atom ($K_0' = 0.013$). On the other hand, the combining constant of quinoline is somewhat higher than that of pyridine ($K_0' = 2.1$). This may be attributed to the large polarizability of quinoline and to the fact that a large part of the benzene ring of quinoline occupies the space *meta* to the annular nitrogen atom, corresponding to the location of the azo group of the immunizing antigen, and can therefore be accommodated by the antibody.

A sketch illustrating the relative closeness of fit of the anti-3AP antibody molecule about various parts of the 3-azopyridine haptenic group is shown in Fig. 2.

BUFFALO 3, N. Y.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, CORNELL UNIVERSITY MEDICAL COLLEGE]

The Synthesis of Lysine-vasopressin[1]

BY VINCENT DU VIGNEAUD,[2] M. FREDERICK BARTLETT AND ALBERT JÖHL

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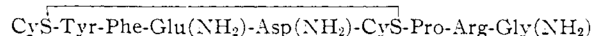
The synthesis of an octapeptide amide with the structure proposed for lysine-vasopressin has been accomplished through the coupling of S-benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine (VIII) with S-benzyl-L-cysteinyl-L-prolyl-N^ϵ-tosyl-L-lysylglycinamide in the presence of N,N'-dicyclohexylcarbodiimide to yield the protected nonapeptide amide, followed by reduction with sodium in liquid ammonia and oxidation. VIII was prepared by the coupling of S-benzyl-N-tosyl-L-cysteinyl-L-tyrosine with L-phenylalanyl-L-glutaminyl-L-asparagine according to the mixed anhydride method. The synthetic octapeptide amide was purified by countercurrent distribution and electrophoresis. Comparison of the chemical, physical and biological behavior of the synthetic product with that of lysine-vasopressin isolated from hog posterior pituitary glands has led to the conclusion that the synthetic product is lysine-vasopressin.

Popenoe, Lawler and du Vigneaud[3] reported the isolation, partial purification and amino acid content of lysine-vasopressin isolated from hog posterior pituitary glands. They found that hydrolysates of lysine-vasopressin contain phenylalanine, tyrosine, proline, glutamic acid, aspartic acid, glycine, lysine and cystine in approximately equimolar quantities and ammonia in a molar ratio of 3 to any one amino acid. This differs from the amino acid content of arginine-vasopressin[4] in the replacement of arginine by lysine.

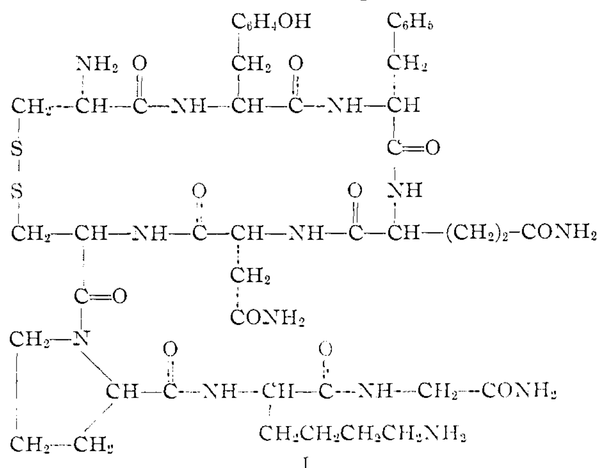
Degradative studies on arginine-vasopressin[5-7] led to the amino acid sequence



CyS-Tyr-Phe-Glu(NH2)-Asp(NH2)-CyS-Pro-Arg-Gly(NH2)

proposed by Popenoe, Lawler and du Vigneaud[3] and also by Acher and Chauvet.[8] The similarity in the results of enzymatic degradation of arginine-vasopressin and lysine-vasopressin and the similarity in the biological behavior of the two vasopressins led du Vigneaud, Lawler and Popenoe[5] to propose structure I for lysine-vasopressin.

The synthesis of an octapeptide having structure I was undertaken by du Vigneaud, Popenoe and Roeske[9] by a pathway which closely paralleled the synthesis of oxytocin.[10] This work led to biologically active material, but in contrast to the synthesis of oxytocin the yield of the active compound was insufficient to allow studies on purification.



