COMPARISON OF THE EFFECTS OF PAPAIN AND VITAMIN A ON CARTILAGE

I. The Effects in Rabbits*

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It was previously shown that an intravenous injection of papain in young rabbits results in prompt depletion of the basophilic component of matrix in all cartilaginous tissues, accompanied by a loss of the structural integrity of cartilage manifested by collapse of the rabbits' ears (1). This effect is demonstrable with crystalline papain protease, and appears to be due to a direct enzymatic action on chondromucoprotein which results in the release of chondroitin sulfate from cartilage (2, 3). In the gross, the ears recover their rigidity within 3 to 5 days, but the normal histologic appearance of cartilage may not be completely restored until 3 weeks (4). Administration of cortisone prevents reconstitution of cartilage matrix (1), presumably by directly interfering with the capacity of cartilage cells to resynthesize the components of cartilage (4).

Fell and Mellanby (5), in a study of the effects of excess vitamin A on organ cultures of embryonic mouse and chicken bones, found that the basophilia disappeared from cartilage matrix within a few days after introducing the vitamin. The end product was a small, deformed structure in which the cartilage cells were packed closely together, although evidently viable and healthy, with little or no basophilic matrix between the cells. They reported that chondroitin sulfate had largely disappeared from the cartilage of rudiments grown in the presence of excess vitamin A (6).

Because of the histological resemblances between the cartilage of rabbits treated with papain, and that of embryonic bone cultures exposed to vitamin A, a study was undertaken to see whether the effects of vitamin A, *in vivo*, would parallel those of papain.

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It has been learned that the administration of vitamin A in large doses to young rabbits usually brings about partial collapse of the rabbits' ears within a few days, attended by loss of basophilia and of the chondroitin sulfate of cartilage matrix. Although the depletion of matrix is less extensive than that previously reported to occur after papain, it has been found that a small dose of papain, sufficient only to cause slight drooping of the ears, produces changes which are remarkably similar to those caused by vitamin A. The present paper is concerned with a description of these events. In a separate communication, it will be shown that the addition of papain protease to embryonic bone cultures brings about changes in cartilage matrix which resemble those previously encountered with vitamin A (7).

Materials and Methods

Rabbits.—Young, hybrid, male, albino rabbits, obtained from several breeding sources, were maintained on commercial pellets and water. The animals weighed approximately 1 kilogram.

Vitamin A.—Solutions of vitamin A palmitate in corn oil were obtained from Merck and Company, Rahway, New Jersey, and from Nutritional Biochemicals Corporation, Cleveland. The vitamin A was administered by intraperitoneal injection or by gastric intubation in amounts described below.

Histology.—The animals were killed by injection of nembutal and the tissues were fixed in 10 per cent buffered formalin. Sections were prepared from ear, trachea, and the distal end of the femur; from several of the animals sections of heart, lung, cornea, skin, aorta, liver, spleen, and kidney were taken as well. Bones were decalcified in dilute formic acid. Sections were stained as routine with hematoxylin and eosin and in some instances with toluidine blue by the method of Kramer and Windrum (8) and with Alcian blue according to the method of Wagner and Shapiro (9). Some sections were stained by the periodic acid–Schiff method.

Sulfated Mucopolysaccharides.—A turbidimetric procedure was used to estimate the concentrations of chondroitin sulfate-like material in the serum of the animals. This was a modification of the method of Vouras and Schubert (10) as previously described (11). From blood obtained by cardiac puncture, a 0.4 ml. sample of serum was dialyzed at 4°C. for 48 hours against 6 liters of distilled water. The contents of the bags were emptied into 10 × 75 mm. tubes and the volume adjusted to 2.2. ml. by the addition of distilled water. The tubes were centrifuged for 20 minutes at 2000 R.P.M. The supernatant was transferred to matched Coleman, Jr., spectrophotometer cuvettes and the apparent absorbance at 615 mμ determined, using distilled water as a blank. Five minutes after the addition of 0.2 ml. of a 0.5 per cent w/v solution of hexamminecobaltic chloride in distilled water, the apparent absorbance was again determined and the result of the test was recorded as the difference between the 2 readings. The solution of hexamminecobaltic chloride was freshly prepared from material twice recrystallized as described previously (10).

Carrier-free sulfur-35 was obtained as sodium sulfate in water. The dosage used was calculated on the basis of data provided by the supplier. In each of the rabbits given sulfur 35, 0.5 mc. was administered intraperitoneally. Serum obtained by cardiac puncture from these animals was subjected to the following procedures: (*a*) 0.1 ml. samples were plated in planchettes of 2.5 cm. diameter and dried by heating at 90°C. for at least 48 hours; (*b*) 0.4 ml. samples were dialyzed and diluted as described above, and 0.1 ml. samples of the dilute serum were obtained before the addition of the cobalt reagent and were plated and dried in planchettes; (*c*) after measurement of the turbidity produced by addition of the hexamminecobaltic

chloride, the cuvettes were centrifuged at 2000 R.P.M. for 20 minutes and 0.1 ml. samples of the supernatant were dried as before. No satisfactory method was devised for directly measuring the sulfur-35 activity in the precipitates produced by the addition of hexamminecobaltic chloride.

Samples were counted in duplicate in a gas flow counter with a micromil end window (Nuclear, Chicago). In one experiment, rabbits were kept in metabolic cages and urine was collected under phenol. Sulfur-35 activity was measured on 0.1 ml. samples of the urine excreted in 24 hours.

Autoradiographs were prepared from paraffin-embedded sections cut at 5 μ using Kodak contrast process ortho film for gross autoradiographs according to the method of Dziewiatkowski (12), and with stripping film AR 10 (Kodak, Ltd.) for microautoradiographs by the method of Pelc (13).

Chondromucoprotein was prepared by the method of Malawista and Schubert (14), from acetone-dried pooled ear cartilage plates obtained from rabbits given sulfur-35. The crude chondromucoprotein was not reprecipitated. Hexosamine was determined by a modification of the method of Elson and Morgan described in reference 15, using glucosamine hydrochloride as the standard. Nitrogen was determined by the Kjeldahl method. The mucoprotein was dissolved in 0.1 M phosphate buffer (pH 7.01) to a concentration of 1 mg. per ml., and 0.1 ml. of this solution was counted for sulfur-35 activity as above.

Crude papain (Nutritional Biochemicals Corp.) was dissolved in 0.05 M phosphate buffer (pH 7) by grinding with mortar and pestle. The insoluble material was removed by filtration through paper. The protein concentration of the solution was determined by the biuret method.

EXPERIMENTAL

Gross Changes Following Vitamin A.—In preliminary experiments, vitamin A was fed to 10 rabbits by stomach tube, in a single dose of 400,000 international units dissolved in 2 ml. of corn oil. Within 2 days the ears of 6 of these animals had become partially collapsed, with curling of the distal third, as illustrated in Fig. 1. Histological studies revealed depletion of the basophilic component of the matrix in articular, epiphyseal, and tracheal cartilage. It was then found that similar changes occurred when vitamin A was injected intraperitoneally, and because of its greater convenience in experiments involving large numbers of rabbits this route of administration was used in all subsequent work. Higher doses were generally employed, in view of reports that vitamin A is less effective when injected than when given by gastric intubation.

