Biochemical Pharmacology, Vol. 24, pp. 2293–2298. Pergamon Press, 1975. Printed in Great Britain.

CALCIUM ETHYLENEDIAMINETETRAACETATE (CaEDTA) TOXICITY—EFFECT OF LYSOSOMAL STABILIZERS AND LABILIZERS ON CaEDTA-INDUCED COLLAGEN DEGRADATION IN THE RAT*†

KATHERINE M. ROGERSON‡ and ARTHUR L. ARONSON
Department of Physiology, Biochemistry and Pharmacology, New York State Veterinary College, Cornell University, Ithaca, N.Y. 14853, U.S.A.

(*Received* 25 *September* 1974; *accepted* 7 *March* 1975)

Abstract—Degradation of collagen accompanies the progression of CaEDTA toxicity in the rat. After the parenteral administration of CaEDTA (6 m-moles/kg/24 hr, constant i.v. infusion for 48 hr), the urinary excretion of hydroxyproline is markedly increased. Radioisotope studies indicated that the source of this enhanced urinary hydroxyproline is the degradation of both mature and immature collagen. The present study was concerned with the effect of known lysosomal stabilizers and labilizers on CaEDTA-induced collagen breakdown. Massive doses of cortisol (50 mg/kg/8 hr, i.m.), as well as other anti-inflammatory agents believed to stabilize lysosomal membranes, were administered together with CaEDTA to rats. The concurrent administration of cortisol partially antagonized the degradative action of CaEDTA as evidenced by a significant reduction in urinary hydroxyproline excretion. Radioisotope labeling experiments traced this reduction in urinary hydroxyproline to inhibition of CaEDTA-induced degradation of mature (insoluble) collagen. In contrast, the concurrent administration of CaEDTA and vitamin A palmitate (250,000 I.U./kg/12 hr, s.c.), a lysosomal labilizer, produced a synergistic increase in urinary hydroxyproline excretion which was the result of increased breakdown of newly formed (soluble, immature) collagen. These results suggest that lysosomal mechanisms play a role in the degradation of collagen produced by CaEDTA.

The usefulness of the calcium chelate of ethylenediaminetetraacetate (CaEDTA) for mobilizing lead as well as many radioactive metals in cases of accidental ingestion in man and animals is well established. Nevertheless, the drug can produce toxic reactions and death. Since treatment with chelating agents must be intensive and prolonged, an understanding of the nature of CaEDTA toxicity is essential to carry out rational therapy.

A number of morphologic and functional changes have been shown to occur after CaEDTA administration in several mammalian organs including the kidney[1], intestine[2–7], adrenal glands, lymph nodes and liver[2, 5, 6, 8]. Except for studies demonstrating inhibition of DNA synthesis in the intestine[9–11], little definitive information is available on the mechanisms by which the drug produces tissue damage.

Recent studies by the present authors showed that collagen degradation was markedly enhanced during the course of CaEDTA toxicity in the rat[12]. Since collagen plays an important role in maintaining the structural and functional integrity of connective tissue, this finding may help to explain some of the tissue damage associated with CaEDTA toxicity.

The purpose of the present work is to study the mechanism by which CaEDTA causes collagen degradation. Since increased concentrations of lysosomal enzymes are reported to occur in cases of rapid collagen breakdown[13], the present investigation is concerned with assessing the importance of lysosomal mechanisms in the process of CaEDTA-induced collagen degradation. This objective was approached indirectly by determining whether known lysosomal stabilizers and labilizers could attenuate and accentuate, respectively, the effect of CaEDTA on collagen breakdown, as measured by their influence on the urinary excretion of hydroxyproline, an imino acid virtually specific for collagen[14].

MATERIALS AND METHODS

Materials. Male, Long Evans rats, weighing 275–350 g at the time of experiment, were purchased from Blue Spruce Farms, Altamont, N. Y.

CaEDTA was prepared either by mixing equimolar quantities of disodium EDTA (Mallinckrodt) and calcium chloride, and adjusting the pH of the solution to 7·4 with NaOH, or by dissolving calcium disodium EDTA (generously supplied by CIBA–GEIGY, Ardsley, N. Y., as Sequestrene Na_2Ca). The infusion solutions were appropriately diluted from a 0·1 M stock solution with 0·9% (w/v) NaCl solution before infusion. Osmolalities of the infusion solutions were adjusted to range between 290 and 310 mOs/liter (Fiske Osmometer).

