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A METHOD FOR THE QUANTITATIVE ESTIMATION OF DRUGS ON THE ISOLATED INTACT TRACHEA

BY

DANA JAMIESON

From the Radiobiological Research Unit, Cancer Institute Board, Melbourne, Australia

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A method is described for the detection and comparison of substances active on tracheal smooth muscle. Several pharmacological agents affecting smooth muscle have been tested using the technique. Sensitivity was high, results reproducible and linear log dose-response relationships obtained over a wide dosage range. The method has been used to estimate the concentrations of adrenaline, acetylcholine and histamine in test solutions of these compounds.

The methods of estimation of pharmacologically active compounds on the smooth musculature of the respiratory tract so far published are unsatisfactory in several ways. *In vivo* preparations involve the use of specialized apparatus. The commonly used methods of Konzett & Rössler (1940) and other similar techniques measure air displacement by the entire lung; according to Graubner, Peters & Wick (1952) this does not give an accurate indication of the effect on the smooth musculature of the respiratory tract. Castillo & de Beer (1947) used the guinea-pig tracheal chain for the testing of broncho-active substances. However, this method is not simple to use and is impractical in the bioassay of drugs owing to the time interval between successive test doses being of necessity very long. We have used a simple volume displacement method to screen and assay various drugs for constrictor and dilator activity on the isolated whole trachea. The method is rapid and sensitive, and elaborate equipment or practice in handling the technique is not required.

Brocklehurst (1958) has demonstrated quantitative differences between the responses of the trachea and bronchi to some pharmacological agents in several species. Our technique measures the effect on the tracheal smooth muscle alone.

METHODS

The tracheal preparation. Adult rats and guinea-pigs were used. An animal having been killed by a blow to the head, the trachea was removed, placed in Krebs-Henseleit solution, and dissected free of extraneous tissue. The trachea was washed through several times with Krebs-Henseleit solution, and one end tightly tied around the shaped end of a length of capillary tubing. Krebs-Henseleit solution was then drawn up through the trachea and into the capillary for about 10 cm, care being taken to exclude air bubbles from the system. In the early experiments the free end of the trachea was then tightly ligatured, and so attached to a support in the organ bath as to apply slight longitudinal tension to the tissue. In later experiments the apparatus shown in Fig. 1 was used to simplify filling of the trachea and

