going procedure to the estimation of ethylamines in mixtures of known composition are given in Table II.

TABLE II

ESTIMATION OF ETHYLAMINES IN MIXTURES OF KNOWN COMPOSITION

Ethylamine	5.0 g. NH_4Cl present		NH_4Cl absent[a]	
	Present, g.	Found, g.	Present, g.	Found, g.
Mono	1.03	0.95	1.03	0.99
Di	0.49	0.48	0.49	0.49
Tri	0.30	0.27	0.30	0.28

[a] Chloroform extraction step omitted.

Table III includes representative data from studies on the ammonolysis of ethyl iodide. Yields are expressed on the basis of 7.51 g. of ethyl iodide employed in each ammonolytic reaction and the total for each run represents the percentage of the starting material accounted for by analysis.

TABLE III

ESTIMATION OF ETHYLAMINES FROM THE AMMONOLYSIS OF ETHYL IODIDE

Ethylamine	Yield, %			
	1	2	3	Average
Mono	46	45	46	46
Di	31	31	32	31
Tri	17	16	17	17
Total	94	92	95	94

Discussion

In contrast to the somewhat fragmentary reports from earlier investigations, the present work shows conclusively that the ammonolysis of ethyl iodide by excess liquid ammonia at 0° is a rapid and vigorously exothermal reaction that is complete in about fifteen minutes and leads to the formation of mono, di, and tri-ethylamines. The absence of tetraethylammonium iodide is not surprising since this product would be expected only if the ethyl iodide were present in excess or if the reaction were slow and tetraethylammonium iodide were of low solubility in liquid ammonia.

With regard to the methods employed in the separation and estimation of ethylamines, carefully conducted experiments involving samples of known composition showed that the procedure gives surprisingly accurate measurement of the secondary amine content of the mixtures. These studies showed also that the results for primary and tertiary amines are always low but too variable from one experiment to another to warrant application of a correction factor without sufficient data to establish a reliable average deviation. A comparison of the two sets of data in Table II shows that the chloroform extraction step (separation of amine hydrochlorides from ammonium chloride) entails a small but significant loss of primary amine. Finally, it should be emphasized that the known mixtures used to evaluate the different procedures were ones which simulated approximately the composition of the reaction mixture. Accordingly, it is not claimed that the procedures described above would lead to equally satisfactory results when applied to mixtures containing widely different mole ratios of mono, di and triethylamine.

Summary

1. The ammonolysis of ethyl iodide by an excess of liquid ammonia under strictly anhydrous conditions at 0° has been shown to be a rapid exothermal reaction that is complete in less than fifteen minutes and yields 46% ethylamine, 31% diethylamine and 17% triethylamine.

2. Methods have been devised for the separation and estimation of ethylamines from mixtures containing a large excess of ammonium salts. The reliability of these methods has been established in terms of samples of composition known to approximate that of the gross product of the ammonolytic reaction.

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

Activity of the Red Pigment from Leguminous Root Nodules[1]

BY HENRY N. LITTLE AND R. H. BURRIS

The root nodules of leguminous plants that are actively fixing nitrogen contain a red pigment. Without the evidence of isolation and characterization, Pietz[2] erroneously suggested that the compound was an oxidation product of 3,4-dihydroxyphenylalanine. Kubo[3] reported that observation of suspensions of crushed nodules showed upon shaking an absorption band at 555 and 575 mμ. He concentrated the pigment by ammonium sulfate fractionation and demonstrated that it was a hemoprotein. He observed that the absorption maxima of a number of derivatives of the nodule pigment closely resembled corresponding derivatives from hemoglobin, and that the pigment yielded hemin crystals apparently identical with those obtained from horse hemoglobin. Addition of the pigment stimulated oxygen uptake by suspensions of *Rhizobium japonicum* oxidizing sodium succinate. Burris and Haas[4] found an absorption maximum for the reduced pigment at 555 mμ. and in addition strong

(1) Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by grants from the Wisconsin Alumni Research Foundation.

(2) J. Pietz, *Zentr. Bakt. Parasitenk.*, II, **99**, 1 (1938).

(3) H. Kubo, *Acta phytochim., Japan*, 11, 195 (1939).

