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EFFECT OF TETRACYCLINE ON COLLAGEN BIOSYNTHESIS IN CULTURED EMBRYONIC BONES

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Abstract—The biosynthesis of collagen was studied with ^{14}C-proline in organ cultures of embryonic ulnae. The rate of conversion of the incorporated ^{14}C-proline to ^{14}C-hydroxyproline in cultured ulnae was optimal up to 6 days, provided that the medium contained ascorbate and was replaced daily. When the medium was not replaced daily, the rate of ^{14}C-proline incorporation was reduced to about 60 per cent, and only a small part of the incorporated ^{14}C-proline was converted to ^{14}C-hydroxyproline. In these conditions, a considerable part of the ^{14}C-proline was found to be in the form of protocollagen, the proline-rich and hydroxyproline-deficient polypeptide precursor of collagen.

The addition of 10, 30 or 100 μg/ml tetracycline to the culture medium reduced the rate of incorporation of ^{14}C-proline into protein. The rate of ^{14}C-hydroxyproline synthesis was reduced even more, and thus a decrease was observed in the conversion of the incorporated ^{14}C-proline to ^{14}C-hydroxyproline. Similar changes were observed when embryonic ulnae from pregnant mice receiving tetracycline were cultured in a medium not containing tetracycline. In experiments with purified protocollagen hydroxylase, tetracycline was found to inhibit the hydroxylation of ^{14}C-proline-labelled protocollagen, the degree of inhibition being dependent on the concentration of ferrous iron in the reaction mixture. Cultivation of the ulnae with tetracycline had no effect on the total amount of protocollagen hydroxylase activity in the bones when the determination was made in the presence of excess of ferrous iron.

The results suggest that tetracycline inhibits collagen synthesis by inhibiting both the incorporation of proline into protocollagen and the conversion of protocollagen to collagen. The latter inhibition is probably due to an interaction of tetracycline with ferrous iron, which is required in the hydroxylation of proline by protocollagen hydroxylase.

TETRACYCLINES have an inhibitory effect on osteogenesis and mineralization in the developing organism (for review, see ref. 1), but the nature of this inhibition is still not fully understood.[1] Several studies have shown that tetracyclines have an unusual affinity for the skeletal system, especially for the areas of bone growth.[2, 3] Interaction occurs between tetracycline and calcium,[2, 4] but it is not clear whether this is an absorption of the antibiotic on the calcium orthophosphate precipitate or a more complex reaction involving collagen fibres as well.[5] The incorporation of ^{45}Ca into foetal bone rudiments *in vitro* has been shown to be inhibited at concentrations comparable to those produced *in vivo* by therapeutic doses,[1, 6] and it has been suggested that the inhibitory effect of tetracyclines on osteogenesis is at least partly due to a direct action on the formation of bone salts.[1]

A close correlation exists between calcium and collagen metabolism in bones,[7, 8] and changes in calcium and collagen metabolism are often parallel.[8] It has been demonstrated that tetracyclines in high concentrations inhibit the uptake of labelled thymidine and proline into cultured bones,[9] and quite recently administration of tetracyclines to pregnant rats in therapeutic doses was shown to inhibit the biosynthesis of collagen and calcification in foetal bones *in vivo*.[10]

Collagen is the major protein of connective tissue, and its composition is unique in that up to 14 per cent of its amino acid content is hydroxyproline, an amino acid found exclusively in collagen. Recent studies on the biosynthesis of collagen have indicated that the hydroxyproline in this protein is synthesized by hydroxylation of the proline in a large polypeptide precursor of collagen, called protocollagen. This hydroxylation reaction is brought about by the enzyme protocollagen hydroxylase, in the presence of ascorbic acid, ferrous iron, α-ketoglutarate and atmospheric oxygen (for review, see refs. 11, 12). In the present study, the mode of action of tetracycline on collagen biosynthesis was investigated in greater detail by using organ cultures of embryonic mouse bones.

