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Characterization of collagenous meshworks by volume exclusion of dextrans

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The volumes from which ^3H-labelled dextrans are excluded by dermal collagenous fibres were calculated by dilution of dextran probes. Five dextrans, of average Stokes' radii 1.72, 2.53, 3.92, 4.54 and 14.24nm, were investigated at concentrations between 0.1 and 3% (w/w). The excluded volume was dependent on dextran concentration only for the two smaller probes. The largest dextran was shown not to bind to the fibres. A plot of the square root of excluded volume against Stokes' radius was linear for the four smallest dextrans, corresponding to the predictions of Ogston's [(1958) *Trans. Faraday Soc.* **54**, 1754–1757] rod-and-sphere model of fibrous exclusion, and suggesting that dextrans of Stokes' radius between 1.72 and 4.54nm were excluded by a cylindrical solid fibre of radius 2.90 ± 0.72nm. Larger molecules were excluded by a structure of much greater size, since the volume exclusion for the largest dextran was only slightly greater than that of the dextran less than one-third its radius. The excluded volume of ^3H_2O fell slightly below the line describing the dextran data, indicating that water had access to most of the volume not occupied by the collagenous fibres.

Several laboratories have reported that meshworks of collagenous fibres exclude other macromolecules from a portion of the solvent volume (Wiederhielm & Black, 1976; Meyer *et al.*, 1977; Pearce & Laurent, 1977). Calculation of the interstitial space available to macromolecules in tissues (Guyton *et al.*, 1975; Comper & Laurent, 1978; Watson & Grodins, 1978) requires volume-exclusion data for the fibres of the tissue of interest. By using a series of labelled dextran probes to study exclusion by dermal collagenous fibres, the size of the component responsible for exclusion has been determined.

Materials and methods

The human dermal collagenous fibres were described previously (Pearce & Laurent, 1977). All experiments were conducted in phosphate-buffered saline (Dulbecco & Vogt, 1954) containing 0.02% (w/v) NaN_3. ^3H_2O (100mCi/g; NET001D, lot no. 537-095; NEN Canada, Lachine, P.Q., Canada) was diluted with phosphate-buffered saline to a specific radioactivity of 1.65μCi/ml.

The weight of collagen was calculated from hydroxyproline analyses, performed by using a micromodification of the procedure of Woessner (1961), with hydroxy-L-proline (A grade; Calbiochem, La Jolla, CA, U.S.A.) as a standard; 1.0μmol of hydroxyproline was taken as equivalent to 0.91mg of collagen (Jackson & Cleary, 1967). The stock dextran solutions were analysed for hexose by a micromodification of the anthrone method (Scott & Melvin, 1953), with D-glucose (lot no. 6093; National Bureau of Standards, U.S. Department of Commerce, Washington, DC, U.S.A.) as a standard. The weight of dextran was calculated as anhydroglucose equivalents. All other chemicals except the dextrans were of commercially available reagent grade.

Radioactivity measurement

A liquid-scintillation spectrometer (Unilux II; Nuclear–Chicago, Des Plaines, IL, U.S.A.) was adjusted to the balance point for ^3H. Glass liquid-scintillation vials (Econovials, NEN Canada) containing 100μl of sample, 400μl of phosphate-buffered saline and 5ml of Aquasol-2 (NEN Canada) were stabilized overnight at 4.6°C before counting for radioactivity. In the equilibration experiments, at least 10^4 counts were accumulated for each sample.

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Table 1. *Characteristics of the dextran fractions*

The values of weight-average molecular weight ($\bar{M}_w$) were provided by Dr. K. Granath, whose lot numbers are given. The concentrations of the stock solutions were determined by hexose analyses. The observed values of Stokes' radius were determined by gel chromatography and the calculated values by using eqn. (2) in the text.

