# Biosynthesis of Interstitial Types of Collagen by Albumin-Producing Rat Liver Parenchymal Cell (Hepatocyte) Clones in Culture†

Ryu-ichiro Hata,* Yoshifumi Ninomiya, Yutaka Nagai, and Yutaka Tsukada

ABSTRACT: We have analyzed collagenous components produced by an albumin-producing rat liver parenchymal cell clone (BB) in culture. The cells secreted over 90% of the [$^3$H]proline-labeled collagenous components into the medium after 18 h of incubation in vitro. Analysis of the components by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and by carboxymethylcellulose chromatography of cyanogen bromide peptides after separation of individual collagen chains showed that the cells produced type I, type I trimer, and type III collagens in the ratio 59:29:4. These components account for over 90% of the collagen isolated. Type I and type I trimer collagens were present in both the medium and cell layer fractions, but type III collagen was found solely in the medium. The cells also produced other minor collagenous components, one of which was quite similar to type V collagen. Analysis of collagen components produced by another liver parenchymal cell clone (BC) and by three subclones derived from BB cells showed that these cloned cells synthesized similar types of collagen. These results support the idea that parenchymal cells play an essential role in the hepatic connective tissue metabolism under normal and pathological conditions.

Recent work on collagen has shown that there are several genetically distinct molecular species in collagen (Martin et al., 1975; Miller, 1976). Type I collagen, $[\alpha 1(I)]_2\alpha 2$, is the most abundant and ubiquitous collagen; type I trimer, $[\alpha 1(I)]_3$, is found in cultured cells as well as tumor or embryonic tissues; type II, $[\alpha 1(II)]_3$, is found in cartilage; type III, $[\alpha 1(III)]_3$, is present in blood vessels, fetal skin, and other soft tissues. In addition to these interstitial types of collagen, basement membranes or fetal membranes also contain collagen components called type IV and type V. Their molecular organizations are supposed to be $[\alpha 1(IV)]_3$ and $\alpha A[\alpha B]_2$, respectively (Kefalides, 1973; Burgeson et al., 1976). The amount and type of collagen produced by individual tissues or cells change during cell differentiation (Reddi, 1976; Hata & Slavkin, 1978; Fessler & Fessler, 1978) and under pathological conditions (Uitto & Lichtenstein, 1976; Lapière & Nusgens, 1976) as well as during cell transformation with viruses and a carcinogen (Hata & Peterkofsky, 1977), though their control mechanisms are still unresolved.

In hepatic fibrosis, a progressive accumulation of collagen fibers is often observed in the perihepatocellular area in addition to the portal and proliferating periductular regions. The cause of the accumulation of collagen fibers and the type of cells responsible for collagen production have not been clarified yet. Recently, we found that hepatocyte clones, which show normal or transformed character, have collagen-producing activity. The major components of the collagen secreted into the medium were partially characterized (Hata et al., 1978b). In this paper, further characterization of the collagen components synthesized by albumin-producing liver parenchymal cell (hepatocyte) clones and subclones is described. The collagen types found in this work resemble those isolated from human liver (Rojkind et al., 1979). A summary of this work was presented at the 51st Annual Meeting of the Japanese Biochemical Society, Kyoto, 1978 (Hata et al., 1978a).

## Experimental Procedures

### *Materials*

Powdered Ham F-12 medium (Flow Laboratories) was purchased from Dai-Nippon Seiyaku Co., Ltd., Osaka, Japan; CS[1] (Flow Laboratories) and FCS (GIBCO) were from Sanko Junyaku, Co., Ltd., Tokyo, Japan. Penicillin G and dihydrostreptomycin were generously donated by Toyo Jozo. Co., Ltd., Shizuoka, Japan; β-aminopropionitrile fumarate was obtained from Tokyo Kasei Co., Ltd., Tokyo, Japan, and sodium ascorbate and CNBr were from Wako Junyaku Co., Ltd., Osaka, Japan. Purified *Clostridium histolyticum* collagenase was obtained from Amano Pharmaceutical Co., Ltd., Nagoya, Japan. Pepsin (1:60000) was from Sigma Chemical Co., Ltd., St. Louis, MO. L-[2,3-$^3$H]Proline (37.1 Ci/mmol) and L-[$^3$H]tryptophan (7.9 Ci/mmol) were purchased from New England Nuclear, Boston, MA, and CM-cellulose (microgranular CM32) was from Whatman Biochemicals, Ltd., Maidstone. Other chemicals used were of reagent grade.

### *Methods*

*Cell Culture and Labeling.* Two liver epithelial clones (BB and BC) derived from JAR-2 suckling rats were generously supplied by the Tissue Culture Laboratory, Yokohama City University School of Medicine, and subclones (BB1S, BB4S, and BB5S) were obtained after plating less than 30 BB cells (at 26th passage) per 60-cm$^2$ Falcon culture dish and isolation by stainless steel cups. Cells were cultured in Ham F-12 medium, supplemented with penicillin G (50 mg/L) and dihydrostreptomycin (50 mg/L), and 10% CS until late log phase. FCS instead of CS was also used for cultures of subclones (BB1S and BB5S). For labeling, cells were rinsed twice and preincubated in 3 mL of Ham F-12 medium supplemented with sodium ascorbate (0.1 mM), β-aminopropionitrile (0.5 mM), and antibiotics. After 30 min, 10 and 100 μCi of

† From the Department of Tissue Physiology, Medical Research Institute, Tokyo Medical and Dental University, Kanda-surugadai, Chiyoda-ku, Tokyo 101, Japan, and the Department of Biochemistry (Y.T.), Hokkaido University, School of Medicine, Sapporo 060, Japan. *Received July 13, 1979.* This work was supported in part by a Grant-in-Aid for Scientific Research (244075) to Y.N. and a Grant-in-Aid for Encouragement of Young Scientists (377111) to R.H. from the Ministry of Education, Science and Culture of Japan. This is the second paper on the molecular mechanisms of liver fibrosis. For the first paper, see Hata et al. (1978b).

[1] Abbreviations used: CM, carboxymethyl; $NaDodSO_4$, sodium dodecyl sulfate; CS, calf serum; FCS, fetal calf serum; MalNEt, *N*-ethylmaleimide; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; CNBr, cyanogen bromide.

0006-2960/80/0419-0169$01.00/0 

[$^3$H]proline per dish were added for the distribution and structural analyses of collagen, respectively, and the samples were labeled for 3, 6, or 18 h under 5% $CO_2$–air at 37 °C.

*Collagen Analysis by Bacterial Collagenase.* Labeled proteins were prepared from the medium (plus 2 mg of chick embryo extract as a carrier) and cell layer as described previously (Peterkofsky, 1972), but to avoid degradation during preparation, dialysis of the medium was omitted and instead precipitation steps were repeated 5 times to remove free radioactive proline.

