This table also gives some idea of the response to repeated doses of the same extract in the same dog. On two different occasions Dog 25 received a given dose of the same extract. The corresponding duplicate responses agree rather well with each other. Actually, however, this is simply another manifestation of the general observation that with doses of 2 to 6 cc. the rise in serum calcium is around 4 to 5 mg.%. Hence any remarks regarding the accuracy of the method will have to pertain to responses which are known to be submaximal.

The conclusion to be reached from these results is that the U. S. P. XI method for Parathyroid Extract is satisfactory for purposes of standardization and certainly is wholly suitable for indicating an absence of parathyroid activity. Certain precautions must be observed: (1) The dose selected must be shown to produce significant but submaximal increases in the serum calcium of the dogs used; (2) for satisfactory accuracy, more than the minimum of five dogs must be used unless precautions are taken to standardize the reactions of the animals. Such standardization might well be accomplished by adopting as a reference standard a stable powder prepared by methods already published (6) and distributed by the Board of Trustees of the U. S. Pharmacopœial Convention.

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CONSTITUENTS IN CASCARA SAGRADA EXTRACT.

3. THE LIPIDS AND GLYCOSIDES.*,[1]

BY MELVIN W. GREEN, C. G. KING AND GEORGE D. BEAL.[2]

The most characteristic compounds, from a pharmaceutical point of view, in cascara bark, senna, frangula, rhubarb and aloes are polyhydroxyanthraquinones. The quinones are present largely as glycosides and it has been generally thought that such glycosides were of major physiological importance. This view has led Beal and Tumminkatti (1), Daels (2) and others to make a thorough study of analytical methods for determining free and combined anthraquinones. Sipple, King and Beal (3) have recently isolated a rhamnoside of emodin from cascara bark, but the compound did not possess striking physiological activity. In fact there is little evidence that the anthraquinones account for the greater part of the purgative activity of the above types of drugs. The present investigation has been directed toward finding further information concerning the active principles in cascara bark, using as a guide a method of assay that was developed in a preliminary part of the study (4).

* The authors are indebted to the Parke, Davis and Company for a research grant and a supply of cascara bark.

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[2] Assistant Director, Mellon Institute of Industrial Research.

EXPERIMENTAL—1. CHEMICAL STUDIES.

Lipids.—A petroleum-ether extract of 570 Gm. of cascara bark yielded 5.5 Gm. of lipids which were saponified and fractionated by the familiar lead soap method of Varrentrapp (5). The results of this separation may be seen in Table I. The apparent small amount of linoleic

TABLE I.—SEPARATION OF THE LIPIDS FROM 500 GM. OF CASCARA BARK.

	Gm.
Saturated fatty acids	2.57
Unsaturated fatty acids	1.93
Oleic acid	1.31
Linoleic acid	0.02
Linolenic acid	0.25
Sterols	0.133

Volatile acids, equivalent to 13.0 cc. of *N* NaOH.

acid was probably due to loss of material into the oleic and linolenic acid fractions. Otherwise the composition of the lipid fraction presented nothing unusual. Rhamnosterol (m. p. 131°) was isolated as the major constituent of the sterol fraction and was further identified by its acetyl derivative (m. p. 119°).

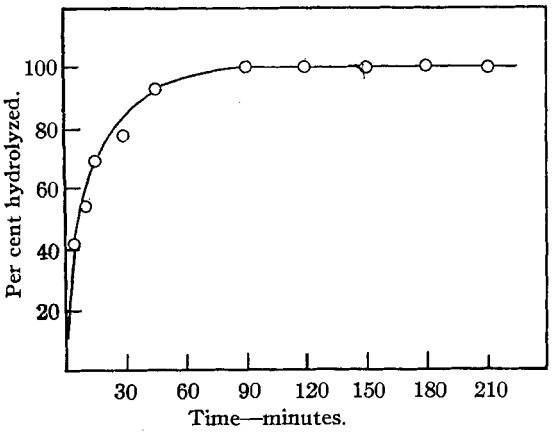

Fig. 1.—Hydrolysis of crude glycoside. Lead-complex from alcoholic extract of bark hydrolyzed at temperature of boiling water-bath in presence of HCl.

The marc from this petroleum-ether extraction was then exhausted with ethanol, from which a resinous precipitate gradually separated on standing. This resinous mass was subjected to steam distillation from which was collected about 5 liters of distillate. The distillate was shaken with large quantities of petroleum ether, yielding an essential oil and methyl hydrocotoin (2,4,6-trimethoxy benzophenone) (m. p. 115°). After the volatile oil had spontaneously evaporated, the methyl hydrocotoin was no longer readily soluble in petroleum ether, but was soluble in ethyl ether, acetone, chloroform and hot alcohol. The volatile oil was not identified.

