BIOLOGICAL ASSAY EMPLOYING EIGHT ISOLATED

TISSUE STRIPS ILLUSTRATED BY THE

ESTIMATION OF ERGONOVINE

By

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<div align="right">Robert E. Thompson</div>

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INTRODUCTION

The usual method of conducting a biological assay on an isolated tissue preparation is to vary the doses of standard and unknown until doses of the two preparations are found which show equal submaximal effect. This method does not lend itself readily to statistical analysis and the error of individual assays cannot therefore be accurately determined. The accuracy of a test of this kind is usually estimated by determining the sensitivity of the preparation to graded doses of the standard, that is, the ability to distinguish between doses, as in the U.S.P. XII method for assaying Posterior Pituitary Injection. (28). The results are often misleading because of chance variations of the responses and because of changing sensitivity of the preparation. It is the object of this paper to describe a new method for conducting a biological assay on an isolated tissue preparation in such a way that the data obtained are suitable for statistical analysis and the error of individual assays can be determined.

Two methods for conducting biological assays on isolated preparations, involving statistical analysis, have appeared in the literature. The first, published in 1940 by Morrell, Allmark and Bachinski (18), concerns the assay of the oxytocic activity of pituitary extract (posterior lobe) on the isolated uterus of the guinea pig. In the procedure described a guinea pig's uterus is divided into eight separate strips. The eight strips are mounted individually in a single tissue

chamber and attached to individual recording levers. Graded doses are administered and the presence or absence of response of the individual strips is recorded. From the data thus obtained calculation of the potency is carried out in the same way as for other "all or none" or "quantal" dosage response data (2) (3) (11) (16). Good results were obtained in the assays of extracts of posterior pituitary of "unknown" strength and the error of individual assays can be calculated.

The second method, published in 1942 by Schild (20), is illustrated by the estimation of histamine on an isolated intestinal strip from the guinea pig. In this procedure only one strip of tissue is used but the experiment is designed to make a statistical analysis possible.

The method of assay employed in the present work was developed and the assays completed without knowledge of Schild's paper (20). A subsequent study of his procedure, however, revealed that the design of his experiments was similar to that developed in the present study. In his experiments, successive groups of four responses are obtained from the repeated administration of four doses to a single tissue strip, while in the present experiments they are obtained from the repeated administration of four doses to a group of eight strips. The analysis for error presents similar problems. His method of eliminating differences among groups of responses from the estimation of error was found to be quite applicable to the data obtained from an assay by the procedure to be described.

The present study was undertaken as a result of observations and theoretical considerations evolving from the application of Morrell, Allmark and Bachinski's (18) "all or none" procedure for posterior pituitary employing guinea pig uterus to the estimation of ergonovine using isolated rabbit's uterus. It was observed that the average height of the responses of the eight uterine strips was rather constant in spite of the variability of the height of response of individual strips. There appeared also to be good regression to graded dosage.

These observations were recognized as similar to the conditions prevailing in a biological assay involving a measurable or quantitative response instead of an "all or none" or quantal response. A number of assays involving the use of a measurable or quantitative response have been developed, in which the extent of the response of individual animals is recorded for a given dose. Gaddum (11) points out "that if the response can be measured quantitatively it will only be necessary to use about half as many animals to attain a given degree of accuracy as would be necessary if the experimental data only indicated the proportion of animals in which the response exceeded some fixed threshold." It is for this reason that the present assays were designed to employ a quantitative response rather than the quantal response as used by Morrell, Allmark and Bachinski (18).

In the present assays the individual uterine strips correspond to the individual animals in other assays which

involve the use of groups of intact animals except that the various doses used are given to the same group of test objects instead of employing a different group for each dose. This procedure eliminates the "sampling error" which is the error due to the chance that groups of test objects of different sensitiveness have been used for the comparison. It, however, introduces the difficulty of changing sensitivity of the test objects at different periods during the assay. The design of the assay and the estimation of error must therefore take into consideration any variation in mean sensitivity of the uterine strips.

The material employed in this study is ergonovine, the physiologically active, water-soluble alkaloid of ergot. The presence of this alkaloid in ergot was suspected by Moir (17) and demonstrated by Thompson (26). It was isolated in pure form, independently, by Thompson (26), Kharasch and Legault (15), Dudley and Moir (9) and Stoll and Burkhardt (23). Its pharmacological action was reported by Thompson (27), Davis, Adair, Chen and Swanson (8), Chen, Swanson, Kleiderer and Clowes (6) and Brown and Dale (4). The alkaloid differs from the water-insoluble group (ergotoxine, ergotamine) in that it has a prompt oxytocic action when given by mouth (26) (25).

The salt, ergonovine maleate, is now official in the United States Pharmacopoeia (28) and is extensively used as a uterine stimulant. Ergonovine maleate is a chemical entity and the chemical tests and physical properties pre-

scribed by the Pharmacopoeia (28) are designed to insure its uniform potency.

The estimation of ergonovine in crude ergot or in impure preparations derived from ergot is complicated by the presence of the water-insoluble group of alkaloids (ergotoxine, ergotamine, etc.), the water-soluble, physiologically inactive isomer of ergonovine (ergometrinine) and possibly other ergometrinine-like substances. A number of methods for the chemical (colorimetric) estimation of ergonovine in crude ergot and its extracts have been proposed (1) (7) (13) (19) (21) but none of them has been definitely shown to measure only ergonovine activity as determined by biological means. They may measure other constituents (ergometrinine-like) and so give higher values than the actual ergonovine content.

Powell, Reagan, Stevens and Swanson (19) described a procedure for preparing an ergonovine solution from crude ergot and its fluidextract which is suitable for biological assay on the isolated rabbit's uterus or for colorimetric assay. They obtained values by the biological and colorimetric methods which agreed quite closely in many cases but on the average the colorimetric method gave higher values than the biological. The results are difficult to compare, however, because the error of individual assays was not determined.

Grove (12) described a procedure for separating ergonovine from mixtures of other known alkaloids of ergot

including ergometrinine. The procedure has not been shown to be suitable for the estimation of ergonovine in ergot or its extracts. Possibly other water-soluble (ergometrinine-like) substances would interfere with its successful application. No comparison with a biological method has been made.

In view of the inadequacy of chemical methods at the present time, it is necessary to measure ergonovine content of crude ergot or its extracts by a biological procedure. The suitability of the rabbit's uterus for measuring ergonovine in the absence of ergotoxine-like alkaloids has been demonstrated by Thompson (27), Swanson, Hargreaves and Chen (24) and Powell, Reagan, Stevens and Swanson (19). The United States Pharmacopoeia XII and the National Formulary VII do not recognize any method for the estimation of ergonovine content in ergot or its preparations.

The present work was undertaken primarily to develop an improved method for conducting a biological assay on an isolated tissue preparation which, with possible minor modifications, should be applicable to such biological assays as ergonovine on the isolated rabbit's uterus, solution of posterior pituitary on the isolated guinea pig's uterus, epinephrine on the rabbit's uterus, or any substance that can be estimated by its measurable effect on a suitable isolated preparation. The ergonovine-rabbit's uterus combination was selected in order to determine the suitability of the procedure for the biological assay of ergonovine. The

procedure should be useful for the estimation of ergonovine in ergot or its extracts as long as this is necessary. It should also be useful in proving or disproving the suitability of various chemical methods which have been or are to be proposed.

EXPERIMENTAL

Material for Assay. One lot of pure ergonovine maleate was obtained from Burroughs Wellcome and Company (U. S. A.) Inc. and another lot was generously supplied by the Eli Lilly Company.

A solution to serve as a standard was prepared for each assay by transferring 5.0 cc. of a stock solution (1 mgm. per cc. in 0.1% aqueous tartaric acid) to a 25 cc. volumetric flask and making to volume with 0.1% aqueous tartaric acid, thus providing a concentration of 0.2 mgm. of ergonovine maleate per cc.

The unknowns were solutions prepared by transferring an accurately measured quantity of the stock solution to a 25 cc. volumetric flask and making to volume with 0.1% tartaric acid solution. To eliminate personal bias the operator was not informed of the concentration of the unknowns until after the assay had been completed and the results calculated. The same calibrated pipette, volumetric flask, and stock solution was used for preparing the standard and unknown for one assay. The stock solutions were stored in a refrigerator in the dark for periods not exceeding three

weeks when not in use.

Animals. Female rabbits were treated with stilbestrol according to the method described by Wick and Powell (30) who found that estrogenic therapy with estrone or stilbestrol "rendered the immature rabbit's uterus more irritable, more reliable in response and thus more utilizable in the assay of ergonovine." The treatment consisted of injecting subcutaneously a total dose of nine gamma of stilbestrol in six fractions, one fraction being injected every other day. The stilbestrol solution was prepared by first dissolving 100 mgm. in 100 cc. of peanut oil and then diluting 1.0 cc. of this solution to 100 cc. with peanut oil, thus making a concentration of ten gamma per cc. Each injection consisted of 0.15 cc. of this solution which contains the required fractional dose of 1.5 gamma. Two days after the last injection the animal was killed and the uterus removed.

Some of the rabbits used in the present work were larger than those used by Wick and Powell (30). It was thought that statistical analysis of the assay data would reveal information as to the suitability of different types of uteri for the procedure to be described. The larger, more mature rabbits usually provided larger, more muscular uteri than the smaller, less mature rabbits. The uteri of all rabbits within the limits chosen were successfully utilized. The weights of the rabbits and the weights of the uteri used in assays 9 to 24 are recorded in Table XXVIII for the purpose of comparison with the expression, σ/b, derived from the

assay data. The rabbits varied in weight from 1.7 Kg. to 3.1 Kg. and the weights of the whole uteri (prepared as described under preparation of muscle) varied from 1.8 Gm. to 7.2 Gm.

All rabbits were purchased as nulliparous females and none of them showed evidence on autopsy of having previously had a litter or of pregnancy. They were kept in individual cages during the stilbestrol treatment.

Preparation of the Muscle. Eight strips of uterine muscle from one horn of a rabbit's uterus were employed for each assay. The animal was sacrificed by a blow on the head and permitted to bleed from the mouth and nostrils. The uterus was removed immediately and placed in Van Dyke and Hasting's solution. All attached tissue was cut away and the horns separated at their junction. The two horns were then weighed in a small, tared beaker containing Van Dyke and Hasting's solution.

Eight strips were prepared by cutting one horn transversely into four segments of approximately equal length. Each segment was opened out by cutting longitudinally along the attachment of the broad ligament. Another longitudinal cut then divided each segment into equal portions. The remaining horn was placed in Van Dyke and Hasting's solution in a refrigerator for preservation. Some uterine horns were used one to three days after removal from the animal.

An alternative procedure for preparing eight strips from a uterus of small diameter would be to use one horn for the preparation of four strips. Each horn may be div-

ided into four segments by cutting transversely and these eight strips used without halving them. This would avoid injuring a relatively large portion of the tissue caused by longitudinally cutting each segment of a small uterus. Strips for only one assay would be obtained from each animal by this procedure.

The eight uterine strips were mounted for recording their activity as indicated in the description and explanation of the apparatus.

Van Dyke and Hasting's Solution. A modified Van Dyke and Hasting's solution was employed. The composition differs from the original (29) in that the magnesium chloride concentration has been reduced to one-half as used by Morrell, Allmark and Bachinski (18). These authors recommend this modification because they found no spontaneous activity of uterine strips suspended in this solution. A further modification was the addition of one gram of anhydrous dextrose per liter. The dextrose was added as a nutrient for the muscle as is customary in working with active isolated tissues (22).

In the present work the solution was prepared from stock solutions A and B--similar to the procedure given by Burn (5) for the preparation of Tyrode's solution. All of the salts were weighed on an analytical balance. C. P. grade chemicals were used. The composition and method of preparation of the solution employed is given by the following scheme:

Solution A	Grams	Solution B	Grams
NaCl	263.60	$Na_2HPO_4 \cdot 12H_2O$	10.08
KCl	18.40	$NaH_2PO_4 \cdot H_2O$	4.60
$CaCl_2 \cdot 2H_2O$	2.649	$NaHCO_3$	100.80
$MgCl_2 \cdot 6H_2O$	7.686		
Distilled water to 2000. cc.		Distilled water to 2000. cc.	

Add 400 cc. solution A and about 7 liters of distilled water to a suitable container. Then add 400 cc. solution B and distilled water to make 8.0 liters. Add eight grams of anhydrous dextrose and adjust the pH to 7.4 by bubbling in carbon dioxide.

The saline was freshly prepared immediately before each assay. Air containing carbon dioxide at a tension of fifty millimeters of mercury was used for supplying oxygen and stirring the solution in the tissue chamber.

Apparatus. The apparatus is essentially the same in principle as that described by Morrell, Allmark and Bachinski (18). Considerable modification in structure was adopted to facilitate the mounting of the uterine strips in the tissue chamber. A 275 cc. tissue chamber was used in place of the 600 cc. chamber employed by Morrell, Allmark and Bachinski (18). This reduces the amount of drug necessary to carry out an assay. This is important for the application of the procedure to the assay of ergonovine in crude ergot and its extracts because it avoids the necessity for extracting samples of impractical amounts of drug. At the same time considerably less saline is required. A more convenient

arrangement was used for withdrawing the frontal writing points between doses; the saline was measured before entering the warming chamber; and the arrangement of the inlet and outlet tubes minimizes the mixing of solution in the warming chamber as the saline is renewed. No difference was observed in the temperatures of the outgoing and ingoing saline in the tissue chamber. This arrangement eliminates the necessity for pressure to force the saline from the warming chamber to the measuring bottle as used by Morrell, Allmark and Bachinski (18).

