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Acceleration of Longitudinal Bone Growth by Intra-Osseous Injection of Papain Protease.* (28372)

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Intravenous injection of papain in immature animals causes profound degenerative changes in all the epiphyseal cartilage plates and arrest of growth(1,2,3). This effect can be produced selectively in the cartilaginous growth plates of a single bone of a limb by injecting the enzyme into the osseous medullary canal and limiting its diffusion by a tourniquet placed proximal to the injection site(4).

During an investigation of the chondrolytic effect of intramedullary injections of papain protease on the epiphyseal cartilage plates of the tibia of young rabbits, it was found that minimal doses of the enzyme would sometimes cause a relative lengthening of the injected bone. The following experiments were performed to study this paradoxical effect of papain.

Materials and methods. Actively growing New Zealand white rabbits, about one kg in weight, were used. They were weighed daily and animals that failed to thrive were excluded from the study.

Papain protease was obtained commercially (Worthington Biochem. Co. and Nutritional Biochem. Co.) in vials containing .03 M cysteine. Activation was also aided by addition of .001 M versene. Phosphate buffer, 0.1 N, pH 7.0, was added to obtain a final enzyme concentration of 1 mg/ml. Assay of each enzyme shipment was performed using iodine^{131}-labelled albumin as a substrate by a modification of the technic of Potter *et al.* (5). Intramedullary injection of the left tibia was performed under intravenous nembutal anesthesia by puncture of the proximal metaphyseal region of the bone, using a No. 22 needle. Injections were given at intervals of 3 or 4 days. A rubber tourniquet on the lower thigh was maintained for 30 minutes following the injection.

A total of 113 animals was used, of which 89 were experimental and 24 control. The experimental animals were subdivided into 5 groups:

1. Seventeen animals received a single intramedullary injection of 0.2 mg of papain protease. Three or more animals were sacrificed serially at 4, 7, 10 and 14 days.

2. Nine rabbits received two 0.2 mg injections of papain protease. Three animals were sacrificed after 10, 14 and 21 days.

3. Forty-one rabbits were administered 3 injections of 0.2 mg papain protease. Thirty-four were sacrificed at 1 month, 7 at 4 months.

4. Fourteen animals received 6 injections of 0.2 mg of papain protease at 3 or 4 day intervals and were sacrificed after 2 months.

5. Eight rabbits were given 3 injections of 0.3 mg of papain protease and were sacrificed at 2 months.

The 24 control animals received a series of 3 intratibial injections, 0.2 ml in volume, containing only buffer solution or 0.2 mg of papain which had been previously inactivated by boiling. Four control animals were sacrificed at 11 days and the remaining 20 at one month.

After sacrifice, the lower limbs were removed by disarticulation at the hip and fixed

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TABLE I. Effect on Tibial Length of Multiple Intramedullary Injections of Papain in Growing Rabbits Followed 1 to 4 Months.

Length of injected tibia compared with contralateral tibia	No. of animals receiving papain	No. of animals receiving control solutions
Shorter by 2 to 11 mm	5	0
−1 mm to 0	1	2
Equal	15	8
+1 mm	8	7
+2 "	8	3
+3 "	13	0
+4 "	6	0
+5 "	6	0
+6 "	1	0
Total	63	20

immediately in neutral formalin. Roentgenograms were made of both lower limbs in 2 planes. The length of each tibia and femur specimen was measured with a caliper. The average of 3 measurements (S.E. ± 0.3 mm) was used. Histological sections, in the coronal plane, were made of the complete epiphysis including its articular surface and the entire epiphyseal plate of the lower and upper tibia and the lower femur of the injected limb and of the upper tibia of the contralateral limb. Five micra sections, stained with hematoxylin and eosin, were made following decalcification by weak acid and embedding in paraffin. Measurement of the thickness of the epiphyseal cartilaginous plates was performed with a ruler in the ocular tube of the microscope which had been standardized with a micrometer.

Results. A. *Gross findings:* 1. *General.* The animals appeared to tolerate the injections well. Although mild swelling in the region of the injection site was occasionally noted, it disappeared within a few days. The contour of the removed tibiae was normal in all but 3 specimens which showed slight bowing of the shaft (Fig. 1-C).

