soils, are of rather infrequent occurrence, and do not seem to retain their chemical identity long.

BUREAU OF SOILS,
U. S. DEPARTMENT OF AGRICULTURE,
WASHINGTON, D. C.

[CONTRIBUTION FROM THE NEVADA AGRICULTURAL EXPERIMENT STATION.]

ON THE COLORING MATTERS IN ALFALFA, ALFALFA INVESTIGATION, III.[1]

BY C. A. JACOBSON.

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That chlorophyll plays one of the most important parts in the physiology of the plant cannot be gainsaid, but whether or not its function is directly connected with the fixation of atmospheric nitrogen by leguminous plants is not known. At any rate a knowledge of the character and quantity of the chlorophyll in alfalfa is indispensable to a study of its metabolic functions.

There is now no longer a question that chlorophyll, the green coloring matter in leaves, is a mixture of two distinct chemical substances. These components of chlorophyll are both green in color, but in solution one is of a darker green than the other.

A difference of opinion has existed among chlorophyll chemists regarding the ratio in which the two components are present,[2] but after an exhaustive study of the chlorophyll of some twelve varieties of leaves, Jacobson and Marchlewski[3] arrived at the conclusion that the components do not occur in a definit ratio in different species of plants, and that the ratio may not be the same in the same species. In a later communication the same authors[4] have presented two methods for determining the two components, neo- and allo-chlorophyll, in the presence of one another.[5]

One of these methods, namely the photographic, was employed for investigating the alfalfa chlorophyll. This method is based upon the fact that the absorption bands of neo- and allochlorophyllan in the ultraviolet part of the spectrum are different and that these bands, which are not influenced to an appreciable extent by impurities, are photographed and the photograph compared with a standard series.

[1] In recent years the chemistry of chlorophyll has been greatly advanced by Prof. Marchlewski's optical methods and so I decided to apply these methods to my chlorophyll products obtained from alfalfa. This investigation was carried out in Prof. Marchlewski's laboratory at Cracow, Austria; and it is with a deep sense of gratitude that I acknowledge his many valuable suggestions and the use of his laboratory appliances.

[2] *Proc. Roy. Soc.*, **21**, 442 (1873); *Ber.*, **41**, 1352 (1908); *Trans. Chem. Soc.*, **77**, 1080 (1900); *Biochem. Z.*, **35**, 413 (1911).

[3] *Am. Chem. J.*, **47**, 221 (1912).

[4] *Ibid.*, **47**, Aug. (1912).

[5] This paper appeared first in the *Bull. Acad. Sci., Cracovie*, Feb., 1912.

The spectra of neo- and allochlorophyll are not as characteristic as those of the corresponding chlorophyllans, so that all chlorophylls investigated were first converted into their nearest acid derivatives. This, however, was unnecessary in the case of the chlorophyll residues of alfalfa brought from Nevada, because the organic acids present in the extracts had already converted all the chlorophyll into chlorophyllan. Air-dried alfalfa hay (*Medicago sativa* L), had been extracted with hot 95% alcohol according to the method described in my first paper.[1] After filtering off the green precipitate there spoken of, the filtrate was evaporated, at a temperature not exceeding 50°, to a sirupy consistency. The products thus obtained from various extractions were added together, bottled and called "chlorophyll residues."

In order to determin the total amount of chlorophyll in these residues, which represented 16.78% of the weight of the dry alfalfa hay, I made use of the extinction method of Malarski and Marchlewski.[2] According to this method, the concentration of any colored solution can be calculated from its extinction coefficient, and the extinction and absorption coefficients of a standard solution of the pure coloring matter.

Pure alfalfa chlorophyllan was prepared by the method of Schunck;[3] 0.7354 gram of this was dissolved in 100 cc. chloroform, and its extinction coefficient determined in a 1 mm. layer by means of the König-Martens spectrophotometer, using sodium light. The following readings were taken.

TABLE I.

Solution left.	Solution right.
82.3 } 165.3°	5.5 } 11.2°
97.0 }	174.3 }
263.0 } 165.6°	185.3 } 11.1°
277.4 }	354.2 }
	5.0 } 11.2°
	173.8 }
	185.2 } 11.2°
	354.0 }
Average 165.4° = α_1.	Average 11.2° = α_2.

The extinction coefficient is obtained by substituting the above values for α_1 and α_2 in the following formula,[4] where d is the thickness of the layer in centimeters.

[1] THIS JOURNAL, **33**, 2048 (1911).

[2] *Biochem. Z.*, **24**, 319 (1910).

[3] *Proc. Roy. Soc.*, **50**, 303 (1891).

[4] In the article referred to (*Am. Chem. J.*, **47**, 230), the reciprocal of d in centimeters was erroneously given, but the calculations were made according to the correct formula. The results were doubled, however, since the solution was diluted with an equal volume of chloroform.

$$\Sigma = \frac{\log \tan \alpha_1/2 - \log \tan \alpha_2/2}{d}.$$

$$\Sigma = 19.0 \text{ (extinction coef.)}.$$

The absorption coefficient A, is calculated according to the following formula, where C is the concentration in grams per liter:

$$A = C/\Sigma.$$

For the above solution A = 0.387.