				Stokes' radius (nm)	
$10^{-3} \times \bar{M}_w$	Lot no.	Specific activity (10^{10} d.p.m./g)	Stock solution (mg/g)	Obs. mean (16–84% range)	Calc.
3.5	PD5558	9.32	31.5	1.72 (1.4–2.0)	1.45
10.4	0094	1.92	29.8	2.53 (1.9–3.3)	2.36
26.5	RMT726	6.29	31.0	3.92 (3.0–5.5)	3.71
36.5	FDR7314	6.29	27.7	4.54 (3.6–5.7)	4.30
485.0	7693	1.92	29.3	—	14.24

Dextrans

Dextran fractions, the weight-average molecular weight ($\bar{M}_w$) of which are shown in Table 1, were a gift from Dr. K. Granath, Pharmacia Fine Chemicals, Uppsala, Sweden. Portions of these dextrans were labelled by end-group reduction with NaB^3H_4 in water as described by D. C. Warrell (The Radiochemical Centre, Amersham, Bucks. U.K.; personal communication to Dr. D. E. Brooks of this Department). After reduction, the excess borohydride was destroyed with acetic acid, the reaction mixture was dialysed against several changes of water, evaporated to dryness in a rotary evaporator and methanol was added and distilled three times to remove residual borate. The residue was taken up in water and chromatographed on either a 26mm × 900mm column of Sephacryl S-200 (Superfine grade) (the two smallest dextrans) or a 50mm × 750mm column of Sepharose 2B (the three largest dextrans). Both media were supplied by Pharmacia (Canada) Ltd., Dorval, P.Q., Canada. The 10ml fractions collected were analysed for hexose and radioactivity. The tubes comprising the peak of both radioactivity and hexose were pooled, concentrated by ultrafiltration by using a UM2 membrane (Amicon Canada Ltd., Oakville, Ont., Canada) and the specific radioactivity calculated from hexose and radioactivity analyses (Table 1). The elution profile of the labelled dextran was compared with that of the untreated material to ascertain if the distributions of molecular sizes had been affected during the preparation. The changes seen after reduction and purification were small and regarded as insignificant.

Stock solutions in phosphate-buffered saline containing approx. 3% (w/w) of each dextran and 3.8×10^4–3.1×10^5 c.p.m./100μl were prepared. Standards for each dextran were obtained by dilution of these stock solutions to approx. 1.0, 0.3, 0.1 and 0.01% (w/w) with phosphate-buffered saline. The concentration of each dilution was calculated from the weights of stock and diluent.

Distribution of molecular sizes of the dextrans

Gel chromatography on the column of Sephacryl S-200 was used to determine the distribution of Stokes' radii for the four smaller dextrans. The column was eluted with phosphate-buffered saline free of azide. Fractions (10ml) were collected. The void volume was determined with 2mg of rectified native dextran (a gift from Dr. K. Granath) and the total volume with 0.82μCi of ^3H_2O, applied together in 1.5ml of phosphate-buffered saline. Globular proteins of known Stokes' radii (the radii in nm, the weights in mg and the sources are given in parentheses) were used to calibrate the column: human serum albumin (3.55nm, 2.5mg, lot no. 346-3; Connaught Medical Research Laboratories, Toronto, Ont., Canada), equine heart cytochrome *c* (1.64nm, 3mg, A grade, lot no. 200212; Calbiochem–Behring Corp., San Diego, CA, U.S.A.), bovine pancreatic α-chymotrypsinogen (2.24nm, 4mg, A grade, lot no. 78; Calbiochem–Behring), human transferrin (4.02nm, 4mg, grade II; Sigma Chemical Co., St. Louis, MO, U.S.A.), human plasma caeruloplasmin (4.50nm, 4mg; a gift from Dr. E. J. Wye, Connaught Laboratories, Willowdale, Ont., Canada). The data were fit by the method of least squares to a plot of $(-\ln K_{av.})^{0.5}$ against Stokes' radius (Siegel & Monty, 1966). The equation of the line was used to prepare a calibration curve on which the average Stokes' radius was plotted against tube number. After calibration of the column, 100μl portions of each stock solution were applied. The fraction of total hexose eluted before and including each tube was plotted against the Stokes' radius for that tube and the points were joined by a smooth curve. The mean Stokes' radius corresponded to a hexose fraction of 0.5. The fractions 0.16 and 0.84 were used as measures of dispersion, since the latter represent 1 S.D. of a normal distribution (Table 1). This approach to the measurement of molecular-size distributions has been discussed in detail elsewhere (Pearce & Grimmer, 1978).

