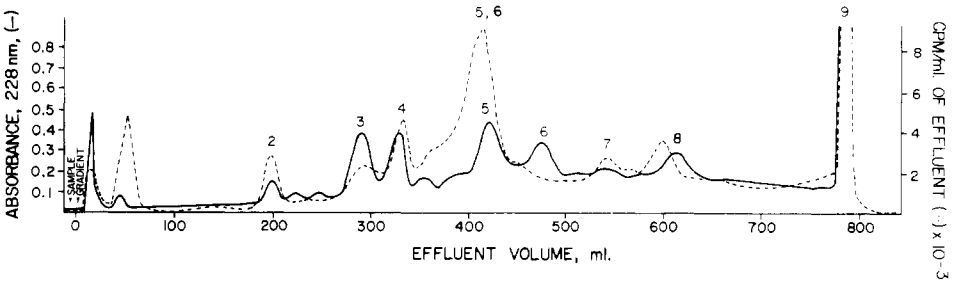

FIGURE 2: CM-cellulose elution pattern of the CNBr peptides derived from the labeled γ component of the collagen isolated from smooth muscle cell cultures. The column (1.5 × 10 cm) was equilibrated with 0.01 M (Na^+) sodium citrate, pH 3.6, and elution was achieved with a linear gradient from 0.0 to 0.17 M NaCl over a total volume of 1000 mL. Carrier CNBr peptides (solid line) were derived from human placental type III collagen and are identified by numbers assigned previously (Chung et al., 1974).

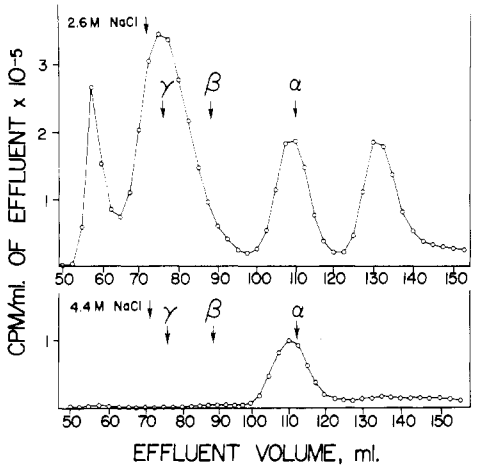

FIGURE 3: Agarose (Bio-Gel A-5m) molecular sieve elution pattern of labeled collagen isolated after precipitation at 2.6 M NaCl (top) and at 4.4 M NaCl (bottom). Collagen was extracted from the combined cell layer and medium of cells at the 8th passage, precipitated as described in the text, and denatured before application to the column. Arrows designate the elution positions of γ, β, and α components.

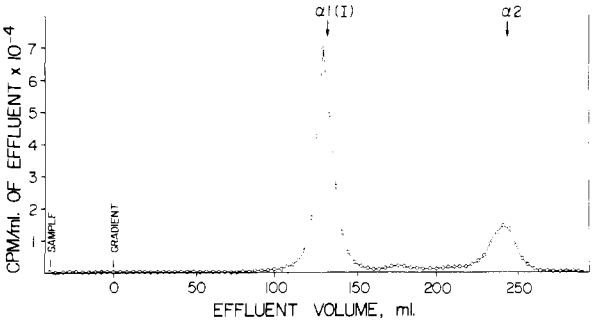

FIGURE 4: CM-cellulose chromatogram of labeled collagen recovered as α chains following agarose chromatography of the 2.6 M NaCl precipitate (Figure 3, top). The column (1.5 × 10 cm) was equilibrated with 0.02 M (Na^+) sodium acetate, pH 4.8, containing 1 M urea and elution was achieved with a linear gradient of from 0.0 to 0.14 M NaCl at a flow rate of 100 mL/h over a total volume of 400 mL. Arrows designate the elution positions of $\alpha 1$(I) and $\alpha 2$ chain present in carrier type I collagen.

