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.

(4) S. Udenfriend, C. T. Clark, J. Axelrod and B. B. Brodie, *J. Biol. Chem.*, **208**, 731 (1954).

(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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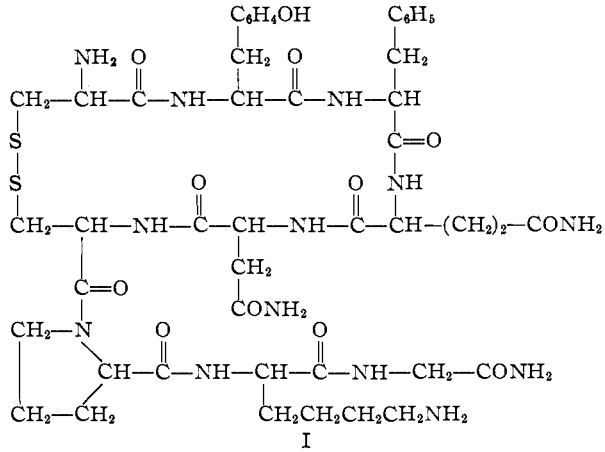
STUDIES ON THE SYNTHESIS OF LYSINE-VASOPRESSIN

Sir:

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



I

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.

of I was also attempted by the coupling of S-benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine[3] with S-benzyl-L-cysteinyl-L-prolyl-ε-tosyl-L-lysylglycinamide (II) according to the pyrophosphite procedure,[4] followed by removal of the protecting groups from the resulting nonapeptide derivative and subsequent oxidation to the disulfide form. This approach yielded a synthetic product assaying approximately 100 pressor units/mg. after purification by electrophoresis at *p*H 5.6 in pyridine acetate and countercurrent distribution between *sec*-butyl alcohol and 0.08 *M* *p*-toluenesulfonic acid.

In addition to these approaches, another method was utilized which has led to material which is as biologically active as the most potent preparation of lysine-vasopressin obtained from natural sources. The key intermediate in this synthesis is S-benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-ε-tosyl-L-lysylglycinamide (III), which has solubility properties favorable for its purification.

III was synthesized by the coupling of S-benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine (IV) with the tetrapeptide amide II. For the synthesis of II, ethyl L-prolyl-ε-tosyl-L-lysylglycinate,[5] m.p. 81–84°, was coupled with S-benzyl-N-carbobenzoxy-L-cysteine[6] by the N,N′-dicyclohexylcarbodiimide method[7] and the resulting protected tetrapeptide ester was converted to the corresponding crystalline amide with ammonia in ethanol; m.p. 101–104°, $[\alpha]^{21}D$ −29.3° (*c* 1, $CHCl_3$) (calcd. for $C_{38}H_{48}O_8N_6S_2$: C, 58.5; H, 6.19; N, 10.8; S, 8.21. Found: C, 58.3; H, 6.34; N, 10.6; S, 8.10); the carbobenzoxy group was then removed with HBr–HOAc.[4] IV was prepared by the coupling of L-phenylalanyl-L-glutaminyl-L-asparagine[8] with S-benzyl-N-tosyl-L-cysteinyl-L-tyrosine[9] by the isobutyl chlorocarbonate mixed anhydride procedure[10] in a yield of 55% after recrystallization; m.p. 203–204°, $[\alpha]^{21}D$ +4.4° (*c* 2.08, dimethylformamide) (calcd. for $C_{44}H_{51}N_7O_{11}S_2 \cdot 1/2H_2O$: C, 57.0; H, 5.65; N, 10.6. Found: C, 57.1; H, 5.66; N, 10.6).

Compounds IV and II were coupled by the N,N′-dicyclohexylcarbodiimide method[7] to give a 39% yield of analytically pure III, m.p. 226–230°, $[\alpha]^{18}D$ −23.0° (*c* 2.11, dimethylformamide) (calcd. for $C_{74}H_{91}O_{16}N_{13}S_4 \cdot H_2O$: C, 56.8; H, 5.99; N, 11.6; H_2O, 1.15. Found: C, 56.7; H, 6.02; N, 11.6; H_2O, 1.02).

(3) V. du Vigneaud, D. T. Gish and P. G. Katsoyannis, *ibid.*, **76**, 4751 (1954).

(4) G. W. Anderson, J. Blodinger and A. D. Welcher, *ibid.*, **74**, 5309 (1952).

