sure to a solid mass. Fractional crystallization from dry methanol separated clivonine hydrochloride, m.p. 263–275° dec., from the sodium chloride. The last traces of clivonine were obtained by dissolving all residues containing alkaloidal material in water, neutralizing the solution with sodium bicarbonate solution and extracting the alkaloid with chloroform–ethanol (4:1). Concentration of this extract and chromatography on alumina gave crude clivonine, m.p. 190–195°. By this procedure, a total of 2.00 g. of clivonine was obtained. A pure sample of clivonine was obtained by recrystallization from ethyl acetate, m.p. 199–200°, $[\alpha]^{23}_{589}$ +41.24°, $[\alpha]^{23}_{436}$ +103.44° (*c* 1.11, chloroform).

Anal. Calcd. for $C_{17}H_{19}NO_5$: C, 64.34; H, 6.04; N, 4.41; NCH_3, 4.73. Found: C, 64.39; H, 5.95; N, 4.41; NCH_3, 2.26. No methoxyl was present.

The ultraviolet absorption spectrum showed maxima at 226 mμ (log ε 4.33), 268 mμ (log ε 3.77) and 308 mμ (log ε 3.76).

Clivonine Hydrochloride.—The crude hydrochloride was recrystallized from methanol–ether (which contained a drop of water) as colorless prisms, m.p. 282–287° dec.

Anal. Calcd. for $C_{17}H_{19}NO_5 \cdot HCl$: C, 57.81; H, 5.70; N, 3.96. Found: C, 57.67; H, 5.74; N, 3.91.

The ultraviolet absorption spectrum showed maxima at 226 mμ (log ε 4.35), 268 mμ (log ε 3.67) and 308 mμ (log ε 3.75).

Clivonine Picrate.—An ethanolic solution of 75 mg. of clivonine was treated with saturated aqueous picric acid until the precipitation of picrate ceased. The resulting solid was recrystallized from aqueous ethanol to give a quantitative yield of derivative, m.p. 250–254° dec.

Anal. Calcd. for $C_{17}H_{19}NO_5 \cdot C_6H_3N_3O_7$: C, 50.55; H, 4.06; N, 10.25. Found: C, 50.55; H, 4.07; N, 10.19.

Clivonine Methopicrate.—A methanolic solution of 129 mg. of clivonine was refluxed overnight with excess methyl iodide. The neutral gum obtained on concentration was triturated with ethanol–ether to give a very hygroscopic solid. The material was converted to the methopicrate in the usual manner and recrystallized from aqueous ethanol, 111 mg., m.p. 285° dec.

Anal. Calcd. for $C_{17}H_{19}NO_5 \cdot CH_3 \cdot C_6H_2N_3O_7$: C, 51.43; H, 4.32; N, 10.00. Found: C, 51.66; H, 4.88; N, 9.89.

Attempted Hydrogenation of Clivonine.—Clivonine absorbed no hydrogen and was recovered unchanged from atmospheric hydrogenation conditions employing either palladium-on-charcoal (10%) or platinum oxide catalysts.

O-Acetylclivonine.—A solution of 110 mg. of clivonine in 2 ml. of acetic anhydride containing twenty drops of pyridine was allowed to stand one day at room temperature and then concentrated under reduced pressure to a colorless gum that was dissolved in chloroform. The solution was washed with dilute sodium bicarbonate solution and water. Concentration of the chloroform solution gave an oil that crystallized on trituration with ethyl acetate. Recrystallization from ethyl acetate gave 87 mg. of colorless needles, m.p. 194–196°. The acetate showed basic properties.

Anal. Calcd. for $C_{19}H_{21}NO_6$: C, 63.50; H, 5.89; N, 3.90. Found: C, 63.74; H, 5.80; N, 3.81.

Tetrahydroclivonine.—Reduction of 313 mg. of clivonine in tetrahydrofuran with an excess of lithium aluminum hydride gave 261 mg. of a colorless oil that was chromatographed on 30 g. of aluminum oxide (Merck). Elution with chloroform removed 60 mg. of unreacted starting material. Elution with 5% ethanolic chloroform gave 200 mg. of colorless oil which could not be crystallized. The ultraviolet spectrum of this oil showed maxima at 238 mμ (log ε 3.58) and 292 mμ (log ε 3.51). A vicinal glycol determination[10] on 68.4 mg. of this oil indicated that 81.6% of the material had a vicinal glycol structure.

Nivaline.—The bulbs of *Galanthus nivalis* L. were purchased from Kipiteiju and Sons, Hillegon, The Netherlands. By the usual extraction method, 9800 g. of fresh bulbs gave 25.6 g. (0.26%) of crude alkaloids. Ethanolic trituration of this material gave 1.76 g. (0.018%) of lycorine, m.p. 250–255° dec. The filtrate was concentrated under reduced pressure to remove the ethanol. The benzene-soluble portion of the residue was chromatographed on aluminum oxide (Merck) to give 4.01 g. (0.04%) of tazettine, m.p. 200–205°. The oils (0.776 g.) eluted prior to the tazettine were rechromatographed. Elution with 5% ethyl acetate in benzene gave 92 mg. of crude nivaline, m.p. 121–125°, which was recrystallized from ethanol to give 73 mg. (0.0007%) of pure nivaline, m.p. 131.5–132.5°, $[\alpha]^{24}D$ +268° (*c* 1.0, ethanol).

