612.744:577.153

CHOLINESTERASE IN VOLUNTARY FROG'S MUSCLE

By A. MARNAY and D. NACHMANSOHN

From the Laboratoire de Physiologie générale, Sorbonne, Paris

(*Received* 18 *January* 1937)

Recent investigations by Dale, Feldberg and their collaborators have shown that impulses reaching motor nerve endings in voluntary muscle fibres cause a liberation of acetylcholine, and suggest that this substance may act as a transmitter of excitation from nerve to muscle. According to previous investigations [Chang & Gaddum, 1933; Plattner & Hintner, 1930], the amount of acetylcholine found in various tissues and the quantity of cholinesterase present can be brought into relation with the existence of the so-called cholinergic transmitter mechanism: the highest concentration of substrate and enzyme was—with a few unexplained exceptions—found in tissues whose main activity is controlled by nerves which are acting, or may be supposed to act, by liberating acetylcholine. As Brown *et al.* [1936] suggest, the acetylcholine liberated is more or less rapidly hydrolysed in order to enable the tissues to return to their previous condition, and therefore the distribution of the enzyme may vary with the kind of tissue. It was therefore of interest to obtain quantitative data of the rate of acetylcholine hydrolysis by voluntary muscle tissue.

Methods

The method developed for these experiments was based on the principle used by Ammon [1934] and Stedman & Stedman [1935]. These authors measured the rate of hydrolysis in blood serum and tissue extracts with a manometric method. In this method the formation of acetic acid formed during hydrolysis is measured by the liberation of CO_2 from bicarbonate added to the solution. The rate of the CO_2 output is proportional to the amount of enzyme present. By this means it is possible to compare the cholinesterase contents of different tissues.

We used a method which differs from that adopted by Ammon and by Stedman & Stedman in the different relation between enzyme and substrate. Amounts of acetylcholine between 0·2 and 0·6 mg. in 2–3 c.c. of total fluid are completely hydrolysed by 0·1 c.c. of horse serum in

60–100 min. The differences between two parallel determinations did not exceed 2–3 p.c. This method made it possible to examine quantitatively the course of acetylcholine hydrolysis in living tissue. Slices or pieces of tissue were suspended in a solution containing acetylcholine. After a suitable time the tissue was removed and an aliquot amount of the fluid taken for the manometric acetylcholine determination. In this way the remaining acetylcholine content was determined and subtracted from the acetylcholine content initially present. The difference represented the amount hydrolysed. Acetylcholine chloride of Hoffmann-La Roche was used in all experiments. All data are calculated for this neutral substance. To obtain the amount of base the figures must be multiplied by 0·8.

Results

Finely cut muscle of the frog

In this way the rate of hydrolysis of different concentrations of acetylcholine by frog's muscle (*Rana esculenta*) has been determined. Table I contains the results of experiments in which pieces of finely cut

Table I

No.	Weight of muscle tissue g.	Period of hydrolysis min.	Initial amount of ACh. mg.	ACh. remaining mg.	ACh. hydrolysed: Whole amount mg.	ACh. hydrolysed: By 100 mg. of tissue µg.	Mean value µg.
1	0·400	50	0·906	0·635	0·271	68	
2	0·410	50	0·906	0·565	0·341	83	66
3	0·504	50	1·140	0·880	0·260	52	
4	0·455	50	1·140	0·857	0·283	63	
5	0·420	100	1·140	0·600	0·540	128	117
6	0·462	100	1·140	0·650	0·490	106	
7	0·487	165	1·140	0·375	0·765	157	148
8	0·393	165	1·140	0·590	0·550	140	
9	1·000	50	2·645	1·370	1·275	127	127
10	1·080	50	2·645	1·280	1·365	126·5	
11	0·670	100	2·645	1·240	1·405	210	209
12	0·700	100	2·645	1·190	1·455	208	
13	0·430	160	2·560	1·430	1·130	262	262
14	0·566	50	5·030	3·980	1·050	186	168
15	0·519	50	5·030	4·260	0·770	149	
16	0·594	100	5·030	3·180	1·850	312	281
17	0·587	100	5·030	3·560	1·470	250	
18	0·535	154	5·030	2·830	2·200	411	373
19	0·597	154	5·030	3·110	1·920	334	
20	2·10	50	14·625	10·950	3·675	175	184
21	2·05	50	14·625	10·700	3·925	192	
22	1·45	100	14·625	9·560	5·065	350	335
23	1·69	100	14·625	9·260	5·395	320	
24	1·22	160	14·625	7·820	6·805	558	544
25	1·19	160	14·625	8·330	6·295	529	

