Gralla, J., Steitz, J. A., & Crothers, D. M. (1974) *Nature (London) 248*, 204.
Gumport, R. I., & Uhlenbeck, O. C. (1981) in *Gene Amplification and Analysis. Vol. 2. Structural Analysis of Nucleic Acids* (Chirikjian, J. G., & Papas, T. S., Eds.) p 314, Elsevier/North-Holland, New York.
Hilbers, C. W., Shulman, R. G., Yamane, T., & Steitz, J. A. (1974) *Nature (London) 248*, 225.
Johnson, R. A., & Walseth, T. F. (1979) *Adv. Cyclic Nucleotide Res. 10*, 135.
Klee, C. B. (1971) *Prog. Nucleic Acid Res. Mol. Biol. 2*, 896.
Koper-Zwarthoff, E. C., Lockhard, R. E., De Weerd, B. A., RajBhandary, U. L., & Bol, J. F. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5504.
Krug, M., & Uhlenbeck, O. C. (1982) *Biochemistry 21*, 1858.
Lockhard, R. E., De Weerd, B. A., Heckman, J. E., MacGee, J., Tabor, M. W., & RajBhandary, U. L. (1978) *Nucleic Acids Res. 5*, 37.
McFarland, G., & Borer, P. N. (1979) *Nucleic Acids Res. 7*, 1067.
McLaughlin, L., Cramer, F., & Sprinzl, M. (1981) *Anal. Biochem. 112*, 60.
Moseman-McCoy, M. I., Lubben, T. H., & Gumport, R. I. (1979) *Biochim. Biophys. Acta 562*, 149.
Nishimura, S. (1979) in *Transfer RNA: Structure, Properties, and Recognition* (Shimmel, P. R., Söll, D., & Abelson, J. N., Eds.) p 551, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Silberklang, M., Gillum, A. M., & RajBhandary, U. L. (1977) *Nucleic Acids Res. 4*, 4097.
Soltis, D., & Uhlenbeck, O. C. (1982) *J. Biol. Chem.* (in press).
Steitz, J. A. (1974) *Nature (London) 24*, 223.
Sugiyama, T., & Nakada, D. (1968) *J. Mol. Biol. 31*, 431.
Thach, R. E., & Doty, P. (1965) *Science (Washington, D.C.) 147*, 1310.
Uhlenbeck, O. C., & Cameron, V. (1977) *Nucleic Acids Res. 4*, 85.
Uhlenbeck, O. C., Borer, P. N., Dengler, B., & Tinoco, I., Jr. (1973) *J. Mol. Biol. 73*, 483.
Vandenberghe, A. (1980) *Anal. Biochem. 107*, 369.
Weith, H. L., Wiebers, J. L., & Gilham, P. T. (1970) *Biochemistry 9*, 4396.

Characterization of the Collagens Synthesized by Chinese Hamster Ovary Cells. Effect of Colcemid and Dibutyryladenosine Cyclic Monophosphate†

Hardy Limeback,* Jaro Sodek, and Jane Aubin

ABSTRACT: The collagens synthesized by Chinese hamster ovary cells have been isolated and characterized. Although these cells produce very small amounts of collagen, at least five distinct collagenous chains could be identified from radiolabeled media and cell extracts after limited pepsin digestion. Two chains were characterized as α_1(V) and α_2(V), based on electrophoretic mobility, resistance to vertebrate collagenase, chromatographic properties on carboxymethylcellulose, and cyanogen bromide peptide patterns. Two smaller collagenous proteins (M_r 34000 and 37000) were also isolated by carboxymethylcellulose chromatography and characterized by cyanogen bromide digestion patterns. These collagens showed similarities to type IV collagen fragments but may be unique to Chinese hamster ovary cells. A colcemid-resistant mutant of Chinese hamster ovary cells designated CM^{R}795 [Ling, V., Aubin, J. E., Chase, A., & Sarangi, F. (1979) *Cell (Cambridge, Mass.) 18*, 423–430] was found to synthesize the same collagen chains but in different proportions. In the wild-type cells colcemid (0.05–0.1 μg/mL) reduced the amount of type V collagen in the culture media but had little effect on the other collagen type, whereas the type V collagen reduction was less pronounced in the CM^{R}795 cells treated with the same concentrations of colcemid. Dibutyryladenosine cyclic monophosphate caused a fibroblast-like "reverse transformation" of the Chinese hamster ovary cells similar to that described previously [Hsie, A. W., & Puck, T. T. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 358–361]. However, collagen synthesis was increased only slightly. Furthermore, no apparent alteration in the types of collagens synthesized was detected.

Various cell types have been used to study the biosynthesis and molecular heterogeneity of collagen. Usually the collagen synthetic pattern is characteristic of the cell type examined and of the tissue of origin. For example, fibroblasts and smooth muscle cells synthesize type I and type III collagens whereas chondrocytes synthesize type II collagen [see Bornstein & Sage (1980) for recent review]. However, the cellular origin of basement membrane collagens, type IV (Kefalides, 1975) and possibly type V[1] (Roll et al., 1980), is unknown; they may be products of epithelial cells, connective tissue cells, or both. Some fibroblasts (Herrmann et al., 1980; Gay et al., 1980) and even bone cells (Aubin et al., 1982) are capable of synthesizing type V collagen in vitro, but synthesis of type

† From the Medical Research Council Group in Periodontal Physiology, Faculty of Dentistry, University of Toronto, Toronto, Ontario, Canada M5S 1A8. *Received January 5, 1982.* This work was supported by the Medical Research Council of Canada and by a grant from the Faculty of Dentistry, University of Toronto.

[1] The nomenclature used for type V collagen chains is described by Bornstein & Sage (1980). α A and α B have been designated α_2(V) and α_1(V), respectively.

0006-2960/82/0421-4720$01.25/0

IV collagen appears to be carried out primarily by epithelial cells (Killen & Striker, 1979). Whether collagen gene expression is under rigid and fixed control or is readily altered by exogenous signals has not been determined.

It is desirable to have homogeneous, stable, clonal populations capable of collagen synthesis to explore some of the questions remaining regarding collagen regulation. The Chinese hamster ovary (CHO)[2] cell line would seem to be a valuable system. CHO cells have been used for many studies including, for example, cell attachment (Kleinman et al., 1978), cell mutations related to drug resistance (Thompson & Baker, 1973), and enzyme (Gottesman et al., 1980) and auxotrophic (Chasin, 1974) markers. In addition, these cells have been found to undergo a "reverse transformation" from epithelioid to a highly elongated fibroblast morphology under the influence of dbcAMP (Hsie & Puck, 1971) or the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (Sisskin & Weinstein, 1980). The mechanism of reverse transformation is of great importance. The degree of microtubule polymerization has been suggested to be altered during dbcAMP-induced reverse transformation (Porter et al., 1974). It has been postulated that the microtubule–microfilament system and adenosine cyclic monophosphate may be important in normal growth regulation through an association with cell surface–genome information transfer and both may be involved when normal cells acquire the transformed phenotype in culture (Puck, 1977; Pastan & Willingham, 1978). Microtubules have been implicated in the secretion of type I collagen to the extracellular milieu (Ehrlich et al., 1974). Whether or not a change in the microtubules and collagen synthesis are causally related to the transformed state is unknown. A mutant CHO cell with altered microtubule function would provide a system for probing some of these questions. First, it may allow one to determine what role, if any, microtubules play in collagen secretion. Second, by manipulating the reverse transformation phenotype, one may analyze the role of collagen synthesis in the expression of this phenotype.

Determination of collagen type is important as a first step in studying CHO cells since cell morphology is not always a reliable indicator of tissue of origin. Though usually considered epithelioid in morphology (Hsie et al., 1971), CHO cells possess many fibroblastic features as well (Puck et al., 1958; Nielson & Puck, 1980). Furthermore, since collagens are involved in attachment of many cells in vitro (Kleinman et al., 1981), it is also of interest to identify the types of CHO collagens synthesized which might be involved in CHO cell attachment or changes in cell attachment during transformation.

