THE NUTRITION OF MATURE AND IMMATURE CARTILAGE IN RABBITS

An Autoradiographic Study

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The fact that articular cartilage contains no blood vessels has been recognised for over 200 years, and for the whole of this period speculation and controversy have surrounded the problem of how this tissue obtains its nutrition. Opinion has been divided on the relative importance of the two most obvious routes: diffusion of joint fluid from the articular aspect, and transudation from the subchondral vessels on the deep face.

The importance of the former route is suggested by the fact that healthy cartilage may be found in articular loose bodies, and that in pathological conditions apparently well nourished cartilage may overlie avascular bone. Conversely, any interference with articular function may lead to regressive changes in the cartilage. These clinical observations are backed up by a wealth of experimental evidence, so that today there must be few who would deny the possibility that at least some nutrition is obtained in this way. The heart of the controversy is the extent to which this source is supplemented by the subchondral route, if at all.

Much of the disagreement about the importance of this latter route is based on the interpretation of the anatomical arrangements at the bone-cartilage interface. Opinions range from the view of Collins (1949) that the deeper calcified layers of the articular cartilage offer a complete barrier to fluid exchange, to the claims of Holmdahl and Ingelmark (1950) that a well developed system of canaliculi exists in this region for this very purpose.

Such a conflict on the anatomical evidence can only be resolved by experiments designed to demonstrate directly the transfer of materials from the subchondral bone to the articular cartilage, and this was done by Ingelmark and Saaf in 1948. They injected finely divided rice particles into the marrow spaces of long bones and were later able to identify these in the articular cartilage. A more physiological version of this experiment was later performed by Ekholm (1951) using radioisotopes. He infused $^{32}$P and $^{198}$Au into the upper tibial epiphysis of rabbits and by comparing this with the effects of intravenous injection was able to demonstrate the direct transfer of isotope from bone to cartilage. He finally concluded that cartilage could obtain its nutrition from both the synovial and the subchondral routes. Similar experiments were performed by Brodin (1955) using a fluorescent marker with identical results.

It is difficult therefore to reconcile all the available evidence. If subchondral nutrition is as important as the above experiments suggest, it is difficult to explain the healthy cartilage which is often seen over areas of avascular necrosis (Bobechko and Harris 1960, Ham and Leeson 1961, Catto 1965). A possible solution is suggested by the finding that subchondral infarction in an immature joint, in contrast to the effect in the adult, leads to a very rapid and marked degeneration in the overlying cartilage (McKibbin and Holdsworth 1966). The anatomical arrangements in these two circumstances are quite different, for while in the adult joint the controversial layer of calcification is well developed, in the immature animal there is no such zone, the bone-cartilage interface being characterised by the vascular invasion which is part of the process of endochondral ossification. The question of subchondral nutrition therefore must be given separate consideration in the mature and the immature animal.

There is no unequivocal evidence in the literature that the transfer of fluid from subchondral bone to cartilage has ever been demonstrated in fully mature animals. Neither
Ingelmark and Saaf (1948) nor Ekholm (1951, 1955) made any statement about the maturity of their animals, although one of the illustrations which Ekholm used to explain the technique of infusion showed a growth plate to be present, so that at least one of the animals was immature. Brodin (1955) was more specific and stated that all his rabbits were only four weeks old. Rabbits continue to grow for a considerable proportion of their life span and special care has to be taken to ensure that animals which on grounds of size, age and sexual maturity might be thought to be fully adult, have in fact complete maturity of the skeleton. The only experimental work in which a clear distinction was drawn between the two types of cartilage is that of Maroudas, Bullough, Swanson and Freeman (1968) in which permeability studies were carried out in mature and immature human cartilage. It was found that penetration from the subchondral bone could be demonstrated only in the child. These studies were, however, carried out in vitro on dead cartilage.

In view of this uncertainty it was decided to conduct experiments based on those of Ekholm (1951, 1955), using two distinct groups of animals, the one being undoubtedly mature with a well developed zone of basal calcification, the other being equally certainly immature. $^{35}$S was substituted for $^{32}$P and $^{14}$C because of the lower energy of its beta radiation.

