ing specificity type. It suggests further that (a) the common sequence around serine is involved in making the otherwise inert CH_2OH side chain reactive and (b) the amino acids responsible for the specificity lie further from the reactive serine, perhaps in adjacent coils. While there must be an intimate steric relation between the amino acids responsible for the catalytic action and those responsible for the specificity, these results indicate that the two may be separable. In this case common sequences related to specificity as well as to the bond-breaking action might be expected, a result which would be of enormous value in relating enzyme structure to function.

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ENZYMATIC INTRODUCTION OF DOUBLE BONDS INTO STEROID RING A

Sir:

The introduction of unsaturation into ring A of steroids is of interest in connection with the aromatization involved in biosynthesis of estrogens,[1] and in the microbiological formation of Δ^1-dehydrosteroids,[2] some of which possess interesting physiological properties. *Pseudomonas testosteroni* (A.T.C.C. No. 11996)[3] can utilize certain steroids as a sole source of carbon and degrade these compounds to CO_2 and H_2O by a series of reactions catalyzed by adaptive enzymes. During the course of a study of the pathway involved in the oxidative degradation of steroids by this microörganism, it was found that early steps were the interconversion of 17β-hydroxyl and 17-ketone functions and the introduction of Δ^1-double bonds and Δ^4-double bonds into steroids of both the 5α-H and 5β-H series. Such reactions have been reported in a variety of microbiological systems.[1,2] The enzymatic interconversion of 17β-hydroxy- and 17-ketosteroids is catalyzed by the diphosphopyridine nucleotide-linked enzyme β-hydroxysteroid dehydrogenase and has been analyzed in detail.[4] The purpose of the present communication is to report on the enzymatic mechanism of the introduction of double bonds into steroids. This constitutes, to our knowledge, the first demonstration of these reactions by means of soluble enzymes.

Under suitable conditions *Ps. testosteroni* carries out efficient conversions of androstane-3,17-dione (I), testosterone, Δ^4-androstene-3,17-dione (II), Δ^1-androstene-3,17-dione (III) and etiocholan-17β-ol-3-one to principally $\Delta^{1,4}$-androstadiene-3,17-dione (IV) and to a lesser extent to $\Delta^{1,4}$-androstadien-17β-ol-3-one (V). In a typical conversion, this microörganism was grown in 450 ml. of a medium containing 0.5% sodium lactate in a mineral base, at 30° and an initial *p*H of 7.0, and at the end of 24 hours 45 mg. of II was added in 2 ml. of acetone and the incubation continued for 24 hours longer. Extraction of the culture with ethyl acetate, followed by chromatography on silicic acid, crystallization from hexane–acetone, and sublimation at 90–115° (0.0005 mm.) afforded 20.5 mg. of IV, m.p. 140.5–141° (no depression on admixture with an authentic sample); $[\alpha]^{25}D$ +123° (*c* 1.04, in $CHCl_3$); λ_{max}^{alc} 243 mμ (ϵ 16,200); sulfuric acid chromogens[5] were identical with a known sample (peaks at 265, 305 and 390 (broad) and a minimum at 288 mμ); (*Anal.* Found: C, 80.08; H, 8.46. Calcd. for $C_{19}H_{24}O_2$: C, 80.24; H, 8.51); infrared analysis showed λ_{max}^{KBr} 5.77 μ (17-ketone), 6.04, 6.17 and 6.25 μ ($\Delta^{1,4}$-3-ketone), identical with an authentic sample. Mixed paper chromatography showed no separation from authentic IV.

In similar experiments, conversions in good yield of I and III to IV were demonstrated and the products rigorously identified by comparison paper chromatography, m.p. and infrared spectra. Evidence for the presence of small amounts of V in each case was obtained. Conversion of 19-*nor*-testosterone by the same system in 72 hours gave a 67% yield of estrone m.p. 253–256°; $[\alpha]^{25}D$ +154° (*c* 0.644, in dioxane); λ_{max}^{alc} 282 mμ (ϵ 1960); on addition of NaOH, λ_{max}^{alc} 243 mμ (ϵ 8000) and 300 mμ (ϵ 2500). The infrared spectrum showed peaks at 5.83 μ (17-ketone), 6.32, 6.18 μ (—C=C—) and 3.06 μ(OH group), and was identical with that of authentic estrone. On paper chromatography the substance migrated as estrone and gave the phenolic reaction with the Turnbull reagent.[6] Estradiol-3,17β was also found in smaller amounts.