Equilibration experiments

For each size of dextran, volume exclusion was determined in triplicate at four approximate concentrations (w/w): 3, 1, 0.3 and 0.1%. Approx. 1 ml of a well-mixed 2% (w/w) slurry of collagenous fibres was pipetted into a tared vial. After centrifugation at 2000 *g* for 10 min at room temperature, the supernatant fluid was removed, leaving a compact pad of collagenous fibres. A record was kept of the weight of all solutions added to, or removed from, the pad; 800 μl of a labelled dextran solution were added and the vial was agitated gently by hand to suspend the fibres. The solution was allowed to equilibrate at room temperature for at least 24 h. The phase containing dextran in equilibrium with the fibres was sampled by transferring with a Pasteur pipette into a tared vessel as much as possible of the essentially collagen-free liquid overlaying the loosely dispersed pad.

The remaining dextran was recovered from the pad. The suspension was centrifuged at 2000 *g* for 10 min at room temperature and the supernatant withdrawn. The pad was washed five times with 500 μl of phosphate-buffered saline with gentle agitation several times over a period of 20 min, centrifuged as before and the washes pooled with the supernatant.

Triplicate 100 μl samples of (1) the phase containing dextran in equilibrium with the fibres, (2) the pool containing the remaining dextran and (3) the five standards all were weighed and counted together to determine dextran concentrations.

Binding experiments

About 1 ml of a 2% (w/w) collagenous slurry was centrifuged at 750 *g* for 10 min at room temperature, the clear fluid above the collagenous pad was removed and 500 μl of labelled dextran ($\bar{M}_w = 4.85 \times 10^5$) were added. After gentle mixing and re-centrifuging the suspension, the supernatant was collected and replaced by an equal weight of phosphate-buffered saline. Weighed portions of six successive washes were counted for radioactivity along with standards to determine the concentration of dextran at each step.

The expected concentration of dextran at dilution i in the absence of binding could be calculated as follows:

$$[\mathrm{Dx}]_i = [\mathrm{Dx}]_{i-1}\left[\frac{W_{s,i-1} - E \cdot W_{\mathrm{coll.}}}{W_{s,i} - E \cdot W_{\mathrm{coll.}}}\right] \qquad (1)$$

where $W_{s,i-1}$ is the weight of solution at step $i-1$ after equilibration and removal of supernatant, in g; $W_{s,i}$ is the weight of solution at step i after the addition of diluent, in g; E is the specific solvent exclusion of collagenous fibres, in g of phosphate-buffered saline per g of collagen; $W_{\mathrm{coll.}}$ is the weight of collagenous fibres present, in g; and $[\mathrm{Dx}]_{i-1}$ is the concentration of dextran in the supernatant at step $i-1$, in g of dextran per g of solution.

Statistical analysis

The lines of best fit to sets of data and the errors of estimate for extrapolated values were calculated as described by Snedecor & Cochran (1967).

Results

Determination of dextran concentration by radioactivity

Phosphate-buffered saline exposed to a slurry of collagenous fibres under conditions similar to those used in the equilibration experiments (described above) and then separated and mixed with labelled dextran gave a radioactivity count identical with that of labelled dextran and phosphate-buffered saline not so exposed. Thus the quenching characteristics of phosphate-buffered saline were not changed by exposure to collagenous fibres.

Replicate counts of the same sample showed the maximum counting error to be 1%. A typical change in concentration attributable to exclusion was approx. 10%. Hence the coefficient of variation of the estimate of excluded volume attributable to counting was $[(0.01)^2 + (0.01)^2]^{0.5}/(0.1)$ or 14%.

The line of best fit of dextran concentration to the c.p.m. of the standards was calculated; for each dextran the correlation coefficient was greater than 0.999. The approximate dextran concentration of an unknown was obtained from the regression equation and the count rate per g of sample. The counting precision was improved by a correction for the slight departure of the standard curve from linearity (Fig. 1). This method of determining dextran concentration was distinctly more reproducible than those that use internal or external standards.

Sizes of the probes

The distributions of Stokes' radii for all but the largest dextran used were determined with a calibrated column of cross-linked allyl-dextran (Sephacryl S-200, Superfine grade; Pharmacia). The central 16–84% of the distribution was used to describe the range of radii. In Table 1 the observed Stokes' radii (r_s) are compared with values calculated from the equation (K. Granath, personal communication):

$$r_s(\mathrm{nm}) = 0.0332\bar{M}_w^{0.463} \qquad (2)$$

The calculated radii agreed reasonably well with those based on gel chromatography. The average radius determined by gel chromatography was used in the calculation of exclusion for all but the largest dextran. Since the largest dextran was excluded from the gel, the value determined from eqn. (2) was used.