**MATERIALS AND METHOD**

$^{35}$S was slowly infused into the bone immediately subjacent to the joint cartilage of the rabbit's tibia, and autoradiographs of this cartilage were later prepared to demonstrate whether or not it had been penetrated by the isotope. These were compared with autoradiographs prepared from the cartilage of the contralateral joint where any isotope present could only have arrived via the general circulation.

*Animals*—Rabbits of Dutch breed were used, fourteen mature and fourteen immature. The mature animals were all over one year old but the criteria of maturity were the subsequent histological demonstration of an absent growth plate and a well developed "tide mark" of calcification in the deeper parts of the cartilage. The immature animals were fourteen to sixteen weeks old. The growth plate was present, and active endochondral ossification was occurring at the junction of the bone and the joint cartilage.

*Isotope*—$^{35}$S as an aqueous solution of sodium sulphate was obtained from the radiochemical centre at Amersham.

*Autoradiographic film*—Kodak AR 50 (coarse grain) and AR 10 (fine grain) stripping film were used.

*Anaesthesia*—Each animal was anaesthetised with urethane 1 gramme per kilogram intraperitoneally, supplemented with intravenous nembutal when required.

*Infusion*—Into the right proximal tibial epiphysis of twelve immature animals, or the corresponding subchondral bone of twelve mature animals, a 21-gauge stiletted needle was placed, using radiographic control. The stilette was withdrawn and 5 millicuries of $^{35}$S, a volume of 1-1.5 millilitres, were infused over one hour. The animal was killed after a further thirty minutes.

In order to place the needle accurately, a small longitudinal incision was made through the skin and fascia overlying the antero-medial aspect of the upper tibia. The periosteum was elevated as far as its attachment to the articular cartilage while the joint space and synovium were maintained intact. The needle tip was placed within two millimetres of the junction of the bone and the joint cartilage—that is, in the epiphysial bone in young animals and immediately beneath the subchondral calcified plate in mature animals. Confirmation of the position was obtained by radiographic examination in two planes (Figs. 1 and 2).

A Palmer constant rate infusion pump was used to infuse the $^{35}$S solution. The isotope was always preceded by a very dilute solution of methylene blue to ensure that there was no leakage in the apparatus or contamination of the extra-articular tissue. Throughout the period of infusion the animals' joints were at rest.
Irrigation—In alternate animals, that is, six mature and six immature, the knee of the infused tibia was irrigated simultaneously with normal saline at twenty drops per minute, begun before, and continued after, the $^{35}$S infusion. A standard intravenous set was used, with two intra-articular needles, one for inflow and the other for drainage. The object of this was to eliminate nutrition by the synovial fluid.

Intra-articular injections—Two millicuries of $^{35}$S were injected into the right knee joint in each of four additional rabbits, two mature and two immature. These animals were killed ninety minutes later.

Preparation of specimens—Twelve rabbits which had had a subchondral infusion and the four animals which had had an intra-articular injection of $^{35}$S were used to provide both paraffin and frozen sections of undecalcified material.

Immediately after death, each animal's knees were opened. Part of the joint cartilage was removed from the femoral and the tibial articlar surfaces on both the injected and the opposite sides. In the immature animals the cartilage peeled away relatively easily, but in the mature animals it was necessary to remove the cartilage together with some fragments of subchondral bone. At least two slices were obtained from each bone. From one slice, cut with a freezing microtome, sections were cut at right angles to the joint surface, floated out on water and deposited unfixed on a glass slide. The other slice was fixed overnight in formalin, embedded in paraffin and sectioned with a sledge microtome. Some of these sections were stained with haematoxylin and eosin; the remainder were used to prepare autoradiographs. Pelc's (1947) procedure for stripping film was used, the AR 50 film being exposed for fourteen days, the AR 10 for forty-two days. Some of the sections were mounted unstained, the remainder were counterstained with neutral red.

