Biochemical Pharmacology, Vol. 26, pp. 1961–1966. Pergamon Press, 1977. Printed in Great Britain.

THE EFFECT OF CERTAIN ANTI-INFLAMMATORY STEROIDS ON COLLAGEN SYNTHESIS *IN VITRO*

HEIKKI SAARNI

Department of Medical Chemistry, University of Turku, Kiinamyllynk. 10, Turku 52, Finland

(*Received* 24 *January* 1977; *accepted* 15 *April* 1977)

Abstract—The effect of some anti-inflammatory steroids (prednisolone, and different derivatives of hydrocortisone, dexamethasone and betamethasone) on the synthesis and extra- and intracellular distribution of collagen was studied using chick embryo tendon cells. The concentrations of steroids varied between 1×10^{-3} M and 1×10^{-7} M. All steroids having an effect decreased the synthesis of collagen and the amount of labeled collagen decreased more in the medium than in the cells. Great variations in the inhibitory activity between different derivatives of steroids were noticed. Hydrocortisone was as inhibitory as hydrocortisone butyrate but both decreased collagen synthesis more than succinate or phosphate derivatives of hydrocortisone. Betamethasone-17-valerate was the most potent inhibitor among the steroids tested, whereas its alcohol and phosphate forms were ineffective. Dexamethasone and its phosphate derivative did not affect collagen synthesis in this system. The inhibitory potency of prednisolone on collagen synthesis was about the same as that of hydrocortisone. No steroid caused an accumulation of labeled collagen into cells and the decrease in the amount of collagen followed the decrease in total amount of labeled proteins. The results suggest that the inhibition of collagen synthesis by anti-inflammatory steroids is a consequence of a general inhibition of protein synthesis.

Synthetic corticosteroids have gained a widespread use in clinical medicine despite their many side effects e.g. the suppression of adrenal glands [1]. When these drugs are applied topically for extended periods various local symptoms appear in dermis as consequence of atrophy i.e. skin thinning, striae, bruising and delayed wound healing [2, 3].

Many theories have been presented in order to explain the genesis of skin atrophy. Steroids have been claimed to inhibit collagen synthesis [4–6] and to promote its degradation [7, 8]. Fibroblast proliferation is also inhibited [9] and this property has been used in screening of new compounds [10, 11]. Inhibition of glycosaminoglycan synthesis is also caused by anti-inflammatory steroids [12, 13].

The effect of anti-inflammatory steroids on collagen synthesis has been generally studied using complex and poorly characterized experimental systems (reviewed by Nacht and Garzon [14]). In the present work freshly isolated chick embryo tendon cells are used. This is a relatively simple system and has been extensively characterized by other authors [15–21]. These cells are well suited for this purpose because about 70 per cent of their protein synthesis is collagen [15]. The time between the addition of radioactive proline and the secretion of collagen chain with labeled hydroxyproline from these cells into the medium is about 20–21 min [16]. Thus the short incubation time (2 hr) used in this system is long enough to detect changes in collagen synthesis. A longer incubation time *in vitro* makes the cells ascorbate-deficient and as a result prolyl hydroxylation becomes the rate-controlling step [22]. Some of the most common steroids used topically (prednisolone and derivatives of hydrocortisone, dexamethasone and betamethasone) were selected and their effects on collagen synthesis were tested.

MATERIALS AND METHODS

White Leghorn chick embryos were obtained from a local hatchery (Munkkila, Paimio, Turku). Tendon cells from 17-day-old embryos were isolated according to the method of Dehm and Prockop [15] as slightly modified by Rönnemaa *et al.* [23]. Cells were then suspended in modified Krebs–Ringer medium (141 mM NaCl, 5.6 mM KCl, 3.0 mM $CaCl_2$, 1.4 mM KH_2PO_4, 1.4 mM $MgSO_4$, 22 mM glucose) buffered with 20 mM *N*-2-hydroxy-ethyl-piperazine-*N*-2-ethansulphonic acid (HEPES, Sigma, St. Louis, U.S.A.), pH 7.4 and containing 0.015% bovine serum albumin (A 4503, Sigma) and 0.1 mM ascorbic acid. Nonradioactive L-proline (14.5 μM) was added to incubation medium to prevent the effects of changes in proline pool size to collagen synthesis.