The apparatus, shown in Figures 1a, 1b and 1c, consists of the 275 cc. tissue chamber B in which the eight uterine strips are suspended. It has an inlet and outlet and is fastened in the constant temperature bath A of about ten liters capacity. This bath is kept at the required temperature (37.8 $\pm$ .2° C.) by two 250 watt heaters (not shown in figures) inserted into the bottom of the bath and connected with a 110 V. A. C. outlet, a stirring paddle SP, and a mercury thermoregulator MT. The eight liters of Van Dyke and Hasting's solution are kept in an aspirator bottle (not shown) on a shelf about three feet above the bath A. The outflow from this bottle is controlled by a stopcock (not shown). As required, the solution is run into the measuring cylinder C, which in this case consists of a condenser jacket of 250 cc. capacity. Any suitable vessel which will measure the required volume sufficiently accurately may be used. If the volume of saline could be measured accurately enough in
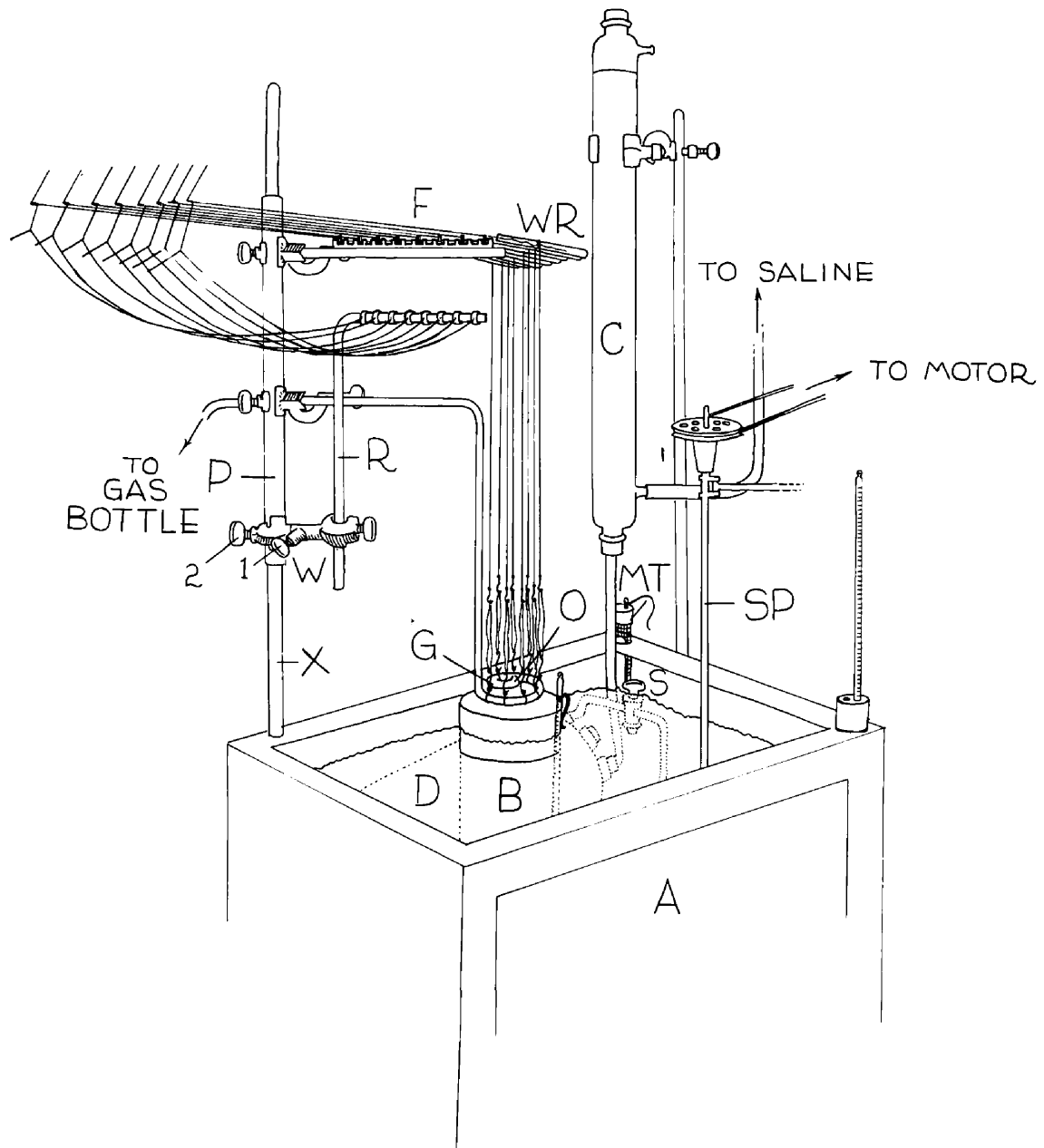
Figure 1a. Multiple Strip Isolated Tissue Bath.
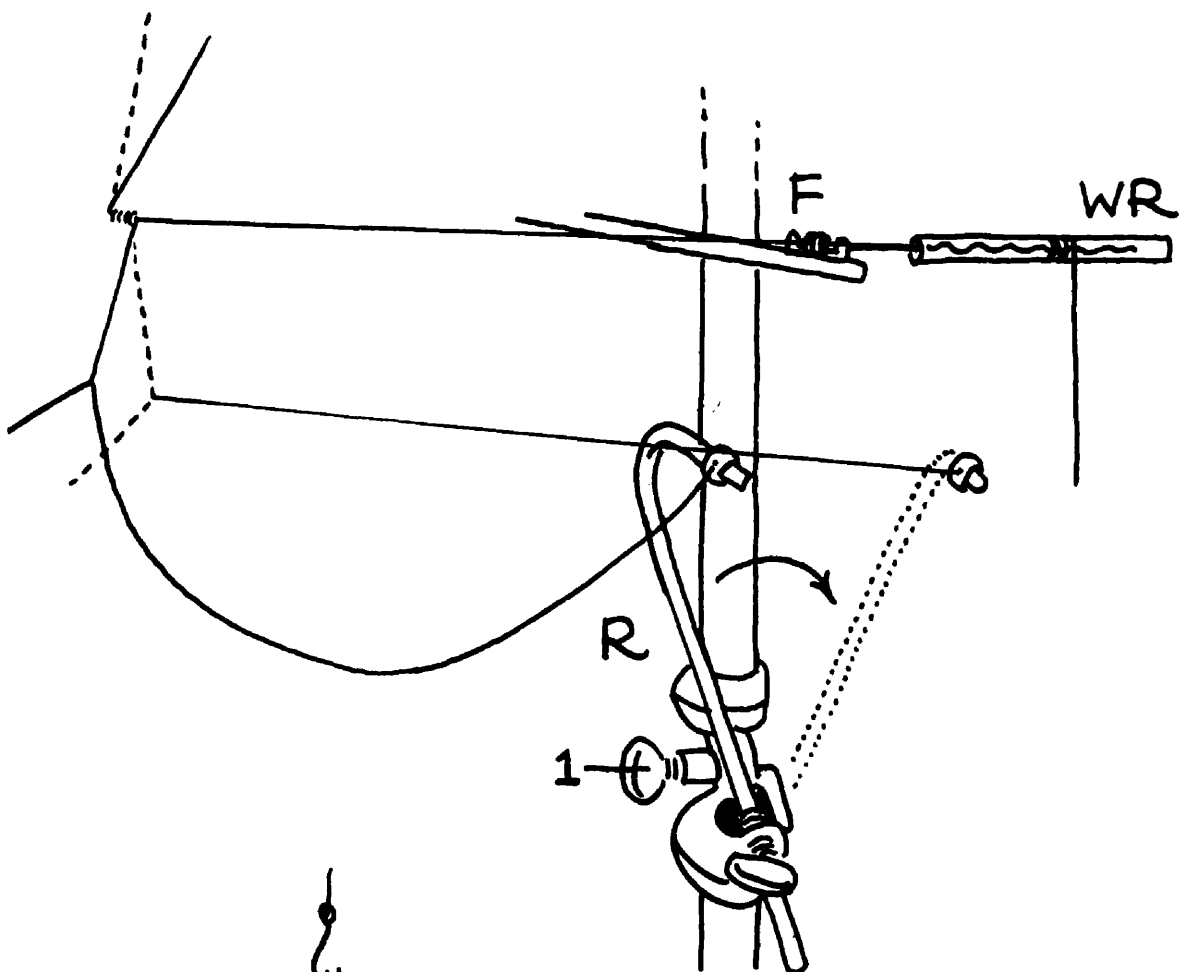
Figure 1b.   Arrangement for One
             Lever.
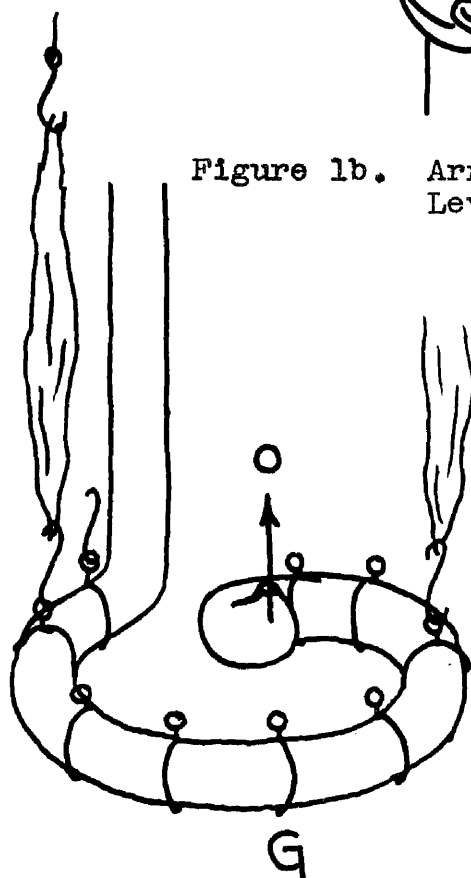
Figure 1c.   Lower End of Tube G Showing Method of Attachment
             of Tissue Strips.

the tissue chamber by filling it to the same mark each time, the measuring device would be unnecesary. With a wide tissue chamber, however, it is better to measure by the smaller meniscus afforded by the measuring cylinder C. By opening the stopcock S the saline is allowed to run into the one liter warming chamber D thus forcing a measured volume of prewarmed solution into the tissue chamber B. The inlet tube of chamber D extends to the bottom of the chamber and the outlet extends just through a rubber stopper. The saline therefore enters D at the bottom and forces 250 cc. of warm saline out before the cold saline diffuses enough to lower the temperature. Clamps and stopcocks are used where necessary to shut off the flow of liquid.

Briefly, the solution is run from the storage bottle to the measuring cylinder C. Opening of stopcock S allows a measured amount of the solution to run into the warming chamber D forcing warm saline into the tissue chamber B. After the contractions have been recorded the solution is drained from an outlet at the bottom of B and fresh solution is added.

The eight recording levers work independently of each other and are held in the frame F which is attached to the metal pipe P. The levers are made of 20 gauge steel wire. They are eleven inches in length from fulcrum to the hinge of the writing point and one to one and one-half inches from fulcrum to the attachment of the uterine strips. The frontal writing points are two and one-half inches from

their hinges to the writing tips. Their upper extensions are of sufficient length and bent slightly to act as counterweights to hold the writing tips lightly against the smoked paper of a kymograph. The hinges of the writing points are made by wrapping the terminal end of a lever around a section of an 18 gauge hypodermic needle ($\frac{1}{4}$ inch long) and inserting the 20 gauge wire writing point through the lumen of the needle section. The upper extension of the writing points are, of course, bent to the proper shape after insertion through the hinge joint. This construction provides a hinge with very little friction and a minimum of side play.

The glass tube G which holds the lower ends of the uterine strips and the device for withdrawing the frontal writing points are also held by the pipe P. The withdrawing device consists of a glass rod R to which threads from each of the writing points are attached. It is held in the swivel clamp W. By loosening setscrew 1 the rod R can be swung back to remove all the writing points from the smoked paper of the kymograph or forward to allow the points to record. Setscrew 2 on clamp W passes through a hole in the pipe P and holds the entire unit (G, R and F) rigid on the stand X. By loosening setscrew 2 the unit may be raised (as in Figure 1a) to facilitate mounting of the uterine strips and lowered to immerse the strips in the saline in B.

The tube G holds the lower ends of the uterine strips and also serves to conduct the air-carbon dioxide mixture into the tissue chamber B. The gas mixture enters from the

pinhole opening O to bubble up in the center of the circular arrangement of the uterine strips without coming in contact with them. The tube G is connected with a gas bottle (not shown) which has a capacity of 19 liters and contains the mixture of air and carbon dioxide. Before an assay is commenced this bottle is evacuated to a negative pressure of fifty millimeters of mercury and the evacuated air replaced by carbon dioxide gas. Water forced into this bottle drives the mixture through the opening O. A fairly rapid and constant rate of bubbling throughout an assay is essential. This is accomplished by forcing water into the gas bottle until there is a positive pressure of about fifty millimeters of mercury. An adjustable screw clamp allows the mixture to pass into the chamber at the required rate of about four bubbles per second. Constancy of the rate of bubbling is maintained by adjusting the flow of water into the gas bottle so that the pressure, indicated by a manometer, remains constant within two or three millimeters of mercury.

Small hooks of nichrome wire, about ten millimeters in length are used in suspending the tissue strips. The upper hook is fastened to a thread for attachment to the tube WR on the muscle lever. The lower hook is made S-shaped. One end is attached to the muscle, the other to the tube G by means of an eye made of nichrome wire, as indicated in Figure 1c.

As each strip is prepared with hooks inserted it is placed in a dish containing Van Dyke and Hasting's solution

until ready for mounting. All eight strips are mounted as rapidly as possible by placing each tube WR in position on a lever and inserting the lower hook into an appropriate eye on G. The whole unit is then lowered (by loosening setscrew 2) until the strips are immersed in the saline. The operation of mounting the strips is accomplished in a period of about two minutes thus avoiding any undue exposure to air.

The tension on each strip is adjusted by moving the glass tube WR toward the fulcrum to increase and away from the fulcrum to decrease the tension. That is, tube WR acts as a weight on the lever adjustable by changing its position. The tube WR must be of sufficient weight to counterbalance the weight of the lever. Four inch sections of glass tubing with an external diameter of seven millimeters and bore of four millimeters diameter are satisfactory. Each recording lever is brought to a horizontal position by turning the tube WR, thereby lengthening or shortening the thread which attaches the strip to the lever. The tube WR is held in a rigid position by friction caused by the undulating portion of the wire lever inserted into the lumen of the tube (see Figure 1b).

Throughout the operation of handling and mounting the uterine strips care must be taken not to injure the tissue by stretching, squeezing, or in any other way.

<u>Procedure</u>. Eight uterine strips were mounted for independently recording their activity as described. After complete relaxation of the strips (about ten minutes) final

adjustments were made on tension and position of the recording levers as previously indicated. The tension should be light but sufficient to allow the writing tips to overcome resistance on the smoked paper. The device for withdrawing the writing points was swung forward to allow the points to record on a slowly moving, flat surface of a long, smoked kymograph paper. A five minute normal tracing was recorded and then a dose of the drug solution was administered beneath the surface of the saline in the tissue chamber by means of a hypodermic syringe of suitable capacity. The activity of the uterine strips was recorded for exactly ten minutes after addition of the drug. The writing points were then removed from the smoked surface and the drugged saline replaced by 250 cc. of fresh saline. The smoked paper was moved to a new position and the uterine strips were allowed to become quiescent during a five minute "rest" period. The writing points were again placed in recording position and the procedure repeated as often as necessary using appropriate doses of standard and unknown.

