ANISOMETRIC TRANSPORT OF IONS AND PARTICLES IN ANISOTROPIC TISSUE SPACES

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ABSTRACT The results of time-lapse measurements and electron microscopic observations on the diffusion of histological dyes, colloidal particles, and heavy metal salts in excised chicken breast tendon are reported. In all cases, the transport was found to be anisometric, the extent of the spreading being much greater parallel than perpendicular to the collagen fibers. The diffusion of colloidal gold was shown to be governed by a random diffusion process, with coefficients of 3 to 5 $\times$ 10^{-7} and 1 to 2 $\times$ 10^{-7} cm^2/sec for the parallel and perpendicular directions, respectively; the anisotropy was attributed to steric hindrance. In the diffusion of uranyl nitrate, a sharp boundary appeared at the leading edge of the diffusate and advanced at a rate proportional to the square root of time. Electron micrographs showed uranyl nitrate clusters localized in space on the surface of the collagen fibrils and tightly bound to the polar amino acid regions of the macromolecule. A model was proposed involving diffusion with attrition, and predicted a sharp boundary advancing proportionally to the square root of time and to the 0.65 power of the initial diffusate concentration. Application of the model to the experimental results for uranyl nitrate gave a diffusion coefficient of 10 $\times$ 10^{-7} and 4 $\times$ 10^{-7} cm^2/sec for the parallel and perpendicular directions, respectively, and a possible explanation of this large difference was advanced. The importance of anisometric transport in anisotropic tissues was indicated.

INTRODUCTION

It is well known that the hairs of mammals, the feathers of birds, and the scales of fish arise in definite space patterns. A study of this last case by Weiss (1) has led him to conclude that the sites of scale formation are at the lattice points of the two intersecting orthogonal systems of connective tissue fibers in the dermis of the fish; one system furnishes the cells while the other provides the additional information necessary to determine the sites of scale formation. The possibility that this

additional information is chemical in nature suggests that perhaps the scales form at the lattice points because of the enhanced transport of chemical entities along the connective tissue fibers. Further, it is by way of the connective tissue that the cells receive their nutrients and the products of metabolism are removed (2, 3). For these reasons, the present work was undertaken in an attempt to explore further the question of anisometric transport.

In order to simplify the investigation, it was decided to select a preparation in which all the connective tissue fibers were oriented parallel to the same direction, thus permitting a study of the transport in this direction and perpendicular to it. The collagen fibers of tendon provide such a configuration, and all the experiments described below were performed on excised chicken breast tendon.

In tendon, the long (essentially infinite) parallel collagen bundles are embedded in a ground substance that includes acid and neutral mucopolysaccharides and comprises only a small fraction of the total dry weight of the tendon (4). The collagen bundles themselves are composed of fibers (2 to 5μ in diameter) which are made up of a hierarchy of successively finer cylindrical elements: fibrils (a few tenths of a micron), filaments (several hundred angstroms), and protofibrils (about 14 A in diameter and 3000 A in length). This last element is actually the monomeric unit of soluble collagen and consists of three helical polypeptide strands twisted about each other in a superhelix (5). Petruska and Hodge (6) have proposed a model in which each of these strands is considered to be a repeating sequence of a smaller subunit.

The primary structure of the collagen macromolecule is known only in part (7), and consists of alternating groups of polar and nonpolar amino acids. It is the polar regions that take up the electron-dense stains such as phosphotungstic acid and uranyl acetate (8) and, as a result, give rise to one of the most striking features of collagen as seen under the electron microscope at medium magnification: the presence of dark banded regions of equal thickness at regular intervals (640 A) along the entire length of the fibril (9, 10). At higher resolutions, these bands appear as a group of well defined subbands so reproducible that they can be used as positive means of identification of the collagen macromolecule (11, 12). Essentially all the basic and acidic amino acids in collagen are present in the banded regions, and it is the latter group of acids that will be invoked in deriving a model to describe the transport processes occurring in tendon.

Transport in collagenous tissues is of considerable interest to the tanning industry. Otto (13) has studied the penetration of dye into leather as a function of the structure of the dye molecule and has found that the process is very sensitive to the number of charged groups and conjugated double bonds but in general not to the size of the molecule. Similar conclusions concerning the importance of dye structure in diffusion through gelatin gels have been reached by Jelley and Pontius (14). In the uptake of phosphotungstic acid, Kühn (15) has been able to distinguish

between firmly bound acid and acid that is loosely bound to the collagen and can be removed by prolonged washing in water.

There have been other studies, some qualitative (2, 16) and some more quantitative (17–20), and these will be discussed together with the experimental results presented below.

