SCIENTIFIC SECTION

THE PHARMACOLOGY OF ERGOT.

(The bibliography will follow the last article of the series.)

PART V. PHARMACOLOGICAL STUDY OF FLUIDEXTRACT OF ERGOT, U. S. P. X.

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The recent reports of other investigators referred to in the preceding articles of this series (37, 38, 39, 41) included extensive studies of the specific alkaloidal activity of crude ergot and its preparations. Owing to the general belief that the amines (histamine, tyramine, etc.) are not specific for the drug and are therefore of little importance, adequate studies of both groups of pharmaco-dynamically active constituents individually and in mixtures as they occur in the crude drug and in its galenicals, seem to be lacking.

Pattee and Nelson (28) and Swanson (34) found that the U. S. P. Cock's Comb Method (24) and the Broom-Clark Isolated Rabbit Uterus Method (13) yielded similar results in estimating the alkaloidal activity of ergot preparations. In marked contrast to the observations of these investigators, the writer found the two methods to yield widely discordant results in certain instances in extensive individual and collaborative assays of U. S. P. Fluidextracts of Ergot. Most of the collaborative studies involved the U. S. P. Cock's Comb Method only and, although the results obtained appeared very discouraging, a careful analysis of these results revealed certain rather striking information, as follows:

Of a total of 50 samples of the Fluidextracts of Ergot investigated, approximately 60 per cent yielded reasonably concordant results in the hands of the bioassayists using the Cock's Comb Method. The results obtained from the remaining 40 per cent of the fluidextracts were at very great variance by the same method, lowest and highest results frequently differing by 200 to 300 per cent. Applying the Broom-Clark (13) Method to all of these samples, the author obtained results agreeing very well with the average results by the Cock's Comb Method in the case of the 60 per cent of fluidextracts. The remaining 40 per cent of the samples, which yielded discordant results by the Cock's Comb Method, gave results by the Broom-Clark Method appreciably higher than those obtained by the Cock's Comb Method.

A careful analysis of the types of fluidextracts involved in these studies revealed that the 60 per cent of samples yielding consistent and concordant results by both methods consisted of fluidextracts of various ages, some very recently prepared and others from four months to two years old. These samples in repeated assays by both methods at 2- or 3-month intervals continued to yield consistent results, showing a gradual decrease in potency as their ages increased.

The 40 per cent of fluidextracts yielding discordant results by the Cock's Comb Method, but consistently higher results by the Broom-Clark Method, consisted

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of fluidextracts which were invariably less than 6 months of age and for the most were freshly prepared at the time of distribution and less than 2 months old at the time of assay. Repeated assay of these fluidextracts by the Cock's Comb Method gave widely varying results even from day to day, making it almost impossible to determine the potency even approximately, as the birds did not respond consistently to varied doses, although the Broom-Clark Method continued to give consistent, though invariably higher, results. As the samples became older assays by the Cock's Comb Method showed an apparent increase in potency instead of an expected decrease. A gradual decrease in potency was shown by the Broom-Clark Method. After attaining an age of 6 to 8 months several of the fluidextracts had apparently doubled or more than doubled their original potency as shown by the Cock's Comb test. The highest value obtained upon the aged products by this method in several instances nearly reached the value shown by the first Broom-Clark Method assays. Strangely, after the samples had aged 6 or 8 months, the two methods gave similar values for a given fluidextract.

These observations indicate that: (1) Certain principles are contained in some Fluidextracts of Ergot of recent preparation in amounts sufficient to destructively interfere with the manifestation of alkaloidal potency by the Cock's Comb Method; (2) These interfering substances do not seriously influence results upon alkaloidal potency by the Broom-Clark Method; and (3) The interfering substances deteriorate to such an extent in 6 months (approximately) that they cease to exist in sufficient amounts to cause serious interference in estimating the alkaloidal potency by the Cock's Comb Method. These indications are substantiated by the experimental results which follow:

METHODS OF ASSAY.

The bio-assay methods used in this study of Fluidextracts of Ergot are the same as those described in Part IV of this series of reports (41) as follows:

(1) The Cock's Comb Method, official in U. S. P. X for the assay of Fluidextract of Ergot. The details of this method are described by Gittinger and Munch (15) and Pattee and Nelson (28).

(2) The Isolated Rabbit Uterus Method of Broom and Clark (13) for the Estimation of the Alkaloidal Activity of Ergot Preparations. The method as employed is described in detail by Pattee and Nelson (28).

(3) The Isolated Guinea-Pig Uterus Method for the estimation of the Non-specific Amine Activity of Fluidextract of Ergot. The details of this method were reported in Part II (38) of this series.

(4) The Pressor Method to Anesthetized Dogs. This method was described in Part IV of this series (41).

These studies were carried out primarily for the purpose of determining the cause of the discordant results obtained by the Cock's Comb Method in the case of freshly prepared fluidextracts previously discussed and, if possible, to develop some means of obviating the observed discrepancies by this method.

The Cock's Comb Method is generally reputed to measure the specific alkaloidal activity of ergot preparations (15, 28, 34, etc.). Yet it has been found that certain aqueous extracts high in non-specific amine activity may exhibit appreciable potency by this method, even when practically devoid of active ergot alkaloids (41), especially if the cockerels employed have previously undergone frequent ergot testing over an extended period.

The results in Tables I and II of Part IV of this series of articles and in Table III of this article definitely show that although the amines (histamine, tyramine, etc.) may exist in sufficient

amounts to cause bluing of the cock's comb if given in the absence of alkaloids, any cyanotic effect of the amines in fluidextracts does not add to that produced by the alkaloids.

The ten Fluidextracts of Ergot listed in Table III were freshly prepared according to the method of the U. S. P. from samples of crude ergot individually differing in alkaloid and amine content. They were tested from time to time, as indicated in Table III, by the Cock's Comb Method.

