

## Effects of $17\beta$ -Estradiol on the Biosynthesis of Collagen in Cultured Bovine Aortic Smooth Muscle Cells<sup>†</sup>

John C. Beldekas,\* Barbara Smith,<sup>‡</sup> Louis C. Gerstenfeld, Gail E. Sonenshein, and Carl Franzblau

**ABSTRACT:** The effect of  $17\beta$ -estradiol ( $E_2$ ) on the biosynthesis of collagen in cultured bovine aortic smooth muscle cells was explored. Cells treated with various concentrations of the hormone for 14 days following subcultivation were subjected to growth studies. The cultures were also evaluated for [ $^{14}C$ ]hydroxyproline formation, the presence of collagenase-susceptible protein, prolyl hydroxylase activity, and procollagen types. There were no effect of  $E_2$  on the growth of these cells. At  $10^{-8}$  M  $E_2$ , the hydroxylation of proline when compared to control cultures was reduced by 25–30%; however, little difference in extractable prolyl hydroxylase activity or total [ $^{14}C$ ]proline incorporation into protein was observed. The

effect on collagen synthesis appears to be dose dependent over concentrations of  $E_2$  ranging from  $10^{-6}$  to  $10^{-12}$  M when measured by collagenase susceptibility. Procollagen typing on diethylaminoethylcellulose displayed reduced amounts of procollagen type I and type III fractions as well as other collagenous components. More importantly, however, the ratio of these two procollagen types was also altered. Similar results were obtained from the medium or cell layer. It is concluded that aortic smooth muscle cells cultured in the presence of  $17\beta$ -estradiol display a decreased production of collagen in addition to altering the ratio of type I to type III procollagen fractions produced.

Several laboratories have demonstrated that aortic smooth muscle cells from a variety of sources can synthesize and accumulate collagen in culture (Faris et al., 1976; Burke et al., 1977; Scott et al., 1977). More recently, these cells have been shown to produce type I and type III collagen as well as less characterized types of collagen (Mayne et al., 1977).

It is well-known that collagen fiber formation is subject to many factors affecting both synthesis and degradation. It has been shown in animal experiments that estrogens, as well as other steroids, greatly influence the distribution of collagen in vascular and nonvascular tissues (Klein, 1969; Fischer, 1972). Wolinsky (1972) has demonstrated that  $17\beta$ -estradiol ( $E_2$ ) can reduce the accumulation of collagen in the aortas of rats which have undergone induced renal hypertension. Other data in the literature are also consistent with the conclusion that the hormone does influence the distribution of collagen in the aorta, possibly by increased turnover and/or degradation or by decreased synthesis.

Since vascular smooth muscle cells are responsible for the bulk of the connective tissue synthesized in large blood vessels, the effect of  $E_2$  on collagen production in cultured cells was examined. This communication suggests that not only is collagen synthesis reduced in the presence of the hormone but also, more importantly, the distribution of procollagen molecules produced by these cells appears to be altered under the influence of the hormone.

### Experimental Procedures

All of the tissue culture materials listed below were purchased from Grand Island Biological Co. (GIBCO), Grand Island, NY. All of the reagents listed below were purchased from Sigma Chemical Co., St. Louis, MO.

**Cell Culture.** Fresh calf aortic arches were removed at the

abattoir and immediately placed in sterile Dulbecco's Vogt medium containing 0.35 g of sodium bicarbonate/L, supplemented with 5% fetal bovine serum, 1% nonessential amino acids (MEM), 1000 units/mL penicillin-G, 0.5 mg/mL streptomycin sulfate, and 0.25  $\mu$ g/mL amphotericin-B (Fungisone) for transportation to the laboratory. The aortas were then cleaned of adhering mesentery, cut along the intercostal arteries, and washed 3 times with medium. The cleaned aortic arches were then placed in sterile petri dishes, intima side down, for approximately 15–30 min. This procedure allows the intima to pull away from the media, making for a more efficient removal of the intima. Once the intima had been removed the tissue was again transferred to sterile petri dishes containing fresh medium, and the inner one-third of the medial layer (closest to the lumen) was stripped from the tissue. The dissected strips were washed 3 times with medium and placed in sterile petri dishes containing Dulbecco's Vogt medium with 3.7 g of sodium bicarbonate/L supplemented with 10% fetal bovine serum, 1% nonessential amino acids (MEM), 100 units/mL penicillin-G, and 100 units/mL streptomycin sulfate. This medium is utilized for much of the subsequent experimental procedures and will be referred to as low antibiotic medium. The dishes were then placed in a humidified 5%  $CO_2$  incubator at 37 °C, overnight. The strips were then placed in the original high antibiotic medium and cut into pieces (explants)  $\sim 1$  mm<sup>3</sup> in size. The explants were transferred to sterile 75-cm<sup>2</sup> plastic flasks and allowed to attach at 37 °C for 40 min. The flasks were then flooded with low antibiotic medium, placed in a humidified 5%  $CO_2$  incubator at 37 °C, and allowed to remain undisturbed for 7 days. Following the initial 7 days, the explants and resulting outgrowth of cells were fed biweekly until subcultivation at the end of the second week in culture. Subcultures were prepared from explants and cells by trypsinization in the same manner as that described by Faris et al. (1976). The cells were seeded in 75-cm<sup>2</sup> plastic flasks at a density of  $1.5 \times 10^6$  cells and fed every 3–4 days with 20 mL of low antibiotic media. Once the cell layer reached confluency, the cells were subcultivated again (second passage) and maintained as described above.

