Br. J. Pharmac. (1971), **41**, 367–378.

Diffusion of labelled substances through isolated rat diaphragm

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Summary

1. The rate of diffusion of [^{14}C] sucrose through rat diaphragm muscle is dependent on the period of preliminary soaking of the muscle in Krebs solution at 36° C. The ratio of the length of the diffusion path through the diaphragm to its geometrical thickness appears to fall from $4{\cdot}39 \pm 0{\cdot}38$ (S.E. of the mean) to $2{\cdot}23 \pm 0{\cdot}18$ after about 6 h of soaking.

2. The area of the diaphragms available for diffusion is apparently constant at 0·3–0·4 of their total area.

3. The measured thickness of the diaphragms increases by about 20% on soaking.

4. Entry of [^{131}I] iodide into the muscle fibres is detected as a fall in both steady state and non-steady state rates of diffusion through the diaphragm.

5. Entry of [^{14}C-methyl] decamethonium into the muscle cells is not evident from such diffusion studies in normal Krebs solution.

Introduction

In order to study the dynamics of neuromuscular blockade in the isolated rat diaphragm (Brookes & Mackay, 1971) it was necessary to determine parameters for diffusion through the muscle interspaces. This has been done by a method similar in principle to that used by Page & Bernstein (1964) for the measurement of diffusion through cat heart muscle.

In the present study it was found that the diffusion properties of isolated rat diaphragm were not constant but changed progressively with time. This aspect is of great interest and has therefore received particular attention. Diffusion of substances known to interact with the tissue and to enter cells was also measured to see whether, and in what way, the observed diffusion parameters would be modified. The three radioactively labelled substances studied were [^{14}C] sucrose, [^{14}C-methyl] decamethonium and [^{131}I] iodide. A preliminary report of some of these results has been given (Brookes & Mackay, 1969).

Methods

The starting point for each of these experiments was a rat phrenic nerve-diaphragm preparation (Bülbring, 1946) modified to take the form of a rectangular

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plane sheet of muscle maintained under a uniform tension of 3–5 g. All of the diaphragms were taken from male albino (Wistar strain) rats weighing 110–170 g and were bathed in Krebs solution (Krebs & Henseleit, 1932) at 36 ± 0·5° C, vigorously gassed with 5% CO_2 in oxygen.

Some of the diaphragms were stimulated by shocks applied alternately to the phrenic nerve and directly to the muscle at a total rate of four min^{-1} for varying periods. Their thickness was measured and the diffusion experiment begun. In these diaphragms the phrenic nerve was cut close to its point of entry into the muscle just before measurement of the thickness. For diaphragms which were not to be stimulated the nerve was cut during dissection.

Measurement of thickness

The thickness of the muscle was measured directly at five or six points spaced around the stump of the phrenic nerve in the centre of the small sheet of diaphragm. These measurements were made with a pair of micrometers mounted in opposition to drive needles through latex covered ports in the side walls of a specially constructed bath. Micrometer readings were made first with the needle points touching each other and then with the points touching the surfaces of the diaphragm which was interposed in a perpendicular plane between them. These measurements were reproducible to within ± 0·005 mm and the standard error of the mean thickness for each diaphragm was always less than 9%. The time taken to measure diaphragm thickness was 15–20 min and during this time the Krebs solution bathing the tissue was at room temperature.

Diffusion measurement

The diaphragm, still suspended under a tension of 3–5 g, was then positioned between the short tubes projecting from the fronts of a pair of Perspex diffusion chambers. The chambers were driven together in their brass holder until the rims of the projecting tubes engaged accurately to clamp between them a disc of diaphragm of area 0·714 cm^2 (Fig. 1). Excess tissue was trimmed away and a strip of

