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DIFFUSION OF ACETYLCHOLINE IN AGAR GELS AND IN THE ISOLATED RAT DIAPHRAGM

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In the course of experiments in which acetylcholine (ACh) released during nerve stimulation was collected from the isolated rat diaphragm (Krnjević & Mitchell, 1960), it became a matter of some importance to know how ACh is distributed within the diaphragm and at what rate it can be expected to diffuse out. The following experiments were therefore undertaken, and, as no information was available about the rate of diffusion of ACh in physiological solutions, estimations were also made of the diffusion coefficient of ACh in gels of Ringer–Locke solution and agar.

METHODS

Diffusion of ACh chloride in agar gels

The method used was derived from one described by Eggleton, Eggleton & Hill (1928) in which measurements were made of the amounts of substances diffusing out of an agar gel. In our first experiments the solution of ACh chloride and agar was poured into a boiling-tube (cross-section about 4 cm^2) and allowed to set, and the rate of diffusion of ACh into physiological saline determined exactly as in the experiments of Eggleton *et al.* (1928). The apparent coefficients of diffusion obtained in this way were unexpectedly high, and, for control, measurements of the diffusion of NaCl into water were made under the same conditions. These also showed very high apparent rates of diffusion (as much as 50 % higher than the values given in the International Critical Tables). Inspection of the diffusion tubes showed that this anomalous diffusion could be ascribed to a substantially greater (+ 10–20 %) area of diffusion at the curved surface of the gel than would be expected from the area of cross-section of the tube. The method was therefore modified as follows.

'Bacto-agar' (Difco Laboratories, Detroit, Michigan) was dissolved in hot Ringer–Locke solution so as to make a 0·5 % (w/v) solution. It was allowed to cool to about 40° C, at which temperature it remained liquid. ACh chloride was then added from a standard solution to give a final concentration in the agar solution of $5{\cdot}0 \times 10^{-5}$ M, the standard solution (5×10^{-3} M) being prepared from a known weight of acetylcholine chloride (Roche Products) dissolved in a solution of 0·1 M-NaH_2PO_4. The Ringer–Locke solution had the following composition (mM): Na^+ 150, K^+ 5·0, Ca^{2+} 2·0, Mg^{2+} 1·0, Cl^- 148, $H_2PO_4^-$ 1·0, HCO_3^- 12·0 and glucose 11, the ionic strength being 0·164.

Diffusion chamber. This consisted of two Perspex tubes. One, with an internal diameter of about 3 cm and length of about 5 cm, was closed at one end; the other (about 4 cm long) was open at both ends and fitted accurately over the open end of the first. Some 25 ml. of the warm solution of ACh and agar was poured carefully into the bottom tube so as to give

a very smooth and flat surface at the open end. When the agar gel had set and reached the desired temperature (by equilibration with a large volume of water in which the chamber was immersed), the upper tube was fitted on. 20·0 ml. portions of Ringer–Locke solution were pipetted into the upper compartment, in contact with the surface of the gel, and kept stirred by bubbles of moist compressed air. They were replaced at intervals so that the external ACh concentration never rose above 1–2 % of that in the agar gel. The amounts of ACh collected were assayed in the conventional manner on a frog rectus preparation, using for comparison dilute solutions of ACh prepared from the same standard. The accuracy of the method of assay was estimated by including ACh solutions of a known concentration in each batch of 'unknowns'. The ACh content of a series of ten such solutions was estimated with a coefficient of variation of $\pm 6{\cdot}2\,\%$. There was also a systematic tendency to underestimate the apparent content by an average of 2·5 %. With this modified method, an estimate of the rate of diffusion of NaCl came to within 5 % of the expected values.