**Addition of Estradiol to Cultures.**  $17\beta$ -Estradiol was dissolved in absolute ethanol at several concentrations. In all experiments a total volume of 10  $\mu$ L of the appropriate hor-

<sup>†</sup> From the Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02118. Received November 14, 1980. This work was supported by Grant HL 13262. G.E.S. is an Established Investigator of the American Heart Association. Portions of this paper are derived from the thesis of J.C.B., presented in partial fulfillment of the requirements for a Ph.D. degree, Boston University, Boston, MA (1979).

<sup>‡</sup> Present address: Veterans Administration Outpatient Clinic, Boston, MA 02108.

hormone solution was added to each 10 mL of medium. The hormone was replenished at each feeding, and in all cases, the estradiol treatment was begun 24 h after the cells were placed in second passage. No attempt was made to remove endogenous  $E_2$  from the fetal calf serum utilized in these experiments. The concentration of  $E_2$  in fetal calf serum is  $\sim 5 \times 10^{-14}$  M (Milo et al., 1976). The same serum lot was used in all the experiments described in this paper.

**Pulse Experiments.** Except when stated, the biosynthesis of protein was studied 14 days after the cells were placed in second passage. The cells were pulsed for either 1 or 24 h with [ $^{14}$ C]proline in proline-free, low antibiotic media. All pulse experiments were carried out in serumless medium supplemented with 50  $\mu$ g/mL sodium ascorbate. The medium for the 24-h pulse contained 1  $\mu$ Ci/mL [ $^{14}$ C]proline (sp act. 291.0 mCi/mmol) while the 1-h pulse was carried out in medium containing 5  $\mu$ Ci/mL [ $^{14}$ C]proline. Following the 24-h pulse, the medium was exhaustively dialyzed against  $H_2O$  and lyophilized. This lyophilized material was evaluated for radioactive hydroxyproline content by amino acid analysis and for the presence of collagenase-susceptible protein as described in the method of Peterkofsky & Diegelmann (1971).

The media from four pooled flasks of cells were amply sufficient for both the hydroxyproline determination and the collagenase susceptibility studies. A minimum of three separate determinations was carried out in all studies. The cell layers were washed 3 times with saline, removed with the aid of a rubber policeman, homogenized, sonicated, dialyzed vs.  $H_2O$ , and then evaluated for the presence of collagenase-susceptible protein. Amino acid analyses were carried out as well. The pooled cell layers from four flasks were quite sufficient for collagenase susceptibility studies as well as amino acid analyses. A minimum of three separate determinations was carried out for each hormone concentration.

In those studies directed at determining procollagen profiles (see below), following a 24-h pulse the media were decanted off and a solution of protease inhibitors was immediately added to yield a final concentration of  $10^{-3}$  M *p*-(chloromercuri)-benzoate (PCMB),  $10^{-5}$  M phenylmethanesulfonyl fluoride (PMSF), and 2 mM EDTA.<sup>1</sup> Following the 1-h pulse the media were decanted off and the cell layer was extracted with 10 mL of a buffer containing 0.05 M Tris, 0.02 M EDTA,  $1 \times 10^{-5}$  M PMSF,  $1 \times 10^{-3}$  M PCMB, 1.0 M NaCl, 0.2% Triton X-100, and 0.5% deoxycholate at pH 7.4. The extraction was carried out 2 times at 4 °C for 16 h; both cell layer extracts were pooled and spun in a refrigerated centrifuge at 1500 rpm for 30 min. The supernatants were then analyzed on DEAE-cellulose for the presence of procollagen fractions as described below. All DNA determinations were carried out according to the method of Dische (1939).

