COMMUNICATIONS TO THE EDITOR

OXIDATION OF STEROIDS BY MICROÖRGANISMS. III. SIDE CHAIN DEGRADATION, RING D-CLEAVAGE AND DEHYDROGENATION IN RING A

Sir:

In recent publications from our own[1] as well as from other laboratories[2] concerned with transformations of steroids by microörganisms, the main emphasis has been on the introduction of one or more hydroxyl groups into the steroid nucleus. We now wish to report some transformations of progesterone and related steroids involving scission of carbon–carbon linkages in the side chain and in ring D, as well as the introduction of a new double bond in ring A.

Fermentation of progesterone with *Streptomyces lavendulae* (Rutgers University No. 3440-14) in a medium containing soybean meal, glucose and soybean oil followed by extraction of the culture filtrate[3] with chloroform afforded after chromatography on alumina $\Delta^{1,4}$-androstadien-3,17-dione (I),[4] m.p. 138–139.5°; $[\alpha]^{23}D$ +115° (*c*, 0.56 in $CHCl_3$); $\lambda_{max.}^{Nujol}$ 5.74 μ (17-ketone), 6.04, 6.16 and 6.24 μ ($\Delta^{1,4}$-3-ketone) (7% yield), identified by comparison with an authentic sample, and $\Delta^{1,4}$-androstadien-17β-ol-3-one (II)[4], m.p. 167–168°; $[\alpha]^{23}D$ +21° (*c*, 1.28 in $CHCl_3$); $\lambda_{max.}^{alc.}$ 243 mμ (ϵ = 16,100); $\lambda_{max.}^{Nujol}$ 2.96 μ (OH), 6.02, 6.18 and 6.24 μ ($\Delta^{1,4}$-3-ketone); (*Anal.* Found: C, 79.65; H, 9.10. Calcd. for $C_{19}H_{26}O_2$: C, 79.66; H, 9.15) (12% yield), which on oxidation with chromic acid was converted to I. The utility of I and II as intermediates in the synthesis of estradiol and estrone is well known.[4] In addition to providing a two step synthesis of estrone and estradiol from progesterone the above bioconversion suggests progesterone, I and II, as possible intermediates in the synthesis of these hormones in the mammalian organism.[5]

Oxidative degradation involving not only elimination of the side chain but cleavage between carbon atoms 13 and 17 as well has been observed with a variety of organisms. Thus, fermentation of progesterone with *Penicillium chrysogenum* (University of Wisconsin No. 49–133)[6] in a medium containing corn steep liquor, $NH_4H_2PO_4$, $CaCO_3$ and soybean oil afforded in 70% yield the known testololactone (III),[7] m.p. 207–209°; $[\alpha]^{23}D$ +43° (*c*, 1.0 in $CHCl_3$); $\lambda_{max.}^{alc.}$ 237 mμ (ϵ = 17,900); $\lambda_{max.}^{Nujol}$ 5.82 μ (lactone carbonyl), 5.99 μ and 6.18 μ (Δ^4-3-ketone); (*Anal.* Found: C, 75.90; H, 8.83. Calcd. for $C_{19}H_{26}O_3$: C, 75.46; H, 8.67) identical with an authentic sample of the substance.[8] Reduction of III with PtO_2 in glacial acetic acid followed by oxidation with chromic acid yielded after chromatography 5α-dihydrotestololactone (IV),[7] m.p. 171–172°; $[\alpha]^{23}D$ −18° (*c*, 0.76 in $CHCl_3$); $\lambda_{max.}^{Nujol}$ 5.82 μ (lactone carbonyl and 3-ketone); (*Anal.* Found:. C, 75.14; H, 8.98. Calcd. for $C_{19}H_{28}O_3$: C, 74.95; H, 9.27) identical with an authentic sample,[8] and the hitherto undescribed 5β-dihydrotestololactone (V), m.p. 202–203°; $[\alpha]^{23}D$ −25° (*c*, 1.0 in $CHCl_3$); $\lambda_{max.}^{Nujol}$ 5.83 μ; (*Anal.* Found: C, 74.94; H, 9.50).

Bioöxidation involving both lactone formation in ring D and dehydrogenation in ring A is less widespread and has been observed with but a small number of organisms. Thus, when progesterone, Reichstein's compound S or testosterone are fermented with *Cylindrocarpon radicola* (A.T.C.C. No. 11011) there results in about 50% yield Δ^1-dehydrotestololactone (VI), m.p. 218–219°; $[\alpha]^{23}D$ −44° (*c*, 1.29 in $CHCl_3$)[9]; $\lambda_{max.}^{alc.}$ 242 mμ (ϵ = 15,800); $\lambda_{max.}^{Nujol}$ 5.83 μ (lactone carbonyl)[10] 6.01, 6.15 and 6.22 μ($\Delta^{1,4}$-3-ketone); (*Anal.* Found: C, 76.29; H, 7.87. Calcd. for $C_{19}H_{24}O_3$: C, 75.97; H, 8.05). The structure of VI is based on the following evidence: Reduction with PtO_2 in glacial acetic acid followed by chromic acid oxidation yielded IV and V. VI forms a semicarbazone, m.p. 250–260° dec.; (*Anal.* Found: N, 11.19. Calcd. for $C_{20}H_{27}N_3O_3$: N, 11.76) showing the ultraviolet spectrum, $\lambda_{max.}^{alc.}$ 244 mμ(ϵ = 12,200), 297 mμ (ϵ = 22,600) char-

(1) D. Perlman, E. Titus and J. Fried, THIS JOURNAL, **74**, 2126 (1952); J. Fried, R. W. Thoma, M. N. Donin, J. Herz, J. R. Gerke and D. Perlman, *ibid.*, **74**, 3962 (1952).

(2) D. H. Peterson, *et al.*, *ibid.*, **75**, 421 (1953), and earlier papers; D. R. Colingsworth, J. N. Karnemaat, F. R. Hanson, M. P. Brunner, M. Mann and W. J. Haines, *J. Biol. Chem.*, **203**, 807 (1953); F. W. Kahnt, Ch. Meystre, R. Neher, E. Vischer and A. Wettstein, *Experientia*, **8**, 432 (1952); O. Mancera, A. Zaffaroni, B. A. Rubin, F. Sondheimer, G. Rosenkranz and C. Djerassi, THIS JOURNAL, **74**, 3711 (1952).