MATERIAL AND METHODS

Embryos of random-bred Swiss mice were used throughout the work. The 0-day of pregnancy was determined by the presence of a vaginal plug. The ulnae of 16-day old embryos with an average length of 11·5 mm and an average dry weight of 0·08 mg were carefully dissected free of muscle and other soft tissues under sterile conditions in a small volume of phosphate-buffered saline.[1] According to previously employed techniques,[1, 13, 14] the ulnae were cultivated on lens paper on a Trowell-type metal screen in petri dishes containing 10 ml of chemically defined protein-free synthetic medium BGJb[15] (for further details, see ref. 1). In most experiments 0·3 mM ascorbic acid, 0·01 mM α-ketoglutarate, and 0·01 mM ferrous iron were added to the medium immediately before use, and the mixture was sterilized by filtration through a Millipore filter. The culture dishes, containing from 5 to 10 ulnae, were kept in 5 per cent CO_2 in air in humified incubators at 37°. Uniformly labelled L-^{14}C-proline, 180 μc/μmole (New England Nuclear Corp.), and Tetracycline-HCl (Pfizer Corp.) were added in 0·1 ml of the medium as indicated in the tables.

After harvesting, the ulnae were immediately washed and homogenized in 4 ml of cold distilled water with a homogenizer of Teflon and glass. In most experiments the homogenates were dialysed against cold running tap-water for 24 hr and, after hydrolysis, assayed for total ^{14}C content[16] and for ^{14}C-hydroxyproline.[17]

In experiments involving the assay of protocollagen hydroxylase activity, the ulnae were homogenized in 1·5 ml of 0·05 M KCl, 0·01 M Tris-HCl buffer, pH 7·8, and the homogenates centrifuged at 15,000 *g* for 30 min at +4°. The activity of protocollagen hydroxylase was assayed as described earlier,[18, 19] in a final volume of 4·0 ml containing 1·0 ml of the 15,000 *g* supernatant of the homogenate as a source for the enzyme; 50,000 dpm of ^{14}C-proline-labelled protocollagen as substrate; 50 mM Tris-HCl buffer, pH 7·8; 2 mM ascorbic acid; 0·5 mM α-ketoglutaric acid; 0·1 mM $FeSO_4$; and 0·05 mg/ml catalase. After incubation for 60 min at 37°, 4 ml of concentrated HCl was added, and the samples were hydrolyzed overnight at 120°. Total ^{14}C content[16] and ^{14}C-hydroxyproline[17] were then assayed in the hydrolysates.

In the experiment in which the synthesis of protocollagen was studied, the ulnae were homogenized in 6 ml of cold distilled water, and solid urea was added to make 7 M.[20] The homogenates were boiled at 100° for 10 min,[20] and centrifuged at 100,000 *g* for 60 min at +4°. The supernatants were dialysed against several changes of 0·1 M KCl, 0·05 M Tris-HCl buffer at pH 7·6. Aliquots of the dialysed supernatants were then used as substrates in incubation with 2 mg of purified protocollagen hydroxylase in a final volume of 4·0 ml as described in preceding paragraph. The protocollagen hydroxylase preparation had been purified through the calcium phosphate gel step.[21, 22]

RESULTS

Effect of culture conditions on collagen biosynthesis in organ culture

Organ cultures of embryonic ulnae have been reported to provide a suitable system for studying the effects of drugs on bone development.[1] In this system, calcification proceeds linearly for several days, and experiments on calcification can be carried out with chemically defined protein-free synthetic media. In experiments on calcification, the daily replacement of this medium seems to be unnecessary in this system.[1]

In the present study, organ cultures of embryonic ulnae were used to investigate the biosynthesis of collagen. In these experiments, ulnae were cultivated for several days, and ^{14}C-proline was added to the medium for the last 24 hr of culture. The amounts of non-dialysable ^{14}C and ^{14}C-hydroxyproline were then assayed in the homogenates of the ulnae. When an ascorbate-containing medium was used for the culture without changing the medium daily, the amount of ^{14}C-hydroxyproline synthesized was found to be very small (Table 1). Additions of α-ketoglutarate and ferrous iron to the medium