**Amino Acid Analysis.** The freeze-dried radiolabeled samples described above were then transferred to glass vials and hydrolyzed in 6 N HCl for 18 h at 110 °C. After hydrolysis, the samples were subjected to analysis on an automated amino acid analyzer equipped with a stream-splitting device. In this manner both radioactivity and ninhydrin were monitored in the column eluents.

**Separation of Procollagens.** The radiolabeled collagenous protein produced in the medium of the [ $^{14}$ C]proline 24-h pulsed cells or in the extracted cell layers of the 1-h pulses were resolved by chromatography on DEAE-cellulose as described in the method of Smith et al. (1972) with a slight modification

in the employment of ammonium sulfate. In these studies 40% ammonium sulfate was used to facilitate precipitation of the collagenous proteins in the medium while the cell layer extracts from the 1-h pulses were chromatographed without prior treatment with ammonium sulfate. Aliquots of several of the samples were subjected to bacterial collagenase digestion before ion-exchange chromatography. Under the conditions we employed the media from five flasks of smooth muscle cells pulsed for 24 h were sufficient to carry out an entire procollagen profile, whereas the cell layers from two flasks of cells pulsed for 1 h were quite sufficient. Fractions of 8.0 mL were collected in all studies, and total radioactivity was determined in each fraction. For determination of radioactive proline and hydroxyproline in the radioactive fractions eluted from the column, pooled fractions were dialyzed against  $H_2O$ , lyophilized, and hydrolyzed in 6 N HCl.

Assessment of the nature of the radioactive collagen in the fractions from the DEAE-cellulose chromatography was obtained by using the electrophoretic technique of Laemmli (1970). Aliquots of pooled radioactive desalted lyophilized fractions from the DEAE-cellulose columns were electrophoresed with and without dithiothreitol (DTT), while other aliquots of the same fractions were treated with 2 mL of a pepsin solution (1.0 mg/100 mL of 0.5 N acetic acid) at 4 °C for 16 h. All samples were evaluated electrophoretically before and after reduction as well as by the discontinuous reduction procedure of Sykes et al. (1976). Cyanogen bromide studies were also carried out as well.

**Prolyl Hydroxylase.** Substrate, reference enzyme preparations, and assay conditions were the same as those described by Dehm & Prockop (1971). To assay for prolyl hydroxylase activity in cell cultures, we extracted the enzyme with 0.1% Triton X-100 in 0.1 M KCl as described by the authors.

**Growth Studies.** For growth studies, cells were seeded in 25-mm<sup>2</sup> petri dishes at a density of 50 000 cells per dish. At 1-day intervals, two dishes were trypsinized and counted in a Coulter Counter or a hemocytometer. When required,  $E_2$  was added in small volumes of ethanol as described above.

## Results

**Growth Studies.** The DNA content of smooth muscle cells placed in second passage was monitored for 14 days in the presence of  $10^{-8}$  and  $10^{-5}$  M  $E_2$ . No difference in DNA content (data not shown) was noted between the hormone-treated and control cell cultures during this 14-day period. Growth curves (cell numbers) also showed no difference during the exponential growth phase up to 7 days. Beyond the exponential phase of growth, the assessment of cell numbers by the Coulter Counter is quite difficult due to clumping of cells.

**Hydroxylation of [ $^{14}$ C]Proline.** Cells grown in the presence of  $10^{-8}$  M  $E_2$  were pulsed with [ $^{14}$ C]proline for 24 h at various times after being placed in second passage, and the formation of [ $^{14}$ C]hydroxyproline was measured. At day 14, as well as at days 21 and 28, significant differences in total hydroxyproline formation (medium plus cell layer) were observed in those cultures grown in the presence of the hormone when compared to the control cultures. At day 14,  $\sim 35\%$  less radioactive hydroxyproline was obtained from the hormone-treated cultures; 82 600 cpm/ $\mu$ mol of leucine was recovered in the control cultures vs. 51 000 cpm/ $\mu$ mol of leucine recovered in the hormone-treated cultures. Since the total incorporation of proline and the formation of hydroxyproline were maximal at day 14 in both the control and hormone-treated cells, all subsequent studies were carried out on cell cultures which had been maintained for 14 days in second passage as described.

<sup>1</sup> Abbreviations used: Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; DNA, deoxyribonucleic acid.

