1.6 g. A sample for analysis was recrystallized from 50% aqueous acetic acid and dried at 130°.

Anal. Calcd. for $C_6H_7N_5S$: C, 39.8; H, 3.9; N, 38.7. Found: C, 39.5; H, 4.0; N, 38.6.

6-Amino-8-methylthiopurine (XXII). Method 1.—To 150 ml. of concentrated ammonium hydroxide was added 15.0 g. of 6-chloro-8-methylthiopurine (VIII). The solution was heated at 110° for 6 hr. The excess ammonia was then evaporated on the steam-bath, and a solid gradually appeared from the hot solution. The volume was reduced to approximately 70 ml. and the solution filtered to yield 10.5 g. of gray solid. The product was purified by reprecipitation from hot dilute sodium hydroxide with acetic acid. The m.p. was 288–290° dec.

Anal. Calcd. for $C_6H_7N_5S$: N, 38.8. Found: N, 38.7.

Method 2.—To 125 ml. of water was added 10 g. of potassium hydroxide and 3.0 g. of 6-amino-8-purinethiol (XXVI). The solution was cooled to 15° and 3.0 g. of methyl iodide added. The solution was stirred at 15–20° for 30 min. The solution was neutralized with acetic acid and filtered to yield 2.1 g. of product. A small sample was recrystallized from a large volume of 50% aqueous ethanol, m.p. 288–290°.

Anal. Calcd. for $C_6H_7N_5S$: N, 38.8. Found: N, 38.7.

The 6-amino-8-methylthiopurine (XXII) thus prepared was judged to be identical with that prepared by method 1 on the basis of ultraviolet absorption spectra and mixed m.p. data.

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[CONTRIBUTION FROM THE DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY, UNIVERSITY OF CALIFORNIA SCHOOL OF MEDICINE]

Dihydrofolic Acid Reductase[1]

BY JAMES M. PETERS AND DAVID M. GREENBERG

RECEIVED MAY 13, 1958

An enzyme has been separated from sheep liver which catalyzes the reduction of dihydrofolic acid to tetrahydrofolic acid. The reduced form of either di- or triphosphopyridine nucleotide serves as the electron donor in this reduction, the most effective nucleotide being determined by the hydrogen ion concentration of the incubation medium. The Michaelis constants for DPNH, TPNH and dihydrofolic acid were determined. A stoichiometric relationship between dihydrofolic acid reduction and nucleotide oxidation has been demonstrated and a synergistic activity between dihydrofolic acid reductase and choline synthetase has been shown to occur when dihydrofolic acid, rather than tetrahydrofolic acid, is employed as the cofactor for choline methyl group formation from formaldehyde. Dihydrofolic acid reductase preparations have an extremely low activity for folic acid reduction, indicating that the enzyme has been separated from the enzyme system which carries out the complete reduction of folic to tetrahydrofolic acid. Studies with sulfhydryl reagents indicate that free sulfhydryl groups are probably not required for enzyme activity.

Introduction

The reduction of folic acid and FH_2[2] to FH_4 by animal and bacterial enzyme systems has been the subject recently of numerous investigations.[3-7] Wright, *et al.*,[3,4] have studied the reduction of folic acid and teropterin (diglutamylfolic acid) in bacteria and have shown that reduction of these compounds to their dihydro derivatives is coupled to pyruvate oxidation. In avian liver Futterman,[5] Zakrzewski and Nichol[6] and Osborn, *et al.*,[7] have demonstrated a requirement for TPNH as the electron donor for folic acid reduction to the dihydro level. The present investigation is concerned with the reduction of FH_2 to FH_4 by an enzyme separated from sheep liver. Both DPNH and TPNH have been reported[5] to function as cofactors for the reduction of FH_2 to FH_4 whereas only TPNH appears to be implicated in the initial reduction stage from folic acid to FH_2.

FH_2 reduction was measured either by the decrease in optical density at 340 mμ of an incubation mixture containing FH_2-reductase, FH_2 and DPNH (or TPNH) or by the production of a diazotizable amine (see later). The purified FH_2-reductase had only slight DPNH- (or TPNH)-oxidizing activity in the absence of FH_2. In order to determine the optimum conditions for the assay, the rate of DPNH and TPNH oxidation as a function of *p*H was measured, and it was found that the rate of FH_2 reduction by DPNH proceeds most favorably at *p*H values below 5.5 whereas reduction by TPNH is favored at *p*H values above 6 (Fig. 1). The rate of FH_2 reduction was also found to be a linear function of the enzyme concentration.