I

Another approach to the synthesis of I was made in this Laboratory through the coupling of S-benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine (II)[11] with S-benzyl-L-cysteinyl-L-prolyl-N^ϵ-tosyl-L-lysylglycinamide (III),[12] followed by removal of the protecting groups from the resulting nonapeptide derivative and oxidation to the disulfide octapeptide amide. A synthetic product which assayed approximately 100 pressor units per mg. after purification by electrophoresis and countercurrent distribution was finally obtained. In the course of this work it was observed that some decomposition occurred in the saponification of S-benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosine ethyl ester and a strong odor of

(1) A preliminary report of part of this work has appeared [M. F. Bartlett, A. Jöhl, R. Roeske, R. J. Stedman, F. H. C. Stewart, D. N. Ward and V. du Vigneaud, THIS JOURNAL, **78**, 2905 (1956)].

(2) This work was supported in part by grants from the National Heart Institute, Public Health Service, Grant H-1675, and Lederle Laboratories Division, American Cyanamid Company.

(3) E. A. Popenoe, H. C. Lawler and V. du Vigneaud, THIS JOURNAL, **74**, 3713 (1952).

(4) R. A. Turner, J. G. Pierce and V. du Vigneaud, *J. Biol. Chem.*, **191**, 21 (1951).

(5) V. du Vigneaud, H. C. Lawler and E. A. Popenoe, THIS JOURNAL, **75**, 4880 (1953); E. A. Popenoe and V. du Vigneaud, *J. Biol. Chem.*, **205**, 133 (1953); **206**, 353 (1954).

(6) R. Acher, J. Chauvet and P. Fromageot, *Biochim. et Biophys. Acta*, **9**, 471 (1952).

(7) P. Fromageot, R. Acher, H. Clauser and H. Maier-Hüser, *ibid.*, **12**, 424 (1953).

(8) R. Acher and J. Chauvet, *ibid.*, **12**, 487 (1953).

(9) This approach was reported in a footnote to a communication by du Vigneaud, Lawler and Popenoe, see ref. 5.

(10) V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts and P. G. Katsoyannis, THIS JOURNAL, **76**, 3115 (1954).

(11) P. G. Katsoyannis and V. du Vigneaud, *ibid.*, **78**, 4482 (1956).

(12) R. Roeske, F. H. C. Stewart, R. J. Stedman and V. du Vigneaud, *ibid.*, **78**, 5883 (1956).

benzylmercaptan was apparent. It also was noted that an alkaline solution of S-benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosine became turbid in a short time and the optical rotation underwent a marked change. Since it was thought that the corresponding tosyl dipeptide might be more stable, S-benzyl-N-tosyl-L-cysteinyl-L-tyrosine ethyl ester (IV) was prepared. The saponification proceeded smoothly without apparent decomposition and the resulting S-benzyl-N-tosyl-L-cysteinyl-L-tyrosine (V) was more stable toward alkali than the carbobenzoxy compound.

In the synthesis of lysine-vasopressin to be described herein, the tosyl group was therefore used as the protecting group of the amino group of S-benzyl-L-cysteinyl-L-tyrosine in a series of reactions leading to the tosyl analog of II. The latter compound then was condensed with III to give the desired nonapeptide intermediate, S-benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-N^{ϵ}-tosyl-L-lysylglycinamide (VI). After this approach had been undertaken, Honzl and Rudinger[13] reported the preparation of S-benzyl-N-tosyl-L-cysteine (VII), IV and V as part of a synthetic route to oxytocin and noted a preference for the use of the tosyl group rather than the carbobenzoxy group under conditions involving alkali and hydrazine.