We report here the determination of the nature of collagens synthesized in epithelioid vs. the fibroblast-like cells in the reverse-transformation system. The collagens of a colcemid-resistant mutant, expressing tubulin with decreased colcemid-binding affinity (Ling et al., 1979; Keates et al., 1981), were also identified so that the specific effects of colcemid on microtubules and the relationship of this to collagen synthesis and secretion could be evaluated. In addition, we have studied the effect of dbcAMP on the collagen synthesis of CHO cells to determine whether reverse transformation of the CHO cells into a fibroblast morphology is accompanied by synthesis of interstitial collagens (types I and III) which are typical of fibroblasts.

Materials and Methods

Cell Cultures. A Chinese hamster ovary (CHO) cell line ($E_{29}Pro^+$) and a colcemid-resistant mutant (CM^R795) were obtained from V. Ling, University of Toronto (Ling et al., 1979; Keates et al., 1981). Cells were grown in α-MEM supplemented with 10% fetal calf serum, 0.3 μg/mL amphoteracin B, 50 μg/mL gentamycin, and 100 μg/mL penicillin. For testing the effects of colcemid and dbcAMP, the cells were subcultured by trypsinization and plated out at appropriate cell densities, using a hemocytometer to determine cell numbers.

Collagen Preparation. Confluent cultures of CM^R795 and $E_{29}Pro^+$ in 75-cm^2 culture flasks (Falcon) were washed twice with 10 mL of serum-free medium and cultured 24 h in a serum-free α-MEM lacking proline and glycine but supplemented with the corresponding U-^{14}C-labeled L-amino acids ([U-^{14}C]glycine, 52.9 mCi/mmol, NEC-047; [U-^{14}C]proline, 250 mCi/mmol, NEC-285, New England Nuclear; 1–2 μCi/mL each). In addition β-aminopropionitrile (50 μg/mL; Sigma) and freshly prepared ascorbic acid (100 μg/mL; Sigma) were added. The media were collected and the cells extracted by shaking with 10 mL of 0.5 M acetic acid overnight at 4 °C. The cellular extracts were freed of cellular debris by centrifugation at 1600*g*. The media and cell extracts were exhaustively dialyzed against 0.5 M acetic acid. Aliquots were saved for amino acid analysis and slab gel analysis. The remaining material was digested with pepsin (50 μg/mL) at 15 °C for 4 h. Type V collagen (1.0 mg) purified from rat placentas was added as carrier and the material dialyzed against 1.2 M NaCl in 0.5 M acetic acid. The precipitates that formed during dialysis were collected by centrifugation at 30000*g* for 30 min, pooled, redissolved in 50 mL of 0.5 M acetic acid, and concentrated by Amicon filtration to 1 mL using an XM-100 filter and 20 psi of N_2 pressure. Dilution to 50 mL and concentration were repeated 3 times to remove any remaining pepsin and most of the low molecular weight peptides that resulted from the pepsin digestion. The concentrated material was dialyzed against the starting buffer (40 mM sodium acetate/1.0 M urea, adjusted to pH 4.8 with acetic acid) prior to separation by carboxymethylcellulose (CM-cellulose) chromatography.

Analysis of Collagen by CM-cellulose Chromatography. Radiolabeled collagens, dialyzed against the starting buffer (40 mM sodium acetate, pH 4.8/1.0 M urea), were denatured by heating at 56 °C for 15 min before application to a column (1.0 × 15 cm) of CM-cellulose (CM-52, Whatman) equilibrated with the starting buffer at 42 °C (Piez et al., 1963). The collagens were eluted from the column at a flow rate of 7.0 mL/h with a linear gradient of 0–120 mM NaCl in starting buffer over a total volume of 200 mL. Radioactive peaks were pooled, dialyzed, and freeze-dried for further analysis by cyanogen bromide digestion, amino acid analysis, and $NaDodSO_4$–polyacrylamide slab-gel electrophoresis.

Cyanogen Bromide (CNBr) Digestion of Radioactive Collagens. Freeze-dried samples prepared from the CM-cellulose column were dissolved in 70% (v/v) formic acid. Solid cyanogen bromide was added to ~0.5% (w/v). After 4 h at 25 °C, the samples were diluted 5-fold with water and freeze-dried prior to analysis by $NaDodSO_4$–polyacrylamide slab-gel electrophoresis.

Amino Acid Analysis. Freeze-dried samples were hydrolyzed in evacuated glass tubes with 6 N HCl at 110 °C for 24 h, dried under vacuum, and dissolved in 0.2 M sodium

[2] Abbreviations: CHO, Chinese hamster ovary; CM, carboxymethyl; CNBr, cyanogen bromide; $NaDodSO_4$, sodium dodecyl sulfate; dbcAMP, dibutyryladenosine cyclic monophosphate; MEM, minimal essential medium: BAPN, β-aminopropionitrile; Tris, tris(hydroxymethyl)-aminomethane.

citrate buffer, pH 2.2. Samples were analyzed on a Beckman 121M amino acid analyzer as previously described (Sodek, 1976) to estimate relative amounts of ^{14}C-labeled 3-hydroxyproline, 4-hydroxyproline, and proline.

Bacterial Collagenase Digestion. Partially purified CHO collagens were dissolved in 25 μL of 50 mM Tris-HCl, pH 7.5, containing 5 mM $CaCl_2$. A 25-μL sample of bacterial collagenase (Worthington) purified on a Sephadex G-200 column and dissolved in the same buffer at a concentration of 1.0 μg/mL was added to the collagens. After a 2-h incubation at 35 °C, the reaction was halted by addition of 50 μL of 2× concentrated sample buffer prior to analysis by $NaDodSO_4$–polyacrylamide slab-gel electrophoresis.

Mammalian Collagenase Digestion. Active mammalian collagenase was prepared from porcine gingival explants maintained in serum-free medium as previously described (Pettigrew et al., 1978). Radiolabeled collagen was prepared by reacting [^{14}C]formaldehyde (NEC-039H, 40–60 mCi/mmol, New England Nuclear) with acid-soluble rat skin type I collagen. This substrate was used to test the activity of the enzyme by using a slab-gel fluorographic assay (Sodek et al., 1981). Samples (25 μL) of active enzyme, dissolved in 50 mM Tris-HCl and 5 mM $CaCl_2$, pH 7.5, were added to 25-μL samples of partially purified CHO collagens in the same buffer; 50 μL of 2× concentrated slab-gel sample buffer was added after 2-h incubation at 25 °C to stop the reaction, and the samples were analyzed by $NaDodSO_4$–polyacrylamide slab-gel electrophoresis.

$NaDodSO_4$–Polyacrylamide Slab-Gel Electrophoresis. $NaDodSO_4$–polyacrylamide slab-gel electrophoresis was performed on discontinuous slab gels (Laemmli, 1970). Fluorograms were prepared (Bonner & Laskey, 1974) and scanned at 550 nm as previously described (Limeback & Sodek, 1979). Relative amounts of radiolabeled collagens were estimated from the fluorograms with a digital integrator (Hommel Electronics, Toronto, Canada) coupled to the film scanning device of a spectrophotometer (Model 240, Gilford Instruments).

Effect of Colcemid. Duplicate 28-cm^2 dishes of $E_{29}Pro^+$ and CM^R795 cells were prepared and labeled as described above. Colcemid (Sigma) was added to the labeling media of the test dishes in concentrations ranging from 0.01 to 0.8 μg/mL. Radiolabeled media and cell extracts were collected after 18-h culture, dialyzed, and pepsin digested. The resulting pepsin-resistant proteins were quantitated by densitometric scanning of the fluorograms prepared after $NaDodSO_4$–polyacrylamide slab-gel electrophoresis. Cell counts were carried out on these separate dishes both before and after the 18-h labeling period.

Effect of dbcAMP. $E_{29}Pro^+$ and CM^R795 cells were grown to confluency in triplicate 25-cm^2 dishes in the presence of α-MEM containing 10% fetal calf serum. The cells were preincubated 24 h with serum-free medium in the presence (test) or absence (control) of 1.0 mM dbcAMP (Sigma). The preincubation medium was replaced with labeling media as described above, and in addition, dbcAMP (1.0 mM) was added to the test dishes. After labeling for 18 h, one set of dishes was used to determine cell numbers, and then radiolabeled media and cell extracts were collected from the remaining dishes and analyzed as described above.

