TABLE II

GROWTH-PROMOTING ACTIVITIES OF HYDROGENATED COMPOUNDS

		Transmission, %		
	μg./10 ml.	Hydrogenated N^{10}-methyl PGA	Hydrogenated aminopterin	Hydrogenated A-methopterin
For *S. faecalis* R.	0.1	100	53	100
	1.0	100	36	100
	10.0	68	25	100
	100.0	66	26	100
For *L. citrovorum 8081*	0.1	90	91	91
	1.0	92	90	93
	10.0	90	77	94
	100.0	78	33	90

The hydrogenated materials were also tested as growth factors for *Streptococcus faecalis* R. and *Leuconostoc citrovorum 8081*. For the former organism, the same basal medium was used as was used for the inhibition studies but no folic acid was added. For the latter organism, the basal medium and technique of Sauberlich[8] were used with the exceptions that no supplementary glycine and alanine were used and a Lumitron colorimeter with a 660 mμ filter was employed. Turbidity was determined after 17 hours. Data are given in Table II. It is quite likely that the growth-promoting activity is due to an impurity in the original compound as suggested by Weygand.

Acknowledgment.—The authors wish to thank Lederle Laboratories for supplying the folic acid, N^{10}-methylpteroylglutamic acid, aminopterin and A-methopterin used in these experiments.

(8) H. E. Sauberlich, *J. Biol. Chem.*, **181**, 467 (1949).

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COMMUNICATIONS TO THE EDITOR

A NEW METHOD FOR THE PREPARATION OF THIO ACIDS AND APPLICATION TO PEPTIDE CHEMISTRY

Sir:

Although Pawlewski[1] demonstrated that thio acids were very active acylating agents, the methods of preparation which have been available heretofore[2] have not been suitable for making the acylaminothio acids which could be useful in peptide synthesis. By passing hydrogen sulfide into a solution of the mixed anhydrides,[3,4,5] RCOO-$COOC_2H_6$, in methylene chloride with an equivalent of triethylamine at −20° and warming to room temperature, we have obtained the thio acids, RCOSH.

In this manner we have prepared, in addition to thioacetic and thiobenzoic acids, *p*-phenylthiobenzoic acid, 88% yield (from the carboxylic acid), m.p. 90–92° (*Anal.* Calcd. for $C_{13}H_{10}OS$: C, 72.89; H, 4.71; S, 14.94. Found: C, 72.86; H, 4.83; S, 15.09); thiohippuric acid, 70% yield, m.p. 98–100° (*Anal.* Calcd. for $C_9H_9NO_2S$: C, 55.39; H, 4.65; N, 7.18; S, 16.40. Found: C, 55.30; H, 4.69; N, 6.79; S, 15.99); phthaloylthioglycine, 45% yield, m.p. 114–116° (*Anal.* Calcd. for $C_{10}H_7NO_3S$: C, 54.30; H, 3.19; S, 14.47. Found: C, 54.52; H, 3.32; S, 14.21).

When thiohippuric acid was warmed to 90–110° in dimethylformamide with *d,l*-alanine in a nitrogen atmosphere, hydrogen sulfide was rapidly evolved and there was obtained a 70% yield of hippuryl-alanine, m.p. 200–201.5°[6] and giving the correct elemental analysis.

Upon treatment of thiohippuric acid with Raney nickel which had been deactivated over acetone[7] there was obtained in one experiment, a 30% yield of hippuraldehyde,[8] isolated as the 2,4-dinitrophenylhydrazone, m.p. 200–202° (*Anal.* Calcd. for $C_{15}H_{13}N_5O_5$: C, 52.48; H, 3.82; N, 20.40. Found: C, 52.63; H, 3.78; N, 20.18).

(1) Br. Pawlewski, *Ber.*, **31**, 661 (1898); **34**, 657 (1901); **35**, 110 (1902).