Hydrocortisone (cortisol) suspension (Cortef), 50 mg/ml, was obtained from the Upjohn Co., Kalamazoo, Mich. The drug was administered i.m. every 8 hr at 50 mg/kg. Dexamethasone (Azium),

* This work was supported by Grants HE-10054 and ES-00769 from the United States Public Health Service.

† A preliminary report of a portion of this work has been published previously (*Pharmacologist* **13**, 547, 1971).

‡ Much of this work was in partial fulfillment of the requirements for the degree of Master of Science, Graduate School, Cornell University, by the senior author.

2 mg/ml, was obtained from the Schering Corp., Bloomfield, N. J. The drug was administered i.m. every 8 hr at 4 mg/kg. Acetylsalicylic acid was obtained from the Aldrich Chemical Co., Cedar Knolls, N. J. A solution of the drug, prepared by dissolving the powder in water immediately before use, was administered p.o. at 50 mg/kg every 12 hr in a volume of 0·1 ml/100 g body weight. Phenylbutazone (generously supplied by CIBA-GEIGY, Ardsley, N. Y.) was prepared immediately before use by dissolving 200 mg in 0·25 ml of 10% (w/v) NaOH solution and adjusting the final volume to 20 ml. The drug was administered orally at 100 mg/kg every 12 hr. Vitamin A alcohol (retinol) was obtained from the Sigma Chemical Co., St. Louis, Mo. A solution of the vitamin was prepared immediately before use by dissolving the crystals in absolute ethanol (20%, v/v, of final volume) and adjusting the final volume with corn oil. All solutions were prepared and administered with minimal exposure to light. The drug was administered p.o. at 300,000 I.U./kg (90 mg/kg) every 12 hr in a volume of 0·3 ml/100 g body weight. Vitamin A palmitate (retinyl palmitate), water dispersible, 275,000 I.U./g, was obtained from Schwartz/Mann, Orangeburg, N. Y. The drug was freshly prepared before use by suspending the material in 0·9% NaCl solution at a concentration of 68,750 I.U./ml. The drug was administered s.c. at 250,000 I.U./kg every 12 hr.

L-Proline-^{14}C (255 μCi/μmole, uniformly labeled) was purchased from the Amersham–Searle Corp., Des Plaines, Ill.

Experimental. The preparation of rats for constant i.v. infusion has been described previously[12]. A control solution of 0·9% NaCl was infused i.v. to all rats for 12 hr before the administration of drugs. When CaEDTA was given, the drug was infused continuously i.v. over a period of 48 hr for a total dose of 12 m-moles/kg. Control rats continued to receive 0·9% NaCl solution during this time. When other drugs were administered (dosage schedules described under Materials), the rats were infused with either NaCl solution or CaEDTA. Urine was quantitatively collected under toluene at 12-hr intervals and stored frozen at −15° until analyzed for hydroxyproline.

Analysis for urinary hydroxyproline. Urinary hydroxyproline was determined by a modification[15] of the method of Prockop and Udenfriend[16]. Total (peptide-bound + free) hydroxyproline was measured after hydrolyzing 1 ml urine in 6 N HCl for 3 hr in an autoclave at 124°.

Determination of the source of urinary hydroxyproline. Hydroxyproline in the mature collagen pool was labeled by injecting a radioactive collagen precursor, L-proline-^{14}C. The urinary excretion of hydroxyproline-^{14}C reflects the degradation of mature collagen approximately 4 weeks later[17, 18]. Male rats weighing 120 g each received a total of 50 μCi in three equal and consecutive daily i.p. injections. After 4 weeks, one rat was infused with CaEDTA and the other with 0·9% NaCl solution; vitamin A palmitate was administered to both rats. In a second experiment, cortisol was administered concurrently with CaEDTA to two rats.

The total radioactivity of hydroxyproline-^{14}C was determined according to the method of Juva and Prockop[19]. Samples were counted with a Beckman LS-100 liquid scintillation counter. The specific activity of hydroxyproline (dis./min/μmole) was calculated by dividing the total radioactivity by the quantity of hydroxyproline present[15].