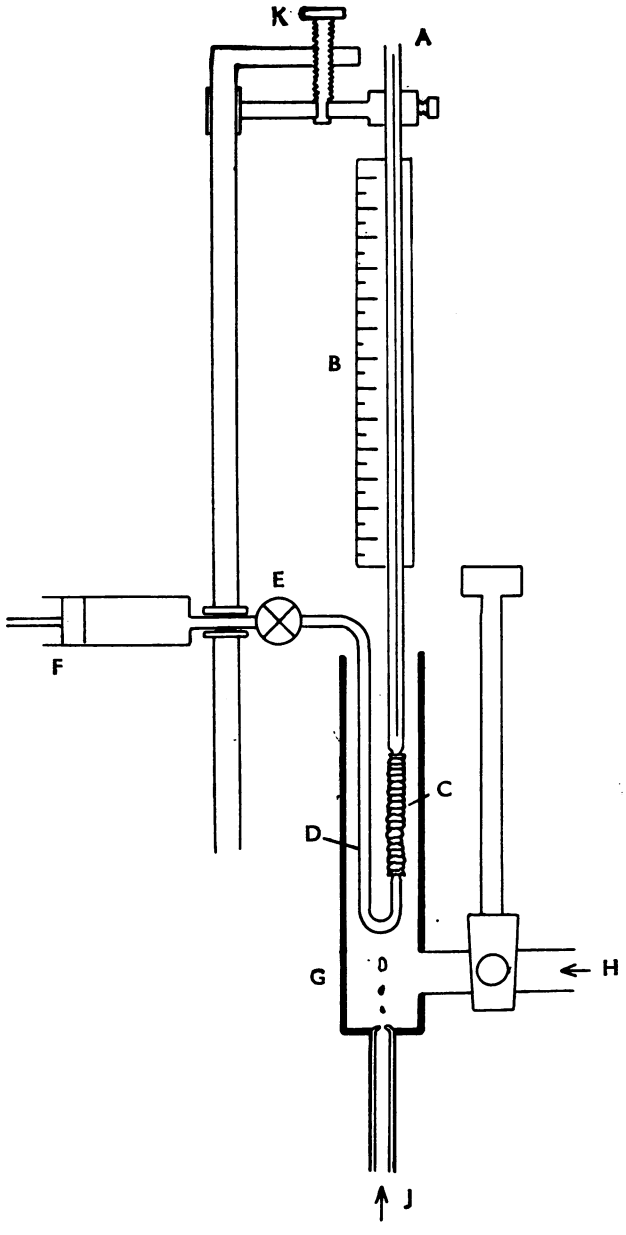

Fig. 1. Diagram of apparatus. A=Capillary tubing, 1 mm internal bore; B=scale, graduated in millimetres; C=trachea; D=U-tube, locating trachea in position and introducing bathing fluid; E=two-way tap; F=syringe; G=10 ml. capacity organ bath; H=inflow of bathing fluid; J=inflow of aerating mixture; K=adjusting screw to apply required longitudinal tension to trachea. The organ bath is immersed in a constant temperature bath maintained at 37° C.

ensure elimination of air bubbles. The trachea was attached as before to one end of the capillary tube, A, and the other end was similarly attached to a U-tube, D, fitted to a stopcock, E. A syringe fitted to the other side of the stopcock was used to flush the system through with Krebs-Henseleit solution until all air bubbles were eliminated. The trachea and U-tube were placed in a 10 ml. organ bath filled with Krebs-Henseleit solution at 37° C and aerated with 95% oxygen and 5% carbon dioxide. The fluid in the manometer was then adjusted to be 7 to 10 cm above the level of the organ bath fluid. A two-way tap between the syringe and U-tube was used to ensure complete sealing of the system at that end. A steady manometer level was reached within 10 min and this level was taken as the baseline for testing both constrictor and dilator drugs. The organ bath fluid was changed by flooding from below, thus avoiding exposure of the trachea to air. The addition of constrictor agents to the organ bath caused increases in column height, measured on a mm scale, B, attached to the capillary tube. The recorded increases in height depend upon the change in volume of the tracheal segment used and the bore of the capillary tubing. A capillary tube of 1 mm internal diameter gave adequate sensitivity and rapidity of movement of fluid in the tubing. The tubing was thoroughly cleaned after each experiment.

Smooth muscle (length c) is seen histologically to be circumferentially disposed in the wall of the trachea and then contraction of this muscle causes narrowing of the lumen. As the length (l) of the trachea is kept constant the volume of fluid displaced (δv) is given by

$$\delta v = \frac{(c_2^2 - c_1^2)l}{4\pi}$$

$$\therefore \quad \delta v \propto \delta c^2$$

The change in volume (δv) in the manometer of constant radius is proportional to the increase in height of the column, δh

$$\therefore \quad \delta h \propto \delta c^2$$

that is, the muscle response is proportional to the square root of the change in height of the manometer fluid and thus $\sqrt{\delta h}$ is plotted against the log dose in constructing dose-response curves.

The trachea was allowed to remain in the organ bath for 10 to 20 min before the application of drugs. After this time the base line remained at or near the initial level for several hours of experimentation and during this time uniform responses were obtained, with little or no tachyphylaxis. Where slight changes in the base line did occur the height of the response was measured from the mean of the manometer fluid height before and after the response.

Compounds. The compounds tested were: acetylcholine, 5-hydroxytryptamine, histamine and barium chloride for constrictor activity; adrenaline and aminophylline for dilator activity; and atropine as an antispasmodic agent.

A contact time of 1.5 to 3.0 min was used with constrictor agents and successive doses were applied each 5 to 8 min. The time intervals used depended on the speed of the response and the subsequent return to the initial manometer level after washing. The organ bath was flushed out at least twice between doses. Once a suitable time interval for dose application was established for a given drug on a preparation this interval was then kept constant throughout the estimation.

RESULTS

Action of drugs on the tracheal preparation

The results obtained were similar to those previously reported using guinea-pig tracheal or bronchial rings (Brocklehurst, 1958; Castillo & de Beer, 1947), but somewhat greater threshold sensitivity was obtained. The sensitivity of this

preparation to the drugs tested is shown in Table 1. Deterioration of preparations occurred after 6 to 8 hr and was accompanied by a progressive slow fall in the base line and a lessened sensitivity. No sudden variations in response occurred and the method offers a rapid, simple means of comparing the potency of bronchoconstrictor

TABLE 1

MINIMAL DETECTABLE CONCENTRATION OF SUBSTANCES (G/ML.) PRODUCING A RESPONSE ON THE TRACHEAL PREPARATION

Compound	Guinea-pig trachea	Rat trachea
Acetylcholine	2×10^{-8}	10^{-8}
5-Hydroxytryptamine	5×10^{-8}	$2{\cdot}5\times10^{-8}$
Histamine	4×10^{-8}	$>10^{-3}$
Adrenaline	10^{-9}	$>10^{-3}$
Aminophylline	10^{-5}	$>10^{-3}$

agents. The responses to the dilator drugs, adrenaline and aminophylline, were more prolonged than those to the constrictor agents tested and the initial base line was not regained until 10 to 15 min after washing out the drug. Results for the individual compounds follow, together with estimations of the potency of unknown solutions of acetylcholine, histamine and adrenaline.

