Cholesterol distribution among plasma lipoproteins in control and cholesterol fed rats

	Control	Control pair fed	Cholesterol fed		
			2	4	7 days
VLDL + LDL	24.6	25.8	81.9	86.8	97.8
HDL	73.4	74.2	18.1	50.7	2.2

Data are expressed as percent of total plasma cholesterol the values were 57.7 ± 5.5 mg/dl in controls, 55.7 ± 3.8 in controls pair fed, 168 ± 43, 389 ± 35 and 905 ± 147 in cholesterol fed animals at 2, 4 and 7 days (mean ± SD, N = 5). VLDL and LDL are calculated together since no apparent peak of LDL can be detected in control and in cholesterol fed rats; only a tail of β VLDL can be found in a region where human LDL are eluted from the column.

Discussion. The purpose of this work was to investigate whether cholesterol feeding resulted in a modulation of the HDL binding sites in the liver. Oram et al. reported that the loading of cultured fibroblasts with cholesterol results in an up-regulation of the binding sites for HDL_3[2]. Our data show that, while short term cholesterol feeding induces a dramatic increase of plasma cholesterol as well as of liver cholesterol in rats, this dietary manipulation does not affect HDL binding to liver membranes.

It has been reported that short term cholesterol feeding decreases the number of β VLDL and LDL receptors in the liver of rabbits and rats[15]. We therefore used β VLDL to show that under our experimental conditions cholesterol feeding results in a down-regulation of the LDL receptor also (fig. 4). The remaining binding of β VLDL can be explained either as binding to the E receptor[16] which is not modulated by cholesterol feeding or as binding that is not inhibited by EDTA[17].

The binding of HDL was not affected by cholesterol feeding while free and esterified cholesterol content of the liver increased. This finding does not support the hypothesis that in vivo loading of the liver with cholesterol results in up-regulation of HDL binding sites. A recent report by Hoeg et al. suggests, however, that in vitro the loading of hepatoma G-2 cells with cholesterol results in an up-regulation of the HDL binding sites[18]. These data disagree with our observation that cholesterol loading of Hepatoma G-2 cells in culture does not modify the HDL binding (Catapano et al., unpublished observations). Furthermore in enterocytes isolated from cholesterol fed rat the binding of HDL is not affected either[19]. We cannot exclude, however, that in vivo HDL binding sites on the liver are indeed up-regulated, but preparation of the membranes results in a loss of the sites induced by cholesterol feeding. This reasoning suggests that two different HDL binding sites may exist. Furthermore our data do not rule out the possibility that the binding of HDL may be up-regulated in peripheral tissues. A third possibility is that intra and extra cellular binding sites for HDL exist and cholesterol loading of the cell promotes their redistribution. Since the membrane preparations we used are a mixture of plasmatic and microsomal membranes the total binding would not be affected. This theory, however, is in disagreement with Oram et al. who found that sites induced by cholesterol loading of the cell are dependent upon protein synthesis[2].

In summary, our data suggest that in vivo in the rat the binding of Apo E-free HDL to liver membranes does not depend upon cholesterol loading of the cells. Whether this HDL binding site is relevant to the catabolism of HDL or to the 'release' of cholesterol by the HDL to the liver is unknown.

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Protein and lysozyme content of adult human nucleus pulposus

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Summary. A radiologically normal human nucleus pulposus was extracted with 4 M guanidinium chloride and the non-collagenous proteins separated from the proteoglycans by dissociative density gradient centrifugation. Lysozyme was identified as a matrix constituent of the normal, mature human nucleus pulposus.
Key words. Lysozyme; nucleus pulposus; intervertebral disc; protein; proteoglycan.

Proteoglycans, type II and minor types of collagen, non-collagenous proteins and traces of elastin are the major macromolecules of the nucleus pulposus of the intervertebral disc[3]. Although the non-collagenous proteins can reportedly comprise up to 45% of the dry weight of the disc[4], their molecular forms and functions remain unknown. We here identify one of the non-collagenous proteins of the normal human disc as lysozyme.

Methods. A radiologically normal human spine (age: 32 years) was obtained at autopsy. The nuclei pulposi (L1/2 through L 4/5) were removed by dissection and extracted by gentle rotation

