at 135–140° for two hours produced 17.3 g. (80.8%) of (VI), a yellow liquid, at 183–185° (8 mm.).

(2) Twenty-nine grams of N,N'-dibutyl-ethylenediamine dithiocarbamate heated at 130–135° for about two hours gave 21.5 g. (85%) of (VI) at 183–184° (8 mm.).

1,3-Dibutyl-trimethylenethiourea (VII).[11]—(1) Starting with 21.4 g. (0.1 mole) of N-formyl-(III) and 4.0 g. of sulfur and heating at 145–150° for two hours and at 175° for one-half hour, 9.2 g. of unconverted N-formyl-(III) was recovered, and 5.8 g. (25%) of (VII) was obtained as a yellow liquid, b. p. 177–178° (3 mm.).

(2) A stirred solution of 37.2 g. (0.2 mole) of (III) in 50 cc. of methanol was treated with a solution of 15.2 g. (0.2 mole) of carbon disulfide in 40 cc. of methanol in the course of fifteen minutes. The solvent was evaporated from the resulting solution and the remaining thick liquid, the dithiocarbamate, was heated at 150–155° until the evolution of gas ceased (about two hours). The reaction mixture then was distilled, yielding 12.4 g. of recovered (III), b. p. 107° (7 mm.), and 14.6 g. (31%) of (VII), b. p. 177–178° (3 mm.).

1,3-Diphenyl-ethylenethiourea.—This product was prepared in 71% yield by heating N-formyl-(V) with sulfur, but in this case little reaction was observed below 195°. (V) did not form a dithiocarbamate on treatment with carbon disulfide and water under reflux at atmospheric pressure for twenty hours.

(11) N-Monosubstituted-trimethylenethioureas have been made by pyrolysis of the dithiocarbamates of the corresponding N-substituted-trimethylenediamines; Goldenring, *Ber.*, **23**, 1171 (1890); Fränkel, *ibid.*, **30**, 2501 (1897).

Acknowledgments.—The author is indebted to Mrs. J. D. Nevins and Mrs. R. C. Schropp of the Monsanto Analytical Laboratory for the analyses reported.

RESEARCH LABORATORIES
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RECEIVED JANUARY 17, 1946

COMMUNICATIONS TO THE EDITOR

CRYSTALLINE VITAMIN A METHYL ETHER

Sir:

In recent years much interest has been shown in the synthesis of vitamin A ethers. However, since no data are available concerning the biological activity of these ethers, we have undertaken the preparation of vitamin A methyl ether from the natural vitamin.

The methyl ether was prepared by the action of dimethyl sulfate on the lithium derivative of the vitamin, which was formed by the reaction of *n*-butyl lithium[1] and crystalline vitamin A alcohol.[2] It was purified by chromatography on activated alumina[3] and was obtained as an orange oil, which crystallized from methanol after several months at −70°, m. p. 31–33°. After three recrystallizations from methanol and two from a 65–70° hydrocarbon fraction [Purified Skelly Solve B],[4] vitamin A methyl ether was obtained as light yellow crystals melting at 33–34°. *Anal.* Calcd. for $C_{21}H_{32}O$: C, 83.95; H, 10.74; OCH_3, 10.34. Found: C, 83.76; H, 11.07; OCH_3, 9.94.

The spectrophotometric curve for crystalline vitamin A methyl ether is identical in all respects with that of vitamin A alcohol, both having absorption maxima at 326 mμ on the Beckman spectrophotometer. The extinction coefficient ($E^{1\%}_{1\,cm.}$) in isopropanol at 326 mμ is 1660. This corresponds to an equivalent extinction coefficient of 1742 for vitamin A alcohol.

Vitamin A methyl ether possesses a biological potency greater than 3,000,000 U. S. P. XII units per gram and is of the same order of activity as crystalline vitamin A alcohol.

The experimental details and complete biological data will appear in a forthcoming paper.

(1) Gilman, Langham and Moore, THIS JOURNAL, **62**, 2327 (1940).
(2) Distillation Products, Inc., Rochester, N. Y.
(3) Aluminum Ore Co., East St. Louis, Illinois.
(4) Purified by treatment with concentrated sulfuric acid and distillation.