TABLE 1. EFFECT OF DAILY CHANGING OF THE INCUBATION MEDIUM ON COLLAGEN BIOSYNTHESIS IN ORGAN CULTURE

Incubation conditions	No.	Total ^{14}C (dpm)	Hypro-^{14}C (dpm)	$\frac{\text{Hypro-}^{14}\text{C}}{\text{Total }^{14}\text{C}} \times 100$
Medium + asc., no change	1.	38,200	880	2·3
	2.	47,000	910	1·9
Medium + asc. + αKG + Fe^{++}, no change	3.	42,700	1220	2·9
	4.	40,500	1200	3·0
Medium + asc. + αKG + Fe^{++}, changed daily	5.	79,200	16,350	20·6
	6.	71,700	14,400	20·1

Cultures containing 7 ulnae each were incubated for 6 days as described in the text. During the last 24 hr incubation medium contained 2 μc ^{14}C-proline. Ascorbic acid, α-ketoglutarate and ferrous iron were added to the medium in concentrations indicated in the text.

only slightly increased the synthesis of ^{14}C-hydroxyproline in these conditions. However, when the medium was replaced daily[14] by fresh medium containing ascorbate, α-ketoglutarate and ferrous iron, about 20 per cent of the total ^{14}C incorporated was converted to ^{14}C-hydroxyproline (Table 1), suggesting that about half[20] of the incorporated ^{14}C is in the collagen. This value is in agreement with those observed in short experiments with chick embryo tibiae,[23] suggesting that the organ cultures used here retained the ability for maximal hydroxylation of the incorporated proline for up to 6 days.

Because changing the medium seemed to increase the rate of ^{14}C-hydroxyproline synthesis more than that of ^{14}C incorporation, it seemed probable that part of the incorporated ^{14}C-proline was in the protocollagen,[20, 24] the large proline-rich and hydroxyproline-deficient polypeptide precursor of collagen. The presence of protocollagen was verified by an experiment in which ulnae were cultivated for 6 days without changing the medium, and ^{14}C-proline was added for the last 6 hr. After cultivation, the homogenates of the ulnae were boiled in 7 M urea, and the dialysed 100,000 *g* supernatants were incubated with purified protocollagen hydroxylase. A marked increase in ^{14}C-hydroxyproline was observed after incubation with the enzyme, indicating that part of the ^{14}C had been in the protocollagen (Table 2).

TABLE 2. HYDROXYLATION BY PURIFIED PROTOCOLLAGEN HYDROXYLASE OF THE PROTEIN IN THE UREA EXTRACTS OF HOMOGENATES OF EMBRYONIC ULNAE CULTIVATED FOR 6 DAYS WITHOUT CHANGING THE MEDIUM

Sample	Total ^{14}C (dpm)	Hypro-^{14}C (dpm)	$\frac{\text{Hypro-}^{14}\text{C}}{\text{Total }^{14}\text{C}} \times 100$ (%)
Control, not incubated with enzyme	34,000	2320	6·8
Incubated with enzyme	34,000	3850	11·3

A culture containing 10 ulnae was incubated for 6 days as described in the text. The cultivation medium contained ascorbate, α-ketoglutarate and ferrous iron, and during the last 6 hr the medium contained 20 μc of ^{14}C-proline. After incubation, the ulnae were homogenized, and the homogenates extracted with 7 M urea at 100° for 10 min.[20] The dialysed 100,000 *g* supernatant was divided in two equal aliquots, and one aliquot was incubated with purified protocollagen hydroxylase as described in Methods.