Table I: Collagenase-Susceptible Protein Produced as a Function of Estradiol Concentration<sup>a</sup>

estradiol concn (M)	media		cell layer	
	counts released/ μg of DNA	% of control	counts released/ μg of DNA	% of control
none (control)	3500	100	2750	100
10 <sup>-10</sup>	2900	82	2420	88
10 <sup>-9</sup>	2730	78	2530	92
10 <sup>-8</sup>	1960	56	1980	72
10 <sup>-7</sup>	1680	48	1292	47
10 <sup>-6</sup>	490	14	550	20

<sup>a</sup> Each data point is the average of three individual determinations.

Table II: Ratio of Media Procollagen Type I to Procollagen Type III after a 24-h Pulse as a Function of Estradiol Concentration

estradiol concn (M)	type I (cpm/ μg of DNA)	type III (cpm/ μg of DNA)	type I/type III
none (control)	421	606	0.70
10 <sup>-12</sup>	492	465	1.06
10 <sup>-10</sup>	402	320	1.26
10 <sup>-9</sup>	439	312	1.41
10 <sup>-8</sup>	473	370	1.28
10 <sup>-7</sup>	376	307	1.22
10 <sup>-6</sup>	321	335	0.96
10 <sup>-5</sup>	320	321	0.99

Table III: Ratio of Cell Layer Procollagen Type I to Procollagen Type III after a 1-h Pulse as a Function of Estradiol Concentration

estradiol concn (M)	type I (cpm/ μg of DNA)	type III (cpm/ μg of DNA)	type I/type III
none (control)	262	376	0.70
10 <sup>-12</sup>	203	230	0.88
10 <sup>-8</sup>	154	130	1.18
10 <sup>-6</sup>	131	128	1.02

**Prolyl Hydroxylase Activity.** Cells grown in the presence or absence of 10<sup>-8</sup> M E<sub>2</sub> were extracted as described, and the activity of prolyl hydroxylase was determined. The total amount of extractable prolyl hydroxylase was equivalent in both the hormone-treated and control cells. Figure 1 shows a plot of enzyme activity vs. protein concentration of the extract obtained from hormone-treated and control cultures. The total quantity of extracted protein from control and hormone-treated cells showed no significant differences as determined by the Lowry procedure (Lowry et al., 1951).

**Collagenase-Susceptible Protein.** For examination of the effect of increasing concentrations of estradiol on collagen synthesis, the cultures were pulsed with [<sup>14</sup>C]proline and the resulting isolated medium and cell layers were treated separately with bacterial collagenase as described. The data in Table I suggest that the effect of estradiol on collagen biosynthesis is concentration dependent. A significant decrease in the production of radioactive collagenase-susceptible protein is demonstrated with increasing concentration of the hormone in both the medium and cell layer.

**Procollagen Determinations: Effect of Estradiol.** The effect of estradiol on specific procollagens produced in the cell

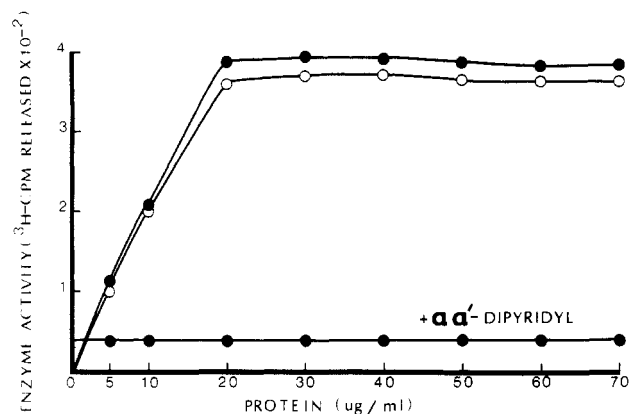


FIGURE 1: Prolyl hydroxylase activity. Second-passage calf aortic smooth muscle cells were grown with 1 × 10<sup>-8</sup> M E<sub>2</sub> or without the hormone for 14 days after seeding as described. Cultures were then extracted with 0.1% Triton X-100 in 0.1 M KCl as described, and the extract was assessed for enzyme activity. Assays at each protein concentration were carried out for 1 h, and the <sup>3</sup>H<sub>2</sub>O released was determined. The α,α'-dipyridyl concentration was 0.2 mM; quantitation of protein was determined by the Lowry procedure (Lowry et al., 1951). Control cultures (●); estradiol cultures (○).