The methods of preparation of VII, IV and V developed independently in the two laboratories were quite similar. As in the work of Honzl and Rudinger,[13] the acid chloride of VII was coupled with ethyl L-tyrosinate to obtain IV. In our work IV was also prepared by the coupling of ethyl L-tyrosinate with VII according to the N,N'-dicyclohexylcarbodiimide method.[14] Saponification of IV gave V in a yield of 95%. V was coupled with L-phenylalanyl-L-glutaminyl-L-asparagine[15] by means of the mixed anhydride procedure with isobutyl chlorocarbonate[16] to give S-benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine (VIII) in crystalline form in 55% yield. Coupling of VIII with the protected tetrapeptide amide III was effected by the N,N'-dicyclohexylcarbodiimide method to obtain the protected nonapeptide amide VI. (A secondary product differing in solubility properties from the protected nonapeptide amide VI was also obtained in this coupling and is being investigated.) The protecting groups were removed from VI by treatment with sodium in liquid ammonia. After evaporation of the ammonia the residue was dissolved in dilute acetic acid and the *p*H was adjusted to 6.5. The sulfhydryl groups in the reduced material were oxidized to the disulfide form by aeration with carbon dioxide-free air, and the solution was concentrated to a small volume and lyophilized. The residue was assayed for pressor activity on the rat. From 300 mg. of VI, reduced and subsequently oxidized, the pressor activity in several experiments ranged from 50,000 to 70,000 units.

(13) J. Honzl and J. Rudinger, *Coll. Czechoslov. Chem. Communs.*, **20**, 1190 (1955).

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

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

(16) J. R. Vaughan, Jr., and J. A. Eichler, *ibid.*, **75**, 5556 (1953).

A product obtained in this manner from 300 mg. of VI and having a pressor activity of approximately 50,000 units was subjected to countercurrent distribution in *sec*-butyl alcohol–0.08 *M* *p*-toluenesulfonic acid[17] for 1450 transfers. Assay for pressor activity indicated that the activity was concentrated in a single peak (*K* 0.64). The *p*-toluenesulfonic acid was removed from the aqueous solutions pooled from the peak area with Amberlite IR-45.[17] The residue obtained after concentration and lyophilization had a pressor activity of approximately 230 units per mg.[18] The activity was not increased by redistribution of the material for 235 transfers (*K* 0.57) in the same solvent system.

In another attempt at purification, material obtained after countercurrent distribution between *sec*-butyl alcohol and 0.08 *M* *p*-toluenesulfonic acid (250 transfers) was submitted to electrophoresis in pyridine–acetate buffer (*p*H 5.6) on a cellulose-supporting medium.[17,19] A slower moving, inactive fraction separated from the pressor component. The material isolated from the segments containing the active fraction possessed an activity of approximately 280 pressor units per mg. Material of equally high activity could be obtained by countercurrent distribution of less pure electrophoretic fractions between *sec*-butyl alcohol and 0.1% acetic acid.

On starch column chromatography of the synthetic material, the expected amino acids were found in molar ratios to each other of approximately 1:1 and ammonia in a molar ratio to any one amino acid of approximately 3:1. Elementary analysis of the material gave values close to those calculated for a diacetate of the hormone. The synthetic material appeared to be a single component on examination by ion-exchange chromatography on IRC-50.[20] The infrared absorption spectra of purified, natural lysine-vasopressin[17,20] and the synthetic material are identical.[21] The natural and synthetic compounds also show the same behavior on countercurrent distribution between *sec*-butyl alcohol and 0.1% acetic acid and have the same electrophoretic mobilities on Whatman No. 1 paper in pyridine–acetate buffer at two *p*H's (5.6 and 4.0).

The synthetic material was assayed for several biological activities against the U. S. P. Standard posterior lobe powder.[18] Repeated assays for pressor activity on the rat,[22] carried out as outlined in the United States Pharmacopeia[23] and by the four-point method of Emmens,[24] gave a value of approximately 280 units per mg. Determination of the avian depressor activity by the method of

(17) D. N. Ward and V. du Vigneaud, *J. Biol. Chem.*, **222**, 951 (1956).

(18) All assay values reported herein are based on an activity of 0.40 unit per mg. for the U. S. Pharmacopeia Posterior Pituitary Standard Powder.

(19) H. G. Kunkel in "Methods of Biochemical Analysis," Vol. I, D. Glick, Ed., Interscience, New York, N. Y., p. 141.

(20) A. Light, R. Acher and V. du Vigneaud, *J. Biol. Chem.*, in press.

