tein components, degree of resolution, and presence of new components. This approach could be of greater usefulness if means were developed for greatly increasing the solubility of protein components in meals subjected to heat damage during processing.

Acknowledgment

The authors are pleased to acknowledge the helpful suggestions of Marjorie Z. Condon in preparation of this paper.

Literature Cited

(1) Briggs, D. R., and Mann, R. L., *Cereal Chem.*, **27,** 243–57 (1950).
(2) Haddon, R., *et al.*, *Cotton Gin and Oil Mill Press*, **52,** No. 9, 18 (1950).
(3) Hayward, J. W., *Feedstuffs*, **22,** No. 30, 30–5, 38, 40–2 (1950).
(4) Horn, M. J., Blum, A. E., Womack, Madelyn, and Gersdorff, C. E. F., *J. Nutrition*, **48,** 231–41 (1952).
(5) Karon, M. L., Adams, M. E., and Altschul, A. M., *J. Phys. Colloid Chem.*, **54,** 56–66 (1950).
(6) Longsworth, L. G., *Chem. Revs.*, **30,** 323–40 (1942).
(7) Mann, R. L., and Briggs, D. R., *Cereal Chem.*, **27,** 258–69 (1950).
(8) Milligan, J. L., and Bird, H. R., *Poultry Sci.*, **30,** 651–7 (1951).
(9) Olcott, H. S., and Fontaine, T. D., *Ind. Eng. Chem.*, **34,** 714–16 (1942).
(10) Pons, W. A., and Guthrie, J. D., *J. Am. Oil Chemists' Soc.*, **26,** 671–6 (1949).
(11) Reiner, M., and Fenichel, R. L., *Science*, **108,** 164–6 (1948).
(12) Research Conference on Processing as Related to Cottonseed Meal Nutrition, Southern Regional Research Laboratory, New Orleans, La., Proceedings, Nov. 13–14, 1950.
(13) Sarett, H. P., and Cheldelin, V. H., *J. Biol. Chem.*, **155,** 153–60 (1944).
(14) Second Conference on Cottonseed Processing as Related to Nutritive Value of the Meal, Southern Regional Research Laboratory, New Orleans, La., Proceedings, Nov. 5–7, 1951.

Received for review February 6, 1953. Accepted April 24, 1953. Presented at the Southwide Chemical Conference, Auburn, Ala., October 23 and 24, 1952. The use of trade names in this article is for identification and implies no endorsement by the Department of Agriculture of the product over similar products not mentioned.

DIGESTIBILITY OF FORAGES

Pigments Involved in the Chromogen(s) Ratio Method

WILLIAM W. G. SMART, JR., FRANK W. SHERWOOD, GENNARD MATRONE, and GEORGE H. WISE

Animal Nutrition Section, Department of Animal Industry, North Carolina Agricultural Experiment Station, Raleigh, N. C.

Although information as to the identity of the pigment or pigments is not essential to employment of the "chromogen(s)" ratio method, knowledge of its properties could lead to improvements in the ratio technique. The purpose of this study was to determine the identities of the pigments involved and to obtain further evidence of validity of the chromogenic method. The pigments contained in the chromogen mixture used as an indicator of digestibility were isolated and identified. Spectral absorption curves of the 85% aqueous acetone extracts from forage and feces displayed absorption maxima indicative of chlorophylls or their degradation products. Cold saponification of these acetone extracts demonstrated the presence of both chlorophyll products and carotenoids. Chromatographic separation of the pigments demonstrated the presence of at least seven pigments in the chromogen extract from hay and feces of animals fed this hay: chlorophyll a, chlorophyll b, pheophytin a, pheophytin b, luteol, violaxanthol, and β-carotene. The chromogen(s) involved in the chromogen ratio method for determining digestibility of forages is essentially a total chloroplast pigment extract. Changes in the chemical structure of these compounds on passage through the digestive tract have little effect on the light absorption of the mixture of pigments at the isosbestic point at 406 mμ.

