in the pharmacology of the veratrum alkaloids[3] and in the clinical use of the hypotensive activity of the tertiary ester alkaloids,[4] it appeared of importance to isolate and study the active principles of *Zygadenus venenosus*.

Heyl and co-workers[5] isolated the first crystalline alkaloid in this series, zygadenine. It appeared unlikely, however, that zygadenine is the most important toxic agent in *Zygadenus venenosus*.[5a]

The probable presence in *Zygadenus venenosus* of ester alkaloids similar to the tertiary alkamine ester alkaloids of the veratrum series has been indicated by the pharmacodynamic properties of the alkaloidal mixtures.[6] We have now isolated two of the ester alkaloids responsible for this activity.

The chloroform-extractable alkaloids of *Zygadenus venenosus* (WATS)[7] were subjected to simplified 8-plate countercurrent distribution patterned on the procedure of Fried, White and Wintersteiner.[4b] Benzene and phosphate buffer of *p*H 7.1 were used as solvents. Veratroylzygadenine separated from a solution of the crude plate 8-fraction in acetone; germine and zygadenine were obtained from the plate 0-fraction.

Veratroylzygadenine crystallized from absolute ethanol as rectangular prisms, m.p. 270–271° dec.; $[\alpha]^{20}D$ −27° (*c* 2.08, chf.); $\lambda_{max.}^{alc.}$ 262, 293 mμ (log ε 4.13; 3.85). *Anal.* Calcd. $C_{36}H_{51}O_{10}N$: C, 65.73; H, 7.82; N, 2.13. Found: C, 65.90; H, 7.86; N, 2.09. Alkaline hydrolysis of veratroylzygadenine yielded veratric acid and a base isomeric with zygadenine, pseudozygadenine. This base was also obtained by similar alkaline treatment of zygadenine. Pseudozygadenine crystallized from ethyl acetate–petroleum ether as needles, m.p. 169–171° dec.; $[\alpha]^{25}D$ −33° (*c* 2.00, chf.). *Anal.* Calcd. $C_{27}H_{43}O_7N$: C, 65.69; H, 8.78; N, 2.84. Found: C, 65.46, 65.79; H, 9.10, 8.69; N, 2.95. Acetylation with acetic anhydride and pyridine gave pseudozygadenine triacetate which crystallized from ether as rhomboids, m.p. 235–236° dec.; $[\alpha]^{23}D$ −33° (*c* 1.89, chf.). *Anal.* Calcd. for $C_{27}H_{40}O_7N(COCH_3)_3$: C, 63.95, H, 7.97; acetyl, 20.84. Found: C, 64.13; H, 8.11; acetyl, 20.69.

The filtrate obtained by removal of veratroylzygadenine from the plate 8-fraction was lyophilized and the residue was dissolved in chloroform and chromatographed on acid-washed alumina. Vanilloylzygadenine crystallized from an ethanol solution of the most difficultly eluted fractions as rods, m.p. 258–259° dec.; $[\alpha]^{20}D$ −27.5° (*c* 2.00, chf.); $\lambda_{max.}^{alc.}$ 264, 294 mμ (log ε 4.07, 3.83). *Anal.* Calcd. $C_{35}H_{49}O_{10}N$; C, 65.30; H, 7.67; N, 2.18; 1 $OCH_3$, 4.82. Found: C, 65.35; H, 7.93; N, 2.29; $OCH_3$, 4.34. Alkaline hydrolysis yielded vanillic acid and pseudozygadenine. Methylation of vanilloylzygadenine with diazomethane gave veratroylzygadenine, identical with an authentic sample by m.p., mixed m.p. and infrared spectrum.

The two ester alkaloids and zygadenine were examined by Professor O. Krayer at Harvard Medical School for their circulatory action in the anesthetized cat, their effect upon the failing heart in the heart-lung preparation of the dog, and their effect upon the amphibian skeletal muscle. In all three types of experiments, the actions of zygadenine were similar to those of cevine, and the actions of veratroylzygadenine and vanilloylzygadenine were similar, quantitatively and qualitatively, to the actions of the cevine ester, veratridine.

This work was supported by grants from Research Corporation and the National Institutes of Health, and the assistance of Eli Lilly and Company and Riker Laboratories, Inc., in gathering and extracting *Zygadenus venenosus* is gratefully acknowledged.

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RECEIVED MARCH 24, 1952

(3) O. Krayer and G. Acheson, *Physiol. Rev.*, **26**, 383 (1946); G. L. Maison, E. Gatz and J. W. Stutzman, *J. Pharmacol. Exptl. Therapy*, **103**, 74 (1951).