(4) R. H. Burris and E. Haas, *J. Biol. Chem.*, **155**, 227 (1944).

absorption at 405 and 423 mμ. for the oxidized and reduced pigments, respectively. They were unable to change the absorption spectrum of the oxidized pigment by evacuation, and reconstructed enzyme systems containing reduced diphosphopyridine nucleotide and triphosphopyridine nucleotide failed to reduce the oxidized pigment.

Keilin and Wang[5] fractionated extracts from soybean nodules with ammonium sulfate, and determined that the absorption curves for the nodule pigment in both the oxygenated and reduced states as well as the carbon monoxide derivative were very similar to the corresponding curves for hemoglobin. Of particular significance was their demonstration that the oxygenated pigment with absorption maxima at 574 and 540 mμ. was reduced by simple evacuation to a compound having a single absorption maximum at 557 mμ. They suggested that the failure of Burris and Haas[4] to observe a change in the absorption spectrum upon evacuation was probably because the pigment was chiefly in the methemoglobin form.

Virtanen[6] has confirmed Kubo's[3] observations on the absorption characteristics of the nodule pigment, and has observed that the red pigment is absent in plants kept for a few days in the dark or infected with strains of bacteria that are ineffective in fixing nitrogen. Virtanen and Laine[7] reported that a filtered preparation of the soybean nodules supported nitrogen fixation by free-living pea organisms, but later Virtanen[8] found that the pigment after purification by ammonium sulfate precipitation did not support such fixation. Virtanen and Laine[9] have speculated that the pigment from nodules participates directly in nitrogen fixation, the fixation being accompanied by a valence change in the iron of the pigment molecule.

In the present work we have been interested in observing the absorption spectra of a number of derivatives of the nodule pigment, checking its behavior upon deoxygenation, and investigating its physiological rôle.

Experimental Methods

For studies of the absorption spectra, nodules of soybean plants were ground in a mortar with water while carbon monoxide was passed through the solution. The pulp obtained after grinding was pressed in cheesecloth by hand and the press juice centrifuged. The supernatant solution was then passed through a Seitz bacterial filter to yield a clear, dark red solution. Tank oxygen was bubbled through this solution for fifteen minutes to obtain the oxygenated form of the pigment. The pigment solutions used for evacuation studies were prepared as above except that no carbon monoxide was used. All pigment solutions were maintained at 5° throughout their preparation. Blood obtained by finger puncture and diluted with 0.1% sodium carbonate was used for a reference as hemoglobin.

The methods of preparing derivatives from the nodule pigment and blood hemoglobin were those described by Heilmeyer.[10] Reduction of the nodule pigment was obtained by placing the solution in a tube sealed to a glass stopcock and evacuating through a Dry Ice trap connected to a "hyvac" pump; evacuation was continued for twenty minutes while the contents of the tube were kept at 30–35°. Under the same conditions blood hemoglobin was reduced more rapidly. Spectral data were taken on a Beckman quartz spectrophotometer using a nominal band width of 2 to 7 Å.

Oxygen uptake was determined with a Warburg respirometer. The Warburg flasks contained 3 ml. total liquid, including 0.02 μM sodium succinate, nodule pigment or hog hemoglobin or water, and bacterial suspension in *p*H 7.5 *M*/15 phosphate buffer. The concentration of hog hemoglobin was 4.7 mg. N/flask, and that for nodule pigment was about 2.5 mg. N/flask. The center well contained 0.15 ml. of 20% potassium hydroxide. The temperature was 30° and the rate of shaking 120 complete oscillations per minute. The results are expressed as Q_{O_2} (N) (l. of oxygen uptake/hour/mg. N).

To prepare pigment for respiration studies, nodules were ground in approximately their own weight of *p*H 7.0 *M*/15 phosphate buffer; about 0.5 mg. of sodium hydrosulfite per gram of nodules was added and carbon monoxide was passed through the mixture during grinding. Ammonium sulfate was added to the press juices to make the concentrations 30 g./100 ml. of solution. The *p*H was adjusted to 7.0 (glass electrode) and the solution centrifuged. The clear supernatant was treated again with ammonium sulfate to make the concentration 45 g. ammonium sulfate/100 ml. of solution. The *p*H was adjusted to 7.0 and the solution centrifuged. The resultant red precipitate was dissolved in a minimal amount of water and dialyzed in a rocking shaker eighteen to twenty-four hours against 3 changes of distilled water saturated with carbon monoxide. The pigment was kept at 5° at all stages of its preparation.