A COMPARATIVELY SIMPLE CHEMICAL METHOD for estimating consumption and digestibility of forages by animals (7) is based on the principle that nonabsorbable material in a forage may be used as a marker and that its concentration in the feces is proportional to the digestibility of the forage. Based on experiments in which data from conventional digestion trials were used as the standard, Reid *et al.* proposed that an 85% acetone-water extract of forage contains a natural marker. This marker was labeled "chromogen(s)" (7), because its identity was unknown. Although information as to the chemical identity of the pigment or pigments is not essential to the employment of this procedure, a knowledge of their properties may lead to improvements in the ratio technique.

In view of the recognized abundance

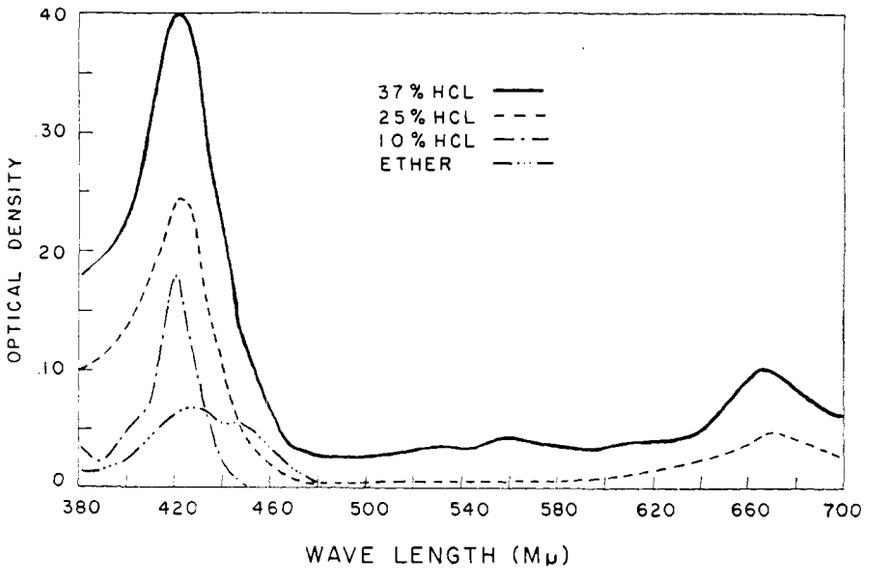

Figure 1. Spectral absorption curves of hydrochloric acid fractions extracted from an ether solution of pigments transferred from an aqueous acetone extract of the feces of a rabbit fed Ladino clover

of chloroplast pigments in the vegetative parts of plants, it was logical to assume that the chromogen(s) extracted from pasture forages and from feces is related to these pigments. The principal chloroplast pigments in all green vegetative tissues are chlorophyll a, chlorophyll b, luteol, and β-carotene (*5, 11*), all of which absorb light in the region of 406 mμ. Chlorophylls are extracted most efficiently from plant tissue by acetone containing 15% water by volume (*12*), the mixture employed to remove chromogen(s) (*7*). Carotenoids also are soluble in this water-acetone mixture.

In the passage of chlorophylls through the digestive tract of the animal, magnesium may be lost, resulting in the formation of phytins. These in turn may lose the phytol group and become phorbides (*2, 3, 8–10*). No further degradation of the chlorophyll molecule in the alimentary canal was indicated in the reports reviewed.

Information on the identity of the plant constituent(s) in the chromogen(s) of forage and of feces should provide a basis for improving the empirical method (*7*) and an insight for circumscribing the area of its application.

Procedure and Results

Experimental materials were samples of forages and of feces collected from digestion trials with rabbits and sheep. Oven-dried clippings of Ladino clover (*Trifolium repens latum*), orchard grass (*Dactylis glomerata*), fescue grass (*Festuca elatior arundinacea*), and various combinations of these forages were used in the rabbit digestion trials, whereas soybean (*Soya max* var. Ogden) and fescue hays were used in the sheep trials. Aqueous acetone extracts of the forages and of the fecal samples were made according to procedures of Reid *et al.* (*7*) and subsequently subjected to various separation and identification procedures (saponification, hydrochloric acid fractionation, and chromatographic analysis). All spectrophotometric measurements were made on a Beckman Model D spectrophotometer using quartz cells having a 1-cm. path length.