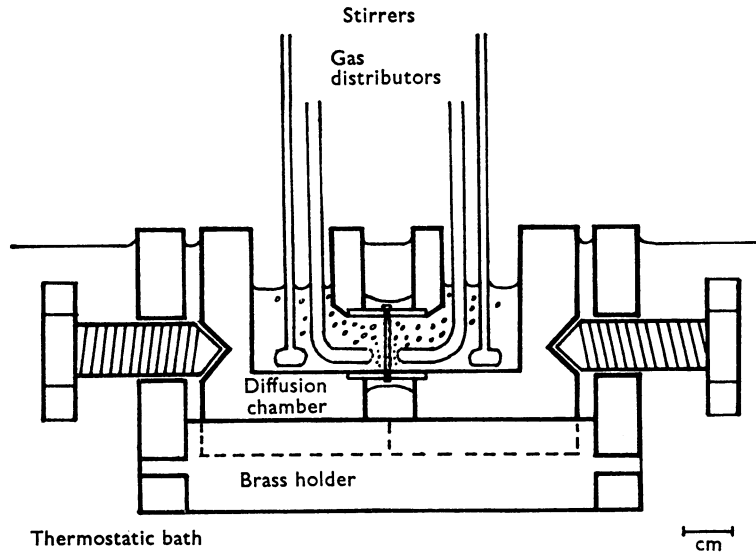

FIG. 1. Perspex diffusion chambers clamped together by bolts in a brass holder.

Parafilm was wrapped around the junction which was then quickly sealed with molten white beeswax at 70° C. The assembly, which had previously been equilibrated at 36° C, was returned to the thermostatic bath and 2 ml of prewarmed Krebs solution was measured by pipette into each chamber. The gas distributor tubes and stirrers were then lowered into position. The solutions were gassed with 5% carbon dioxide in oxygen, presaturated with water vapour at room temperature. Into the ends of the gas distributor tubes were sealed sintered glass plugs through which a spray of fine bubbles could be directed close to both surfaces of the muscle. The time taken to clamp the diaphragm and begin gassing the solutions was no more than 4 minutes. A further 10–20 min was then allowed before adding to the pleural side of the diaphragm 1 ml of Krebs solution containing the radioactive tracer. Sufficient Krebs solution was immediately added to the peritoneal side to equalize the hydrostatic pressures across the diaphragm. The temperature of the solutions inside the chambers was $36 \pm 0{\cdot}5$° C, and the initial volumes (3 ml) reached approximately the level shown in Fig. 1.

For [^{14}C] sucrose and [^{14}C-methyl] decamethonium steady state diffusion was usually reached within 40 min of adding the tracer. In most cases 0·1 ml samples were withdrawn from both chambers five times (at 10 min intervals) starting from the fortieth minute. For diffusion of [^{131}I] iodide, sampling was begun sooner and was at 5 min intervals. The initial radioactivity on the pleural side was generally 1–2 μCi/ml. The activity on the peritoneal side never rose above 4% of that on the pleural side during the course of an experiment.

The radioactivity of the 0·1 ml samples, or of the same volume of suitable dilutions in Krebs solution, was measured using a liquid scintillation counter (Panax Equipment Ltd., type SC-LP) with a scaler and automatic timer (Panax Equipment Ltd., type A.C. 300/6). Five millilitres of a dioxane phosphor (Panax Equipment Ltd., type D.E.M.) was added to each sample and two or three successive counts of 10^4 were timed. Appropriate corrections were made for the background count and the dead-time of the counter, but no corrections were made for quenching or for the decay of [^{131}I] iodide, both of which were considered negligible. For each experiment the total amount of activity (Q_t) which had diffused across the diaphragm was plotted against time, t (Fig. 3a and b).

Materials

The radioactive substances used were [U-^{14}C] sucrose of approximately specific activity 35 μCi/mg, [^{14}C-methyl] decamethonium bromide of specific activity 12 μCi/mg (The Radiochemical Centre, Amersham) and a carrier-free aqueous solution of [^{131}I] sodium iodide of approximate specific activity 20 μCi/ml (Department of Medical Physics, Leeds General Infirmary). Other substances used were (+)-tubocurarine chloride (Koch-Light Laboratories Ltd.), decamethonium iodide (gift from Allen and Hanburys Ltd.), and gallamine triethiodide (gift from May & Baker Ltd.).

