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Structural and metabolic changes in articular cartilage induced by iodoacetate

Jane Dunham*, Sibylle Hoedt-Schmidt† and D.A. Kalbhen†
*Unit of Cellular Pharmacology and Toxicology, Robens Institute of Health and Safety, University of Surrey, UK, and †Institute of Pharmacology and Toxicology, University of Bonn, FR Germany

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Summary. The chemically induced injury to articular cartilage, caused by two successive intra-articular injections of sodium iodoacetate, has been used in studies on the effects of anti-inflammatory and of potentially chondroprotective agents. It has been assumed that the injurious effects are caused by inhibition of the glycolytic pathway. In the present study this inhibition has been shown to be greater than expected from *in vitro* studies, and to influence equally other oxidative pathways. However, the response is clearly not a simple one in that some of the surface chondrocytes, and synovial lining cells in close proximity to the cartilage, show virtually no inhibition.

Keywords: iodoacetate, articular cartilage, quantitative cytochemistry, oxidative enzymes, proteoglycans

It is well established that the intra-articular injection of iodoacetate in the hen, rat and guinea-pig induces loss of the articular cartilage. This chemically induced injury has been used as a model for studying the possible effects of non-steroidal anti-inflammatory agents and of potentially chondroprotective agents (Kalbhen & Blum 1977; Kalbhen 1989; Williams & Brandt 1984). It has generally been assumed that the effect of iodoacetate has been primarily on glycolytic respiration seeing that it is a very potent inhibitor of the activity of glyceraldehyde-3-phosphate dehydrogenase, which is focal to both the Embden–Meyerhof pathway and ultimately to the pentose shunt oxidative systems. However, the mechanism of the adverse effect induced by the intra-articular injection of iodoacetate has not been elucidated. The object of the present study was to investigate structural and oxidative mechanisms that might be influenced by such injections.

Material and methods

Male rats of the Sprague–Dawley strain weighing between 300 and 600g were used. The animals were anaesthetized with ether and the left knee of each animal was injected intra-articularly with 0.8mg sodium iodoacetate in 0.05ml saline on day 1 and day 2 of the experiment. The animals were killed at times from 24 h to 9 weeks after the second iodoacetate injection.

Correspondence: Jane Dunham, Unit of Cellular Pharmacology and Toxicology, Robens Institute of Health and Safety, University of Surrey, Guildford, Surrey GU2 5XH, UK.

Both knee joints were dissected out and the patellas marked. They were dipped in 5% aqueous PVA for 5 min before chilling to −70% by precipitate immersion in n-hexane (BDH grade low in aromatic hydrocarbons) surrounded by a mixture of industrial spirit and crushed CO_2 ice. The joints were mounted onto microtome chucks with the patella uppermost with 5% PVA as the adhesive. They were then sectioned at 10 μm on a Brights' cryostat fitted with a tungsten tipped carbide knife.

The sections were then either reacted for one of several enzyme activities or stained for structural and histological investigations.

Assays for enzyme activity

The following concentrations of substrate and cofactors were used for assaying enzyme activity (Chayen & Bitensky 1991).

Glucose-6-phosphate dehydrogenase (G6PD): 5 mM glucose 6-phosphate, monosodium salt; 3 mM NADP (pH 8.0). Lactate dehydrogenase (LDH): 5 mM sodium lactate; 2.5 mM NAD (pH 8.0). Glyceraldehyde-3-phosphate dehydrogenase (G3PD): 5 mM fructose-1,6-diphosphate trisodium salt; 1.5 mM NAD; 10 units/ml aldolase (pH 8.5). Malate dehydrogenase [MalD): 10 mM L-malic acid disodium salt with either 1 mM NAD (pH 6.9) or 3 mM NADP (pH 7.5). Glutamate dehydrogenase (GlutD): 20 mM L-glutamic acid monosodium salt with either 1 mM NAD or 3 mM NADP (pH 8.0). Glycerophosphate dehydrogenase (Glyc. D): 14 mM DL-α-glycerophosphate, disodium salt, 1 mM NAD (pH 7.6). Hydroxyacyl dehydrogenase (HOAD): 50 mM hydroxybutyrylcysteamine with 1 mM menadione, pH 8.5. Succinate dehydrogenase (SDH): 50 mM sodium succinate (pH 7.8).