(3) Extraction of the dried mycelium with acetone yielded a reduction product of progesterone, m.p. 170–172°; $[\alpha]^{20}D$ +81° (*c*, 0.93 in $CHCl_3$); $\lambda_{max.}^{alc.}$ 240 mμ (ϵ = 15,900); $\lambda_{max.}^{Nujol}$ 2.91 μ (OH), 5.98 and 6.20 μ (Δ^4-3-ketone), which was identified as Δ^4-pregnen-20β-ol-3-one by reoxidation to progesterone and comparison with an authentic sample of the hydroxy ketone kindly furnished by Dr. R. B. Turner (*cf.* P. Wieland and K. Miescher, *Helv. Chim. Acta*, **32**, 922 (1949), and R. B. Turner and D. M. Voitle, THIS JOURNAL, **73**, 2283 (1951)).

(4) H. H. Inhoffen, G. Zuehlsdorff and Huang-Minlon, *Ber.*, **73**, 451 (1940); H. H. Inhoffen, *Angew. Chem.*, **59**, 207 (1947).

(5) Evidence for or against such a hypothesis could be adduced by extending the perfusion studies using radioactive acetate recently reported by N. T. Werthessen, E. Schwenk and C. Baker, *Science*, **117**, 380 (1953), to labelled progesterone, I and II.

(6) This reaction has also been accomplished using organisms of the genera *Aspergillus* and *Mucor*, *e.g.*, *A. flavipes* (A.T.C.C. No. 11013) and *M. mucedo* (A.T.C.C. No. 7941).

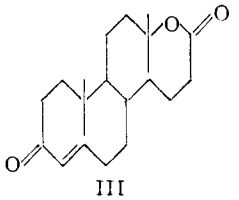
(7) H. Levy and R. P. Jacobsen, *J. Biol. Chem.*, **171**, 71 (1947). These authors left open the question, whether the lactones described by them possess structures exemplified by III, VI and VII or the alternate structures, in which ring scission has occurred between carbon atoms 16 and 17. Evidence favoring the formulation given in this paper has since been accumulated by various authors and is well summarized in reference 8 of a recent publication by G. M. Picha, THIS JOURNAL, **74**, 703 (1952). To this may be added the observation made in our own laboratories that the hydroxy acid derived from VI remained unchanged during treatment with chromic acid indicating that it possesses a tertiary hydroxyl group rather than a primary one as required by the alternate formulation of the lactone.

(8) We are indebted to Dr. H. Levy for kindly furnishing the samples of testololactone, 5α-dihydrotestololactone and estrololactone.

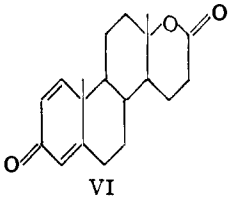
(9) The difference in molecular rotation between Δ^1-dehydrotestololactone and testololactone $\Delta[M]_D^{VI-III}$ −257°. The average contribution for the 1,2-double bond in $4\Delta^{1,4}$-3-ketones is −231°.

(10) This band shifts to 5.76μ when the spectrum is taken in carbon disulfide solution; *cf.* R. N. Jones, P. Humphries and K. Dobriner, THIS JOURNAL, **72**, 956 (1950).

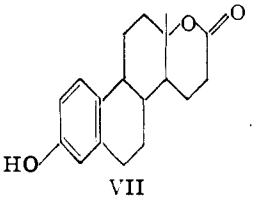
acteristic of $\Delta^{1,4}$-3-semicarbazones.[4] Pyrolysis of VI at 550–600° in mineral oil[11] furnished Westerfeld's lactone (estrololactone)[12,13] (VII), identified after conversion into the acetate, m.p. 149–151°; $\lambda_{max.}^{alc.}$ 267 mμ(ϵ = 1080) and 275 mμ(ϵ = 950); $\lambda_{max.}^{Nujol}$ 5.70 μ (phenolic acetyl), 5.80 μ (lactone carbonyl), by comparison with an authentic sample of the latter.[8]



III



VI



VII

The biochemical conversion of the β-oriented acetyl side chain in progesterone to a 17β-hydroxyl group parallels the degradation of 20-ketosteroids by peracids, which likewise proceeds with retention of configuration at C_{17}.[14] Similarly, the formation of ring D lactones has its chemical parallel in the reaction sequence: progesterone $\xrightarrow{RCO_3H}$ testosterone $\xrightarrow{-H_2}$ androstenedione $\xrightarrow{RCO_3H}$ III. A bioöxidation mechanism involving these same intermediates is not out of the question since testosterone is readily converted into VI by *C. radicola*.

(11) E. B. Hershberg, M. Rubin and E. Schwenk, *J. Org. Chem.*, **15**, 232 (1950).

(12) W. W. Westerfeld, *J. Biol. Chem.*, **143**, 177 (1943).

(13) R. P. Jacobsen, *ibid.*, **171**, 61 (1947).

(14) T. F. Gallagher and T. Kritchevsky, THIS JOURNAL, **72**, 882 (1950); R. B. Turner, *ibid.*, **72**, 878 (1950).

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RECEIVED OCTOBER 19, 1953

THE SYNTHESIS OF ERYTHRINANE[1]

Sir:

The purpose of this communication is to report on a simple method of probable general applicability for the elaboration of the entire ring system of the *Erythrina abyssinica* Lam. alkaloids, the constitution of which has been elucidated recently by Prelog and his co-workers.[2] The conversion of 2-carbethoxycyclohexanone to erythrinane in five steps and about 12% over-all yield is described. It is anticipated that a number of substituted erythrinanes will now be easily accessible for chemical and pharmacological studies.

(1) The term "erythrinane" is proposed to designate the basic ring system of the Erythrina alkaloids.

(2) M. Carmack, B. C. McKusick and V. Prelog, *Helv. Chim. Acta*, **34**, 1601 (1951); H. G. Khorana, G. W. Kenner and V. Prelog, *ibid.*, **34**, 1969 (1951). Concerning the structure of β-erythroidine, the exhaustive investigations of Boekelheide and his collaborators (ref. 7 and accompanying papers) should be consulted.

