shown here that even at the threshold level of angiotensin infusion, angiotensin causes, apparently in and of itself, a renal ischemia. Hence, it may be tentatively argued, angiotensin, if it does get into the systemic circulation, does not correct renal ischemia but, if anything, depresses renal blood flow. (Part of its pressor effect is due to the fact that it constricts, among others, the renal vascular bed(4,5). In renal ischemia, then, its secretion would be, if anything, damaging. This general suggestion receives support from the persistent and continuing failure to obtain a consistent and wholly satisfactory proof for the presence of angiotensin in systemic blood, or even in renal venous blood, during the experimental renal hypertensions which follow partial constriction of the renal artery, etc. (6). It is, however, perhaps present in lymph (7,8).

Summary and conclusions. The threshold dose for both the pressor effect and the renal ischemic effect of angiotensin was found to be, in the dog, 3 mμg/kg/min. It is deduced that angiotensin, if it is produced in sufficient quantity to raise the blood pressure, would aggravate, not benefit, a pre-existing renal ischemia.

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Inhibition of Bone Collagen Synthesis by Salicylates.* (29725)

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Salicylates have frequently been employed in the treatment of connective tissue diseases (1,2). Several different salicylates have been used as analgesic and antipyretic agents in treatment of rheumatoid arthritis, and p-aminosalicylic acid has been used extensively in treatment of pulmonary tuberculosis. Many anti-inflammatory drugs, including certain salicylates, have been found to inhibit the metabolism of cartilage and other connective tissues both *in vivo* and *in vitro*(3).

Recent studies from our laboratory have dealt with the interaction between parathyroid hormone and bone metabolism. Therefore, we have been interested in possible inhibitors of bone formation as a means by which bone resorption could be studied apart from the simultaneous influences of formation.

The *in vivo* and *in vitro* use of radioactive amino acid precursors of bone collagen as indicators of bone and bone collagen formation is well documented(4-6). In the studies reported here, radioproline uptake into bone matrix was used to assess the formation of bone collagen, and evidence is presented which demonstrates that this formation is inhibited by salicylates.

Materials and methods. A. *Incubation of rat femora. Procedure.* Adult male Holtzman rats, 150-200 g, were used in these studies. Femoral metaphyseal bone chips were prepared by the method of Borle, Nichols, and Nichols(7). The preparations from both femora were pooled for each animal and incubated for 4 hours at 37°C with constant shaking in 3.0 ml pooled rat serum (pH 7.4).

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All samples were gassed with a mixture of 95% oxygen and 5% CO_2.

P-aminosalicylic acid was added at the beginning of the incubation from a stock solution (1.63 M) of the sodium salt of this compound (pH 7.4), usually in a 0.1 ml or less volume, to give a calculable, final molarity in the incubation medium. Acetylsalicylic acid (aspirin) was added directly to the incubation medium to give the desired molarity. Tritiated (H^3) L-proline†—10 μc in a 0.1 ml volume—was also added prior to the incubation.

To minimize possible variations, control and experimental femora were always incubated simultaneously in aliquots of the same preparation of pooled serum.

Analysis. After incubation, the bone chips were fractionated into a "cellular" fraction and a "collagen" fraction according to the method of Flanagan and Nichols(8). The collagen fraction was extracted and solubilized by conversion to gelatin(9); the samples were extracted in 4.0 ml H_2O at 127°C in an autoclave (15 lb pressure) for 3 hours, the extraction was repeated, the 2 extractions were pooled, and the samples were then brought to constant volume and an aliquot taken for determination of radioactivity. Analysis of the residue left after extraction showed no detectable radioactivity. The cellular fraction was also brought to constant volume and an aliquot taken for counting.

Total radioactivity in each fraction was determined by counting the samples in a Nuclear-Chicago ambient temperature liquid scintillation counter. The solvent system used consisted of 2,5-diphenyloxazole and 5-phenyloxazole, and all samples were corrected for background. Results are expressed as counts per minute per milligram dry weight ("collagen") of the bone chips.