(2) R. Connor, "Organic Sulfur Compounds," p. 835 in Gilman's "Organic Chemistry," Vol. I, Second Edition, John Wiley and Sons, Inc., New York, N. Y., 1943; S. Sunner and T. Nilson, *Svensk. Kem. Tid.*, **54**, 163 (1942) [*C. A.*, **38**, 3249 (1944)]; B. Tchoubar and Tetellier-Dupre, *Bull. soc. chim. France*, 792 (1947).

(3) R. A. Boissonnas, *Helv. Chim. Acta*, **34**, 874 (1951); T. Wieland and H. Bernhard, *Ann.*, **572**, 190 (1951); J. R. Vaughan and R. L. Osato, THIS JOURNAL, **74**, 676 (1952).

(4) T. Wieland, W. Schäfer and E. Bokelmann, *Ann.*, **573**, 99 (1951), prepared $RCOSC_6H_5$ by addition of C_6H_5SH to the mixed anhydride.

(5) H. Adkins and Q. E. Thompson, THIS JOURNAL, **71**, 2242 (1949), prepared thiobenzoic acid by passing H_2S into dibenzoyl sulfide in pyridine.

(6) T. Curtius and E. Lambotte, *J. prakt. Chem.*, [2] **70**, 114 (1904).

(7) G. B. Spero, A. V. McIntosh and R. H. Levin, THIS JOURNAL, **70**, 1907 (1948).

(8) J. Bougault, E. Cattelain and P. Chabrier, *Bull. soc. chim.*, [5] **5**, 1699 (1938), have reported the conversion of thioacetic acid to acetaldehyde.

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RECEIVED JUNE 23, 1952

THE SYNTHESIS AND REACTIONS OF N-ACYL THIOL AMINO ACIDS

Sir:

Recent evidence that enzymatic acylations involve thiolacid derivatives as activated intermediates[1] has stimulated interest in similar thiol analogs of amino acids as possible participants in the physiological synthesis of peptides. By two

(1) For example, acetyl coenzyme A is considered to be a key intermediate in biological acetylations; F. Lynen, E. Reichert and L. Rueff, *Ann.*, **574**, 1 (1951); T. C. Chou and F. Lipmann, *J. Biol. Chem.*, **196**, 89 (1952).

methods, we have prepared N-acylated thiol amino acids and have found them to be active acylating agents[2] for amines and amino acid derivatives under mild conditions.

Interaction of phenaceturic acid, triethylamine and ethyl chlorocarbonate[3,4] in methylene chloride solution at $-10°$, followed by treatment with excess hydrogen sulfide, led to a 72% yield of thiolphenaceturic acid,[5] m. p. 116.5–118.0° (dec.). *Anal.* Calcd. for $C_{10}H_{11}NO_2S$: C, 57.39; H, 5.30; N, 6.69. Found: C, 57.45; H, 5.33; N, 6.75. A solution of thiophenaceturic acid and aniline in 50–50 ethanol–phosphate buffer of *p*H 7.5 (0.1 *M*) deposited 78% of phenaceturanilide, m.p. 163–164°, in 18 hours at room temperature.

Phthaloylthioglycine was prepared by a similar procedure in 61% yield, m.p. 116.5–118.0°. *Anal.* Calcd. for $C_{10}H_7NO_3S$: C, 54.29; H, 3.19; N, 6.33. Found: C, 54.49; H, 3.33; N, 6.36. Treatment of phthaloylglycyl chloride with sodium hydrosulfide in dimethylformamide solution also afforded phthaloylthioglycine in good yield.

A solution in methylene chloride of phthaloylthiolglycine, glycine methyl ester hydrochloride and triethylamine reacted at room temperature to give phthaloylglycylglycine methyl ester. The addition of iodine-potassium iodide[6] to an aqueous solution (0–5°) of phthaloylthiolglycine and glycine methyl ester hydrochloride containing excess sodium bicarbonate produced an immediate precipitate of the peptide derivative.

(2) Thiolacetic acid has been reported previously to react readily with amines, B. Pawlewski, *Ber.*, **31**, 661 (1898).

(3) R. A. Boissonnas, *Helv. Chim. Acta*, **34**, 874 (1951).

