TABLE IV

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

	Micro-organism alone Q_{O_2} (N) p_{O_2} 0.01	0.21	+ Hog hemoglobin % stimulation 0.01	0.21	+ Heated hog hemoglobin % stimulation 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

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(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).

presence of magnesium and a small quantity of mercuric chloride, using rather large volumes of a mixture of toluene and ether as solvent to prevent resin formation. Verley[6] has cyclized citrylideneacetic acid and several of its derivatives to cyclic isomers using sirupy phosphoric acid containing 6–8% of sulfuric acid as cyclizing agent. The structure of the cyclic products was not determined. Young, Andrews and Cristol have recently described[7] the preparation of pure β-cyclocitrylideneacetic acid by hypochlorite oxidation of β-ionone. A similar oxidation of α-ionone gave a very small yield of crude acid which was not further purified.

The present work was undertaken to reinvestigate the use of citral in the Reformatsky reaction and to determine the major products obtained on cyclization of ethyl citrylideneacetate. We have condensed citral with ethyl bromoacetate under the usual conditions of the Reformatsky synthesis. An intermediate hydroxy-ester was obtained which readily lost water on heating with potassium bisulfate to give ethyl citrylideneacetate in 60% yield. Citrylideneacetic acid was obtained by hydrolysis of the ester with alcoholic potassium hydroxide. The acid was obtained as a light yellow, mobile oil of a pleasant, fruity odor. Pure α- and β-cyclocitrylideneacetic acids were prepared by potassium hypochlorite oxidation of the corresponding ionones. β-Cyclocitrylideneacetic acid was obtained as a colorless, crystalline solid, while the α-isomer was obtained as a colorless, extremely viscous sirup. Neither cyclic acid possessed a marked odor. The ultraviolet absorption spectra of the above compounds are shown in Fig. 1. Citrylideneacetic acid and its β-cyclic isomer, containing almost identical conjugated systems, exhibit very similar absorption with maxima at 274 and 277 mμ, respectively. α-Cyclocitrylideneacetic acid, in which this conjugation is broken, exhibits its maximum absorption at a considerably shorter wave length, <210 mμ.

Ethyl citrylideneacetate was found to be extremely resistant both to acid hydrolysis and to cyclization by the usual acidic reagents. Refluxing the ester with 20% aqueous sulfuric acid led to a small yield of an acid product and to 68.5% recovery of unchanged ethyl citrylideneacetate. Treatment of the ester under reflux with 20% sulfuric acid in a mixture of equal parts of acetic acid and water gave a sirupy acid product in 17.2% yield and 38.5% recovery of an ester. Examination of the absorption spectra of the acid product and of the recovered ester (Fig. 2), however, indicates that both were very largely cyclized to α-cyclocitrylideneacetic acid and the corresponding ester. It is impossible in the mixtures of isomers obtained to distinguish the open-chain compounds from the β-cyclic isomers by comparison of absorption spectra. It is assumed, however, from consideration of the cyclization behavior of similar compounds, such as pseudoionone[2] and geranic acid,[8] that cyclization to the β-isomer did not occur under the mildly acid conditions employed, and that the absorption in the region 270–80 mμ is due to the presence of uncyclized material or small amounts of unidentified impurities rather than to the β-isomer. Attempts to im-

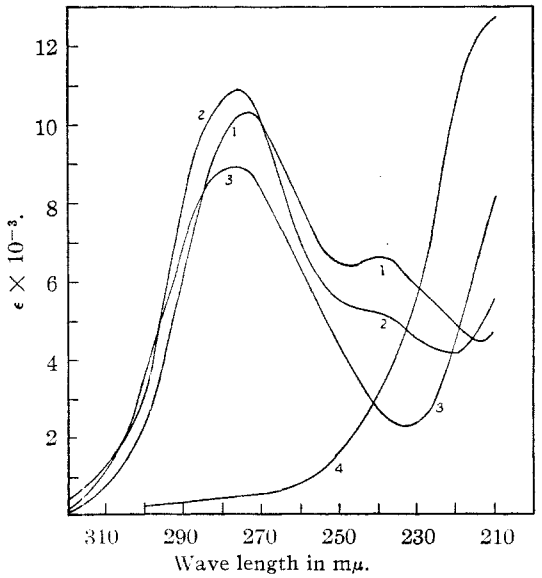


Fig. 1.—Absorption spectra of: 1, citrylideneacetic acid; 2, ethyl citrylideneacetate; 3, β-cyclocitrylideneacetic acid; 4, α-cyclocitrylideneacetic acid.