Through the condensation of the potassio derivative of 2-carbethoxycyclohexanone with 2-bromoethylphthalimide in boiling toluene, there was obtained as a viscous oil, 2-carbethoxy-2-(β-phthalimidoethyl)-cyclohexanone which, without purification, was hydrolyzed with boiling concentrated hydrochloric acid to 2,3,4,5,6,7-hexahydroindole (35% over-all yield), [b.p. 80° (19 mm.)]; *Anal.* Calcd. for $C_8H_{13}N$: N, 11.37. Found: N, 11.28. *Picrate*,[3] m.p. 132–133° [*Anal.* Calcd. for $C_{14}H_{16}O_7N$: C, 47.72; H, 4.57. Found: C, 47.85; H, 4.45]. Over Adams catalyst in ethanol, the latter base absorbed 0.98 molar proportion of hydrogen to yield octahydroindole isolated as the picrate, m.p. 135–137°, which proved identical with a sample prepared according to the literature.[4] Treatment of hexahydroindole with phenylacetyl chloride under the Schotten-Baumann conditions afforded in 80% yield, *2-(β-phenylacetamidoethyl)-cyclohexanone* (I), m.p. 53–54°, (*Anal.* Calcd. for $C_{16}H_{21}O_2N$: C, 74.09; H, 8.16. Found: C, 73.96; H, 8.18.); maxima at 2.92 μ (NH—), 5.85 μ (C=O), 6.02 and 6.60 μ (—CONH—) and 6.70 μ (phenyl) in the infrared region. When heated for twenty-four hours at 100° in excess polyphosphoric acid, (I) was converted in 60% yield to *8-oxoerythrinane*[5] (II), m.p. 132–133° (*Anal.* Calcd. for $C_{16}H_{19}ON$: C, 79.62; H, 7.93; N, 5.80. Found: C, 79.71; H, 8.02; N, 5.62.), whose structure was deduced from the following evidence: (a) its infrared spectrum shows a single peak at 6.14 μ (besides the phenyl bands) which is characteristic of a disubstituted amide carbonyl group; (b) it is unaffected by prolonged heating in concentrated hydrochloric acid, a behavior inconsistent with an acyclic amide structure but consistent with a lactam structure[6]; (c) when treated with lithium aluminium hydride in boiling ether it is converted in 70% yield to the corresponding base *erythrinane*, a colorless oil, b.p. 195° (bath temp.) (0.1 mm.) (*Anal.* Calcd. for $C_{16}H_{21}N$: C, 84.52; H, 9.31. Found: C, 84.38; H, 9.17.); *picrate:* m.p. 184–185° (*Anal.* Calcd. for $C_{22}H_{24}O_7N_4$: N, 12.27. Found: N, 12.29); *methiodide:* m.p. 201–203° (*Anal.* Calcd. for $C_{17}H_{24}NI$: I, 34.3. Found: I, 34.1.). The infrared spectrum of the free base lacks the band of (II) at 6.14 μ; (d) on vigorous oxidation with nitric acid it yields 4-nitrophthalic acid isolated as its anhydride and further characterized as its anil derivative; both proved identical with authentic specimens.

Therefore, (II) is the only reasonable structure accommodating the evidence and further work in the methoxylated series is contemplated.

The biogenetic implications of the facile conversion of (I) to (II) are obvious and in keeping with Boekelheide's recent suggestion.[7]

Several attempts to ring close N-(β-phenylethyl)-

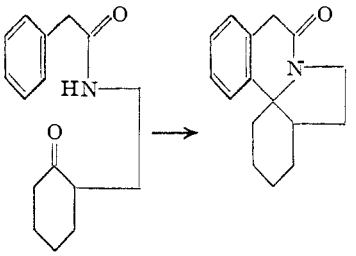
(3) Boiling points and melting points are uncorrected.

(4) R. Willstätter and D. Jaquet, *Ber.*, **51**, 767 (1918), report 137–138° as the m.p. of octahydroindole picrate.

(5) For the numbering of the ring system, see ref. 2.

(6) The opened form of the lactam undoubtedly exists in the hot acid mixture but cannot be isolated presumably because of spontaneous ring closure during the process of isolation. A similar apparent refractoriness of a lactam to boiling hydriodic acid can be found in the morphinane series (M. Gates, R. B. Woodward, W. F. Newhall and R. Kunzli, THIS JOURNAL, **72**, 1141 (1950)).

(7) V. Boekelheide, *et al.*, *ibid.*, **75**, 2550 (1953).



(I) (II)

hexahydroindole [b.p. 123–124° (0.4 mm.); *Anal.* Calcd. for $C_{16}H_{21}N$: C, 84.52; H, 9.31. Found: C, 84.33; H, 9.45; oily salts; obtained by treating β-phenylethyl bromide with hexahydroindole] to erythrinane, led only to unchanged starting material.

DEPARTMENT OF BIOCHEMISTRY
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LAVAL UNIVERSITY, QUEBEC

RECEIVED OCTOBER 22, 1953

(8) Reed and Carnrick Co., 155 van Wagener Ave., Jersey City 6, N. J.

ENZYMATIC REDUCTION OF β-ASPARTYL PHOSPHATE TO HOMOSERINE

Sir:

The enzymatic formation of β-aspartyl phosphate from L-aspartate and ATP[1] recently has been reported.[2] A synthetic preparation of this substance now has been obtained, and found to be enzymatically reduced to homoserine in a TPN-dependent system obtained from yeast. The phosphorylation and subsequent reduction of the β-carboxyl group of aspartate to a hydroxyl group appear, in view of recent isotope and genetic work, to represent intermediate steps in the biosynthesis of threonine. The latter was demonstrated by such experiments to arise from aspartate via homoserine.[3,4]

The starting material for BAP[1] synthesis was carbobenzoxy-L-aspartyl-α-benzyl ester β-chloride.[5] To replace the chloride with phosphate this compound was shaken in ether with monosilver phosphate,[6] and the carbobenzoxy and benzyl groups were then removed by hydrogenation over palladium black in cold potassium bicarbonate solution. Because of its extreme lability the product was not further purified. It was characterized by the β-asparthydroxamic acid, identified chromatographically, formed on reacting with hydroxylamine. Enzymatic identification was made by its ability to transfer phosphate to ADP.[2] This substance is approximately 30% hydrolyzed in 30 minutes at 30° in aqueous solutions from *p*H 4 to 10. In such solutions at 15° it is relatively stable for several hours.

The enzyme preparation used in these experiments was an extract of baker's yeast partially purified by heating to 55°, discarding the precipitate, and dialyzing 15 hours against cold potassium bicarbonate buffer.