Results

Collagen Synthesis by $E_{29}Pro^+$ and CM^R795 Chinese Hamster Ovary Cells. Amino acid analysis of the media and cell extracts of CHO cells after a 24-h labeling period showed that these cells synthesized low amounts of collagenous proteins

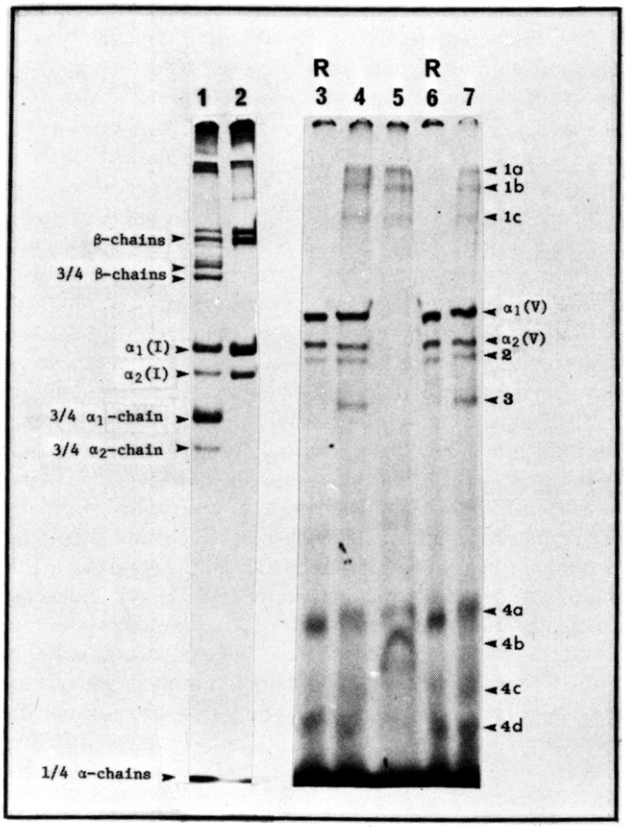

FIGURE 1: Slab-gel fluorography of pepsin-resistant proteins from CHO cells. ^{14}C-Labeled proteins from CHO cells were pepsin digested before separation on a 7.5% polyacrylamide slab gel. The resulting bands were visualized by fluorography. Samples in the lanes marked R were exposed to 0.05 M mercaptoethanol for 2 h and then 0.1 M iodoacetamide for 1 h before separation on the gel. The major CHO proteins are numbered 1–4, and the migration of the CHO type V collagen is indicated. (Lane 1) Type I methyl-^{14}C-labeled collagen standard digested with porcine gingival collagenase; (lane 2) type I methyl-^{14}C-labeled collagen standard; (lane 3) CHO collagens after porcine gingival collagenase digestion, reduction, and alkylation; (lane 4) CHO collagens after porcine gingival collagenase digestion; (lane 5) CHO collagens after bacterial collagenase digestion; (lane 6) CHO collagens after reduction and alkylation; (lane 7) CHO collagens untreated.

(Table I). Usually the protein synthesized by CM^R795 cells had 25–50% more radiolabel than the $E_{29}Pro^+$ cells and the ratio of collagen to noncollagen protein synthesis was also increased in the colcemid-resistant cells. Significant amounts of collagen were found to be secreted into the media of both cell types: the media were enriched 2–3 times with collagen relative to noncollagen protein, but the amount of radiolabeled protein found in the cell layers varied from 60 to 90% of the total radiolabeled protein, depending on the cell-layer densities. A large proportion of the total hydroxyproline synthesized by both cell types was 3-hydroxyproline, indicating synthesis of a collagen with characteristics of type IV collagen. Furthermore, the levels of 3-hydroxyproline relative to 4-hydroxyproline were 2–3 times higher in the cell layers than in the media.

Analysis of CHO Collagens by $NaDodSO_4$–Polyacrylamide Slab-Gel Electrophoresis. The pepsin-resistant proteins from the radiolabeled media pool of $E_{29}Pro^+$ and Cm^R795 cells were visualized by fluorography after $NaDodSO_4$–polyacrylamide slab-gel electrophoresis (Figure 1). Several bands of various molecular weights were observed. Two bands were identified as type V α_1 and α_2 chains. Three high molecular weight bands (1a–c) were completely resistant to bacterial collagenase digestion but were reduced by mercaptoethanol. The re-

Table I: Amino Acid Analysis of CHO Clones[a]

cells	cell fraction	expt	cell no.[b] ($\times 10^{-6}$)	dpm of protein/10^6 cells[c] ($\times 10^{-5}$)	3-hydroxyproline (dpm)	4-hydroxyproline (dpm)	proline (dpm)	4-hydroxyproline/proline	total[d] label in collagen (%)	% hydroxyproline as 3-hydroxyproline
$E_{29}Pro^+$	medium	1			33	228	21 900	0.012	0.45	13
		2			29	116	24 700	0.0064	0.22	20
	cell extract	1	4.9	2.0	63	123	22 200	0.0084	0.31	34
		2	2.0	5.6	92	113	36 400	0.0056	0.21	45
CM^R795	medium	1			62	395	23 700	0.019	0.73	14
		2			33	170	14 600	0.014	0.53	16
	cell extract	1	3.9	2.8	55	89	24 100	0.0061	0.22	38
		2	2.3	7.2	62	117	35 010	0.0051	0.19	35

[a] In two different experiments cultures of $E_{29}Pro^+$ and CM^R795 cells were labeled with [^{14}C]proline and [^{14}C]glycine for 24 h. The media and cell extracts were then dialyzed, acid hydrolyzed, and analyzed for amino acids. [b] Cell numbers were determined from triplicate samples plated identically to those used for collagen analysis. [c] Estimated by combining the nondialyzable radioactivity from the cell-layer extracts and the media. [d] Calculated according to the methods of Smith & Niles (1980) where Hyp = 3-Hypro + 4-Hypro.

Table II: Summary of $NaDodSO_4$-Polyacrylamide Slab-Gel Electrophoresis Results of CHO Proteins

pepsin-resistant band	estimated[a] M_r	reduction by mercaptoethanol	digestion by bacterial collagenase	digestion by porcine gingival collagenase
1a	320 000	+[b]	–[c]	–[c]
1b	270 000	+	–	–
1c	310 000	+	–	–
α_1(V)	110 000	–	+++	–
α_2(V)	98 000	–	+++	–
2	91 000	–	++	–
3	75 000	+	+++	–
4a	37 000	–	+	–
4b	34 000	–	ND[d]	–
4c	29 000	–	+++	–
4d	26 000	–	+++	–

[a] Molecular weights were determined from a standard curve constructed by using β_1(I), $^3/_4$ β_{11}(I), α_1(I), $^3/_4$ α_1(I), and $^1/_4$ α_1(I) as molecular weight standards. [b] (+) Complete reduction; (–) no reduction. [c] (+++) Complete digestion; (++) partial digestion; (+) incomplete digestion; (–) no digestion. [d] ND, not determined.

maining proteins were susceptible to bacterial collagenase digestion, but bands 2 and 4a were incompletely digested. Band 3 was also susceptible to reduction by mercaptoethanol, but the bands labeled α_1(V), α_2(V), 2, and 4a–d were unaffected by reduction. All the proteins described above were resistant to digestion by porcine gingival collagenase in amounts capable of digesting at least 25 μg of methyl-^{14}C-labeled rat skin type I collagen in 2 h at 25 °C. These results are summarized in Table II.

The bands labeled α_1(V) and α_2(V) migrated in the same positions as the α chains of type V collagen isolated from rat placentas. Band 2 migrated slightly faster than the α_1(I) band and band 3 slightly slower than the $^3/_4$ fragment of the α_1(I) band. When type I collagen precursors, α chains, and vertebrate collagenase digestion products were used as molecular weight standards, the molecular weights of the various CHO collagenous proteins could be estimated by $NaDodSO_4$–polyacrylamide slab-gel electrophoresis (Table II).

The relative amounts of high and low molecular weight collagenous components showed some variation in different experiments (compare, for example, Figures 2, 6a, and 8a). However, the CM^R795 cells consistently produced less band 2 and more type V collagen chains than the $E_{29}Pro^+$ cells.

Collagen Purification: Salt Fractionation. Radiolabeled proteins from pepsin-digested $E_{29}Pro^+$ and CM^R795 media, partially purified by precipitation in 1.2 M NaCl, were analyzed by slab-gel fluorography (Figure 3). The noncollagenous high molecular weight bands, 1a–c, and the collagenous bands, 3, 4c, and 4d, were not found in the precipitate, whereas the type V α chains as well as bands 2, 4a, and 4b were precipitated.