Changes in alkaloidal content were determined at the same intervals by the Broom-Clark Isolated Rabbit Uterus Method (28), and changes in the amine content by the Isolated Guinea-Pig Uterus Method (38).

TABLE III.—CHANGES OCCURRING IN FLUIDEXTRACTS OF ERGOT, U. S. P., AS OBSERVED BY BIO-ASSAY METHODS.

Fluidextracts, U. S. P. process. No.	Age of fluidextract at time of assay.	Potency by U. S. P. Cock's Comb Method (15). In Terms of U. S. P. Standard Fluidextract. *Per cent.*	Potency by Broom-Clark Method (28) for alkaloidal activity. *Per cent.*	Total amines, calculated as histamine; by Isolated Guinea-Pig Uterus Method for amines of fluidextracts (38). *Per cent.*
20	1 day	130–150	140–150	0.012
	1 month	130–150	125–135	0.006
	3 months	120–135	120	0.004
	6 months	100–120	110	0.004
	9 months	100	100	0.004
21	1 day	200–250	225	0.010
	1 month	200–250	200–225	0.006
	3 months	175–200	200	0.006
	6 months	150–175	170–180	0.004
	9 months	150	150–160	0.004
22	1 day	130–200	250	0.045
	1 month	180–225	225	0.027
	3 months	180–200	200–210	0.012
	6 months	160–170	160–170	0.009
	9 months	100–120	120–130	0.005
23	1 day	50–100	110–120	0.065
	1 month	80–100	100	0.027
	3 months	60–80	70–80	0.015
	6 months	50–60	60–70	0.009
	9 months	40–60	50–60	0.007
24	1 day	100–350	425–450	0.150
	1 month	200–300	400–425	0.097
	3 months	250–330	375–400	0.042
	6 months	275–300	330–350	0.023
	9 months	250–275	280–300	0.012
25	1 day	400–450	440–450	0.027
	1 month	350–400	400–420	0.013
	3 months	300–350	330–350	0.009
	6 months	250–300	260–280	0.008
	9 months	200–250	230–250	0.005
26	1 day	150–300	370–400	0.087
	1 month	200–300	340–360	0.066
	3 months	250–300	300–325	0.027
	6 months	225–280	250–270	0.015
	9 months	200–250	200–225	0.010
27	1 day	130–150	150	0.008
	1 month	120–140	140–150	0.008
	3 months	120–140	130–140	0.005

	6 months	100–125	120–130	0.006
	9 months	80–100	100	0.006
28	1 day	75–350	400–420	0.125
	1 month	200–350	370–380	0.095
	3 months	200–330	330–350	0.066
	6 months	200–300	300–325	0.045
	9 months	200–250	250–270	0.037
29	1 day	100–200	200–220	0.075
	1 month	130–175	180–190	0.045
	3 months	140–175	160–170	0.033
	6 months	130–160	150	0.027
	9 months	120–140	130–140	0.015

TABLE IV.—COCK'S COMB ASSAY OF FLUIDEXTRACT NO. 24 IMMEDIATELY AFTER PREPARATION.

Bird no.	Threshold dose. *Cc./Kg.*	Intensity* of reaction from threshold dose.	Weight of bird. *Kg.*	Multiple of threshold dose.	Dose of F. E. No. 24 injected. Per Kg. *Cc.*	Per bird. *Cc.*	Intensity of reaction* at end of 1 hr.	1½ hrs.	Results: Percentage potency in terms of U. S. P. standard based on observation at end of 1 hr.	1½ hrs.
554	0.5	122	1.630	1/5	0.100	0.163	012	001	<500	<500
1564	0.5	123	1.650	1/5	0.100	0.165	012	002	<500	<500
7193	0.7	123	1.580	1/4	0.175	0.276	112	122	<400	400
7210	0.5	123	1.610	1/4	0.125	0.201	122	123	400	400
2772	0.5	122	1.720	1/3	0.166	0.285	012	223	<300	>300
7471	0.6	123	1.650	1/3	0.200	0.330	123	223	300	>300
7124	0.4	023	1.510	2/5	0.160	0.242	123	233	250	>250
2721	0.6	123	1.550	2/5	0.240	0.372	123	223	250	>250
1790	0.5	022	1.690	1/2	0.250	0.423	233	233	>200	>200
7369	0.6	123	1.730	1/2	0.300	0.519	012	233	<200	>200
1482	0.7	123	1.660	3/4	0.525	0.872	102	233	<133	>133
7372	0.6	123	1.690	3/4	0.450	0.760	333	333	>133	>133
7466	0.5	023	1.500	1	0.500	0.750	013	133	<100	>100
6923	0.5	122	1.530	1	0.500	0.765	223	333	>100	>100
7302	0.5	123	1.570	1 1/4	0.625	0.981	233	333	> 80	> 80
6984	0.6	123	1.540	1 1/4	0.750	1.255	333	333	> 80	> 80
7545	0.7	123	1.760	1 1/2	1.050	1.848	123	132	66	> 66
2836	0.5	122	1.690	1 1/2	0.750	1.268	123	023	66	< 66
7382	0.6	123	1.490	2	1.200	1.788	123	223	50	> 50
7116	0.5	123	1.520	2	1.000	1.520	223	333	> 50	> 50

* The number code used to record the intensity of the cock's comb cyanosis is derived as follows:

1 indicates a barely perceptible reaction; *2*, a pronounced but sub-maximal effect; and *3*, a maximal reaction. The numerical expressions of intensity in Tables IV and V indicate readings from the anterior to the posterior portions of the comb. The anterior of the comb is usually affected less than the posterior. Thus "123" denotes the intensity usually produced by a "threshold" dose, *i. e.*, a satisfactory reaction, reading from anterior to posterior; "223" a proportionately greater reaction; and "333" a maximal discoloration from anterior to posterior, etc. An "023" reading, similarly, denotes no effect in the anterior third of the comb; a satisfactory cyanosis of the central portion, and a maximal reaction in the posterior third of the comb.

