THE EXTRACTION AND ASSAY OF CRUDE ERGOT.

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(*Continued from page 856, September 1932.*)

A DETAILED DESCRIPTION OF A SATISFACTORY TECHNIQUE FOR APPLYING THE ISOLATED RABBIT UTERUS METHOD OF BROOM AND CLARK (5).

Burn and Ellis (6) have described in detail the technique they employ in carrying out this method. The experience of the present writer confirms in general their entire description, to which the reader is directly referred.

The comments which follow are primarily intended for those who have experienced difficulties in securing accuracy and dependability with the method, and are more or less supplementary to the earlier descriptions by Broom and Clark (5), Burn and Ellis (6), Pattee and Nelson (7), Swanson (8) and Thompson (9). The slight modifications in technique and particularly the interpretation of results which will be described, remove serious objections to the original as a routine method for estimating the alkaloidal activity of Fluidextract of Ergot.

I. THE APPARATUS.

The principles upon which the method is based require that the apparatus be designed so that two similar uterine strips, mounted in two separate chambers, can be kept under identical conditions for the duration of the assay.

Although the apparatus to be employed need not be expensive or elaborate, there are certain points of construction and arrangement which are of paramount importance. The usual type of apparatus used for isolated smooth muscle experiments has been found quite satisfactory. Dependability, accuracy and speed are increased if the following conditions are met:

(*a*) *Temperature Control.*—The temperature of the bath should be maintained at 37.5° C., as constantly as practicable. Equipment which would maintain this temperature with no fluctuation whatever, would be unnecessarily expensive. In the experience of the writer, a fluctuation of plus or minus 0.25° C. does not detract from the accuracy of the method. This should not be construed as indicating that this degree of fluctuation has no effect upon the quantitative response of the uterine strips. It must be noted that both tissue chambers are in the same bath, and that slight changes in temperature of the bath affect both uterine strips in the same manner to the same degree. Consequently, the percentage expression of potency of an unknown Ergot preparation, in terms of the Standard preparation, will not be altered. Several inexpensive types of temperature regulators, preferably electrically controlled, are available, which provide the constancy indicated.

(*b*) *The Tissue Chambers.*—Tissue chambers, each accurately graduated to a volume of 50.0 cc., have been found satisfactory both from the standpoint of accuracy and also of economy in the amount of Locke-Ringer solution required. The height of the chambers should be such that the graduation is not more than an inch from the top. This permits the addition of accurate doses of test fluids to the top of the liquid in the chambers without the danger of droplets striking and adhering to the glass walls. It is essential that both chambers be of the same size and shape because of the influence these factors have upon the rate of diffusion of drugs through the fluid surrounding the uterine strips. The chambers may be conveniently designed to drain and refill from the bottom.

(*c*) *The Oxygen Supply.*—The oxygen should be led through a sodium bicarbonate solution (approximately 5%), then to the tissue chambers in such a manner that the bubbles are discharged from the very bottom of the chambers. These bubbles, in addition to supplying oxygen to the tissues, facillitate the diffusion of the drug solutions throughout the fluid surrounding the tissues. At the same time, the arrangement should be such that the bubbles do not actually strike the tissues in order to avoid stimulation from this source. The oxygen should issue into the two tissue chambers at the same rate, in bubbles of the same size, as nearly as possible. This is

controlled by making uniform the glass jets through which the oxygen is led into the chambers, and by the use of screw clamps on the rubber tubes leading the oxygen from the sodium bicarbonate solution to the tissue chambers.

The rate of oxygen supply depends to some extent upon the time the uterine strips employed have been kept in the refrigerator. Strips freshly taken require less oxygen than those having been kept in the cold for one or more days. The rate *must* be the same in both chambers.