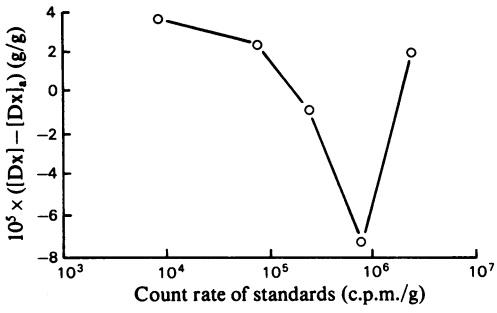

Fig. 1. *Typical correction curve for the calculation of dextran concentration*

Quintuplicate standards, prepared by weighing, were counted for radioactivity at five concentrations of dextran. The average c.p.m. at each concentration was used to calculate a line of best fit. The equation of the line was used in turn to calculate the approximate dextran concentration of each standard, $[Dx]_a$. The plot shows the difference between the true dextran concentration, [Dx], and $[Dx]_a$ for the various standards, shown here for the dextran of $\bar{M}_w = 10400$. The concentration of an unknown was calculated from an average c.p.m. of replicate samples by using the line of best fit to obtain $[Dx]_a$ and the correction curve to obtain a value for addition to $[Dx]_a$ to give a more accurate estimate of dextran concentration.

Recovery

All the radioactivity added to the vials was recovered in the equilibrium phase plus the pooled washes of the pad. For the dextrans of various average molecular sizes, the percentage recoveries and their S.D. values were: 1.72nm, 101.0±1.4; 2.53nm, 99.7±0.5; 3.92nm, 100.4±0.9; 4.54nm, 101.6±1.2; 14.24nm, 99.7±1.8. These recoveries represent a marked improvement over those obtained in past studies (Pearce & Laurent, 1977).

Methods of calculating exclusion

Two semi-independent methods were used, both utilizing the concentration of dextran in the phase in equilibrium with the fibres. Both assume the dextran to be present in the accessible volume at the equilibrium concentration. The first method relied on knowledge of the total weight of fluid and dextran in the system. Division of the total mass of dextran added by the concentration of dextran in the equilibrium phase gave the mass of fluid occupied by the dextran. The difference between the total fluid mass and that occupied by the dextran was the mass of fluid from which dextran was excluded. Division of the latter by the weight of collagenous fibres and the density of phosphate-buffered saline (1.01g/ml) gave the excluded fluid volume per unit weight of the fibres.

In principle, the second method was similar to the first. The dextran concentration in the pooled washes multiplied by the weight of the pool gave the weight of dextran in the pad. This weight, divided by the concentration in the equilibrium phase, gave the fluid mass in the pad occupied by the dextran. Since the total fluid mass after removal of the equilibrium phase was known, the difference between the two was the fluid mass from which the dextran was excluded. Division of this fluid mass by the weight of the fibres and the density of phosphate-buffered saline gave the fluid volume excluded by a unit weight of the fibres.

The excluded fluid volume calculated by either of these methods represented the volume of fluid outside the collagenous fibres inaccessible to the dextrans. Dextrans were excluded also from the volume of the collagenous fibres themselves. The partial specific volume of monomeric tropocollagen is 0.66ml/g (Davison & Drake, 1966; Elden, 1968). This value, assumed to apply to the collagenous fibres, was added to all values of fluid volume exclusion to determine total volume exclusion.

An analysis of variance indicated that the excluded volumes calculated by the two methods gave identical results ($P = 0.88$ by an F-test). The two sets of results have been pooled in the data that follow.

Volume exclusion and dextran concentration

Five dextrans of differing size, each at four concentrations, were used to measure the volume exclusion by collagenous fibres (Fig. 2). In all experiments the concentration of collagen was close to 20mg/ml. Table 2 shows the parameters of the lines of best fit of excluded volumes to dextran concentrations. The volume exclusions at infinite dilution were calculated from these lines (Table 2). Only the two smaller dextrans showed significant effects of concentration.