In standardizing cockerels, record is made of the effective dose, and also the intensity of reaction to that dose, since all combs do not react the same in all respects.

DISCUSSION OF RESULTS IN TABLE III.

The fluidextracts enumerated were very carefully and freshly prepared by the method of the U. S. P. and stored in amber-colored, tightly-sealed bottles in a cool room. The samples

were further protected from light by placing the bottles in a covered wooden box. Each fluidextract was physiologically assayed for amine and alkaloid potency immediately after preparation and at the various time intervals indicated in the table. Undue exposure of the fluidextracts to air was avoided by withdrawing the liquid with a hypodermic syringe, inserting the needle through the stopper. The crude drug samples entering into the preparation of the fluidextracts were purposely selected to show the influence of high and low proportions of amines upon the manifestation of alkaloidal potency by the Cock's Comb and Broom-Clark Isolated Rabbit Uterus Methods.

The potency values of the various fluidextracts by the Cock's Comb and Broom-Cark Methods are expressed in terms of the U. S. P. X Standard Fluidextract, the standard being taken as 100 per cent. In certain instances the results obtained by the Cock's Comb Method are indefinite. For instance, in the case of Fluidextract No. 24, when assayed immediately after preparation, different cockerels gave inconsistent results, varying from an apparent potency of 50 to 350 per cent of the U. S. P. standard, even though the birds had been carefully standardized in the usual manner (15, 28). At the same time, the Broom-Clark Method showed a potency of 440 to 450 per cent consistently in several assays.

To show the inconsistencies observed, the detailed cock's comb assay of freshly prepared Fluidextract No. 24 is given in Table IV.

The cock's comb results in Table IV for Fluidextract No. 24 of Table III are inconsistent, different cockerels showing a potency varying from 50 to 400 per cent U. S. P. Standard. The highest value shown by this cock's comb test agrees fairly well with that obtained by the Broom-Clark Method (Table III). The probability of error, however, is very great, as most of the birds show a potency appreciably less than 300 per cent, variations being so great as to actually prevent an estimation of the potency, even though 20 birds were used for the single sample. In attempting to reach an explanation for the inconsistent cock's comb results it was observed that the only apparent difference between this particular fluidextract and the fluidextracts yielding definite and consistent results by the same method lies in the unusually high amine activity. The results in Table III show that the non-specific amine concentration bears a direct relation to the magnitude of the inconsistencies met in the use of the Cock's Comb Method for estimating the specific alkaloidal activity of Fluidextract of Ergot. It is also shown that the amine activity deteriorates more rapidly than that of the alkaloids. As the amine activity deteriorated, the Cock's Comb Method yielded much more consistent results, most of the freshly prepared fluidextracts reaching a reasonably stabilized condition (as indicated by consistent cock's comb results) in approximately 4 months, even in samples originally having an extraordinarily high amine content.

In Article IV of this series (41) it was shown that the active non-specific amine fraction often exists in ergot in concentrations high enough to cause bluing of the cock's comb. The results in Tables III and IV show that any cyanosis produced by these amines does not add to that produced by the ergot alkaloids, for, if the effect were additive, results by the Cock's Comb Method would be higher than those obtained by the Broom-Clark Method which measures only the alkaloidal content. As the apparent potency shown by the Cock's Comb Method is never higher, but frequently lower than that shown by the Broom-Clark Method in the case of fluidextracts high in amine content, it is only logical to assume that the amines may exist in amounts sufficient to interfere destructively in the manifestation of alkaloidal potency as observed by the Cock's Comb Method. The success that has attended the use of the Cock's Comb Method in commercial application is undoubtedly due to the fact that many samples of crude ergot do not contain enough amines to cause interference in the assay of the resulting fluidextracts. Even those fluidextracts which do contain interfering quantities of amines when freshly prepared will lose the greater part of this amine activity before final assay and subsequent distribution if they are aged for six months as is specified in the U. S. P. for the Standard Fluidextract.

As is generally known, considerable lack of uniformity exists in commercial Fluidextracts of Ergot. This non-uniformity seems to be due largely to the interference of the non-specific amines in the estimation of specific alkaloidal activity by the official Cock's Comb Method. It is due also in part to the unstable nature of the specific alkaloids.

The individual pharmaco-dynamically active constituents of ergot are available in the pure state. If the conclusions based upon the foregoing observations on the destructive amine interference in assaying certain freshly prepared fluidextracts by the Cock's Comb Method are

correct, it should be possible to show this interference by studying the effects of these individual constituents singly and in mixtures using the Cock's Comb Method. This study, therefore, was undertaken.

MANIFESTATIONS OF ACTIVITY OF THE INDIVIDUAL ACTIVE CONSTITUENTS OF ERGOT AS SHOWN BY THE COCK'S COMB METHOD.

1. The Specific Ergot Alkaloids.

Two alkaloids, ergotamine and ergotoxine, are responsible for practically the entire alkaloidal activity of ergot. These two alkaloids exhibit identical pharmaco-dynamic reactions. Therefore, either one may be regarded as entirely representative of the alkaloidal activity of ergot. Both have been extensively used in these studies. As the results in all instances were identical, however, only those obtained from ergotamine will be discussed.

The ergotamine solution was prepared by dissolving crystalline ergotamine tartrate in water in a concentration such that 1.0 cc. of the solution represented 0.5 mg. of ergotamine base. (Ergotamine tartrate used corresponds to approximately 84.5% ergotamine base.) A bare trace of hydrochloric acid was added to facilitate solution. This particular concentration was selected because it closely approximates that of U. S. P. Fluidextract of Ergot with respect to alkaloidal activity.

Injections were made deep into the pectoral or breast muscles in the usual manner. Other details of technique as described in the U. S. P. were observed.

In experiments involving over 250 injections to 50 carefully standardized white Leghorn cockerels of U. S. P. specifications, the following results were obtained:

(*a*) Ergotamine, in suitable doses, consistently produced the characteristic bluing or cyanosis of the cock's comb in all instances.