Forty-six rabbits were given daily injections of 1 million units of vitamin A and killed for histological study at intervals ranging from 24 hours to 7 days. Of these, 31 (67 per cent) showed curling of the tips of their ears at some stage during the period of observation. This change (Fig. 1), was characterized by loss of the rigidity and erectness of the distal 1 to 2 cm. of the ears. It never involved the more proximal portions of the ear, in contrast to the complete collapse which usually follows an injection of 5 mg. of crude papain in rabbits of this size. In the majority of animals affected, curling occurred within 72 hours. In some it appeared as early as 24 hours, in others as late as 6 days. Recovery of the erect state of the ears occurred in 5 of the animals after 72

hours, while vitamin A was still being administered. Weights were recorded regularly throughout the experiment, and most animals either failed to gain weight or lost weight. The rabbits given vitamin A for 5 days or longer usually developed loss of hair, especially in the region of the mouth and paws.

Control animals consisted of 11 rabbits kept under the same conditions and given daily intraperitoneal injections of corn oil without vitamin A, and 11 litter mates which received no treatment. None of these animals showed the abnormalities of cartilage observed in the treated group.

Histologic Changes.—Histologic sections were prepared from tissues in 30 rabbits injected with vitamin A. Three animals were killed 24 hours after one injection; these showed no changes in cartilage. In most of the rabbits killed 2 or more days after the first injection, there were characteristic alterations in cartilage, affecting especially articular and epiphyseal cartilage. Examination of ear cartilage was unsatisfactory because of the variability of staining properties in normal rabbits and only equivocal depletion of ear cartilage was seen in vitamin A–treated rabbits. Sections from the distal end of the femur showed reduction or disappearance of basophilic, metachromatic, and Alcian blue staining of the articular cartilage and of the epiphyseal plates associated with thinning of these structures (Figs. 2 to 7). The cartilage cells appeared to be intact but were smaller than normal and showed little or no basophilia or metachromasia. In sections stained by the periodic acid–Schiff method, the cells showed reduction in the amount of PAS-positive material. The cartilaginous cores of the metaphyseal bony trabeculae and of the trabeculae adjacent to the articular cartilage stained normally with hematoxylin, toluidine blue, and Alcian blue. The frequency and severity of the changes in cartilage increased with the number of days of treatment. It should be noted, however, that in 8 of 23 rabbits killed after 5 days of injections, there were no changes in cartilage.

The earliest alterations seen in the epiphyseal plates were present in 2 of the 4 animals killed 2 days after the first injection and consisted of loss of basophilic staining and slight thinning of the plates (Fig. 5). In animals killed after 5 days, there were usually more severe changes in the epiphyseal plates. In some animals, vascularization of the zone of proliferating cartilage extended in from the metaphysis (Fig. 6). In others, the epiphyseal plate was reduced to a thin layer of completely eosinophilic, non-metachromatic tissue with little or no arrangement of cartilage cells into columns (Fig. 7). This was associated with increased numbers of metaphyseal bony trabeculae immediately adjacent to the epiphyseal plate (Fig. 7).

In all the rabbits that showed changes in the epiphyseal plates, there was also depletion and slight thinning of articular cartilage (Figs. 2 and 3).

Changes were present in the tracheal cartilage in 8 of the 23 rabbits that were killed 2 or more days after the first injection and consisted of loss of basophilic staining of certain areas of cartilage matrix. Where the cartilage plate was thin, its entire thickness was sometimes involved, but usually the depletion was seen only in the peripheral portions of cartilage with the central area remaining normally basophilic (Figs. 8 and 9). Sections of the tracheal cartilage from control rabbits occasionally showed absence of basophilic staining toward the periphery, but in 8 of the vitamin A–injected animals the lack of basophilia was far more extensive than that seen in any of the control animals.

Sections of heart, lung, liver, spleen, kidney, aorta, skin, and cornea were prepared from 10 experimental animals. The only abnormalities found were slight fatty change in the liver of 3 animals and metastatic calcification in the kidney of 2 animals.

Measurement of Cobalt-Precipitable Material in the Serum of Vitamin A–Treated Rabbits.—The histologic changes in cartilage described above indicated

that chondroitin sulfate was lost from cartilage. In order to see if this was associated with release of chondroitin sulfate into the blood, determinations of cobalt-precipitable material in serum were made, by the method described above, in 22 rabbits injected daily with 1 million units of vitamin A, in 11 rabbits injected daily with corn oil, and in 11 untreated controls.

The upper limit of normal, based on measurements in 50 rabbits kept under similar conditions and bled repeatedly, was found by this method to be 0.065 absorbance units.

This level was not exceeded at any time in the 11 untreated, control animals tested in these experiments, nor in 11 rabbits given daily intraperitoneal injections of corn oil.

TABLE I

Incidence of Increased Cobalt Turbidity in Serum of Rabbits Given Daily Injections of Vitamin A, 1 Million Units

No. of days after 1st injection of vitamin A	No. of rabbits tested	Increased cobalt turbidity*		Controls‡
		No.	Percentage	
0	19	0	0	0/17
1	15	3	20	0/13
2	22	8	36	0/18
3	18	9	50	0/12
4	7	6	86	0/7
6	17	10	59	0/13

* Absorbance increment greater than 0.065.

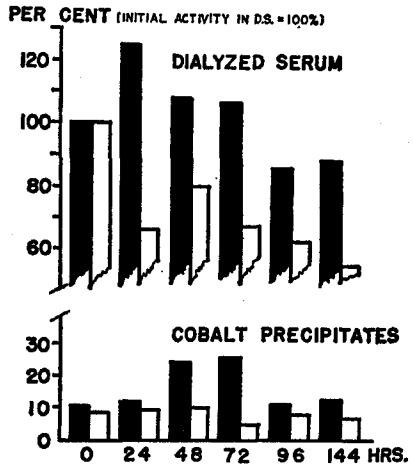
‡ Daily injections of corn oil, 11 rabbits. No treatment, 11 rabbits.

In the rabbits injected daily with vitamin A, however, there was a progressive increase in the incidence of abnormally elevated serum cobalt turbidity. Between the 3rd and the 6th day after the first injection of vitamin A, 25 of 33 samples showed increased serum cobalt turbidity (Table I). While additional data are necessary to establish the precise quantitative significance of these values in terms of chondroitin sulfate, on the basis of *in vitro* experiments in which known amounts of chondroitin sulfate were added to a variety of sera (11), it is estimated that on the average there was a 10-fold increase and in some cases a 50-fold increase in the serum level of chondroitin sulfate at 48 and 72 hours. When beef nasal septum chondroitin sulfate was added to normal rabbit serum at a concentration of 1 mg./ml. and carried through the above turbidimetric procedure, the absorbance at 615 mμ was 0.150 units.

