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CHOLINE ESTERASE IN VOLUNTARY MUSCLE

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THE theory of chemical transmission of motor nerve impulses to voluntary muscle—suggested by Dale and his co-workers—encountered as a chief difficulty, that the chemical process involved must occur with a flashlike suddenness. The acetylcholine (ACh.) liberated at the nerve endings must be removed in the very brief limits of the refractory period [Brown *et al.* 1936]. The necessity for so great a rapidity is a main difference between the transmission of nerve impulses in quickly reacting cells like the fibres of striated muscle and the transmission from the autonomic system to the effector cells.

The problem has been attacked by measuring the rate of ACh. hydrolysis by choline esterase in voluntary muscle [Marnay & Nachmansohn, 1937*a*]. These experiments indicated that the concentration of the enzyme in muscle tissue is not high enough to remove during the refractory period such an amount of ACh. as may be liberated by a single motor nerve impulse. It was calculated that the time necessary for the hydrolysis of 50 p.c. of such an amount was many thousand times longer than the refractory period, at a concentration of the enzyme as found in muscle tissue. The conclusion was drawn that, if the disappearance of ACh. at the end-plates during the refractory period was due to the activity of the esterase, a concentration of the enzyme at the nerve endings is necessary which is many thousand times as high as that found in muscle tissue.

In this paper it will be shown that a high concentration at the end-plates, such as postulated in the previous paper, does in fact exist. The rapidity of the hydrolysis of ACh. is, therefore, as high as is required by the hypothesis that this substance is involved in the transmission of motor nerve impulses to voluntary muscle.

METHODS

In the previous experiments chopped muscle pieces or slices of tissue were put into Ringer solution containing acetylcholine. After a suitable time an aliquot amount of the suspension fluid was withdrawn and used for the manometric determination of ACh. Some of the experiments described in this paper were carried out by this method. In most of the experiments, however, the tissue was ground with quartz. The rate of hydrolysis can thus be determined directly by measuring the output of CO_2 liberated from the bicarbonate of the solution by the acetic acid formed. Under these conditions the output of CO_2 corresponds to the amount of ACh. hydrolysed, whereas in intact cells other metabolic processes interfere. The procedure was as follows: a weighed amount of muscle was thoroughly ground in 1 c.c. of Ringer solution with a small quantity of quartz in a small porcelain mortar. The suspension was transferred to a Warburg vessel, the mortar being washed twice with 1 c.c. of Ringer solution. The ACh. solution was run into the side bulb. After equilibrium had been attained in the thermostat, acetylcholine was tipped in. In the suspensions from some tissues—especially nerves—there sometimes occurs a slight uptake of CO_2 continuing for a long time (up to several hours) in the absence of ACh., so that a control vessel with tissue extract without ACh. is necessary.

If a solution or suspension in the manometric vessels contains substances binding acids, as proteins or secondary phosphates or other buffers, the output of CO_2 does not correspond exactly to the CO_2 or to the acids formed: a fraction of the acid is bound by the buffer substances [Warburg, 1925]. The amount of retained CO_2, the "retention", must be determined and a factor must be added to the normal constant of the vessel to obtain the real amount of acid formed.

Suspensions of ground muscle in Ringer solution contain such retaining substances. The retention varies a little for the different tissues and the different conditions as temperature, pH, etc. For frog's muscle at 19° the following values for $\left(\frac{\Delta u}{\Delta p}\right)_{\text{acid}}$ have been found if 100 mg. were ground up in 3 c.c. of Ringer solution: 0·035, 0·044, 0·038, 0·022 and 0·037; mean value 0·035. The factor is proportional to the weight of muscle: for 200 mg. ground in 3 c.c. of Ringer solution, the factor rises to the mean value of 0·079 (0·080, 0·093, 0·106, 0·076, 0·053 and 0·070 are the values found).

The Ringer solutions had the same composition as described in our previous paper. All experiments were carried out in nitrogen containing

5 p.c. CO_2. The initial concentration of ACh. was always 1 mg. per c.c. The frogs used were *Rana esculenta*.

Phosphates and phosphorylated substances were determined with the hydrolytic method of Lohmann [1928]. In these experiments the muscles were cut and immediately frozen in liquid air.

