

Chondrocytic monoamine oxidase activity in the development of natural murine osteoarthritis

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Summary. Most of the male STR/ORT mice develop osteoarthritis (OA) involving the medial tibial plateau. A peculiarity of two chondroprotective drugs is the presence of a nitrogen atom so that cleavage of the molecule could generate a molecule that might act as an inhibitor of monoamine oxidase (MAO). Direct examination showed abnormal localization of MAO in the potentially osteoarthritic cartilage indicating possible abnormal response to catecholamines. In normal cartilage, the direct effect of excessive concentration of adrenaline caused considerable oedema, as measured by microscopic interferometry. It is therefore suggested that the excess of water found in the matrix of osteoarthritic cartilage may be related to disturbance of the MAO activity.

Keywords: osteoarthritis, monoamine oxidase activity, microscopic interferometry, quantitative cytochemistry

Most male STR/ORT mice develop osteoarthritis (OA) of the medial tibial plateau by the age of 12 months, as assessed radiologically (Walton 1977). In the present colony of this strain of mice, damage to this cartilage was found histologically by around 30 weeks of age (Dunham *et al.* 1988). The immediate possibility of how the cartilage matrix becomes damaged involved damage to the lysosomes of the chondrocytes allowing leakage of proteolytic enzymes into the matrix as has been suggested to occur in other systems (Weissmann 1971). Preliminary results (unpublished), using the lysosomal fragility test (Bitensky & Chayen 1977), indicated

that the lysosomal membranes of the chondrocytes remained normal.

Another possibility involved a defect in the local metabolism of catecholamines. This arose from the findings of Cohen *et al.* (1965) that the shedding of the endometrium, that is found at menstruation in the human, was associated with leakage of the mitochondrial monoamine oxidase (MAO) activity from the mitochondria of the glandular epithelium into the general cytoplasm and even into the extracellular spaces. Interest in this possibility was enhanced by the fact that the two 'chondroprotective' non-steroidal anti-inflammatory drugs, that appear to have some

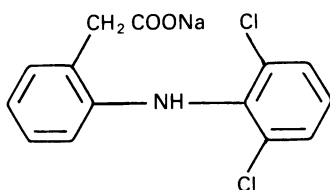


Fig. 1. The structure of diclofenac sodium.

effect against osteoarthritis (OA), have a nitrogen atom held within the molecule. In one of these, diclofenac sodium, cleavage of the molecule around the central nitrogen atom, would liberate molecules that could mimic substrates for monoamine oxidase and could act as competitive inhibitors (Fig. 1). Consequently, it seemed relevant to test whether the MAO activity in the chondrocytes of the potentially affected medial tibial cartilages of the STR/ORT mice was abnormal relative to that found in such cells in the lateral tibial cartilages of the same mice.

Materials and methods

Male STR/ORT mice, of 25–29 weeks of age, were used in the first study. This involved ten that were fed by gavage with the anti-inflammatory agent, diclofenac sodium (2 mg/kg), 5 days each week beginning when the mice were between 5 and 8 weeks old, and eight that were fed only with the vehicle (polyethylene glycol 400 in a 5% aqueous solution of dextrose). For the second study, the age of the mice varied from 15 to 39 weeks; 12 were fed with diclofenac sodium and 12 with the vehicle alone. For comparison, male mice of the CBA strain of similar ages, that do not normally become osteoarthritic, were also studied. The mice were killed by asphyxiation in nitrogen. Each STR mouse that had been treated with diclofenac sodium was killed together with an STR mouse of the same age treated with vehicle alone and the tissues were processed together. The knee joints were removed intact together with the associated muscle and the patella was marked with indelible

ink to assist orientation for sectioning. They were dipped briefly in a 5% aqueous solution of polyvinyl alcohol (GO4/140, Wacker Chemicals Ltd, Walton, Surrey, UK) and then chilled to -70°C with industrial spirit and solid carbon dioxide (Chayen *et al.* 1973). They were stored dry at -70°C and sectioned within 7 days with the patella uppermost. Sections were cut at $10\ \mu\text{m}$ in a Bright's bone-cutting cryostat, fitted with automatic drive to ensure sections of constant thickness, and with the cabinet temperature of -25°C ; the tungsten carbide knife was cooled by having CO_2 -ice packed around its haft. The sections were flash-dried on to glass slides. These procedures have been described fully by Chayen *et al.* (1973).