A. R. HANZE
THE UPJOHN COMPANY T. W. CONGER
NUTRITION DIVISION E. C. WISE
KALAMAZOO, MICHIGAN D. I. WEISBLAT

RECEIVED JUNE 17, 1946

AMINOMETHYLATION OF THIOPHENE

Sir:

During the course of formylation studies with thiophene it was noted that in the presence of ammonium chloride and formaldehyde thiophene appeared to undergo a reaction to give water-soluble amine hydrochlorides. From the reaction mixture was isolated 2-thenylamine (2-aminomethylthiophene) (I), b. 58° (5 mm.), $n^{20}D$ 1.5589; secondary di-(2-thenyl)-amine, b. p. 150–152° (10 mm.), $n^{22}D$ 1.5914; and a third amine (III). Amine III is polymeric in nature and is believed to contain methylol groups. The hydroxyl number of III produced by the reaction of one mole of thiophene with four moles of 37% formaldehyde and one mole of ammonium chloride at the reflux was 475, indicating that methylol groups may be substituted around the thiophene in all remaining positions. Other analysis obtained on the product were as follows: 20.6% sulfur and 7.3% nitrogen. With the use of aqueous 37% formaldehyde in excess III is obtained exclusively and molecular weights of 600–750 are the usual order. The use of trioxymethylene with a few per cent. by weight acetic acid (to promote depolymerization at lower temperatures) gave

products that were insoluble in all common solvents and no molecular weights have been determined.

The yields of I, II and III are 40, 20 and 40%, respectively, based on the thiophene reacted, when two moles of thiophene was treated with one mole of formaldehyde and three moles of ammonium chloride. The excess reactants were recoverable. Attempts to improve the yields of I and II are being made.

Hexamethylenetetramine was found to react with thiophene in the presence of concentrated hydrochloric acid to give 7% of I, 25% of II and 68% of III on a weight per cent. basis.

Superficially, at least, this reaction appears to be similar to the Mannich reaction with ketones. It differs in that free amine bases and formaldehyde appear not to react and that primary and secondary amine hydrochlorides do not react as rapidly as ammonium chloride.

A preliminary study of the reaction with thiophene derivatives indicates wide applicability. Full details of the reaction with such derivatives as 2- and 3-methylthiophene, 2-chlorothiophene, and 2-*t*-butylthiophene will be reported in a later communication.

SOCONY-VACUUM LABORATORIES HOWARD D. HARTOUGH
DIVISION OF SOCONY-VACUUM OIL CO., INC.
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EVERETT H. MURRAY, JR.

RECEIVED JUNE 14, 1946

MICRO-ANALYSIS OF MIXTURES (AMINO ACIDS) IN THE FORM OF ISOTOPIC DERIVATIVES

Sir:

A mixture is treated with a reagent containing a stable or radioactive isotope to form quantitatively a stable derivative of the desired constituent. An overwhelming excess, W, of the unlabelled derivative (the carrier) is added and purified to constant concentration, C_c. If C_r is the isotopic concentration of pure isotopic derivative prepared with the same reagent, the amount of derivative present is $W(C_c/C_r)$.

The method has much higher sensitivity than the familiar isotope dilution technique,[1] being theoretically operable at the level of trade substances. Furthermore, the use of racemic carriers avoids errors due to partial racemization. One isotopic reagent suffices for the analysis of many compounds.

As the labelled reagent we used *p*-iodophenyl sulfonyl chloride (PIPSYLchloride), prepared from radioactive iodide ion and *p*-diazobenzenesulfonic acid, followed by treatment with phosphorus pentachloride. A 5–10-fold excess reacts quantitatively with amino acids (glycine, alanine, isoleucine) as indicated by the disappearance of amino nitrogen.

β-Lactoglobulin was analyzed for glycine as follows: 0.3 ml. of an acid hydrolysate (1.13 mg. protein), 20 mg. of PIPSYLchloride, and excess sodium carbonate were shaken in a Folin tube at 90° for ten minutes. The walls were washed down, 5 mg. of labelled reagent added and the procedure repeated. One ml. of ammonia was added. The mixture, together with an acetone solution of some solid reaction products, was added to 200 mg. of normal PIPSYLglycine in ammonia, acidified, extracted with *n*-butanol, and iodobenzenesulfonate ion removed by passing the butanol over Duolite C3 (ion exchange resin). Ligroin was added and the carrier extracted into alkali and purified by repeated precipitation by acid, solution in ammonia, and treatment with activated charcoal, the amount at any stage being estimated spectrophotometrically at 2500 Å. and its radioactivity measured in solution with a Geiger counter. Values obtained at stages of purification corresponding to carrier recoveries of about 12.5, 10 and 7.5 were 1.59, 1.52 and 1.54% glycine for one analysis and 1.52, 1.52 and 1.50% for another. Rittenberg and Foster reported 1.5%.[1]

