emulsifying efficiency of cholesterol in petrolatum.

4. A comparison of the emulsifying ability of cholesterol, epicholesterol, 3α-(hydroxymethyl)-Δ^5-cholestene, and 3α-carboxy-Δ^5-cholestene in petrolatum has been made.

5. Recognition has been made of the nature of the quantal response occurring in the application of the Powers-Cataline method of comparing emulsifying systems.

6. The Powers-Cataline method of comparing emulsifying systems has been expanded and modified to permit a statistical evaluation of the results.

REFERENCES

(1) Cataline, E. L., Sinsheimer, J. E., and Worrell, L., THIS JOURNAL, **43**, 558(1954).
(2) Powers, J. L., Leask, H. B., and Warner, R. S., *ibid.*, **29**, 14(1940).
(3) Cataline, E. L., Reinish, F., and Jefferies, S. F., *ibid.*, **34**, 33 (1945).
(4) Bliss, C. I., "The Statistics of Bioassay," Academic Press Inc., New York, 1952. p. 536.
(5) Munch, J. C., and Garlough F. W., THIS JOURNAL, **24**, 619(1935).
(6) Shackell, L. F., *J. Gen. Physiol.*, **5**, 783(1923).
(7) Shackell, L. F., *et al.*, *J. Pharmacol. Exptl. Therap.*, **24**, 53(1924).
(8) Shackell, L. F., *ibid.*, **25**, 275(1925).
(9) Trevan, J. W., *Proc. Roy. Soc. London*, **101B**, 483 (1927).
(10) Litchfield, J. T., and Wilcoxon, F., *J. Pharmacol. Exptl. Therap.*, **96**, 99(1949).
(11) Janistyn, H., *Fette u. Seifen*, **47**, 351, 405(1940).
(12) Langmuir, I., Shaefer, V. J., and Sobotka, H., *J. Am. Chem. Soc.*, **59**, 1751(1937).

The Effect of Age and of Heat on the "Ferment" in *Cascara sagrada**

By M. CHRISTINE GOSSELIN and CHARLES W. BAUER†

The hydrolytic agent in *Cascara sagrada* has been isolated and a method of analysis for the hydrolytic agent in different lots of *Cascara sagrada* has been worked out and evaluated. The agent has been isolated as an impure residue which is a black, shiny, glass-like, sticky substance with a melting point of about 220°.

IN 1888 Meier and Webber (1) reported that freshly harvested *Cascara sagrada* contained a "ferment" that produced a "griping effect" when this drug was used by human beings as a laxative. This griping effect was not produced by the aged *Cascara sagrada* which "did not contain the ferment." These workers did not identify the ferment, nor did they give the age or sex of any of the experimental subjects. Furthermore they failed clearly to outline their experimental procedure for evaluation by future investigators.

In 1904 Jowett (2) identified the ferment as emulsin since he found that it would readily hydrolyze amygdalin. He made a concentrated extract of this ferment and found that it did not produce a griping effect when it was administered to dogs and to human beings. He did not explain how he determined the absence of a griping effect.

The work of Meier and Webber was responsible for the statement in the monograph under "Cascara Sagrada" in the *United States Pharmacopeia:* "Cascara Sagrada should be collected at least one year before being used for medicinal preparations."

It is well known that the fresh bark gives a troublesome precipitate, that is not given by the aged bark, in the making of the official Cascara Sagrada Fluidextract.

It is a general assumption that the reason for the aging of *Cascara sagrada* before being used for medicinal preparations is not to avoid a troublesome precipitate in making the fluidextract, but to prevent the griping effect of the ferment. This assumption is based on a proposal made in 1950 to the members of the United States Pharmacopoeial Revision Committee

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that *Cascara sagrada* be heated at 100° for one hour in order to destroy the ferment and thus avoid the necessity of aging.

This proposal met with vigorous opposition because it was not supported by experimental evidence that heat would destroy the ferment. It was suggested that an investigation of the character of this "ferment" be made.