CM-cellulose Column Chromatography. Elution of the partially purified CHO collagens after application to a CM-cellulose column is shown in Figure 2. Four major peaks were recovered; less than 10% of the total radiolabeled imino acids in the unbound material (peak 1) was hydroxyproline, whereas the bound material (peaks 2–4) contained primarily collagenous protein as judged by the amino acid analysis of the individually pooled peaks (Table III). The CM-cellulose peaks were analyzed by slab-gel fluorography (Figure 3). Peak 1 contained protein fragments of less than 15 000 molecular weight (slab-gel pattern not shown), peak 2 contained the collagenous proteins labeled bands 4a and 4b, peak 3 contained primarily α_2(V), and a small amount of band 2 and peak 4

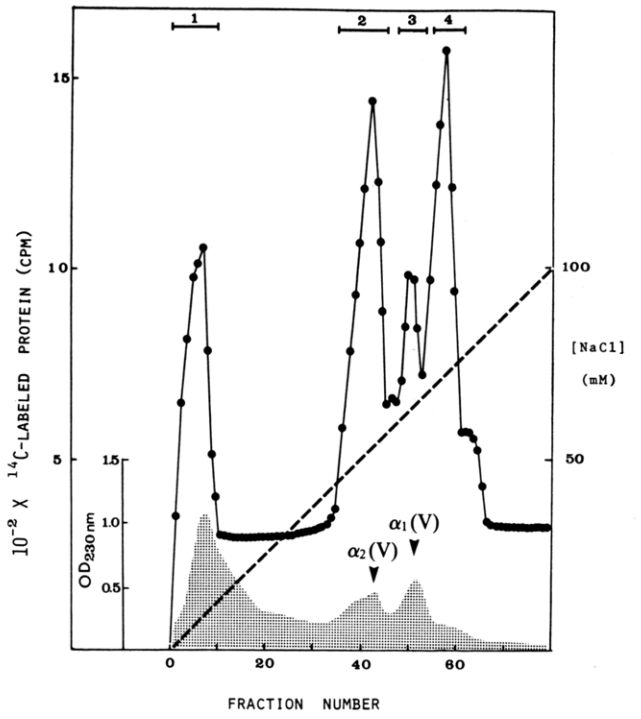

FIGURE 2: CM-cellulose separation of CHO collagens. Partially purified CHO collagens from the pooled 1.2 M NaCl precipitates of $E_{29}Pro^+$ and CM^R795 medium pepsin digests were separated on a CM-cellulose column equilibrated with 40 mM sodium acetate, pH 4.8/1.0 M urea, and eluted with a 0–120 mM NaCl linear gradient at 42 °C over a total volume of 200 mL. Partially purified rat placenta type V collagens were separated on the column under identical conditions: the resulting profile is represented by the shaded area. Four major CHO peaks were obtained, and the material in each peak was pooled for further analysis. The fractions pooled are indicated by the soild bars. (●) ^{14}C-Labeled CHO protein.

Table III: Amino Acid Analysis of CHO Collagens Purified on CM-cellulose

CM-cellulose peak	principle collagen component	4-hydroxyproline/proline	3-hydroxyproline (%)	M_r[a] principle band(s)
1		0.1	0	<15 000
2	low M_r fragments (type IV)			34 000
		1.3	5	37 000
3	α_2(V)	1.2	<1	98 000
4	α_1(V)	1.4	ND[b]	110 000

[a] Molecular weights were estimated from slab-gel electrophoresis using type I α_1 chain as a standard. [b] ND, not determined.

contained primarily α_1(V). The elution position of the CHO type V α chains from the CM-cellulose was similar to rat placenta type V collagen α chains except that the elution of the former was slightly delayed. The type V from both sources eluted after α_1(I) and before α_2(I) separated on the column under similar conditions.

CNBr-Digestion Pattern of Purified CHO Collagens. CNBr-digested pooled peaks 2–4 obtained from the CM-cellulose column (Figure 3) were analyzed by fluorography (Figure 4). The pattern produced by each collagen component was unique and unlike typical patterns produced by the CNBr digestion of type I–III collagens. The material in band 2 was codigested with α_2(V) by CNBr, but because there is little of band 2 present relative to α_2(V), its contribution to the resulting CNBr pattern is minor. The pattern obtained, therefore, represents the CNBr pattern of the CHO α_2(V)

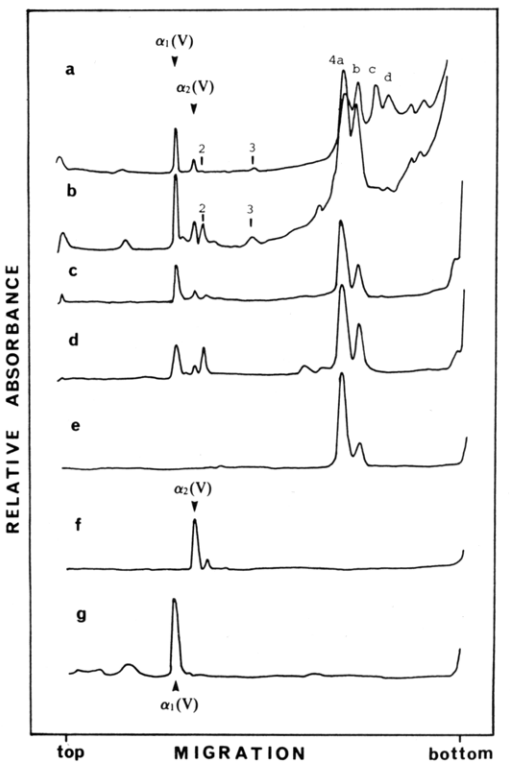

FIGURE 3: Densitometric scans of partially purified CHO collagens. The pepsin-resistant proteins from the $E_{29}Pro^+$ and CM^R795 cells were partially purified by 1.2 M NaCl precipitation, pooled, concentrated by ultrafiltration, and separated on a CM-cellulose column (Figure 2). Each fraction was run on 7.5% polyacrylamide slab gels. Fluorographs were prepared and scanned at 550 nm. The migration positions of the CHO type V collagen chains are indicated. (a) $E_{29}Pro^+$ medium pepsin-resistant proteins; (b) CM^R795 medium pepsin-resistant proteins; (c) proteins from (a) precipitated by 1.2 M NaCl in 0.5 M acetic acid; (d) proteins from (b) precipitated by 1.2 M NaCl in 0.5 M acetic acid; (e) CM-cellulose peak 2 collagen chains; (f) CM-cellulose peak 3 collagen chains; (g) CM-cellulose peak 4 collagen chains.

chain. Digestion of bands 4a and 4b by CNBr produced fragments unrelated to type I–III and V CNBr fragments, and therefore, they were probably the result of digestion of a unique collagen chain.

Amino Acid Analysis of Purified CHO Collagens. The pooled peaks from the CM-cellulose separation of the CHO collagens (Figure 2) were analyzed for their hydroxyproline and proline content (Table III). The three main collagenous components, peaks 2–4, had hydroxyproline to proline ratios of greater than 1.0, indicating that very little of the material was noncollagenous. Furthermore, as much as 5% of the total radiolabeled hydroxyproline in bands 4a and 4b (CM-cellulose peak 2) was 3-hydroxyproline, which suggests that these collagen chains may be related to type IV collagen.