*C. histolyticum* collagenase, prepared by diethylaminoethylcellulose chromatography and gel filtration, was used for the collagen analysis (Peterkofsky & Diegelmann, 1971). This enzyme (10 or 20 μg in 250 μL of reaction mixture) released no radioactivity from [$^3$H]tryptophan (an amino acid lacking in collagen) labeled chick embryo proteins but released a constant amount (78.8 ± 1.8%) of radioactivity from partially purified [$^3$H]proline-labeled chick embryo collagen during 0.5–18 h of incubation at 37 °C in the presence of MalNEt; 1.5 h of incubation was routinely used.

Assays were always performed in duplicate, and mean values were used. Errors were less than 12% of the values described.

*Preparation of Collagen for Structural Analysis.* Carrier rat skin type I and type III collagens were added (final concentration each ~200 μg/mL) to the pooled medium plus the first wash of the cell layer with or without protease inhibitors (final concentration: PMSF, 1 mM; MalNEt, 10 mM; EDTA, 25 mM), and the medium was precipitated by adding ammonium sulfate (176 mg/mL) at 4 °C.

The precipitate was suspended in and dialyzed against 0.5 M acetic acid and diluted with 0.5 M acetic acid to make up 1.1 mg each of carrier type I and type III collagens per mL of the solution. One-tenth volume of pepsin (1 mg/mL of 0.5 M acetic acid) was added, and digestion was performed for 6 h at 6–8 °C. After addition of 0.1 volume of 1 M Tris-HCl buffer, pH 7.6, the solution was titrated to pH 8 with 2 M NaOH to inactivate pepsin. Cell layer collagens were prepared similarly after sonication.

*Differential Salt Fractionation of Collagen.* In some experiments medium collagens were separated in the presence of rat skin type I and III collagens and bovine type II collagens (~500 μg/mL each) by successive dialysis against 1.0, 1.7, 2.6, and 4.4 M NaCl in 0.05 M Tris-HCl, pH 7.6, at 4 °C. The precipitates formed after dialysis at each concentration were collected separately by centrifugation (Chung & Miller, 1974; Trelstad et al., 1976). The supernatant after dialysis against 4.4 M NaCl was dialyzed against diluted acetic acid and then lyophilized.

*CM-cellulose Chromatography.* Radioactive samples were dissolved in and dialyzed against 0.06 M sodium acetate (pH 4.8) containing 4 M urea. An aliquot was saved for collagen assay with collagenase to calculate the amount of collagen applied. Rat skin type I and/or type III collagens (1–2 mg) were added as internal markers and carriers. After denaturation at 50 °C for 10 min, samples were applied to a 1.0 × 10 cm column of CM-cellulose at 43 °C and washed with 50 mL of the buffer. Collagen chains were separated by using a linear gradient of 0–0.1 M NaCl over a total volume of 200 mL at 40 mL/h, and 2-mL fractions were collected as described (Hata et al., 1978b). The recovery of collagenous components was over 85% except for the supernatant at 4.4 M NaCl (~50%; see Figure 3E). The CNBr peptides were chromatographed on an identical column to separate collagen components. The column was washed with starting buffer (0.02 M sodium citrate–0.02 M NaCl) and then developed with a salt concentration gradient (0.02–0.18 M NaCl) with a total volume of 400 mL at 80 mL/h. Two-milliliter fractions were collected (Epstein et al., 1971; Hata & Slavkin, 1978).

Table I: Production of Collagen and Serum Proteins by Rat Liver Parenchymal Cell Clones in Culture[a]

| cell line | doubling time (h) | albumin[b] (ng/mL) | α-fetoprotein[b] (ng/mL) | labeling time (h) | collagen[c] (%) | collagen present in the medium[d] (%) |
|---|---|---|---|---|---|---|
| BB | 23 | 18 | <1 | 3 | 1.40 ± 0.22 | 55 ± 4 |
| | | | | 6 | 1.64 ± 0.12 | 68 ± 3 |
| | | | | 18 | 1.92 ± 0.14 | 91 ± 1 |
| BC | 23 | 37 | <1 | 3 | 1.75 ± 0.18 | 48 ± 4 |
| | | | | 6 | 1.92 ± 0.23 | 56 ± 5 |
| | | | | 18 | 2.39 ± 0.07 | 58 ± 7 |

[a] Cells were cultured in Ham F-12 containing 10% CS until late log phase (5 to 6 days) and then further cultured for 3 days in the fresh medium. The medium was collected and spun down. [b] Albumin and α-fetoprotein were measured by radioimmunoassay (Nishi et al., 1974). Average of two experiments. [c] Late log phase cells were labeled for 3, 6, or 18 h with 10 μCi/3 mL [$^3$H]-proline as described under Methods, and the values were calculated by assuming that collagen has an imino acid content 5.4 times higher than that of other proteins (Peterkofsky & Diegelmann, 1971). Average of two separate experiments. [d] Average of two separate experiments.

*CNBr Digestion.* Labeled and carrier collagens (10–15 mg) were dissolved in 2 mL of 70% formic acid, and cleavage at methionyl residues was achieved as described (Hata & Slavkin, 1978).

*NaDodSO$_4$–Polyacrylamide Gel Electrophoresis.* Collagen fractions were examined by disc or slab gel electrophoresis. In both cases, samples before or after collagenase digestion were dissolved in 30 μL of 0.5 M Tris-HCl buffer, pH 6.8, 20 μL of 9 M urea–0.3% NaDodSO$_4$, and 10 μL of bromophenol blue–glycerol with or without 10 μL of 0.1 M DTT and heated at 80 °C for 10 min. Electrophoresis was performed by using a 3% stacking gel, pH 6.8, containing 3 M urea, a 5% separating gel, pH 8.8, containing 3 M urea, and an electrode buffer, pH 8.5, as described (Hayashi & Nagai, 1979). After electrophoresis, the disc gels were stained, sliced, and counted (Hata & Peterkofsky, 1978). Slab gels were dehydrated with dimethyl sulfoxide (30 min × 3), immersed in a 2,5-diphenyloxazole solution for 3 h, and dried for fluorescence autoradiography (Bonner & Laskey, 1974; Laskey & Mills, 1975). The dried gels were exposed to X-ray films at −70 °C for 1–60 days, and the amounts of radioactive collagen components were estimated by densitometry at 500 nm of the developed films. Linearity between radioactivity and density of the developed film was tested by using purified [$^3$H]proline-labeled α1 chains.

*Counting of Radioactive Samples.* Samples were dissolved in Bray's solution or in toluene–Omnifluor (in case of counting of gel slices) and counted by a liquid scintillation spectrometer (Beckman LS9000).