Results

Diffusion theory

The amount of substance diffusing through a homogeneous membrane which separates two reservoirs, when concentration C_1 of the substance is introduced into

one of the reservoirs at zero time and the concentration in the second reservoir remains close to zero, is described by the equation

$$Q_t = DC_1 t/b - C_1 b/6 - (2C_1 b/\pi^2) \sum_{1}^{\infty} [(-1)^n/n^2]\exp(-Dn^2\pi^2 t/b^2) \quad \ldots\ldots(1)$$

where Q_t is the total amount of diffusing substance which has passed through the membrane in time t, b is the membrane thickness and D is an appropriate diffusion coefficient. This solution of the diffusion equation was given by Barrer (1939) who further showed that as t approaches infinity equation (1) approaches the line

$$Q_t = (DC_1/b)\ (t - b^2/6D) \quad \ldots\ldots(2)$$

This line intersects the t axis at a positive time L (the ' time lag ') given by

$$L = b^2/6D \quad \ldots\ldots(3)$$

The slope of the line defined by equation (2) is the ' steady-state ' flux, F, of diffusing substance through the membrane, where

$$F = DC_1/b \quad \ldots\ldots(4)$$

When applying equation (1) to diffusion through a non-homogeneous membrane such as rat diaphragm muscle, D must be considered to be only an apparent diffusion coefficient since its value will be dependent on the detailed structure of each diaphragm. The area available for diffusion can be taken to be a fraction, α, of the

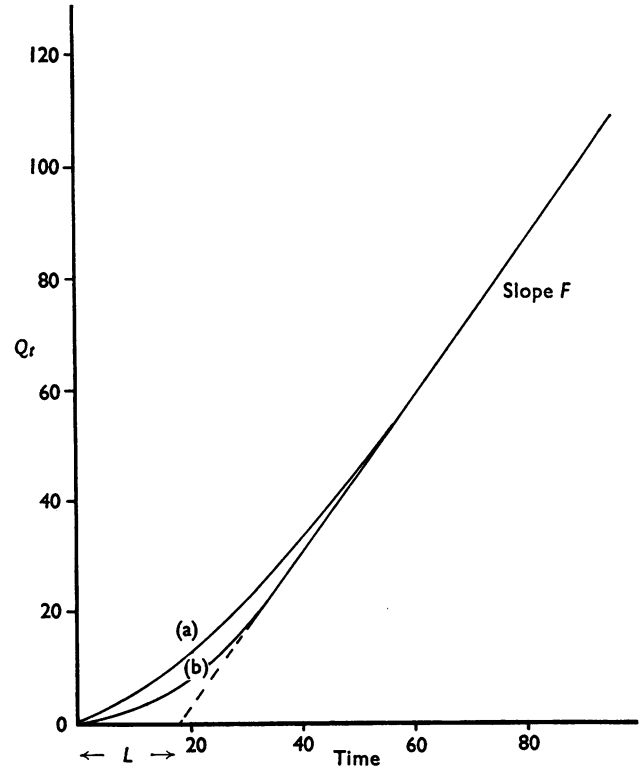

FIG. 2. The total amount of substance (Q_t) which has diffused through the diaphragm is plotted against time t. Note that though these curves are clearly different they have the same intercept L on the time axis and the same final slope F. Curve (b) approaches linearity at a shorter time than does curve (a).