Less than one-hundredth per cent. of *d*(−)alanine was found in the β-lactoglobulin hydrolysate using PIPSYL *d*(−)alanine carrier. Seven and four-tenths per cent. of alanine was found when racemic carrier was employed. Chibnall reported 6.7%[2]; Brand, 6.2%.[3] Four and seven-tenths per cent. alanine was found in insulin. Chibnall reported 4.6%.[2] When 113 micrograms of alanine was added to a β-lactoglobulin hydrolysate containing 105 micrograms, 215 micrograms was found.

In two analyses, <0.2 and <0.5% isoleucine were found in human hemoglobin, confirming the low values previously reported.[4,5] The isotope concentration of the carrier diminished so slowly that only a few tenths per cent. of the carrier remained when the values were calculated.

The systematic application of this method to protein analysis is in progress.

DEPARTMENTS OF PSYCHIATRY AND CHEMISTRY[6]
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NEW YORK, N. Y. R. KEITH CANNAN

RECEIVED JUNE 19, 1946

(1) D. Rittenberg and G. L. Foster, *J. Biol. Chem.*, **133**, 737 (1940).

(2) A. C. Chibnall, *J. Int. Leather Trades Chem.*, **30**, 1 (1946).

(3) E. Brand, *et al.*, THIS JOURNAL, **67**, 1524 (1945).

(4) E. Brand and J. Grantham, *ibid.*, **68**, 721 (1946).

(5) A. Albanese, *J. Biol. Chem.*, **157**, 613 (1946).

(6) Aided by a grant from the John and Mary Markle Foundation

STREPTOMYCES ANTIBIOTICS. IX. DIHYDROSTREPTOMYCIN

Sir:

Streptomycin has been catalytically hydrogenated to dihydrostreptomycin which is active against *B. subtilis in vitro* and *S. schottmülleri in vivo*.

Streptomycin trihydrochloride was hydrogenated in aqueous solution with a platinum cata-

lyst at atmospheric pressure. About one molar equivalent of hydrogen was absorbed. The product obtained by drying the filtered solution from the frozen state was a white granular solid which showed $(\alpha)^{25}D$ −88.7° (*c*, 1.0 in water), and had an activity of about 750 units/mg. as compared with 800 units/mg. for streptomycin.

Dihydrostreptomycin trihydrochloride was converted to the trihelianthate as described for streptomycin.[1] A sample of the trihelianthate after recrystallization three times melted at 215–225° (dec.), activity about 400 units/mg. *Anal.* Calcd. for $C_{21}H_{41}N_7O_{12}(C_{14}H_{15}N_3O_3S)_3$:[2] C, 50.46; H, 5.79; N, 14.94. Found: C, 50.14; H, 5.83; N, 15.08.

Conversion of dihydrostreptomycin trihelianthate to the trihydrochloride as described for streptomycin[1] gave a white powder, m. p. 185–190° (dec.), $(\alpha)^{25}D$ −89.5° (*c*, 0.98 in water), activity about 750 units/mg. Potentiometric titration of this sample gave an equivalent weight of 690; calcd. mol. wt. 693; pK_A 7.75. *Anal.* Calcd. for $C_{21}H_{41}N_7O_{12}\cdot 3HCl$: C, 36.40; H, 6.40; N, 14.15. Found: C, 36.50; H, 6.21; N, 13.91.

The presence of a free or potential carbonyl group in streptomycin was demonstrated by the formation of an oxime and semicarbazone.[3] Streptomycin was inactivated[3] by hydroxylamine in aqueous pyridine solution at *p*H 4. Dihydrostreptomycin is not inactivated by hydroxylamine under these conditions, which is evidence that the carbonyl group in the streptobiosamine moiety is the functional group which was reduced. Acid hydrolysis of dihydrostreptomycin yields streptidine; hence, the reduction involves only the streptobiosamine moiety.