## Results and Discussion

*Collagen Synthesis by Liver Parenchymal Cell Clones and Subclones.* Two liver parenchymal clones produced and secreted serum albumin but not α-fetoprotein (Table I). Under these assay conditions we found that transformed and tumorigenic liver parenchymal cell (M) and its clones produced α-fetoprotein but not albumin and that mesenchymal cell clones produced neither of them [Hata et al. (1978b) and data not shown]. The results prove that these clones were derived from liver parenchymal cells (hepatocytes). The increase in the relative content of collagen during the time course of

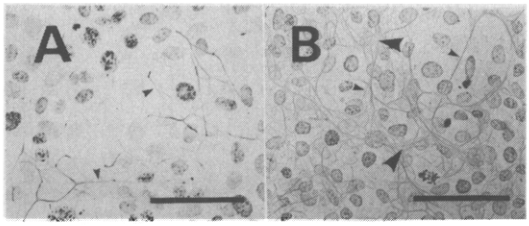


FIGURE 1: Micrographs of cloned liver cell lines BB and BB1S cultured on slide glasses for 2 weeks without subculture. Silver impregnation of BB cells at the 38th passage (A) and of BB1S at the 7th passage (B). Note the presence of thin reticular fibers (small arrowheads) and thick, reddish collagen fibers (large arrowheads) between the cells. The bar indicates 50 $\mu$m.

Table II: Characterization of Subclones of Liver Parenchymal Cell (BB)[a]

| cell line | cell type | albumin (ng/mL) | α-fetoprotein (ng/mL) | collagen synthesized (%) | collagen present in the medium (%) |
|---|---|---|---|---|---|
| BB1S [2][b] | epithelial | 32.4 | <1 | 2.91 | 89.4 |
| BB1S [2][c] | epithelial | 25.4 | <1 | 2.65 | 91.2 |
| BB4S [2][b] | epithelial | 20.4 | <1 | 1.32 | 59.3 |
| BB5S [2][b] | epithelial | 25.4 | <1 | 1.48 | 72.0 |
| BB5S [2][c] | epithelial | 23.2 | <1 | 1.28 | 68.9 |

[a] See footnote *a* of Table I. The figure in brackets is the passage level of the subclones. [b] Cultured in Ham F-12 + 10% calf serum and labeled for 18 h as described under Methods. [c] Cultured in Ham F-12 + 10% fetal calf serum and labeled for 18 h as described under Methods.

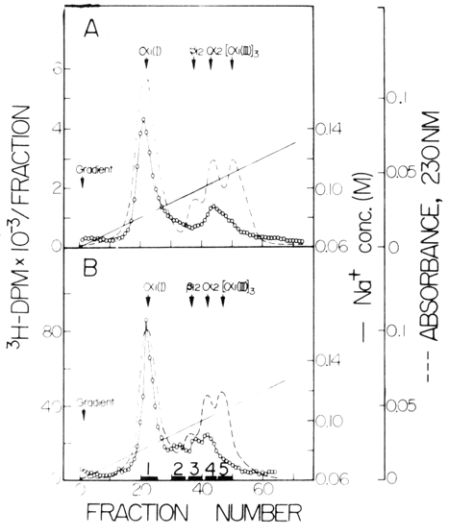


FIGURE 2: CM-cellulose chromatograms of pepsin-resistant [$^3$H]-proline-labeled proteins from medium (A) and cell (B) fractions. BB cells were cultured in medium containing [$^3$H]proline for 18 h, and the medium was precipitated with $(NH_4)_2SO_4$ (176 mg/mL at 4 °C), dissolved in 0.5 M acetic acid, and treated with pepsin (100 $\mu$g/mL) in the presence of carrier type I and type III collagens (1 mg/mL each). Aliquots of radioactive collagens were chromatographed with carrier type I (2 mg) and type III (1 mg) collagens as described under Methods. The cell fraction was treated with pepsin as described above, and then radioactive collagens were precipitated with 4.4 M NaCl at pH 7.5. The precipitate was dissolved in the starting buffer and chromatographed as described under Methods. Arrows indicate elution positions of carrier rat collagen chains. The effluent solution was divided into five fractions as shown in (B) and saved for further analysis.

incubation (Table I) showed a continuous synthesis of collagen during the labeling period. The relative rates of collagen produced in two clones were similar, but the relative amount of collagen accumulated in the medium increased with BB cells but not with BC cells. This suggests that the rate of procollagen processing to collagen in BC cells is higher than BB cells, which is consistent with histological observations that deposition of collagen was only found in BC cells and not in BB cells in an earlier work (Sakakibara et al., 1976). Deposition of collagen and/or reticular fibers was observed in the BB cell culture (Figure 1A) and in the BB1S cell culture (Figure 1B), but the amount was less than that of BC cells or its subclones under similar culture conditions (Hata et al., unpublished experiments). We isolated subclones from BB cells at the 26th passage stage. All the subclones produced serum albumin and collagen but not $\alpha$-fetoprotein. These results showed the stability of the nature of the cell functions as to production of albumin and collagen even though the relative rate of collagen synthesis and processing of procollagen might be variable (Table II).

*Isolation of Collagen Components Produced by BB Cells.* After precipitation of procollagen by ammonium sulfate (176 mg/mL) from the combined medium and the washing of the cell layer, we always found ~15% of collagenase digestible material in the supernatant but not any collagen-like band on the fluorogram of the fraction by $NaDodSO_4$–polyacrylamide gel electrophoresis (data not shown). This indicates that the enzyme-digestible material in the fraction is collagen degradation products released during the culture process with [$^3$H]proline. The material may not be the degradation products of collagen during the preparation, because its relative content was constant regardless of the presence or absence of protease inhibitors during preparation of procollagens. Bienkowski et al. (1978) reported the degradation of newly formed collagen during lung tissue culturing in vitro by analyzing dialyzable hydroxyproline. The collagenous material remaining in the supernatant at 30% ammonium sulfate saturation in this experiment seems to have a larger molecular weight than that of Bienkowski et al. (1978) (~200), since it was trichloroacetic acid precipitable. This fraction was not characterized further and was omitted from the calculation of the relative abundance of each collagen component.

The ammonium sulfate precipitate (procollagen) was treated with pepsin at 6–8 °C for 6 h in the presence of carrier type I and type III collagens and then subjected to differential salt precipitation. The total recovery of collagen in all the fractions was more than 90% of the collagenous material before the enzyme treatment, indicating that there was little degradation of collagen components during pepsin treatment and fractionation. Figure 2 shows the separation of collagen from the medium and cell layer fractions by CM-cellulose chromatography. Components corresponding to $\alpha$1, $\alpha$2, and [$\alpha$1(III)]$_3$ were observed in the medium fraction (Figure 2A). In the cell layer (Figure 2B), there were two small peaks eluted between $\alpha$1 and $\alpha$2 in addition to the peaks coinciding with $\alpha$1 and $\alpha$2. Each peak was separately collected, dialyzed, and lyophilized for further analysis.