The Effect of Vitamin A Administration on Incorporated Sulfur-35.—It has been established that when sulfur-35 is administered to animals as inorganic sulfate, a large percentage of the injected activity is excreted within a few days.

The retained sulfur-35 is incorporated mainly in sulfated mucopolysaccharides (16, 17), such as the chondroitin sulfate of cartilage (16). In order to study the effect of vitamin A administration on incorporated sulfur-35, the following experiment was performed:

10 rabbits were each given 0.5 millicurie of carrier-free sulfur-35 as $Na_2S^{35}O_4$ intraperitoneally. Five days later, daily injections of 1 million units of vitamin A were initiated in 7 of these animals and continued for 5 days. The remaining 3 rabbits were given a similar course of injections of corn oil. Serum levels of sulfur-35 were measured immediately before treatment and daily thereafter for 7 days, at which time the rabbits were sacrificed, and histologic sec-



TEXT-FIG. 1. Sulfur-35 activity in dialyzed serum and cobalt precipitates. Mean Values for vitamin A–treated rabbits (black) and controls injected with corn oil (open). Daily injections of vitamin A and corn oil were initiated at 0 hours, and were continued for 5 days.

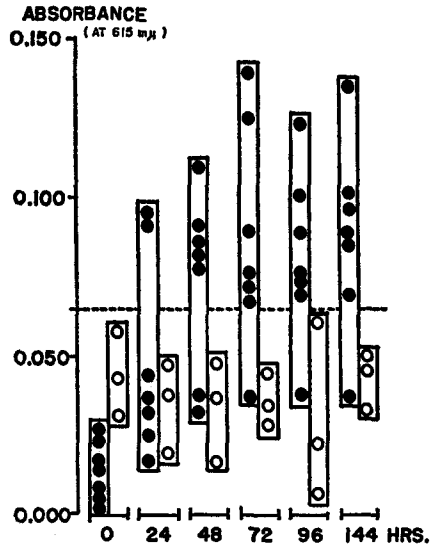
Sulfur-35 activity in material precipitated by hexamminecobaltic chloride was calculated from the amount of activity lost from dialyzed serum following removal of the precipitates. All values are expressed as percentages of the activity in dialyzed serum (D.S.) at 0 hours.

tions and autoradiographs prepared. Activity was determined in whole serum, dialyzed serum and in the supernatants following removal of the material precipitated by hexamminecobaltic chloride.

From the sulfur-35 activity in blood and urine prior to administration of vitamin A or corn oil, it was apparent that the amount of the isotope retained by different animals varied considerably. For this reason the data have been expressed as percentages of the activity in dialyzed serum at the start of the treatment. The averages calculated from these percentages are therefore not weighted in favor of animals which retained larger amounts of sulfur-35. In all samples of serum from both treated and control groups, little or no reduction of sulfur-35 activity occurred during dialysis, the maximum loss being 20 per cent, and this only from initial samples. The euglobulin precipitated by dialysis with distilled water had negligible sulfur-35 activity.

Twenty-four hours after the first injection of vitamin A, the sulfur-35 activity of dialyzed serum increased in 6 of the 7 rabbits, to an average for the whole group of 125 per cent of the initial activity (Text-fig. 1). Some increase was still apparent in the 48 and 72 hour samples of dialyzed serum from the vitamin A–treated rabbits.

In contrast, there was no similar increase at any stage in serum from the control animals, and in addition the ultimate level of activity in this group was considerably below that of the vitamin A–treated rabbits.



TEXT-FIG. 2. Hexamminecobaltic chloride turbidity of rabbit serum following administration of vitamin A. Turbidity was measured before and after vitamin A 1 million units daily in 7 rabbits (black circles) and corn oil 1 ml. daily in 3 rabbits (open circles).

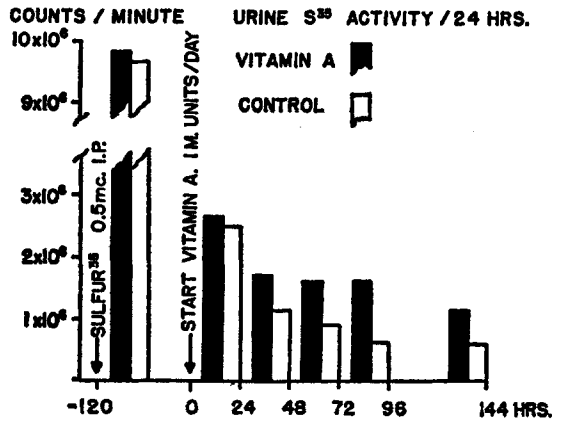
Increased amounts of cobalt-precipitable material were present in the serum of 6 of the 7 vitamin A–treated rabbits at 72, 96, and 144 hours after the start of treatment. Of these 6, 5 also showed significant elevation at 48 hours and 2 at 24 hours. No significant increase was observed at any stage in serum from the control group (Text-fig. 2).

Initially, the sulfur-35 activity removed by precipitation with hexamminecobaltic chloride was about 10 per cent of the total in dialyzed serum in the control and experimental groups. At 48 and 72 hours this increased to about 25 per cent in the vitamin A–treated animals, but did not change in the controls (Text-fig. 1).

Urine was collected from the 3 control rabbits and 2 of the experimental animals. Sulfur-35 activity was measured in 0.1 ml. aliquots from each 24 hour collection, and the total output calculated. Shortly following administration of

the isotope, a large amount of radioactivity was present in the urine of all 5 rabbits. This rapidly declined in the next 6 days, the last 2 of which were in the experimental period. Thereafter, the 2 vitamin A–treated rabbits excreted nearly twice as much sulfur-35 as the 3 controls (Text-fig. 3).

The Isolation of Chondromucoprotein from Rabbits with Incorporated Sulfur-35.—Following sacrifice of these animals, the cartilage plates of the ears were stripped of skin and underlying tissues, diced, and dried in acetone. The chondromucoprotein obtained was weighed and analyzed for hexosamine and nitrogen. Sulfur-35 activity was measured in samples. The results are presented in Table II. While the amount of extracted chondromucoprotein per gram of



TEXT-FIG. 3. Urinary output of sulfur-35 in experimental and control groups. Means of total sulfur-35 activity in 24 hour collections of urine from vitamin A–treated rabbits (black columns) and controls (open columns). Initial value is the average daily output between administration of sulfur-35 and first injection of vitamin A or corn oil.

dry cartilage was essentially the same in both control and experimental animals, the cartilage from the control group yielded slightly more hexosamine and less nitrogen than that from vitamin A–treated rabbits. The sulfur-35 activity of chondromucoprotein from the control animals was nearly twice that of vitamin A–treated rabbits.

Histologic and Autoradiographic Findings.—Of the 7 rabbits treated with vitamin A in this experiment, 6 showed moderate or severe depletion of matrix with loss of basophilic, metachromatic, and Alcian blue–positive material in epiphyseal plates and in articular cartilage in sections taken from the lower end of the femur. There was moderate or severe thinning of the epiphyseal plates in all of the affected animals.