In this investigation the effect of age and of heat on the ferment in different lots of *Cascara sagrada* has been made. It has been found that the ferment in *Cascara sagrada* readily hydrolyzes starch. This ferment has amylase-like activity, but it is not destroyed by aging nor by moist heat at 100° in five minutes.

EXPERIMENTAL

Source of Cascara sagrada Used in This Investigation.—The *Cascara sagrada* used in this investigation came in four lots: three of them from the State of Washington,[1] and one from Massachusetts.[2] The latter was collected in Massachusetts in order to obtain a convenient supply for quick delivery. Samples of *Cascara sagrada* collected in 1949, 1950, and 1951 in the State of Massachusetts were not available.[3]

Procedure A

Preliminary Investigation.—Preliminary investigation during 1950–1951 showed that potato starch was readily hydrolyzed by the ferment in *Cascara sagrada*. All the experiments reported below, in which starch was used as the substrate, were run on the same batch of potato starch. All the experimental comparative observations to determine whether the ferment was destroyed by being aged were made in the same year, 1952.

Pulverization.—The bark could be easily ground to a suitable powder by an electric drug grinder. The powder was not sifted because all portions of the powder gave approximately the same results when they were assayed.

Drying.—The powdered bark was dried in one of two ways: *Method I.*—The ground bark was air-dried at room temperature for seven days, during which the freshly harvested bark lost approximately 37% of its weight. During the same interval loss from lots which had been in storage for a year or more was negligible.

Method II.—The ground bark was oven-dried at 100° for seven days. The freshly harvested bark lost about 53% of its weight in this time. The lots which had been in storage for a year or more lost approximately 8% of their weight during oven-drying.

Preparation of Filtrate for Analysis.—Filtrates were prepared from the air-dried and from the oven-dried samples in one of two ways: *Method I.*—One gram from each lot of *Cascara sagrada* was macerated in 50 cc. of water for one hour. The macerated material was then filtered through Whatman No. 1 filter paper, and the filtrate was assayed for amylase-like activity.

Method II.—One gram from each lot of *Cascara sagrada* was added to 50 cc. of water and was brought to boiling. It was allowed to simmer for five minutes. The source of heat was then removed and the mixture was allowed to macerate for one hour. It was then filtered as described above and the filtrate was assayed for amylase-like activity.

This procedure gave four different filtrates for each lot of *Cascara sagrada:* (*a*) air-dried at room temperature and macerated at room temperature, (*b*) air-dried at room temperature and brought to boiling before maceration, (*c*) oven-dried at 100° and macerated at room temperature, and (*d*) oven-dried at 100° and brought to boiling before maceration.

Preparation of Digestion Mixture and the Control.—In the digestion mixture the following ingredients were used: *Cascara sagrada* filtrate 5 cc.; physiological salt solution, 2 cc.; phosphate buffer solution, *p*H 7, 2 cc.; 1% solution of potato starch, freshly prepared, 5 cc.; and distilled water 6 cc.

In the control the *Cascara sagrada* filtrate was omitted, but 11 cc. of distilled water was used to bring the volume to 20 cc.

Preparation and Use of Special Iodine Test Solution.—One cc. of 0.1 *N* iodine test solution was diluted with distilled water to make 125 cc.; 1 cc. of this special iodine test solution was placed in the depression of a white spot plate. The end point was considered to be reached when one drop of the digestion mixture added to 1 cc. of this test solution was free of a blue color.

The digestion mixture and the control were each placed in 125-cc. Erlenmeyer flasks. The flasks were placed in a constant temperature water bath at 40° ± 1°. Each flask contained a dropper pipet. One drop from each mixture was tested at one-minute intervals until no blue color was obtained. A comparison of the hydrolytic activity of the amylase-like activity of *Cascara sagrada* collected at different times and prepared in four different ways may be seen in Table I.