Binding of dextran to collagenous fibres

The calculation of volume exclusion as described above ignores the possibility of dextran binding to collagenous fibres. However, once a value for volume exclusion was known, any interaction between the fibres and dextran could be assessed. Fig. 3 compares the calculated (eqn. 1) with the observed dextran concentrations for duplicate experiments using the largest dextran. The line representing agreement of the calculated and observed concentrations fitted the data. Had significant binding of dextran to the fibres or entrapment due to centrifugation occurred, the data would show deviation from this line, especially at low concentrations. In the range of concentrations employed, the largest dextran and collagenous fibres did not interact. A smaller dextran capable of deeper

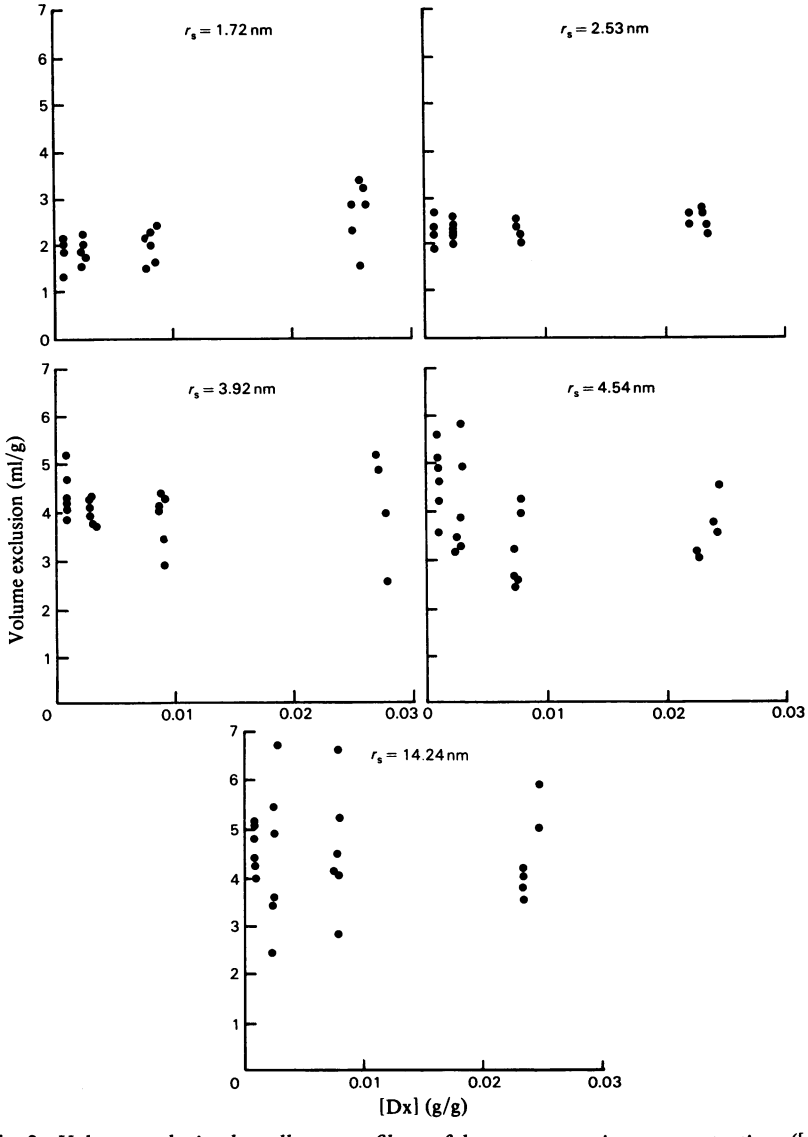

Fig. 2. *Volume exclusion by collagenous fibres of dextrans at various concentrations* ([*Dx*])

penetration of the collagenous meshwork might have shown binding not detectable with the large dextran used. However, the complete lack of evidence for binding suggested that further experiments would be unenlightening.