About 2×10^6–3×10^6 cells in 3.0 ml of the medium were incubated in 25 ml polypropylene tubes with air as the gaseous phase. After a 15 min preincubation at 37°, 5 μCi of L[G-^{3}H]proline (635 mCi/mmole, Radiochemical Centre, Amersham, England) were added to the medium and the incubation was continued for 120 min. The incubation was stopped by cooling the tubes to 0° in an ice bath and by adding 0.27 mg of cycloheximide (Sigma) and 0.4 mg of alfa-alfa-bipyridyl (Merck, Darmstadt, Germany) in 0.2 ml of medium. At the end of the incubation an aliquot of 200 μl was taken from each tube for the measurement of cell viability by Trypan Blue exclusion test. The remaining cells were separated from the medium by centrifugation at 350 *g* for 12 min. The cell pellet was washed once with the medium containing 0.01% cycloheximide and 0.015% alfa-alfa-bipyridyl. This second supernatant was discarded. The cells were then suspended in distilled water and sonicated for 30 sec. The cell homogenates and media were dia-

lyzed against running tap water overnight and the amount of radioactive hydroxyproline was measured by the method of Juva and Prockop [24].

The steroids used included: hydrocortisone (Merck), hydrocortisone monosodium phosphate (Lääke Oy, Turku, Finland), hydrocortisone sodium succinate (Orion, Mankkaa, Finland), hydrocortisone-17-butyrate (Gist-Brocades, Delft, Holland), prednisolone (Merck), dexamethasone (Orion), dexamethasone monosodium phosphate (Orion), betamethasone (Glaxo, Greenford, Middx., England), betamethasone disodium phosphate (Glaxo) and betamethasone-17-valerate (Glaxo). The phosphates and the succinate derivative were dissolved directly into the incubation media. The others were first dissolved in ethanol and amounts of steroids required per tube were added into tubes. Ethanol was then removed by evaporation in air stream before adding the medium and the cells. The steroids were added at the beginning of the preincubation. The controls were incubated without drug and their number was the same as the number of samples at each drug concentration [4–6]. The statistical significance of the results was calculated using analysis of variance and the Scheffe's *S*-test.

RESULTS

The viability of the cells was good and no increase in cell membrane permeability to Trypan blue was detected in any concentration of steroids used after a 2-hr incubation.

Effect of steroids on total collagen synthesis. The two water-soluble derivatives of hydrocortisone, hydrocortisone sodium succinate and hydrocortisone monophosphate, differed in their inhibitory potency on collagen synthesis measured as proline incorporation into hydroxyproline (Fig. 1). Hydrocortisone succinate inhibited the synthesis of collagen in this system by 90 per cent at the concentration of 1×10^{-3} M while the inhibition by hydrocortisone phosphate was 56 per cent. At concentrations lower than this, both steroids had no effect. The other watersoluble steroids studied, dexamethasone and betamethasone phosphate, did not affect collagen synthesis at the tested concentrations.

Hydrocortisone inhibited collagen synthesis by 46 per cent at 1×10^{-4} M and 20 per cent at 1×10^{-5} M concentrations (Fig. 2). Hydrocortisone butyrate and prednisolone at 1×10^{-4} M concentration inhibited the synthesis of collagen by 24 and 35 per cent, respectively. At concentrations lower than this they had no effect. Dexamethasone and betamethasone had no effect on collagen synthesis while betamethasone-17-valerate decreased it by 90 per cent at 1×10^{-4} M and by 20 per cent at 1×10^{-5} M concentrations. Doses lower than these were without any inhibitory effect.