For testing for monoamine oxidase activity, the reaction medium contained 5 mM epinephrine bitartrate (adrenaline bitartrate; Sigma) and nitroblue tetrazolium (1 mg/ml, Sigma) in 0.05 M phosphate buffer. The final pH was 7.5 (Chayen *et al.* 1973). The reaction time was 30 min at 37°C , with the medium replaced with freshly prepared medium after 15 min. For specificity studies, iproniazid (Sigma) was added at either 0.01 or 0.001 M. As further controls, similar tissue from CBA mice of 15–33 weeks of age was also tested.

The coloured reaction product was measured in ten individual cells, or adjacent areas of equivalent size in the matrix in duplicate sections, by means of a Vickers M85A scanning and integrating microdensitometer at 585 nm, with a $\times 40$ objective and scanning spot of $0.4\ \mu\text{m}$. By suitable calibration, the results were expressed in terms of units of absolute extinction (mean integrated extinction, MIE $\times 100$).

For measuring dry mass in the tissues, sections ($10\ \mu\text{m}$ thick) were mounted on carefully cleaned and dried glass slides. Immediately before use they were immersed in a 20% aqueous solution of Polypep 5115 (Sigma). They were examined with a Zeiss microscopic interferometer (Chayen 1967) with a $\times 10$ interference objective and cor-

Table 1. The number of mice in which the monoamine oxidase activity in the chondrocytes of the tibial plateau occurred in discrete granules related to the total number of mice examined

	Lateral cartilage	Medial cartilage
Control	8/8	2/8
Treated with diclofenac sodium	10/10	8/10

responding condenser. A green filter (550 nm) gave monochromatic light.

For the in-vitro studies, tibias from three CBA mice were mounted on lens tissue that covered a metal mesh table, as used in conventional organ maintenance culture

(Trowell 1959) and placed inside a culture pot. For these studies the culture pot was left uncovered. Trowell's T₈ medium (Gibco) was poured over three; the other three were exposed to this medium containing 10^{-3} M adrenaline (Sigma). This was repeated every 10 min for 30 min. The tibias were then chilled, sectioned at 10 μ m, and mounted in a 20% aqueous solution of Polypep 5115.

Results

Localization of monoamine oxidase activity

The monoamine oxidase activity was examined in the tibial cartilage of four male CBA mice (ages 15, 19, 31 and 33 weeks), of eight untreated STR/ORT male mice and of ten STR/ORT male mice that had been treated