B. *Organ culture of mouse embryonic radii.*‡ *Procedure.* Embryos removed from mice on the 15th day of pregnancy were used in these experiments. Radii were removed from the embryos and cultivated according to the method of Gaillard(10) in a medium comprised of 15% serum and 85% Hanks' physiological salt solution. Both H^3- and C^{14}-proline† were utilized with variation only in the counting procedure. Radii were cultivated for 5 to 72 hours with radioproline. Sodium p-aminosalicylic acid (20% stock solution) was diluted with Hanks' solution and added to the incubation medium to give the desired final molarity.

Analysis. Radii were washed after incubation, dissolved in a drop of HCl, and evaporated to dryness. The samples were counted in a Packard liquid scintillation counter.

In a few studies the effect of p-aminosalicylic acid addition on uptake of radioproline by the embryonic radius was compared to the effect of puromycin, a known inhibitor of protein synthesis, in order to provide a comparative inhibitor standard.

In one experiment, TPN, which has been shown to reverse the action of parathyroid extract in this system(11), was added to a final concentration of 2×10^{-4} M.

The results are expressed as the percentage inhibition produced by addition of the various concentrations of p-aminosalicylate and puromycin.

Results. A. *Incubation of rat femora.* The results are presented in Table I. All concentrations of p-aminosalicylate except 1.36×10^{-2} M produced a marked reduction in H^3-proline uptake into collagen. In all cases of reduced uptake into collagen, there was a parallel, equally marked, suppression of radioproline uptake into the cellular fraction. All concentrations of acetylsalicylic acid used resulted in a significantly reduced H^3-proline uptake into collagen. Although much less suppression was noted in the cellular fraction, acetylsalicylic acid proved to be a much stronger inhibitor of collagen synthesis than p-aminosalicylic acid, since the former gave significant inhibition at 2.33×10^{-3} M

† Source of radioisotopes: A) Rat studies: Nuclear Chicago Corp., H^3-proline 200 mc/mM. B) Mouse studies: Philips Duphar; C^{14}-proline 33 mc/mM, H^3-proline 141 mc/mM.

‡ The organ culture studies were carried out by R. V. Talmage while holding a PHS special fellowship and were conducted in the laboratory of Pieter J. Gaillard, State University, Leiden, Holland. Dr. Gaillard's advice and assistance are gratefully acknowledged.

TABLE I. Effect of p-Aminosalicylate and Acetylsalicylic Acid on Uptake of H^3-Proline by Incubated Rat Femora.*

Treatment†	Animals	Counts/min/mg dry wt Cellular fraction	Collagen fraction
Control	19	2283 ± 84	823 ± 32
p-Aminosalicylate:			
2.33×10^{-1} M	1	731	4
1.26×10^{-1} M	3	601 ± 81	18 ± 18
5.26×10^{-2} M	3	914 ± 89	5 ± 3
2.67×10^{-2} M	3	1045 ±155	55 ± 13
1.36×10^{-2} M	3	2124 ± 76	594 ±219
Acetylsalicylic acid			
2.33×10^{-1} M	3	1803 ±282	0
5.26×10^{-2} M	4	1088 ± 75	0
1.36×10^{-2} M	6	1784 ± 88	107 ± 9
5.26×10^{-3} M	4	2156 ± 62	447 ± 27
2.33×10^{-3} M	4	2872 ±272	658 ± 22

* Results are expressed as mean ± S.E.

$$\left(\text{S.E.} = \sqrt{\frac{\Sigma d^2}{n(n-1)}}\right)$$

† Concentrations (M) expressed in molarity.

while the latter was effective to 2.67×10^{-2} M.

B. *Organ culture of mouse embryonic radii.* The results of this series of experiments are given in Table II. Again a marked inhibition of radioproline uptake by the bone matrix was found in radii cultured with p-aminosalicylic acid. This inhibition was not reversed by addition of TPN (2×10^{-4} M). For purposes of comparison it is interesting to note that the minimal level of p-aminosalicylate required for significant inhibition was in the range of 10^{-2} M (in both the mouse embryo and the adult rat), while puromycin, a more potent suppressor, was effective (in the mouse embryo) at concentrations approaching 10^{-6} M.