culture media was also examined. Media from cells grown in the presence of 10<sup>-12</sup>, 10<sup>-8</sup>, and 10<sup>-6</sup> M E<sub>2</sub> yielded radioactive elution profiles from DEAE-cellulose chromatography as shown in Figure 2. Data from control cells are also included for reference. The recoveries of radioactivity from the columns ranged from 68 to 72%. For examination of the profile of procollagens produced, intracellular, short-term pulses of 1-h duration were carried out as described under Experimental Procedures. The column conditions were exactly the same for both the medium and the cell layer; however, as noted, the cell layer extracts did not undergo ammonium sulfate fractionation before chromatography on DEAE-cellulose. Recoveries from these columns varied from 41 to 45%, most likely due to the excessive noncollagen protein produced in the cell layer and the omission of the salt fractionation step. The procollagen profiles from the cell layers of the 1-h pulsed cultures treated with 10<sup>-12</sup>, 10<sup>-8</sup>, and 10<sup>-6</sup> M E<sub>2</sub> and the control cells are given in Figure 3. The procollagen profiles obtained from the cell layer after short-term pulses agree quite well with the data obtained in Figure 2 from the media after long-term pulses. It is apparent from these data that there is an overall decrease in total radioactivity in the procollagen fractions obtained from estradiol-treated cells. Table II displays the total radioactivity expressed as a function of DNA obtained in the procollagen type I and type III fractions and the ratio of these two values from the 24-h pulse studies. Table III gives similar results for the cell layers obtained from the 1-h pulse studies. It should be noted that no nondialyzable radioactive hydroxyproline was detected in the medium of the 1-h pulses suggesting that no detectable radioactive collagen was present in the medium after the 1-h time period.

**Characterization of Procollagen Fractions from DEAE-cellulose Chromatography.** (A) Content of Proline and Hydroxyproline. The procollagen fractions obtained from the

Table IV: Radioactive Proline and Hydroxyproline Content of Procollagen Fractions from DEAE-cellulose

estradiol concn (M)	procollagen type I fraction			procollagen type III fraction		
	Pro (total cpm)	Hyp (total cpm)	Hyp (cpm)/ Pro (cpm)	Pro (total cpm)	Hyp (total cpm)	Hyp (cpm)/ Pro (cpm)
none (control)	114 048	92 576	0.81	102 550	89 500	0.87
10 <sup>-12</sup>	110 000	91 916	0.84	82 588	72 776	0.88
10 <sup>-8</sup>	91 476	77 616	0.85	83 952	71 104	0.85
10 <sup>-6</sup>	97 528	74 580	0.76	82 984	77 528	0.93