Preliminary doses were administered to match the effects of standard and unknown approximately and to determine the doses necessary to cause most (preferably all) of the strips to respond. Several types of information were utilized in matching the effects of the two solutions. The presence or absence of effect on some strips, the time of onset of action in all strips-which responded, the character of the individual tracings, and the height of the recorded activity

may all be utilized in determining doses of standard and un-
known which produce approximately equal effects. Rarely were
more than two to four preliminary doses required in choosing
suitable doses for the assay. All doses used in an assay
should, of course, produce submaximal responses. At least
two preliminary doses should be given so that the tissue
strips may become acclimated to the experimental conditions
before actually commencing the assay. During this period,
in addition to choosing suitable doses for the assay, any
necessary adjustments of the apparatus may be made. Once
the actual assay is started, however, further adjustments
should not be made.

After doses of the standard and unknown were found
which showed approximately equal submaximal effects, graded
doses were given in the manner to be described in order to
obtain data suitable for statistical analysis. Further de-
tails of the procedure will become evident in subsequent
pages since their development constitutes a portion of this
study. When the assay was completed the kymograph record
was varnished and allowed to dry. The maximum heights from
the normal base line, of the tracings of the recorded activ-
ity of individual strips were then measured to the nearest
millimeter by means of a millimeter scale.

The distance between the writing points on the apparatus
is sufficient to allow the fifteen minute recording of the
activity of the individual strips using a slowly moving
smoked paper ($1\frac{1}{2}$ millimeters per minute).

With the five minute "normal", ten minute "action" and five minute "rest" periods, the doses were given at exactly twenty minute intervals. The five minute "normal" recording was made to show the condition of the individual strips just preceding the administration of a dose of the drug and provided suitable base lines from which to measure the heights of the tracings from individual strips. The ten minute period for the action of the drug was chosen in order to record the maximum effect of a given dose. With the doses usually employed, especially the low doses, many of the strips showed greater activity (height) five to ten minutes after administration of the dose. The five minute "rest" period was employed to allow the strips adequate time to become quiescent before beginning the next recording.

RESULTS

Fourteen experiments were performed in order to determine the best procedure for conducting an assay. There were several variables in these experiments including the order of administration of doses of standard and unknown, logarithmic interval between low and high doses, number of doses, and the number of points determined on the regression line. The data for these experiments, obtained by measuring the maximum heights of the recorded activity of the uterine strips, are given in Tables I to XIV inclusive.

A definite procedure, with regard to the above mentioned variables, was adopted on the basis of information revealed

by the preliminary experiments and ten additional assays were conducted in order to determine the suitability of this procedure. The data for these assays appear in Tables XV to XXIV.

Tables I to XXIV contain the pertinent data for each assay and are arranged to show the design of each experiment. They give the number of doses employed, column 1; the dosage order, column 2 (S = standard, U = unknown); the doses in cc. employed, column 3; and the height of the recorded responses of the individual strips in millimeters, columns 4 to 11 (strip numbers 1 to 8). The symbols in the final column in the tables have the following significance:

Tables I to VIII:

O - preliminary orienting doses and responses not employed in the calculation of potency.

A - doses and responses employed in the calculation of potency.

M - a large dose to produce maximal responses, administered to determine whether or not the responses constituting the actual assay are submaximal.

Tables IX to XXIV:

O - same significance as in tables I to VIII.

I, II, III - Doses and responses for "tests" I, II and III respectively, conducted at different sensitivity levels but all combined in one calculation of potency and estimate of error.

M - same significance as in tables I to VIII.

TABLE I

Assay 1

Tracing 93042

Standard - .2 mgm./cc.

Unknown - .173 mgm./cc.
(86.5% of Standard)

Rabbit No.: 11

Weight: 2.0 Kg.

Wt. uterus: 2.6 Gm.

Dose No.	Prepn.	Dose cc.	Response in millimeters Strip number								
			1	2	3	4	5	6	7	8	
1	S	1.00	0	23	30	24	23	27	12	0	O
2	U	1.00	1	16	38	15	16	20	10	0	
3	S	.70	1	22	30	2	15	16	7	0	
4	U	.85	0	23	31	3	16	15	4	0	
5	S	.84	0	29	38	6	16	16	9	0	
6	U	1.00	0	28	40	6	8	22	9	0	
7	U	1.20	0	25	47	7	9	22	6	0	
8	S	1.00	0	24	41	4	9	25	5	0	
9	U	1.45	6	22	45	7	10	30	9	0	
10	S	1.20	6	15	37	3	10	26	7	0	A
11	U	1.75	20	19	39	8	10	18	7	6	
12	S	1.45	11	18	31	15	12	21	8	5	
13	S	2.20	19	19	43	22	15	18	10	31	
14	U	2.60	20	29	53	33	24	23	10	23	
15	S	3.30	26	44	48	45	40	28	17	28	
16	U	3.90	22	35	46	37	29	25	14	24	
17	S	10.00	40	60	55	62	32	28	20	25	M

Estimated Potency - 88.4% of standard.

Per cent. true value - 102.2%.

TABLE II

Assay 2

Tracing 10242

Standard - .2 mgm./cc.

Unknown - .268 mgm./cc.
(134.% of Standard)

Rabbit No.: 12

Weight: 1.9 Kg.

Wt. uterus: 2.6 Gm.

Dose No.	Prepn.	Dose cc.	Response in millimeters Strip number								
			1	2	3	4	5	6	7	8	
1	S	.50	6	12	7	14	13	11	5	27	O
2	U	.50	9	12	6	11	21	11	7	26	
3	S	.75	13	13	9	17	34	19	16	34	
4	U	.75	18	21	19	38	42	27	22	46	
5	S	1.10	37	29	57	45	46	42	43	52	
6	U	1.10	30	50	87	87	60	52	60	50	
7	S	1.70	38	50	89	66	80	57	55	54	
8	U	1.70	46	55	113	95	66	58	80	57	A
9	S	2.60	44	53	91	84	72	35	63	40	
10	U	2.60	47	46	94	110	56	57	70	42	
11	S	3.90	55	48	97	111	74	60	70	50	
12	U	3.90	55	52	107	110	74	59	80	50	
13	S	10.00	69	60	124	133	87	62	90	48	M

Estimated Potency - 140.1%

Per cent. true value - 104.5%

TABLE III

Assay 3

Tracing 10342

Standard - .5 mgm./cc.

Unknown - .235 mgm./cc.
(47.0% of Standard)

Rabbit No.: 13

Weight: 2.1 Kg.

Wt. uterus: 5.3 Gm.

Dose No.	Prepn.	Dose cc.	Response in millimeters Strip number								
			1	2	3	4	5	6	7	8	
1	S	.20	18	21	13	17	48	39	3	31	O
2	U	.20	0	0	3	21	0	0	0	24	
3	S	.10	0	1	5	0	11	0	0	24	
4	U	.15	0	0	3	0	0	0	19	0	
5	U	.30	0	2	4	0	26	26	0	21	A
6	S	.15	0	2	0	0	16	20	0	21	
7	U	.45	0	2	2	0	24	21	0	34	
8	S	.23	0	0	0	0	23	16	16	0	
9	U	.68	0	0	0	14	27	16	0	22	
10	S	.34	0	0	1	0	28	23	0	0	
11	U	1.00	0	41	0	12	19	53	0	13	
12	S	.50	0	0	0	12	0	13	0	16	
13	U	1.50	0	49	0	55	36	13	0	21	
14	S	.75	0	45	1	77	51	52	2	26	
15	U	2.30	51	81	59	103	86	88	36	67	
16	S	1.20	47	89	45	104	99	74	52	50	
17	U	3.50	25	103	42	131	92	75	72	42	M

Estimated Potency - 54.6% of standard.

Per cent. true value - 116.2%.

TABLE IV

Assay 4

Tracing 11642

Standard - .2 mgm./cc.

Unknown - .16 mgm./cc.
(80.0% of standard)

Rabbit No.: 21

Weight: 1.8 Kg.

Wt. uterus: 2.3 Gm.

Dose No.	Prepn.	Dose cc.	Response in millimeters Strip number								
			1	2	3	4	5	6	7	8	
1	U	.50	0	0	0	0	0	0	0	0	O
2	S	.50	5	0	0	28	0	0	0	0	
3	S	1.00	25	0	0	25	0	11	28	21	
4	U	1.00	16	0	0	34	0	2	1	0	
5	U	2.00	30	1	6	63	0	25	2	31	
6	S	2.00	33	5	18	66	7	24	88	30	
7	U	4.00	40	10	25	73	5	33	77	8	
8	S	4.00	41	16	23	64	22	30	82	46	A
9	U	1.00	0	0	0	25	7	0	0	0	
10	S	1.00	0	0	0	14	6	0	0	0	
11	U	2.00	10	0	7	23	10	18	39	0	
12	S	2.00	20	2	8	33	18	16	80	2	
13	U	4.00	39	4	26	53	29	31	83	21	
14	S	4.00	46	5	16	51	41	28	81	31	
15	U	8.00	41	5	24	52	47	28	83	34	M

Estimated potency - 76.7% of standard.

Per cent. true value - 95.9%

TABLE V

Assay 5

Tracing 112142

Standard - .2 mgm./cc.

Unknown - .324 mgm./cc.
(162.0% of standard)

Rabbit No.: 25

Weight: 2.1 Kg.

Wt. uterus: 4.1 Gm.

Dose No.	Prepn.	Dose cc.	Response in millimeters Strip number								
			1	2	3	4	5	6	7	8	
1	S	.50	0	8	0	0	2	3	0	1	O
2	S	1.00	30	37	16	18	56	10	15	8	
3	U	1.00	57	55	35	56	62	20	30	18	
4	U	.25	0	0	15	0	36	1	3	0	
5	S	.70	34	14	24	45	32	5	6	6	A
6	U	.35	33	17	26	34	37	3	0	4	
7	S	1.40	46	52	34	61	50	12	9	13	
8	U	.70	51	44	32	54	40	11	8	8	
9	S	2.80	61	53	39	71	48	21	20	18	
10	U	1.40	57	51	34	77	58	12	14	10	
11	S	5.60	63	58	40	72	44	24	31	22	M*
12	U	1.40	69	55	36	66	57	10	9	10	A
13	S	2.80	58	51	29	68	51	15	17	13	
14	U	.70	54	42	17	48	58	8	0	4	
15	S	1.40	67	59	16	61	59	18	0	9	
16	U	.35	30	54	7	25	42	0	0	4	
17	S	.70	43	46	11	36	44	1	0	3	

Estimated Potency - 162.0% of standard.

Per cent. true value - 100.0%

* "Maximal" dose administered at this point instead of at the end of the assay as usual. All the A values were used in one calculation of potency.

TABLE VI

Assay 6

Tracing 112742

Standard - .2 mgm./cc.

Unknown* - .50 mgm./cc.
(250.0% of standard)

Rabbit No.: 26

Weight: 2.3 Kg.

Wt. uterus: 4.1 Gm.

Dose No.	Prepn.	Dose cc.	Response in millimeters Strip number								
			1	2	3	4	5	6	7	8	
1	S	1.00	0	0	0	0	0	0	0	0	
2	U	1.00	22	12	54	25	37	58	9	45	
3	U	.50	7	7	42	27	28	41	2	30	O
4	S	1.00	7	10	35	11	21	20	2	31	
5	U	.30	0	0	5	0	4	0	0	0	
6	S	1.00	1	12	33	3	23	27	4	25	
7	U	1.00	4	10	26	3	15	19	2	21	
8	S	2.00	15	26	43	13	20	47	4	37	
9	S	2.00	13	10	36	15	24	53	3	27	
10	U	4.00	50	28	42	19	15	40	7	43	A
11	U	4.00	51	32	41	18	16	43	7	44	
12	S	4.00	50	29	38	21	15	40	11	36	
13	S	4.00	49	26	42	20	8	40	16	37	
14	U	10.00	60	56	51	34	30	74	25	39	M

Estimated Potency - 229.0% of standard.

Per cent. true value - 91.2%.

* Unknown solution diluted 1:2.5 for actual assay (doses under A).

TABLE VII

Assay 7

Tracing 12142

Standard - .2 mgm./cc.

Unknown - .196 mgm./cc.
(98.0% of standard)

Rabbit No.: 28

Weight: 2.5 Kg.

Wt. uterus: 3.9 Gm.

Dose No.	Prepn.	Dose cc.	Response in millimeters Strip number								
			1	2	3	4	5	6	7	8	
1	S	.50	23	50	34	20	40	35	39	2	O
2	U	.50	27	54	45	29	51	28	37	8	
3	S	.60	36	69	54	35	56	40	46	10	
4	S	.50	Tried stationary drum--unsatisfactory.								
5	S	.50	28	62	49	25	56	31	2	0	
6	U	.50	28	60	55	19	15	40	35	3	
7	U	1.00	36	70	76	40	65	61	47	18	
8	S	1.00	45	75	79	40	67	63	40	20	A
9	S	2.00	65	75	92	59	81	77	58	41	
10	U	2.00	56	69	78	49	73	49	54	26	
11	U	2.00	57	71	70	50	77	45	60	29	
12	S	2.00	59	72	81	53	78	58	53	35	
13	S	1.00	61	72	89	32	65	65	46	29	*
14	U	1.00	57	82	88	31	71	65	36	32	
15	S	10.00	141	119	140	95	88	122	92	85	M

Estimated potency - 80.0% of standard.

Per cent. true value - 81.6%.

* These data were not employed in the calculation of potency because the temperature of the bath failed to remain constant.

TABLE VIII

Assay 8

Tracing 122942

Standard - .2 mgm./cc.

Unknown - .304 mgm./cc.
(152.0% of standard)

Rabbit No.: 32

Weight: 3.1 Kg.

Wt. uterus: 5.0 Gm.

Dose No.	Prepn.	Dose cc.	Response in millimeters Strip number								
			1	2	3	4	5	6	7	8	
1	S	1.00	0	7	13	0	0	0	0	0	} 0
2	U	1.50	146	120	103	120	9	7	114	98	
3	S	1.50	0	1	110	0	0	0	0	0	
4	U	.80	0	0	38	0	0	0	0	0	
5	S	2.40	8	18	121	4	0	2	3	3	
6	S	3.80	179	147	126	141	0	2	114	4	
7	U	1.90	0	1	0	3	0	0	0	74	} A
8	U	3.80	188	150	132	108	0	84	106	113	
9	S	3.80	188	145	12	0	0	0	33	30	
10	S	5.00	199	150	126	73	0	108	44	108	
11	U	2.40	154	141	1	1	0	0	0	24	
12	U	4.00	190	160	136	97	61	8	101	90	
13	S	15.00	210	156	143	163	84	123	119	120	M

Estimated potency - 135.0% of standard.

Per cent. true value - 88.8%.

TABLE IX

Assay 9

Tracing 112442

Standard - .2 mgm./cc.

