BIOPHYSICAL AND PHYSIOLOGICAL INVESTIGATIONS ON CARTILAGE AND OTHER MESENCHYMAL TISSUES

III. THE DIFFUSION RATE OF VARIOUS SUBSTANCES IN NORMAL BOVINE *NUCLEUS PULPOSUS**

by

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INTRODUCTION

The central part of normal intervertebral discs is occupied by a gelatinous pad, called the *Nucleus Pulposus***, surrounded by a tendinous ring, the *Annulus Fibrosus*, and the vertebral bodies. According to a recent report[12] on the ultrastructure of *N.P.*, this represents an immature connective tissue rich in water and mucoid material, probably a chondroitin sulphate-protein compound. In this three-dimensional lattice gel a very fine network of collagenous fibrils is enclosed. The tissue is devoid of vessels and it is therefore assumed that diffusion from the surrounding vascularized mesenchymal tissues plays a dominant rôle for its nutrition (cf.[11]). Both for theoretical and clinical reasons discussed elsewhere[2], this diffusion mechanism is of interest and forms the scope of the present report.

Previous electron microscope studies[12] indicate that for vascular transport we have to deal with a large number of interfibrillar pathways which are partly occupied by mucoid material. The magnitude of these diffusion pathways or "pores" (cf.[3]) cannot be determined by electron microscopy. However, by means of diffusion studies using molecules of different size it is possible to obtain a rough estimate of the average "effective" size of pores. It should be stressed that this diffusion method can only be used in a static system. Direct information is not obtainable as to the possibility of transport of liquids in this tissue during dynamic conditions. For this and other theoretical reasons pertaining to a variety of factors influencing diffusion, the present results are only expected to give information on the actual rate of diffusion of substances tested so far under the conditions mentioned above.

* This is the third report of a program of joint investigations on the physiology and nutrition of some mesenchymal tissues contracted between the Caroline Institute (Depts. of Orthopedic Surgery and Cancer Research Division of Radiumhemmet) and the Institute of Biochemistry at the University of Upsala.

** Abbreviated *N.P.*

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MATERIAL AND METHODS

Fresh pieces of *N.P.* from just sacrificed calves were cut in the freezing microtome (Unicam, Cambridge) in 40 micron thick sections and stored on object slides at −8° C. Diffusion measurements were then performed in the interferometer apparatus[8]. The sections were transferred without damage by means of a few drops of distilled water from the object slides to the aluminium coated glass plate of the Fabry-Perot etalon. The sections were allowed to swell until a thickness of a little more than 40 microns was reached whereafter they were placed between the glass plates of the etalon. The section thickness is accurately determined by the distance between the glass plates[8]. Some excess water forms a thin stripe along the margins of the section.

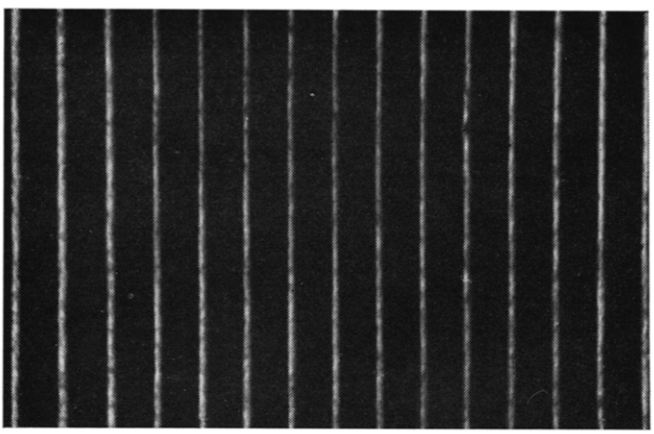

Fig. 1. Interference fringes in an optically homogeneous medium

The concentration of the diffusing substances to be tested was determined by weight and in most cases also controlled by means of the refractive index, which determination after correction gave the real concentration value (c_s). This correction is influenced by two factors. Some of the solute diffuses into the *N.P.* section and some of the water mentioned above has been pressed out from the same section, which in both cases will dilute the solution. This method postulates full convection in the solution[8], which is fulfilled if the boundary between the section and the solution is always vertical. When the solute diffuses in horizontal direction into the tissue the solution just outside the border of the tissue becomes a little more diluted and thus it is lighter than the surrounding solution. This causes a more or less constant stream of the solution upwards along the border of the tissue. This movement can be seen in the interferometer apparatus.

