Brit. J. Pharmacol. (1959), **14**, 158.

ASSAY OF SUXAMETHONIUM AND LAUDEXIUM ON THE FROG RECTUS ABDOMINIS

BY

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(RECEIVED NOVEMBER 26, 1957)

Suxamethonium, like acetylcholine, causes contracture of the isolated rectus abdominis muscle of the frog, whereas laudexium antagonizes this effect. These actions may be used to assay both drugs with reasonable speed and fiducial limits, provided that changes in responsiveness of the preparation during the course of assay are met by suitable design and analysis. Small quantities of suxamethonium and laudexium may be assayed in certain body fluids.

As chemical methods of estimating suxamethonium and laudexium are not specific or sensitive enough to study their keeping properties or *in vivo* metabolism, we have sought suitable methods of bioassay. We considered that *in vitro* methods would be preferable to *in vivo*, for reasons of speed, economy of material, precision and Home Office regulations. The isolated rat phrenic nerve diaphragm method of Mogey, Trevan, and Young (1949) cannot be employed as the rat is particularly insensitive to laudexium (Collier and Macauley, 1952). This led us to explore the use of the frog rectus abdominis preparation, with which Norton and de Beer (1954) had estimated suxamethonium, and de Jalon (1947) and Mahfouz (1949) tubocurarine.

The method of Norton and de Beer (1954), using *Rana pipiens*, entailed a recovery time of 60 to 90 min. between successive doses to the same preparation. Using *Rana temporaria*, we have developed a general method for the assay of suxamethonium, with a recovery period of 4.5 to 5 min. This method is described below, together with its extension to the assay of laudexium.

METHODS

Preparation.—All experiments were performed with *Rana temporaria*, of British or Continental origin. Continental frogs were rather more sensitive to acetylcholine and recovered more readily from laudexium. The rectus abdominis muscle was suspended in a 10 ml. organ bath at room temperature in an oxygenated Ringer solution of the following composition: NaCl 7.0 g.; KCl 0.14 g.; $CaCl_2$ (anhydrous) 0.12 g.; $NaHCO_3$ 0.2 g; glass distilled water to 1 l.

The movements of the muscle were recorded on smoked paper by an isotonic lever with a frontal writing point. This lever, which magnified the muscular movement about tenfold, applied a load of 2 to 3 g. to the muscle.

Chemicals.—Specimens of suxamethonium chloride and laudexium methylsulphate were set aside as reference standards. The chlorides of choline, succinylmonocholine, suxamethonium, acetylcholine and tubocurarine, and the methylsulphate of laudexium were used throughout, and weights are given in terms of base. These drugs were dissolved in Ringer solution and doses given in volumes not exceeding 0.5 ml. Doses of drugs added to the organ bath are expressed as weights in 10 ml.

Estimation in Body Fluids. — In estimating suxamethonium in rabbit blood, eserine sulphate was added both to the blood and to the standard suxamethonium in Ringer solution to give a concentration of 10 μg./ml. Laudexium was extracted from human urine by the method that Mahfouz (1949) used for tubocurarine. The urine was evaporated to dryness, the residue extracted with absolute ethanol, the alcoholic extract then evaporated to dryness and the residue taken up in Ringer solution.

Statistical Methods. — Analysis of variance and covariance and assessment of potencies with fiducial limits were carried out by standard statistical methods for parallel line assays. Fiducial limits corresponded to $P=0.95$.

RESULTS

Suxamethonium

Action of Drug.—Choline, succinylmonocholine and suxamethonium stimulate the frog rectus muscle (Fig. 1). The potency of suxamethonium was about 500 times that of choline and about 100 times that of the monocholine.

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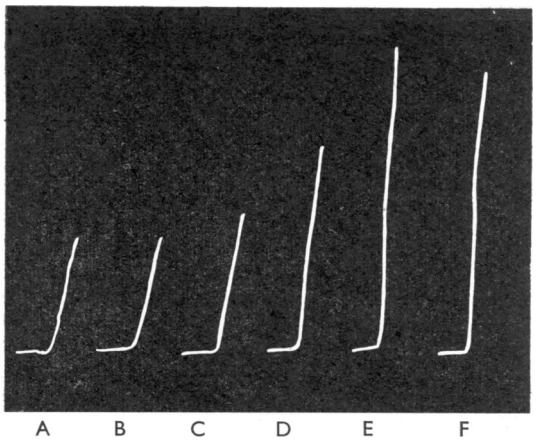

FIG. 1.—Frog rectus abdominis muscle in a 10 ml. bath. Responses to 1.5 min. of exposure to choline, succinylmonocholine, and suxamethonium. At **A**, 5 mg. and **D**, 7.5 mg. choline; at **B**, 1 mg. and **E**, 1.5 mg. succinylmonocholine; at **C**, 10 μg. and **F**, 15 μg. suxamethonium.