Effect of steroids on the amounts of collagen in cells and media. The amount of radioactive collagen in the medium was 2 and 33 per cent of the control amounts at 1×10^{-3} M concentrations of hydrocortisone sodium succinate and hydrocortisone monophosphate (Fig. 3). Radioactive collagen in the cells was 29 per

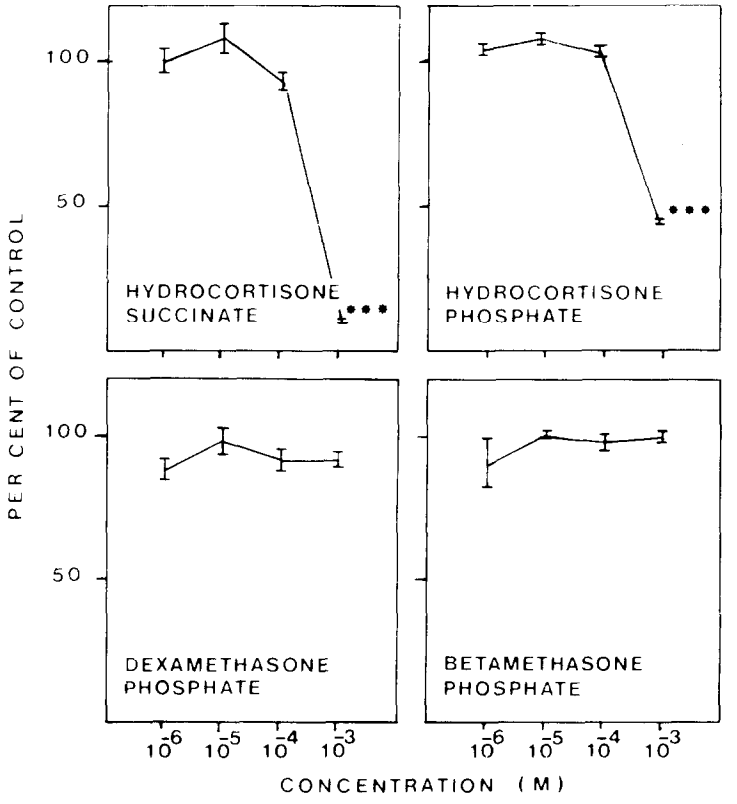

Fig. 1. Effect of some anti-inflammatory steroids on the incorporation of [^{3}H]proline into collagen at steroid concentrations 1×10^{-3} M to 1×10^{-6} M. The data are given as per cent of the controls +S.E., $n = 4$–6. *P < 0.05, **P < 0.01, ***P < 0.001.

