Electron microscopic stereological study of collagen fibrils in bovine articular cartilage: volume and surface densities are best obtained indirectly (from length densities and diameters) using isotropic uniform random sampling

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ABSTRACT

Results obtained by the indirect zonal isotropic uniform random (IUR) estimation were compared with those obtained by the direct point and interception counting methods on vertical (VS) or IUR sections in a stereological study of bovine articular cartilage collagen fibrils at the ultrastructural level. Besides comparisons between the direct and indirect estimations (direct IUR vs indirect IUR estimations) and between different sampling methods (VS vs IUR sampling), simultaneous comparison of the 2 issues took place (direct VS vs indirect IUR estimation). Using the direct VS method, articular cartilage superficial zone collagen volume fraction (V_v 41%) was 67% and fibril surface density (S_v 0.030 nm^2/nm^3) 15% higher ($P < 0.05$) than values obtained by the indirect IUR method (V_v 25% and S_v 0.026 nm^2/nm^3). The same was observed when the direct IUR method was used: collagen volume fraction (V_v 40%) was 63% and fibril surface density (S_v 0.032 nm^2/nm^3) 21% higher ($P < 0.05$) than those obtained by the indirect IUR technique. Similarly, in the deep zone of articular cartilage direct VS and direct IUR methods gave 50 and 55% higher ($P < 0.05$) collagen fibril volume fractions (V_v 43 and 44% vs 29%) and the direct IUR method 25% higher ($P < 0.05$) fibril surface density values (S_v 0.025 vs 0.020 nm^2/nm^3) than the indirect IUR estimation. On theoretical grounds, scrutiny calculations, as well as earlier reports, it is concluded that the direct VS and direct IUR methods systematically overestimated the V_v and S_v of collagen fibrils. This bias was due to the overprojection which derives from the high section thickness in relation to collagen fibril diameter. On the other hand, factors that during estimation tend to underestimate V_v and S_v, such as profile overlapping and truncation ('fuzzy' profiles), seemed to cause less bias. As length density (L_v) and collagen fibril diameter are minimally biased by the high relative section thickness, the indirect IUR method, based on utilisation of these estimates, is here regarded as representing a 'gold standard'. The sensitivity of these 3 methods was also tested with cartilage from an in vitro loading experiment which caused tissue compression. In the superficial zone of articular cartilage V_v and S_v of collagen fibrils increased ($P < 0.05$). This difference in the stereological estimates was only detected by the indirect IUR estimation but not by the direct VS or direct IUR methods. This indicated that the indirect IUR estimation was more sensitive than the direct VS or direct IUR estimations. On the basis of these observations, the indirect zonal IUR estimation can be regarded as the technique of choice in the electron microscopic stereology of cartilage collagen.

Key words: Stereology; vertical section sampling; overprojection; truncation; isector.

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INTRODUCTION

Articular cartilage is a specialised form of connective tissue which forms a smooth and resilient covering on articulating surfaces of diarthrodial joints. Cartilage tissue has to withstand large contact forces transmitted through the joint during motion. The tensile stiffness and strength of the articular cartilage is provided by the collagen network. Of this collagen, 90–95% belongs to type II collagen. Other collagen types contributing to the fibrils are types IX and XI (Buckwalter & Mankin, 1997). Benninghoff (1925) demonstrated, by aid of the polarised light microscope, the tangential, transitional and radial zones of the articular cartilage. The orientation, thickness and amount of collagen fibrils varies within these zones and also with distance from the chondrocytes (Clarke, 1974; Minns & Steven, 1977). Aspden & Hukins (1981) suggested that collagen fibrils were oriented in relation to the mechanical demands put on the articular cartilage. The average diameter of the collagen fibrils, measured from fibril profiles of electron micrographs, increases from the superficial to deep zones of the uncalcified cartilage in rabbits (Paukkonen & Helminen, 1987), cattle (Hedlund et al. 1993), dogs (Arokoski et al. 1996) and man (Weiss et al. 1968; Hwang et al. 1992).

Due to the complex architecture of the cartilage collagen network, stereological analysis at the ultrastructural level is a powerful tool to investigate the collagen fibrils. In stereology, specimen sampling plays crucial role (Weibel, 1979). In order to assess the number of animals, number of blocks per animal, number of sections per block, and number of fields per section, it is important to know the variation for the material of interest. The number of animals in the group should be at least 5, and up to 5 blocks per animal and up to 5 sections per block should be sufficient (Cruz-Orive & Weibel, 1990). The number of counts (points, intersections, or profiles) per animal usually does not need to exceed 200 (see nomogram in Gundersen & Jensen, 1987; and Gundersen et al. 1988*a*, *b*; Cruz-Orive & Weibel, 1990) and the magnification of the fields of vision should be limited to the minimum that allows resolution of the objects of interest (Mayhew, 1991). Going higher than this threshold magnification serves only to increase field-to-field variation.

The sampling procedure has to be randomised at every stage because biological objects tend to vary with spatial position and orientation. If content does not vary with position, the object is said to be homogenous (Mayhew, 1991). Articular cartilage is an example of homogenous tissue in terms of its cytoarchitecture and matrix as a whole. If content does not vary with orientation, the object is said to be isotropic (Mayhew, 1991). Articular cartilage is not isotropic because it is layered and, within its layers, i.e. zones, collagen fibrils are oriented either parallel with, at random or perpendicular to the cartilage surface. For all stereological parameters, section position must be randomised, i.e. all parts of the specimen must have the same chance to be selected for examination (Uniform Random). For the estimation of certain quantities, e.g. surface (S_v) and length (L_v) densities, section orientation must also be randomised (Isotropy) (Mayhew, 1991). Traditional methods in stereology assume isotropic uniform random (IUR) sampling of the specimens for estimation of S_v and L_v. Baddeley et al. (1986) advocated the use of vertical section sampling for layered, anisotropic tissues such as cartilage. In this vertical section technique, sections are cut perpendicular to an a priori chosen horizontal plane, i.e. section planes are not IUR in 3-D. This IUR in 3-D is, however, achieved by using cycloid lines (Baddeley et al. 1986; Mayhew, 1991). Besides estimation of volume fraction (V_v) with a point grid, the cycloid test system on vertical sections made possible the estimation of S_v, without use of isotropic uniform random sections. Also it is possible to estimate L_v of lineal, truly 1-D features with cycloid shaped test lines on vertical slices (Gokhale, 1989).

Finally, the fields of view from the sections under the microscope and measurements from the fields must also fulfil the rule of random sampling. The simplest sampling system is the one where the field sampling is systematic and independent of the content—and of the observer (Gundersen et al. 1988*a*). Systematic random sampling is well illustrated by Gundersen & Jensen (1987). This technique, in which the position of the first area is selected at random and, by using a certain pattern, determines the position of all other areas in the sample (Mayhew, 1991), diminishes remarkably the error variance of the estimate and so increases the efficiency of the estimation procedure.

