TURNOVER OF THE ORGANIC MATRIX OF CARTILAGE AND BONE AS VISUALIZED BY AUTORADIOGRAPHY

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ABSTRACT

Tibiae and humeri were removed from suckling rats at intervals of time after intraperitoneal injection of C^{14}-L-phenylalanine, C^{14}-L-leucine, S^{35}-sulfate, or Ca^{45} Cl_2. Autoradiograms of sections of the bones were prepared. Ca^{45} was removed from sections treated with dilute acetic acid; neither the concentration of S^{55} nor that of C^{14} was thereby markedly decreased. The S^{35} was removed from the demineralized sections on incubation in a solution of testicular hyaluronidase; the C^{14} was not. These results are interpreted as indicating that most of the S^{35} was present in the bones as chondroitin sulfate and that most of the C^{14} in the bones was present as protein. In the epiphyses, the C^{14} was initially concentrated in the proliferating and hypertrophic chondrocytes, as was the S^{35}. Secretion of S^{35}- and C^{14}-labeled materials into the matrix followed. Thereafter, however, although the S^{35}-labeled material (chondroitin sulfate) persisted in the matrix, albeit at a diminished concentration, and was incorporated into metaphyseal bone, the C^{14}-labeled material (protein) was almost completely removed from the matrix. When rats were given repeated doses of 17-β-estradiol benzoate so as to inhibit resorption of their metaphyses, repeated doses of S^{35}-sulfate were discerned as strata of S^{35} in their metaphyses. This was not the case if the rats received repeated doses of C^{14}-L-phenylalanine or C^{14}-L-leucine. On the basis of the results in these experiments it is suggested that although a portion of the chondroitin sulfate produced by the chondrocytes of the epiphyseal plate is retained and becomes part of the cores of metaphyseal spicules of bone, the protein of the proteinpolysaccharide is somehow removed before calcification of the cartilage ensues.

Following the isolation of chondroitin sulfate-S^{35} (1, 2) from the cartilage of rats given S^{35}-labeled inorganic sulfate, autoradiographic studies showed that, at short intervals of time after administration, sulfur-35 was present predominantly in the chondrocytes and later in the matrix as well (3, 4). An attempt to determine whether the S^{35} visualized in the chondrocytes was present therein as chondroitin sulfate led to the conclusion that this was indeed the case (5). The labels from C^{14}-bicarbonate (6) and S^{35}-methionine (7) were also initially taken up by chondrocytes, wherein they were concentrated before being secreted into the matrix. Materials thus labeled were not removed from the cartilage on incubation with hyaluronidase, as was the case if S^{35}-sulfate was administered. Demineralization was equally ineffective. Consequently, the carbon-14 and sulfur-35 derived from bicarbonate and methionine, respectively, were considered to have been incorporated into components of the cartilage

other than chondroitin sulfate, probably proteins (8, 9).

Sylvén (10) observed that calcified cartilage did not stain metachromatically, and concluded that chondroitin sulfate was somehow removed from cartilage prior to calcification. Subsequently, however, it was suggested that chondroitin sulfate was still present in calcified cartilage but was not detectable on the basis of metachromasia because of the mineral; after demineralization of the tissue the metachromatic reaction could be elicited (11, 12). Additionally, Dziewiatkowski (13, 14) and Dziewiatkowski *et al.* (15, 16) demonstrated autoradiographically and chemically the presence of S^{35}-labeled components in metaphyseal trabeculae which were derived from the matrix of the epiphyseal plate; this material contained uronic acid and hexosamine and migrated as chondroitin sulfate when examined electrophoretically and chromatographically (15, 16). It was suggested, therefore, that chondroitin sulfate or materials related to it were somehow involved in calcification (16).

Chondroitin sulfate has been shown to be present in cartilage as part of macromolecules, so-called proteinpolysaccharides, in which the protein moiety is not collagen (17, 18). By the use of S^{35}-sulfate and lysine-1-C^{14}, Gross *et al.* (19) found that the protein moiety and chondroitin sulfate of the proteinpolysaccharide in costal cartilage of rats were catabolized at comparable rates. More recently, proteinpolysaccharides and collagen were isolated from slices of bovine costal cartilage which had been incubated in a buffered solution of salts containing either C^{14}-L-phenylalanine, C^{14}-L-leucine, or S^{35}-sulfate (20). The proteinpolysaccharides and collagen were thereby labeled, the more so the longer the slices were incubated. The rate at which the protein of the complex macromolecules was labeled with C^{14} paralleled the rate at which the chondroitin sulfate in the molecules was labeled with S^{35}. Autoradiograms of the cartilage slices revealed that most of the radioactivity, whether of C^{14} or of S^{35}, up to 4 hours of incubation, was primarily confined to the chondrocytes. The conclusion was that both the polysaccharide moiety and the protein were synthesized intracellularly and concurrently.

As an extension of the *in vitro* studies of the synthesis of proteinpolysaccharides by bovine costal cartilage, the same labeled materials, S^{35}-sulfate, C^{14}-L-leucine, and C^{14}-L-phenylalanine, were injected into young rats, and the disposition of the radioisotopes in their bones was examined autoradiographically in the hope of gaining further insight as to the role of the ground substance of cartilage in calcification. In addition, for comparative purposes, calcium-45 was used. The results of these experiments are described herein.

