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

ROBERT D. CAMPO, Ph.D., and DOMINIC D. DZIEWIATKOWSKI, Ph.D.

From The Rockefeller Institute. Dr. Campo's present address is the Department of
Biochemistry, Temple University School of Medicine, Philadelphia

ABSTRACT

Tibiae and humeri were removed from suckling rats at intervals of time after intraperitoneal
injection of C14-L-phenylalanine, C14-L-leucine, S35-sulfate, or Ca4 C12. Autoradiograms of
sections of the bones were prepared. Ca4 was removed from sections treated with dilute
acetic acid; neither the concentration of S35 nor that of C14 was thereby markedly decreased.
The S35 was removed from the demineralized sections on incubation in a solution of testicu-
lar hyaluronidase; the C14 was not. These results are interpreted as indicating that most of
the S35 was present in the bones as chondroitin sulfate and that most of the C14 in the bones
was present as protein. In the epiphyses, the C14 was initially concentrated in the prolifera-
ing and hypertrophic chondrocytes, as was the S35. Secretion of S35- and C14-labeled materials
into the matrix followed. Thereafter, however, although the S35-labeled material (chondroi-
tin sulfate) persisted in the matrix, albeit at a diminished concentration, and was incorpo-
rated into metaphyseal bone, the C14-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 S35-sulfate were discerned
as strata of S35 in their metaphyses. This was not the case if the rats received repeated doses
of C14-labeled phenylalanine or C14-labeled 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 protein-polysaccharide is somehow removed before calcification of
the cartilage ensues.

Following the isolation of chondroitin sulfate-S35
(1, 2) from the cartilage of rats given S35-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 S35 visualized
in the chondrocytes was present therein as
chondroitin sulfate led to the conclusion that this
was indeed the case (5). The labels from C14-
bicarbonate (6) and S35-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 S35-sulfate was
administered. Demineralization was equally
ineffective. Consequently, the carbon-14 and
sulfur-35 derived from bicarbonate and methi-
onine, respectively, were considered to have been
incorporated into components of the cartilage
synthesis of protein and polysaccharides by bovine costal cartilage, the same labeled materials, concurrently.

As an extension of the in vitro studies of the synthesis of protein polysaccharides 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	extsuperscript{1} or C^{14}-L-leucine	extsuperscript{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 mc of carrier-free S^{35}-sulfate	extsuperscript{2} or 15 mc of Ca^{45}Cl_{2}	extsuperscript{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 ± 14 gm. All the rats in each group received 2 mg of 17-β-estradiol benzoate	extsuperscript{3} in 0.2 ml of corn oil	extsuperscript{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 mc of C^{14}-L-phenylalanine intraperitoneally, those in the second group received 10 mc of C^{14}-L-leucine, and those in the third group received 120 mc 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 sucking 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 μm and were mounted on albuminized slides.

Several sections of each bone were demineralized

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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.
 Autoradiograms were prepared as follows: contact (13) using Kodak Contrast Process Ortho Film, coated (22) using Kodak NTB3 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 S35 and Ca45-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 Ca45-labeled bones in order to produce images of an intensity comparable to those obtained with S35 and Ca45.

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 C14-l-phenylalanine and C14-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 C4-L-phenylalanine, S35-sulfate, or Ca45, during the interval 24 to 72 hours after injection, are shown in Fig. 1. The already familiar characteristic distributions of S35 (3, 13), Fig. 1 d to f, and Ca45 (24, 25), Fig. 1 g to i, differ in many respects from that of the C14-L-phenylalanine, Fig. 1 a to c. The concentration of C14 is greater in the periosteal region than in the epiphysial plate; the converse relationship is seen after S35-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 C45-bicarbonate, and by Bélanger (3, 9), who used S35-sulfate and S35-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 C14 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 C14 in the medullary cavity decreased; by 72 hours (Fig. 1 c), the concentration of C14 in the diaphyses was greater than that in the hemopoietic tissue. A comparable uptake of S35 or Ca45 by components in the medullary cavity was not seen (Fig. 1 d and g).

