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THE RELEASE OF HISTAMINE BY D-TUBOCURARINE FROM THE ISOLATED DIAPHRAGM OF THE RAT

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Alam, Anrep, Barsoum, Talaat & Wieninger (1939) have shown that, when the dog's gastrocnemius is perfused with defibrinated blood, injections of curare into the perfusion fluid cause a release of histamine from the muscle. These findings have been confirmed by Schild & Gregory (1947).

The present communication deals with a simple diffusion method by means of which the release of histamine from striated muscle by D-tubocurarine may be demonstrated and studied quantitatively. When the rat's diaphragm, which has a relatively high histamine content, is placed in Ringer's solution containing an adequate amount of curarine, most of the histamine contained in the muscle becomes diffusible and is released into the surrounding fluid. It will be shown that, above a certain concentration of curarine, the rate at which histamine diffuses from the muscle becomes constant and is presumably only limited by the rate of diffusion of histamine through muscle. On this assumption a 'diffusion constant' of histamine through muscle has been calculated and its magnitude has been found to be of the same order as that of urea as determined by Conway & Fitzgerald (1942).

METHODS

The diffusion method. Rats weighing 200–400 g. were killed by a blow on the head and bled. The diaphragm was then dissected out, washed in cold Tyrode solution to remove traces of blood, cleaned of connective tissue and divided. Usually the two lateral portions of the diaphragm, which are similar in thickness and shape, were used as control pairs. Strips of muscle weighing 150–300 mg. were attached to platinum hooks fused into the tip of capillary glass tubes, transferred to warm Tyrode solution, and thence into the experimental solution containing D-tubocurarine dissolved in Tyrode solution at 37°, and stirred by a stream of oxygen. After a measured time the muscle was removed from the solution which was then ready for assay. The muscle may be placed in further experimental solutions until no more histamine is released. At the end of the experiment the histamine remaining in the muscle may be extracted with trichloracetic acid (Code, 1937) or by boiling with saline (Feldberg & Kellaway, 1937).

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The volume of experimental solution was usually 2–5 c.c. and the ratio of fluid to muscle weight of the order of 10 to 1. The mean thickness of the diaphragm muscle was 0·75 mm. as computed from weight and surface. The latter was estimated by drawing the contour of the flat muscle on a piece of paper and weighing the paper.

Histamine assays. The solutions were assayed without further treatment. Histamine determinations were done on the guinea-pig's ileum using a statistical method (Schild, 1942). A small organ bath of about 4 c.c. volume was used. Histamine values are expressed in terms of histamine acid phosphate/3.

Effect of curarine on histamine assay. The presence of D-tubocurarine in the solutions slightly modifies the histamine response of the guinea-pig's ileum. D-Tubocurarine itself has no effect, but it often slightly potentiates the response to a given dose of histamine. Fig. 1. shows that the addition of 0·8 mg. D-tubocurarine to 0·02 μg. histamine slightly increases the response. At other times, when larger doses were used, or when the preparation was fatigued, curarine slightly depressed the histamine response. In order to obviate this interference, the same amount of curarine as was present in the test solution was added as a rule to the histamine standards. We are indebted to the Wellcome Foundation for a supply of crystalline D-tubocurarine, which was readily soluble in Tyrode solution.

Effect of benadryl and atropine. A concentration of benadryl of 1:100 million inhibited the effect of the active substance released by D-tubocurarine quantitatively to the same extent as that of histamine (Fig. 2A). This suggests that the released substance is either histamine or closely related to it (Schild, 1947). Atropine in a concentration sufficient to abolish the effect of acetylcholine produced a barely perceptible depression of the effects of the released substance and of histamine (Fig. 2B).

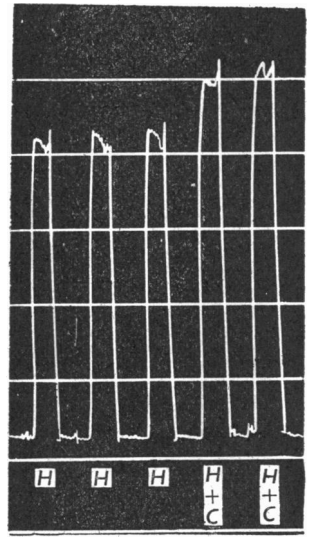

Fig. 1. Potentiation of histamine effect by D-tubocurarine. Contraction of guinea-pig ileum. H = histamine alone (0·02 μg.); $H + C$ = same dose of histamine given together with 0·8 mg. D-tubocurarine.