(*d*) *The Recording Levers.*—The two recording levers must be of light material (aluminum wire is satisfactory), of identical weight, with the fulcrum at the same point on each lever, arranged in such a manner as to magnify the uterine contraction about six times, but identical for each lever. Each lever should be counter-balanced so that any necessary increase of tension on the strips may be accurately accomplished identically on each strip by a movable light weight on the long arm of each of the levers.

(*e*) *The Tension on the Uterine Strips.*—It is obviously impossible to express in any precise manner the amount of tension to place upon the uterine strips. It must be pointed out, however, that the weights on the levers must be only sufficient to cause the writing points to fall with the relaxation of the uterine strips. If the Locke-Ringer solution, temperature and oxygen supply are satisfactory, and the tissues have not been abused prior to, or during, mounting, it is wholly unnecessary to try to force relaxation of the strips by increasing the tension upon them. In short, as little tension as possible should be employed, and it should be similar upon each similar strip.

(*f*) *The Locke-Ringer Solution.*—The ordinary mammalian Locke-Ringer solution used in Pituitary assays or similar isolated mammalian tissue work has been found satisfactory. C.P. salts should be used; distilled water, of recent distillation should be employed. The writer has not found it necessary to use water which has been double-distilled from glass, but has, without exception, successfully used water which was single-distilled from an ordinary large metal still. It has further been observed from a great many tests, that the saline solution may be used with or without magnesium. As to whether or not the Locke-Ringer solution should be freshly prepared on the day of use, it is important to point out that it has never been found necessary to discard any of the saline solution, and has been used with no evidence of difficulty after having attained an age of three weeks in this laboratory. Reasonably fresh preparation of the saline is to be recommended, however, since it imposes no hardship, but it is certainly unnecessary to discard any amount remaining at the end of the day in laboratories having routine assays to perform.

(*g*) *General Comment on Apparatus.*—To combine speed with accuracy in carrying out this method, the entire apparatus must be well and carefully constructed with a view toward a design which makes exchanging used uterine strips for new ones a simple matter requiring less than five minutes.

II. THE SELECTION OF SUITABLE UTERI.

This appears to be a part of the technique which has caused much difficulty in other laboratories. It is well known that the uterus of the rabbit normally undergoes certain very marked changes. The changes which come into immediate evidence are size, tonus, irritability and contractility. Three factors, aside from individual variation in all animals, are responsible for these changes. *First*, the development of the uterus, both as to size and activity, progresses as the animal attains maturity. *Second*, the size and activity of the uterus is very greatly influenced by the œstrus cycle. *Third*, pregnancy exerts a marked influence on the size and activity. Therefore, the size and activity of an isolated uterus depends upon the age of the rabbit, whether pregnant or not, and the stage of œstrus at the time the uterus is removed from the animal.

Satisfactory uteri may be obtained from any of the usual breeds of rabbits available on the market. This laboratory has experienced no difficulty in securing suitable rabbits. We have found it necessary to impose only two restrictions in purchasing, *i. e.*, *first*, that they be non-pregnant females, and *second*, that they be practically full-grown. Dependable sources of supply are numerous. We have come to prefer virgin uteri, although this is not essential. Multiparous uteri of older rabbits are less sensitive to minute gradations of drug dosage than the uteri of younger animals, and therefore the younger are to be preferred. It is significant that, in the use of over a hundred rabbits during the past two years, all were used successfully. In case the source of supply cannot guarantee non-pregnancy, the rabbits should be kept for about one month before using, in order to permit pregnant females to litter. Two weeks, or more, after littering, they are ready for use.

In selecting a rabbit for use, an attempt must be made to avoid any which are actually in œstrus, although finding it in this condition after killing is no reason for discarding it. Only one method for this purpose, which is sufficiently practical as a routine procedure, is available. It involves macroscopic examination of the vaginal orifice (which should show absence of exudation and unusual redness or inflammation). Although the stage of œstrus has a marked influence on the irritability of the uteri, and the character of their response to epinephrine, particularly with respect to spontaneous rhythmical contractions which seriously interfere with the interpretation of results by older procedures, the new method of interpretation described below entirely obviates any errors due to this cause, and makes possible the successful use of uteri whether they are quiescent or not.