luted with carrier $\alpha 1$(I) and $\alpha 2$ chains (data not shown). The two additional peaks eluted between $\alpha 1$(I) and $\alpha 2$ suggesting the possible synthesis of the A and B chains, which previous work had shown to elute in this region of the chromatogram (Chung et al., 1976; Burgeson et al., 1976). Burgeson et al. (1976) described a procedure for isolating molecules containing A and B chains from other native collagens which involved differential salt precipitation at close to neutral pH. In their work collagens containing A and B chains remained in solution after precipitation of other collagens by dialysis against 2.6 M NaCl and could subsequently be precipitated by dialysis against 4.0 M NaCl. We used a similar procedure to separate collagens containing A and B chains. For this purpose we added carrier type I collagen which was first precipitated by dialysis against 2.6 M NaCl and carrier type II collagen which was subsequently precipitated by dialysis against 4.4 M NaCl. The results of such an experiment are shown in Figure 3. Following denaturation of the precipitated collagens, agarose gel chromatography revealed that the 2.6 M NaCl precipitate contained the γ component, some α chains, and CP45. However, the material precipitating at 4.4 M NaCl contained only α chains.

The α chains present in the 2.6 M NaCl precipitate were further characterized by CM-cellulose chromatography. Figure 4 shows a representative CM-cellulose elution pattern of these chains after chromatography with carrier type I collagen. Material was observed chromatographing as both $\alpha 1$ and $\alpha 2$ chains with an $\alpha 1/\alpha 2$ chain ratio of 3.1. Further characterization of the peaks as $\alpha 1$(I) and $\alpha 2$ was achieved by preparing CNBr peptides in the presence of carrier type I collagen isolated from human placenta. Close agreement was observed between the radioactivity profiles and the CNBr peptides derived from $\alpha 1$(I) and $\alpha 2$ present in the carrier collagen (Figure 5). The identification of CNBr peptides from human type I collagen was based on a previous publication (Epstein et al., 1971).

The α chains recovered from agarose chromatography of the 4.4 M NaCl precipitate were also characterized by CM-cellulose chromatography in the presence of additional carrier type I collagen (Figure 6). Three prominent peaks of radioactivity were observed which were designated I, II, and III. Further characterization of each of these peaks was achieved by the preparation of CNBr peptides in the presence of carrier type I collagen, and fractionation by CM-cellulose chromatography (Figure 7). Peak I initially coeluted with carrier $\alpha 1$(I) chains and the profile of CNBr peptides confirmed that it was $\alpha 1$(I). Peak II eluted in roughly the expected position for the A chain, and comparison of the CNBr peptide profile with the profile published by Burgeson et al. (1976) strongly suggested that it was the A chain. Likewise, peak III eluted at a similar position to the B chain and comparison of the CNBr peptide profiles with the profile of Burgeson et al. (1976) strongly suggested that it was B. The collagen precipitating between 2.6 M NaCl and 4.4 M NaCl therefore consists predominantly of $\alpha 1$(I), A and B with the synthesis of very little $\alpha 2$. The isolation of $\alpha 1$(I) without stoichiometric quantities of $\alpha 2$ may reflect the synthesis of the type I trimer, chain composition $[\alpha 1(I)]_3$, which has previously been detected among the collagens synthesized by several types of cultured cells (Mayne et al., 1975; Narayanan and Page, 1976; Daniel, 1976; Little