Dose Cycle.—The curve illustrated in Fig. 2 was obtained with exposures of the rectus muscle to suxamethonium for 2 min. followed by recovery in Ringer solution for 5 min. A dose cycle of 6 min. (1.5 min. exposure and 4.5 min. recovery) was sufficient for most preparations, and was used on many occasions.

Dose/Response Curve.—When a series of doses of different magnitude was given and the resulting contractures plotted as ordinates against log dose, a typical sigmoid curve was obtained (Fig. 2).

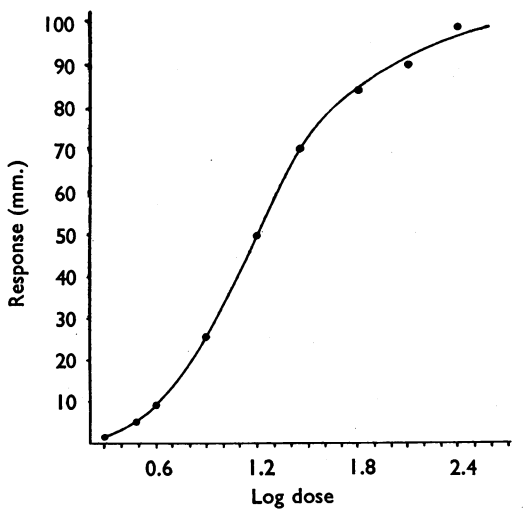

FIG. 2.—Dose response curve for suxamethonium using the frog rectus abdominis muscle. Abscissa, log dose in μg. of suxamethonium in a 10 ml. bath.

The central portion of the curve, between about 25% and 75% maximal response, was rectilinear and thus provided a basis for linear regression analysis.

Response Fall-off.—When dose cycles of 6 or 7 min. were used, the response of the preparation fell off with repeated doses. In the typical experiment illustrated in Fig. 3, sixteen doses, each of 15 μg. of suxamethonium, were given in a 6 min. cycle. The sixteenth response was 49% of the first, and regression analysis showed that the fall-off was practically rectilinear. After the 16 doses had been given the recovery period was extended to 28.5 min., making a 30 min. cycle, and 3 further doses were administered. The nineteenth response was 81% of the first.

The question arose whether the response to one dose was affected by the magnitude of the preceding response. To answer this, we performed the experiment illustrated in Fig. 4, in which alternate pairs of low and high doses of suxamethonium were given. Regression analysis showed that, within each dose group, the plot of response against time yielded a rectilinear fall-off over the assay period, although the slope of the regression line through the high doses was the steeper indicating that the true nature of the fall-off was probably exponential. Analysis also showed that the regression line through the first responses of each pair in a given dose group was parallel to that through the second responses of each pair in the same group. This showed that the size of one response did not affect that of the next.

A second experiment using alternate pairs of doses consisting of 10.0 μg. and 5.0 μg. of suxamethonium yielded similar results. These findings will be discussed in greater detail later.

Assay Design and Analysis.—Using either 6 or 7 min. dose cycles, we adopted a (2+2) pattern of assay, in which high and low doses of test and standard preparations were given in orders based on a 4×4 randomized block, a 4×4 Latin square or a 25 dose serially balanced design (Finney and Outhwaite, 1956).

The protocol of a typical assay based on a Latin square is given in Table I. The results of this assay and of assays of similar solutions of suxamethonium on randomized block and serially balanced designs are summarized in Table II.