The amount of collagen component corresponding to type III explains only 5% of the medium collagen (Figure 2A and Table III). This figure is lower than that observed before (15%) (Hata et al., 1978b). The apparent discrepancy is due to the difference in labeling times between the previous (3 h) and present (18 h) experiments (Hata et al., unpublished experiments). Factors affecting the relative contents of collagen components synthesized during labeling are under investigation.

*Characterization of the Medium Collagen Components.* Collagens in the medium were fractionated by differential salt precipitation (Table III) and analyzed by CM-cellulose

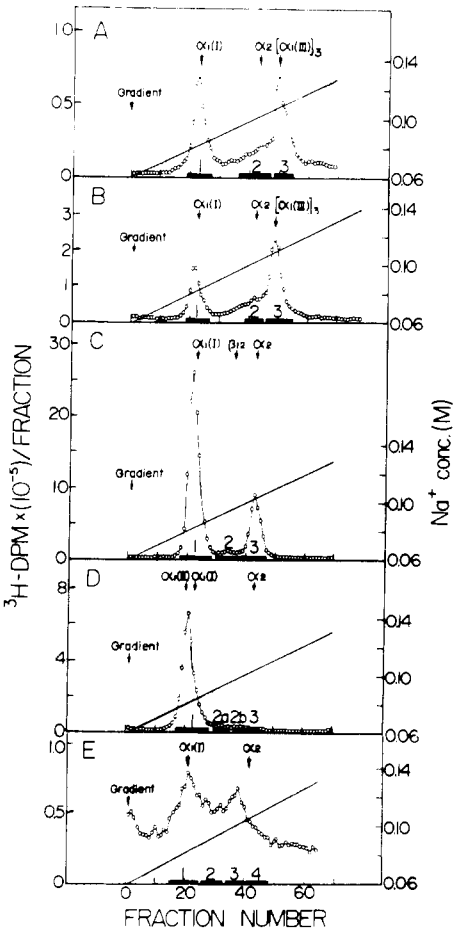


FIGURE 3: CM-cellulose chromatograms of labeled collagen from the medium after fractionation by differential salt precipitation with NaCl at neutral pH as described under Methods. Precipitate at 1.0 M NaCl (A); precipitates between 1.0 and 1.7 M (B); precipitates between 1.7 and 2.6 M (C); precipitates between 2.6 and 4.4 M NaCl (D); supernatant at 4.4 M NaCl (E). Peaks were collected and saved for analysis (see Figures 4 and 5).

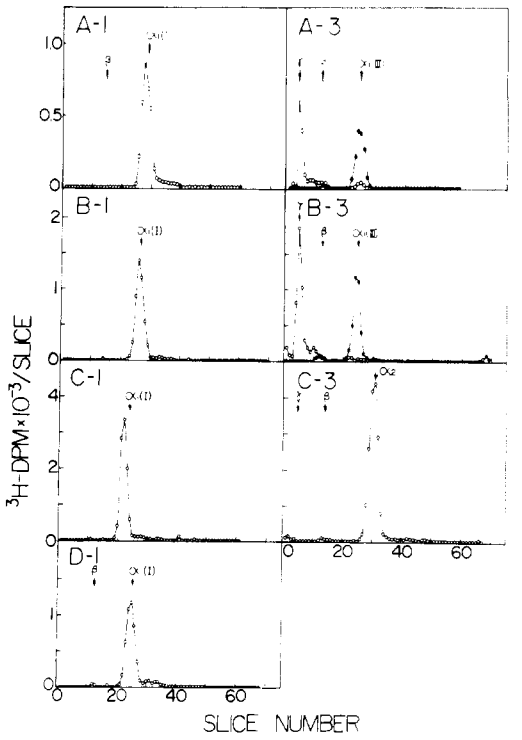


FIGURE 4: NaDodSO$_4$–polyacrylamide gel electrophoresis of collagen from the fractions of CM-cellulose chromatography. (A-1 and A-3) Fractions 1 and 3 of Figure 3A; (B-1 and B-3) fractions 1 and 3 of Figure 3B; (C-1 and C-3) fractions 1 and 3 of Figure 3C; (D-1) fraction 1 of Figure 3D. Electrophoresis was performed in the absence (○) or in the presence (●) of 1 mM DTT. After staining and destaining procedures, 1-mm gel slices were prepared and counted as described under Methods. Arrows show the migration positions of carrier rat skin collagen chains.

chromatography. Precipitates at 1.0 and 1.7 M NaCl concentrations contained collagen components corresponding to carrier rat skin $\alpha$1(I) and [$\alpha$1(III)]$_3$ (Figures 3A-1, 3A-3, 3B-1 and, 3B-3). The $\alpha$1 components from the 1.0 and 1.7 M NaCl precipitates also coincided with rat skin $\alpha$1(I) on NaDodSO$_4$–polyacrylamide gel electrophoresis (Figures 4A-1 and 4B-1), and the A-3 and B-3 components in Figure 3 migrated as $\gamma$ before reduction and comigrated with $\alpha$1(III) after reduction (Figures 4A-3 and 4B-3). $\alpha$1 fractions of the 2.6 and 4.4 M NaCl precipitates (Figures 3C-1 and 3D-1) also corresponded to $\alpha$1 chains on NaDodSO$_4$–polyacrylamide gel electrophoresis (Figures 4C-1 and 4D-1), and the $\alpha$2 fraction of the 2.6 M NaCl precipitate (Figure 3C-3) coincided with the $\alpha$2 chain on NaDodSO$_4$–polyacrylamide gel electrophoresis (Figure 4C-3). Each component (A-1–D-1 in Figure 3) was further identified by comparing the CNBr peptide patterns with those of carrier collagens (Figure 5). Elution profiles of radioactive collagen chains in A-1, B-1, and C-1 showed quite similar patterns to those of carrier $\alpha$1(I) chains from rat skin, and those in A-3 and B-3 showed quite similar patterns to those of carrier [$\alpha$1(III)]$_3$ from rat skin. Fraction C-3 was identified as $\alpha$2 (Figure 5C-3). The elution profile of $^3$H-labeled CNBr peptides in D-1 was quite different from the peptides of carrier $\alpha$1(II) but corresponded well with $\alpha$1(I) (Figure 5D-1). Fluorescence autoradiograms of fractions A-2 and B-2 in Figure 3 after NaDodSO$_4$–polyacrylamide gel electrophoresis indicated the presence of the $\alpha$2 component as the major band regardless of the absence or presence of DTT (data not shown). From the data described above (see Figure 3), we concluded that the 1.0 M NaCl and 1.7 M NaCl precipitates contain type I (A-1 + A-2 and B-1 + B-2) and type III (A-3 and B-3). Precipitates at 2.6 and 4.4 M NaCl are mainly composed of type I (C-1 and C-3) and $\alpha$1 type I trimer (D-1), respectively. The ratio of $\alpha$1/$\alpha$2 in the 2.6 M NaCl precipitate was 3.09. Since the amino acid content (proline plus hydroxyproline) in the $\alpha$1(I) chain is 15% higher than that in the $\alpha$2 chain (Piez, 1967), the $\alpha$1/$\alpha$2 ratio of [$^3$H]proline-labeled type I collagen should be 2.3. From the difference between the values of 3.09 and 2.3, the relative amounts of type I and type I trimer collagens recovered in the 2.6 M NaCl precipitate were estimated to be 47.9 and 12.4% of the total collagen isolated, respectively.