Unknown - .448 mgm./cc.
  (224.0% of standard)

Rabbit No.: 26

Weight: 1.9 Kg.

Wt. uterus: 2.1 Gm.

Dose No.	Prepn.	Dose cc.	Response in millimeters Strip number								
			1	2	3	4	5	6	7	8	
1	S	.50	0	62	15	3	13	45	45	0	
2	U	.25	0	52	21	15	22	23	34	16	0
3	S	.25	0	29	15	20	16	21	8	5	
4	U	.10	0	20	5	8	14	2	0	0	
5	S	.20	0	18	5	0	9	0	0	0	
6	U	.20	0	30	8	2	21	25	27	0	I
7	S	.40	0	27	6	2	27	20	26	0	
8	U	.40	1	45	20	5	36	23	24	16	
9	S	.80	0	34	13	1	39	23	37	10	
10	U	.80	8	58	38	60	54	31	36	21	II
11	S	1.60	0	30	23	55	52	25	27	17	
12	U	1.60	34	70	67	69	73	30	42	25	M
13	S	1.60	2	33	22	2	46	19	20	4	
14	U	.80	0	30	27	2	46	15	18	4	
15	S	.80	0	5	12	2	5	14	9	6	III
16	U	.40	0	31	13	1	24	6	6	7	

Estimated potency - 242.7% of standard.

Per cent. true value - 108.3%.

Limits of error of estimate (P-.99) - 177.2% to 332.4%.

TABLE X

Assay 10

Tracing 12242

Standard - .2 mgm./cc.

Unknown - .248 mgm./cc.
(124.0% of standard)

Rabbit No.: 28

Weight: 2.5 Kg.

Wt. uterus: 4.2 Gm.

Dose No.	Prepn.	Dose cc.	Response in millimeters Strip number								
			1	2	3	4	5	6	7	8	
1	S	1.00	46	10	60	64	34	72	24	18	O
2	U	1.00	62	25	81	56	29	90	22	23	
3	S	1.00	44	20	96	58	29	79	27	32	I*
4	U	1.00	53	33	91	67	27	81	22	25	I
5	U	1.00	44	21	81	63	26	77	19	29	II
6	S	1.00	45	20	85	65	33	88	30	27	II
7	S	2.00	46	36	95	71	59	89	33	41	I
8	U	2.00	72	37	100	67	50	96	33	43	I
9	U	2.00	64	42	95	64	42	85	26	30	II
10	S	2.00	51	24	90	66	38	77	30	31	II
11	S	10.00	88	53	100	99	67	102	61	65	M

Estimated potency - 112.9% of standard.

Per cent. true value - 91.0%.

* In this assay all low doses, 3 to 6, were given first followed by all high doses, 7 to 10. Only two "tests" comprise the assay.

TABLE XI

Assay 11

Tracing 12542

Standard - .2 mgm./cc.

Unknown* - .288 mgm./cc.
(144.0% of standard)

Rabbit No.: 30

Weight: 2.7 Kg.

Wt. uterus: 7.2 Gm.

Dose No.	Prepn.	Dose cc.	Response in millimeters Strip number								
			1	2	3	4	5	6	7	8	
1	S	1.0	116	99	35	95	0	82	79	0	
2	U	1.0	150	155	60	108	124	10	98	74	
3	U	.5	0	0	0	0	0	0	0	0	
4	S	1.0	0	0	0	74	0	0	1	1	0
5	S	1.3	40	57	0	93	0	0	77	1	
6	U	.7	0	0	0	86	0	0	73	0	
7	S	1.5	55	65	50	102	84	61	90	54	
8	S	3.0	154	109	70	113	127	95	110	78	I
9	U	3.0	122	102	61	100	83	69	102	43	
10	U	1.5	33	45	35	48	13	31	85	12	
11	U	1.5	15	36	38	55	0	3	74	22	
12	U	3.0	176	126	70	113	122	100	100	77	
13	S	3.0	177	129	63	102	101	82	103	83	II**
14	S	1.5	0	87	32	68	3	9	86	8	
15	S	6.0	191	142	70	95	141	98	100	85	M

Estimated potency - 127.5% of standard.

Per cent. true value - 88.5%.

Limits of error of estimate (P-.99) - 103.6% to 156.9%

* Unknown solution diluted 1:1.5 for actual assay (I and II)

** Only two "tests" comprise the assay.

TABLE XII

Assay 12

Tracing 12942

Standard - .2 mgm./cc.

Unknown - .088 mgm./cc.
(44.0% of standard)

Rabbit No.: 31

Weight: 2.2 Kg.

Wt. uterus: 2.2 Gm.

Dose No.	Prepn.	Dose cc.	Response in millimeters Strip number								
			1	2	3	4	5	6	7	8	
1	S	1.00	44	50	8	21	37	28	23	24	O
2	U	1.00	20	37	13	18	41	36	27	20	
3	S	.80	14	57	19	18	53	44	25	21	
4	S	1.30	40	64	24	31	55	48	30	21	
5	U	1.585	29	44	25	21	46	28	25	19	I
6	U	1.00	23	12	24	17	32	20	8	5	
7	S	1.30	48	60	32	25	42	52	33	20	
8	S	.80	29	51	29	26	52	53	22	16	
9	U	1.00	26	12	28	14	42	39	19	9	II
10	U	1.585	23	41	28	22	48	53	32	22	
11	U	1.00	26	55	23	13	26	23	13	10	
12	U	1.585	41	81	37	24	40	43	32	23	
13	S	.80	53	65	39	20	51	60	40	21	III
14	S	1.30	65	88	46	25	59	69	35	23	
15	S	5.00	98	131	77	55	85	104	58	63	M

Estimated potency - 46.1% of standard.

Per cent. true value - 104.7%.

Limits of error of estimate - (P-.99) - 34.1% to 62.5%

TABLE XIII

Assay 13

Tracing 121242

Standard - .2 mgm./cc.

Unknown - .188 mgm./cc.
(94.0% of standard)

Rabbit No.: 31

Weight: 2.2 Kg.

Wt. uterus: 2.2 Gm.

Dose No.	Prepn.	Dose cc.	Response in millimeters Strip number								
			1	2	3	4	5	6	7	8	
1	S	1.00	10	2	2	0	7	4	4	0	O
2	U	1.50	30	5	4	3	24	9	14	2	
3	S	1.50	33	6	5	4	40	17	17	6	
4	S	3.00	31	11	9	7	53	29	26	9	
5	U	3.00	31	9	9	7	56	26	25	9	I
6	U	1.50	30	9	7	4	40	21	14	6	
7	S	3.00	38	11	16	11	46	29	27	11	
8	S	1.50	37	8	8	5	47	23	25	4	
9	U	1.50	30	14	10	7	46	16	17	3	II
10	U	3.00	51	19	16	7	68	32	35	11	
11	U	1.50	30	12	4	5	45	15	16	2	
12	U	3.00	54	17	13	7	58	22	26	6	
13	S	1.50	25	8	4	4	48	12	10	2	III
14	S	3.00	58	20	13	9	67	22	26	5	
15	S	10.00	76	39	37	24	86	42	50	37	M

Estimated potency - 106.3% of standard.

Per cent. true value - 113.1%

Limits of error of estimate - (P-.99) - 86.7% to 130.3%.

TABLE XIV

Assay 14

Tracing 1543

Standard - .2 mgm./cc.

Unknown - .108 mgm./cc.
(54.0% of standard)

Rabbit No.: 33

Weight: 2.4 Kg.

Wt. uterus: 6.8 Gm.

Dose No.	Prepn.	Dose cc.	Response in millimeters Strip number								
			1	2	3	4	5	6	7	8	
1	S	1.00	130	140	135	155	150	107	150	140	O
2	U	.50	48	82	87	88	54	45	70	72	
3	S	.40	63	91	89	97	120	53	90	108	I
4	S	.634	94	97	90	105	160	74	126	130	
5	U	.951	82	90	72	107	150	64	85	108	
6	U	.60	24	13	34	63	87	40	55	65	
7	S	.634	57	106	73	86	125	71	102	115	II
8	S	.40	53	86	71	74	92	58	107	117	
9	U	.60	53	100	70	105	111	52	125	100	
10	U	.951	70	117	108	106	161	79	147	136	
11	S	.40	67	157	126	97	180	70	146	130	III
12	S	.634	116	158	138	137	200	88	159	167	
13	U	.60	76	111	146	117	186	64	153	130	
14	U	.951	100	173	146	155	200	90	166	168	
15	S	5.00	180	200	158	203	200	175	160	195	M

Estimated potency - 62.9% of standard.

Per cent. true value - 116.5%.

Limits of error of estimate -(P-.99) - 56.9% to 69.5%.

TABLE XV

Assay 15

Tracing 1643

Standard - .2 mgm./cc.

Unknown - .108 mgm./cc.
(54.0% of standard)

Rabbit No.: 33

Weight: 2.4 Kg.

Wt. uterus: 6.8 Gm.

Dose No.	Prepn.	Dose cc.	Response in millimeters Strip number								
			1	2	3	4	5	6	7	8	
1	S	.40	0	2	10	0	19	13	1	0	} 0
2	U	.60	15	6	45	52	50	74	39	0	
3	S	.40	51	15	50	68	62	78	69	0	
4	U	.60	52	12	56	70	49	76	69	0	
5	S	.634	114	57	91	79	62	116	82	56	} I
6	U	.951	58	44	80	71	58	74	77	52	
7	U	.60	21	17	36	61	41	36	47	24	
8	S	.40	31	57	88	52	53	77	52	29	
9	U	.951	80	87	109	62	98	116	70	60	} II
10	S	.634	95	106	113	63	98	116	75	55	
11	S	.40	63	90	114	66	62	123	70	40	
12	U	.951	151	122	124	67	106	140	89	77	
13	U	.60	60	104	116	66	62	84	66	36	} III
14	S	.634	186	132	125	73	106	143	100	117	
15	S	5.00	197	146	140	125	168	160	160	180	M

Estimated potency - 58.5% of standard.

Per cent. true value - 108.3%.

Limits of error of estimate - (P-.99) - 53.1% to 64.4%.

TABLE XVI

Assay 16

Tracing 11243

Standard - .2 mgm./cc.

Unknown - .372 mgm./cc.
           (186.0% of standard)

Rabbit No.: 34

Weight: 1.8 Kg.

Wt. uterus: 4.5 Gm.

Dose No.	Prepn.	Dose cc.	Response in millimeters Strip number								
			1	2	3	4	5	6	7	8	
1	S	.50	0	0	0	0	0	0	0	0	0
2	U	1.00	144	90	120	47	70	5	57	45	
3	S	1.00	12	0	0	44	0	0	0	0	
4	S	1.50	69	34	81	52	73	0	31	29	
5	U	.80	68	0	94	47	67	0	31	5	I
6	S	2.377	85	92	123	59	95	29	65	102	
7	U	1.268	84	99	105	64	95	3	40	65	
8	U	.80	0	0	33	2	2	0	21	8	
9	S	1.50	70	0	40	0	59	0	27	7	II
10	U	1.268	93	64	147	63	80	42	39	90	
11	S	2.377	78	77	51	71	51	0	45	48	
12	S	1.50	55	0	5	2	7	0	45	21	
13	U	1.268	115	94	145	73	104	49	63	69	III
14	U	.80	85	35	5	0	25	0	47	53	
15	S	2.377	106	90	125	78	106	55	69	118	
16	S	10.00	200	167	150	104	155	72	109	155	M

Estimated potency - 187.1% of standard.

Per cent. true value - 100.6%

Limits of error of estimate - (P-.99) - 167.0% to 209.5%.

TABLE XVII

Assay 17

Tracing 11343

Standard - .2 mgm./cc.

Unknown - .372 mgm./cc.
(186.0% of standard)

Rabbit No.: 34

Weight: 1.8 Kg.

Wt. uterus: 4.5 Gm.

Dose No.	Prepn.	Dose cc.	Response in millimeters Strip number								
			1	2	3	4	5	6	7	8	
1	S	1.50	0	0	0	4	0	4	7	27	} 0
2	S	2.00	0	0	20	44	0	43	22	66	
3	S	2.00	4	5	29	49	0	41	18	61	} I
4	U	1.00	3	0	28	38	0	45	30	57	
5	S	3.168	31	30	45	58	33	69	56	87	
6	U	1.585	36	34	54	46	27	62	46	71	
7	U	1.00	0	3	11	36	0	48	46	61	} II
8	S	2.00	57	4	58	44	12	70	60	81	
9	U	1.585	84	54	59	57	41	81	71	87	
10	S	3.168	76	58	81	65	51	56	57	79	
11	S	2.00	53	51	66	58	0	65	43	49	} III
12	U	1.585	78	53	70	63	53	64	58	75	
13	U	1.00	0	36	5	66	0	62	46	56	
14	S	3.168	107	67	80	78	69	90	72	93	
15	S	13.00	180	118	129	104	96	110	109	122	M

Estimated potency - 170.8% of standard.

Per cent. true value - 91.8%.

Limits of error of estimate - (P-.99) - 150.5% to 193.8%

TABLE XVIII

Assay 18

Tracing 11943

Rabbit No.: 36

Standard - .2 mgm./cc.

Weight: 1.7 Kg.

Unknown - .136 mgm./cc.
(68.0% of standard)

Wt. uterus: 2.9 Gm.

Dose No.	Prepn.	Dose cc.	Response in millimeters Strip number								
			1	2	3	4	5	6	7	8	
1	S	.50	0	7	0	0	16	0	0	12	
2	U	.50	35	0	64	16	59	56	0	57	O
3	S	1.00	63	103	143	20	84	90	73	93	
4	U	1.00	29	73	118	19	74	66	74	61	
5	S	.80	28	70	90	26	77	68	60	82	
6	U	1.585	23	120	59	48	67	103	94	92	I
7	S	1.268	57	100	124	68	78	102	107	94	
8	S	.80	33	35	55	53	33	73	52	88	
9	U	1.00	48	13	13	53	24	59	45	54	
10	S	1.268	65	22	56	66	50	76	85	92	II
11	U	1.585	70	32	31	88	49	76	58	77	
12	U	1.00	63	1	5	59	15	51	0	62	
13	S	1.268	74	50	81	80	58	80	69	80	
14	S	.80	63	0	1	66	45	62	0	43	III
15	U	1.585	75	86	90	83	32	77	69	80	
16	S	10.00	117	161	175	117	116	138	128	110	M

Estimated potency - 72.5% of standard.

Per cent. true value - 106.6%.

Limits of error of estimate - (P-.99) - 61.6% to 85.3%

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TABLE XIX

Assay 19

Tracing 12043

Standard - .2 mgm./cc.

Unknown - .136 mgm./cc.
(68.0% of standard)

Rabbit No.: 36

Weight: 1.7 Kg.