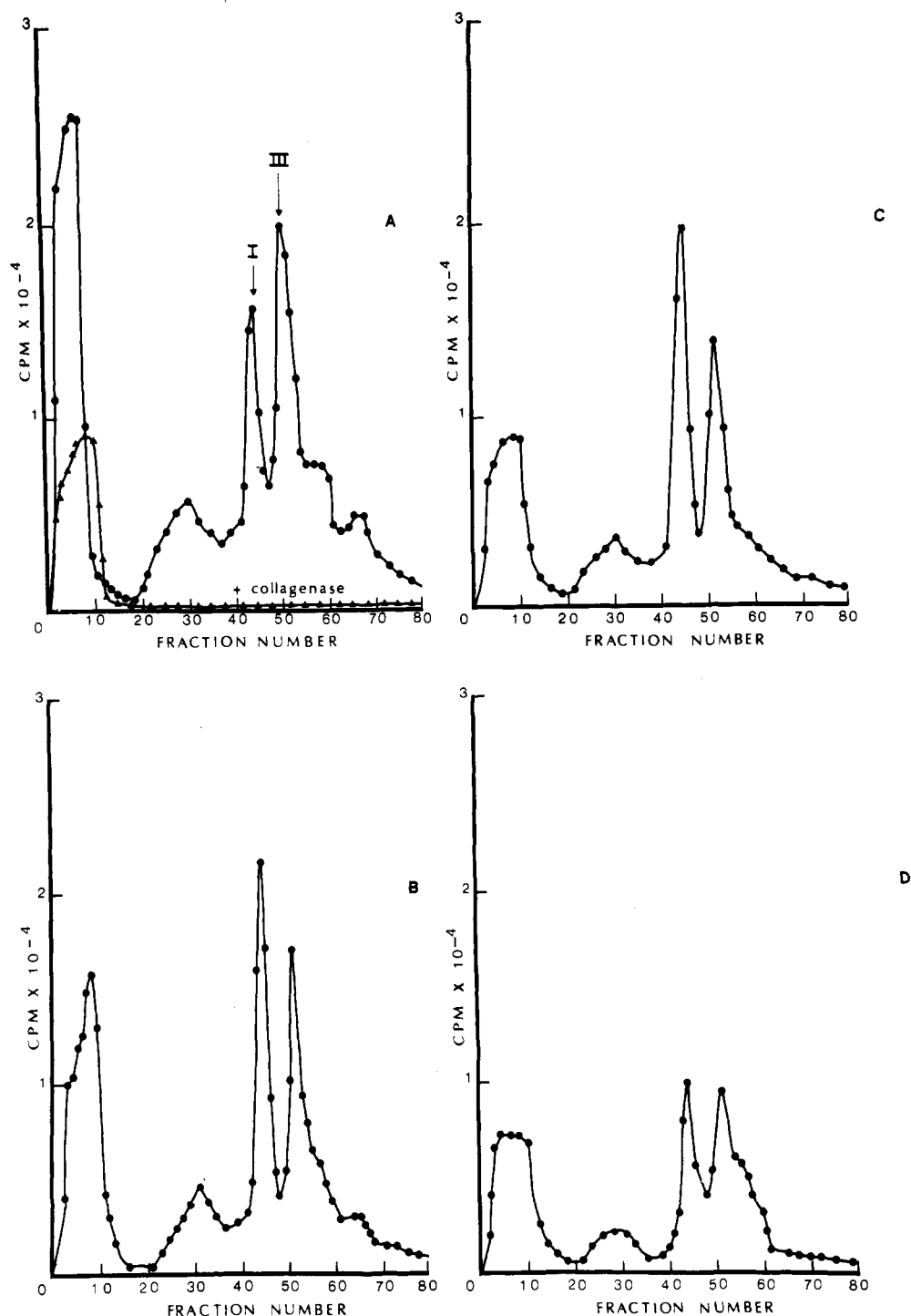


FIGURE 2: DEAE-cellulose profiles of procollagens in medium from control and estradiol-treated cells. Second-passage calf smooth muscle cells were pulsed with [ $^{14}\text{C}$ ]proline for 24 h. The medium was collected, and the procollagens were resolved on DEAE-cellulose as described. (I) refers to the region where procollagen type I elutes from the column, and (III) refers to the region where procollagen type III elutes. (A) represents the control cultures; also included is the effect of collagenase prior to chromatography. (B) represents cultures maintained for 14 days at an estradiol concentration of  $1 \times 10^{-12}$  M, (C) at  $1 \times 10^{-8}$  M, and (D) at  $1 \times 10^{-6}$  M. Each chromatograph represents data from equivalent aliquots of culture medium. (See text for details.)

DEAE-cellulose chromatography of both control and estradiol-treated cell media were pooled separately and subjected to amino acid analyses and radioactivity determinations as shown in Table IV. The ratios of radioactivity in the proline and hydroxyproline fractions are consistent with the presence of collagenous material.

(B) *Electrophoresis*. Pooled fractions from the DEAE-cellulose column corresponding to type I and type III procollagen were electrophoresed before and after reduction on 5% polyacrylamide gels. No differences in processing of precursors between  $\text{E}_2$ -treated and control cultures were noted.

Equal radioactive aliquots of the procollagen fractions obtained from hormone-treated and control cultures were also subjected to a limited pepsin digestion and then resolved on a 5% polyacrylamide gel as well. Other aliquots were reduced after 3 h of electrophoresis with DTT. Figure 4 displays the results of the electrophoretogram of both control and hormone-treated cells. Procollagen type I fractions appear to contain only procollagen type I while procollagen type III fractions contain primarily procollagen type III. The same procollagen type I and III fractions were subjected to CNBr digestion and electrophoresed as well. Data obtained (not shown) confirm