MATERIALS AND METHODS

Ten microcuries of C^{14}-L-phenylalanine[1] or C^{14}-L-leucine[1] were injected intraperitoneally into twenty-two 8- to 10-day-old suckling rats of the Sprague-Dawley strain. The average weight was 16 gm. Two or more rats from each group were killed by decapitation 4, 24, 48, 72, and 96 hours after injection. Comparable experiments were performed on 13-day-old rats (average weight 20 gm) into which 80 μc of carrier-free S^{35}-sulfate[2] or 15 μc of $Ca^{45}Cl_2$[2] were injected.

Rats at 21 days of age were segregated without regard to sex into three groups of 8 animals each. The average weight of the rats was 46 $\pm$ 14 gm. All the rats in each group received 2 mg of 17-β-estradiol benzoate[3] in 0.2 ml of corn oil[4] once a week for 4 weeks (15). At 24 hours after injection of hormone, each of the rats in one of the groups received 10 μc of C^{14}-L-phenylalanine intraperitoneally, those in the second group received 10 μc of C^{14}-L-leucine, and those in the third group received 120 μc of S^{35}-sulfate. At 4 and 24 hours after the last injection of the radioactive tracers, representative animals from each group were killed by decapitation.

Immediately after death, the humeri and tibiae were removed from the rats and fixed in 10 per cent formalin (*v/v*). The bones from rats injected with $Ca^{45}Cl_2$ were fixed for 48 hours in 80 per cent ethanol saturated with $MgCO_3$. The whole bones from suckling rats were thus fixed; those obtained from the estrogenized rats were split lengthwise before fixation. The tissues were then dehydrated in ethanol, starting with a 30 per cent solution, cleared in xylene, and embedded in paraffin (64°C mp). Sections were cut at a thickness of 7 μ and were mounted on albuminized slides.

Several sections of each bone were demineralized

[1] Uniformly labeled C^{14}-L-phenylalanine (10.2 mc/mM) and C^{14}-L-leucine (6.2 mc/mM) were purchased from Nuclear-Chicago Corporation.

[2] S^{35}-sulfate and $Ca^{45}Cl_2$ were obtained from the Oak Ridge National Laboratory.

[3] The 17-β-estradiol benzoate was purchased from Mann Research Laboratories.

[4] Mazola is the brand name for corn oil from Corn Products Company.

by immersion in a 4 per cent solution of acetic acid (*v/v*) for 30 minutes, pH 2.4. After thorough washing in distilled water, some of the demineralized sections were incubated for 3 hours at 37°C in a 0.1 per cent solution of testicular hyaluronidase[5] in 0.85 per cent NaCl (21). Control sections were incubated under identical conditions in a solution of NaCl. After incubation the sections were washed repeatedly in distilled water.

Autoradiograms were prepared as follows: contact (13) using Kodak Contrast Process Ortho Film, coated (22) using Kodak NTB_3 emulsion, and stripping film (23) using British Kodak AR-10 Scientific Plates. The exposure of the film or emulsion to the sections varied considerably depending on the radioactive tracer which had been used. With S^{35}- and Ca^{45}-labeled tissues, only a few days or weeks were required to obtain an adequate autoradiographic image. On the other hand, the emulsions had to be exposed for months to the C^{14}-labeled bones in order to produce images of an intensity comparable to those obtained with S^{35} and Ca^{45}.

Subsequent to autoradiography, histological sections which had been used to produce the contact autoradiograms were stained with toluidine blue (13). The sections covered with liquid emulsion or stripping film were not stained. Photomicrographs of the latter sections were prepared by the use of a Leica camera attached to a Leitz Ortholux microscope fitted with phase contrast optics. Photographs of contact autoradiograms were obtained as follows: The original autoradiograms were placed in an enlarger and printed on Kodak Lantern Slide Plates. The plates were then used as negatives to produce prints.

Autoradiographic reactions that were given by the C^{14}-L-phenylalanine and C^{14}-L-leucine were qualitatively identical. As a result, anything that is said about one applies equally to the other.

RESULTS AND DISCUSSION

The changes in the distribution of radioactivity in the tibiae of rats given C^{14}-L-phenylalanine, S^{35}-sulfate, or Ca^{45}, during the interval 24 to 72 hours after injection, are shown in Fig. 1. The already familiar characteristic distributions of S^{35} (3, 13), Fig. 1 *d* to *f*, and Ca^{45} (24, 25), Fig. 1 *g* to *i*, differ in many respects from that of the C^{14}-L-phenylalanine, Fig. 1 *a* to *c*. The concentration of C^{14} is greater in the periosteal region than in the epiphyseal plate; the converse relationship is seen after S^{35}-sulfate. In either case, under higher magnification, at 24 hours after injection the radioactive elements were seen just beneath and in the periosteum. 48 hours later they were at a greater distance from the periosteum. Similar observations have been reported by Greulich (8), who used C^{14}-bicarbonate, and by Bélanger (3, 9), who used S^{35}-sulfate and S^{35}-methionine. The apparent "migration" of the labeled components of the diaphyses toward the endosteum is the result of appositional growth in which relatively non-radioactive bone is laid down adjacent to the periosteum (8).