Diffusion of ACh through diaphragm muscle

Rats weighing 300–400 g were anaesthetized with ether, and their entire diaphragms were then removed in one piece, still attached to the costal margin. After soaking in cold Ringer–Locke fluid, well aerated with 5 % CO_2 in O_2, the muscular portion of one half of the diaphragm was stretched lightly and tied over the smooth end of a hollow Perspex cylinder (internal diameter 1·28 cm). Excess tissue was trimmed off, and the cylinder, occluded by the disk of diaphragm muscle, was dipped into a small beaker containing 5·0 ml. of Ringer–Locke fluid kept agitated by bubbles of 5 % CO_2 in O_2. A solution of ACh chloride in Ringer–Locke fluid (which varied between 5×10^{-5} and 5×10^{-4} M) was pipetted into the lumen of the cylinder until the fluid level inside equalled that in the beaker outside. A similar gas mixture stirred the internal solution (volume about 1·5 ml.), which was replaced by fresh ACh solution every 2–5 min. The external solution was changed at intervals of 10–15 min, allowing a sufficient amount of ACh to diffuse through to give an adequate concentration for assay. To prevent destruction of ACh by cholinesterases in the tissue, eserine sulphate was added to the solutions ($1{\cdot}5 \times 10^{-5}$ M); alternatively, the rats before death were given intraperitoneal injections of di-isopropylphosphorofluoridate (DFP) in doses of 5 μmole/100 g body weight.

Estimation of thickness of diaphragms. 20 μ frozen sections of the muscles used in diffusion studies were cut and examined immediately, either unstained or after light colouring with haematoxylin and eosin.

ACh space within the diaphragm muscle. The diaphragms were removed from 300 g rats anaesthetized with ether and, after a preliminary 20–30 min period of soaking in cold Ringer–Locke solution well aerated with 5 % CO_2 in O_2, the muscles were dissected free from ribs and connective tissue, lightly blotted with filter paper and quickly weighed (usual weight 200–300 mg). They were allowed to soak in well-aerated and well-stirred Ringer–Locke fluid, containing 5×10^{-4} M ACh chloride, for periods of some 60–120 min; after this they were blotted with filter paper as before, and transferred to a beaker containing 5·0 ml. of well-stirred Ringer–Locke solution. 1-ml. samples of this solution were removed for assay after set intervals, the volume of Ringer–Locke fluid being maintained approximately constant by the addition of known amounts of fresh solution from time to time as required.

In the first experiments of this kind destruction of ACh was prevented by eserine sulphate ($1{\cdot}5 \times 10^{-5}$ M); in later experiments the rats were given an intraperitoneal injection of DFP (5 μmole/100 g body weight). A further modification in later experiments was that the diaphragms were not cut away from the ribs until *after* the period of soaking in ACh. A number of samples were treated with strong alkali (1/5th volume of N/3-NaOH) and then neutralized to exclude the possibility of a release of an interfering substance from the muscle.

RESULTS

Diffusion of ACh from a Ringer–Locke-agar gel into Ringer–Locke solution

When diffusion takes place from a semi-infinite source into a well-stirred solution in which the concentration is maintained at zero, the amount of substance diffusing out is proportional to the square root of time. It can be shown (Stefan, 1871; Crank, 1956) that the total quantity (Q) which has diffused out after time t should be

$$Q = 2AC\sqrt{(Dt/\pi)}, \tag{1}$$

where A is the area across which diffusion takes place, C the initial uniform concentration in the source, and D the appropriate diffusion coefficient.