All the reaction media contained 30% polyvinyl alcohol in 0.5M glycylglycine buffer pH 8.0 except for SDH (0.1 M phosphate buffer, pH 7.8; no PVA) and hydroxyacyl dehydrogenase (20% PVA in 0.5M glycyl glycine buffer, pH 8.0). All media contained 3.75 mM (3.0 mg/ml) nitroblue tetrazolium except for SDH (1mg/ml NBT) and HOAD (1 mg/ml neotetrazolium chloride). Phenazine methosulphate was added to all reaction media just prior to use at a concentration of 0.7 mM.

Measurement of enzyme activity. Enzyme activity was measured in individual chondrocytes and in synovial lining cells using a Vickers M85A scanning and integrating microdensitometer (585 nm wavelength, ×40 objective and scanning spot size of 0.5 μm in the plane of the section). Twenty cells, from duplicate sections, were measured from each particular articular cartilage. The results were expressed in units of extinction.

Histological and structural observations

For histology the sections were stained with toluidine blue or with haematoxylin–eosin. For structural observations Alcian blue (0.05% in 0.025 M acetate buffer, pH 5.8) in the presence of 0.5 M $MgCl_2$ was used to stain the cartilage proteoglycans (Scott & Dorling 1965) and measured by microdensitometry. Results were expressed as percentage inhibition of Alcian blue stainability in the injected knee when compared to the control knee. Full details of all these methods are given in Chayen and Bitensky (1991).

Results

Histological changes

The response of individual animals to iodoacetate injection was very variable but generally the first histological evidence of pathological changes in the cartilage was seen around 5 days by which time the matrix staining was significantly reduced. There was patchy loss of nuclear staining with haematoxylin in those regions which showed enzyme inhibition (see later). From 4 weeks, occasional large round atypical chondrocytes were seen staining with haema-

toxylin. Clusters of chondrocytes were seen. Between 6 and 9 weeks large calcified chondrocytes occurred in the deeper zones of the cartilage. Six weeks after treatment with iodoacetate, fibrillation of the articular surfaces was evident in several animals and at 9 weeks detached pieces of cartilage could be seen in the joint space. In one animal, 9 weeks after injection the articular cartilage of the femoral groove had been totally replaced by fibrocartilage.

From 6 weeks after the injections of iodoacetate, the sub-chondral bone showed extensive hyperplasia of the osteoblasts so that the trabecular spaces were filled with cell clusters which extended into the cartilage.

Synovial tissue also showed highly increased cellularity both of the lining cells and fibroblasts, but there was no acute or chronic cellular infiltrate.

Direct effect of iodoacetate on sections

It was first considered advisable to check how well iodoacetate inhibited active groups when applied directly to sections of normal healthy joints. Sections (10 μm) were reacted with the appropriate reaction medium either with or without 10^{-3}M sodium iodoacetate in the medium. The activities in the chondrocytes of the patella cartilage and in the associated muscle were measured. The results (Fig. 1) showed around 60% inhibition of the glyceraldehyde-3-phosphate dehydrogenase (G3PD) activity in both tissues, around 20% inhibition of the lactate dehydrogenase (LDH) activities, and less than 10% inhibition of the succinate dehydrogenase (SDH) activities. The inhibition of the glucose-6-phosphate dehydrogenase (G6PD) activity in the chondrocytes was less (19%) than that of this activity in muscle (41%).

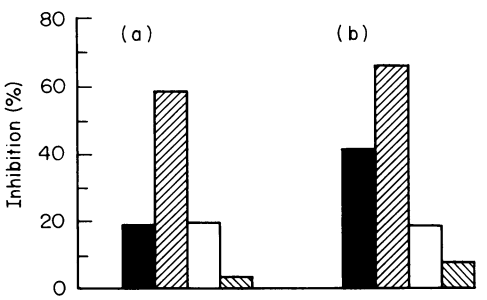

Fig 1. The effect of sodium iodoacetate (10^{-3}M), when included in the reaction medium, on the enzyme activity in cells. ■, G6PD; ▨, G3PD; □, LDH; ▧, SDH. a, Patella; b, muscle.

The influence of a range of concentrations of iodoacetate included in the reaction medium for the two most affected enzymes was then tested. This showed that even with concentrations of 100 mM, the G3PD activity in the chondrocytes was inhibited by 80% and that of G6PD by 40%. In the associated muscle 100mM iodoacetate caused almost complete inhibition of the G3PD activity.