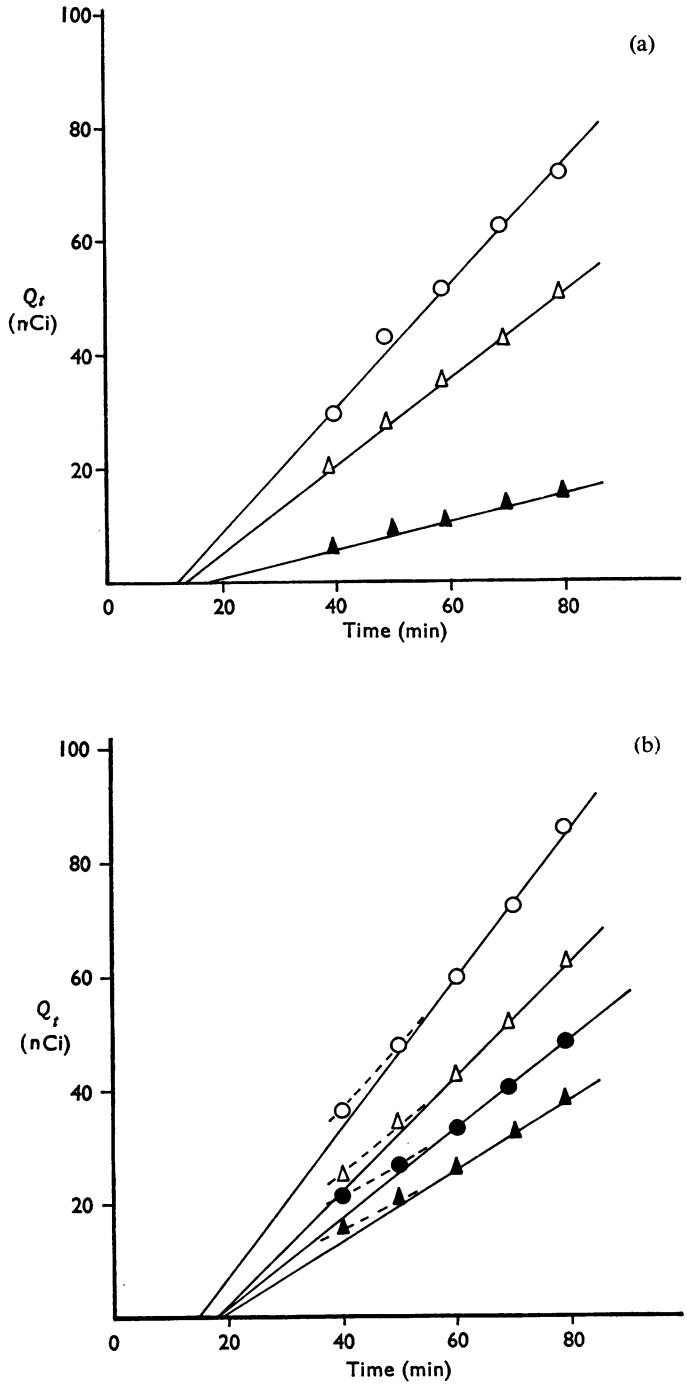

FIG. 3. (a) Diffusion of [^{14}C] sucrose through each of three diaphragms which, after dissection and thickness measurement, had been left in Krebs solution at 36° C for 0·1 h before starting the diffusion experiment. The total amount of activity (Q_t) which has diffused through each diaphragm is plotted against time. Note that in each case linearity was reached within 40 min of starting the experiment. (b) As for (a) except that each diffusion experiment was started as soon as the thickness of the diaphragm had been measured. Note that in these cases linearity was not reached until about 60 min after starting the experiment.

geometrical area of the diaphragm; and the mean path of the diffusing molecules can be considered to be longer than the geometrical thickness of the diaphragm by factor, k (the tortuosity factor), due to the tortuous route taken by the molecules around obstacles in the tissue. Equations (3) and (4) then become

$$L=b^2k^2/6D \quad \text{.....(5)}$$

and $$F=\alpha DC_1/bk \quad \text{.....(6)}$$

where D is now the diffusion coefficient of the substance in free solution. Equation (1) can be expected to fit the experimental plot of Q_t versus t only if the diffusion parameters remain fairly constant during the course of a diffusion experiment. However the steady state flux F and the time lag L do not completely determine the curve of Q_t versus t (see Fig. 2). According to Crank (1956) if equation (1) is valid then the plot of Q_t versus t should become linear when $D't/b^2$ is equal to about 0·45. This can be used to test for any serious deviations from equation (1), which might arise from changes in the membrane diffusion parameters during the diffusion experiment. It also seems likely that any marked non-uniformity of the diaphragm, on the macroscopic scale, would produce deviations from equation (1) which might be detected by the test described above. In the present experiments good linear plots of Q_t against t were generally obtained and the plots became linear at the appropriate times, with specific exceptions which will be discussed (see, for example, Figs. 3a and 3b). Equations (5) and (6) were used to calculate the tortuosity factor, k, and fractional available area, α, from the intercept, L, on the t axis and the slope, F, of the straight line plots of Q_t against t. It should be noted that the apparent 'non-steady state' diffusion coefficient, $D'(=D/k^2)$, obtained from the time lag is not usually identical with the apparent 'steady state' diffusion coefficient, $D''(=\alpha D/k)$, obtained from the flux.