When the ulnae were cultivated with daily replaced medium containing ascorbate but no additional ferrous iron and α-ketoglutarate, and ^{14}C-proline was added for the last 24 hr, the rate of ^{14}C-hydroxyproline synthesis in most experiments did not notably differ from that observed when all three of the above-mentioned substances were added (Table 3). However, in some experiments slightly reduced ratios of ^{14}C-hydroxyproline to total ^{14}C were observed when α-ketoglutarate and ferrous iron were not added (not shown in the table). When a medium containing 0·01 mM instead

TABLE 3. EFFECT OF ASCORBATE, α-KETOGLUTARATE, AND FERROUS IRON ON COLLAGEN BIOSYNTHESIS IN ORGAN CULTURE. THE MEDIUM WAS CHANGED DAILY

Incubation conditions	No.	Total ^{14}C (dpm)	Hypro-^{14}C (dpm)	$\frac{\text{Hypro-}^{14}\text{C}}{\text{Total }^{14}\text{C}} \times 100$
Medium + 0·3 mM asc. + αKG + Fe^{++}	1.	72,800	16,000	22·0
	2.	91,500	19,600	21·4
Medium + 0·3 mM asc.	3.	73,700	15,900	21·6
	4.	87,000	18,300	21·0
Medium + 0·01 mM asc.	5.	75,000	6940	9·3
	6.	59,600	4320	7·3

Cultures containing 7 ulnae each were incubated for 6 days as described in the text. During the last 24 hr the cultivation medium contained 2 μc ^{14}C-proline.

of 0·3 mM ascorbate was used, less than 10 per cent of the incorporated ^{14}C was converted to ^{14}C-hydroxyproline, even though the medium was replaced daily (Table 3). This suggests that added ascorbate was an essential requirement for optimal collagen synthesis in the organ culture system used.

Effect of tetracycline on collagen biosynthesis in organ culture

The effect of tetracycline on collagen biosynthesis was studied by experiments in which tetracycline was added to the medium, and ulnae were cultivated for 6 days, with daily replacement of the medium. This stage of cultivation was chosen because bones were found to synthesize collagen still actively, and studies on the effects of tetracycline on calcification had indicated that definite effects are observed after this period. Ascorbate, α-ketoglutarate and ferrous iron (experiments I and II, Table 4) or ascorbate only (experiment III, Table 4) were added to the medium in these experiments. Incorporation of ^{14}C-proline into non-dialysable protein during the last 24 hr of culture was found to be retarded by 10, 30 and 100 μg/ml tetracycline (Table 4).

TABLE 4. EFFECT OF TETRACYCLINE ON COLLAGEN BIOSYNTHESIS IN ORGAN CULTURE

Sample	No.	Total ^{14}C (dpm)	Hypro-^{14}C (dpm)	$\frac{\text{Hypro-}^{14}C}{\text{Total }^{14}C} \times 100$ (%)
Experiment I				
Control	1.	72,800	16,000	22·0
,,	2.	91,500	19,600	21·4
Tetracycline, 10 μg/ml	3.	52,000	10,100	19·4
,,	4.	66,000	13,800	20·7
Experiment II				
Control	1.	80,300	15,700	19·6
,,	2.	78,800	16,000	20·4
Tetracycline, 30 μg/ml	3.	68,700	11,700	17·0
,,	4.	63,100	11,900	18·9
Experiment III				
Control	1.	35,800	6800	19·0
,,	2.	42,000	8100	19·3
Tetracycline, 100 μg/ml	3.	26,900	3600	13·4
,,	4.	27,900	3900	14·0

Cultures containing 7 ulnae each in experiments I and II, and 5 ulnae each in experiment III were incubated for 6 days as described in the text, and the medium was replaced daily. In experiments I and II, ascorbate, ferrous iron and α-ketoglutarate were added to the medium; in experiment III only ascorbate was added. During the last 24 hr the media contained 2 μc ^{14}C-proline.