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

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

(23) "The Pharmacopeia of the United States of America," 15th revision, Mack Printing Co., Easton, Pa., 1955, p. 776.

(24) C. W. Emmens, "Hormone Assay," Academic Press Inc., New York, N. Y., 1950, p. 18.

Coon[25] indicated a potency of approximately 30 units per mg., and the synthetic product showed an oxytocic activity on the isolated rat uterus, according to the method of Burn,[26] of approximately 4 units per mg. These biological activities agree with those of our best sample of natural lysine-vasopressin within the limits of the assay procedures.

These comparisons of the properties of the synthetic product with those of lysine-vasopressin isolated from hog posterior pituitary glands have led us to conclude that the synthetic product is lysine-vasopressin and that structure I represents that of the hormone.

Experimental[27,28]

S-Benzyl-N-tosyl-L-cysteine (VII).—S-Benzyl-L-cysteine[29] (20 g.) was dissolved in 120 ml. of 3 *N* sodium hydroxide and 450 ml. of water with stirring. A solution of *p*-toluenesulfonyl chloride (32 g.) in ether (100 ml.) was added. The solution was stirred at room temperature for 3 hr. The *p*H was checked at regular intervals, and sodium hydroxide pellets were added if the solution was not alkaline. After 3 hr. the aqueous phase was separated and washed once with ether. The solution was then slowly acidified with 50% hydrochloric acid. The crystalline product was separated by filtration and washed with water until the filtrate was neutral; yield 27.0 g. (78%); m.p. 125–126°; $[\alpha]^{21}D$ +11.3° (*c* 2.00, 95% ethanol). A sample was recrystallized from ethanol–water and acetone–water for analysis. The m.p. remained unchanged.

Anal. Calcd. for $C_{17}H_{19}O_4NS_2$: C, 55.9; H, 5.24; N, 3.83. Found: C, 55.7; H, 5.32; N, 3.82.

S-Benzyl-N-tosyl-L-cysteinyl-L-tyrosine Ethyl Ester (IV).—VII (11 g.) was dissolved in peroxide-free tetrahydrofuran (50 ml.) and the solution was cooled in ice-water. Ethyl L-tyrosinate, prepared from the corresponding hydrochloride (7.5 g.) and triethylamine (4.1 ml.) in tetrahydrofuran (50 ml.), and N,N'-dicyclohexylcarbodiimide (6.9 g.) were added with stirring. Stirring and cooling were continued for 4 hr. The N,N'-dicyclohexylurea was then filtered off, the filtrate was evaporated to dryness *in vacuo* and the residual oil was dissolved in ethyl acetate. The ethyl acetate solution was washed successively with water, 1 *N* hydrochloric acid, water, 5% sodium bicarbonate and water and then dried over sodium sulfate. After the solution was evaporated to a small volume, hexane was added dropwise over a period of about 2 hr. The protected dipeptide ester crystallized in fine needles (14.5 g.). The product was recrystallized from ethyl acetate–hexane and melted at 109–110°; $[\alpha]^{19}D$ +3.71° (*c* 2.39, 95% ethanol).

Anal. Calcd. for $C_{25}H_{32}O_6N_2S_2$: C, 60.4; H, 5.79; N, 5.03. Found: C, 60.4; H, 5.94; N, 5.06.

S-Benzyl-N-tosyl-L-cysteinyl-L-tyrosine (V).—A solution of 2 g. of IV in 6 ml. of acetone was cooled in ice. Sodium hydroxide (2 *N*, 6 ml.) was added in small portions over a period of 20 minutes. The reaction mixture was allowed to stand at room temperature for 40 minutes. The solution was then diluted with water (10 ml.) and acidified with concentrated hydrochloric acid under cooling with ice. The product precipitated as an oil which could be easily solidified with scratching. It was filtered off, washed with water and dissolved in 5% sodium bicarbonate. The solution was extracted with ethyl acetate, and the protected dipeptide was precipitated by acidification with concentrated hydrochloric acid. The material was crystallized from ethanol–water; yield 1.80 g. (95%); m.p. 155–156°; $[\alpha]^{18}D$ +28.2° (*c* 2.11, absolute ethanol), $[\alpha]^{23}D$ +23.6° (*c* 2.85, 95% ethanol).