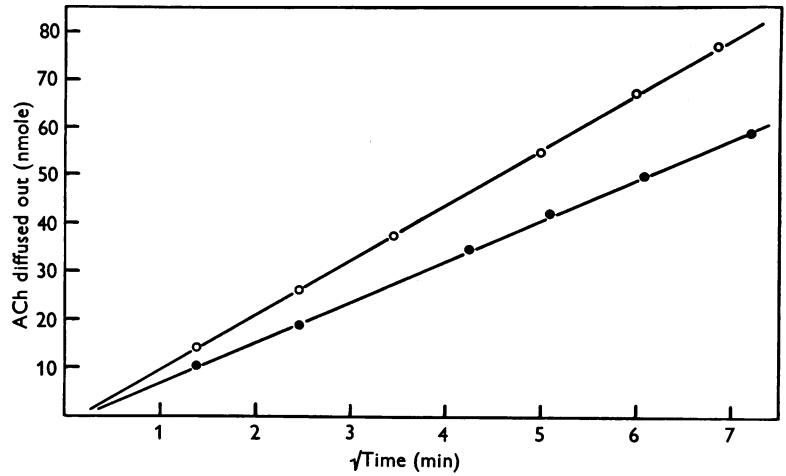

Fig. 1. Diffusion of ACh from a Ringer–Locke-agar gel into well-stirred Ringer–Locke solution. Ordinates indicate the total amount of ACh that had left the gel at a given time. Straight lines were drawn by eye, through points obtained during one experiment at 21° C (●) and another at 37·5° C (○).

In practice, finite systems behave as semi-infinite sources so long as the concentration does not fall appreciably below C at some distance from the boundary. For a system which is several centimetres deep, this condition would be expected to hold for many hours, but in any case it was advisable to find out whether the simple relation between Q and $\sqrt{t}$ really held before making further use of this equation.

We found in our experiments that plots of Q against $\sqrt{t}$ gave good straight lines in the time during which observations were made (up to 2 hr). Two examples of such graphs are shown in Fig. 1. There were small but systematic differences between observations made on different occasions,

no doubt because of unavoidable small variations in such factors as the area of diffusion, the concentration of ACh, and the temperature. In all cases, however, there was a linear relation between Q and $\sqrt{t}$, which justified the use of Equation (1) for determining the coefficient of diffusion (D); and the only feature worthy of comment is that, at room temperature, there was a consistent tendency for the straight lines to cut the abscissae a short distance on the positive side of zero (as in Fig. 1).

Diffusion at room temperature. In all, eleven experiments were performed at room temperature (20, range $\pm$ 1° C) using the same diffusion chamber (with a diffusion area of 6·80 cm^2), and the same concentration of acetylcholine chloride (5×10^{-5} M); from these experiments 58 pairs of values of Q and $\sqrt{t}$ were obtained. These values were all grouped together, and a regression coefficient was calculated for the regression of Q on $\sqrt{t}$. The line of best fit so obtained is described by Equation (2), where $\sqrt{t}$ is given in min$^{\frac{1}{2}}$ and Q in nmole:

$$Q = 9{\cdot}32\sqrt{t} - 3{\cdot}54, \tag{2}$$

the S.E. of the coefficient being $\pm$ 0·20. From this relation between Q and $\sqrt{t}$, the diffusion coefficient in Ringer–Locke solution was calculated to be $9{\cdot}8(\pm 0{\cdot}42) \times 10^{-6}$ cm^2/sec, for 20 $\pm$ 1° C.

Diffusion at 36·5° C. For comparison, four experiments were performed under similar conditions but at 'body' temperature, i.e. 36·5 $\pm$ 1·5° C. Twenty-one pairs of values of Q and $\sqrt{t}$ gave a line described by the following equation:

$$Q = 11{\cdot}44\sqrt{t} + 0{\cdot}95, \tag{3}$$

in which the regression coefficient had a S.E. of $\pm$ 0·36. The consequent value for the diffusion coefficient in Ringer–Locke solution at 36·5 $\pm$ 1·5° C was $14{\cdot}8\ (\pm 0{\cdot}93) \times 10^{-6}$ cm^2/sec.

Diffusion of ACh through the rat diaphragm

Four experiments were performed, special attention being paid to the steady-state conditions rather than to the kinetics of diffusion. The aim was to find the apparent coefficient of diffusion on the assumption that the entire surface of the diaphragm exposed to the ACh solution was available for diffusion. The coefficient was calculated from

$$Q = AD' \frac{dc}{dx}, \tag{4}$$

where Q is the amount of ACh diffusing per unit time, dc/dx the uniform gradient of concentration across the diaphragm, A the area of the muscle exposed (1·29 cm^2), and D' the apparent diffusion coefficient. The concentration of ACh in the solution on the upper surface of the muscle (C_0)

