Biotransformations of Δ^4-3-Ketosteroids by the Fungus *Rhizopus arrhizus*

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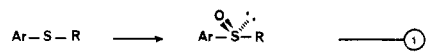
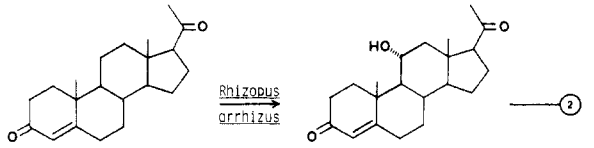
In recent decades natural products chemists have seen an expansion in their area of research which has resulted in a shift in emphasis away from the traditional pursuits of isolation and structure determination. To these have now been added the study of biosynthesis and, more recently, biotransformation and enzyme mechanisms.

The technology of biotransformation,[1] using either isolated enzymes or intact microorganisms to perform specific chemical reactions, enables the organic chemist to carry out many reactions in a highly regio- or stereospecific manner. For example, the asymmetric oxidation of alkyl aryl sulfides to sulfoxides (eq 1) is



readily performed by active or resting cultures of fungi,[2] and the enzyme-catalyzed reduction of ketones, producing chiral alcohols, has been extensively documented.[1] Although recent developments in synthetic organic chemistry have provided many reagents of high stereo- and regioselectivity, the problem of remote functionalization, such as the introduction of a hydroxyl group at C-11 of the pregnane skeleton en route to the corticosteroids (eq 2), remains one which is most practically solved by biotransformation.

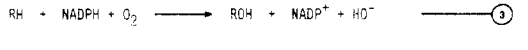


The discovery of the reaction of eq 2 in the early 1950s by Murray and Peterson[3] began two decades of extensive development of oxidative biotransformation technology applied to steroidal substrates, with the result that micoorganisms were identified which can introduce the hydroxyl function into a steroid molecule at any position and with almost any desired stereochemistry.[4] Many of these biotransformations have their counterpart in mammalian metabolism,[5] where hydroxylation provides a route for both the activation of steroid prehormones and the oxidative degradation of steroids and other exogenous organic compounds.

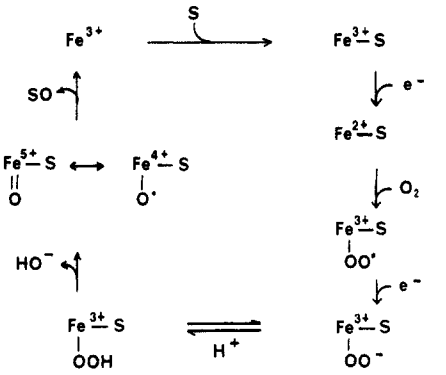
Hydroxylation Mechanisms

In contrast with the rapid growth of practical biotransformation technology in this area, the study of the mechanism of the enzymes responsible for hydroxylation reactions proceeded more slowly and was for many years limited to purely biochemical problems such as cofactor requirements and cellular location. That these enzymes were slow to yield their secrets cannot be attributed to lack of interest or effort on the part of biochemists, but was due to the complexity of the enzymes and to the severe practical difficulties associated with their study.

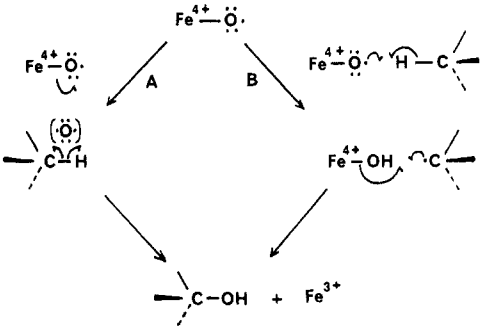
Among the enzymes which perform hydroxylation at carbon are the cytochrome P-450 dependent monooxygenases.[6] In fungal and mammalian systems these are membrane-bound multienzyme complexes which function according to the stoichiometry of eq 3. These