Absorption Spectra Of Acetone Extracts Spectral absorption curves for aqueous acetone extracts were studied over a range from 380 to 700 mμ in order to include the secondary maxima and the two primary maxima of chlorophyll products. The absorption curves of all the aqueous acetone extracts from the sheep and the rabbit digestion trials were similar. All extracts from dried forages and feces exhibited two principal maxima, one in the blue region from 410 to 430 mμ and the other in the red region between 660 and 670 mμ. In contrast, extracts from fresh forages exhibited a sharp maximum in the blue region at 430 mμ and one in the red region at 660 mμ, indicating the presence of chlorophyll degradation products in dried forage and feces. The three secondary maxima, which have been reported between 500 and 620 mμ (*1, 6*), were not apparent in the dilutions of extracts which were most convenient for making readings at the isosbestic point (*7*), but in more concentrated solutions three lesser maxima at approximately 505, 535, and 610 mμ became evident. The location of the maxima, both primary and secondary, in all the extracts indicates that either chlorophyll or closely related products is a part of the chromogen(s).

Saponification Separation Because chromogen(s) of the aqueous acetone extracts may contain carotenoids, this class of pigments was separated from chlorophylls and related compounds by saponification (*12*). In this process, the pigments of the aqueous acetone extracts underwent changes characteristic of the phase test for chlorophyll. The yellow ether solutions of the unsaponifiable fractions from forage and feces absorbed light between 380 and 500 mμ, having peaks between 400 and 450 mμ. There was no light absorption beyond 500 mμ. These characteristics are indicative of the presence of carotenoids.

For comparison the total pigment content of aliquots of the aqueous acetone extracts of forages and feces was transferred to ether. Aliquots of the aqueous acetone extracts were diluted with water and shaken with successive portions of diethyl ether until all visible color had been removed. The ether solutions of the pigments were washed with water and made up to volumes equal to those of the original aqueous acetone extracts. Similarly, the volumes of the ether solutions of the unsaponifiable fractions were adjusted to equal those of the aqueous acetone extracts from which they had been prepared. Optical density readings on these solutions showed that the unsaponifiable fraction accounted for approximately 20 to 25% of the total light absorbed at 406 mμ.

Hydrochloric Acid Fractionation of Extracted Pigments As the previous studies indicated that chlorophyll products were the predominant pigments present, the ex-

Table I. Hydrochloric Acid Fractions of Aqueous Acetone Extracts of Pigments from Sheep Digestion Trials

HCl Concentration, %	Optical Density at 406 mμ		Proportion of Total Light Absorption	
	Hay	Feces	Hay, %	Feces, %
5	0.008	0.009	0.3	0.4
10	0.045	0.041	1.7	1.7
25	0.188	0.184	7.3	7.7
37	1.800	1.690	70.5	71.2
Remaining in ether	0.517	0.450	20.2	19.0

Table II. Identity of Pigments Isolated from Chromatograms of 85% Acetone Water Extracts from Fescue Hay

Band No.	Color	Absorption Maxima, mμ		Pigment
		Observed[a]	Reported[b]	
1	Green	.....	.....	Unresolved mixture
2	Red	438–655	438–655	Pheophytin b
3	Olive-green	410–665	410–665	Pheophytin a
4	Green	430–660	429–660	Chlorophyll a
5	Orange-yellow	415–442–472	417–442–472	Violaxanthol
6	Yellow	420–442–472	420–447 472	Luteol
7	Yellow	450–476	449–478	β-Carotene

[a] In diethyl ether.
[b] Maxima reported for chlorophylls and pheophytins (*13*) and for carotenoids (*5*).

tracts transferred to ether were subjected to hydrochloric acid fractionation (*2*, *12*) in order to gain information on the changes in pigment that take place in passage through the digestive tract. Hydrochloric acid fractionations were made on the feces of rabbits and sheep and on the corresponding forages fed to the sheep.