A typical experiment is shown in Fig. 3. In this experiment the muscle was left for nine successive periods of 5 min. in solutions of 1:1000 D-tubocurarine, and a total of 13·7 μg. histamine per g. of tissue was released. In control experiments of the same duration the total amount of histamine diffusing from the muscle was of the order of 0·4 μg./g. of tissue (less than one-thirtieth). Similar changes of the bathing solution at frequent intervals were practised in the majority of the longer experiments in order to maintain the full diffusion gradient of histamine.

RESULTS

Histamine release and concentration of curarine

The concentrations of curarine which produce a release of histamine are very much higher than those required for curarization at the neuromuscular junction. This point will be discussed in detail in a subsequent paper. It is necessary to distinguish between the effect of concentration of curarine on the *rate* of histamine release and on the *total amount* released until liberation is completed. The effect on the total amount is difficult to determine since the experiments cannot be indefinitely prolonged, the effect on rate, however, is very pronounced. An example of this is shown in Fig. 4 which represents two parallel experiments in which concentrations of 1:1000 and 1:4000 curarine were used.

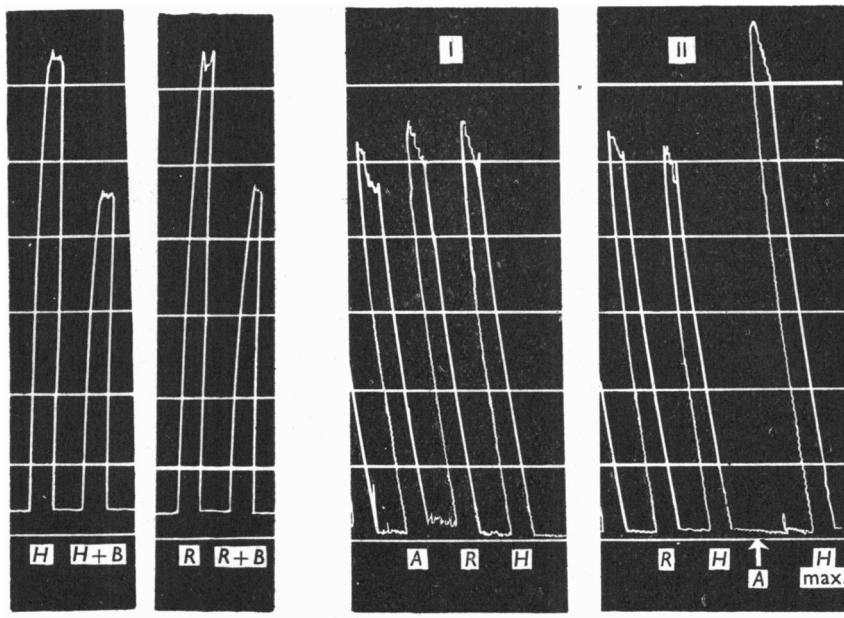

Fig. 2 A. Fig. 2 B.

Fig. 2A. Effect of (1:100 million) benadryl (*B*) on contraction produced by histamine (*H*) and substance (*R*) released by curarine. B. Effect of (1:5 million) atropine on released substance (R), histamine (H) and acetylcholine (*A*). Between I and II atropine was added to the Tyrode solution.

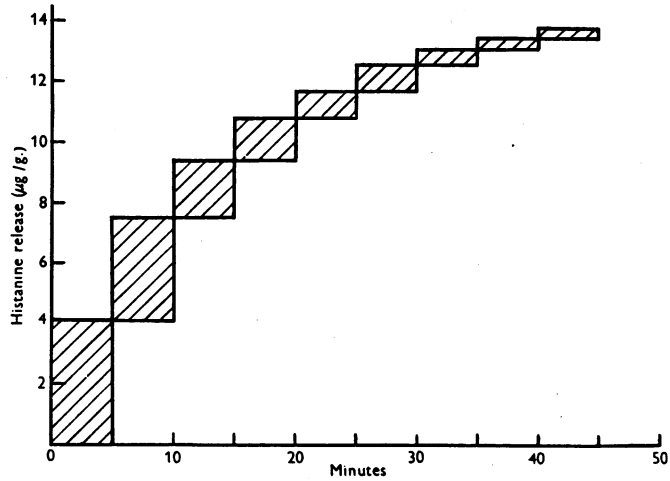

Fig. 3. Total histamine released from a diaphragm which was left during successive periods of 5 min. in solutions of D-tubocurarine (1:1000) in Tyrode solution.