III. PERFORMANCE OF TEST.

The actual mechanics of performing the test in this laboratory is as described by Burn and Ellis (6), except that we use 50-cc. instead of 100-cc. tissue chambers, and consequently, doses of epinephrine and Ergot alkaloids indicated by the above authors, are only half as great. It is important to point out that, after much experimentation, we no longer wash out the drugged solution before applying the final epinephrine trials. This washing technique was first recommended by Broom and Clark (5), then Pattee and Nelson (7) and later by the present writer (9), for the purpose of removing non-specific amine (histamine, etc.) augmentation of the epinephrine response.

The following is the technique adopted in this laboratory for evaluating the alkaloidal activity of Fluidextract of Ergot by this method:

The uterus is carefully removed from the rabbit in the usual manner, and placed immediately in normal saline solution. A 1-cm. segment of one horn is clipped off with a fine sharp scissors, and this segment is halved longitudinally, also with the fine scissors. This is accomplished by making the first cut, full length, along the mesometrium, and laying it out flat. If the segment is very large and muscular, a longitudinal portion is trimmed off equally on each side, discarded, and the remainder divided in equal halves, also longitudinally. If the rabbit was of the more desirable age (not too old), and virgin, the segment is simply divided into two equal halves. The attempt in all cases must be to have the two strips identical, both as to size and character of tissue. A little experience soon results in familiarity with characteristic lines of demarcation in the uterine segments, which permits of obtaining strips which always respond with a sufficient degree of similarity, and frequently will show almost exactly the same type of spontaneous activity as well as identical response to a given dose of epinephrine. The two similar strips are then mounted in the chambers, by means of either metal or glass hooks, care being taken to avoid stretching or pinching of that part of the strip between the hooks.

The weights are then adjusted on the long arm of the two recording levers so that the writing points will overcome the slight resistance on the kymograph and fall with the relaxation of the strips. If the strips have been properly cut and mounted, they will be of equal length when both are relaxed. If the uterus was taken on the same day of the test, the assay may be started immediately. If the uterus employed had been kept in the ice chest for more than one day, it is usually necessary to wait from five to fifteen minutes before applying the first epinephrine dose, the waiting period increasing with the time that the uterus had been kept in the refrigerator.

With freshly taken tissues, a dose of 0.2 cc. of a 1:10,000 solution of epinephrine is then added to each chamber. Roughly, the response should begin well within one minute, and persist at its height for not less than two minutes, and not more than five minutes. If such is not the case, the drugged solution is washed out, refilled and the next dose increased or decreased as indicated. If all conditions are properly controlled, the response of both strips will be similar as to character and magnitude. It is not essential that the epinephrine contractions be of exactly the same height. Although a definite dose is indicated above, this must not be construed as meaning that all strips respond correctly to the same dose. This dose is merely suggested as a start for the inexperienced. Subsequent doses can then be increased or decreased, as indicated by the response. Tissues not freshly taken on the day of the test require higher doses of epinephrine. If a dose as great as 0.6 cc. of a 1:10,000 epinephrine solution does not produce reasonably prompt response, the tissues are no longer fit for use, due either to becoming too old in the refrigerator, or to improper storage. Attempts to use uteri after they have attained an age of more than three days in the refrigerator is usually found to result in an unjustified waste of time. Where con-

siderable numbers of Ergot preparations are tested, a single uterus, which yields 8 to 16 pairs of strips, is invariably used up before the passage of three days.