Wt. uterus: 2.9 Gm.

Dose No.	Prepn.	Dose cc.	1	2	3	4	5	6	7	8	
						Strip number					
1	S	1.00	0	0	47	0	0	0	0	1	0
2	S	1.50	5	10	75	0	30	4	17	13	
3	S	1.50	17	26	95	5	48	45	46	30	
4	U	2.00	32	28	98	18	60	67	48	36	I
5	S	2.377	48	35	120	57	81	102	75	63	
6	U	3.17	57	42	117	56	70	97	53	60	
7	U	2.00	44	38	110	40	21	105	17	85	
8	S	1.50	51	32	120	84	38	117	32	75	II
9	U	3.17	60	37	122	94	51	117	80	95	
10	S	2.377	69	47	129	99	77	128	75	94	
11	S	1.50	43	57	86	83	33	112	54	85	
12	U	3.17	77	78	118	89	92	136	81	95	III
13	U	2.00	50	57	100	62	69	116	66	81	
14	S	2.377	78	79	108	69	56	106	80	83	
15	S	15.00	140	126	153	120	130	175	120	115	M

Estimated potency - 76.6% of standard.

Per cent. true value - 112.6%

Limits of error of estimate - (P-.99) - 68.98% to 85.17%

TABLE XX

Assay 20

Tracing 2943

Standard*- .2 mgm./cc.

Unknown - .044 mgm./cc.
    (22.0% of standard)

Rabbit No.: 37

Weight: 1.9 Kg.

Wt. uterus: 3.1 Gm.

Dose No.	Prepn.	Dose cc.	Response in millimeters Strip number								
			1	2	3	4	5	6	7	8	
1	S	.50	0	10	0	0	0	0	7	0	
2	U	1.00	0	0	0	0	0	0	0	0	
3	U	2.00	0	0	0	0	0	0	0	0	0
4	U	4.00	0	3	1	0	0	0	7	0	
5	S	4.00	0	0	0	0	0	0	8	0	
6	S	6.31	14	27	3	7	9	15	21	5	
7	U	6.31	0	4	2	2	1	2	11	2	
8	S	10.00	6	29	7	19	16	22	19	12	I
9	U	10.00	1	11	4	6	5	5	15	6	
10	U	6.31	0	1	2	1	0	2	1	4	
11	S	6.31	0	2	1	0	0	2	8	2	
12	U	10.00	9	19	5	3	1	6	11	4	II
13	S	10.00	10	27	5	6	.1	7	13	3	
14	S	6.31	0	15	0	0	0	0	4	2	
15	U	10.00	6	24	3	2	3	2	10	4	
16	U	6.31	0	14	0	0	0	0	5	0	III
17	S	10.00	13	43	5	7	3	3	17	9	
18	S	40.00	134	86	63	118	80	81	65	82	M

Estimated potency - 17.3% of standard. % true value - 78.6
Limits of error of estimate - (P-.99) - 13.5% to 22.3%
* Standard diluted 1:4 for doses 5 to 18.

TABLE XXI

Assay 21

Tracing 21043

Standard - .2 mgm./cc.

Unknown - .192 mgm./cc.
(96.0% of standard)

Rabbit No.: 37

Weight: 1.9 Kg.

Wt. uterus: 3.1 Gm.

Dose No.	Prepn.	Dose cc.	Response in millimeters Strip number								
			1	2	3	4	5	6	7	8	
1	S	1.00	0	1	0	0	0	0	0	0	0
2	U	1.50	1	2	2	6	10	1	0	0	
3	S	2.00	1	2	4	15	20	2	3	2	
4	S	3.00	3	2	13	21	24	7	7	3	
5	U	2.50	0	2	11	22	21	3	3	0	
6	S	4.75	13	2	34	22	29	11	8	8	I
7	U	3.96	1	3	27	24	23	10	7	1	
8	U	2.50	0	2	16	13	17	6	2	0	
9	S	3.00	0	2	15	15	14	7	2	0	II
10	U	3.96	0	2	17	15	18	7	2	1	
11	S	4.75	0	2	16	16	17	10	3	1	
12	S	3.00	0	1	7	11	6	5	0	0	
13	U	3.96	0	1	11	11	12	5	0	0	III
14	U	2.50	0	1	6	8	0	2	0	0	
15	S	4.75	0	2	8	11	12	5	1	1	
16	S	20.00	16	4	57	18	27	22	15	13	M

Estimated potency - 95.7% of standard.

Per cent. true value - 99.7%

Limits of error of estimate - (P-.99) - 72.3% to 126.5%.

TABLE XXII

Assay 22

Tracing 21143

Standard - .2 mgm./cc.

Unknown - .28 mgm./cc.
(140.0% of standard)

Rabbit No.: 38

Weight: 2.3 Kg.

Wt. uterus: 3.8 Gm.

Dose No.	Prepn.	Dose cc.	Response in millimeters Strip number								
			1	2	3	4	5	6	7	8	
1	S	1.00	28	15	12	0	0	4	0	3	0
2	U	1.50	32	34	30	4	9	28	14	14	
3	S	1.50	57	64	23	5	7	21	11	12	I
4	U	1.50	68	69	26	10	10	31	17	16	
5	S	2.38	76	102	44	14	17	43	22	16	
6	U	2.38	84	97	32	13	21	50	23	25	
7	U	1.50	65	91	34	8	7	22	16	26	II
8	S	1.50	72	72	24	14	11	33	11	18	
9	U	2.38	66	77	31	8	17	35	23	35	
10	S	2.38	59	63	26	12	23	37	31	18	
11	S	1.50	48	60	22	10	11	14	15	15	III
12	U	2.38	58	77	24	7	12	32	21	20	
13	U	1.50	57	60	18	8	11	12	21	13	
14	S	2.38	60	85	23	7	12	14	18	18	
15	S	10.00	76	108	47	8	32	48	40	56	M

Estimated potency - 115.6% of standard.

Per cent. true value - 82.6%

Limits of error of estimate - (P-.99) - 91.5% to 146.1%.

TABLE XXIII

Assay 23

Tracing 21243   Rabbit No.: 38

Standard - .2 mgm./cc.   Weight: 2.3 Kg.

Unknown - .152 mgm./cc.   Wt. uterus: 3.8 Gm.
           (76.0% of standard)

Dose No.	Prepn.	Dose cc.	Response in millimeters Strip number								
			1	2	3	4	5	6	7	8	
1	S	2.00	22	11	21	9	30	17	14	26	O
2	U	2.00	28	3	21	6	50	14	18	44	
3	S	2.00	45	13	33	17	48	23	23	40	
4	U	2.50	56	17	38	24	60	31	32	47	I
5	S	3.17	68	44	58	51	56	58	51	69	
6	U	3.96	63	33	50	46	62	46	38	57	
7	U	2.50	60	30	39	22	57	31	30	48	
8	S	2.00	58	28	40	26	62	40	35	47	II
9	U	3.96	63	67	53	42	53	37	41	65	
10	S	3.17	60	48	51	33	59	55	41	68	
11	S	2.00	65	32	44	21	57	52	40	65	
12	U	3.96	52	36	55	40	80	39	44	63	III
13	U	2.50	38	15	43	15	63	37	41	55	
14	S	3.17	50	25	38	20	78	33	37	64	
15	S	12.00	72	61	67	46	93	63	52	85	M

Estimated potency - 79.0% of standard.

Per cent. true value - 103.9%

Limits of error of estimate - (P-.99) - 68.9% to 90.6%

TABLE XXIV

Assay 24

Tracing 3243

Standard - .2 mgm./cc.

Unknown - .288 mgm./cc.
(144.0% of standard)

Rabbit number: 39

Weight: 1.8 Kg.

Wt. uterus: 1.8 Gm.

Dose No.	Prepn.	Dose cc.	Response in millimeters Strip number								
			1	2	3	4	5	6	7	8	
1	S	1.00	23	0	2	10	21	17	13	1	
2	U	1.00	51	40	22	44	51	29	44	13	0
3	S	1.50	51	40	25	55	50	26	45	16	
4	S	1.20	16	44	23	39	32	28	36	7	
5	U	.80	12	36	18	38	20	28	31	2	
6	S	1.90	15	34	23	47	27	23	42	9	I
7	U	1.27	9	28	16	39	28	24	48	7	
8	U	.80	1	10	6	18	12	8	8	2	
9	S	1.20	4	19	8	29	12	10	20	2	
10	U	1.27	8	16	10	44	18	22	31	4	II
11	S	1.90	5	10	10	25	11	6	13	2	
12	S	1.20	0	4	3	8	3	2	2	0	
13	U	1.27	2	10	6	27	13	8	15	2	
14	U	.80	0	4	3	4	5	1	5	1	III
15	S	1.90	7	10	6	19	12	5	15	3	
16	S	6.00	19	33	17	48	28	36	43	17	M

Estimated potency - 142.2% of standard.

Per cent. true value - 98.8%.

Limits of error of estimate - (P-.99) - 105.8% to 191.1%.

MATHEMATICAL TREATMENT OF RESULTS

The height of recorded contraction is a quantitative response of the type which Irwin termed a "continuous variate" (14). That is, the extent of the response of each uterine strip to a particular dose is actually determined, in contrast to the "all or none" or "quantal" response in which only the presence or absence of an effect would be recorded.

The chief difference between the present procedure and the usual ones involving a continuous variate is that the different doses are given to the same test objects instead of employing a separate group for each dose. The original intention, was to use the method of least squares, as given by Irwin (14), for the calculation of potency and, further, to determine a suitable means for calculating the limits of error of an individual assay, either by Irwin's formula 12 (14) which may be used for data involving different groups of test objects, or some modification of it. In the procedure adopted (Assays 15 to 24), however, the data are more efficiently analyzed for error by applying Schild's method of calculation (20).

For the estimation of potency of the unknown in terms of standard in assays 1 to 8 (Tables I to VIII) the following mathematical procedure, adapted from Irwin (14), was applied:

Let X_s = logarithm of the dose (by volume) for the standard preparation.

X_u = logarithm of the dose (by volume) for the unknown preparation.

$\bar{y}_s$ = average response of the eight uterine strips receiving the dose X_s.

$\bar{y}_u$ = average response of the eight uterine strips receiving the dose X_u.

n_s = number of uterine strips receiving dose X_s.

n_u = number of uterine strips receiving dose X_u.

S - indicates summation.

Then calculate:

$$X_s = \frac{S(n_s X_s)}{S(n_s)}, \qquad \bar{y}_s = \frac{S(n_s \bar{y}_s)}{S(n_s)}, \qquad X_u = \frac{S(n_u X_u)}{S(n_u)}, \qquad \bar{y}_u = \frac{S(n_u \bar{y}_u)}{S(n_u)}$$

Two straight lines having the same slope b but differing in position are fitted by least squares to the observations for each assay. The dosage-response relationships for the standard and unknown are

$$Y - \bar{y}_s = b(X_s - X_s)$$
$$\text{and } Y - \bar{y}_u = b(X_u - X_u)$$

with

$$b = \frac{S\left[n_s \bar{y}_s (X_s - X_s)\right] + S\left[n_u \bar{y}_u (X_u - X_u)\right]}{S\left[n_s (X_s - X_s)^2\right] + S\left[n_u (X_u - X_u)^2\right]}$$

The horizontal distance between the two straight lines gives the logarithm of the potency ratio (M):

$$M = \left(\frac{\text{Potency of unknown}}{\text{Potency of standard}}\right) = X_s - X_u + \frac{\bar{y}_u - \bar{y}_s}{b}$$

In the usual application of the above formulae the standard deviation of M is given by

$$\sigma_M^2 = \frac{E^2}{b^2} + \frac{(y_u - y_s)^2 \, \sigma_b^2}{b^4}$$

where

$$E^2 = \sigma^2 \left(\frac{1}{S(n_s)} + \frac{1}{S(n_u)} \right)$$

$$\sigma_b^2 = \frac{\sigma^2}{S\left[n_s(X_s - \bar{X}_s)^2 \right] + S\left[n_u(X_u - \bar{X}_u)^2 \right]}$$

and σ^2 is the variance in response of test objects receiving the same dose. It is estimated by taking the sum of squares of deviations from the dose means and dividing by $(S(n_s) + S(n_u) - r)$, where r is the total number of doses used.

The above method for estimating the standard deviation of the logarithm of the potency ratio (σ_M) is unsuitable for determining the limits of error of an assay by the present procedure because the calculation of σ^2 does not give the true variance in response of the uterine strips. When the response is measured in millimeters (or any other unit) and σ^2 is estimated as the variance in response of uterine strips receiving the same dose, then the differences in magnitude of the recordings caused by inequalities in the length of the eight strips, inequalities in the magnification of the responses, difference in sensitivity of the eight strips and changing sensitivity as the experiment progresses would give an unduly large value to σ^2. Differ-

ences in the magnitude of the responses should not be included in the estimate of error because the various doses are given to the same group of test objects.

Schild (20) assayed solutions of histamine on the isolated intestine (single strip) of the guinea pig. By the repeated randomized administration of only four doses (low and high doses of standard and low and high doses of unknown) successive groups of four responses were obtained at different levels of sensitivity. This procedure largely eliminates the effect of changing sensitivity from the experimental comparisons, but as stated by Schild (20) "It is essential, however, that the difference between groups should be eliminated not only from the experimental comparison but also from the estimate of error, by the methods of analysis of variance described ...". The design of the assay developed in the present study is similar in that only four doses are repeated in successive "tests" at different levels of sensitivity to largely eliminate the effect of changing sensitivity from the experimental comparison. Successive groups of four responses were obtained (Tables XV to XXIV) but by the use of eight strips instead of one, eight groups were recorded each time the four doses were repeated. Elimination of the difference among groups from the estimate of error becomes still more essential because of the additional factors, such as difference in sensitivity, inequalities in length of strips and inequalities in the magnification of the responses by the recording levers,

which cause these differences.

The statistical method described by Schild (20) for the estimation of potency and analysis for error in histamine assays on single strips was employed for the assays on eight strips by the procedure adopted for ergonovine (assays 15 to 24). The same method was also applied to the preliminary assays 9 to 14. Assays 1 to 8 are not of a suitable design to make the method of analysis applicable.

The following outline of the calculations involved was adopted from Schild (20). The logarithm of the ratio of the potencies of the standard and unknown doses administered is given by

$$M = \frac{\bar{y}_u - \bar{y}_s}{b}$$

where $\bar{y}_u - \bar{y}_s$ is the difference between the mean responses to unknown and standard and b is the slope of the regression line. When the doses of standard and unknown are not the same the logarithm of the ratio of the potency of the unknown in terms of the standard is given by

$$M = \bar{X}_s - \bar{X}_u + \frac{\bar{y}_u - \bar{y}_s}{b}$$

where $\bar{X}_s - \bar{X}_u$ is the difference between the mean logarithms of the doses of the standard and unknown respectively. This is, of course, the same as the previous formula for M, p. 48.