Scheme I
Catalytic Cycle of Cytochrome P-450 Dependent Monooxygenases



Scheme II
Possible Routes for Hydroxylation at Saturated Carbon



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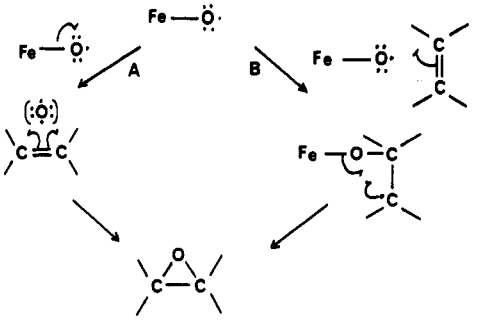
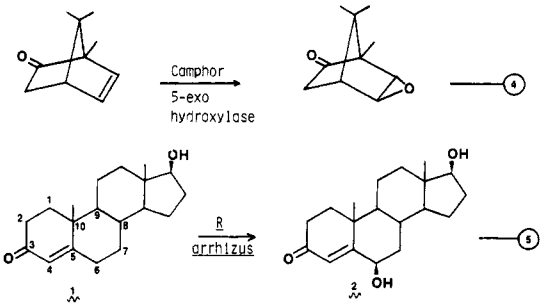
Scheme III
Possible Routes for Epoxidation at Olefinic Carbon



enzymes, in addition to performing steroid hydroxylation, are thought to be responsible for the oxidative metabolism of a wide range of organic substrates including the polycyclic aromatic hydrocarbons[6] and halocarbon solvents.[7] Their catalytic cycle (Scheme I) was deduced largely from a study of the soluble camphor hydroxylating enzyme of *Pseudomonas putida*. Although P-450 dependent monooxygenases from different sources show differences in electron-transport cofactor requirements, the available data suggest that the overall mechanistic features of these enzymes are largely independent of source.[8]

In recent years, the route by which the activated oxygen complex of Scheme I reacts with a C–H bond has received much attention. Two routes have been proposed (Scheme II), a concerted insertion of electrophilic oxygen ("oxene") into a C–H bond[9] and a radical abstraction–recombination process.[10] Evidence for both is available, but the work of Groves,[11] among others,[12] lends credence to the nonconcerted radical abstraction pathway B of Scheme II.[13]

Although the conversion of saturated C–H to hydroxyl in the absence of any activating features of the substrate is a characteristic cytochrome P-450 dependent enzymic reaction, these enzymes also perform related reactions such as the epoxidation of olefins (eq 4)[14] and the hydroxylation of ketones at positions adjacent or vinylogous to carbonyl (e.g., eq 5).[4] Possible



routes for epoxidation parallel those for hydroxylation and are presented in Scheme III. In hydroxylations adjacent to carbonyl, the possibility of activation of the substrate toward attack by the oxidant allows the consideration of mechanistic routes which are denied to the hydroxylation at remote, saturated carbon represented by eq 1.

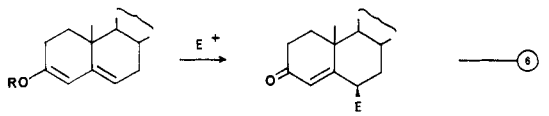
C-6β Hydroxylation

The reaction of eq 5, the C-6β hydroxylation of testosterone (1), is one of the major routes for testosterone metabolism in the mammalian liver[15] and is also carried out efficiently by many microorganisms. It is the commonest enzymic hydroxylation of the ubiquitous Δ^4-3-ketosteroids.[4] The enzyme preparations obtained from mammalian sources have properties characteristic of cytochrome P-450 dependent monooxygenases[15,16]. Steroid hydroxylating enzymes of fungal origin have been found to exhibit similar inhibition properties in cell-free studies[17] and to function with cytochrome P-450 characteristic stoichiometry in cases where intact cells were used.[18]

Our study of the reaction of eq 5 has employed the fungus *Rhizopus arrhizus* ATCC 11145, an efficient C-6β hydroxylator of Δ^4-3-ketosteroids.[4] The initial aims were twofold: first, to define the mechanism of hydroxylation with respect to the question of substrate activation, and second, to define the mechanism of interaction of (activated) substrate and oxidizing species according to the routes of Schemes II and III.

Our work has been based on product–substrate relationships and has been carried out using intact resting cells of *R. arrhizus*. The difficulties inherent in obtaining cell-free hydroxylase preparations from fungi have been alluded to above; no P-450-dependent steroid hydroxylase has yet been obtained in homogeneous form from a fungal source. In the absence of a stable enzyme preparation of rigorously defined nature, a whole cell system presents a practical alternative to the use of ill-defined, unstable cell-free preparations.

