utilization of the monose, in the apple, on the contrary, the ratio of the specific activity of the sucrose to the glucose was 0.043 after 22 hours, a condition remote from complete equilibration.

Because of the inequality of the labeling, it must be concluded that both halves of the sucrose do not arise entirely from a common precursor. On the basis of the present information it would be premature to speculate on this point at length. Two obvious mechanisms by which an unequal distribution of label might arise are:

The conversion of glucose-C^{14} to the precursor of the glucose moiety may be more rapid than the interconversion of glucose to fructose (or fructose derivative), thus increasing the relative contribution of any endogenous precursor of the fructose moiety that might be present. Whether the endogenous precursor is simply free fructose (segregated in a pool distinct from the bulk of the fructose) or a fructose derivative is immaterial.

Transglycosidation could cause unequal labeling, although invertase has been reported absent from the apple (*14*).

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Received for review September 20, 1954. Accepted October 26, 1954. Mention of products or equipment does not constitute endorsement by the Department of Agriculture over others of a similar nature not mentioned.

FEED DIGESTIBILITY

Use of Copper Derivatives of Chlorophylls in Ratio Method for Estimating Digestibility of Forages

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The "chromogen" ratio method was found unsatisfactory for use in determining digestion coefficients in studies with switch cane (*Arundinaria sp.*). A slight modification of the analytical procedure, treating samples with 0.1*M* copper chloride in 1*N* hydrochloric acid, overcame the difficulties. This proposed modification will be useful in cases where the forage under study is relatively low in chlorophyll and its derivatives.

THE "CHROMOGEN(S)" of Reid's ratio method for determining the digestibility of forages are not a single entity but a mixture of pigments, composed predominantly of chlorophylls and pheophytins and small amounts of carotenoids (*2, 5*). Because the procedure lacks specificity in the sense of involvement of either a single pigment or a fixed ratio of pigments, conceivably this method might fail to yield valid digestion coefficients when the test forage is low in chloroplast pigments and high in pigments that can be lost or changed during passage through the digestive tract (*1*). In view of the importance of indirect methods for determining digestibility in pasture research and the soundness of the basic idea underlying the chromogen(s) method, it seemed desirable to study possible modifications of Reid's method to increase its specificity and useful range.

Experimental Work

Previous experience (*5*) has shown that the chromogen ratio method may be applied successfully to the estimation of digestibility of grass and legume hay by rabbits; therefore, four tortoise Dutch rabbits were used as experimental animals in this study. The test forage used was the edible leaves of switch cane (*Arundinaria sp.*), a plant which is grazed the year around by sheep and cattle on the coastal plain of North Carolina. Winter leaves were used in the first trial and spring leaves, collected after active growth had started, in the second.

Each diet was composed of 100 parts of ground switch cane leaves, 25 parts of glucose (Cerelose), and 7 parts of refined cottonseed oil (Wesson oil). The trials consisted of a 7-day preliminary period and a 7-day collection period. The samples of feed and of feces from these digestion trials were used in comparing digestion coefficients obtained by three methods of analysis: conventional, Reid's chromogen, and copper derivatives of chlorophyll.

In the first trial in which the winter collection of switch cane leaves was used, the chlorophyll derivatives were determined as sodium copper chlorophyllins according to a method of the Cerophyll Laboratories (*3*). This procedure offered promise as a substitute for Reid's method (*4*) for some plant species, but as many extra time-consuming steps were introduced, one of the main advantages of the chromogen method was lost.

An attempt to simplify the procedure led to the development of the following method.

Two grams of feed and of fecal samples were allowed to stand 2 to 4 hours in 20 ml. (or enough to cover the sample) of approximately 0.1*M* cupric chloride in 1*N*

hydrochloric acid. The samples were then extracted with 85% acetone in water as in Reid's procedure and the extracts were allowed to stand overnight (12 hours or more). These solutions were then diluted and read in a Beckman Model D spectrophotometer at 406 mμ. Solutions with cupric chloride in acetone, equivalent to those in the samples, were used as blanks.

Results

As indicated by the digestion coefficients of dry matter in Table I and by the data on chromogen recovery in Table II, the copper chlorophyll method was superior to that of Reid (*4*) for determination of digestibility of either winter or spring leaves of *Arundinaria*. The difference between these methods is particularly evident in the results from the winter leaves. In this trial only 55% of the ingested chromogen was recovered in the feces, whereas approximately 100% of the copper-derivatives of chlorophyll was recovered.

The modified method was tested further with feed and fecal samples obtained from a previous rabbit digestion trial in which Reid's chromogen method had given good results with soybean forage. As may be seen in Table I, both indicator methods gave digestion coefficients similar to the coefficient by the conventional procedure, and as indicated in Table II, recovery data are similar.