A relatively high concentration of C^{14} from phenylalanine (or leucine) was made out in the hemopoietic tissue of the medullary cavity at 24 hours after injection (Fig. 1 *a*). Thereafter the concentration of C^{14} in the medullary cavity decreased; by 72 hours (Fig. 1 *c*), the concentration of C^{14} in the diaphyses was greater than that in the hemopoietic tissue. A comparable uptake of S^{35} or Ca^{45} by components in the medullary cavity was not seen (Fig. 1 *d* and *g*).

In the metaphyses the C^{14} of the amino acids was deposited so that at 4 hours after injection the greatest concentration was in the region abutting on the epiphyseal plate (Fig. 2 *a*, arrow). This stratum of C^{14} was subsequently displaced toward the medullary cavity (Fig. 2 *b* and *c*, arrows), so that by 72 hours it was no longer seen, except in the diaphysis in the region of incorporated metaphysis (Fig. 2 *d*, arrow). These observations in regard to C^{14} of the amino acids are to some extent reminiscent of those made by Bronner (26) after the administration of Ca^{45} to young rats. However, since on immersion of the sections in dilute acetic acid the Ca^{45} was removed completely but the C^{14} was not, most of the C^{14} seen in the metaphyses is considered to be part of the organic components instead of the mineral. Greulich (8), too, observed a similar phenomenon in day-old rats given C^{14}-bicarbonate, except that in these very young rats the band of reactivity progressed down the spicules more rapidly. In view of the fact that osteoblasts in large number are present in the subepiphyseal region of the metaphyses, as is also the case subperiosteally, it may be that the C^{14}-labeled materials visualized in both loci shortly after administration of the C^{14}-labeled amino acids are products elaborated by the osteoblasts.

C^{14} was also visualized in the epiphyses. Here, at 4 hours after injection, more C^{14} was present in the region of the proliferative and hypertrophic

[5] Bovine testicular hyaluronidase (300 U.S.P. units per mg) was purchased from Worthington Biochemical Corporation.

chondrocytes than in the region of greatly enlarged and degenerate chondrocytes (Fig. 2 *f*, *a*). Similar distributions of S^{35} from methionine (27), H^3 from histidine (28), and C^{14} from proline (29) have been reported. From the series of autoradiograms in Fig. 2 *a* through *e*, it is apparent that subsequently, up to 96 hours after injection, although the concentration of C^{14} in the epiphyseal plate in the region of the proliferative and hypertrophic chondrocytes did decrease, there was concurrently no striking increase in the concentration of C^{14} in the region of the degenerate chondrocytes. Furthermore, there was no increase in the concentration of C^{14} subepiphyseally in the interval of time 24 to 96 hours. In contrast, previous observations (3, 4) have indicated that S^{35} of inorganic sulfate, although present in the region of the hypertrophic chondrocytes shortly after administration, was later found also in high concentration in the region of the degenerate chondrocytes (compare Fig. 1 *a* to *c* with *d* to *f*), and by 72 hours it could be made out subepiphyseally in the spicules of bone of the metaphyses (Fig. 1 *f*).

Very little C^{14} was seen in and around the degenerate chondrocytes in the regions of developing secondary ossification centers at 4 hours. In the mature, hypertrophic cells, and in the matrix surrounding them, however, the C^{14} from the amino acids was concentrated (Fig. 2 *a*). In more fully developed ossification centers (Fig. 2 *b* to *e*), a relatively high concentration of C^{14} was seen; the C^{14} was present chiefly in the blood cells of the ossification center.

The region of the perichondral collar, from which chondrocytes may originate (30), and which is coextensive with the band of proliferative and hypertrophic chondrocytes in the epiphyseal plate, also accumulated much C^{14}, indeed, even more than the proliferative and hypertrophic chondrocytes (Fig. 2 *a*).

From observation at a higher magnification, 24 hours after injection of S^{35}-sulfate, S^{35} was seen in the matrix and in the proliferating and hypertrophic chondrocytes (Fig. 3 *a*). Within the following 48 hours the proliferating cells should become degenerate cells (31). An examination of the region of degenerate cells at this time, 72 hours after injection of S^{35}-sulfate, revealed that almost all of the S^{35} had been secreted into the matrix (Fig. 3 *b*). However, the concentration of S^{35} in the septa of the matrix around the degenerate cells (Fig. 3 *b*) was not so great as that in the matrix around the hypertrophic cells 48 hours