Glycosides.—The alcohol-soluble extractives were hydrolyzed with hydrochloric acid and from the clarified hydrolysate rhamnose and dextrose were recovered. These sugars were identified chiefly by their osazones. The total quantity of sugars was determined by the Scales titration (6) and the dextrose was then fermented out with yeast. After fermentation, the sugars were again determined to provide an approximation of the rhamnose present. Only a very small quantity of rhamnose was fermented by the yeast under controlled conditions with known mixtures of glucose and rhamnose. By this means, the ratio of dextrose to rhamnose was found to be approximately 1:1. Supplementary fermentation tests with the organism B. rhamnosi-fermentans[1] also indicated that the second sugar was rhamnose, although we did not find such rapid gas production with rhamnose as one would expect from the results recorded by Castellani (7). Rotation values for the clarified sugar solutions also agreed with the values for glucose and rhamnose. The Seliwanoff test for ketoses was only faintly positive, and the mucic acid test was

[1] We wish to express our appreciation to Dr. A. Castellani for supplying a pure culture of B. rhamnosifermentans for fermentation tests. The qualitative differentiation of rhamnose from arabinose, xylose and the hexoses, on the basis of gas production by this organism, was in essential agreement with the results recorded previously.

negative, indicating that fructose and galactose were not present in significant quantities. The Kiliani test for desoxy sugars was also negative.

The rate of acid hydrolysis of a crude glycoside preparation was studied by determining the total sugars liberated at successive time intervals. The rapidity of hydrolysis (Fig. 1) indicated that the sugars were probably present in a true glycosidic linkage rather than in an ether linkage as suggested by Leger (8) for barbaloin from aloes. Complete hydrolysis occurred in less than 90 minutes, while in the case of barbaloin, Leger found that 8 hours were required to effect complete hydrolysis. Gardner and co-workers (9) obtained similar curves for the hydrolysis of synthetic glycosides of polyhydroxy-anthraquinones.

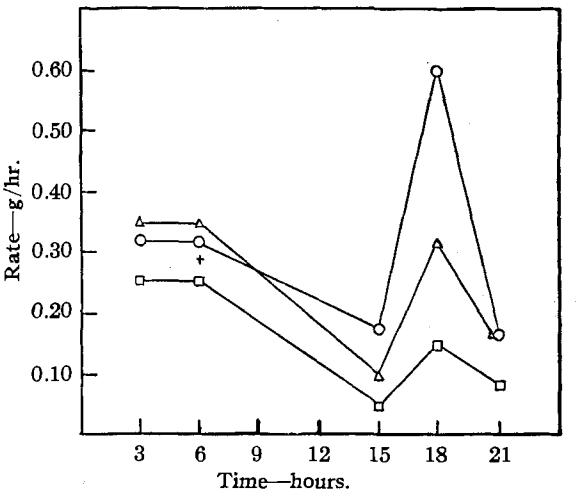

Fig. 2.—Effect of oxidation at elevated temperature on fluidextract of cascara.

○ Fluidextract (1.0 g.). □ Control.
△ Oxidized fluidextract (1.0 g.). + Cascara fed.

To obtain the aglycones, the hydrolytic residue was shaken with chloroform, the chloroform evaporated, and the residue taken up in toluene. By this means was obtained isoemodin, which was more soluble in toluene than emodin. Keimatsu and Hirano (10) have proved the formula of isoemodin by synthesis to be 3,5,8-trihydroxy-2-methyl-anthraquinone. Emodin is 1,6,8-trihydroxy-3-methyl-anthraquinone. The isoemodin was recrystallized several times from glacial acetic acid and acetylated with acetic anhydride in the presence of sodium acetate. The acetyl derivative was recrystallized from alcohol to a constant melting point. In addition, the hydranthrone was prepared by reduction of the anthraquinone with hydriodic acid. Table II gives the melting points and analytical data for these compounds.

TABLE II.—ANALYSIS OF CASCARA BARK CONSTITUENTS.

	M. P.* °C.	C %.	H %.	Ash %.
Isoemodin, calcd.	(212)	66.65	3.73	...
Found	212	66.41	3.97	...
Isoemodin acetate, calcd.	(New)	63.62	4.06	...
Found	184–185	63.40	3.86	...
Isoemodin hydranthrone, calcd.	(215–220)	74.19	4.71	...
Found	214–217	74.11	4.68	...
Methyl hydrocotoin, calcd.	(115)	70.56	5.92	...
Found	115	70.29	5.58	...
Solids from fluidextract		49.0	4.05	3.4
Solids from 48-hr. dialysate		50.3	5.3	4.2

* Values in parentheses represent published data.