Let $S(y_s)_1$ denote the sum of all the responses (heights of recorded contraction) from the larger dose of the standard and $S(y_s)_2$, $S(y_u)_1$, $S(y_u)_2$ represent corresponding sums of

responses of the smaller dose of the standard and larger and smaller dose of the unknown. Let N denote the number of groups of four responses and d the logarithmic interval between low and high doses, then

$$\bar{y}_u - \bar{y}_s = \frac{S(\bar{y}_u)_1 + S(\bar{y}_u)_2 - S(\bar{y}_s)_1 - S(\bar{y}_s)_2}{2N}$$

and

$$b = \frac{S(\bar{y}_u)_1 + S(\bar{y}_s)_1 - S(\bar{y}_u)_2 - S(\bar{y}_s)_2}{2Nd}$$

Let

$$S(\bar{y}_u)_1 + S(\bar{y}_u)_2 - S(\bar{y}_s)_1 - S(\bar{y}_s)_2 = A$$

and

$$S(\bar{y}_u)_1 + S(\bar{y}_s)_1 - S(\bar{y}_u)_2 - S(\bar{y}_s)_2 = B$$

then the expression for M, when standard and unknown doses are not the same, becomes

$$M = \bar{X}_s - \bar{X}_u + \frac{A}{B} d \quad .$$

A typical analysis of variance, for illustration, is computed from the data of ergonovine assay 19 (Table XIX) by application of the same mathematics employed by Schild (20). The variate is the recorded maximum height of contraction produced by the addition of the indicated volumes (doses) of ergonovine solution (Table XXV). Five distinct sources of variation are isolated (Table XXVI); for each source, column 1, the sum of squares of deviations from the mean is computed, column 2, which divided by the appropriate

TABLE XXV. Data from Ergonovine Assay 19
Rearranged for Computation.

Strip No.	Group No.	U_1 3.17	S_1 2.38	U_2 2.00	S_2 1.50	Sum in mm.
Test 1						
1	1	57	48	32	17	154
2	2	42	35	28	26	131
3	3	117	120	98	95	430
4	4	56	57	18	5	136
5	5	70	81	60	48	259
6	6	97	102	67	45	311
7	7	53	75	48	46	222
8	8	60	63	36	30	189
Test 2						
1	9	60	69	44	51	224
2	10	37	47	38	32	154
3	11	122	129	110	120	481
4	12	94	99	40	84	317
5	13	51	77	21	38	187
6	14	117	128	105	117	467
7	15	80	75	17	32	204
8	16	95	94	85	75	349
Test 3						
1	17	77	78	50	43	248
2	18	78	79	57	57	271
3	19	118	108	100	86	412
4	20	89	69	62	83	303
5	21	92	56	69	33	250
6	22	136	106	116	112	470
7	23	81	80	66	54	281
8	24	95	83	81	85	344
Sum		1974	1958	1448	1414	6794

degrees of freedom (df), column 3, gives the mean square, column 4.

TABLE XXVI

Analysis of Variance of Ergonovine Assay 19.

Source of Variation	Sum of squares	Degrees of freedom	Mean square
Between groups	67,306	23	2926
Between S and U	26	1	26
Regression	11,926	1	11,926
Deviation from parallelism	3.4	1	3.4
Error	6,411.6	69	92.9
Total	85,673.	95	

If S_1, S_2, ..., S_N are the sums of the four responses in each group (sum of each line as given in the last column of Table XXV), and $S = S_1 + S_2 + ... + S_N$, then the following statistics are computed to obtain the values in Table XXVI.

(1) The correction term, $C = S^2/4N = 6794^2/4(24) = 480,817$.

(2) The sum of the squares of all items,

$$= S(X^2) = 57^2 + 48^2 + ... + 81^2 + 85^2 = 566,490.$$

(3) The total sum of squares

$$= S(X^2) - C = 566,490 - 480,817 = 85,673.$$

(4) The sum of squares for groups

$$= (S_1^2 + S_2^2 + ... + S_N^2)/4 - C = (154^2 + 131^2 + ... + 344^2/4$$
$$- 480,817 = 67,306;$$

and the corresponding mean square by division by $(N - 1)$, the corresponding degrees of freedom: $67,306/(24 - 1) = 2,926$.

(5) The sum of squares for

(a) variation between standard and unknown

$$= A^2/4N = (1974 + 1448 - 1958 - 1414)^2/4(24) = 26.$$

(b) regression

$$= B^2/4N = (1974 + 1958 - 1448 - 1414)^2/4(24) = 11,926.$$

(c) deviation from parallelism

$$= S(\bar{y}_u)_1 + S(\bar{y}_s)_2 - S(\bar{y}_u)_2 - S(\bar{y}_s)_1{}^2/4N$$

$$= (1974 + 1414 - 1958 - 1448)^2/4(24) = 3.4$$

For each of these three sources of variation, a, b, c, only a single degree of freedom is available and their mean square is thus numerically equal to their sum of squares.

(6) The sum of squares for error

$$= \text{total} - (\text{groups} + \text{standard v. unknown} + \text{regression} + \text{parallelism})$$

$$= 85673 - (67,306 + 26 + 11,926 + 3.4) = 6411.6$$

The mean square for error is obtained by dividing by $(3N-3)$ which is the corresponding degrees of freedom:

$$6411.6/(72-3) = 92.9.$$

Tests of significance may be made by comparing the ratio F (larger mean square/smaller mean square) with tabulated values of F as described by Schild [20]. The main purpose of isolating the sources of variation, indicated in Table XXVI, is to eliminate from the calculation of the limits of error the difference among groups caused by changing sensitivity and difference in sensitivity, length of strips and magnification of responses.

The estimation of potency and the limits of error of the estimate are calculated for this example as follows:

$$M = \bar{X}_s - \bar{X}_u + \frac{A}{B}d$$

$$= -.1249 + \frac{50}{1070} \times .2$$

$$= -.1155 = \bar{1}.8845$$

100(antilog M) = Per cent. potency of unknown in terms of standard.

Therefore

100(antilog $\bar{1}.8845$) = 76.6% of standard.

Per cent. of the true value is obtained by dividing the estimated potency by the actual potency.

So

76.6/68.0 x 100 = 112.6% of true value.

The per cent. error is given by

112.6% - 100% = + 12.6% error.

The standard error of M is given by

$$s_M = 2 \, \sigma \, d \, \sqrt{N} \, \frac{\sqrt{(A^2 + B^2)}}{B^2}$$

where σ is the square root of the error mean square in the analysis of variance (Table XXVI). Therefore

$$s_M = 2 \times \sqrt{92.9} \times .2 \times \sqrt{24} \times \frac{\sqrt{2500 + 1144900}}{1144900}$$

$$= .01776$$

The limits of error of the estimate of potency are given by M $\pm$ $s_M t$. The value of t for the desired probability is obtained from Fisher's t table (10) for (3N-3) degrees of freedom, the same number as for error in the analysis of

variance. The value of t for P = .99 and 69 df is practically the same as for an infinite df, i. e. 2.576.

In this example

$$s_M t(P-.99) = .01776 \times 2.576 = .0457$$

and the P = .99 limits of error of the assay are

$$- .1155 + .0457 = -.0698 = \bar{1}.9303$$

$$\text{Antilog } \bar{1}.9303 = 68.98\%$$

and

$$-.1155 - .0457 = -.1612 = \bar{1}.8387$$

$$\text{Antilog } \bar{1}.8387 = 85.17\%.$$

This means for a probability of 0.99 that there is only one chance in a hundred that the true potency falls outside the limits of 68.98% and 85.17%.

The results of the estimation of potency and analysis of variance of assays 15 to 24 are given in Table XXX. Similar calculations for potency and limits of error for assays 9 to 14 were made and the results included in Table XXX.

Figure 2 is a graphical presentation of the data from assay 19. Figure 2a shows the scatter of the regression lines for standard and unknown from individual strips caused chiefly by the differences in the magnitude of the responses in different groups. Figure 2b shows the mean regression line for standard and unknown.
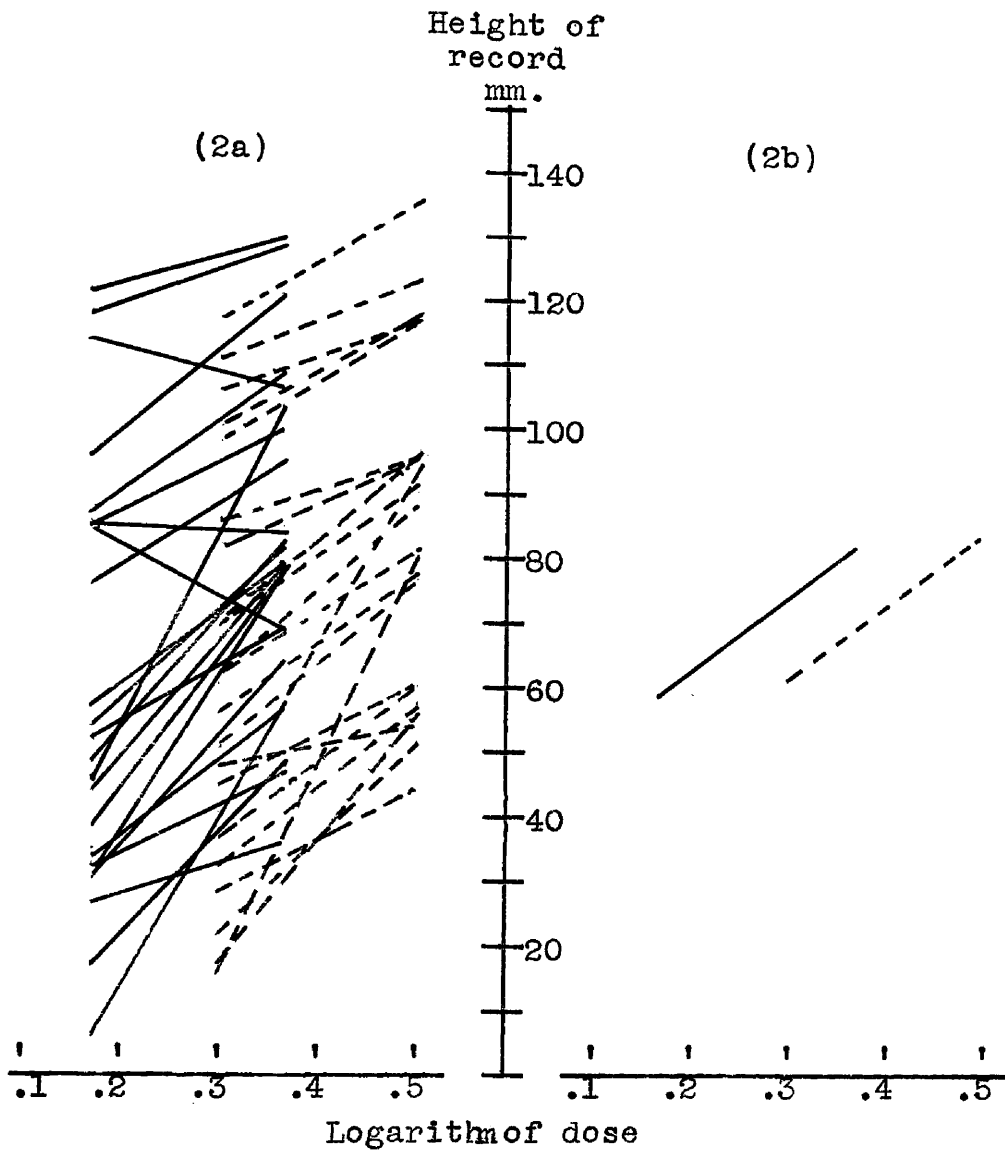
Figure 2. Graphical Presentation of Data

from Assay 19.

Figure 2a shows the regression lines from individual strips

Standard = _____, Unknown = ------.

Figure 2b shows the mean regression curves for standard, ——,

and unknown, -----.

Though the method of analysis effectively eliminates differences among groups from the estimation of error, another factor was observed which might have an effect of the estimated potency ratio and also on the estimation of limits of error. It was found that the responses in some groups were quite small compared to those in other groups. When this is the case the small responses would have relatively little effect on the mean or total response from which A and B are calculated. For example, in a hypothetical series of groups:

| | Response in milli- | | | | | |
| | meters for strips No. | | | | Sum | |
Dose	1	2	3	4	mm.	
U_1	4	6	8	100	118	
S_1	4	6	8	80	98	A = 40
U_2	2	3	4	90	99	B = 38
S_2	2	3	4	70	79	

assume that standard and unknown are of equal potency. The first three strips measure the potency accurately but have too little effect on the total because of the large responses of strip 4. The potency calculated from these figures, when d = .2, would be: M= 40/38 x .2 = .2105. 100(antilog M) = 100 x 1.634 = 163.4% of standard instead of the true value of 100.%.

To give the responses from each strip an approximately equal weight in the totals let the highest response for each strip equal 100 and let each of the other values in the same group have its proportional value. The values

then become

Dose	Response in milli-meters for strips No. 1	2	3	4	Sum mm.	
U_1	100	100	100	100	400	
S_1	100	100	100	80	380	A = 40
U_2	50	50	50	90	240	B = 120
S_2	50	50	50	70	220	

and the erratic responses of strip 4 have much less effect on the result. The estimated potency now is: M = 40/120 x .2 = .0667. 100(antilog M) = 100 x 1.165 = 116.5% of the true value instead of 163.4% as calculated previously.

To determine whether or not conversion of the responses in this manner would have any practical effect in evaluating the potency, the responses constituting the assays in Tables IX to XXIV were converted to "percentage highest response in each group" (%HR) and the data treated as before. For example the data in Table XXV (assay 19) were converted to %HR and are given in Table XXVII. The results of assays 9 to 24 after conversion of the responses to %HR and subsequent analysis are given in Table XXXI. Table XXXII gives the results by both methods of recording the responses for comparison.

TABLE XXVII. Data from Table XXV (Assay 19) converted to % highest response (%HR).