Typical absorption curves for hydrochloric acid fractionation of an extract of feces from a rabbit fed Ladino clover are shown in Figure 1. For each concentration of acid used, the proportion of pigments removed from fecal samples from rabbits was similar for all forages fed (orchard grass, fescue grass, and Ladino clover). The largest proportion of the pigments was extracted with 37% hydrochloric acid and decreasing amounts with 25% hydrochloric acid and 10% hydrochloric acid, but none by 5% hydrochloric acid. According to Willstätter (*12*) the 37% hydrochloric acid fraction should contain only the pheophytins; the 25%, the phorbides; the 10%, the chlorines; and the 5%, the products originating from hemoglobin or bile.

The hydrochloric acid fractionation procedure also was applied to ether solutions of the pigments in aqueous acetone extracts of soybean hays and of feces from sheep fed these hays. The relative distribution of the pigments in the various fractions, as measured by light absorption at 406 mμ, is shown in Table I for a soybean hay and a fecal sample. These results are typical of all obtained from the sheep digestion trials.

About 80% of the substances absorbing light at 406 mμ were found in the hydrochloric acid fractions, the remainder being insoluble in any concentration of hydrochloric acid. The pigments contained in the 37% hydrochloric acid fraction (pheophytins) accounted for about 70% of the total light absorption at 406 mμ; the 25% hydrochloric acid fraction (pheophorbides), for approximately 8%; and the 10% and 5% hydrochloric acid fractions together, for about 2%.

The similarity between the results from the feed and the feces indicated that only slight degradation of the chlorophyll products in the artificially dried hay took place during passage through the alimentary tract of either sheep or rabbits, at least in respect to light absorption at the isosbestic point.

Chromatographic Separation of Extracted Pigments

As the data from the saponification experiments and the hydrochloric acid fractionations indicated that the pigments of aqueous acetone extracts are a mixture consisting of chlorophylls and their degradation products and of carotenoids, the next step was to isolate and identify the individual pigments comprising the mixture. In view of the possibility that the pigments from previous experimental samples might have undergone some changes in storage, fresh samples from a digestion trial with sheep fed fescue hay were used. Chromatographic separations also were made on samples of fresh fescue from grazing paddocks and on feces from sheep grazing thereon.

Because chromatographic separation of plant pigments in polar aqueous acetone extracts was unsatisfactory, the pigments of these extracts were transferred to diethyl ether for subsequent chromatographic separation. Magnesium oxide (adsorptive powdered magnesia No. 2641, Westvaco Chemical Corp.) mixed with an equal weight of Supercel effected good resolution with ether extracts of either feed or fecal samples. Ether solutions of the pigments in either hay or fecal extracts were resolved into seven bands (see Table II).

The magnesia columns were removed intact from their glass containers; the individual bands were mechanically separated, and each band was eluted from the magnesia by aqueous acetone, transferred to ether, and rechromatographed on a magnesium oxide column until only a single band was obtained. The identification of the pigments was based on the similarity of the observed spectral absorption curves to those reported for the carotenoids (*4*) and for the chlorophyll derivatives (*13*). When fresh fescue grass was used instead of fescue hay, only five bands were observed. Bands 2 and 3 (Table II) were absent.

The presence of chlorophyll b, which has absorption spectra maxima at 452 and 642 mμ (*13*), was demonstrated only by washing the column with diethyl ether containing 5% acetone. The new pigment band was observed between what had formerly been designated as bands 2 and 3. The pigment had absorption maxima identical with those reported for chlorophyll b.

Even though data from isolation and spectral identification of chromatographic bands were limited to fescue, the seven bands from extracts of dried Ladino clover, orchard grass, and soybean hay appeared to be similar to those for fescue.