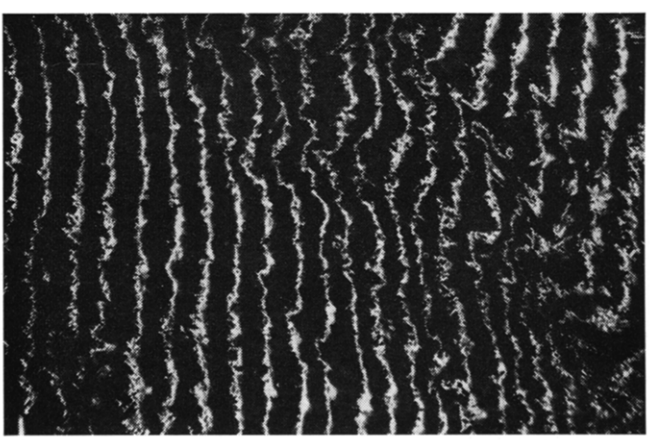

Fig. 2. Distorted interference fringes in a 40 micron section of *N.P.*

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When the medium between the plates is optically homogeneous the interference fringes are equidistant straight lines (Fig. 1). But in the case of *N.P.* which presents a certain degree of optical inhomogeneity they become distorted to some extent (Fig. 2). This fact, however, does not prevent interference measurements, but causes a relative error of about 20% in single diffusion measurements. It is consequently necessary to make many independent observations. The magnitude of the accidental errors is demonstrated in Table I showing a representative series of observations on the diffusion of sucrose ($c_s = 13.8\%$; + 19° C). The time (t) is measured from the moment of contact between the section and the solution. The distance (x) is measured from the border of the tissue section with an eye-piece micrometer. The displacement of an interference fringe is measured in micrometer scale units (0.0456 mm), denoted by (s).

CALCULATIONS

Under the boundary conditions which are valid for this apparatus[8] FICK's second law can be written

$$c = c_s \left(1 - \frac{2}{\sqrt{\pi}} \int_0^{\xi} e^{-y^2}\, dy \right) \qquad (1)$$

$$\text{where } \xi = \frac{x}{2\sqrt{Dt}} \qquad (2)$$

The concentration c is proportional to the interference fringe displacement s. From equations (1) and (2) the value of the diffusion constant D can be calculated (Table I). Deviations from FICK's second law in this material cause D to increase with increasing values of x (Fig. 3). To be able to compare the diffusion rates of different substances the value of D at $x = 1.5$ mm (D^1) was always determined from a D-x-diagram as in Fig. 3. All D^1-values were corrected to 20° C; symbol D^1_{20}. The temperature correction formula[13] was

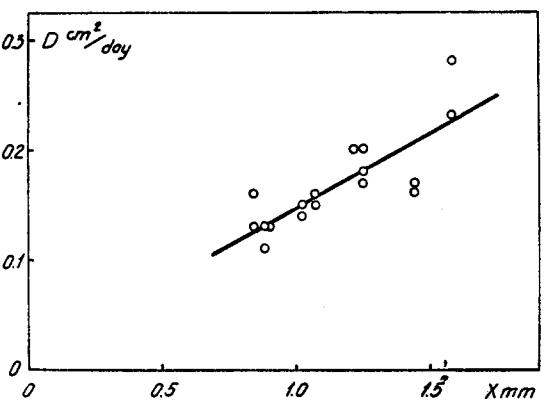

Fig. 3. The diffusion constant (D) as a function of the distance (x) from the border of the *N.P.* section

$$D^1_{20} = D_T \frac{293 \cdot \eta_T}{T \cdot \eta_{293}} \qquad (3)$$

where η is the viscosity of the solvent. This means that the diffusion constant increases about 3% per centigrade.

TABLE I

SUCROSE DIFFUSING INTO BOVINE NUCLEUS PULPOSUS

Interference fringe displacement (s) as a function of time (t) and distance from the border of the tissue (x).

t min	x mm	s scale units	ξ	D cm²/day
20	0.84	3	0.873	0.16
30	0.84	3.5	0.807	0.13
30	1.02	2.5	0.946	0.14
30	1.21	2.5	0.946	0.20
40	0.88	4	0.748	0.13
40	1.02	3.5	0.807	0.15
40	1.25	3	0.873	0.18
50	0.88	5	0.644	0.13
50	1.07	4	0.748	0.15
50	1.25	4	0.748	0.20
50	1.44	2.5	0.946	0.17
50	1.58	3.5	0.807	0.28
60	0.88	5	0.644	0.11
60	1.07	5	0.644	0.16
60	1.25	4	0.748	0.17
60	1.44	3	0.873	0.16
60	1.58	3.5	0.807	0.23

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TABLE II

DIFFUSION CONSTANTS OF VARIOUS SUBSTANCES IN BOVINE NUCLEUS PULPOSUS

The symbol k_1 signifies the ratio between D^1_{20} and the diffusion constant in water