In each case 0·5 g. of muscle tissue was left in 10 c.c. of solution, of which small samples were withdrawn periodically for assay. The difference is mainly one of rate and not of final amount released, e.g. the relative activity of the two solutions is 1:5 after 10 min. but only 1:1·2 after 90 min.

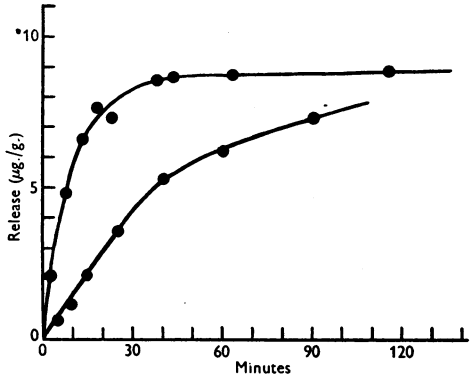

Fig. 4. Rates of release of histamine with concentrations of 1:1000 (upper curve) and 1:4000 D-tubocurarine (lower curve).

The initial rate of release. The simplest way of determining the effect of curare concentration on the rate of histamine release is to measure the initial rates. Fig. 4 shows that the time-release curves are exponential, but that during the initial stages they are approximately linear. Our main object was to find out whether the rate attained a maximum or whether it would continue to increase with increasing concentrations of curarine. Table 1 shows that the initial rates

TABLE 1. Initial rates of histamine release

Average of two experimental periods of 3 and 5 min. duration

Conc. D-tubocurarine (mg./c.c.) ...	Nil	0·25	0·5	0·75	1·0	2·0	3·0	4·0
Histamine released (μg./g.) ...	0·1	0·35	1·7	2·2	3·7	4·8	3·5	5·0

increase rapidly within the range of concentrations of 0·25–1·0 mg./c.c. curarine, but that above this range they become approximately constant. Since the greatest amount released in these experiments is less than half the amount that would be released over longer periods, it follows that the rate of release attains a limiting value beyond which a further increase in the concentration of curarine produces little or no effect. The probable reasons for this limitation are discussed in the next section.

Histamine release as a diffusion process

The release of histamine from the isolated diaphragm by curarine is a complex process, the rate of which presumably depends on many factors. Two obvious limiting factors must be the rates of diffusion of curarine and histamine through muscle. Although curarine presumably diffuses more slowly than histamine,

since it is a larger molecule, yet the time taken up by its diffusion may be rendered negligible by the simple device of increasing its concentration in the surrounding fluid. The greater the concentration in the surrounding fluid the less time is needed to build up an adequate concentration at the site of action. The time for diffusion of histamine cannot in the same way be made negligible,

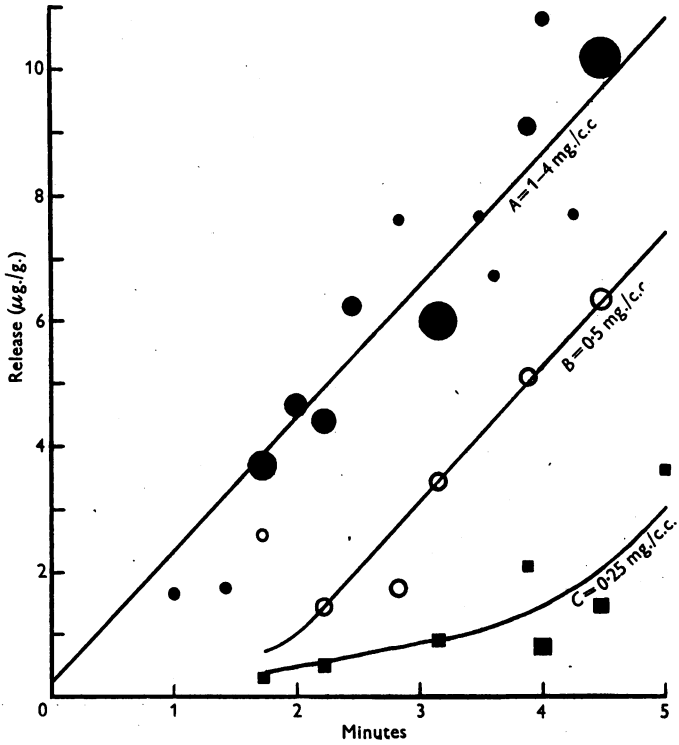

Fig. 5. Summary of 106 individual measurements of histamine release by curarine. Large circles represent mean values, the area of each circle being proportional to the number of observations. Black circles: histamine release with concentrations of curarine of 1 mg./c.c. or more; white circles: 0·5 mg./c.c.; squares: 0·25 mg./c.c. The slope of line *A* has been used for the calculation of the diffusion constant of histamine. The root mean square deviation of individual points from the regression line *A* is $\pm 2{\cdot}5$ μg./g.