Cell-free extracts of *Ps. testosteroni* were prepared from cultures grown on 0.5% sodium lactate as described above. After 24 hours growth, II was added to give a final concentration of 0.01% and incubation continued for 24 hours longer. Cells were harvested and washed by centrifugation. The washed cells efficiently converted I to IV. After rupture by sonic oscillation in a 9 KC Raytheon oscillator for 20 minutes, the sonicate also converted I to IV vigorously.

The unwashed residue from centrifugation of the sonicate for 20 minutes at 10,000 × *g* was also active in this conversion. The supernatant alone was inactive and its activity could be restored by adding phenazine methosulfate (PMS), an oxidation reduction dye which reacts with certain flavoprotein enzymes[7] but not by the addition of di- and triphosphopyridine nucleotides. The supernatant upon the addition of PMS still introduced Δ^1- and Δ^4-double bonds after centrifuging for 30 minutes at 105,000 × *g*. The enzymatic activities were demonstrated in a variety of ways including paper chromatographic analysis of products and ultraviolet spectrophotometry of products formed from I.

A convenient and specific assay of the Δ^1-dehydrogenase activity was the measurement of estrone formation from Δ^4-estrene-3,17-dione with the Folin

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(2) S. H. Eppstein, P. D. Meister, H. C. Murray and D. H. Peterson, *Vitamins and Hormones*, **14**, 359 (1956).

(3) P. Talalay, M. M. Dobson and D. F. Tapley, *Nature*, **170**, 620 (1952).

(4) (a) P. Talalay and M. M. Dobson, *J. Biol. Chem.*, **205**, 828 (1953); (b) P. Talalay and P. I. Marcus, *ibid.*, **218**, 675 (1956).

(5) A. Zaffaroni, *Rec. Prog. in Hormone Research*, **8**, 51 (1953).

(6) G. M. Barton, R. S. Evans and J. A. F. Gardner, *Nature*, **170**, 249 (1952).

(7) T. P. Singer, E. B. Kearney and V. Massey, in O. H. Gaebler, "Enzymes: Units of Biological Structure and Function," Academic Press, New York, N. Y., 1956, p. 417.

reagent.[8] Table I demonstrates the progression of this reaction with time and demonstrates the requirement for PMS.

TABLE I

CONVERSION OF Δ^4-ESTRENE-3,17-DIONE TO ESTRONE BY Δ^1-DEHYDROGENASE

		μM Phenol (as estrone) Incubation time, min. 0	45	60	90
(1)	Complete system[a]	0.10	0.42	0.48	0.63
(2)	Enzyme omitted[b]	.11	.10	.09	.09
(3)	Estrone synthesized, (1) minus (2)	..	.32	.39	.54
(4)	PMS omitted[c]	.03	.05	.04	.05
(5)	Steroid omitted	.01	.02	.01	.02

[a] The reactions were carried out in air with agitation at 30° in 4 ml. systems containing 115 μM phosphate buffer, *p*H 7.2, and the following additions in the complete system. 0.2 ml. enzyme (supernatant from 105,000 × *g* centrifugation, see text) containing 4.3 mg. protein; 1.84 μM Δ^4-estrene-3,17-dione in 0.1 ml. acetone (or 0.1 ml. acetone only when steroid was omitted); and 3.1 μM phenazine methosulfate (added last). At the end of the reaction the mixture was acidified with 0.3 ml. of concd. HCl, extracted three times with a total of 12 ml. of CH_2Cl_2, the extract dried over Na_2SO_4, and a 5-ml. aliquot evaporated to dryness and analyzed by the Folin reaction.[8] A standard curve was prepared using estrone. [b] A small blank which did not increase on incubation was always found when PMS and steroid were mixed either in the absence of enzyme or in the presence of acid-inactivated enzyme. [c] The sample of Δ^4-estrene-3,17-dione was found to be contaminated with about 1–2% phenol, presumably estrone.