The chlorophyllan from 3.05 grams of the residue was then extracted with chloroform and the solution made up to 250 cc. The extinction coefficient of this solution was determined in the same way and found to be 1.28. By substituting these values in the formula the concentration of chlorophyllan in grams per liter was found to be 0.49536, which is equivalent to 4.06% of the crude chlorophyll residue or 0.68% of the dry alfalfa hay.

In order to compare the exact locations of the absorption bands of alfalfa chlorophyllan, in the visible part of the spectrum, with those of other chlorophyllans obtained in pure condition, the following measurements were taken.

TABLE II.—SPECTRUM OF ALFALFA CHLOROPHYLLAN CONCENTRATION : 0.0004 GRAM PER CC. $CHCl_3$.

Thickness of layer.	1 mm.	3 mm.	5 mm.
Band I	λ = 676.0 — 653.0$\mu\mu$	λ = 680.5 — 647.5$\mu\mu$	λ = 682.8 — 641.6$\mu\mu$
Band II	607.2 — 595.8	611.2 — 595.0	614.2 — 592.7
Band III	.....	567.7 — 558.4	568.4 — 555.6
Band IV	540.7 — 531.9	541.8 — 532.4	543.4 — 531.6
Band V	513.2 — 504.2	513.3 — 498.2	513.2 — 494.1

Thickness of layer.	7 mm.	10 mm.
Band I	λ = 686.5 — 641.5$\mu\mu$	λ = 690.0 — 636.8$\mu\mu$
Band II	616.0 — 590.8	619.5 — 588.8
Band III	569.4 — 553.5	575.1 — 555.0
Band IV	543.7 — 530.0	545.8 — 527.7
Band V	513.7 — 494.1	517.0 — 492.7

Order of intensity of the bands:

$$I > V > IV > II > III.$$

From the results here given it will be seen that the location of the five absorption bands is identical, within the limits of experimental error, with those of nettle chlorophyllan.[1]

A photograph was taken of the ultraviolet absorption bands of the purified alfalfa chlorophyllan, and is represented on the accompanying plate. The concentration was 0.00004 gram per cubic centimeter chloroform solution, and the thickness of layers 2, 4, 6, 8, 10 and 12 mm. The copper spectrum above, shows the location of the bands. By comparing

[1] *Am. Chem. J.*, **47**, 221 (1912).

this spectrograph with those in the standard series,[1] it is seen that the alfalfa chlorophyllan contains 66% neo- and 34% allochlorophyllan. It is of course evident that this only means that the particular lot of alfalfa with which I was working gave these results, and that the same species of *Medicago*, grown under different conditions, would in all probability give different values. Later on I shall try to investigate the influence of the conditions of growth upon the production of one or the other of these chlorophyll constituents.

Chlorophyll in leaves is accompanied to a larger or smaller extent by carotin and xanthophylls. These are known as the yellow coloring matters, although the former is a red crystallin substance.

To determin the amount of yellow coloring matters in the chlorophyll residue from alfalfa, 51.981 grams of the latter were treated with about 2.5 grams potassium hydroxide and some alcohol. After stirring for a while the solution was evaporated on a water bath to a thick sirup, after which about 150 cc. water was added, and the whole transferred to a separatory funnel and extracted with ether. The extractions were continued until the ether separated colorless. The ether portions were added together and the solvent evaporated, leaving a residue weighing 0.8638 gram.

Besides carotin and xanthophylls, there may have been other substances removed by the ether, but the color of the latter and of the residue indicated that no chlorophyll product had been removed. The amount of the yellow coloring matters present in the chlorophyll residues from alfalfa is therefore 1.66%, or 0.28% of the weight of the dry alfalfa hay.

To summarize the foregoing it may be said that the chlorophyll from alfalfa closely resembles that from nettle leaves. The lot with which I was working contained 66% neochlorophyll and 34% allochlorophyll. However, this ratio may vary in other lots, depending upon the conditions of growth. In air-dried alfalfa hay there is 0.68% of chlorophyll and 0.28% yellow coloring matters. The latter do not influence the chlorophyll absorption bands in the concentration most suitable for photographing, but when the concentration is five times as strong, they show two regions of absorption in the ultraviolet.

[CONTRIBUTION FROM THE NEVADA AGRICULTURAL EXPERIMENT STATION.]

A DELICATE METHOD FOR DETERMINING MINUTE QUANTITIES OF CHLOROPHYLL.[2]

BY C. A. JACOBSON.

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After finishing the work on the two methods for determining the ratio

[1] *Am. Chem. J.*, **47**, Aug. (1912).

[2] The experimental work involved in the present paper was also carried out in Prof. Marchlewski's laboratory at Cracow.