The hemoglobin was prepared from hog blood according to the method of Welker and Williamson.[11] The heated pigments were placed in boiling water for one minute and then cooled with running water.

The suspensions of nodule bacteria were obtained by grinding freshly picked nodules from soybeans, filtering through cheesecloth, and centrifuging down the cells in the resulting juice. The cells thus obtained were washed three times with *p*H 7.5 *M*/15 phosphate buffer and then resuspended in the same buffer. The "cultured" rhizobia were harvested from forty-eight hour cultures of *Rhizobium trifolii* (Wis. strain 209) grown on 2% agar containing the liquid yeast extract and the salts of medium 79 of Fred and Waksman.[12] *Escherichia coli* (A. T. C. C. 4157) and *Torula canadensis* (Wis. strain 3) were grown for twenty-four hours on nutrient agar and glucose nutrient broth, respectively. The suspensions were washed three times and then suspended in *p*H 7.5 *M*/15 phosphate buffer at a concentration to give an oxygen uptake of 150 to 250 μl./hour/flask.

For studies at reduced oxygen tension, suitable mixtures of oxygen were introduced into the Warburg flasks either by the displacement method or by the evacuation method.[13] Experiments were run for thirty to sixty minutes at reduced oxygen pressures; the flasks were then removed from the manometers, equilibrated with air, replaced, and the rate of respiration in the normal air atmosphere determined.

(5) D. Keilin and Y. L. Wang, *Nature*, **155**, 227 (1945).

(6) A. I. Virtanen, *Nature*, **155**, 747 (1945).

(7) A. I. Virtanen and T. Laine, *Suomen Kemistilehti B*, **18**, 39 (1945).

(8) A. I. Virtanen, *ibid.*, **19**, 48 (1946).

(9) A. I. Virtanen and T. Laine, *ibid.*, **18**, 38 (1945)

(10) L. Heilmeyer, "Spectrophotometry in Medicine," A. Hilger, Ltd., London, 1943.

(11) W. H. Welker and C. S. Williamson, *J. Biol. Chem.*, **41**, 75 (1920).

(12) E. B. Fred and S. A. Waksman, "Laboratory Manual of General Microbiology," McGraw-Hill Co., New York, N. Y., 1928.

(13) W. W. Umbreit, R. H. Burris and J. F. Stauffer, "Manometric Techniques and Related Methods for the Study of Tissue Metabolism," Burgess Publishing Co., Minneapolis, Minn., 1945, p. 43.

Experimental Results and Discussion

In agreement with other workers, the absorption characteristics of the nodule pigment have proved very similar to those of hemoglobin. Pigment prepared by the method of Keilin and Wang[5] has shown a behavior upon oxygenation and deoxygenation strictly in accord with their observations. Figure 1 shows typical absorption curves for the oxygenated and reduced nodule pigment as well as its carbon monoxide derivative.

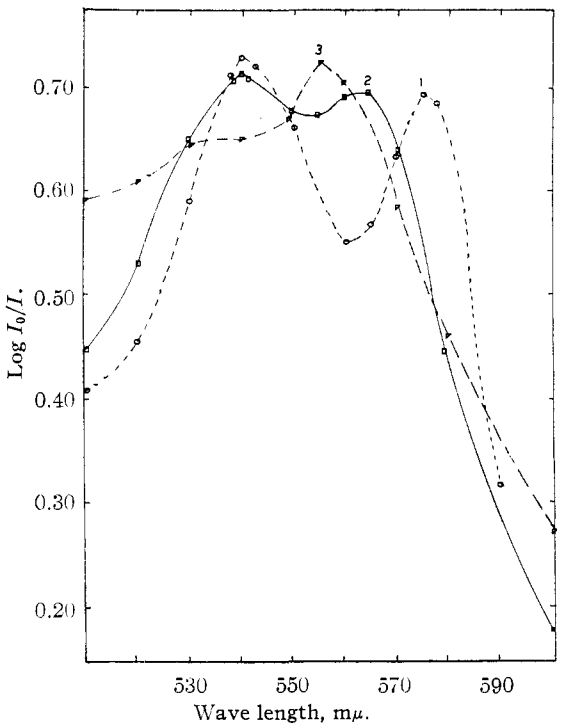

Fig. 1.—Absorption curves for the pigment from soybean nodules: (1) oxygenated pigment, (2) carbon monoxide derivative, (3) pigment reduced by evacuation.