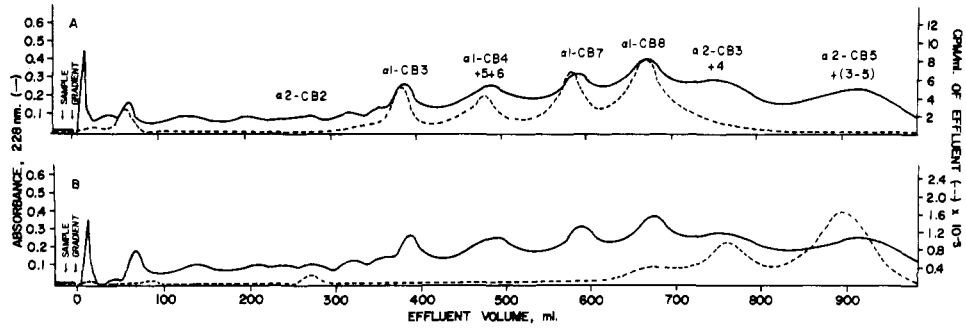

FIGURE 5: CM-cellulose elution patterns of the CNBr peptides derived from labeled $\alpha 1$ and $\alpha 2$ chains present in the 2.6 M NaCl precipitate and isolated initially by molecular sieve chromatography followed by CM-cellulose chromatography of the intact chains. (A) Labeled $\alpha 1$ chains with carrier type I collagen isolated from human placenta; (B) labeled $\alpha 2$ chains with carrier type I collagen.

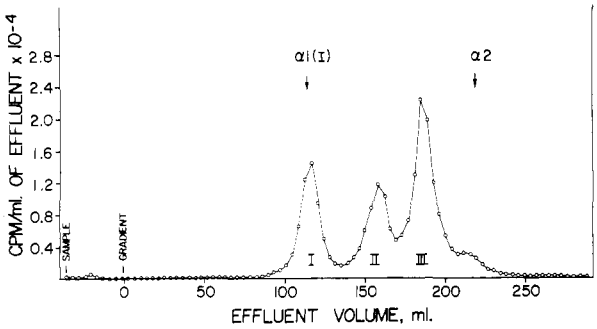

FIGURE 6: CM-cellulose chromatogram of labeled collagen recovered as α chains following agarose chromatography of the 4.4 M NaCl precipitate (Figure 3, bottom). Arrows designate the elution positions of $\alpha 1$(I) and $\alpha 2$ chains present in carrier type I collagen.

et al., 1977; Benya et al., 1977). However, in the present series of experiments it has not been possible to separate completely the type I trimer either from type I collagen or from collagens containing the A and B chain by differential salt precipitation.

Characterization of CP45. Initial experiments to further characterize CP45 isolated by agarose gel chromatography resulted in very poor yields after passage over a desalting column (Bio-Gel P-2). For this reason, CP45 was routinely prepared by dialysis against 0.5 M acetic acid and subsequent lyophilization. Figure 8 shows a CM-cellulose elution pattern of CP45 after chromatography with carrier type I collagen. The label can be observed to chromatograph as a single peak eluting after the $\alpha 2$ chains present in the carrier collagen. Figure 9 shows the fractionation of CNBr peptides prepared from CP45 in the presence of carrier type I collagen. A unique profile of radioactivity is observed which does not correspond to that for peptides obtained from $\alpha 1$(III) (Figure 2), $\alpha 1$(I) and $\alpha 2$ (Figure 5), or A and B (Figure 7). Several additional experiments were performed with CP45. Attempts to fractionate CP45 by polyacrylamide gel electrophoresis resulted in a single peak of radioactivity after sectioning and counting the gels. Digestion of CP45 with bacterial collagenase followed by agarose gel chromatography (Bio-Gel A-1.5m) of the reaction mixture showed complete degradation of CP45 to fragments of low molecular weight. Incubation of CP45 in conditions leading to reduction and alkylation of disulfide bridges did not result in a change in molecular weight upon subsequent agarose gel chromatography (Bio-Gel A-5m).

Characterization of Human Skin Fibroblast Collagens. Previous studies have shown that cultured smooth muscle cells synthesize a relatively high proportion of type III collagen when compared with fibroblast populations (Rauterberg et al., 1977; Burke et al., 1977; Mayne et al., 1977). During the present series of experiments cultured human skin fibroblasts were also examined with regard to collagen biosynthesis by the procedures described above for the monkey aortic smooth muscle cells. A representative elution pattern obtained after agarose gel chromatography of labeled collagen extracted from

