the procedure of Franchimont and Klobbie[3] for the preparation of 1,2-dinitraminoethane from 1,3-dinitro-1,-3-diazacyclopentanone-2. The crude yield of 1,4-dinitraminobutane (m. p. 159.6–160.5° with decomposition) was 91.8%. Two crystallizations from water (25 cc. per g.) raised the melting point to 162.2° dec. Dekkers[5] prepared this compound from N,N¹-dinitro-N,N¹-dicarbomethoxytetramethylenediamine and reported a melting point of 163°.

1,3-Dinitraminopropane.—1,3-Dinitraminopropane (m. p. 67.2–68°) was prepared in 93% yield from 1,3-dinitro-1,3-diazacyclohexanone-2[1] by the procedure described above for the preparation of 1,3-dinitraminobutane. Franchimont and Klobbie[4] have reported a melting point of 67° for 1,3-dinitraminopropane.

Summary

A series of nitration products has been prepared from 4(or 5)-methyl-2-nitramino-1,3-diazacyclopentene-2,4(or 6)-methyl-2-nitramino-1,3-diazacyclohexene-2, and 2-nitramino-5-hydroxyl-1,3-diazacyclohexene-2. The first compound was the only one that gave a 1-nitro derivative.

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF WISCONSIN]

Properties of the Red Pigment from Soybean Nodules[1]

BY HENRY N. LITTLE[2]

A red pigment resembling animal hemoglobin has been observed in the nodules of leguminous plants. This pigment has been termed leghemoglobin by Virtanen, whereas Keilin and co-workers have applied the name hemoglobin. The more general term hemoprotein has been used in this paper. Kubo[3] found that the pigment was present in the nodules of all the leguminous plants he tested. Virtanen[4] reported that only plants with nodules containing visible amounts of the pigment actively fixed nitrogen; when the plants matured and the pigment disappeared from the nodules, nitrogen fixation ceased. Virtanen, *et al.*,[5] also observed a positive correlation between the hemin content of the nodules and the effectiveness of the bacterial strain used for inoculation. Keilin and Smith[6] concluded from the universal occurrence of the nodule "hemoglobin" in actively fixing nodules and the inhibition of nitrogen fixation by very low partial pressures of carbon monoxide that this pigment must be linked in some way to symbiotic nitrogen fixation.

The evidence to date concerning the nature of hemoprotein has been largely limited to the observation that the absorption peaks of this pigment and its derivatives agree well with those of the corresponding derivatives of animal hemoglobin. In addition Kubo[3] has reported that the form of the hemin crystals derived from nodule hemoprotein was similar to that from horse hemoglobin. Keilin and Wang,[7] and later Little and Burris,[8] observed that the oxygenated nodule hemoprotein could be deoxygenated by evacuation.

The nodule pigment has never been obtained in a crystalline state. Keilin and Wang[7] prepared pigment of 40–50% purity based on heme content. Virtanen[9] obtained pigment preparations having an iron and heme content corresponding to 80–85% purity.

The present paper reports a study of the purification of the pigment, its reversible splitting and reconstitution, and the nature of the porphyrin which it yields.

Experimental Methods

The nodule hemoprotein was prepared from soybean nodules by ammonium sulfate fractionation. After removal from the plant, the nodules were chilled and at all stages thereafter the preparation was maintained at 0–5°. The nodules were ground in approximately their own weight of *p*H 7.0, 0.1 *M* phosphate buffer; about 0.5 mg. of sodium hydrosulfite per gram of nodules was added and carbon monoxide was passed through the mixture during the grinding. The pulp was pressed by hand in cheesecloth. This extraction was repeated once or twice using one-half the original volume of buffer each time. Enough solid ammonium sulfate was added to the combined press juices to bring the solution to 1.9 *M*; the *p*H was adjusted to 7.0 and the solution centrifuged. The precipitate was discarded and to the supernatant solution was added enough solid ammonium sulfate to bring the concentration to 2.7 *M*; the *p*H was again adjusted to 7.0 and the solution centrifuged. The small amount of precipitate was discarded and the supernatant solution was made to 3.4 *M* ammonium sulfate. After standing one hour the solution was centrifuged. The resultant red precipitate was resuspended in water and dialyzed against numerous changes of distilled water. This pigment was used in further attempts at purification. In general about 1–2 mg. of crude nodule hemoprotein was obtained from each gram of fresh nodules.

The ratio of hemin content of the hemoprotein to dry weight, expressed as per cent. hemin, was used to determine changes of purity in different stages of purification. The hemin content was estimated by forming the pyridine hemochromogen, determining the optical density at 527 mμ with a Beckman spectrophotometer and comparing the absorption with a standard curve based on recrystallized hemin. To form the hemochromogen, 2 ml. of pyridine and 1 ml. of 3% ammonium hydroxide were added to 5

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ml. of the solution containing hemin. A few crystals of sodium hydrosulfite was added and the optical density was determined after five minutes.