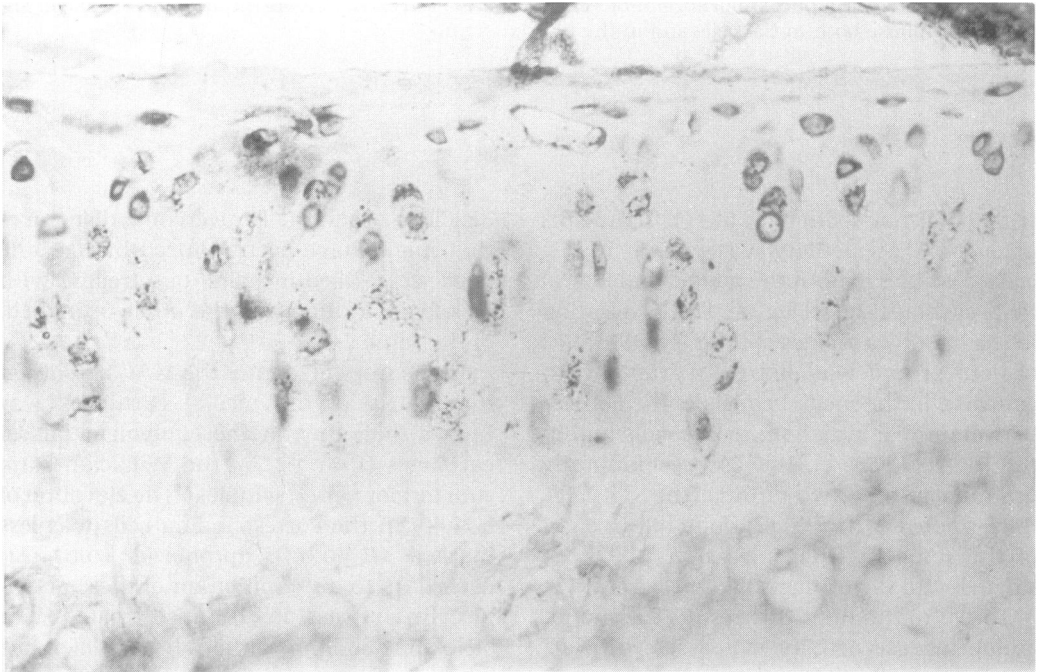


Fig. 2. A section of a normal joint reacted for monoamine oxidase activity. The activity is predominantly in discrete granules. $\times 360$.

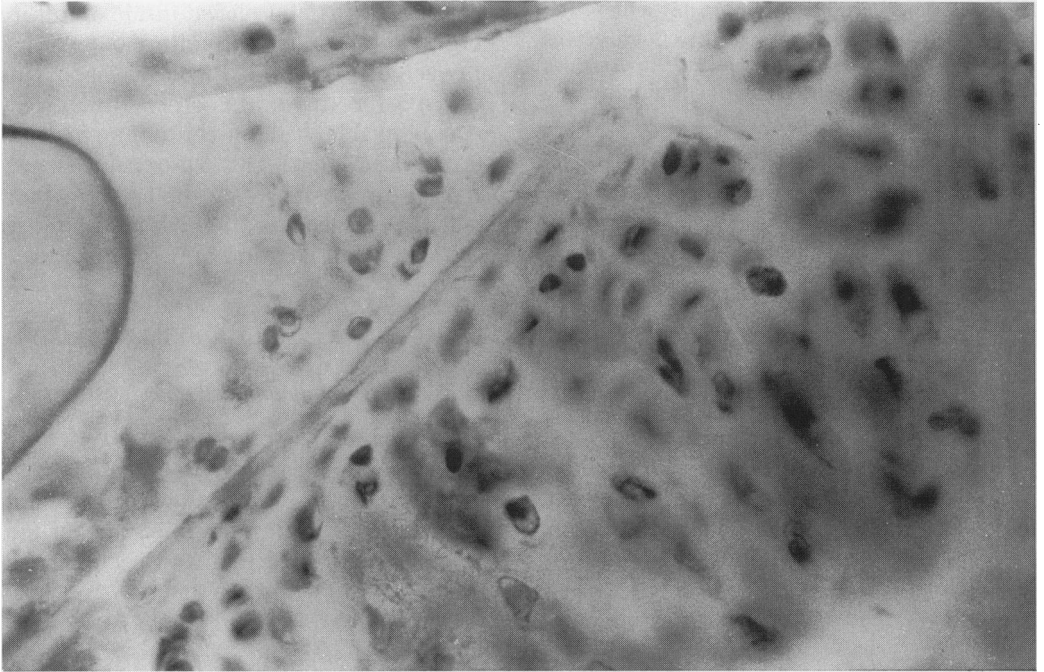


Fig. 3. Monoamine oxidase activity in the affected medial tibial plateau of a joint from an STR/ORT mouse treated with vehicle alone. Small fibrillations can be seen at both extremities of the articular cartilage. The activity is diffuse both in the cells and in the matrix. $\times 360$.

with diclofenac sodium (Table 1). In the CBA mice the MAO activity was present in discrete granules in both the medial and lateral tibial cartilages (as in Fig. 2). The localization in the lateral cartilages of the STR/ORT mice of both groups was discretely granular. In contrast, in the medial cartilage the activity was mainly diffuse, both in the cells and in the matrix (Fig. 3). It was predominantly granular in only two of the eight STR/ORT mice treated with vehicle alone but in eight of the ten STR/ORT mice that had been treated with diclofenac sodium (Fig. 4).