Gross autoradiographs showed that in these 6 animals there was only a faint image corresponding to the epiphyseal plate and over articular cartilage, in contrast to the 3 control rabbits which showed an intense image over these

structures (Figs. 10 to 17). The 7th animal in the vitamin A group showed a normal distribution of sulfur-35 in these areas and normal staining properties of cartilage. In the 6 vitamin A–treated animals with changes in cartilage, there was a zone of increased density in the metaphysis and a dense image just beneath the articular cartilage. These findings were studied by microautoradiographs which are illustrated in Figs. 12, 13, 16, and 17. There was marked reduction in the number of sulfur-35 granules over the articular cartilage and over the epiphyseal plates. The image overlying the cartilaginous cores of the metaphyseal trabeculae showed the same intensity as in controls (Fig. 18); this together with the increased number of metaphyseal trabeculae in the vitamin A–treated group accounted for the zone of increased density seen in this region in the gross autoradiographs. There was also a normal amount of sulfur-35 in the cores of newly formed bone immediately beneath the articular

TABLE II

Comparison of Crude Chondromucoprotein Obtained from Control and Vitamin A—Treated Rabbits, 1 Million Units Daily

	Control	Vitamin A
Yield of chondromucoprotein*	65.00 mg.	77.50 mg.
Yield of residue*	871.00 mg.	866.00 mg.
Hexosamine, *per cent*	10.0	8.6
Nitrogen, *per cent*	6.5	8.1
Nitrogen/hexosamine ratio	8.3	12.0
Counts per minute S^{35}*	23,500.	13,700.

* Per gram of dry cartilage.

cartilage and this was responsible for the image seen in this area in the gross autoradiographs.

The Production by Small Doses of Papain of Changes in Cartilage Resembling Those Seen Following Vitamin A.—The changes seen in cartilage following vitamin A treatment differed from those usually observed in young rabbits after the injection of 5 mg. of crude papain, in that with papain there is complete collapse of the rabbits' ears and complete disappearance of basophilic staining in all cartilaginous tissue throughout the body. An investigation was undertaken to see whether smaller doses of papain would produce changes in cartilage more nearly resembling those brought about by vitamin A.

Accordingly, 18 rabbits averaging 1000 gm. were treated as follows: 5 received a single intravenous injection of 0.5 mg. of crude papain and were killed 2 days later; 5 rabbits were given 3 daily injections of 0.25 mg. of crude papain and killed 1 day after the last injection; 5 rabbits were given 5 daily injections of 0.12 mg. of crude papain and killed 1 day after the last injection. Three rabbits were used as controls. Histologic sections were prepared from ear, trachea, and distal end of the femur and stained with hematoxylin and eosin, toluidine blue, and Alcian blue.

The alterations in cartilage in the 3 groups of papain-treated rabbits were remarkably similar to those seen after vitamin A administration. There was almost complete loss of basophilic, metachromatic, and Alcian blue–positive material in articular cartilage in every experimental animal (Fig. 19). The epiphyseal plates were narrowed and showed diminution or absence of the usual staining properties of cartilage in all of the papain-injected rabbits (Fig. 20). In some rabbits, vascularization extended from the metaphysis into the zone of proliferating cartilage. These changes in epiphyseal plates were indistinguishable from those seen in vitamin A-treated rabbits showing moderate thinning of epiphyseal plates. The cartilaginous cores of the bony trabeculae adjacent to epiphyseal plates and articular cartilage stained normally in the papain-injected animals, as in the rabbits given vitamin A. Sections of trachea from the rabbits injected with papain showed only slight and irregular depletion of cartilage matrix in 6 animals, which was similar to that seen in vitamin A–injected rabbits. Sections of ear showed only equivocal loss of basophilic staining.

DISCUSSION

On the basis of the experiments reported here it is evident that depletion of cartilage matrix can be produced in intact animals by the administration of large amounts of vitamin A. This effect has not previously been described in intact animals, but the changes in cartilage are similar to those observed in organ cultures in the presence of excess vitamin A.

There are several independent lines of evidence showing that chondroitin sulfate is lost from the cartilage of rabbits given large amounts of vitamin A. These include the diminution or disappearance of basophilic, metachromatic, and Alcian blue—positive material from cartilage, especially in certain areas (articular and epiphyseal cartilage); the loss of previously incorporated sulfur-35 from these sites, as shown by autoradiography; and the appearance in the blood of cobalt-precipitable material presumed to be chondroitin sulfate. In addition, the finding of increased amounts of non-dialyzable sulfur-35 activity in the serum, the relative increase in the amount of sulfur-35 in the urine, the reduction in the sulfur-35 activity, and the increased nitrogen/hexosamine ratio of extracted crude chondromucoprotein support this interpretation.

Evidence indicating that the cobalt turbidity technique is a relatively specific method for measuring serum chondroitin sulfate levels above 250 μg./ml. has been presented elsewhere (11).

In the present investigation it was found that after the administration of vitamin A, a marked increase in non-dialyzable sulfur-35 activity preceded the appearance in serum of elevated levels of cobalt-precipitable sulfur-35 activity. Maximum sulfur-35 activity was present in the cobalt-precipitable material in serum obtained 72 hours after the first injection of vitamin A.

Samples of sera obtained after 72 hours continued to have increased amounts of cobalt-precipitable material, but the sulfur-35 activity of these precipitates fell progressively to insignificant levels at 144 hours.

These findings are open to several interpretations. The initial increase in sulfur-35 activity in dialyzed serum may be due to the liberation from cartilage of components of chondroitin sulfate that are not capable of forming a complex with hexaminecobaltic chloride. Alternatively, non-precipitable material containing sulfur-35 may be liberated from other tissues at this stage. The cobalt-precipitable sulfur-35 activity seen at 48 and 72 hours most probably represents chondroitin sulfate liberated from cartilage. A similar increase of cobalt-precipitable material containing sulfur-35 activity has been observed in association with depletion of cartilage matrix produced by papain (11). The low sulfur-35 activity of cobalt precipitates at 96 and 144 hours could be due to the liberation from cartilage of the most recently synthesized chondroitin sulfate, which would contain relatively little sulfur-35.

Although most of the rabbits given 5 daily injections of 1 million units of vitamin A showed striking changes in cartilage, some of the rabbits receiving such treatment showed no changes in cartilage in any location.

Acute hypervitaminosis A in the rabbit affects cartilage of joint surfaces and epiphyseal plates more regularly and severely than in ear and trachea. It is noteworthy that repeated injections of minute amounts of papain produce depletion of cartilage matrix in the same areas. With larger doses of papain, depletion of matrix occurs in all cartilaginous tissue throughout the body.

