anol was heated under reflux for one hour. Three milliliters of glacial acetic acid was added to the warm, yellow reaction solution, followed by sufficient hot water to induce crystallization. After cooling, the solution was filtered and the light yellow crystalline precipitate recrystallized from absolute ethanol. 3-Benzyl-6,7-diphenyl-4(3*H*)-pteridinone (I) (0.26 g., 25%) separated as colorless platelets from the warm ethanol; m.p. 248°. A mixed melting point with an authentic sample of I[1] showed no depression. Addition of a small amount of water to the ethanol filtrate and further cooling caused the separation of 0.19 g. of 3-amino-N-benzyl-5,6-diphenylpyrazinamide (III); m.p. 187°.

The mother liquor from the original reaction mixture above was diluted with an equal volume of water. A heavy, tacky yellow solid separated which was collected by filtration and extracted with 20 ml. of hot 1 *N* sodium hydroxide. Acidification of the filtrate precipitated 0.195 g. of unreacted 6,7-diphenyl-4(3*H*)-pteridinone (II), while repeated recrystallizations of the base-insoluble solid yielded an additional 0.11 g. (total yield 29%) of pure III.

In a second experiment, a mixture of 40 ml. of freshly prepared, anhydrous methanol, 0.793 g. (0.00264 mole) of 6,7-diphenyl-4(3*H*)-pteridinone, 0.301 ml. (0.00262 mole) of benzyl chloride and 0.148 g. (0.00264 mole) of potassium hydroxide was heated under reflux for 24 hours. By the end of this time, the reaction mixture was only faintly basic. Addition of a few drops of acetic acid to acidity followed by water caused the crystallization of light yellow crystals; yield 0.530 g.; m.p. 230–238°. Repeated recrystallizations from methanol gave 0.21 g. (20%) of pure I melting sharply at 248°.

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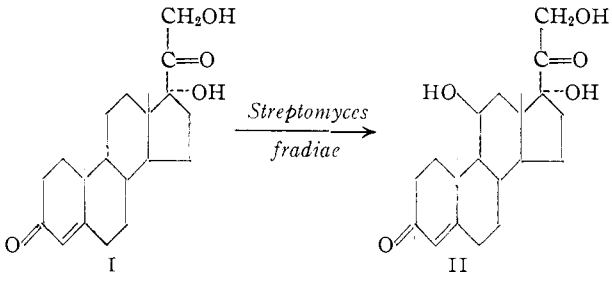
COMMUNICATIONS TO THE EDITOR

A PARTIAL MICROBIOLOGICAL SYNTHESIS OF ADRENAL CORTEX HORMONES

Sir:

It appears that either a hydroxyl group, having the *beta* configuration, or a ketone at the 11-position of the steroid nucleus is an obligatory structural requirement for the so-called carbohydrate-regulating hormone activity of the adrenal steroids, corticosterone, 11-dehydrocorticosterone, 17-hydroxycorticosterone and 11-dehydro-17-hydroxycorticosterone. Since both 11-desoxycorticosterone and 11-desoxy-17-hydroxycorticosterone are essentially devoid of this type of bioactivity, as measured by the Rat Liver Glycogen Deposition Assay,[1] it is possible to detect the introduction of an 11-β-hydroxyl group or an 11-keto group into these compounds by means of this assay.

We wish to report evidence for the microbiological oxygenation of these latter two steroids, with particular emphasis on the conversion of 11-desoxy-17-hydroxycorticosterone (Reichstein's compound S) (I) to 17-hydroxycorticosterone (Kendall's compound F, hydrocortisone) (II) by *Streptomyces fradiae*, Waksman's strain 3535.



Several species of *Streptomyces* were incubated with 100-mg. quantities of 11-desoxycorticosterone and 11-desoxy-17-hydroxycorticosterone. The quantitative measurement of glycogen deposition activity in the resulting beers[2] and calculation of this bioactivity in terms of a theoretical conversion to corticosterone and 17-hydroxycorticosterone gave values which varied from 1.4 to 5.8%.