Fraction No.	Density (g/ml)	% of dry wt of extracts	% of total protein in extract	% of total uronate in extract	µg lysozyme per mg dry wt of fraction
D1	1.56	44.7	22.6	56.8	0.004
D2	1.55	13.7	9.7	13.8	0.037
D3	1.54	5.9	5.2	5.8	0.050
D4	1.51	9.2	10.0	7.5	0.097
D5	1.49	16.1	19.4	12.2	0.172
D6	1.43	10.3	29.1	3.8	0.536

at 4 °C for 24 h with 4 M guanidinium chloride, 0.05 M sodium acetate, pH 5.8 containing 0.01 M N-ethylmaleimide[5], 0.01 M EDTA, 0.1 M 6-aminocaproic acid, 5 mM benzamidine-HCl[6] and 1 mM phenyl-methylsulphonyl fluoride[7]. The extract was clarified by filtration through a glass-wool plug in a Pasteur pipette that had been thoroughly washed with guanidinium chloride. Caesium chloride was added to the extract to yield a density of 1.5 g/ml and the proteins separated from the proteoglycans by centrifugation at 100,000 at 12 °C for 64 h as described in detail by Cox et al.[8]. The contents of tubes were separated into 6 fractions of equal volume by means of a Buchler Auto Densi-Flow. They were then dialyzed at 4 °C against 0.05 M sodium acetate, pH 5.8, containing the proteinase inhibitors, then against water and finally freeze-dried. Weighed aliquots of the fractions were dissolved in 0.06 M Na phosphate buffer, pH 6.3, for analysis of their lysozyme activity.

Lysozyme activity was measured by a turbidometric technique with lyophilized *Micrococcus lysodeikticus* as substrate[9]. The bacterium was suspended at 400 µg/ml in 0.06 M phosphate buffer, pH 6.3. Enzyme activity was determined by plotting the initial slope of the change in absorbance at 645 nm against the logarithm of the lysozyme.

The standard was human lysozyme purified from the urine of a patient with monomyelocytic leukemia[10]. The stability of the lysozyme activity in the D6 fraction to acid and alkali was assessed by mixing aliquots of the fractions with equal volumes of either buffer, 0.01 M HCl or 0.01 M NaOH, heating to boiling for 90 s and then reassaying the activity[11].

The density gradient fractions were examined by SDS-borate pore gradient polyacrylamide gel electrophoresis[12].

Results and discussion. The spine was classified as normal by the absence of Schmorl's nodes, disc narrowing, osteophytes, chondrocalcinosis and eburnation of the bone plate. The guanidinium chloride extracted 47% of the dry weight of the nucleus pulposus. About 26% of the dry weight of the extract was located in the low buoyant density D5 and D6 fractions where protein usually bands. Gel electrophoresis (not shown) of reduced D6 fractions revealed a range of bands with apparent molecular weights as high as 219 kDa. Two closely spaced bands with molecular weights about 14.5 kDa were particularly prominent in the gels. Lysozyme activity (table) was concentrated in the least dense (D6) fraction. The enzyme activity was stable to acid but unstable to alkali under the test conditions described. The test samples had activities in arbitrary units as follows: heated, 0.298; acid-heated, 0.300; alkali-heated, 0.007. Lysozyme comprised about 0.5% of the total protein and 0.1% of the total dry weight of the extracted material.

Jolles et al.[13] have listed the properties that define a lysozyme: (a) lyse suspensions of M. lysodeiktieus; (b) low molecular weight ('about 15,000'); (c) basic protein; stable at acid pH values at higher temperatures; (d) lability at alkaline pH values. The activity we detected in discs satisfies criteria (a), (c) and (d) and is consistent with (b). Moreover, lysozyme activity as assessed by the procedures we adopted in this study is abolished by antiserum to the purified enzyme[14].

This is the first report of lysozyme in the mature nucleus pulposus. Economou[15] detected lysozyme in the discs of puppy dogs, the cells of which are notochordal. It is noteworthy that we detected the enzyme in discs that were radiologically non-pathological and it must therefore be considered a normal matrix constituent of the tissue.

Lysozyme is a bacteriolytic enzyme that lyses the cell walls of susceptible organisms by hydrolysis of 1,4 glycosidic linkages betweeen N-acetylmuramic acid and N-acetylglucosamine[16]. It is a highly cationic protein of molecular weight 14.5 kDa. Lysozyme is present in mammals in the secretions of organs that are exposed to airborne bacteria, such as tears, saliva, sputum and milk[16]. Neutrophils and monocytes have particularly high concentrations of lysozyme. The enzyme is usually lysosomal or granular in most tissues. Cartilage contains intriguingly high levels of lysozyme[17] where it is almost exclusively extracellular[18]. The origin of the lysozyme in the nucleus is unknown. The nearest blood vessels to the nucleus lie in the outermost lamellae of the annulus fibrosus and in the verterbral sites immediately adjacent to the cartilage end-plate. If the source of the lysozyme is extra nuclear, the enzyme would have to penetrate either the annulus or the end-plate to arrive in the tissue. Either route is probably feasible for a cationic, small molecular weight protein such as lysozyme.

There is no known substrate for lysozyme in normal disc cartilage and its function in these tissues is an enigma. Although the enzyme has been implicated in the mineralization of cartilage[18], the nucleus pulposus seldom calcifies and the discs studied were normal in that respect. The enzyme can bind to cartilage proteoglycans[16] and it may as a consequence influence the intermolecular interactions of these macromolecules and/or the water-binding capacity of the disc. The elucidation of the origin and function of lysozyme in the disc and cartilage remains a challenge in connective tissue biology.

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