

## The Effect of Sodium Fluoride on the Rates of Synthesis and Degradation of Bone Collagen in Tissue Culture\* (33472)

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Despite a number of studies on patients and experimental animals, the effect of fluoride on bone remodelling is not yet clear. The ingestion of large amounts of fluoride by experimental animals (500 ppm of fluoride in the drinking water) was reported to stimulate bone resorption (1), and in addition to cause the formation of exostotic bony outgrowths (2). On the other hand, several studies showed that the administration of relatively high doses of sodium fluoride (up to 100 mg/day) to patients with osteoporosis or Paget's disease of bone, leads to an increased retention of calcium, increased mineralization of bone, and relief of bone pain (3-7). Other studies suggested, partly on the basis that fluoride increases the crystallinity, perfection, and size of the mineral crystallites in bone (8-10) that the first effect of fluoride is to decrease bone resorption. This would tend to lower serum calcium, which in turn would stimulate the secretion of parathyroid hormone, with the result that both bone formation and resorption would be eventually increased (11-13).

Tissue culture techniques were used by Goldhaber (14) who reported that low concentrations of fluoride in the tissue culture medium inhibit bone resorption, without any apparent effect on bone formation, but that at higher concentrations of fluoride, bone formation and bone resorption are both inhibited. The present study extends these morphological observations by directly measuring

the rates of bone collagen synthesis and degradation using the incorporation of proline-<sup>3</sup>H to hydroxyproline-<sup>3</sup>H, and the increase in total hydroxyproline as an index of bone collagen synthesis, and the release of <sup>3</sup>H-labeled hydroxyproline and hydroxyproline into the tissue culture medium as an index of bone collagen resorption.

*Experimental Methods.* The calvaria of 5-day-old Swiss albino mice of the Webster Strain were removed aseptically and the frontal and parietal bones were dissected free and placed on rectangular glass coverslips (15, 16). The tissue culture system was similar to that previously reported (15). Sodium fluoride was added to the media of the experimental groups at various concentrations, from  $1 \times 10^{-6}$  M to  $1 \times 10^{-3}$  M. Chromatographically pure proline-<sup>3</sup>H was added to the tissue culture media as previously described (15). Equal amounts of proline-<sup>3</sup>H were added to the medium of each calvarium in any one experiment, although the amount varied from one experiment to another. All cultures were gassed with a mixture of 50% O<sub>2</sub> and 50% N<sub>2</sub>. The media were changed at 2-day intervals. The total time of the experiments varied from 6 to 14 days.

At the end of each 2-day period, the media were collected, aliquot samples were hydrolyzed in triple-distilled 6 N HCl, at 105° for 24 hr; and hydroxyproline and proline were separated by resin column chromatography (17). Hydroxyproline was measured colorimetrically (18) and hydroxyproline-<sup>3</sup>H was measured in an automatic liquid scintillation counter. Calvaria, at zero times and at the end of the experimental periods, were weighed after drying for 48 hr in a vacuum oven at 50°. They were then either directly hydrolyzed in an excess of triple-distilled 6 N HCl at 105° for 24 hr, or demineralized first

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TABLE I. The Effect of Sodium Fluoride on the Rate of Incorporation of Hydroxyproline-<sup>3</sup>H by Bone in Tissue Culture, and on the Rate of Release of Hydroxyproline-<sup>3</sup>H into the Tissue Culture Media.<sup>a</sup>

Fluoride concentration in media ( <i>M</i> )	Hydroxyproline- <sup>3</sup> H recovered in (cpm/calvaria)		Total Hydroxyproline- <sup>3</sup> H synthesized (cpm/calvaria) (3)	Hydroxyproline- <sup>3</sup> H recovered in the media (%) (2):(3)	Ratio of hydroxyproline- <sup>3</sup> H-labeled collagen synthesized to that degraded (3):(2)
	(1) Calvaria	(2) Media			
Incubation (14 days)					
None	4028	6745	10,773	63	1.59
1 × 10 <sup>-6</sup>	4748	7547	12,295	62	1.63
1 × 10 <sup>-5</sup>	3755	7262	11,017	66	1.52
5 × 10 <sup>-4</sup>	2173	4780	6953	69	1.45
1 × 10 <sup>-3</sup>	931	2302	3233	71	1.41
Incubation (14 days)					
None	8594	21,975	30,569	72	1.39
1 × 10 <sup>-5</sup>	6524	29,600	36,124	82	1.22
1 × 10 <sup>-4</sup>	7950	24,500	32,450	76	1.32
Incubation (6 days)					
None	1560	2786	4346	64	1.56
1 × 10 <sup>-6</sup>	1604	2989	4593	65	1.54
1 × 10 <sup>-5</sup>	1982	2961	4943	60	1.67
1 × 10 <sup>-4</sup>	2005	3192	5197	61	1.63
5 × 10 <sup>-4</sup>	1948	2117	4065	52	1.92