Strip No.	Group No.	U_1 3.17	S_1 2.38	U_2 2.00	S_2 1.50	Sum in mm.
Test 1 1	1	100	84	67	30	281
2	2	100	83	67	62	312
3	3	97	100	82	79	358
4	4	98	100	32	9	239
5	5	86	100	74	59	319
6	6	95	100	66	44	305
7	7	71	100	64	61	296
8	8	95	100	57	48	300
Test 2 1	9	87	100	64	74	325
2	10	79	100	81	68	328
3	11	94	100	85	93	372
4	12	95	100	40	85	320
5	13	66	100	27	49	242
6	14	91	100	82	91	364
7	15	100	94	21	40	255
8	16	100	99	90	79	368
Test 3 1	17	99	100	64	55	318
2	18	99	100	72	72	343
3	19	100	91	85	73	349
4	20	100	78	70	93	341
5	21	100	61	75	36	272
6	22	100	78	85	82	345
7	23	100	99	82	67	348
8	24	100	87	85	89	361
	Sum	2252	2254	1617	1538	7661

DISCUSSION

Effect of Changing Sensitivity. The nature of the re-gression curves, Figures 3a and 3b, suggest that their de-viation from a straight line may be due chiefly to changing mean sensitivity of the eight uterine strips as the assay progresses. That there is usually such a change is clearly shown by the shifting position of the regression curves in Figure 3c and by the sensitivity curves in Figure 4. In Figure 3c, if there were no change in mean sensitivity the four doses repeated in successive tests would produce the same regression curves. That is, curves 1 - 2, 6 - 5 and 9 - 10 for the standard would be superimposed. This would also be true of curves 4 - 3, 7 - 8 and 11 - 12 for the unknown. Due to changing sensitivity the curves differ in position as can be seen from the figure.

The curves in Figure 4 illustrate the changing sensi-tivity of the strips which occurred in thirteen assays. If there were no change in mean sensitivity of the uterine strips to the drug these curves would all be straight lines parallel to the abscissas. The change in sensitivity is not constant. In some assays there is a marked increase, in others there is a decrease--sometimes there is both decrease and increase in the same assay. In most cases, however, although the rate of change differs it is usually in one direction and approaches linearity.

The chief difficulty in the development of a suitable procedure for conducting the assay is to eliminate the

Assay 1   Assay 2   Assay 14
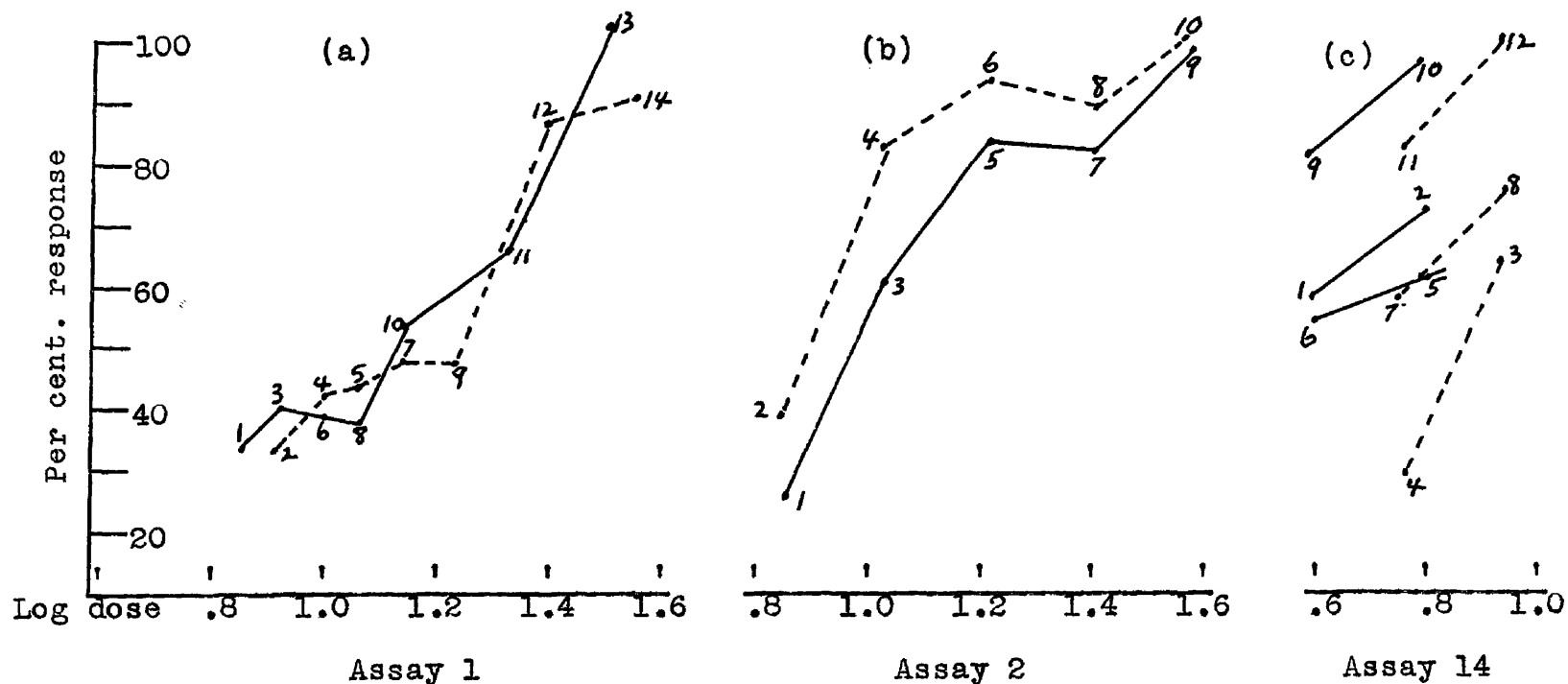
The average response of eight strips, as percentage of greatest mean respønse, is plotted against the logarith of the dose. Standard = ———, unknown = ------.

The numbers on the curves give the order of administration of doses. In (c) only four doses were administered in three successive tests.

Figure 3. Examples of Regression Curves.
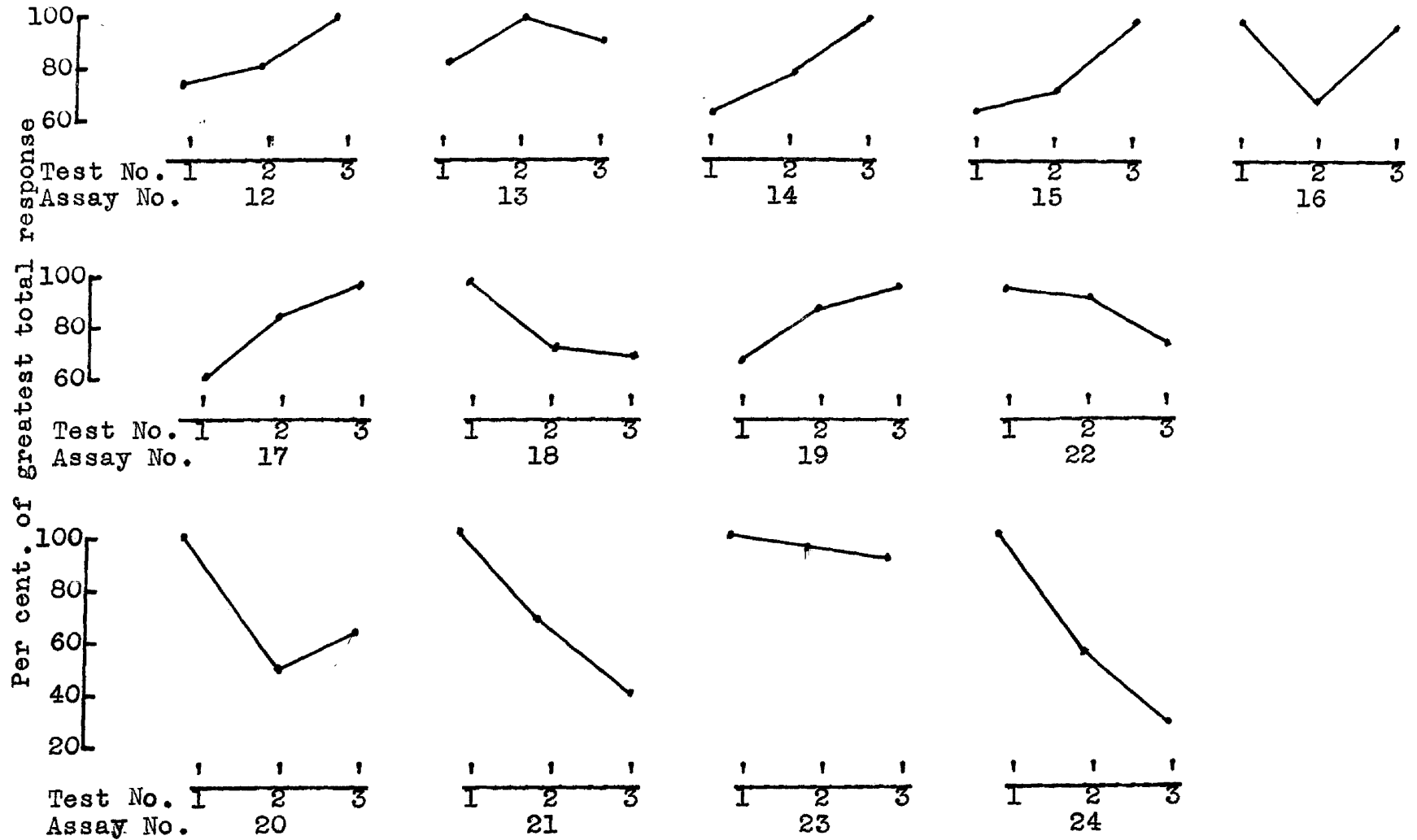
Each curve represents the assay given by number. Each point on a curve represents the total response from the four doses applied in a single "test". Three "tests" comprise each assay.

Figure 4. Curves Showing Changing Sensitivity.

effect of changing sensitivity. The logarithm of the potency ratio is given by $M = \bar{X}_s - \bar{X}_u + \bar{y}_u - \bar{y}_s/b$ and the changing mean sensitivity of the uterine strips must therefore be considered from the standpoint of its effect on these terms. Since $\bar{y}_u$ is the mean response from all doses of the unknown and $\bar{y}_s$ is the mean response of all doses of the standard, $\bar{y}_u - \bar{y}_s$ would have its true value only if the standard and unknown doses were given at the same level of sensitivity. This is most nearly approached when doses of standard and unknown are given alternately as in assay 2 (Table II). Under this condition the doses of the two solutions to be compared are separated by only one dose interval, 20 minutes in these assays, and the effect of changing sensitivity is limited to that which occurs during this short interval.

In order to determine b for each experiment it is necessary to use at least two different doses of standard and two different doses of unknown. To obtain the true value of b, low and high doses must be given at the same level of sensitivity. This condition is most effectively satisfied by administering one low and one high dose of one preparation successively as in assay 14 (Table XIV). This "dose order", however, makes it impossible to alternate standard and unknown doses, which must be done to eliminate the effect of changing sensitivity on $\bar{y}_u - \bar{y}_s$. Any error in the determination of b would not usually have much effect on the estimation of potency because the effects of standard and unknown can be quite closely matched by the administration of

preliminary orienting doses and, since $M = X_s - X_u + \bar{y}_u - \bar{y}_s/b$, an error in b would have less effect as $\bar{y}_u$ and $\bar{y}_s$ became equal. The b value is, however, a component of the formula which defines the limits of error of the assay and should be as accurate as possible.

The Order of Administration of Doses. The procedure adopted as suitable for conducting an assay was chosen on the basis of the above considerations of the effect of changing sensitivity. The object is to obtain critical data over the shortest possible range of changing sensitivity, that is, during the shortest possible time. This is done by giving the minimum number of doses (four) that will provide data for the calculation of potency and error of the assay. The doses are administered in the following order in the first "test": $S_2 \, U_2 \, S_1 \, U_1$ where 1 indicates the high dose and 2 indicates the low dose. To obtain greater accuracy and to further neutralize the effect of changing sensitivity the doses are repeated in a second "test" with S and U in reverse order: $U_2 \, S_2 \, U_1 \, S_1$ and in a third "test" in the order $S_2 \, U_1 \, U_2 \, S_1$, reversing S and U and the order of low and high doses within the "test". A complete assay then consists of three separate "tests" carried out at different levels of sensitivity of the uterine strips.

To summarize, the doses of standard and unknown which are found by preliminary orienting tests to produce approximately equal effects and to cause most (preferably all) of the strips to respond comprise the two low doses. The

high doses are automatically fixed since they are larger by a definite dose interval. A logarithmic interval of .2 was found suitable in the assays 15 to 24. A logarithmic interval of .30, that is doubling the dose, could be used as in some of the preliminary assays (10, 11, 13). The four doses are administered in three successive "tests" as follows:

Test 1	Test 2	Test 3
S_2 U_2 S_1 U_1	U_2 S_2 U_1 S_1	S_2 U_1 U_2 S_1

This is similar to Schild's procedure (20) except that the doses are repeated on eight strips instead of one and the doses are not given in random order. When eight strips are used "randomization" occurs by virtue of the variability of the responses of the different strips to a given dose. A "planned" order of administration of doses is desirable in this case to experimentally minimize the effect of changing mean sensitivity within "tests".

In view of the nature of the sensitivity curves in figure 4, it may be that the above "dosage order" does not represent the optimal procedure. Most of the curves show an almost linear change in sensitivity of the uterine strips for a given assay. Reversal of the low and high doses in "test 2" above should more effectively neutalize the effect of changing sensitivity on the determination of b. On the other hand, the order of doses in "test 3" would seem more satisfactory because it assumes a linear change in sensitivity only during the period of one "test" both with respect to S and U and the low and high doses. Probably the

nearest approach to the complete elimination of the effect of changing sensitivity would be to administer the doses as follows:

$$\overbrace{S_2 \ U_1 \ U_2 \ S_1}^{\text{Test 1}} \quad \overbrace{U_2 \ S_1 \ S_2 \ U_1}^{\text{Test 2}} \quad \overbrace{S_2 \ U_1 \ U_2 \ S_1}^{\text{Test 3}}$$

More tests could be conducted to increase the accuracy of an assay.

Further improvement could probably be effected by reducing the time interval between doses, thereby reducing the time necessary to complete a "test" and therefore the time during which changing sensitivity would affect the result. The ten minute period allowed for the action of a given dose in these assays could probably be reduced by several minutes. The time for the "normal" recording could also be reduced somewhat. If the time interval between doses were reduced from twenty minutes to fifteen minutes, one "test" could be completed in sixty minutes instead of eighty. Other drug-tissue combinations, such as solution of posterior pituitary on isolated guinea pig uterus may permit of an even further reduction in the time interval.

Suitability of the Uteri Used. All uteri were successfully employed but considerable difference in the amount of variation in response to the drug was encountered in uteri from different animals. This variation is a biological phenomenon inherent in the tissue itself and cannot be controlled. The quantity σ, which is the standard deviation of the responses of the tissue strips to a given dose, is a measure of this variation. However, the numerical value

TABLE XXVIII

Comparison of the Variability of the Responses
(σ/b) of Strips from Different Uteri.