Diffusion through rat diaphragm

[^{14}C] *Sucrose.* Sucrose was chosen to characterize extracellular diffusion in the diaphragm because of its negligible rate of penetration into muscle fibres and because it has a molecular weight of the same order as the common neuromuscular blocking cations. Parameters for the diffusion of [^{14}C] sucrose were first determined in two groups of four diaphragms. One group had been stimulated at four shocks min^{-1} in Krebs solution at 36° C for a period of 4 h during which time the rate of action of gallamine was observed. In the second group of diaphragms which had been stimulated for 5·3–6·3 h, the rate of action of tubocurarine had been studied (Brookes & Mackay, 1971). The mean tortuosity factors found for these groups were $2{\cdot}73 \pm 0{\cdot}14$ (S.E. of mean) and $2{\cdot}23 \pm 0{\cdot}18$, respectively and although the difference between them is not statistically significant the possibility remained that the tortuosity of the diffusion path through isolated diaphragm might be showing a decrease with time. The mean k for a group of three diaphragms which were soaked only for a brief time (shown as 0·1 h in Fig. 4) before measurement of thickness was $4{\cdot}39 \pm 0{\cdot}38$ (S.E. of mean), and this is significantly greater than the mean for the first two groups combined ($P<0{\cdot}001$). In a further pair of groups, soaked for 0·5–1 h (four diaphragms) and approximately 0·7 h (three diaphragms), the influence of stimulation at four shocks min^{-1} (see **Methods**) was examined. The mean k for the unstimulated group was $3{\cdot}60 \pm 0{\cdot}11$ (S.E. of mean) and for the stimulated group was $2{\cdot}89 \pm 0{\cdot}22$, and these means are significantly different ($P<0{\cdot}05$). The mean

values of α for these five groups of diaphragms range between 0·31 and 0·42, and do not show significant differences.

In a final group of four diaphragms (designated 0 h in Fig. 4) an attempt was made to obtain diffusion data with the muscle as nearly as possible in its freshly excised condition. In practice there was a minimum delay of about 40 min between excision of the diaphragm and the addition of tracer to the diffusion chamber. The experimental plot of Q_t against t is shown for each of these diaphragms in Fig. 3b. In contrast with the other groups (see Fig. 3a) a linear flux is not reached here until after the third sampling. As was mentioned earlier linearity should be reached when $D't/b^2$ is equal to about 0·45, which, in this group of experiments, is when t is equal to 40–50 minutes. The observed deviation from theory in this group could possibly be explained if rapid changes in the diaphragm were taking place during measurement of diffusion. The mean value of k was $4{\cdot}77 \pm 0{\cdot}09$ (S.E. of mean) and the mean value of α was $0{\cdot}54 \pm 0{\cdot}07$ (S.E. of mean) but these estimates must obviously be subject to serious error.

A summary of these results for the diffusion of [^{14}C] sucrose through rat diaphragm is given in Fig. 4.

Diaphragm thickness. In the above experiments, diffusion of [^{14}C] sucrose was studied in a total of twenty-two diaphragms. Of these, eleven were soaked in Krebs solution at 36° C and stimulated at a rate of four shocks min^{-1} for periods