In an experiment of somewhat larger scale 5.0 g. of I was incubated with *Streptomyces fradiae*, strain 3535, for 7 hours at 24° in rotary shaker flasks, using a medium containing dextrose, soybean meal and distillers' solubles. The total volume of the beer was 15 liters. A neutral hormone concentrate which was obtained from the beer by a standard procedure,[3,4] weighed 4.86 g. and possessed total bioactivity equivalent to 140 mg. of 17-hydroxycorticosterone. Evaluation of this material by paper chromatography[5,6,7] indicated the presence of II, a trace of 11-dehydro-17-hydroxycorticosterone and unreacted I.

One-half of the neutral hormone concentrate (2.43 g.) was subjected to automatic partition column chromatography.[8] The three adrenal steroids mentioned above were found in individual bands in the resulting chromatogram. The "17-hydroxycorticosterone band" weighed 110 mg. First crop crystals from acetone (22.8 mg.) were identified as II. Evidence for this characterization was afforded by long-term paper chromatography in which fermentation product moved at a rate identical with authentic II. In addition, a mixture of the fermentation product and authentic II could not be resolved under any of several conditions. Furthermore, the data from infrared spectroscopy,[9] as shown in Fig. 1, provided additional evidence for the identification of the crystalline product from

(1) M. L. Pabst, R. Sheppard and M. H. Kuizenga, *Endocrinology*, **41**, 55 (1947).

(2) We are indebted to Dr. K. J. Olson and his staff for the bioassays reported herein.

(3) M. H. Kuizenga, A. N. Wick, D. J. Ingle, J. W. Nelson and G. F. Cartland, *J. Biol. Chem.*, **147**, 561 (1943).

(4) W. J. Haines, R. H. Johnson, M. P. Goodwin and M. H. Kuizenga, *ibid.*, **174**, 925 (1948).

(5) A. Zaffaroni, R. B. Burton and E. H. Keutmann, *Science*, **111**, 6 (1950).

(6) R. B. Burton, A. Zaffaroni and E. H. Keutmann, *J. Biol. Chem.*, **188**, 763 (1951).

(7) W. J. Haines and N. A. Drake, *Fed. Proc.*, **9**, 180 (1950).

(8) W. J. Haines, N. A. Drake, C. D. Alway and M. P. Brunner, *Abstracts of Papers*, 118th Meeting Am. Chem. Soc., Chicago, Illinois, Sept. 1950, p. 11-M.

(9) We are indebted to Dr. J. L. Johnson and his staff for the infrared data reported herein.

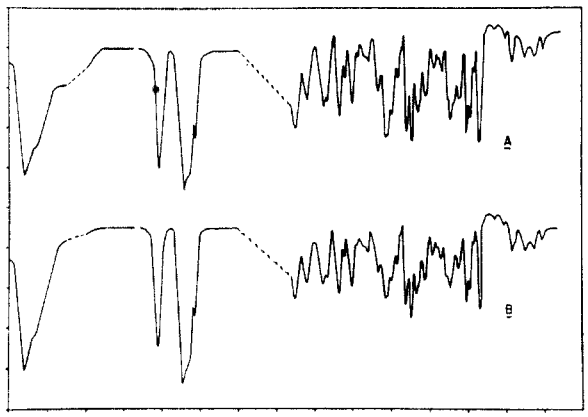


Fig. 1.—Infrared absorption spectra (Nujol mulls), Perkin Elmer spectrophotometer, model 12C: Curve A, authentic 17-hydroxycorticosterone; Curve B, crystalline product from *Streptomyces fradiae* conversion of 11-desoxy-17-hydroxycorticosterone.

the *Streptomyces fradiae* conversion as 17-hydroxycorticosterone.

The authors wish to acknowledge the helpful interest shown in this work by Dr. J. S. Evans.[10]

(10) Attention is directed to the microbiological oxidation of steroids at carbon 11, using fungi of the order *Mucorales*, as reported by Peterson and Murray, THIS JOURNAL, **74**, 1871 (1952).

