acidification of the alkaline solution with hydrochloric acid, tributylacetic acid separated as an oil which solidified upon standing; yield 8 g. (80%); m. p. 33.5–35.5°.

Acknowledgment.—The authors wish to express their appreciation to Mrs. Rosemarie Fricano for her technical assistance during the course of this investigation. We are also grateful to Dr. Richard Tislow and Mrs. Annette La Belle of our Biological Laboratories for the data on the antispasmodic activities reported herein.

Summary

1. The conversion of trialkylacetamides to trialkylacetic acids has been investigated using a number of different hydrolytic agents. Of the methods studied, the reaction of alkyl nitrites in organic solvents in the presence of gaseous hydrogen chloride gave consistently high yields of trialkylacetic acids.

2. Several new trialkylacetic acids are described.

3. Trialkylacetic acids having a total of 15–20 carbon atoms show musculotropic and neurotropic antispasmodic activity.

BLOOMFIELD, NEW JERSEY RECEIVED MARCH 26, 1948

[CONTRIBUTION FROM THE SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH]

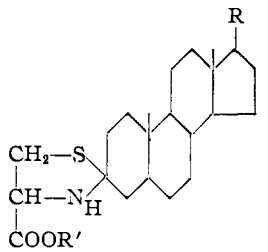
Spiro (Steroid) Thiazolidines[1]

BY SEYMOUR LIEBERMAN, PAUL BRAZEAU AND LUCIE B. HARITON

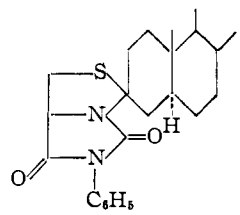
In a preliminary note[2] the preparation of several spiro (steroid) thiazolidines was reported, and the possible role such structures may have in the action of steroid hormones was discussed.

Schubert[3] in a series of papers has described the thiazolidines resulting from the condensation of cysteine with various aldehydes. Ratner and Clarke[4] studied the formation and properties of thiazolidine-4-carboxylic acid, and it has been announced[5] that the various penicillins are thiazolidine derivatives. This paper describes the thiazolidines formed by the reaction of 1(+)-cysteine with a number of 3-ketosteroids.

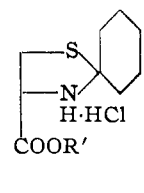
When an alcoholic (methanol or ethanol) solution of cholestanone was added to an aqueous alcoholic solution of 1(+)-cysteine hydrochloride buffered with potassium acetate, a heavy precipitate formed in a few minutes. The product (I) melted unsharply between 220 and 230° and was insoluble in water and very slightly soluble in organic solvents. The solubility together with the high melting point suggested that the product exists as a dipolar ion. The compound produced no color with sodium nitroprusside in the presence of ammonia or sodium bicarbonate whereas in the presence of sodium carbonate, a positive test for the free SH grouping was obtained. The compound was readily oxidized by a solution of iodine producing cystine and cholestanone. These reactions indicated that the product was (I), a thiazolidine analogous to those prepared by Schubert.[3] It was characterized as its N-acetyl derivative, m. p. 266–267°. Spiro [cholestane-3,2′-thiazolidine-4′-carboxylic acid ethyl ester] (II) prepared from cholestanone and cysteine ethyl ester hydrochloride in buffered solution reacted similarly to the free acid and when treated with phenyl isocyanate, the product isolated was the N-phenylhydantoin (III), m. p. 216–217°.



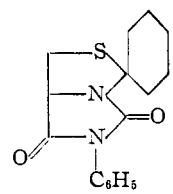
I	R = C_8H_{17}	; R′ = H
II	R = C_8H_{17}	; R′ = C_2H_5
IV	R = O	; R′ = H
IVa	R = O	; R′ = C_2H_5
V	R = $COCH_3$	; R′ = H
Va	R = $COCH_3$	; R′ = C_2H_5
VI	R = ...OH	; R′ = H
VIa	R = ...OH	; R′ = C_2H_5
VII	R = —OH	; R′ = H



III



VIII R′ = H
IX R′ = C_2H_5



X

Spiro-thiazolidines were prepared from the following 3-ketosteroids: androstanedione-3,17 (IV and IVa), pregnanedione-3,20 (V and Va), androstanol-17α-one-3 (VI and VIa) and its 17β isomer androstanol-17β-one-3 (VII). The products formed may exist in two possible steric modifications since the thiazolidine ring is at right angles to the plane of the steroid nucleus. Consequently one isomer can be considered to have the sulfur atom of the heterocyclic ring above the plane of the nucleus, while the sulfur atom of the second isomer would be below the plane of the nucleus. However, only one isomer has been obtained from the compounds investigated.