<sup>a</sup> Results represent the average values obtained from four calvaria at each of the fluoride concentrations and of the control samples.

in 0.5 *M* ethylenediaminetetraacetic acid, pH 8.3, before hydrolysis. Hydroxyproline and hydroxyproline-<sup>3</sup>H were measured after separation from proline as described. Preliminary experiments showed that there was no detectable hydroxyproline in the tissue culture medium prior to incubation or after incubation with dead calvaria, so that its presence in the media could be used as indication of active bone collagen resorption. Also as previously reported (15, 19, 20), the hydroxyproline and hydroxyproline-<sup>3</sup>H in the medium after incubation were in the form of small peptides and as the free amino acid, indicating that the collagen was degraded and not merely solubilized.

**Results.** The effect of various concentrations of fluoride in the tissue culture medium on bone collagen synthesis, and on the rate of degradation of that fraction of bone collagen synthesized during the experimental period (hydroxyproline-<sup>3</sup>H was released into the media), is shown in the data from three

typical experiments (Table I). It is clear that the rate of bone collagen synthesis in tissue culture under the conditions used, is approximately the same in the control samples without fluoride as with fluoride in the media at concentrations of 1 × 10<sup>-6</sup> *M* to 1 × 10<sup>-4</sup> *M*. At the end of 14 days, however, at fluoride concentrations of 5 × 10<sup>-4</sup> *M* and 1 × 10<sup>-3</sup> *M*, there was a decided decrease in the rate of bone collagen synthesis as evidenced by a decrease in the amount of proline-<sup>3</sup>H converted to hydroxyproline-<sup>3</sup>H.

The rate of degradation of the fraction of the bone collagen synthesized during the experimental period (new or recently synthesized bone collagen) was roughly the same in the control samples and in the samples incubated in the presence of fluoride at concentrations of 1 × 10<sup>-6</sup> *M* to 1 × 10<sup>-4</sup> *M*. At the end of 6 days of incubation, the resorption of the newly synthesized collagen was slightly decreased at fluoride concentrations of 5 × 10<sup>-4</sup> *M*, while in other experiments carried

TABLE II. The Effect of Sodium Fluoride on the Rate of Bone Collagen Synthesis and Degradation in Tissue Culture.

Fluoride concentration in media ( <i>M</i> )	Hydroxyproline recovered ( $\mu\text{g}/\text{calvaria}$ )			Collagen synthesized ( $\mu\text{g}$ hydroxyproline/calvarium) (4)	Total collagen degraded and recovered in media (%) (2):(3)	% of preincubation collagen degraded <sup>a</sup>	Ratio of collagen synthesized to collagen degraded
	(1)	(2)	(3)				
	Calvaria	Media	Total calvaria and media				
Incubation (14 days)							
None	42.8	60.4	103.2	63.2	58.5	51.5	1.05
$1 \times 10^{-6}$	50.4	75.6	126.0	86.0	60.0	52.6	1.00
$1 \times 10^{-5}$	53.0	70.4	123.4	83.4	57.2	37.5	1.01
$5 \times 10^{-4}$	53.7	32.2	85.9	45.9	37.4	<1	1.42
$1 \times 10^{-3}$	56.4	18.2	74.6	34.6	24.4	0	1.35
Incubation (6 days)							
None	33.9	39.2	73.1	33.1	53.6	45.0	0.84
$1 \times 10^{-6}$	34.5	33.7	68.2	28.2	49.4	38.5	0.83
$1 \times 10^{-5}$	41.4	29.6	71.0	30.0	41.7	27.5	1.04
$1 \times 10^{-4}$	44.7	24.7	69.4	29.4	35.6	17.0	1.20
$5 \times 10^{-4}$	47.2	13.6	60.8	20.8	22.3	7.0	1.53

<sup>a</sup> Calculated from the data of this table and from the hydroxyproline-<sup>3</sup>H data of Table I.

out for 14 days, it was slightly increased at fluoride concentrations of  $5 \times 10^{-4}$  *M* and  $1 \times 10^{-3}$  *M*.