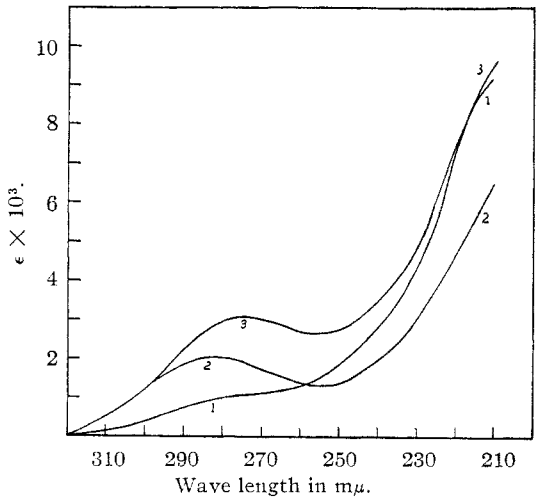


Fig. 2.—Absorption spectra of: 1, citrylideneacetic acid cyclized by 10% sulfuric acid in phosphoric acid; 2, cyclic ester from treatment of ethyl citrylideneacetate with 20% sulfuric acid in water–glacial acetic acid; 3, cyclic acid from treatment of ethyl citrylideneacetate with 20% sulfuric acid in water–glacial acetic acid.

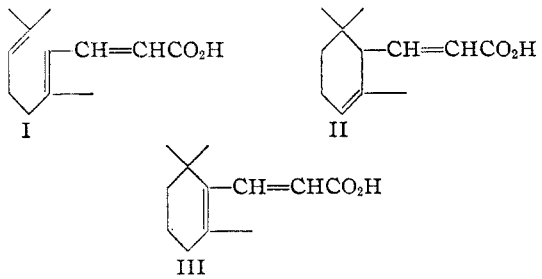
(6) Verley, German Patent 153,575; *Chem. Zentr.*, **75**, II, 677 (1904).

(7) Young, Andrews and Cristol, THIS JOURNAL, **66**, 520 (1944).

(8) Semmler, *Ber.*, **26**, 2725 (1893); Tiemann, *ibid.*, **33**, 3705 (1900).

prove the extent of hydrolysis or cyclization by varying the relative proportions of water, acetic acid, and sulfuric acid were without avail.

Ethyl citrylideneacetate was isomerized by a 10% solution of sulfuric acid in sirupy phosphoric acid to a cyclic ester which on subsequent hydrolysis with alcoholic potassium hydroxide yielded cyclocitrylideneacetic acid as a viscous sirup. The absorption spectrum of this acid (Fig. 2) indicates that it is principally α-cyclocitrylideneacetic acid. The low absorption in the region 270–280 mμ precludes the presence of more than small amounts of either the β-cyclic or the open-chain isomer. An equal concentration of sulfuric acid in glacial acetic acid effected no cyclization of ethyl citrylideneacetic acid after two days at room temperature, while this reagent at reflux temperature yielded only a resinous product from which no definite compound could be isolated. It has already been noted in the discussion above that aqueous solutions of sulfuric acid effected no cyclization of ethyl citrylideneacetate even at reflux. Various attempts to cyclize ethyl citrylideneacetate with concentrated solutions (50% or greater) of sulfuric acid in water, glacial acetic acid or phosphoric acid in the hope of obtaining the β-cyclic isomer led only to unworkable tars.