(1) The authors gratefully acknowledge the assistance of the Jane Coffin Childs Memorial Fund for Medical Research, the Commonwealth Fund, the Whiting Foundation, the Alfred P. Sloan, Jr., Research Fund, the Baird Foundation and the Pardee Foundation.

(2) Lieberman, *Experientia*, II, 411 (1946).

(3) Schubert, *J. Biol. Chem.*, **111**, 671 (1935); **114**, 341 (1936); **121**, 539 (1937); **130**, 601 (1939).

(4) Ratner and Clarke, THIS JOURNAL, **59**, 200 (1937).

(5) *Science*, **102**, 627 (1945).

Experimental[6,7,8]

Spiro[cholestane-3,2′-thiazolidine-4′-carboxylic Acid] (I).—The conditions employed for the preparation of this compound are essentially the same as those of Schubert[3] for the condensation of cysteine with simple aldehydes (Procedure I). One gram of cholestanone-3 was dissolved by heating in 37 ml. of ethanol. After cooling, 10 ml. of 50% ethanol solution containing 418 mg. of l(+)-cysteine hydrochloride and 250 mg. of potassium acetate were added. In a few minutes the precipitation of the thiazolidine began; the yield was 1.2 g. In common with most other amino acids, it did not have a sharp, constant melting point; it sintered at 210° and melted unsharply from 220–230°. Comparable yields can be obtained by using pyridine as the solvent. The product was extremely insoluble in organic solvents including acetic acid, pyridine, dioxane and was not recrystallized.

A suspension of the compound in alcohol made alkaline with sodium bicarbonate or ammonia did not produce a color with sodium nitroprusside. However, when made alkaline with sodium carbonate, a positive test for the free sulfhydryl group was obtained. A suspension of the thiazolidine in methanol rapidly decolorized an aqueous iodine solution. The precipitate remaining after the oxidation was cholestanone melting at 128–129°.

The **N-acetyl** derivative was prepared by suspending 250 mg. of the thiazolidine (I) in 4 ml. of pyridine containing 0.4 ml. of acetic anhydride. After standing for two hours, the white precipitate was collected by filtration; it weighed 180 mg. and melted at 264–267°. After recrystallization from large volumes of either ethyl acetate or acetone, it melted at 266–267°; $[\alpha]^{24}D$ +11.4 ± 2° (pyridine). The neutralization equivalent was 570; calculated 591. The compound was recovered unchanged after acidification of the alkaline solution.

Anal. Calcd. for $C_{32}H_{53}O_3NS$: C, 72.29; H, 10.03; N, 2.63; S, 7.58. Found: C, 72.25; H, 10.02; N, 2.82; S, 7.39.

When cholestanone was treated with N-acetylcysteine or N-phenylureidocysteine or methionine, no condensation was observed, indicating that both the amino and sulfhydryl groups must be unsubstituted to effect reaction. When the tripeptide, glutathione, was substituted for cysteine, no evidence of reaction was observed.

Spiro[cholestane-3,2′-thiazolidine-4′-carboxylic Acid Ethyl Ester] (II).—This compound was prepared in pyridine solution from 250 mg. of cholestanone and 120 mg. of cysteine ethyl ester hydrochloride (Procedure II). The solution was kept at room temperature overnight and then the product was precipitated by the careful addition of water. It melted at 140–142° and weighed 311 mg. On recrystallization from ethanol, long needles melting at 148–150° were obtained, $[\alpha]^{22}D$ −30.9 ± 2° (benzene). The substance was readily oxidized with iodine and was decomposed by sodium carbonate as evidenced by a positive sodium nitroprusside test.

Anal. Calcd. for $C_{32}H_{55}O_2NS$: C, 74.23; H, 10.70; N, 6.18. Found: C, 74.35; H, 10.85; N, 6.33.