Anal. Calcd. for $C_{18}H_{19}NO_5$: C, 65.64; H, 5.82; N, 4.25; OCH_3, 9.42; NCH_3, 4.56. Found: C, 65.88; H, 5.84; N, 4.36; OCH_3, 9.34; NCH_3, 3.30.

The ultraviolet absorption spectrum showed maxima at 230 mμ (log 4.43), 269 mμ (log ε 3.75) and 308 mμ (log ε 3.77).

A similar processing of 26,900 g. of *Hymenocallis occidentalis* (le Conte) Kunth gave lycorine (0.007%), tazettine (0.03%) and nivaline (0.0002%).

Dihydronivaline.—A solution of 48 mg. of nivaline in 20 ml. of ethanol absorbed 3.05 ml. of hydrogen when reduced at atmospheric pressure and room temperature with 40 mg. of 10% palladium-on-charcoal catalyst. The dihydro derivative was an oil.

BETHESDA, MARYLAND

COMMUNICATIONS TO THE EDITOR

REDUCED TRIPHOSPHOPYRIDINENUCLEOTIDE REQUIREMENT FOR THE ENZYMATIC FORMATION OF 3-HYDROXYKYNURENINE FROM L-KYNURENINE

Sir:

Two recent reviews on the metabolism of tryptophan[1,2] have stressed the lack of information concerning the formation of 3-hydroxykynurenine from kynurenine. In an effort to account for the absence of the usual tryptophan metabolites from cat urine[3] certain *in vitro* studies were carried out. This letter describes a cell-free system occurring in liver mitochondria of cats and rats which forms 3-hydroxykynurenine from L-kynurenine.

The disappearance of substrates and the appearance of the metabolites were followed by ascending paper chromatography on Whatman No. 1 filter paper. The spots were identified by comparison with authentic compounds according to the criteria in Table I.

Washed mitochondria equivalent to 200 to 400 mg. wet weight of liver was incubated aerobically at 37° for two hours in 0.05 *M* phosphate buffer at *p*H 7.4 containing 5.0 μmoles of citrate, 0.67 μmole of triphosphopyridinenucleotide (TPN), 33.0 μmoles of nicotinamide and 2.5 μmoles of L-kynurenine. The final volume was 1.5 ml. It was not necessary to deproteinize before spotting the

(1) C. E. Dalgliesh in Advances in Protein Chemistry, **10**, 86 (1955).

(2) A. H. Mehler in "Amino Acid Metabolism," edited by W. D. McElroy and H. B. Glass, The Johns Hopkins Press, Baltimore, Md., 1955, p. 898.

(3) R. R. Brown and J. M. Price, *J. Biol. Chem.*, **219**, 985 (1956).

TABLE I[a]

Compound	R_F in MeOH, BuOH, Bz, H_2O 2:1:1:1 containing 1% AcOH	1% 15 N NH_4OH	Ultraviolet fluorescence	DSA[b]	Ekman's reagent[b]
L-Kynurenine	0.48	0.50	GB		M
D-Kynurenine	.37	.48	GB		M
3-Hydroxy-L-kynurenine	.38	.25[e]	GY	R	Y[f]
3-Hydroxy-D-kynurenine	.30	.25[e]	GY	R	Y[f]
Kynurenic acid	.60	.60	V[c]		
Xanthurenic acid	.60	.18[e]	LB[d]	R	
Anthranilic acid	.92	.60	BV		M
N^{α}-Acetyl-L-kynurenine	.78	.62	GB		M
N^{α}-Acetyl-D-kynurenine	.78	.62	GB		M

[a] Abbreviations: MeOH, methanol; BuOH, *n*-butanol; Bz, benzene; AcOH, acetic acid; DSA, diazotized sulfanilic acid; G, green; B, blue; Y, yellow; V, violet; L, light; R, red; M, magenta. [b] These reagents were prepared and used according to C. E. Dalgliesh, *Biochem. J.*, **52**, 3 (1952). [c] Changes to light blue after ultraviolet irradiation. [d] Changes to yellow with ammonia vapor. [e] R_F is variable in the presence of salts. [f] Becomes yellow with nitrous acid.

incubation mixture (20 μliters) on the paper.

