is very desirable. It is not offered as a final answer to the problem but as a procedure worthy of trial.

In its use careful technic is essential—not only in regard to the staining of the blood but especially with respect to the counting of the reticulocytes. In the testing of a preparation it would seem important that the responsibility for the counts should rest upon one worker. Inasmuch as an occasional pigeon apparently fails to "react" it is important to utilize a sufficient number of birds. Experience only will tell how many, but we would suggest ten or twelve. In some birds the increase may begin later than the ninth or tenth day resembling the so-called delayed type of reaction.

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THE STANDARDIZATION OF ERGOT—A MODIFICATION OF SMITH'S QUANTITATIVE COLORIMETRIC ASSAY.*[1]

BY ASA N. STEVENS.

In view of the chemical nature of ergot and its preparations physiological methods of standardization have long been considered more satisfactory. None of the usual alkaloidal assay procedures have been found adequate. More recent investigations into the chemistry of ergot and the application of certain color reactions have given considerable promise that advantage might be taken of a more rapid and a more accurate colorimetric chemical method of standardization.

It is the object of this paper, therefore, to present some data accumulated over a period of about two years, using a modified colorimetric chemical assay method together with the Cock's Comb (Official U. S. P. X) and the Reversal Uteri (Broom and Clark (2)) methods All the comparative figures that are presented in tabulated form have been obtained from samples that were assayed by the indicated method within a period of one week.

DEVELOPMENT.

The observation that certain color reactions can be obtained with the constituents of ergot is not new. Tanret (6) observed color changes when concentrated sulphuric acid was poured on ergotinine. Later, Keller (3) improved the reaction by dissolving the alkaloid in glacial acetic acid, adding a trace of ferric chloride and pouring the sulphuric acid underneath. He obtained an immediate intensely blue color.

Van Urk (8) using a modification of the general reaction for indol derivatives, mixed 1 cc. of a one per cent solution of para-dimethyl-amino-benzaldehyde with an equal volume of an alcoholic solution of ergot alkaloid and poured sulphuric acid down the side of the tube. Smith (5) further modified van Urk's method by mixing 2 cc. of a one per cent tartaric acid solution of the alkaloid with 1 cc. of

* Scientific Section, A. PH. A., Toronto meeting, 1932.
[1] A contribution from the Analytical Laboratories, Eli Lilly & Company.

M/60 para-dimethyl-amino-benzaldehyde in concentrated sulphuric acid and allowed sunlight to develop the blue color.

Smith's (5) quantitative modification of van Urk's original color reaction has been the subject of this investigation. Briefly, Smith's (5) procedure for the fluidextract of ergot is as follows:

"Five cc. of the fluidextract are evaporated on the water-bath under a current of air or *in vacuo* to remove the alcohol. Excess heating should be avoided. The thick, syrupy residue is transferred quantitatively with the aid of about 50 cc. H_2O to a separatory funnel. The aqueous suspension is rendered slightly alkaline with NH_4OH to a distinct blue reaction with litmus. About 2 cc. of 1:10 concentrated NH_4OH will generally suffice. The solution is than extracted with four successive portions of ether, using 40 cc. in the first, 25 cc. in the second and 15 to 20 cc. in the third and fourth. The ethers are then united, returned to the separatory funnel and washed two or three times with 25 cc. H_2O and a few drops of NH_4OH. This treatment removes most of the yellow alkali-soluble pigment which is present in greater or less amount in most ergots. One or two additional washings with water will remove the excess alkali. The washed ether is made up to 100 cc. and may be kept in this condition, if well stoppered and protected from light, for many weeks.

"To complete the determination, 50 cc. of the ether containing the alkaloids of 2.5 cc. of the fluidextract of ergot are extracted in a separatory funnel three times with an aqueous solution of one per cent tartaric acid, using 10, 10 and 5 cc., respectively. The acid solution is freed from ether by evaporating it on the water-bath under the electric fan to about 15 cc. and is made up to volume (20 cc.).

"Two tubes of 1 and 2 cc., respectively, of this solution are prepared and to the first 1 cc. H_2O added. To both tubes 1 cc. of *M*/60 para-dimethyl-amino-benzaldehyde in concentrated H_2SO_4 are added. The tubes are all exposed to direct sunlight for 30 minutes. They are then compared with standards containing 0.06, 0.08 and 0.10 mg. of ergotamine tartrate in a colorimeter. From these results the number of mg. per cc. of ergot alkaloids can be calculated."