Table I shows the equivalent disappearance of BAP and formation of homoserine in the complete enzyme system. No homoserine appears when the BAP or enzyme is destroyed by brief heating prior to addition. On prolonged incubation complete transformation of BAP to homoserine occurs.

TPN and glutamate markedly increase the extent of the reduction, presumably through the TPN-linked yeast glutamic dehydrogenase[7] which is very active in the protein preparation. Glutamate may be replaced by glucose plus the glucose dehydrogenase of ox liver.[8] Though the latter reduces both DPN and TPN, only TPN serves to reduce BAP in its presence.

TABLE I

Homoserine was qualitatively identified by paper chromatography in water-saturated phenol with 0.3% NH_3, and in the two alternate solvent systems recommended by Redfield.[9] It was further identified by conversion to its DNP-derivative,[10] which was chromatographed on paper.[11] Quantitative estimation of homoserine was made by eluting the DNP-homoserine from paper chromatograms and analyzing spectrophotometrically at 362 mμ. BAP was determined as acyl phosphate.[12] The complete system contained in 1.0 ml. 100.0 μM. TEA-chloride buffer, 0.16 μM. TPN (63% pure), 5.0 μM. TEA-glutamate, 2.7 μM. BAP, and 0.27 ml. of enzyme. The *p*H was 7.9 and the incubation was at 15° for 60 minutes.

Experiment	BAP utilized, μM.	Homoserine formed, μM.
Complete system	1.4	1.4
Omit TPN	0.4	0.4
Omit glutamate	0.5	0.5
Heated enzyme used (100°, 5 min.)	0.0	−0.1
Heated BAP used (100°, 10 min., neutral solution)	...	0.1

The enzyme preparation used here causes a rapid splitting of BAP in the presence of potassium arsenate, a point of similarity with 3-phosphoglyceraldehyde dehydrogenase which also catalyzes a reduction and an arsenolysis of an acyl phosphate.[13,14] Both arsenolysis and reduction are inhibited by iodoacetate.

We are grateful to Drs. J. P. Greenstein and A. Meister for gifts of several compounds, and for assistance during the synthesis of BAP.

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES
NATIONAL INSTITUTES OF HEALTH SIMON BLACK
PUBLIC HEALTH SERVICE NANCY G. WRIGHT
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(1) Abbreviations used are BAP (β-aspartyl phosphate), ADP (adenosine diphosphate), ATP (adenosine triphosphate), TPN (triphosphopyridine nucleotide), DPN (diphosphopyridine nucleotide), DNP (dinitrophenyl), and TEA (triethanolamine).

(2) S. Black and N. M. Gray, THIS JOURNAL, **75**, 2271 (1953).

(3) A. M. Delluva, *Arch. Biochem. Biophys.*, **45**, 443 (1953).

(4) M. L. Hirsch and G. N. Cohen, *Compt. rend.*, **236**, 2338 (1953).

(5) M. Bergmann, L. Zervas and L. Salzmann, *Ber.*, **66B**, 1288 (1933).

(6) F. Lipmann and L. C. Tuttle, *J. Biol. Chem.*, **153**, 571 (1944). Excess H_3PO_4 greatly enhances solubility. A mixture of 1 g. Ag_3PO_4 + 0.5 ml. 85% H_3PO_4 was used.

(7) E. Adler, G. Gunther and J. E. Everett, *Z. physiol. Chem.*, **255**, 27 (1938).

(8) H. J. Strecker and S. Korkes, *J. Biol. Chem.*, **196**, 769 (1952). Use of this enzyme was suggested by Dr. E. R. Stadtman. A preparation was supplied by Dr. B. L. Horecker.

(9) R. R. Redfield, *Biochim. et Biophys. Acta*, **10**, 344 (1953).

(10) The conversion of amino acids to DNP-derivatives was by an unpublished method used in Dr. O. H. Lowry's laboratory and communicated to us by Dr. J. L. Strominger.

(11) K. R. Rao and H. A. Sober, submitted for publication. We thank the authors for making information on the chromatography of DNP-homoserine available to us prior to publication.

(12) F. Lipmann and L. C. Tuttle, *J. Biol. Chem.*, **159**, 21 (1945).

(13) J. Harting and S. Velick, *Federation Proc.*, **11**, 226 (1952).

(14) E. Racker and I. Krimsky, *J. Biol. Chem.*, **198**, 731 (1952).

LIPOIC ACID DEHYDROGENASE: THE FUNCTION OF E. COLI FRACTION B*

Sir:

The oxidative decarboxylation of pyruvate by extracts of *Escherichia coli* is dependent on two enzyme fractions referred to as A and B which in the presence of pyruvate form acetyl-phosphate, provided transacetylase and orthophosphate are present, or citrate, provided condensing enzyme and oxaloacetate are present.[1] From knowledge of the reactions catalyzed by phosphotransacetylase[2] and the condensing enzyme[3] the function of *E. coli* fractions A and B were deduced as the catalysis of the reaction

$$\text{Pyruvate} + \text{DPT}^+ + \text{DPN}^+ + \text{CoA} \rightleftharpoons \text{Acetyl-CoA} + \text{CO}_2 + \text{DPNH} + \text{DPT}^+ \quad (1)$$

Subsequently further purified fractions A and B were shown to catalyze reaction 1 in the presence of substrate amounts of DPN and CoA.[4,5] Fraction A has been shown to contain the carboxylase enzyme; *i.e.*, catalyzes $C^{14}O_2$ exchange into pyruvate[6] in the presence of DPT, and the oxidative decarboxylation of pyruvate in the presence of DPT and ferricyanide[4] (Green[7], *et al.*, assay). Further evidence of the function of Fraction B was obtained[5] from its activation of the alpha-ketoglutarate carboxylase (Fraction A′) for DPN reduction in the presence of DPT and CoA according to the reaction

$$\alpha\text{-ketoglutarate} + \text{DPT}^+ + \text{DPN}^+ + \text{CoA} \rightleftharpoons \text{succinyl-CoA} + \text{CO}_2 + \text{DPNH} + \text{DPT}^+ \quad (2)$$

Since the transacetylase enzymes show a high degree of specificity and the only reaction common to all keto-acid dehydrogenases is the DPN reduction, it was suggested[4] that coli fraction B is the dehydrogenase which functions via the reoxidation of lipoic acid as follows

$$\text{Lip(SH)}_2 + \text{DPN}^+ \rightleftharpoons \text{LipS}_2 + \text{DPNH} + \text{H}^+ \quad (3)$$