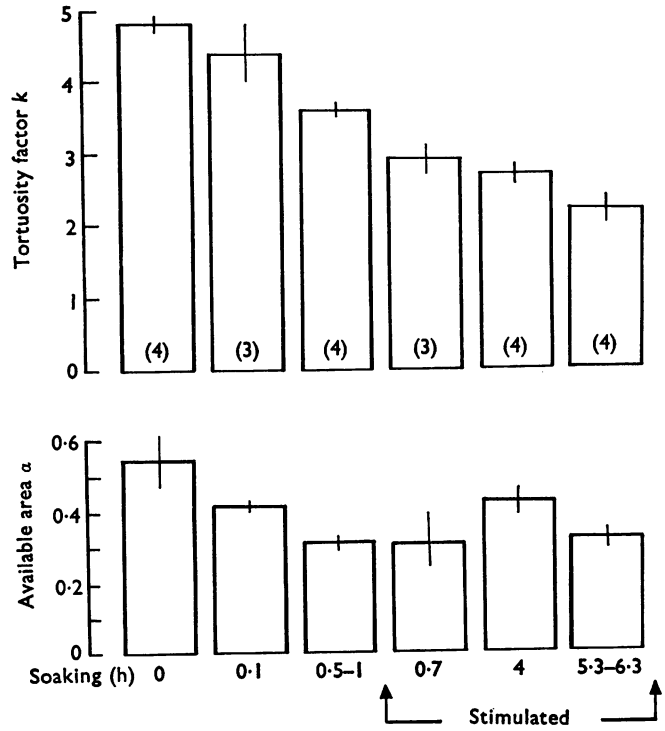

FIG. 4. Mean tortuosity factors (k) and available areas (α) determined by measurement of the diffusion of [^{14}C] sucrose through six groups of diaphragms which were soaked in Krebs solution for increasing periods of time before the measurement of diffusion. The number of diaphragms in each group is given in brackets, and the vertical bars indicate standard error of the mean. In those groups shown as 'stimulated' the diaphragms received single shocks at the rate of four min^{-1} during the period of soaking.

of from 0·7 to 6·3 h before measurement of their thickness. The mean thickness of these eleven diaphragms was $0{\cdot}529 \pm 0{\cdot}018$ mm (S.E. of mean). The remaining eleven diaphragms, which were soaked for not more than 1 h and were not stimulated, had a mean thickness of $0{\cdot}437 \pm 0{\cdot}012$ mm. These means are significantly different ($P<0{\cdot}001$). The mean weights of the rats from which the diaphragms were taken were $139{\cdot}0 \pm 3{\cdot}8$ g (S.E. of mean) (stimulated diaphragms) and $133{\cdot}8 \pm 3{\cdot}5$ g (unstimulated diaphragms), and these means were not significantly different ($P>0{\cdot}3$).

[^{14}*C-methyl*] *decamethonium.* There is evidence for fairly rapid cell penetration and binding of decamethonium in the end-plate of skeletal muscle (Taylor, Creese, Nedergaard & Case, 1965; Waser, 1960) although transmembrane exchange of decamethonium-like compounds may be slow (Creese, Taylor & Tilton, 1963). It was anticipated that uptake of a proportion of the labelled cations diffusing through rat diaphragm in the present experiments might be detected as an apparent increase in tortuosity factor. However, the mean k determined in a group of three dia-

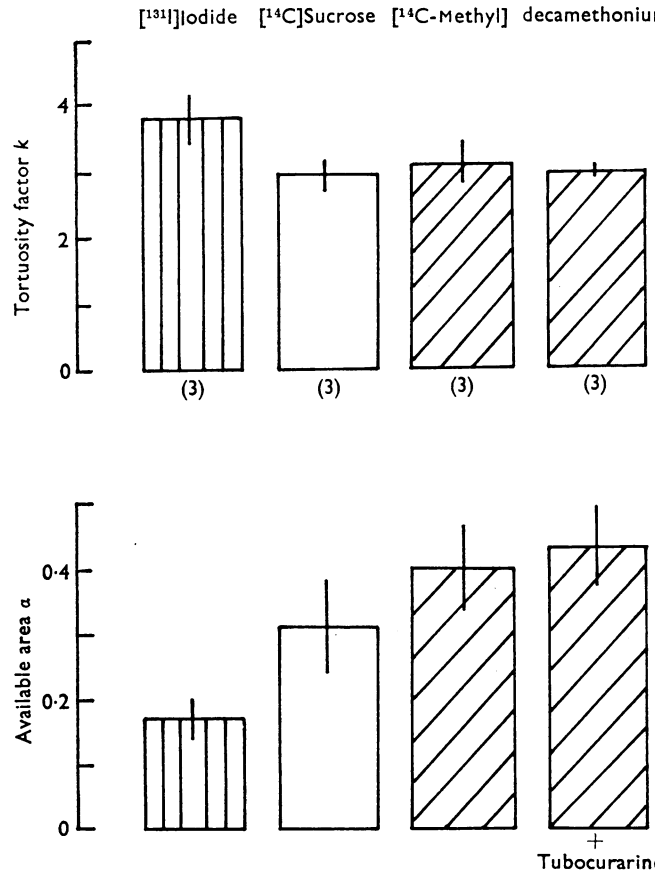

FIG. 5. Mean tortuosity factors (k) and available areas (α) determined by measurement of the diffusion of [^{131}I] iodide, [^{14}C] sucrose and [^{14}C-methyl] decamethonium through diaphragms which were stimulated at four shocks min^{-1} in Krebs solution for 45 min before the measurement of diffusion. The number of diaphragms in each group is given in brackets, and the vertical bars indicate standard error of the mean where appropriate. As indicated, the effect was also examined of modifying the solution in the diffusion chambers by addition of tubocurarine (10 μg/ml).