varied between 5×10^{-5} and 5×10^{-4} M in different experiments; the ACh concentration in the external solution, in contact with the lower surface, was not allowed to rise above 1–2 % of C_0. It was assumed that during steady-state diffusion the gradient of concentration was C_0/L, L being the thickness of the diaphragm measured in frozen sections (see below). Q was estimated by assaying samples of the external solution for ACh. Q appeared to reach a steady level within 30 min, and although there were small variations after that, they did not exceed 10–25 % over a further period of 1–3 hr. For calculating D', values of Q were averaged out over a period of at least 1 hr, beginning 30 min after the start of the experiment. The resulting values of D' ranged from 1·15 to $1{\cdot}6 \times 10^{-6}$ cm²/sec, with a mean of $1{\cdot}4 \times 10^{-6}$ cm²/sec (at a temperature of $20 \pm 1°$ C).

Thickness of rat diaphragms. Seventeen measurements were made of the thickness of diaphragms from six rats, all of body weight about 300 g. There was as much variation of thickness within one muscle as there was between different diaphragms. The mean thickness in frozen sections was 0·94 mm, with an S.D. of ± 0·036 mm.

Kinetics of diffusion of ACh from the diaphragm

The outward movement of ACh (at $20 \pm 1°$ C) from diaphragms previously equilibrated for 1–2 hr with 5×10^{-4} M ACh is shown on a semi-log. scale in Figs. 2 and 3. The four muscles of Fig. 3 had been cut from the ribs before soaking in ACh, whereas the five muscles of Fig. 2 had not. In both groups a straight line was drawn through the later, slow phase of outward movement and extrapolated to zero time. It seems that a certain proportion of the ACh in the muscle was unable to diffuse freely, either because it had entered muscle fibres, or because it was bound to some tissue component. In muscles not cut away from the ribs before soaking in ACh, the fraction of 'slow' ACh amounted to about 0·15; in those muscles which were cut away from the ribs the fraction was about 0·38. The initial phase of quick outward movement of ACh is also shown in each figure on an expanded time scale. These points were determined by subtraction of the slow component, and a theoretical diffusion curve for a plane sheet was fitted to the points of Fig. 2 by using appropriate values of Dt/l^2 (where $2l$ is the thickness of the tissue) given by Hill (1928, Fig. 5). The same curve was also drawn through the fast points in Fig. 3; although it fitted only approximately, this was about as good a fit as could be obtained (for any value of D/l^2) with the diffusion equation for a plane sheet, presumably because of the distortion caused by diffusion from the cut end of the fibres. It may be noted that one half of the 'free' ACh was lost in about 1·5 min, and that the later, straight portion of this diffusion curve had a half-time of 2·4 min. If one takes the value of $9{\cdot}8 \times 10^{-6}$ cm²/sec for D, which was

obtained as already described, the apparent value of l (the diffusion distance) is 1·4 mm.