it must always remain a limiting factor, and we have assumed that when the concentration of curarine in the surrounding fluid is sufficiently high, histamine diffusion becomes the main release rate determining factor. This is borne out by the results shown in Fig. 5.

Fig. 5 shows the quantities of histamine released by various concentrations of curarine plotted against the square root of time. In a simple diffusion process the points should lie along a straight line passing through the origin. Regression line *A* is the mean square regression calculated from seventy-six original ob-

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servations on histamine release using concentrations of curarine of 1 mg./c.c. or more. It will be seen that the regression line passes almost exactly through the origin and that the experimental points lie along a straight line. Lines *B* and *C* drawn through points corresponding to lower concentrations of curarine do not pass through the origin and have a complex shape which need not be discussed here. These results suggest that, provided the concentration of curarine is sufficiently high, histamine release can be treated as a simple diffusion process starting practically at zero time, the rate of which is determined by the rate of diffusion of histamine through muscle. An apparent diffusion coefficient of histamine through muscle may thus be calculated from the slope of regression line *A*. For this purpose, however, it is necessary first to express diffusion in terms of diffusing surface rather than in terms of weight, and to determine the diffusion head of histamine, or the concentration of diffusible histamine within the muscle.

The cencentration of diffusible histamine in the muscle

Histamine is presumably held within the cell in an indiffusible form. The clearest proof of this is found in some experiments by one of us (H.O.S., unpublished), which show that histamine when added to muscle permeates freely through tissue water. If intracellular histamine were freely dissolved in the cell water it would probably diffuse out just as easily. Bound histamine may be localized in certain parts of the cell, but when it is released it may be assumed to acquire rapidly a uniform concentration throughout the muscle. This assumption is based on the view discussed in detail by Conway & Kane (1934) that muscle can be regarded as a uniform diffusion mass and that diffusion is conducted through the tissue elements and not along the interspaces. Otherwise histamine might conceivably diffuse into the surrounding tissue space from a high concentration head situated near the cell surface without ever attaining equilibrium within the fibres.

The quantity of histamine that can be released by curarine need not necessarily be the same as the quantity that can be extracted by chemical procedures which destroy the cell structure, it might conceivably be either more (through formation of histamine) or less. Table 2 shows that, in fact, the maximum quantity that is released by curarine is always less than the total that can be extracted from a control muscle. In this respect curarine seems to differ from ammonia which releases all histamine present in the cell (Schild, 1949). In some of these experiments the muscles were left in curare solution for periods up to 3 hr. and then extracted. In all cases an appreciable amount of histamine could be extracted at the end of the experiments, averaging 35% of the original amount present in the controls or 30% if only the experiments of longest duration are considered. The quantities released and left behind add up approximately to the original activity.

It may be argued that insufficient time was allowed for complete diffusion. It can be calculated, however, that given the initial diffusion rate (and a simple diffusion process) over 99% should have been released at the end of 3 hr. It can be concluded therefore that only about 70% of the total histamine present in muscle is released by curarine.

The apparent diffusion coefficient of histamine through muscle

This may now be estimated from the equation

$$P = 2c_0\sqrt{\frac{kt}{\pi}},$$

where P is the number of μg. of histamine diffusing across 1 cm.^{2} of the outer surface of the muscle, c_0 the initial concentration of diffusible histamine in μg./c.c., k the diffusion coefficient (cm.^{2}/min.) and t the time of diffusion in minutes. Since the various constants were not determined in each individual experiment the following average values have been assumed:

Initial concentration of diffusible histamine (c_0) 11·7 μg./g.