Results

Choline esterase in frog's sartorius. The nerveless pelvic end of frog's sartorius has often been used for investigations of the properties of muscle tissue [Lucas, 1907; Lucas & Mines, 1907; Lapicque, 1931; Rushton, 1931]. To secure evidence for an accumulation of choline esterase at the nerve endings, the enzymic activity of the nerve-free part was compared with that of the part containing nerve endings.

The amount of acetylcholine hydrolysed per unit of weight in 1 hr. by a quarter of the sartorius containing nerve endings is about threefold the corresponding quantity for the pelvic end [Marnay & Nachmansohn, 1937*b*]. As the volume of the end-plates constitutes only a very small fraction of the whole muscle, such an increase of the total hydrolytic power indicates a very high concentration of the enzyme in this small volume.

The difference of the concentration of enzyme between the nerveless part and that containing nerve endings can be shown in an even more striking manner. The distribution of the nerve endings in frog's sartorius has been studied recently in a very complete manner by Pézard & May [1937]. These authors found that only about an eighth of the pelvic end is really nerveless. About one-fifth contains practically no nerve endings. The second fifth is very rich in nerve endings and then the frequency decreases considerably. The second fifth contains about twice as many nerve endings as the third fifth.

The comparison of the time-course of hydrolysis between 1/8 to 1/6 of the pelvic end and the second and third fifth shows a plain parallelism between richness in nerve endings and rate of hydrolysis. The second fifth, containing the greatest number of nerve endings, hydrolyses acetylcholine about 6 times as quickly as the nerveless part, whereas the third fifth hydrolyses only about 3 times as quickly (see Fig. 1). Table I summarizes the results of four experiments, each made with the corresponding parts of four muscles. These experiments were carried out with ground tissue.

The rate of hydrolysis by the sciatic nerve is about six- to sevenfold that in the muscle (nerveless part). There is practically no difference

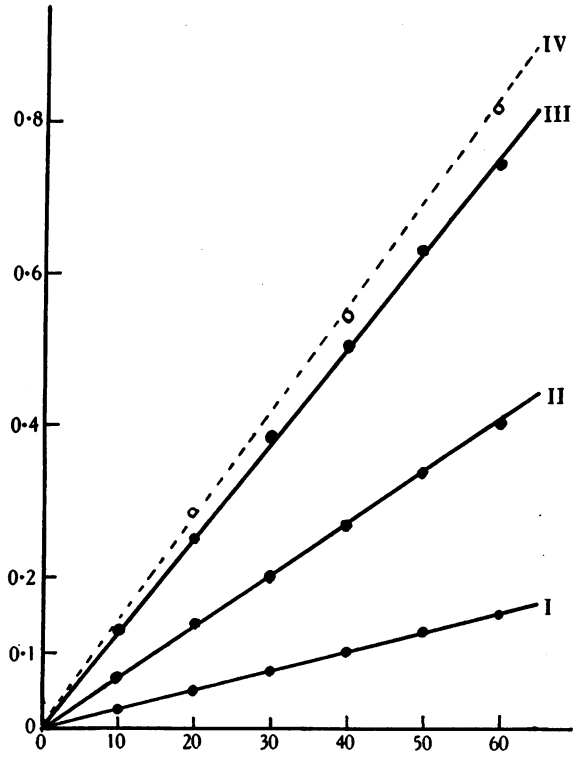

Fig. 1. Rate of hydrolysis of ACh. by different parts of frog's sartorius. Abscissa: time in min. Ordinate: mg. ACh. hydrolysed by 100 mg. of tissue.