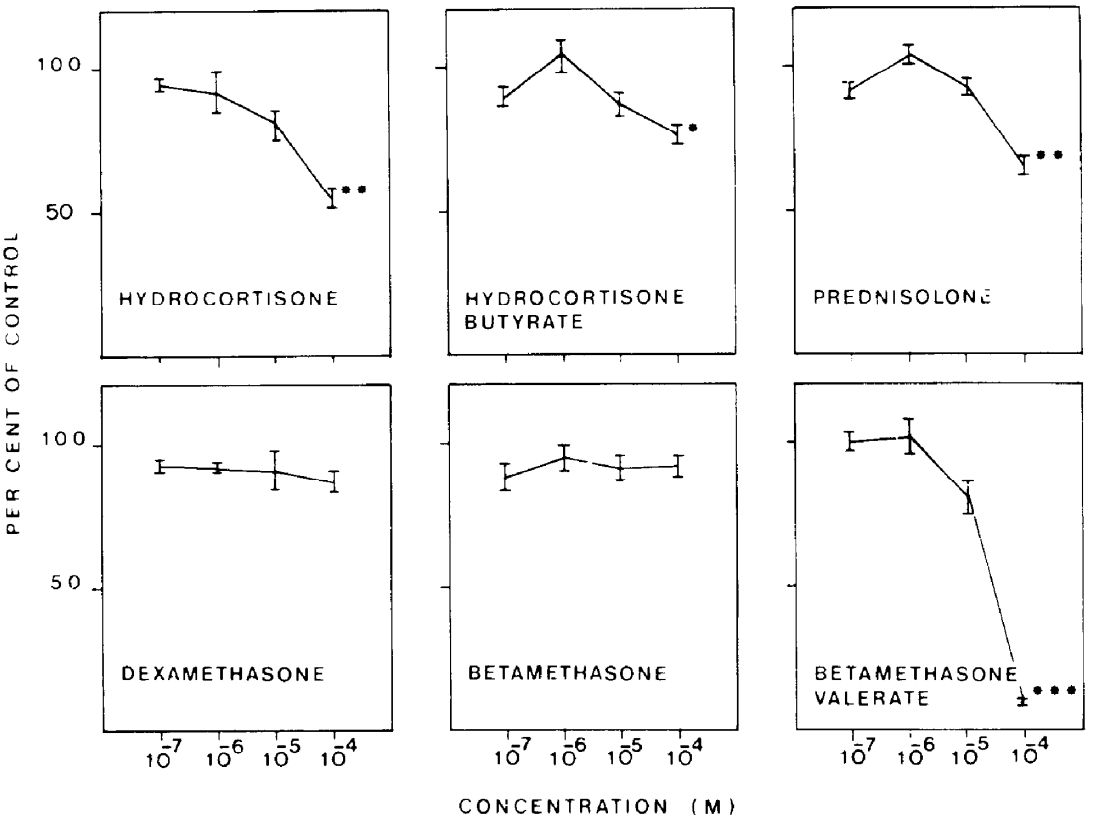

Fig. 2. Effect of some anti-inflammatory steroids on the incorporation of [^{3}H]proline into collagen at steroid concentrations 1×10^{-4} M to 1×10^{-7} M. The data are given as per cent of the controls ±S.E., $n = 4$–6. *P < 0.05, **P < 0.01, ***P < 0.001.

cent and 83 per cent, respectively. At lower concentrations these steroids did not alter the intra- or extracellular amounts of labeled collagen. Hydrocortisone in concentrations of 1×10^{-4} M and 1×10^{-5} M inhibited the labeling of media collagen by 49 per cent and 20 per cent and the intracellular collagen by 46 per cent and 15 per cent, respectively (Fig. 4). Hydrocortisone butyrate at 1×10^{-4} M concentration decreased the labeling of media collagen by about 33 per cent but had no effect on the amounts of intracellular collagen. Prednisolone at its only effective concentration (1×10^{-4} M) decreased the amount of extracellular collagen by 43 per cent and that of intracellular by 17 per cent. Betamethasone-17-valerate was the most potent steroid and suppressed the radioactivities of media and cellular collagens by 97 per cent and 70 per cent, respectively, at a concentration of 1×10^{-4}.

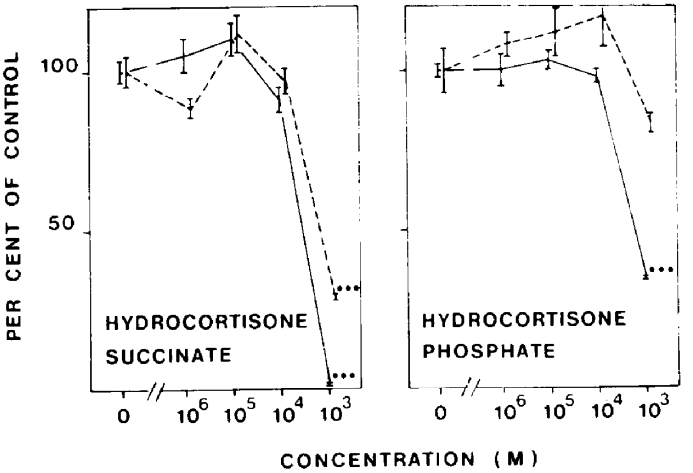

Fig. 3. Effect of some steroids on the amount of radioactive collagen in media and cells at concentrations 1×10^{-3} M to 1×10^{-6} M. The values are given in per cent of controls ±S.E., $n = 4$–6. *P < 0.05, **P < 0.01, ***P < 0.001. —— = medium, ----- = cells.

