ent study shows that cottonseed oil emulsion containing other non-ionic detergents are capable of releasing histamine and perhaps 5-hydroxy-tryptamine from tissue cells in rats.

Summary. Two triglyceride emulsions prepared for intravenous use in man have been examined for ability to release histamine from tissue mast cells in rat peritoneal cavity. The emulsions themselves had no direct action upon smooth muscle. Intraperitoneal injection of these materials caused rupture of the mast cells. The fluid recovered from the peritoneal cavity produced bronchiolar constriction in the pithed guinea pig and contraction of guinea pig ileum *in vitro,* indicating a significant histamine content. It is suggested that histamine release may play an important role in development of the occasional "anaphyloctoid" response following intravenous infusion of triglyceride emulsions in patients.

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Effects of Irradiation *in vitro* on Calcifying Mechanism of Epiphyseal Cartilage.* (25942)

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The calcifying mechanism of vertebrate bone, responsible for deposition of apatite crystals in the organic matrix, has been associated with a specific type of ground substance (1), mucopolysaccharide(2), a specific form of collagen(3,4,5) or collagen in combination with mucopolysaccharide(6). Proteins and polysaccharides have been found to undergo changes when irradiated with x-rays at sufficiently high dose levels(7,8). Thus, it was considered likely that irradiation could be used as a tool for study of the calcifying mechanism. The present investigation is a study of effects of irradiation *in vitro* on calcifiability and metachromatic staining properties of epiphyseal cartilage of rachitic rats. The metachromatic staining was undertaken on fresh sections to observe the system unaltered by histological technics. The effect of irradiation was also studied on rachitic specimens in which the initial phase of calcification had begun. Such sections will be referred to in this paper as nucleated.† Rachitic epiphyseal cartilage was used because it is a convenient system for study of the calcifying mechanism *in vitro* and has been used extensively for studying the intimate mechanism responsible for mineralization(9,10,11). When rachitic epiphyseal cartilage is incubated in serum of normal young animals, or in inorganic solutions containing physiological concentrations of calcium and phosphate, mineralization occurs only in the hypertrophic zone with the same histological distribution as occurs *in vivo.*

Materials and methods. Albino rats, 21 days old, were weaned and maintained for 3 weeks on a modified rachitogenic diet No. 2, U.S.P. The animals were then sacrificed by

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† Nucleated section refers to presence of nuclei of crystallization, which are initial submicroscopic aggregates of Ca and P which can serve as a center about which deposition of bone mineral may occur under appropriate conditions(13).

a blow on the head. Fresh sections including epiphysis and metaphysis, 0.1-0.3 mm thick, were cut free-hand in a frontal plane from proximal part of the tibias. A minimum of 7 intact sections was obtained from the tibias of each animal. Each section was treated by one of the following procedures: (a) Unirradiated section stained with silver by the von Kossa method to demonstrate initial state of calcification at time of sacrifice. (b) Unirradiated section incubated in calcifying solution for 18 hours, then stained with silver, to demonstrate maximum calcifiability under the conditions used. (c) Irradiated sections incubated for 18 hours in calcifying solution, then stained with silver. (d) Unirradiated sections heated at 65° for 15 minutes, incubated in calcifying solution for 18 hours, and stained with silver. This heat treatment results in inactivation of the calcifying mechanism, yet does not interfere with subsequent growth of crystals if nuclei of crystallization are already present(11). (e) Unirradiated section incubated in calcifying solution for 30 minutes to produce nuclei of crystallization, then treated as in (d). This is a control of the nucleation test. (f) Unirradiated and (g) irradiated sections stained with toluidine blue. The *in vitro* calcifying solution contained 9 mg % Ca and 5 mg % P. Its preparation and the technics used for *in vitro* calcification have been described(12). Degree of calcification was assessed by microscopic examination of the silver stained specimens. Sections were irradiated on moistened filter paper placed in glass vials which were set in cylindrical lead shields mounted on a Philips x-ray diffraction unit. The open end of the vial faced the x-ray tube. The radiation used was the unfiltered output of a copper target x-ray tube operated at 40 Kv and 18 ma. Dosage rate at the position of the specimen was 880,000 R/hr as measured with a Victoreen R-meter. While specimens were irradiated, control sections were kept on moist filter paper. The animals were divided into 2 groups depending on whether or not nuclei of crystallization had developed, as demonstrated by procedure (d). Epiphyseal cartilage showed little or no calcification by procedure (a), even when nucleation had occurred. Animals with nucleation usually had a fluctuating weight record. Formation of such nuclei is believed to occur during a period of increased serum inorganic phosphate concentration associated with loss of body weight(13). To study the effect of irradiation on the mucopolysaccharide-protein complex of cartilage, sections (f) and (g) were compared after staining supravitally for 15 minutes in 0.2 ml of a solution containing 0.01% toluidine blue in 0.01 M acetate buffer at pH 3.4. Final pH was 4.4-4.8 due to buffer capacity of the tissue. After staining, the sections were rinsed very briefly in distilled water and examined immediately.