When a satisfactory response from a given dose of epinephrine has been obtained (we rarely find it necessary to make more than two trials), the chambers are drained and refilled to the mark, and an accurately measured dose of the test Ergot preparation (properly diluted) is added to one chamber, and exactly one minute later a dose of the Standard is added to the other. The proper dose of the present U. S. P. Standard Fluidextract of Ergot should be between 0.3 and 0.6 cc. of a 1:20 dilution, depending upon the amount of epinephrine used in securing proper response of the uterine strips, which is usually between 0.2 and 0.6 cc. of a 1:10,000 solution. To illustrate, if a dose of 0.3 cc. of the epinephrine solution is used in a given test, the dose of the Standard Fluidextract dilution should be about 0.4 cc., for the first determination. In general, the dose of Ergot to use increases with the dose of epinephrine required, but it must not be assumed that a definite ratio can be adopted for all different uterine strips.

Ten minutes after the first Ergot dose was added, the selected dose of epinephrine is added to the first chamber, and exactly one minute later the same dose of epinephrine is added to the second. If significant response is not then obtained within two minutes after the addition of the epinephrine, twice the previous epinephrine dose is added to each chamber, *accurately maintaining the one-minute interval between the additions, again without washing*, since no results can be obtained in the absence of significant response from both strips. If response *was* obtained from the first additions of epinephrine, the response is allowed to subside, and, without washing, another dose of epinephrine is added to each, the size of the dose to each depending upon the magnitude of the response obtained from the first additions, and maintaining as before the one-minute interval between the additions. If this response was slight in either strip, the dose of epinephrine should be increased by, for example, one and one-half to twofold, because it must be remembered that the Ergot paralysis becomes greater with the passage of every minute, and were the original epinephrine dose again applied, the resulting response is less than before, even to the point of complete abolition of response.

Following this procedure, one obtains in the first determination, information as to whether a given dose of an unknown Ergot preparation is stronger, weaker or the same as a given dose of the Standard preparation, the greatest potency being shown by the greatest degree of inhibition of the epinephrine response, or, conversely, the lesser potency being shown by the greater epinephrine response. No attempt is made at this point to calculate the potency of the preparation, since no dependable calculation is possible with the data thus obtained. Additional separate determinations must be performed until a dose of the unknown preparation is found which is the exact equivalent of a given dose of the standard preparation employed. Fresh pairs of uterine strips must be used for each separate determination, because we find it impossible to remove, by washing, all traces of the Ergot activity within a reasonable length of time. We work for a rather great degree of paralysis, using doses which inhibit the epinephrine response 50 to 75% in ten minutes.

As one gains experience, it is possible to make the dose selections for the subsequent determinations with an accuracy which makes it unnecessary to carry out more than three separate determinations upon a single sample, and yet obtain accuracy sufficient for all practical purposes. Thus, in no case is it necessary to consume more than two hours for a complete assay, and in many cases a considerably shorter time is required. The writer finds in almost all cases, that three determinations upon a single sample can be carried out in approximately one hour, and yet an accuracy and dependability is attained which can be equalled by no other available biological method.

IV. INTERPRETATION OF RESULTS.

The interpretation of results obtained by this method resolves itself into determining which of the two similar uterine strips was paralyzed the most by the doses of Ergot applied respectively to them, or, in the final determination upon a sample, whether or not the degree of paralysis upon each uterine strip was actually the same, so that from the respective doses of unknown and Standard employed, the potency of the unknown can be calculated in terms of the Standard.

The hitherto accepted method of ascertaining the magnitude of the respective degrees of paralysis in the two strips was simply to measure the heights of the epinephrine responses before and after the Ergot effect, then calculating the percentage inhibition of the epinephrine response