muscle from the upper half of the hindlegs were suspended in 5 c.c. of Ringer solution, containing varying amounts of acetylcholine. The Ringer solution used throughout these experiments contained in 1 litre 6·25 g. NaCl, 0·2 g. $CaCl_2$ and 0·15 g. KCl. To 100 c.c. of this solution 10 c.c. of a 1·1 p.c. $NaHCO_3$ solution were added. The experiments were carried out in Erlenmeyer flasks, a gas mixture containing 5 p.c. CO_2 and 95 p.c. N_2 being bubbled through the solution during the experiment to ensure a constancy of *p*H at 7·2.

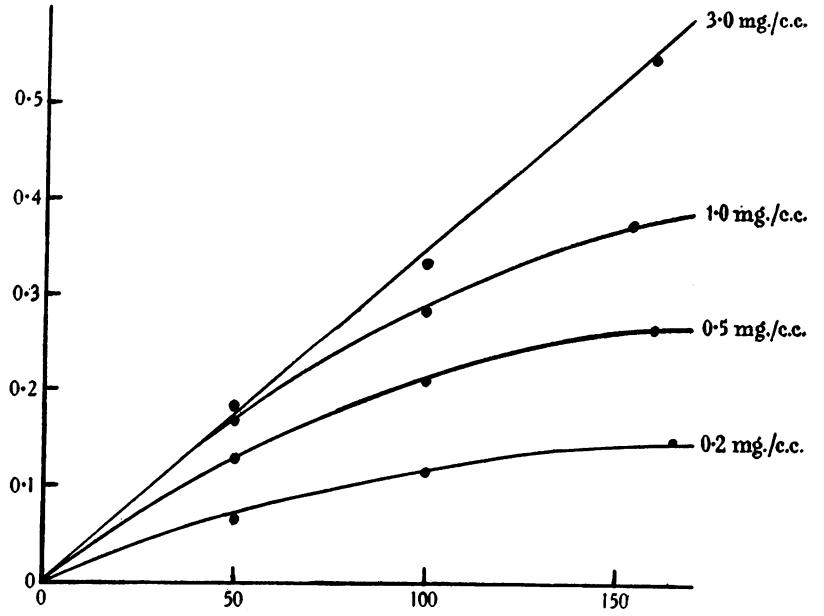

Fig. 1. Time course of acetylcholine (ACh.) hydrolysis by finely cut frog muscle (*Rana esculenta*) for different concentrations. *p*H = 7·2. Temp. 18°. Abscissa: time in min. Ordinate: mg. ACh. hydrolysed by 100 mg. of muscle.

The results of these experiments are also seen in Fig. 1 in which the time course of the hydrolysis for different concentrations of acetylcholine is shown. With two exceptions each point represents the mean of two single determinations. It can be seen that only the initial concentration of 3 mg. per c.c. gave an uniform rate of hydrolysis for the period studied. With lower initial concentrations the rates slowed down during the observations. Since the initial rates of the two highest concentrations—1 and 3 mg. per c.c. respectively—were practically identical, it follows that at this initial concentration saturation was reached.