Acetylcholine. Dose-response curves were obtained for acetylcholine on 8 rat and 8 guinea-pig preparations. In both species the response to acetylcholine was rapid; after a short latent period the manometer fluid rose rapidly and reached maximum height in approximately 1 min. A measurable response (1 mm rise) was obtained with 10^{-8} acetylcholine on 3 rat preparations, while about 10 times this concentration was needed for the remaining rat tracheal preparations to give a similar rise. Doses giving a final concentration of 10^{-7} to 10^{-6} gave suitable responses for assay work. The guinea-pig trachea was approximately half as sensitive to acetylcholine as the rat trachea in these experiments (Table 1). A typical dose-response curve for acetylcholine on the guinea-pig trachea is shown in Fig. 2.

5-Hydroxytryptamine. In the rat 5-hydroxytryptamine proved almost as potent as acetylcholine. Where these drugs were compared on the same trachea 5-hydroxytryptamine was found as active as acetylcholine in 2 cases and approximately one half as active in another 3 trials. The contractile response to 5-hydroxytryptamine was a little slower than that to acetylcholine, therefore the contact time and time interval between doses were extended accordingly. A dose-response curve for 5-hydroxytryptamine on the rat trachea is shown in Fig. 2.

Similar results were obtained with the guinea-pig trachea although this tissue was more sensitive to acetylcholine than 5-hydroxytryptamine in every case, and approximately double the dose of 5-hydroxytryptamine was needed to produce responses similar to those of acetylcholine. The time intervals between successive drug challenges used for the rat trachea were also adequate for the guinea-pig preparations.

Histamine. Rat trachea was quite refractory to histamine, even in concentrations of 10^{-3}. Guinea-pig trachea responded well to histamine, with a sensitivity similar to that of 5-hydroxytryptamine.

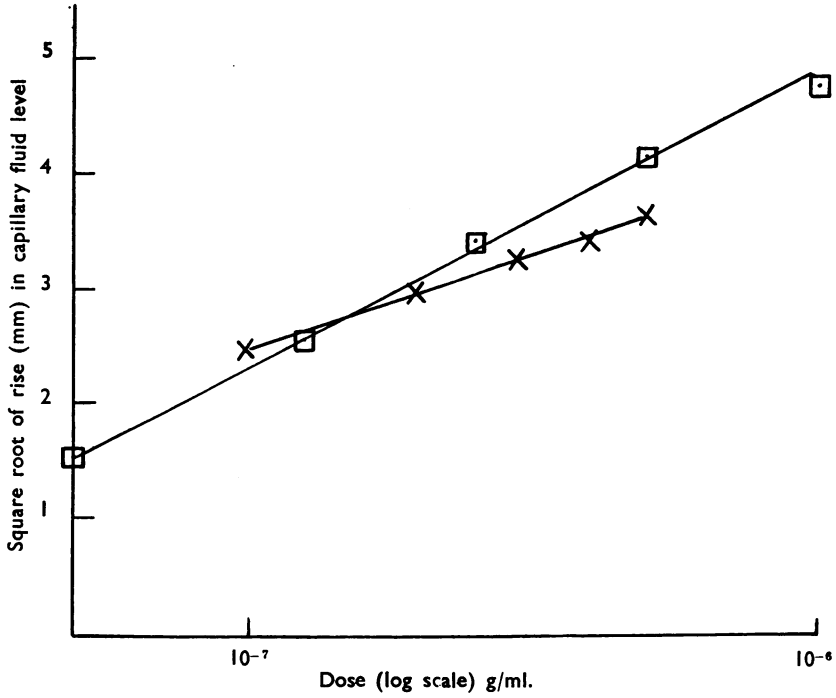

Fig. 2. Log dose-response lines obtained for 5-HT on a rat tracheal preparation, and for ACh on a guinea-pig tracheal preparation.

