THE ASSAY OF ACETYLCHOLINE ON THE SUPERFUSED FROG RECTUS MUSCLE

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A method of superfusion for assaying acetylcholine on the eserinised frog rectus muscle applying each dose in a volume of 0·2 ml. is described. The sensitivity of the superfused preparation is compared with that of the preparation in a 5 ml. bath and is 10 to 20 times more sensitive than the bath preparation. Results of 10 parallel assays (2 + 2) suggest that the method of superfusion gives a reliable and accurate assay of acetylcholine.

The estimation of acetylcholine in biological materials requires a method of high sensitivity when the volume and concentration likely to be found is very small. In recent years the technique of superfusion has been increasingly used for assaying minute quantities of active substances such as histamine and 5-hydroxytryptamine on isolated preparations. The present investigation was undertaken with the object of applying the method of superfusion to the assay of acetylcholine on the frog rectus muscle. In order to assess the sensitivity, reliability and the accuracy of the superfusion method, parallel assays by the usual bath method were also made using muscles from the same frogs. The results show that the superfusion method is highly sensitive and that the assay can be made using only 1/20 to 1/10th of the dose required for the bath method with a dose volume of only 0·2 ml.

METHODS

One rectus abdominis muscle of the pithed frog was suspended in a 5 ml. bath and the other was suspended in air enclosed in a wide glass tube. Frog Ringer solution from a reservoir placed about 30 inches above the bench was used to supply both preparations. Each litre of frog Ringer solution contained NaCl 6·5 g.; KCl 0·14 g., $CaCl_2$ 0·12 g.; $NaHCO_3$ 0·2 g.; NaH_2PO_4 0·01 g. and glucose 2 g. The fluid in the bath was continuously aerated. Eserine salicylate was added to the Ringer solution to give a concentration of 10^{-5} (as salicylate).

The rate of flow for superfusing the muscle was controlled by a screw clip on the rubber tube and the flow interrupted or continued by turning the stopcock in the glass tube. A flow rate of 80 drops per minute was employed.

Frontal writing levers were used and adjusted to give a 10 fold magnification and a tension on the muscle equivalent to 2·0 g.

The procedure of administration of drug solution to the superfused preparation was similar to that of Gaddum[1]. Superfusion was stopped

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10 seconds before the application of a dose. The doses were administered in a volume of 0·2 ml. from a 0·2 ml. blood pipette. The pipette was rested near the capillary end of the glass tube touching the thread and the drug solution was allowed to fall dropwise down the thread and flow over the surface of the muscle. The drug solution was left in contact with the muscle for 90 seconds, after which the superfusion was restarted.

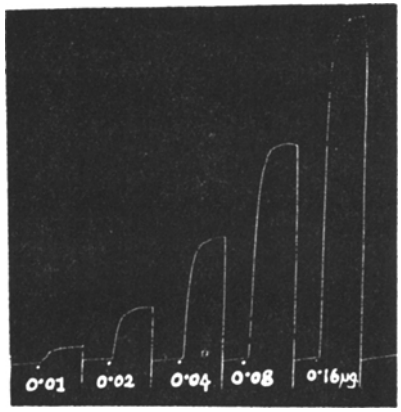

FIG. 1. Frog rectus muscle superfused with Ringer's solution (without eserine). Records of contractions due to different doses of acetylcholine solution (as acetylcholine chloride). Muscle allowed to contract for 90 seconds.

Assay Procedure

The muscles were prepared and left for half to one hour, then doses of acetylcholine were applied regularly at 6 to 10 minute intervals. The acetylcholine was dissolved in frog Ringer solution and added to both preparations in a constant volume of 0·2 ml. When the response had become regular the assay was made by Schild's method[2]. The approximate concentration of the unknown solution was estimated by the bath method, diluted if necessary and four responses obtained at each of two doses, the ratio of the high to low dose being 2 : 1. In assaying by the superfusion method these concentrations had to be further diluted 10 to 20 times. Four responses were obtained at each dose level for standard and unknown solutions in each assay, the doses being randomly distributed in a 4 × 4 Latin square design.

RESULTS

Response of Superfused Frog Rectus Preparation to Acetylcholine Solution

Figure 1 shows the type of contracture produced by different doses of acetylcholine in superfused frog rectus muscle not sensitised by eserine. The contracture starts as soon as the drug solution comes in contact with the surface of the muscle, reaches a maximum within 20–30 seconds and remains at this level until the superfusion is restarted. Relaxation commences soon afterwards and is complete within 3–6 minutes. In the eserinised preparation, the contracture does not reach the maximum even in 90 seconds (see Fig. 3).