Effect of Colcemid on Collagen Synthesis by CHO Cells. In two separate experiments the effect of colcemid on the secretion of the CHO collagens was studied in the colcemid-sensitive clone, $E_{29}Pro^+$, and the colcemid-resistant clone, CM^R795. In preliminary experiments, it was found that 0.01 μg/mL colcemid added for 18 h had very little effect on cell division in either cell type but that cell division in $E_{29}Pro^+$ was inhibited with colcemid concentrations of 0.05 μg/mL or greater and 0.10 μg/mL or greater for the CM^R795 cells. The effect of the colcemid on CHO collagen secretion and cell numbers is summarized in Figure 5. Type V collagen and the low molecular weight fragment 4a were quantitated from fluorographs of the pepsin-digested radiolabeled proteins separated by electrophoresis. Bands α_1(V), α_2(V), 3, and 4c

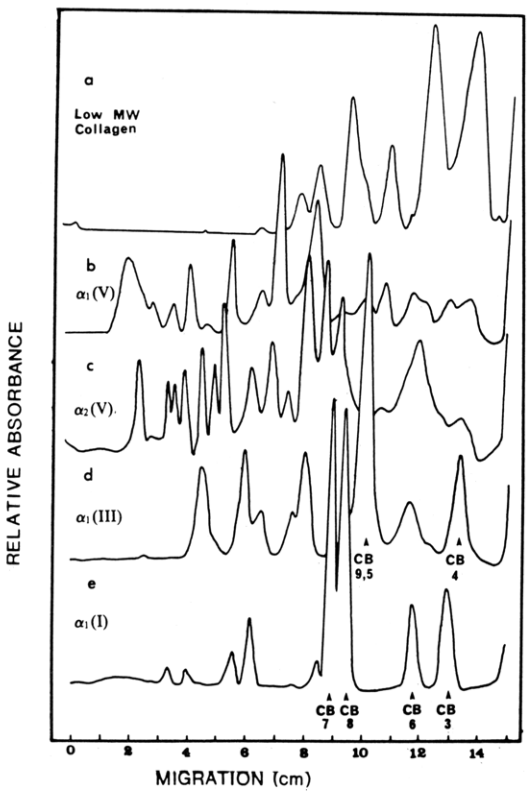

FIGURE 4: Densitometric scans of the CNBr digests of CHO collagens. CHO collagens purified on the CM-cellulose column (Figure 2) were digested with CNBr and analyzed by $NaDodSO_4$–polyacrylamide slab-gel electrophoresis and fluorography using a 10% polyacrylamide slab gel. The fluorographs were scanned at 550 nm. The migration positions of the known type I and III collagen CNBr peptides are shown. (a) CHO low molecular weight collagens, bands 4a and 4b from CM-cellulose peak 2; (b) CHO α_1(V) from CM-cellulose peak 4; (c) CHO α_2(V) from CM-cellulose peak 3; (d) α_1(III) from ^{14}C-labeled monkey periodontal ligament fibroblasts; (e) α_1(I) from ^{14}C-labeled rat periodontal ligament.

were specifically reduced in the media relative to the total protein secreted, but the low molecular weight band 4a was unaffected (Figure 6). Furthermore, there was little effect on the collagen that was extracted from the cell layers, indicating that only the secretion of the CHO collagens was affected. At the low concentrations of colcemid, the decrease in secretion of type V collagen was less pronounced in the CM^R795 cells.

Effect of dbcAMP on Collagen Synthesis by CHO Cells. Addition of dbcAMP to cultures of $E_{29}Pro^+$ and CM^R795 cells caused alterations in morphology (Figure 7). Both cell types demonstrated reverse transformation when treated with dbcAMP by becoming more elongated and spindle shaped and by aligning into organized arrays parallel to the long axis of the cell. The morphology change was more pronounced at subconfluent cell densities and occurred within 2 h after the addition of the dbcAMP.

The radiolabeled collagens produced by wild-type and colcemid-resistant cells in the absence and presence of dbcAMP and at two different cell densities were analyzed by slab-gel fluorography (Figure 8). At subconfluent densities, fluorographic quantitation showed a less than 50% increase in the secretion of the typical CHO collagen α chains in the test cells compared to controls (Table IV). The increase in collagen-chain secretion was greater at high cell densities (Figure 8e,f), but in terms of overall collagen synthesis, there was only a 13% increase in the hydroxyproline to proline ratio and a 34% increase in the total collagen synthesis per 10^6 cells (Table IV).

Discussion

Both wild-type $E_{29}Pro^+$ and a colcemid-resistant mutant CM^R795 of CHO cells synthesized collagen in relatively small amounts. The percent of total [^{14}C]proline label in collagen was estimated to range from approximately 1% for the $E_{29}Pro^+$ to approximately 2% for the CM^R795 cells, based on the amino acid analysis of the media and cell extracts (Table I). Corrected for the amount of proline in collagen vs. that of other proteins, the actual percentages of collagen synthesized are very low. This level of collagen synthesis is much less than that found in fibroblasts but is similar to the levels found in a transformed epithelial cell line (Smith & Niles, 1980). The amount of collagen that accumulated in the cell layers during the 24-h labeling period depended on the cell density of the cells; at high cell densities more collagen was found in the cell layers, and at subconfluent densities up to half of the total collagen was found in the medium.

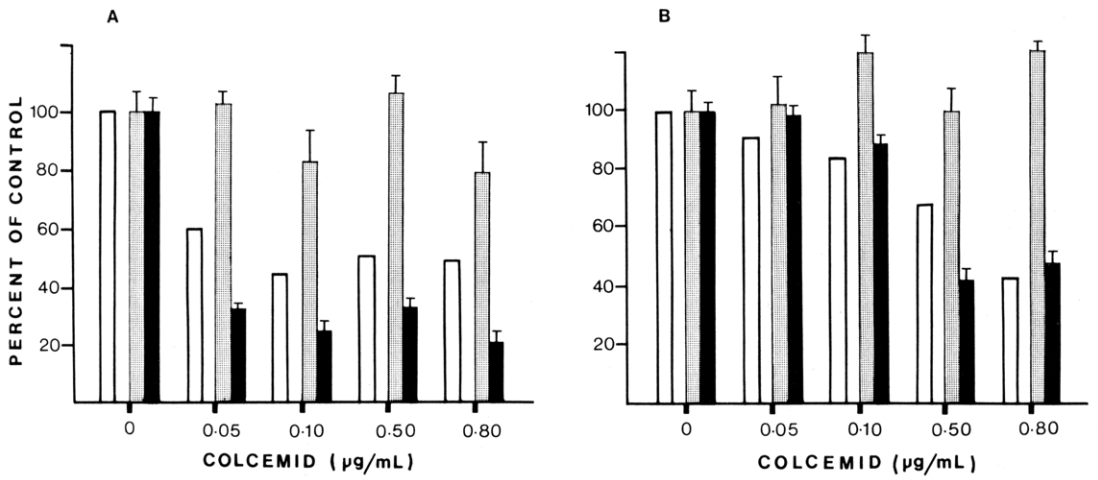

FIGURE 5: Summary of the effect of colcemid on collagen secretion of CHO cells. Radiolabeled media from $E_{29}Pro^+$ and CM^R795 cells treated with various colcemid concentrations were pepsin digested and analyzed by slab-gel fluorography. The collagens were quantitated densitometrically from the fluorographic images (Figure 6). Control values of the total number of cells per dish after the 18-h labeling period and the ratio of integration units in collagen over total protein were taken to be 100%. All test values were then expressed as a percentage of the control. Error bars for values obtained from duplicate dishes treated identically are included. Cell number values are expressed as averages from triplicate determinations. Open bars, cell numbers; shaded bars, type IV (band 4a)/total protein; solid bars, type V/total protein. (A) $E_{29}Pro^+$ cells; (B) CM^R795 cells.