This method was applied and the results obtained are given in Table I together with the physiological results that were obtained by the method of Broom and Clark.

TABLE I.

Sample No.	Chemical % Activity.	Reversal Uteri Method (Broom and Clark) % Activity.	Difference %.
1	160	150	+10
3	80	100	−20
4	80	100	−20
5	80	100	−20
6	80	100	−20
7	80	100	−20
8	50	40	+10
9	20	40	−20
10	28.8	55	−26.2
12	40	50	−10
13	40	50	−10
14	24	50	−26
15	20	30	−10
17	30	37.5	− 7.5
18	64	75	−11
19	80	90	−10

It will be observed that the chemical results (Table I) were, for the most part, lower than those obtained by the Broom and Clark method. These low re-

sults may be explained as being due to variations in technic, for example, the formation of emulsions and the fact that ether is a rather difficult solvent to measure accurately. It is possible that some of these factors may be eliminated and closer agreement obtained by making certain modifications in the procedure.

ETHER EXTRACTION.

With this thought in mind the first ether extraction was made by means of a Watkins' (9) extractor, instead of using a separatory funnel. The alkaloids were then shaken from the total ether extract into one per cent tartaric acid solution, the final dilution being made to 40 cc. instead of 20 cc. Table II gives some of the results obtained by using the Watkins' extraction apparatus.

TABLE II.

Sample No.	Chemical % Activity.	Reversal Uteri Method (Broom and Clark) % Activity.	Difference %.
20	92	100	− 8
21	74	85	−11
22	40	40	0
23	20	30	−10
24	40	50	−10
25	50	60	−10
26	50	50	0
27	90	100	−10

The results in Table II show that closer agreement was obtained.

ALCOHOL EVACUATION.

Since the initial evacuation of alcohol on the water-bath might lead to destruction of the alkaloid, and the transfer from one vessel to another is a source of error, it seemed advisable to eliminate this step. Consequently, 5 cc. of the fluidextract was pipetted directly into the Watkins' extractor, made alkaline with ammonia and extracted with ether. Some of the results are tabulated in Table III, which follows:

TABLE III.

Sample No.	Chemical % Activity.	Reversal Uteri Method (Broom and Clark) % Activity.	Difference %.
30	50	50	0
31	24	30	− 6
32	48	50	− 2
33	100	100	0
34	92	100	− 8
35	120	110	+10
36	104	90	+14
37	100	110	−10
38	87	90	− 3

The figures in Table III show that the initial alcohol evacuation is unnecessary and it was therefore discontinued.

THE ADDITION OF THE SULPHURIC ACID SOLUTION.

At the beginning of this investigation a difficulty arose which was of the greatest importance. In mixing the para-dimethyl-amino-benzaldehyde in con-

centrated sulphuric acid with the alkaloids in the one per cent tartaric acid solution, the mixture becomes hot and the blue color develops immediately, becoming deeper on exposure to sunlight. The color developed in this way gave unsatisfactory results and was of poor quality. In a number of instances an assay was repeated three or four times before a satisfactory color was obtained. Maintaining the tubes at a temperature of about 10° C. while adding the sulphuric acid solution solved this difficulty. In this case the blue color does not develop immediately, but does appear readily in direct sunlight or under a carbon arc. The color so obtained was found to be of greater intensity and of better quality. The figures in Table IV show the difference in results between the cooled and uncooled solutions.

TABLE IV.

Sample No.	Uncooled %.	Cooled %.	Difference %.
A	50	67	17
B	84	88.8	4.8
C	80	88.8	8.8
D	53	67	14
E	80	88.8	8.8
F	93.5	106	12.5
G	113	120	7
H	100	100	0
I	88.8	100	11.2
J	54	80	26

Note that the cooled solutions give higher results, varying from 0 per cent to 26 per cent.

THE MODIFIED PROCEDURE.

In consideration of the foregoing results, Smith's method has been modified as follows:

An aliquot portion of the sample to be tested is pipetted into a Watkins' extractor and diluted to about 50 cc. with distilled water. This aqueous suspension is then made faintly alkaline to litmus with three per cent ammonia. A large excess should be avoided because, in some instances, it tends to destroy the alkaloid. A small piece of litmus paper is placed in the aqueous suspension as an indicator. Ether is then added and the alkaline solution extracted for four hours on a water-bath using just enough heat to cause continuous evaporation

At the end of this time the apparatus is dismantled and the total ether extract transferred to a separatory funnel. This is washed with successive 25-cc. portions of distilled water containing a few drops of three per cent ammonia *as many times as are necessary* to remove any yellow color. One or two additional washings with distilled water should be used to remove any excess alkali.