2. *Effect on bone length.* a. Tibia. Significant differences in length of the tibiae were noted only in the animals followed for more than one month. The results of the comparative measurements of the 2 tibiae at autopsy in these animals are summarized in Tables I and II.

A minimal relative elongation of the injected tibia amounting to 2 mm occurred in 3 of the 20 animals in the control group presumably due to the trauma of 3 intraosseous injections. The other control animals showed no difference between the 2 tibiae greater than 1 mm. Among the 63 long-term experimental animals, 26 (41%) had a relative elongation of the injected tibia of 3 to 6 mm. The gain in this group of 26 averaged 3.8 mm, or 3.5% of 10.8 cm, the average length of an adult rabbit tibia. Thirty-two experimental animals (51%) had no relative gain of the injected tibia, or elongation of less than 2 mm. There were 5 animals with slight to severe retardation of growth of the injected bone (2 to 11 mm).

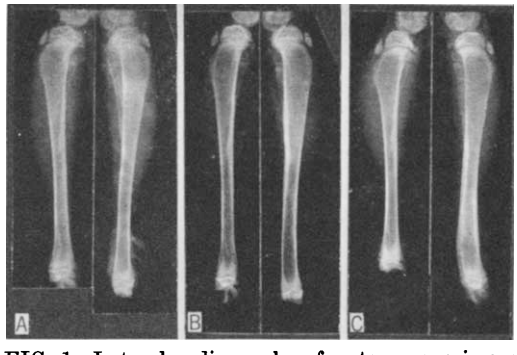

FIG. 1. Lateral radiographs of autopsy specimens of both tibiae of 3 rabbits. Each animal received a series of 3 intratibial injections of 0.2 mg papain protease into the upper metaphysis of the left tibia and was sacrificed at 1 mo. The relative increase in length of left tibia over right tibia measured: A—5 mm, B—5 mm, C—6 mm. C shows minimal posterior bowing of the distal third of the shaft.

TABLE II. Effect on Tibial Length of Varying Quantity of Papain Injected.

Enzyme dose (mg)	No. of injections	Duration of exp (mo)	No. of animals	No. with tibia increment of 3 to 6 mm	Retardation of injected tibia
.2	3	1	34	11	1 with 2 mm shortening
.2	3	4	7	5	0
.2	6	2	14	7	1 with 2 mm and 2 with 4 mm shortening
.3	3	2	8	3	1 with 11 mm shortening

TABLE III. Effect on Epiphyseal Plates of a Single Injection of 0.2 mg Papain Protease into Upper Metaphysis of Left Tibia.

No. of rabbits	Duration of exp (days)	Histological findings
4	4	One or both tibial cartilage plates increased in thickness 20 to 100%
4	7	Three tibiae showed a 20 to 30% increase in epiphyseal plate thickness of one plate
6	10	Two had 20% increase in thickness of lower tibial epiphyseal plate
3	14	One upper tibial plate showed 30% increase in thickness

Significant retardation, however, was found only in the animals on a total papain dosage schedule greater than 0.6 mg (Table II). Two had a relative shortening of 4 mm. One showed 11 mm shortening.

Measurements of the tibiae of the animals receiving 1 or 2 injections of 0.2 mg of papain prior to sacrifice during the first 3 weeks disclosed no differences between the 2 sides exceeding 2 mm.

b. Femur. The femur of the injected limb was found to be 2 mm longer than the contralateral femur in 6 of 63 experimental animals followed for more than one month. Among the control animals, the comparative lengths of the femurs disclosed no differences greater than 1 mm.

3. *Roentgenographic findings.* Two-plane roentgenograms of both lower limbs made at autopsy disclosed a normal osseous trabecular pattern of the injected bones (Fig. 1). The thickness of the cartilaginous epiphyseal plates appeared to be considerably increased in the specimens removed 4 days following one injection of enzyme.