Synthesis of ^{14}C-hydroxyproline was retarded even more than total ^{14}C incorporation, and a decrease was observed in the ratio of ^{14}C-hydroxyproline to total ^{14}C (Table 4). In the experiment with 100 μg/ml tetracycline, the absolute amount of ^{14}C-hydroxyproline synthesized was only about half of that in the control culture.

Because the ratio of ^{14}C-hydroxyproline to total ^{14}C was found to be decreased in ulnae incubated with tetracycline, experiments were carried out to study whether tetracycline had a direct inhibitory effect on the protocollagen hydroxylase reaction.

Ferrous iron has been shown to be an essential requirement for the protocollagen hydroxylase reaction,[25] and because tetracycline binds bivalent cations[4] it seemed possible that tetracycline might inhibit this reaction by chelating ferrous iron. Therefore, experiments were carried out in which ^{14}C-proline-labelled protocollagen was incubated with purified protocollagen hydroxylase in the presence of three different concentrations of ferrous iron. Inhibition of hydroxylation of the ^{14}C-proline-labelled protocollagen was observed in the presence of tetracycline, and the degree of inhibition was dependent on the concentrations of tetracycline and of ferrous iron in the reaction mixture (Table 5). The results suggest that tetracycline can inhibit collagen biosynthesis by binding the ferrous iron required.

TABLE 5. EFFECT OF TETRACYCLINE ON THE HYDROXYLATION OF ^{14}C-PROLINE-LABELLED PROTOCOLLAGEN BY PURIFIED PROTOCOLLAGEN HYDROXYLASE

Sample	Fe^{++} concn. (mM)	^{14}C-hypro formed (dpm)	(%) of Control
Control	0·01	3400	100
Tetracycline 10 μg/ml	0·01	3050	90
,, 100 μg/ml	0·01	250	7
Control	0·005	3370	100
Tetracycline 10 μg/ml	0·005	2900	86
Control	0·001	3250	100
Tetracycline 10 μg/ml	0·001	2140	66
,, 100 μg/ml	0·001	60	2

^{14}C-proline-labelled protocollagen was incubated with purified protocollagen hydroxylase as described earlier.[18,19] Incubation was carried out with three different concentrations of ferrous iron in the incubation mixture, and tetracycline was added to the incubation mixture in the concentrations indicated. The results are expressed as dpm ^{14}C-hydroxyproline synthesized in an aliquot of 50,000 dpm of ^{14}C-proline-labelled substrate.

The mechanism by which tetracycline inhibits collagen biosynthesis was further studied by determining the amount of protocollagen hydroxylase activity in ulnae cultivated for 3 and 6 days in the presence of 100 μg/ml tetracycline. No decrease was observed in the enzyme activity compared to the controls when the hydroxylation reaction was carried out in the presence of excess[18] of ferrous iron, ascorbate and α-ketoglutarate (Table 6). This suggests that tetracycline did not reduce the amount of the enzyme protocollagen hydroxylase in the ulnae.

To study whether the effect of tetracycline on collagen biosynthesis can be demonstrated in cultured bones of embryos from mothers receiving tetracycline, pregnant mice were injected with 40 and 100 mg/kg tetracycline during days 9–15 of pregnancy. On the 16th day of pregnancy, embryonic ulnae were prepared, and cultivated for 9 hr in medium without added tetracycline. ^{14}C-Proline was added to the medium for the last 6 hr of culture. The administration of 40 mg/kg tetracycline to pregnant mice for 7 days had no effect on the biosynthesis of collagen in subsequent cultures of embryonic ulnae, but with doses of 100 mg/kg a decrease was observed both in total ^{14}C-proline incorporation and in ^{14}C-hydroxyproline synthesis (Table 7). A slight decrease was also observed in the ratio of ^{14}C-hydroxyproline to total ^{14}C in these cultures.