The concept of efficiency means low variability in the estimate after spending moderate amounts of time for the analysis. Another concept in stereology is unbiasedness, meaning lack of systematic deviation from the true value (Gundersen et al. 1988*a*). Precision depends on sample size but bias does not (Mayhew, 1991). Nonuniform, nonrandom, or nonisotropic sampling cause bias. One additional source of bias derives from the use of so-called 'thick sections' in the

estimation procedure. When the relative section thickness (G)

$$G = t/d, \quad (1)$$

where t is the section thickness and d the dimension of the structural component of interest (e.g. diameter of the collagen fibril), is high, the overprojection (also called the Holmes effect; Holmes, 1927), truncation, and overlapping cause bias to the direct estimation of V_v and S_v (Gundersen, 1979). Usually, when G is 0.1–0.2 or less, the section practically represents an infinite 2-D section plane of the object and the bias is negligible (Weibel & Paumgartner, 1978). At the ultrastructural level, however, where section thickness varies between 30 and 80 nm and the diameter of collagen fibrils varies from 5–15 to 120 nm (Zambrano et al. 1982; Hedlund et al. 1993), the relative section thickness is high, varying from 0.25 to 16, and the stereological probe, instead of representing a 2-D section plane, is a 3-D 'thick' section having a volume. In this case the bias caused by the overprojection of the fibrils, overlapping of the projected fibrils, and truncation, i.e. decreased resolution and contrast of the fibril profiles, have to be taken into consideration (Gundersen, 1979; Cruz-Orive, 1983). The finite section thickness causes an increase in the amount of collagen fibrils in this stereological section probe. This brings about overprojection of fibril profiles in the observation plane and leads to overestimation of both V_v and S_v, when estimated directly from the observation plane using point and cycloid/line test systems (Holmes, 1927; Gundersen, 1979; Cruz-Orive, 1983). On the other hand, fibril overlapping in this 3-D stereological probe hides profiles of structures in the observation plane. A third origin of bias, truncation, is best illustrated by spherical features. In 'thick' sections, profiles of spherical features will be lost if their diameter is less than the resolution threshold diameter. Also those features are lost whose spherical cap subtends an angle less than, or equal to, twice the capping angle (see Cruz-Orive, 1983). In practical stereology of tubules, e.g., of collagen fibrils, in 'thick' sections, this truncation effect is called the 'fuzzy profile' phenomenon and it makes distinction between the fibril profile and background difficult, since contrast of an obliquely sectioned fibril is reduced towards the ends of the elongated profile. Both the fibril overlapping and the 'fuzzy profiles' underestimate both V_v and S_v, when estimated directly using point and cycloid/line test systems (Gundersen, 1979). Besides section thickness and sectioning angle, technical details such as specimen contrast and resolution affect the ability to identify ultrastructural components of interest (Mayhew & Reith, 1988). The problem of oblique sectioning and identification of the component of interest is discussed in work by Mayhew & Reith (1988), who presented practical ways to correct S_v of cytomembranes when loss of membrane images results from oblique sectioning.

There are also other techniques to calculate the bias caused by the finite section thickness (overprojection, overlapping, and truncation) on an estimate of V_v and S_v (Cahn & Nutting, 1959; Miles, 1976; Weibel & Paumgartner, 1978; Cruz-Orive, 1983). However, requirements which need to be fulfilled before these correction factors can be used are not suitable for an anisotropic structure such as an articular cartilage collagen network. In addition, none of these models correct biased data for all these effects. Also the magnitude of the 'fuzzy bias' is difficult to predict, due to the variation of tissue properties between animals, area of the joint, variations in specimen processing, section staining and photographic conditions.

There are stereological studies on collagen fibrils of bovine (Hedlund et al. 1993), human (Curtin & Reville, 1995), dog (Arokoski et al. 1996; Panula et al. 1998) and rat (Itayem et al. 1997) articular cartilage at the ultrastructural level. Noonan et al. (1998) made stereological studies on collagen fibrils of miniature pig growth cartilage. In none of these stereological studies, however, was the effect of finite section thickness and the bias arising therefrom, taken into consideration. Because V_v and S_v estimations were carried out by using direct test systems either on systematic, vertical, or isotropic uniform random sections, the results were biased when considered from the theoretical point of view.

In this paper we compare the results from V_v and S_v stereological analyses at the ultrastructural level of articular cartilage collagen fibrils obtained either by the direct point and intercept counting method with point and cycloid or point and line test systems (1) on vertical sections (VS) or (2) on IUR sections, or (3) by the indirect, zonal estimation from collagen fibril L_v and diameters on isotropic uniform random sections (IUR) using formulae proposed by Gundersen (1979). It is worth noting that here the comparisons have been made between the direct and indirect stereological estimators and also between 2 different sampling protocols. Both of these issues take place simultaneously in comparisons between direct VS and indirect IUR methods. Two other comparisons (direct VS vs direct IUR estimation and direct IUR vs indirect IUR estimation) affect only one issue.

The theoretical model behind the indirect IUR method assumes collagen fibrils to be open-ended, straight cylinders or tubules with infinite length. Also it is assumed that collagen fibril diameters and lengths vary independently, or one of them is a constant. These assumptions are practically fulfilled in the case of articular cartilage collagen fibrils at the ultrastructural level. The majority of collagen fibrils are straight, especially in the superficial and deep zones of articular cartilage (Zambrano et al. 1982). Also the lengths of fibrils are practically infinite when compared with the diameters of the fibrils, i.e. the fibril structure is much longer than it is wide (ratio of diameter to length of the collagen fibril is practically zero). Based on this infinity of the length of collagen fibrils at the ultrastructural level, we can assume the length of the fibril to be a constant (i.e. ∞ in practical terms). The indirect estimation using the IUR sections, which results in indirect determination of V_v and S_v of the collagen fibrils, is based on the utilisation of an estimate of the average collagen fibril diameter and L_v of the collagen fibrils, which is the number of collagen fibril profiles, N_A, in the area frame multiplied by 2. The infinite length (in practical terms) of the collagen fibrils at the ultrastructural level enables unbiased estimation of L_v independent of variations in section thickness and so prevents bias due to overprojection (Gundersen, 1979). Collagen fibril profile counting is independent of their size, shape, and degree of fuzziness (Gundersen, 1978; 1979), i.e. both clear and 'fuzzy profiles' can be counted and so truncation does not cause bias. Profile counting is also much less interfered with by overlapping than is estimation of V_v or S_v. The average collagen fibril diameter can be estimated unbiasedly in terms of practical stereology at the middle of the long axis of the collagen fibril profile. Since the estimates are computed separately for each zone, the critical information concerning the zones and distance from cartilage surface is preserved.