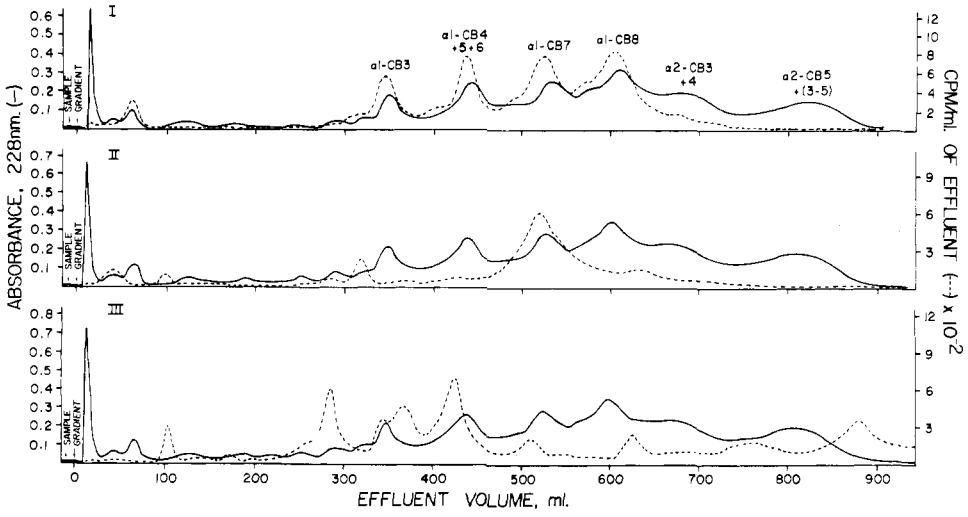

FIGURE 7: CM-cellulose elution patterns of the CNBr peptides derived from peaks I, II, and III present as the α-chains of the 4.4 M NaCl precipitate and isolated initially by molecular sieve chromatography followed by CM-cellulose chromatography. The carrier is type I collagen isolated from human placenta.

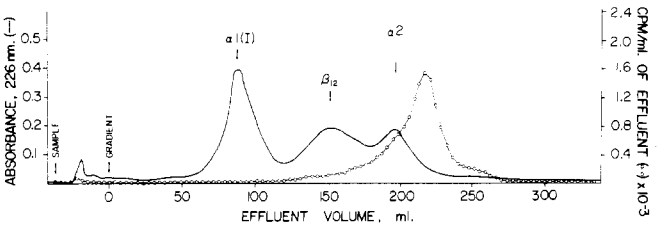

FIGURE 8: CM-cellulose chromatogram of labeled collagen isolated as CP45 from the combined cell layer and medium of smooth muscle cell cultures after initial fractionation by agarose gel chromatography (Figure 1). Carrier collagen (solid line) is type I collagen isolated from human fetal skin.

the combined cell layer and medium of cultured skin fibroblasts showed that approximately 90% of the total radioactivity elutes as α chains, with the remaining 10% eluting as a γ component (data not presented). Further analyses of both peaks were performed showing that the γ component consists entirely of disulfide-bonded $\alpha 1$(III) chains, whereas the α component consists of $\alpha 1$(I) and $\alpha 2$ chains with an $\alpha 1/\alpha 2$ chain ratio of either 2.1 determined by CM-cellulose chromatography or 2.0 determined by polyacrylamide gel electrophoresis.

Discussion

The present results demonstrate that five different collagen chains as well as a smaller collagenous fragment can be isolated from cultured monkey aortic smooth muscle cells. The components are distinguishable by their chromatographic properties on CM-cellulose as well as by the CM-cellulose profiles of peptides generated on cleavage with CNBr. Smooth muscle cells will continue to synthesize all six collagen components over at least eight passages of the cells, and the cells can be stored in liquid nitrogen and will continue to show the same behavior after recovery. Monkey smooth muscle cells also synthesize a relatively higher proportion of the total collagen as type III (about 50% of the total *extracted* collagen after initial fractionation by agarose gel chromatography) when compared with a fibroblast population. This behavior also occurs for eight passages of the cells, although the relative proportion of type III does fall if later passages are examined, and type I collagen becomes the predominant collagen synthesized (Mayne, Vail, and Miller, unpublished observations). The present results were obtained from smooth muscle cells derived from a single rhesus monkey aorta, and assayed for collagen biosynthesis at each passage. However, less extensive analyses have also been carried out with cells derived from two additional monkeys and have shown essentially the same results.