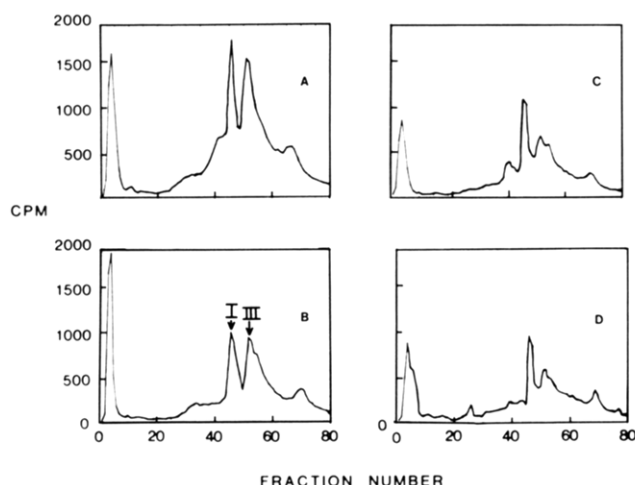


FIGURE 3: DEAE-cellulose profiles of procollagens in the cell layer from control and estradiol-treated cultures. Second-passage calf smooth muscle cells were pulsed with [ $^{14}\text{C}$ ]proline for 1 h as described. The cell layer was then extracted, and the procollagens were resolved on DEAE-cellulose as described. (I) refers to the region where procollagen type I elutes from the column, and (III) refers to the region where procollagen type III elutes. (A) represents the control cultures. (B) represents cultures maintained for 14 days at an  $\text{E}_2$  concentration of  $1 \times 10^{-12}$  M. (C) represents cultures maintained at an  $\text{E}_2$  concentration of  $1 \times 10^{-8}$  M. (D) represents cultures maintained at an  $\text{E}_2$  concentration of  $1 \times 10^{-6}$  M. Each chromatograph represents data from equivalent aliquots obtained from five pooled flasks per concentration.

that the two radioactive pooled fractions contain procollagen type I and procollagen type III, respectively.

#### Discussion

Smooth muscle cells in culture have been shown to produce both procollagen type I and type III in the medium (Burke et al., 1977; Barnes et al., 1976; Mayne et al., 1977, 1978). The data presented herein confirm this and suggest that the addition of estradiol to the cultured calf cells has a profound effect on the quantity and distribution of the procollagen molecules produced in the medium. As shown in Figure 2 and Tables II and III, estradiol added to the medium over the 14-day period causes the cells in culture to alter the ratio of procollagen type I fractions relative to procollagen type III

fractions produced. The data suggest that at low concentrations of estradiol ( $10^{-10}$  M) a major effect of the hormone treatment is a decrease in the ability of the cell to produce normal quantities of procollagen type III fractions, while at the higher concentrations ( $10^{-6}$  M) of estradiol both procollagen type I and type III are decreased. Examination of the intracellular product produced after short-term pulses (1 h) and evaluation of the procollagen profiles from the medium (24-h pulse) display essentially similar results, supporting the conclusion that the decreased level of procollagen type III in the media is due to a decrease in synthesis rather than an alteration in secretion.

The question of whether the ratio of type I/type III collagen is also affected by the hormone treatment is pertinent. The observed decrease in procollagen type III with hormone treatment might well be explained by an increased rate of processing of procollagen type III to type III collagen. Thus in the course of our studies we might have expected a significant increase in the presence of type III collagen. In the procollagen profile studies, the presence of type I and type III collagens, which are not absorbed to the DEAE-cellulose column and appear primarily in the forepeak, was evaluated. We could not observe significant type I or type III collagen in this fraction when it was monitored by gel electrophoresis. The fact that there was an observed decrease in the amount of radioactivity in this fraction with the hormone treatment cannot be explained by changes in the ratio of type III to type I collagen. The forepeak fraction from these smooth muscle cells appears to contain collagenous peptides and single chains of collagen along with significant amounts of noncollagenous material.

The results obtained in this study regardless of whether one examines the medium or the intracellular products strongly support the premise that estradiol is directly effecting, in a dose-dependent manner, the production of extracellular collagen. From the data available, it appears that the procollagens produced under the influence of the hormone are chemically equivalent to the procollagens produced in the absence of the hormone. For example, hydroxylation of proline occurs to the same extent in those procollagen fractions obtained from either hormone-treated or control cell cultures. The procollagen fractions from DEAE-cellulose columns showed no difference