Table II presents the data from two typical experiments in which the hydroxyproline contents of the media and of the calvaria were measured chemically. By combining these data with those of the hydroxyproline-<sup>3</sup>H distribution in the media and calvaria, and the average hydroxyproline content of a calvarium at zero time ( $\sim 40\mu\text{g}$  of hydroxyproline), it is possible to calculate how much of the bone collagen which was present in the calvarium at the start of the experimental period, was degraded.

The data in Table II also clearly show that the amount (and rate) of bone collagen synthesis is not significantly decreased until the fluoride concentration in the tissue culture medium reaches  $5 \times 10^{-4}$  *M* at which concentration it is decreased approximately 35%. At a fluoride concentration of  $1 \times 10^{-3}$  *M*, collagen synthesis is diminished by about half. Histological examination showed no morphological changes in the cells at fluoride concentrations of  $5 \times 10^{-4}$  *M*, but massive

cell death and necrosis at fluoride concentrations of  $1 \times 10^{-3}$  *M*.

The most striking findings occurred in the amount and rate of collagen degradation at fluoride concentrations of  $1 \times 10^{-5}$  *M* and higher, and the fact that this decrease occurred almost entirely in that portion of the collagen present in the calvaria *prior* to incubation (older collagen). In fact, at the end of 14 days of incubation in fluoride concentrations of  $5 \times 10^{-4}$  *M* and  $1 \times 10^{-3}$  *M*, essentially none of the older collagen which was originally present in the calvaria, was degraded. This was verified in several other experiments.

*Discussion.* In the tissue culture system employed in these experiments, the addition of sodium fluoride to the tissue culture media at various concentrations from  $1 \times 10^{-6}$  *M* to  $1 \times 10^{-4}$  *M* either does not significantly change the rate of bone collagen synthesis, or in some instances actually increases it slightly. These data confirm the histological observations of Goldhaber (14) who used the same tissue culture system, and is consistent with the results of *in vivo* animal experiments