The unusual *p*H–activity curves (Fig. 1) suggest the possibility that two enzymes are involved in FH_2 reduction, one which utilizes DPNH as cofactor and another which utilizes TPNH. The findings recorded in Table I indicate that both activities probably are associated with one enzyme.

TABLE I

EFFECT OF NUCLEOTIDE SATURATION UPON THE ACTIVITY OF DIHYDROFOLIC ACID REDUCTASE

Incubations were carried out at room temperature in 1 cm. Corex cuvettes for 20 minutes. Nucleotide oxidation was measured by the change in optical density at 340 mμ and was corrected for the optical density change in controls containing no FH_2. Each cuvette contained 0.01 ml. of FH_2-reductase and 0.133 μmole of FH_2 in 3 ml. of 0.1 *M* sodium phosphate buffer, *p*H 5.7.

DPNH added, μmoles	TPNH added, μmoles	Nucleotide oxidized,[a] μmoles
0.32	0	0.044
0.32	0.14	.043
0	0.14	.041

[a] Each value represents the average of duplicate determinations.

(1) Aided by research grants from the National Cancer Institute (CY-3175), United States Public Health Service, and the American Cancer Society, California Division (151).

(2) Abbreviations used are: FH_2, dihydrofolic acid; FH_4, tetrahydrofolic acid; DPN and TPN, di- and triphosphopyridine nucleotides; DPNH and TPNH, reduced nucleotides.

(3) B. E. Wright and M. L. Anderson, THIS JOURNAL, **79**, 2027 (1957).

(4) B. E. Wright, M. L. Anderson and E. C. Herman, *J. Biol. Chem.*, **230**, 271 (1958).

(5) S. Futterman, *ibid.*, **228**, 1031 (1957).

(6) S. F. Zakrzewski and C. A. Nichol, *Biochim. Biophys. Acta*, **27**, 425 (1958).

(7) M. J. Osborn, M. Freeman and F. M. Huennekens, *Proc. Soc. Exptl. Biol. Med.*, **97**, 429 (1958).

The Michaelis constants for DPNH at *p*H 5.0, $K_m = 1.5 \times 10^{-5}$ *M*, for TPNH at *p*H 7.1, $K_m = 1.0 \times 10^{-5}$ *M*, for FH_2 at *p*H 5.0 (DPNH as cofactor), $K_m = 6.2 \times 10^{-6}$ *M*, and for FH_2 at *p*H 7.1 (TPNH as cofactor), $K_m = 1.0 \times 10^{-5}$ *M*, were determined by the Lineweaver–Burk reciprocal plot method.[8]

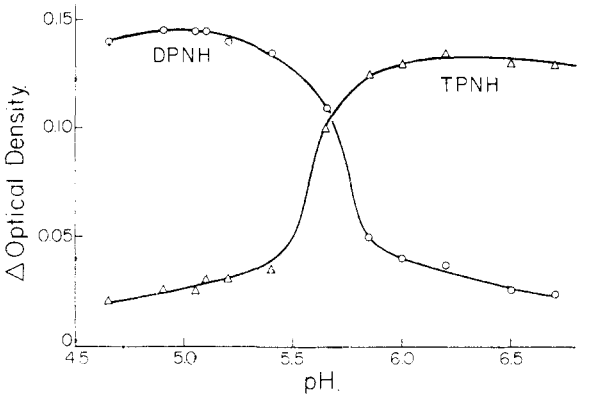


Fig. 1.—*p*H–activity curve of FH_2-reductase: 15-minute incubations were carried out at room temperature in 1 cm. Corex cuvettes. Cuvette contents: 3.0 ml. of 0.1 *M* sodium acetate buffer (*p*H 4.6–5.6) or 0.1 *M* sodium phosphate buffer (*p*H 5.8–6.7), 0.06 μmole of FH_2, 0.02 ml. of purified FH_2-reductase and 0.3 μmole of DPNH (O), or TPNH (Δ). Decrease in optical density (Δ, O.D.) was measured at 340 mμ. The values obtained were corrected for the slight decrease in optical density which occurred in the absence of FH_2.