The **hydrochloride** of spiro[cholestane-3,2′-thiazolidine-4′-carboxylic acid ethyl ester] was prepared by boiling 50 ml. of ethanol containing 250 mg. of cholestanone and 129 mg. of cysteine ethyl ester hydrochloride for fifteen minutes. The solution was evaporated to dryness and the residue extracted with ether and water to remove unchanged reactants. The insoluble residue weighed 211 mg. and melted at 215–228°. It was recrystallized from ethanol for analysis, m. p. 225–229°; $[\alpha]^{24}D$ −2.5 ± 2° (benzene).

Anal. Calcd. for $C_{32}H_{56}O_2NSCl$: C, 69.30; H, 10.19; Cl, 6.40; S, 5.77. Found: C, 69.32; H, 10.26; Cl, 6.46; S, 5.89.

The compound reduced iodine readily and when treated with pyridine, it was converted to the free base II, m. p. 143–145° (no depression in m. p. when mixed with II).

N-Phenylhydantoin of spiro[cholestane-3,2′-thiazolidine-4′-carboxylic acid] (III).—Phenyl isocyanate (0.24 ml.) was added to a solution of 620 mg. of II in 4 ml. of pyridine. After standing overnight, the solution was concentrated to dryness in vacuum, and the residue was extracted several times with boiling ligroin (b. p. 90°). The ligroin extract was concentrated to a small volume, and on cooling the product crystallized as needles which melted at 195–198° and weighed 593 mg. Two recrystallizations from ethanol gave a sample melting at 216–217°; $[\alpha]^{24}D$ +30.8 ± 1° (benzene).

Anal. Calcd. for $C_{37}H_{54}O_2N_2S$: C, 75.20; H, 9.21; N, 4.74; S, 5.43. Found: C, 75.21; H, 9.26; N, 4.95; S, 5.69.

Spiro[17-keto-androstane-3,2′-thiazolidine-4′-carboxylic Acid] (IV).—This compound was prepared in 67% yield from androstanedione-3,17 using Procedure I; m. p. 217–220° (from methanol); $[\alpha]^{24}D$ −19.4 ± 2° (pyridine).

Anal. Calcd. for $C_{22}H_{33}O_3NS$: C, 67.48; H, 8.49; N, 3.58; S, 8.19. Found: C, 67.03; H, 8.55; N, 3.63; S, 8.27.

Spiro[17-keto-androstane-3,2′-thiazolidine-4′-carboxylic Acid Ethyl Ester] (IVa).—This substance was prepared in 50% yield from androstanedione-3,17 and cysteine ethyl ester hydrochloride according to Procedure I; m. p. 206–207° (ethanol), $[\alpha]^{28}D$ −6.2 ± 2° (benzene).

Anal. Calcd. for $C_{24}H_{37}O_3NS$: C, 68.40; H, 8.89; N, 3.34. Found: C, 68.62; H, 8.64; N, 3.99.

Spiro[20-keto-pregnane-3,2′-thiazolidine-4′-carboxylic Acid] (V).—This substance was prepared in 59% yield from pregnanedione-3,20 by Procedure I; m. p. 149–152° (methanol); $[\alpha]^{24}D$ −8.0 ± 3° (pyridine).

Anal. Calcd. for $C_{24}H_{37}O_3NS \cdot CH_3OH$: C, 66.48; H, 9.15; N, 3.10; S, 7.09. Found: C, 66.31, 66.25; H, 9.32, 8.94; N, 3.26; S, 7.70.

Spiro[20-keto-pregnane-3,2′-thiazolidine-4′-carboxylic Acid Ethyl Ester] (Va).—This compound was prepared in 43% yield from pregnanedione-3,20 and cysteine ethyl ester hydrochloride by Procedure I; m. p. 119–121° (methanol); $[\alpha]^{24}D$ +5.6 ± 2° (ethanol).

Anal. Calcd. for $C_{26}H_{41}O_3NS$: C, 69.75; H, 9.23. Found: C, 69.79; H, 9.27.

Spiro[17α-hydroxy-androstane-3,2′-thiazolidine-4′-carboxylic Acid] (VI).—This substance was prepared in 57% yield from androstanol-17α-one-3 using Procedure I, m. p. 225–227° (methanol); $[\alpha]^{26}D$ −69.0 ± 2° (pyridine).

Anal. Calcd. for $C_{22}H_{35}O_3NS \cdot CH_3OH$: C, 64.91; H, 9.24. Found: C, 65.56; H, 8.89.