In the complete system, much of the L-kynurenine disappeared and spots corresponding to 3-hydroxy-L-kynurenine, kynurenic acid and xanthurenic acid appeared. No anthranilic acid nor 6-hydroxykynurenic acid was observed. In the absence of either TPN or citrate, 3-hydroxykynurenine was not readily detectable. Reduced TPN (4.0 μmoles per flask) was just as effective as TPN plus citrate in stimulating 3-hydroxykynurenine formation. Succinate, fumarate or malate was not as effective as citrate in supporting hydroxylation. Reduced diphosphopyridinenucleotide (DPNH) or DPNH plus TPN was not as good as TPNH. Anaerobic conditions or boiling the mitochondria inhibited the reaction completely.

Ion exchange chromatography was used to isolate the reaction products in spectroscopically pure form. From an incubation of 7.5 μmoles of L-kynurenine with cat liver mitochondria in the complete system, 4.26 μmoles of 3-hydroxykynurenine, 0.11 μmole of kynurenic acid, and 0.11 μmole of xanthurenic acid were produced and 2.95 μmoles of unreacted kynurenine remained. These reaction products chromatographed on ion exchange resins exactly like the corresponding authentic compounds. From pooled incubation mixtures containing an estimated 14 mg. of 3-hydroxykynurenine, 6.7 mg. of 3-hydroxykynurenine was obtained as beautiful yellow needles. The ultraviolet spectrum of this material in 0.1 *M* phosphate buffers revealed peaks at 223, 260 and 369 mμ at *p*H 2.0; at 228, 267 and 368 mμ at *p*H 7.4; and 248, 286 and 393 mμ at *p*H 12.0, corresponding exactly to the spectrum of synthetic 3-hydroxy-DL-kynurenine. The spectra of the isolated kynurenic acid and xanthurenic acid were also identical with spectra of authentic compounds at each of these *p*H values.

The extent to which the added kynurenine was accounted for and the absence of detectable quantities of 6-hydroxykynurenic acid suggests that little if any hydroxylation occurred at the 5-position of kynurenine.

The hydroxylation system was specific for L-kynurenine since D-kynurenine, N^{α}-acetyl-L-kynurenine, N^{α}-acetyl-D-kynurenine, kynurenic acid, and anthranilic acid were not attacked. This is in contrast to the non-enzymatic systems described by Udenfriend, Clark, Axelrod and Brodie[4] and by Dalgliesh.[5] The enzyme system resembles the one which oxidizes non-aromatic rings in certain steroids.[6,7]

Acknowledgments.—We are indebted to Dr. L. M. Henderson, Division of Biochemistry, Noyes Laboratory of Chemistry, University of Illinois, Urbana, Illinois, for a gift of 3-hydroxy-DL-kynurenine, and to Drs. G. C. Mueller and G. Rumney of the McArdle Memorial Laboratory for gifts of reduced triphosphopyridinenucleotide. This work was supported in part by the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

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(5) C. E. Dalgliesh, *Biochem. J.*, **58**, xlv (1954).

(6) M. L. Sweat and M. D. Lipscomb, THIS JOURNAL, **77**, 5185 (1955).

(7) A. C. Brownie and J. K. Grant, *Biochem. J.*, **62**, 29 (1956).

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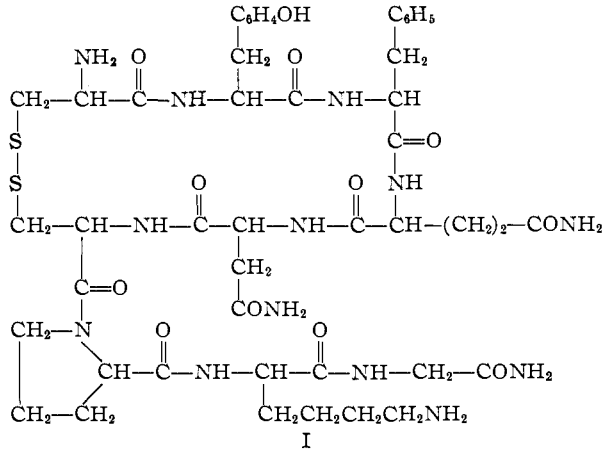
RECEIVED MARCH 22, 1956

STUDIES ON THE SYNTHESIS OF LYSINE-VASOPRESSIN

Sir:

du Vigneaud, Lawler and Popenoe[1] proposed Structure I for lysine-vasopressin, the hormone isolated from hog posterior pituitary glands. A



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synthesis of biologically active material according to this structure was announced by du Vigneaud, Popenoe and Roeske (footnote, ref. 1).[2] Synthesis

(1) V. du Vigneaud, H. C. Lawler and E. A. Popenoe, THIS JOURNAL, **75**, 4880 (1953).

(2) This synthetic approach paralleled the method employed for the synthesis of oxytocin [V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts and P. G. Katsoyannis, *ibid.*, **76**, 3115 (1954)], with ϵ-tosyllysine (ϵ-*p*-toluenesulfonyllysine) replacing leucine and phenylalanine replacing isoleucine in the series of reactions. However, the yield of pressor-antidiuretic material was very low. Confirmation of these results has now been obtained and this method of synthesis has given biologically active material in somewhat higher yield.