The changes occurring in the epiphyseal plates following vitamin A treatment are more complicated than those involving cartilage in other locations. Two processes appear to be involved in the production of marked narrowing of the epiphyseal plate: one, the depletion of cartilage matrix; and two, the concurrent vascularization and ossification of the zone of proliferating cartilage. As a result, the epiphyseal plate is reduced to a thin zone of non-basophilic cartilage abutting directly on metaphyseal bony trabeculae. The ossification of part of the epiphyseal plate, together with absence of continued growth of this structure, accounts for the increased number of bony trabeculae seen in the metaphysis. The reason for the vascularization and ossification of the zone of proliferating cartilage is not clear. It might be the result of accelerated ossification secondary to changes in the cartilage matrix or simply the result of the continuation of the normal rate of ossification in the face of diminished or absent growth of the epiphyseal plate.

It is interesting to compare the results of the present study with previous observations on hypervitaminosis A in experimental animals. Most of the earlier studies have been made in the rat, in which the most conspicious changes reported are spontaneous hemorrhages and bony abnormalities characterized by resorption of bone and pathological fractures (18–20). Thinning and ossi-

fication of the epiphyseal plates have been observed in rats given large doses of vitamin A, but apparently no description of depletion of cartilage matrix in this or other locations has been recorded. Hypervitaminosis A in the rabbit has been less extensively studied, but in the few reports concerning this species, there is no description of changes in cartilage matrix (21).

The experiments reported here provide no direct information about the fundamental mechanism whereby vitamin A effects depletion of cartilage matrix, but the results suggest two general possibilities. The depletion might be caused by the inhibition of synthesis in the presence of normal degradation; or by increased breakdown, either by acceleration along normal pathways or by an entirely abnormal means of destruction. The data indicate that increased breakdown is the more likely mechanism. The appearance of increased amounts of material presumed to be chondroitin sulfate in the blood, demonstrated by the cobalt method and sulfur-35, supports this interpretation. Moreover, the depletion of previously incorporated sulfur-35 from articular and epiphyseal cartilage, as demonstrated by autoradiography, is also indicative of increased breakdown. It has been shown in earlier work that cortisone, which inhibits synthesis of chondroitin sulfate, produces histological changes in cartilage (4); these changes are substantially different from those which follow vitamin A treatment. No experiments have been performed to investigate the possibility of a change in the rate of synthesis of cartilage matrix accompanying the breakdown seen after vitamin A administration. There is no evidence to show whether the increased breakdown is due to acceleration of normal processes or to an entirely different mechanism.

The fact that the histological alterations in cartilage produced by vitamin A were remarkably similar to those produced by small doses of papain suggests the possibility that an endogenous proteolytic enzyme is activated by vitamin A under these experimental conditions. While these effects on cartilage were produced by the administration of massive doses of the vitamin and may, therefore, be unrelated to the normal role of vitamin A, the possibility remains that these changes are the result of an exaggeration of normal processes. It is possible that the changes in cartilage are mediated by mechanisms involved in the normal metabolism of vitamin A.

SUMMARY

The administration of large amounts of vitamin A to rabbits has been shown to result in depletion of cartilage matrix. The normal basophilic, metachromatic, and Alcian blue staining properties of the matrix are lost, especially in articular and epiphyseal cartilage. The cartilage cells remain intact, but are reduced in size.

These changes sometimes appeared as early as 48 hours after the initiation of daily injection of 1 million units of vitamin A, and were usually well estab-

lished by 5 days. Some rabbits failed to show changes in cartilage, even after 5 daily injections.

Increased amounts of material presumed to be chondroitin sulfate were present in the sera of vitamin A–treated rabbits, usually by 72 hours after the first injection. This was demonstrated by a turbidimetric procedure using hexamminecobaltic chloride.

In rabbits given sulfur-35 ($Na_2S^{35}O_4$) 5 days before the initiation of vitamin A treatment, it was shown that sulfur-35 was lost from articular and epiphyseal cartilage. This was associated with an increase in the non-dialyzable sulfur-35 in both serum and in the cobalt-precipitable material. These rabbits also excreted more sulfur-35 than rabbits not given vitamin A. There was a reduction in sulfur-35 activity in chondromucoprotein extracted from the ear cartilage of vitamin A–treated rabbits.

The changes are interpreted as indicating that the administration of large amounts of vitamin A to rabbits results in removal of chondroitin sulfate from cartilage matrix.

The administration of small amounts of crude papain causes histologic changes in cartilage that are remarkably similar to those seen in vitamin A–treated rabbits.

The possibility is suggested that the changes in cartilage produced by administration of vitamin A to rabbits may be the result of activation of a proteolytic enzyme or enzymes, with properties similar to those of papain.

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EXPLANATION OF PLATES

PLATE 53

FIG. 1. Rabbit given 2 daily injections of 1 million units of vitamin A. This shows the maximal extent of ear collapse seen in the rabbits injected with vitamin A. × ¾.

FIG. 2. Section of articular cartilage of distal end of femur from control rabbit. There is intense metachromatic staining of the cartilage. Toluidine blue stain. × 100.

FIG. 3. Section of articular cartilage from vitamin A—injected rabbit. There is absence of metachromatic staining of articular cartilage, but there is normal staining of the cartilaginous cores of the underlying bony trabeculae. Toluidine blue stain. × 100.