Minor components in the 2.6 and 4.4 M NaCl precipitates were further analyzed. Fractions C-2, D-2a, and D-2b in Figure 3 were all eluted between $\alpha$1 and $\beta$12 by rechromatography on a CM-cellulose column (Figure 6). Fractions 1 and 2 in Figure 6A showed several bands on NaDodSO$_4$–polyacrylamide gel electrophoresis in either the presence or the absence of DTT (Figure 7, 1a, 1b, 2a, and 2b). Therefore, we could not identify the type of collagen in this fraction, though all the proteins corresponding to each band were collagenase digestible (Figure 7, 1c and 2c). Fractions 3, 4, and 5 in Figure 6 contained main bands corresponding to $\alpha$A and $\alpha$B chains (Figure 7, 3a–c, 4a–c, and 5a–c; Burgeson et al., 1976), respectively, although their separation was incomplete. Elution positions of the two chains on CM-cellulose chromatography (Figure 6) also support the above classification even though their isolation and further characterization are necessary for identification.

Table III: Distribution of Several Types of Collagens in the Cell Layer and Medium Subfractions from BB Cell Culture[a]

| fraction[b] | after CMC | collagen component identified by: CMC chromatog | rechromatog on CMC | $NaDodSO_4$ electrophoresis | CNBr peptide analysis | type of collagen | rel amount[c] (%) |
|---|---|---|---|---|---|---|---|
| medium | | | | | | | |
| 1.0 M NaCl ppt (4.8%) | A-1 | α1 | α1 | α1 | α1(I) | I | 2.6 |
| | A-2 | α2 | | α2 | | | |
| | A-3 | $[\alpha 1(III)]_3$ | $[\alpha 1(III)]_3$ | γ (−DTT)<br>α1(III) (+DTT) | $[\alpha 1(III)]_3$ | III | 2.2 |
| 1.7 M NaCl ppt (3.8%) | B-1 | α1 | | α1 | α1(I) | I | 1.9 |
| | B-2 | α2 | | α2 | | | |
| | B-3 | $[\alpha 1(III)]_3$ | $[\alpha 1(III)]_3$ | γ (−DTT)<br>α1(III) (+DTT) | $[\alpha 1(III)]_3$ | III | 1.9 |
| 2.6 M NaCl ppt (62.8%) | C-1 | α1 | | α1 | α1(I) | I | 47.9 |
| | | | | | | I trimer | 12.4 |
| | C-2 | α1–α2 | β12[d] | NI[e] | | NI | 2.5 |
| | C-3 | α2 | | α2 | α2 | | |
| 4.4 M NaCl ppt (16.3%) | D-1 | α1 | | α1 | α1(I) | I trimer | 13.8 |
| | D-2a | α1–β12[d] | α1–β12[d] | αA | | V (αA + αB) | 1.3 |
| | D-2b | α1–β12[d] | β12[d] | αB | | | |
| | D-3 | α2 | α2 | α2 | | I | 1.2 |
| 4.4 M NaCl sup (3.6%) | E-1 | α1 | α1 | α1 | | NI | 3.6 |
| | E-2 | α1–α2 | α1–β12[d] | α1 | | NI | |
| | E-3 | α1–α2 | β12[d] | α1 | | NI | |
| | E-4 | α2 | β12[d]–α2 | α1 | | NI | |
| cell | | | | | | | |
| 4.4 M NaCl ppt (8.7%) | F-1 | α1 | | α1 | | I | 5.0 |
| | F-2 | αA | | αA, X | | I trimer | 2.9 |
| | F-3 | αB | | αA, αB, X | | V (αA + αB) | 0.6 |
| | F-4 | α2 | | α2, αB, X | | X | 0.2 |
| | F-5 | | | α2, α1(III) | | | |

[a] Pepsin-treated [$^3$H]proline-labeled collagens were fractionated by differential salt precipitation, and the relative quantity of collagenous material was estimated by an assay with bacterial collagenase. Collagen components were first separated by CM-cellulose (CMC) chromatography, pH 4.8, and then further analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis and/or CM-cellulose chromatography of CNBr peptides at pH 3.6. Some samples were further purified by rechromatography on CM-cellulose columns before CNBr digestion. Proportions of collagen components were determined by measuring the area of each component on the first CM-cellulose chromatography. In case of collagen components in the cell layer, the proportion was estimated by densitometry after fluorescence autoradiography. A ratio of 2.3 was used for α1/α2 of [$^3$H]proline-labeled type I collagen (Piez, 1967) to estimate the amount of type I trimer collagen. [b] Numbers in parentheses mean the percent of total collagen components present in each fraction. [c] Components comprising less than 0.2% of the total collagen were omitted from the calculation. [d] Indicates the elution position of carrier collagen components and does not necessarily mean the presence of β component. [e] NI, not identified.

The supernatant at 4.4 M NaCl contained heterogeneous materials (Figure 3E). Rechromatography of each fraction (E-1, E-2, E-3, and E-4) showed a peak at the position corresponding to that on the first CM-cellulose chromatography (data not shown), and only faint bands were observed on $NaDodSO_4$–polyacrylamide gel electrophoresis (not shown). Further characterization of these components was not performed because of the scarcity of the materials.

*Characterization of Cell Layer Collagen.* Preliminary experiments on the direct analysis of the pepsin-solubilized cell layer fraction by CM-cellulose chromatography showed high background counting. Therefore, the 4.4 M NaCl precipitate at pH 7.5 was employed for routine analysis of collagen. The supernatant at 4.4 M NaCl contained ~10% of the collagenase-digestible material in the cell layer, which accounts for less than 1% of the total collagen recovered in both the medium and cell layer. Since no characteristic bands corresponding to collagen components were observed by $NaDodSO_4$–polyacrylamide gel electrophoresis, it was not included in the recovery of collagen.

The precipitate at 4.4 M NaCl was fractionated into five fractions by CM-cellulose chromatography (Figure 2B), as termed F-1–F-5 in Table III, and analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis. As shown in Figure 8, F-1 contained exclusively the α1 chain. F-2 showed a band slightly slower than α1 but corresponding to αA or α1(III) (Figure 8, column F-2a). DTT did not affect the migration of this component (Figure 8, column F-2b). From the elution position on CM-cellulose chromatography, it was determined to be αA. F-3 contained αA and αB. The elution profile on CM-cellulose also supports the observation. F-4 was composed of α2 and αB chains. The ratio of αA/αB, estimated by densitometry of F-2–F-4, was ~2.