RESULTS

Effect of lysosomal stabilizers on the increased urinary excretion of hydroxyproline associated with CaEDTA administration. When either cortisol (50 mg/kg) or dexamethasone (4 mg/kg) was administered i.m. every 8 hr concurrently with CaEDTA (6 m-moles/kg/24 hr, constant i.v. infusion), the urinary excretion of hydroxyproline was markedly reduced when compared to CaEDTA infusion alone (Fig. 1). When the steroids were given alone, the urinary excretion of hydroxyproline was not altered. This is in accord with previously published data[20] demonstrating the inability of corticosteroids alone to affect the urinary excretion of hydroxyproline in mature rats.

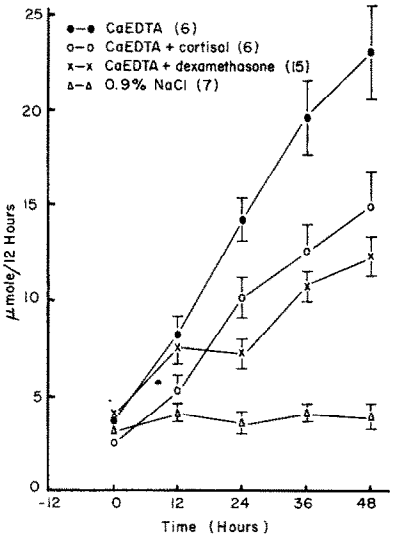

Fig. 1. Urinary excretion of hydroxyproline during the infusion of CaEDTA alone and the effect of the concurrent administration of either cortisol (50 mg/kg/8 hr, i.m.) or dexamethasone (4 mg/kg/8 hr, i.m.). All rats were infused i.v. with 0·9% NaCl solution for 12 hr. CaEDTA was then infused i.v. beginning at time 0 for 48 hr at a rate of 6 m-moles/kg/24 hr; control rats continued to receive 0·9% NaCl solution during this time. Quantitative urine collections were made every 12 hr. Number of rats per group is stated in parentheses; points represent means ± S. E.

Since the degradation of both mature and immature collagen is enhanced by CaEDTA[12], it was of interest to determine whether one collagen pool was more affected than the other. Thus, CaEDTA and cortisol were given concurrently to two rats treated with L-proline-^{14}C 4 weeks previously. A moderate increase (2-fold) in urinary hydroxyproline-^{14}C occurred (upper curves of Fig. 2); however, the increase was not as marked as that measured for total urinary hydroxyproline excre-

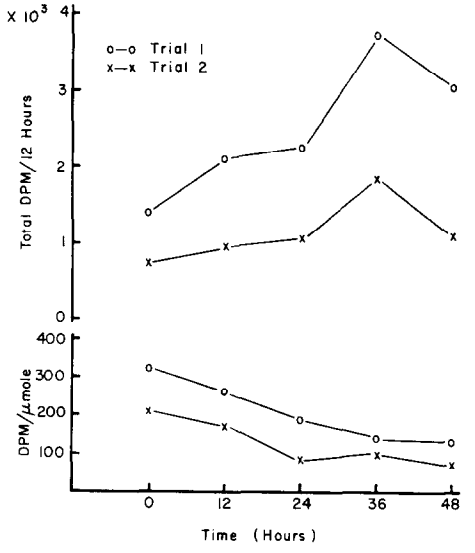

Fig. 2. Effect of the concurrent administration of CaEDTA and cortisol on the urinary excretion of hydroxyproline-^{14}C in two rats. Trials 1 and 2 represent the data from each rat. The upper graph illustrates the excretion of total urinary hydroxyproline-^{14}C. The lower graph illustrates the specific activity (dis./min/μmole) of total urinary hydroxyproline. Proline-^{14}C was administered i.p. 4 weeks prior to the experiment. Starting at 0 time, cortisol was administered at 50 mg/kg/8 hr i.m. and CaEDTA was infused continuously i.v. at 6 m-moles/kg/24 hr for 48 hr.

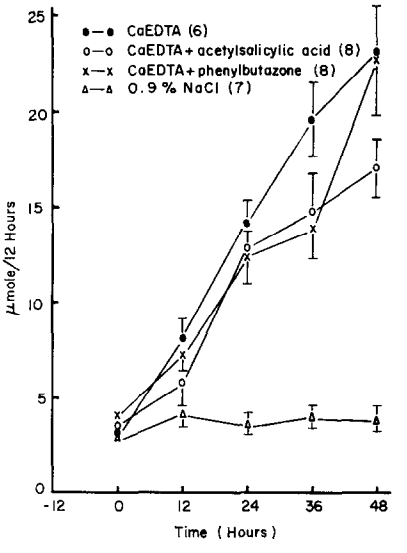

Fig. 3. Urinary excretion of hydroxyproline during the infusion of CaEDTA alone and the effect of the concurrent administration of either acetylsalicyclic acid or phenylbutazone (both drugs given p.o. at 100 mg/kg/24 hr). All rats were infused i.v. with 0·9% NaCl solution for 12 hr. CaEDTA was then infused i.v. beginning at time 0 for the next 48 hr at 6 m-moles/kg/24 hr; control rats continued to receive 0·9% NaCl solution during this time. Quantitative urine collections were made every 12 hr. Number of rats per group is stated in parentheses; points represent means ± S. E.