(*b*) As in the case of Fluidextract of Ergot, the intensity of the cock's comb reaction produced by ergotamine was directly proportional to dosage within maximum and minimum limits.

(*c*) Different cockerels varied with respect to the intensity of reaction produced by a given dose of ergotamine. Once the threshold dose (15, 28) had been established, however, the individual cockerels responded consistently to that dose for two to four months, if a 2-week rest period between injections (24) was given. Although cockerels varied in susceptibility to ergotamine, most of those used in these studies exhibited a barely perceptible cyanosis from the intramuscular injection of 0.10 to 0.20 mg. of ergotamine base per kilogram body weight. A pronounced but sub-maximal bluing was produced by 0.15 to 0.30 mg., and a maximal effect was usually observed when doses of 0.30 mg. or more per kilogram body weight were injected, observations being recorded approximately one hour from the time of injection.

(*d*) Ergotamine, in doses just sufficient to produce a perceptible cyanosis of the comb in one hour, gave no other characteristic significant symptoms in the cockerels. Respiration and pulse were not affected to any degree discernible by simple observation.

The bluing of the combs became evident in approximately 30 minutes in most instances, registering greatest effect in approximately one hour. The posterior lobe of the combs showed greater discoloration than the anterior. After persisting at this intensity for an additional one-half to 1 hour, the bluing gradually lessened. Some birds returned to apparent normality in five or six hours, although in most instances a longer period was necessary, occasionally as much as four or five days. Slightly greater doses, producing a pronounced but sub-maximal cyanosis of the comb, however, in some instances caused a slight manifestation of general symptoms. The characteristic bluing of the comb began to appear in the posterior lobe in approximately 30 minutes, increasing in intensity and gradually spreading toward the anterior, showing greatest

effect in 45 to 90 minutes, the time varying with different birds. At this point respiration was mildly stimulated in a few instances, but no symptoms other than the cyanosis of the comb were significantly in evidence. The discoloration usually persisted at this intensity for two hours or more before gradually receding. Return of the combs to normal required from four to ten days, the time for complete recovery varying greatly for individual birds. The posterior lobes of the comb always showed a more intense bluing than the anterior portions. The anterior portions usually cleared in 1 to 2 days, while the posterior lobes cleared much more slowly, occasionally requiring 15 to 20 days for recovery but usually attaining normality in less than two weeks. Still greater doses, just sufficient to produce a maximal bluing of the comb (0.3 to 0.5 mg. per Kg.), frequently caused the appearance of a preliminary respiratory and cardiac stimulation in 15 or 20 minutes, which gradually gave way to symptoms of slight depression, although no evidence of tremors, muscular incoördination or weakness resulted. The characteristic cyanosis of the comb appeared in the posterior lobes in 15 to 20 minutes, gradually increasing in intensity and spreading toward the anterior portions until a maximal reaction was observed in 45 to 90 minutes. This intensity usually persisted for two to six hours, after which the normal color began to return in the anterior portions of the combs. Several days were usually required for complete recovery of the combs, although in many instances more than 10 days were necessary.

(*e*) By using carefully standardized white Leghorn cockerels, *i. e.*, those whose threshold dose had been accurately determined, it was found possible to detect variations in dosage of as little as 0.05 mg. per Kg. of Ergotamine by the U. S. P. Cock's Comb Method. Accepting the values of Nelson and Pattee (28) and Swanson (34) of approximately 0.5 mg. of ergot alkaloids per cc. in terms of ergotamine or ergotoxine for U. S. P. Fluidextract of Ergot, a variation of ±0.05 mg. ergot alkaloids per cc. corresponds to a variation of ±0.1 cc. of fluidextract. Evaluating the U. S. P. Fluidextract as 100%, with the U. S. P. dose requirement of 0.5 cc. per Kg. to cockerels, a ±0.1-cc. variation corresponds to ±20% U. S. P. X Potency.

Thus, with a simple solution of pure ergot alkaloids, the U. S. P. X Cock's Comb Method could be depended upon to yield estimates of alkaloidal potency within ±20% of the potency of the U. S. P. Fluidextract in all assays, and in many instances even a greater accuracy was observed, frequently with only a ±10% U. S. P. potency variation.

(*f*) Continued usage of the cockerels every two weeks for 6 to 8 months, the time covered by the experiments here described, caused no apparent change in their response to ergot alkaloids, with the exception of slight changes in threshold reactions, necessitating restandardization of the birds every two or three months. The cockerels invariably were found to increase in susceptibility from continued use.

2. *Histamine.*

Histamine is generally conceded to be the most important of the amines associated with ergot from the standpoint of pharmaco-dynamic activity and occurrence in the drug. This has been confirmed by the experimental results of this investigation.

With this information at hand, and the fact that certain aqueous extracts of ergot high in amine content but practically devoid of alkaloids frequently exhibited appreciable potency by the Cock's Comb Method (see Part IV) (41), there arose the possibility that histamine probably sometimes existed in amounts sufficient to cause bluing of the cock's comb. In spite of the fact that the Cock's Comb Method is reputed to measure only the alkaloidal activity of ergot preparations, histamine alone often produced upon the comb of cockerels an effect closely resembling that produced by ergot alkaloids.

From the very beginning of these experiments it was noted that, although the general symptoms produced by intramuscular injections of histamine to various cockerels were fairly consistent, the bluing or cyanosis of the comb was produced much more easily and to a greater degree of intensity on birds that had been used for ergot testing every two weeks for from 6 months to 1 year than on new birds

which had received but a few injections of ergot preparations. The results obtained from both types of cockerels will therefore receive consideration.

