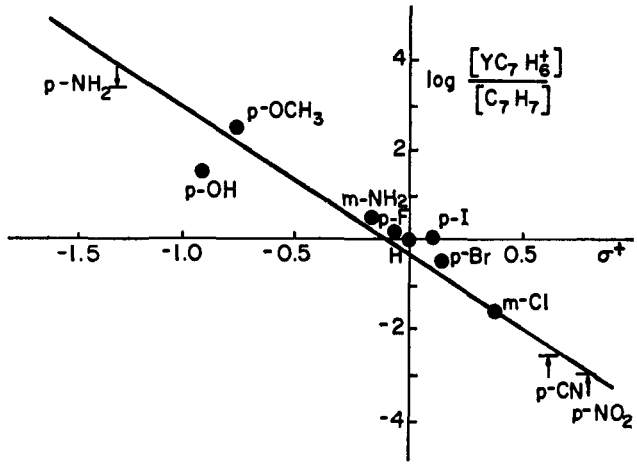

Figure 1. Correlation of the abundance of $YC_7H_6^+$ with that of $C_7H_7^+$ in the spectra of substituted 1,2-diphenylethanes, $YC_6H_4CH_2$-$CH_2C_6H_5$. The values indicated by a line with an arrow are upper or lower limits; in these cases the abundance of one of the conjugate ions was too low for accurate determination. The data were measured at 15.3 eV using a Hitachi RMU-6D mass spectrometer as described previously.[2]

reaction resembles that of the product.[4,11] Further, the substituent effect on the stability of the ion product far exceeds that on the radical product; *e.g.*, in p-NH_2-$C_6H_4CH_2CH_2C_6H_5$, although the electron-donating group should stabilize the benzyl radical, the abundance of the conjugate $C_7H_7^+$ ion is still negligible because of the competitive stabilization of the p-$NH_2C_6H_4CH_2^+$ ion. This is similar to polar effects observed in free-radical reactions.[12]

Following the reasoning of Brown,[6] the difference of a factor of nearly 1000 in $[NH_2C_7H_6^+]/[C_7H_7^+]$ for the *meta* and *para* isomers can only be explained by different transition states for the respective decompositions. With the strong evidence for the close similarity of the transition state and product ion structures, this is consistent with a benzylic structure for the ground-state $NH_2C_7H_6^+$ products, and not the tropylium structure. Any effect of differences in the distributions of energy values in the molecular ions of the isomers should be eliminated.[1,6]

The correlation of σ^+ with log Z/Z_0, where $Z = [YC_7H_6^+][YC_6H_4CH_2CH_2C_6H_5^+]$, is poor, as expected because the distribution of the energy values of the molecular ions is changing with Y. However, it appears that the ionization potential is an approximate measure of this effect, so that the results are much better correlated by a two-term equation. Such techniques for the quantitative measurement of the basic factors yielding substituent effects[1] will be discussed in the full paper.[13]

(11) G. S. Hammond, *J. Am. Chem. Soc.*, 77, 334 (1955).
(12) R. Breslow, "Organic Reaction Mechanisms," W. A. Benjamin, Inc., New York, N. Y., 1966, p 220.
(13) We thank the National Institutes of Health, Grants GM12755 and FR00354, for generous support of this work.
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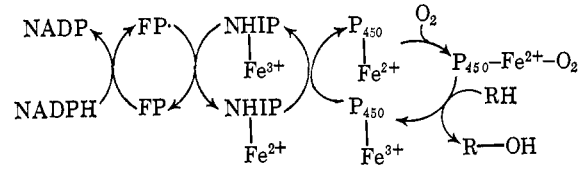
The Roles of Reduced Nicotinamide–Adenine Dinucleotide Phosphate in Steroid Hydroxylation

Sir:

The C-11β hydroxylation of deoxycorticosterone (DOC) by adrenal cortex mitochondria requires reduced nicotinamide–adenine dinucleotide phosphate (NADPH)[1] as the electron donor and incorporates ^{18}O from molecular oxygen into DOC.[2] Since there is nearly a 1:1 correlation between oxygen consumed and DOC hydroxylated,[3] the steroid 11β-hydroxylase of adrenocortical mitochondria falls within the external mixed-function oxidase classification of Mason[4] or the monooxygenase terminology of Hayaishi.[5] Since enzymatic hydroxylation of steroids has been found to proceed with retention of configuration[6-8] and appear to follow the rule of Bloom and Shull,[9] it is likely that enzymatic hydroxylations occur by stereospecific displacement of hydrogen by an electrophilic species such as OH^+. Largely due to the elegant studies of Cooper, *et al.*,[10-12] on the one hand and Kimura's group[13-15] on the other, the mitochondrial steroid 11β-hydroxylase has been resolved into three components: adrenodoxin reductase or flavoprotein (FP), adrenodoxin or nonheme iron protein (NHIP), and cytochrome P_{450} (P_{450}) or hemoprotein. On the basis of reconstitution experiments, Scheme I was proposed[12] for the roles of these components in electron transfer and steroid hydroxylation.

Scheme I



According to Scheme I, reduced cytochrome P_{450} reacts with substrate and molecular oxygen; one atom of the "activated" oxygen molecule is utilized to oxidize the hemoprotein (P_{450}) while the other atom of oxygen reacts with the substrate molecule and results in the introduction of one oxygen atom as a hydroxyl group into the steroid molecule. The function of NADPH is *solely* to provide the reducing equivalents for $P_{450}(Fe^{2+})$ *via* the NADPH–cytochrome P_{450} reductase electron-

(1) D. C. Sharma, E. Forchielli, and R. I. Dorfman, *J. Biol. Chem.*, 237, 1495 (1962).
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(3) W. Cammer and R. W. Estabrook, *ibid.*, 122, 721 (1967).
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(6) M. Hayano, M. Gut, R. I. Dorfman, O. K. Sebek, and D. H. Peterson, *J. Am. Chem. Soc.*, 80, 2336 (1958).
(7) S. Bergstrom, S. Lindstredt, B. Samuelson, E. J. Corey, and G. A. Gregoriou, *ibid.*, 80, 2337 (1958).
(8) E. J. Corey, G. A. Gregoriou, and D. H. Peterson, *ibid.*, 80, 2338 (1958).
(9) B. M. Bloom and G. M. Shull, *ibid.*, 77, 5767 (1955).
(10) T. Omura, R. Sato, D. Y. Cooper, O. Rosenthal, and R. W. Estabrook, *Federation Proc.*, 24, 1181 (1965).
(11) T. Omura, E. Sanders, D. Y. Cooper, O. Rosenthal, and R. W. Estabrook in "[Non-Heme Iron Proteins," A. San Pietro, Ed., Antioch Press, Yellow Springs, Ohio, 1965, p 401.
(12) T. Omura, E. Sanders, R. W. Estabrook, D. Y. Cooper, and O. Rosenthal, *Arch. Biochem. Biophys.*, 117, 660 (1966).
(13) K. Suzuki and T. Kimura, *Biochem. Biophys. Res. Commun.*, 19, 340 (1965).
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Table I. Stereochemistry of $NADPT_B$ Oxidation[a]

Reaction mixture	Spec act. of NADPH recovered, counts/(min μmol)	Spec act. of NADP recovered, counts/(min μmol)
$NADPT_B$ + NHIP[b] + FP	4.92×10^5	5.93×10^4
$NADPT_B$ + FP + NHIP + P_{450}	4.90×10^5	4.14×10^4
$NADPT_B$ + FP + NHIP + P_{450} + DOC[c]	4.83×10^5	5.09×10^4
$NADPT_B$ + FP + NHIP + P_{450} + Cort.	4.95×10^5	4.09×10^4

[a] The reaction mixture contained 29 μg of FP (specific activity 3×10^3 counts/(min μmol)), 200 μg of NHIP, 2 mg of P_{450} (supernatant fraction, obtained by sonication of P_3 for 5 min, followed by centrifugation for 100 min at 144,000*g*), 2.48 μmol of $NADPT_B$ (specific activity 7.46×10^5 counts/(min μmol): B. Kadis, *J. Am. Chem. Soc.*, **88**, 1846 (1966)), 0.06 μmol of DOC, and 5 μmol of $MgCl_2$, in a total volume of 3 ml of 0.05 *M* glycylglycine buffer. The contents were incubated for 30 min at 37°. NADP and NADPH were isolated by DEAE-cellulose chromatography (E. Pastore and M. Friedkin, *J. Biol. Chem.*, **236**, 2314 (1961)). [b] 2 mg of NHIP was used instead. [c] In this experiment, 0.03 μmol of corticosterone was formed.