It should be noted the collagenase-labile components migrating slower than the γ component were observed in the fractions F-2, F-3, and F-4 on the fluorogram by $NaDodSO_4$–polyacrylamide gel electrophoresis. Their migration positions were shifted to the β region (molecular weight ~200 000) upon reduction. These components, tentatively termed as X, may not be procollagen of type I or type III, because collagenous materials were first isolated from cell layers by pepsin treatment and the size of the subunit of the component after reduction (molecular weight ~200 000) was even higher than intact pro-α chains (molecular weight ~150 000). Type X could be a precursor of so-called basement membrane collagens because the molecular weight of the collagens is larger even after pepsin treatment. Fraction F-5 contained trace amounts (less than 0.1% of total collagen) of α1(III) and α2 on $NaDodSO_4$–polyacrylamide gel electrophoresis (data not shown).

In summary, type I, type I trimer, and type III collagens were 58.6, 29.1, and 4.1% of the labeled collagens isolated from BB cell cultures, respectively. These interstitial types of collagens account for 92% of the collagen isolated. Components similar to type V, which account for 1.9% of total collagen, also were found (Table III).

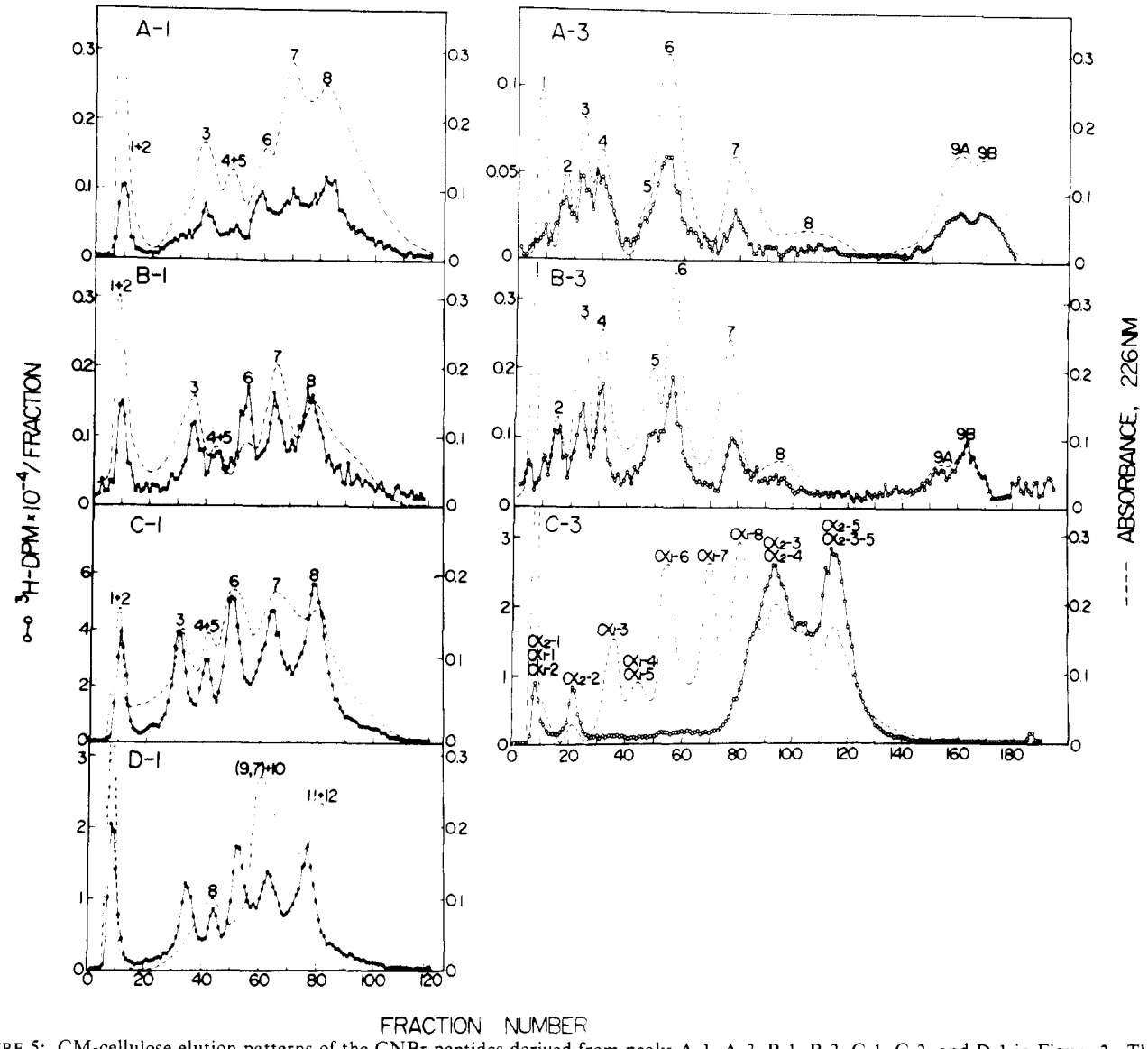


FIGURE 5: CM-cellulose elution patterns of the CNBr peptides derived from peaks A-1, A-3, B-1, B-3, C-1, C-3, and D-1 in Figure 3. The peak fractions employed are the same as those in Figure 4. 10 mg of $\alpha$1(I) (A-1, B-1, and C-1), [$\alpha$1(III)]$_3$ (A-3 and B-3), or $\alpha$1(II) (D-1) chain or 15 mg of rat skin type I collagen (C-3) was added before CNBr digestion. Elution patterns of radioactivity (O) and CNBr peptides of carrier collagen chains (---) are shown. Each peak is numbered as assigned previously (Butler et al., 1967; Miller & Lunde, 1973; Hata et al., unpublished experiments).

*Characterization of Collagen from BC Cells or Subclones of BB.* Collagen components produced by another hepatocyte clone (BC) were partially characterized by CM-cellulose chromatography after differential salt precipitation of the medium collagen or after precipitation at 4.4 M NaCl in case of the cell layer collagen. Elution profiles of these fractions on CM-cellulose chromatography resembled those of BB cells (Figures 2B and 3A–3E), and NaDodSO$_4$–polyacrylamide gel electrophoresis also showed quite similar results to those from BB cell culture (not shown). Relative contents of type I, type I trimer, and type III collagen components in the isolated collagenous material were 67.6, 11.3, and 6.9%, respectively. Type V like component was also observed in the cell layer (not shown).