(*a*) *Old Cockerels of U. S. P. Specifications Regarding Type and Weight.*—These birds had been used every two weeks for 6 to 12 months for testing ergot preparations. Results reported include only those obtained from 20 birds having a threshold (15, 28) of 0.5 cc. U. S. P. Standard Fluidextract of Ergot per kilogram body weight, to obviate the necessity of tabulating all results from birds showing different susceptibility or tolerance.

As in the consideration of ergot alkaloids, three ranges of doses of histamine, determined by the intensity of the comb cyanosis, have been studied.

Intramuscular doses of less than 0.5 mg. per Kg. were invariably ineffective in producing any change in the appearance of the combs. Slight symptoms of general depression became evident in a minority of instances. Doses of 0.75 mg. of histamine per Kg. reacted as follows:

The first perceptible effect usually became evident in 20 to 30 minutes. The combs began to blanch, respiration and pulse became a bit more rapid. As time elapsed the blanching of the combs increased and began to show bluing. Respiration and pulse became slower, with symptoms of general depression, muscular weakness and incoördination. Greatest effect was usually observed approximately 40 minutes from the time of injection. No symptoms were significantly in evidence except for the general depression and the blanching or bluing of the combs. Two hours from the time of injection invariably found all cockerels restored to apparent normality, with the combs clear and red.

Cockerels receiving 1.0 mg. histamine per Kg. reacted in a similar manner except that all symptoms developed slightly sooner and attained a greater intensity. The bluing and blanching of the combs became clearly evident; the preliminary brief stimulation of pulse and respiration quickly gave way to marked general depression and muscular weakness, coupled with incoördination and tremors. These effects usually had reached their greatest intensity in 1 to $1^1/_2$ hours after injection, after which they began to recede. In 3 or 4 hours all cockerels had apparently returned to normal, with their combs a bright red.

Doses of 1.5 mg. of histamine reacted in a comparable manner, although to a proportionately greater intensity, showing a maximal effect in approximately 45 minutes. The combs became intensely blanched and blued, and a very marked general depression was manifest, with prostration in several instances and severe muscular weakness, incoördination and tremors in all instances. Recovery usually began within two hours and all birds had apparently returned to normal with a clear comb within an over night period.

Thus, it was observed that histamine was capable of producing a cyanosis of the cock's comb which was comparable to that produced by the specific ergot alkaloids, although other symptoms were vastly different. Since it has been shown (41) that histamine frequently exists in crude ergot to the extent of 1.0 mg. per gram of drug, and that the total amount of histamine and other amines is completely extracted by the menstrua employed in the manufacture of ergot preparations, it is evident that an appreciable potency may be observed by the Cock's Comb Method, even though no alkaloids are present. The amine potency, however, could not equal that of the U. S. P. Fluidextract in any of the samples examined because it would require 1.0 cc. per Kg., *i. e.*, twice the volume specified in the official method, to produce the desired reaction, indicating only 50% of the U. S. P. X potency for fluidextracts. A cock's comb potency of 100% U. S. P. X for any fluidextract would therefore indicate the presence of an appreciable quantity of ergot alkaloids.

(*b*) *New Cockerels.*—This group of birds comprised birds of the same type as those in (*a*), but they had never undergone ergot testing except for the several injections of Standard Fluidextract necessary to establish their threshold doses.

Although it is usually accepted that continued usage of cockerels for ergot testing does not alter their response to the active constituents of the drug, aside from slight changes in susceptibility, these studies have shown that the combs of new cockerels are not affected by histamine nearly as easily as those of birds used over an appreciable period of time, even though the response of both types was identical for the alkaloids or the Standard Fluidextract. The symptoms of depression and muscular weakness could be produced by the usual doses, but the combs

occasionally showed no appreciable effect, even in doses of 1.5 mg. per Kg. In most instances, however, at least a slight blanching or bluing of the comb was produced.

3. *Mixtures of Ergot Alkaloids and Histamine.*

The assay results obtained from fluidextracts high in amine content (Tables I, III and IV) indicated that the active amines interfered with the manifestation of alkaloidal potency by the Cock's Comb Method, *i. e.*, the amine fraction was

TABLE V.—RESPONSE OF SIX COCKERELS* TO ERGOTAMINE AT 2-WEEK INTERVALS FOR 10 WEEKS AND THE DESTRUCTIVE INTERFERENCE OF HISTAMINE ADMIXTURE UPON THE MANIFESTATION OF ALKALOIDAL ACTIVITY.

Cockerel no.	Ergot constituent injected Feb. 26, 1929.	Dose per Kg. *Mg.*	Intensity of comb reaction at end of one hour.	Ergot constituent injected Mar. 12, 1929.	Dose per Kg. *Mg.*	Intensity of reaction at end of one hr.
1	Ergotamine	0.20	123	Ergotamine	0.20	123
2	Ergotamine	0.20	112	Ergotamine	0.20	112
3	Ergotamine	0.225	123	Ergotamine	0.225	123
4	Ergotamine	0.25	223	Ergotamine	0.25	223
5	Ergotamine	0.30	223	Ergotamine	0.30	233
6	Ergotamine	0.40	333	Ergotamine	0.40	333
	March 26, 1929.			April 9, 1929.		
1	Ergotamine plus Histamine	0.20 0.20	123	Ergotamine plus Histamine	0.20 0.20	012
2	Ergotamine plus Histamine	0.20 0.30	012	Ergotamine plus Histamine	0.20 0.30	123
3	Ergotamine plus Histamine	0.225 0.40	002	Ergotamine plus Histamine	0.225 0.40	012
4	Ergotamine plus Histamine	0.25 0.50	022	Ergotamine plus Histamine	0.25 0.50	122
5	Ergotamine plus Histamine plus Tyramine	0.30 0.60 0.20	123	Ergotamine plus Histamine plus Tyramine	0.30 0.60 0.20	012
6	Ergotamine plus Histamine plus Tyramine	0.40 0.75 0.20	122	Ergotamine plus Histamine plus Tyramine	0.40 0.75 0.20	012
	April 23, 1929.			May 7, 1929.		
1	Ergotamine	0.20	123	Ergotamine	0.20	122
2	Ergotamine	0.20	122	Ergotamine	0.20	123
3	Ergotamine	0.225	123	Ergotamine	0.225	223
4	Ergotamine	0.25	223	Ergotamine	0.25	223
5	Ergotamine	0.30	233	Ergotamine	0.30	223
6	Ergotamine	0.40	333	Ergotamine	0.40	333

* These cockerels had been used every two weeks for four months or more in testing ergot preparations. The tests were made on the following dates: February 26, 1929; March 12, 1929; March 26, 1929; April 9, 1929; April 23, 1929; May 7, 1929.

antagonistic to the alkaloidal fraction as indicated by the comb cyanosis or discoloration, or, that the apparent potency is often lower than the actual alkaloidal potency.