(4) J. R. Vaughan and R. L. Osato, THIS JOURNAL, **74**, 676 (1952).

(5) A similar method has been used to prepare N-acyl thiol amino acid esters; T. Wieland, W. Schäfer and E. Bokelmann, *Ann.*, **573**, 99 (1951).

(6) G. Alliger, G. E. P. Smith, Jr., E. L. Carr and H. P. Stevens, *J. Org. Chem.*, **14**, 962 (1949).

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RECEIVED AUGUST 4, 1952

THE BETA-LUCIFERIN OF *CYPRIDINA*[1]

Sir:

The production of light by the marine ostracod crustacean, *Cypridina hilgendorfii*, is a result of interaction between oxygen, the enzyme luciferase, and either of two amorphous substances of unknown nature which have been designated α- and β-luciferin.[2,3,4] We wish to report evidence that these luciferins are chromopolypeptides.

The most highly purified preparations of the luciferins are oxygen-sensitive orange-yellow resins from which no bioluminescent substance can be sublimed and from which it has not been possible to obtain crystalline fractions.[4] At 65° in high vacuum, α-luciferin is converted to B-luciferin; the transformation is reversed in dilute acid. The infrared spectrum of films of β-luciferin lacks fine structure but contains strong absorptions at 3250, 2825, 1680, 1625, and 1510 cm.$^{-1}$, which collectively indicate the amide bond as it occurs in peptides[5,6,7] or in cyclic ureides.[8,9] Accordingly, an attempt was made to degrade β-luciferin by hydrolysis and, although 0.5 *N* hydrochloric acid does not attack the molecule appreciably at 100°, de-oxygenated 4 *N* acid slowly degrades it at 125° with loss of activity. β-Luciferin does not give a ninhydrin test but the product of its hydrolysis contains a number of ninhydrin-positive substances. These have been presumptively identified by two-dimensional paper chromatography as the amino acids: glycine, threonine, proline, lysine, aspartic acid, glutamic acid, and leucine, isoleucine, or phenylalanine. The hydrolysate contains an unidentified ninhydrin-positive substance and a yellow pigment readily separable from the amino acid fraction. In addition, when β-luciferin is chromatographed on paper with either hydrogen-saturated *n*-butanol or *i*-amyl alcohol (R_f 0.8 and 0.65, respectively, determined by the position of light-emitting areas after wetting the chromatogram with luciferase) the N-chloroamide test,[10] the retention test,[11] and hydrolysis of eluted substance show that the position of luciferin activity coincides with the position of a polypeptide. Our preparations of β-luciferin contain in addition polypeptide (R_f 0 in both solvents) which does not possess luminescent activity in the presence of luciferase but which may be related to luciferin since it and the active polypeptide have identical amino acid compositions. Only the active polypeptide is yellow and accordingly belongs to the class of pigmented polypeptides hitherto encountered in *Actinomyces*.[12,13] Such substances thus occur in higher organisms, and the bioluminescent reaction between *Cypridina* luciferin and luciferase is a naturally-occurring phase in the metabolism of these compounds.

(1) This investigation was supported by a grant from the Research Corporation, New York.

(2) E. N. Harvey, "Living Light," Princeton University Press, Princeton, N. J., 1940; "Bioluminescence," Academic Press, New York, N. Y.

(3) R. S. Anderson, *J. Gen. Physiol.*, **19**, 301 (1935).

(4) H. S. Mason and E. F. Davis, *J. Biol. Chem.*, **197**, 41 (1952).

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(7) I. M. Klotz, P. Griswold and D. M. Gruen, THIS JOURNAL, **71**, 1615 (1949).

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(9) E. R. Blout and M. J. Fields, THIS JOURNAL, **72**, 479 (1950).

(10) H. N. Rydon and P. W. G. Smith, *Nature*, **169**, 922 (1952).

(11) F. A. Robinson, K. L. A. Fehr and W. Dickinson, *Biochem. J.*, **51**, 298 (1952).