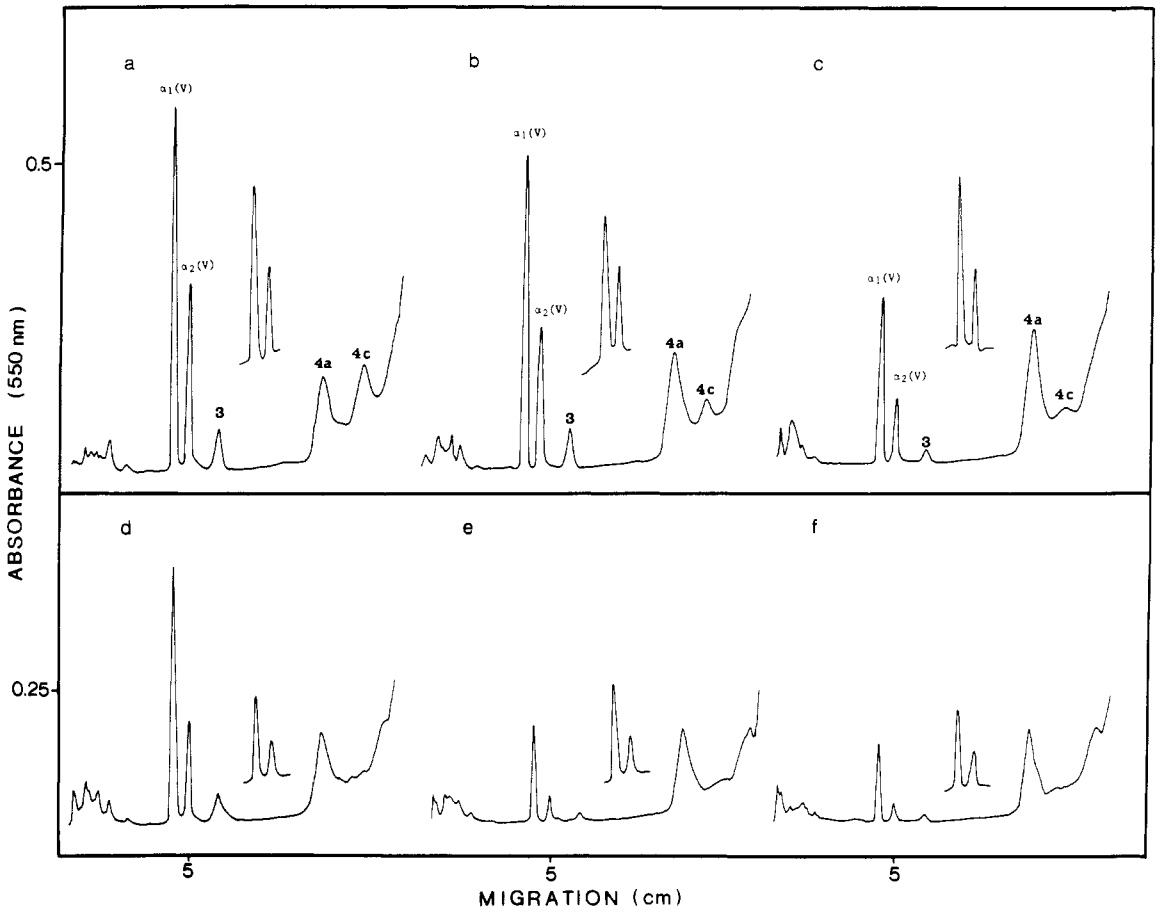

FIGURE 6: Effect of colcemid on the secretion of CHO collagens. $E_{29}Pro^+$ and CM^R795 cells were labeled in the absence (control) and presence of colcemid (test). The media and cell extracts were pepsin digested, separated by $NaDodSO_4$–polyacrylamide slab-gel electrophoresis under nonreducing conditions, and visualized by fluorography. Complete densitometric scans of the images produced by the medium pepsin digests are shown above. The type V α chains and the still unidentified collagens 3, 4a, and 4c are labeled. Included with each medium scan is the portion of the cell-layer scan containing the type V collagen. (a) Control CM^R795 collagens; (b) CM^R795 collagens from cells treated with 0.10 μg/mL colcemid; (c) CM^R795 collagens from cells treated with 0.80 μg/mL colcemid; (d) control $E_{29}Pro^+$ collagens; (e) $E_{29}Pro^+$ collagens from cells treated with 0.10 μg/mL colcemid; (f) $E_{29}Pro^+$ collagens from cells treated with 0.80 μg/mL colcemid.

We have provided the first characterization of the collagens synthesized by CHO cells. That one of the collagens is type V collagen was based on the following observations. First, the type V α chains precipitated in the presence of 1.2 M NaCl/0.5 M acetic acid; this is the salt fraction which normally contains type V collagen (Rhodes & Miller, 1978). Second, the migration positions of the CHO type V α chains on $NaDodSO_4$–polyacrylamide slab gels were the same as the type V α chains from human placenta (Hong et al., 1979; Sage & Bornstein, 1979) and rat placenta. Third, the CHO type V collagen was not affected by reduction and was resistant to digestion by a mammalian collagenase that digested type I collagen. Fourth, elution of the CHO type V α chains from CM-cellulose was similar to that of other type V collagens (Burgeson et al., 1976; Sage & Bornstein, 1979). Fifth, the CNBr peptide patterns of the CHO type V α chains were unique and unlike those typical of the interstitial collagens. The CHO α_1(V) CNBr peptide pattern obtained in this study most resembled that described by Burgeson et al. (1976) for human α_1(V). Although conclusive identity of collagen type depends on the CNBr patterns (Bornstein & Sage, 1980), variable electrophoretic patterns have been obtained even from the same species (Burgeson et al., 1976; Hong et al., 1979; Sage & Bornstein, 1979; Kumamoto & Fessler, 1980).

Several other bacterial collagenase sensitive proteins were synthesized by the CHO cells (Table II). The collagenous protein labeled band 2 coeluted with the CHO α_2(V) on CM-cellulose and was estimated by slab-gel electrophoresis to have a molecular weight of 91 000 by using type I α_1 collagen chains as molecular weight standards. The data presented here indicate that band 2 is unrelated to the interstitial collagens (types I–III). First, the slab-gel migration position was slightly faster than that of α_1 chains of the collagen types I–III and α_2(V). Second, band 2 was unaffected by reduction and coeluted with α_2(V) on CM-cellulose. Third, CNBr fragments typical of collagen types I–III could not be detected when the CNBr peptides of a mixture of α_2(V) and band 2 were analyzed by slab-gel fluorography.

Another CHO collagen, band 3, revealed unique properties. This collagen was not purified in this study because it was produced in small amounts relative to the other collagen chains and did not precipitate in the 1.2 M NaCl/0.5 M acetic acid solution. We have been unable to find any collagen chain described in the literature with similar properties; these are (a) its solubility in 1.2 M NaCl/0.5 M acetic acid, (b) its molecular weight of 75 000, and (c) its reducibility by mercaptoethanol.

Bands 4a and 4b were insoluble in 1.2 M NaCl/0.5 M acetic acid and separated from the type V α chains on CM-cellulose as a single peak. Amino acid analysis showed that they contained some 3-hydroxyproline and had ratios of 4-hydroxyproline to proline greater than 1. In addition, these two fragments were likely part of a higher molecular weight aggregate since they did not pass through the XM 100 filter

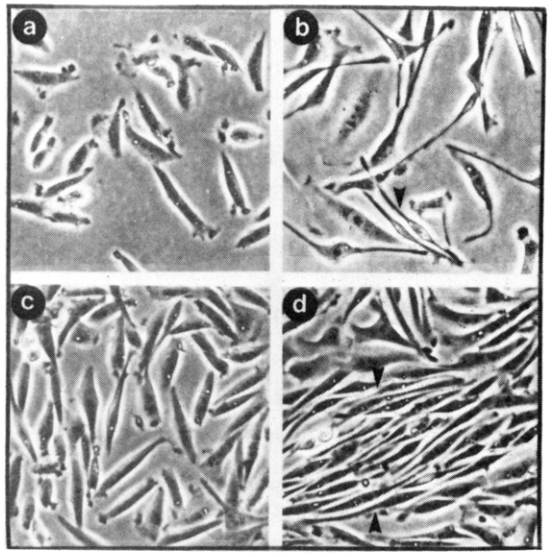

FIGURE 7: Effect of dbcAMP on the morphology of $E_{29}Pro^+$ CHO cells. The addition of dbcAMP (1.0 mM) to the CHO cells from clone $E_{29}Pro^+$ caused the cells to have a more fibroblast-like appearance. The CM^R795 cells behaved in a similar manner under the influence of dbcAMP (not included in this figure). (a) Low density $E_{29}Pro^+$ cells in control α-MEM; (b) low density $E_{29}Pro^+$ in α-MEM containing 1.0 mM dbcAMP; (c) high-density $E_{29}Pro^+$ cells in control α-MEM; (d) high-density $E_{29}Pro^+$ cells in α-MEM containing 1.0 mM dbcAMP. The arrow in (b) illustrates two elongated and spindle-shaped cells, and the cells between the arrows in (d) show an ordered parallel alignment not evident in the controls.

under nondenaturing conditions. Although no new bands were produced by reduction of bands 1a–c and 3, there appeared to be an increase in 4a and 4d after the reduction. The CHO collagens 4c and 4d may be related to band 3 because they remained in solution in 1.2 M NaCl/0.5 M acetic acid. The relative amounts of the various CHO low molecular weight collagens varied slightly with each pepsin digest. It is now well established that type IV collagen has a particular susceptibility to pepsin degradation, and a great variety of pepsin digest products from type IV procollagen have been described (Crouch et al., 1980; Foidart et al., 1981; Tryggvason et al., 1980; Timpl et al., 1979). In addition many collagen fragments of M_r 40 000 or less have been isolated from pepsin-digested basement membranes (Furuto & Miller, 1980; Laurain et al., 1980; Kresina & Miller, 1979). Taken together, these findings suggest that the collagens in bands 3 and 4a–d are pepsin-digest fragments derived from a CHO collagen of unusual properties reminiscent of other type IV collagens.