Since the product of the reduction, FH_4, has been reported to undergo non-enzymic decomposition when exposed to air to yield *p*-aminobenzoylglutamic acid,[5,6,9] it was of interest to determine if the reduction could be assayed by diazotization of the resulting decomposition product. The method was that of Blakley[9] modified by the use of an aqueous solution of N-(1-naphthyl)-ethylenediamine dihydrochloride[10] instead of ethanolic N-ethyl-1-naphthylamine. The concentration of the resulting diazo compound was measured optically at 545 mμ. Figure 2 shows the relative diazotizability of folic acid, FH_2 and FH_4 by this method and indicates that reduction of the pyrazine ring of folic acid, even to the dihydro level, causes instability of the bond between carbon atom No. 9 and nitrogen atom No. 10. In order to adapt this method for the assay of FH_2-reductase it was necessary to determine the change in diazotizable amine which could be measured when 1 μmole of FH_2 was reduced to 1 μmole of FH_4. A stoichiometric relationship between nucleotide oxidation, diazotizable amine production and FH_2 reduction is shown in Table II. FH_4 was prepared by the method of May, *et al.*,[11] or O'Dell, *et al.*,[12] and, when freshly dissolved in buffers which were 0.02

(8) H. Lineweaver and D. Burk. THIS JOURNAL, **56**, 658 (1934).
(9) R. L. Blakley, *Biochem. J.*, **65**, 331 (1957).
(10) A. C. Bratton and E. K. Marshall, *J. Biol. Chem.*, **128**, 537 (1939).
(11) M. May, T. J. Bardos, F. L. Barger, M. Lansford, J. M. Ravel, G. L. Sutherland and W. Shive, THIS JOURNAL, **73**, 3067 (1951).
(12) B. L. O'Dell, J. M. Vandenbelt, E. S. Bloom and J. J. Pfiffner, *ibid.*, **69**, 250 (1947).

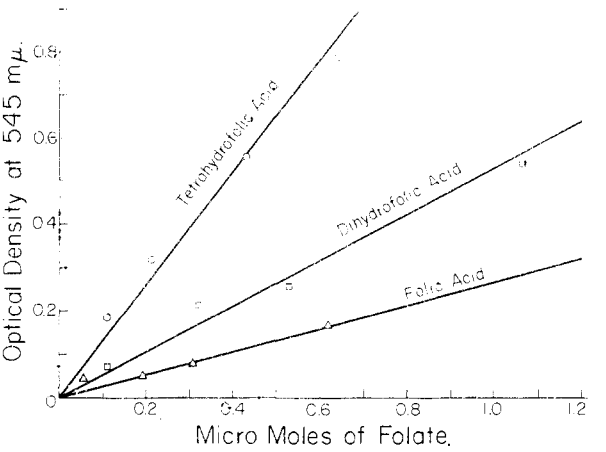


Fig. 2.—Estimation of folic acid, FH_2 and FH_4 as diazotizable amine: Δ, folic acid; □, FH_2; O, FH_4.

M with respect to 2-mercaptoethanol, had an absorption maximum at about 300 mμ which gradually (*ca.* 60 minutes) shifted to about 280 mμ. The resulting material was slightly active as FH_2 in oxidizing DPNH and TPNH.

TABLE II

STOICHIOMETRY OF DIHYDROFOLIC ACID REDUCTION, NUCLEOTIDE OXIDATION AND DIAZOTIZABLE AMINE PRODUCTION

Incubations were carried out at room temperature in 1 cm. Corex cuvettes until the rate of optical density change at 340 mμ in the samples was approximately equal to the controls (*ca.* 1 hr.). Nucleotide oxidation was measured by the change in optical density at 340 mμ between the control and the corresponding sample using a molar extinction coefficient of 6.22×10^6.[a] Three ml. of 0.1 *M* sodium acetate buffer, *p*H 5.0, or 0.1 *M* sodium phosphate buffer, *p*H 7.1, and 0.02 ml. of purified FH_2-reductase were added to each cuvette. The initial nucleotide concentration in each case was 0.1 μmole per ml.

Substrate	DPNH oxidized (*p*H 5.0), μmoles	TPNH oxidized (*p*H 7.1), μmoles	FH_2 reduced,[b] μmoles
None	0	0	...
FH_2, 0.061 μmole	0.057	0.050	0.052
FH_2, 0.123 μmole	.119	.114	.103
FH_2, 0.184 μmole	.160	.149	.142
Folic acid, 0.110 μmole	.002	.007	...
Citrovorum factor, 0.141, μmole	0	.001	...