This is more clearly shown in Fig. 2 in which the initial concentrations of acetylcholine are plotted against the amounts of substrate hydrolysed by 100 mg. of muscle. The points giving the hydrolysis after equal times are connected by curves. It can be seen, e.g. that the amounts hydrolysed in 50 min. are approximately the same for initial concentrations of 1 and 3 mg. per c.c. respectively.

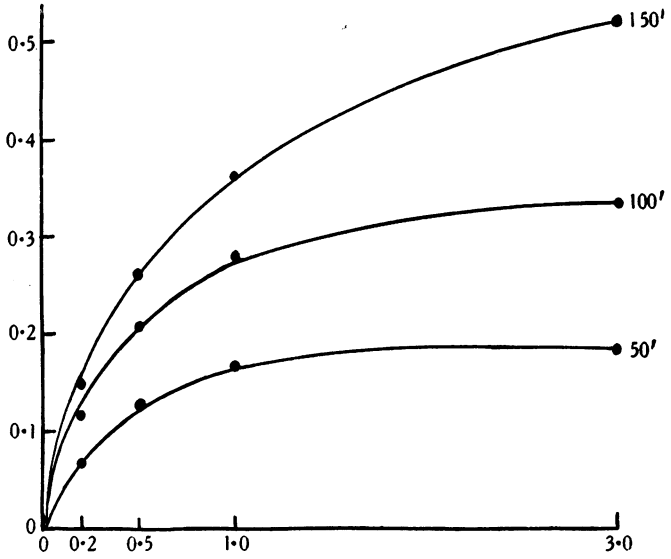

Fig. 2. Rate of acetylcholine (ACh.) hydrolysis by finely cut muscle tissue for different periods in relation to the concentration of the substrate. Abscissa: concentration of ACh. in mg. per c.c. Ordinate: mg. of ACh. hydrolysed by 100 mg. of tissue.

Intact frog's muscle

In experiments in which the effect of acetylcholine on the metabolism of muscle was studied it was found that this effect persisted for many hours [see Marnay & Nachmansohn, 1936]. This suggested that the rate of acetylcholine hydrolysis in intact muscle was slower than could be expected from data so far available in the literature, and it was therefore of interest to determine the rate of decomposition of acetylcholine in the intact muscle with the method described. Since it was desired to follow the time course of hydrolysis in a single experiment and to obtain several points on the hydrolysis curve with tissue from one animal, the muscles chosen for these experiments were the gracilis and semitendinosus of the two hindlegs. These muscles had been found in previous experiments on metabolism to be very similar in weight and properties.

TABLE II

Weight of muscle mg.	Period of hydrolysis min.	Initial amount of ACh. μg.	ACh. remaining μg.	ACh. hydrolysed (μg.) Whole amount	ACh. hydrolysed (μg.) By 100 mg. of muscle
420	55	1087	905	182	43
410	100	1087	765	322	78
410	210	1087	575	512	125

In the experiments shown in Table II each muscle was incubated in 5 c.c. bicarbonate Ringer solution containing 0·2 mg. acetylcholine per c.c. A gas mixture containing 95 p.c. N_2 and 5 p.c. CO_2 again ensured a *p*H of 7·2. The results are shown by the unbroken line in Fig. 3. For