Early responses to the injections of iodoacetate

One day after the second injection, the results in different rats and in the different articular cartilages were rather variable. For example (Table 1) in the first rat there was 95% inhibition of the G3PD activity in the chondrocytes of the patella but only 11% inhibition in those of the femur. In the second rat the G3PD activity in the chondroycytes of the patella was inhibited by only 20%; that of LDH was not inhibited.

In the first of the rats 2 days after the injections of iodoacetate (Table 2) there was 91% inhibition of G3PD activity in most of the chrondrocytes of the patella but only 53% inhibition in the superficial, spindle-shaped chrondrocytes. Although the LDH activity of the chondrocytes of the patella was 77% inhibited, those of the femoral groove showed only 38% inhibition. The chondrocytes of the femur of the second rat showed only about 20% inhibition of G6PD and G3PD activities, with no inhibition of the mitochondrial succinate dehydrogenase (SDH) activity.

Five days after the injections of iodoacetate

Table 1. Enzyme activity (%) inhibition) in chondrocytes from different regions of the joint in three animals 24 hours after injection of indoacetate

	G6PD	G3PD	LDH	SDH
(i)				
Patella	72	95	59	52
Femoral groove	33	96	11	30
Femur	—	11	22	23
Tibia	20	44	45	19
(ii)				
Patella	30/13*	20/28*	0	17
Femoral groove	0	39	4	28
Femur	0	46	6	24
Tibia	0	47	0	45
(iii)				
Patella	38	76	—	—
Femoral groove	10	75	—	—

* Enzyme activity in superficial cells.

in three rats, most of the chondrocytes had no activity of either G6PD or G3PD. However, in the femoral groove in two of the rats there were peculiar large cells, generally towards the articular surface, in which both activities were virtually the same as those found in the normal chondrocytes of the equivalent uninjected knee.

In two rats tested 2 weeks after the injections of iodoacetate, there was virtually complete inhibition of all four oxidative enzymes with normal activities in a few cells either at the surface (zone 1: Dunham *et al*, 1986) or in spindle-shaped cells at the margins of the cartilages. Such activities were also found in a few large cells deep in the femoral groove.

Four weeks after the injections of iodoacetate

The results in the rats 4 weeks after the injections of iodoacetate were remarkably variable (Table 3). In the first rat there was no inhibition of G6PD activity in two sites; no inhibition of G3PD or LDH in two sites; and only slight inhibition of the activities in the chondrocytes of the patella and of the femoral groove. Similarly, in the fourth rat, whereas the G3PD activity in the chondrocytes of the femur was totally uninhibited, it was totally inhibited in those of the tibial cartilage.

Six weeks after the injections of iodoacetate

Similar results were obtained in three rats 6 weeks after the injections of iodoacetate. Some of the features are illustrated in Figs 2–4. In the femoral groove of two of these, there was no activity of the following enzymes: G6PD, G3PD, hydroxyacyl dehydrogenase (HOAD, as a putative marker of fatty acid oxidation), glycerophosphate and malate dehydrogenase. In one of these, the activity of glutamate dehydrogenase (with either NAD or NADP as coenzyme) also proved negative. In the third rat, the superficial cells of the femoral groove showed activities com-

Table 2. Enzyme activity (% inhibition) in chondrocytes 48 hours after injection of indoacetate

	G6PD	G3PD	LDH	SDH
(i)				
Patella	72/10*	91/53*	77/34*	43/0*
Femoral groove	11	93	38	0
Femur	—	—	56	—
(ii)				
Patella	79	84	78	50
Femoral groove	86/13*	72/28*	77/9*	25
Femur	22	19	—	0
Tibia	57	83	—	17

* Enzyme activity in superficial cells.

Table 3. Enzyme activity (% inhibition) in chondrocytes 4 weeks after injection of indoacetate

	G6PD	G3PD	LDH	SDH
(i)				
Patella	0	17	15	0
Femoral groove	0	0	0	16
Femur	27	0	0	0
(ii)				
Patella	100	100	100	100
Femoral groove	100	100	100	100
Femur	100/57	100/21	23	43
Tibia	38	26	33	0
(iii)				
Femur	48	100/8*	100/30*	100/39*
Tibia	92/32*	100/0*	100/0*	100/0*
(iv)				
Femur	32	0	9	0
Tibia	100/58	100/23	100/0*	54

* Residual enzyme activity only in cells towards femoral notch.

parable to those found in the untreated side for G6PD, G3PD, HOAD and malate dehydrogenases. Even in those cartilages which showed no measurable enzymatic activities, the closely apposed synovial lining cells had strong activity (Table 4). The synovial tissue was hyperplastic and more cellular than normal, with closely packed fibroblasts but with no sign of either acute or chronic inflammatory cell infiltrate.