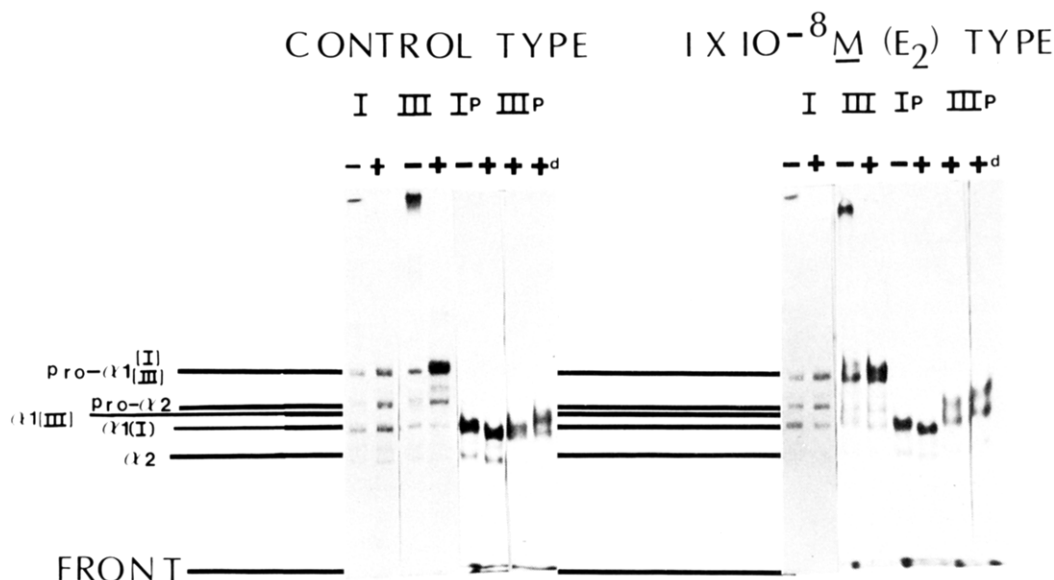


FIGURE 4: Electrophoretogram of pooled procollagen fractions. Fractions from the DEAE-cellulose column were resolved on a 5% polyacrylamide gel (see text and Figure 2 for details of the method). Both control and hormone-treated ( $1 \times 10^{-8}$  M  $\text{E}_2$ ) fractions were subjected to pepsin digestion prior to electrophoresis (P). Half of the samples were reduced with DTT (+) and delayed reduced with DTT (+d).

in their electrophoretic pattern even though the amount of the various procollagen fractions obtained on a per cell basis was reduced significantly by the hormone treatment. Similarly, CNBr digestion of these same procollagen fractions showed no differences in the electrophoretic pattern of the procollagen obtained from control or hormone-treated cells.

The reduction of hydroxyproline formation cannot be explained by a decrease in the content of cellular prolyl hydroxylase, although the data cannot rule out the possibility of a direct effect of the hormone on the intracellular activity of the enzyme. The collagenase-susceptible data supports the conclusion that collagen synthesis, not hydroxylation of the nascent collagen, is the major effect of the hormone.

Wolinsky (1973a,b) showed that in vivo administration of estradiol decreased the accumulation of collagen in the rat aorta. He also found that estrogens prevented the increased accumulation of connective tissue which occurs when male rats are made hypertensive either by clipping the renal artery or by inducing the disease with drugs. Previous studies have also shown that estrogens have a collagen-lowering effect (Fischer, 1971; Wolinsky, 1972; Henneman, 1968), although these studies were not carried out in cell cultures. Cembrano et al. (1960) reported that estradiol resulted in a lower accumulation of collagen in the aorta, while Henneman (1968) found that estradiol caused a decrease in activity of specific hydroxyproline in guinea pig aorta 30 h after an injection of [ $^{14}\text{C}$ ]-proline. On the other hand, estradiol also has been shown to increase collagen content in the uterus and other tissues (Kao et al., 1964; Woessner, 1969). Kowalewski et al. (1971) reported that estradiol caused both an increased synthesis and a breakdown of collagen in the bones of rats. Fischer & Swain (1978) have indicated that the turnover of elastin in the aortas is greater than that of collagen under the influence of estradiol and suggested that elastin may be even more important than collagen in the development of the disease. It is apparent from all of the above studies that there is a lack of agreement on the effects of the hormone. It may well be that the hormone affects different tissues in different ways since several cell types are usually involved in such tissues (Fischer, 1973). In preliminary studies, we have found that collagen synthesis in IMR-90 human diploid fibroblasts is not affected significantly by treatment with estradiol. Since the major portion of the procollagens produced by the fibroblast is procollagen type I, this result is not unreasonable in light of our present findings.

Although one should be cautious in relating in vitro cell cultures studies to in vivo observations, it is interesting to note that natural menopause is usually characterized by a general decrease in estrogen concentration and the decline continues for some time after the menopause. Such changes have been correlated to an increased incidence of cardiovascular disease. In addition, the effect of estradiol may reflect an increased relative production of a more soluble collagen, namely, type

I collagen. In general, all of the data presented in this paper suggest that the ability of smooth muscle cell cultures to produce procollagen can be altered by appropriate estradiol treatment.

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