When medium collagens from the culture of subclones of the BB clone were analyzed by CM-cellulose chromatography after pepsin digestion (not shown), elution profiles were again similar to that of the maternal clone (BB) (Figure 2A) whether they were cultured in the presence of CS or FCS, but the relative amount of type III collagen in a subclone (BB1S) (15%) was higher than that (5%) in the medium collagen from BB cells labeled for 18 h. These results indicate that collagen-producing capacity of liver parenchymal cell clones is a quite stable character, though relative rates in production of each collagen component are variable.

*General Discussion.* In this experiment we quantitatively analyzed collagen components produced by hepatocyte clones by using salt fractionation, chromatography, and NaDodSO$_4$–polyacrylamide gel electrophoresis together with protease-free collagenase digestion. This is additional evidence that cloned cells produce several genetically different types of collagens, as reported previously (Hata & Peterkofsky, 1977, 1978). After 18 h of culture, over 90% of the collagenous materials were recovered in the medium fraction (Table I). After pepsin digestion and NaCl precipitation, 91.3% of the collagen components were found in the medium and the remaining 8.7% in the cell layer fraction (Table III). Type I and type I trimer collagens were distributed both in the medium and cell layer fractions, but the relative amount of type V collagen was higher in the cell layer (6.3% of cell layer collagens compared to 1.4% of medium collagens). This is in clear contrast to type III collagen, which was found almost

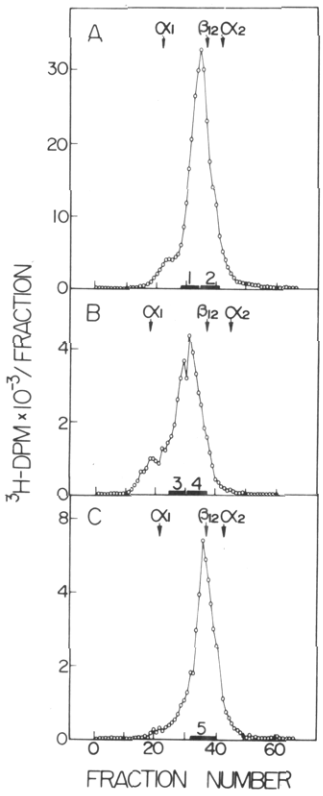


FIGURE 6: CM-cellulose rechromatography of minor fractions in Figure 3. Fraction 2 in Figure 3C (A) and fractions 2a (B) and 2b (C) in Figure 3D were pooled separately and rechromatographed with 1 mg of carrier rat skin type I collagen at pH 4.8. Arrows indicate the elution positions of type I collagen chains. Peak fractions were pooled as indicated.

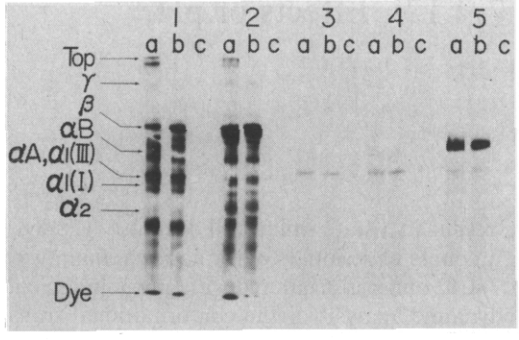


FIGURE 7: Fluorescence autoradiograms of [$^3$H]proline-labeled collagen chains isolated by rechromatography of collagen components as in Figure 6. Fractions 1, 2, 3, 4, and 5 in Figure 6 were electrophoresed in the absence (a) or presence (b) of DTT or after collagenase digestion (c). Arrows indicate the migration positions of carrier rat skin collagen chains.

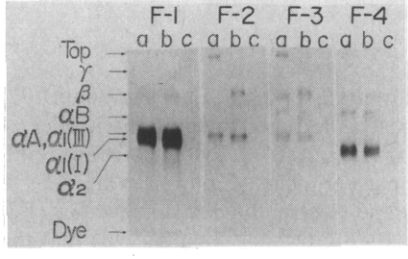


FIGURE 8: Fluorescence autoradiograms of [$^3$H]proline-labeled cell layer proteins after pepsin digestion followed by CM-cellulose chromatography. Aliquots of the effluent fractions 1 (F-1) to 4 (F-4) in Figure 2B were electrophoresed in the absence (a) or presence (b) of DTT or after collagenase digestion (c). Arrows indicate the migration positions of carrier collagen chains.

exclusively in the medium fraction. Thirty percent of the collagen isolated from BB cells was type I trimer collagen. Since the first report on the finding of type I trimer collagen in bromodeoxyuridine-treated chondrocyte cultures (Mayne et al., 1975), this collagen component has been found in cultured cells or tissues such as inflammed human gingiva (Narayanan & Page, 1976) and embryonic teeth (Hata & Slavkin, 1978; Munksgaard et al., 1978). A precursor form of that collagen was also reported (Little et al., 1977; Crouch & Bornstein, 1978). Production of type I trimer collagen is not an artifact of in vitro culture, because this collagen was also found in tumors in vivo (Moro & Smith, 1977) and in embryonic chick tendons and calvaria (Jimenez et al., 1977). These data suggest a relationship between production of type I trimer and cell growth because cultured cells proliferate faster than the cells in vivo. Growth rates of cells are also high in tumor and embryonic tissues. In this connection, the presence of type I trimer in human cirrhotic liver and the absence in normal liver (Rojkind et al., 1979) are quite suggestive, because the turnover rate of hepatocytes, which account for about 80% of the liver cells, is generally low. The average doubling time in normal liver would be over 100 days when calculated from the life span of the cells (MacDonald, 1961). The turnover of cells in cirrhotic liver, where degeneration and regeneration of hepatocytes occur, is much faster, especially in the early phase of cirrhosis. The hepatocyte clones (BB and BC) used in this experiment grow very rapidly (doubling time 23 h, Table I). The finding of type I, type III, type I trimer, and so-called basement membrane collagens (types IV and V) in human liver (Rojkind et al., 1979) is also interesting because these components are quite similar to the components that we have found, even though the relative amounts of the various types of collagens are different. The difference could be attributable to the fact that analyses were of accumulated collagen in human liver and of newly formed collagen in culture, though other factors affecting the proportions of collagen types such as culture conditions and degrees of passage may not be excluded. Our recent experiments on acidic glycosaminoglycans, other connective tissue components, showed that liver parenchymal cell clones (BB and BC) produced heparan sulfate as the major component and dermatan sulfate, chondroitin sulfate, and hyaluronic acid as minor components. These results are also quite similar to those of whole liver (Y. Ninomiya, R. Hata, and Y. Nagai, unpublished experiments). Regulation mechanisms of the metabolism of collagen and acidic glycosaminoglycan components in hepatocyte clones are now under investigation to clarify the role of hepatocyte in the metabolism of the liver under normal and pathological conditions.

## Acknowledgments

We thank Hironobu Sunada and Yukiko Sakai for their skilled technical assistance and Kazuyo Oda for typing the manuscript. We also acknowledge Drs. Toshihiko Hayashi and Hisae Hori for critical readings of the manuscript.