The washed ether is extracted three times with one per cent aqueous tartaric acid solution, using 10-, 10- and 5-cc. portions, respectively. The aqueous tartaric acid solution is then freed from ether by gentle heating on a water-bath and diluted to 40 cc.

Two tubes containing 1 and 2 cc., respectively, of this solution are prepared. To the first, 1 cc. of distilled water is added. Both tubes are then immersed in

cold water (about 10° C.) and to each tube 1 cc. of $M/60$ para-dimethyl-amino-benzaldehyde in concentrated sulphuric acid is *slowly* added. *Care should be taken to keep the tubes cold.* The tubes are then stoppered and either exposed to bright sunlight for two hours or a carbon arc for forty-five minutes. A blue color develops if the sample tested is active.

The tubes are then compared in a colorimeter with standards containing known quantities of ergotamine tartrate. From this data the quantities of the alkaloid in the sample tested can be calculated.

About a year ago, E. E. Swanson had occasion to study the deterioration of fluidextract of ergot under varying conditions. To different samples of this fluidextract, containing either hydrochloric acid or tartaric acid there was added one of the following salts: sodium acid phosphate; sodium hyposulphite; or sodium hydrosulphite. After a period of aging their activity was determined using the modified procedure already described. The results appear in Table V.

TABLE V.

Sample No.	Chemical % Activity.	Reversal Uteri Method (Broom and Clark) % Activity.	Difference %.
142	26	23	+ 3
143	88 8	67	+21.8
144	106	109	− 3
145	100	100	0
146	67	100	−33
147	68	64	+ 4
148	80	95	−15
149	62	86	−24
150	88.8	82	+ 6.8
151	88.8	80	+ 8.8
152	67	91	−24
153	88.8	80	+ 8.8
154	106	88	+18
155	100	100	0
156	100	85	+15
157	100	80	+20
158	80	70	+10
159	110	83	+27
Original activity	120	120	0
after aging	81	80	+ 1

The results thus far recorded have applied only to the fluidextract. The following table (VI) gives the results obtained with solutions of a pure ergotamine salt that were manipulated in such a manner as to bring about their gradual deterioration. These samples originally contained 0.05% of the pure salt of ergotamine in each cc. of hydro-alcoholic solution. There were also added to some of the samples one of the acids, and one of the salts mentioned in connection with Table V.

TABLE VI.

Sample No.	Chemical % Activity.	Reversal Uteri Method (Broom and Clark) % Activity.	Difference %.
113	*−10	*7	....
114	−10	13	....
115	−10	10	....

TABLE VI. (*Continued.*)

Sample No.	Chemical % Activity.	Reversal Uteri Method (Broom and Clark) % Activity.	Difference %.
116	10	32.5	−22.5
117	5	25	−20
118	10	22	−12
119	40	58	−18
120	50	72	−22
121	30	47	−17
122	−10	18	....
123	60	60	0
124	25	18	+ 7
125	−10	8	....
126	−10	35	....
127	20	41	−21
128	−10	4.7	....
129	54.5	63	− 8.5
130	−10	6	....
131	20	10	+10
132	−10	−10	0
133	−10	−10	0
134	56	43	+13
135	50	40	+10
136.	48.4	34	+14.4
137	46	38	+ 8
138	44.4	43	+ 1.4
139	−10	−10	0
140	45	50	− 5

* Minus signs used in these columns express values that are below the indicated figures, for example: "−10" means that the sample was found to be less than ten per cent active.

It will be noted that all the samples indicated in Tables V and VI have deteriorated, nine of them varying more than ± 20% from their corresponding physiological assay results.

THE MODIFIED PROCEDURE, REVERSAL UTERI AND COCK'S COMB METHODS.

Table VII shows the results obtained on some samples of the fluidextract of ergot using the modified procedure, the Cock's Comb (Official U. S. P. X) and the Broom and Clark methods.

TABLE VII.

Sample No.	Chemical % Activity.	Reversal Uteri Method (Broom and Clark) % Activity.	Cock's Comb U. S. P. X % Activity.
1	90	100	100
2	100	110	100–120
3	110	100	100
4	100	95	97
5	116	100	114
6	96	100	91
7	120	100–115	100
8	110	85–90	100
9	100	100	120
10	110	115	100–106
11	100	115	110

12	50	50	70
13	100	100	100
14	10–15	25	—40
15	35	40	50–60
16	65	100	80

DISCUSSION.