Hydroxylation of Δ^4-3-ketosteroids at C-6β cannot be classified as "remote functionalization", since products of this type are available by chemical procedures of high stereospecificity, involving electrophilic addition to a $\Delta^{3,5}$-dienol ester or ether (eq 6). Typical electrophiles



include peracids, giving alcohols such as **2**,[19] and *N*-halo derivatives.[20] The axial stereochemistry of addition arises from a stereoelectronic effect based upon preferred interaction of reagent orbitals in the transition state leading to an axial product.[20,21]

This chemical analogy hints at a mechanism for the enzymic reaction in which the product stereochemistry

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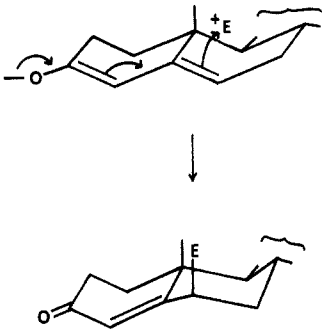
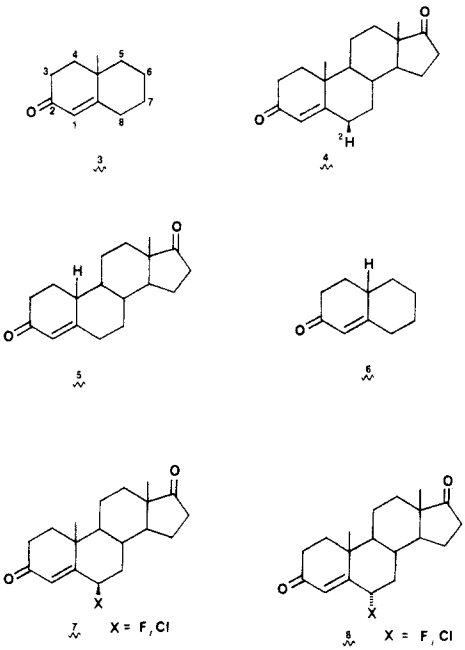
Scheme IV
Stereoelectronically Controlled
Axial Addition at C-6 of a Dienol Ether or Ester

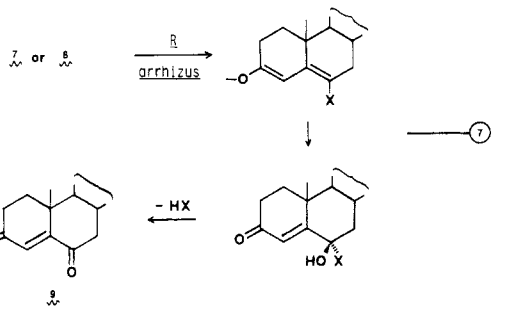


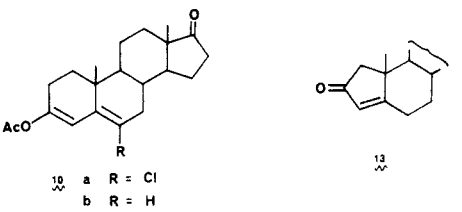
may be controlled by the electrophilic oxygenation of a $\Delta^{3,5}$-dienol equivalent at C-6, analogous to the reaction of eq 6. This proposal, first made by Ringold,[22] has been verified by subsequent work in our laboratories. Hydroxylation of the flexible bicyclic enone **3** at both C-8α and C-8β,[21] the C-6β hydroxylation of the 6β-deuterium labeled substrate, **4**, with some retention of deuterium label at C-6α,[23] and the isolation from incubation of the 19-norsteroid, **5**, and its analogue, **6**,



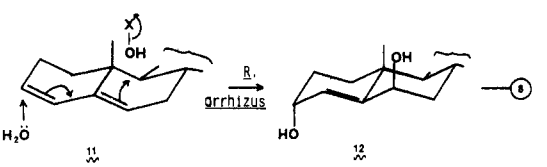
with *R. arrhizus* of C-10β hydroxylated products, in addition to C-6β hydroxyl derivatives,[23,24] all suggest a route for the enzymic reaction via an enolic intermediate.