Lipoic acid had previously been shown to be essential in alpha-keto acid and dicarbonyl cleavage by independent experiments using *Streptococcus faecalis*,[8] *Tetrahymena geleii*,[9] and more recently with a mutant of *E. coli*.[10] Lipoic acid was previously suggested to act[5] as acceptor for the "aldehyde-"[6] generated by keto acid decarboxylation with consequent oxidation to acyl, acetyl or succinyl of equations 1 and 2, to form thio esters of lipoic acid. The transfer of the acyl from lipoic acid to coenzyme A—by lipoic transacylases—would regenerate reduced lipoic acid which in turn would be reoxidized via DPN as electron acceptor (lipoic dehydrogenase). Reed and DeBusk[11] have outlined a reaction sequence similar to that given previously,[4] with the added suggestion that lipoic acid functions as lipothiamide pyrophosphate, the amide of lipoic acid with the 4-aminopyrimidyl group of thiamine.

Lipoic dehydrogenase can be measured directly by following DPN reduction (reaction 3) or more conveniently by following the disappearance of thiol groups in the presence of pyruvate, lactic dehydrogenase and catalytic amounts of DPN according to the reaction

$$\text{Lip(SH)}_2 + \text{Pyruvate} \xrightleftharpoons{\text{(DPN)}} \text{LipS}_2 + \text{Lactate} \quad (4)$$

The substrate and cofactor dependencies of reaction (4), measured by disappearance of thiol groups, is shown in Table I.

TABLE I

A DPN LINKED LIPOIC ACID DEHYDROGENASE

	Additions	Lip(SH)$_2$ Oxidation μM/30′
1.	Complete System[a]	7.7
2.	Complete System − DPN, 0.5 μM	0.4
3.	Complete System − Pyruvate, 20 μM	0.4
4.	Complete System − Lactic Dehydrogenase, 2000 U	0.2
5.	Complete System − Fraction B, 140 γ (S.A. 200[b] μM)	0.0

[a] Additions 2 through 5, plus; 10 μM reduced lipoic acid (Lip(SH)$_2$), 100 μM phosphate buffer, *p*H 6.0. [b] S.A. = μM lipoic acid oxidized/hr./mg. Protein.

Table II shows the lipoic dehydrogenase activity of fraction B over a 200-fold range of purity as compared to its activation of the dismutation reaction in the presence of excess fraction A, which contains lipoic acid. As the data show, the lipoic dehydrogenase activity of fraction B is approximately twice, (1.8×), its dismutation activity. A similar correlation with the rate of DPN reduction in the presence of enzymes A and B plus DPN, DPT and

TABLE II

PURIFICATION OF LIPOIC DEHYDROGENASE: COLI FRACTION B

	Fraction	Protein, g.	Lipoic DeH Sp. Act.[d]	Lip.[a] DeH × 10^3	Units of Pyr.[b] Dism. × 10^3	Ratio L/D
1.	Cell Extract	6.3	4.85	30.6	18	1.7
2.	1 + 25–75 $AmSO_4$[c]	6.0	3.21	19.1	10	1.82
3.	2 + Protamine	2.4	6.95	16.5	8.8	1.88
4.	3 + 60–100 $AmSO_4$	0.52	34.6	18.0	10.4	1.73
5.	4 + 50–60 $AmSO_4$	.15	94.5	13.6	8.2	1.67
6.	5 + Ca_3PO_4 Gel Eluate	.09	695	6.5	3.8	1.7

[a] Unit = 1 μM 6,8 dimercaptoöctanoic acid, Lip (SH)$_2$, oxidized/hr. System, Table I. [b] Unit (Fraction B) = 1 μM CO_2 hr. evolved in the presence of excess fraction A. Dismutation system; 200 μM K phosphate, *p*H 6; 50 μM K pyruvate; 1 μM $MnSO_4$; 0.5 μM DPN; 0.2 μM DPT; 0.1 μM CoA; 10 μM cysteine; 2000 units lactic dehydrogenase; 10 units P-transacetylase; 10 units (Sp. Act. 340); Fraction A. (contains lipoic acid; no rate increase on adding lipoic acid). [c] Per cent. saturation with ammonium sulfate at 0°, precipitate used. [d] Units/mg. protein.

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(1) S. Korkes, A. delCampillo, I. C. Gunsalus and S. Ochoa, *J. Biol. Chem.*, **193**, 721 (1951).

(2) E. R. Stadtman, *J. Cell. and Comp. Physiol.*, **41**, 89 (1952).

(3) J. R. Stern and S. Ochoa, *J. Biol. Chem.*, **161**, 191 (1951).

(4) I. C. Gunsalus. Address delivered to 2nd Intern. Congr. Biochem., Paris, July 1952; see also E. C. Slater, *Ann. Rev. Biochem.*, **22**, 19 (1953).

(5) L. P. Hager, J. D. Fortney and I. C. Gunsalus, *Fed. Proc.*, **12**, 213(1952).

(6) S. Korkes, (See Ochoa-Symposium sur le Cycle Tricarboxylique, 2nd Intern. Congr. Biochem., Paris, July, 1952, pp. 80 and 81).

(7) D. E. Green, P. K. Stumpf and K. Zarundnaya, *J. Biol. Chem.*, **167**, 811 (1947).

(8) D. J. O'Kane and I. C. Gunsalus, *J. Bact.*, **54**, 20 (1947); *ibid.*, **56**, 499 (1948); M. I. Dolin, *Fed. Proc.*, **12**, 198 (1953).

(9) V. C. Dewey and G. W. Kidder, *Proc. Soc. Exptl. Biol. and Med.*, **80**, 302 (1952).

(10) L. J. Reed and B. C. DeBusk, *J. Biol. Chem.*, **199**, 873 (1952).

(11) L. J. Reed and B. C. DeBusk, THIS JOURNAL, **75**, 1261 (1953)

CoA, also occurs, *i.e.*, reaction 1. Thus over a 200-fold range of purity, the lipoic dehydrogenase activity of *E. coli* fraction B, at least in high substrate concentration, is sufficient to account for the total rate of oxidative decarboxylation of pyruvate. These data confirm the previous suggestions[4,5] that the B fraction of *E. coli* is a dehydrogenase enzyme common to the keto acid systems and that it links lipoic acid to DPN.