The present results both compare and contrast with an earlier study with guinea pig aortic smooth muscle cells (Mayne et al., 1977). In this previous study the synthesis of a high proportion of type III collagen and the synthesis of CP45 were only observed if the first passage of the cells from the initial outgrowth was examined. For later passages the cells showed a more fibroblastoid behavior with low type III relative to type I and little or no synthesis of CP45. With monkey smooth muscle cells this change in the relative proportions of collagen chains also eventually occurred but only after several passages of the cells. These results with both guinea pig and monkey cells showing changes in behavior with increasing time in culture may explain the earlier results of others analyzing the proportions of collagen types synthesized by smooth muscle cell cultures in which either no type III collagen (Layman and Titus, 1975) or a relatively low proportion of type III collagen (Layman et al., 1977; Leung et al., 1976; Barnes et al., 1976; Rauterberg et al., 1977) was observed. The present results do, however, compare favorably with the high proportion of type III collagen reported by Burke et al. (1977) in which monkey smooth muscle cells were also analyzed, but from a different species (*Macaca nemestrina* vs. *Macaca mullata* used in our studies).

Burke et al. (1977) in their recent study with monkey smooth muscle cell cultures compared several methods of estimating the relative proportions of collagen types and concluded that quantitation of type I and type III procollagens by DEAE-cellulose chromatography gave the most reliable results. It was also suggested that the use of pepsin to convert procollagens to collagens resulted in a relatively greater loss of type III collagen. Incubation with pepsin was carried out in these studies at 15 °C, rather than at 4–7 °C used in our experiments. We have carried out preliminary experiments comparing the collagens extracted at two different concentrations of pepsin (100 μg/mL and 1 mg/mL) both at 4–7 °C, and could not observe any marked difference in the amounts of type I and type III collagens which were extracted. A greater yield of CP45 was, however, observed at the higher concentration of pepsin.

Rauterberg et al. (1977) in a recent analysis of cultured human smooth muscle cells observed that type III collagen was present only in the medium, whereas type I collagen was present both in the cell layer and in the medium. In the present series of experiments occasionally the cell layer and the medium were analyzed separately. Type III collagen was found only in the medium, whereas $\alpha 1$(I), $\alpha 2$, A, B and CP45 were all present in roughly equal proportions in the cell layer and in the medium.

The present results, and earlier publications (Rauterberg et al., 1977; Burke et al., 1977; Mayne et al., 1977) show that, although fibroblasts and smooth muscle cells both synthesize type I and type III collagens, differences must exist in the control mechanisms which determine the relative proportions in which these collagens are synthesized. The present study shows that such controls may persist over many generations of the cells, and similar results have recently been reported by Hance and Crystal (1977) who, working with rabbit lung fibroblasts, observed a constant relative proportion of type I and type III collagens over as many as 20 replicative cycles. The

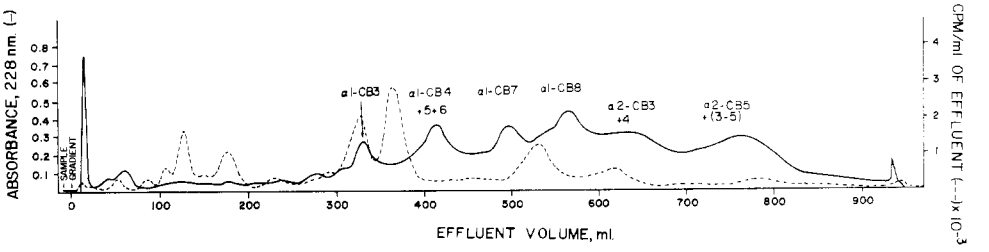

FIGURE 9: CM-cellulose elution pattern of the CNBr peptides derived from labeled CP45 obtained after initial fractionation by agarose gel chromatography. Carrier CNBr peptides were prepared from human placental type I collagen.

molecular basis of such controls is unknown, but an understanding of such control mechanisms may be very important in understanding the pathogenesis of atherosclerotic lesions, since it has been claimed that changes in the relative proportions of type I and type III collagens occur when the collagens of normal human aortic media are compared with atherosclerotic intima (McCullagh and Balian, 1975). Atherosclerotic lesions are usually considered to arise from the migration of smooth muscle cells from the media to the intima together with their continued proliferation (Ross and Glomset, 1976).