tion. Consequently, the specific activity of urinary hydroxyproline (lower curves of Fig. 2) decreased.

The protective effect of nonsteroidal anti-inflammatory agents on CaEDTA-induced collagen degradation was somewhat ambiguous. When acetylsalicylic acid was administered together with CaEDTA, a significant degree of protection was apparent after hr 24 of infusion, almost to the same degree as with cortisol (Fig. 3 compared to Fig. 1). Some protection occurred with phenylbutazone up to hr 36 of infusion, but was no longer evident by the end of 48 hr (Fig. 3).

Effect of lysosomal labilizers on the increased urinary excretion of hydroxyproline associated with CaEDTA administration. Vitamin A palmitate produced an increase in urinary hydroxyproline excretion even more striking than CaEDTA (Fig. 4). The initial rate of increase was especially prominent. When vitamin A palmitate was administered concurrently with CaEDTA, the effect on enhanced urinary hydroxyproline excretion was synergistic; a 25-fold increase over controls was measured by hr 48 of infusion (Fig. 4). However, equimolar doses of vitamin A alcohol p.o. produced no measurable difference in urinary hydroxyproline excretion compared to controls (Fig. 4). Also, when vitamin A alcohol was given with CaEDTA, the effect on urinary hydroxyproline excretion was the same as that anticipated for CaEDTA alone. The inability of vitamin A alcohol in the present study to enhance urinary hydroxyproline excretion may be due to insufficient dosage. Vitamin A alcohol was administered orally at a dosage approximately equimolar to the palmitate form (90 mg/kg) based on a report that this dosage regimen resulted in

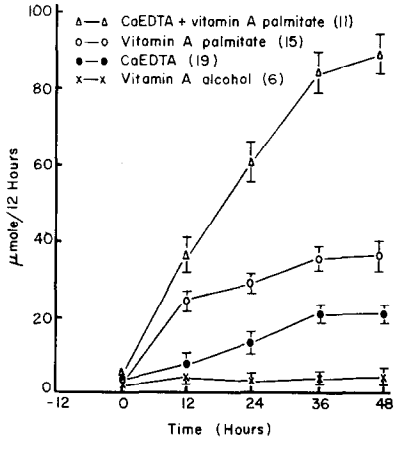

Fig. 4. Urinary excretion of hydroxyproline during the administration of either CaEDTA or vitamin A preparations alone and the effect of the concurrent administration of CaEDTA and vitamin A palmitate. All rats were infused i.v. with 0·9% NaCl solution for 12 hr. CaEDTA-treated rats then were infused i.v. (6 m-moles/kg/24 hr for 48 hr) beginning at time 0. Other rats continued to receive 0·9% NaCl solution as well as vitamin A treatment (vitamin A palmitate, s.c. at 250,000 I.U./kg/12 hr; vitamin A alcohol, p.o. at 300,000 I.U./kg/12 hr). Quantitative urine collections were made every 12 hr. Number of rats per group is stated in parentheses; points represent means ± S. E.

labilization of lysosomes in the rabbit[21]. We were unable to measure any effect on the integrity of rat liver or kidney lysosomes with this dosage[22]. Another recent study[23] showed that oral ad-

ministration of vitamin A alcohol to rats induced marked lysosomal disruption at 175 and 350 mg/kg, but was without appreciable effect at doses of 50 or 100 mg/kg.

Proline-^{14}C was administered 4 weeks preceding treatment with either vitamin A palmitate alone, or vitamin A palmitate plus CaEDTA in order to ascertain the collagen pool affected by the vitamin. The results (upper curves, Fig. 5) show that treatment with the vitamin alone had no effect on the urinary excretion of hydroxyproline-^{14}C. The increase in radioactivity occurring with the concurrent administration of the vitamin and CaEDTA was similar to that anticipated for CaEDTA alone[12]. The marked decrease in the specific activity (lower curves, Fig. 5) is a reflection of a markedly elevated excretion of the imino acid without a corresponding increase in hydroxyproline-^{14}C.