[a] B. L. Horecker and A. Kornberg, *J. Biol. Chem.*, **175**, 385 (1948). [b] FH_2 reduction was measured as the change in diazotizable amine present in the supernatant of the deproteinized reaction mixture. Deproteinization was accomplished by the addition of 1 ml. of 15% trichloroacetic acid immediately after the final reading of the optical density at 340 mμ. Only the samples containing DPNH and FH_2 were assayed by diazotization.

FH_2-reductase was prepared from frozen sheep livers which were partially thawed and then homogenized with 3 volumes of 0.1 *M* sodium phosphate buffer, *p*H 7. The crude homogenate was centrifuged to remove the bulk of insoluble material. The soluble proteins were precipitated by the addition of solid ammonium sulfate. No adjustment of the *p*H was made after the addition of ammonium sulfate. The fraction which pre-

cipitated between 50 and 75% saturation was dissolved in about 25 ml. of water and dialyzed overnight against 6 liters of water at 0-4°. Precipitated protein was removed by centrifugation. A second precipitation with ammonium sulfate was carried out and the 50-75% fraction was again suspended in water and dialyzed overnight. The dialyzed ammonium sulfate fraction was adjusted to 1% protein concentration by the addition of water and negatively adsorbed on calcium phosphate gel[13] (gel:protein ratio = 0.5). Positive adsorption on calcium phosphate gel (gel:protein ratio = 1.0) was followed by elution with 10-ml. aliquots of 0.1 *M* sodium phosphate buffer, *p*H 6. The eluted protein fraction was dialyzed to remove phosphate and then adsorbed on sodium carboxymethyl-cellulose[14] in 0.02 *M* sodium acetate buffer, *p*H 5.0. Elution from the cation exchanger was achieved with 0.1 *M* sodium phosphate buffer, *p*H 7.2. All of the fractions were stable for several days when stored in the frozen state. The carboxymethyl-cellulose fraction lost about 30% of its enzymic activity when stored overnight at 0–4°. The purification of sheep liver FH_2-reductase is summarized in Table III.

TABLE III

SUMMARY OF PURIFICATION OF SHEEP LIVER DIHYDROFOLIC ACID REDUCTASE

Fraction	Protein concn.,[a] mg./ml.	Enzyme, units[b]/ ml.	Specific activity, units/mg.
Ammonium sulfate, 1st pptn.	42.0	8,570	204
Ammonium sulfate, 2nd pptn.	53.5	17,100	319
Gel fraction	7.7	6,340	824
Carboxymethyl-cellulose fraction	2.4	5,080	2120

[a] Protein concentration was determined from the ultraviolet absorption by the method of O. Warburg and W. Christian, *Biochem. Z.*, **310**, 384 (1941–42). [b] One unit of FH_2-reductase is defined as that amount which will oxidize 1 mμmole of DPNH in 20 minutes when incubated at room temperature in the presence of 0.1–0.2 μmole of FH_2 and 0.3 μmole of DPNH in 3 ml. of 0.1 *M* sodium acetate buffer, *p*H 5.0 (corrected for controls containing no FH_2).

FH_4 has been implicated in the *de novo* synthesis of choline methyl groups from formaldehyde.[15,16] A preparation of choline synthetase was found to be essentially free from FH_2-reductase activity. Also, FH_2-reductase, prepared as described above, showed no choline synthetase activity.[17] The synergistic activity between these two enzyme preparations (Table IV) is further evidence that FH_4 is the product of FH_2-reductase activity. The mechanism of formaldehyde reduction by FH_4 in choline methyl group synthesis is being investigated in the Laboratory by Dr. Venkataraman.