I. Nerveless pelvic end.
II. Third fifth.
III. Second fifth.
IV. Sciatic nerve (broken line).

TABLE I. Choline esterase in frog's sartorius

	Nerveless end		Second fifth		Third fifth		Nerve (sciatic)	
No.	Muscle weight mg.	ACh. hydrolysed by 100 mg. in 60 min. mg.	Muscle weight mg.	ACh. hydrolysed by 100 mg. in 60 min. mg.	Muscle weight mg.	ACh. hydrolysed by 100 mg. in 60 min. mg.	Weight mg.	ACh. hydrolysed by 100 mg. in 60 min. mg.
1	97	0·152	114	0·745	137	0·402	54·5	0·893
2	83	0·135	111·5	0·850	132	0·432	56	0·705
3	90·5	0·131	98·5	0·890	102	0·485	66	0·807
4	102	0·123	121	0·695	134	0·298	38	0·868
5	—	—	—	—	—	—	36	0·816
Mean value		**0·135**		**0·795**		**0·404**		**0·818**

between the rate of hydrolysis by the nerve and the part rich in nerve endings. $\left(\frac{\Delta u}{\Delta p}\right)_{acid}$ for 100 mg. of nerve tissue ground in 3 c.c. of Ringer solution is the same as that for muscle tissue: 0·034 and 0·036 are the values found; mean value 0·035.

Diffusion through muscle tissue. In the previous paper it was shown that the time-course of hydrolysis of acetylcholine by intact frog's muscle of 400 mg. fresh weight does not differ considerably from chopped muscle pieces of about 30 mg. fresh weight. These experiments seemed to indicate that a diffusion of acetylcholine through muscle tissue occurs rather quickly.

The rate of hydrolysis by sartorius containing nerve endings is only a little higher than that by the nerve-free part of the muscle if small muscle pieces are used. Table II shows that the amount of acetylcholine

TABLE II. Difference of enzyme activity between ground tissue and muscle pieces

	(*a*) Pieces of about 30 mg. fresh weight		(*b*) Ground tissue	
No.	Weight mg.	ACh. hydrolysed by 100 mg. in 120 min. mg.	Weight mg.	ACh. hydrolysed by 100 mg. in 120 min. mg.
A. Nerveless end				
1	189	0·238	97	0·308
2	230	0·283	83	0·279
3	263	0·312	90·5	0·269
4	198	0·116	102	0·246*
5	255	0·169	127	0·260
6	152	0·210	—	—
7	165	0·266	—	—
8	224	0·139	—	—
9	200	0·195	—	—
	Mean value	**0·214**		**0·272**
B. Second fifth				
1	188	0·250	114	1·490†
2	157	0·344	111·5	1·700†
3	187	0·266	98·5	1·780†
4	—	—	121	1·390†
	Mean value	**0·285**		**1·590**

* Calculated from a value found for 60 min.
† Calculated from the values for 60 min. given in Table I.

hydrolysed by pieces rich in nerve endings (second fifth of sartorius) is only about 30 p.c. higher than by pieces of the nerve-free part (mean value 0·285 mg. ACh. hydrolysed by 100 mg. in 120 min. against 0·214

mg.). The difference appears however if the muscle pieces are ground: the amount hydrolysed by 100 mg. of nerve-free tissue rises only from 0·214 to 0·272 mg., whereas the amount hydrolysed by muscle containing nerve endings rises from 0·285 to 1·590 mg.

The difference in the rate of hydrolysis between ground muscle and muscle pieces of the nerve-free part indicates that the rate of hydrolysis is delayed by diffusion through muscle tissue. As the enzymic activity at the nerve endings is much higher than that in muscle tissue itself, the diffusion to these spots is probably not quick enough to saturate their enzyme with substrate and therefore the delay in the rate of hydrolysis brought about by diffusion is much greater in muscle containing nerve endings. If such muscles are ground, the enzyme of the nerve endings comes in close contact with the substrate and works now at the maximal rate. This may explain the big increase of the rate of hydrolysis.

The experiments with muscle pieces were carried out under exactly the same conditions as the experiments with ground muscle. The pieces were put into Warburg vessels containing 3 c.c. of Ringer solution. The ACh. concentration was the same (1 mg. per c.c.). The vessels were shaken during 2 hr. in the thermostat and then the ACh. that remained and the initial amount determined as previously described. The time of 2 hr. was chosen in order to obtain more exact results because the absolute amount hydrolysed after 1 hr. would not be great enough, as the initial amount (3 mg.) is comparatively high in view of the small quantity of muscle used.