This supposition was confirmed by the use of the C-6 substituted substrates, **7** and **8**.[25] In the case of **7** (X = Cl), starting material was recovered in which the stereochemistry of substitution at C-6 was inverted. (**8** X = Cl). From **7** (X = F), the major product was the 6-ketone, **9**, presumably formed by the route of eq 6. The formation of similar products from the $\Delta^{3,5}$-6-chloro substrate, **10a**, is consistent with the intermediacy of the latter type of functionality; other work has demonstrated that $\Delta^{3,5}$-dienol acetates such as **10b** are efficiently and directly converted to 6β-hydroxy-Δ^4-3-ketosteroids by *R. arrhizus*[26].

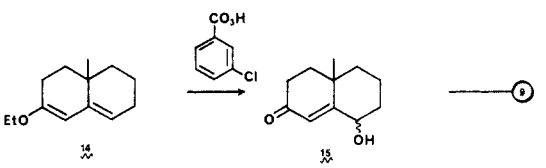




The observation that the unsubstituted $\Delta^{3,5}$-diene, **11**, is converted mainly to the diaxial 3α,6β-diol, **12**, by *R. arrhizus*[27] may be explained by the mechanism of eq 8, which presumes that the stereochemistry of substitution is dictated by the stereoelectronic preference for axial additions to a π electron system. If the stereochemistry of the normal 6β hydroxylation reaction is similarly a function of stereoelectronically controlled axial addition at C-6 to a $\Delta^{3,5}$-dienol intermediate, analogous to the reaction of eq 6, then the stereochemistry of product formation should be dependent upon the conformation of ring B of the substrate.



Malholtra and Ringold[28,29] have shown that the transition state for electrophilic addition to a $\Delta^{3,5}$-dienol resembles the keto form. The C-6–O bond is almost complete, and the C-5–C-6 bond has lost most of its π electron density with ring B close to a chair conformation and the C-6β bond axial. This is presented in Scheme IV. The stereospecific hydroxylation of the conformationally constrained Δ^4-3-ketosteroids (**1**) and the corresponding A-norsteroids, **13**,[30] at the axial 6β position can thus be rationalized. This interpretation is also consistent with the lack of stereoselectivity observed in the peracid oxidation of **14** (eq 9)[21] and the enzymic hydroxylation of **3**. The flexible B ring of products **15** may have either the 8α or 8β position axially disposed, depending on conformation.



(22) Hayano, M. In "Oxygenases"; Hayaishi, O., Ed.; Academic Press: New York, 1962; p 225.
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(26) Holland, H. L.; Auret, B. J. *Tetrahedron Letters* **1975**, 3787.
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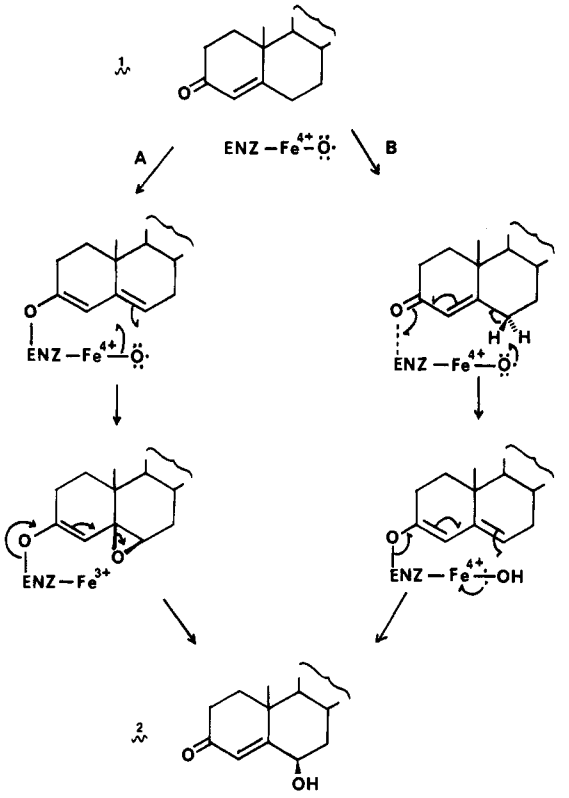
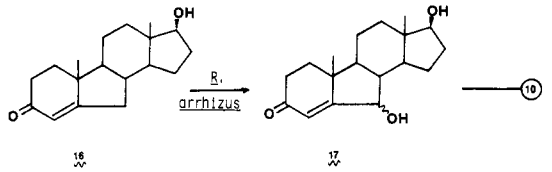
Scheme V
Possible Routes for Enzymic 6β Hydroxylation