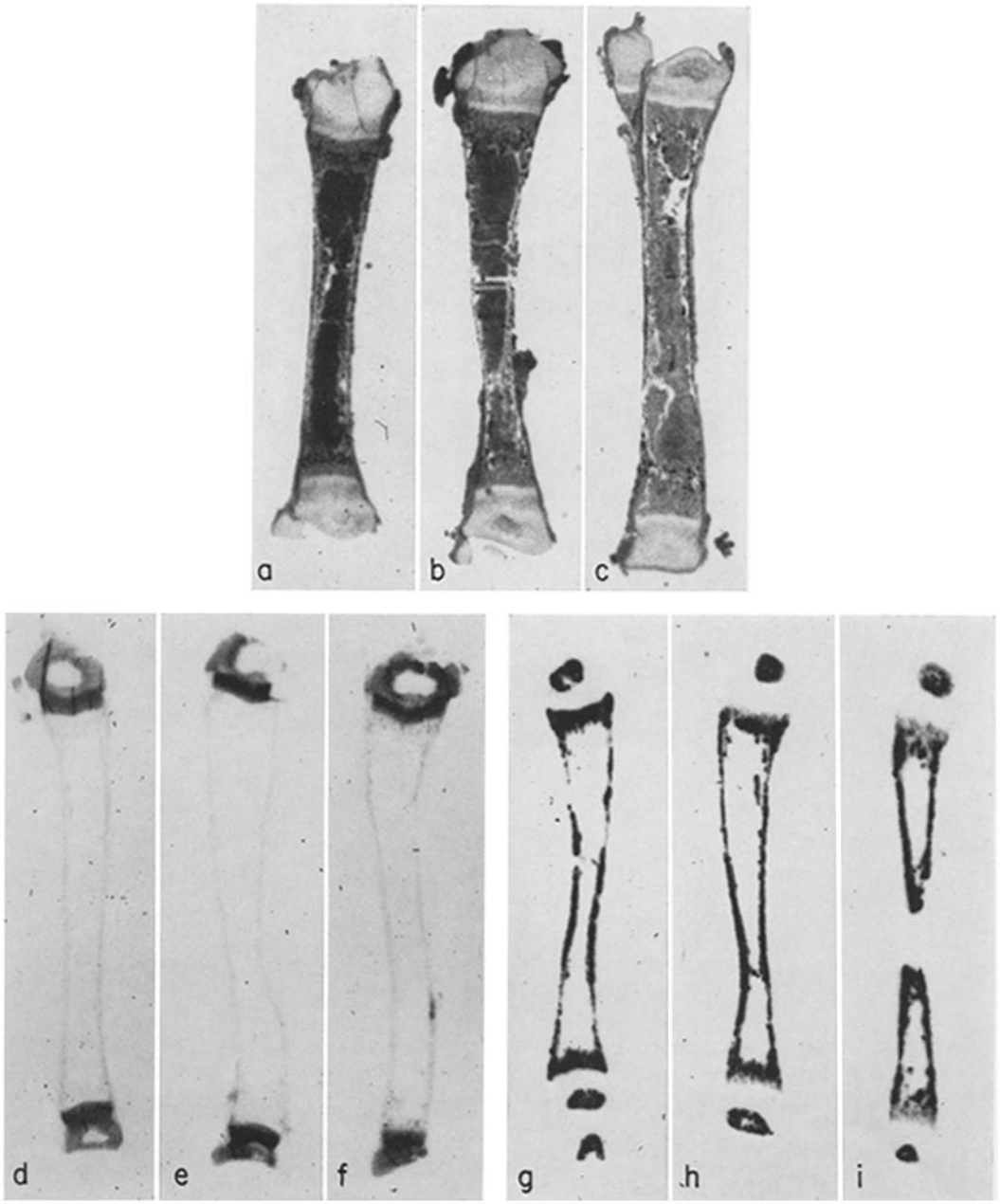
FIGURE 1

Contact autoradiograms of undemineralized sections of tibiae from suckling rats. The consecutive sets of letters refer to sacrifice at 24, 48, and 72 hours after injection of radioactive material.

a to *c*. The rats at 8 to 10 days of age received 10 μc of C^{14}-L-phenylalanine intraperitoneally. A persistent difference in the concentration of C^{14} in different regions of the epiphyseal plate can be seen. Here the C^{14} was concentrated at some distance from the metaphysis. Although the concentration of label subsequently decreased in this region, it did not increase perceptibly in the plate adjacent to the metaphysis. At all times the concentration of C^{14} in the periosteal region was greater than that in the epiphyses. The materials of the marrow were initially, relatively to other regions of the bone, intensely labeled. By 72 hours, however, the concentration of C^{14} in the region of the periosteum was greater than that in the medullary cavity. Comparable autoradiograms were produced by sections which were demineralized in dilute acetic acid and then incubated in a solution of hyaluronidase. × 5.

d to *f*. The rats at 13 days of age received 80 μc of S^{35}-sulfate intraperitoneally. In contrast to the results after C^{14}-labeled amino acids, the entire epiphyseal plate was labeled. At 72 hours after injection some of the S^{35} was discernible in the metaphyses. Comparable autoradiograms were obtained when sections demineralized in dilute acetic acid were used. If, in addition, the sections were incubated in a solution of testicular hyaluronidase, the S^{35} was removed. × 4.