having occurred in each strip. This method of interpretation is quite satisfactory, *provided that the uterine strips are of the quiescent type and do not exhibit any appreciable amount of spontaneous or rhythmical activity.* If the strips do exhibit spontaneous or rhythmical movement to a considerable degree, one will be led into a most serious error by the above method of interpretation, simply because of the fact that spontaneous contractions may add greatly to height of the epinephrine response. This has been one of the most serious objections to the entire method, because it is possible to obtain only a decidedly unsatisfactory percentage of uteri which show a lack of interfering spontaneous contractions. It is only a relatively short period during the œstrus cycle of the rabbit that the uterus is relatively inactive, and there is, as yet, no method of ascertaining that period which justifies itself from the practical standpoint. It is, however, possible to detect, by simple examination of the vaginal orifice, the most active period of the uterus. If abnormal redness or evidence of exudation is apparent, the particular rabbit should not be used for several days. This has been our experience, and we know it has been the experience of others as well, in spite of the fact that all of the kymograph tracings appearing in the literature to illustrate the method were produced by using satisfactorily quiescent uteri, which permitted accurate interpretations of results by the ordinary manner indicated above. If only the relatively quiescent type of uterus could be employed, it can readily be understood why the method would be adopted with extreme hesitancy by laboratories having many assays to perform.

Regarding the accuracy or discrimination afforded by the method in assaying Fluidextract of Ergot for alkaloidal activity, we have found no difficulty in always avoiding an error of more than plus or minus 10%, when the method of interpretation described below is used, no matter how much spontaneous activity was shown by the uterine strips. On the other hand, we are convinced that an error as high as 50% is possible by the hitherto described method of interpretation (comparing only the *heights* of epinephrine responses) when the uteri exhibit appreciable spontaneous activity.

To permit the successful use of all uteri, obtained as indicated above, this laboratory has adopted the following method of interpreting the degree of paralysis of the uterine strips, or, more specifically, the inhibition of the epinephrine response, the same method applying to results obtained from either quiescent uteri or those showing varying amounts of spontaneous activity.

Instead of measuring the total heights of the epinephrine responses, *i. e.*, the distance between the base line and the highest point reached by the epinephrine response, varying parts of such distances being due to spontaneous contractions, we measure the height of the sustained curve only, or, the height of the actual sustained tonus increase. It will be attempted to clarify this point by illustrating with kymograph tracings which show the use of uterine strips with varying degrees of spontaneous movement. It will be noted that the speed of our kymograph drum is approximately 1 cm. per minute. This relatively fast speed is selected because we consider it highly essential to record clearly all details of response without "piling" contractions and relaxations upon one another. Character, height and duration of the epinephrine response may thus serve as criteria in ascertaining the degree of paralysis by comparing the epinephrine response before and after the Ergot action.

Figure 1 shows the use of uterine strips that are devoid of spontaneous or rhythmical movement. This is the type reproduced by other writers. It is very simple with such uteri to be able to detect whether or not there is a difference in the degree of Ergot paralysis in the two strips by using height of contractions as the sole criterion. It is immediately obvious that the Ergot paralysis is greatest in the lower tracing.

Figure 2 shows the use of uterine strips having a somewhat greater degree of rhythmical or spontaneous movement than in Fig. 1, although the amount is not enough to seriously interfere with accurate interpretation of comparative degree of paralysis, by the usual method. The Ergot paralysis is greater in the lower tracing, whether one compares either heights alone or the sustained tonus increase.

In Fig. 3 the amount of rhythmical or spontaneous movement is still greater than in Fig. 2. Here, it will be seen, one would be led into error were the maximum heights to serve as the sole criterion for determining percentage inhibition in each strip. The degree of paralysis here is greatest in the upper tracing, but the actual difference was not conclusively evident until the second epinephrine trial (after the Ergot was added) was made. Any difference in paralysis increases with the passage of time. The epinephrine dose was, of course, increased similarly for strip in

making the second epinephrine trial, because the response in the first trial is low. Had the original epinephrine dose been employed in the second trial, it is quite probable that no sustained tonus increase would have resulted in the upper tracing, and only a slight response in the lower.

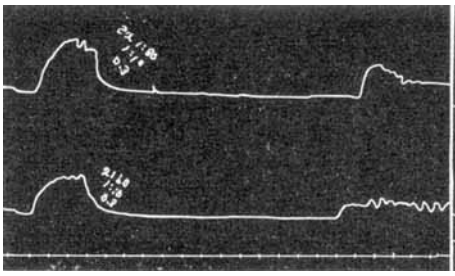

Fig. 1.