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RECEIVED APRIL 16, 1952

SYNTHETIC PREPARATION OF LIPOIC ACID

Sir:

Alpha lipoic acid, a catalytic agent, possessing pyruvate oxidation factor activity[1] has been obtained in crystalline form, and identified as a cyclic disulfide containing an *n*-octanoic acid carbon chain.[2,3] Physical data have been reported[3] which may be interpreted as follows: (a) *pKa* 4.7; no sulfur atom attached to carbon α or β to the carboxyl group, (b) lack of resolved methyl at 3.4μ; carbon 8 of the octanoic acid chain is probably substituted, (c) polarographic half-wave potential and hydrogen ion reduction potentials more nearly correspond to the values for 6-membered than to 5- or 7-membered disulfide rings, (d) $[\alpha]^{20}D$ +96.7; at least 1 center of asymmetry is indicated.

The following synthetic approach was used to confirm the presence of an 8-membered carbon chain in lipoic acid and to gain further insight into the location of the sulfur atoms. The 4-(α-tetrahydrofuryl)-butyric, 3-α-(α'-methyltetrahydrofuryl)-propionic, and 3-(α-tetrahydropyranyl)-propionic acids were prepared. These ether-acids were treated with hydrobromic acid and thiourea[4] to give thiouranium salts which were hydrolyzed without isolation to unstable dithioloctanoic acids, presumably 5,8-, 4,7- and 4,8-dithioloctanoic acids, respectively.

After spontaneous air oxidation in dilute solution, the preparations were assayed for biological activity in the pyruvate oxidation factor assay.[5] In one experiment, a 1-g. sample of each ether-acid was treated with one gram of thiourea and one milliliter of 40 per cent. hydrobromic acid in a sealed tube at 120° for ninety minutes, followed by hydrolysis with twenty-five milliliters of concentrated ammonium hydroxide at 120° for forty-five minutes in the presence of a trace of ferrous sulfate. The yields of pyruvate oxidation factor activity, "lipoic acid," were as follows

4-(α-Tetrahydrofuryl)-butyric acid	20,000 units
3-α-(α'-Methyltetrahydrofuryl)-propionic acid	500 units
3-(α-Tetrahydropyranyl)-propionic acid	250

Under similar conditions with a twelve-hour heating period, 0.5 g. of 4-(α-tetrahydrofuryl)-butyric acid gave 1,200,000 units of activity. These observations favor one of the optical isomers of the cyclic disulfide derived from 5,8-dithioloctanoic acid as the structure of α-lipoic acid.

The active material, obtained from 4-(α-tetrahydrofuryl)-butyric acid, in these and similar preparations, showed a behavior in the bioautographic[6] and counter-current[7] procedures characteristic of α-lipoic acid; including the formation of a more polar material[2] referred to as "β-lipoic acid." In the pyruvate oxidation factor assay, an excess (5 units) of the synthetic preparations and of crystalline α-lipoic acid obtained from liver each activated the assay maximally. Increasing levels of crystalline α-lipoic acid and of the synthetic preparations gave similar activity-concentration curves characterized by a K_m approximating 10^{-8} mole/liter by the dried cell assay method.[8]

(5) I. C. Gunsalus, M. I. Dolin and L. Struglia, *J. Biol. Chem.*, **194**, 849 (1952).

(6) L. J. Reed, *et al.*, *J. Biol. Chem.*, **192**, 851 (1951).

(7) I. C. Gunsalus, L. Struglia and D. J. O'Kane, *ibid.*, **194**, 859 (1952).

(8) I. C. Gunsalus and G. H. F. Schnakenberg, unpublished work.

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RECEIVED APRIL 16, 1952

ZYGADENUS ALKALOIDS. I. VERATROYLZYGADENINE AND VANILLOYLZYGADENINE, TWO NEW HYPOTENSIVE ESTER ALKALOIDS FROM ZYGADENUS VENENOSUS

Sir:

The plant species *Zygadenus venenosus* has long been known to possess principles which are poisonous to livestock.[1] Some fifty years ago, the observation was made that these active principles possess pharmacological activity resembling that of the veratrum alkaloids.[2] In view of the recent interest

(1) U. S. Dep. Agr. Bull. 125 (1915); 1210 (1924); 1376 (1926).

(2) R. Hunt, *Am. J. Physiol.*, **6**, XIX (1902).