In the remaining twelve rabbits, six mature and six immature, the upper tibia and lower femur were removed on each side and decalcified for two weeks in formol citrate solution. Paraffin sections were then made of the ends of these bones and autoradiographs prepared as above.
RESULTS

Haematoxylin and eosin stained sections from all the animals confirmed that each had been assigned to its correct group and was unquestionably mature or immature according to the criteria outlined above.

The material obtained from four animals was technically unsatisfactory, so that a total of twelve mature and twelve immature rabbits remained for comparison.

FIG. 3
Fine grain autoradiograph of a decalcified section from the injected tibia of an adult rabbit. (× 37.) Although isotope can be seen in the marrow spaces, none is present in the articular cartilage.

FIG. 4
Fine grain autoradiograph of a decalcified section from the injected tibia of an immature rabbit. (× 37.) Isotope is present in the marrow spaces and also in the cartilage.

In the rabbits which were used to provide decalcified sections no isotope was ever seen in the adult group (Fig. 3). Some isotope was seen in the cartilage of all the immature animals but the quantity was small and in some cases represented a mere trace. Such isotope as was
present was seen to be closely related to the chondrocytes (Fig. 4). Abundant isotope was present in the growth plate of these animals in association with the columns of ossifying cells.

In the material which had not been subjected to decalcification there was a much more marked difference between the two groups. In all of the immature animals there was dense blackening on the autoradiograph of the cartilage from the tibia on the injected side. The isotope appeared to be evenly distributed over the cartilage without apparent association with the chondrocytes (Fig. 5). In the mature group no isotope was ever seen in the cartilage (Fig. 8), although it could be detected in the small fragments of bone which were adherent to the cartilage.

In the immature animals isotope was also seen in the articular cartilage of the ipsilateral femur although the amount present was always much less than that seen in the tibia (Fig. 6). There was also some isotope in the cartilage from the contralateral femur and tibia although this was still less in amount (Fig. 7). The fact that the joint had been irrigated appeared to make no difference to the distribution of the isotope.

No isotope was ever identified in the cartilage from any of the other joints examined in the mature animals.

In the animals which had received an intra-articular injection of $^{35}$S the isotope was found in quantity in the joint cartilage of both the mature and the immature group (Figs. 9 and 10).

**DISCUSSION**

The results of these experiments appear to confirm that in an immature animal fluid can pass freely from subchondral bone to joint cartilage. The isotope was found in all the articular cartilage examined but the quantity found was much greater in the tibial cartilage on the side of the infusion, arguing in favour of a direct transfer. Irrigation of the joint, which must severely limit the amount of any synovial nutrition on this side, did not appear to diminish the amount of isotope deposited, suggesting that the subchondral route is the principal source of nutrition in these circumstances. This conclusion is in accord with the finding that the removal of the subchondral circulation leads to a cessation of proliferation and degeneration of immature cartilage (McKibbin and Holdsworth 1966) and represents the same findings as in the experiments of Ekholm (1951).
The results obtained from adult animals on the other hand suggest that this transfer can no longer take place since no isotope was ever seen in the cartilage in spite of its presence deep to the calcified zone (Fig. 3). This is in sharp contrast to the appearance when isotope was introduced to adult articular cartilage from its articular face (Fig. 9), when it was found to have permeated its entire thickness. It may be concluded therefore that adult cartilage is entirely dependent on the synovial route for its nutrition, which is consistent with the clinical finding that apparently healthy cartilage can exist over an area of dead bone.

Figure 7—Section prepared from the contralateral tibia of the same animal as in Figure 5. (×37.) Isotope is present in the cartilage but is much less than on the injected side. Figure 8—Coarse grain autoradiograph of an undecalcified section from the injected tibia of a mature rabbit. (×37.) No isotope is present in the cartilage, but some is present in the fragments of bone adhering to its deep surface.