We also tested the sensitivity of the direct VS, direct IUR and indirect IUR estimation techniques. This was performed by estimating collagen V_v and S_v from an in vitro-loaded and control cartilage explants. The in vitro-loading was used here as an experimental model, which altered cartilage matrix properties, i.e. diminished cartilage thickness and caused local compression of the collagen network (Király et al. 1998).

MATERIALS AND METHODS

Untreated articular cartilage specimens

Bovine knee joints from 10 (1–2-y-old) animals were obtained from a local slaughterhouse. Knee joints were opened 1–2 h after death. Glistening and healthy looking articular cartilage was accepted for specimen preparation. From each joint 8 articular cartilage plugs (diameter 3 mm) were prepared from the medial patellar surface of the femur (Fig. 1). The plugs were dissected free from the subchondral bone using a razor blade. The plugs from each animal were then collected on a petri dish with 0.1 M cacodylate buffer (pH 7.4), and 4 plugs were picked at random for vertical sectioning (VS) and another 4 for zonal isotropic uniform random (IUR) sampling. From the precise centre of the specimen collection area, one specimen with the subchondral bone attached was taken for light microscopic determination of cartilage thickness (Fig. 1).

In vitro-loading of articular cartilage plugs

Cartilage plugs were mechanically loaded in vitro as described earlier (Király et al. 1998). Briefly, the loading was carried out with an apparatus developed for cyclic compression of cartilage explants (Parkkinen et al. 1989). Cartilage plugs (diameter 3 mm), chosen at random for loading and taken from the medial patellar surface of the femur of bovine knee joints ($n = 9$) were completely immersed in the Dulbecco's MEM/F-12 (1:1) incubation medium (Gibco BRL, Life Technologies, Paisley, Scotland) with 10 mmol/l Hepes (Sigma Chemical Company, St Louis, MO, USA), antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin; Flow Laboratories, Irvine, Scotland), 70 µg/ml ascorbate (Sigma Chemical Company, St Louis, MO, USA) and 3 mmol/l glutamine (Flow Laboratories, Irvine, Scotland), were loaded with a plane-ended stainless steel loading head at a 4.1 MPa cyclic pressure at 0.5 Hz frequency at 37 °C for 4 h. Unloaded control plugs, chosen at random from the same animals were incubated in the same medium for 4 h.

Preparation of samples for electron microscopy

Cartilage plugs were fixed in 2% glutaraldehyde buffered with 0.1 M cacodylate (pH 7.4), postfixed in 1% osmium tetroxide, dehydrated in an ascending series of ethanol solutions and embedded in LX-112 epoxy resin (Ladd Research Industries, Burlington, VT, USA). For VS sampling, one ultrathin vertical section per block through the whole depth of cartilage

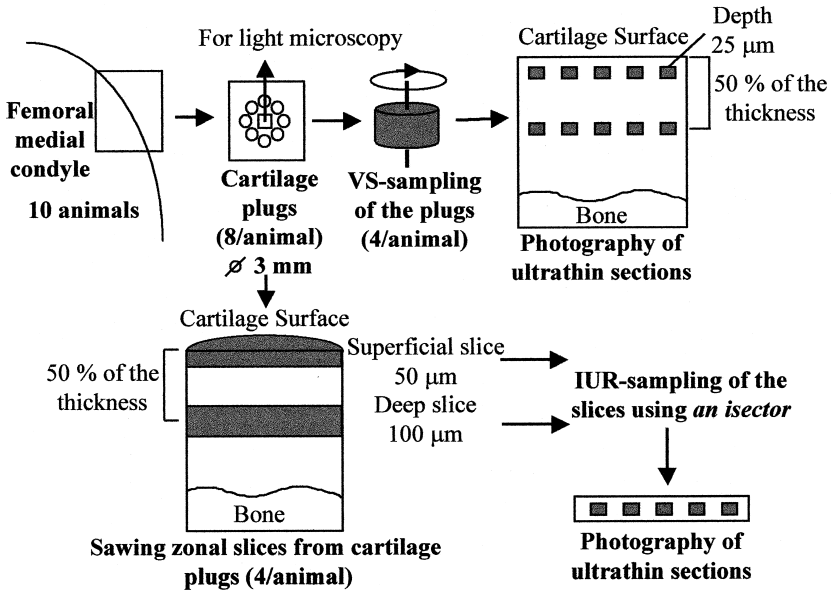

Fig. 1. Schematic presentation on collection and processing of specimens from bovine knee joints for vertical section (VS) and zonal, isotropic, uniform random (IUR) sampling.

was cut, normal to the cartilage surface, with a silver-grey interference colour by a Reichert-Jung Ultracut E ultramicrotome (Reichert-Jung, Vienna, Austria) (Fig. 1). Cartilage plugs for zonal isotropic uniform random sampling were embedded in horizontal moulds. After polymerisation, surplus Epon from the cartilage surface was trimmed off with a Leica Reichert Ultratrimmer (Leica, Vienna, Austria) and an LKB 11800 Pyramitome (LKB, Bromma, Sweden). Then, with a Leitz Wetzlar 1600 sawmicrotome (Ernst Leitz, Wetzlar, Germany), a 51.3 μm ± 1.8 (mean ± S.E.M.) thick cartilage slice was cut from the cartilage surface (superficial zone) and another 107.5 μm ± 1.4 thick slice halfway between the cartilage surface and cartilage-bone junction (deep zone). The slices were re-embedded in Epon using the isector (Nyengaard & Gundersen, 1992). This created a spherical specimen either of the superficial or the deep zone of articular cartilage (Fig. 1). The spherical specimens were rolled on the table and re-embedded in moulds at random orientations. One ultrathin section per tissue slice block was cut from these tissue slices with a silver-grey interference colour. The ultrathin vertical and IUR sections were stained first manually with 1% tannic acid (Mallinckrodt, Paris, KY, USA) (Afzelius, 1992) and thereafter with uranyl acetate and lead citrate using a Leica Reichart Ultrostainer (Leica, Vienna, Austria).

Cartilage specimens for light microscopy were fixed in 4% formaldehyde buffered with 0.1 M phosphate to pH 7.0, decalcified for 12 d in 10% EDTA, dehydrated in an ascending series of ethanol solutions and embedded in Paraplast Plus (Sherwood Medical, St Louis, MO, USA). Sections (5 μm) were cut using an LKB 2218 Historange Microtome (LKB, Bromma, Sweden). Cartilage thickness was measured for each animal from these sections by light microscopy.