(4) (a) E. Meilman and O. Krayer, *Circulation*, **1**, 204 (1950); (b) J. Fried, H. L. White and O. Wintersteiner, THIS JOURNAL, **72**, 4621 (1950).

(5) (a) F. W. Heyl, F. E. Hepner and S. K. Loy, *ibid.*, **35**, 258 (1913); (b) F. W. Heyl and M. E. Herr, *ibid.*, **71**, 1751 (1949).

(6) S. Vaffe and S. M. Kupchan, *Federation Proc.*, **9**, 326 (1950).

(7) Plant gathered in Washington in June, 1950. We are grateful to Dr. Reed Rollins, Grey Herbarium, Harvard University, for confirming the identity of the plant.

## IDENTIFICATION OF THE CARBON SKELETON OF α-LIPOIC ACID

*Sir:*

It has been reported[1] recently that α-lipoic acid, a catalytic agent required for oxidative decarboxylation of pyruvic acid by certain lactic acid bacteria, is a monocarboxylic acid, *pKa* 4.7, containing a disulfide linkage and possessing the empirical formula $C_8H_{14}O_2S_2$. Infrared absorption spectrum failed to show the presence of any carbon–carbon double bonds. These data indicate that α-lipoic acid is a cyclic disulfide. The *pKa* value indicates that the sulfur atom is not attached to the carbon atoms α- or β- to the carboxyl group. Comparison of the polarography of α-lipoic acid with that of several dithiols and cyclic and linear disulfides also indicated its structure to be that of a cyclic disulfide. The presence of a six-membered ring in α-lipoic acid was suggested by the following observation. The catalysis of hydrogen ion reduction at the dropping mercury electrode by the reduced form of α-lipoic acid resembled that of 1,4-dithiols more than 1,3- or 1,5-dithiols.

Even with these limitations, the number of structures possessing the empirical formula of α-lipoic acid is considerable. It was therefore of paramount importance to determine the nature of the carbon skeleton. Assuming the cyclic disulfide nature of the molecule, much information could be obtained by the desulfurization of the substance. A 3-mg. sample was subjected to treatment with Raney nickel to remove the sulfur atoms.[2] The product was isolated as the crystalline silver salt. A comparison of the X-ray powder diagrams of this material with that of silver *n*-caprylate[3] revealed that the two samples were identical. The silver

(1) L. J. Reed, B. G. DeBusk, I. C. Gunsalus and G. H. F. Schnakenberg, THIS JOURNAL, **73**, 5920 (1951).

(2) R. Mozingo, D. E. Wolf, S. A. Harris and K. Folkers, *ibid.*, **65**, 1013 (1943).

(3) F. W. Matthews, G. G. Warren and J. H. Michell, *Anal. Chem.*, **22**, 514 (1950).

salt of α-lipoic acid, prepared in a similar manner, was amorphous.

Biosynthetic studies[4] provided the initial evidence favoring the straight chain carbon skeleton. A mutant strain of *Escherichia coli*, which can synthesize α-lipoic acid but which requires a more complex form of this factor for growth, produces appreciably more α-lipoic acid in the presence of acetate or more particularly 2,4,6-octatrienoic acid. This effect was observed in resting cell suspensions and was favored by the presence of cysteine. *n*-Caprylic acid and pyruvate were inactive in these tests.

Further confirmation of this structure was obtained by comparing the infrared spectrum of α-lipoic acid with that of various fatty acids. The infrared spectrum revealed no resolved methyl absorption at high dispersion in the 3.4 λ region.

It has thus been established that the carbon skeleton of α-lipoic acid is the straight chain $C_8$ acid, and α-lipoic acid is therefore the intramolecular disulfide of a dimercapto-*n*-octanoic acid, unsubstituted in the α- and β-positions.

The optical activity of crystalline α-lipoic acid was found to be $[\alpha]^{25}D$ +96.7° (1.88% in benzene.

(4) L. J. Reed and B. G. DeBusk, unpublished results.

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## ENZYMATIC PYROPHOSPHORYLATION OF COENZYME A BY ADENOSINE TRIPHOSPHATE[1]

*Sir:*

It is well known that acetate may be enzymatically activated by a reaction chain involving adenosine triphosphate (ATP) and coenzyme A (CoA). Acetyl-CoA was found to be the product of this reaction,[2] the mechanism of which became of particular interest since Lynen, *et al.*,[3,4] have shown acetyl-CoA to be an acetyl mercaptoester.