It is believed that at least two and probably three separate enzymes are involved in these dehydrogenating reactions (Δ^1-, Δ^4-5α- and Δ^4-5β-dehydrogenases), and that probably these enzymes are flavoproteins.

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(9) Dr. D. H. Peterson and colleagues have observed the transformation of 19-*nor*-testosterone to estrone and estradiol-3,17β by *Septomyxa affinis* (personal communication).

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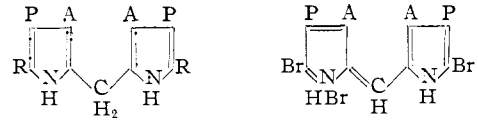
DIPYRRYLMETHANES

Sir:

Although dipyrrylmethane itself and nuclear carboxy, acyl, and hydroxy derivatives are known, those without such groups or their vinylogs are not, being presumed to be very unstable.[1] It now appears that some dipyrrylmethanes of the latter type, which may be intermediates in the biosynthesis of porphyrins, may be obtained by and undergo conventional pyrrole reactions without fission or oxidation at the bridge.

For example, I (R = H) forms nearly colorless micro-prisms without visible absorption, m.p. 199° dec., Ehrlich's reaction strongly positive cold. (Calcd. for $C_{19}H_{22}N_2O_8$: C, 56.15; H, 5.46; N, 6.89; eq. wt., 101.6. Found: C, 56.35; H, 5.57; N, 6.80; eq. wt., 103.2.) It was obtained in 80% yield from I (R = COOH)[2] with 10% sodium hydroxide for four hours at 170°, and also from II[2] with sodium amalgam. Diazomethane converted I (R = H) into its tetramethyl ester III, m.p. 105°, (Calcd. for $C_{23}H_{30}N_2O_8$: C, 59.73; H, 6.54; N, 6.06. Found: C, 59.58; H, 6.59; N, 6.10) which gave IV (80%), the ester of I (R = CHO), m.p. 203°, with hydrogen cyanide and hydrogen chloride (Calcd. for $C_{25}H_{30}N_2O_{10}$: C, 57.91; H, 5.83; N, 5.40. Found: C, 57.85; H, 5.73; N, 5.34).



A = CH_2·COOH; P = CH_2CH_2·COOH

Uroporphyrin II[2] was obtained from I (R = H) with formic acid and hydrogen bromide–acetic acid at 100° (~ 20%, methyl ester, m.p. *ca.* 310°, degraded to coproporphyrin II methyl ester, m.p. 284–286°) and also from III with IV in methanolic hydrogen bromide at 20° followed by warming with aqueous sodium hydroxide (~ 25%, methyl ester, m.p. *ca.* 313–315°, degraded to coproporphyrin II methyl ester, m.p. 285–286°). Under these last conditions neither III nor IV separately gave any porphyrin.

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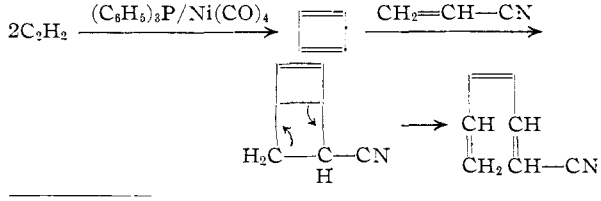
A MECHANISM STUDY OF THE 2,4,6-HEPTATRIENENITRILE SYNTHESIS FROM ACRYLONITRILE AND ACETYLENE

Sir:

The existence of transition-metal complexes of cyclobutadiene as intermediates in reactions of acetylene has been suggested recently.[1] For example, it was proposed that the cycloöctatetraene synthesis from acetylene in the presence of nickel cyanide catalyst involves the intermediate complex $Ni(CN)_2 \cdot C_4H_4$. As an extension of this concept, it seemed reasonable to postulate that the heptatrienenitrile synthesis from acrylonitrile and acetylene[2] could be pictured as



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