Electron microscopy and stereological analysis of the articular cartilage collagen

Ultrathin sections were photographed with a JEOL-1200EX transmission electron microscope (JEOL, Tokyo, Japan) at 80 kV. Five micrographs per section (1 section per block, 4 blocks per animal) were taken from the superficial zone of vertical sections utilising a systematic random sampling pattern (Weibel, 1979) at a depth of 25 μm from the cartilage surface at a magnification of ×50000, and 5 micrographs per section (1 section per block, 4 blocks per animal) from the deep zone, halfway between the cartilage surface and the cartilage-bone junction, with the systematic random sampling pattern at a magnification of ×20000 (Fig. 1). Five micrographs from both the superficial (4 blocks per animal) and deep (4 blocks per animal) zonal IUR ultrathin sections were obtained along a central line of the section with a systematic random sampling pattern at a magnification of ×50000 (Fig. 1). The minimum distance from the chondrocyte was adjusted to 10 μm to make sure that the micrographs were taken from the inter-territorial matrix. The magnification of the microscope was verified with a cross grating replica (54864 lines/inch, Bal-Tec, Liechtenstein).

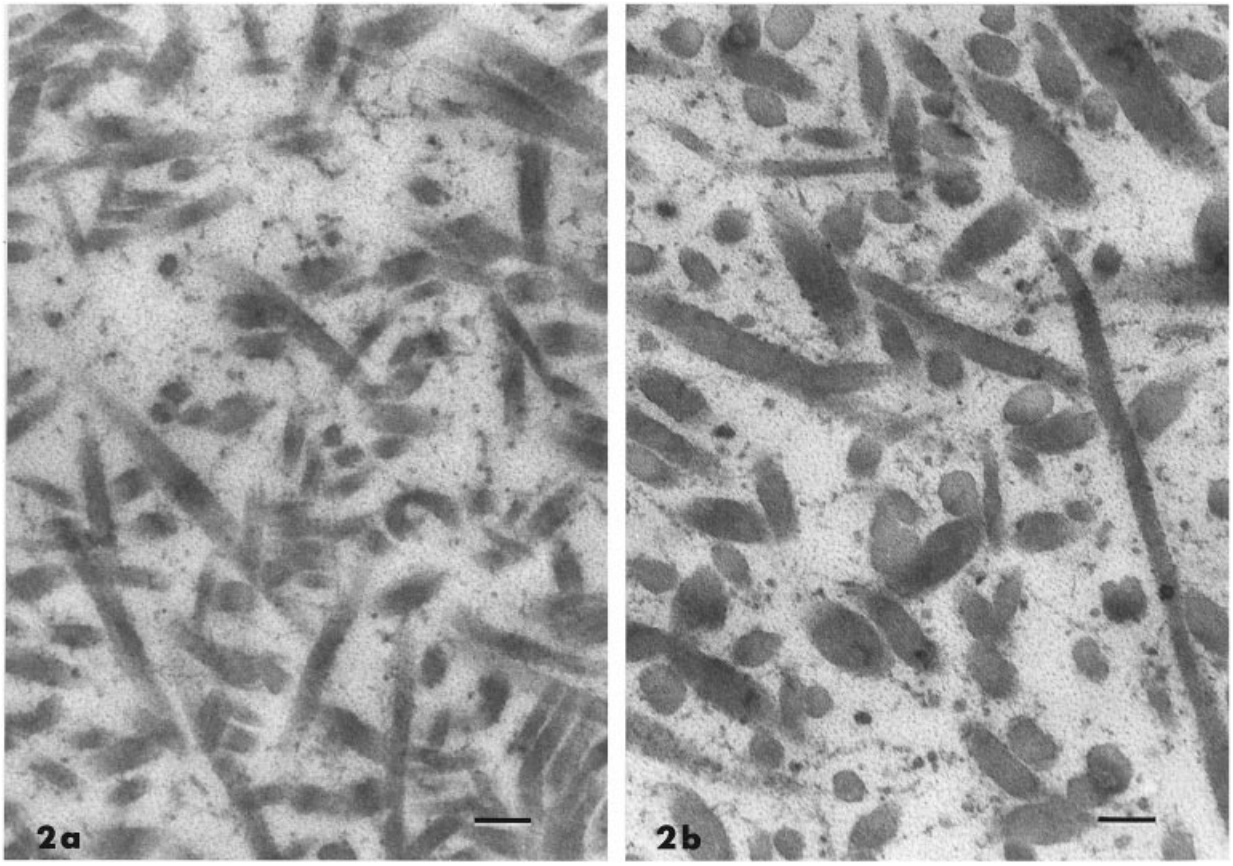

Fig. 2. Two IUR sampled micrographs from the interterritorial matrix of the superficial (*a*) and deep (*b*) zones of the articular cartilage. Measurements were carried out from TEM negatives of the micrographs. Sections were contrasted with tannic acid, uranyl acetate, and lead citrate. Bar, 100 nm.

The number of superficial and deep zone micrographs per animal (n = 10) in untreated tissue specimens totalled 40 (4 cartilage plugs, 5 micrographs for the superficial and 5 for the deep zone of each plug) for both VS and zonal IUR sampling. For in vitro-loaded and unloaded control plugs, the number of superficial and deep zone micrographs per animal (n = 9) totalled 20 (2 cartilage plugs, 1 loaded and 1 control, 5 micrographs for the superficial and 5 for the deep zone in each plug) for both VS and zonal IUR sampling. The total number of micrographs analysed in this study was thus 1160.

The TEM negatives were digitized and stored as 8-bit greyscale images by using Apple Power Macintosh 7100/80 computer, equipped with Photometrics 250 thermal stabilized CCD-camera (Photometrics, Tucson, AZ, USA) and IPLab image processing software (v. 2.5.5, Signal Analytics, Vienna, VA, USA). Final pixel sizes were 1.1 nm and 2.7 nm in digitized images at magnifications of $\times 50000$ and $\times 20000$, respectively.

Estimates of V_v and S_v were obtained in 3 ways: directly from point and intersection counts made on vertical sections (direct VS method), directly from point and intersection counts made on zonal IUR sections (direct IUR method), and indirectly from collagen length density and diameter estimates taken from zonal IUR sections (indirect IUR method).

Direct VS and direct zonal IUR stereological measurements with point and cycloid or point and line test systems on VS and zonal IUR micrographs, respectively, were performed using NIH-Image (v. 1.62, National Institutes of Health, Bethesda, MD, USA) image analysis software equipped with point, cycloid, and line test system macros (Russ, 1995). V_v of the collagen fibrils on both the VS and zonal IUR micrographs was estimated by using a grid with 80 points in 1.8 μm^2 frame in the superficial zone. In the deep zone of articular cartilage, V_v of the collagen fibrils was estimated by using a grid with 80 points in 10.5 μm^2 and in 1.8 μm^2 frames on VS and zonal IUR micrographs, respectively. S_v of the collagen fibrils on VS micrographs was estimated by using a cycloid grid (Baddeley et al. 1986) with a length of 4.3 μm in 1.8 μm^2 frame in the superficial zone and 10.4 μm in 10.5 μm^2 frame in the deep zone of articular cartilage. On the zonal IUR micrographs S_v of the collagen fibrils was estimated by using a line grid with a length