The properties of the hydrolytic agent in the different lots of *Cascara sagrada* when assayed by this method may be listed as follows: (*a*) it is stable to heat; (*b*) it is not lost on storage; (*c*) it is partially inactivated when heated at 100°; and (*d*) the partially inactivated hydrolytic agent is reactivated by moist heat.

The hydrolytic agent is not a typical enzyme if one accepts the proposition that an enzyme is readily destroyed by moist heat (3). However, lysozyme is often classified as an enzyme and it is very stable to moist heat (4).

Procedure B

In this procedure photometric methods were used in an attempt to compare activity. These methods were not successful because of the deep colors of the filtrates from the *Cascara sagrada* samples. Dilutions made to overcome this interference simul-

[1] The lots of *Cascara sagrada* collected in 1949, 1950, and 1951 were supplied through the courtesy of Parke, Davis and Company.

[2] The lot of *Cascara sagrada* collected in 1952 was obtained from the medicinal plant garden of the Massachusetts College of Pharmacy.

[3] All of the samples of *Cascara sagrada* used in this investigation were authenticated by Professor Heber W. Youngken and Professor Maynard W. Quimby of the Massachusetts College of Pharmacy.

TABLE I.—THE EFFECT OF STORAGE AND OF HEAT ON THE HYDROLYTIC AGENT IN DIFFERENT LOTS OF CASCARA SAGRADA

Year Collected	Age of Bark	Time in Minutes for the Complete Hydrolysis of Starch: Air-Dried at RT for Seven Days — Macer. at RT	Air-Dried at RT for Seven Days — Boiled before Macer.	Oven-Dried at 100° for Seven Days — Macer. at RT	Oven-Dried at 100° for Seven Days — Boiled before Macer.
1952	Fresh	<1	<1	<1	<1
1951	1 yr.	<1	<1	7	<1
1950	2 yr.	<1	<1	36	<1
1949	3 yr.	...	<1	90	<1
Control		...	...	...	...

taneously reduced the active principle content to a point too minute for measurement by photometric means.

Procedure C

A Study of the Hydrolytic Activity of the Different Lots of Cascara sagrada by Serial Dilutions of the Filtrate.—It was obvious from the previous assay that air-drying at room temperature for seven days was satisfactory for the purposes of this investigation. It was equally obvious that the dried bark showed its maximum hydrolytic activity after the aqueous mixture was brought to boiling before maceration. Hence in the following studies the bark was first air-dried at room temperature and the aqueous mixture was brought to a boil for five minutes and was allowed to macerate for one hour before being filtered.

Preparation of Filtrate and of Serial Dilutions.—An amount of 5.562 Gm. of powdered air-dried *Cascara sagrada* was added to 50 cc. of distilled water. This mixture was brought to a boil and was allowed to simmer for five minutes. The mixture was macerated at room temperature for two days. The mixture was centrifuged, then filtered through folded gauze. The filtrate obtained from each lot of *Cascara sagrada* was diluted with water to obtain ten different concentrations for each lot. Then 5 cc. of each dilution was assayed as outlined in *Procedure A*. The concentrations of *Cascara sagrada* represented in 5 cc. of the diluted filtrate used in each assay are shown in Table II.

Composition of Digestion Mixture Containing Diluted Filtrate.—Five cubic centimeters of the diluted filtrate was added to a digestion mixture

TABLE II.—CONCENTRATIONS OF CASCARA SAGRADA REPRESENTED IN DILUTED FILTRATE USED IN THE ANALYSIS

Dilution Number	Filtrate Used, cc.	Distilled Water Added, cc.	Total Volume	*Cascara sagrada* in 5 cc. Used in Analysis, Gm.
1	9	1	10	0.500
2	8	2	10	0.444
3	7	3	10	0.389
4	6	4	10	0.333
5	5	5	10	0.277
6	4	6	10	0.222
7	3.5	6.5	10	0.195
8	3	7	10	0.167
9	2	8	10	0.111
10	1	9	10	0.056

previously prepared as shown under *Procedure A*. The mixture was shaken for about five seconds. Then one drop of this mixture was added to an iodine test solution. In this analysis there was no color produced in the more concentrated solutions, but a blue color was obtained in the more highly diluted solutions of each lot of *Cascara sagrada*.