Support for the presence of the inhibitive effect of salicylates *in vivo* is derived from a few experiments in which adult rats were fed 0.5 g of the sodium salt of p-aminosalicylic acid (in a 2.0 ml volume, pH 7.4) by stomach tube once a day for 4, 7, and 14 days. These animals were then injected intraperitoneally with 50 μc H^3-proline, and sacrificed 30 minutes after isotope injection. Microradioautographs of the distal femoral metaphysis were processed according to conventional methods(12), and the per cent labeled osteoblasts of the animals treated with p-aminosalicylate were compared to control femora from animals receiving no p-aminosalicylate treatment (Table III). While the metaphyseal osteoblast labeling from the 7 and 14 day-treated animals was somewhat variable, a definite suppression of H^3-proline uptake by these collagenetic cells was detectable in these animals compared to the controls. Since microradioautographic examination also indicated histological differences in the osteoblasts from animals with p-aminosalicylate administration, electron microscopic examination of 14 day-treated and control

TABLE II. Effect of p-Aminosalicylic Acid and Puromycin on Radioproline* Uptake by Embryonic Mouse Radii.†

Concentration in medium (molarity)	Duration of organ culture 5 Hr	24 Hr	48 Hr	72 Hr
p-Aminosalicylic acid:				
1×10^{-2} M	—	20 ± 14%(4)‡	—	—
2×10^{-2} M	—	33 ± 7 (5)	40 ± 14% (4)	—
4×10^{-2} M	33 ± 8%(6)	—	—	33 ± 5% (3)
1×10^{-1} M	62 ± 6 (4)	59 ± 5 (6)	—	—
2×10^{-1} M	—	90 ± 1 (6)	—	—
1×10^{-1} M plus TPN (2×10^{-4} M)	69 (2)	—	—	—
Puromycin:				
1×10^{-5} M	—	—	100%	—
1×10^{-6} M	—	33 ± 14%(4)	—	—
2×10^{-7} M	—	11 ± 8 (4)	—	—

* Results were obtained using both H^3- and C^{14}-proline.

† Results are expressed as % inhibition (mean ± S.E.): % inhibition = 100% − [experimental radius/control radius (same embryo) × 100].

‡ No. in parentheses = No. of animals.

animals was performed. Fig. 1 shows a typical osteoblast from a control animal; Fig. 2 is an osteoblast from the metaphysis of a rat treated with p-aminosalicylate. Large, abundant, osmiophilic inclusions were consistently found in the femoral osteoblasts of the treated rats.

Discussion. Besides being used in the treatment of tuberculosis, both p-aminosalicylic acid and salicylic acid have been found to be goitrogens(13,14). However, since we have demonstrated a direct *(in vitro)* effect of both p-aminosalicylate and acetylsalicylic acid on bone collagen, any possible role of the goitrogenic effect appears to be negated. The possible effect of acetylsalicylic acid on dermal rat collagen has been investigated (15), and no significant influence was detected.

Certain salicylates have been reported to be suppressors of connective tissue metabolism. These agents inhibit S^{35} incorporation into rat rib cartilage, apparently by uncoupling oxidative phosphorylation(3). The data derived from our studies strongly support this. Both p-aminosalicylate and acetylsalicylic acid effectively inhibit bone collagen formation, and autoradiographic and electron microscopic evidence indicate functional alteration of the collagenetic, femoral osteoblasts.

Summary. In vitro studies with rat femora and mouse embryonic radii showed a reduced uptake of radioproline into the bone collagen when p-aminosalicylic acid (approximately 10^{-2} M) and acetylsalicylic acid (aspirin; approximately 10^{-3} M) were added to the incubation medium. Autoradiographic examination of rat femora from p-aminosalicylate treated animals also indicated reduced H^3-proline uptake by the osteoblasts compared to control animals. Electron microscopic examination of metaphyseal bone from p-aminosalicylate treated and control rats showed cytological alteration of the osteoblasts after p-aminosalicylate treatment.

TABLE III. H^3-proline Uptake *in vivo* by Osteoblasts in p-Aminosalicylate Treated Rat Femur. Per cent cells labeled at 30 min after injection of radioproline.*

Days treatment with p-aminosalicylate		Metaphyseal labeling
0 = Controls	(8)†	85.9 ± 1.14%
4	(3)	82.3 ± 2.10
7	(2)	55.7
14	(6)	75.9 ± 1.70

* Each average = 300 or more cells counted; expressed as mean ± S.E.
† No. in parentheses = No. of animals.