We studied the ATP-CoA-acetate reaction with an enzyme obtained from yeast extract by protamine and ammonium sulfate fractionation. The generation of acetyl-CoA was followed by the use of hydroxylamine as chemical acetyl-acceptor,[2] determining acethydroxamic acid, according to Lipmann and Tuttle.[5] An important lead toward the understanding of the mechanism was obtained when it was found that acethydroxamic acid may accumulate without an equivalent liberation of inorganic phosphate from ATP. In the presence of fluoride, ATP was found to be converted to an acid-labile phosphate, identified eventually as inorganic pyrophosphate, and to adenosine monophosphate (AMP). Fluoride preserves the pyrophosphate by inhibiting pyrophosphatase, which is a contaminant of our enzyme. A balance experiment is shown in Table I.

ATP was determined by the hexokinase-hexosemonophosphate-dehydrogenase-TPN procedure of Kornberg,[6] AMP spectrophotometrically according to Kalckar[7] using Schmidt's deaminase. Pyrophosphate was determined by manganese precipitation according to Kornberg.[6] The pyrophosphate was further identified by the use of a five times recrystallized pyrophosphatase,[8] kindly supplied to us by Dr. Kunitz.

TABLE I

Each vessel contained: 29 μM ATP, 250 μM acetate, 860 μM $NH_2OH$ (*p*H 6.5), 80 μM glutathione, 160 μM potassium fluoride, 640 μM tris-(hydroxymethyl)-aminomethane buffer (*p*H 7.4), and 32 μM $MgCl_2$ in 3.2 ml. total volume. Each vessel contained 0.32 ml. of the yeast enzyme.

| CoA, units | Incubation time, min. | ATP, μM | AMP, μM | Acethydroxamic acid, μM | Pyrophosphate, μM |
|---|---|---|---|---|---|
| 0 | 0 | 29.0 | 0.2 | 0 | 0 |
| | 150 | 22.9 | 1.2 | 2.7 | 0 |
| 290 | 0 | 28.7 | 2.8 | 0 | 0 |
| | 150 | 0.4 | 19.7 | 32.5 | 24.9<br>23.1[a] |

[a] Value determined with pyrophosphatase.

In view of these results, the ATP-CoA-acetate reaction is formulated as a two-step reaction

$$\text{ATP} + \text{CoA} \rightleftharpoons \text{AMP} + \text{CoA-pyrophosphate} \quad (1)$$

$$\text{CoA-pyrophosphate} + \text{acetate} \rightleftharpoons \text{acetyl-CoA} + \text{pyrophosphate} \quad (2)$$

The exchange of pyrophosphate for acetyl in acetyl-CoA as indicated by reverse reaction (2) was confirmed in the following experiment:

TABLE II

Each sample contained, per 5 ml.: 1.8 μM acetyl-CoA, 200 μM potassium pyrophosphate or arsenate or phosphate, 200 μM magnesium chloride, 100 μM potassium fluoride, 1 ml. yeast enzyme. Incubate 30 minutes at 37°. The *p*H was 7.1.

| | μM acetyl-CoA[a] |
|---|---|
| Initial | 1.8 |
| Incubated with pyrophosphate | 0.5 |
| Substituted arsenate for pyrophosphate | 1.85 |
| Substituted phosphate for pyrophosphate | 1.8 |

[a] Determined as acethydroxamic acid.

When, after partial conversion of acetyl-CoA to CoA-pyrophosphate, excess acetate was added and reincubated, acetyl-CoA reformed. The equilibrium between acetyl-CoA and pyrophosphate is in favor of the acetyl compound. Nevertheless, if acetyl phosphate with Stadtman's transacetylase[9] is used as acetyl "feeder," CoA, pyrophosphate,

(1) This work was supported by the National Cancer Institute of the National Institutes of Health, Public Health Service; the Atomic Energy Commission; and the National Foundation for Infantile Paralysis.

(2) T. C. Chou and F. Lipmann, *J. Biol. Chem.*, **196**, 89 (1952).
(3) F. Lynen and E. Reichert, *Angew. Chem.*, **63**, 47 (1951).
(4) F. Lynen, E. Reichert and L. Rueff, *Ann.*, **574**, 1 (1951).
(5) F. Lipmann and L. C. Tuttle, *J. Biol. Chem.*, **158**, 505 (1945).
(6) A. Kornberg, *ibid.*, **182**, 779 (1950).
(7) H. M. Kalckar, *ibid.*, **167**, 445 (1947).
(8) M. Kunitz, *J. Gen. Physiol.*, **35**, 423 (1952).
(9) E. R. Stadtman, G. D. Novelli and F. Lipmann, *J. Biol. Chem.*, **191**, 365 (1951).