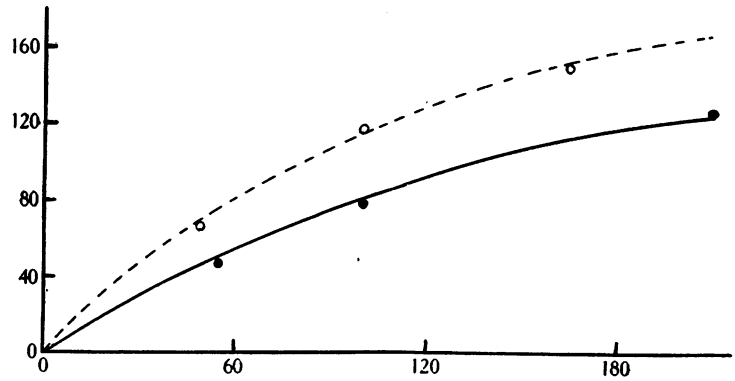

Fig. 3. Time-course of acetylcholine (ACh.) hydrolysis by intact muscle of *Rana esculenta*. Weight of muscle 420 and 410 mg. respectively. Concentration of ACh. 0·2 mg. per c.c. The broken line gives comparatively the time course of hydrolysis by finely cut muscle for the same initial concentration of ACh. Abscissa: time in min. Ordinate: μg. of ACh. hydrolysed by 100 mg.

comparison, the curve found with the same acetylcholine concentration in finely cut muscle (Fig. 1) is drawn in as a broken line. It is seen that the rate of hydrolysis is only slightly smaller in the intact muscle. This shows that the figures obtained for finely cut muscle can be taken, without great error, as indicating the rate of acetylcholine hydrolysis in intact muscle.

Other organs

The same method has also been applied in a few experiments to the determination of the esterase activity of various guinea-pig organs. The Ringer solution for these tissues contained [see Krebs & Henseleit, 1932]: NaCl 0·154 *M*, 100 parts; KCl 0·154 *M*, 4 parts; $CaCl_2$ 0·11 *M*, 3 parts; $MgSO_4$ 0·154 *M*, 1 part; $NaHCO_3$ 0·154 *M*, 10 parts. The

slices were suspended in 5 c.c. of this solution. These experiments were carried out in Warburg vessels kept in a thermostat at 37°. The concentration of acetylcholine was always 0·2 mg. per c.c. The substance was added after adjustment of the temperature. For the rest the procedure was the same as described above.

TABLE III

No.	Tissue	Weight of tissue mg.	Period of hydrolysis min.	Initial amount of ACh. mg.	ACh. remaining mg.	ACh. hydrolysed (mg.) Whole amount	By 100 mg. of tissue	Mean value
1	Brain cortex	80·3	30	0·837	0·231	0·606	0·755	**0·755**
2	,,	32·3	60	0·966	0·535	0·431	1·335	
3	,,	39·2	60	0·949	0·414	0·535	1·365	**1·353**
4	,,	42·45	60	0·949	0·372	0·577	1·360	
5	,,	18·15	120	0·876	0·438	0·438	2·410	
6	,,	22·10	120	0·876	0·373	0·503	2·270	**2·340**
7	Renal cortex	109·0	30	0·980	0·793	0·183	0·168	
8	,,	62·9	30	0·966	0·857	0·109	0·174	**0·171**
9	,,	92·5	60	0·980	0·716	0·264	0·285	
10	,,	177·0	60	0·837	0·402	0·435	0·246	**0·266**
11	,,	78·5	120	0·980	0·628	0·352	0·442	
12	,,	47·6	120	0·966	0·777	0·189	0·396	**0·419**
13	Liver	130·0	30	0·980	0·752	0·228	0·175	
14	,,	96·9	30	0·966	0·780	0·186	0·192	
15	,,	407·0	30	0·837	0·298	0·539	0·132	**0·155**
16	,,	440·0	30	0·837	0·313	0·524	0·119	
17	Liver	157·0	60	0·980	0·648	0·332	0·212	
18	,,	120·9	60	0·966	0·715	0·251	0·208	**0·210**
19	,,	149·0	120	0·980	0·519	0·461	0·310	
20	,,	75·0	120	0·966	0·652	0·314	0·418	**0·364**
21	Uterus	74·25	30	0·876	0·311	0·565	0·760	
22	,,	17·30	120	0·876	0·472	0·404	2·330	
23	,,	17·75	120	0·876	0·550	0·326	1·840	**2·085**
24	Pregnant uterus	38·8	62	0·837	0·115	0·722	1·850	
25	,,	13·2	120	0·837	0·439	0·398	3·000	
26	Heart	162·45	60	0·949	0·623	0·326	0·205	
27	,,	132·50	60	0·949	0·678	0·271	0·203	**0·204**
28	Voluntary muscle	350·0	30	0·861	0·229	0·632	0·180	
29	,,	201·0	60	0·861	0·304	0·557	0·277	
30	,,	117·0	120	0·861	0·193	0·668	0·550	
31	Adrenals	94·2	60	0·915	0·663	0·252	0·270	
32	,,	91·45	60	0·915	0·619	0·296	0·325	**0·298**