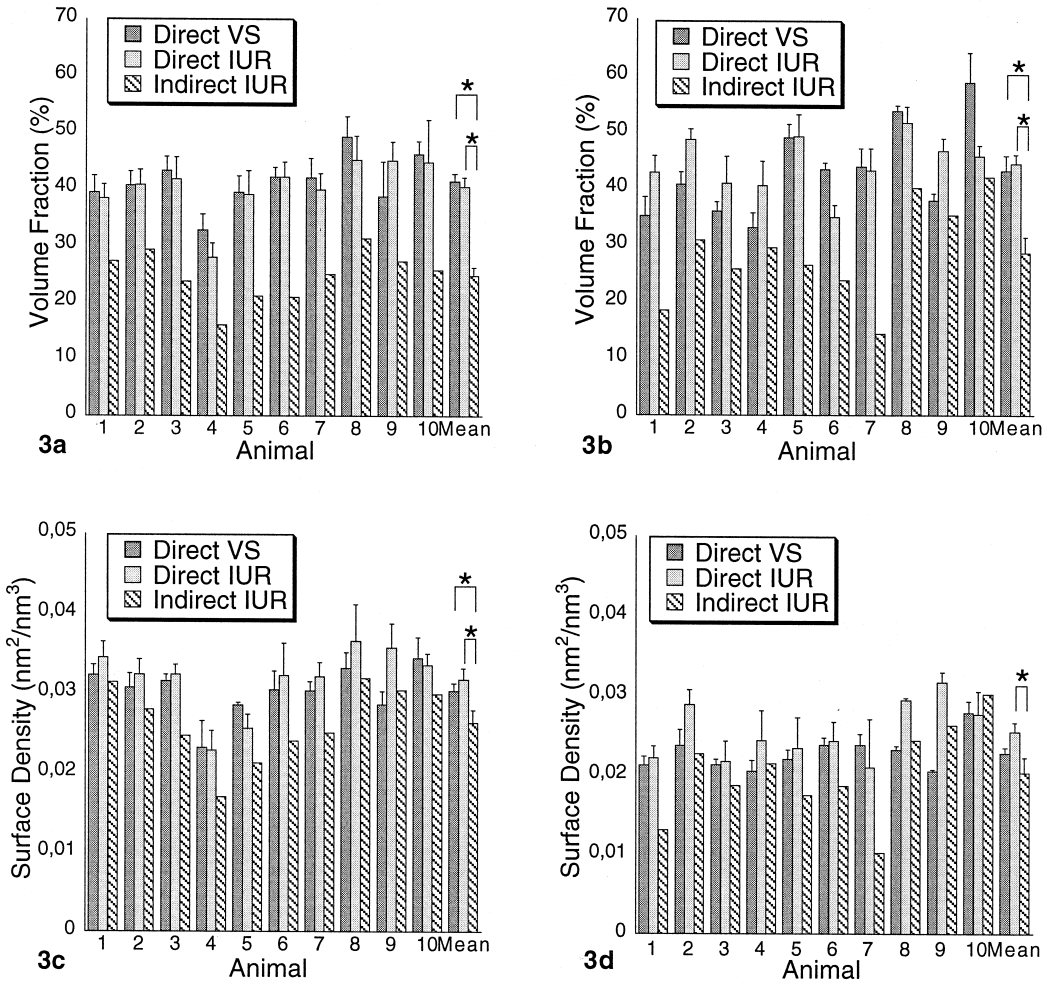

Fig. 3. Collagen fibril volume fraction, V_v (%), in the superficial (*a*) and deep (*b*) zones of the articular cartilage and collagen fibril surface density, S_v (nm^2/nm^3), in the superficial (*c*) and deep (*d*) zones of the articular cartilage determined by the direct vertical section (VS), direct isotropic, uniform random (IUR), and indirect IUR estimations. The mean value (±S.E.M.) for each animal (n = 10) and the group mean are shown. N.S., not significant, *, $P < 0.05$ after Bonferroni correction.

of 5.4 μm in 1.8 μm^2 frame in the superficial and deep zones of articular cartilage.

The ultrathin VS sections were aligned so that the vertical direction was parallel to the short sides of the micrographs. The minor axes of cycloid test lines were parallel to the vertical direction. Test systems were overlaid systematically on micrographs which were photographed with systematic random sampling. The test systems were thus placed at random with respect to the features in the microstructure (Russ, 1995).

Indirect zonal IUR stereological measurements on the zonal IUR micrographs were made with PRISM (v. 3.5, Analytical Vision, Raleigh, NC, USA) image analysis software. V_v and S_v of the collagen fibrils were calculated indirectly by using an estimate of L_v of the collagen fibrils:

$$L_v = 2*N_A, \quad (2)$$

where N_A = number of collagen fibril profiles in the area frame. The average collagen fibril diameter, E(d), and the average second power of the collagen fibril diameter, $E(d^2)$, were also utilised when computing V_v and S_v for each animal using the equations:

$$V_v = \pi/4*E(d^2)*L_v \quad (3)$$

and

$$S_v = \pi*E(d)*L_v \quad (4)$$

(Gundersen, 1979). The area frame used to count the number of the collagen fibril profiles in the superficial zone was 0.64 μm^2 and in the deep zone 1.28 μm^2. The unbiased counting rule proposed by Gundersen (1977) was used to count the number of collagen fibril profiles. To determine the average collagen fibril diameter, E(d), and the average second power of the collagen fibril diameter, $E(d^2)$, the minimum diameter of each collagen fibril profile in 0.12 μm^2 area of the superficial zone and in 0.26 μm^2 area of the deep zone of articular cartilage was measured from all zonal IUR micrographs. Area frames were overlaid system-

atically on micrographs which were photographed with systematic random sampling (Russ, 1995). The relative standard error of the mean (R.S.E.M.) was calculated for all parameters of the direct VS, direct IUR, and indirect IUR estimations.

The in vitro-loaded and control cartilage explants, sampled and processed for both vertical and zonal IUR sections, were photographed in the same way as described above. The micrographs were coded, randomised, and the same analyses were made without knowledge of tissue treatment.

Statistical analysis

For statistical analysis of the parameters (V_v, S_v, L_v, the average collagen fibril diameter, E(d), and the cartilage thickness, *t*), the 2-tailed nonparametric Wilcoxon's matched-pairs signed-ranks test was made with SPSS (v. 6.1.1., SPSS, Chicago, IL, USA) software. Due to multiple comparisons between parameters obtained with the direct VS, direct IUR, and indirect IUR methods, the Bonferroni adjustment was applied when necessary (Miller, 1991).