Anal. Calcd. for $C_{26}H_{28}O_6N_2S_2$: C, 59.1; H, 5.34; N, 5.30. Found: C, 59.1; H, 5.35; N, 5.33.

S-Benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine (VIII).—A solution of 2.64 g. of V in 25 ml. of peroxide-free tetrahydrofuran was cooled to −10°. Triethylamine (0.70 ml.) and isobutylchlorocarbonate (0.65 ml.) were added, and the solution was stirred at −15° for 7 minutes. A solution of L-phenylalanyl-L-glutaminyl-L-asparagine[15] (2.04 g.) in water (10 ml.) and triethylamine (0.75 ml.), cooled to the freezing point, was then added to the mixed anhydride over a period of 1 minute. The solution was stirred at −10° for 5 minutes and at room temperature for 30 minutes. Water was added until the mixture became turbid. It was then acidified with 50% hydrochloric acid to *p*H 2, diluted to a volume of 100 ml. with water and cooled at 0° for 4 hr. The precipitate was separated by filtration and washed with water until the *p*H of the washings was greater than 4. The dried material was triturated with ethyl acetate; yield 2.84 g. (62%); m.p. 203–205°.

Another reaction was performed with the same quantities. The products from both runs were combined and dissolved in peroxide-free 90% tetrahydrofuran. The solution was concentrated to one-third volume under reduced pressure and the protected pentapeptide VIII was obtained in crystalline form; yield 5.0 g. (55%); m.p. 203–205°; $[\alpha]^{21}D$ +4.4° (*c* 2.08, dimethylformamide).

Anal. Calcd. for $C_{44}H_{51}O_{11}N_7S_2 \cdot {}^1/_2H_2O$: C, 57.0; H, 5.65; N, 10.6. Found: C, 57.1; H, 5.66; N, 10.6.

S-Benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-N$^\epsilon$-tosyl-L-lysylglycinamide (VI).—VIII (1.84 g.) and S-benzyl-L-cysteinyl-L-prolyl-N$^\epsilon$-tosyl-L-lysylglycinamide[12] (1.29 g.) were dissolved in peroxide-free 90% tetrahydrofuran (100 ml.) at room temperature. N,N'-Dicyclohexylcarbodiimide (0.62 g.) was added to the ice-cooled solution. Stirring and cooling were maintained for 4 hr. The reaction mixture was then stirred for 20 hr. at room temperature. The solution was concentrated to a volume of about 20 ml. *in vacuo* and cooled for 1 hr. The resulting precipitate was separated by filtration and washed with 90% tetrahydrofuran. On extraction of this material with dimethylformamide (20 ml.) N,N'-dicyclohexylurea separated and was filtered off. The crude protected nonapeptide VI precipitated from the filtrate on the addition of 80 ml. of ethyl acetate followed by cooling overnight. The material was separated by filtration and washed with ethyl acetate; yield 1.30 g. (42%); m.p. 224–226°. The compound was dissolved in formic acid (5 ml.), precipitated by the addition of water (20 ml.), collected on a filter pad, washed with water and dried over P_2O_5; yield 1.21 g. (39%), m.p. 226–230°; $[\alpha]^{18}D$ −23.0° (*c* 2.11, dimethylformamide).

Anal. 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.

Reduction of the Protected Nonapeptide and Subsequent Oxidation.—VI (300 mg.) was dissolved in liquid ammonia (100 ml.), previously distilled from sodium. The solution was stirred with a magnetic stirrer. A piece of glass tubing filled with sodium was inserted into the flask through a side arm. The end of the sodium stick was intermittently pushed below the surface of the liquid until a permanent blue color remained for 1 to 2 minutes. Ammonium chloride was added in order to discharge the blue color. The liquid ammonia was allowed to evaporate at atmospheric pressure and the residue was finally dried *in vacuo*. The reduction product was dissolved in 1% acetic acid (30 ml.) and quantitatively washed into a beaker with distilled water. The volume of the solution was increased to 300 ml. with distilled water and the *p*H was adjusted from 4.5 to 6.5 with ammonium hydroxide. A slow stream of CO_2-free air was passed into the stirred solution. After 1 hr. the mixture gave a negative reaction to the nitroprusside test. The solution was concentrated to a small volume on a rotary evaporator[30] at a bath temperature of 30° and then lyophilized. The residue contained approximately 60,000 units of pressor activity. The reduction and aeration were repeated a number of times with essentially the same results.