Table III gives a survey of the data obtained with brain cortex, renal cortex, liver, striated muscle (hindleg), muscle of uterus, heart muscle (ventricle) and adrenals. The weights given in the table are fresh weights,

these being derived, with a few exceptions, from the dry weights by multiplying by 5.

Fig. 4 shows the curves of hydrolysis obtained from brain, kidney and liver. The few observations with other tissues indicate that the activity

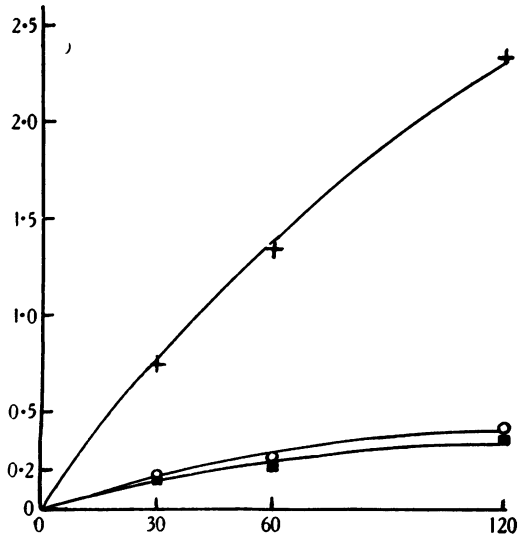

Fig. 4. Time course of acetylcholine (ACh.) hydrolysis by slices of some organs of the guinea-pig. Concentration of ACh. 0·2 mg. per c.c. pH = 7·2. Temp. 37°. + + + brain cortex; ooo renal cortex; ■■■ liver. Abscissa: time in min. Ordinate: mg. ACh. hydrolysed by 100 mg. tissue.

of the uterus is not very different from that of brain tissue and that the data for heart muscle, adrenals and striated muscle lie close to those found for liver and kidney.

Discussion

We have used our data as a basis for calculation of the rate of acetylcholine disappearance under conditions which are of interest in connexion with the chemical transmitter theory of neuromuscular excitation. According to Brown *et al.* [1936] one of the chief difficulties which this theory encounters when applied to the excitation of ganglion cells and voluntary muscle fibres is the brief refractory periods of these quickly reacting cells. The same authors have suggested that a persistent supraliminal concentration of acetylcholine at the end plates will produce a repetitive response of the muscle. The fact that a single motor impulse evokes only a single response of the muscle fibre would accord-

ingly entail, on the chemical theory, a fall in the local concentration of acetylcholine to a subliminal value within the very brief limits of the refractory period. They suggest, therefore, in order to explain so rapid a fall, that the esterase is concentrated at the nerve endings.

Now the dose required to produce in a cat's gastrocnemius a contraction tension similar to that of the twitch from maximal stimulation of the motor nerve is about 2 μg. On the other hand the largest amount of acetylcholine collected by Dale *et al.* [1936] from this muscle during stimulation of its nerve with maximal shocks corresponded only to one-hundredth of that dose—viz. about 0·02 μg. per shock. But as, on the one hand, only a part of the dose injected will reach the motor end plates rapidly, and on the other the quantities collected represent certainly less than the whole of the amount liberated by nerve impulses, the amount actually liberated must lie between 2 μg. and 0·02 μg.