TABLE 6. ACTIVITY OF PROTOCOLLAGEN HYDROXYLASE IN ULNAE CULTURED WITH TETRACYCLINE WHEN ASSAYED WITH EXCESS OF FERROUS IRON

Culture period	Group	No.	^{14}C-hydroxyproline formed (dpm)
3 *days*			
(Experiment I)	Control	1.	5140
	,,	2.	6000
	Tetracycline, 100 μg/ml	3.	5320
	,,	4.	6070
6 *days*			
(Experiment II)*	Control	1.	2820
	Tetracycline, 100 μg/ml	2.	4710
	,,	3.	3240

Cultures containing five ulnae each were incubated for 3 or 6 days, as described in the text, with added ascorbate, α-ketoglutarate and ferrous iron. After incubation, the ulnae were homogenized, and the 15,000 *g* supernatants of the homogenates were incubated with ^{14}C-proline-labelled protocollagen as described in the text. The results are expressed as dpm ^{14}C-hydroxyproline synthesized in an aliquot of 50,000 dpm of ^{14}C-proline-labelled protocollagen substrate.

* Because of variations in the substrate activity of the various ^{14}C-proline-labelled protocollagen preparations, the values for ^{14}C-hydroxyproline synthesis are comparable only within the same experiment.

TABLE 7. EFFECT OF ADMINISTRATION OF TETRACYCLINE TO PREGNANT MICE ON SUBSEQUENT COLLAGEN BIOSYNTHESIS IN EMBRYONIC ULNAE IN CULTURE

Group	No.	Total ^{14}C (dpm)	Hypro-^{14}C (dpm)	$\frac{\text{Hypro-}^{14}\text{C}}{\text{Total }^{14}\text{C}} \times 100$
Experiment I				
Control	1.	83,700	15,500	18·5
,,	2.	87,300	16,100	18·5
,,	3.	87,200	17,400	20·0
,,	4.	108,000	24,800	23·0
Tetracycline, 40 mg/kg	5.	79,100	15,200	19·3
,,	6.	89,000	17,100	19·2
,,	7.	93,800	18,700	19·9
,,	8.	97,000	19,400	20·0
Experiment II				
Control	1.	81,700	16,600	20·3
,,	2.	86,500	18,100	20·9
Tetracycline, 100 mg/kg	3.	64,600	11,600	18·0
,,	4.	67,600	11,900	17·6

Pregnant mice were injected with tetracycline during days 9–15 of pregnancy. On the 16th day organ cultures of embryonic ulnae were prepared and cultivated for 9 hr without added tetracycline. ^{14}C-proline, 6 μc in experiment I, and 8 μc in experiment II, was added for the last 6 hr of culture. Each culture contained seven ulnae in experiment I, and five ulnae in experiment II.

DISCUSSION

Organ cultures of embryonic mouse ulnae seemed to provide a suitable system for studying the effects of drugs on collagen biosynthesis. The cultures retained a maximal rate of conversion of the incorporated ^{14}C-proline to ^{14}C-hydroxyproline for up to 6 days, and about half of the label incorporated was present in the collagen. A chemically defined synthetic medium could be used for the culture, but daily replacement[14] of the medium was found to be essential for the optimal rate of hydroxylation

of the incorporated ^{14}C-proline to ^{14}C-hydroxyproline. When the same medium was used throughout the 6 days of cultivation, ^{14}C-proline incorporation proceeded at about 60 per cent of the rate observed with daily changes of fresh medium. The rate of ^{14}C-hydroxyproline synthesis was very low, however, and a considerable part of the ^{14}C-proline incorporated was found to be in the protocollagen,[20, 24] the large proline-rich and hydroxyproline-deficient polypeptide precursor of collagen. The hydroxylation of proline in protocollagen to hydroxyproline in collagen requires ferrous iron, α-ketoglutarate, ascorbate and atmospheric oxygen (for review, see refs. 11, 12). The factor limiting the hydroxylation of ^{14}C-proline was found to be ascorbate in the present experiments, whereas the bones seemed to contain sufficient or close to sufficient amounts of ferrous iron and of α-ketoglutarate for maximal rate of ^{14}C-hydroxyproline synthesis. The dependence of collagen synthesis on added ascorbate has been demonstrated earlier by several workers in various experimental conditions (e.g. refs. 27–31).