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MICROBIOLOGICAL TRANSFORMATIONS OF STEROIDS. IX. DEGRADATION OF C_{21} STEROIDS TO C_{19} KETONES AND TO TESTOLOLACTONE

Sir:

In continuing our studies on microbiological transformations of steroids,[1,2,3] we wish to report the degradation of C_{21} compounds, particularly progesterone (I), to C_{19} compounds: *e.g.*, 4-androstene-3,17-dione (II), 6β-hydroxy-4-androstene-3,17-dione (III) and testololactone (IV).[4] These results are given in the table.

BIOCONVERSION OF C_{21} TO C_{19} STEROIDS

Substrate	Product	Microörganism
Progesterone (I)	Androstenedione (II) + other oxygenated steroids	*Gliocladium catenulatum*, *Penicillium lilacinum* Thom, *Aspergillus flavus*
	6β-Hydroxyandrostenedione (III)	*Gliocladium catenulatum*
	Testololactone (IV) + other oxygenated steroids	*Aspergillus flavus*, *Penicillium adametzi*
17α-Hydroxyprogesterone (VI)	Testololactone (IV) + other oxygenated steroids	*Aspergillus flavus*
Other substrates	(5)	

Gliocladium catenulatum (A.T.C.C. 10523) converts I to II and III, while a strain of *Penicillium lilacinum* Thom[6] converts I to II plus other oxygenated steroids. The fermentation and extraction techniques used have been previously described.[1,2]

Turfitt[7] has shown that degradation of the side chain of 4-cholestenone to a C_{20} compound, 3-keto-4-etiocholenic acid, is accomplished by *Proactinomyces*.

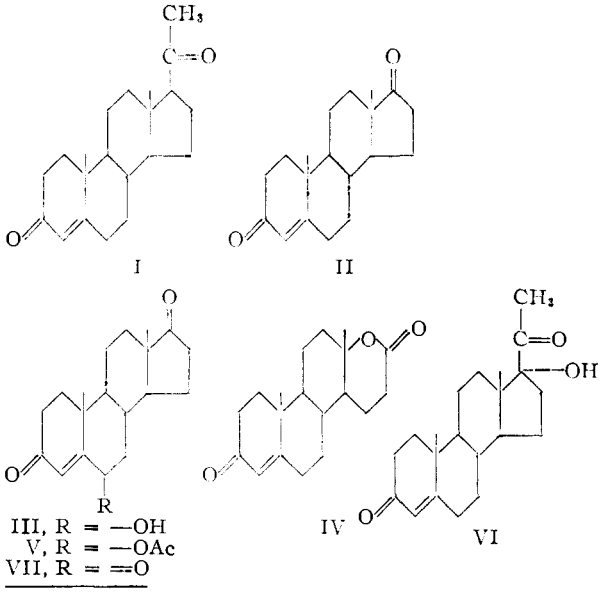
In these microbiological transformations, we have found that it is possible to degrade the side chain of C_{21} steroids to C_{19} compounds by a simple and novel one step process without the necessity of protecting the sensitive 3-keto-Δ^4 system.

The methylene chloride extractives of the beer from the *Gliocladium* were chromatographed over alumina to yield II, m.p. 174–176°, $[\alpha]^{23}D$ +194° (*c* 0.8695 in $CHCl_3$), (*Anal.* Calcd. for $C_{19}H_{26}O_2$: C, 79.68; H, 9.15. Found: C, 79.53; H, 8.84 and III, m.p. 190–192°, $[\alpha]^{23}D$ +107° (*c* 0.6685 in $CHCl_3$) (*Anal.* Calcd. for $C_{19}H_{26}O_3$: C, 75.46; H, 8.67. Found: C, 75.39; H, 8.47). Compound III yielded a monoacetate (V), m.p. 202–205°, $[\alpha]^{23}D$ +114° (*c* 0.9353 in $CHCl_3$) (*Anal.* Calcd. for $C_{21}H_{28}O_4$: C, 73.22; H, 8.19. Found: C, 72.97; H, 8.01). By similar techniques the beer from the *Penicillium* yielded II from I.

The physical constants of III and V are identical with those reported by Ehrenstein.[8] Oxidation of III produced 4-androstene-3,6,17-trione (VII), m.p. 220–224°, whose infrared spectrum was identical with an authentic sample.

Microbiological transformation of progesterone (I) and 17α-hydroxyprogesterone (VI) with *Aspergillus flavus* yielded testololactone (IV), m.p. 210–212°, $[\alpha]^{23}D$ +43° (*c* 1.00 in $CHCl_3$), (*Anal.* Calcd. for $C_{19}H_{26}O_3$: C, 75.46; H, 8.67. Found: C, 75.61; H, 8.51); androstenedione (II) and other oxygenated steroids. Similarly I was transformed to IV by *Penicillia*. The product obtained by these bioconversions was identical in all physical properties with IV obtained by chemical means,[4] the structure of which has been definitely established.[9]

When the fermentation is interrupted before the substrate is completely transformed, II as well as IV can be isolated; however, when the substrate is



(1) D. H. Peterson, H. C. Murray, S. H. Eppstein, P. D. Meister, L. M. Reineke, A. Weintraub and H. M. Leigh, THIS JOURNAL, **75**, 421 (1953), and earlier references.

(2) H. C. Murray and D. H. Peterson, U. S. Patent 2,602,769, *C.A.* **46**, 8331 (1952).

(3) Microbiological transformations of steroids have also been reported by D. Perlman, *et al.*, THIS JOURNAL, **74**, 2126 (1952); J. Fried, *et al*, *ibid.*, **74**, 3962 (1952); O. Mancera, *et al.*, *ibid.*, **74**, 3711 (1952); and F. W. Kahnt, *et al*, *Experientia*, **8**, 422 (1952).

(4) H. Levy and R. P. Jacobsen, *J. Biol. Chem.*, **171**, 61 (1947).

(5) Studies have indicated that desoxycorticosterone and Reichstein's Compounds S can be converted to 4-androstene-3,17-dione (II) or testololactone (IV) and other oxygenated steroids by these cultures as well as by various *Aspergilli* and *Penicillia*.