Dihydrostreptomycin is not inactivated by cysteine under conditions[4] which cause the inactivation of streptomycin: thus, it appears that a mechanism involving the reaction of the carbonyl group with the amino and/or mercapto groups of cysteine is involved in the activation.

Dihydrostreptomycin is not degraded to maltol[5] when treated with alkali.

Tests by Dr. H. Robinson and Mr. O. Graessle of the Merck Institute for Therapeutic Research have shown that single doses of 85 units of dihydrostreptomycin trihydrochloride as contrasted with 45 units of streptomycin trihydrochloride–calcium chloride double salt were required to protect 50% of the mice against one lethal dose of *Salmonella schottmülleri*.

RESEARCH LABORATORIES ROBERT L. PECK
MERCK AND CO., INC. CHARLES E. HOFFHINE, JR.
RAHWAY, N. J. KARL FOLKERS

RECEIVED JUNE 22, 1946

(1) Kuehl, Peck, Hoffhine, Graber and Folkers, THIS JOURNAL **68**, in press (1946).

(2) Results to be published on streptomycin degradation products (Kuehl, Flynn, Brink and Folkers) are in best agreement with the formula $C_{21}H_{39}N_7O_{12}$ for streptomycin.

(3) Brink, Kuehl and Folkers, *Science*, **102**, 506 (1945).

(4) Denkewalter, Cook and Tishler, *ibid.*, **102**, 12 (1945).

(5) Schenck and Spielman, THIS JOURNAL, **67**, 2276 (1945).

OXIDATION OF LIGNIN SULFONIC ACIDS BY PERIODIC ACID

Sir:

It doubtless has occurred to many investigators that the degradation of cellulose in wood by periodic acid oxidation might provide a mild and facile method of obtaining lignin, provided that ligneous substances are not attacked by the reagent. Wald, Ritchie and Purves reported[1] the isolation of lignin by the action of periodic acid. We are moved therefore to make a preliminary report on our study of periodic acid oxidation of lignin sulfonic acids and other isolated lignins in progress in this Laboratory for more than a year. Periodic acid has been found to oxidize lignin sulfonic acids, including samples scrupulously freed of carbohydrate material originating from the wood pulping process. The purification processes employed to remove carbohydrates are: (a) fermentation with yeast; (b) diffusion of fermented sulfite waste liquor in Northup-Anson type sintered glass diffusion cells for sixty-two days; (c) preparation of barium ligno-sulfonate soluble in 40% acetone–water solution, insoluble in 70% acetone–water solution; (d) dialysis of fermented sulfite waste liquor against running water for one hundred and sixty-eight hours[2]; (e) purification by a quinoline extraction method, precipitation from quinoline solution of quinolinium ligno-sulfonates by the addition of ether and re-solution of the quinolinium salts in aqueous ammonium hydroxide.

Table I lists the various preparations and the equivalent weights of lignin sulfonic acids per mole of periodate reduced.

TABLE I

Preparation	% Methoxyl content of ammonium salt	Equiv. wt./mole of periodate reduced
a	8.5	200
b	10.1	304
c	9.7	347
d	13.0	525
e	11.9	525

It is apparent that preparations of low methoxyl content contain extraneous periodate-reducing substances, probably carbohydrates; but as refinement improves, the approach of the methoxyl content to a limiting value is accompanied by a similar constancy in the periodate equivalent weight. We believe samples (d) and (e) to be substantially free of carbohydrate material. The equivalent weights of 525 for preparations (d) and (e) correspond approximately to a ratio of two methoxyl groups for each linkage oxidizable by periodic acid.

Periodic acid oxidation of lignin sulfonic acids prepared by method (e) yields a barium salt,

(1) W. H. Wahl, P. F. Ritchie and C. B. Purves, abstract No. 8, p. 4C, Division of Cellulose Chemistry, Abstracts of Papers 109th Meeting, American Chemical Society, Atlantic City, New Jersey, April 8, 1946.

(2) This material and its analysis were kindly supplied by Dr. Quentin Peniston of this Laboratory.

difficulty soluble in water. In contrast the barium salt of the unoxidized starting material is extraordinarily soluble. The methoxyl content of the unoxidized barium salt was 10.4% with a barium content of 12.5%. The barium salt of the oxidized material has a methoxyl content of 6.4% with barium content of 17.2%. This indicates the cleavage from the anion of a methoxyl-containing fragment during periodate oxidation. From the oxidized material an oxime has been prepared containing 2.7% nitrogen.