Attempts were made to separate and to measure the relative contribution of each pigment to the total light absorption at 406 mμ by determining optical densities of solutions of the seven eluted bands. The results from one rabbit digestion trial, in which the animal was fed dried Ladino clover, and from one

Table III. Relative Amounts of Pigments in 85% Aqueous Acetone Eluates from Bands of Chromatograph Columns

(As measured by optical density at 406 mμ)

Pigment	Optical Density[a]		% Color[b]		Optical Density[a]		% Color[b]	
	Fescue hay	Sheep feces	Fescue hay	Sheep feces	Ladino hay	Rabbit feces	Ladino hay	Rabbit feces
Carotene	0.069	0.093	1.8	2.7	0.120	0.193	3.9	5.4
Luteol	0.438	0.518	11.7	15.3	0.600	0.618	19.8	17.3
Violaxanthol	0.268	0.100	7.1	2.9	0.093	0.100	3.0	2.8
Chlorophyll a	1.558	0.404	41.6	11.9	0.708	0.400	23.4	11.2
Pheophytin a	0.550	1.500	14.7	44.5	0.709	1.520	26.1	42.6
Pheophytin b	0.630	0.620	16.8	18.4	0.518	0.602	17.1	16.8
Unresolved mixture	0.230	0.135	6.1	4.0	0.190	0.135	6.2	3.7

[a] Optical density determined at 406 mμ in aqueous acetone.
[b] % color = % of total light absorption at 406 mμ.

sheep digestion trial, in which the animal was fed a diet of fescue hay, are shown in Table III.

The results of the chromatographic separations indicate that the chromogen(s) is a mixture of at least seven pigments, chlorophylls a and b, pheophytins a and b, and three carotenoids.

Discussion

Aqueous acetone extracts of forages contain a mixture of the chloroplast pigments. The principal ones in the extracts studied include chlorophyll a, chlorophyll b, pheophytin a, pheophytin b, β-carotene, luteol, and violaxanthol. The chlorophylls and/or their degradation products account for approximately two thirds to four fifths of the light absorption at 406 mμ and the carotenoids, approximately one fifth to one third.

Chromatographic adsorption on magnesium oxide under the conditions used did not separate chlorophyll b as a distinct band. The presence of this pigment was demonstrated in only a few extracts, but it probably was present in all, since chlorophyll b is the second most abundant pigment in the chloroplasts of higher plants (*5*).

The separation and identification processes used did not reveal the presence of either a true chromogen or any pigments that did not originate from plant chloroplastids. These studies indicated the presence of five major pigments in fresh fescue. In artificially dried fescue the chlorophylls were partially converted to pheophytins, and on passage through the digestive tracts of animals a further conversion of chlorophylls to pheophytins took place.

The intensity of light absorption at all chlorophyll maxima is altered, except at the principal maximum in the blue region at 430 mμ, as the chlorophylls are degraded to phytins and phorbides. As the chlorophyll molecule is degraded, this maximum at 430 mμ is shifted slightly toward the shorter wave lengths but without marked changes in intensity of light absorption (*1*, *6*).

In all forages tested, there was an appreciable proportion of chlorophyll a converted to pheophytin a in the digestive tracts of animals (Table III). The chlorophylls apparently are sufficiently stable so that the changes that occur in the alimentary tract do not affect the part of the molecule that is involved in the absorption of light at the isosbestic point of 406 mμ.

This point for both the sheep and the rabbit digestion trials varied from 390 to 425 mμ. The true isosbestic point in any digestion trial apparently is dependent upon the amount of conversion of the chlorophylls to pheophytins and upon the amount of carotenoids present. Thus, the establishment of an isosbestic point for each type of forage and for each species of animal probably would reduce the discrepancy between digestibility coefficients determined by the chromogen(s) method and those obtained by the conventional methods.

The lability of the chromogen(s) to light is probably one of the more serious disadvantages of this method. Chlorophylls are easily photo-oxidized. The carotenoids are also light-labile in pure solutions but are more labile in the presence of chlorophylls (*5*).

The hydrochloric acid fractionation indicated that the chromogen(s) extracted from the feces of either rabbits or sheep contained pheophorbides, but the chromatographic isolation of pigments did not substantiate this observation. If pheophorbides were present, they probably were contained in band 1 (unresolved mixture) in tract amounts. This observation indicates that a further change in the chlorophyll pigments occurs during hydrochloric acid fractionation.

There were differences in the results obtained from rabbits and those obtained from sheep when hydrochloric acid fractionation was used. With both species of animals the 37% hydrochloric acid fraction contained the largest amount of pigment, but the 25% and 10% hydrochloric acid fractions from the feces of rabbits contained more of the pigment than did those from the feces of sheep. It is not known whether these differences were due to a difference in hays fed the animals or to differences between species of animals.