Dog's gastrocnemius. It can also be shown with dog's gastrocnemius that the distribution of choline esterase in voluntary muscle is not even, but differs according to the distribution of the nerve endings. Small pieces of 30–50 mg. can be obtained in which no nerves can be found as can be controlled microscopically.[1] By following nerve ramifications as far as possible with a magnifying glass it is possible to obtain pieces containing nerve endings. Although the nerve endings constitute only a very small fraction of these pieces, the rate of hydrolysis is about twice as high as in the nerve-free pieces. This is shown in Table III. If the pieces are too big, as in No. 2*e*, the amount hydrolysed is only a little increased, the part played by the nerve endings becoming too small. On the other hand in one experiment (No. 3*c*) it was possible to obtain a very small piece containing nerve endings which yielded a particularly high rate of hydrolysis.

[1] We are much obliged to Dr Couteau from the Laboratoire de Biologie Expérimentale, who has carried out the microscopical examinations for these experiments.

TABLE III. Dog's gastrocnemius (ground with quartz)

No.	Part of muscle	Muscle weight mg.	ACh. hydrolysed by 100 mg. in 60 min. mg.
1 (*a*)	Nerveless	24	0·58
(*b*)	Nerveless	31	0·48
(*c*)	Containing nerve endings	20·5	1·05
2 (*a*)	Nerveless	34	0·34
(*b*)	Nerveless	42·5	0·36
(*c*)	Nerveless	45	0·36
(*d*)	Containing nerve endings	17	0·81
(*e*)	Containing nerve endings	87	0·47
3 (*a*)	Nerveless	30	0·49
(*b*)	Containing nerve endings	18	0·97
(*c*)	Containing nerve endings	6	1·35

The experiments were made with ground muscle at a temperature of 37°. The retention of CO_2 was determined with 50 mg. of muscle ground in 3 c.c. of Ringer solution. With this amount $\left(\frac{\Delta u}{\Delta p}\right)_{acid}$ is 0·044.

Phosphorylated substances in frog's sartorius. It seemed to be of interest to investigate if there are other substances which are not evenly distributed in frog's sartorius, or if this distribution is peculiar to choline esterase. The concentration of phosphates and phosphorylated substances has been determined, as these compounds have so many different functions in the glycolysis. Table IV shows that no differences

TABLE IV. Distribution of phosphorylated substances in frog's sartorius (mg. P_2O_5 per g. muscle)

No.	Weight g.	Phos-phagen	0 min.	7 min.	30 min.	Adenyl-pyro-phos-phate	180 min.	Total P_2O_5	Hexose-mono-phos-phate	Hexose-di-phos-phate
1 (*a*)	0·473	0·69	2·09	2·49	2·51	0·38	3·23	3·48	0·35	0·66
(*b*)	0·497	0·76	2·17	2·61	2·65	0·40	3·31	3·55	0·34	0·64
2 (*a*)	0·421	0·55	1·94	2·35	2·43	0·33	3·04	3·34	0·42	0·65
(*b*)	0·471	0·48	1·88	2·36	2·46	0·38	3·01	3·23	0·32	0·65
3 (*a*)	0·469	0·73	1·83	2·58	2·65	0·68	3·14	3·29	0·21	0·57
(*b*)	0·533	0·60	1·96	2·78	2·83	0·77	3·18	3·37	0·27	0·37

(*a*) Nerveless end. (*b*) Second fifth.

of concentration exist between the nerveless pelvic end and the second fifth particularly rich in nerve endings. The concentration of phosphagen is rather low and indicates that a fraction of the initial amount is hydrolysed, probably during the cutting of muscle as would be expected. The directly determined phosphate however, which is the sum of phosphagen phosphate and inorganic phosphate, is equal in the two parts (column

0 min.). A difference in the initial amounts of phosphagen is therefore not probable.

Lizard's muscle. The lizard is an animal capable of very quick movements, and its nerve endings, described by Rouget [1862] as "plaques terminales", are quite different from the nerve endings in frog's muscle. They resemble the end-plates in mammalian muscle and are even larger. In view of these special functional and structural properties it seemed to be of interest to know if there is a difference between the concentration of choline esterase in this muscle and that in other muscles.