phragms which had been stimulated for 45 min was $3{\cdot}08 \pm 0{\cdot}33$, and this is very close to the corresponding value for diffusion of [^{14}C] sucrose. Tubocurarine antagonizes entry of decamethonium into muscle fibres (Taylor *et al.*, 1965), but diffusion of [^{14}C-methyl] decamethonium was not influenced by the presence of tubocurarine, 10 μg/ml, in a further group of three experiments (mean $k = 3{\cdot}02 \pm 0{\cdot}10$ (S.E. of mean)). Diffusion of [^{14}C-methyl] decamethonium was also measured through freshly excised diaphragms which received no preliminary soaking. The measured flux was erratic in two experiments and the third experiment yielded a tortuosity factor of 3·19.

[^{131}I] *Iodide.* The iodide ion enters muscle fibres slowly (Hill & Macpherson, 1954) and it was of interest to know whether this entry would significantly influence the estimated values of k and α. Throughout these experiments Krebs solution was modified by replacing chloride 1–10 M equiv/l. with an equivalent concentration of iodide. In a group of three diaphragms which had been stimulated for 45 min the mean k was $3{\cdot}76 \pm 0{\cdot}38$ (S.E. of mean) and the mean α was $0{\cdot}17 \pm 0{\cdot}03$ (S.E. of mean) (Fig. 5).

Estimation of experimental error. For each diffusion experiment the linear regression of Q_t upon t was calculated and estimates of the slope, F, and intercept, L, on the t axis, were obtained each with a standard error of the estimate (Bliss, 1967). A standard error of the mean thickness of each diaphragm was also available (see **Methods**). Since there is a quantity term on each side of equation (6) the errors in determining the amount of radioactivity were assumed to cancel. The errors in the diffusion coefficients in free solution, D, for sucrose, decamethonium cation and iodide anion were not estimated (see below). A standard error of each estimate of k and α was therefore derived from the standard errors of the time lag (L), the slope (F) and the thickness (b) by accepted procedures. These errors of estimate of k were scattered in the range 4·0 to 18·5% and the errors for α were from 2·0 to 17·9%, although one atypical result was included for which both errors were about 30%.

Diffusion coefficients in free solution

Values were required for the diffusion coefficients D (in water at 36° C) of sucrose, iodide anion and decamethonium cation. Estimates have also been made of the diffusion coefficients of gallamine and tubocurarine cations, for the work to be described in a later paper (Brookes & Mackay, 1971). The values of D obtained

TABLE 1. *Diffusion coefficients in water at* 36° C

Substance or ion	Molecular or ionic weight	Limiting equivalent conductance (mhos)	Diffusion coefficient $cm^2s^{-1} \times 10^6$	Source
Sucrose	342·3	—	6·84	American Institute of Physics Handbook, 2nd ed., McGraw-Hill Book Co., Inc.
Iodide anion	—	94·0	25·9	Robinson & Stokes (1965).
Decamethonium cation	258·5	55·8	7·70	Conductance measurements (see text).
Gallamine cation	510·8	63·7	5·86	
Tubocurarine cation	624·8	44·0	6·07*	

* (but see text)

and the origin of each value are shown in Table 1. The diffusion coefficients of decamethonium, gallamine and tubocurarine were determined from their limiting equivalent conductances which were found experimentally by standard methods using a simple immersion cell and conductance bridge (Mullard Ltd., type E.7566). Some difficulty was experienced in measuring the low conductances of dilute solutions of tubocurarine and the accuracy of the estimated diffusion coefficient was therefore in doubt. The approximate rule which states that diffusion coefficient is inversely proportional to the square root of molecular weight (Thovert's rule) was used as a check on the experimentally determined coefficients. Using the diffusion coefficient of sucrose as a reference, the expected values for decamethonium, gallamine and tubocurarine cations were calculated to be $7{\cdot}87 \times 10^{-6}$, $5{\cdot}60 \times 10^{-6}$ and $5{\cdot}06 \times 10^{-6}$ cm^2s^{-1}, respectively. From Table 1 it can be seen that there is good agreement with the experimental values except in the case of tubocurarine for which the experimental diffusion coefficient is greater than would be expected on the basis of its ionic weight. From conductance measurements del Castillo & Katz (1957) estimated the diffusion coefficient of tubocurarine to be $3{\cdot}6 \times 10^{-6}$ cm^2s^{-1} at 18° C. If the quantity $T/D\eta$ is assumed to be constant (where T is the absolute temperature, D the diffusion coefficient and η the viscosity of water) then their experimental estimate of D at 18° C gives a predicted value of $5{\cdot}70 \times 10^{-6}$ cm^2s^{-1} at 36° C. It is not possible to decide which estimate of D for tubocurarine is the most reliable without the benefit of accurate experimental data but the value $5{\cdot}06 \times 10^{-6}$ cm^2s^{-1} seems the most reasonable.