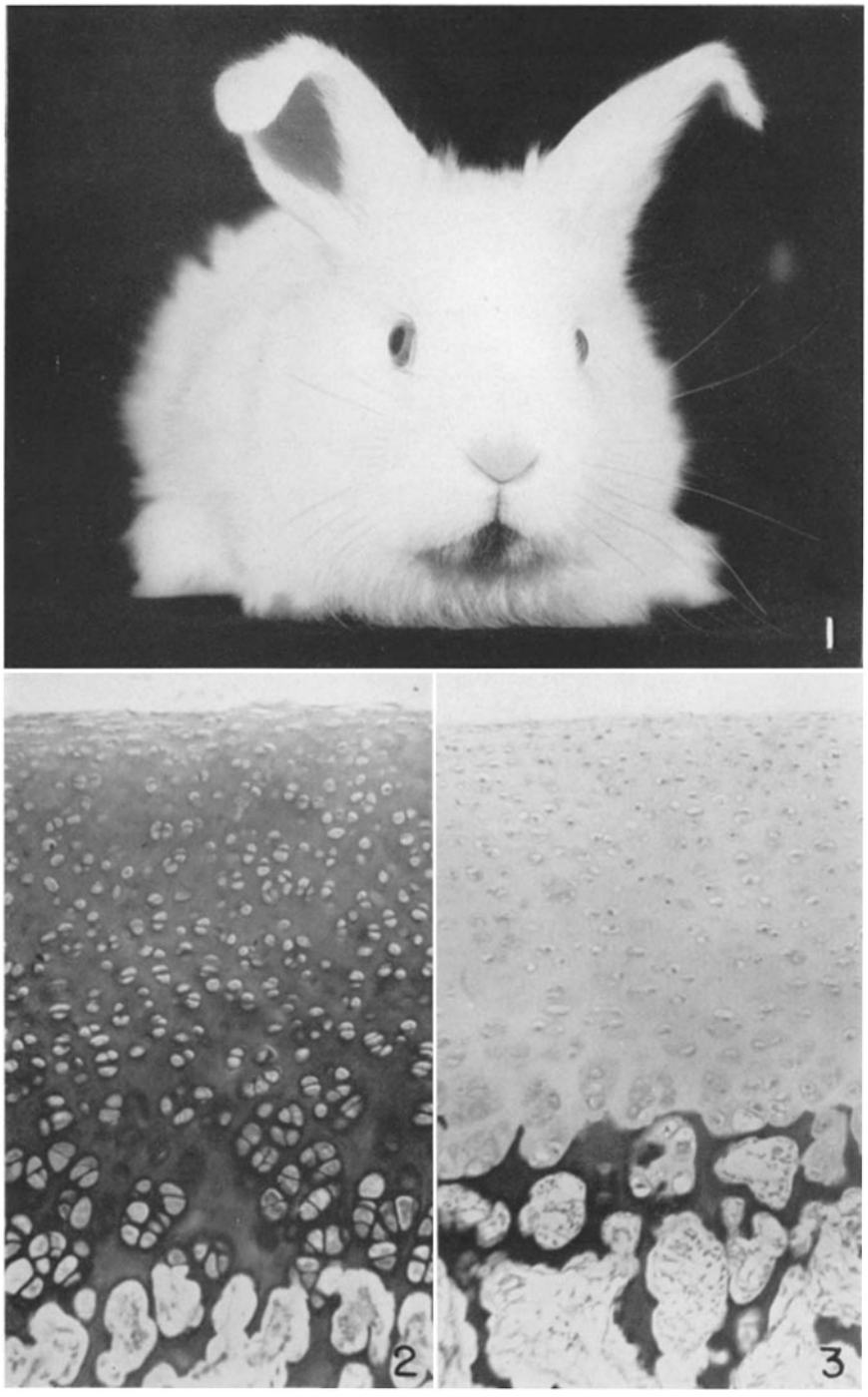

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PLATE 54

FIG. 4. Epiphyseal plate of distal end of femur from a control rabbit. Hematoxylin and eosin stain. × 80.

FIGS. 5, 6, 7. Epiphyseal plates from 3 rabbits given 5 daily injections of vitamin A. Hematoxylin and eosin stain. × 80.

FIG. 5. There is moderate thinning of the cartilage plate and diminution of basophilic staining.

FIG. 6. There is loss of basophilic staining of the portion of the plate toward the epiphysis and vascularization of the zone of proliferating cartilage.

FIG. 7. The epiphyseal plate is reduced to a thin layer of tissue which shows no basophilic staining. There is increase in the number of metaphyseal bony trabeculae.

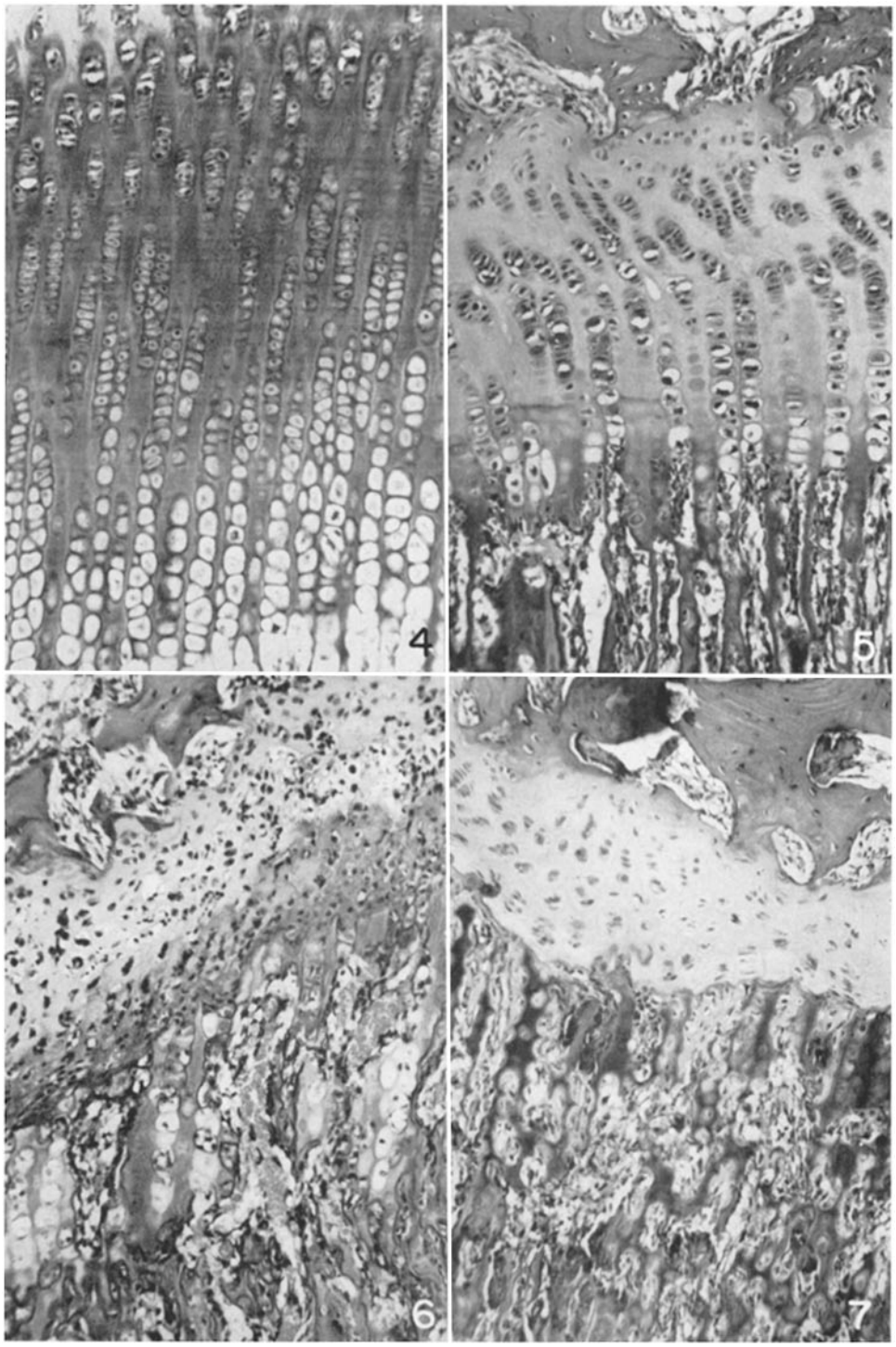

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PLATE 55

FIG. 8. Tracheal cartilage from control rabbit. Hematoxylin and eosin stain. × 80.

FIG. 9. Tracheal cartilage from rabbit given 5 daily injections of 1 million units of vitamin A. Basophilic staining is absent except for the central portion in the upper part of the field. Hematoxylin and eosin stain. × 80.