In experiments involving the injection of mixtures of ergot alkaloids or amine-free fluidextracts, with histamine or alkaloid-free Aqueous Extracts in various proportions, this amine-interference has been repeatedly demonstrated.

Table V shows the consistent response of six cockerels to ergotamine at 2-week intervals and the effect of histamine in diminishing the response of the combs to ergotamine. Final repetitions of the ergotamine doses at 2-week intervals are recorded to show that changes in the reactions of the birds were not so variable as to cause the discrepancies noted in the injection of mixtures of amines and alkaloids.

The proportion of histamine to ergotamine that will produce a partial neutralization of effect on the cock's comb has been found exceedingly variable. In several instances, birds were injected with doses of ergotamine and histamine in proportions such that no effect was apparent at the end of an hour. Use of these same proportions in other experiments failed to show such marked antagonism between the two substances, although the manifestation of alkaloidal activity was usually diminished to some extent. In no instance was histamine capable of adding to the cyanosis produced by the alkaloids.

These observations have been duplicated by the addition of alkaloid-free aqueous extracts high in amine content to fluidextracts rich in alkaloidal activity but which were practically amine-free. While the amine content of ergot is exceedingly variable, it was found sufficiently high in a number of instances to cause marked interference in estimating the alkaloidal activity of ergot preparations by the U. S. P. X Cock's Comb Method, especially if the cockerels employed had been subjected to ergot testing for an appreciable period. It is believed that this factor of error explains the discrepancies observed in the cock's comb results recorded in this and preceding articles of this series, since it was observed that histamine caused interference even though the concentration present was not high enough to cause bluing of the combs in the absence of ergot alkaloids.

If a sufficient number of carefully standardized cockerels are employed, and all other details of the official method are properly observed, a correct interpretation of the results obtained reveals much information as to the nature and activity of Fluidextract of Ergot, as follows:

If results are not consistently accurate to within $\pm 20\%$ it is highly probable that appreciable quantities of amines are present. Symptoms other than the comb effect also must be observed. If the combs of different birds respond consistently to graded dosage, it is highly probable that the activity of the fluidextract is due entirely to the alkaloids present. Should a preparation produce comb cyanosis only from doses much greater than the predetermined "threshold dose," and the comb reaction is associated with symptoms of marked depression (described under Histamine), it is probable that the effect is due largely to the histamine present. Such a condition is never encountered with the U. S. P. Fluidextract, but occasionally is manifest by aqueous or semi-solid extracts of ergot.

It is a fact that any fluidextract prepared by the U. S. P. method contains appreciable quantities of ergot alkaloids if a satisfactory reaction is obtained from threshold doses of the preparation, regardless of the amine content. Any interference caused by the possible presence of appreciable quantities of amines will result in a low apparent alkaloidal potency as shown by the official method. *In other words, a sample of crude ergot meeting the U. S. P. biological requirements always contains at least as much alkaloidal activity as is indicated by the cock's comb test, and, if interfering quantities of amines also are present, the actual alkaloidal content will be greater than the amount indicated by the test.*

THE BROOM-CLARK ISOLATED RABBIT UTERUS METHOD.

A study of the Broom-Clark Isolated Rabbit Uterus Method, together with the details of its technique, was first reported by the originators (13). Further studies have been reported by Nelson and Pattee (1) Pattee and Nelson (28), Swanson (34), Rothlin (31) and others. A detailed description of the technique

involved need not be repeated here. Briefly, the method depends upon the ability of the active alkaloids of ergot to inhibit the response of suitable strips of isolated rabbit uterus to constant epinephrine concentrations. As the amines associated with ergot are not capable of causing this inhibition of the epinephrine response, the method serves to estimate the alkaloidal activity of ergot preparations. Pattee and Nelson (28), however, found that the amines may cause interference by accentuating the epinephrine response, even though they are not present in sufficient amounts to cause contraction of the tissue by themselves. This, of course, would cause the results to indicate a slightly lower alkaloidal potency than was actually present because a greater concentration of ergot alkaloids would be