RESULTS

Direct VS and direct IUR estimations yielded higher ($P < 0.05$ after Bonferroni adjustment) V_v values for collagen fibrils in the interterritorial matrix of the superficial zone than the value determined by the indirect IUR method (Fig. 3*a*). V_v was $41\% \pm 1.4$ (mean ± S.E.M.) after direct VS, $40\% \pm 1.6$ after direct IUR, and $25\% \pm 1.4$ after indirect IUR estimation. In the interterritorial matrix of the deep zone of articular cartilage, V_v of collagen fibrils was also higher ($P < 0.05$ after Bonferroni adjustment) in the direct VS and direct IUR estimations when compared with the indirect IUR method. V_v after direct VS estimation was $43\% \pm 2.7$, $44\% \pm 1.6$ after direct IUR, and $29\% \pm 2.8$ after indirect IUR estimation (Fig. 3*b*). V_v obtained by direct VS and direct IUR methods did not differ from each other in the superficial or deep zones (Fig. 3*a*, *b*).

S_v of collagen fibrils in the interterritorial matrix of the superficial zone of articular cartilage, determined by the indirect IUR estimation, was $0.026\ nm^2/nm^3 \pm 0.0015$. By using the direct VS and direct IUR estimations, S_v was higher ($P < 0.05$ after Bonferroni adjustment) in both cases: $0.032\ nm^2/nm^3 \pm 0.0014$ after direct IUR and $0.030\ nm^2/nm^3 \pm 0.0010$ after direct VS method (Fig. 3*c*). S_v of the collagen fibrils in the interterritorial matrix of the superficial zone after direct VS method did not differ when compared with S_v obtained by the direct IUR method (Fig. 3*c*). In the interterritorial matrix of the deep zone, S_v of collagen fibrils between the direct IUR and indirect IUR methods did also differ ($P < 0.05$ after Bonferroni adjustment), and was $0.020\ nm^2/nm^3 \pm 0.0019$ after the indirect IUR method and $0.025\ nm^2/nm^3 \pm 0.0012$ after direct IUR estimation. S_v of collagen fibrils in the interterritorial matrix of the deep zone after direct VS method did not differ when compared with S_v obtained either by the direct IUR or indirect IUR methods (Fig. 3*d*).

Collagen fibril diameter, measured from IUR sampled sections, was $34.5\ nm \pm 0.7$ in the interterritorial matrix of the superficial and $50.5\ nm \pm 1.2$ in the interterritorial matrix of the deep zone (Fig. 4*a*). The difference between these diameter values was

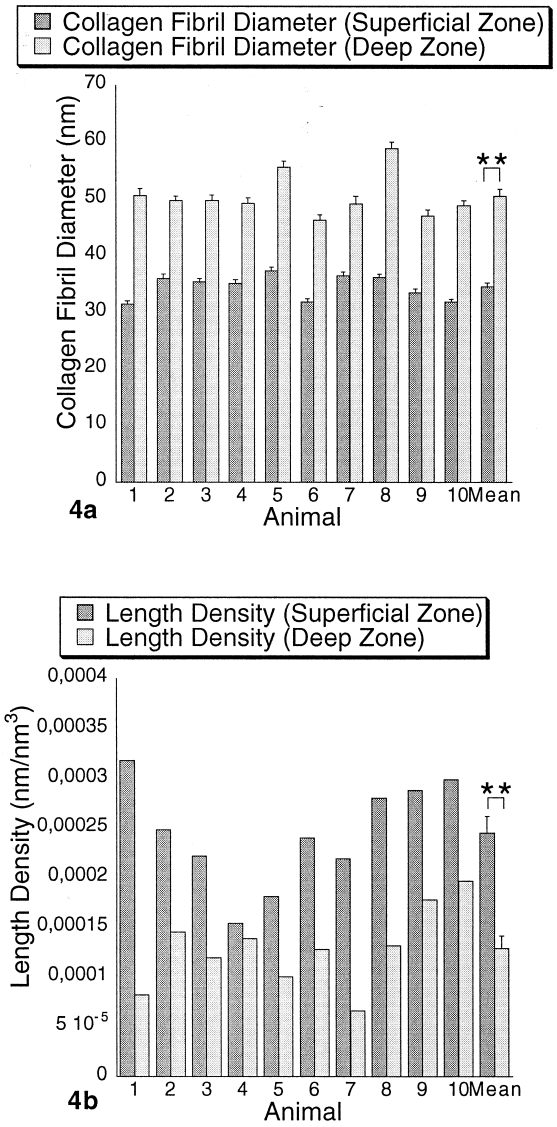

Fig. 4. Average collagen fibril diameter (*a*) and length density, L_v, (*b*) in the interterritorial matrix of the superficial and deep zones of the articular cartilage, measured from IUR sampled micrographs. The mean value (±S.E.M.) for each animal (n = 10) and the group mean are shown, **, $P < 0.01$.