A lack of stereospecificity similar to that noted for the microbial hydroxylation of the bicyclic enone **3** (vide supra) is once again encountered in hydroxylation of B-nortestosterone (**16**) (eq 10), where 6α and 6β prod-

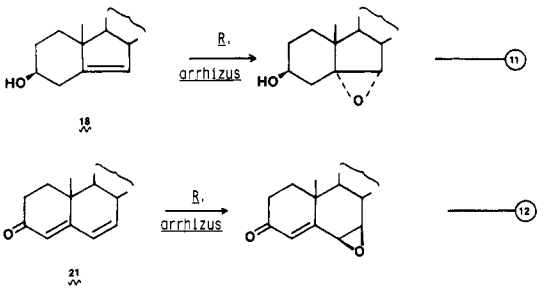


ucts (**17**) are formed in equal amounts.[31] In this case, the conformation of the five-membered B ring is such that both α and β positions at C-6 are stereochemically equivalent with respect to the plane of the O–C-3–C-4–C-5 enone system, so that developing electron density at C-6 from the interaction of a $\Delta^{3,5}$-enol with an electrophilic oxidizing species will occur equally favorably from both faces.

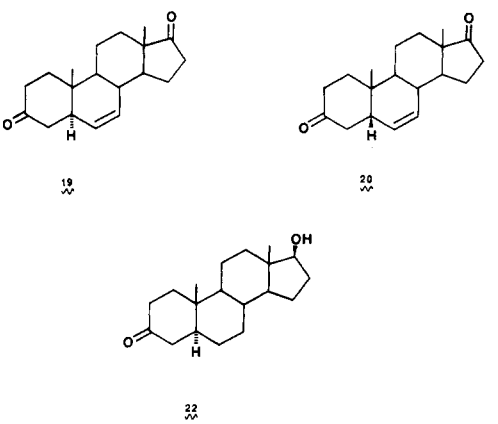
C-6 Epoxidation

The epoxidation of steroid olefins by microorganisms which introduce an axial hydroxyl group into the analogous saturated steroids is a well-known phenomenon,[13,32] such reactions usually occurring with a common stereochemistry.[32] The available evidence suggests that both epoxidation and hydroxylation are carried out by the same enzyme system,[14,33] although the corresponding saturated and unsaturated substrates may not always be able to induced both enzyme activities simultaneously.[33]

Although *R. arrhizus* does not epoxidize normal Δ^5-steroids,[27] the B-nor-Δ^5-steroid **18** is specifically epoxidized with α stereochemistry[31] (eq 11), are the Δ^6-5α-



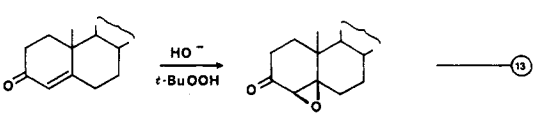
and Δ^6-5β-ketosteroids **19** and **20**.[34] In contrast, $\Delta^{4,6}$-dien-3-ones **21** are epoxidized at the 6,7 position with β stereochemistry (eq 12).[34] These data, together with the results discussed above, now enable conclusions to be drawn about the mechanism of interaction of substrate and oxidizing species (cf. Schemes II and III). Also relevant is the observation that the 5α-saturated steroid **22** is hydroxylated at the C-6α position by *R. arrhizus*.[35]



Mechanisms for C-6 Hydroxylation and Epoxidation

The routes presented in Scheme IV embody the concerted (path A) and stepwise (path B) mechanistic proposals for hydroxylation and epoxidation presented in Schemes II and III and discussed above. Only those mechanisms which are consistent with the cycle of Scheme I and with the observed retention of oxygen label at C-3[35] have been considered in Scheme V. In either mechanism of Scheme V, the initial hydrogen abstraction is apparently controlled by the stereoelectronic properties of the substrate[28,29] and occurs predominantly[23] but not exclusively[25] from the β side. Thus C-6β-substituted Δ^4-3-ketosteroids are metabolized by *R. arrhizus* to products similar to those obtained from the α isomers.[25]