Discussion

The extracellular space in the rat diaphragm tends to increase with time, even in thin diaphragms maintained *in vitro* under favourable conditions (Creese & Northover, 1961). The muscle fibres themselves do not seem to undergo a detectable change in volume (Creese, 1954 ; Creese, D'Silva & Hashish, 1955). In this context the present findings, that the thickness of the diaphragms increases with time and that the tortuosity of the diffusion channels through the extracellular compartment decreases greatly, are explicable at least on a qualitative basis. The evidence suggests that the changes taking place in the extracellular compartment were most rapid during the first hour or two after excision of the diaphragms, and were accelerated by stimulation at the very slow rate (four min^{-1}) used throughout this work. Although the diffusion parameters α and k vary with time these variations seem to have been too slow to cause any serious deviations from equation 1 (during any diffusion experiment) except for freshly excised diaphragms (see Fig. 3a and b).

The estimated tortuosity factor for diffusion of [^{14}C] sucrose fell from $4{\cdot}39 \pm 0{\cdot}38$ (S.E. of the mean) in muscles only briefly soaked in Krebs solution, to $2{\cdot}23 \pm 0{\cdot}18$ in muscles stimulated for about 6 hours. This corresponds to an approximately 4-fold increase in the apparent diffusion coefficient D' determined from the time lag. There was no consistent trend towards increase or decrease with time of the fractional available area α. Goodnight & Fatt (1961) have pointed out that dead-end space, made up of blind channels through which there is no flux during steady state diffusion, will prolong the transient phase of non-steady state diffusion through a porous medium. Thus, in the present work, differences in the values of k obtained from the time lag equation (5) could conceivably result from changes in the tortuosity of the diffusion channels or changes in the dead-end volume. It is also quite

possible that substitution of these values of k into the flux equation (6) in order to calculate α has led to overestimation of the fractional available area. If the observed constancy of α in the range 0·3–0·4 is real, this is presumably because the physical disposition of the surface layers of muscle fibres is relatively unaffected while degenerative changes proceed more rapidly in the interior of the muscle (Law, 1967).

Consideration must be given to the effect on the diaphragms of clamping before measurement of diffusion, although this cannot be reliably assessed. It is hard to imagine that this procedure would not hasten the degenerative changes which must be responsible for the decreasing values of k. On the other hand one is impressed by the sensitivity of the method to subtle changes in the treatment of the diaphragms prior to clamping.

The tortuosity factors for diffusion of [^{14}C] sucrose and [^{14}C-methyl] decamethonium (alone and in the presence of tubocurarine 10 μg/ml) through diaphragms which had been stimulated for about 45 min, were close to 3·0 and were not significantly different from one another. It is clear, therefore, that an uptake of the diffusing tracer which was confined to the end-plate region has not been detected as an apparent rise in k. However, the more general penetration of [^{131}I] iodide through the membrane of muscle fibres has produced an apparent increase of the mean k to $3{\cdot}76 \pm 0{\cdot}38$ (S.E. of mean) and an apparent reduction of the mean α to $0{\cdot}17 \pm 0{\cdot}03$ (S.E. of mean). The fall in α is much greater than can be accounted for by use of the large value of k in equation (6).

We are grateful to Professor D. R. Wood for helpful criticism of the first draft of this paper, and to the Medical Research Council and the Smith, Kline and French Foundation for financial support.

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(*Received March* 12, 1970)