Colcemid, a mitotic inhibitor that binds tubulin [for a review, see Dustin (1978)], had a concentration-dependent effect on collagen secretion in the CHO cells. Inhibition of cell division was observed with low colcemid concentrations (0.05 and 0.1 μg/mL) in the colcemid sensitive parent, $E_{29}Pro^+$, but not the colcemid-resistant mutant, CM^R795. At these concentrations, colcemid had the effect of specifically reducing the type V collagen recovered in the medium of the $E_{29}Pro^+$ cells whereas the reduction was much less pronounced in the CM^R795 cells. In contrast, the secretion of the low molecular weight type IV like chain (4a) was generally unaffected relative to the other CHO proteins under the influence of colcemid in either of the cell types. The effective concentrations of colcemid in the colcemid-resistant vs. the wild-type cells are consistent with the degree of resistance of these cells and other aspects of their phenotypes (Ling et al., 1979; Aubin et al., 1980; Keates et al., 1981) and suggest that the drug is eliciting the response by an effect on the microtubules. These results

Table IV: Effect of dbcAMP on CHO Collagen Secretion[a]

cells	approx. cell density (cells/100 μm^2)	dbcAMP concn (mM)	total dpm/10^6 cells[b] ($\times 10^{-5}$)	type V collagen[c] total integration units		$\alpha_1(V)/\alpha_2(V)$	total dpm		collagen[b] (%)
				$\alpha_1(V)$	$\alpha_2(V)$		hydroxyproline	proline	
CM^R795	30	0	2.7	5650	2050	2.7			
				5200	1960				
		1.0	2.7	7860	3330	2.3			
				7290	3120				
$E_{29}Pro^+$	30	0	2.0	5130	1980	2.7			
				5200	1800				
		1.0	2.7	6870	2590	2.4			
				7620	3420				
	90	0	5.0	2000	900	2.2	311	66 730	0.17
				2200	1000				
		1.0	5.9	4600	1600	2.6	342	64 480	0.20
				5300	2200				

[a] $E_{29}Pro^+$ and CM^R795 cells labeled 18 h in the presence or absence of dbcAMP. The media and cell layer extracts of duplicate dishes were pepsin digested and analyzed by slab-gel fluorography. [b] Estimated by combining nondialyzable dpm from the cell-layer extracts and the media. [c] Total units of integration contained in each scan peak (Figure 8). Only the medium collagens are shown for the low-density cells; the cell-layer collagens could not be quantitated by slab-gel fluorography. The values shown for the high-density cells were obtained by combining medium and cell-layer extract values. [d] Calculated as according to Smith & Niles (1980).

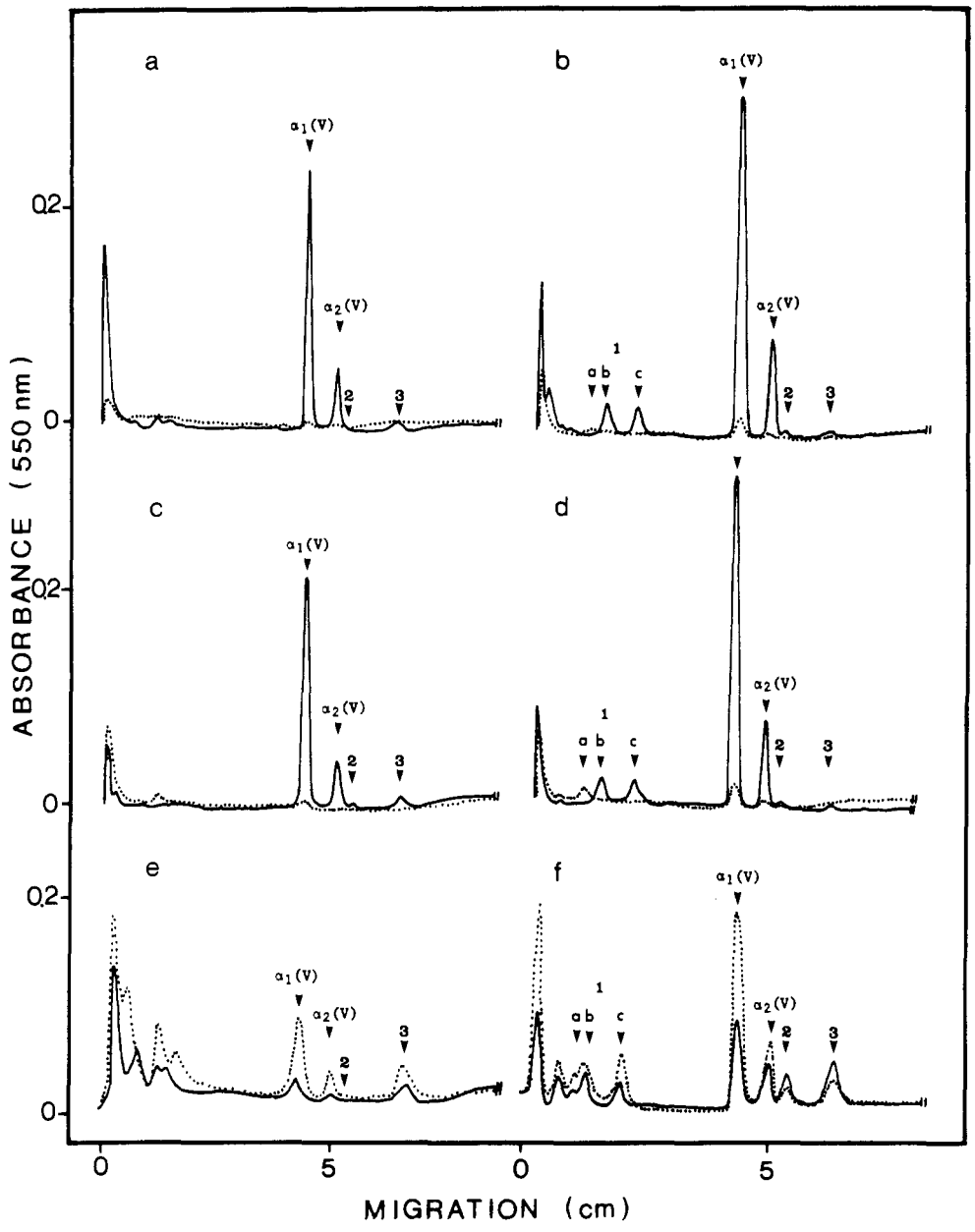

FIGURE 8: Effects of dbcAMP on collagen synthesis by CHO cells. $E_{29}Pro^+$ and CM^R795 cells were cultured at two different densities and labeled in the absence (control) and presence (test) of 1.0 mM dbcAMP. Samples from the media and cell extracts containing 40 000 cpm were pepsin digested, separated by $NaDodSO_4$–polyacrylamide slab-gel electrophoresis under nonreducing conditions, and visualized by fluorography. Partial densitometric scans of the images produced by the medium and cell-extract samples are shown. The type V α chains and the other CHO collagens 1, 2, and 3 are identified. The lower portions of the scans which include the low molecular weight collagen fragments have been omitted for convenience. (a) Collagens from low-density CM^R795 cells; (b) collagens from low-density CM^R795 cells treated with 1.0 mM dbcAMP; (c) collagens from low-density $E_{29}Pro^+$ cells; (d) collagens from low-density $E_{29}Pro^+$ cells treated with 1.0 mM dbcAMP; (e) collagens from high-density $E_{29}Pro^+$ cells; (f) collagens from high-density $E_{29}Pro^+$ cells treated with 1.0 mM dbcAMP. (—) Medium collagens; (···) cell layer extract collagens.

suggest that secretion of type V may be dependent on the microtubule system but that the secretion of the other CHO type IV like collagen may not. While microtubule-binding agents have been suggested to disrupt the secretion of type I collagen (Ehrlich et al., 1974), no information about the mechanism of type IV and V collagen secretion were available previously. That they may utilize different pathways of secretion is an intriguing possibility.