g to *i*. The rats at 13 days of age received 15 μc of $Ca^{45}Cl_2$ intraperitoneally. All mineralized regions of the bones were thereby labeled. With time, the concentration of Ca^{45} in the region of the metaphysis adjacent to the cartilage plate decreased. On treatment of the sections with dilute acetic acid, the Ca^{45} was completely removed. × 4.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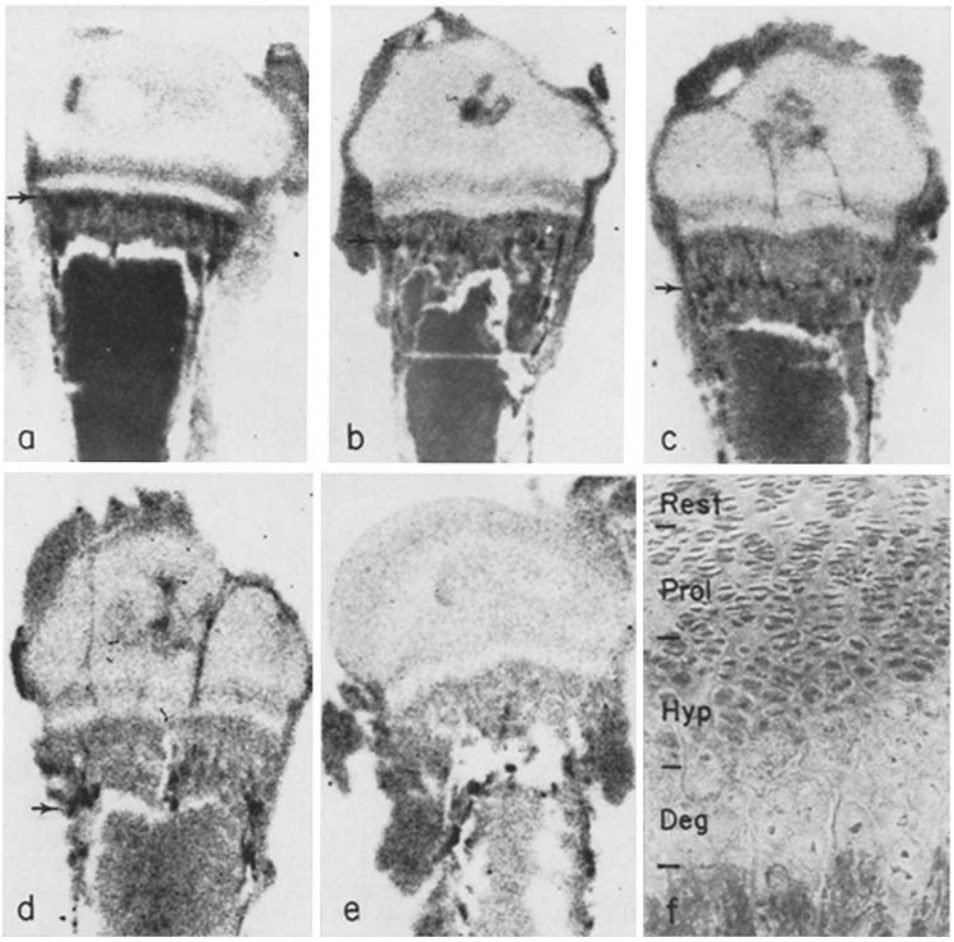


FIGURE 2

a to *e*. Contact autoradiograms of demineralized sections of the proximal ends of humeri from suckling rats (*a*) 4 hours, (*b*) 24 hours, (*c*) 48 hours, (*d*) 72 hours, and (*e*) 96 hours after intraperitoneal injection of C^{14}-L-phenylalanine. A band of C^{14} in the metaphysis, just underneath the epiphyseal plate, can be seen in *a*. Later (*b* and *c*) it is seen at a distance from the epiphyseal plate. The band in question is indicated by arrows. It can also be seen that a relatively unreactive zone of the epiphyseal plate, a zone of very hypertrophic and degenerate chondrocytes, does not markedly increase in reactivity with time. The highest concentration of C^{14} in the epiphyseal plate was initially in the zone of the proliferating and hypertrophic chondrocytes. With time, the concentration of C^{14} did decrease in this zone. × 9.

f. A coated autoradiogram of the epiphyseal plate of a humerus removed 4 hours after injection of C^{14}-L-phenylalanine. It can be seen that at this time the highest concentration of C^{14} was present in the proliferating and mature, well hypertrophied chondrocytes. This autoradiogram is shown to delineate the position of the band of C^{14} across the epiphyses as seen in Fig. 2 *a* through *e*. As the concentration of C^{14} in this region of the epiphyseal plate decreased with time, there was no marked increase in the concentration of C^{14} in the region of very hypertrophic and degenerate chondrocytes. × 86.

previously (Fig. 3 *a*). This may be taken as suggesting that a portion of the chondroitin sulfate had been somehow removed from the cartilage. A similar suggestion that there may be a decrease in the concentration of chondroitin sulfate has been made by Bélanger (3) on the basis of his autoradiographic study.

In the epiphyses of humeri and tibiae of rats, 24 hours after the administration of C^{14}-labeled amino acids, C^{14} also was seen in the proliferating and hypertrophic chondrocytes and in the matrix around them (Fig. 3 *c*). In contrast, however, to the results obtained by the use of S^{35}-sulfate, within the following 48 hours most of the C^{14} was removed from the matrix but was still present in the degenerate chondrocytes (Fig. 3 *d*). One suspects that, at the time the C^{14}-labeled amino acids were presented to the animals, these degenerate cells were young and were actively synthesizing not only components of the matrix but cytoplasmic proteins as well. Such structural, cellular proteins with the C^{14} in them then persisted in the cells.