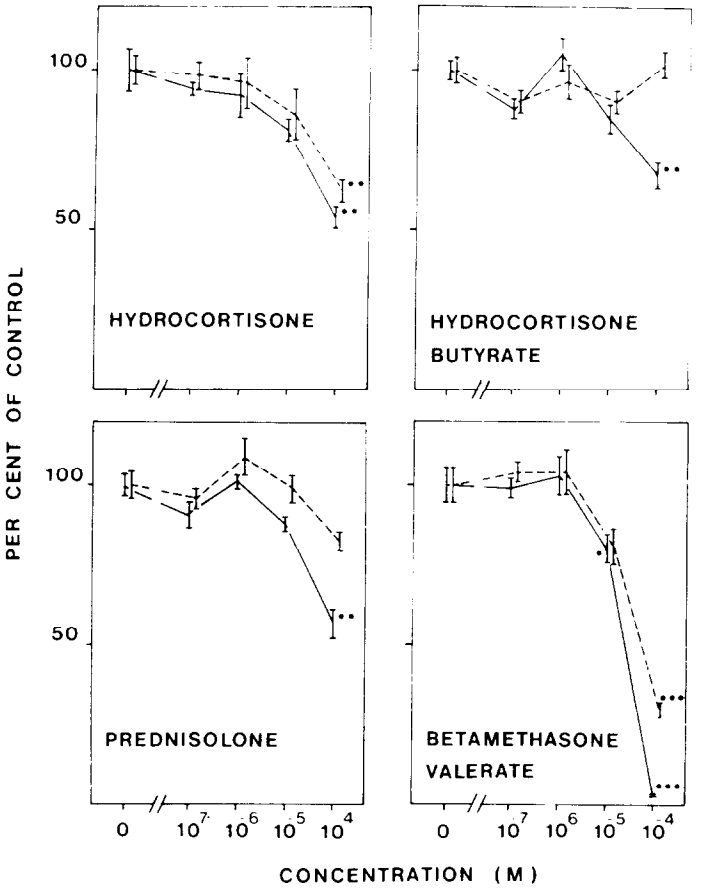

Fig. 4. Effect of some steroids on the amount of radioactive collagen in media and cells at concentrations 1×10^{-4} M to 1×10^{-7} M. The values are given in per cent of controls ±S.E., $n = 4$–6. *P < 0.05, **P < 0.01, ***P < 0.001, —— = medium, ----- = cells.

The ratio of labeled collagen in the medium vs cells in the different concentrations of steroids is shown in Table 1. This ratio was changed only at the highest or the two highest steroid concentrations. The ratio diminished from 1, calculated to controls to 0.05 by hydrocortisone succinate and to 0.41 by hydrocortisone phosphate at a concentration of 1×10^{-3} M. Betamethasone-17-valerate and hydrocortisone caused a change in this ratio from 1 to 0.10 and 0.49 at a concentration of 1×10^{-4} M. With other concentrations and other steroids the ratio diminished to 0.34 or less.

The total incorporated radioactivity versus radioactivity in hydroxyproline in the cells. There was no increase in the incorporated radioactivity in the cells as compared with controls. The ratio between the total incorporated radioactivity and that in hydroxyproline was calculated in order to detect whether unhydroxylated collagen accumulated in the cells. The ratio remained the same in the presence of all the tested steroids.

DISCUSSION

The results indicate that derivatives of hydrocortisone have a different potency in decreasing the rate of collagen synthesis. Hydrocortisone as free alcohol was as potent an inhibitor as prednisolone and these both were more potent than hydrocortisone monosodium phosphate or sodium succinate. The active form

Table 1. Effect of steroids on the ratio of labeled collagen in the medium vs cells. The ratio in controls was calculated as 1.0

Drug	Hydrocortisone succinate	Hydrocortisone phosphate	Hydrocortisone	Hydrocortisone butyrate	Prednisolone	Betamethasone valerate
Control	1.00	1.00	1.00	1.00	1.00	1.00
Concn. (M)						
10^{-7}	NT*	NT	0.94	0.97	0.92	0.96
10^{-6}	1.12	0.95	0.97	1.10	0.93	0.99
10^{-5}	1.02	0.94	0.85	0.94	0.89	0.98
10^{-4}	0.89	0.88	0.49	0.66	0.68	0.10
10^{-3}	0.05	0.41	NT	NT	NT	NT

* NT = not tested.

of both of these water soluble steroids is believed to be hydrocortisone and the variation in potency may reflect the rate at which they are converted to this form. Hydrocortisone butyrate inhibited collagen synthesis slightly less than hydrocortisone. Betamethasone-17-valerate was the most potent inhibitor of collagen synthesis among the steroids studied.