Tetracycline was found to have an inhibitory effect on collagen biosynthesis in the organ cultures studied. A decrease was observed in the incorporation of ^{14}C-proline into protein in the ulnae in the presence of tetracycline, and as a consequence the rate of ^{14}C-hydroxyproline synthesis was reduced. It is possible that this effect of tetracycline represents a more general effect of the antibiotic on the incorporation of amino acids into protein, not limited to collagen synthesis. However, tetracycline was found to have an additional inhibitory effect on the later stages in the biosynthesis of collagen, and it seems probable that this effect is more specifically limited to collagen synthesis. The antibiotic was found to inhibit the synthesis of ^{14}C-hydroxyproline to a greater extent than the incorporation of ^{14}C-proline into protein, suggesting an inhibition of the conversion of protocollagen to collagen. Because ferrous iron is required in this reaction and because tetracycline binds bivalent cations,[4] it seemed possible that tetracycline might inhibit protocollagen hydroxylase reaction by binding ferrous iron. Even though the control bones seemed to contain enough intracellular ferrous iron for optimal hydroxyproline synthesis, it can be expected that binding of this iron will inhibit hydroxyproline formation. Such inhibition of collagen biosynthesis in bones in organ culture has been demonstrated in an earlier study,[14] when a specific iron chelator, α,α′-dipyridyl, was added to the medium. Our present experiments with purified protocollagen hydroxylase demonstrated that tetracycline can inhibit protocollagen hydroxylase reaction through this mechanism, and it seems possible that the same mechanism was involved in intact ulnae. On the other hand, when the protocollagen hydroxylase activity of ulnae cultured with tetracyline was assayed in the presence of excess ferrous iron, no decrease in activity was observed, indicating that the decrease in the synthesis of ^{14}C-hydroxyproline was not due to decreased amounts of the enzyme in the bones.

In addition to retarding both incorporation of proline into protocollagen and conversion of protocollagen to collagen, tetracycline may have some further effects on collagen metabolism which were not studied here. Calcium and other metal cations have been shown to affect the rate of conversion of newly-synthesized soluble collagen into insoluble form,[32] and if tetracycline binds these cations, a change in the rate of this reaction might be observed. In addition, tetracycline has been shown to interact with collagen fibrils,[33] and this interaction may affect the metabolism of the fibrils.

Changes similar to those observed in the present study on organ cultures in the presence of tetracycline have been recently reported in rat embryos *in vivo*,[10] suggesting that the inhibitory effects of tetracycline reported here may also be exerted in embryonic bones *in vivo*. In addition, changes similar to those occurring in ulnae cultured with tetracycline were observed in the present study in embryonic ulnae from pregnant mice receiving tetracycline, when the ulnae were cultivated for 9 hr without added tetracycline. However, a very high dose of tetracycline was required in this experiment to demonstrate the inhibitory effect. This may be due to partial reversal of the effect of tetracycline during the culture period without the antibiotic, but other explanations are not excluded.

The results of the present study clearly indicate that the inhibitory effects of tetracycline on bone development are not limited to calcium metabolism but the antibiotic has direct effects on collagen metabolism as well. It seems possible, therefore, that at least part of the inhibition in calcification is secondary to the decreased rate of synthesis of the organic matrix of the bone. However, as a clear inhibition of the calcification can be obtained with 5 μg/ml tetracycline,[1] but 30 μg/ml was required in the present study for a definite inhibition of ^{14}C-hydroxyproline synthesis, it seems that the drug exerts direct effects both on collagen biosynthesis and on mineralization.