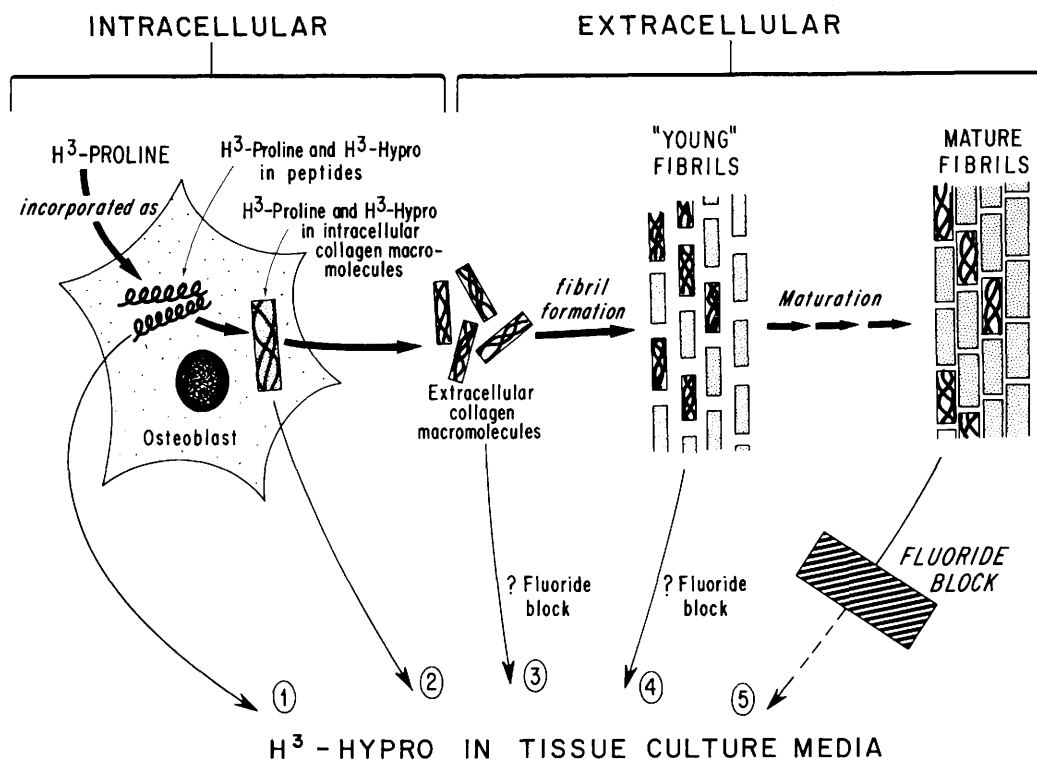


FIG. 1. Schematic diagram to indicate the possible sources of hydroxyproline found in the tissue culture medium during active bone formation and resorption. Fluoride concentrations of  $5 \times 10^{-4} M$  or greater in the tissue culture medium prevent the resorption of most if not all of the older, more mature collagen and possibly some of the newly synthesized collagen macromolecules which have been recently incorporated into structural elements of the tissue. It is not clear whether the large percentage ( $\sim 60-70\%$ ) of the newly synthesized hydroxyproline-<sup>3</sup>H found in the media even in the presence of  $5 \times 10^{-4} M$  fluoride represents (1) the release of intracellular, peptide-bound hydroxyproline-<sup>3</sup>H; (2) degradation products of intracellular collagen macromolecules; (3) degradation of newly synthesized but extracellular collagen macromolecules, either not yet incorporated into the structural elements of the tissue, or soon after incorporation into fibrils, or (4) comes from a combination of these sources. It emphasizes the point that at least a certain fraction of urinary hydroxyproline may be more an index of the rate of bone collagen *formation* than bone collagen *degradation*.

(3-7; 11-13) although in the latter instances, the mechanism was felt to be an indirect one, secondary to increased parathyroid hormone secretion, and not the result of a direct action of fluoride on the bone cells.

The morphological observations of Goldhaber (14) concerning the effect of relatively high concentrations of fluoride were also confirmed: a moderate to marked decrease in the rate of collagen synthesis at fluoride concentrations of  $5 \times 10^{-4} M$  and  $1 \times 10^{-3} M$ .

The most striking findings concerned the effect of fluoride on bone collagen degradation. Although there was little or no effect of fluoride even at high concentrations on the rate at which the newly synthesized collagen was degraded, there was a very considerable decrease in the rate of degradation of that fraction of the collagen which was present in the bone prior to incubation in tissue culture. Although bone collagen synthesis was reduced at the higher concentrations of fluoride, bone collagen resorption was much more

affected than bone collagen synthesis, so that the net result was an increase in bone mass at the end of the experimental periods, directly proportional to the concentration of fluoride in the media.

The failure of sodium fluoride at any concentration to significantly alter the rate at which newly synthesized collagen was degraded, even at concentrations where little or none of the older collagen was resorbed, is similar in many respects to the results found when thyrocalcitonin was added to the tissue culture medium (21). In this instance as well, the thyrocalcitonin inhibited the resorption of the older collagen, but had little effect on the degradation of newly synthesized collagen.

This is consistent with the proposal (15) that a large proportion of the proline which is hydroxylated to hydroxyproline presumably after its incorporation into peptide chains, is rapidly released into the tissue culture medium. It is not clear from these and previous experiments (15) whether this represents peptides released and possibly degraded prior to their incorporation into the fully formed collagen macromolecules, degradation of intracellular collagen macromolecules, or degradation of newly synthesized bone collagen before or immediately after its incorporation into the structural elements of the tissue (Figure 1). The present experiments which suggest that this fraction may amount to as much as 60-70% of the total H<sup>3</sup>-hydroxyproline synthesized, a figure close to that found in bone *in vivo* (22), emphasizes that urinary hydroxyproline excretion, or a fraction thereof, may be more a reflection of the rate of bone collagen synthesis than of bone collagen degradation. It is also possible that the older, more heavily calcified bone collagen is preferentially protected as a result of changes in the bone mineral induced by the sodium fluoride (8-10).

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