Experimental

Synthesis of Ethyl Citrylideneacetate.—A mixture of 32.5 g. (0.5 g. atom) of zinc, 100 cc. of dry, thiophene-free benzene, and a small crystal of iodine was placed in dry one-liter flask fitted with a mechanical stirrer, a dropping funnel, and a reflux condenser. The flask contents were protected from atmospheric moisture by suitably placed calcium chloride tubes. The benzene was heated to boiling, the stirrer was started, and a solution of 83.5 g. (0.5 mole) of ethyl bromoacetate and 76.1 g. (0.5 mole) of freshly distilled citral in 200 cc. of dry, thiophene-free benzene was added slowly from the dropping funnel. Heating was continued until a vigorous reaction began within about five to ten minutes. The remainder of the solution was added during about thirty minutes without further heating. The reaction mixture was then refluxed over a hot-plate for one hour.

The reaction mixture was cooled to room temperature, decanted from a small portion of unreacted zinc, and vigorously stirred with 500 g. of a 15% acetic acid solution for two hours. The benzene layer was drawn off, and the aqueous layer was twice extracted with small portions of benzene. The benzene extracts were combined with the main benzene solution, washed several times with water, and dried over sodium sulfate. The drying agent was filtered off, and the benzene was removed by distillation. When most of the benzene had been removed, 50 g. of freshly fused and powdered potassium bisulfate was added to the residue. The bath temperature was then maintained at 160–170° while the pressure was slowly reduced to a final value of about 100 mm. Water elimination, vigorous at first, seemed to be complete in this time. The residue was taken up in benzene and dried over sodium sulfate. The drying agent was filtered off, the benzene was removed by distillation at atmospheric pressure, and the residue was distilled under reduced pressure through a modified Widmer column to yield 66.7 g. (60.0%) of ethyl citrylideneacetate, b. p. 129–144° (2 mm.) (141–156° (7 mm.)), $n^{25}D$ 1.5011. *Anal.* Calcd.: sapn. equiv., 222. Found: sapn. equiv., 222. The compound showed an absorption maximum at 277 mμ, ε 11,030.

Citrylideneacetic Acid.—Ethyl citrylideneacetate, 50 g. (0.225 mole) was heated under reflux for two hours with a solution of 11.8 g. (0.21 mole) of potassium hydroxide in 100 cc. of 95% ethyl alcohol. The alcohol was then distilled from a water-bath, the last portions under slightly reduced pressure. The residue was taken up in 200 cc. of water, made slightly acidic with glacial acetic acid, then just basic with sodium bicarbonate. The aqueous solution was then repeatedly washed with ether, and the acid hydrolysis product was finally liberated by careful acidification with glacial acetic acid. The product was taken up in ether and dried over sodium sulfate. After removal of the drying agent and ether, the residue was distilled under reduced pressure through a modified Widmer column to yield 22.3 g. (54.5% of the theoretical on potassium hydroxide) of citrylidene acetic acid, b. p. 140–143° (1 mm.), $n^{25}D$ 1.5170. Neutral equivalent determinations showed this product to be about 82% pure. The combined products from several such runs were dissolved in 10% sodium bicarbonate solution, and the solution was repeatedly washed with ether. Liberation of the acid by acidification with glacial acetic acid, subsequent drying in ether solution and redistillation through a modified Widmer column gave pure citrylideneacetic acid in about 25% over-all yield from the ester, b. p. 138–142° (0.4 mm.), $n^{25}D$ 1.5211. *Anal.* Calcd.: neut. equiv., 194. Found: neut. equiv., 201. This compound showed an absorption maximum at 274 mμ, ε 10,170.

α-Cyclocitrylideneacetic Acid.—A solution of potassium hypochlorite was prepared according to directions of Newman and Holmes.[9] A solution of 10 g. (0.052 mole) of pure α-ionone in 75 cc. of methanol was cooled to 5° in an ice-bath and vigorously stirred while 120 ml. of the potassium hypochlorite solution (approximately 0.184 mole of potassium hypochlorite) was added dropwise during thirty minutes. Stirring was continued overnight at room temperature. The reaction mixture was distilled from a hot-plate until about 100 cc. of distillate had passed over. The residue was cooled to room temperature and washed several times with ether. The acid product was then liberated by acidification with hydrochloric acid, taken up in ether, and dried over sodium sulfate. The drying agent and ether were removed, and the residue was distilled under reduced pressure from a small Claisen flask to yield 7.5 g. (74%) of α-cyclocitrylideneacetic acid, b. p. 143–150° (1 mm.), $n^{25}D$ 1.5080. Calcd.: neut. equiv. 194. Found: neut. equiv., 195. This product showed an absorption maximum at <210 mμ, ε 12,530. Young, Andrews and Cristol reported[7] absorption maximum, <212.5 mμ, ε 10,100.