Substance	c_s%	t° C	D^1 cm²/day	D^1_{20} cm²/day	D cm²/day in water at 20° C	k_1	References
NaCl	11.1	19	0.40	0.41			
		20	0.47	0.47			
			Mean	0.44	1.14	0.39	5
$CaCl_2$	8.0	19	0.40	0.41			
		21	0.42	0.41			
			Mean	0.41	1.00	0.41	5
Na_2SO_4	12.2	20	0.42	0.42			
		20	0.32	0.32			
			Mean	0.37	0.67	0.55	5
Urea NH_2CONH_2	19.4	20	0.55	0.55			
		19	0.45	0.46			
			Mean	0.51	0.98	0.52	5
Glycine NH_2CH_2COOH	13.0	19	0.37	0.38			
		18	0.27	0.29			10 and 7 mean value
		19	0.32	0.33			
			Mean	0.33	0.78	0.42	
Acetamide CH_3CONH_2	12.1	20	0.51	0.51			
		18	0.34	0.36			
		18	0.37	0.39			
			Mean	0.42	0.86	0.49	5
Glucose $C_6H_{12}O_6$	17.2	19	0.22	0.23			
		21	0.29	0.28			
		20	0.29	0.29			
			Mean	0.27	0.45	0.60	4
Sucrose $C_{12}H_{22}O_{11}$	13.8	19	0.20	0.21			
		19	0.21	0.22			
			Mean	0.21	0.36	0.58	5 and 4

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TABLE II (continued)

Substance	c_s%	t° C	D^1 cm²/day	D^1_{20} cm²/day	D cm²/day in water at 20° C	k_1	References
Raffinose $C_{18}H_{32}O_{16}$	9.0	20	0.074	0.074			
		20	0.098	0.098			
		19	0.074	0.076			
			Mean	0.08	0.30	0.28	5
Glucosamine hydrochloride $C_6H_{11}O_5NH_3Cl$	11.1			<0.05	0.58	<0.09	
Glucurono-lactone	18.8	21	0.27	0.26			
		19	0.23	0.24			
			Mean	0.25	0.78	0.32	

RESULTS

Large series of measurements have been performed and the average values are compiled in Table II. It can be seen from Table I that the D values show a tendency to decrease with increasing time. Such a tendency has been observed in most series.

Diffusion rate in water

The diffusion constants in water at 20° C and in most cases at the same concentrations as those during the measurements are also given in Table II. The ratios (k_1) between D^1_{20} and the corresponding diffusion constants in water were calculated. Most diffusion constants in aqueous solutions were taken from the literature, but measurements had to be made in two cases. For this purpose a CLAESSON diffusion cell[1] at 20.0° C was used and the concentration gradient was determined by the scale method of LAMM[6]. The pertinent diffusion constants are given in Table III.

TABLE III

DIFFUSION CONSTANTS IN WATER

Substance	Concentration	D_A 10^7 cm²/sec	D_m 10^7 cm²/sec
Glucosamine hydrochloride*	0.37%	67.1	67.0
Glucurono-lactone**	0.46%	90.9	90.0

* From S.A.F. Hoffman-La Roche & Co.
** From Sigma Chemical Co., contains less than 4% impurities.

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Evaluation of errors

The main errors are supposed to originate from variations in the tissue material, section thickness and the previously mentioned optical inhomogeneity of the material. Because of the movement of the interference fringes in the solution lack of sufficient convection in the solution outside the tissue is supposed to give only a small error. The tissue sections most likely contain some salts diffusing into the solution against the main concentration gradient. This factor seems, however, to be of minor importance due to the following fact. When finely minced pieces of *N.P.* were extracted with water during 24 hours and the extract was tested with silver nitrate no precipitate appeared. Thus, the *N.P.* contains less than 0.01% sodium chloride in a diffusible state. Only minor temperature corrections were necessary because all measurements were made between 18–21° C.

DISCUSSION

In most experiments the k_1-values were found to be about 0.5. This means that the diffusion rate of low molecular substances in *N.P.* is generally about half of that in water. This decrease can be compared with the decrease in diffusion constants in gelatin gels according to FRIEDMAN[3], who found diffusion constants of this magnitude in gels with a comparable water content of about 83.5%[9]. According to FRIEDMAN, the average pore diameter in such gelatin gels would be about 14 Å. If a comparison is valid the average effective pore size in *N.P.* would be of this magnitude.