Pathway A proposes a direct, concerted epoxidation of the Δ^5 bond with β stereochemistry, followed by rearrangement of the resulting epoxide to product. Although ample chemical precedent for the latter step exists[37] (eq 13), the evidence presented above and



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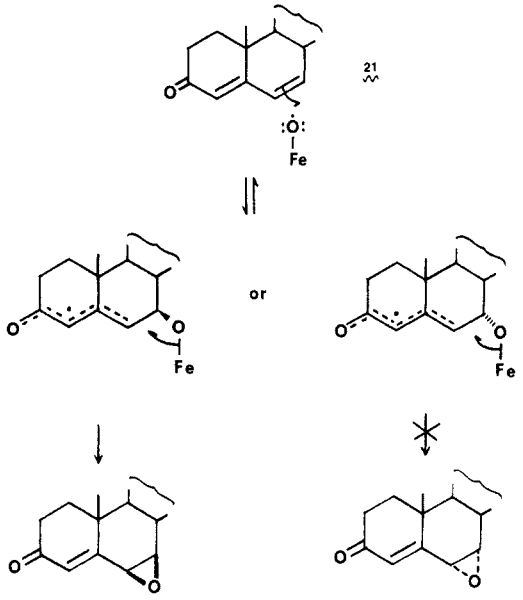
(36) Holland, H. L.; Taylor, G. J. *Can. J. Chem.* **1980**, *58*, 2326.

Scheme VI
Possible Routes for Epoxidation
$\Delta^{4,6}$-Dienones by *R. arrhizus*



chemical precedent[38] militate against the initial epoxidation of the Δ^5 bond occurring from the β face. Inspection of models provides no clear steric reason for the epoxidation of path A to occur specifically β, when in the absence of stereoelectronic factors, both chemical and enzymic oxidation at C-6 occur only from the α side.

A stepwise mechanism of hydroxylation (Scheme V, path B), in contrast, is now subject to the stereoelectronic constraints inherent in the addition of the oxidizing species to the $\Delta^{3,5}$-dienol and would proceed under stereoelectronic rather than enzymic control of stereochemistry to yield **2**.

A similar mechanism (Scheme VI) is also required to account for the enzymic β epoxidation of $\Delta^{4,6}$-dienones (eq 12),[34] in light of the observed chemical and enzymic epoxidation at the Δ^6 position of **19** and **20** with α stereochemistry.[30] The stepwise mechanism of oxidation of **21** (Scheme VI) proposes that the addition of the iron–oxygen complex occurs initially at C-7, giving resonance stabilized intermediates. By analogy with additions at C-5 of Δ^4-3-ketosteroids,[39] the 7α intermediate would experience severe 1,3-diaxial interactions with the C-14 and -15 hydrogens when correctly oriented toward epoxide ring closure, whereas the 7β isomer would not. Product therefore results only from the latter stereochemistry of intermediate. The formation of the 6,7β-epoxide is consistent with this explanation and has a precdent in the chemical oxidation of Δ^4-3-ketosteroids to give predominantly 4,5β-epoxides.[39]

Summary

The data discussed in this Account are consistent with the mechanism for the microbial C-6β hydroxylation of Δ^4-3-ketoandrostanes presented in path B, Scheme V. The wider implications of this proposal for the mechanism of cytochrome P-450 hydroxylations are twofold: first, our data clearly support a stepwise rather than concerted mechanism for both the hydroxylation and epoxidation reactions; second, we present an example of an enzymic oxidation in which the product stereochemistry is controlled by the stereoelectronic properties of the substrate, rather than by any steric control imposed upon the substrate by the enzyme. The latter possibility is one which has only recently come to the attention of bioorganic and biochemists[40] and is one worthy of further attention.

(37) de la Mare, P. B. D.; Wilson, R. D. *J. Chem. Soc, Perkin Trans. 2* **1977**, 975.
(38) Reference 20, p 71.
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