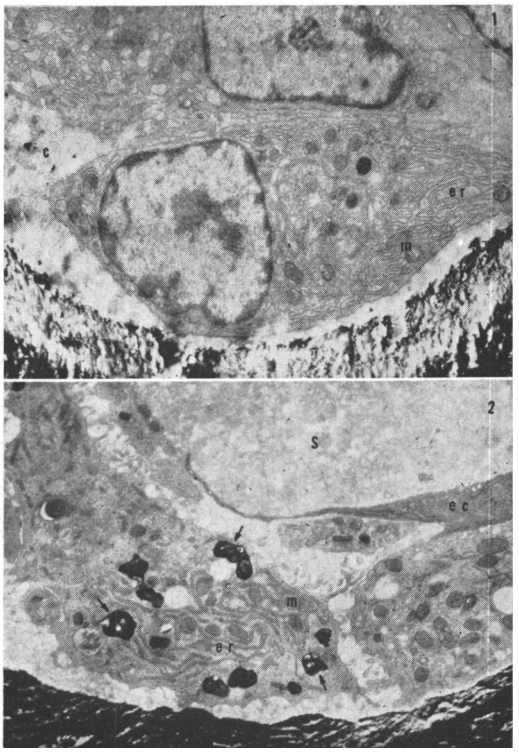

FIG. 1. Osteoblasts found in femur of a non-treated rat. The cytoplasm contains extensive granular endoplasmic reticulum (er), several mitochondria (m), and a few membrane bound particles. There are no large osmiophilic inclusions. Non-calcified collagen (c) is distinguishable around the cell. Bone tissue is at bottom of figure. × 4,000.

FIG. 2. Osteoblasts found in femur from p-aminosalicylate treated rats. Note large osmiophilic inclusions (arrows) scattered between mitochondria (m) and granular endoplasmic reticulum (er). At top of micrograph is a sinusoid (s) lined with endothelial cells (ec). The calcified bone matrix borders bottom of picture. × 4,000.

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Papain Induced Chemical Changes in Adult Rabbit Cartilage Matrix.* (29726)

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Intravenous administration of papain to baby rabbits has been reported by Thomas (1) to produce marked softening of the ear cartilage which results in the collapse and drooping of the ears. These gross changes are accompanied microscopically by a loss of basophilia and metachromasia(2) of the cartilage matrix and chemically by a decrease in the chondromucoprotein (CMP) content of the matrix(3).

Previous reports(1,2) have indicated that animals weighing more than 1,200 g do not respond to this action of papain. Spicer and Bryant(2) noticed an occasional accumulation of metachromatic material in lymphatics of the ears following near lethal doses of papain. In a personal communication Thomas indicated findings similar to those of Spicer and Bryant.

Recent work from this laboratory has demonstrated that in spite of minimal or absent gross and histological changes, larger rabbits referred to as "adolescent," showed marked chemical changes in the matrix following papain(4). These animals weighed 3.1 $\pm$ 0.3 kg and demonstrated partially open epiphyseal plates by X-ray examination; hence, the term "adolescent."

This report deals with the action of papain on the cartilage of fully mature or "adult" rabbits. Maturity was determined on the basis of completely closed epiphyseal plates as demonstrated by X-ray of the upper end of the tibia. All animals in this group weighed between 3.6 and 4.1 kg.

Materials and methods. A total of 20 male and 22 female New Zealand white rabbits were used in these experiments. Of this group, 7 males and 9 females were used as controls; 13 males and 13 females were injected with papain. These animals were given by the ear vein an injection of 35 mg of papain per kg in NaCl solution. The papain was purified‡ but not crystalline. Controls received an equivalent amount of 0.15 M NaCl. The papain solution was prepared as follows: 2 g of powdered papain were mixed with 100 ml of 0.15 M NaCl. The mixture was placed on a magnetic stirrer and stirred for 30 minutes. The resulting turbid, yellowish solution was transferred to presterilized Boerner centrifugal filters, equipped with bacteriological filter pads, and centrifuged for 20 minutes at 800 $\times$ *g*. The filtrate was a clear, straw-colored solution. It was transferred under aseptic conditions into presterilized bottles and stop-

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