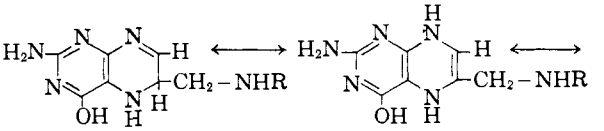
TABLE IV

SYNERGISTIC ACTIVITY BETWEEN DIHYDROFOLIC ACID REDUCTASE AND CHOLINE SYNTHETASE

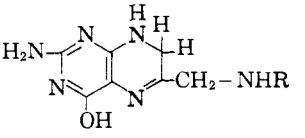
	Enzyme fraction	Folate cofactor	Choline methyl group synthesis[a] c.p.m. per mg. protein	Total FH_2-reductase units
(1)	FH_2-reductase, 47.2 mg.	FH_2	259	15,000
(2)	Choline synthetase, 9.9 mg.	FH_2	414	20
(3)	1 + 2, 14.3 mg.	FH_2	2004	3,755
(4)	Choline synthetase, 9.9 mg.	FH_4	2465	20

[a] The assay for choline methyl group synthesis from C^{14}-formaldehyde is described elsewhere.[15]

The thymine methyl group apparently is synthesized in a manner similar to the synthesis of choline methyl groups. Results from this Laboratory[18,19] have demonstrated that a cyclic oxidation of FH_4 to FH_2 and reduction of FH_2 to FH_4 by pyridine nucleotide is involved in the formation of the thymine methyl group from formaldehyde. Reduction of pyridine nucleotides by tetrahydrofolate compounds has been reported.[20,21] From these observations it seems reasonable to conclude that FH_4 is an intermediary source of hydrogen atoms for thymine methyl group formation as well as a coenzyme for the transfer of one-carbon units. Theoretically the oxidation of FH_4 could produce any of 3 possible tautomers of FH_2, given by the equations.[22] Carbon atom no. 6 in FH_4 is asymmetric and therefore only 50% of synthetic FH_4



5,6-FH_2 5,8-FH_2



7,8-FH_2

should show enzymic activity. Humphreys and Greenberg[19] have shown this to be the case in thymine methyl synthesis. Synthetic citrovorum factor (N^5-formyl-FH_4; leucovorin) and anhydrocitrovorum factor (5,10-methenyl-FH_4) have also been reported to show only 50% enzymic activity.[21,23] 5,6-FH_2 would be expected to show

(13) D. Keilin and E. F. Hartree, *Proc. Roy. Soc.* (*London*), **B124**, 397 (1938).

(14) M. B. Rhodes, P. R. Azari and R. A. Feeney, *J. Biol. Chem.*, **230**, 399 (1958).

(15) R. Venkataraman and D. M. Greenberg, THIS JOURNAL, **80**, 2;25 (1958).

(16) J. A. Stekol, S. Weiss and E. I. Anderson, *ibid.*, **77**, 5192 (1955).

(17) The preparation and assay of choline synthetase and the choline synthetase assay of the FH_2-reductase preparations were done by Dr. Venkataraman.

(18) D. M. Greenberg and G. K. Humphreys, *Federation Proc.*, **17**, 234 (1958).

(19) G. K. Humphreys and D. M. Greenberg, *Arch. Biochem. Biophys.*, in press.

(20) L. Jaenicke, *Biochim. et biophys. Acta*, **17**, 588 (1955).

(21) J. M. Peters and D. M. Greenberg, *J. Biol. Chem.*, **226**, 329 (1957).

(22) R = benzoyl-L-glutamic acid.

(23) J. C. Rabinowitz and W. E. Pricer, Jr., THIS JOURNAL, **78**, 4176 (1956).

the same property whereas 5,8-FH_2 and 7,8-FH_2, with symmetry at the C6-position, would not. From the data of Table II it can be seen that synthetic FH_2 shows greater than 50% enzymic activity, indicating that either the 5,8- or 7,8-configuration represents the enzymatically active form.

Inhibition experiments on FH_2-reductase were performed with aminopterin, *p*-chloromercuribenzoate and *o*-iodosobenzoate. Inhibition by aminopterin was found to be essentially the same as reported by Osborn, *et al.*,[7] while no inhibition by sulfhydryl reagents could be observed.

Experimental

Apparatus and Materials.—A Beckman model DU spectrophotometer and 1 cm. Corex cuvettes were used for the determination of nucleotide oxidation. A Beckman model B spectrophotometer was used for the estimation of diazotizable amine production. A Beckman model G *p*H meter was used for *p*H determinations.

FH_2 was prepared by the method of Futterman[5] and was estimated by its extinction coefficient in 0.01 *N* NaOH (E_{283} = 21,000). FH_2 could be stored as a suspension in 0.005 *N* HCl at 0° for periods up to two weeks. Nucleotides were products of the Sigma Chemical Company, St. Louis, Mo.