The experiments were carried out with ground muscle tissue of the hindlimb and the tail of the green lizard (*Lacerta viridis*). The retention of CO_2 after grinding 100 mg. of these muscles in 3 c.c. of Ringer solution is the same as that of frog's muscle. $\left(\frac{\Delta u}{\Delta p}\right)_{acid}$ under these conditions was found to be 0·018, 0·037, 0·038; mean value 0·031 (for frog's muscle 0·035).

Table V gives the data obtained. The amount hydrolysed in 1 hr. by 100 mg. of muscle is indeed very high, 3–5 times that by frog's muscle and more than twice that by mammals at 37° (cat, dog and guinea-pig).

TABLE V. Lizard's muscle

No.	Part	Muscle weight mg.	ACh. hydrolysed by 100 mg. in 60 min. mg.
1	Tail and hind limb	41	1·60
		44	1·44
		57	1·35
2	Hind limb	42	2·62
		50	2·77
	Tail	47	2·74
		39	2·72
3	Hind limb	35	2·72
		44	2·83
4	Hind limb	47	3·40
		48	2·58
5	Hind limb	55	2·92

Whilst 100 mg. of frog's muscle split 0·4–0·6 mg. ACh. in 1 hr. at 18° and 100 mg. of guinea-pig's muscle 0·8–1·0 mg. at 37°, 100 mg. of lizard's muscle at 20–22° split 1·5–3·0 mg. ACh. in the same time.

Eserine completely inhibits the esterase in a concentration of $1:10^{-5}$ g. per c.c. of Ringer solution.

Discussion

The main result of these experiments is the fact that choline esterase is not uniformly distributed in muscle tissue but is concentrated at the nerve endings or in their neighbourhood. This conclusion is based chiefly upon the observation that 100 mg. of nerveless muscle tissue—frog's sartorius—split only 0·135 mg. of ACh. in 1 hr., whereas 100 mg. of the same muscle containing nerve endings split 0·4–0·8 mg. during the same time. It is improbable that the increased esterase activity is due to the presence of nerve fibres. The nerve fibre of the frog (sciatic) contains only 6 times as much esterase as the nerveless muscle tissue. Even if the volume of nerve fibres should constitute 2–3 p.c. of the total volume of the muscle (and probably the fraction is much smaller), the total amount of esterase of muscle would only be increased by 12–18 p.c. Actually the esterase activity of muscle with nerve endings is 300–600 p.c. that of the nerve-free part. It may be argued that the myelin sheath contains no enzyme at all and that therefore the concentration of the esterase in neuro-fibrils may be more than 6 times as high as that in muscle tissue, as the rate of hydrolysis was determined by grinding a part of the sciatic nerve enveloped by myelin. But even assuming 2 or 3 times the amount of esterase in the neuro-fibrils, the concentration would not be sufficient to explain the great difference between the nerve-free end and the part containing nerve endings. The improbability of such an interpretation is shown by the fact that there is no difference between the part rich in nerve endings and the sciatic nerve. If the great hydrolytic power of this part of the muscle were due to the neuro-fibrils, it would be necessary to assume that the fraction of volume taken by the neuro-fibrils in this part of the muscle and in the sciatic is approximately the same, and this is obviously impossible. It can therefore be concluded that there is a special accumulation of the enzyme between nerve and muscle, i.e. in the end-plates. This interpretation is emphasized by the striking parallelism between the hydrolytic power and the number of nerve endings.

It is impossible at present to give exact data of the fraction which the volume of end-plates constitutes in the whole organ. But let us assume that the fraction constitutes one-thousandth. An increase of the total esterase activity to 3–6 times, due to the concentration of enzyme in this fraction, would mean that at these spots the enzyme concentration must be increased 3000–6000 times. The nerve endings probably occupy less than 1/1000 of the total volume. Some estimations, although rough

and approximative, suggest that their volume is less than 1/5000. For this value a 3–6 times increased esterase activity would correspond to a concentration which is 15,000–30,000 times higher than in nerveless muscle tissue.