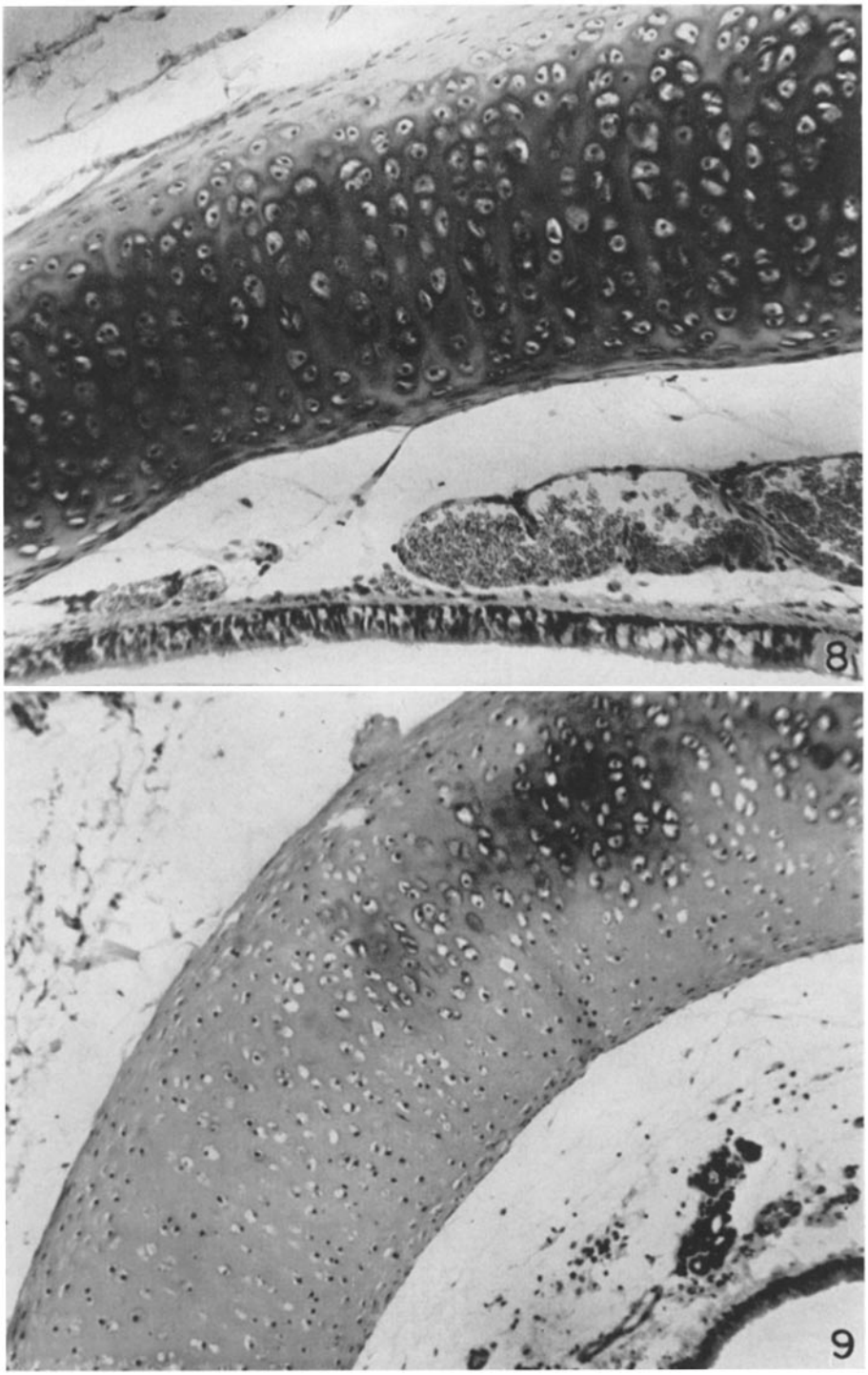

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PLATE 56

FIG. 10. Gross autoradiograph of distal end of the femur from a control rabbit given sulfur-35 12 days previously. An image is present over the articular cartilage, epiphyseal plate, metaphyseal trabeculae, and the trabeculae beneath the articular cartilage. × 4.

FIG. 11. Gross autoradiograph of distal end of the femur from rabbit given sulfur-35 12 days previously and beginning on the 5th day, 5 daily injections of 1 million units of vitamin A. There is only a very faint image over the articular cartilage and over the epiphyseal plate. There is an intense image in the metaphysis and just beneath the articular cartilage. × 4.

FIG. 12. Microautoradiograph in region of arrow shown in Fig. 10. Numerous granules are present over the articular cartilage. Hematoxylin stain. × 500.

FIG. 13. Microautoradiograph in region of arrow shown in Fig. 11, at the junction between articular cartilage and underlying bony trabeculae. There is an intense image over the cartilaginous cores of the bony trabeculae shown in the lower part of the figure, but the number of granules diminishes over the articular cartilage above. Hematoxylin stain. × 500.