EXPERIMENTAL METHODS

The breast tendons of freshly killed chickens were excised, cut into rectangular strips about 9 mm by 8 mm and placed in isotonic Ringer's solution for several hours or overnight. Each tendon was then removed, threaded through the centers of its large faces with a thin surgical suture, and placed in a holder. One end of the suture was dipped into a small container filled with an aqueous solution of the diffusate, and the entire system was sealed by a microscope slide and clamped onto a rotating stage device that permitted up to eight samples to be positioned under the microscope and photographed in succession, one at a time. Thus time-lapse motion pictures of several tendons were obtained concurrently, and direct comparisons were possible between various diffusates.

Before being placed in the holders, some of the specimens were dipped in celloidin, and then the experiments were carried out with the tendons immersed in paraffin oil. This was done in order to inhibit dehydration of the samples and permit longer runs. Results obtained for the same diffusate but without the celloidin and oil showed no substantial difference during the period before dehydration became too great. This is in accordance with previously published results (2).

The diffusates used fall into three main categories: histological dyes, heavy metal ions, and colloidal particles. In the main, three histological dyes were used—acid fuchsin, Nile Blue A, and neutral red. The first is an acid dye and the others are basic dyes, but no qualitative difference was observed in the results obtained. The diffusion of several heavy metal salts was studied, such as silver nitrate, potassium permanganate, lead citrate, phosphotungstic acid, and uranyl nitrate. Data obtained with the uranyl ion seem to be of most interest, and these will be reported in detail. Colloidal gold[1] and f2 bacteriophage (21) have also been investigated. Both of these are particles of about 150 to 200A in diameter and are readily identified under the electron microscope (22, 23).

After the diffusion had proceeded for several hours, the samples were removed and, in the case of the dyes, cleared and mounted. With the metal ions and colloidal particles, the tissues were fixed in osmium tetroxide or glutaraldehyde, dehydrated in alcohol, and embedded in Epon 812. They were then sectioned at thicknesses ranging between 500 and 600A.

The time-lapse motion pictures were analyzed with a microdensitometer to obtain the density of diffusate as a function of distance along the tendon fibers for various elapsed times. The actual spread at any one time was then taken to be the separation on the microdensitometer tracing between the positions of most rapid density change, corresponding to maximum contrast. Occasionally the positions of maximum contrast were determined visually by projecting the motion pictures on a screen and measuring with a ruler. Essentially the same results were obtained by the two methods, but in the case

[1] Inactivated, 2mg/ml, with 3mg/ml gelatin, 4mg/ml ascorbic acid, 10mg/ml sodium acetate, and 0.9% benzyl alcohol as preservative.

of colloidal gold the visual contrast was poor (see below). These results were then plotted as a function of the square root of time, and the procedure repeated for the direction perpendicular to the fibers. Finally the curves were compared with theoretical curves based on the model presented below.

RESULTS

Fig. 1 is a photograph of four tendons following the spread of neutral red (*a*), acid fuchsin (*b*), and Nile Blue A (*c*) and (*d*). The results are qualitatively quite reproducible, having been obtained in more than a hundred tendons and over a wide range of pH and relative humidity. In all cases studied the extent of the

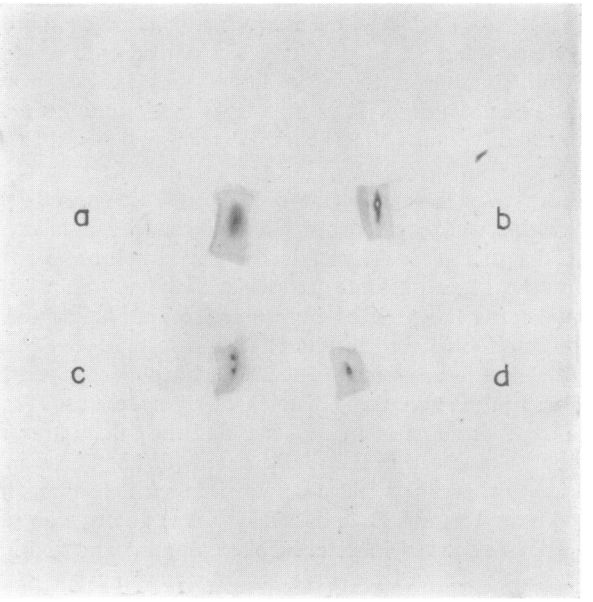

FIGURE 1 A photograph of four tendons following the spread of (*a*) neutral red, (*b*) acid fuchsin, (*c*) and (*d*) Nile Blue A. The extent of the spreading can be seen to be much greater parallel to the collagen fibers than perpendicular to them. (Initial magnification 1.0, final magnification 1.5.)

spreading is much greater parallel to the collagen fibers than perpendicular to them.

When the films obtained from the diffusion of (un-ionized) colloidal gold were analyzed on the microdensitometer, the optical density of each frame was found to decrease gradually from the center of the image outwards. This is just what one would expect for a random diffusion process—the slope of the density versus distance curve decreasing monotonically from a large (negative) value at the origin to zero at infinity. In order to permit a comparison of these results with theoretical calculations, an arbitrary but fixed optical density (relative to its value at the origin)

was chosen and the position x of this density level was measured as a function of time t. A typical plot of x versus $\sqrt{t}$ is shown in Fig. 2. The value of x at $t = 0$ represents in each case the size of the hole made by the suture; it is somewhat greater parallel to the fibers owing to the natural tendency of the tendon to yield more in that direction. The departure from linearity in this region is similarly explained.