It is possible to calculate from the data given the approximate amount of ACh. which can be hydrolysed in the refractory period at the nerve endings of a single frog's sartorius of 300 mg. On the average 100 mg. of muscle containing nerve endings split at least about 0·4 mg. ACh. in 1 hr. 0·135 mg. is the amount hydrolysed by nerveless muscle tissue in this time. As the difference is essentially due to the esterase accumulated at the nerve endings, about 0·265 mg. ACh. can be hydrolysed there by 100 mg. muscle in 1 hr. This means that an amount of about 0·001 μg. ACh. can be hydrolysed at the nerve endings of a frog's sartorius of 300 mg. in 5 msec. (the refractory period). As about 600 nerve endings exist in a sartorius [Pézard & May, 1937] the amount which can be hydrolysed in a single motor nerve ending during the refractory period is about 0·000002μg. This amount corresponds to 8.10^9 molecules.

Two conclusions can be drawn from our experiments: first, the chemical changes at the end-plates of voluntary muscle can occur with the flashlike suddenness necessary for a chemical transmission of nerve impulses; secondly, a concentration of a specific enzyme at the nerve endings, many thousand times as high as that in the surrounding muscle tissue, must have a functional significance, and suggests—without anticipating the function—that ACh. intervenes in the transmission. The considerable difference of the concentration of choline esterase between frog's and lizard's muscle can also be interpreted in favour of its functional significance.

Summary

1. The rate of hydrolysis of acetylcholine by the nerveless pelvic end of a frog's sartorius was compared with that of the parts containing nerve endings. 100 mg. of the nerve-free ground muscle tissue split only 0·13 mg. of ACh., whereas 100 mg. of the same muscle containing nerve endings split 0·4–0·8 mg., according to the richness in nerve endings.

2. These experiments indicate the presence of a concentration of choline esterase at the end-plates which is many thousand times as high as that found in muscle tissue, enabling the muscle to split the ACh. liberated by the nerve impulses during the refractory period. The chemical changes can, therefore, occur with the rapidity necessary for the assumption of a chemical transmission of nerve impulses in such quickly reacting cells as fibres of voluntary muscle.

3. From the data given it can be calculated that about 0·001μg. ACh. can be hydrolysed at the nerve endings of a frog's sartorius weighing 300 mg. during the refractory period. In a single motor nerve ending about 0·000002 μg. can be hydrolysed in this period, that is 8.10^9 molecules.

4. In the dog's gastrocnemius also, the choline esterase is unevenly distributed in muscle tissue.

5. The rate of hydrolysis by ground muscle is only a little higher (about 30 p.c.) than by muscle chopped into pieces of about 30 mg. The difference between the esterase activity of ground muscle and of muscle pieces increases considerably if the tissue contains nerve endings, the rate of hydrolysis being several times as high in the ground muscles. This difference is probably due to the delay occasioned by diffusion of ACh. through muscle tissue to the nerve endings, the enzyme there being unsaturated with substrate if muscle pieces are used.

6. Phosphates and phosphorylated substances have been estimated both in the nerve-free end of frog's sartorius and the part rich in nerve endings, with a view to discovering whether there are other substances showing an unequal distribution. No difference of concentration has been found between the two parts of muscle.

7. The rate of hydrolysis of ACh. by lizard's muscle is three- to five-fold that produced by frog's muscle and two- to threefold that produced by mammalian muscle at 37°.

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REFERENCES

Brown, G. L., Dale, H. H. & Feldberg, W. (1936). *J. Physiol.* **87**, 394.
Lucas, K. (1907). *Ibid.* **36**, 113 and 253.
Lapicque, L. (1931). *Ibid.* **72**, 265.
Lucas, K. & Mines, G. R. (1907). *Ibid.* **36**, 334.
Lohmann, K. (1928). *Biochem. Z.* **194**, 308.
Marnay, A. & Nachmansohn, D. (1937*a*). *J. Physiol.* **89**, 359.
Marnay, A. & Nachmansohn, D. (1937*b*). *C.R. Soc. Biol., Paris*, **124**, 942.
Pézard, A. & May, R. M. (1937). *Ibid.* **124**, 1081.
Rouget (1862). *C.R. Acad. Sci., Paris*, **55**, 548.
Rushton, W. A. H. (1931). *J. Physiol.* **72**, 265.
Warburg, O. (1925). *Biochem. Z.* **164**, 481.