The lack of inhibition of collagen synthesis by dexamethasone and betamethasone as well by their phosphate derivatives was unexpected. These steroids are soluble in water in the concentrations used [25, 26]. This suggests that they may have to be changed to some other form before being inhibitory. Problems of plasma membrane penetration, nuclear binding etc. may be involved.

Many authors claim, that anti-inflammatory steroids decrease accumulation of collagen in the test systems used. Cutroneo *et al.* [27] reported that hydrocortisone, methylprednisolone and triamcinolone decreased the activity of prolyl hydroxylase in rat liver and granulation tissue *in vivo*. On the other hand prolyl hydroxylation is postulated to be one of the rate-limiting steps in collagen synthesis [28]. Triamcinolone in rat skin *in vivo* decreased, however, prolyl hydroxylase only 35–40 per cent after three days treatment and this was not enough to account for the 77 per cent decrease in hydroxyproline formation [29]. In the present experimental system procollagen chains will accumulate in the cells if prolyl hydroxylase is inhibited by alfa-alfa-bipyridyl [15, 19, 21]. In the present study no accumulation of unhydroxylated [^{3}H]proline labeled protein in the cells was found due to exposure to steroids. Another possible reason for the decreased amount of extracellular collagen would be the inhibition of collagen secretion from cells. This has also been suggested to be one of the possible effects of anti-inflammatory drugs [6, 30]. The ratio of labeled hydroxyproline in medium vs cells was found to be decreased in the presence of anti-inflammatory steroids. It correlated to the decrease in total collagen synthesis and was probably due to the slowered synthesis of cellular proteins. Thus, the present results do not suggest rate limiting inhibition of collagen hydroxylation or secretion by anti-inflammatory steroids. It is interesting to note that others have found inhibition of collagen secretion with colchicine and vinblastine which leads to an intracellular accumulation of collagen [15, 19].

The effect of steroids on catabolism of collagen has been studied by Houck *et al.* [7, 8]. They showed, that hydrocortisone and prednisolone induce a detectable collagenolytic enzyme activity in rat skin and human fibroblast culture after a 3-hr exposure which was suppressed by inhibitors of protein synthesis, namely actinomycin-D and cycloheximide. However, no increase in collagen degradation was reported by other authors in rat skin after hydrocortisone treatment [4, 31]. In the present 2-hour incubation, induction of collagenolytic activity seems improbable.

Chick embryo tendon cells synthesize only type I collagen while skin fibroblasts synthesize both type I and III collagen [32]. However, collagen synthesis by chick tendon cells responded to steroids in a similar way to collagen synthesis by human skin fibroblasts in culture in that inhibition was only seen at very high concentrations [33].

The inhibitory action of steroids on collagen and other protein synthesis may reflect their potency to produce skin atrophy in topical use. Betamethasone-17-valerate is known to produce more skin atrophy than hydrocortisone or prednisolone. The skin atrophy obtained by hydrocortisone and hydrocortisone butyrate is reported to be of the same magnitude [34]. The relative anti-inflammatory potencies of hydrocortisone butyrate and betamethasone-17-valerate are claimed to be the same in clinical applications [35]. In this study the inhibition of collagen synthesis, however, had quite a different magnitude. This suggests that the relations of the anti-inflammatory effect and the inhibition of collagen and other protein synthesis are to be studied further.

The concentrations of steroids which are required to inhibit collagen synthesis in this system are very high and can hardly be reached with systemic administration. On the other hand, concentrations in skin during topical therapy have never been measured accurately and it is possible that concentrations high enough to inhibit collagen synthesis might occur. Furthermore, as shown by Uitto and Mustakallio [36] higher concentrations of corticosteroids are needed to inhibit collagen synthesis *in vitro* than *in vivo*.

Acknowledgements—The author would like to acknowledge the encouragement and support of Professor V. K. Hopsu-Havu. He is thankful to Assoc. Professor R. Penttinen for the comments on the manuscript and Professor E. Kulonen, in whose laboratory this work was carried out. This investigation was supported by a grant from Suomalainen Lääkäriseura Duodecim.