2. PHYSIOLOGICAL STUDIES.

The authors have previously described a method of assay for cascara (4), using guinea pigs as test animals. The degree of catharsis was determined by the rate of fecal output (expressed in Gm. per hour), the feces being collected and weighed every three hours except during the over night period of 9 hours before catharsis

began. Control animals were given 1 or 2 cc. of water to eliminate differences due to the handling of the animals. Food cups were withdrawn during the assay period to keep the peristaltic stimulation of food at a minimum and to avoid appetite differences subsequent to dosage. The fluidextract of the U. S. P. XI was used as a standard cascara preparation against which the various fractions were measured. A single dosage was given after a six-hour period (generally 4 to 10 P.M.) had permitted matching the animals for comparable and normal groups.

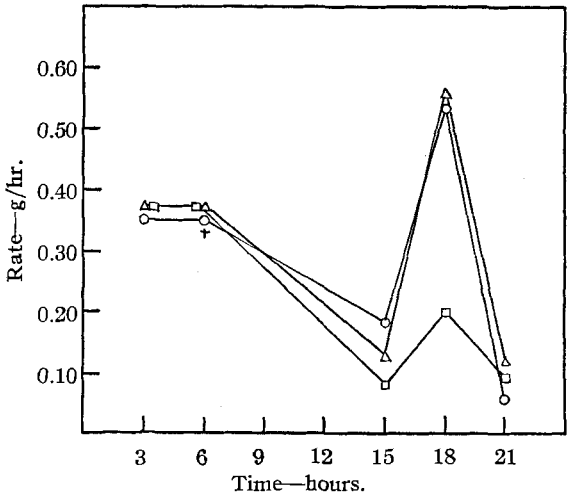

Fig. 3.—Effect of acid hydrolysis on fluidextract.
○ Fluidextract (1.0 g.). □ Control.
△ Hydrolysate (1.0 g.). + Cascara fed.

Oxidation of the fluidextract by bubbling air through it for three hours at the temperature of a boiling water-bath showed an approximate loss of 50% as shown in Fig. 2.

Since it had been thought that the purgative activity was due to the presence of the previously mentioned glycosides, a portion of the fluidextract was completely hydrolyzed at p_H 3.0 for two hours and the hydrolysate fed. Figure 3 indicates that the effect of hydrolysis was negligible. The positive control material (fluidextract) was held at the same temperature at a p_H of 6.8. Statistical analysis of the two curves based on the probable error of differences indicated that the differences were not significant.

Petroleum ether, alcohol and ethyl acetate were used as bark extractants and the corresponding fractions were assayed for cathartic value. None of these fractions were as active as the fluidextract in doses one to five times larger than the standard when based upon the quantity of bark extracted. Mixtures of two or more fractions, in their natural proportions, were more effective than single fractions, but a mixture of all fractions failed to account for more than 60–70% of the fluidextract activity.

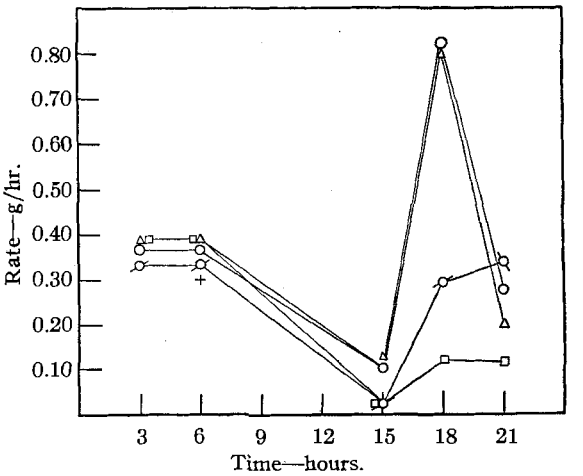

Fig. 4.—Assay of 48 hours dialysate and isoemodin.
○ Fluidextract (1.0 g.).
△ Dialysate (1.0 g.). ○ isoemodin (75 mg.).
□ Control.
+ Cascara fed.