transfer system. This accessory role of NADPH has also been ascribed to other microsomal P_{450} mixed-function oxidase[16,17] and microbial hydroxylases[18] and appears to be the popular view. Nevertheless, this mechanism is difficult to reconcile with the following lines of experimental evidence.

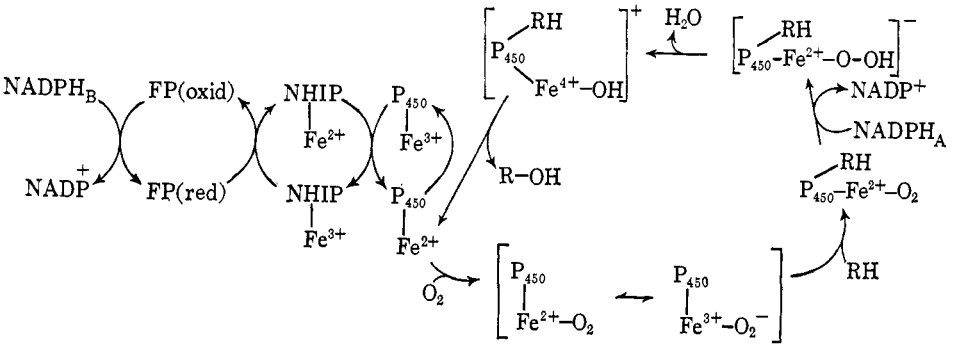
(1) The reduction of adrenodoxin by NADH can also be catalyzed by adrenodoxin reductase (FP) but at a considerably slower rate.[13] According to Scheme I, the inability of NADH to support 11β hydroxylation must be due to the slow rate of electron transfer from NADH to adrenodoxin which is unable to keep cytochrome P_{450} in the ferrous level of oxidation, required for hydroxylation. We have now raised the concentration of FP so that the rate of reduction of NHIP by NADH is identical with that of NADPH. Under the adjusted conditions, again no significant quantities of conticosterone was obtained with the NADH system.

(2) Since NADPH and DOC combine with different enzymes according to the above scheme, a double-reciprocal plot of $1/V$ (V = μmol of DOC hydroxylated/min) *vs.* 1/[NADPH] at varied fixed concentrations of DOC should yield a series of parallel lines or Ping-Pong kinetics.[19] However, the results of Figure 1 clearly show that a series of intersecting lines are obtained, indicating that NADPH and DOC either combine with the same enzyme or with a different enzyme form but connected by reversible steps. The lines intersect left of the *y* axis, suggesting that NADPH also combines with a different enzyme such as FP.

(3) The flavoprotein (FP) (Y_{-2} fraction)[12] was further chromatographed on hydroxyapatite to give a preparation with specific activity of 3×10^3 (mμmol of 2,6-dichlorophenol indophenol reduced per mg of protein). The stereochemistry of $NADPT_B$ oxidation by the purified flavoprotein was investigated. Table I shows that adrenodoxin reductase preferentially removes the H_B hydrogen with at least 83% stereospecificity. The stereochemistry of $NADT_B$ oxidation was then examined with the complete 11β-hydroxylase components in the presence and absence of DOC. It was found that the specific activity of recovered NADP in the presence of DOC was 5.09×10^4 counts/(min μmol) and 4.14×10^4 counts/(min μmol) in the absence of DOC. No increase in the specific activity of NADP was noted upon the addition of corticosterone. *This result clearly shows that DOC-dependent oxidation of NADPH involves the removal of the H_A hydrogen.* Similar experiments have been performed with $NADPT_A$, and the results coincide with those of $NADPT_B$.

All these results are consistent with a mechanism of the type earlier proposed by Hayano[20] which may be envisaged as shown in Scheme II.