Assay No.	Weight Rabbit Kg.	Weight Uterus Grams	o/b
9	1.9	2.1	.2500
10	2.5	4.2	.1516
11	2.7	7.2	.1361
12	2.2	2.2	.1587
13	"	" *	.1672
14	2.4	6.8	.0822
15	"	"	.0769
16	1.8	4.5	.0936
17	"	"	.0985
18	1.7	2.9	.1331
19	"	"	.0864
20	1.9	3.1	.1622
21	"	"	.2074
22	2.3	3.8	.1854
23	"	"	.1412
24	1.8	1.8	.2424

* Strips were prepared from the remaining horn
of the same uterus employed in the preceding
assay.

of σ will vary from one assay to another depending on the length of the tissue strips used, magnification of responses and other undetermined factors. Comparison of the values of σ from different assays therefore yields no useful information. The variability of the responses of the tissue strips used in different assays can, however, be reduced to a comparable expression by dividing σ by the slope b of the regression curve. The value of σ/b then provides an absolute measure of the variability of the responses of the tissue strips used in a given assay (20).

Small values for σ/b indicate small variations and, conversely, large values indicate greater variation. The result of an assay, when σ/b is small, is more accurate for a given number of responses. Therefore, a uterus which yields a small σ/b value is more suitable than those giving high values.

Inspection of Table XXVIII, compiled for assays 9 to 24, reveals that in general the heavier (more muscular) uteri usually obtained from the larger rabbits are more suitable for this method of assay because they give smaller σ/b values. While correlation is not significant there is a trend in this direction.

Discussion of Results of Assays. The results of the entire series of assays (Tables XXIX and XXX) indicate that an estimate of potency can be made by any one of several procedures so far as order of administration of doses, logarithmic interval between low and high doses and

TABLE XXIX

Results of Assays 1 to 8*
(see Tables I to VIII).

Assay No.	True potency % standard.	Estimated potency.% std.	Per cent. true potency	Per cent. error.
1	86.5	88.4	102.2	+ 2.2
2	134.0	140.1	104.5	+ 4.5
3	47.0	54.6	116.2	+ 16.2
4	80.0	76.7	95.9	- 4.1
5	162.0	162.0	100.0	0.0
6	250.0	229.0	91.2	- 8.8
7	98.0	80.0	81.6	- 18.4
8	152.0	135.0	88.8	- 11.2

* No analyses of variance were made for these assays.

TABLE XXX. Results of Assays 9 to 24 (Tables IX to XXIV).

Assay No.	True potency % Std.	Est'd potency % Std.	$s_M t$ (P-.99)	Limits of error of estimate % (P-.99)	Per cent. true value (Est'd/true)	Per cent. error	σ/b	Significance of deviation from parallel.*
9	224.0	242.7	.1365	177.2 - 332.4	108.3	+ 8.3	.2500	O
10	124.0	112.9	.0992	89.8 - 141.8	91.0	- 9.0	.1516	S
11	144.0	127.5	.0902	103.6 - 156.9	88.5	- 11.5	.1361	O
12	44.0	46.1	.1314	34.1 - 62.5	104.7	+ 4.7	.1587	S
13	94.0	106.3	.0884	86.7 - 130.3	113.1	+ 13.1	.1672	O
14	54.0	62.9	.0435	56.9 - 69.5	116.5	+ 16.5	.0822	HS
15**	54.0	58.5	.0421	53.1 - 64.4	108.3	+ 8.3	.0769	O
16	186.0	187.1	.0492	167.0 - 209.5	100.6	+ 0.6	.0936	O
17	186.0	170.8	.0549	150.5 - 193.8	91.8	- 8.2	.0985	O
18	68.0	72.5	.0706	61.6 - 85.3	106.6	+ 6.6	.1331	O
19	68.0	76.6	.0457	68.98 - 85.17	112.6	+ 12.6	.0864	O
20	22.0	17.3	.1092	13.5 - 22.3	78.6	- 21.4	.1622	O
21	96.0	95.7	.1213	72.3 - 126.5	99.7	- 0.3	.2074	O
22	140.0	115.6	.1015	91.5 - 146.1	82.6	- 17.4	.1854	O
23	76.0	79.0	.0595	68.9 - 90.6	103.9	+ 3.9	.1412	O
24	144.0	142.2	.1283	105.8 - 191.1	98.8	- 1.2	.2424	HS

*F test (20). H-highly significant, P>.99; S-significant, P>.95; O-not significant, P<.95
** Assays 15 to 24 were made by the procedure adopted (see page 66).

the number of points on the regression line are concerned. The average error for the twenty four assays is 8.71% ± 5.95%. The results of the last ten assays, 15 to 24 (Table XXX, column 7), conducted by the procedure adopted as most suitable for reasons stated previously, show an average error of 8.05% ± 7.21%. Seven of the ten assays gave errors of less than 10.%. The largest error obtained in any assay was 21.4% (assay 20). The true potency of the unknown in this assay (22.0% of standard) is, however, within the computed P = 0.99 limits of error (13.5% to 22.3%).

The limits of error of the estimated potencies, calculated by the method described, provide a good indication of the reliability of the results of individual assays. The true potencies of the unknowns in assays 15 to 24 (Table XXX, column 2), with the exception of assay 19, are within the computed P = .99 limits of error (Table XXX, column 5). The true potency of the unknown in assay 19 (68.0%) is barely outside the computed limits of error (68.98% to 85.17%).

The $s_M t$ values for P = .99 (Table XXX, column 4) from which the limits of error are calculated (M ± $s_M t$) range from .0421 in assay 15 to .1283 in assay 24. The limits of error for individual assays therefore vary between 90.76% to 110.2% and 74.42% to 134.4% of the true values. Because the limits of error for different assays are not constant it is necessary to compute these limits for each assay in order to determine the reliability of the result.

The values of σ/b (Table XXX, column 8) vary from

.0769 in assay 15 to .2424 in assay 24. These values indicate the extent of the variability of the uterine strips in these assays. They are rather large and vary over a considerable range which is not unusual for this type of assay. Any improvement in technique, such as selection of certain types of uteri or increased efficiency of the mecahnical arrangement of the apparatus which would reduce the value of σ/b, would increase the accuracy of the assays.

The method of assay is based on the assumption that the response to the drug increases linearly with the logarithm of the dose. The analysis of variance for the assay in its present form does not include a test for deviation from linearity but the test for deviation from parallelism is an indirect test of linearity and is sufficiently stringent to indicate that in the procedure adopted the assumption of linearity is not unwarranted. When there is a significant deviation from parallelism the results of an assay should not have as much weight as when the lines are parallel. This would probably be important in the application of the procedure to the assay of ergonovine in crude ergot where samples of ergonovine solutions might be contaminated with the antagonistic ergotoxine-like alkaloids and so give a regression curve not parallel with the regression curve of the ergotoxine free standard solution.

The regression lines for standard and unknown show significant deviations from parallelism in assays 10 and 12 (Table XXX, column 9) and highly significant deviations

from parallelism in assays 14 and 24. In only one assay (assay 24), of the series 15 to 24, was there a significant deviation from parallelism indicating that the procedure adopted for this series is more suitable for carrying out the assay than those employed in assays 9 to 14.

All of the assays analyzed (9 to 24) showed highly significant regression (F test (20)) making a quantitative estimate of the potency possible. All of these assays also showed highly significant differences among groups of responses showing the necessity for eliminating these differences from the estimate of error.

The results of assays 9 to 24, computed after converting the data to percentage of the highest response in each group (%HR) are given in Table XXXI and appropriate values are compared in Table XXXII. The mean values and standard deviations of $s_M t$ (P = .99), per cent. error and σ/b are somewhat smaller after conversion of the responses. Deviations from parallelism are somewhat less. The results of some individual assays are changed considerably (assays 12, 17, 20) but the mean values are so nearly the same that it is concluded that conversion of the responses in this manner does not produce sufficient improvement in the accuracy of the assays to justify the additional calculations involved in converting the responses.

TABLE XXXI. Results of Assays 9 to 24 after Conversion of the Responses from Millimeters to Percentage Highest Response in Each Group (%HR).

Assay No.	True potency % Std.	Est'd potency % Std.	$s_M t$ (P-.99)	Limits of error of estimate % (P-.99)	Per cent. true value (Est'd/true)	Per cent. error	σ/b	Significance of deviation from parallel.*
9	224.0	237.8	.1198	180.5 - 313.3	106.2	+ 6.2	.2205	0
10	124.0	108.1	.1600	74.8 - 156.2	87.2	- 12.8	.2043	0
11	144.0	126.0	.0763	103.4 - 146.9	87.5	- 12.5	.1141	0
12	44.0	49.1	.0997	39.0 - 61.8	111.6	+ 11.6	.1286	S
13	94.0	105.7	.0590	92.3 - 121.1	112.4	+ 12.4	.1116	0
14	54.0	61.2	.0701	52.1 - 71.9	113.3	+ 13.3	.1310	S
15**	54.0	59.1	.0477	53.0 - 66.0	109.4	+ 9.4	.0878	0
16	186.0	190.8	.0448	172.1 - 211.5	102.6	+ 2.6	.0850	0
17	186.0	176.8	.0412	160.8 - 194.4	95.1	- 4.9	.0759	0
18	68.0	70.7	.0742	59.6 - 83.8	104.0	+ 4.0	.1361	0
19	68.0	77.0	.0559	67.7 - 87.6	113.2	+ 13.2	.1061	0
20	22.0	19.8	.0545	17.6 - 22.4	90.0	- 10.0	.0951	0
21	96.0	95.3	.0760	80.0 - 113.5	99.3	- 0.7	.1293	0
22	140.0	112.0	.0907	90.9 - 138.0	80.0	- 20.0	.1673	0
23	76.0	79.0	.0775	.66.1 - 94.5	103.9	+ 3.9	.1458	0
24	144.0	145.1	.0916	117.5 - 179.1	100.8	+ 0.8	.1737	S

* and ** Same as in Table XXX.

TABLE XXXII. Comparison of Results Obtained by Recording Response in Millimeters (mm.) and Percentage Highest Response (%HR) from Tables XXX and XXXI.

Assay No.	$s_M t$ (mm.)	$s_M t$ (%HR)	% error (mm)	% error (%HR)	σ/b (mm.)	σ/b (%HR)	Sig. Dev. Parallel. (mm)	Sig. Dev. Parallel. (%HR)
9	.1365	.1198	+ 8.3	+ 6.2	.2500	.2205	O	O
10	.0992	.1600	- 9.0	- 12.8	.1516	.2043	S	O
11	.0902	.0763	- 11.5	- 12.5	.1361	.1141	O	O
12	.1314	.0997	+ 4.7	+ 11.6	.1587	.1286	S	S
13	.0884	.0590	+ 13.1	+ 12.4	.1672	.1116	O	O
14	.0435	.0701	+ 16.5	+ 13.3	.0822	.1310	HS	S
15	.0421	.0477	+ 8.3	+ 9.4	.0769	.0878	O	O
16	.0492	.0448	+ 0.6	+ 2.6	.0936	.0850	O	O
17	.0549	.0412	- 8.2	- 4.9	.0985	.0759	O	O
18	.0706	.0742	+ 6.6	+ 4.0	.1331	.1361	O	O
19	.0457	.0559	+ 12.6	+ 13.2	.0864	.1061	O	O
20	.1092	.0545	- 21.4	- 10.0	.1622	.0951	O	O
21	.1213	.0760	- 0.3	- 0.7	.2074	.1293	O	O
22	.1015	.0907	- 17.4	- 20.0	.1854	.1673	O	O
23	.0595	.0775	+ 3.9	+ 3.9	.1412	.1458	O	O
24	.1283	.0916	- 1.2	+ 0.8	.2424	.1737	HS	S
Mean	.0857	.0774	8.98	8.64	.1483	.1320		
Std.Dev.	.0343	.0307	5.78	5.50	.0541	.0418		

Time and Material Required for an Assay. An assay involving three "tests" as described can be completed conveniently in one day. The time required is governed by the time interval between doses and the total number of doses employed, including the preliminary orienting doses and the "maximal" dose. In the assays reported (15 to 24) the dose interval was twenty minutes and the total number of doses varied from 15 to 18. The actual performance of an assay was completed therefore in from 5 to 6 hours. Additional time is required for preparing the fresh Van Dyke and Hasting's solution and preparing and mounting the uterine strips for each assay.

The amount of ergonovine required for completion of an assay of three "tests" varies depending on the sensitivity of the uterine strips. The total amount of ergonovine required for either standard or unknown never exceeded 5.25 milligrams (assay 21). Usually considerably less was required. In assay 15, for instance, less than 1 milligram of sample was sufficient. An additional amount of standard, about 2 to 4 milligrams, must be provided to determine maximum response of the tissues on completion of the assay.

When this assay procedure is applied to crude ergot it must be remembered that the ergonovine content varies from zero to about twenty milligrams per one hundred grams of drug (19). Therefore an adequate sample would amount to a minimum of twenty-five grams or more according to the suspected ergonovine content.

SUMMARY

1. A new method for conducting a biological assay on isolated tissue is described. Results are computed mathematically and the limits of error for individual assays are calculated. The method involves the use of eight tissue strips and their responses are measured quantitatively. The order of administration of doses is designed to practically eliminate the effect of changing mean sensitivity of the tissue strips.

2. The suitability of the method of assay is demonstrated by the estimation of the potencies of "unknown" solutions of pure ergonovine maleate in comparison with solutions of the same material of known concentration. The average error of ten assays was 8.05% ± 7.21%. The computed P = 0.99 limits of error for individual assays varied between 90.76% to 110.2% and 74.42% to 134.4%.

3. An attempt was made to increase the accuracy of the assays by converting the measured responses of individual tissue strips to percentage of the highest response of _the strip in a test. It was concluded that conversion of the responses in this manner produced no improvement in the accuracy of the results.

4. Eight tissue strips were prepared for each assay from one horn of the uterus of a rabbit which previously received estrogenic therapy with stilbestrol. All animals sacrificed provided uterine strips which were suc-

cessfully employed in an assay.

5. An assay can be conveniently completed in one day by this method.

6. A sample to be assayed should contain at least one to five milligrams of ergonovine depending on the sensitivity of the uterine strips. About five milligrams are necessary to be certain of having sufficient sample to complete an assay.

7. The method is recommended for the biological assay of ergonovine in ergot or ergot preparations from which solutions free of the ergotoxine group can be prepared. The method can be used to determine whether or not chemical methods for the estimation of ergonovine in ergot or its preparations actually measure the biological activity of the drug.

8. The method is recommended for study as to the application of the general procedure for the biological assay of other substances on isolated preparations, such as Posterior Pituitary Injection U.S.P.XII on guinea pig's uterus.

9. An apparatus of improved design, for recording independently the contractions of eight tissue strips immersed in one chamber in a constant temperature bath, is described.

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