Dialysis of the fluidextract (without alcohol) gave the most encouraging results for a means of separating active from inactive ingredients. The most active preparation was obtained from the material which dialyzed through a Visking artificial sausage skin membrane in 48 hours. This material was found to be at least equivalent in activity to the fluidextract when fed on the basis of total solids (Fig. 4). Under these conditions the dialysate fed was equivalent to 1.8 Gm. of bark, while the fluidextract was equivalent to 1.0 Gm. of bark. The material retained by the membrane exhibited some activity but it was very low compared to the dialysate. The rest of the activity was presumably lost during evaporation. It is also evident in Fig. 4 that 75 mg. of isoemodin, a quantity much above that in 1 Gm. of bark, exerted only a mild catharsis. Dialysis of glucose and citric acid through the membrane indicated that glucose came to an approximate equilibrium in about 20 hours and the citric acid reached a similar state in about 5 hours. Neither the fluidextract nor the dialysate contained appreciable quantities of nitrogen as determined by the micro procedure of Koch and McMeekin (11) on the solids from 1 Gm. of bark. Table II shows the carbon, hydrogen and ash content of the solids from the fluidextract and the 48-hour dialysate.

DISCUSSION.

The relatively low activity of the separate alcohol, petroleum ether and ethyl-acetate extractives cannot be adequately explained at the present time. Possibly all of the activity was not extracted by the respective solvents, but this is unlikely because the marc after exhaustion by these solvents yielded only a negligible quantity of water extractives. Again, a change may have taken place during the processing of the crude extracts. After the solvent had been completely removed by evaporation under reduced pressure below 60°, the residue often did not redissolve in the solvent originally used. An aqueous solution of these fractions always contained considerable suspended matter, and in the case of the ethyl acetate fraction, the material was practically insoluble in water. It may be that the physico-chemical nature of the partially purified material was changed so greatly that the active compounds were absorbed too quickly from the anterior portion of the intestine and thus did not reach the colon in sufficient concentration to exert a normal effect. The fact that the dialysate most nearly approximated the original material is an argument against the latter view, however.

From the results of feeding of the anthraquinones and the fluidextract minus the anthraquinones, it is evident that these compounds contribute to the cathartic value of cascara in either the free or the combined state, but they do not account for all or the greater part of the activity.

From the observed rate of dialysis it is probable that the active constituents of cascara are of relatively low molecular weight. Since dialysis offers a convenient way of removing the bulk of the resinous material without losing appreciable activity, we intend to study the dialysate in more detail.

SUMMARY.

Methyl hydrocotoin has been found as a new ingredient in cascara and the presence of isoemodin and rhamnosterol has been verified. Rhamnose and dextrose were found in the approximate ratio of 1:1. The rate of hydrolysis of these sugars

from their glycosides indicates that they are present in a true glycosidic linkage rather than in an ether linkage as reported for barbaloin. From the results of animal feedings, it was found that dialysis gave the most expedient way of separating inert material from the active ingredients in cascara fluidextract. The lipid fraction from cascara bark was practically inert. Isoemodin was only slightly active in a 75-mg. dosage. The ethyl acetate and alcohol extracts from bark were less active than the standard fluidextract, on either a total solids basis, or on a basis of bark extracted.

Bubbling air through the fluidextract for three hours at boiling water-bath temperature caused a loss in activity of approximately 50 per cent.

The activity of the fluidextract was not altered appreciably by (*a*) complete hydrolysis of the glycosides present or (*b*) extraction of the free anthraquinones.

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THE STANDARDIZATION OF ERGOT—A COMPARISON OF RESULTS OBTAINED BY THE COLORIMETRIC, THE COCK'S COMB AND THE BROOM AND CLARK METHODS OF ASSAY.*

BY ASA N. STEVENS.[1]

Van Urk (1) was among the first to report a color reaction between the ergot alkaloids and para-dimethyl-amino-benzaldehyde. Smith (2) later modified van Urk's procedure by directly mixing a one per cent tartaric acid solution of the alkaloids with concentrated sulfuric acid containing para-dimethyl-amino-benzaldehyde. Allport and Cocking (3) then modified Smith's reagent by altering the acid concentration and introducing ferric chloride as a catalyst. The Allport and Cocking reagent is now used by a majority of workers and has been adopted by the British Pharmacopœia, 1932, Addendum 1935 (4), in the estimation of the ergot alkaloids.

Smith (2) was perhaps the first to propose a quantitative colorimetric assay for the alkaloids in fluidextract of ergot. Other methods of the same general nature appear in the British Pharmacopœia, 1932 (5) and the Methods of Analysis of the Association of Official Agricultural Chemists, Fourth Edition, 1935. In each of these methods only those alkaloids which are sparingly soluble in water are deter-

* Scientific Section, A. PH. A., New York meeting, 1937.

[1] Control Laboratories, Eli Lilly and Company.