To try to quantify this result, the monoamine oxidase activity was assessed in 12 untreated STR/ORT male mice of between 15 and 39 weeks of age and 12 male mice of equivalent ages that had been treated with

diclofenac sodium. To overcome differences that might have been induced by age, the mice were killed in pairs, one treated with diclofenac sodium and the other untreated, of the same age.

In the untreated mice the MAO activity in the matrix of the medial cartilages was higher than that in the equivalent lateral cartilages ($P=0.01$ by the Wilcoxon rank sum test for paired samples). The elevation of activity in the corresponding cells was less marked ($P=0.05$). Iproniazid (0.01 M) caused up to 44% inhibition of these activities. In contrast, in the mice treated with diclofenac sodium, although the difference between the activity in the matrix was just significant ($P=0.05$) there was no difference in the activity of the cells of the medial as

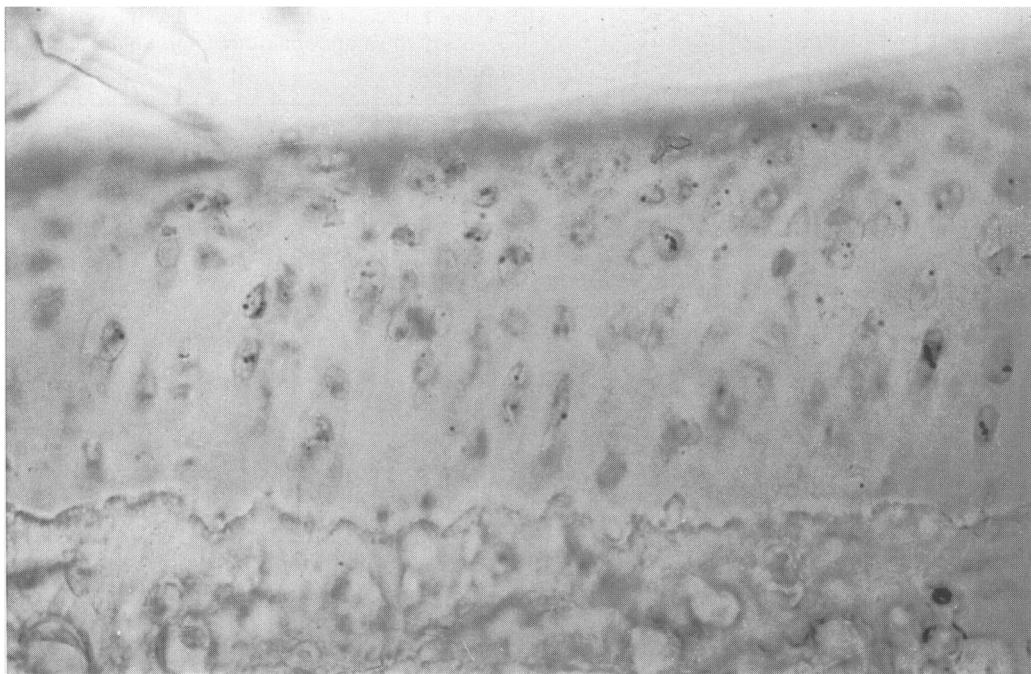


Fig. 4. The monoamine oxidase activity in a section of the medial tibial plateau of an STR/ORT mouse treated with diclofenac sodium. The activity is predominantly granular. $\times 360$.

against that found in the corresponding lateral cartilage.