Three guinea-pigs were sensitized by the subcutaneous injection of 15 mg of crystalline egg albumin. Three weeks later tracheal preparations from these animals were tested with egg albumin, using histamine as a control. Concentrations of 10^{-6} to 10^{-5} egg albumin produced a rapid contraction after about a minute delay, equal to the contractions produced by approximately 10^{-6} final concentration of histamine.

Barium chloride. A final concentration of 0.1% barium chloride produced a maximal constrictor response in both the rat and guinea-pig trachea.

Atropine. Atropine alone had no action on the isolated intact trachea from rat or guinea-pig. However, its interaction with acetylcholine or 5-hydroxytryptamine on this preparation was similar to that seen with intestinal muscle. For example, on a rat tracheal preparation a final concentration of 5×10^{-8} atropine in the perfusion fluid almost abolished the contraction to 2×10^{-7} acetylcholine, but did not affect the response to 5-hydroxytryptamine (10^{-6}). Increasing the concentration of atropine to 5×10^{-7} completely inhibited the response to 10 times the initial dose of acetylcholine, and reduced the 5-hydroxytryptamine response to one tenth of the initial value. This is similar to the effect of atropine on intestinal muscle where contractions due to acetylcholine are inhibited by lower doses of atropine than are those due to 5-hydroxytryptamine (Robertson, 1953 ; Rapport & Koelle, 1953). The effect of atropine was slowly reversible. The contraction caused by barium chloride on the isolated trachea was unaffected by the presence of atropine in the bath.

Adrenaline. Only the guinea-pig trachea responded to adrenaline. Very small concentrations of adrenaline (10^{-9}) could be detected on good preparations and graded responses to different doses were obtained (Fig. 3). However, although the

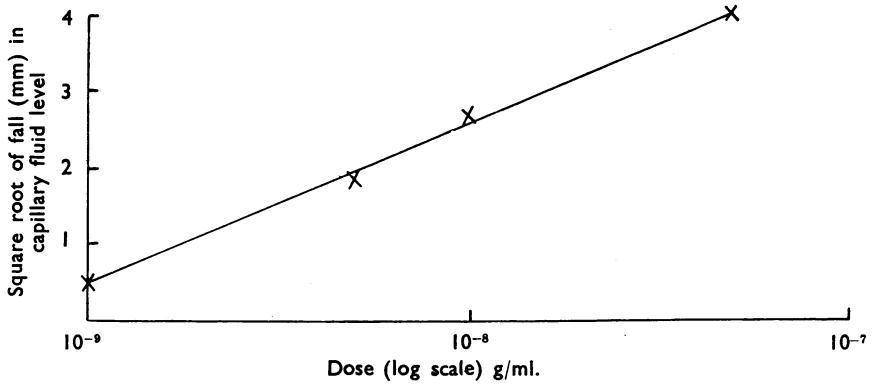

Fig. 3. Log dose-response line obtained for adrenaline on a guinea-pig tracheal preparation.

response was rapid in onset the maximal relaxation of the muscle was not completed until after some 5 min contact with the drug in 5 experiments, and 3 min in a further 3 trials. After washing, recovery took a further 10 to 15 min.

In the rat, adrenaline in concentrations up to 10^{-3} failed to cause any dilatation, nor did it inhibit the response to the bronchoconstrictor agents tested in this species.

Aminophylline. The rat trachea failed to respond to aminophylline also. In the guinea-pig aminophylline caused dilatation in concentration of the order of 10^{-5} to 10^{-4}. Its time course of action was similar to that of adrenaline on this preparation.

The assay of unknown solutions on the isolated trachea

The responses of the isolated trachea to pharmacological agents indicated that it was a suitable preparation for the bioassay of drugs affecting the smooth muscle of the respiratory tract. The responses to any given dose remain uniform for some hours and a linear relationship was found between the response and the logarithm of the dose. The threshold sensitivity of the preparation to active agents was high and well within the physiological range.

Assay procedure. Several trial doses of the reference standard were given to determine the doses necessary to produce suitable and consistent responses within the linear log dose-response range, between 10% and 90% of the maximum response. Dilutions were made to obtain a standard high dose and a standard low dose. Trial doses of the unknown solution were also given and the concentration of the unknown solution was then adjusted to be as nearly equivalent to that of the standard solution as possible. The unknown solution had been previously diluted from the stock standard by a co-worker. Two doses of unknown dilution equal in volume to those of the standard were selected and used as the unknown high and low dose. The ratio of high dose to low dose was the same for both the standard and unknown solution.

These doses of standard and unknown solution were then added to the organ bath in a Latin square arrangement to eliminate as much error as possible from variations in the sensitivity of the preparation during the course of the assay.