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DEPARTMENT OF BIOLOGY
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LIPOIC ACID–CONJUGASE

Sir:

Lipothiamide pyrophosphate (LTPP), the amide of lipoic acid (LA) and thiamin pyrophosphate (TPP), is required for the oxidative decarboxylation of pyruvate and α-ketoglutarate by cell-free extracts of an *Escherichia coli* mutant.[1a] It has now been demonstrated that cell-free extracts of wild-type *E. coli* contain an enzyme, lipoic acid conjugase,

(1) (a) L. J. Reed and B. G. DeBusk, THIS JOURNAL, **74**, 3964 (1952); (b) **74**, 3457 (1952).

which forms LTPP from TPP and bound LA. The loss of this enzyme is apparently the critical change resulting from mutation of the parent strain. This conclusion is based upon studies of pyruvate oxidation systems, resolved and partially purified by the method of Korkes, *et al.*,[2] from the wild and mutant strains of this organism.

Pyruvate disinutation requires transacetylase, lactic dehydrogenase, orthophosphate, diphosphopyridine nucleotide, coenzyme A, TPP, and two enzyme fractions from the wild strain (designated A_W and B_W)[2] (Table I). However, the enzyme fraction B_W and the cofactor TPP can be replaced by a single substance, LTPP, indicating that the basic pyruvate oxidase system, which is activated by the coenzyme LTPP, is present only in fraction A. The apoöxidase fraction from the mutant strain, A_M, can likewise be activated either by LTPP, or by TPP plus B_W, but *neither* apoöxidase fraction is activated by TPP plus B_M, the fraction from the mutant corresponding to fraction B_W of the wild strain.

TABLE I

PYRUVATE DISMUTATION WITH PURIFIED ENZYME FRACTIONS

Components[a]	μM Products in 90 min. Carbon dioxide	Acetyl phosphate	Lactate
A_W + TPP	0	0	0.2
A_W + B_W + TPP	3.6	3.4	3.7
A_W + TPP + LA	0	0	0.2
A_W + LTPP	4.7	4.4	4.7
A_W + Incubated[b] (B_W + TPP)	3.5	3.3	3.4
A_W + Control[c]	0.1	0.2	0.2
A_M + TPP	0.1	0.1	0.1
A_M + B_W + TPP	3.6	3.3	3.5
A_M + LTPP	4.7	4.4	4.8
A_M + Incubated[b] (B_W + TPP)	3.5	3.2	3.4
A_M + Control[c]	0	0	0.1
A_W + B_M + TPP	0.1	0.1	0.3
A_W + B_M + TPP + LA	0.1	0.1	0.3
A_M + B_M + TPP	0.1	0.1	0.1

[a] Present at following levels: enzyme fractions, 2.0 mg. protein; TPP, 100γ; LA, 10γ; LTPP, 24γ of crude synthetic preparation[1b]; final volume, 2 ml. Supplements and experimental conditions as previously described.[1a] [b] Incubated 90 min. at 25°, boiled 10 min., and supernatant added to A_W or A_M. [c] B_W incubated and boiled prior to contact with TPP.

Incubation of fraction B_W alone with TPP produces a heat stable product, presumably LTPP, which can subsequently activate the apoöxidase of either strain; however, heating fraction B_W prior to its contact with TPP results in an incubation mixture having no coöxidase activity. Fraction B_W must furnish lipoic acid conjugase as well as lipoic acid, presumably bound to the conjugase, or less likely to a contaminating protein, by a union not dissociable by dialysis.

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RECEIVED AUGUST 6, 1952

(2) S. Korkes, *et al.*, *J. Biol. Chem.*, **193**, 721 (1951).