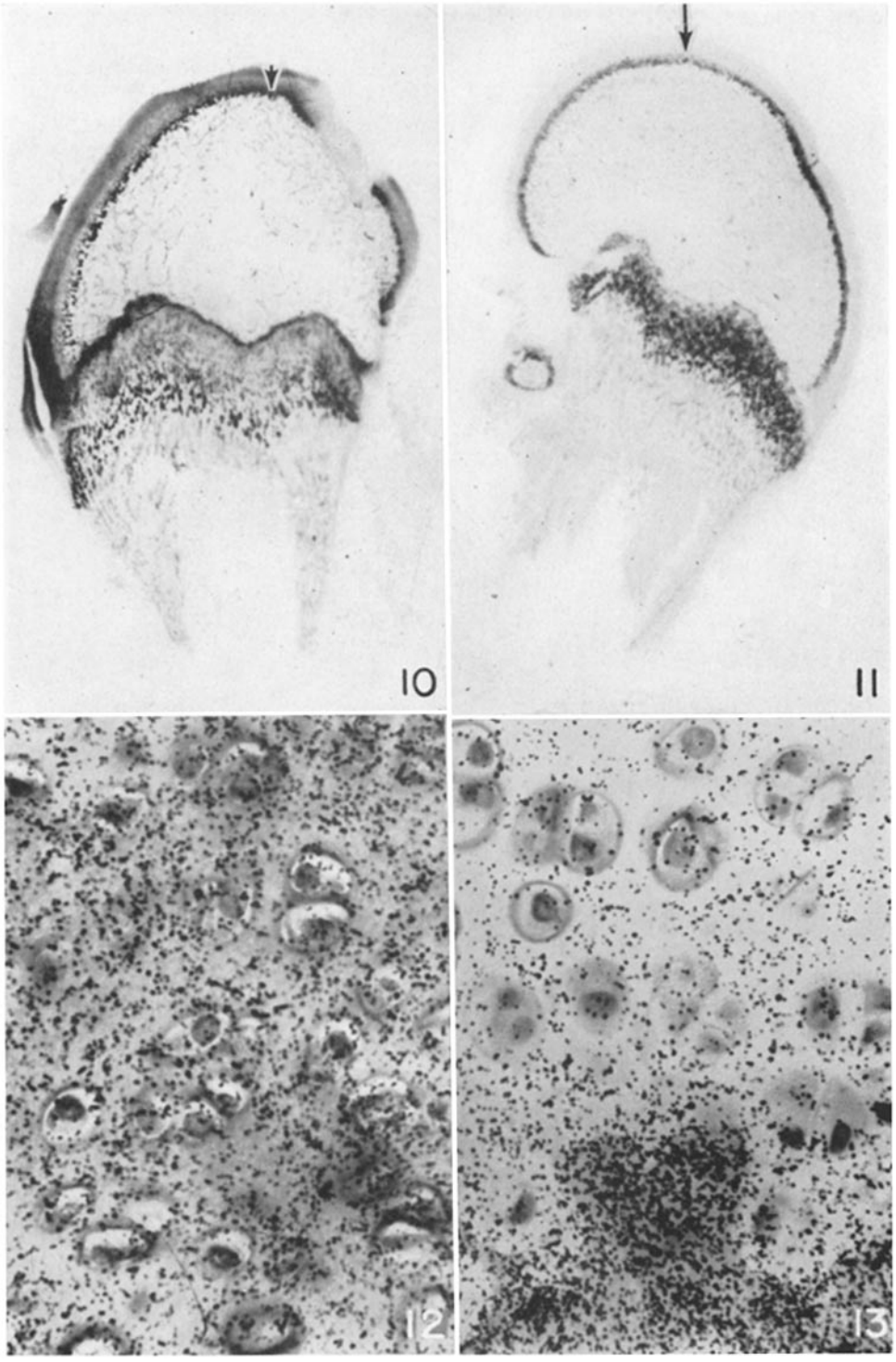

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PLATE 57

FIG. 14. Gross autoradiograph from distal end of the femur from control rabbit. × 4.

FIG. 15. Gross autoradiograph from vitamin A–treated rabbit. × 4.

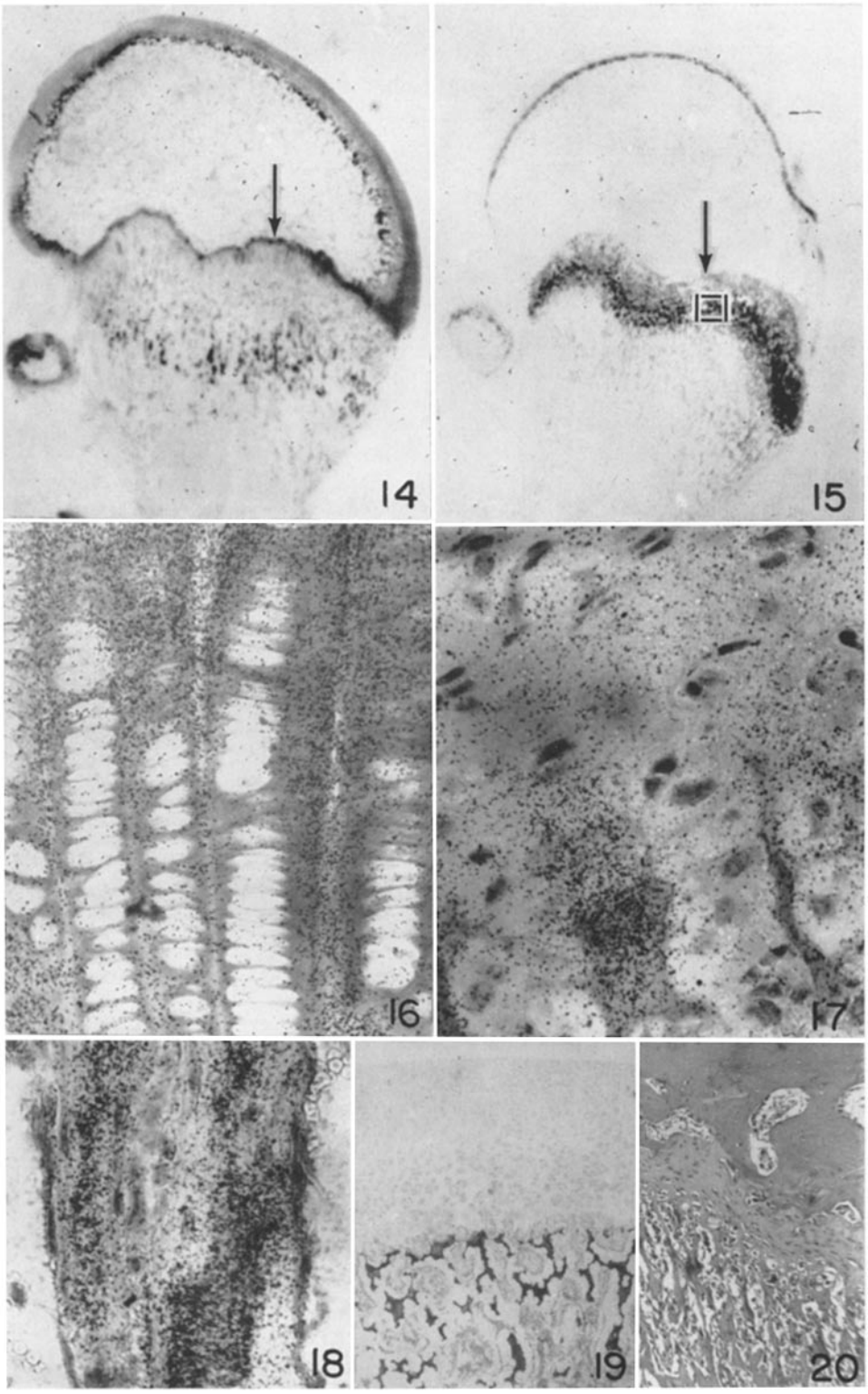
FIG. 16. Microautoradiograph from region of arrow shown in Fig. 14. Granules are seen over the matrix of the epiphyseal plate. Hematoxylin-stained section. × 300.

FIG. 17. Microautoradiograph from region of arrow in Fig. 15. There are a few granules over what remains of the epiphyseal plate (above) and an intense image over the metaphyseal trabeculae below. Hematoxylin-stained section. × 300.

FIG. 18. Microautoradiograph from region of square shown in Fig. 15. The intense image seen in this zone results from the high activity in the cartilaginous cores of the bony trabeculae. Hematoxylin-stained section. × 300.

FIG. 19. Articular cartilage of distal femur from rabbit injected with 0.5 mg. of crude papain and sacrificed 2 days later. Metachromatic staining is absent in the articular cartilage but normal in the underlying trabeculae. Toluidine blue stain. × 70.

FIG. 20. Epiphyseal plate of distal femur from rabbit injected with 0.5 mg. of crude papain and sacrificed 2 days later. The cartilage is thin and devoid of basophilic staining. Hematoxylin and eosin stain. × 70.



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