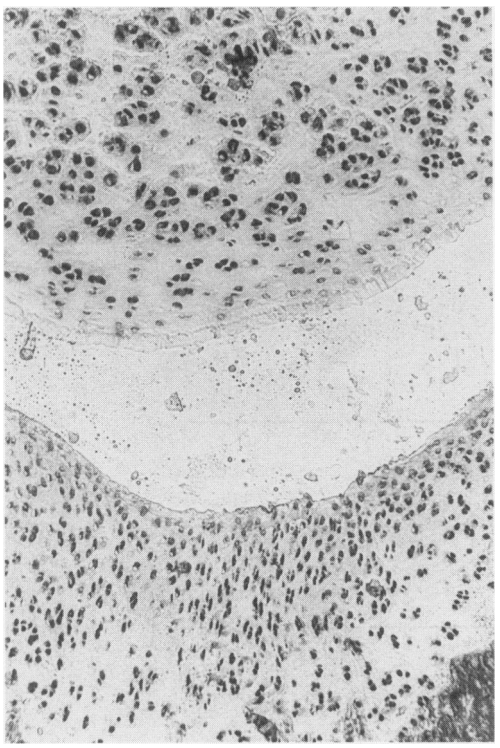

Fig. 2. Glyceraldehyde-3-phosphate dehydrogenase activity in the chondrocytes of the patella (top) and femoral groove (bottom) in the control knee of a 6-week experimental rat. × 25.

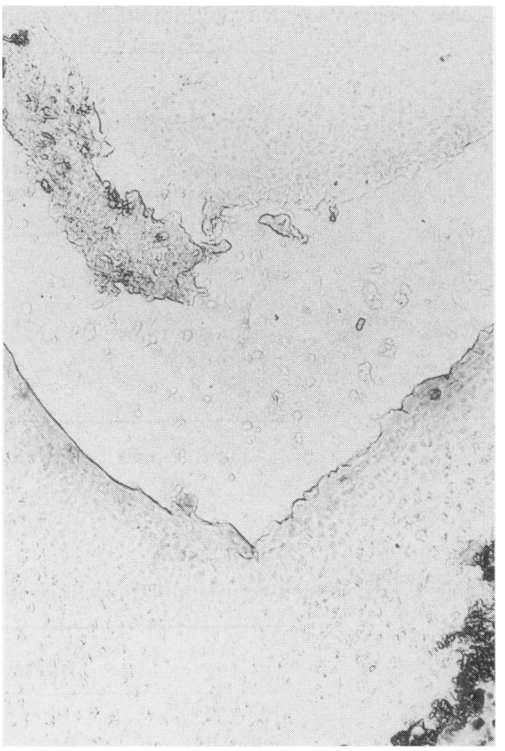

Fig. 3. Total inhibition of glyceraldehyde-3-phosphate dehydrogenase activity in the chondrocytes of the patella and femoral groove 6 weeks after injection of iodoacetate into the joint. × 25.

Similar results were obtained in five rats that had been examined 9 weeks after the injection of the iodoacetate. Even extending the reaction time for LDH activity from the normal 5 min to 40 min did not show activity in those regions which had no activity when tested with the normal reaction time. Similar results were found with tests for G6PD and G3PD activities.

Nuclear changes

In many of the cartilages of the joints treated with iodoacetate, there was virtually no enzymatic activity. The obvious question was whether or not this implied that the chondrocytes were dead. When sections of metabolically inactive cartilage were stained with toluidine blue or with haematoxylin–eosin, the regions where nuclei should have been showed no nuclear staining. However, under phase contrast illumination, the lacunae did not appear to be empty. It was therefore necessary to try to establish whether the nuclei were still intact.