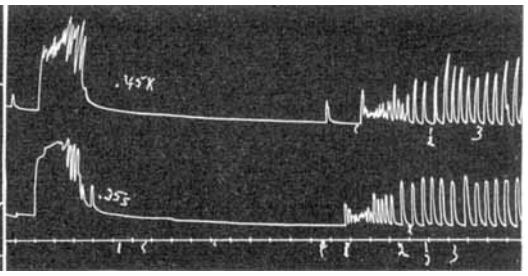

Fig. 2.

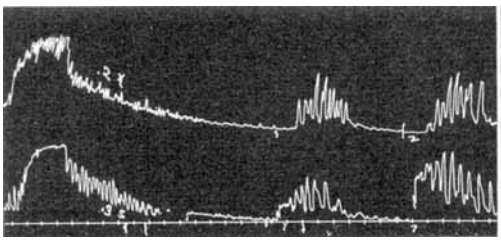

Fig. 3.

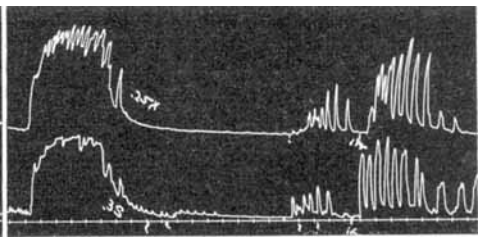

Fig. 4.

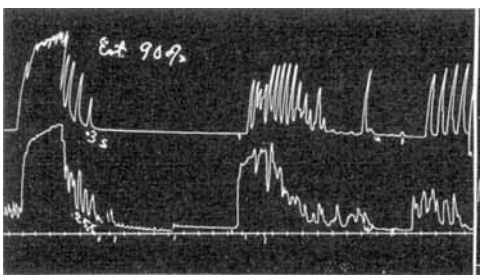

Fig. 5.

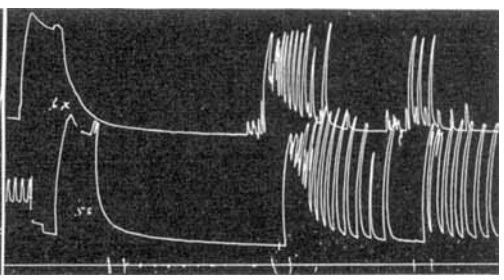

Fig. 6.

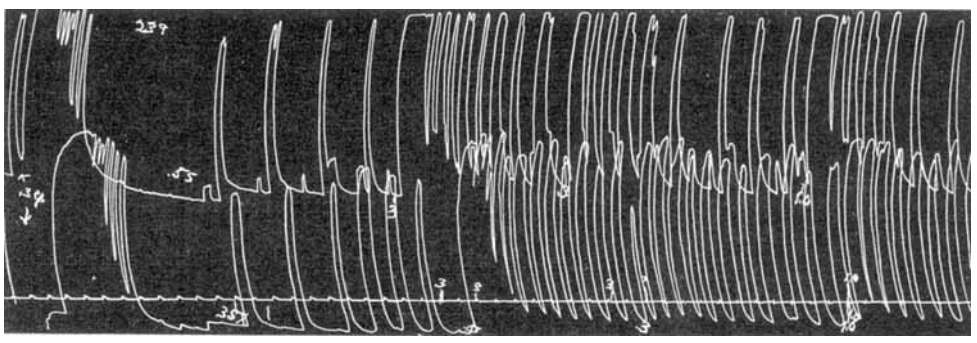

Fig. 7.

In Fig. 4, rhythmical interference is still greater than in Fig. 3, although by comparing the sustained tonus increases, it is readily apparent that the paralysis is the greater in the lower strip, both in the first and second epinephrine trials (after the Ergot effect).