Four responses to each of the four doses were obtained, and the concentration of the unknown solution and the limits of error of the experiment calculated according to the general statistical analysis described by Schild (1942) with a modification for the Latin square design. This order of dosage imposes further restrictions upon the assay (Schild, 1942); thus in the analysis of variance the source of variation for "between groups" in Schild's analysis is divided into 2 sources of variation, one between blocks and one between order of doses. The remainder of the statistical analysis is identical with that used by Schild (1942).

The square roots of the responses obtained in the above manner for the assay of acetylcholine on the rat trachea are shown in Table 2, and Table 3 gives the analysis of variance obtained from the data in Table 2. The regression is highly

TABLE 2

RESPONSES TO DOSES OF ACETYLCHOLINE APPLIED TO THE TRACHEA IN A LATIN SQUARE ARRANGEMENT

S_H=standard high dose, S_L=standard low dose. U_H=unknown high dose, U_L=unknown low dose. Each group of four, in order of dose, constitutes one row, and the first numerals in each group (i.e. nos. 1, 5, 9, 13) constitute one column, giving a total of 4 columns and 4 rows

Order of doses	Dose	$\sqrt{\text{Rise in mm}}$ of fluid level in capillary
1	S_H	2·74
2	S_L	1·73
3	U_H	2·64
4	U_L	1·58
5	S_L	1·58
6	S_H	2·45
7	U_L	1·73
8	U_H	2·55
9	U_H	2·35
10	U_L	1·58
11	S_H	2·24
12	S_L	1·22
13	U_L	1·22
14	U_H	2·12
15	S_L	1·41
16	S_H	2·24

TABLE 3

ANALYSIS OF VARIANCE OF DATA OF TABLE 2

Source of variation	Sum of squares	Degrees of freedom	Mean square	F ratio
Between rows	0·0250	3	0·0083	
Between columns	0·4671	3	0·1557	
Between standard and unknown	0·0016	1	0·0016	
Regression	3·3124	1	3·3124	163·17
Deviation from parallelism	0·0020	1	0·0020	0·098
Error	0·1221	6	0·0204	
Total	3·9302	15		

significant while deviations from parallelism are very slight. The mean square for blocks is significant at the 5% level, but that for the dose order is not significant.

Assays have also been performed on the guinea-pig trachea using unknown concentrations of histamine and adrenaline. In the analysis of variance for these assays also the F ratio for regression and deviation from parallelism passed at the 1% level, showing the validity of the assay.

Table 4 summarizes the experimental details used in these assays and the experimental error. In every case the true potency fell well within the limits of error of the found potency.

TABLE 4

ASSAYS PERFORMED ON THE ISOLATED TRACHEAL PREPARATION

Drug	Standard high dose μg/ml.	Standard low dose μg/ml.	Contact time min	Interval between successive doses min	True activity of unknown solution μg/ml.	"Found" activity of unknown solution μg/ml.	Limits of error (P=0·95) μg/ml.
Acetycholine	3·0	1·5	1·5	6·0	16·39	15·24	13·45–17·38
Histamine	10·0	5·0	2·5	9·0	42·86	46·01	37·88–55·88
Adrenaline	0·1	0·05	3·0	13·0	0·857	0·813	0·574–1·152

The assay of constrictor agents using this experimental design was found to be practicable and to give a reasonable degree of accuracy. The assay of adrenaline proved more laborious, owing to the length of the recovery time after washing out each dose. This limited the number of doses which could be given before the preparation deteriorated, thus limiting the accuracy of the assay.

DISCUSSION

The isolated intact trachea has been used in a reliable and sensitive method for screening and comparing drugs active on the smooth musculature of the respiratory tract. The early experiments of Bert (1870) and Williams (1840) used isolated lungs and trachea exposed in air and attached to a manometer, but since then most work on broncho-active substances has been done either on intact preparations using the Konzett & Rössler (1940) method or similar techniques, or on strips of bronchial muscle as described by Trendelenburg (1912) and modified for small animals by using the tracheal chain preparation (Castillo & de Beer, 1947). Although widely used this latter method has the disadvantages of being time-consuming and not easy to handle. The isolated whole trachea fitted with a capillary tubing appears to react to the compounds tested in the same manner as the tracheal chain preparation, but sensitivity is more than doubled. The maximum displacement of fluid, which corresponds to roughly half the measured total capacity of the trachea, is large in view of the presence of the cartilaginous rings. Owing to the ease of manipulation experiments may be quickly performed and the method is suitable for use on small laboratory animals.

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