Spiro[17α-hydroxy-androstane-3,2′-thiazolidine-4′-carboxylic Acid Ethyl Ester] (VIa).—This compound was prepared in 83% yield from androstanol-17α-one-3 and cysteine ethyl ester hydrochloride using Procedure II; m. p. 170–171° (methanol), $[\alpha]^{25}D$ −44.3 ± 2° (pyridine).

Anal. Calcd. for $C_{24}H_{39}O_3NS$: C, 68.36; H, 9.32; S, 7.61. Found: C, 68.12; H, 9.19; S, 7.79.

Spiro[17β-hydroxy-androstane-3,2′-thiazolidine-4′-carboxylic Acid] (VII).—This substance was prepared in 74% yield from androstanol-17β-one-3 by Procedure I; m. p. 218–220° (methanol); $[\alpha]^{24}D$ −96.0 ± 3° (pyridine).

(6) The melting points were taken in a Hershberg melting point apparatus and are correct to about ±1°. For rotation, 5 to 10 mg. of the compounds were dissolved in 2.00 ml. of solvent and the rotations were measured in a 1-dm. tube. Prior to analysis the samples were dried in vacuum (10 mm.) at 100° for at least eighteen hours.

(7) The microanalyses reported herein, except those noted, were done through the courtesy of Dr. A. Elek, The Rockefeller Institute for Medical Research, New York.

(8) The authors wish to express their gratitude to Dr. C. R. Scholz, Ciba Pharmaceutical Co., Summit, N. J., and to Dr. Erwin Schwenk, Schering Corp., Bloomfield, N. J., for their generosity in making available most of the steroids used in this investigation.

Anal. Calcd. for $C_{22}H_{35}O_3NS$: C, 67.13; H, 8.96. Found: C, 66.75; H, 9.12.

Spiro[cyclohexane-1,2′-thiazolidine-4′-carboxylic Acid Hydrochloride] (VIII).—Two and a half grams of cysteine hydrochloride was suspended in 10 ml. of ethanol containing 1.64 ml. of cyclohexanone. During the course of three hours, the amino acid hydrochloride dissolved completely. The solution was filtered, concentrated to a small volume and, after cooling, 953 mg. of the product crystallized. By the addition of ligroin to the filtrate, an additional 1.348 g. of product was obtained.

The product was insoluble in acetone, ether and ligroin, but soluble in water and ethanol. It was oxidized by iodine and gave a negative sodium nitroprusside test in sodium bicarbonate solution. However, positive tests were obtained with dilute solutions of ammonia, sodium carbonate or sodium hydroxide. A water solution of the hydrochloride possessed no odor but after the addition of dilute alkali, the odor of cyclohexanone was easily discernible. The product crystallized from ethanol:ether (1:3) in the form of plates which melted unsharply with decomposition between 140–150° (Kofler block); $[\alpha]^{17}D$ −76.7 ± 0.3° (ethanol).

Anal. Calcd. for $C_9H_{16}O_2NSCl$: C, 45.47; H, 6.78. Found: C, 45.59; H, 6.76.[9]

The **N-benzoyl derivative** of spiro[cyclohexane-1,2′-thiazolidine-4′-carboxylic acid] was prepared by suspending 0.1 g. of VIII in 0.2 ml. of pyridine and 5 ml. of dry ether containing 10 drops of benzoyl chloride. After two hours the ether was evaporated, water was added to the residue and the mixture was extracted with chloroform. The chloroform extract was dried with sodium sulfate and the solution concentrated to dryness *in vacuo*. The residue was dissolved in acetone and the product crystallized by the addition of ether. Several recrystallizations from acetone:ether gave rods melting at 205–206° (Kofler block).

Anal. Calcd. for $C_{16}H_{19}O_3NS$: C, 62.92; H, 6.27. Found: C, 62.96; H, 6.25.[9]

Spiro[cyclohexane-1,2′-thiazolidine-4′-carboxylic Acid Ethyl Ester Hydrochloride] (IX).—A suspension of 283 mg. of cysteine ethyl ester hydrochloride in 0.17 ml. of cyclohexanone was heated on a steam-bath for five minutes. During this time, the reaction mixture solidified, m. p. 184–188°. The solid residue crystallized from ethanol as plates, m. p. 189–192° (dec.); $[\alpha]^{24}D$ −73.2 ± 2° (ethanol).

Anal. Calcd. for $C_{11}H_{20}O_2NSCl$: C, 49.69; H, 7.58. Found: C, 49.82; H, 7.56.