(5) This tripeptide ester was prepared from α-carbobenzoxy-ε-tosyl-L-lysine, m.p. 85–88°, and ethyl glycinate, removal of the carbobenzoxy group, and coupling of the product with carbobenzoxy-L-proline by the *o*-phenylene chlorophosphite procedure [G. W. Anderson and R. W. Young, THIS JOURNAL, **74**, 5307 (1952)] followed by removal of the carbobenzoxy group with HBr–HOAc.

(6) C. R. Harington and T. H. Mead, *Biochem. J.*, **30**, 1598 (1936).

(7) J. C. Sheehan and G. P. Hess, THIS JOURNAL, **77**, 1067 (1955).

(8) E. A. Popenoe and V. du Vigneaud, *ibid.*, **76**, 6202 (1954).

(9) This compound was prepared independently by a method similar to that recently published by J. Honzl and J. Rudinger [*Collection Czechoslov. Chem. Communs.*, **20**, 1190 (1955)]. They also reported the synthesis of a product of high oxytocic activity from a nonapeptide derivative containing a tosylcysteine residue.

(10) J. R. Vaughan, Jr., and J. A. Eichler, THIS JOURNAL, **75**, 5556 (1953).

The N-tosyl and S-benzyl groups were removed from III (300 mg.) by reduction with sodium in liquid ammonia and the resulting product was oxidized to the disulfide form by aeration in aqueous solution at *p*H 6.4. The yield of pressor activity[11] ranged in several experiments from 50,000 to 70,000 units (per 300 mg. of III). After concentration and lyophilization, the product was purified by countercurrent distribution between *sec*-butyl alcohol and 0.08 *M* *p*-toluenesulfonic acid followed by electrophoresis in pyridine acetate buffer (*p*H 5.6) on a cellulose-supporting medium.[12,13] The purified material had a pressor activity of 250–290 units/mg.[14] when assayed as usual against the U.S.P. Standard Powder.[15] The ratios between pressor, antidiuretic, milk-ejecting and avian depressor activities for the synthetic material are the same as those for natural lysine-vasopressin.[12,14,16] Starch column chromatography[17] of a hydrolysate of this material showed the eight amino acids to be present in molar ratios to each other of approximately 1:1 and ammonia in a ratio to any one amino acid of approximately 3:1. The natural hormone and the synthetic product showed the same infrared spectrum.[18] They showed the same behavior on countercurrent distribution between *sec*-butyl alcohol and 0.08 *M* *p*-toluenesulfonic acid and also had the same electrophoretic mobility on Whatman No. 1 paper in pyridine acetate buffer at *p*H 5.6 and 4.0.

(11) J. Dekanski, *Brit. J. Pharmacol.*, **7**, 567 (1952).

(12) D. N. Ward and V. du Vigneaud, *J. Biol. Chem.*, in press.

(13) H. G. Kunkel in "Methods of Biochemical Analysis," Vol. 1, D. Glick, Ed., Interscience Publishers, Inc., New York, p. 141.

(14) A sample of natural lysine-vasopressin with approximately the same pressor activity as the synthetic material was kindly supplied by Dr. Albert Light of this laboratory. This material was obtained by purification by countercurrent distribution followed by chromatography on Amberlite IRC-50.

(15) "The Pharmacopeia of the United States of America," fifteenth revision, Mack Printing Co., Easton, Pa., 1955, p. 776. These assay values are based on an activity of 0.4 U.S.P. Posterior Pituitary Unit/mg. for the Standard Powder.

(16) For the ratios between these four activities in natural lysine-vasopressin, see H. B. van Dyke, S. L. Engel and K. Adamsons, Jr., *Proc. Soc. Exptl. Biol. Med.*, **91**, 484 (1956). The ratios of antidiuretic and milk-ejecting activities to pressor activity for the synthetic material were determined by Professor H. B. van Dyke and Mr. S. L. Engel of the College of Physicians and Surgeons.

(17) S. Moore and W. H. Stein, *J. Biol. Chem.*, **178**, 53 (1949).

(18) The authors wish to thank Dr. Julian R. Rachele of this laboratory for determination of the infrared spectra.

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THE PREPARATION OF ACID-STABILIZED SUBHALIDES FROM MOLTEN METAL-METAL HALIDE SOLUTIONS[1]

Sir:

The formation of slightly stable subhalides by the solution of certain metals in their fused halides

(1) Work was performed in the Ames Laboratory of the Atomic Energy Commission.