Purification of the Biologically Active Compound.—A product obtained as already described from 300 mg. of VI and containing approximately 50,000 units of pressor activity

(25) J. M. Coon, *Arch. intern. pharmacodynamie*, **62**, 79 (1939).

(26) J. H. Burn, *Quart. J. Pharm. Pharmacol.*, **4**, 517 (1931); J. H. Burn, D. J. Zinney and L. G. Goodwin, "Biological Standardization," 2nd Ed., Oxford University Press, London, 1950, p. 180.

(27) Capillary melting points were determined for all compounds and are corrected.

(28) The authors are indebted to Mr. Joseph Albert of this laboratory for carrying out the analyses.

(29) J. L. Wood and V. du Vigneaud, *J. Biol. Chem.*, **130**, 110 (1939).

(30) L. C. Craig, J. C. Gregory and W. Hausmann, *Anal. Chem.*, **22**, 1462 (1950).

was placed in the first 9 tubes of an all-glass countercurrent distribution apparatus[31] and distributed for 1450 transfers in the system *sec*-butyl alcohol–0.08 *M* *p*-toluenesulfonic acid.[17] The pressor activity was concentrated in a single peak (*K* 0.64). The modified Folin color curve[32] approximated the activity curve closely, except for the indication of the presence of a small amount of other material, possibly due to decomposition. The contents of 36 tubes included in the peak were placed in a separatory funnel, and ether was added to facilitate separation of the phases. The aqueous layer was withdrawn, the organic phase was washed twice with water and the washings were added to the aqueous phase. The *p*-toluenesulfonic acid was removed by passage of the solution through a column of Amberlite IR-45 (acetate).[17] The eluate (approximately 16,000 pressor units) was concentrated on a rotary evaporator[30] and lyophilized. The weight of the dry residue was 69 mg.; 1.16 mg. of this material was dissolved in 0.25% acetic acid (50 ml.) and tested for pressor activity. The activity was found to be approximately 230 units per mg. A sample was redistributed in *sec*-butyl alcohol–0.08 *M* *p*-toluenesulfonic acid for 235 transfers (*K* 0.57). The biologically active product was isolated in the same way and the specific activity was found to be approximately the same as before the additional distribution step.

In another purification, 2.0 g. of crude material (approximately 150,000 pressor units) resulting from the sodium–liquid ammonia reduction of the protected nonapeptide was used. After distribution between *sec*-butyl alcohol and 0.08 *M* *p*-toluenesulfonic acid for 250 transfers (*K* 0.82), the material (1.2 g.) had a pressor activity of 125 to 135 units per mg. and contained considerable inorganic contamination. A portion of this material (170 mg., 21,000–23,000 pressor units) was subjected to electrophoresis on a cellulose-supporting medium in pyridine–acetate buffer (*p*H 5.6; ionic strength, 0.4 *M*).[17,19] The material was dissolved in 1 ml. of buffer and placed 2 cm. from the anode end of a previously prepared cellulose block (10 cm. × 46 cm. × 10 cm.). A current of 400 volts and 70–80 milliamperes was applied for 43 hr. The block was then cut into segments (2 cm.) starting from the origin. The buffered solution was pressed from each segment and the cellulose was washed once with 1% acetic acid (7 ml.). The pressor activity, modified Folin color and weight of aliquots of each segment were determined. The activity was found to be concentrated in segments 11, 12 and 13. A second compound having no pressor activity appeared in small quantities in segments 2 to 9 with a peak in segments 6 to 7. The solutions from segments 11, 12 and 13 were lyophilized separately, the weight of material isolated being 14, 24 and 14 mg., respectively. Assays on a sample from segment 12 gave an activity of approximately 280 pressor units per mg. Samples from the other two segments were found to have approximately the same activity.