Neither the modified procedure, nor the reasons for modification need be discussed here. The data presented with each step should suffice. It would be well to discuss, however, Tables V and VI in the light of some recent reports.

Wokes (7) states that the Maurice Smith Tests "Fail to distinguish between 'active' and 'inactive' alkaloids" and draws the conclusion that the method as at present applied cannot be considered a satisfactory one for directly estimating the physiological activity of ergot preparations.

Barger (1) states that

"Since all these chemical methods of alkaloidal assay estimate the very slightly active ergotinine along with the real active principles, their validity has been doubted..... As regards ergotamine, Stoll is of the opinion that this alkaloid is only formed artificially by the reagents in some extraction processes..... Thompson has put forward the view that there is only one alkaloid in ergot, which according to the method of extraction may be isolated as ergotinine, ergotamine or ergotoxine."

Rice (4) says that

"Individual laboratories, working upon the same samples of fluidextract have generally found that the colorimetric method has yielded results consistent with those obtained by physiological means. However, merely on a theoretical basis, the method may not be entirely trustworthy since it is known that the physiologically inactive alkaloids of ergot give the color reaction."

It has been decided by the sub-committee for the revision of the British Pharmacopœia to recommend the introduction of a colorimetric assay for ergot. Wokes (7) gives the reason in these words:

"The colorimetric method measures the physiologically active alkaloid with greater accuracy than the biological method measures the physiologically active alkaloid. Tests on over twenty different extracts of ergot showed that the two methods run fairly parallel and it appears probable that any variation which may occur in the proportions of 'active' and 'inactive' alkaloid is less than the possible error of the biological method."

The samples in Tables V and VI did lose potency and therefore must have contained the degradation products of the alkaloids in the fluidextract and in the solutions of the ergotamine salt. This being true, it leads one to believe that the physiologically inactive alkaloids are not degradation products of the active alkaloids, or high results should have been obtained. Furthermore, one would suspect that the physiologically inactive alkaloids do not occur in ergot.

Up to the present time our laboratory has assayed, colorimetrically, more than two hundred and twenty-five samples of ergot preparations by the modified procedure. These preparations include commercial preparations placed upon the market under trade names, as ampules or liquid extracts, as well as the official fluidextract of ergot. Of this number one hundred and eighty-seven have been checked against the Reversal Uteri or Cock's Comb methods. Of these, fifteen have fallen outside the limits of $\pm 20\%$ when compared with the corresponding physiological test.

CONCLUSION.

1. A modified colorimetric procedure has been presented based upon Smith's Quantitative Colorimetric Reaction.

2. Data obtained while comparing the modified colorimetric procedure, the Cock's Comb and the Broom and Clark methods of physiological standardization have been given.

3. The presence of hydrochloric and tartaric acids, sodium hydrosulphite, sodium acid phosphate or sodium hyposulphite does not interfere with the modified colorimetric procedure.

The author is indebted to Mr. Clarence E. Powell for the Broom and Clark and to Mr. C. C Hargreaves for the Cock's Comb assay results appearing in this paper. He also wishes to express his appreciation to Mr. W. J. Rice and Mr. E. J. Hughes for their friendly criticism.

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THE BUFFER CAPACITY OF TINCTURE OF DIGITALIS.*

BY JOHN C. KRANTZ, JR.

INTRODUCTION.

Hydrogen-ion concentration and buffer capacity of various extractive preparations have received the attention of the pharmacist and physical chemist during the past few years. The buffer capacity of the extractive preparations of ergot was studied by Wokes and Elphick (1) in 1930. In addition, Thompson (2) in his comprehensive study of ergot mentioned the buffer value of ergot in his efforts to adjust the hydrogen-ion concentration of the fluidextract. In studying the work of Joachimaglu and Bose (3), the author (4 and 5) observed the rather striking buffer capacity of the extractive substance obtained in the percolation of digitalis leaves with hydroalcoholic menstrua.

Wokes (6) examined the relationship between the total solids of various tinctures of digitalis and their potency and found the relationship to be of no significance. With Munch (7) the author showed that there existed a great variation in the potency of tinctures prepared with absolute alcohol and those prepared

* Scientific Section, A. PH. A., Toronto meeting, 1932.