For studies of the reversible splitting and reconstitution of animal hemoglobin and nodule hemoprotein, the protein from these two sources was prepared by the method described by Anson and Mirsky.[10] The animal hemin from beef blood was prepared and recrystallized by the method of Delory[11] and dissolved in 0.1 *N* sodium hydroxide. Nodule hemin was extracted with chloroform from an aqueous solution of nodule hemoprotein containing 60% acetone and 0.02% hydrochloric acid. This chloroform solution was reduced to dryness *in vacuo* and the crude hemin dissolved in 0.1 *N* sodium hydroxide. To obtain the recombined pigments, the nodule hemin solution was added to an aqueous beef globin solution and the beef hemin solution was added to the nodule pigment protein solution. A few crystals of sodium hydrosulfite were added, the solution shaken and the spectrum of oxygenated reconstituted pigment observed in the Beckman spectrophotometer. Additional sodium hydrosulfite was added and the spectrum of the reduced compounds was determined.

The methyl ester of the porphyrins derived from nodule hemoprotein was prepared by the method of Grinstein.[12] For comparison, the dimethyl ester of protoporphyrin was prepared in the same manner from beef hemoglobin. These esters were dissolved in chloroform and their absorption spectra determined in the Beckman spectrophotometer.

For the production of mesoporphyrins, the methyl esters of the porphyrins of nodule hemoprotein and beef hemoglobin were reduced with hydriodic acid. To 1 ml. of a glacial acetic acid solution of the porphyrin methyl ester was added two drops of hydriodic acid (sp. gr. 1.5). This mixture was heated at 119° for one minute and then 1 ml. of a solution saturated with sodium acetate and containing 10% sodium sulfite was added. The ester was extracted with a small portion of ether and then transferred to 5% hydrochloric acid. The acid was saturated with sodium acetate and again extracted with ether. The ester was extracted a second time with 5% hydrochloric acid and was finally recovered in ether by saturating the acid with sodium acetate. This ether solution was washed several times with water and then evaporated to dryness. The residue was dissolved in chloroform and the absorption spectrum determined in the Beckman spectrophotometer.

Results and Discussion

The percentage of hemin in nodule hemoprotein precipitated between 2.3 and 3.4 *M* ammonium sulfate from aqueous extracts of nodules was consistently 3.20–3.62. Further refractionation of this pigment at different *p*H's and ammonium sulfate concentration produced little increase in purity over this value. Table I indicates the effect of *p*H on the purity of the nodule hemoprotein when reprecipitated with 2.7 *M* ammonium sulfate. It is evident that varying the *p*H between 5.0 and 8.0 did not markedly affect the purity of the pigment although more complete precipitation was obtained at the higher *p*H's. At *p*H 4.0, however, a small amount of pigment was precipitated in the absence of ammonium sulfate. The per cent. hemin in three such precipitates was 3.88, 4.08 and 4.22. These values indicated an increase in purity over other fractions. However, since the fraction represented so small a portion of the total crude pigment and since the amount of crude pigment was limited, further work on this fraction was impractical.

TABLE I

PERCENTAGE HEMIN IN PIGMENT WHEN REPRECIPITATED WITH 2.7 *M* AMMONIUM SULFATE AT DIFFERENT *p*H'S

*p*H	Hemin, % Initial	Final	Recovery, %
5.0	3.58	3.20	..
6.0	3.50	3.20	18
6.5	3.50	3.41	37
7.0	3.46	3.28	86
7.5	3.50	3.58	85
8.0	3.50	3.41	86

The results obtained by reprecipitating the pigment at various ammonium sulfate concentrations are indicated in Table II. In general, the pigment fractions precipitated by successive narrow increments of added ammonium sulfate were no more pure than the pigment precipitated between the limits of 2.3 and 3.4 *M* ammonium sulfate. Although the fraction precipitated between 2.7 and 3.0 *M* ammonium sulfate was slightly more pure, the recovery was considerably lowered so as to render impractical further fractionation by this method. Precipitation of the pigment a third time with ammonium sulfate produced no further purification and resulted in poor yields.