TABLE I. Effect of Irradiation on *In Vitro* Calcifiability of Rachitic Epiphyseal Cartilage.

Irradiation	Degree of calcification* Non-nucleated sections	Nucleated sections
Control	4.0	4.0
100,000 R and below	4.0 (10)	
250,000 R	2.9 (11)	3.2 (10)
500,000 R	1.1 (15)	3.0 (10)
1,000,000 R	.2 (19)	2.3 (10)
2,000,000-4,000,000 R		1.8 (6)

* Figures represent avg for number of sections indicated in parentheses. For each animal, degree of calcification of control sections treated as in (b) was assigned an arbitrary value of 4. Degree of calcification of experimental sections was graded by comparison of area of calcified cartilage with that of control (b), and assigned a value from 0 to 4.

Results. Effect on in vitro calcifiability. The effect of *in vitro* irradiation on calcifiability of rachitic epiphyseal cartilage is summarized in Table I. Preliminary experiments indicated that doses of up to 100,000 R had no detectable effect on calcifiability. In the absence of nuclei of crystallization there was a small decrease of calcifiability following 250,000 R, there was a marked decrease after 500,000 R and almost a complete loss following 1,000,000 R. Calcifiability was affected to a lesser extent in sections which were nucleated before irradiation. After irradiation of 2,000,000 to 4,000,000 R, nucleated sections still retained almost half their ability to calcify.

Effect on toluidine blue staining. In unirradiated sections (f) the small cells of the proliferative zone of epiphyseal cartilage were

filled with coarse granules exhibiting β-metachromasia (purple staining), whereas the large cells in matured and hypertrophic zones were filled with fine γ-metachromatic (pink-staining) granules. Capsules of the cells of the proliferative zone were not well stained, whereas capsules of the hypertrophic cells showed a γ-metachromasia. Under the conditions used, staining of the cartilage matrix (except for the capsules) was negligible.

After irradiation with 250,000 R, there was a loss of metachromatic granules from some of the cells of the proliferative region and a small, but more general decrease in staining in the hypertrophic region. After irradiation with 500,000 R, granules were lost from most of the cells of the proliferative zone and from some of the hypertrophic cells. In general, staining was more diffuse, and in some areas the matrix stained. After irradiation with 1,000,000 R, neither cartilage cells nor matrix stained.

These results can be correlated with the color of the residual staining solution after removal of tissue. The toluidine blue solutions used on sections treated with 1,000,000 R were purple whereas those used on sections treated with 500,000 R and 250,000 R were blue with a purple tinge. The toluidine blue solutions used on control sections showed little if any change in color.

Discussion. The calcifying mechanism of rachitic epiphyseal cartilage was inactivated by *in vitro* irradiation at high dosages. There was less inactivation in specimens nucleated before irradiation. Inactivation of the calcifying mechanism by heat or chemical means was reduced or prevented in sections with nuclei of crystallization(11). These results may be interpreted as indicating that a critical phenomenon in calcification is some change in the intercellular matrix capable of inducing formation of submicroscopic nuclei of crystallization. This nucleating mechanism appears to be most sensitive to x-ray, heat, and chemical inactivation. Once nuclei have been formed, the remainder of calcifying process is more resistant to inactivation. The moderate reduction in calcifiability of nucleated sections following irradiation may indicate that the nuclei have some sensitivity to x-irradiation. Another possibility is that the nuclei were not present in sufficient quantity before irradiation to effect complete calcification on subsequent incubation in calcifying solution.