The question that was particularly pertinent to the present study was to quantify whether, in osteoarthritis, the MAO activity became more diffuse, even extending into the matrix rather than being restricted solely to the mitochondria. Hence the feature that required to be analysed was the amount of the activity that was found to be in the matrix of the medial cartilage relative to that found in the cells, and how this ratio differed from that found in the lateral cartilage of the same animal. This also overcame the problem of variations that occur in the activities in different animals. The same calculation was applied to the values obtained from two CBA mice. The results of this calculation in the tibias of the CBA mice were close to 1.0,

as were those from the STR/ORT mice that had been treated with diclofenac sodium (Fig. 5). In contrast, the results from the untreated STR/ORT mice were appreciably higher than those in the treated STR/ORT mice, as assessed by the Chi-squared test (testing activities of above and below 1.1, $0.01 > P > 0.001$). Thus it appears that there was significantly more activity in the matrix of the cartilage of the untreated STR/ORT mice than in that of the mice treated with diclofenac sodium.

These results indicated that the MAO of the untreated STR/ORT mice was relatively freed from its normal location. Under such conditions, although it can be demonstrated by the cytochemical reaction, with NBT as the hydrogen acceptor, it is unlikely to be operative in life if, in becoming dislocated

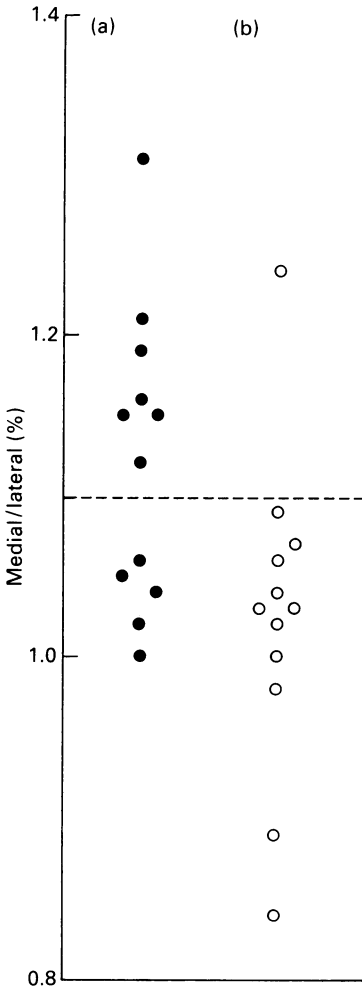


Fig. 5. The monoamine oxidase activity in the cells related to that in the matrix, in the medial as against that in the lateral cartilages of either a, untreated STR/ORT mice or of b, those that had been treated with diclofenac. $0.01 > P > 0.001$.

from the mitochondria, it also lacks the FAD with which it is normally associated within the mitochondria (Tipton 1986) and the

Table 2. Effect of adrenaline on dry mass (10^{-12} g/ μm^2) of the matrix of intact mouse tibial articular cartilage