Dimensions of the excluding structure

Since two particles cannot occupy the same space at any given time, the volume available to a particle is restricted by the presence of other particles. The collagen–dextran system has been represented geometrically by a cylinder-and-sphere model (Ogston, 1958; Laurent, 1970; Ogston *et al.*, 1973; Meyer *et al.*, 1977). If r_f is the radius of a rod of collagen whose length per unit mass is L, and if r_s is the radius of a dextran molecule, then the volume that the centre of the dextran molecule cannot occupy, that is, the excluded volume, $V_{exc.}$, is defined by:

$$V_{exc.} = \pi L(r_f + r_s)^2 \quad (3)$$

From eqn. 3, $(V_{exc.})^{0.5}$ is related linearly to r_s, if r_f and L are constant. Fig. 4 shows such a plot, the values of $V_{exc.}$ extrapolated to infinite dilution (Table 2) being used to eliminate effects of dextran concentration. A linear relationship was apparent for all but the largest dextran. The values of $V_{exc.}$ for the

four smallest dextrans, extrapolated by a line of best fit to $(V_{exc.})^{0.5} = 0$, gave a radius, r_f, of 2.90nm ± 0.72nm (standard error of estimate) for the solid rod equivalent to the collagenous fibres. The standard error of estimate of r_f was calculated as the standard error of estimate of $(V_{exc.})^{0.5}$ at $r_s = 2.90$nm divided by the slope of the line.

Volume exclusion of water

Katz & Li (1973) suggested that water can penetrate freely into the intermolecular volume of the microfibrillar structure of collagen. Hence water should not be excluded from any volume other than that actually occupied by the collagenous fibres. To test this assumption and the validity of the methods employed here, the volume exclusion of ^3H_2O by collagenous fibres was measured by the substitution of ^3H_2O for dextran in some equilibration experiments.

The binding of water to the fibres complicated the interpretation of these experiments. Two water molecules are believed to stabilize the triple helix by firm binding to three amino acids, corresponding to approx. 0.12g water per g of collagen (Grigera & Berendsen, 1979; Ramachandran & Ramakrishnan, 1976). The ^3H_2O was considered freely interchangeable with this water. When calculating exclusion in

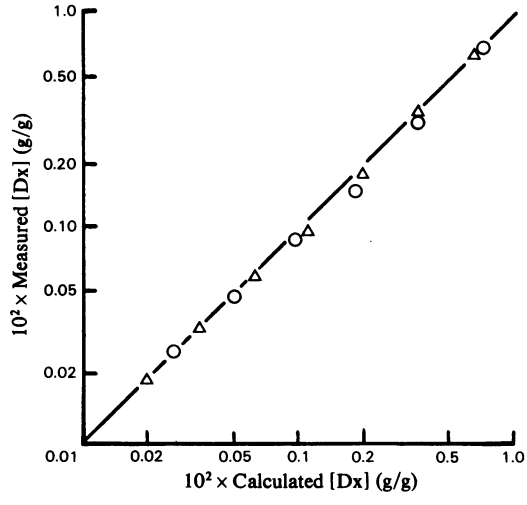

Fig. 3. *Test for binding of a dextran ($\bar{M}_w = 4.85 \times 10^5$) to collagenous fibres*

The dextran concentrations, [Dx], of a series of washes of collagenous fibres, measured by counting each wash for radioactivity, are compared with a value calculated from the dilution of the solvent by weight. Data for two experiments (○, △) are compared with a line representing equivalent concentrations.

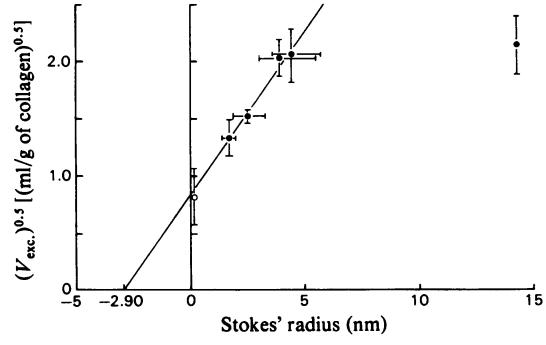

Fig. 4. *Volume exclusion by collagenous fibres of various dextrans and water*

The square roots of the estimated excluded volumes at infinite dilution and their standard errors of estimate (bars at end of vertical lines through each point) for five dextrans (●) and ^3H_2O (○) were both taken from Table 2. The Stokes' radii and the limits encompassing 16–84% of the size distribution (bars at end of horizontal lines through each point) were taken from Table 1. The calculated line of best fit for the four smallest dextrans is shown with its intercept $(V_{exc.})^{0.5} = 0$ (corresponding to $-r_f$).