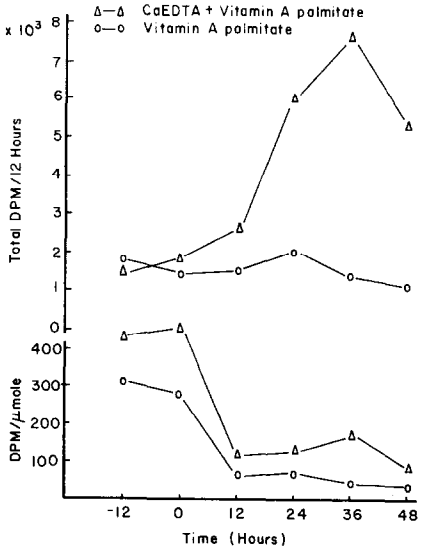

Fig. 5. Effect of vitamin A palmitate alone and the concurrent administration of vitamin A palmitate and CaEDTA on urinary hydroxyproline-^{14}C excretion. The upper graph illustrates the excretion of total urinary hydroxyproline-^{14}C. The lower graph illustrates the specific activity (dis./min/μmole) of total urinary hydroxyproline. Proline-^{14}C was administered i.p. 4 weeks prior to the experiment. Vitamin A palmitate was administered beginning at time 0 at 250,000 I.U./kg/12 hr s.c. for a total of 48 hr and CaEDTA was infused continuously i.v. at 6 m-moles/kg/24 for 48 hr.

DISCUSSION

The data indicated that known lysosomal stabilizing agents such as cortisol[21, 24–26] or acetylsalicylic acid and phenylbutazone[27–31] antagonized the action of CaEDTA in increasing urinary hydroxyproline excretion. Conversely, vitamin A, an agent known to labilize lysosomes[32–35], markedly enhanced this effect of CaEDTA. Thus, cortisol reduced and vitamin A palmitate increased the ability of CaEDTA to induce collagen degradation. Support for this contention stems from the fact that urinary hydroxyproline originates from the degradation of collagen or its immediate precursors[36–38].

The collagen pool affected by CaEDTA and lysosomal stabilizers or labilizers was investigated using a radioisotope labeling technique[17] which provided the basis for distinguishing effects on the degradation of immature (newly synthesized, soluble) and mature (insoluble) collagen. When proline-^{14}C is administered to rats, it is incorporated into collagen precursors, whereupon it undergoes hydroxylation to hydroxyproline. Newly synthesized collagen gradually undergoes polymerization to form mature collagen. The urinary excretion of hydroxyproline-^{14}C reflects the degradation of mature collagen approximately 4 weeks after the administration of proline-^{14}C[18].

Using the technique described above, we previously demonstrated that CaEDTA induced the degradation of mature and immature collagen about equally[12]. When CaEDTA was administered to rats 4 weeks after the injection of proline-^{14}C, enhanced urinary excretion of hydroxyproline and hydroxyproline-^{14}C occurred with no change in specific activity. If only immature collagen were degraded, the specific activity in the urine would have decreased; by contrast, if only mature collagen were degraded, the specific activity would have increased.

The decreased urinary excretion of hydroxyproline measured when cortisol was administered concurrently with CaEDTA was associated with a decreased urinary excretion of hydroxyproline-^{14}C. Considering that cortisol reduced the urinary excretion of hydroxyproline associated with CaEDTA administration by a factor of 2 (i.e. a 6-fold increase in the urinary excretion effected by CaEDTA alone compared with only a 3-fold increase when cortisol was administered concurrently), and that the specific activity of urinary hydroxyproline also was reduced by a factor of 2, it seems reasonable to conclude that cortisol almost exclusively antagonized the CaEDTA-induced degradation of mature collagen but was without discernible effect on the CaEDTA-induced degradation of immature collagen.

When vitamin A palmitate was administered alone, a marked increase in urinary hydroxyproline occurred with no measurable effect on urinary hydroxyproline-^{14}C excretion. This indicated that the vitamin induced the degradation of immature collagen with no discernible effect on mature collagen. This finding sheds some light on the collagen pool affected by the concurrent administration of CaEDTA and vitamin A palmitate. When the two drugs were administered together, there was a synergistic increase in total urinary hydroxyproline excretion. Since the urinary excretion of hydroxyproline-^{14}C was about the same as that expected for CaEDTA alone, it would appear that the synergistic increase in urinary hydroxyproline was derived from the immature collagen pool.