4. *Histological findings.* Significant changes were present chiefly in the short-term experiments (Tables III and IV). The tibial epiphyseal plates of a normal growing rabbit have a characteristic pattern on frontal section. The height of the cartilage columns measure about 0.4 mm. Four days after a single intra-osseous injection of 0.2 mg of papain protease, the treated tibia of all the animals studied showed thickening of part or all of one or both epiphyseal plates (Table III). Portions of the plate measured as much as 0.85 mm in height. During the next 10 days this finding became progressively less pronounced.

TABLE IV. Results of 2 Injections, 3 Days Apart, of 0.2 mg Papain Protease into Upper Metaphysis of Left Tibia.

No. of rabbits	Duration of exp (days)	Histological findings
3	10	Marked thickening (100%) of one or both plates with splitting through hypertrophic zone
3	14	Negative
3	21	"

Six days after the second of 2 spaced injections of papain protease, one or both tibial plates had uniformly doubled in thickness (Table IV). There was transverse splitting through the hypertrophic zone (Fig. 2). These changes did not persist more than a few days. Similarly treated animals sacrificed 10 and 17 days after the second injection showed no evidence of abnormality on microscopic examination.

Among the 63 injected animals sacrificed 1 to 4 months after the start of the experiment 7 showed permanent changes in the epiphyseal plates of the injected tibia. All were among the animals receiving 0.3 mg dose of enzyme or a total of 6 injections. The microscopic abnormalities consisted of V-shaped deformity of the epiphyseal cartilage plate, persistent cartilaginous rests in the metaphysis, or small areas of the plate in which the cartilage columns were reduced to a few rows of resting cells. In the animal with tibial shortening of 11 mm both tibial epiphyseal cartilage plates had central defects involving more than half its area, where no cartilage cells were seen. The rim of cartilage plate that remained appeared, however, to be functional.

Discussion. Stimulation of longitudinal growth in a single long bone following a fracture has been noted in children(6) and in young animals(7). This finding has been commonly ascribed to the effect of hyperemia on the epiphyseal cartilage plates of the broken bone. There is evidence supporting this view in the fact that growth in one limb may be accelerated by ipsilateral lumbar

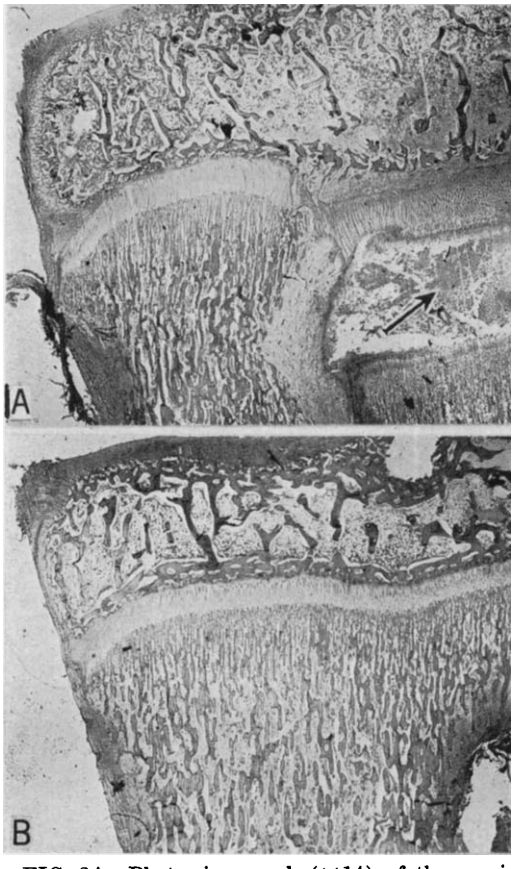

FIG. 2A. Photomicrograph (×14) of the proximal epiphyseal region of a rabbit's tibia which had been injected with 0.2 mg papain protease on the 1st and 5th day. Rabbit was sacrificed on 10th day. The central part of the plate is double the usual thickness. An arrow indicates a gap in the hypertrophic zone which is occupied by hemorrhage. A thick layer of fibrous tissue is noted to the left of the hemorrhagic area, extending distally from a region of the plate which had separated producing a step.