## References

Bienkowski, R. S., Cowan, M. J., McDonald, J. A., & Crystal, R. G. (1978) *J. Biol. Chem. 253*, 4356–4363.

Bonner, W. M., & Laskey, R. A. (1974) *Eur. J. Biochem. 46*, 83–88.

Burgeson, R. E., El Adli, F. A., Kaitila, I. I., & Hollister, D. W. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2579–2583.

Butler, W. T., Piez, K. A., & Bornstein, P. (1967) *Biochemistry 6*, 3771–3779.

Chung, E., & Miller, E. J. (1974) *Science 183*, 1200–1201.

Crouch, E., & Bornstein, P. (1978) *Biochemistry 17*, 5499–5509.
Epstein, E. H., Jr., Scott, R. D., Miller, E. J., & Piez, K. A. (1971) *J. Biol. Chem. 246*, 1718–1724.
Fessler, J. H., & Fessler, L. I. (1978) *Annu. Rev. Biochem. 47*, 129–162.
Hata, R., & Peterkofsky, B. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2933–2937.
Hata, R., & Peterkofsky, B. (1978) *J. Cell. Physiol. 95*, 343–352.
Hata, R., & Slavkin, H. C. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2790–2794.
Hata, R., Ninomiya, Y., & Nagai, Y. (1978a) *Seikagaku 50*, 1006.
Hata, R., Ninomiya, Y., Nagai, Y., Sakakibara, K., & Tsukada, Y. (1978b) *Proc. Jpn. Acad., Ser. B 54*, 391–396.
Hayashi, T., & Nagai, Y. (1979) *J. Biochem. (Tokyo) 86*, 453–459.
Jimenez, S. A., Bashey, R. I., Benditt, M., & Yankowski, R. (1977) *Biochem. Biophys. Res. Commun. 78*, 1354–1361.
Kefalides, N. A. (1973) *Int. Rev. Connect. Tissue Res. 6*, 63–104.
Lapière, C. M., & Nusgens, B. (1976) in *Biochemistry of Collagen* (Ramachandran, G. N., & Reddi, A. H., Eds.) pp 377–447, Plenum Press, New York and London.
Laskey, R. A., & Mills, A. D. (1975) *Eur. J. Biochem. 56*, 335–341.
Little, C. D., Church, R. L., Miller, R. A., & Ruddle, F. H. (1977) *Cell 10*, 287–295.
MacDonald, R. A. (1961) *Arch Intern. Med. 107*, 335–343.
Martin, G. R., Byers, P. H., & Piez, K. A. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol. 42*, 167–191.
Mayne, R., Vail, M. S., & Miller, E. J. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4511–4515.
Miller, E. J. (1976) *Mol. Cell. Biochem. 13*, 165–192.
Miller, E. J., & Lunde, L. G. (1973) *Biochemistry 12*, 3153–3159.
Moro, L., & Smith, B. D. (1977) *Arch. Biochem. Biophys. 182*, 33–41.
Munksgaard, E. C., Rhodes, M., Mayne, R., & Butler, W. T. (1978) *Eur. J. Biochem. 82*, 609–617.
Narayanan, A. S., & Page, R. C. (1976) *J. Biol. Chem. 251*, 5464–5471.
Nishi, S., Kobayashi, K., & Hirai, H. (1974) *Proc. Jpn. Cancer Ass. 33*, 161.
Peterkofsky, B. (1972) *Arch. Biochem. Biophys. 152*, 318–328.
Peterkofsky, B., & Diegelmann, R. (1971) *Biochemistry 10*, 988–994.
Piez, K. A. (1967) *Treatise Collagen 1*, 207–252.
Reddi, A. H. (1976) in *Biochemistry of Collagen* (Ramachandran, G. N., & Reddi, A. H., Eds.) pp 449–478, Plenum Press, New York and London.
Rojkind, M., Giambrone, M., & Biempica, L. (1979) *Gastroenterology 76*, 710–719.
Sakakibara, K., Saito, M., Umeda, M., Enaka, K., & Tsukada, Y. (1976) *Nature (London) 262*, 316–318.
Trelstad, R. L., Catanese, V. M., & Rubin, D. F. (1976) *Anal. Biochem. 71*, 114–118.
Uitto, J., & Lichtenstein, J. R. (1976) *J. Invest. Dermatol. 66*, 59–79.

# Conformational Stability of Ribosomal Protein L7/L12: Effects of pH, Temperature, and Guanidinium Chloride†

Carl A. Luer and Kin-Ping Wong*

ABSTRACT: The effects of pH, temperature, and guanidinium chloride on the conformation of ribosomal protein L7/L12 have been investigated in order to understand the stability of this protein dimer. The results indicate that many of the molecular forces stabilizing the conformation of the dimer are disrupted at low pH or high temperature. These acid- and thermal-denatured states, however, still retain considerable secondary structure. Approximately half of the $\alpha$-helical content present in the native protein remains intact at pH below 2 and at temperatures above 90 °C. Further denaturation of the acid-denatured protein by 6 M guanidinium chloride results in a state which still contains ~20% $\alpha$ helix. Similar amounts of residual conformation remain when the native L7/L12 dimer is denatured with guanidinium chloride. Thermodynamic analysis of the conformational transitions studied indicates that none is compatible with a simple two-state process. The complexity of these denaturation data and the structural characterizations of the various denatured states are consistent with the possible existence of structural domains in the protein molecule possessing different conformational stabilities.

Protein L7/L12, from the large subunit of *Escherichia coli* ribosomes, is an extremely elongated (Wong & Paradies, 1974; Österberg et al., 1976; Luer & Wong, 1979), highly $\alpha$-helical protein (Möller et al., 1970; Dzionara, 1970; Boublik et al., 1973; Gudkov et al., 1978a,b; Luer & Wong, 1979) which is known to exist as a dimer in solution (Möller et al., 1972; Wong & Paradies, 1974). Evidence is accumulating from reconstitution experiments (Caldwell et al., 1978; Koteliansky et al., 1978) and electron microscopic studies (Boublik et al., 1976; Lake, 1976; Strycharz et al., 1978) that the elongated dimer may exist in the functionally active ribosome. This suggests that studies on the L7/L12 dimer in solution may provide relevant structural information which can be applied to the growing knowledge of the biomolecular components of the ribosome.

† From the Department of Biochemistry, University of Kansas Medical Center, Kansas City, Kansas 66103. *Received April 24, 1979.* This investigation was supported by Research Grant GM 22962 and in part by Grant HL 18905 from the National Institutes of Health, U.S. Public Health Service, Bethesda, MD.

* Correspondence should be addressed to this author. He is a National Institutes of Health Career Development Awardee (GM 70628).

0006-2960/80/0419-0176$01.00/0 