Sensitivity of Superfused Preparations

The sensitivity of the preparations to acetylcholine varied moderately with different frogs. The smallest effective dose for the superfused preparation was 0·0025 to 0·005 μg. of acetylcholine per 0·2 ml. dose; that for the 5 ml. bath was 0·05 to 0·1 μg.

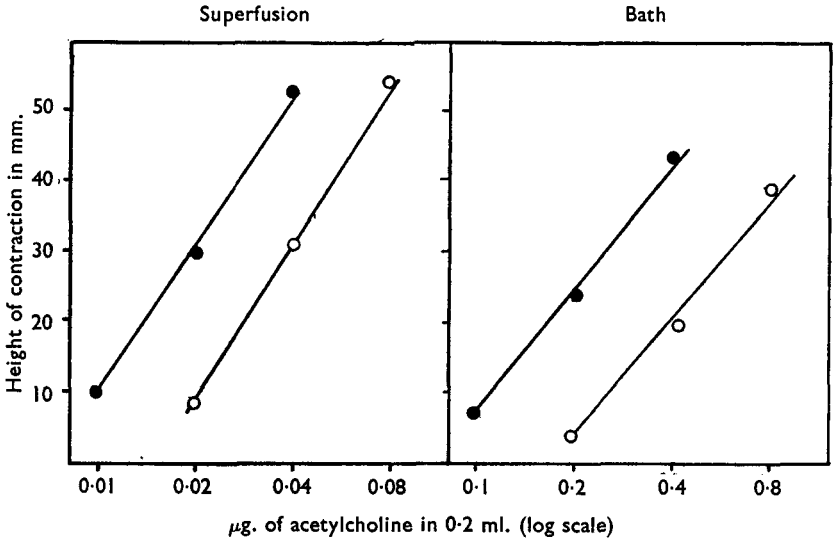

FIG. 2. Log dose response curve of frog rectus muscle obtained by superfusion and bath method. Closed circle—preparations sensitized with eserine; open circle—not sensitised with eserine. Each point represents the mean value obtained from six muscles.

TABLE I

SUMMARY OF ACETYLCHOLINE ASSAY RESULTS BY BATH (5 ML.) AND SUPERFUSION METHODS

No. of Assay	Method	Amount of acetylcholine μg./ml.		Limits of error P = 0·05	Standard Deviation (s)	Slope (b)	λ (s/b)
		Actual	Found				
1	Bath	4	4·16	92·6–107·9	2·34	78·45	0·029
	Superfusion		4·4	86·8–115·1	1·86	47·36	0·039
2	Bath	3	3·3	93·9–106·4	3·61	120	0·0308
	Superfusion		3·25	94·2–105·9	3·4	119·1	0·0286
3	Bath	8	9·3	76·4–130	6·13	71·84	0·086
	Superfusion		7·6	92·3–108·3	1·76	73·92	0·024
4	Bath	3	3·08	86·6–115·6	2·83	67·27	0·043
	Superfusion		3·15	77·6–125	3·22	42·85	0·075
5	Bath	2·5	2·55	88·9–112·4	2	64·78	0·0308
	Superfusion		2·60	95·5–104·7	1·27	41·52	0·0306
6	Bath	0·725	0·72	87·1–114·4	2·48	41·5	0·060
	Superfusion		0·69	87·8–113·9	4·72	69·35	0·068
7	Bath	2·5	2·65	82·2–121·1	3·23	63·5	0·0508
	Superfusion		2·32	86·0–116·3	3·64	79·3	0·046
8	Bath	2·75	2·78	85·3–117·8	3·39	84·7	0·046
	Superfusion		3·0	86·2–116·1	5·8	83·8	0·070
9	Bath	0·77	0·863	89·0–111·7	1·63	52·45	0·031
	Superfusion		0·813	81·4–122·7	3·35	52·82	0·063
10	Bath	2·75	2·8	95·9–104·7	1·83	75·58	0·024
	Superfusion		2·81	82·6–121·1	4·93	80·56	0·061

Figure 2 shows the dose response curves of eserinised and noneserinised preparations in the superfusion and bath methods. The dose response was found to be linear within the range of 0·01 to 0·04 μg. (eserinised) and 0·02 to 0·08 μg. (noneserinised). The bath method gave linearity of response within the range of 0·1 to 0·8 μg. (eserinised). The assays (2 + 2) were carried out using these dose ranges.