An exact mathematical analysis of this problem with the appropriate boundary conditions has been carried out only for the one dimensional case (24). To a first approximation, then, we consider the transport of colloidal gold in tendon as consisting of two mutually independent diffusion processes, one parallel and the other perpendicular to the direction of the fibers. We then obtain (25) for the diffusion

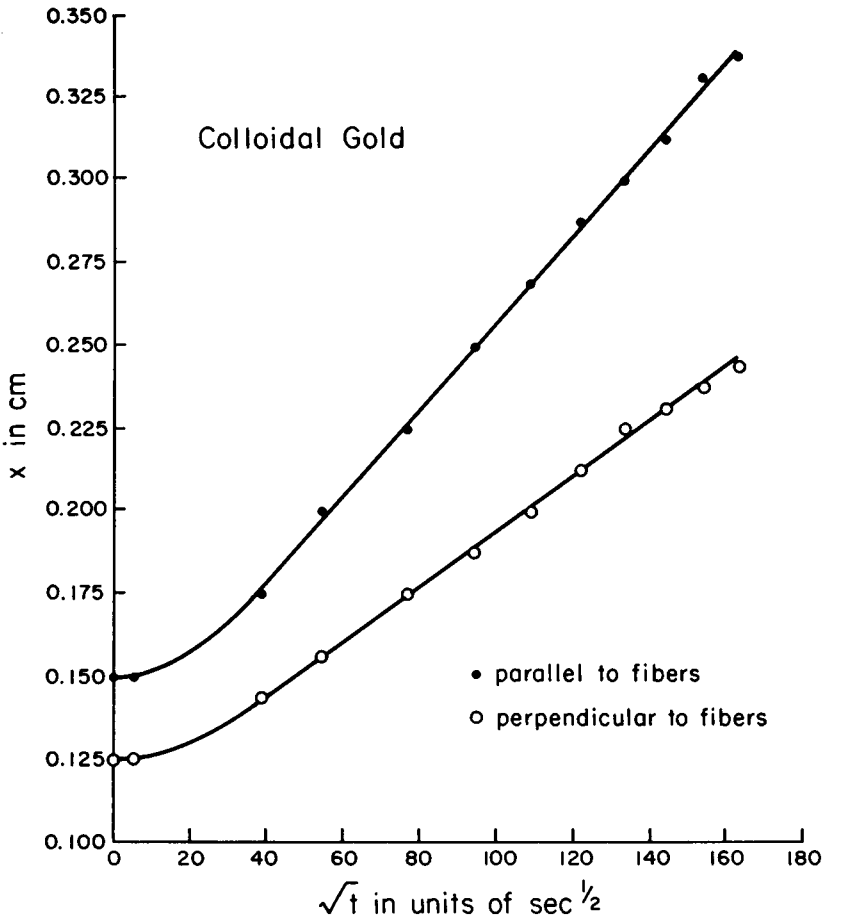

FIGURE 2 A plot of the diffusion of colloidal gold as a function of the square root of time for the directions parallel (solid circles) and perpendicular (open circles) to the collagen fibers. The slopes of the curves give values of the diffusion coefficient in the range 3 to 5 $\times$ 10^{-7} cm^2/sec and 1 to 2 $\times$ 10^{-7} cm^2/sec for the two directions.

coefficients, values in the range 3 to 5 $\times$ 10^{-7} cm^2/sec and 1 to 2 $\times$ 10^{-7} cm^2/sec for the parallel and perpendicular directions, respectively. The lack of precision in these values arises from the uncertainty in the determination of the precise relative optical density at which the measurements were made. The ratio of the two coefficients, however, is independent of this determination and equal to 2.4.

Time-lapse motion pictures were also made of several hundred tendons following the introduction of aqueous uranyl nitrate solutions by the methods described above. In contrast to the case of colloidal gold, with the uranyl ion (UO_2^{++}) the optical density of the films (after some time had elapsed) showed a sharp gradient that with time advanced outward from the center of the image but did not become diffuse. Such behavior cannot be explained on the basis of a random diffusion process alone, and in the following section a model is presented which predicts a sharp boundary as well as other features observed experimentally.

A plot of the distance x of the sharp boundary from the center of the tendon as a function of the square root of time t is shown in Fig. 3. Comparison with the predictions of the model (dashed curves), again using the approximation that the transport processes in the two directions are mutually independent, yields for the diffusion coefficients the values 10 $\times$ 10^{-7} cm^2/sec and 4 $\times$ 10^{-7} cm^2/sec for the parallel and perpendicular directions, respectively. It should be mentioned that for low values of t, the fact that the boundary is not very well defined causes these experimental points to be somewhat uncertain. Similar uncertainty exists in the theoretical results, and the present accuracy of the computations does not permit extending the dashed curves beyond the region shown in the figure. At extreme values of t, on the other hand, the experimental curves begin to drop slightly below the theoretical ones, and this is probably due to the onset of dehydration of the tendon.