(1) L. J. Reed, B. G. DeBusk, I. C. Gunsalus and C. S. Hornberger, Jr., *Science*, **114**, 93 (1951).

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

(3) L. J. Reed, Q. F. Soper, G. H. F. Schnakenberg, S. F. Kern, H. Boaz and I. C. Gunsalus, *ibid.*, **74**, 2383 (1950).

(4) R. L. Frank and P. V. Smith, *ibid.*, **68**, 2103 (1946).

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. Yaffe 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. C. 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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RECEIVED MARCH 29, 1952

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 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).

and AMP will yield considerable quantities of ATP by a reversal of reactions (2) and (1).

Experiments with pigeon liver extract have shown that the mechanism of the ATP-CoA-acetate reaction there is the same as described here. Preliminary studies of the properties of CoA-pyrophosphate have been made. The compound is stable to acid at room temperature and may be heated at neutral reaction with only slight loss of activity. Further characterization by use of chromatography is in progress.

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(10) Fellow of the Atomic Energy Commission.

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ACTIVATION OF PURIFIED GLUTAMIC-ASPARTIC APOTRANSAMINASE BY CRYSTALLINE PYRIDOXAMINE PHOSPHATE

Sir:

Several years ago, Snell[1,2] suggested that reversible interconversion between pyridoxamine and pyridoxal might be involved in the mechanism of enzymatic transamination. There is now ample evidence that pyridoxal phosphate (PALPO) is a coenzyme of transaminase. However, Umbreit, *et al.*,[3] found that although impure preparations of PALPO and pyridoxamine phosphate (PAMPO) activated crude glutamic–aspartic apotransaminase of *S. faecalis*, only PALPO was effective with purified pig heart glutamic–aspartic apotransaminase. These findings were interpreted to indicate that either the proposed mechanism of transamination was incorrect, or, less likely, that the mechanisms of transamination for the heart and bacterial systems were different. Because of the significant implications of these results in terms of the mechanism of transamination, we have performed similar experiments using purified pig heart apotransaminase,[4] crystalline PAMPO,[5] and crystalline sodium PALPO.[6]

The present studies demonstrate that PAMPO[7] as well as PALPO activates purified pig heart transaminase. In general agreement with O'Kane and Gunsalus,[4] it was found that simultaneous mixing of PALPO, enzyme, and substrates resulted in low activity, and that pre-incubation of enzyme and PALPO was necessary for appreciable activation with low concentrations of PALPO. As described in Table I, PALPO, but not PAMPO, significantly activated transamination with a pre-incubation period of five minutes. When the enzyme was incubated with PALPO or PAMPO for longer periods before the addition of substrates, equivalent activity was observed with both phosphate esters at the same concentrations. Dialysis (*p*H 7.5, 5°, 48 hours) of the enzyme-coenzyme mixtures before addition of substrate did not affect the activity. Thoroughly dialyzed PALPO-reconstituted enzyme preparations contained 1 to 3% of the added PALPO[8] as determined at *p*H 5.5[9] with tyrosine apodecarboxylase.[10] Similar values for PALPO binding were obtained in experiments with PALPO labeled with P^{32}. On the other hand, P^{32}-PAMPO-reconstituted preparations retained only about 0.1% of the added radioactivity,[8] and no PALPO was detected[10] either in dialyzed PAMPO-reconstituted preparations or in the concentrated dialysates.

TABLE I

ACTIVATION OF PIG HEART APOTRANSAMINASE BY PYRIDOXAL PHOSPHATE AND PYRIDOXAMINE PHOSPHATE

Duration of incubation of enzyme with coenzyme prior to addition of substrates, min.	Transaminase activity[a] (microliters per 15 minutes) With pyridoxal phosphate	With pyridoxamine phosphate
0	32.0	12.7
5	93.0	17.6
10	118	37.2
30	119	75.2
60	112	116
90	111	123
120	118	120
120[b]	124	126
120[c]	70.0	62.0
120[d]	205	213

[a] The enzyme preparation (50γ) was incubated with 5γ of coenzyme in 1.7 cc. of 0.059 *M* potassium phosphate buffer (*p*H 7.5) in the main compartment of a Warburg vessel at 37°. At the indicated intervals, a side arm containing 100 micromoles of L-aspartate and 200 micromoles of sodium α-ketoglutarate (0.5 cc.) was tipped. After 15 minutes 0.5 cc. of aniline–50% citric acid (1:1) was added from a second side arm, and the evolved carbon dioxide was recorded. There was no activity in the absence of coenzyme. [b] Dialyzed for 48 hours at *p*H 7.5. [c] Concentration of coenzyme = 2.5γ. [d] Concentration of coenzyme = 10.0γ.