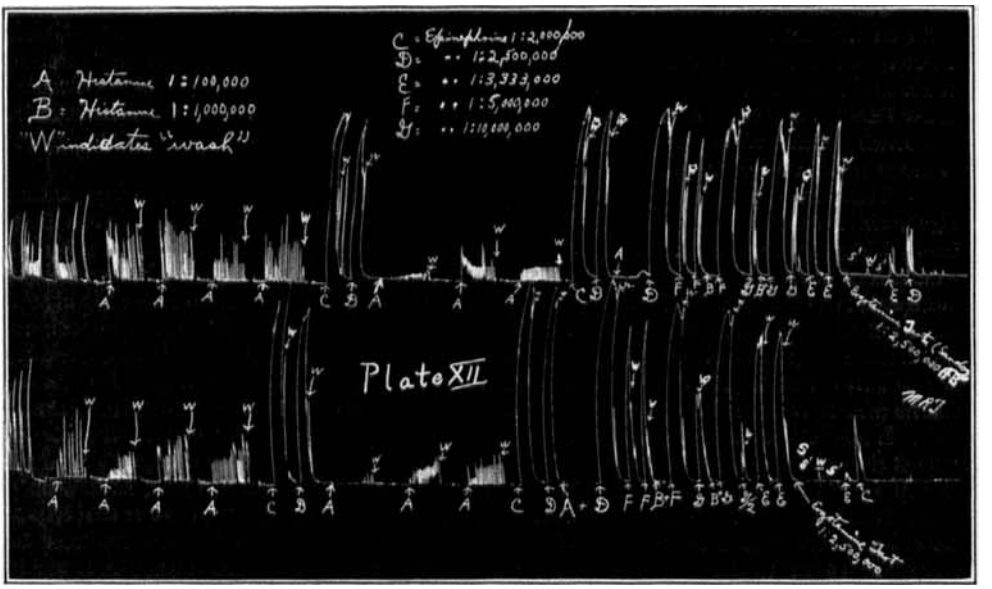

Plate XII.—The response of two similar strips of isolated Rabbit Uterus to histamine; epinephrine; epinephrine in the presence of stimulant and non-stimulant concentrations of histamine; and the similar inhibition produced by ergotamine alone (lower tracing), and in mixture with histamine (upper tracing) observed when both strips are washed at the end of five minutes. Note the inhibiting influence of epinephrine upon the response to histamine, and the effect of histamine admixture in accentuating the epinephrine response.

required to produce a given inhibition of the amine-accentuated epinephrine response of the uterine strips than would be required were the amines not present. Pattee and Nelson observed that the amine interference could be obviated by washing after permitting the alkaloids to act for five minutes, refilling the chambers with the saline solution and observing the final epinephrine response at the end of another five minutes.

The results obtained in the author's investigation tend to confirm these observations and are typified by the record in Plate XII. A careful study of this tracing shows, on two similar strips of isolated rabbit uterus: (*a*) the rhythmic contractions produced by stimulating concentrations of histamine; (*b*) the tonic contractions produced by epinephrine; (*c*) a loss of response to histamine after the administration of epinephrine; (*d*) the gradual return of response

to histamine; (*e*) the ability of histamine in stimulant and non-stimulant concentrations to accentuate the epinephrine response; (*f*) the constant response of both strips to epinephrine in similar concentrations; (*g*) the similarity of the inhibition produced by ergotamine in the presence of histamine (upper tracing) and the inhibition produced by the same concentration of ergotamine in the absence of histamine (lower tracing) obtained by observing the washing technique described by Pattee and Nelson (28).

This method has been used in estimating the alkaloidal content of all types of ergot preparations in these studies and, by employing a washing technique similar to that used and described by Pattee and Nelson (28), consistent results were obtained, irrespective of the amine content. The details of the method were described by the above authors. Without repeating the details of the method the writer has found that greatest accuracy may be obtained by observing the following precedure:

(*1*) Subject both of the similar strips to various concentrations of epinephrine until a concentration is found which will produce, upon several repetitions, constant and appreciable but sub-maximal contractions.

(*2*) Adjust the weights on the levers so that contractions of equal magnitude are obtained from each strip. This, of course, necessitates the use of levers of equal length, with the fulcrums at equal distances from the points of the strip attachments.

(*3*) After obtaining identical and constant sub-maximal response of both strips to a determined epinephrine concentration, and after all other factors (oxygen supply, temperature, etc.) are identical for each strip, permit the set-up to stand unmolested for exactly five minutes. Then drain and refill both strip chambers and allow them to stand exactly five minutes more. At this point again determine the epinephrine response. If the response of both strips is still identical, all is in readiness for the addition of the properly diluted ergot preparations. Place the standard preparation (either a solution of one of the alkaloids or the U. S. P. Standard Fluidextract) in one chamber and the unknown preparation in the other, carefully noting the time in each case. Allow the ergot preparations to act exactly five minutes on each strip, then wash, and let stand for exactly five minutes more. Then repeat the predetermined dose of epinephrine and observe the response. If a contraction is produced in both strips, repeat the epinephrine dose as a check. The second response is usually less than the first because the time which has elapsed permits the furtherance of the ergot alkaloid effect. Failure of the strips to contract shows that the ergot concentration administered was too great and the epinephrine response has been completely abolished. In such a case, add a larger dose of epinephrine, not that the response thus obtained is accurately significant, but it often serves to indicate roughly how much to decrease the concentration of the ergot preparation in the next experiment.

(*4*) Only one trial on a single pair of strips is possible because of the persistence of the effect of the ergot alkaloids, regardless of the amount of washing to which the strips are subjected. Therefore, fresh uterine strips are required for each determination.

(*5*) The concentration of ergot employed should be sufficient to produce not more than a 75 per cent inhibition nor less than a 50 per cent inhibition.

(*6*) An assay is complete only when a quantity of an unknown preparation which will produce the same inhibition as a definite amount of the standard preparation is determined.

This method is somewhat tedious and difficult of technique. After a reasonable amount of experience is attained, however, it has been found exceedingly accurate in estimating the specific alkaloidal activity of ergot preparations, provided a sufficient number of trials are made upon a single sample. Pattee and Nelson (28) and Swanson (34) found the method to be accurate within $\pm 10\%$, which is considerably more accurate than the present official method. The writer has found the method accurate to this degree in all instances. A $\pm 10\%$ variation in potency corresponds to a variation of approximately $\pm 0.005\%$ of ergot alkaloids, based on the strength of U. S. P. Fluidextract found by Pattee and Nelson (28). Plus or minus 0.005% ergot alkaloids corresponds to 0.05 mg. per cc. A variation of this size in the concentration of ergot alkaloids in an ergot preparation is easily detectable by the Isolated Rabbit Uterus Method.

PRESSOR METHODS TO ANESTHETIZED DOGS.