The occasional appearance of a clear region preceding the advancing front of uranyl nitrate, similar in shape and sharpness, may be due to the diffusion of hydrogen ions from the solution (pH = 4.7) into the tendon in advance of the uranyl ions that later replace them (26, 27) on the binding sites, as absorption of acid is known to cause collagen to become transparent (28).

In order to try to correlate the behavior of colloidal gold and of uranyl nitrate with microscopic processes, the tendons were prepared for electron microscopy as described in the previous section. Fig. 4 shows a typical electron micrograph (final magnification 32,400) of a near-longitudinal section of tendon following the diffusion of colloidal gold. [The large interfibrillar spaces may be, in part, a dehydration artifact (28, 29).] The gold particles appear to be randomly distributed throughout the interfibrillar spaces and to exhibit no apparent preference for any particular sites along the collagen fibrils. Similar results have been obtained with f2 bacteriophage diffusion.

Figs. 5 and 6 are electron micrographs of a longitudinal and an oblique section

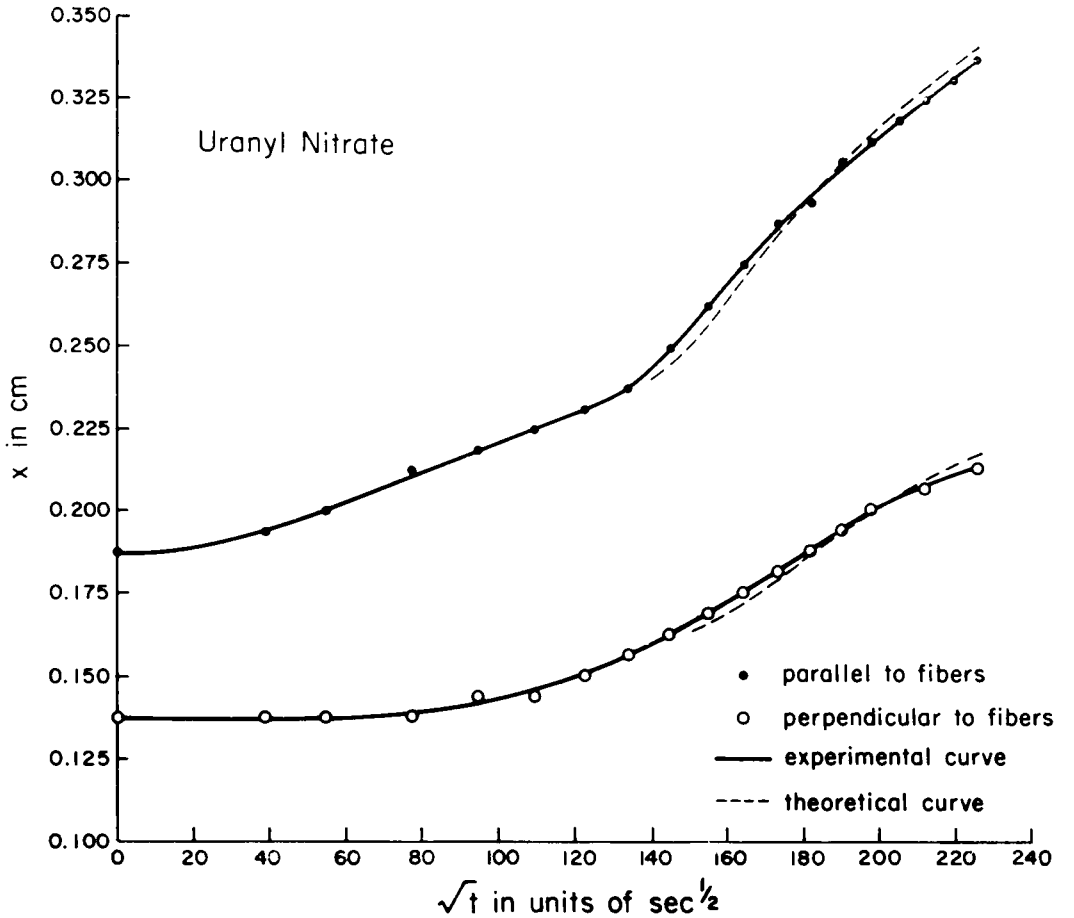

FIGURE 3 A plot (solid lines) of the diffusion of uranyl nitrate as a function of the square root of time for the directions parallel (solid circles) and perpendicular (open circles) to the collagen fibers. Theoretical curves from the diffusion with attrition model are also shown (dashed lines), and correspond to diffusion coefficients of 10×10^{-7} cm²/sec and 4×10^{-7} cm²/sec for the two directions.

of tendon following the spread of uranyl nitrate (final magnification is 32,400 in each case). One immediately observes that the uranyl ions are concentrated along the surfaces of the collagen fibrils and are almost completely absent from the interfibrillar spaces. Furthermore, the clusters are predominantly localized at the positions of the banded regions of the collagen fibrils and are not present in the interband segments. The absence of clusters from the interfibrillar spaces may be due to the removal of loosely held ions during the dehydration process, but if so, it only serves to emphasize the fact that the ions along the fibril surfaces are bound firmly to the collagen macromolecule.