The spectra of the oxygenated pigments and the carbon monoxide derivatives were determined 6 times each with consistent results. The spectrum of the pigment reduced by evacuation was also measured with the Beckman spectrophotometer; in addition reduction by evacuation as evidence by reversible disappearance of the 2 bands produced by oxygenation was observed numerous times with a Zeiss hand spectroscope. Table I gives the absorption maxima of various derivatives of hemoglobin and the nodule pigment as determined with the Beckman spectrophotometer; the values were consistently obtained (±1 mμ) and each was determined on two or more pigment preparations. The values determined for the blood hemoglobin control agree well with those cited by Heilmeyer.[10]

The physiological rôle of the pigment from leguminous root nodules has been a subject for speculation, but the only experimental data suggesting a function are in Kubo's[3] report that it stimulates respiration of the root nodule bacteria. Since the nodule pigment yields derivatives closely similar to those from hemoglobin and can be reduced by evacuation, it is plausible that its function may be analogous to that of hemoglobin in the animal, namely, that of furnishing an available source of oxygen at reduced oxygen pressures. Accordingly, the effect of hemoglobin and nodule pigment on the respiration of bacteria was determined.

TABLE I

ABSORPTION MAXIMA OF HOG HEMOGLOBIN, THE PIGMENT FROM SOYBEAN NODULES, AND THEIR DERIVATIVES

Compound	Absorption maximum in mμ. Hog blood	Soybean nodule pigment
Oxyhemoglobin	577, 541	575, 540
Reduced hemoglobin, $Na_2S_2O_4$	555	555
Evacuation	555	555
Carboxyhemoglobin	569, 540	565, 539
Fluoromethemoglobin	605	605
Cyanhemoglobin	541	541
Acid methemoglobin	629, 502	627

From the results of several experiments summarized in Table II it is evident that hog hemoglobin consistently caused a significant increase in the oxygen uptake of bacteria respiring on a sodium succinate substrate at low oxygen tensions; heat inactivated the hemoglobin. The stimulation was not observed at the p_{O_2} of air. With the pigment from nodules a similar but not as marked stimulation is apparent (Table III). The smaller increase effected by the nodule pigment may be attributed to a lower amount per flask of the nodule pigment than of the hog hemoglobin, a partial denaturation of the pigment during extraction, or to the possible higher affinity of the nodule pigment for oxygen. Table IV shows that the

TABLE II

THE EFFECT OF HOG HEMOGLOBIN ON THE OXIDATION OF SODIUM SUCCINATE BY BACTERIA FROM SOYBEAN NODULES

Bacteria alone Q_{O_2} (N)		+ Hemoglobin % stimulation		+ Heated hemogobin % stimulation	
p_{O_2} 0.01	0.21	0.01	0.21	0.01	0.21
95	457	83	− 5	..	..
85	385	84	10	−22	3
175	370	62	−12	..	..
178	346	105	4	0	4
152	357	103	16	..	..
61	302	154	−16	− 9	..

TABLE III

THE EFFECT OF PIGMENT FROM SOYBEAN NODULES ON THE OXIDATION OF SODIUM SUCCINATE BY BACTERIA FROM SOYBEAN NODULES

Bacteria alone Q_{O_2} (N)		+ Pigment % stimulation		+ Heated pigment % stimulation	
p_{O_2} 0.01	0.21	0.01	0.21	0.01	0.21
103	162	33	12	8	11
87	193	28	−11	0	7
265	575	49	13	−13	8

TABLE IV

THE EFFECT OF HOG HEMOGLOBIN ON THE RESPIRATION OF PURE-CULTURE MICROÖRGANISMS

	Micro-organism alone Q_{O_2} (N)		+ Hog hemoglobin % stimulation		+ Heated hog hemoglobin % stimulation	
	p_{O_2} 0.01	0.21	0.01	0.21	0.01	0.21
Rhizobium trifolii	126	422	132	24	−13	14
Escherichia coli	190	690	142	10	6	10
Torula canadensis	68	149	91	−1	−20	−2

stimulation induced by hemoglobin was not confined to the rhizobia taken directly from the nodules, but was also apparent with *R. trifolii*, *E. coli* and *T. canadensis* from laboratory cultures.