Check on Method.—Assay of a solution containing 50 mg./ml. suxamethonium in Ringer solution gave a value of 52.30 mg./ml. with fiducial limits of 49.27 to 55.54 mg./ml. (94.2 to 106.2%). A

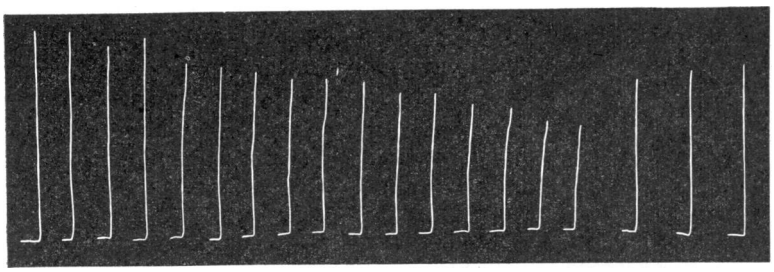

FIG. 3.—Frog rectus abdominis muscle in 10 ml. bath. Effect of repeated doses of 15 μg. of suxamethonium. From the first to the sixteenth dose, a dose cycle of 6 min. (1.5 min. exposure and 4.5 min. recovery) was used. From the sixteenth to the nineteenth dose a 30 min. cycle (1.5 min. exposure and 28.5 min. recovery) was employed.

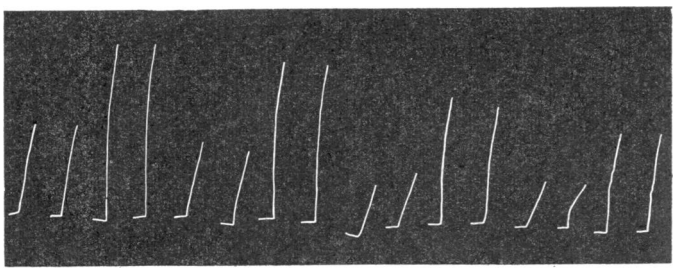

FIG. 4.—Frog rectus abdominis muscle in 10 ml. bath. Effect of repeated alternate pairs of doses of 5.0 μg. and of 7.5 μg. of suxamethonium. A dose cycle of 6 min. (1.5 min. exposure and 4.5 min. recovery) was used throughout.

TABLE I

PROTOCOL OF AN ASSAY OF SUXAMETHONIUM BY MEANS OF THE FROG RECTUS PREPARATION, USING A LATIN SQUARE DESIGN

Values indicate height of response in mm. SL=0.15 ml. and SH=0.20 ml. of 50 μg./ml. solution of standard. TL=0.15 ml. and TH=0.20 ml. of 1 in 2 dilution of test preparation.

	SL	SH	TL	TH	Total
	55	83	49	73	260
	SH	SL	TH	TL	
	78	51	67	39	235
	TL	TH	SH	SL	
	36	60	66	37	199
	TH	TL	SL	SH	
	58	34	38	62	192
Total	227	228	220	211	886

second experiment of the same type yielded similar results. A solution assayed by the rectus method at 29.00 mg./ml. with limits of 27.11 to 31.03 mg./ml. (93.5 to 107.0%) gave a value of 28.25 mg./ml. by the mouse rotating drum method of Collier, Hall and Fieller (1949).

Estimation in Urine and Blood.—Suxamethonium was added to human urine to give a concentration of 10 μg./ml. Assayed against suxamethonium dissolved in Ringer solution, this sample gave a value of 9.10 μg./ml., with limits of 8.21 to 10.01 μg./ml. (90.1 to 110.0%). Suxamethonium was added to eserinized rabbit blood to give a concentration of 10 μg./ml. and assayed against standard suxamethonium in eserinized Ringer solution. This gave a value of 11.14 μg./ml., with limits of 9.81 to 12.64 μg./ml. (88.1 to 113.5%). Both the above assays were performed with 16 dose randomized block designs.

Laudexium

Action of Drug.—Laudexium, like tubocurarine and unlike suxamethonium, failed to stimulate the frog rectus muscle, even when 1 mg. was added to the bath. Laudexium also resembled tubocurarine in antagonizing the stimulant action of acetylcholine and suxamethonium on this preparation. In the experiment illustrated in Fig. 5, 0.5 μg. of laudexium reduced the response to acetylcholine rather more effectively than did an equal dose of tubocurarine. De Jalon (1947) has pointed out that the inhibition by tubocurarine of the response to acetylcholine persists even after washing the tubocurarine out of the bath. A similar effect could be seen after laudexium.