The electrophoresis of the material previously distributed between *sec*-butyl alcohol and 0.08 *M* *p*-toluenesulfonic acid (250 transfers) was repeated on a larger scale (60,000 pressor units). Material (55 mg.) having a pressor activity of approximately 280 units per mg. was obtained from the peak segments. The material obtained from the solution extracted from the trailing side of the peak was found to have a somewhat lower specific activity and was purified by countercurrent distribution in *sec*-butyl alcohol–0.1% acetic acid. Two components separated after 600 transfers, one having no pressor activity (*K* 0.017) and the other (*K* 0.66) containing all the activity and about 70% of the weight of the material before distribution. Pressor assays indicated an activity of approximately 280 units per mg.

Amino acid analysis[33] of an hydrolysate of the material showed the following amino acid content, expressed in molar ratios (with the ratio for phenylalanine arbitrarily taken as 1): phenylalanine 1.00, tyrosine 0.88, proline 0.91, glutamic acid 1.03, aspartic acid 1.08, glycine 1.05, lysine 0.98, cystine 0.68 and ammonia 3.28.

The specific rotation of the synthetic material was $[\alpha]^{20}_D$ $-47.5°$ (c 0.99, H_2O).

A sample of the hygroscopic product was dried at room temperature *in vacuo* over P_2O_5 for 18 hr.

Anal. Calcd. for $C_{46}H_{65}N_{13}O_{12}S_2 \cdot (C_2H_4O_2)_2$: C, 51.0; H, 6.25; N, 15.5. Found: C, 50.5; H, 6.27; N, 14.9.

(31) L. C. Craig, W. Hausmann, E. H. Ahrens, Jr., and E. J. Harfenist, *Anal. Chem.*, **23**, 1236 (1951).

(32) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

(33) S. Moore and W. H. Stein, *ibid.*, **178**, 53 (1949).

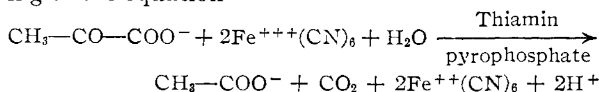
NEW YORK 21, N. Y.

COMMUNICATIONS TO THE EDITOR

ACTIVATION OF A FERRICYANIDE LINKED PYRUVATE OXIDASE BY α-TOCOPHEROL ESTERS

Sir:

A purified soluble enzyme and particulate fraction obtained from cell extracts of an acetate requiring mutant of *Escherichia coli* combine to catalyze the ferricyanide linked oxidative decarboxylation of pyruvate to acetate and CO_2 according to the equation[1,2]



$$CH_3{-}CO{-}COO^- + 2Fe^{+++}(CN)_6 + H_2O \xrightarrow[\text{pyrophosphate}]{\text{Thiamin}} CH_3{-}COO^- + CO_2 + 2Fe^{++}(CN)_6 + 2H^+$$

In an attempt to obtain soluble enzyme preparations from the particulate fraction, acetone-dried particles were prepared by extracting aqueous suspensions of the particles with 20 volumes of acetone at $-20°$. The dried particles obtained by this procedure were essentially inactive; however, the acetone residue obtained by removal of the acetone under reduced pressure, activated the soluble enzyme in the absence of the particulate fraction. By analogy to the work of Nason and Lehman[3,4] on the activation of the diphosphopyridine nucleotide–cytochrome C reductase system by α-tocopherol, α-tocopherol and various vitamin E derivatives, as well as other fat soluble vitamins and oxidation–reduction coenzymes were tested for their ability to replace the factor or factors present in the acetone residue. Among a large number of compounds tested, only α-tocopherol phosphate and α-tocopherol succinate were active. These results are summarized in Table I. The rate of pyruvic acid oxidation is a function of the concentration of the α-tocopherol ester or acetone residue as shown in Fig. 1.

(1) L. P. Hager, *Federation Proc.*, **16**, 190 (1957).

(2) L. P. Hager, *J. Biol. Chem.*, in press.

(3) A. Nason and I. R. Lehman, *Science*, **122**, 19 (1955).

(4) A. Nason and I. R. Lehman, *J. Biol. Chem.*, **222**, 54 (1956).