Scheme II



Implicit in this sequence is a dual role of NADPH. (a) NADPH serves in an accessory capacity, keeping P_{450} in the ferrous level of oxidation. This NADPH-requiring reaction is steroid independent and serves to reduce autoxidized P_{450}-Fe^{3+} back to P_{450}-Fe^{2+}, this being the form of hemoprotein presumably required for oxygen complexation. (b) NADPH is directly involved in the steroid hydroxylation reaction to generate the highly reactive hydroperoxo complex, P_{450}-Fe^{2+}-O-OH. There is no over-all valence change of the P_{450} on completion of one stoichiometic cycle of the reaction. The order of addition of oxygen, substrate (RH), and NADPH remains to be established.

(16) H. S. Mason, J. C. North, and M. Vanneste, *Federation Proc.*, 24, 1172 (1965).
(17) L. Ernster and S. Orrenius, *ibid.*, 24, 1190 (1965).
(18) M. Katagiri, B. N. Ganguli, and I. C. Gunsalus, *J. Biol. Chem.*, 243, 3542 (1968).
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(20) M. Hayano in "Oxygenases," O. Hayaishi, Ed., Academic Press Inc., New York, N. Y., 1962, p 181.

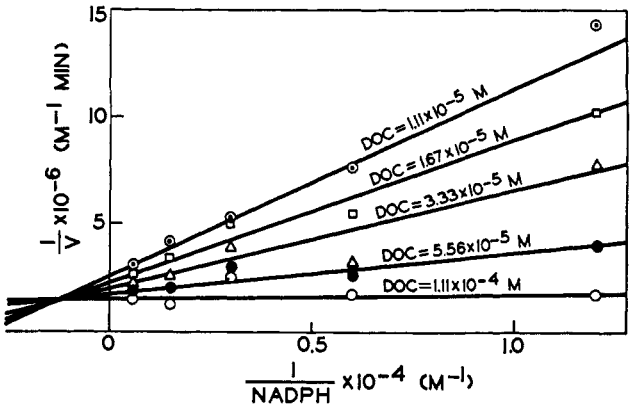

Figure 1. Double-reciprocal plots of $1/V$ *vs.* 1/[NADPH] at several fixed concentrations of DOC. The system consists of 5 mg of P_2 fraction (P_{450}), 0.2 mg of R_1 fraction (NHIP), 0.2 mg of Y_2 fraction (FP) (see ref 12 for details of fractionation), and 5 μmol of $MgCl_2$, in a total volume of 3 ml of 0.1 *M* phosphate buffer, pH 7.5. The reaction mixture was incubated for 10 min at 37°. Corticosterone was assayed by a radiochromatographic method.

The proposed mechanism avoids the cogent objection that a ferrous ion–oxygen complex should not be reactive enough to enter directly into oxidations such as those of unactivated carbon–hydrogen bonds. It seems reasonable that a much more highly reactive species is required for this. Postulation of reduction of an iron–oxygen complex with formation of a reactive species is consistent with studies of the autoxidation of Fe^{2+}, whose second-order dependence on ferrous ion concentration suggests rate-determining reduction of a ferrous ion–oxygen complex.[21,22] It is likely that proposed scheme is of general physiological significance, common to all external mixed-function oxidases.[23]

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Biogenetic Relationship between Methyl Triacetic Lactone and Stipitatic Acid

Sir:

Recent isolations of methyl triacetic lactone[1] (3,6-dimethyl-4-hydroxy-2-pyrone, **1**), triacetic lactone,[2,3] and tetraacetic lactone[2] from higher fungi which simultaneously produce tropolones or phenols have provoked speculation[1-3] on the role of mutual progenitor poly-β-ketides[4] in the formation of these metabolites. We wish to report data which demonstrate that the extended, presumably enzyme-bound, acetate–polymalonate precursor to **1** is probably involved in the formation of stipitatic acid (**5**), and that the origin of the augmented methyl or methyl-derived carbons in *both* metabolites appears to arise from the same single-carbon transfer pool. After growth in the presence of sodium formate-^{14}C, cultures of *Penicillium stipitatum* NRRL 1006 afforded samples of **1** and **5** which were isolated and degraded as outlined below. Intermediates in the stepwise degradation of **1** (Figure 1A) were shown to have uv, ir, and mass spectra and elemental analyses consonant with their structures; the ultimate 3-methyl-5-pyrazolecarboxylic acid (**4**) ex-