The observations presented in this paper are important for an understanding of the nature of CHO cells. Since only type V and type IV like collagens are synthesized, they would appear to originate from basement membrane associated tissues. CHO cells have a variety of properties common in transformed cells (Puck, 1977), including very low synthesis of fibronectin (Nielson & Puck, 1980), and have been used extensively to examine cell attachment to collagen (Kelinman et al., 1978). The CHO clones that we examined appeared to undergo a reverse transformation under the influence of 1.0 mM dbcAMP as was previously described in the CHO-K1 cells (Hsie et al., 1971). However, the morphological change was not accompanied by changes in the types of collagens synthesized. Although CHO cells are induced by dbcAMP to express many fibroblast-like characteristics, which include the sudden onset of fibronectin synthesis (Nielson & Puck, 1980; Rajaraman et al., 1980), the concept that they have transformed into a general fibroblast phenotype as a result of the dbcAMP treatment must be modified in view of the above findings.

The function of type V collagen is still speculative. Type V collagen remains closely associated with the cell surfaces of Chinese hamster lung cells (Haralson et al., 1980) and skin fibroblasts (Gay et al., 1980) and is thought to be a component of a membranelike exocytoskeleton (Gay et al., 1981). This characteristic may be cell specific since other cells in culture (Mayne et al., 1978; Limeback & Sodek, 1979; Linsenmayer & Little, 1978), in addition to the cells in this investigation, have been shown to secrete type V collagen into their media. In view of the colcemid effects presented in this paper, type V collagen may be packaged and secreted like the interstitial collagens in secretory vesicles that are dependent on the microtubule system. Type IV collagen, on the other hand, with its much slower secretion time than type I collagen (Bornstein & Sage, 1980) and its apparent resistance to the effects of colcemid, may be packaged and secreted by a different mechanism. Although our studies have not demonstrated the exact molecular composition of the presumptive native type IV and the secreted type V collagen molecules, the CHO type V collagen was consistently recovered in approximately 2 to 1 ratios of α_1(V) to α_2(V), suggesting that most of the secreted type V collagen would have the composition $[\alpha_1(V)]_2\,\alpha_2(V)$.

Acknowledgments

Special thanks are due to Dr. V. Ling for the generous gift of the $E_{29}Pro^+$ and CM^R795 clones and for very helpful comments. Dr. Hsin-Min Wang prepared the porcine gingival collagenase and the methyl-^{14}C-labeled collagen substrate. We also thank Susan Barnes for her expertise in the management of the CHO cells, Celina Diu for typing the manuscript, and Susan Mandell for the rat placenta type V collagen.

References

Aubin, J. E., Tolson, N., & Ling, V. (1980) *Exp. Cell Res. 126*, 75–85.

Aubin, J. E., Heersche, J. N. M., Merrilees, M. J., & Sodek, J. (1982) *J. Cell Biol. 92*, 452–461.

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

Bornstein, P., & Sage, H. (1980) *Annu. Rev. Biochem. 49*, 957–1003.

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

Chasin, L. A. (1974) *Cell (Cambridge, Mass.) 2*, 37–41.

Crouch, E., Sage, H., & Bornstein, P. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 745–749.

Dustin, P. (1978) in *Microtubules* pp 167–225, Springer-Verlag, New York.

Ehrlich, H. P., Ross, R., & Bornstein, P. (1974) *J. Cell Biol. 62*, 390–405.

Foidart, J.-M., Tryggvason, K., Robey, P. G., Liotta, L. A., & Martin, G. R. (1981) *Collagen Relat. Res.: Clin. Exp. 1*, 137–150.

Furuto, D. K., & Miller, E. J. (1980) *J. Biol. Chem. 255*, 290–295.

Gay, R. E., Buckingham, R. B., Prince, R. K., Gay, S., Rodnan, G. P., & Miller, E. J. (1980) *Arthritis Rheum. 23*, 190–196.

Gay, S., Rhodes, R. K., Gay, R. E., & Miller, E. J. (1981) *Collagen Relat. Res.: Clin. Exp. 1*, 53–58.

Gottesman, M. M., LeCam, A., Bukowski, M., & Pastan, I. (1980) *Somatic Cell Genet. 6*, 45–61.

Haralson, M. A., Mitchell, W. M., Rhodes, R. K., Kresina, T. F., Gay, R., & Miller, E. J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5206–5210.

Herrmann, H., Dessau, W., Fessler, L. I., & Von der Mark, K. (1980) *Eur. J. Biochem. 105*, 63–74.

Hong, B.-S., Davison, P. F., & Cannon, D. J. (1979) *Biochemistry 18*, 4278–4282.

Hsie, A. W., & Puck, T. T. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 358–361.

Hsie, A. W., Jones, C., & Puck, T. T. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1648–1652.

Keates, R. A. B., Sarangi, F., & Ling, V. (1981) *Proc Natl. Acad. Sci. U.S.A. 78*, 5638–5642.

Kefalides, N. A. (1975) *J. Invest. Dermatol. 65*, 85–92.

Killen, P. D., & Striker, G. E. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3518–3522.

Kleinman, H. K., Murray, J. C., McGoodwin, E. B., & Martin, G. R. (1978) *J. Invest. Dermatol. 71*, 9–11.

Kleinman, H. K., Klebe, R. J., & Martin, G. R. (1981) *J. Cell Biol. 88*, 473–485.

Kresina, T. F., & Miller, E. J. (1979) *Biochemistry 18*, 3089–3097.

Kumamoto, C. A., & Fessler, J. H. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 6434–6438.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Laurain, G., Delvincourt, T., & Szymanowicz, A. G. (1980) *FEBS Lett. 120*, 44–48.

Limeback, H. F., & Sodek, J. (1979) *Eur. J. Biochem. 100*, 541–550.

Ling, V., Aubin, J. E., Chase, A., & Sarangi, F. (1979) *Cell (Cambridge, Mass.) 18*, 423–430.

Linsenmayer, T. F., & Little, C. D. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3235–3239.

Mayne, R., Vail, M. S., & Miller, E. J. (1978) *Biochemistry 17*, 446–452.

Nielson, S. E., & Puck, T. T. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 985–989.

Pastan, I., & Willingham, M. (1978) *Nature (London) 274*, 645–650.

Pettigrew, D. W., Ho, G., Sodek, J., Brunette, D. M., & Wang, H. M. (1978) *Arch. Oral Biol. 23*, 767–777.

Piez, K. A., Eigner, E. A., & Lewis, M. S. (1963) *Biochemistry 2*, 58–66.

Porter, K. R., Puck, T. T., Hsie, A. W., & Kelley, D. (1974) *Cell (Cambridge, Mass.) 2*, 145–162.

Puck, T. T. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4491–4495.

Puck, T. T., Cieciura, J. J., & Robinson, A. (1958) *J. Exp. Med. 108*, 945–955.

Rajaraman, R., Sunkara, S. P., & Rao, P. N. (1980) *Cell Biol. Int. Rep. 4*, 897–906.

Rhodes, R. K., & Miller, E. J. (1978) *Biochemistry 17*, 3442–3448.

Roll, F. J., Madri, J. A., Albert, J., & Furthmayr, H. (1980) *J. Cell Biol. 85*, 597–616.

Sage, H., & Bornstein, P. (1979) *Biochemistry 18*, 3815–3822.

Sisskin, E. E., & Weinstein, I. B. (1980) *J. Cell. Physiol. 102*, 141–153.

Smith, B. D., & Niles, R. (1980) *Biochemistry 19*, 1820–1825.

Sodek, J. (1976) *Biochem. J. 160*, 243–246.

Sodek, J., Hurum, S., & Feng, J. (1981) *J. Periodontal Res. 16*, 425–433.

Thompson, L. H., & Baker, R. M. (1973) in *Methods in Cell Biology*, pp 210–280, Academic Press, New York.

Timpl, R., Bruckner, P., & Fietzek, P. (1979) *Eur. J. Biochem. 95*, 255–263.

Tryggvason, K., Robey, P. G., & Martin, G. R. (1980) *Biochemistry 19*, 1284–1289.