REFERENCES

1. L. Axelrod, *Medicine* **55**, 39 (1976).
2. E. Schöpf, *Ann. clin. Res.* **7**, 353 (1975).
3. I. B. Sneddon, *Drugs* **11**, 193 (1976).
4. K. I. Kivirikko, O. Laitinen, J. Aer and J. Halme, *Biochem. Pharmac.* **14**, 1445 (1965).
5. J. Uitto, P. Teir, P. Peltokallio and K. K. Mustakallio, *Acta endocr.* (Kbh) suppl. **155**, 146 (1971).
6. N. Blumenkrantz and G. Asboe-Hansen, *Acta endocr.* (Kbh) **83**, 665 (1976).
7. J. C. Houck, Y. M. Patel and J. Gladner, *Biochem. Pharmac.* **16**, 1099 (1967).
8. J. C. Houck and V. K. Sharma, in *Inflammation Biochemistry and Drug Interaction* (Eds A. Bertelli and J. C. Houck) p. 85. Excerpta Medica Foundation, Amsterdam (1969).
9. A. G. Ruhmann and D. L. Berliner, *Endocrinology* **76**, 916 (1965).
10. R. Marks, D. Pongsehirun and T. A. Saylan, *Brit J. Dermatol.* **88**, 69 (1973).
11. J. Brotherton, *Endocrinology* **49**, XV (1971).
12. K. Karzel and R. Domenjoz, *Pharmacology* **2**, 302 (1969).
13. H. Boström, *Opusc. Med.* **13**, 202 (1968).
14. S. Nacht and P. Garzon, *Adv. Steroid Biochem. Pharmac.* **4**, 157 (1974).
15. P. Dehm and D. J. Prockop, *Biochim. biophys. Acta* **240**, 358 (1971).
16. P. Dehm and D. J. Prockop, *Biochim. biophys. Acta* **264**, 375 (1972).
17. S. A. Jimenez, P. Dehm and D. J. Prockop, *FEBS Lett.* **17**, 245 (1971).
18. S. A. Jimenez, *J. biol. Chem.* **249**, 4480 (1974).
19. R. Harwood, M. E. Grant and D. S. Jackson, *Biochem. J.* **152**, 291 (1975).

20. R. Harwood, M. E. Grant and D. S. Jackson, *Biochem. J.* **156,** 81 (1976).
21. S. A. Jimenez, P. Dehm, B. R. Olsen and D. J. Prockop, *J. biol. Chem.* **248,** 720 (1973).
22. D. J. Prockop, R. A. Berg, K. I. Kivirikko and J. Uitto, in *Biochemistry of Collagen* (Eds G. N. Ramachandran and A. H. Reddi) p. 163. Plenum Press, New York and London (1976).
23. T. Rönnemaa, K. Juva and E. Kulonen, *Atherosclerosis* **21,** 315 (1975).
24. K. Juva and D. J. Prockop, *Analyt. Biochem.* **15,** 77 (1966).
25. *The Merck Index* 8th edit. p. 334, Merck & Co., Inc., Rahay, NJ, U.S.A. (1968).
26. Unpublished data of the author.
27. K. R. Cutroneo, D. Costello and G. C. Fuller, *Biochem. Pharmac.* **20,** 2797 (1971).
28. E. Mussini, J. J. Hutton Jr. and S. Udenfriend, *Science, N.Y.* **157,** 927 (1967).
29. K. R. Cutroneo and D. F. Counts, *Molec. Pharmac.* **11,** 632 (1975).
30. E. Kulonen and M. Potila, *Biochem. Pharmac.* **24,** 1671 (1975).
31. Q. T. Smith and D. J. Allison, *Endocrinology* **77,** 785 (1965).
32. K. I. Kivirikko and L. Risteli, *Med. Biol.* **54,** 159 (1976).
33. N. S. Doherty and H. Saarni, *J. Pharm. Pharmac.* **28,** 656 (1976).
34. I. Sneddon, *Br. J. Dermatol.* **89,** 505 (1973).
35. J. Thormann and H. Brodthagen, *Dermatology* **152,** suppl. 1, 209 (1976).
36. J. Uitto and K. K. Mustakallio, *Biochem. Pharmac.* **20,** 2495 (1971).