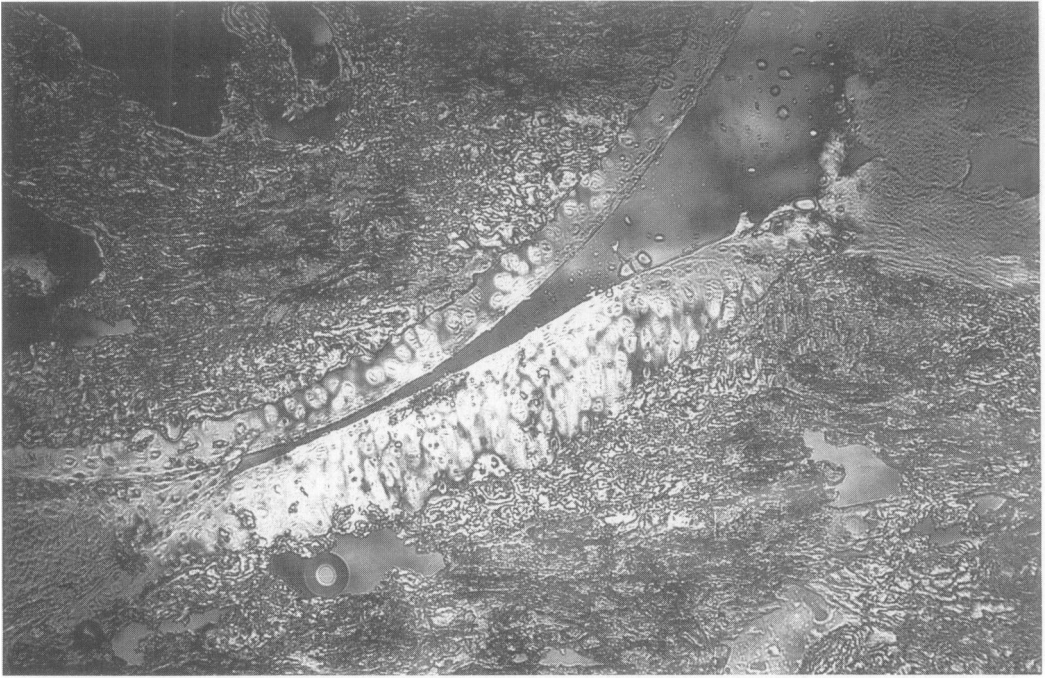
	Control (with T ₈)	Treated (with 10^{-3} M adrenaline in T ₈)
1st Expt.		
Section 1	0.28	(i) 0.02
Section 2	0.28	(ii) 0.07
Section 3	0.28	(iii) 0.04
		(iv) 0.04
2nd Expt.	0.27	0.05
3rd Expt.	0.29	0.05

related mitochondrial oxidizing system. Under such circumstances, it will not metabolize catecholamines such as adrenaline. It was therefore pertinent to test the effect of adrenaline in normal mouse cartilage at a sufficiently high concentration that it would swamp the influence of endogenous MAO.

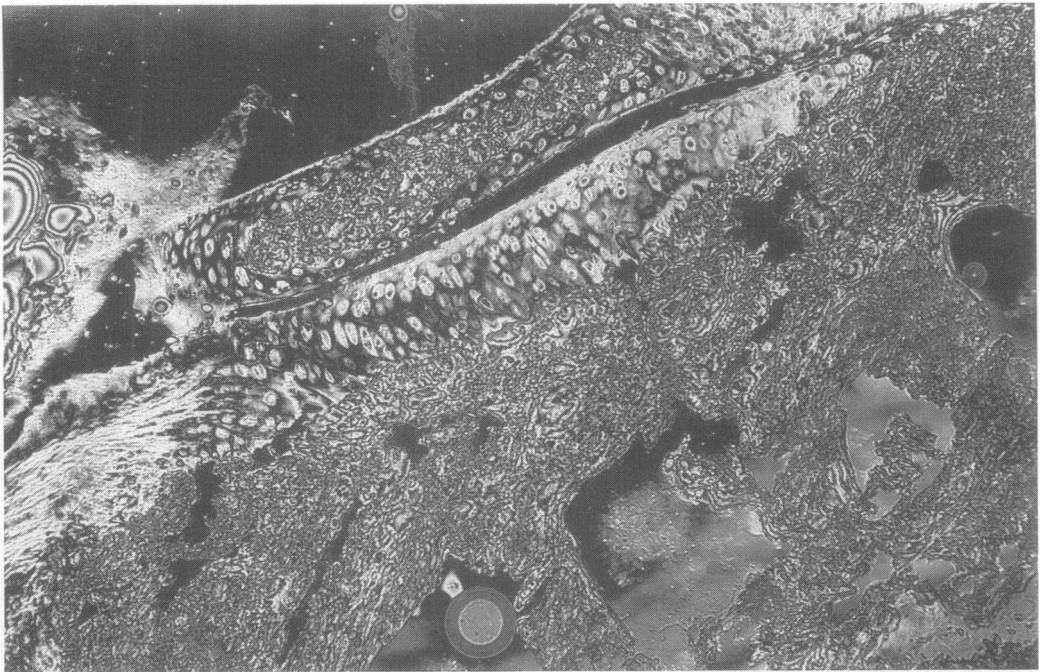
To this end tibias from three CBA mice were treated either with Trowell's T₈ medium alone, or T₈ medium containing 10^{-3} M adrenaline. Sections ($10 \mu\text{m}$ thick), mounted in 20% aqueous Polypep 5115, were examined by interference microscopy.