β-Cyclocitrylideneacetic Acid.—β-Cyclocitrylideneacetic acid was prepared by hypochlorite oxidation of pure β-ionone by a procedure similar to that described above for the α-isomer, except that a shorter reaction period (four hours) was employed. Oxidation of 10 g. (0.052 mole) of β-ionone gave 9.5 g. of a crude, yellow, semi-solid acid product, which after one crystallization from aqueous alcohol and three crystallizations from dilute acetic acid gave 5.0 g. (49.5%) of cyclocitrylideneacetic acid, colorless needles, m. p. 105.5–107.0° (uncor.). Calcd.: neut. equiv., 194. Found: neut. equiv., 195. This product showed an absorption maximum at 277 mμ, ε 8,954. Young, Andrews and Cristol reported[7] for this compound, m. p. 106–108°, absorption maximum 277 mμ, ε 9,240.

(9) Newman and Holmes, "Organic Syntheses," **17**, 66 (1937).

Attempted Hydrolysis of Ethyl Citrylideneacetate.—A mixture of 20 g. (0.09 mole) of ethyl citrylideneacetate, 48 cc. of water, and 6.4 cc. of sulfuric acid was vigorously refluxed for twenty hours. The ester layer became quite darkly colored during this period. The reaction mixture was poured into 200 cc. of water and extracted with 200 cc. of ether used in small portions. The dark red-brown ether solution was then extracted with four 25-cc. portions of 5% aqueous sodium hydroxide. These extracts were combined and acidified with phosphoric acid to yield a crude acid product, which was taken up in ether and dried over sodium sulfate. Distillation of the acid reaction product gave 1.8 g. of a light yellow oil, b. p. 142–151° (1 mm.), $n^{25}D$ 1.5038. This produc twas not investigated further. Distillation of the neutral fraction gave 13.7 g. (68.5%) of unchanged ethyl citrylideneacetate, b. p. 135–150° (3.5 mm.), $n^{25}D$ 1.5002, absorption maximum 277 mμ, ε 12,310.

A mixture of 20 g. (0.09 mole) of ethyl citrylideneacetate, 40 g. of water, 40 g. of glacial acetic acid and 20 g. of sulfuric acid was heated to reflux for five hours. The dark reaction mixture was diluted with 200 cc. of water and worked up as described above. Distillation of the acid reaction product gave 3.0 g. (17.2%) of a light yellow oil, b. p. 140–150° (2 mm.), $n^{25}D$ 1.5091. This products howed an absorption maximum at 277 mμ, ε 3,100. Examination of the complete absorption curve (Fig. 2) indicates that this product consists principally of α-cyclocitrylideneacetic acid. Calcd.: neut. equiv., 194. Found: neut. equiv., 210 (92.3% acid purity). Distillation of the neutral reaction product gave 7.7 g. (38.5%) of a light yellow ester, b. p. 127–145° (5 mm.), $n^{25}D$ 1.4952. This ester showed an absorption maximum at 282 mμ, ε 2,250. Examination of the complete absorption curve (Fig. 2) indicates that the product consists principally of ethyl α-cyclocitrylideneacetate. Calcd.: sapn. equiv., 222. Found: sapn. equiv., 209.