The **N-phenylhydantoin** of spiro[cyclohexane-1,2′-thiazolidine-4′-carboxylic acid] (X) melted at 143–145° (ethanol); $[\alpha]^{22}D$ −54.4 ± 2° (benzene).

Anal. Calcd. for $C_{16}H_{18}O_2N_2S$: C, 63.54; H, 6.00. Found: C, 63.83, 63.84; H, 6.06, 6.04.

Spectroscopic Experiments.—A Beckman photoelectric spectrophotometer equipped with a hydrogen discharge tube and 1-cm. cells was used. The molecular extinction coefficient of cyclohexanone at the maximum, 283 mμ, was observed to be 15.7. In the presence of an equimolar amount of *l*(+)-cysteine hydrochloride and twice that amount of potassium acetate, a 70% ethanol solution of cyclohexanone showed $\epsilon_{283} = 7.7$, indicating that 51% of the cyclohexanone had reacted with the cysteine. On the other hand, under similar conditions isophorone ($\epsilon_{234} = 12{,}600$), acetophenone ($\epsilon_{241} = 11{,}870$) and progesterone ($\epsilon_{241} = 17{,}000$) did not react with cystine since there was no decrease in extinction coefficient.

The molar extinction coefficient of benzalacetophenone at the maximum 312 mμ is 24,300. After a 10^{-3} *M* solution in 75% ethanol was kept for twenty-four hours with an equimolar amount of cysteine hydrochloride and 2×10^{-3} *M* potassium acetate, the extinction coefficient was unchanged. However, when the concentration of reactants was increased to 0.020 *M*, a crystalline reaction product precipitated within ninety minutes. After twenty hours, the concentration of the unreacted benzalacetophenone in the supernatant solution was determined spectroscopically and found to be 0.0075 *M*, indicating that 62.5% of the ketone had reacted.

(9) Microanalyses done by F. Weiser, Basel, Switzerland.

Discussion

The extension of this condensation reaction to steroids having the carbonyl group on carbon atoms other than C_3 has been attempted but no thiazolidine formation has been observed. The 17-ketosteroids, dehydroisoandrosterone, androsterone acetate, estrone and the 20-ketones, Δ^5-pregnenol-3β-one-20, its 21-benzal derivative, and the ketols, Δ^5-pregnenediol-3β,21-one-20, and its 21-acetoxy derivative did not react under the experimental conditions. Compounds with ketone groups in other positions were not extensively investigated but the 6-ketone, 3-hydroxy-6-ketocholanic acid and the 12-ketone, methyl 3-acetoxy-12-ketocholanate likewise failed to form a derivative. These results indicate that the fusion of the two ring systems in the thiazolidine (IV–Va) formed from the diketones androstanedione-3,17 and pregnanedione-3,20 must be at C_3 of the steroid nucleus.

An unexpected finding was the failure of the α,β-unsaturated 3-ketosteroids to react with cysteine or its ethyl ester. Testosterone, its propionate and benzoate, vinyltestosterone, ethynyltestosterone and Δ^4-cholestenone-3, did not react with cysteine or its ethyl ester under several conditions. Progesterone, desoxycorticosterone and Δ^4-androstenedione-3,17 likewise did not form thiazolidines. From these results it appears likely that the formation of spiro(steroid)thiazolidines can be employed to separate and distinguish saturated 3-ketosteroids from α,β-unsaturated 3-ketones and steroids possessing a carbonyl group elsewhere in the molecule.

In order to study the anomalous behavior of unsaturated ketones, several simple model compounds were investigated. Cyclohexanone readily reacts with cysteine or cysteine ethyl ester to yield the expected thiazolidines (VIII, IX and X), whereas the unsaturated cyclohexenone derivative, isophorone (Δ^2-3,5,5-trimethylcyclohexenone) did not react. Similarly, mesityl oxide, phorone, benzalacetone, furfuralacetophenone, as well as acetophenone,[3] ω-hydroxyacetophenone, benzophenone and benzoin did not yield thiazolidines under the conditions employed. On the other hand, crystalline products were obtained when the α,β-unsaturated carbonyl compounds cinnamaldehyde, benzalacetophenone and acrylophenone were treated with cysteine. The structures of these products are being investigated.