The extent to which a given dose of laudexium reduced the contracture varied with the dose of agonist (acetylcholine or suxamethonium) used. It is evident from Fig. 5 that the lower the dose of agonist, the more sensitive the preparation to laudexium. With too small a dose of agonist, however, repeated responses to a given dose of laudexium proved inconsistent. On the basis of a number of experiments, we decided to use for assay purposes just sub-maximal doses of agonist (1 to 5 μg. of acetylcholine or 3 to 15 μg. of suxamethonium, according to the sensitivity of the preparation).

Arrangement of Doses Within a Cycle.—As already noted, even after laudexium has been washed out of the bath, it depresses the response to agonist. We call this the "hangover" effect. Taking this effect into account, doses within a cycle were arranged in the following sequence: *agonist, wash, laudexium, agonist, wash, agonist,*

TABLE II

EFFECT OF DESIGN ON PRECISION OF ASSAY OF SUXAMETHONIUM BY MEANS OF THE FROG RECTUS PREPARATION

Three designs were used to assay similar solutions.

Design of Assay	Potency (μg./ml.)	95% Fiducial Limits (μg./ml.)	95% Fiducial Limits (%)	Slope (b)	Variance (s^2)	Index of Precision (s/b)
Latin square	92·81	91·23–94·39	98·3–101·7	26·00	1·50	0·047
Randomized block	92·41	87·60–97·40	94·8–105·4	21·38	11·40	0·158
Serially balanced	91·75	86·43–97·44	94·2–106·2	29·83	24·63	0·166

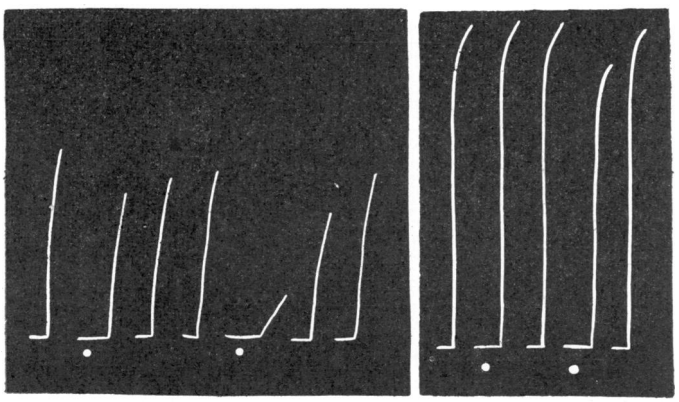

FIG. 5.—Frog rectus abdominis muscle in 10 ml. bath. Successive responses to 2 min. exposure to acetylcholine, **A** to **G**, 1.5 μg. (lever magnification × 15), **H** to **M**, 6 μg. (lever magnification × 5). At white dots at **B** and **J**, 0.5 μg. of tubocurarine and at **E** and **L**, 0.5 μg. of laudexium were added 1.5 min. before acetylcholine.

wash, etc. The sequence *agonist, wash, etc.*, was continued until no further increase in response to agonist could be seen, that is, until the hangover effect was apparently removed. The last response to agonist was termed a stabilized response and became the first dose of the next cycle. If the interpolated doses of agonist were omitted or reduced to one, the response to agonist in the presence of laudexium disappeared after a few doses of laudexium.

Each exposure of the preparation to agonist lasted 1.5 min. Laudexium was added 1.5 or 2 min. before agonist, giving a total exposure to laudexium of 3 or 3.5 min.

Choice of Metameter.—The stabilized response to agonist and the immediately following response to agonist in the presence of laudexium were measured. The % reduction was generally taken as response metameter. We have also used the metameter of response to agonist in the presence of laudexium (or reduced response). The results of using both metameters are compared in Table V and discussed below.

Dose/Response Curve.—When the logarithm of the laudexium dose was plotted against % reduction, we obtained a typical sigmoid curve with a central rectilinear portion, corresponding to a reduction of 20 to 80%. For assay purposes, doses of laudexium were chosen to produce a response within the rectilinear portion.

Using reduced response as alternative metameter, we obtained a sigmoid dose/response curve with a negative slope. The central portion of this curve was also rectilinear within the limits of 80 to 20% response. It was therefore possible to use either metameter in analysing results.

Effect of Fall-off in Response to Agonist.—We have already described the fall-off in response to suxamethonium with repeated doses. Acetylcholine produced the same effect, though to a lesser extent, as can be seen from the totals of columns and rows in Table III. This table also shows that, while the reduced response metameter falls off as the assay proceeds, that of % reduction rises slightly.