The chondrocytes around secondary centers of ossification undergo changes similar to those shown by cells in rows within the epiphyseal plate; they become, in turn, hypertrophic and degenerate (32). The disposition of S^{35} and C^{14} around secondary centers of ossification was likewise found to be similar to that seen in the epiphyseal plate. Both S^{35} and C^{14} were present in the proliferating and hypertrophic cells and in the matrix around them 24 hours after administration (Fig. 3 *d*). After 72 hours, however, a difference was again made out: whereas almost all of the S^{35} was in the matrix around degenerate cells, C^{14} was almost exclusively present in the cells of this zone (Fig. 3 *e*). The suggestion made above, that most of the C^{14}-labeled components elaborated in the cells and then secreted into the matrix are somehow removed from the matrix, is reinforced.

The above observations on the turnover of the C^{14} of the amino acids in the epiphyseal cartilage differ from those reported by Greulich (8) as regards the C^{14} of bicarbonate, in that he found the cartilage cells of the epiphyses to be devoid of C^{14} at 72 hours after injection. Instead, the C^{14} was seen only in the matrix at this time, including that around the degenerate chondrocytes. The reason for this discrepancy is not apparent.

On treatment of the sections of bone with dilute acetic acid, although Ca^{45} was removed as one would expect, most of the S^{35} and C^{14} was not removed. On incubation of the demineralized sections in a solution of hyaluronidase, the S^{35} was removed but not the C^{14}. These results suggest that the S^{35} was a part of chondroitin sulfate A or C but that the C^{14} was not. The C^{14}, as phenylalanine and leucine or derivatives thereof, was probably incorporated into the protein moiety of proteinpolysaccharides and collagen, as well as into structural cytoplasmic proteins. Support for this inference derives from the fact that proteinpolysaccharides and collagen, labeled with these amino acids, have been isolated from bovine costal cartilage which had been incubated in media containing them (20) and that proteinpolysaccharides have been isolated from the epiphyses of rats into which these labeled compounds were injected (33). Bone, too, has been shown to contain protein (34) as well as sulfated mucopolysaccharides (15, 16, 35).

To verify or disprove the suggestion stemming from the examination of autoradiograms of bones from suckling rats given C^{14}-labeled amino acids, namely, that most if not all C^{14}-labeled materials elaborated by the chondrocytes of the epiphyseal plate and secreted into the matrix were removed from the matrix before it was calcified, experiments with estrogenized rats were undertaken. The resorption of the metaphyses in weanling rats is inhibited if they are given massive doses of estradiol benzoate repeatedly. If, into rats so treated, S^{35}-sulfate is also repeatedly injected, each dose of the S^{35} can be visualized autoradiographically as a stratum in the metaphyses at those ends of bones which are growing in length. This is shown in Fig. 4 *a*. These strata are a reflection of the chondroitin sulfate which was synthesized in the epiphyseal plate shortly after each dose of S^{35}-sulfate was administered. The S^{35}, as seen in such autoradiograms of bones from estrogenized rats, is not removed to an appreciable extent on demineralization with dilute acetic acid. The S^{35} is, however, removed from demineralized sections of the bones on incubation in a solution of testicular hyaluronidase. These results are in accord, therefore, with the finding that most of the S^{35} thus deposited in the metaphyses of estrogenized rats is part of chondroitin sulfate or material akin to it (16).

In the concurrent experiments, in which C^{14}-phenylalanine or C^{14}-leucine was administered to estrogenized rats, strata of C^{14}-labeled material,

which might have originated in the epiphyseal plate, comparable to those of S^{35} were not seen in the metaphyses (Fig. 4 *b*). One possible explanation of these results is that, although the chondrocytes of the epiphyseal plate synthesize proteinpolysaccharides (20, 33) and secrete them into the matrix, the protein is somehow removed before the chondroitin sulfate, in part at least, is incorporated into spicules of metaphyseal bone. Since practically no C^{14} was seen in the matrix around degenerate chondrocytes, as noted above, this is a most probable explanation. There is, however, an alternative possibility: since the concentration of C^{14} in the metaphyses and bone marrow, at all times after administration, was many times greater than that in the epiphyseal plate, any C^{14}-labeled protein originating in the epiphyseal plate might not have been discerned in the metaphyses. As a check on this latter possibility, the isolation of proteinpolysaccharides from the metaphyses of the estrogenized and suckling rats, given C^{14}-labeled amino acids or S^{35}-sulfate, was undertaken by methods previously described (20). Although the procedure was successful when the epiphyseal cartilage of the suckling rats was used, no proteinpolysaccharides were isolated from the samples of metaphyseal bone. Admittedly the samples were small. Because of this, further attempts were made using large pools of the metaphyses from estrogenized rats which had not received isotopically labeled compounds, from the metaphyses of suckling rats, and from the metaphyses of calves. All attempts were equally unsuccessful, even if the samples were demineralized by prior dialysis against versene, an acetate buffer, or by electrodialysis. Instead, materials resembling chondroitin sulfate were obtained.