In all experiments the diffusion constants have shown some increase with increasing distance from the border of the *N.P.* section. This deviation from FICK's second law, discussed previously by PAULSON AND SNELLMAN[8], may be explained by the assumption that *N.P.* contains pores with widely differing effective diameters. The smallest effective pore diameter would then be of the same order of magnitude as the diffusing molecule.

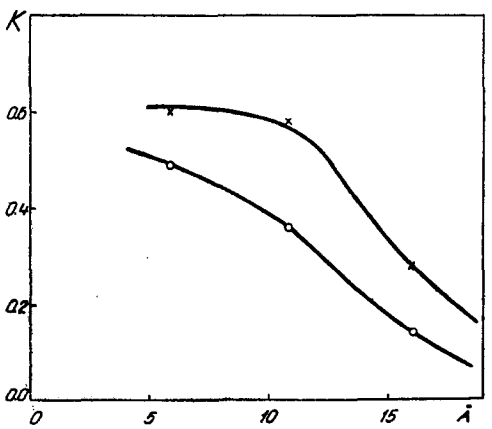

Fig. 4. In this diagram the ratio k of the diffusion constant in *N.P.* and the diffusion constant in water for glucose, sucrose, and raffinose have been plotted as a function of increasing molecular length. The upper curve represents observations made on a distance of $x = 1.5$ mm from the border of the *N.P.* section; in the lower curve the distance was $x = 0.8$ mm.

A comparison between the three saccharides of different molecular weight is of special interest (Fig. 4). No significant interaction, such as adsorption, can be expected between any one of these sugars and the constituents of *N.P.* In Fig. 4 the ratio between the diffusion constant in *N.P.* and in water is plotted against the length of the diffusing sugar molecule. It is concluded that *N.P.* contains many pores permeable to sucrose but not to raffinose, which pores consequently would have an effective size of about 15 Å.

Unpublished extraction and electrophoretic studies[9] on the interfibrillar mucoid of *N.P.* indicate that the polysaccharide component contains ester sulphate, probably chondroitin or other related sulphate. Anyhow, the mucoid contains to all appearance hexosamine and uronic acid. This mucoid material must influence the diffusion rate

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and it is of special interest to observe the low k_1-value of glucuronolactone and the very low one of glucosamine. These molecules are considerably smaller than sucrose which has a high k_1-value (Table II). In the case of glucuronolactone and glucosamine, adsorption—or perhaps salt linkage formation—seem to interfere. Actually, the suggested adsorption of glucosamine almost prevents diffusion measurements in *N.P.*

These diffusion data will thus provide more detailed information pertaining to the ultrastructure of normal *N.P.* in the region below 50 Å (cf.[12]).

ACKNOWLEDGMENT

Our thanks are due to Professors A. TISELIUS AND S. CLAESSON, Upsala, for the privilege of carrying out this work in their laboratories. The costs have been defrayed by grants from "H.M. King Gustaf Vth's 80th Anniversary Fund", and from the Board of the Caroline Institute, Stockholm.

SUMMARY

By means of an interferometric method the diffusion rate of a number of salts and organic compounds has been determined in fresh sections of normal *Nucleus pulposus* from the intervertebral discs of young calves. Small and medium sized molecules diffuse only about half as rapidly in *Nucleus pulposus* as in water. *Nucleus pulposus* presents a gel structure with a variety of interfibrillar pores of different sizes. Available data indicate the average effective pore size to be about 15 Å.

RÉSUMÉ

A l'aide d'une méthode interférométrique nous avons déterminé la vitesse de diffusion d'un certain nombre de sels et de composés organiques dans des coupes fraîches de *Nucleus pulposus* normal du disque intervertébral de jeunes veaux. Les molécules petites et moyennes diffusent dans le *Nucleus pulposus* à peu près deux fois plus lentement que dans l'eau. Le *Nucleus pulposus* présente la structure d'un gel contenant un grand nombre de pores interfibrillaires de différente largeur. Les données disponibles indiquent que la largeur effective moyenne des pores est de 15 Å.

ZUSAMMENFASSUNG

Mit Hilfe einer interferometrischen Methode wurde die Diffusionsgeschwindigkeit einer Anzahl von Salzen und organischen Verbindungen in frischen Schnitten von normalem *Nucleus pulposus* aus der Zwischenwirbelscheibe von jungen Kälbern bestimmt. Kleine und mittelgrosse Moleküle diffundieren in *Nucleus pulposus* nur ungefähr halb so schnell als in Wasser. Der *Nucleus pulposus* zeigt eine Gel-Struktur mit zahlreichen interfibrillären Poren von verschiedener Grösse. Die zur Verfügung stehenden Angaben weisen darauf hin, dass die durchschnittliche Porengrösse ungefähr 15 Å ist.

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Received December 7th, 1950