Supplemental chemical studies were conducted in order to ascertain some of the chemical characteristics of the copper derivatives of chlorophyll. Spectral absorption curves of copper chlorophyll solutions from feed and from feces were similar throughout the visible range of the spectrum (380 to 700 mμ). Good recovery data could be obtained when solutions were read at 406 mμ and also at 650 mμ (Table II). This maximum in the red region of the spectrum is out of the range where common carotenoids would interfere, but sensitivity is lost when this maximum is used.

The copper derivatives of chlorophyll, once formed, are extremely stable compounds (*6*). In this study they were found to be stable in both acid and alkali solutions as well as to light. They remained stable at room temperature for periods up to 1 week (Table II).

Transfer of the copper chlorophyll pigments to ether, followed by chromatographic separation on a 1 to 1 magnesia–SuperCel mixture, distinguished at least two components. One pigment moved off the column with diethyl ether; the second was removed by pyridine but not by a number of other common solvents. Pigment I had absorption maxima at 422 and 650 mμ, while pigment II had maxima at 410 and 650 mμ.

The copper-stabilized acetone extracts obtained from the switch cane leaves contained no detectable carotenoids. When either the mixed carotenoids isolated from these leaves or pure β-carotene was dissolved in acetone, these pigments were destroyed within 4 hours after treatment with the cupric chloride solution in hydrochloric acid.

Table I. Dry Matter Digestion Coefficients

Method	*Arundinaria* Leaves, %		Soybean Hay, %
	Winter	Spring	
Conventional	41.5 ± 0.1[a]	41.1 ± 0.9	55.4 ± 1.0
Chromogen	−5.9 ± 2.6	29.3 ± 1.5	53.4 ± 1.3
Cu chlorophylls	42.4 ± 1.1	41.1 ± 0.7	55.0 ± 0.8
Na Cu chlorophyllins	39.4 ± 1.3		

[a] Standard errors, $\sqrt{\frac{s^2}{n}}$

Table II. Mean Recovery of Chromogen from Feces

Chromogen	*Arundinaria* Leaves, %		Soybean Hay, %
	Winter	Spring	
Total chromogen in acetone extract	55.2 ± 3.7[a]	82.9 ± 2.67	98.8 ± 3.2
Na Cu chlorophyllins	97.2 ± 4.0	...	...
Cu chlorophylls			
Without standing	...	89.1 ± 5.14	...
After standing 12 hours	101.0 ± 2.5	99.3 ± 2.55	99.0 ± 2.9
After standing 1 week	...	102.0 ± 3.27	...
406 mμ	...	100.2 ± 2.48	...
650 mμ	...	99.4 ± 2.04	

[a] Standard error, $\sqrt{\frac{s^2}{n}}$

Discussion

The chromogen method of Reid *et al.* (*4*) works well when chlorophylls and their higher degradation products are the principal constituents of the aqueous-acetone extract. When a considerable portion of the pigments in the aqueous-acetone solution consists of pigments other than chlorophyll derivatives, poor results may be obtained with this method. The low recoveries of chromogen in trials with plants low in chlorophyll, such as switch cane, could be due to the loss of carotenoids or other pigments that are metabolized or destroyed in their passage through the animal body. As the chlorophylls and their derivatives apparently are rather stable in the alimentary tract (*5*), a chemical method which is specific for chlorophyll derivatives should be better than the less specific chromogen method. The success of the copper chlorophyll method bears out this assumption.

The solutions of copper chlorophyll pigments were read at 406 mμ, even though this is not the maximum for these solutions. As the spectral curves for both feed and feces are similar, it is possible that the absorption maximum at 415 mμ could have been used; however, it was not done in this case.

Summary

The chromogen ratio method was found unsatisfactory for use in determining digestion coefficients in studies on switch cane forage, but a simple modification of the analytical procedure overcame the objection. This modification involved introducing copper into the porphyrin ring of chlorophyll and pheophytins, thus stabilizing these pigments and at the same time largely eliminating other pigments, particularly the carotenoids. Probably the proposed modification will be the method of choice whenever the forage under study contains relatively small amounts of chlorophyll and its derivatives.

Acknowledgment

This investigation was supported in part by funds from the Animal and Poultry Husbandry Research Branch, Agricultural Research Service, U. S. Department of Agriculture. The authors are indebted to G. O. Kohler, Cerophyll Laboratories, Kansas City, for his method for chemical assay of chlorophyll derivatives in cerophyllin D and similar products.

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Received for review October 9, 1954. Accepted October 28, 1954. Published with approval of the Director of Research as paper No. 596 in the Journal Series.