The data are consistent with the hypothesis that activation requires enzyme–coenzyme combination, and demonstrate that after suitable pre-incubation equal concentrations of PALPO and PAMPO produce equivalent activity. The observation that P^{32} binding was greater with P^{32}-PALPO than with P^{32}-PAMPO suggests that most of the PALPO is bound at non-functional sites, or, less likely, that PAMPO enzyme combination involves some dissociation of the phosphate group. Conversion of PAMPO to PALPO *prior* to addition of substrates was not demonstrated by these studies, and such a reaction appears unlikely. Interconversion of PAMPO and PALPO probably occurs in the presence of substrates after enzyme-coenzyme combination has been established. Conversion of PAMPO to PALPO by transamination with pyruvate and

(1) E. E. Snell, *J. Biol. Chem.*, **154**, 313 (1944).

(2) E. E. Snell, THIS JOURNAL, **67**, 194 (1945).

(3) W. W. Umbreit, D. J. O'Kane, and I. C. Gunsalus, *J. Biol. Chem.*, **176**, 629 (1948).

(4) D. E. O'Kane, and I. C. Gunsalus, *ibid.*, **170**, 425 (1947).

(5) E. A. Peterson, H. A. Sober and A. Meister, THIS JOURNAL, **74**, 570 (1952).

(6) E. A. Peterson, H. A. Sober, and A. Meister, *Federation Proceedings*, 11, 268 (1952).

(7) The crystalline PAMPO preparation did not activate tyrosine apodecarboxylase indicating the absence of PALPO.

(8) These studies were carried out under conditions similar to those described in Table I, with 0.625 to 200γ of coenzyme, 220γ of enzyme preparation, and a pre-incubation period of 120 minutes.

(9) PALPO can be released from the enzyme preparation with 0.05 *M* sodium acetate buffer of *p*H 5.5.

(10) W. W. Umbreit, W. D. Bellamy, and I. C. Gunsalus, *Arch. Biochem.*, **7**, 185 (1946).

other α-keto acids in *Clostridium welchii* has been described.[11] Although the mechanism of coenzyme binding and function in the heart system requires further study, the evidence supports the hypothesis that both PAMPO and PALPO play significant roles in enzymatic transamination.

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(11) A. Meister, H. A. Sober and S. V. Tice, *J. Biol. Chem.*, **189**, 577 (1951).

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BOOK REVIEWS

Principles of Chemical Thermodynamics. First Edition. By MARTIN A. PAUL, Ph.D., Professor of Chemistry, Harpur College, State University of New York. McGraw-Hill Book Company, 330 West 42nd Street, New York 18, N. Y. 1951. viii + 740 pp. 16 × 23.5 cm. Price, $7.50.

In the preface the author states: "This book is intended to serve a course in chemical thermodynamics which may accompany or immediately follow the introductory course in physical chemistry, at either the senior undergraduate or the first-year graduate level. The emphasis throughout is on general principles and their origins, with specific applications to a limited number of fields which are primarily the concern of the chemist." Actually a one-year course in physical chemistry would seem to be a prerequisite for the study of this text. This book, in common with other recently published texts in chemical thermodynamics, is fairly satisfactory in the presentation of those applications considered by Lewis and Randall, but is entirely unsatisfactory in the discussion of the fundamentals of thermodynamics. The author gives for the most part correct equations and presents some of the mathematical methods employed in thermodynamics, although the derivation of thermodynamic relations, particularly the problem of change in independent variables, should be discussed more fully. The main trouble with the text is a dearth of precise definitions, lack of logic, and vagueness in interpretation. There is a noticeable tendency toward the long and sometimes ambiguous discussion that is apt to confuse the student.