The Pressor Methods to anesthetized dogs are so well known that they need not be described here. A review of the literature reveals many attempts to apply

Plate XIII.—The effect produced by the intravenous administration of 0.2 cc. of the freshly prepared Fluidextract (by the U. S. P. Method to dog). Note the initial fall in blood pressure followed by a slight rise and another fall, indicating that a very appreciable quantity of Histamine has found its way into this preparation and that it has almost totally prevented a manifestation of alkaloidal response. By aging this preparation, the Histamine effect steadily diminished until finally the effect produced was entirely analogous to that of the pure specific alkaloid Ergotamine. Time in seconds.

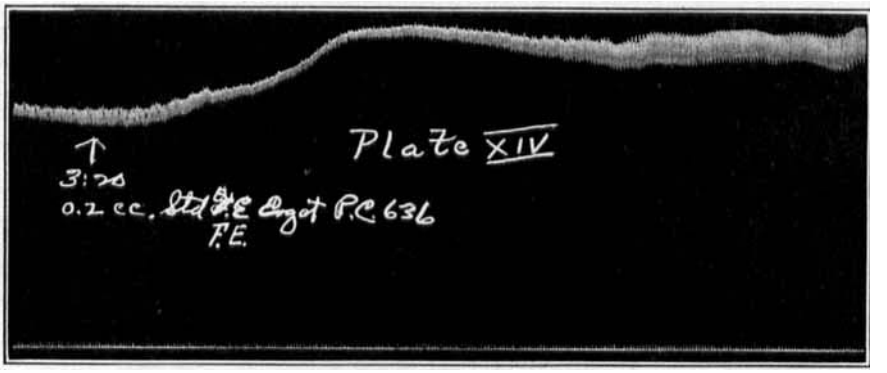

Plate XIV.—The effect produced upon the blood pressure of a dog by the intravenous administration of 0.2 cc. of Standard Fluidextract of Ergot. Note the absence of Histamine effect and the similarity between this rise in blood pressure and that shown in Plate VIII of Part IV (41).

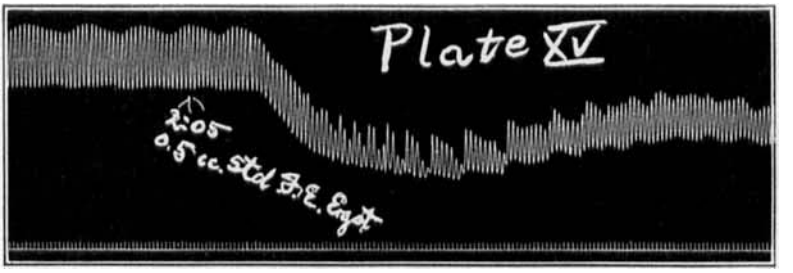

Plate XV.—Showing a reversal of the normal rise in blood pressure resulting when a dose of 0.5 cc. of the Standard Fluidextract of Ergot is preceded by a dose of Histamine or Aqueous Extract of Ergot. Histamine is capable of reversing the effect of the ergot alkaloids.

these methods in assaying ergot preparations. While the pressor reactions produced by the intravenous administration of the various constituents of ergot

are characteristic, the appearance of these constituents in mixtures, such as usually exists in fluidextracts, practically precludes their application quantitatively for the following reasons:

(*a*) The water-soluble amine fraction of the active constituents of ergot often exists in ergot preparations (especially fluidextracts) in amounts sufficient to cause a fall in blood pressure which may mask the pressure-raising effect of the specific alkaloids (Plate XIII), a condition comparable to that observed when a mixture of histamine and ergotamine is administered (Plate XI of Article IV (41)).

(*b*) The active ergot alkaloids produce a rise in blood pressure (Plate VIII, Article IV (41)). Repeated administration of identical doses of ergot alkaloids results in diminishing response, even when the doses are given at intervals of an hour.

(*c*) Injection of a preparation containing histamine results in a fall in blood pressure. If, after the pressure has returned to normal, an effective dose of pure ergot alkaloids is administered, a fall in pressure results instead of the rise usually produced. Thus, histamine, in amounts frequently existing in ergot, is capable of reversing the effect of the ergot alkaloids. This is illustrated in Plate XIV, which shows the rise in blood pressure produced by a fluidextract containing practically no active amines, and in Plate XV, which shows the effect produced by the same preparation after a dose of aqueous extract of ergot had been given. The same observations have been duplicated by administering pure histamine before ergotamine.

As histamine and other amines have been found to exist in varying proportions in most of the samples of crude ergot entering this country (41), this method cannot be depended upon to accurately determine either the amine or alkaloidal content of Fluidextracts of Ergot because of the antagonistic manifestation of their effects upon blood pressure.

The assay of crude ergot necessitates the application of a method to a freshly prepared fluidextract. Such a preparation has been found to contain practically all of the amines and alkaloids present in the parent drug. Pressor methods are therefore contra-indicated in this connection.

Although aging of fluidextracts causes a rapid loss of amine activity, thereby eliminating the greater part of the interference in several months, pressor methods cannot be regarded as being nearly as accurate or as applicable as the Isolated Rabbit Uterus Method or the Cock's Comb Method, because of the great variations observed in test animals and the diminishing pressor response produced by repeated dosage. The effects produced by the first dose are often all that may be considered as significant.

Pressor studies, however, are exceedingly valuable in determining the presence or absence of amines, or the qualitative nature of ergot preparations, because of the characteristic differences in reaction produced by the amines and the alkaloids.

(To be continued)

GLEDITSCHIA TRIACANTHOS LINNÉ.*—A PRELIMINARY REPORT ON THE CHEMISTRY OF THE FRUIT.

BY LOYD E. HARRIS.

This plant, commonly known as Honey Locust, probably first came into prominence in 1878, when Lautenbach[1] reported the presence of an alkaloid which he called gleditschine.

* Read by title, Scientific Section, A. PH. A., Rapid City meeting, 1929.

[1] *Phila. Med. Times*, 1878 ("U. S. Disp.," 20, page 1410).