Let us assume that 1 μg. is liberated in the gastrocnemius of the cat by a single nerve impulse, and let us further assume that this liberation occurs in 10 mg. of muscle. It can be calculated from the data above that 5 min. must pass before 50 p.c. of 1 μg. of acetylcholine are destroyed by 10 mg. of voluntary frog's muscle tissue at 18°. As in the warm-blooded muscle at 37° (see Table III) the hydrolysis is about 4 times quicker, the time necessary to hydrolyse 0·5 μg. would be 75 sec. The refractory period of the frog's skeletal muscle is about 5 μsec. and in mammalian muscle even shorter.

The time necessary for hydrolysing 50 p.c. of 1 μg. ACh. in the gastrocnemius of a cat is therefore at least 15,000 times longer than the refractory period of the muscle. The assumption of 1 μg. acetylcholine liberated in 10 mg. is the most favourable. Up to 200 mg. the values are approximately the same, above 200 mg. or below 10 mg. the rate of hydrolysis would be even lower. The esterase activity of nervous tissue (brain cortex) is about six times greater than that of voluntary muscle. The same concentration of enzyme may be admitted in the case of the motor nerves. Furthermore, in the muscle pieces diffusion may be an inhibiting factor which does not intervene at the surface of the nerve endings. In any case, however, a very high concentration of esterase at the end plates is necessary for the hydrolysis, within the limits of the refractory period, of the acetylcholine liberated during the stimulation. It will be of interest therefore to bring some evidence for such a high concentration of esterase at the end plates.

Quastel *et al.* [1936] have recently published a paper in which they determined for several tissues of the guinea-pig the $Q_{CO_2}^{N_2}$, i.e. the anaerobic

liberation of CO_2 by 1 mg. dry weight of tissue in 1 hour, in the presence of acetylcholine. Their procedure differs from that described in the present paper, in that they did not determine the amounts of acetylcholine metabolized directly but assumed that the CO_2 liberation was an adequate measure of the rate of acetylcholine hydrolysis. In some preliminary experiments made before that paper was published, it was observed that the CO_2 liberation of tissue slices after the addition of acetylcholine did not correspond exactly with the disappearance of acetylcholine as found by the method used in this paper. The CO_2 formation was less than was to be expected from the amounts of acetylcholine hydrolysed. Moreover, the authors used concentrations different from those used in our experiments, so that the results obtained are not quite comparable. This point too needs further investigation.

SUMMARY

1. A method is described for determinating small amounts of acetylcholine (0·2–0·6 mg.).

2. The time course of hydrolysis of acetylcholine by finely cut voluntary muscle of the frog (*Rana esculenta*) is determined for different concentrations of substrate.

3. A comparison is made with the rate of hydrolysis by the intact muscle of the frog.

4. The rate of acetylcholine hydrolysis by some tissues of the guinea-pig is determined.

5. The difficulty is discussed of assuming a hydrolysis of acetylcholine within the refractory period of voluntary muscle.

The authors are indebted to Prof. L. Lapicque for the hospitality given to them in his laboratory.

REFERENCES

Ammon, R. (1934). *Pflügers Arch.* **233**, 486.
Brown, G. L., Dale, H. H. & Feldberg, W. (1936). *J. Physiol.* **87**, 394.
Chang, H. C. & Gaddum, J. H. (1933). *Ibid.* **79**, 255.
Dale, H. H., Feldberg, W. & Vogt, M. (1936). *Ibid.* **86**, 353.
Krebs, H. A. & Henseleit, K. (1932). *Z. physiol. Chem.* **210**, 33.
Marnay, A. & Nachmansohn, D. (1936). *C. R. Soc. Biol.*, Paris, **122**, 1265.
Plattner, F. & Hintner, H. (1930). *Pflügers Arch.* **225**, 19.
Quastel, J. H., Tennenbaum, M. & Wheatley, A. J. M. (1936). *Biochem. J.* **30**, 1668.
Stedman, E. & Stedman, E. (1935). *Ibid.* **29**, 2107.