(6) Oxidation of 14α-hydroxyprogesterone to 14α-hydroxy-4-androstene-3,17-dione by this microörganism was reported by our group at 123rd Meeting Am. Chem. Soc., Los Angeles, California, March 15–19, 1953 (Division of Biological Chemistry, Abstract 5C).

(7) G. E. Turfitt, *Biochem. J.*, **42**, 376 (1948).

(8) C. P. Balant and M. Ehrenstein, *J. Org. Chem.*, **17**, 1587 (1952).

(9) M. F. Murray, R. L. Pederson, B. A. Johnson and A. C. Ott, THIS JOURNAL, to be published.

completely utilized, II could not be found. This evidence suggests that the reaction to form IV proceeds through II.

In previous papers from these laboratories,[1,2,3,10] 6 and 11 hydroxylation and reduction of the double bond in the A ring by microörganisms have been described. The present communication describes the formation of 4-androstene-3,17-dione from C_{21} steroids. It is interesting to note that these metabolic end products from steroidal substrates are similar to those produced by higher vertebrates.[11,12,13]

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(10) D. R. Colingsworth, M. P. Brunner and W. J. Haines, THIS JOURNAL, **74**, 2381 (1952).

(11) W. J. Haines, "Recent Progress in Hormone Research," Vol. VII, Academic Press, Inc., New York, N. Y., 1952, p. 255.

(12) J. von Euw and T. Reichstein, *Helv. Chim. Acta*, **25**, 988 (1942); **24**, 879 (1941).

(13) H. L. Mason and W. W. Engstrom, *Physiol. Rev.*, **30**, 321 (1950).

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HYDROLYTIC POLYMERIZATION OF ZIRCONIUM(IV)[1]

Sir:

Granér and Sillén[2] suggested that in the hydrolysis of Bi(III) a continuous series of particles is formed, all in equilibrium with each other and ranging in size from monomers to "infinitely" large polymers, the exact distribution depending on acidity and concentration. Recently Connick and Reas[3], in an attempt to interpret solvent extraction data on Zr(IV), advanced the same hypothesis of continuous polymerization and equilibrium between the species and postulated the existence of high molecular weight particles in acidic solutions of Zr(IV). Other workers[4,5,6] drew the conclusion that only low molecular weight polymers are formed in strongly acidic media. High molecular weight polymers, not in equilibrium with the more "normal" species, are apparently formed under considerably drastic conditions (*e.g.*, lower acidity or after boiling).[7]

Since the assumption that high molecular weight polymers are in equilibrium with low molecular weight polymers and monomers appears rather improbable and since measurement of the acidity of $ZrCl_4$ solutions[8] made it unlikely that an infinite series of polymers exists at high acidities, equilibrium ultracentrifugations of Zr(IV) were carried out in chloride and perchlorate solutions.[9] The data were recently augmented and reanalyzed by a modification of the method of Lamm[10] which was suggested to us by Professor George Scatchard, details of which will be published separately. In this computation the charge of the polymer units was considered, and estimates of this charge were obtained by ultracentrifugations under a variety of conditions. Centrifugation of 0.05 *M* Zr(IV) solutions in 1 *M* HCl–1 *M* MCl (where M was Li, Na and Cs) revealed the existence of only one principal species of Zr(IV) with an apparent degree of polymerization of 3.0 and charge $Z' < 1$ per monomer unit. At considerably higher and lower acidities (3 *M* HCl and 0.1 *M* HCl–1.9 *M* NaCl) mixtures were found with apparent degree of polymerization varying between *ca.* 2 to 2.6 (3 *M* HCl) and 4 to 5.4 (0.1 *M* HCl).

Similar low degrees of polymerization were found in ultracentrifugations in perchlorate solutions (1 *M* $HClO_4$–1 *M* $NaClO_4$). In this medium the zirconium particles appeared to carry a considerable charge and hence preliminary estimation of the degree of polymerization is somewhat more uncertain than for the chloride solutions. The most probable degree of polymerization for 0.05 and 0.12 *M* Zr(IV) solutions in this medium was 3, with an outside possibility that it may be as high as 4.5. There was no indication of an increase in degree of polymerization with concentration. These results may be compared with the (weight average) degrees of polymerization (N_w) estimated by Connick and Reas.[3] These authors report $N_w = 18$ for a considerably more dilute solution (0.03 *M* Zr(IV)–1 *M* $HClO_4$–1 *M* $LiClO_4$) and a value of N_w between 10 and 300 at a higher acidity (0.17 Zr(IV)–2*M* $HClO_4$).

The results of the ultracentrifugation experiments described here thus indicate that Zr(IV) in strongly acidic solutions (M $H^+ > 0.1$) does not show continuous polymerization with high molecular weight products but rather forms only low molecular weight polymers with trimers apparently predominating at acidities near 1 *M*.

(1) This document is based on work performed for the Atomic Energy Commission at the Oak Ridge National Laboratory: "Hydrolytic Behavior of Metal Ions. II," previous paper, THIS JOURNAL, **72**, 3901 (1950).

(2) F. Granér and L. G. Sillén, *Acta Chem. Scand.*, **1**, 631 (1947).

(3) R. E. Connick and W. H. Reas, THIS JOURNAL, **73**, 1171 (1951).

(4) M. Adolf and W. Pauli, *Kolloid Z.*, **29**, 173 (1921).

(5) G. Jander and K. F. Jahr, *Kolloid-Beih.*, **43**, 295 (1936).

(6) B. A. Lister and L. A. McDonald, *J. Chem. Soc.*, 4315 (1952).

(7) See, *e.g.*, R. Ruer, *Z. anorg. u. allgem. Chem.*, **43**, 282 (1905).

(8) K. A. Kraus and S. Y. Tyree, Jr., Report ORNL-499 (Sept. 1949).

(9) J. S. Johnson and K. A. Kraus, Reports ORNL-607 (1949), ORNL-1053 (1951).

(10) O. Lamm, *Arkiv Kemi, Mineral. Geol.*, **17A**, No. 25 (1944).