Figure 9—Coarse grain autoradiograph of an undecalcified section from a mature rabbit following an intra-articular injection of isotope. (×37.) Abundant isotope is present in the cartilage. Figure 10—Coarse grain autoradiograph of an undecalcified section from an immature rabbit following an intra-articular injection of isotope. (×37.) Abundant isotope is present in the cartilage.
It seems therefore possible to reconcile some of the conflicting views which have been expressed on this problem by realising that a clear distinction must be drawn between developing joint cartilage and adult articular cartilage. The former is an actively growing tissue with considerable metabolic demands and it seems not unreasonable to expect it to obtain the bulk of its nutrition from the abundant blood vessels which invade its deep surface. Adult cartilage is in an entirely different situation, being sealed off from the bone by its calcified basal layer, and having much lower metabolic demands which can be satisfied by the rather ineffectual process of diffusion of synovial fluid (Bywaters 1937). This conclusion is further supported by the in vitro findings of Maroudas et al. (1968).

There is, however, an important distinction between these experiments and those of Ekholm (1951) which requires comment, namely, the substitution of the isotope $^{32}$S for $^{32}$P and $^{198}$Au. This was done to take advantage of the lower energy $\beta$ radiations of $^{32}$S which both reduces the radiation hazard and improves the accuracy of localisation on the autoradiograph. It was feared that a high energy $\beta$ emitter in the fragments of subchondral bone present on the cartilage might appear to lie within the cartilage itself.

However, the substitution of this isotope does introduce a complication, for whereas $^{32}$P and $^{198}$Au probably do not enter extensively into chemical union with the cartilage but rather act as passive markers of the movement of tissue fluids, $^{32}$S is actively metabolised by the chondrocytes and is laid down in the matrix as chondroitin sulphate. It could therefore be argued that the isotope which was seen in the immature cartilage had in fact been involved in chondrocyte metabolism, and the fact that no isotope was seen in the adult cartilage merely reflects the differing rates of metabolism between these tissues.

The fact that there was such an absolute distinction between the two groups makes this explanation unlikely since no trace of isotope was ever seen in the adult cartilage. This is in sharp contrast to the response to an intra-articular injection of isotope where the quantity subsequently found in the cartilage was comparable in the two groups (Figs. 9 and 10).

Further evidence for this is afforded by the appearances of the decalcified material. The amount of isotope found here was very much less even in the immature group and that which remained was located only in the close vicinity of chondrocytes. This suggests that in the process of decalcification the isotope had been leached out by the decalcifying fluid, leaving only that part which had been permanently incorporated by the cartilage matrix. Since this was in fact only a small amount it must be concluded that the much greater amount of isotope seen in the undecalcified sections represents largely freely diffusible isotope whose presence is unrelated to the cartilage metabolism.

Another feature of the experiments which requires comment was the finding of a considerable quantity of isotope in the ipsilateral femur and the contralateral joint.

The preliminary injection with methylene blue showed that the injected fluid left the bone by a variety of vessels, but prominent among these were some which ran along the cruciate ligaments to the other side of the joint where they joined in the subchondral circulation of the femur. It is likely that much isotope passed directly by this route. The isotope which appeared on the contralateral side can only have arrived via the general circulation. It might perhaps have been expected that some isotope would have been found in the adult cartilage having been derived from the general circulation via the synovial fluid, but this was not so. It may have been that the short time of the experiment did not allow the secretion of a sufficient quantity of synovial fluid to effect a significant transfer of isotope particularly since the joints were immobile, for the importance of mechanical factors in promoting synovial nutrition has been demonstrated (Fisher 1922, Bennett and Bauer 1937). The fact that irrigation had no effect on the distribution of the isotope in the immature animals also suggests that no significant synovial fluid transfer occurred during the course of these experiments.
SUMMARY

1. The source of nutrition of articular cartilage still remains a subject of controversy.
2. Experiments are described in which an attempt to demonstrate the direct transfer of fluid from the subchondral bone has been made using $^{35}$S and an autoradiographic technique. These experiments were based on ones originally performed by Ekholm (1951), except that two distinct groups of animals were used: immature rabbits and adult rabbits whose skeletons were mature.
3. The transfer of fluid to the cartilage could be demonstrated only in the immature rabbits.
4. It is suggested that some of the conflicting opinions which have been advanced on this subject stem from a failure to distinguish between mature and immature joint cartilage. Subchondral nutrition is a feature only of the immature animal.

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REFERENCES