(1) (a) P. E. Brenneisen, T. E. Acker, and S. W. Tanenbaum, *J. Am. Chem. Soc.*, 86, 1264 (1964); (b) T. E. Acker, P. E. Brenneisen, and S. W. Tanenbaum, *ibid.*, 88, 834 (1966).
(2) R. Bentley and P. M. Zwitkowitz, *ibid.*, 89, 676, 681 (1967).
(3) (a) T. M. Harris, C. M. Harris, and R. J. Light, *Biochim. Biophys. Acta*, 121, 420 (1966); (b) R. J. Light, T. M. Harris, and C. M. Harris, *Biochemistry*, 5, 4037 (1966).
(4) A. J. Birch, *Science*, 156, 202 (1967).

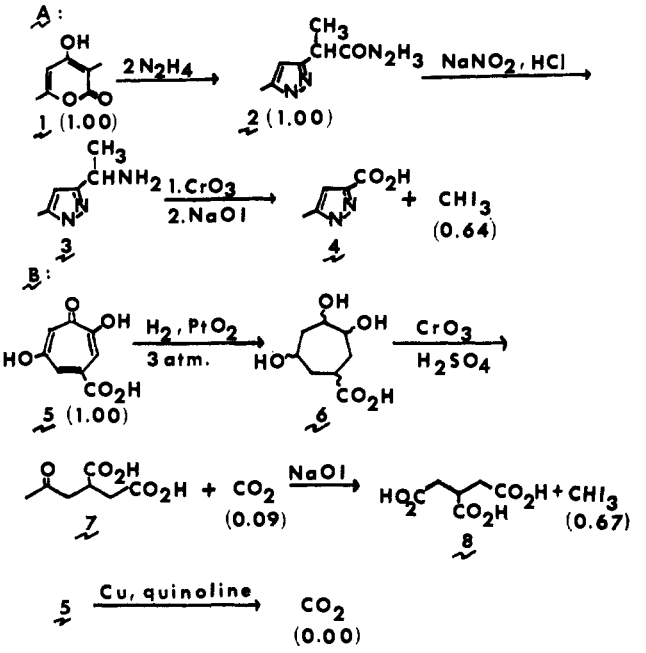

Figure 1. Partial degradations of methyl triacetic lactone and of stipitatic acid.

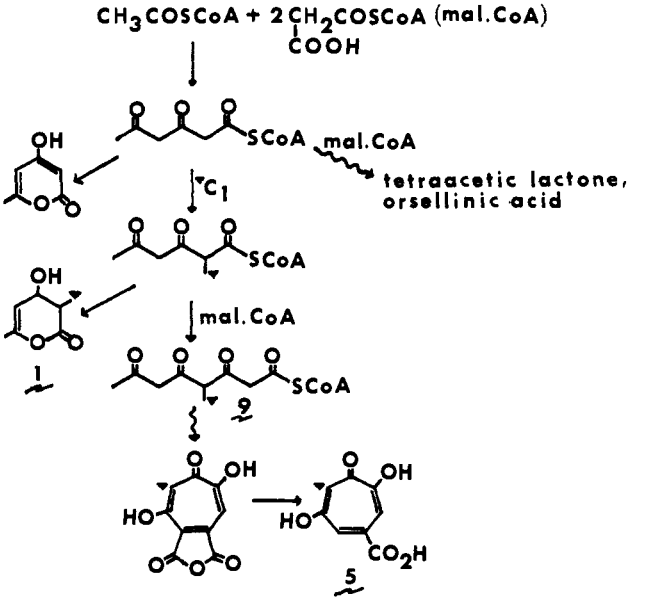

Figure 2. Proposed biosynthetic interrelationships among fungal polyketides and their metabolic congeners. In actuality other 4′-phosphopantetheine carriers, such as ACP, may be involved in oligoketide transfers.