Assay Design and Analysis.—Using the above arrangement of doses within a cycle, we adopted a (2+2) pattern of assay with high and low doses of test and standard laudexium. These were given in orders based on a randomized block design (usually 4 × 4) or a 4 × 4 Latin square. The protocol of a typical Latin square assay of laudexium is given in Table III. Table IV contains the analysis of covariance and the results of these analyses are summarized in Table V. Assays usually gave fiducial limits lying within the requirements for tubocurarine of the *British Pharmacopoeia* (1953).

Check on Method.—A solution containing 12 mg./ml. laudexium in Ringer solution assayed at 11.65 mg./ml., with fiducial limits of 11.28 to 12.03 mg./ml. (96.8 to 103.3%). Other experiments

yielded similar results. The solution assaying at 11.65 mg./ml. by the frog rectus gave a value of 11.9 mg./ml. by the mouse rotating drum method.

Estimation in Urine.—We investigated whether the method of Mahfouz (1949) could be applied to laudexium. We added laudexium to human urine to give a concentration of 10 μg./ml., and in one experiment, based on an 8 dose randomized block design, we found 10.86 μg./ml. with limits of 8.80 to 13.85 μg./ml. (78.3 to 127.6%). Two further experiments gave similar results.

TABLE III

PROTOCOL OF AN ASSAY OF LAUDEXIUM BY MEANS OF THE FROG RECTUS PREPARATION USING A LATIN SQUARE DESIGN

ACh=response to 2 μg. of acetylcholine in mm. ACh+La=response to acetylcholine in presence of laudexium in mm. SL=0.1 ml. and SH=0.2 ml. of 10 μg./ml. solution of standard preparation. TL=0.1 ml. and TH=0.2 ml. of 1 in 2,000 dilution of test preparation. % Redn.=% reduction of response.

	SH	TH	TL	SL	Total
ACh	40	42	39	41	162
ACh+La	17	13	35	30	95
% Redn.	57·5	69·0	10·3	26·8	163·6
	TL	SL	SH	TH	
ACh	38	39	40	36	153
ACh+La	36	32	10	11	89
% Redn.	5·3	17·9	75·0	69·4	167·6
	TH	SH	SL	TL	
ACh	36	35	36	33	140
ACh+La	10	10	31	25	76
% Redn.	72·2	71·4	13·9	24·2	181·7
	SL	TL	TH	SH	
ACh	38	35	34	36	143
ACh+La	32	31	11	10	84
% Redn.	15·8	11·4	67·6	72·2	167·0
Total ACh	152	151	149	146	598
,, ACh+La	95	86	87	76	344
,, % Redn.	150·8	169·7	166·8	192·6	679·9

TABLE V

EFFECT OF CHOICE OF RESPONSE METAMETER AND METHOD OF ANALYSIS ON RESULTS OF TWO ASSAYS OF LAUDEXIUM, USING A LATIN SQUARE DESIGN

Assay No.	Response Meta-meter	Method of Analysis	Potency Ratio	95% Fiducial Limits (Ratio)	95% Fiducial Limits (%)
1	% Reduction	Variance	1·408	1·191–1·664	84·6–118·2
		Covariance	1·443	1·231–1·690	85·3–117·1
	Reduced response	Variance	1·557	1·267–1·914	81·4–122·9
		Covariance	1·329	1·116–1·584	84·0–119·2
2	% Reduction	Variance	0·967	0·879–1·063	90·9–110·0
		Covariance	1·015	0·923–1·117	90·9–110·0
	Reduced response	Variance	1·000	0·917–1·091	91·7–109·1
		Covariance	0·969	0·882–1·067	90·9–110·1

DISCUSSION

Contaminants.—Since choline and succinylmonocholine are possible contaminants of suxamethonium, their very low potency on the frog rectus is important. For example, if as much as 10% succinylmonocholine were present in a specimen of suxamethonium, the sample would assay at 90.1%, as against 90% if the contaminant were inert. With choline, the bias would be even less, and we therefore consider that contamination with these two substances is unlikely to bias estimates of potency.

Response Fall-off and Assay Design.—An assay method is required which allows a sufficiently large number of doses to be fitted into a working day, at the same time compensating for any fall-off by a suitable method of design and analysis.