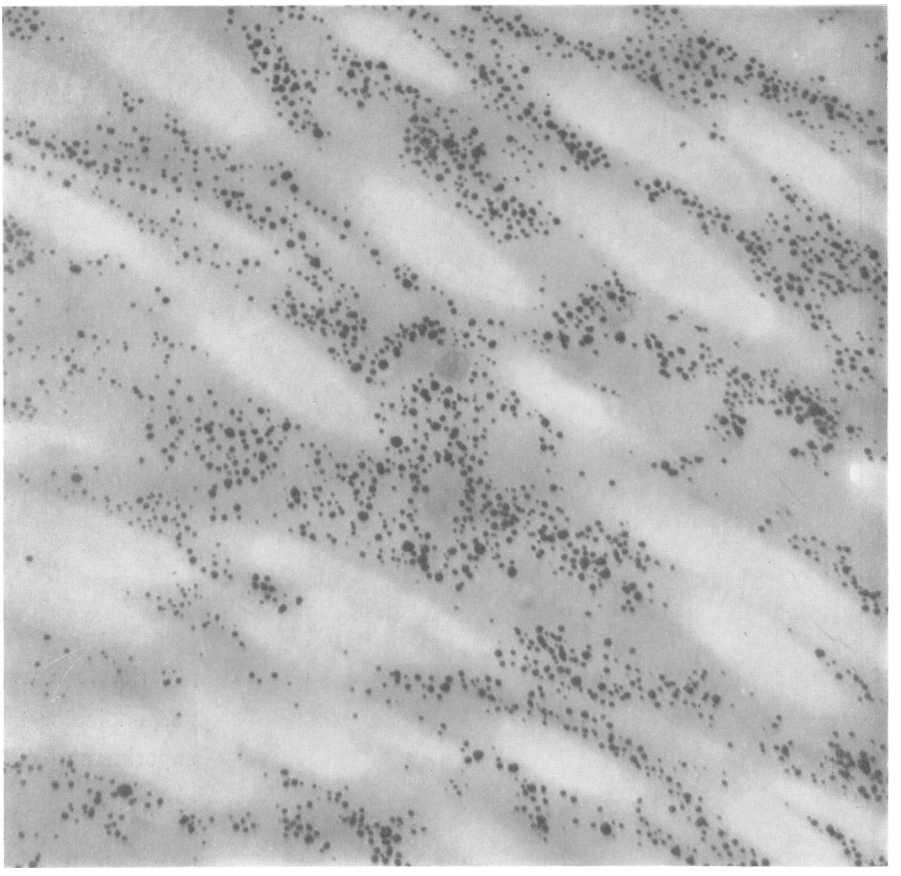

FIGURE 4 Electron micrograph of a near-longitudinal section through excised chicken breast tendon following the introduction and diffusion of colloidal gold. Fixation in osmium tetroxide (Sjös 1%) and embedding in Epon 812. (Initial magnification 14,000, final magnification 32,400.)

Essentially the same results have been obtained with most of the heavy metal salts listed in the previous section, and in no case have there been any observations contrary to the general features described above.

MODEL

In order to explain the presence of a sharp boundary in the uranyl nitrate diffusion experiments, we make use of the observation that there is a strong interaction between the diffusate and particular sites within the diffusant. The model proposed is one of diffusion with attrition of the diffusate, and it has been treated before in an approximate way (30, 31). It envisages a system of uniformly spaced binding