This value was obtained from Table 2 assuming 70% of the average histamine content of 16·7 μg./g. to be diffusible. This average may be too low owing to the

TABLE 2. Histamine content of rat's diaphragm before and after exposure to D-tubocurarine

Conc. D-tubocurarine (mg./c.c.)	Duration exp. (min.)	Histamine		
		Release (μg./g.)	Extract end of exp. (μg./g.)	Extract control (μg./g.)
2·0	20+10	11·6	8·0	—
Nil	0	—	—	15·9
Nil	20+10	—	—	24·0
2·0	20+10	7·0	8·7	—
Nil	0	—	—	24·2
2·0	30+90	11·2	4·7	—
Nil	120	—	—	14·5
8·0	30	10·5	9·1	—
Nil	30	—	—	17·0
Nil	60	—	—	14·0
4·0	30+30	—	3·9	—
2·0	30+30	—	5·8	—
1·0	30+30	—	7·5	—
Nil	—	—	—	19·3
Nil	—	—	—	18·3
1·0	90+90	13·5	4·9	—
Nill	0	—	—	14·8
1·0	90+90	9·3	2·6	—
Nil	0	—	—	11·0
1·0	90+90	11·9	4·4	—
Nil	0	—	—	15·6
1·0	90+90	—	4·1	—
Nil	0	—	—	11·6
		Means		
		10·7	5·8	16·7
		16·5		

inclusion of a rather atypical set of experiments with small rats (last group of Table 2). If these are omitted the average histamine content becomes 18·4 μg. and $c_0 = 12{\cdot}8$. This value would account better for the continuing linearity of regression line *A* in Fig. 5, since the highest values would still be less than 80% of the maximum.

Ratio of surface to weight: this was determined directly in a number of diaphragms, the average ratio being 27·2:1.

Ratio $P/\sqrt{t}$: this was obtained from regression line *A* (Fig. 5) after substituting corresponding surface for weight.

Substituting these values $K = 3{\cdot}9 \times 10^{-5}$ at 37°, or $3{\cdot}3 \times 10^{-5}$ using the larger value of c_0.

This may be compared with a diffusion coefficient of about 6×10^{-5} for urea for a diaphragm of the same thickness (Conway & Fitzgerald, 1942). Histamine thus seemingly diffuses through muscle about half as rapidly as urea.

The effect of temperature on the rate of release of histamine by curarine

The effect of three different temperatures on the rate of release is shown in Fig. 6. Each point is the mean of four observations. The results have again been plotted against the square root of time, in a manner appropriate to a diffusion process. The temperature coefficient for 10° of a diffusion process is of the order of 33%. The ratio of the slopes of two consecutive regression lines in Fig. 6 should therefore be approximately $\sqrt{1{\cdot}33} = 1{\cdot}15$. In fact, the ratio of slopes at 37 and at 27° is 1·19, but the ratio of 27 and 17° is 2·5. It is thus probable that within the range of temperatures of 27–37° diffusion is still the dominant factor and that the effect of temperature on diffusion can fully account for the slower histamine release at 27°. Below this range the effect of temperature appears to be much more complicated and may be due to an effect on the diffusion of curarine and/or the chemical process by which curarine releases histamine.

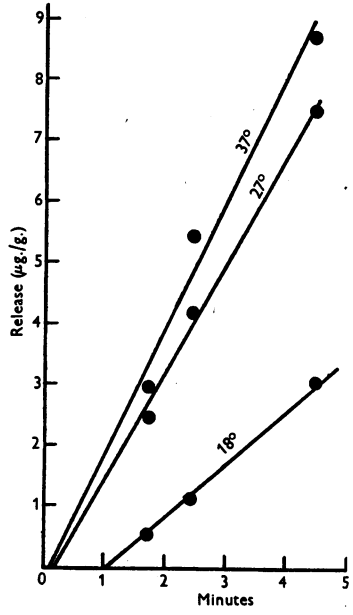

Fig. 6. Effect of temperature on rate of release of histamine (see text).

DISCUSSION

These experiments show that when rat's diaphragm is placed in a solution of curare approximately two-thirds of the histamine contained in it suddenly changes from an indiffusible into a diffusible state. It then diffuses out at a rate conforming to a simple diffusion process, the coefficient of diffusion being

about half that of urea. The rest of the histamine which may be contained in some histologically distinct structure is not released by curare, although it can be released by other substances such as ammonia.

The effect of curare on histamine release depends critically on the concentration; if this is reduced to a quarter of the optimal, the rate of release becomes many times slower, and at a tenth of the optimal, it is only just perceptibly greater than in the controls. It will be shown in a subsequent paper that an extremely slow steady release of histamine occurs even in Tyrode solution alone, and it is possible that the effect of curare is greatly to accelerate a normally occurring process.