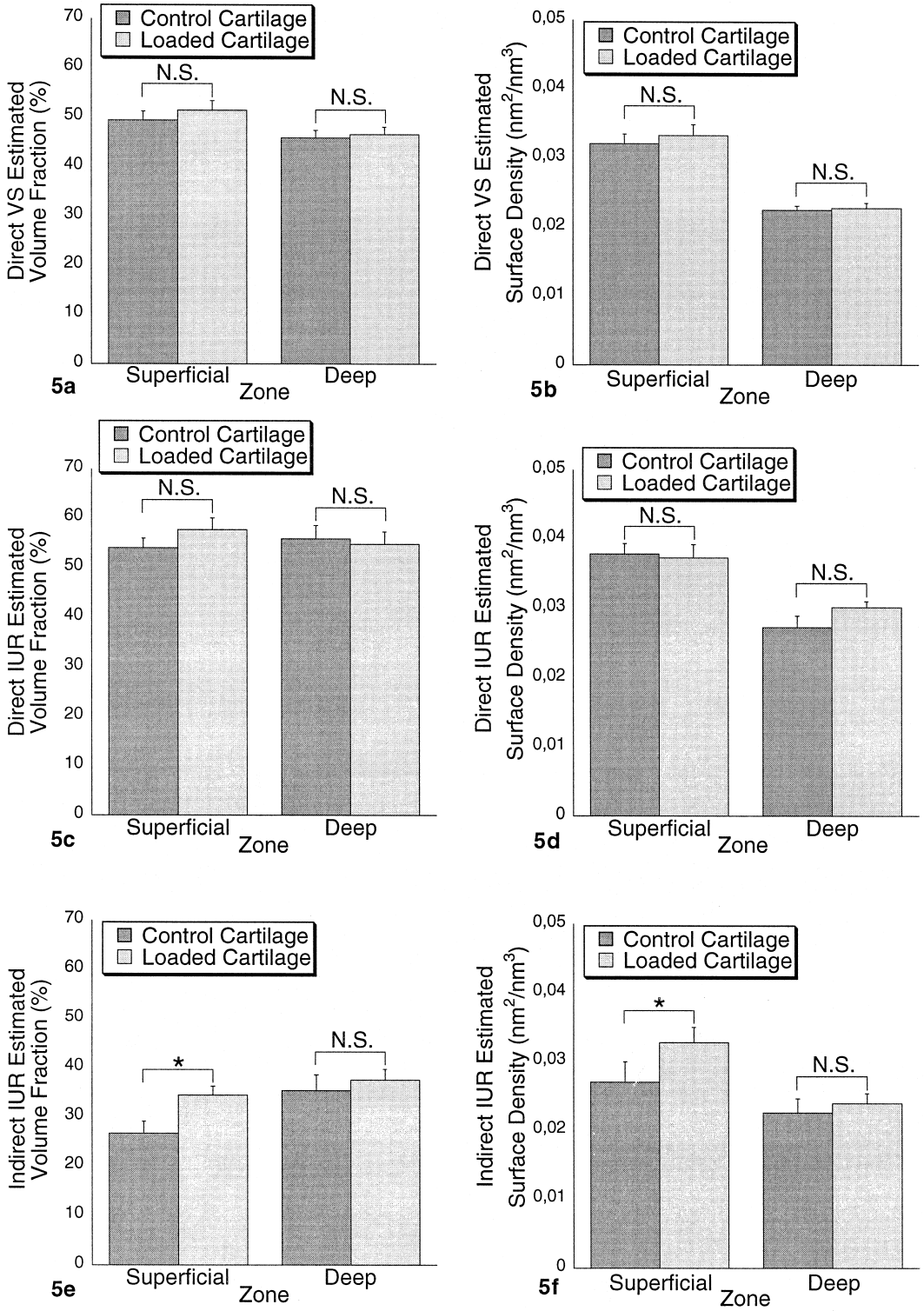

Fig. 5. Effect of the 4 h in vitro loading on the collagen volume fraction, V_v, (*a*, *c*, *e*) and surface density, S_v, (*b*, *d*, *f*) determined by using the direct vertical section (VS) (*a*, *b*), direct, isotropic, uniform random (IUR) (*c*, *d*), and indirect IUR (*e*, *f*) estimations (mean ± S.E.M.). N.S., not significant; *, $P < 0.05$.

significant ($P < 0.01$). Variation in fibril diameter between animals was quite low. Instead, L_v showed considerable variation between animals. L_v was 244.9 μm/μm³ ± 16.6 in the interterritorial matrix of the superficial zone and 128.8 μm/μm³ ± 12.6 in the interterritorial matrix of the deep zone (Fig. 4*b*). The difference between these L_v values was significant ($P < 0.01$).

The in vitro loading caused compression of the uncalcified cartilage from 1401.9 μm ± 48.0 to

1124.8 μm ± 50.2 in the cartilage tissue plugs ($P < 0.01$). This compression caused an increase in collagen V_v and S_v in the interterritorial matrix of the superficial zone, determined by the indirect IUR estimation ($P < 0.05$) (Fig. 5*e*, *f*). The alterations were not observed when the direct VS or direct IUR estimations were used (Fig. 5*a–d*). With 10 animals the relative standard error of the mean (R.S.E.M.) of all parameters was under 10% in the direct VS, direct IUR, and indirect IUR estimations, although they were somewhat higher in the indirect IUR method (for direct VS method 3.1–6.2%, for direct IUR method 3.5–4.6%, and for indirect IUR method 5.7–9.8%). The indirect IUR estimation was more time consuming to perform than direct VS or direct IUR estimations.

DISCUSSION

The differences ($P < 0.05$) in V_v and S_v values after utilising the direct VS or direct IUR estimations compared with those obtained by the indirect IUR estimation in the superficial and deep zones of articular cartilage (Fig. 3*a–d*) most probably arose from the high relative section thickness and the overprojection, which caused overestimation of V_v and S_v obtained by the direct VS and direct IUR methods. This conclusion can be proved in a very practical way. The relationship between V_v, S_v, and collagen fibril diameter, d, can be written as follows [from equations (3) and (4)]:

$$V_v/S_v = d/4. \quad (5)$$

If a method yields good estimates of V_v and S_v, it can be expected that the values would give a sensible prediction of the mean fibril diameter. By calculating collagen fibril diameters from V_v and S_v obtained by each method and by comparing the predicted and observed diameters with each other we can get an idea of the internal consistency of the methods. For indirect IUR method the predicted fibril diameters were 37.6 and 56.5 nm, for the direct IUR method 50.9 and 69.8 nm, and for the direct VS method 54.5 and 75.8 nm for the superficial and deep zones, respectively. When these estimates are compared with the observed fibril diameters, which were 34.5 and 50.5 nm for the superficial and deep zones, respectively, we find quite good agreement between the predicted and observed diameters obtained by the indirect IUR method but not with those obtained by the direct IUR or direct VS methods. On basis of its internal consistency, the indirect IUR method can be regarded as 'a gold standard' in the EM stereological assay of the collagen network of cartilage. In any case it can be stated that the indirect IUR method is the least biased of the 3 methods judged from both theoretical and practical standpoints.

One indication of bias observed after application of the direct VS and direct IUR estimations was the quite large variation in overestimation of parameter values between animals (Fig. 3*a–d*). This was probably due to variation in the 'fuzziness' (truncation) of the fibril profiles between animals. The 'fuzziness' of profiles depends on variation in specimen processing, section staining and photographic conditions.

There was no difference between collagen V_v of the superficial (25%) and deep (29%) zones after indirect IUR estimation. Instead, the collagen fibril S_v decreased ($P < 0.05$) from superficial (0.026 nm^2/nm^3) to deep (0.020 nm^2/nm^3) zone after the indirect IUR estimation (Fig. 3*c*, *d*). This is in harmony with a previous study (Hedlund et al. 1993) and coincides with an increase ($P < 0.01$) of the collagen fibril diameter from surface (34.5 nm) to the deep (50.5 nm) zone of cartilage. This increase in fibril diameter is in harmony with earlier observations (Fig. 4*a*) (Paukkonen & Helminen, 1987; Hedlund et al. 1993). Decrease ($P < 0.01$) of collagen fibril L_v from surface (244.9 $\mu m/\mu m^3$) to the deep (128.8 $\mu m/\mu m^3$) zone of cartilage causes a decrease in S_v. As to V_v, a decrease L_v is masked by the abrupt increase in the second power of collagen diameter.

The efficiency of the indirect IUR method was lower than that of the direct VS or direct IUR methods. This was noticed by slightly higher R.S.E.M. values of the parameter means by indirect IUR method than by the direct VS or direct IUR methods. The indirect IUR method was also more time consuming than the direct VS or direct IUR methods. The best stereological methods are those which have no bias or are minimally biased and are efficient (Gundersen et al. 1988*a*). Unbiasedness is the most important of these issues, due to the fact that the amount of bias cannot be affected by increasing the sample size as it is possible with efficiency (Gundersen & Jensen, 1987; Mayhew, 1991). The indirect IUR method, being the least biased of these 3 methods, is the best of them, regardless of time consumed or precision achieved.