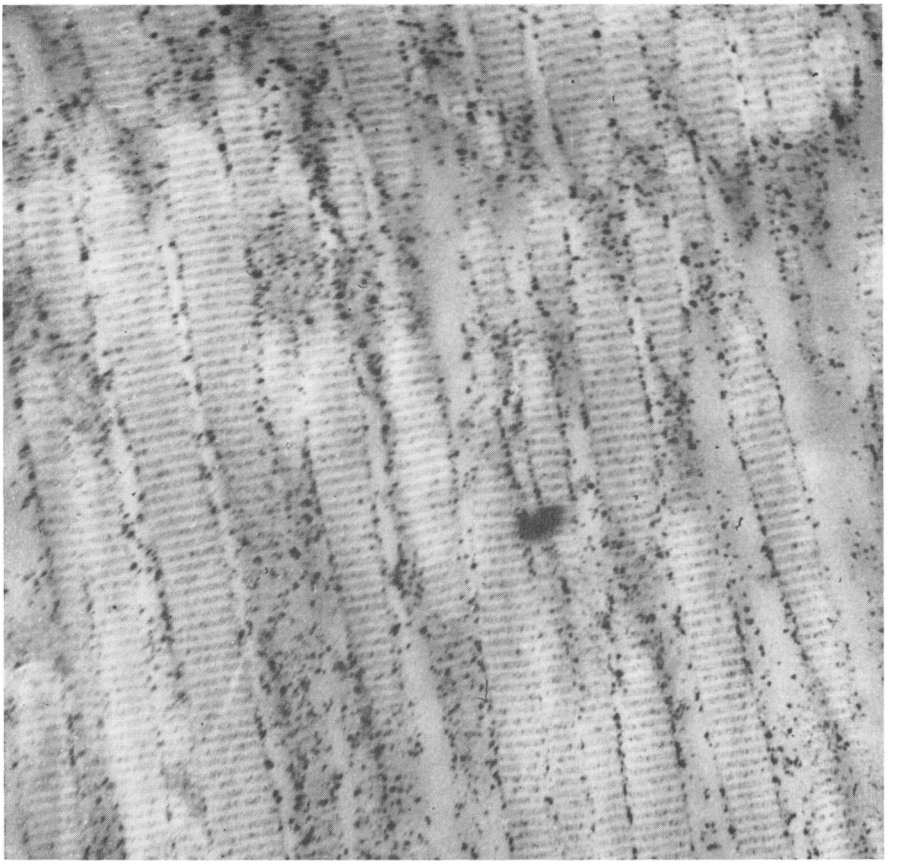

FIGURE 5 Electron micrograph of a longitudinal section through excised chicken breast tendon following the introduction and diffusion of uranyl nitrate solution. Fixation in osmium tetroxide (Sjös 1%) and embedding in Epon 812. (Initial magnification 8100, final magnification 32,400.)

sites of concentration m that bind the diffusate ions (concentration c) with a bimolecular interaction constant k, so that

$$\partial c/\partial t = D \frac{\partial^2 c}{\partial x^2} - kcm, \tag{1}$$

where we have assumed diffusion in one dimension (x), t is the time, D the diffusion coefficient in the x direction, and the second term on the right represents the loss in freely diffusing ions due to binding. The number of available binding sites also decreases with time, according to the equation

$$\partial m/\partial t = -kcm. \tag{2}$$

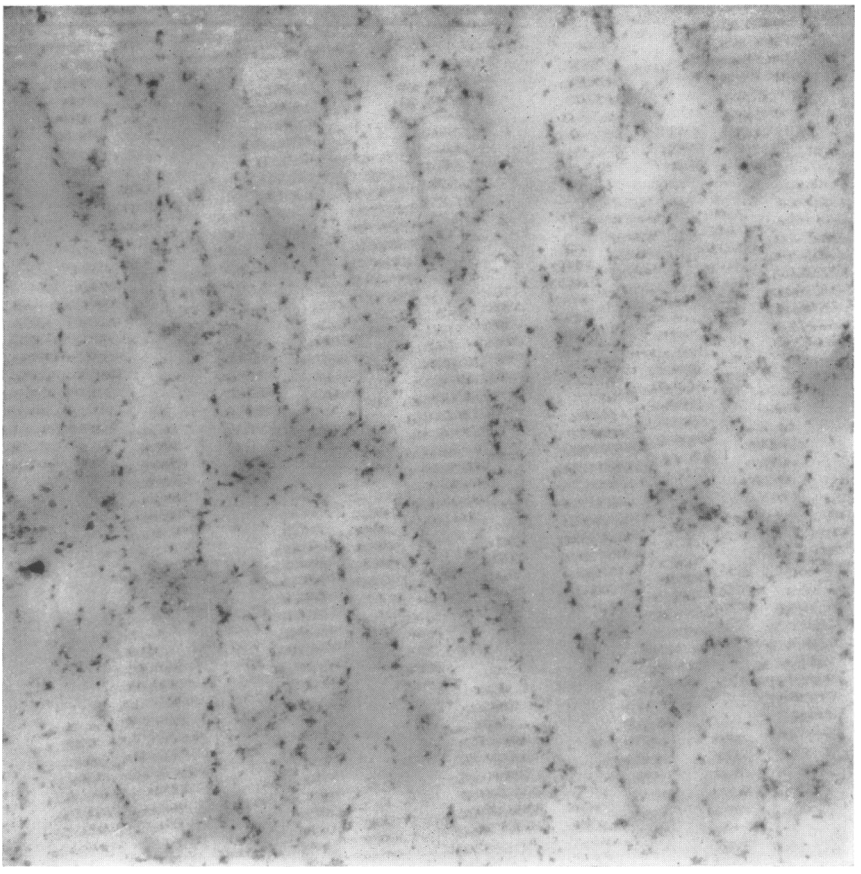

FIGURE 6 Electron micrograph of an oblique section through excised chicken breast tendon following the introduction and diffusion of uranyl nitrate solution. Fixation in osmium tetroxide (Sjös 1%) and embedding in Epon 812. (Initial magnification 14,000, final magnification 32,400.)