Sections (10 μm) were cut, including the femoral groove and the patella, from one of the rats 9 weeks after the administration of iodoacetate. They showed no nuclear staining. Other sections (10 μ) were mounted in 5% Polypep 5115 on quartz slides and examined with a × 32, 0.4 NA objective with the Zeiss Universal ultraviolet and visible microspectrophotometer. The full

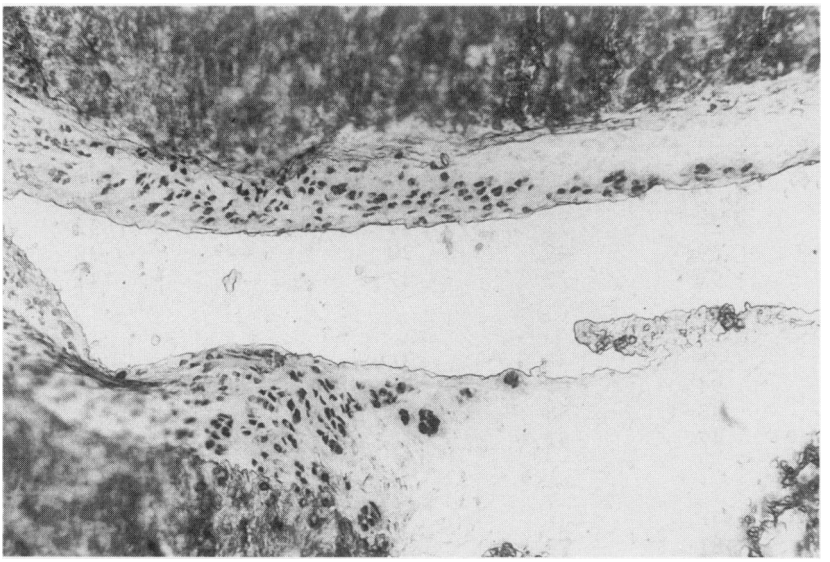

Fig 4. An example of the variability of the effect of iodoacetate in the joint. Both patella (top) and femoral groove (bottom) show enzyme activity in the peripheral cells and some superficial cells of the patella are also somewhat active. The chondrocytes beneath the fibrillation of the femoral groove are devoid of enzyme activity as are those in the deeper zone of the patella. × 28.

ultraviolet absorption spectrum, specifically of the nuclear region, was measured from 310 to 245 nm and showed appreciable specific absorption at around 265 nm, as expected for nucleoprotein (Walker 1958). Serial sections were treated with ribonuclease; there was no loss of this absorption. Consequently there did appear to be nuclei in these lacunae, even though they did not stain with conventional stains.

Matrix staining

There was virtually no immediate effect of the injection of iodoacetate on the Alcian blue-staining of the cartilage (with 0.5 M $MgCl_2$). However, 5 days after the injections and thereafter, the results generally showed an extensive loss of staining which was patchy throughout the individual joint (Table 5). For example, 5 days after the injections there was no loss of staining in the femoral groove of one rat, byt 44% loss in the patella. Six weeks after the injections of the indoacetate, there was almost complete loss of staining (over 80%) in the patella region of three animals and considerable loss in the cartilage of the femoral groove.

Effect on sulphydryl groups

Sections of the joints from two rats, 4 weeks after the injection of iodoacetate, were treated for sulphydryl groups by the ferric–ferrocyanide method (Chayen & Bitensky 1991). Sections from the uninjected joints showed normal staining; there was no measurable stain in the sections of the injected joints.

Discussion

Initially it was assumed that the injection of iodoacetate into the joint of rats involved a simple response caused by the inhibition of molecules rich in sulphydryl groups. Of such active molecules the most obvious was glyceraldehyde-3-phosphate dehydrogenase

Table 4. The response of a number of oxidative enzymes in chondrocytes of the femoral groove (Fg) and synovium (Syn) in three treated knee joints 6 weeks after injection of indoacetate and in control joints

	G6PD	G3PD	HOAD	GlyPD	MalD	GlutD
(i)						
IO (Fg)	0/57*	0/67*	0/21*	—	0/31*	—
IO (Syn)	76	71	34	—	32	—
C (Fg)	59	49	20	—	32	—
C (Syn)	91	72	37	—	41	—
(ii)						
IO (Fg)	0	0	0	0	0	0
IO (Syn)	94	67	59	46	34	57
C (Fg)	45	58	13	21	38	—
C (Syn)	47	52	33	46	23	—
(iii)						
IO (Fg)	0	0	0	0	0	—
IO (Fg)	81	75	32	31	32	—
C (Fg)	43	54	23	21	37	—
C (Syn)	65	62	29	31	25	—

Values are mean integrated extinction × 100/60 min reaction time except G6PD and G3PD which were 30 min.