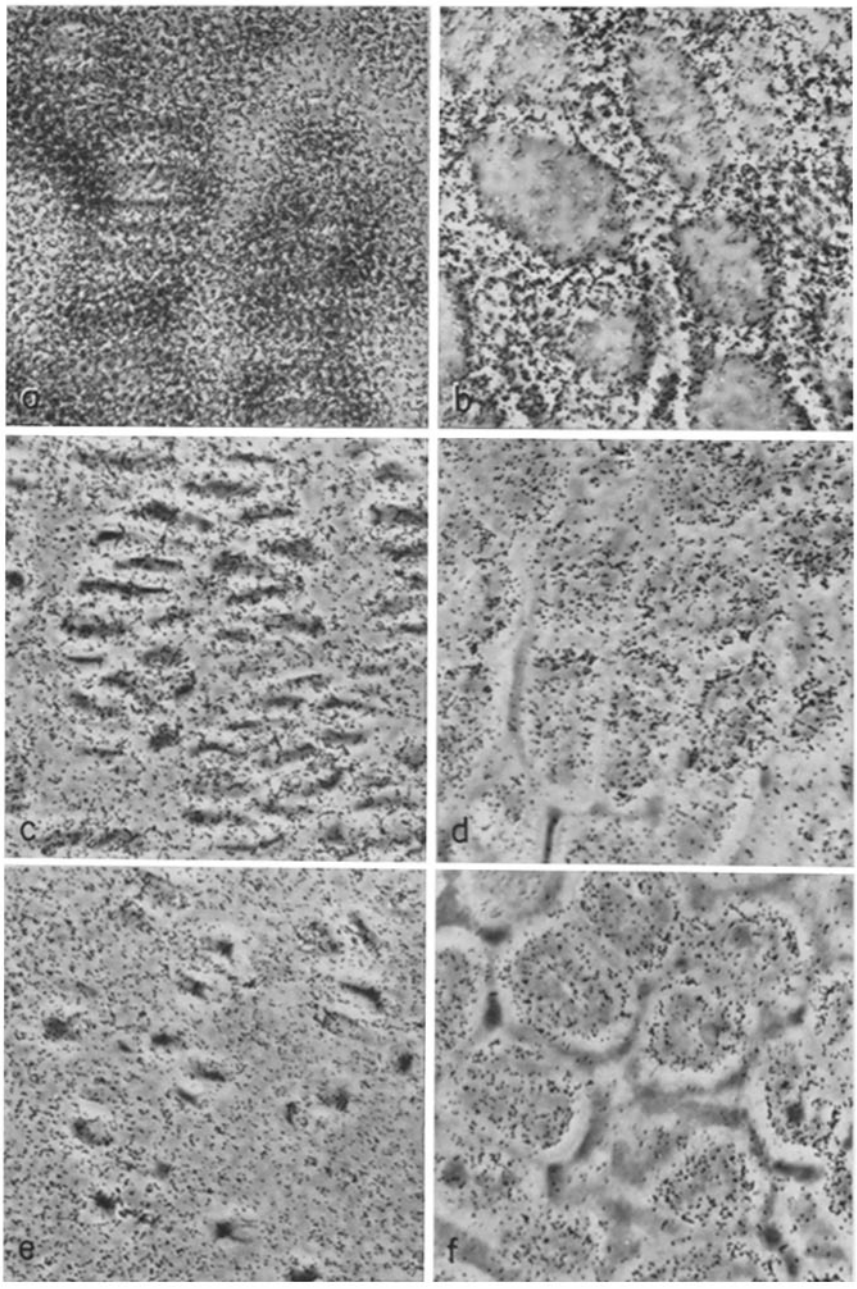
Since in the suckling rats C^{14} was found in high concentration subepiphyseally (Fig. 2 *a*), within 4 hours of the administration of C^{14}-labeled amino acids, a similar stratum of C^{14} was sought in the metaphyses of the estrogenized rats. No such evidence for doses other than the last was seen. A slightly greater concentration of C^{14} in the region of the metaphyses adjacent to the epiphyseal plate than in the rest of the metaphyses was discerned (Fig. 4 *b*), whether the bones were removed at 4 or 24 hours after the last dose of isotope. This stratum of C^{14}, however, was not displaced away from the epiphyseal plate at 24 hours as was seen in the suckling rats, possibly because the estrogenized rats were growing less rapidly. The rate of displacement is very likely a reflection of endochondral growth. As noted above, the distance over which such a band of C^{14} activity was displaced in the metaphyses of 10-day-old rats was shorter than the distance in 1-day-old rats (8). Additionally, estradiol benzoate may have promoted the maturation of the epiphyseal plate of the proximal end of the tibia beyond the stage normal for animals of this age. In rats given repeated massive doses of estradiol benzoate the epiphyseal plate at the proximal end of the tibia was found to be narrower than the plate in untreated littermates; it was like that of much older animals (15).

A compensatory change in the volume of matrix between chondrocytes occurs as the chondrocytes become hypertrophic: the matrix is elongated and stretched into thinner septa. To account for this alteration in the matrix, Dodds (36) proposed that either new material was added to the matrix or there was a rearrangement of the existing matrix. Jackson (37) found that the chondrocytes of avian epiphyses become hypertrophic in a matrix whose content of hexosamine has been reduced. As evidenced in the present experiments, the continuing hypertrophy of chondrocytes in rat epiphyses is accompanied by a decrease in the concentration of chondroitin sulfate and protein in the matrix.