Table 2. *Effect of dextran concentration on excluded volume*

The data are shown in Fig. 2. The lines of best fit of volume exclusion to dextran concentration were calculated. For each dextran of Stokes' radius r_s, the slope, b, the standard deviation, s_b, the number of points used in the calculations, n, and the probability, P, of the slope differing from zero are given. The estimated excluded volumes at infinite dilution, $\hat{Y}$, and their standard deviations, $s_{\hat{y}}$, were also calculated.

r_s (nm)	$b \pm s_b$ (n) $\left(\frac{\text{ml of solvent/g of collagen}}{\text{g of dextran/g of solution}}\right)$	P	$\hat{Y} \pm s_{\hat{y}}$
1.72	34.55 ± 9.04 (21)	0.001	1.86 ± 0.43
2.53	10.70 ± 4.47 (20)	0.00025	2.41 ± 0.19
3.92	−4.97 ± 14.72 (22)	0.5	4.22 ± 0.65
4.54	−34.01 ± 23.16 (23)	0.15	4.28 ± 0.97
14.24	−3.20 ± 25.06 (24)	0.5	4.65 ± 1.11

these experiments, the solvent weight was corrected for the 2.4 mg of bound water per 20 mg of collagen. The calculated fluid volume exclusion, without the addition of the specific volume of the collagen, was 0.02 ± 0.41 ml/g of collagen in six experiments, a value of essentially zero, as expected. The recovery of ^3H_2O was 99.8% ± 1.2%. In Fig. 4, the r_s of water was taken to be 0.16 nm (Katz & Li, 1973). At this value, the excluded volume was close to, but beneath, the line calculated for the dextrans.

Discussion

The largest dextran (r_s = 14.24 nm) behaved in a manner obviously different from water and the four smaller dextrans (Fig. 4). This discrepant behaviour can be explained by a restriction of the Ogston (1958) model. If the centre-to-centre spacing of two collagen rods is less than $2(r_f + r_s)$, a dextran sphere could not enter some of the space between the rods. Thus the two rods would act together to exclude the dextran and the volume exclusion per g of collagen would be less than expected from the exclusion exerted by individual fibres. The low value of $V_{exc.}$ for the large dextran is probably the result of this restriction. If sufficient data were available for dextrans of Stokes' radius greater than 4.54 nm, the dimension of the next level in the hierarchial organization of the fibres would be measureable. Other workers have recognized many levels of structural organization in collagenous fibres (Miller, 1975; Baer *et al.*, 1975; Kastelic *et al.*, 1978).

The data for the four smaller dextrans suggests an excluding structure in the fibre meshwork of a diameter most probably 5.8 nm (2 × 2.9 nm), with a possible range of 3.0–8.6 nm ($P \sim 0.95$, corresponding to two standard errors of estimate). This observation does not relate simply to the size of collagenous fibres measured by other methods. Certainly the excluding species appears to represent more than one tropocollagen molecule, the accepted diameter of which ranges from 1.2 to 1.5 nm (Baer *et al.*, 1975). By using the hexagonal-packing model proposed by Hulmes & Miller (1979) and assuming a symmetrical organization, the fibril responsible for exclusion most probably represents a four- or five-layered structure containing 14–23 tropocollagen molecules in cross-section. The structure responsible for our data corresponds reasonably ($P \sim 0.44$) to the 8 nm unit present in collagenous fibrils (Parry & Craig, 1979). If so, the preparation must represent a substructure of the 25 nm fibril they observed in foetal human skin by using electron microscopy.

The decrease in apparent excluded volume seen with increasing dextran concentration (Table 2 and Fig. 2) appears to represent molecular compression of either the dextran or collagenous fibre or both, of the type described by Ogston & Preston (1979).

The collagenous fibres of connective tissue are associated intimately with a large fraction of the body's interstitial fluids. Knowledge of the relationship between collagen organization, macromolecular size and volume exclusion is necessary for an understanding of distribution and transport of fluid in the body. Collagenous fibres are usually considered to be thermodynamically inactive, serving mainly to provide the tissues with structural integrity (Meyer *et al.*, 1976). However, such fibres also restrict the interstitial volume accessible to plasma proteins, yielding protein concentrations in the accessible space above those anticipated if the proteins are distributed in the entire interstitial space. Recent attempts to explain transport of interstitial plasma protein have recognized the importance of volume exclusion in their formulation, even though appropriate data are not available (Reeve, 1977; Rothschild *et al.*, 1979).

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