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DPNH-CYTOCHROME REDUCTASE, A FERRO-FLAVOPROTEIN[1]

Sir:

Within the past year several flavoprotein enzymes have been shown to contain heavy metals as part of their prosthetic groups. Copper was identified as a constituent of butyryl CoA dehydrogen-

(1) Paper IV in a series entitled Studies on Diphosphopyridine Nucleotide-Cytochrome c Reductase. For paper III see L. P. Vernon, H. R. Mahler and N. K. Sarkar, *J. Biol. Chem.*, **199**, 598 (1952).

ase[2] and molybdenum of xanthine oxidase,[3] and nitrate reductase.[4] In view of these observations, the strong inhibition of DPNH-cytochrome reductase by such metal complexing agents as pyrophosphate[1] and 8-hydroxyquinoline[5] has led us to investigate the possible presence of a metal ion as part of the prosthetic group of the enzyme which had been identified previously as a flavoprotein of somewhat unusual properties.[6]

The presence of iron in the enzyme can be unambiguously demonstrated as follows: the enzyme is isolated and purified in the usual manner, the protein is removed by centrifugation after precipitation with 5% (w./v., final concentration) trichloroacetic acid, and the supernatant solution is analyzed for iron by a micro-adaptation of the *o*-phenanthroline method.[7] Four preparations showing specific activities (units per mg. protein) of 18.0, 34.0, 78.0 and 190 (corresponding to estimated enzymatic purities of 9, 17, 40 and 95%) had an iron content of 0.250, 0.453, 1.05 and 2.67 μg. Fe per mg. protein. Thus the ratio enzyme units per μg. Fe is constant and equal to 73 ± 2. A similar constancy is observed when the flavin:iron ratios are compared; *e.g.*, our two best preparations contained 6.9 and 11.7 mμmoles flavin per mg. (as a flavin adenine dinucleotide[8]), respectively, and had an iron content of 28.0 and 47.8 mμmoles per mg. The ratio iron:flavin equals 4.1 ± 0.1. Calculation of the molecular weight, assuming four gram atoms of iron per mole of enzyme, leads to a value of 80,000, in good agreement with previous determinations based on sedimentation constant and flavin content.[6]

Samples of the enzyme show varying distributions of the metal between its two valence states (Table I). The substrate, DPNH, is capable of reducing all the iron to the ferrous form (experiments 1b, 2b and 3b), while ferricytochrome c, even in considerable excess, does not convert all the iron initially present in the ferrous form to the ferric state (experiments 3c and d). It is apparent, however, that not only the flavin[6] but the iron as well is capable of being reduced by the substrate and oxidized by the acceptor.

TABLE I

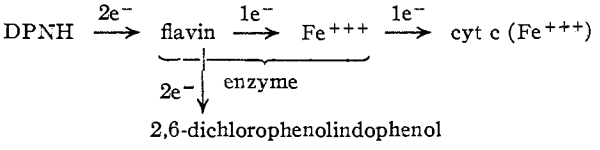
VALENCE STATE OF IRON UNDER DIFFERENT CONDITIONS

Experiment	Conditions	Fe^{++},[a] %	Fe^{+++},[a] %
1a	Enzyme 2 R_5[b] (2.8 γ Fe in 2.8 mg. protein), no additions	0.0	100
b	Same, incubated anerobically with 0.1 μ mole DPNH	92	8
2a	Enzyme 4 R_5 (1.7 γ Fe in 0.63 mg.), no additions	60	40
b	Same, with DPNH	95	5
3a	Enzyme 4 R_4 (2.5 γ Fe in 1.6 mg.)	88	12
b	Same, with DPNH	100	0.0
c	Same, with 200 γ cytochrome c	44	56
d	Same, with 1000 γ cytochrome c	48	52

[a] Fe^{++} estimated without added reducing agent; hydroxylamine hydrochloride is then added and total iron determined. Fe^{+++} is the difference total $-Fe^{++}$. [b] The code designations of various enzyme fractions are described in ref. 6.

Prolonged incubation of the enzyme with *o*-phenanthroline and DPNH or dialysis against 8-hydroxyquinoline leads to a pronounced lowering of the iron content, and of the enzymatic activity, as measured by cytochrome c reduction. When 2,6-dichlorophenolindophenol is substituted for cytochrome c, however (diaphorase reaction[1,6]), little or no inhibition is observed. Full enzymatic activity can be restored by addition of ferric (but not ferrous) iron at a concentration of 5×10^{-4} *M*.

The observations just presented are in accord with the following working hypothesis which is similar to the one previously proposed for butyryl CoA dehydrogenase[2]



Diaphorase,[9] the flavoprotein which catalyzes the reduction of dyes but not of cytochrome c by DPNH, contains less than one-tenth the concentration of iron per mg protein found in a sample of cytochrome reductase of comparable purity, although the flavin content of the two enzymes is similar. Diaphorase may therefore constitute a transformed cytochrome reductase, where the removal of iron has led to certain changes in both structure and function of the enzyme molecule.

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(2) H. R. Mahler, THIS JOURNAL, **75**, 3288 (1953).

(3) (a) D. E. Green and H. Beinert, *Biochim. Biophys. Acta*, **11**, 599 (1953); (b) E. C. DeRenzo, E. Kaleita, P. Heytler, J. J. Oleson, B. L. Hutchings and J. H. Williams, THIS JOURNAL, **75**, 753 (1953); (c) D. A. Richert and W. W. Westerfeld, *J. Biol. Chem.*, **203**, 915 (1953).

(4) D. J. D. Nicholas, A. Nason and W. D. McElroy, *Nature*, **172**, 34 (1953).

(5) H. R. Mahler, unpublished observations.

(6) H. R. Mahler, L. P. Vernon, N. K. Sarkar and R. A. Alberty, *J. Biol. Chem.*, **199**, 585 (1952).

(7) E. B. Sandell, "Colorimetric Determination of Traces of Metals," Interscience, New York, N. Y., 1944, p. 273. Hydroxylamine hydrochloride was used as a reducing agent. All the enzyme-bound iron is estimated under these conditions.

(8) The absorption coefficient of the dinucleotide[1] was taken to be 9.83×10^6 cm.^{2} × mole^{-1} at 450 mμ in the acid extract (E. Dimant, D. R. Sanadi and F. M. Huennekens, THIS JOURNAL, **74**, 5440 (1952)).

(9) J. G. Dewan and D. E. Green, *Biochem. J.*, **32**, 626 (1938); (b) H. von Euler and H. Hellstrom, *Z. physiol. Chem.*, **252**, 31 (1938); (c) F. M. Straub, *Biochem. J.*, **33**, 789 (1939).

(10) Supported by a grant-in-aid of the American Cancer Society, as recommended by the Committee on Growth, National Research Council.