VERATROBASINE AND GERALBINE, TWO NEW ALKALOIDS ISOLATED FROM VERATRUM ALBUM[1]

Sir:

After separation of the ester alkaloids, protoveratrine and veralbidine,[2] and the alkamines jervine and rubijervine from the mixture of alkaloids contained in *Veratrum album*, the mother liquor was divided into two fractions, one of which contained the markedly basic alkaloids, and the other the weakly basic alkaloids. From the fraction containing the first group an unknown alkaloid could be crystallized out of a solution in ethyl acetate.[3] This was purified as its hydrochloride which is only slightly soluble in water. After cleavage of the salt with dilute ammonia the pure base was obtained. This we intend to call *veratrobasine*. The new base crystallizes from methanol in large prisms, which turn yellow from 270° upward, and melt at 285–288°, with decomposition. Its optical rotation in pure alcohol is $[\alpha]^{20}D$ −76.6°, and in pyridine $[\alpha]^{20}D$ −126°. When veratrobasine was dissolved in 84% sulfuric acid (2 mg. of the base in 10 cc. acid) an intensely orange fluorescent solution was obtained. The solution kept this color for over 24 hours. *Anal.* Calcd. for $C_{24}H_{37}O_3N$: C, 74.44; H, 9.63; N, 3.62. Found: C, 74.46, 74.38; H, 9.81, 9.57; N, 3.66, 3.85.

In possessing only 24 carbon atoms, the new alkaloid is significantly different from the other alkamines so far obtained from *Veratrum album* and *Veratrum viride*, which all have 27 carbon atoms. Veratrobasine has one $N\text{-}CH_3$ group, the first to be found in the veratrum alkaloids: $N\text{-}CH_3$ calcd. 3.85, found 3.94.

The base also contains two active hydrogen atoms: calcd. 0.508, found 0.51.

The infrared absorption spectrum shows no band typical of ketones; the ultraviolet absorption spectrum, however, shows a definite maximum at 252 mμ (log ε 2.14).

From the fraction containing the weakly basic alkaloids, a further new alkaloid could be crystallized from ethyl acetate. This new alkaloid, which we have called *geralbine*, crystallizes from aqueous acetone in large prisms, and from a mixture of ethyl acetate and ether (1:1) in rectangular plates which melt at 221–223° with slight yellowish discoloration. In contrast to the other alkaloids isolated from *Veratrum*, geralbine exhibited no measurable rotation in pure alcohol, chloroform or pyridine. When dissolved in 84% or pure sulfuric acid a light yellow solution was obtained which had not lost its color after twelve hours. In crystalline form geralbine is fairly stable but when dissolved in alcohol or chloroform, the solution turns yellow within a few hours. *Anal.* Calcd. for $C_{22}H_{33}O_2N$: C, 76.91; H, 9.68; N, 4.07. Found: C, 76.73, 76.78; H, 9.79, 9.78; N, 3.96, 3.98.

Geralbine hydrochloride crystallizes from methanol–ether in fine needles, and melts at 270°. *Anal.* Calcd. for $C_{22}H_{34}O_2NCl$: C, 69.55; H, 9.02; Cl, 9.35. Found: C, 69.96; H, 9.23; Cl, 9.35, 9.37.

(1) Second communication, A. Stoll and E. Seebeck, *Helv. Chim. Acta*, **35**, 1270 (1952).

(2) A. Stoll and E. Seebeck, *Science*, **115**, 678 (1952).

(3) A detailed description of the process used to isolate the two new alkaloids will be published later in *Helv. Chim. Acta*.

Like veratrobasine, geralbine has one N-CH_3 group: calcd. 4.38%, found 3.96%.

In the infrared spectrum geralbine shows a band typical of ketones at 1715 cm.$^{-1}$.

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RECEIVED AUGUST 12, 1952

STRUCTURE OF HYALURONIC ACID. THE GLUCURONIDIC LINKAGE

Sir:

The isolation of a crystalline disaccharide[1] from the biologically important polysaccharide hyaluronic acid[2] has recently been reported. The glucuronic acid–glucosamine disaccharide, designated hyalobiuronic acid, has now been shown to be D-glucopyruronosido<β-1,3>D-glucosamine. This structure follows from transformation of the disaccharide to D-glucopyranosido<β-1,2>D-arabinose, a new compound whose structure follows in turn from its preparation from laminaribiose (glucopyranosido<β-1,3>glucose[3]) by an application of the Zemplén[4] degradation.