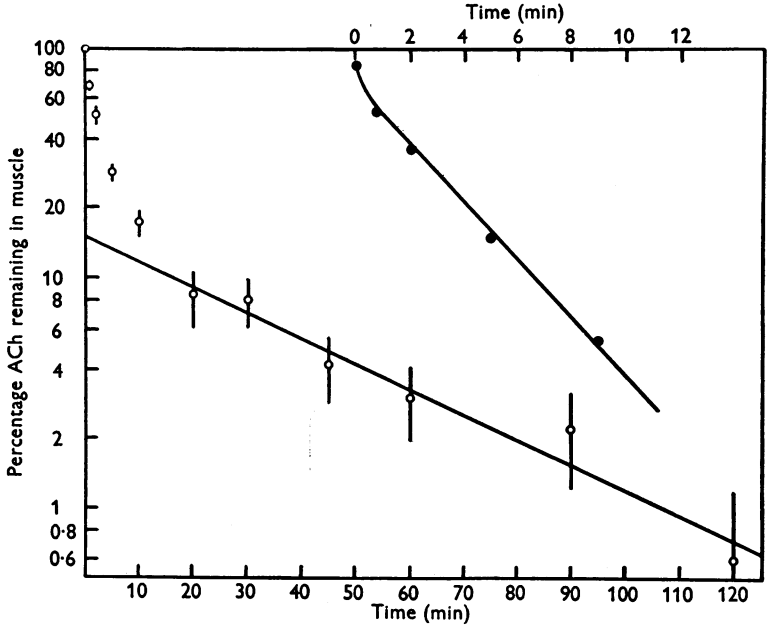

Fig. 2. Rate of loss of ACh from rat diaphragms after prolonged soaking in $5–10^{-4}$ M ACh. Each point (○) is mean of 5 observations and vertical lines show $\pm 1 \times$ S.E. Straight line was drawn by eye through last six points. Fast initial component (●) was obtained by subtraction of straight line, and is shown on expanded time scale in upper right quadrant. A diffusion curve was fitted to these points by using value of 0·002 for D/l^2. Semi-log. scale.

ACh space inside the diaphragm

After soaking diaphragms in 5×10^{-4} M ACh chloride for 1–2 hr at about 20° C, the ACh diffused out into an ACh-free solution as shown above. In our initial experiments we relied on eserine to prevent destruction of ACh, but found that an appreciable amount of ACh was being lost. We therefore injected DFP into the rats before removing the diaphragms, and also added eserine to the soaking solution; this prevented any apparent loss of ACh in subsequent experiments.

From the total amount of ACh which diffused out of these muscles, it is clear that added ACh is not distributed freely throughout the muscle. In five diaphragms which remained attached to the ribs while soaking in ACh the mean ACh space was 29·0 (S.E. $\pm$ 2·4) ml./100 g muscle. In four muscles cut away from the ribs before soaking in ACh the mean ACh space was substantially greater, 37·7 $\pm$ 0·8 ml./100 g.

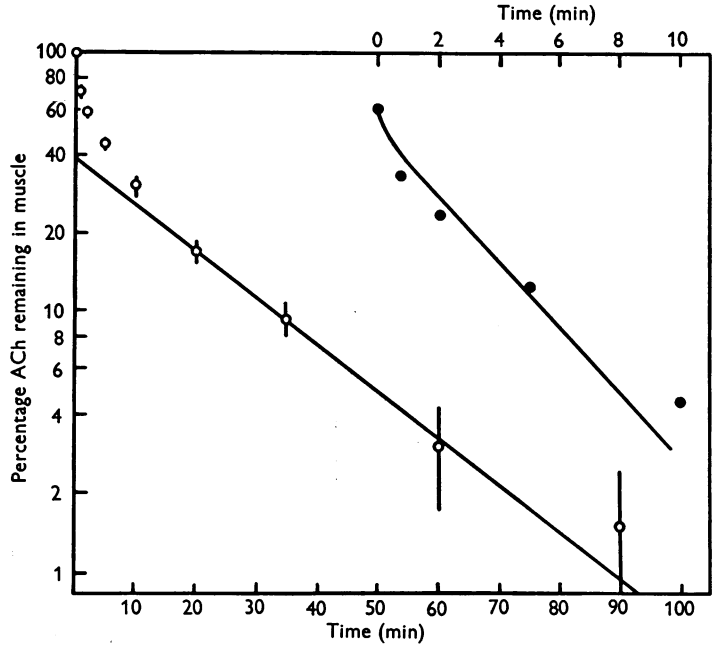

Fig. 3. Rate of loss of ACh from rat diaphragms cut away from rib margin before prolonged soaking in 5×10^{-4} M ACh. Each point (○) is mean of 4 observations with $\pm 1 \times$ S.E. Fast initial component (●), obtained by subtraction, is shown on expanded time scale, with diffusion curve calculated by using $D/l^2 = 0{\cdot}002$. Semi-log. scale.

DISCUSSION

Diffusion of ACh in agar gels

There is good reason to believe that dilute agar gels do not slow down diffusion appreciably. Stiles & Adair (1921), studying the penetration of NaCl into 0·5 % agar gels, found no evidence of slowing caused by the agar particles. In fact, diffusion seemed to be slightly faster than the reported diffusion of NaCl in pure water; but, as they pointed out, this difference was probably illusory, arising from uncertainty about the real concentration of Na and Cl ions in the region of diffusion in the two cases.