Cyclization of Ethyl Citrylideneacetate.—A mixture of 10 g. of concentrated sulfuric acid and 90 g. of 85% phosphoric acid was cooled to 3–4° in an ice-bath and mechanically stirred while 20 g. (0.09 mole) of ethyl citrylideneacetate was added dropwise during fifteen minutes. The cooling bath was then removed, and stirring was continued while the temperature rose to 25° during thirty minutes. The dark reddish brown reaction mixture was poured into 500 cc. of water, and the yellow, oily reaction product was taken up in ether, washed with water, and dried over sodium sulfate. Removal of the drying agent and ether followed by distillation under reduced pressure gave 10.1 g. (50%) of ethyl cyclocitrylideneacetate, b. p. 106–126° 0.4 mμ, $n^{25}D$ 1.4879. Hydrolysis of this ester with alcoholic potassium hydroxide according to the procedure described above gave 5.8 g. (66% of the theoretical from the cyclized ester) of cyclocitrylideneacetic acid, b. p. 136–140° 0.4 mμ, $n^{25}D$ 1.5070. The absorption curve for this product (Fig. 2) indicates that it is α-cyclocitrylideneacetic acid containing practically none of the open-chain or β-cyclic isomers. Calcd.: neut. equiv., 194. Found: neut. equiv., 225 (86.4% acid purity).

Ethyl citrylideneacetate, 15 g. (0.068 mole), was stirred at room temperature with a solution of 6.0 g. of concentrated sulfuric acid in 60 g. of glacial acetic acid. On working up this mixture as described above, 8.6 g. (57.4%) of ethyl citrylideneacetate was recovered, unchanged as evidenced by its refractive index and absorption curve, $n^{25}D$ 1.5015, absorption maximum, 277 mμ, ε 11,000.

A number of similar experiments were performed in an attempt to cyclize ethyl citrylideneacetate and the free acid using such strongly acidic reagents as 100% phosphoric acid and high concentrations of sulfuric acid (50% and greater) in water, acetic acid and 85% phosphoric acid. All such attempts led to undistillable tars.

Absorption Spectrum Measurements.—Determinations of ultraviolet spectra were made on the Beckman Spectrophotometer, Model DU. The solvent was 95% ethanol.

Summary

1. The Reformatsky reaction has been applied to the synthesis of ethyl citrylideneacetate, and this ester has been hydrolyzed to citrylideneacetic acid.

2. Pure α- and β-cyclocitrylideneacetic acids have been prepared by hypochlorite oxidation of the corresponding ionones.

3. The ultraviolet absorption spectra of these compounds have been determined.

4. A study has been made of the cyclization of ethyl citrylideneacetate by various acidic reagents. Most satisfactory results were obtained using small concentrations of sulfuric acid in sirupy phosphoric acid as cyclizing agent; this procedure yielded a product consisting principally of ethyl α-cyclocitrylideneacetate. No conditions of cyclization were found which would yield the β-cyclo ester as the major product.

ATLANTA, GEORGIA RECEIVED JULY 29, 1946

[CONTRIBUTION FROM THE RESEARCH LABORATORIES, THE UPJOHN COMPANY]

An Improved Synthesis of β-Alanine. III. The Addition of Ammonia to Acrylonitrile at 50–150°[1]

BY JARED H. FORD, SAUL R. BUC[2] AND J. WARD GREINER

In a previous paper from these Laboratories[3] it was shown that the reaction of ammonia with acrylonitrile gave higher yields of β-aminopropionitrile with aqueous than with liquid ammonia.[4,5] Although it was demonstrated that higher temperatures favored the formation of the primary amine our previous studies were restricted to the temperatures spontaneously attained in closed vessels after mixing the reactants at room temperature. Since this gave the undesired secondary amine, di-(β-cyanoethyl)-amine, as the major product of the reaction, it seemed advisable to extend the studies to include higher temperatures.

Experimental

Preliminary experiments at moderate pressures (less than 75 p. s. i.) were made in a 36-inch length of 3-inch Pyrex pipe, the top of which was closed by a rubber stopper that was held in place by a steel plate bolted to

(1) Part II, Ford, THIS JOURNAL, **67**, 876 (1945).

(2) Present address: General Aniline and Film Corporation, Easton, Pennsylvania.

(3) Buc, Ford and Wise, THIS JOURNAL, **67**, 92 (1945).

(4) Hoffmann and Jacobi, U. S. Patent 1,992,615.

(5) Whitmore, Mosher, Adams, Taylor, Chapin, Weisel and Yanko, THIS JOURNAL, **66**, 725 (1944).