Fig. 2 shows that fall-off is reduced by extending the recovery period (see, too, Norton and de Beer, 1954). We consider it more efficient, however, to give 16 doses in a 6 min. cycle and

TABLE IV

ANALYSIS OF COVARIANCE OF THE LAUDEXIUM ASSAY PRESENTED IN TABLE III

M.S.′ and F' are mean square and variance ratios of the responses adjusted for covariance. N.S.=Not significant.

Source	d.f.	S.S. (x^2)	xy	S.S. (y^2)	M.S.	F	M.S.′	F'
Preparations	1	9·00	15·8250	27·82563				
Regression	1	0·00	0·0000	11,486·48602				
Parallelism	1	2·25	9·4875	40·00563	40·00563	1·10668 (N.S.)	5·30271	<1·0 (N.S.)
Doses	3	11·25	25·3125	11,554·31188				
Rows	3	75·25	−45·0125	48·15187	16·05062	0·44401 (N.S.)		
Columns	3	5·25	−31·4875	222·43188	74·14396	2·05106 (N.S.)		
Error	6	12·00	30·5250	216·89375	36·14895		27·84915	
Total	15	103·75	−20·6625	12,041·78938				

Source	Ex^2	Exy	Ey^2	d.f.	Ey'^2	M.S.′
Parallelism	2·25	9·4875	40·00563	1	5·30271	5·30271
Error	12·00	30·5250	216·89375	5	139·24579	27·84915
Parallelism+error	14·25	40·0125	256·89938	6	144·54850	

compensate for fall-off. This becomes particularly important in the laudexium assay when several cycles of *agonist, wash,* may be required to be interpolated between doses of laudexium.

Analysis of the 25-dose serially balanced design of Finney and Outhwaite (1956) failed to reveal a residual effect from a previous dose other than the fall-off described. This is in confirmation of our observations on the assays involving repeated alternate pairs of high and low doses of suxamethonium.

For these reasons we believe that the 4×4 Latin square design is basically the most efficient for the suxamethonium assay, although a somewhat more refined analytical technique than the classical methods for dealing with parallel line assays may be required.

In the laudexium assay we preferred acetylcholine as agonist, because the fall-off was less than with suxamethonium. The response to acetylcholine in the presence of laudexium fell off less than that to acetylcholine alone, both trends being approximately linear over the assay period. This is in further confirmation of the experiment involving repeated alternate pairs of doses of suxamethonium where a similar effect was observed (see Fig. 4). This leads to the slight rise in the percentage reduction metameter already mentioned.

In this assay, as the change in response with time is only relatively slight we have found it advantageous to use a 4×4 randomized block design. In some earlier assays, we obtained rather wide fiducial limits and for a time changed over to Latin square design obtaining better limits. We now feel that this improvement was coincidental and have adopted randomized blocks as a routine.

In a few laudexium assays we attempted to reduce further the error variance of the Latin square by covariance analysis, using the stabilized response to acetylcholine as concomitant variate. We found no consistent gain in precision (Table V) and, in view of the computational labour involved, do not use this method as a routine.

The alternative metameter of reduced response, previously defined for this assay, showed considerable fall-off with time. Using this metameter and applying covariance analysis with the same concomitant variate as above we obtained in some assays a slight gain in precision and rather wider limits in others (Table V).

For the above reasons we have adopted the percentage reduction in response as metameter and are now using randomized block design. As in the suxamethonium assay it may be advantageous to use slightly more rigorous methods of analysis than the classical methods for parallel line assays. These will be discussed in a later publication.

Hangover in the Laudexium Assay.—As we have described, the hangover effect can be largely overcome by interpolating doses of agonist after laudexium has been washed from the bath. Since recovery from hangover operates in the opposite direction to fall-off, the choice of a stabilized response to agonist depends to some extent on the operator. We therefore sought an alternative method of compensating for hangover. One such method would be to omit interpolated doses of agonist and to apply the serially balanced design of Finney and Outhwaite (1956). As we have already described, omission of interpolated doses of agonist leads to disappearance of the response in the presence of laudexium and therefore this method could not be used.

We wish to thank Mrs. P. L. Hutchings and Mrs. G. M. J. Hayes for assistance with experiments, and the Directors of Messrs. Allen and Hanburys Ltd. for permission to publish this paper.

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