The relatively high value for D which we have obtained ($9{\cdot}8 \times 10^{-6}$ cm^2/sec) provides further reason for believing that diffusion was not appreciably slower in our experiments than it would have been in the absence of agar. Fatt (1954) has calculated a value for D of 8×10^{-6} cm^2/sec from an estimate of the limiting equivalent conductance of ACh^+ at 18° C. At the ionic strength of Ringer–Locke solution one would expect this figure to be reduced by approximately 10 %. There seems to be no obvious explanation why our figure is somewhat higher than this. It is unlikely that Cl^- could

much accelerate the movement of ACh^+ in the presence of relatively high concentrations of other ions, which would prevent the generation of any appreciable diffusion potentials.

The temperature coefficient of D between 20 and 36·5° C was about 2 % per degree, which is of the usual order of magnitude.

A slightly puzzling feature of the diffusion experiments at room temperature was that the straight line which could regularly be drawn through graphs of Q against $\sqrt{t}$ rather consistently tended to cross the abscissae at some positive value of t. Reference to Equation (2), which describes the combined results, shows that at zero time Q was not zero but $-3{\cdot}54$ (with a S.E. of $\pm 1{\cdot}31$); the probability of obtaining such a large deviation from zero by chance is only 0·01 (experiments at $36{\cdot}5 \pm 1{\cdot}5$° C did not show a significant initial delay of diffusion; the value of Q for zero time was $0{\cdot}95 \pm 1{\cdot}11$).

It is of some interest that a similar delay at the beginning of diffusion was observed by Stiles (1920), whilst studying the penetration of NaCl into an agar gel. It seems that the surface of a gel exposed to the air has a somewhat reduced permeability, which slows initial diffusion when a solution is poured on to the gel.

Outward diffusion of ACh from the diaphragm

Not all the ACh which entered the muscle diffused out freely. A small fraction (some 10–20 % of the total) only left the muscle with a half-time of about 30 min (Fig. 2). The remainder, however, moved out at a rate consistent with free diffusion in a system 50 % thicker than the diaphragms (1·4:0·94 mm). This would be in agreement with the possibility that ACh in the main does not enter the muscle fibres and that the fast component of outward movement represents extracellular diffusion slowed by closely packed impermeable cylinders. Theoretical considerations would lead one to expect the diffusion path to be lengthened by $\frac{1}{2}\pi$. Such good agreement with expectation is likely to be fortuitous; previous authors have found the apparent extracellular diffusion path in muscle of ions such as Na^+ increased by substantially more than $\frac{1}{2}\pi$ (Harris & Burn, 1949; Keynes, 1954; Creese, 1954), probably because other factors also interfere with free diffusion of ions in muscle. The rate of diffusion of ACh during the rapid phase of outward movement was 1·7 and 3·0 times faster than the outward diffusion of ^{131}I-labelled decamethonium and curare, respectively, observed by Creese, Taylor & Tilton (1959) in the rat diaphragm at 38° C.

It can be concluded that at least 4/5 of the ACh added to a diaphragm diffuses out quickly, but it may, nevertheless, take some 7 min for the extracellular ACh to be reduced to 1/10 of the initial amount. This may be of some significance for the function of muscle, particularly when the de-

struction of ACh liberated during activity is prevented by saturation of cholinesterases or through the action of drugs (cf. the eserine 'slow wave' of Eccles, Katz & Kuffler, 1942). Furthermore, even if ACh is destroyed by hydrolysis, there will be a temporary accumulation of choline which may have some physiological action (Hutter, 1952; del Castillo & Katz, 1957).

Diffusion of ACh through the diaphragm

The very low diffusion coefficient of ACh calculated from its movement through the diaphragm ($1{\cdot}4 \times 10^{-6}$ cm^2/sec) also suggests that ACh was unable to diffuse freely through the muscle fibres. If we assume that the diffusion path through the full thickness of the diaphragm was increased by 50 %, the apparent value of D becomes $2{\cdot}1 \times 10^{-6}$ cm^2/sec. It would then follow that the area available for diffusion was only about 0·21 of the total area.