The crystalline glucuronido-glucosamine is produced from umbilical cord hyaluronic acid in yields as high as 61% by the combined enzymatic and acid hydrolysis earlier described,[1] in somewhat lower yield by direct acid hydrolysis. The picture[2] of the polysaccharide as a chain of alternating glucosamine and glucuronic acid residues must therefore be essentially correct. Also, the β-1,3-linkage now found in the disaccharide is apparently the predominating if not sole glucuronidic linkage in the polysaccharide.

In earlier structural investigations, a methylated glucopyruronoside derivative has been isolated in trace quantity on methanolysis of the methylated polysaccharide,[5] and various workers have inferred from the periodic acid consumption of the polysaccharide and its derivatives the presence of 1,3-,[6a] 1,4-[6b] or mixed 1,3- and 1,4-[6c] glucuronidic linkages.

With cold weak methanolic hydrogen chloride the glucuronidoglucosamine (I) gives an amorphous methyl ester hydrochloride (II). Acetylation of this gives heptaacetylglucuronido-glucosamine, methyl ester (III, 65% yield from I), obtained as needles, m.p. 120°, $[\alpha]^{22}D$ + 25° (chloroform), containing one ethanol of crystallization incompletely lost on drying at 110°. Found (crystals): CH_3O, 8.97; N, 2.08; loss on drying, 5.1. Found (dried substance): CH_3O, 6.05; N, 2.10; C, 48.49; H, 5.77; CH_3CO, 45.9; mol. wt., 668. With ketene the glucuronido-glucosamine (I) gives the amorphous N-acetyl derivative, $[\alpha]^{28}D$ −32° (water). Found: N, 3.28; uronic acid (CO_2), 48.2; hexosamine, 44.4. Treatment with cold weak methanolic hydrogen chloride, followed by acetylation, gives the heptaacetyl methyl ester (III) described above.

The methyl ester hydrochloride (II), on oxidation with yellow mercuric oxide, followed by sodium borohydride reduction, gives glucosido-glucosaminic acid (20% yield from I), needles, $[\alpha]^{30}D$ −34° (water; *c*, 0.9). Found: neut. equiv. (formol), 355. Degradation of this amino acid with ninhydrin gives a glucosido-arabinose, isolated as the heptaacetate (IV), needles m.p. 198–199° (micro-block), $[\alpha]^{23}D$ −47° (chloroform). Found: C, 49.92; H, 6.00. This acetate gives a melting point depression with Zemplén's[4] heptaacetyl-glucosido<β-1,3>arabinose, $[\alpha]_D$ −17°, and gives no depression with heptaacetylglucosido<β-1,2>-arabinose (IV) from laminaribiose.

Synthetic laminaribiose[3,7] is treated with hydroxylamine. The resulting glass with acetic anhydride and sodium acetate at 110° gives octa-acetyllaminaribionitrile, m.p. 140–141°, $[\alpha]^{30}D$ +3° (chloroform). Found: N, 2.10. Reaction of the nitrile with sodium methoxide and acetylation of the product gives heptaacetyl-glucopyranosido<β-1,2>D-arabinose (IV), m.p. 199.5–200° (micro-block), $[\alpha]^{30}D$ −46° (chloroform). Found: C, 49.20; H, 5.62; CH_3CO, 47.8.

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(2) K. Meyer, *Physiol. Reviews*, **27**, 335 (1947).

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(4) G. Zemplén, *Ber.*, **59**, 1254 (1926).

(5) M. A. G. Kaye and M. Stacey, *Biochem. J.*, **48**, 249 (1951).

(6) (a) R. W. Jeanloz and E. Forchielli, *J. Biol. Chem.*, **190**, 537 (1951); (b) K. H. Meyer, J. Fellig and E. H. Fischer, *Helv. Chim. Acta*, **34**, 939 (1951); H. Masamune, Z. Yosizawa and T. Isikawa, *Tohoku J. Exp. Med.*, **55**, 166 (1952); (c) G. Blix, *Acta Chem. Scand.*, **5**, 981 (1951).

(7) We are indebted to Prof. E. L. Hirst for seed material.