To test the variability of the measurements, the dry mass in the articular cartilage in serial sections from a tibia that had been treated with T₈ medium alone was measured by interference microscopy (Table 2). Four sections were measured from a tibia that had been exposed to adrenaline. The results with the other two sets of tibias gave comparable results. Consequently it follows that exposure to adrenaline had decreased the mass per unit area in the matrix of the articular

Fig. 6. a, Section of tibial articular cartilage of the control disarticulated knee joint of a mouse, examined in green light by interference microscopy. The background is black (having relatively little dry mass); the amount of dry mass is shown by the degree of white. The initial magnification on the negative of the film was $\times 40$; the magnification of the print is $\times 160$. b, Section of the articular cartilage of the other disarticulated knee joint from the same mouse after the joint had been exposed to adrenaline. There is decreased mass, as shown by the increased amount of black appearance. Conditions as for a.



(a)



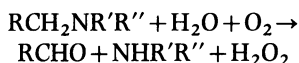
(b)

cartilage from around 0.28 pg/cm² to about 0.05 pg/cm² (after correction for the refractive index of the mounting fluid). This implies that there has been a considerable swelling of the cartilage due to uptake of water. The loss of concentration of dry mass is shown in Fig. 6, where the mass is indicated by the amount of white (light) that can be seen relative to the dark background.

Discussion

These results show firstly that the MAO activity in the medial tibial cartilage of the male STR/ORT mice was abnormally located, extending beyond the chondrocytes into the surrounding matrix; and secondly, that the administration of diclofenac sodium largely restored the normal location of this enzymatic activity. It is now necessary to consider the possible implication of these findings.

Fractionation studies of normal tissues have shown that MAO is normally tightly bound to the outer membrane of mitochondria and requires to have FAD closely bound to it (Tipton 1986). It is generally believed to catalyse the following reaction:



where R' and R'' are H or CH₃ (Glover & Sandler 1986). When present in mitochondria, the potentially damaging hydrogen peroxide that is produced can be removed by associated peroxidases. However, when the MAO acts in the matrix of the cartilage, where such peroxidases are not present, the hydrogen peroxide can act directly on the components of the matrix.

However, MAO activity requires oxygen as the final hydrogen acceptor. If this is not available inside the matrix of the cartilage, it is unlikely that MAO within the matrix could be operative. The present results indicate that the essentially mitochondrial location of the enzyme is lost in the medial tibial cartilage of the untreated male STR/ORT mice. (The enzymatic activity can be demonstrated

histochemically because the NBT acts as a substitute for oxygen.) Under these conditions, catecholamines, such as adrenaline, would not be metabolized and would therefore be free to cause oedema.

Diclofenac sodium largely restored the appearance of the granular location of MAO. It also decreased the availability of the enzyme that might be located in the matrix. It seems possible that part of the effect of diclofenac sodium might arise from its cleavage into two molecules, one of which would have a terminal NH₂ group and so act as a competitive inhibitor of MAO in the matrix. Consequently, diclofenac sodium might have two functions, first, as the intact molecule, in protecting the mitochondria, and second, as a cleaved molecule, in inhibiting free MAO activity.

In the light of these results, it appears that abnormal activity and distribution of chondrocytic MAO needs to be added to the spectrum of abnormalities that occurs in osteoarthritis (Dunham *et al.* 1989) and that the administration of diclofenac sodium is able to diminish this abnormality.

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