Distribution of ACh in the diaphragm

Our results show that the ACh recoverable from muscles not separated from their ribs, after prolonged soaking, corresponds to a total ACh space of 29 ml./100 g of intact muscle. It is difficult to separate the diaphragm from its insertion into the costal margin without cutting the muscle fibres; this procedure usually causes the fibres to twitch and, when followed by soaking in ACh, results in a higher value for the ACh space, 37·7 ml./100 g (cf. also the very different estimates of the inulin space in the diaphragm made by Creese, 1954, and Pappius & Elliott, 1956). Extrapolating the slow phase of outward movement to zero time has shown that about 85 % of the ACh is free to diffuse out from intact muscles. The extracellular or 'free' ACh space in the diaphragm is therefore $0{\cdot}85 \times 29$ or about 25 ml./100 g of muscle, in excellent agreement with the mean value of 26·4 ml./100 g obtained by Creese (1954) for the inulin space in the isolated diaphragm. It may be objected that it is not justifiable to extrapolate in this way to zero time, because the rate of movement from the slow component should initially be much reduced by the high extracellular level of ACh (cf. Keynes, 1954, and Dainty & Krnjević, 1955). Inspection of the results suggests that a value of about 12 % (instead of 15 %) might be a better approximation to the true slow fraction of ACh, but the scatter of data hardly justifies a more detailed analysis. In any case the exact value of the slow fraction is not at all critical; it could be anywhere between 0 and 25 % without materially affecting the conclusion that the free ACh space agrees quite well with the inulin space.

The belief that ACh remains mostly outside the cells is thus supported both by the slowness of its diffusion through the diaphragm, and its apparent distribution within it. This is, of course, not wholly unexpected; direct measurements of the entry of labelled ACh into the squid giant axon

have shown only extremely slow penetration (Rothenberg, Sprinson & Nachmansohn, 1948), and the demonstration by del Castillo & Katz (1955) that ACh depolarizes the end-plate membrane only if applied to the outside, and not the inside, of the muscle fibre is not consistent with a high degree of permeability.

'Slow' ACh

Few definite statements can be made about the nature of the slow component of ACh diffusion in the diaphragm, except that it is extremely unlikely to be ACh released spontaneously by the nerve endings. In the diffusion experiments over a period of 1–2 hr the slow component corresponded to 50–70 nmole of ACh; spontaneous release would not be expected to amount to much more than 0·1 nmole in that time (Krnjević & Mitchell, 1960), and even maximal activation of the phrenic nerve would release less than 2 nmole in 2 hr (Straughan, 1960). It seems probable that the slow component represents some penetration of the muscle fibres, but we have not excluded the possibility that it may be caused at least partly by binding of ACh to some extracellular tissue component, or even to the surface of the fibres, perhaps at the receptor sites.

SUMMARY

1. The rate of movement of ACh from a Ringer–Locke-agar gel into well-stirred Ringer–Locke solution corresponds to a diffusion coefficient of 9·8 (S.E. $\pm$ 0·42) $\times 10^{-6}$ cm^2/sec at 20 $\pm$ 1° C.

2. Under steady-state conditions the diffusion of ACh across the rat diaphragm, from a solution containing a high concentration to one containing virtually none, occurs at a rate which is only 1/7 of that expected from diffusion in agar gels.

3. After soaking the rat diaphragm in solutions of ACh (5×10^{-4} M) for 1–2 hr, 80–90 % of the ACh in the muscle ('free ACh') escapes at the rate expected from simple diffusion; the remaining 10–20 % leaves much more slowly with a half-time of about 30 min.

4. The total ACh space in the soaked muscles is 29 (S.E. $\pm$ 2·4) ml./100 g; the 'free ACh' space is about 25 ml./100 g and agrees well with previous estimates of the inulin space.

5. It is concluded that all but a small proportion of the added ACh remains in the extracellular space of the muscle; the rest may enter the fibres or may be 'bound' to some component of the tissue.

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