A NEW BIOLOGICAL METHOD FOR THE ASSAY OF DEPOLARIZING SUBSTANCES USING THE ISOLATED SEMISPINALIS MUSCLE OF THE CHICK

BY

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The isolated semispinalis cervicis muscle of a 3 to 10 days old chick has been found to provide a very useful assay method for depolarizing substances. There are three main advantages of this preparation over that of the rectus abdominis of the frog: (a) quick relaxation without any artificial stretching of the muscle, (b) greater sensitivity to depolarizing drugs, and (c) no falling off in sensitivity for at least 2 hr.

A particular type of avian muscle is very sensitive to substances possessing an acetylcholine-like action at the neuro-muscular junction. For example, in adult fowls and chicks an intravenous injection of a minute dose of decamethonium or suxamethonium causes a rigid extension of the limbs and retraction of the head (Buttle and Zaimis, 1949). This is a peripheral effect, the muscles reacting to the depolarizing drugs with a true contracture (Zaimis, 1954, 1959). Because of this and in the hope of obtaining a sensitive and specific method for the quantitative determination of depolarizing drugs, the isolated leg and neck muscles of the chick were investigated. Of all the muscles, the semispinalis cervicis and the biventer cervicis muscles have been found to provide the best preparations as they are thin, very sensitive to depolarizing drugs, and easy to dissect.

The method was demonstrated for the first time to the Pharmacological Society in July, 1954, and a preliminary communication was given to the Biological Methods Group of the Society for Analytical Chemistry in June, 1954.

METHOD

Chicks usually obtained soon after hatching were housed at a temperature of 35 to 38° and kept on a diet of chick pellets and water.

After the chick had been anaesthetized with ether a longitudinal incision was made in the skin at the back of the neck from the base of the skull to the region of the thoracic vertebrae; the neck was then slightly flexed by means of a small pad of cotton wool. In the middle of the neck there are two pairs of thin muscles which arise from the second thoracic vertebra, run upwards side by

side, and are inserted into the medial part of the occipital bone round the area of the nuchal crest. The superficial pair is formed by the two biventer cervicis, the deep one by the two semispinalis cervicis muscles. Either the biventer cervicis or the semispinalis can be used for this assay. Our preference, however, was given to the semispinalis because it could be dissected with the least possible damage. In order to expose the semispinalis, the biventer muscle of one side was lifted gently after cutting its proximal tendon (Fig. 1). The semispinalis muscle was then freed from the oblique muscles of the neck and a thread was tied round each end. Finally, the muscle was mounted as quickly as possible in a bath containing oxygenated Tyrode solution. In the present study a 5 ml. bath was used; the magnification was about 4 and the tension was 1 g.

The sensitivity of the muscle was at its best in chicks between 3 and 10 days old. In chicks less than 3 days old the muscle was very delicate and difficult to handle, whilst in chicks older than 10 days the sensitivity diminished and the differentiation between doses was less good. Differences in sex and breed did not appear to influence the response of the muscle. It was found that careful and gentle dissection was essential and that a muscle dissected from an anaesthetized chick was more sensitive than one from a recently killed animal.

Tyrode, Locke, de Jalon and Krebs solutions were tested. The sensitivity of the muscle was greatest in Tyrode solution at a temperature of 40 to 40.5°. While a temperature higher than 41° decreased the magnitude of the response without altering the time required for complete relaxation, a temperature below 39° affected both these factors; the response became less regular and the time required for complete relaxation increased markedly.

Pure oxygen and air were equally satisfactory for the oxygenation of the Tyrode solution provided that the air was finely dispersed.

FIG. 1.—Chick; dissection of the back of neck to demonstrate (A) the biventer cervicis muscle retracted to show (B) the semispinalis cervicis muscle.

RESULTS

Substances Mimicking Acetylcholine

Decamethonium.—The semispinalis cervicis muscle responded with a sustained contracture to decamethonium and the magnitude of the response increased linearly with the logarithm of the dose (Fig. 2). The onset of the contracture was almost immediate with all but very small doses of the drug, when there was a latent period of 5 to 15 sec. The rate of development of the contracture was rapid at first but slowed down as the peak tension was approached; usually a plateau was reached in less than 1 min. The muscle remained in contracture until the bath was filled with fresh Tyrode. Relaxation was rapid and complete in less than 2 min. without any artificial stretching of the muscle. An interval of 4 min. between doses was almost always adequate.

Fig. 3 shows the result of an assay of a solution of decamethonium from which Tables I and II have been constructed. Two doses of "Standard" solution and two doses of "Test" solution were used in each assay, the doses being administered in the form of a Latin square. For the calculation of the ratio of potency and the analysis of variance of the results the method described by Schild (1942) was used with the difference that owing to the Latin square arrangement a further set of three degrees of freedom was set aside for "dose order." The value of M (log. ratio potency of unknown to standard) was estimated to be -0.0497 ± 0.003 . The ratio of potency was therefore 0.892 and the potency of the test solution of decamethonium = $0.892 \mu\text{g./ml.}$ The limits of error of the estimate = $0.877-0.907$ ($P=0.05$). The index of precision, $\lambda=s/b=0.005$. In order to test the accuracy of the method the same solution of decamethonium of known strength was assayed on six fresh muscles, and the results and limits of error of the estimate of these assays are given in Table III. In each case there was a highly significant difference in activity between the two solutions and a highly significant regression between the smaller and larger doses making a quantitative estimate of activity possible. The low F value obtained for "groups" in every assay indicates that the sensitivity of the preparation did not change during the experiment.