In the metaphyses the C14 of the amino acids was deposited so that at 4 hours after injection the greatest concentration was in the region abutting on the epiphysial plate (Fig. 2 a, arrow). This stratum of C14 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 C14 of the amino acids are to some extent reminiscent of those made by Bronner (26) after the administration of Ca45 to young rats. However, since on immersion of the sections in dilute acetic acid the Ca45 was removed completely but the C14 was not, most of the C14 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 C45-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 subepiphysial region of the metaphyses, as is also the case subperiosteally, it may be that the C14-labeled materials visualized in both loci shortly after administration of the C45-labeled amino acids are products elaborated by the osteoblasts.

C14 was also visualized in the epiphyses. Here, at 4 hours after injection, more C14 was present in the region of the proliferative and hypertrophic...
chondrocytes than in the region of greatly enlarged and degenerate chondrocytes (Fig. 2 f, a). Similar distributions of S\textsuperscript{35} from methionine (27), H\textsuperscript{3} from histidine (28), and C\textsuperscript{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\textsuperscript{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\textsuperscript{14} in the region of the degenerate chondrocytes. Furthermore, there was no increase in the concentration of C\textsuperscript{14} subepiphysally in the interval of time 24 to 96 hours. In contrast, previous observations (3, 4) have indicated that S\textsuperscript{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 subepiphysally in the spicules of bone of the metaphyses (Fig. 1 f).

Very little C\textsuperscript{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\textsuperscript{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\textsuperscript{14} was seen; the C\textsuperscript{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\textsuperscript{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\textsuperscript{35}-sulfate, S\textsuperscript{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\textsuperscript{35}-sulfate, revealed that almost all of the S\textsuperscript{35} had been secreted into the matrix (Fig. 3 b). However, the concentration of S\textsuperscript{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 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 C14-L-phenylalanine. A band of C14 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 C14 in the epiphyseal plate was initially in the zone of the proliferating and hypertrophic chondrocytes. With time, the concentration of C14 did decrease in this zone. X 9.

f. A coated autoradiogram of the epiphyseal plate of a humerus removed 4 hours after injection of C14-L-phenylalanine. It can be seen that at this time the highest concentration of C14 was present in the proliferating and mature, well hypertrophied chondrocytes. This autoradiogram is shown to delineate the position of the band of C14 across the epiphyses as seen in Fig. 2 a through e. As the concentration of C14 in this region of the epiphyseal plate decreased with time, there was no marked increase in the concentration of C14 in the region of very hypertrophic and degenerate chondrocytes. X 86.
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 C14-labeled amino acids, C14 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 S35-sulfate, within the following 48 hours most of the C14 was removed from the matrix but was still present in the degenerate chondrocytes (Fig. 3 d). One suspects that, at the time the C14-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 C14 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 S35 and C14 around secondary centers of ossification was likewise found to be similar to that seen in the epiphyseal plate. Both S35 and C14 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 S35 was in the matrix around degenerate cells, C14 was almost exclusively present in the cells of this zone (Fig. 3 e). The suggestion made above, that most of the C14-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 C14 of the amino acids in the epiphyseal cartilage differ from those reported by Greulich (8) as regards the C14 of bicarbonate, in that he found the cartilage cells of the epiphyses to be devoid of C14 at 72 hours after injection. Instead, the C14 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 Ca45 was removed as one would expect, most of the S35 and C14 was not removed. On incubation of the demineralized sections in a solution of hyaluronidase, the S35 was removed but not the C14. These results suggest that the S35 was a part of chondroitin sulfate A or C but that the C14 was not. The C14, 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 C14-labeled amino acids, namely, that most if not all C14-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, S35-sulfate is also repeatedly injected, each dose of the S35 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 S35-sulfate was administered. The S35, as seen in such autoradiograms of bones from estrogenized rats, is not removed to an appreciable extent on demineralization with dilute acetic acid. The S35 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 S35 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 C14-phenylalanine or C14-leucine was administered to estrogenized rats, strata of C14-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 C14-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 sucking 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
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 S³⁵-sulfate or (b) 10 μc of C¹⁴-L-phenylalanine. They were sacrificed 24 hours after the last dose of radioactive materials. It can be seen in a that repeated doses of S³⁵-sulfate are reflected as strata of S³⁵ in the metaphyses. Similar strata of C¹⁴-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 S³⁵ 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 C¹⁴ 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. X8.

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