It was shown in *Procedure A* that the lots of *Cascara sagrada* collected in 1951 and in 1952 contained a more active hydrolytic agent than the 1949 and 1950 lots when the different lots were oven-dried at 100° for seven days and the bark was assayed after maceration without the use of moist heat. (See Table I.) The results obtained by *Procedure C* not only confirm but also extend the interpretations that can be made from the findings recorded in Table I. In *Procedure A* there was no difference in the hydrolytic activity in the air-dried lots of *Cascara sagrada* that were boiled before maceration. In this dilution method it is shown that the 1951 lot of the drug contained more of the hydrolytic agent than did either the 1949 or the 1950 air-dried lots.

Procedure D

Titration Method.—To determine the amount of *Cascara sagrada* required to hydrolyze a definite amount of starch in a given period of time a titration method was tried.

Preparation of Filtrate.—Five grams of each lot of *Cascara sagrada* was added to 50 cc. of distilled water. This mixture was brought to a boil and allowed to macerate for two days at room temperature. Following filtration, as outlined before, the filtrate of each lot was assayed.

Preliminary Assay.—The approximate number of cc. of each filtrate required to hydrolyze completely the 1% starch solution in the digestion mixture at room temperature was determined.

Assay Procedure.—Eight determinations were made on each filtrate. For the 1949 lot 2.3 cc. of filtrate was added to the digestion mixture rapidly from a 10-cc. buret. Then one drop at a time was added; and after each drop of filtrate was added to the digestion mixture, one drop was removed and added to an iodine test solution. For the 1950 lot 2.2 cc. of the filtrate was rapidly added; and then one drop at a time was removed as above. For the 1951 lot 1.4 cc. was the starting amount. The results of this method of analysis are shown in Table IV.

The results obtained by this method of analysis confirm the previous findings shown in Tables I and III. The 1951 lot had greater activity than

TABLE III.—HYDROLYTIC ACTIVITY OF DILUTED FILTRATE OF DIFFERENT LOTS OF CASCARA SAGRADA

Dilution Number	Year *Cascara sagrada* Was Collected 1949	1950	1951
1	No color	No color	No color
2	No color	No color	No color
3	No color	No color	No color
4	No color	No color	No color
5	No color	No color	No color
6	No color	No color	No color
7	End point	End point	No color
8	Deep blue	Deep blue	No color
9	Deep blue	Deep blue	End point
10	Deep blue	Deep blue	Deep blue
Control	Deep blue	Deep blue	Deep blue

either the 1949 or the 1950 lot. The results shown in Table IV have been submitted to statistical evaluation and found to be significant. The analysis of variance shows a real variation between lots. The criterion of likelihood shows a real uniformity within lots (5).

ISOLATION OF THE HYDROLYTIC AGENT IN CASCARA SAGRADA

Procedures to isolate the hydrolytic agent were carried out on three different lots of *Cascara sagrada*.

Procedure.—About 45 grams of the air-dried powder of *Cascara sagrada* was added to 200 cc. of distilled water, brought to a boil, and allowed to simmer for five minutes. Then the mixture was allowed to macerate at room temperature for four days.

The macerated mixture was filtered through folded gauze. This yielded approximately 100 cc. of filtrate. The filtrate was heated to 70°, and 80 cc. of alcohol was added. A slight precipitate formed, but was easily separated by filtration. The precipitate was discarded since it possessed only slight hydrolytic activity.