Complete accounts of these experiments and other studies now in progress on the periodate oxidation of lignin sulfonic acids and other lignins will be given in future communications.

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RECEIVED JUNE 10, 1946

ON THE PEPTIDE NATURE OF VITAMIN Bc CONJUGATE FROM YEAST

Sirs:

Evidence has been presented to support the view that vitamin Bc conjugate consists of vitamin Bc linked to an ultraviolet-transparent nitrogeneous moiety.[1] The non-vitamin Bc portion of the molecule has been found to consist of six molecules of 1(+)-glutamic acid in peptide linkage.

Following hydrolysis (18% hydrochloric acid for sixteen hours at 100°) 59.6% of the total N reacted as α-amino acid N,[2] which was accounted for as glutamic acid nitrogen by microbiological assay.[3,4] From 298 mg. of conjugate methyl ester 220 mg. of 1(+)-glutamic acid hydrochloride was isolated. $[\alpha]^{24}D$ +25.4° (5.1% solution in 1 *N* hydrochloric acid; C, 32.75; H, 5.6; N, 7.8, 7.6. Calcd.: C, 32.7; H, 5.5; N, 7.6. Under comparable hydrolytic conditions 20.1% of the total nitrogen of vitamin Bc reacted as α-amino acid nitrogen and as glutamic acid nitrogen by microbiological assay.

The ratio of the $E^{1\%}_{1\,cm.}$ values of vitamin Bc to the conjugate was determined as 2.72:1. With the molecular formula of vitamin Bc established as $C_{19}H_{19}O_6N_7$ (mol. wt. 441.4)[5] the above ratio suggests a minimum molecular weight for the conjugate of 1200 (found by diffusion 1400).[6] A molecule consisting of one molecule of the vitamin in peptide linkage with a peptide chain consisting of six 1(+)-glutamic acid residues would have the molecular formula $C_{49}H_{61}O_{24}N_{13}$ (1216.1). This formulation is in agreement with elementary analytical findings some of which have been reported.[1] That the conjugate is not a mixture of peptides with an average of 7 glutamic acid residues is evidenced by its homogeneous behavior on electrophoresis.[6] Following the suggested nomenclature of Angier, *et al.*,[7] vitamin Bc conjugate may be designated pteroylhexaglutamylglutamic acid.

The conjugate is essentially inactive microbiologically, whereas the fermentation *L. casei factor* reported by Angier, *et al.*,[7] to yield 3 moles of glutamic acid has high microbiological activity.

Demonstration of the peptide nature of the conjugate identifies the conjugase enzymes[8] which split the microbiologically active compound from the conjugate as peptidases. Since conjugases do not liberate vitamin Bc from the conjugate methyl ester[1] they can be further classified as carboxypeptidases.

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RECEIVED JUNE 20, 1946

(1) Pfiffner, Calkins, O'Dell, Bloom, Brown, Campbell and Bird, *Science*, **102**, 228 (1945).

(2) Van Slyke, *J. Biol. Chem.*, **16**, 121 (1913).

(3) Hier, Graham, Freides and Klein, *ibid.*, **161**, 705 (1945).

(4) We wish to thank Dr. O. D. Bird for conducting the microbiological determinations.

(5) Our analytical data allowed a choice between $C_{19}H_{19}O_6N_7$ and $C_{21}H_{20}O_6N_8$ as the probable molecular formula while our degradation results ruled out the latter formulation. Angier, *et al.* (*Science*, **103**, 667 (1946)) demonstrated by degradation and synthesis the structure of the liver *L. casei factor* to be N-[4-[[(2-amino-4-hydroxy-6-pteridyl)-methyl]amino]-benzoyl]-glutamic acid and suggested the name pteroylglutamic acid. A sample of the synthetic compound was generously supplied us by the Lederle Laboratories, and we found it to be identical with the compound which we isolated from liver and yeast and tentatively called vitamin Bc (*Science*, **97**, 404 (1943)).

(6) We wish to thank Dr. J. M. Vandenbelt for the ultraviolet absorption, diffusion and electrophoresis determinations.

(7) Angier, *et al.*, *Science*, **103**, 667 (1946).

(8) Bird, Binkley, Bloom, Emmett and Pfiffner, *J. Biol. Chem.*, **157**, 413 (1945).