Other Depolarizing Drugs.—Table IV shows the results obtained with suxamethonium and other substances possessing an acetylcholine-like action at the neuromuscular junction. The activity quoted for suxamethonium is the mean result of three estimations using decamethonium as the standard;

TABLE I

	Decamethonium $\mu\text{g./ml.}$	Dose ml.	Dose $\mu\text{g.}$	Responses mm.	Mean
A	1.0	0.60	0.600	32 32 32 32	32
B	1.0	0.45	0.450	14 15 16 15	15
C	0.9	0.60	0.540	25 26 24 25	25
D	0.9	0.45	0.405	9 9 8 9	8.75

TABLE II

THE ANALYSIS OF VARIANCE OF THE ASSAY RESULTS SHOWN IN TABLE I

The log. ratio of potency, M , = -0.0497 with limits of error ± 0.0074 . The asterisked values of F are reciprocals.

Source of Variance	Sum of Squares	Degrees of Freedom	Variance	$F = V_1/V_2$	P
1. Groups	0.69	3	0.23	2.40*	Not significant
2. Standard and unknown ..	175.56	1	176	318	<0.001
3. Regression	1,105.62	1	1,106	2,000	<0.001
4. Dev. from parallelism ..	0.56	1	0.56	1.01	Not significant
5. Dose order	0.69	3	0.23	2.40*	„ „
6. Residual error	3.32	6	0.553		
Total	1,286.44	15			

the regression lines for these two substances did not differ significantly from parallel (in each case $P > 0.1$).

The semispinalis cervicis muscle is very sensitive to depolarizing drugs; for example, concentrations

of decamethonium and suxamethonium as low as $0.1 \mu\text{g./ml.}$ in the bathing fluid may be readily estimated. Furthermore, even less specific substances, such as carbachol and nicotine produced responses which could be easily measured.

TABLE III

ESTIMATION OF THE POTENCY OF A TEST SOLUTION OF DECAMETHONIUM CONTAINING $0.9 \mu\text{G./ML.}$ ON EACH OF SIX SEPARATE CHICK MUSCLE PREPARATIONS

Expt. No.	Estimated Potency $\mu\text{g./ml.}$	Limits of Error ($P=0.05$)	Dose Ratio	λ
1	0.908	0.878-0.939	5:4	0.009
2	0.892	0.877-0.907	4:3	0.005
3	0.889	0.864-0.915	4:3	0.009
4	0.896	0.868-0.924	4:3	0.011
5	0.889	0.865-0.913	5:4	0.009
6	0.885	0.859-0.910	6:5	0.009

TABLE IV

THE ACTIVITY OF VARIOUS DEPOLARIZING SUBSTANCES COMPARED WITH DECAMETHONIUM (=100)

All values refer to the bases.

Substance	Method of Assessment	Activity
Suxamethonium	Latin square assay	56-62 (mean = 60)
Adipylcholine	Direct comparison	42
Acetylcholine	„ „	<1
Carbachol ..	„ „	<2
Nicotine ..	„ „	<2

FIG. 2.—The log dose-response curve of decamethonium assayed on the isolated semispinalis cervicis muscle of the chick. $S=0.59$, $b=93.89$, $\lambda=0.006$.

Acetylcholine.—The response of the muscle to acetylcholine was unpredictable. In some preparations contracture was obtained with 5 μg . while in others 50 μg . produced no effect at all. However, in the presence of anticholinesterase drugs the sensitivity of the muscle increased and the responses became more predictable (Tyler, 1960).

Competitive Antagonists of Acetylcholine.—Competitive antagonists of acetylcholine antagonize the action of depolarizing drugs. Fig. 4 shows the

In every experiment a potentiation of the response of the muscle to decamethonium was observed after tubocurarine had been washed out. Such an effect was not apparent following the administration of hexamethonium.

The Administration of Biological Fluids.—It was found that biological fluids containing depolarizing drugs could also be assayed provided they were approximately neutral. In acid ($\text{pH} < 6$) or alkaline

FIG. 3.—The estimation of potency of a decamethonium solution of unknown concentration on the isolated semispinalis cervicis preparation. A=0.60 ml. and B=0.45 ml. of a standard solution containing 1 μg ./ml. of decamethonium. C=0.60 ml. and D=0.45 ml. of the test solution. Dose interval=3 min. 45 sec.

FIG. 4.—Contractions of the isolated semispinalis cervicis muscle produced by decamethonium 0.5 μg . Tubocurarine (1 to 4 μg .) (a), and hexamethonium (50 to 200 μg .) (b), were added to the bath 1 min. before the decamethonium at the points indicated. The last contraction was produced by 0.6 μg . decamethonium.

effect of tubocurarine and hexamethonium; the response of the muscle to 0.5 μg . decamethonium may be reduced by approximately 50% by the previous administration of 2 μg . tubocurarine or of 100 μg . of hexamethonium. Fig. 5 shows the regression line relating log dose of tubocurarine and the percentage reduction of the response of the muscle to decamethonium.

($\text{pH} > 8$) biological fluids the sensitivity of the muscle decreased very rapidly. In the alkaline fluids the decrease in sensitivity was sometimes preceded by a brief period of potentiation. Biological fluids tested included human blood and plasma, cat plasma, horse serum, and human urine. The administration of whole blood tended to reduce the rate of development of the contracture.

FIG. 5.—Log dose-response curve of tubocurarine assayed by its inhibitory effect on the decamethonium induced contractions of the isolated semispinalis cervicis muscle.

DISCUSSION

The semispinalis cervicis muscle of the chick, like the rectus abdominis muscle of the frog, responds by contracture to acetylcholine and to substances possessing an acetylcholine-like action at the neuromuscular junction. This muscle offers advantages over the rectus abdominis because (a) relaxation following contracture is much more rapid and occurs without any artificial stretching of the muscle; (b) the muscle is more sensitive to substances mimicking acetylcholine; and (c) there is no falling off in sensitivity for at least 2 hr.

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