In Fig. 5, note that in this tracing, taking sustained tonus increase as the criterion, the paralysis is much the greatest in the upper strip. Note also in the final epinephrine trial on each strip, that the heights attained by the contractions are greater in the upper strip than in the lower, yet the sustained tonus increase is much less in the upper strip.

Figures 6 and 7 show the successful use of strips taken at still more active periods, consequently a very high degree of irritability and a great amount of rhythmical spontaneous movement. Note that height of contractions alone offers no clue as to the degree of Ergot paralysis, since the height of epinephrine contractions is just as great after the Ergot had acted, as before the Ergot action. By noting the sustained epinephrine responses, however, it is immediately evident that the Ergot paralysis is greatest in the upper tracing of Fig. 6, and in the lower tracing of Fig. 7.

As an aid to the inexperienced, it will be found to be time well spent to study the response of epinephrine alone upon uterine segments, showing varying degrees of spontaneous movement for the purpose of ascertaining exactly how the tissues actually manifest their response to different doses of epinephrine, before attempting to judge degree of paralysis caused by Ergot alkaloids. Then it is advisable to compare the epinephrine-inhibiting action of different known doses of the same Ergot preparation, for the purpose of obtaining a clear understanding of just how known differences in Ergot potency manifest themselves.

What has been stated in the above description refers to the application of this method as a means of estimating the alkaloidal activity of Fluidextract of Ergot, U. S. P. X, or preparations prepared in a pharmaceutically similar manner, and using as the standard of comparison either the official Standard Fluidextract of Ergot, or freshly prepared solutions of ergotamine tartrate, ergotamine methanesulphonate, or ergotoxine ethanesulphonate. The basic principles involved in the application of the method for this purpose, require that a given dose of *the alkaloids present in a test preparation act with essentially the same degree of rapidity as the alkaloids present in an equivalent dose of the preparation used as the standard of comparison.* This requirement is adequately met by tests involving U. S. P. Fluidextracts of Ergot, or preparations manufactured in a similar manner. Specific attention is called to the point, however, because of the fact that one type of alkaloidal Ergot preparation, manufactured by a method not similar to the official method for the Fluidextract, has been encountered whose epinephrine-inhibiting activity is very perceptibly faster than that of the U. S. P. Fluidextract. Consequently it was found that the degree of paralysis, for example, was the same for this preparation as for the Standard Fluidextract at the end of five minutes of action on the uterine strips, but after ten minutes the paralysis was much greater from the unknown preparation than from the Standard Fluidextract. Many experiments, using different time intervals, were carried out, until there remained no doubt that there was a great difference in the rate of action between these two types of preparations. As indicated above, such a condition has not been observed thus far in the testing of preparations manufactured by a method similar to that of the official Fluidextract, and consequently this factor has not been found to be a source of significant error in testing Fluidextract of Ergot.

As to a comparison of the above with other available methods for estimating the alkaloidal activity of Fluidextract of Ergot, the following points are worthy of mention:

1. The method complies with one of the most important requirements for any quantitative biological method, in that it provides for a direct comparison of the potency of an unknown with a standard preparation, thereby avoiding errors due to individual, daily and seasonal variation in susceptibility of the test objects.

2. The method provides for discrimination, or sensitivity to graded dosage, which is sufficient for all practical purposes, and which can be equalled by no other available biological method in our hands.

3. The fact that several separate trials are made upon a single preparation, reduces the probability of a serious error to a minimum.

4. The time required to carry out an adequate number of trials to constitute a complete assay, compares very favorably with the most rapid of other methods.

5. The method provides for subjecting the test preparation to direct assay without the necessity of subjecting it to any chemical procedure which might alter the identity of the easily changeable alkaloids of Ergot.

6. The method necessitates no extensive facilities in the way of providing for the care of large numbers of test animals, and is consequently a procedure of very modest expense.

(*To be continued*)