From the present data, it also appears possible that cultured smooth muscle cells synthesize type I trimer, chain composition $[\alpha 1(I)]_3$. Consistently, an $\alpha 1(I)/\alpha 2$ chain ratio in excess of 2:1 was observed with these cells although human skin fibroblast cultures, like other normal fibroblast populations (Mayne et al., 1976; Narayanan and Page, 1976; Daniel, 1976), synthesize type I collagen with an $\alpha 1(I)/\alpha 2$ chain ratio very close to 2.0. Previous studies have shown that the type I trimer is more soluble than type I collagen during differential salt precipitation (Mayne et al., 1975; Narayanan and Page, 1976; Benya et al., 1977). In the present experiments, CM-cellulose chromatography of the collagens remaining in solution at concentrations higher than 2.6 M and precipitating at 4.4 M NaCl showed the presence of relatively large proportions of $\alpha 1(I)$ chains accompanied by little or no corresponding $\alpha 2$ chains (Figure 6). Despite several attempts, it has not been possible to separate completely the type I trimer from either type I or from collagens containing A and B chains by differential salt precipitation.

The present results suggest that smooth muscle cells of the aortic media are competent to synthesize several different collagen types. Previous studies in which collagens have been isolated from intact aortas have shown the presence of type I and type III collagens (Chung and Miller, 1974; Trelstad, 1974; Rauterberg and von Bassewitz, 1975; McCullagh and Balian, 1975; Epstein and Munderloh, 1975; Scott et al., 1977), and also the B chain (Chung et al., 1976). However, the A chain and CP45 have not been demonstrated so far in the intact aorta. Burgeson et al. (1976), working with human placental membranes, isolated A and B in a constant ratio of 1:2 and observed that during differential salt precipitation both A and B chains were present in the collagen which precipitated within a narrow range of salt concentrations. These results were taken as being indicative of a native structure $[A][B]_2$. However, the data of Chung et al. (1976) suggested that the ratio of A and B chains in a given preparation was dependent on the tissue source of the collagen. We have also observed ratios of A to B very close to 1:2 (e.g., Figure 6). However, this has not been a consistent observation and more often equal incorporation into A and B occurred. Moreover, if later passages of the cells were analyzed (9–12 passages), the cells appear to synthesize the A chain with little or no corresponding B chain after initial differential salt precipitation and CM-cellulose chromatography of the 4.4 M NaCl precipitate (Mayne, Vail, and Miller, unpublished observations). The data of Chung et al. (1976) as well as the present results are therefore consistent with the notion that A and B chains originate in separate molecules.

In previous studies (Chung et al., 1976; Burgeson et al., 1976), it was suggested that A and B may be components of basement membranes, and the recent demonstration of indirect immunofluorescence staining of the endomysium of striated muscle with antibodies against a preparation of collagen containing the A and B chains isolated from human placenta would support this suggestion (Duance et al., 1977). Electron microscopic studies of cultured smooth muscle cells have shown the accumulation of large amounts of amorphous basement membrane-like material (Ross, 1971, 1972). The function of CP45 is also unknown, and it too may be a component of basement membrane structure. Additional experiments are, however, required to determine the chemical composition of this component and its molecular configuration before a morphological role can be assigned.

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References

Barnes, M. J., Morton, L. F., and Levene, C. I. (1976), *Biochem. Biophys. Res. Commun. 70,* 339.

Benya, P. D., Padilla, S. R., and Nimni, M. E. (1977), *Biochemistry 16,* 865.

Burgeson, R. E., El Adli, F. A., Kaitila, I. J., and Hollister, D. W. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 2579.