* Enzyme activity in superficial cells only.

IO, Iodoacetate injected.

C, Control.

(G3PD) with cysteine (Cys-149) at the active site of the enzyme causing marked inhibition by iodoacetate (Harris & Waters 1976). Indeed it was this enzymatic activity that was most inhibited *in vitro*, being 80% inactivated in the chondrocytes subjected to 100 mM iodoacetate. For the in-vivo study, the standard concentration of iodoacetate injected into such joints was 80 mM. This would be the maximum concentration achieved in the joints immediately after injection, assuming there was no dilution by any joint fluid. For all that, the inhibition locally was as great as 95% for G3PD but 72% for G6PD (Table 1). Similar results were found 2 days after the injections (Table 2) and by 4 weeks in two animals there was 100% inhibition of all four enzymes tested (Table 3) even though, *in vitro*, the other enzymes were less susceptible to iodoacetate (Fig. 1).

The inhibitory effect of the injection was very variable in the different regions of each joint so that not all cartilages, or even regions of the same cartilage, were equally affected. It was noticeable that less inhibition was found peripherally even though this would be the region most likely to be the site of drainage. Equally, the variability cannot be explained by diffusion seeing that it was the superficial chondrocytes that were frequently less affected. Moreover, the enzymes of the synovium, that is in close contact with the periphery of the cartilage and is as accessible to the joint space as is the cartilage, were apparently unaffected by the iodoacetate.

As would be expected, the sulphydryl groups were inhibited. In the light of the findings of Kalkert (1985) that radiolabelled iodoacetate is cleared from such joints within 48 hours, the prolongation of the inhibition

Table 5. The loss (%) of Alcian blue staining (in the presence of 0.5M MgC_2) in the cartilage matrix of a number of different knee joints at various times after injection of iodoacetate

	Patella	Femoral groove
24h		
(i)	0	0
(ii)	17	0
48h		
(i)	0	0
5 days		
(i)	44	0
(ii)	23	15
4 weeks		
(i)	48	57
(ii)	51	60
6 weeks		
(i)	83	62
(ii)	91	87
(iii)	92	53
9 weeks		
(i)	80	73
(ii)	72	56
(iii)	59	0

to at least 4 weeks after the injections was surprising.

Following the injection of iodoacetate, the enzymatic reactions showed many regions apparently devoid of the main oxidative enzymatic activities. It was conceivable that such chondrocytes had switched their oxidative metabolism to other routes, such as the ultilization of glycerophosphate (Lehninger 1965) or of glutamate (Moreadith & Lehninger 1984) or of malate (Greenhouse & Lehninger 1977). While such substrates produced responses in the chondrocytes of the non-injected joint, no activity was found in the chondrocytes of the injected joint. The obvious assumption was that these chondrocytes were dead. This was supported by the lack of nuclear staining with toluidine blue or with haematoxylin. However, phase contrast microscopy indicated that the lacunae were not empty. Ultraviolet microspectrophotometry showed the presence of material that had the absorption characteristics normally associated with the nucleic acids; treatment with ribonuclease did not diminish this absorption indicating that it was probably due to DNA. The persistence of DNA in otherwise apparently inert chondrocytes, and the loss of glycosaminoglycans from the matrix are in agreement with the biochemical findings of Kalbhen and Blum (1977) and Kalbhen (1978) which showed that the glycosaminoglycans were lost much more rapidly than the DNA in such cartilage in animals treated with iodoacetate.

These results were perturbing in that they indicated greater inhibition of all the oxidative enzymes studied, not solely of G3PD, but, more surprisingly, that the inhibition did not seem to follow any pattern. Thus the peripheral chondrocytes (of zone 1: Dunham *et al*, 1986), which would be most immediately exposed to the iodoacetate, often retained enzymatic activities while chondrocytes in the lower regions showed total inhibition. Moreover, the synovial lining cells, lying in close proximity to the surface of the cartilage and therefore likely to be exposed to the full concentration of the iodoacetate, showed no inhibition or even apparently enhanced activity. It appears, therefore, that the effect of intra-articular injection of iodoacetate *in vivo* is far more complex than that of merely exposing cells to this enzymatically inhibitory substance.

Acknowledgements

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