The data reported in this study are consistent with recently published studies indicating that steroidal and nonsteroidal anti-inflammatory agents inhibit collagen degradation. Beta-methasone was shown to inhibit collagen degradation in rat carrageenin granuloma[39]. Acetylsalicylic acid has been shown to inhibit the degradation of cartilage matrix[28]. The finding that triamcinolone inhibits

the degradation of mature collagen [40] is of special interest, since in the present study cortisol also appeared to antagonize only the CaEDTA-induced degradation of mature collagen. On the other hand, excess vitamin A is known to cause the dissolution of cartilage matrix and connective tissue *in vivo* [32, 33]. The effect of vitamin A has been attributed to a rupturing of lysosomal membranes and the consequent release of lysosomal proteases [41]. Enhanced collagenase activity also has been demonstrated in vitamin A-induced resorption of bone [42].

We have shown that CaEDTA, as well as vitamin A palmitate, induces an activation of lysosomes *in vivo* under the same conditions that cause collagen degradation [22]. Moreover, cortisol antagonized both the activation of lysosomes and the breakdown of collagen induced by CaEDTA. The correlation between collagen degradation and activation of lysosomes *in vivo* suggests that the two events may be causally related. Lysosomes have been shown to participate in collagen breakdown; electron micrographic studies have demonstrated the digestion of collagen fibrils within lysosomes [43–45].

Although both CaEDTA and vitamin A palmitate increase lysosomal enzyme activity *in vivo* [22], it is clear that their action on collagen breakdown is different. The equally enhanced degradation of mature and immature collagen resulting from CaEDTA administration is suggestive of enhancement of existing pathways of collagen degradation. In this regard it may be noteworthy that CaEDTA produces an effect on collagen breakdown qualitatively identical to parathyroid hormone [46]. In contrast, vitamin A palmitate only enhanced the degradation of newly synthesized collagen.

Chelation of metals undoubtedly is involved in the action of CaEDTA; chelates of greater stability, e.g. Cr^{-}, Zn^{-} or CoEDTA, neither produce collagen degradation [12] nor alter the distribution or total amount of cathepsin D *in vivo* (unpublished observations). Certain metals have been implicated in the action of lysosomal collagenolytic cathepsins. For example, zinc, copper and iron have been shown to inhibit [47, 48] and EDTA to activate [48] cathepsins capable of digesting soluble as well as insoluble collagen *in vitro*. Thus, chelation of inhibiting metals constitutes a plausible mechanism for the activation of these enzymes after their release from lysosomes during CaEDTA administration. Although the low pH optimum of these enzymes would preclude them from acting freely outside the cell, it has been suggested that the pH at the surface of connective tissue cells *in vivo* may be sufficiently low for significant lysosomal collagenolytic activity [49]. This possibility is supported by histologic studies demonstrating the presence of cathepsin D at extracellular sites where proteoglycans have been degraded and lost from tissues [50].