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REFERENCES

1. L. Saxén, *J. exp. Zool.* **162**, 269 (1966).
2. R. A. Milch, D. P. Rall and J. E. Tobie, *J. natn. Cancer Inst.* **19**, 79 (1957).
3. G. Bevelander, H. Nakahara and G. K. Rolle, *Devl. Biol.* **2**, 298 (1960).
4. J. Sternberg, *Int. J. appl. Radiat. Isotopes* **17**, 497 (1966).
5. G. A. N. Finerman and R. A. Milch, *Nature, Lond.* **198**, 486 (1963).
6. L. Saxén, *Science* **149**, 870 (1965).
7. G. Shtacher and H. E. Firschein, *Am. J. Physiol.* **213**, 863 (1967).
8. O. Laitinen, *Acta endocr., Copenh. suppl.* 120 (1967).
9. I. C. Bennett, W. R. Proffit and L. A. Norton, *Nature, Lond.* **216**, 176 (1967).
10. J. Halme and J. Aer, *Biochem. Pharmac.* **17**, 1479 (1968).
11. S. Udenfriend, *Science* **152**, 1335 (1966).
12. D. J. Prockop and K. I. Kivirikko, *Ann. intern. Med.* **66**, 1243 (1967).
13. R. S. Bhatnagar, Ph.D. Dissertation, Duke University, Durham, N.C. (1964).
14. R. S. Bhatnagar, K. I. Kivirikko and D. J. Prockop, *Biochim. biophys. Acta* **154**, 196 (1968).
15. J. D. Biggers (Ed. E. N. Willmer) *Cells and Tissues in Culture—Methods, Biology and Physiology*, p. 197. Academic Press, London and New York (1965).
16. D. J. Prockop and P. S. Ebert, *Anal. Biochem.* **6**, 263 (1963).
17. K. Juva and D. J. Prockop, *Anal. Biochem.* **15**, 77 (1966).
18. K. I. Kivirikko and D. J. Prockop, *Proc. Natl. Acad. Sci. U.S.* **57**, 782 (1967).
19. T. Takeuchi, K. I. Kivirikko and D. J. Prockop, *Biochem. biophys. Res. Commun.* **28**, 940 (1967).
20. K. I. Kivirikko and D. J. Prockop, *Biochem. J.* **102**, 432 (1967).
21. K. I. Kivirikko, H. J. Bright and D. J. Prockop, *Biochim. biyohys. Acta* **151**, 558 (1968).
22. J. Halme and K. I. Kivirikko, *FEBS Letters*, **1**, 223 (1968)
23. R. S. Bhatnagar and D. J. Prockop, *Biochim. biophys. Acta* **130**, 383 (1966).
24. D. J. Prockop and K. Juva, *Proc. Natn. Acad. Sci. U.S.* **53**, 661 (1965).
25. J. Hurych and M. Chvapil, *Biochim. biophys. Acta* **97**, 361 (1965).
26. J. J. Hutton, A. L. Tappel and S. Udenfriend, *Biochem. biophys. Res. Commun.* **24**, 179 (1966).
27. B. S. Gould, *Vitamins Hormones (NY)* **18**, 89 (1960).
28. W. van B. Robertson, *Ann. N.Y. Acad. Sci.* **92**, 159 (1961).
29. J. J. Jeffrey and G. R. Martin, *Biochim. biophys. Acta* **121**, 269 (1966).

30. J. J. REYNOLDS, *Exp. Cell Res.* **47**, 42 (1967).
31. W. A. PECK, S. J. BIRGE and J. BRANDT, *Biochim. biophys. Acta* **142**, 512 (1967).
32. E. HEIKKINEN, K. IVASKA and E. KULONEN, *Scand. J. Clin. Lab. Invest. suppl.* **101**, 5 (1968).
33. R. JACOBS, W. H. HARRIS, E. P. KATZ and M. GLIMCHER, *Biochim. biophys. Acta* **86**, 579 (1964).