TABLE II

PERCENTAGE HEMIN IN PIGMENT WHEN REPRECIPITATED BETWEEN VARIOUS SALT CONCENTRATIONS

Interval of $(NH_4)_2SO_4$ concn., *M*	% Hemin of original sample	% Hemin of precipitate	% Recovery
2.3–3.4	..	3.40	..
	..	3.45	..
	..	3.28	..
2.3–2.7	3.45	3.28	66
	3.49	3.58	85
	3.49	3.41	86
2.7–3.0	3.45	3.62	26
	3.49	3.79	19
	3.49	3.62	16

A comparison of the nodule pigment with animal hemoglobin was made by preparing solutions of the protein and heme from each of these two sources. Although the individual components in concentrations used for recombination gave no or very diffuse absorption bands, when a solution of animal globin was mixed with one of nodule heme, or when a solution of nodule pigment protein was mixed with one of animal heme, the sharp absorption bands indicated in Fig. 1 were observed. A summary of the absorption maxima of these curves is given in Table III. Included in this table for comparison are values for hog hemoglobin and nodule hemoprotein. It is evident that the protein and heme moieties of nodule hemoprotein and animal hemoglobin may be interchanged to yield compounds possessing absorption maxima similar to the original pigments.

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TABLE III

ABSORPTION MAXIMA OF NATIVE AND RECOMBINED HEMOPROTEINS

Compound	Absorption maxima in mμ		
	Oxygenated		Reduced ($Na_2S_2O_4$)
Hog hemoglobin[8]	577	541	555
Soybean hemoprotein[8]	575	540	555
Beef globin + nodule heme	577	539	554
Nodule pigment protein + beef heme	575	540	556

That iron protoporphyrin is widespread in naturally occurring hemoproteins is indicated by a survey[13] of the porphyrins found in vertebrate and invertebrate hemoglobins, myoglobins, catalase and peroxidase. In the established cases the porphyrin of these compounds has been found to be iron protoporphyrin IX. Klüver[14] has recently found that protoporphyrin was liberated from the heme compounds of red soybean nodules when appropriate procedures were employed for splitting off the iron. The prosthetic group of nodule hemoprotein might therefore reasonably be expected to be protoporphyrin. Support for this suggestion is seen in Table IV. This table presents

TABLE IV

ABSORPTION MAXIMA OF PORPHYRIN DERIVATIVES

Derivative	Maxima in mμ			
Me Ester of Protoporphyrin				
From beef hemoglobin	630	575	540	505
From nodule hemoprotein	632	576	542	507
Me Ester of Mesoporphyrin				
From beef hemoglobin	623	570	535	500
From nodule hemoprotein	624	569	534	500

a comparison of the absorption maxima of the methyl ester of the porphyrin prepared from nodule hemoprotein with that prepared from beef hemoglobin. Also presented are the absorption maxima of these two methyl esters after reduction with hydriodic acid. The presence of a vinyl group in the porphyrin ester from nodule hemoprotein was indicated by the reduction of this ester to one having absorption maxima similar to the mesoporphyrin ester derived from the animal hemoglobin. It is evident that the absorption maxima of the porphyrins derived from the nodule source agreed favorably with those isolated from the animal source. This evidence taken with that from the recombination studies indicates strongly the identity of the porphyrin from nodule hemoprotein with protoporphyrin.

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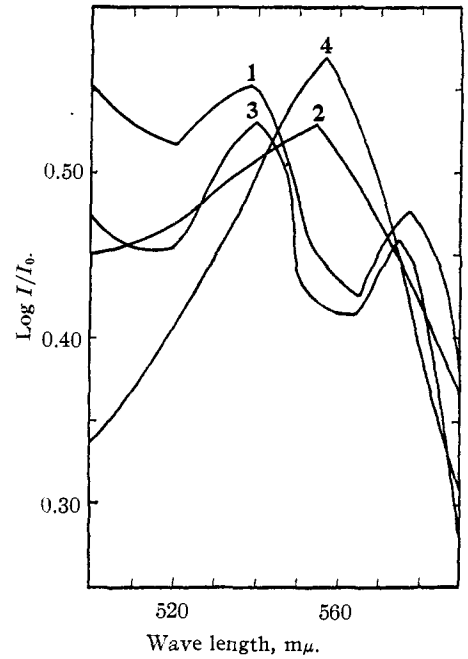

Fig. 1.—Absorption curves of the compounds formed by interchange and recombination of the heme and protein from nodule hemoprotein and animal hemoglobin: 1, oxygenated compound reconstituted from beef globin and nodule heme; 2, reduced ($Na_2S_2O_4$) compound of curve 1; 3, oxygenated compound reconstituted from beef heme and nodule pigment protein; 4, reduced ($Na_2S_2O_4$) compound of curve 3.

Summary

1. Fractionation of nodule hemoprotein with ammonium sulfate yielded pigments containing 3.20–3.62% hemin.

2. The protein and heme moieties of nodule hemoprotein and animal hemoglobin may be isolated and interchanged to yield compounds possessing absorption maxima similar to the native pigments.

3. Properties of the porphyrin esters isolated from the nodule pigment as well as the recombination studies indicated that protoporphyrin is the prosthetic group of nodule hemoglobin.

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