A dark gray precipitate was obtained when 200 cc. of alcohol was added to the clear filtrate, at room temperature. This precipitate was removed by centrifuging at 1800 r. p. m. The supernatant fluid was filtered through folded gauze. The precipitate showed only slight hydrolytic activity and was discarded.

The clear filtrate was then heated on a water bath until practically alcohol-free. Then distilled water was added to bring the volume to 100 cc. To this mixture an equal volume of a saturated solution of ammonium sulfate was added. This mixture was stored for two days, at room temperature, in an evaporating dish (toluene was added as a preservative). A dark, oily-like precipitate formed which was removed by filtration. This precipitate showed only slight hydrolytic activity and was discarded.

The filtrate was diluted with distilled water to make a volume of 100 cc. This dilution was divided into four 25-cc. portions and each was subjected to dialysis. After two days of dialysis all of the ammonium sulfate was washed out of the mixture.

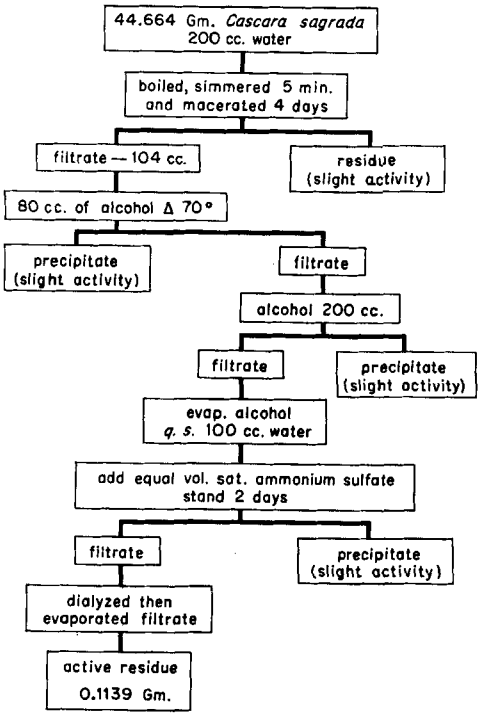

Fig. 1.—Flow chart for isolation of hydrolytic agent from *Cascara sagrada*, 1951 lot.

TABLE IV.—ASSAY RESULTS OF DIFFERENT LOTS OF CASCARA SAGRADA BY TITRATION METHOD OF ANALYSIS OF FILTRATE

Trial Number	Filtrate of Each Lot of *Cascara sagrada* for Complete Hydrolysis of Starch, cc. 1949	1950	1951
1	2.35	2.95	2.05
2	2.40	2.20	1.85
3	2.75	2.50	1.80
4	2.90	2.25	1.60
5	2.45	2.55	1.70
6	2.50	2.85	1.55
7	2.90	2.95	2.00
8	2.80	2.55	1.45
Lot mean ($\bar{X}$)	2.63	2.60	1.75
Lot range (R)	0.55	0.75	0.60

The solutions in the dialyzing tubes were then evaporated to dryness on a water bath. A black, shiny, glass-like substance was obtained. It had a melting point of about 220°. The precipitates from the different lots showed approximately the same degree of hydrolytic activity. The hydrolytic activity of this residue is approximately ten times greater than that of the lot of *Cascara sagrada* from which it was obtained. An example of the steps involved in this procedure is shown in Fig. 1.

SUMMARY

1. The hydrolytic agent in *Cascara sagrada* has the following properties: (*a*) it readily hydrolyzes starch; (*b*) it is not lost on aging; (*c*)

it is not destroyed by moist heat at 100°; (*d*) it is partially inactivated by dry heat at 100° after a period of seven days of heating but is reactivated by boiling water; and (*e*) it is soluble in water.

2. A method of analysis for the hydrolytic agent in different lots of *Cascara sagrada* has been worked out and evaluated as follows: (*a*) The results have been treated statistically and have been found to be reliable. (*b*) The 1951 lot had greater activity than either the 1950 or the 1949 lot. (*c*) The 1950 and 1949 lots showed no significant difference. (*d*) There was no significant difference in the results of the assays of the same lot.