REFERENCES

1. H. Foreman, in *Metal Binding in Medicine* (Eds. M. J. Seven and L. A. Johnson), p. 160. Lippincott, Philadelphia (1960).
2. A. L. Aronson, P. B. Hammond and A. C. Strafuss, *Toxic. appl. Pharmac.* **12**, 337 (1968).
3. F. A. Ahrens and A. L. Aronson, *Fedn Proc.* **28**, 740 (1968).
4. F. A. Ahrens and A. L. Aronson, *Toxic. appl. Pharmac.* **18**, 10 (1971).
5. K. M. Weber, *Z. ges. exp. Med.* **150**, 354 (1969).
6. K. M. Weber, *Strahlentherapie* **137**, 708 (1969).
7. V. B. C. Braide and A. L. Aronson, *Toxic. appl. Pharmac.* **30**, 52 (1974).
8. M. D. Reuber, *Toxic. appl. Pharmac.* **11**, 321 (1967).
9. K. M. Weber, F. Bohne and U. Rabe, *Eur. J. Pharmac.* **1**, 117 (1970).
10. F. Bohne, *Strahlentherapie* **143**, 106 (1972).
11. D. M. Taylor and J. D. Jones, *Biochem. Pharmac.* **21**, 3313 (1972).
12. A. L. Aronson and K. M. Rogerson, *Toxic. appl. Pharmac.* **21**, 440 (1972).
13. J. F. Woessner, Jr., in *Tissue Proteinases* (Eds. A. J. Barret and J. T. Dingle), p. 291. American Elsevier, New York (1971).
14. D. J. Prockop and K. I. Kivirikko, in *Treatise on Collagen* (Ed. B. S. Gould), Vol. 2, p. 231. Academic Press, New York (1968).
15. K. I. Kivirikko, O. Laitenen and D. J. Prockop, *Analyt. Biochem.* **19**, 249 (1967).
16. D. J. Prockop and S. Udenfriend, *Analyt. Biochem.* **1**, 228 (1960).
17. D. J. Prockop, *J. clin. Invest.* **43**, 453 (1964).
18. O. Laitinen, *Acta Endocr., Copenh.* (suppl.) 120 (1967).
19. K. Juva and D. J. Prockop, *Analyt. Biochem.* **15**, 77 (1966).
20. K. I. Kivirikko, O. Laitinen, J. Aer and J. Halme, *Biochem. Pharmac.* **14**, 1445 (1965).
21. G. Weissmann and L. Thomas, *J. clin. Invest.* **42**, 661 (1963).
22. K. M. Rogerson and A. L. Aronson, *Biochem. Pharmac.* **00**, 000 (0000).
23. L. J. Ignarro, *J. Pharmac. exp. Ther.* **182**, 179 (1972).
24. G. Weissmann and J. T. Dingle, *Expl. Cell Res.* **15**, 207 (1961).
25. G. Weissmann and L. Thomas, *J. exp. Med.* **116**, 433 (1962).
26. G. Weissmann and L. Thomas, *Recent Prog. Horm. Res.* **20**, 215 (1964).
27. D. P. Simmons and O. D. Chrisman, *Arthritis Rheum.* **8**, 960 (1965).
28. O. D. Chrisman, G. A. Snook and T. C. Wilson, *Clin. Orthop.* **84**, 193 (1972).
29. W. S. Miller and J. G. Smith, *Proc. Soc. exp. Biol. Med.* **122**, 634 (1966).
30. K. Tanaka and Y. Iizuka, *Biochem. Pharmac.* **17**, 2023 (1968).
31. L. J. Ignarro, *Biochem. Pharmac.* **20**, 2847 (1971).
32. H. B. Fell and L. Thomas, *J. exp. Med.* **111**, 719 (1960).
33. J. T. Dingle, J. A. Lucy and H. B. Fell, *Biochem. J.* **79**, 497 (1961).
34. J. A. Lucy, J. T. Dingle and H. B. Fell, *Biochem. J.* **79**, 500 (1961).
35. J. T. Dingle, *Biochem. J.* **79**, 509 (1961).
36. S. Lindstedt and D. J. Prockop, *J. biol. Chem.* **236**, 1399 (1961).
37. D. J. Prockop, B. Peterkofsky and S. Udenfriend, *J. biol. Chem.* **237**, 1581 (1962).
38. K. I. Kivirikko, *Acta physiol. scand.* (suppl.) 219 (1963).
39. H. Nakagawa and S. Tsurufuji, *Biochem. Pharmac.* **21**, 839 (1972).
40. R. Rudolph and L. Klein, *J. surg. Res.* **14**, 435 (1973).
41. J. T. Dingle, I. M. Sharman and T. Moore, *Biochem. J.* **98**, 476 (1966).
42. J. M. Evanson, *J. Bone Jt Surg.* **52B**, 182 (1970).
43. D. Brandes and E. Anton, *J. Geront.* **24**, 55 (1969).

44. P. F. Parakkal, *J. Cell Biol.* **41**, 345 (1969).
45. P. F. Parakkal, *J. Ultrastruct. Res.* **40**, 284 (1972).
46. E. D. Harris and A. Sjoerdsma, *J. clin. Endocr. Metab.* **26**, 358 (1966).
47. A. J. Anderson, *Biochem. J.* **113**, 457 (1969).
48. D. J. Etherington, *Biochem. J.* **127**, 685 (1972).
49. A. J. Anderson, *Biochem. Pharmac.* **17**, 2253 (1968).
50. A. R. Poole, R. M. Hembry and J. T. Dingle, *J. Cell Sci.* **14**, 139 (1974).