2 + 2 *Assays*

Assays were made simultaneously on superfused and bath preparations and the results of ten such parallel assays are given in Table I. The same standard and unknown solutions were used for both methods except that in the case of the superfusion method, they were diluted 10 to 20 times. The first five assays were made on noneserinised preparations and the last five assays were made one hour after eserinisation.

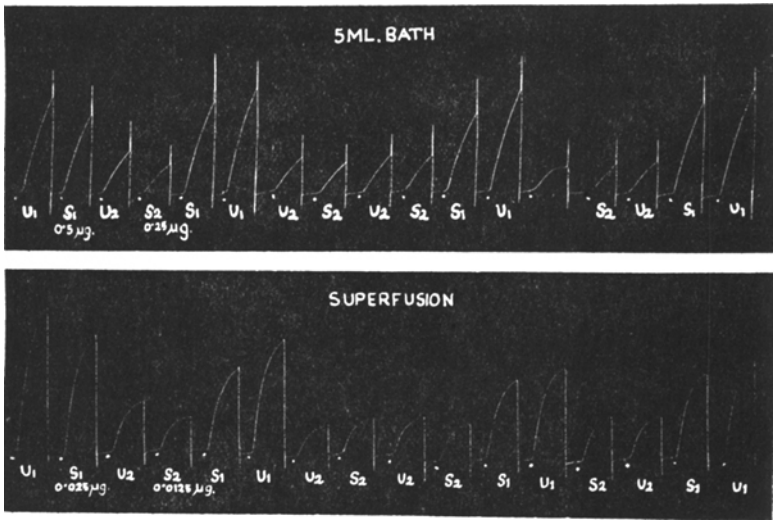

FIG. 3. Record of a parallel assay (2 + 2) with acetylcholine solution on frog rectus eserinised muscles by bath and superfusion methods (assay No. 10, Table I). In bath method—$S_1 = 0{\cdot}5$ μg.; $S_2 = 0{\cdot}25$ μg.; $U_1 = 0{\cdot}55$ μg.; $U_2 = 0{\cdot}275$ μg. In superfusion method—$S_1 = 0{\cdot}025$ μg.; $S_2 = 0{\cdot}0125$ μg.; $U_1 = 0{\cdot}0275$ μg.; $U_2 = 0{\cdot}01375$ μg.

A typical parallel assay on eserinised preparations is shown in Figure 3. The actual concentrations of the test solutions were unknown to the operator until the assay had been completed.

It will be seen from the Table that the accuracy of the assay and the limits of error were much the same for both bath and superfusion methods, though the sensitivity of the superfused preparation was 10 to 20 times greater than the bath preparation. The index of precision (λ) was found to be less than 0·05 in five assays by the method of superfusion. The estimates of the assays and their errors were calculated by the method of analysis of variance. In all the assays the quantity "t" for parellelism

was less than 2·18 for 12 degrees of freedom thus indicating no significant deviation from parallelism.

Discussion

Kwiatkowski[3] used the superfused frog rectus for assaying acetylcholine by injecting acetylcholine into the superfusing fluid. In these circumstances the dose of acetylcholine is immediately diluted so that the senstitivity is greatly reduced. Cambridge and Holgate[4] found that a graded response to acetylcholine could be obtained from a superfused frog rectus if the solution containing it were applied directly to the muscle. They found the optimum dose was 2·5 ml. of solution containing 0·3 to 0·8 μg./ml. at a rate of 1 drop per second for 50 seconds. The present investigation shows that the concentration of the acetylcholine solution and the dose volume may be reduced. The concentration of acetylcholine in the solutions assayed in our superfusion experiments ranged from 0·1 to 0·4 μg./ml. in noneserinised preparations and from 0·05 to 0·2 μg./ml. in eserinised preparations. The volume of the dose was also lower—0·2 ml.

Statistical evidence is adduced to show that the method of superfusion and bath method are of equal accuracy when used under comparable conditions. In the case of the superfusion method assayable responses could be obtained with 1/10 to 1/20th of the dose required in the bath method.

References

1. Gaddum, *Brit. J. Pharmacol.*, 1953, **8**, 321.
2. Schild, *J. Physiol.*, 1942, **101**, 115.
3. Kwiatkowski, *ibid.*, 1941, **100**, 147.
4. Cambridge and Holgate, *Brit. J. Pharmacol.*, 1954, **9**, 370.