The staining of fresh sections of epiphyseal cartilage with toluidine blue differed markedly from that seen in similar tissues prepared by usual histological methods (*e.g.*, fixation in formalin, Carnoy, or Rossman solutions, followed by embedding in paraffin). With the toluidine blue solution used in our investigation, the matrix of fresh cartilage did not stain, whereas that of fixed cartilage usually stained. Conversely the metachromatic granules were clearly seen in fresh tissues, but were broken up or absent in fixed tissues. The histological treatment resulted in a disruption of the granules and possibly, diffusion of their material into the matrix. In addition, the histological treatment may have freed bound electronegative groups in the matrix which may have been present but masked.

In unirradiated sections (f) stained with toluidine blue, the difference in appearance of the granules in the cells of the proliferative and hypertrophic zones was striking. As the chondrocyte matures and hypertrophies, the relatively large β-metachromatic granules abruptly differentiate into small γ-metachromatic granules.

Metachromasia is presumably due to presence of free electronegative surface charges (14). The change in type of metachromasia of the granules probably reflects an increase in number of free sulfate or carboxyl groups in the mucopolysaccharides, and/or a redistribution of these groups more favorable for metachromasia.

The occurrence of metachromasia in the staining solutions coincident with loss of granules in the irradiated sections suggests that the metachromatic substance was not destroyed by irradiation, but became soluble in the staining solutions. This could be due to breaking of mucopolysaccharide-protein bonds, depolymerization, and increased permeability of the cell membrane.

The effects of irradiation on γ-metachromatic granules and capsules of the calcifiable hypertrophic zone occurred in the same dose

range as the effect on calcifiability. This is in contrast to the effects on β-metachromatic granules of the non-calcifiable proliferative zone which were practically complete after lower doses of irradiation that only partly inactivated the calcifiability. The appearance of the γ-metachromasia in the tissue when it becomes calcifiable, and the similar sensitivity of the γ-metachromatic material and calcifiability to irradiation, suggest that a relationship may exist between them.

Summary. 1. X-irradiation of rachitic epiphyseal cartilage with 250,000 R resulted in small decrease in calcifiability, loss of β-metachromatic granules from some cells of the proliferative zone, and a slight general decrease in staining of the hypertrophic zone. X-irradiation with 500,000 R resulted in marked decrease in calcifiability, loss of most of the β-metachromatic granules from the proliferative zone, and loss of γ-metachromatic granules from some cells of the hypertrophic zone. X-irradiation with 1,000,000 R resulted in almost complete loss of both calcifiability and stainability with toluidine blue. 2. Toluidine blue staining of fresh sections of epiphyseal cartilage was employed for demonstration of metachromatic substances. 3. Once the process of calcification had been initiated, the inactivating effect of irradiation on calcifiability was considerably reduced. 4. A change in mucopolysaccharide resulting in increase of free electronegative groups is a factor associated with calcifiability of epiphyseal cartilage.

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Estrogen Antagonisms: Inhibition of Estrone-Induced Uterine Growth and Vaginal Smear Effects with Testosterone Derivatives. (25943)

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19-Nortestosterone derivatives are highly potent antagonists of uterine growth and vaginal smear effects of estrogens(1). Lengthening of the chain at carbon 17 increased potency in straight-chained forms to ethyl, beyond which potency decreased, and unsaturation affected potency which seemed increased. Data are now available on natural forms of certain of these materials, allowing comparison of structure-activity relationships between 19-nor and natural derivatives of testosterone.

Materials and methods have been described (1). Briefly, in uterine weight studies, various doses of test compound were administered mixed with 0.3 μg of estrone in 0.3 ml of corn oil. 0.1 ml of the mixture was injected daily for 3 days and mice were sacrificed 24 hr after final injection. At autopsy the cleaned, blotted uteri were weighed on a torsion balance. Dose of test compound that was esti-