Since a hemoprotein always appears to be present in the root nodules of leguminous plants that are actively fixing nitrogen, it is attractive to speculate that it plays a direct role in nitrogen fixation. This, however, ignores the weight of evidence suggesting a close similarity between the nitrogen fixing mechanism of leguminous plants and the free-living azotobacter.[14] In limited experiments with fresh cell-free extracts of *Azotobacter vinelandii* we have been unable to find a pigment analogous to the hemoprotein of root nodules. On the other hand, the nitrogen fixing system of the azotobacter is sensitive to carbon monoxide and the organisms have a relatively high requirement for iron as a nutrient.

Although there is no specific evidence to support a direct function of the hemoprotein in nitrogen fixation, its stimulation of respiration at low oxygen tensions suggests that it may function indirectly by aiding in the rapid release of energy within the nodule. The soil inhibits gas exchange at the surface of the nodules, and it is logical to suppose that oxygen is a limiting factor in the respiration of the bacteria packed in the active tissue of root nodules. The oxygen uptake by crushed nodules is considerably faster than by intact nodules, and Allison, *et al.*,[15] have accumulated other evidence for the limited oxygen supply in nodules. Although it is not clear how the pigment itself would pick up oxygen, the hemoprotein may well function in an indirect manner by supplying oxygen to stimulate oxidation within the nodule, rather than by combining directly with molecular nitrogen and effecting its fixation.

(14) R. H. Burris and P. W. Wilson, "Ann. Rev. Biochem.," Annual Reviews Inc., Stanford Univ. P. O., Calif., **14**, 685 (1945).

Summary

1. The absorption maxima of the oxygenated pigment from the root nodules of soybeans were found to be 575 and 540 mμ, whereas those for oxygenated hemoglobin were 577 and 541 mμ. The absorption maxima of the reduced forms of these two pigments was 555 mμ. The carboxyhemoglobin, the fluorohemoglobin, the cyanhemoglobin, and the acid methemoglobin also showed close agreement in absorption maxima between hemoglobin and the nodule pigment.

2. The reduction of the nodule pigment by evacuation was observed spectrophotometrically.

3. Hemoglobin stimulated the rate of oxygen uptake by the rhizobia taken directly from soybean nodules and by washed suspensions of pure cultures of *Rhizobium trifolii*, *Escherichia coli* and *Torula canadensis* at low oxygen tensions but not at the p_{O_2} of air. The nodule pigment from soybeans caused a similar but less marked stimulation of the respiration of nodule bacteria.

(15) F. E. Allison, C. A. Ludwig, S. R. Hoover and F. W. Minor, *Botan. Gaz.*, **101**, 513 (1940).

MADISON, WISCONSIN RECEIVED DECEMBER 6, 1946

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF THE GEORGIA SCHOOL OF TECHNOLOGY]

The Isomeric Citrylideneacetic Acids

BY E. EARL ROYALS[1]

In continuation of a program of research[2] dealing with citral and ionone derivatives, we wish to report a study of citrylideneacetic acid (I) and its cyclic isomers, α- (II) and β- (III) cyclocitrylideneacetic acids. The ethyl and methyl esters of citrylideneacetic acid have been prepared[3] by heating citral with mono-esters of malonic acid in the presence of pyridine. Alkaline hydrolysis of the esters gave citrylideneacetic acid. Verley stated[3] that ethyl citrylideneacetate may be hydrolyzed, but is not cyclized, by refluxing with dilute sulfuric acid. No details of this experiment were given. The literature contains only a single reference to application of the Reformatsky reaction to the synthesis of ethyl citrylideneacetate; Tetry[4] reported the reaction of citral with ethyl iodoacetate without solvent in the presence of zinc. He obtained a liquid product boiling over a wide temperature range from which a small fraction of material was isolated giving the correct elementary analysis for ethyl citrylideneacetate. More recently, Cherbuliez and Heger have reported[5] a synthesis of ethyl citrylideneacetate by a modification of the Reformatsky reaction. They condensed citral with ethyl chloroacetate in the

(1) Present address: Department of Chemistry, Emory University, Emory University, Georgia.

(2) Royals, *Ind. Eng. Chem.*, **38**, 546 (1946).

(3) Verley, *Bull. soc. chim.*, [3] **21**, 416 (1899); v. Braun and Rudolph, *Ber.*, **67**, 280 (1934).

(4) Tetry, *Bull. soc. chim.*, [3] **27**, 600 (1902).

(5) Cherbuliez and Heger, *Helv. Chim. Acta*, **15**, 191 (1932).