Little can be said at this stage about the mechanism by which curare produces this effect. Rocha e Silva (1943) has shown that histamine can form peptide linkages with certain amino-acids and has suggested that these might be chemical models of the way histamine is bound within the cell. It would be tempting to assume that curare and other bases which release histamine (diamidines, MacIntosh & Paton, 1947) did so by displacing histamine from its cellular 'receptors'. There is at present no positive evidence to support this assumption, but it can be shown that when histamine is released by curarine the amount of curarine involved would not only be sufficient to replace all the histamine on a molecular basis but usually exceeds it by a factor of 10. We have performed a series of experiments in which curarine was injected into the perfusate of rat's hindlimbs perfused with Tyrode solution. In this type of experiment, repeated injections of curare cause a repeated release of histamine, and very large totals may eventually be released. Table 3 summarizes some of

TABLE 3. Perfusion of rat's hindlimbs with Tyrode solution
Quantities of histamine released by single injections of D-tubocurarine

	D-Tubocurarine injected (mg.)	Histamine released (μg.)	Mol. curarine / Mol. histamine
R1 1st injection	3	12·4	31
2nd injection	3	16·8	23
R2	3	18·9	20
R3	2	5·0	51
R5	6	23·4	33
R7	3	16·4	23
R9 1st injection	6	35·6	22
2nd injection	6	31·1	25

these data and shows in each case the molar ratio of curarine injected to histamine released. The most favourable ratios were obtained when 0·3 c.c. of D-tubocurarine (1:100) were injected into the artery, but even then the ratio of molecules of curarine injected to molecules of histamine released was 20 or more. Relations of similar magnitude are found when instead of quantities, concentrations are compared. For instance, the concentration of bound histamine within the cell is of the order of N/5000, but the optimal concentration of

curarine for histamine release is of the order of N/1000, a fivefold difference in favour of curarine. It may be concluded that chemical interaction between the two substances is feasible in view of the magnitudes involved, but that there is no positive evidence to suggest a stoichiometric relation between histamine released and curarine taken up. On the other hand, these results do not disprove a stoichiometric relationship, since the number of molecules of curarine taken up by the cell may be only a fraction of those injected.

As an alternative hypothesis, curarine may be producing 'tissue damage', or more concretely it may be activating certain enzymes which then release histamine. Gross histological damage at the neuromuscular junction following the injection of curare has in fact been described by Herrera (1936) and certain other less drastic but characteristic changes following the injection of curare have been described by Rojas, Szepsenwol & Resta (1939). On the other hand, McIntyre (1947) states that King & Willard were unable to find any significant change in the size of motor end-plates in rats after curarization with 1:1000 D-tubocurarine in Ringer solution. This may be a similar case to that of cholinesterase which was found to be inhibited by certain impure curare solutions but unaffected by pure D-tubocurarine (McIntyre & King, 1943). Dr R. E. Davies (Sheffield) has kindly tested for us the effect of D-tubocurarine on the oxygen consumption of the diaphragm using Warburg's technique. No decrease of tissue respiration was found in a solution of 1:1000 D-tubocurarine in Krebs Ringer. This excludes any gross interference with the respiratory functions of the cell. An interesting effect of curarine on potassium diffusion in frog's muscle was observed by McIntyre & King (1944) who found that tubocurarine (1:1000) approximately doubled the rate at which potassium diffuses from muscle.

SUMMARY

1. When the isolated diaphragm of a rat is placed in Tyrode solution containing 1:1000 D-tubocurarine at 37°, two-thirds of the histamine contained in the muscle diffuse rapidly into the surrounding fluid.

2. If the concentration of curarine is reduced the rate of release of histamine is greatly retarded, but if the concentration is increased the rate of release is not increased further.

3. A quantitative study of the rate of release suggests that, at the maximum rate, the liberation of histamine from diaphragm muscle can be treated as a simple diffusion process and reasons are given for assuming that the rate is determined by the rate of diffusion of histamine through muscle. On this assumption a 'diffusion coefficient' of histamine through muscle has been calculated. Its magnitude appears to be of the same order as that of urea.

4. The effect of temperature on the rate of release has been determined. The magnitude of the effect suggests that between 27 and 37° a simple diffusion process prevails.

5. Histamine is also released when rat's hindlimbs are perfused with Tyrode solution and curarine is injected into the perfusate. Under these conditions at least 20 molecules of D-tubocurarine are required to release 1 molecule of histamine.

6. Possible mechanisms by which curarine may cause a release of histamine are discussed.

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