One general criticism is that the author does not distinguish between the process and the change in state. He does not seem to have defined the concept of the change in state of a system. He writes far too few changes in state and those that he does give are not sufficiently defined.

After a long chapter on temperature and its measurement, the author introduces the first law by a discussion of the mechanics of conservation and non-conservative systems. This is an unfortunate starting point since the term work is used in thermodynamics in a different sense from that in which it is used in mechanics. Quantity of heat is correctly defined (p. 55). However, the author employs the ambiguous terms work done on or by the system and heat received by the system, which may lead to confusion. Apparently the author does not define the concept of the boundary, the location of which is frequently of paramount importance in discussing work and heat. The first law is announced as the conservation of energy, and then the equation $\Delta U = Q - W$ appears during a discussion of the internal energy of a system. This would seem to be the wrong order of presentation. If it is the author's intention to define work and heat first, then he should state the first law in terms of these quantities and proceed to prove the existence of the energy function.

The next two chapters deal with the thermal behavior of simple systems and with thermochemistry. The author discusses open systems (pp. 75–78) apparently without the realization of all of the implications of Eq. (3–12). Equation (3–15) is incorrect since the substance i has energy which it carries across the boundary of the system. The same error does not occur in the treatment of partial molal enthalpy. The writing of a change in state would have made the error obvious. Equations (3–16) and (4–36) would hardly be called Gibbs–Duhem equations. Early in the chapter on the application of the first law, the author introduces a second law equation. This is a dangerous pitfall for the student and one into which the author falls. In the chapter on thermochemistry a different convention regarding the sign of quantity of heat is used. Under the paragraph heading "Heat of a Chemical Reaction" (p. 132) we find the typically vague statements: "The heat of a chemical reaction is defined in general as the quantity of heat evolved when a given quantity of the reaction takes place, as represented by its chemical equation. For example, the combustion of methanol is represented by the thermochemical equation

$$CH_3OH(l) + \frac{3}{2} O_2(g) = 2H_2O(l) + CO_2(g)$$

$$Q^\circ_{25^\circ C} = 173.64 \text{ kcal.}$$

The change of ΔH (of a chemical change in state at constant pressure and temperature) with temperature is not discussed for the case that a change in aggregation-state of a reactant or product occurs.

The author does not use a Carnot cycle to derive the second law equation but a cycle consisting of two isothermals and two isometrics, with an ideal gas as the working fluid. In the derivation he makes use of the relation proved in an earlier chapter from a second law equation that the constant volume heat capacity of an ideal gas is independent of the pressure! From the discussion under "Maximum Work and Thermodynamic Reversibility" (pp. 192–199) the student is apt to draw the incorrect conclusion that a maximum work function exists, although the author does not actually state this. The derivations of the Clausius inequalities are not satisfactory. In the discussion of the conditions of equilibrium in an isolated system the author follows the German treatment rather than the more general methods of Gibbs. Thus his Eq. (5–39), $(\Delta S)_{U,V} \leqq 0$, contains the unnecessary subscript V.

A chapter on the thermodynamic behavior of simple systems is followed by a long one on solutions (including gaseous solutions) and heterogeneous mixtures. The treatment of gas mixtures is not rigorous and the student may well draw the incorrect conclusion that the equation (p. 325) for the fugacity coefficient of a gas in a mixture can be derived from Dalton's law (p. 310) or Amagat's law (p. 311). The discussion of liquid solutions would be improved if the relation of the activity to the choice of standard state and of activity coefficient to the choice of reference state were treated in greater detail. This portion of the text contains some excellent tables on the computation of activity coefficients and the effect of the choice of the reference state on the numerical values of activity coefficients.

In the chapter on chemical equilibrium the author introduces the ideal gas assumption too early in the argument in some of the examples discussed and he does not consider the effect of pressure on the activity of a condensed phase. This leads to some peculiar misconceptions. Thus he states that his Eq. (8-1-8) for the effect of temperature on the dissociation pressure of $CaCO_3$ "bears a close relationship to the Clausius-Clapeyron equation for the vapor pressure of a pure liquid or solid." If rare had been taken