Since in our experiments the string is kept saturated with the diffusate solution (concentration c_o), we have as boundary conditions that

$$\begin{aligned} c(0, t) &= c_o \\ c(\infty, t) &= 0 \\ c(x, 0) &= 0 \quad \text{for} \quad x > 0 \\ m(x, 0) &= m_o \\ m(0, t) &= 0 \quad \text{for} \quad t > 0, \end{aligned} \tag{3}$$

where m_o is the density of sites prior to binding.

The expressions (1) and (2) are simultaneous nonlinear differential equations

and cannot be solved in analytical form. Their solution, subject to boundary conditions, expression (3), has been programmed for a high speed digital computer and will be published elsewhere (32). Here we quote only the relevant results. The curves of c as a function of x for various values of t show monotonically decreasing (negative) slopes as one proceeds outward from the origin. This is not too surprising and is actually not very different from what one obtains with normal, random diffusion under these boundary conditions. But c is not the variable that is observed in the experiments—what one really sees is the total concentration of diffusate, *both* free (c) and bound ($m_o - m$). And when this parameter, $c + m_o - m$, is calculated as a function of x, one obtains an exceedingly rapid drop over a very small interval of x. This is the sharp boundary. One can then proceed to investigate the advance of this boundary with time, and the calculations show that everywhere but for small values of t (where the computation is not sufficiently accurate to yield reliable results), the position of the boundary remains sharp and advances approximately proportionally to the square root of time. The exact value of the proportionality constant depends on D and on the ratio c_o/m_o. If we assume one site per free carboxyl group in the collagen macromolecule, then for the concentration of uranyl nitrate used (0.6 g/ml), we get a ratio of 0.5. The corresponding theoretical curves have been drawn in Fig. 3 for $D = 10 \times 10^{-7}$ cm^2/sec in the direction parallel to the fibers and 4×10^{-7} cm^2/sec perpendicular to the fibers. The fit to the experimental results is good. Only a very crude estimate of the order of magnitude of the interaction constant is possible from this type of analysis. Such an estimate gives values of 10^{-3} M^{-1} sec^{-1} and 10^{-4} M^{-1} sec^{-1} for the parallel and perpendicular directions, respectively.

If the slopes of the linear portions (at large t) of the theoretical x versus $\sqrt{t}$ curves are plotted against the ratio c_o/m_o, a best fit to this plot shows that the slope is very nearly proportional to the 0.65 power of the ratio and hence of the initial concentration c_o.

DISCUSSION

The anisometric transport observed in the dye diffusion experiments (Fig. 1) agrees with data reported by Weiss (16) on the spread of dye in fish skin. McMaster and Parsons (2) studied the movements of various dyes in the mouse ear, and they too found that the dyes traveled along the connective tissue fibers. These qualitative results suggest that the use in nature of such fibers as pathways along which substances move through tissues to and from cells, is a very efficient one.

The transport experiments performed with colloidal gold (diameter 150 to 200A) can be interpreted on the basis of a random diffusion process. This is supported by electron microscopic evidence (Fig. 4) that shows the distribution of the gold particles to be random throughout the interfibrillar region of the tendon and unrelated to the fine structure of the collagen fibrils. The range of values determined

for the diffusion coefficient is 3 to 5 $\times$ 10^{-7} cm^2/sec and 1 to 2 $\times$ 10^{-7} cm^2/sec for the directions parallel and perpendicular to the fibers, respectively (Fig. 2). Armstrong (19) has obtained very similar results (2.3 $\times$ 10^{-7} cm^2/sec) for the diffusion of mimosa tannin in bovine skin, and calculates the diameter of the diffusate to be about 180 A.

The diffusion coefficient in the perpendicular direction is smaller by a factor of 2.4. This may be due, at least in part, to steric hindrance by the fibrils of the large colloidal particles used, which would explain the absence of gold from the exposed surfaces of the sectioned collagen fibrils. Maurice (20), investigating the diffusion of mollusk hemaglobin (diameter 185 A) in the corneal stroma of ox, finds somewhat less steric hindrance in the perpendicular direction, but he too concludes that the collagen fibrils are the structures limiting diffusion.

In all the diffusion experiments with uranyl nitrate, a sharp boundary was always seen to appear after a time at the advancing edge of the diffusate. Many similar observations have been described before. Thus McMaster and Parsons (2) reported that the dye seemed to appear suddenly and to grow in intensity with time, which is just the effect one would expect after sufficient time had elapsed and the sharp boundary became evident, while Slather and Lauffmann (17) and Armstrong (19) describe the existence of sharp advancing boundaries in tanning experiments.