Burke, J. M., Balian, G., Ross, R., and Bornstein, P. (1977), *Biochemistry 16,* 3243.

Chung, E., and Miller, E. J. (1974), *Science 183,* 1200.

Chung, E., Keele, E. M., and Miller, E. J. (1974), *Biochemistry 13,* 3459.

Chung, E., Kinsey, R. W., and Miller, E. J. (1975), in Extracellular Matrix Influences on Gene Expression, Slavkin, H. C., and Greulich, R. C., Ed., New York, N.Y., Academic Press, p 285.

Chung, E., Rhodes, R. K., and Miller, E. J. (1976), *Biochem. Biophys. Res. Commun. 71,* 1167.

Daniel, J. C. (1976), *Cell Differ. 5,* 247.

Duance, V. C., Restall, D. J., Beard, H., Bourne, F. J., and Bailey, A. J. (1977), *FEBS Lett. 79,* 248.

Epstein, E. H., Jr. (1974), *J. Biol. Chem. 249,* 3225.

Epstein, E. H., Jr., and Munderloh, N. H. (1975), *J. Biol. Chem. 250,* 9304.

Epstein, E. H., Jr., Scott, R. D., Miller, E. J., and Piez, K. A. (1971), *J. Biol. Chem. 246,* 1718.

Faris, B., Salcedo, L. L., Cook, V., Johnson, L., Foster, J. A., and Franzblau, C. (1976), *Biochim. Biophys. Acta 418,* 93.

Furthmayr, H., and Timpl, R. (1971), *Anal. Biochem. 41,* 510.

Hance, A. J., and Crystal, R. G. (1977), *Nature (London) 268,* 152.

Jarmolych, J., Daoud, A. S., Landau, J., Fritz, K. E., and McElvene, E. (1968), *Exp. Mol. Pathol. 9,* 171.

Layman, D. L., and Titus, J. L. (1975), *Lab. Invest. 33,* 103.

Layman, D. L., Epstein, E. H., Jr., Dodson, R. F., and Titus, J. L. (1977), *Proc. Natl. Acad. Sci. U.S.A. 74,* 671.

Leung, D. Y. M., Glagov, S., and Mathews, M. B. (1976), *Science 191,* 475.

Little, C. D., Church, R. L., Miller, R. A., and Ruddle, F. H. (1977), *Cell 10,* 287.

Mayne, R., Vail, M. S., and Miller, E. J. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 4511.

Mayne, R., Vail, M. S., Mayne, P. M., and Miller, E. J. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 1674.

Mayne, R., Vail, M. S., Miller, E. J., Blose, S. H., and Chacko, S. (1977), *Arch. Biochem. Biophys. 181,* 462.

McCullagh, K. A., and Balian, G. (1975), *Nature (London)*

258, 73.
Narayanan, A. S., and Page, R. C. (1976), *J. Biol. Chem. 251,* 5464.
Piez, K. A. (1968), *Anal. Biochem. 26,* 305.
Rauterberg, J., and von Bassewitz, D. B. (1975), *Hoppe-Seyler's Z. Physiol. Chem. 356,* 95.
Rauterberg, J., Allam, S., Brehmer, U., Wirth, W., and Hauss, W. H. (1977), *Hoppe-Seyler's Z. Physiol. Chem. 358,* 401.
Ross, R. (1971), *J. Cell Biol. 50,* 172.
Ross, R. (1972), in The Pathogenesis of Atherosclerosis, Wissler, R. W., and Geer, J. C., Ed., Baltimore, Md., Williams & Wilkin, p 147.
Ross, R. (1973), in Biology of Fibroblast, Kulonen, E., and Pikkarainen, J., Ed., New York, N.Y., Academic Press, p 627.
Ross, R., and Glomset, J. A. (1976), *N. Engl. J. Med. 295,* 369 and 420.
Scott, D. M., Harwood, R., Grant, M. E., and Jackson, D. S. (1977), *Connect. Tissue Res. 5,* 7.
Seyer, J. M., Hutcheson, E. T., and Kang, A. H. (1976), *J. Clin. Invest. 57,* 1498.
Trelstad, R. L. (1974), *Biochem. Biophys. Res. Commun. 57,* 717.
Trelstad, R. L., Kang, A. H., Toole, B. P., and Gross, J. (1972), *J. Biol. Chem. 247,* 6469.