The 5% hydrochloric acid extract, which should contain any pigments that originate from bile or hemoglobin (*2*), did not remove any of the color from ethereal solutions of aqueous acetone extracts from the feces of either rabbits or sheep. The absence of color in this fraction as well as the failure to obtain nonchloroplast pigments in the chromatographic separations is evidence that pigments of animal origin do not interfere with the chromogen(s) method.

Summary

The spectral absorption curves of acetone-water extracts of Ladino clover, orchard grass, fescue grass, and soybean hay and feces of rabbits and sheep fed these forages displayed absorption maxima indicative of chlorophylls and of chlorophyll degradation products. The principal maxima were in the blue region of the spectrum (410 to 430 mμ) and in the red region (660 mμ).

Cold saponification with alcoholic potassium hydroxide followed by extraction with ether, and fractionation with successively increasing concentrations of hydrochloric acid showed that the aqueous acetone extracts of forages and of feces contained both carotenoids and chlorophylls or their degradation products or a combination.

Chlorophyll a, chlorophyll b, luteol, violaxanthol, and β-carotene were isolated from aqueous acetone extracts of fresh fescue. In addition to these five pigments, samples of the artificially dried fescue hays and of the feces contained two additional pigments, pheophytins a and b.

The chromogen(s) involved in the chromogen-ratio method for determining digestibility of forages is principally a mixture of carotenoids, the chlorophylls, and the pheophytins. Changes in the chemical structure of these compounds on passage through the digestive tract have little effect on the light absorption of the mixture of pigments at the isosbestic point of 406 mμ.

This study indicates that the chromogen(s) ratio technique is applicable only where diets contain sufficient amounts of the chloroplast pigments.

Literature Cited

(1) Aronoff, S., *Chem. Revs.*, **47,** 175 (1950).
(2) Brugsch, J. T., and Sheard, C., *J. Lab. Clin. Med.*, **24,** 230 (1938).
(3) Fischer, H., and Stadler, F., *Z. physiol. Chem.*, **239,** 167 (1936).
(4) Karrer, P., and Jucker, E., "Carotenoids," trans. and revised by E. A. Brande, Amsterdam, Elsevier Publishing Co., 1950.
(5) Rabinowitch, E. I., "Photosynthesis and Related Processes," Vol. II, Part I, New York, Interscience Publishers, 1951.
(6) Rabinowitch, E. I., *Revs. Mod. Phys.*, **16,** 225 (1944).
(7) Reid, J. T., Woolfolk, P. G., Richards, C. R., Kaufman, R. W., Loosli, J. K., Turk, K. L., Miller, J. I., and Blaser, R. E., *J. Dairy Sci.*, **33,** 60–71 (1950).
(8) Rothemund, P., *J. Am. Chem. Soc.*, **56,** 2400 (1934).
(9) Rothemund, P., and Inman, O. L., *Ibid.*, **54,** 4702 (1932).
(10) Seybold, A., and Egle, K., *Z. physiol. Chem.*, **257,** 49 (1939).
(11) Strain, H. H., *Ann. Rev. Biochem.*, **13,** 591 (1944).
(12) Willstätter, R., and Stoll, A., "Investigations on Chlorophyll," trans. by F. N. Schertz and A. R. Mertz, Lancaster, Pa., Science Press Printing Co., 1928.
(13) Zscheile, F. P., and Comar, C. L., *Botan. Gaz.*, **102,** 463 (1941).

Received for review January 21, 1953. Accepted March 11, 1953. Presented in part before the Southeastern Regional Meeting of the AMERICAN CHEMICAL SOCIETY, *Alabama Polytechnic Institute, Auburn, Ala., October 25, 1952. Taken from data submitted to the faculty of North Carolina State College in partial fulfillment of the requirements for the Ph.D. degree in animal industry by W. W. G. Smart, Jr. Published with approval of the Director of Research as Paper No. 451 in Journal Series.*