Because some of the unsaturated carbonyl compounds failed to produce crystalline reaction products with cysteine or its ester, spectroscopic evidence of reaction was sought. In the presence of an equimolar amount of cysteine the molecular extinction coefficient of cyclohexanone at the max-

imum, 283 mμ, was markedly depressed, since the carbonyl group disappears by reaction with cysteine. However, the absorption of isophorone at the maximum, 234 mμ was not decreased by cysteine, and no reaction between acetophenone or progesterone and cysteine could be demonstrated spectroscopically. A solution of benzalacetophenone showed no decrease in molecular extinction coefficient at the maximum, 312 mμ, in the presence of an equimolar amount of cysteine when the concentration of ketone was 10^{-3} M but when the concentration of the reactants was increased to 0.020 M, a crystalline reaction product precipitated from the solution. This result indicates an equilibrium wherein the reactants are completely dissociated at high dilutions. In view of this equilibrium it can be suggested that the failure to isolate thiazolidines from α,β-unsaturated ketosteroids was not due to the inability of these substances to react with cysteine but was caused by an unfavorable equilibrium or an improper choice of experimental conditions.

The condensation of carbonyl compounds with cysteine to yield thiazolidines raises the interesting question as to what extent carbonyl compounds could react with other –SH compounds, including sulfhydryl-containing-proteins. In this connection it is interesting to note that Middlebrook and Phillips[10] have already demonstrated that protein-thiazolidines are formed when formaldehyde reacts with the cysteinyl residues of modified wool. They have secured evidence that the thiazolidine structure exists in a combined form in modified wool-protein treated with formaldehyde and have isolated thiazolidine-4-carboxylic acid from similarly treated wool. Furthermore, regarding the possible participation of protein-thiazolidines in biochemical systems, it may be pointed out that several sulfhydryl-dependent enzymes are inhibited by aldehydes and ketones[11,12,13] and also that Geiger and Conn[14] have suggested that certain antibiotics react with and inhibit sulfhydryl-containing compounds essential for the metabolism of susceptible bacteria. The latter investigators[14] observed that some simple α,β-unsaturated ketones, notably benzalacetophenone and acrylophenone, possess bacteriostatic and fungistatic properties and postulated that the unsaturated ketones react with the –SH compounds by means of a 1,4 addition to produce β-alkylthio ketones. The evidence presented, however, does not exclude the possibility that thiazolidines were formed. It may also be possible that carbonyl compounds condense with proteins to yield products which may be considered α-amino thioethers $\left(-NH-\underset{|}{\overset{|}{C}}-S-CH_2-\right)$. In this case the free amino group necessary for the condensation could be furnished by a neighboring peptide chain and need not be on the carbon atom adjacent to that bearing the –SH group. McLeod and Robinson[15] have prepared α-amino thioethers by the condensation of formaldehyde with simple amines and mercaptans.

Is it therefore conceivable that steroid hormones conjugate with proteins by virtue of their ability to condense with free –SH groups? If so, they could then function as prosthetic groups or they could be transported in blood as analogous protein conjugates. Moreover, water-soluble thiazolidine polypeptides could constitute another form of conjugation in which steroid ketones are excreted in the urine. The diketones, androstanedione-3,17, etiocholanedione-3,17, allopregnanedione-3,20 and pregnanedione-3,20 have been isolated from human urine[16] and since these compounds lack hydroxyl groups, the known modes of conjugation, sulfate or glucuronidate formation, can not apply to them.

Summary

Spiro(steroid)thiazolidines have been prepared by the condensation of cysteine or its ethyl ester with several saturated 3-ketosteroids. Attempts have been made to synthesize similar derivatives from α,β-unsaturated 3-ketosteroids, and also from steroids having a carbonyl group on carbon atoms other than C_3. A suggestion has been made regarding the possible role condensation products of this type may play in biochemical processes.

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(11) Murray, *ibid.*, **23**, 292 (1929).

(12) Weinstein and Wynne, *J. Biol. Chem.*, **112**, 649 (1936).

(13) Mann and Quastel, *Biochem. J.*, **34**, 414 (1940).

(14) Geiger and Conn, THIS JOURNAL, **67**, 112 (1945).

(15) McLeod and Robinson, *J. Chem. Soc.*, **119**, 1470 (1921).

(16) Lieberman, Dobriner, Hill, Fieser and Rhoads, *J. Biol. Chem.*, **172**, 263 (1948).

(17) Original manuscript received May 1, 1947.