V_v of collagen fibrils in the interterritorial matrices of the superficial (25%) and deep zones (29%) of bovine articular cartilage after the indirect IUR method were much lower than the values 56% for the superficial and 91% in the deep zones reported earlier by Hedlund et al. (1993). The differences between

results reported here and by Hedlund et al. (1993) can be explained, at least to some extent, by the differences ($P < 0.05$) of results obtained by the indirect IUR, the direct VS, and direct IUR estimations (Fig. 3*a*, *b*). Also Arokoski et al. (1996) reported quite large V_v values for collagen in canine articular cartilage (87.6 % in the superficial and 94.3 % in the deep zone). On the other hand, Curtin & Reville (1995) (43.2 % in the superficial and 42.8 % in the deep zone) and Panula et al. (1998) (35 % in the superficial zone) reported V_v values of human and canine articular cartilage, respectively, which were closer to the values reported here, but were still higher. Another explanation for these observations might be, besides the overestimation due to overprojection, that the collagen network varies significantly in different areas of the joint. This is indeed the case in the canine knee joint as clearly demonstrated by polarised light microscopy (Arokoski et al. 1996; Panula et al. 1998). The collagenous network is far more extensive and makes the cartilage more stiff in, e.g. the canine lateral femoral condyle as compared with the lateral tibial condyle (Jurvelin et al. 1988; Arokoski et al. 1996).

Although the S_v gradient changes in the same fashion as in the previous study (Hedlund et al. 1993), i.e. S_v of the collagen fibrils decreases towards basal parts of the cartilage, the absolute values of this study (0.026 nm^{-1} in the superficial and 0.020 nm^{-1} in the deep zone) differ dramatically from their values, which were $7.4*10^{-5}$ cm^{-1} ($7.4*10^{-12}$ nm^{-1}) and $6.5*10^{-5}$ cm^{-1} ($6.5*10^{-12}$ nm^{-1}) in the interterritorial superficial and deep zones, respectively. In our opinion, these absolute values are not explained by a difference in sampling design and in estimation method or by variations of the collagen network between different areas and parts of the joint, but probably result from an error in the sign of the multiplier 10^{-5}. If 10^5 is used instead of 10^{-5}, S_v values in the work by Hedlund et al. would be 0.074 nm^{-1} and 0.065 nm^{-1} in the superficial and deep zones, respectively. These values would be of the same order of magnitude as values presented here, estimated by all 3 methods.

V_v varied in the superficial zone from 16 % to 31 % and in the deep zone from 14 % to 42 % between the animals, determined with the indirect IUR estimation (Fig. 3*a*, *b*). This large biological variation probably affects biological properties of cartilage and its ability to withstand stress. The variation was mainly due to differences in L_v of the collagen fibrils. L_v varied from 154 $\mu m/\mu m^3$ to 318 $\mu m/\mu m^3$ and from 66 $\mu m/\mu m^3$ to 197 $\mu m/\mu m^3$ in the superficial and deep zones, respectively (Fig. 4*b*). In both zones the collagen fibril diameter was quite constant from animal to animal (Fig. 4*a*). Hedlund et al. (1993) reported collagen fibril diameter in bovine articular cartilage to be thinner than in our observations, both in the superficial and deep zones, the average being in the interterritorial matrix 17 nm of the superficial and 38 nm of the deep zone of the articular cartilage. Other reports on fibril diameter support higher values for collagen fibrils than those of Hedlund et al. (1993) (Weiss et al. 1968; Paukkonen & Helminen, 1987; Hwang et al. 1992).

In vitro loading caused compression in the cartilage explants and increased ($P < 0.05$) collagen V_v and S_v in the superficial zone of the articular cartilage (Fig. 5*e*, *f*). The finding was supported by results from a recent study where quantitative polarised light microscopy was used to investigate the collagen network (Király et al. 1998). The authors reported that the area-integrated retardation (AIR) values (emanating from the amount and arrangement of collagen fibrils of cartilage) of the superficial zone of cartilage were significantly increased in loaded cartilage. It is reasonable to think that the mechanical loading compressed, i.e. diminished the volume of the superficial zone, and thus caused an increase in AIR, V_v, and S_v estimates emanating from collagen fibrils. Because there was a difference ($P < 0.05$) between the control and loaded cartilages only after using the indirect IUR technique and not after the direct VS or direct IUR estimations, the conclusion is that the indirect IUR method is more sensitive than the direct VS and direct IUR estimations. The overall explanation to this would be that the combined effects of overestimation due to overprojection and underestimation due to profile overlapping and 'fuzzy' profiles masked collagen network compression.

To conclude, the indirect IUR estimation for the stereological investigation of articular cartilage collagen fibrils at the at the ultrastructural level appears to be less biased and more sensitive than the direct VS or direct IUR estimations. Therefore, in our opinion, it should always be used when possible. There are, however, some situations when the use of indirect zonal IUR estimation can be inefficient. One situation arises when it is not possible to separate the different zones of the articular cartilage for analysis, e.g. when cartilage of small animal species such as the mouse is investigated. In mouse the thickness of articular cartilage is less than 100 μm. Of course, it is possible to take the whole cartilage thickness of a single IUR sampled specimen, but differences in collagen fibril diameter, V_v, and S_v between the superficial and deep zones of the articular cartilage might be quite large and the variation in the estimators in and between the

animals would be more than the absolute mean difference to be detected between 2 or more groups, namely, alterations in articular cartilage can take place in one cartilage zone only and these changes might not be revealed if average parameters for the whole cartilage are estimated. In these situations it might be possible to use vertical slices, i.e. 3-D probes perpendicular to an a priori chosen horizontal plane with constant thickness, to estimate L_v of the lineal features on projection images by using cycloid-shaped test lines with the minor axis perpendicular to the vertical axis (Gokhale, 1989). Collagen V_v and S_v can then be calculated by using formulae (3) and (4). However, this method proposed by Gokhale assumes features to be truly 1-D lineal filaments, i.e. their cross-section is a point. In case of the collagen fibrils of articular cartilage this statement is not always true, namely the cross sectional area of the collagen fibrils on 'thick' sections can be too large. Also overlapping and truncation complicate the interpretation of the central line or 'spine' of the fibrils. Instead, counting the number of fibril profiles with unbiased counting frame (Gundersen, 1977) is not affected by truncation ('fuzzy' profiles) because there is symmetry of the decision process (Gundersen, 1979). This symmetry is based on the fact that N_A is independent of profile size and shape (Gundersen, 1978). In case of an obliquely sectioned fibril profile on 'thick' section some bias may arise when the observer has to decide whether the central line of the fibril in projected profile is a point, short line, or long line. Also congestion of a collagen fibrils in projects of 'thick' sections may complicate the interpretation of intersections between the test system and profile central lines due to strong overlapping effect and may cause underestimation of L_v

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