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[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF ENGELHARD INDUSTRIES, INC.]

Use of Ruthenium Tetroxide as a Multi-purpose Oxidant

BY LEWIS M. BERKOWITZ AND PAUL N. RYLANDER

RECEIVED AUGUST 19, 1958

Ruthenium tetroxide is a powerful oxidizing agent and readily attacks a variety of functional groups and aromatic systems. Aldehydes are oxidized to acids, alcohols to aldehydes or ketones, olefins to aldehydes, amides to imides and ethers to esters.

Osmium tetroxide has been used frequently for the oxidation of organic compounds, especially the hydroxylation of olefins,[1] but ruthenium tetroxide has been used practically not at all. *A priori* the two compounds might be expected to behave very similarly since ruthenium appears directly above osmium in the periodic table. Ruthenium would appear to offer several practical advantages in that it is less volatile, less toxic, less expensive, and more readily available than osmium. Further examination of the reaction of ruthenium tetroxide seemed worthwhile especially in regard to delimiting the scope of its usefulness.

Only two prior references to the reactions of ruthenium tetroxide have been made. Djerassi and Engle[2] oxidized several sulfides to sulfoxides and sulfones and oxidized phenanthrene to 9,10-phenanthrenequinone. They also reported that ether, benzene and pyridine, the solvents usually found useful for osmium tetroxide oxidations, reacted violently and instantaneously with ruthenium tetroxide. The only other reference to reactions of ruthenium tetroxide is to an unsuccessful oxidation of a very hindered olefin.[3]

Ruthenium tetroxide is so much more powerful an oxidizing agent than osmium tetroxide that it cannot be used without a solvent. Carbon tetrachloride and alcohol-free chloroform are satisfactory solvents for most reactions.[2] Paraffins, ketones, esters and water may also be used, although the tetroxide reacts slowly with the oxygenated compounds. A solution of the tetroxide in carbon tetrachloride is reported[2] to have remained unchanged for a year. In the present work, the tetroxide was made as needed by a simple procedure and either collected on a condenser or distilled directly into a solvent.

Ruthenium tetroxide at 0° oxidizes secondary alcohols very rapidly to ketones, and primary alcohols to aldehydes or acids. *t*-Butyl alcohol reacts very slowly, possibly through dehydration. Cyclohexanol, menthol and 3β-cholestanol were all cleanly oxidized to their respective ketones. Benzyl alcohol gave benzaldehyde without difficulty. This is especially interesting since benzene is reported to explode on contact with ruthenium tetroxide[2] and even in dilute carbon tetrachloride solution benzene is oxidized with reasonable rapidity, although qualitatively more slowly than alcohols. It was not found possible to convert aliphatic alcohols to aldehydes. · *n*-Hexyl alcohol gave only caproic acid even when an excess of the alcohol was used. Infrared analysis of the crude reaction mixture revealed no aldehyde at all.

The reaction of *trans*-1,2-cyclohexanediol with ruthenium tetroxide was accomplished using water as a solvent. The product, whose infrared spectrum showed a carbonyl band at 5.78 μ, gave a 2,4-dinitrophenylhydrazone identical with that of 1,2-cyclohexanedione. The product was either 1,2-cyclohexanedione or 2-hydroxycyclohexanone, both of which give the same 2,4-dinitrophenylhydrazone.[4]

Aldehydes are oxidized rapidly by ruthenium tetroxide to acids. *n*-Heptaldehyde gave heptanoic acid and benzaldehyde gave benzoic acid. The latter case provides another example of a functional group being oxidized preferentially to the aromatic nucleus.

Ruthenium tetroxide reacts very rapidly with olefins in a manner quite unlike osmium tetroxide. Osmium tetroxide gives hydroxylation of the

(1) H. Gilman, "Organic Chemistry, an Advances Treatise," Vol IV, J. Wiley & Sons, Inc., New York, N. Y., 1953, p. 1180.

(2) C. Djerassi and R. R. Engle, THIS JOURNAL, **75**, 3838 (1953).

(3) P. D. Bartlett and M. Stiles, *ibid.*, **77**, 2806 (1958).

(4) J. C. Sheehan, R. C. O'Neill and M. A. White, *ibid.*, **72**, 3376 (1950).