The widespread presence of these sharp boundaries is inconsistent with a random diffusion process, and the electron micrographs (Figs. 5 and 6) indicate that the uranyl clusters are localized in space on the surface of the collagen fibrils and are tightly bound to the banded regions. For these reasons the diffusion with attrition model (30–32) was suggested as a possible means of interpreting the experimental results. A mathematical analysis of this model does indeed predict the presence of a sharp boundary, except possibly when the elapsed time is small, and further calculations (32) indicate that this boundary should advance at a rate that is approximately proportional to the square root of time. Such indeed is the case in the present experiments and in many others that have been reported (17–20, 33, 34), although in a random diffusion process the position of a particular diffusate concentration (relative to its value at the origin) will also advance proportionally to the square root of time. But the simultaneous existence of both a sharp boundary and an advance that is proportional to the square root of time, is significant evidence in favor of the applicability of the diffusion with attrition model.

A fit of the theoretical curves to the experimental results (Fig. 3) gives values for the diffusion coefficient of 10 $\times$ 10^{-7} cm^2/sec and 4 $\times$ 10^{-7} cm^2/sec for the directions parallel and perpendicular to the collagen fibers, respectively. Almost identical results have been obtained by Maurice (20) who studied the transport of fluorescein in the corneal stroma of rabbit. He reports values of 10 $\times$ 10^{-7} cm^2/sec and 3.3 $\times$ 10^{-7} cm^2/sec for the two directions, but maintains that his experimental

data were not really accurate enough to distinguish between isotropic and anisotropic mechanisms.

The small size of the uranyl ion precludes the possibility that the factor of 2.5 between the coefficients in the two directions is the result of steric hindrance perpendicular to the fibers—the electron micrographs show the extent to which the uranyl clusters penetrate the fibrillar structure. What may cause this large factor is suggested by nuclear magnetic resonance studies on native and partially dried tendon. In both cases, Berendsen (35) found that proton interactions take place dominantly in the fiber direction and that water molecules may form chains in the fiber direction that have long range order. But whatever the precise mechanism, the fact that ions do move faster *along* the collagen fibers could play an important role in avascular transport.

An analysis of the theoretical curves depicting the position of the boundary as a function of the square root of time shows that the slopes of the curves at larger values of t are very nearly proportional to the 0.65 power of the ratio of the initial concentration of the diffusate to the total concentration of binding sites. This compares favorably with results reported in tanning experiments (17, 19), where the rate of advance of the boundary was found to be proportional to the square root of the initial concentration.

The exceedingly low values obtained for the interaction constant, 10^{-3} M^{-1} sec^{-1} in the parallel direction and 10^{-4} M^{-1} sec^{-1} in the perpendicular direction, may indicate that activated complexes are involved in the binding reaction and that considerable rearrangement of the protein is necessary before the uranyl ion becomes firmly bound.

CONCLUSIONS

The transport of histological dyes, colloidal particles, and heavy metal salts has been investigated in excised chicken breast tendon using time-lapse techniques and electron microscopy.

In all dyes observed, the transport was found to be anisometric, the extent of the spreading being much greater parallel to the collagen fibers than perpendicular to them.

A quantitative study of the diffusion of colloidal gold particles 150 to 200 A in diameter showed that the results could be explained on the basis of a random diffusion process, the diffusion coefficient lying in the range 3 to 5 $\times$ 10^{-7} cm^2/sec for the direction parallel to the fibers and 1 to 2 $\times$ 10^{-7} cm^2/sec for the perpendicular direction; the anisotropy was attributed to steric hindrance in the latter direction.

In measurements performed on the tendons following the introduction of uranyl nitrate solution, a sharp boundary was seen to appear at the advancing edge of the diffusate. This boundary moved outwards from the center of the tendon (where the diffusate was introduced and maintained at a constant concentration), proceed-

ing at a rate proportional to the square root of time and always remaining well defined. Electron micrographs indicated that uranyl clusters were localized in space on the surfaces of the collagen fibrils and were tightly bound to the polar amino acid regions of the macromolecule.

To explain these results, a model was proposed in which random diffusion is coupled with interaction between the diffusate and the diffusant. The model predicts a sharp boundary that advances proportionally to the square root of time and to the 0.65 power of the initial diffusate concentration.

A fit of the theoretical curves to the experimental results for uranyl diffusion gave values for the diffusion coefficient of 10×10^{-7} cm^2/sec and 4×10^{-7} cm^2/sec for the parallel and perpendicular direction, respectively. An estimate of the order of magnitude of the interaction constant was also obtained. A possible explanation for the existence of this large ratio between the coefficients in the two directions was advanced, and it was pointed out that anisometric transport could play an important role as nutrients are carried to cells and metabolic products are removed by way of anisotropic tissues.

Investigations were carried out as part of a research program under the direction of Dr. Paul Weiss, supported in part by Grant No. CA-06375 from the National Cancer Institute (National Institutes of Health of the United States Public Health Service).

Received for publication 24 August 1965.

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