Interactions of Small Ligands with *Busycon canaliculatum* and *Limulus polyphemus* Hemocyanins as Studied by Ultraviolet Spectrophotometry and ^1H and ^{19}F Nuclear Magnetic Resonance†

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ABSTRACT: We have used UV (ultraviolet) spectrophotometric and ^1H and ^{19}F NMR (nuclear magnetic resonance) techniques to study the interaction of small ligands with *Busycon canaliculatum* and *Limulus polyphemus* hemocyanins. Addition of thioacetamide (up to 0.02 M) to oxyhemocyanin causes a relatively rapid drop in absorbance at 340 nm, a primary reaction, followed by a considerably slower secondary process. The primary reaction between thioacetamide and *Busycon* hemocyanin commonly takes 15–30 min. The ^{19}F NMR signal of 0.125 M KF is broadened by oxyhemocyanin from both *Limulus* and *Busycon* species, and the extent of broadening is linearly dependent on hemocyanin concentration (more marked dependence for *Limulus* than for *Busycon*). Glycine, a ligand which binds to oxyhemocyanin at the copper active site without expelling oxygen, also causes a decrease in the ^{19}F line width as a function of glycine concentration which is analyzed in terms of an apparent dissociation constant (0.15 M) for glycine–hemocyanin (*Limulus*) complex(es). Addition of thioacetamide (up to 0.1 M) to solutions of oxyhemocyanin containing 0.125 M KF causes a considerable decrease in the line width of the ^{19}F signal from its paramagnetically broadened value, again with a biphasic time dependence. Our results can be interpreted in terms of thioacetamide exerting an allosteric effect, inducing a conformational change in hemocyanin leading to an altered active-site configuration, incapable not only of retaining oxygen, but also incapable of binding fluoride ion as strongly. However, at this stage, reduction of paramagnetic Cu(II) to diamagnetic Cu(I) cannot be rigorously excluded as an alternative.

For over a century the nature and function of the copper in the respiratory proteins known as hemocyanins have puzzled researchers. These huge, oxygen-carrying molecules, with molecular weights in the range of 10^6 and 10^7, occur in arthropods and molluscs, not localized in blood cells, but freely dissolved in the hemolymph (van Holde & van Bruggen, 1971). The oxygenated protein is a striking, deep blue and considerable current effort is being directed toward defining the nature(s) of the copper atoms in oxyhemocyanins (Bannister & Wood, 1972; Lontie & Vanquickenborne, 1974; Freedman et al., 1976).

One approach has been the use of small ligands which bind to hemocyanin. Proton magnetic resonance studies have shown that, with glycine derivatives, there is selective, paramagnetic line broadening of the ligand signals by oxyhemocyanin (Ke et al., 1973), providing evidence that at least part of the active site is copper(II). Ligands of hemocyanin can be divided into two classes: (a) those whose binding does not lead to expulsion of oxygen from oxyhemocyanin (e.g., glycine (Ke et al., 1973)); (b) those whose binding is accompanied by conversion of the protein to deoxyhemocyanin (e.g., thiourea (Rombauts, 1968), thioacetamide (Lee et al., 1977)).

In this paper we report studies on members of each class of ligands as complementary probes of hemocyanin activity, using both arthropod (*Limulus polyphemus*) and molluscan (*Busycon canaliculatum*) species of hemocyanin. In particular we use glycine (NMR line broadening), thioacetamide (spectrophotometric analysis of ligand binding) and fluoride ion ($^{19}F^-$ NMR line broadening).

Fluoride ions are known to bind to *Helix pomatia* hemocyanin leading to the formation of methemocyanin (Witters & Lontie, 1975) as detected by measurement of the absorbance at 340 nm (a copper-oxygen absorption band of oxyhemocyanin): it is worth noting that, by these criteria, fluoride ion was

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