FIG. 2B. Portion of proximal epiphysis of injected tibia of another rabbit which had received the same treatment as animal in 2A. This animal was not sacrificed until the 21st day. The epiphyseal plate appears completely normal.

sympathectomy(8), by unilateral diathermy (9) or femoral arterio-venous fistula(10). This concept has provided the theoretical basis for the attempts of numerous investigators to stimulate longitudinal growth of a single bone in children and laboratory animals by direct trauma to the bones. The methods employed have included periosteal stripping, drilling of the metaphysis, and insertion into the marrow of various metallic or organic materials(11). Although many of these technics have produced growth stimulation of the treated bone in occasional individual trials, results generally were unpredictable. Most subjects showed either no gain or a retardation effect as a result of the procedure. The possibility of stimulating bone growth by intra-osseous injections was investigated by Meisenbach who published the only documented report describing this type of experiment that could be found in the literature(12). He injected various irritants into the region of the upper tibial epiphyseal plate of young rabbits with negative results except for 3 animals which showed retardation of growth of the treated tibiae after injection of pure formalin.

From the results of the various reported experiments on growth stimulation, it appears that application of a mild tissue irritant in the region of an epiphyseal plate does accelerate longitudinal growth. This effect, however, is readily reversed causing growth retardation if the optimal degree of irritation is exceeded by a small margin. The failure to obtain growth stimulation in a higher percentage of subjects treated by implantation of local irritants may be related to the narrow limits of tolerance of the epiphyseal cartilage plate. The stimulation of bone growth following the intra-osseous injection of papain protease must be due to the reversible traumatic effect of the enzyme on the epiphyseal growth cartilage.

The histological findings in the first 2 weeks following intra-osseous papain injections indicate that an interruption of the normal process of endochondral ossification precedes the growth spurt in the injected bone. Transverse splitting through the hypertrophic zone causes a piling up of the other layers of cartilage cells and thickening of the plate. This marked disorganization of the regular cellular pattern of the plate was promptly repaired in all but a few animals. Although the acceleration of bone growth noted is most likely a non-specific effect of proteolytic digestion of elements of the cartilage plate or bone marrow, the interruption of the normal sequence of endochondral ossification resulting from the splitting of the

plate through the hypertrophic zone may also be a factor.

In this experiment, intra-osseous injection of papain caused significant acceleration of bone growth in 26 (41%) of the 63 animals followed for more than one month (Table II). Comparison of these results with the reported effects of various surgical implantations and other operative procedures(11, 13) on bone performed in laboratory animals shows that the highest percentage of animals with significant acceleration of longitudinal bone growth was noted after intra-osseous injection of papain.

Summary. A series of injections of papain protease, in doses of 0.2 or 0.3 mg, was administered into the proximal tibial metaphysis of 89 immature rabbits and the effect on the epiphyseal cartilage plates and on longitudinal growth observed for periods up to 4 months. Twenty-six of 63 animals followed 1 to 4 months had a relative elongation of the injected tibia of 3 to 6 mm. Significant retardation and moderate damage to the epiphyseal cartilage plates of the injected tibia occurred in 3 animals on the higher dosage schedule. Histological studies of the tibial epiphyses a few days after one or two injections of papain disclosed transverse splitting through portions of the hypertrophic zone and a marked increase in thickness of the cartilage plates.

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Interaction of Aldosterone with Liver and Kidney Cell Membranes and Subcellular Fractions of Kidney. (28373)

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The binding of steroid hormones to serum proteins is being actively investigated in an effort to clarify problems of transport as well as of regulation and mechanism of their action. This subject has been reviewed(1,2). The possibility that a correlation exists between the binding of steroid hormones to target tissues and the mechanism of action of such steroids has led to studies of steroid interactions with various tissues and their biological components(3-11).

We have previously reported(12) the investigation of aldosterone binding by serum proteins and the displacing effect of spirolactones and other steroids. It was considered of interest to study the interaction of aldosterone with possible binding sites of its target tissue, the kidney. The binding of aldosterone to kidney and liver cell membranes and to kidney subcellular fractions was determined and compared with the binding of other steroid hormones.

Methods. Male Sprague-Dawley rats weighing 250-300 g and large male New Zealand rabbits were used in these experiments. The animals were sacrificed by a blow on the

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