Calcifiability of cartilage has been ascribed to

FIGURE 3

Coated autoradiograms of regions in the epiphyseal plates of humeri removed (*a*) 24 hours and (*b*) 72 hours after S^{35}-sulfate, and (*c*) 24 hours and (*d*) 72 hours after C^{14}-L-phenylalanine. Similarly prepared autoradiograms of regions around secondary centers of ossification in humeral epiphyses removed at 24 hours and 72 hours after C^{14}-L-phenylalanine are shown as *e*) and *f*), respectively. At 24 hours after injection, S^{35} and C^{14} can be seen in the proliferating and maturing cells as well as in the matrix surrounding them (*a*, *c*, and *e*). At 72 hours after injection, in the zone of degenerate cells, the S^{35} is present predominantly in the matrix (*b*), but the C^{14} is almost entirely localized intracellularly (*d* and *f*). Photographed using phase contrast optics. × 620.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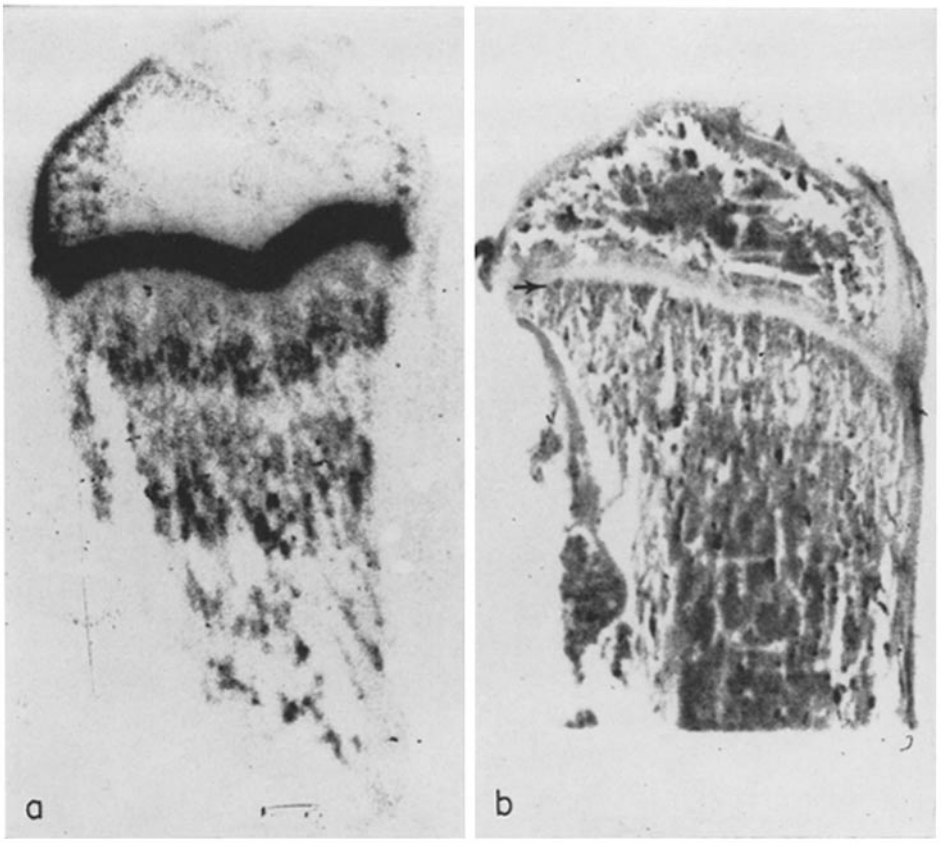

FIGURE 4

Reproductions of contact autoradiograms of demineralized sections of the proximal ends of tibiae from rats into which 17-β-estradiol benzoate, 2 mg each time, was injected at weekly intervals for 4 weeks. 24 hours after each dose of steroid the rats received (*a*) 120 μc of S^{35}-sulfate or (*b*) 10 μc of C^{14}-L-phenylalanine. They were sacrificed 24 hours after the last dose of radioactive materials. It can be seen in *a* that repeated doses of S^{35}-sulfate are reflected as strata of S^{35} in the metaphyses. Similar strata of C^{14}-labeled material, which might have originated in the epiphyseal plate and therefore at distances from the epiphyseal plate comparable to the distances at which the strata of S^{35} are located, were not seen in the metaphyses (*b*). The arrow in *b* is intended to draw attention to the relatively higher concentration of the C^{14} in the region of the metaphysis abutting on the epiphyseal plate than in the rest of the metaphysis. This zone of reactivity may be due to the activity of osteoblasts. ×8.

changes in the ground substance, the matrix. Mineralization has been associated with a loss of sulfate (38), the degree of polymerization of components in the matrix (39), and the extent to which the matrix is hydrated (40). Chondroitin sulfate has been suggested as a local factor which promotes calcification (41). Alternatively, this polysaccharide, or the protein moiety of protein-polysaccharides, has been considered as a possible inhibitor of calcification (42). The answer cannot as yet be detailed. The observations herein reported, however, do support the thesis that the cartilage matrix is altered before mineral can be deposited in and on it.

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