3. The hydrolytic agent has been isolated as an impure residue. This residue is a black, shiny, glass-like, sticky substance with a melting point of about 220°.

REFERENCES

(1) Meier, H. F., and Webber, J. Leroy, *Am. J. Pharm.* **60**, 87(1888).
(2) Jowett, H. A. D., *Proc. Am. Pharm. Assoc.*, **52**, 288 (1904).
(3) Gortner, R. A., *Outlines of Biochemistry*, 2nd ed., John Wiley & Sons, Inc., New York, 1938, p. 925.
(4) Fleming, A., *Proc. Roy. Soc.*, **B93**, 306(1922).
(5) Gosselin, R. A., Personal communication, 1953

A Note on the Flavoring of Methionine and Choline Combinations*

By HERBERT M. GROSS

MEDICINAL PRODUCTS containing methionine and the choline salts in combination with inositol, thiamine, riboflavin, and liver are abundant in the physician's medicamentorum. These formulations provide the lipotropic principles necessary to reverse fatty liver infiltration and prevent hepatic destruction. While the individual characteristics of these substances pose a flavoring problem, the combination of these ingredients is nauseating. The necessity for an appealing flavor to disguise this objectionable taste is obvious.

EXPERIMENTAL

A formulation containing 400 mg. choline dihydrogen citrate, 100 mg. methionine, 10 mg. inositol, 2 mg. thiamine hydrochloride, 2 mg. riboflavin, 10 mg. niacinamide, 5 μg. cyanocobalamin, and 470 mg. liver extract per 4 cc. (one teaspoonful) was prepared. This aqueous vehicle, exemplifying several commercial preparations, contained 10% glycerin as solubilizer and 0.1% methyl *para*-hydroxybenzoate as a preservative. After preliminary tasting of the raw formulation, it was apparent that some sweetening agent would be necessary. Cyclamate sodium was used in a 0.01% concentration as an adjunct to eliminate the bitterness of saccharin and the use of sucrose which reportedly is incompatible with cyanocobalamin (1). The glycerin in the formula also contributed to the sweetness.

The raw formulation had both an offensive, putrid odor and a nauseating, bitter, fishy taste. The addition of 3% polysorbate 80 to the formulation had a marked influence on the reduction of the nauseating odor. This substance contributed a soapy factor to the taste, but the therapeutic indications of lipotropic activity (2) were felt to overshadow this disadvantage.

Several manufacturers of flavoring materials were contacted and their suggested samples were combined with the lipotropic solution at a 1% concentration in a comparative study of their odor- and taste-masking properties.

Hamann (3) states that flavor has three components: odor, taste, and consistency, and he reports that consistency has a direct influence on palatability. The effectiveness of flavor enhancement by viscosity functions on the principle that the viscid disguising flavor, due to the tenacity caused by viscosity, is diminished at a slower rate than the solution time of the bitter principle. The viscosity of this preparation was adjusted by introducing 0.5% carboxymethylcellulose (medium viscosity).

A panel comprised of 18 adults, male and female, indicated the tabulated preferences. Each subject tested the samples on two occasions. The necessity for a trained flavor panel in testing this formulation was diminished by its inherent unpleasantness. Little difficulty was encountered with the nauseous obviousness of odor and taste, and once instructed in the method of determining taste and aftertaste, reproducible ratings were possible with an inexperienced panel. Odor coverage was first tested, then a small sample was tasted by swishing it about in the mouth and finally spitting out the sample. Both immediate and aftertastes were considered in this method outlined by Crocker (4). The mouth was rinsed between samples with lukewarm water. Since the formulation had a brown color, no artificial colors were added to the samples so as to limit any psychological effect of color on taste.

* Received December 7, 1953, from the Department of Pharmaceutical Development, Commercial Solvents Corporation, Terre Haute, Ind.