(e) 75% methanol and then glacial acetic acid, tan powder (0.12% P). Fractions (b) and (c) were placed in benzene, mixed with silica gel, and stripped to dryness. The residue was applied to a 20 × 1.6-cm column of silica gel (in pentane). Distinct, colored fractions were eluted with benzene in pentane: (a) 100 ml each of 40%, 45%, and 50% mixture, brown solid, 0.47% P; (b) 100 ml of 60% mixture, brown solid, 0.64% P. Elution with chloroform in benzene followed: (c) 200 ml of 25% mixture, brown wax, 0.81% P; (d) 100 ml of 50% mixture, brown wax, 0.56% P. All color was finally removed from the column with 2-propanol, giving a brown wax (0.21% P). Thin-layer chromatography on cellulose (without binder) in 2-propanol-benzene (1:19) moved the phosphorus in fraction (b) with the colored material, near the solvent front. For fraction (d), the bulk of the colored material moved to R_F 0.5, and phosphorus was concentrated there. Location of phosphorus was accomplished by analysis of sections of each plate. Each section was eluted with 2-propanol-benzene (1:1), and the eluate was directly evaporated onto the paper for the Schöniger analysis.

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Catalysis of the Oxidation of Norethynodrel by Horseradish Peroxidase*

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ABSTRACT: Autoxidation of 17α-ethynyl-17β-hydroxy-5(10)-estren-3-one (norethynodrel) occurs slowly in aqueous solution. The initial products of the reaction are 17α-ethynyl-10β-hydroxy-19-nortestosterone and 17α-ethynyl-10β-hydroperoxy-19-nortestosterone. The oxidation is rapidly catalyzed by horseradish peroxidase in the presence of hydrogen peroxide and manganese ion.

Hemoglobin also catalyzes the reaction, although the ratio of the 10β-hydroperoxy- to the 10β-hydroxy-metabolites is lower when hemoglobin is substituted for peroxidase in the incubation medium.

In studies on the metabolism of orally administered 17α-ethynyl-17β-hydroxy-5(10)-estren-3-one (norethynodrel) in the rabbit (Arai *et al.*, (1962) and in the human (Layne *et al.*, 1963), we have shown that hydroxylation at position 10 plays a major part in the metabolism of this steroid. Incubation of norethynodrel with blood (Arai *et al.*, 1962) led to the formation of 17α-ethynyl-10β-hydroxy-19-nortestosterone and of another unidentified ketone. The latter has since been found to be 17α-ethynyl-10β-hydroperoxy-19-nortestosterone, which strongly suggested that a peroxidative reaction was involved in the *in vitro* conversion of norethynodrel by blood, and possibly also in the *in vivo* metabolism of this steroid. The present paper reports the finding that norethynodrel and related $\Delta^{5(10)}$ steroids are rapidly oxidized by horseradish peroxidase at *p*H 7.4 in the presence of hydrogen peroxide and manganese ion. The effect of several factors on the rate of oxidation

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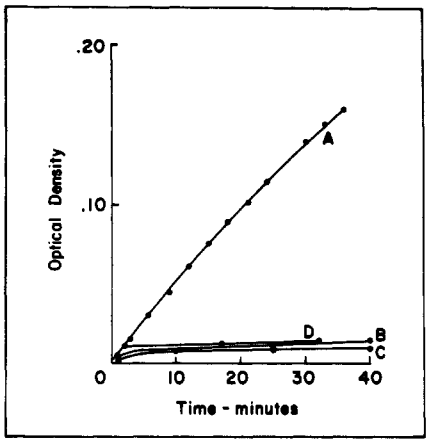

FIGURE 1: Oxidation of 17α-ethynyl-17β-hydroxy-5(10)-estren-3-one. Measured by the appearance of some of the products of the reaction, the oxidation is shown as the plot of optical density at 242 mμ in water against time in minutes. Curve A is the complete incubation system (see text). The velocity was calculated from the slope of the straight line obtained in the first 10 minutes of reaction. Curve B is the result observed when catalase is added to the complete system on an equal weight basis to the peroxidase. Curve C is the reaction when $MnCl_2$ is omitted, and curve D depicts the effect of omitting the peroxidase.

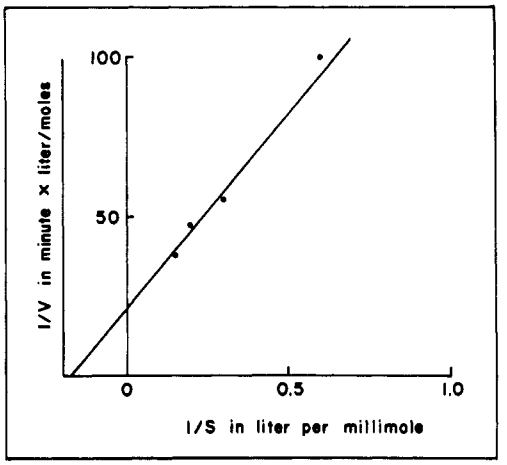

FIGURE 2: Measurement of the Michaelis constant (K_m) for oxidation of 17α-ethynyl-17β-hydroxy-5(10)-estren-3-one catalyzed by horseradish peroxidase at 37°.

has been studied and some of the end products have been identified.

Materials and Methods

Horseradish peroxidase (Reinheitzahl 3.0) and hemoglobin (bovine, grade A) were purchased from California Corp. for Biochemical Research. Samples of 17β-hydroxy-5(10)-estren-3-one, its 17α-ethyl- and 17α-ethynyl- derivatives, and 17α-ethynyl-17β-hydroxy-10β-hydroperoxy-4-estren-3-one (17α-ethynyl-10β-hydroperoxy-19-nortestosterone) were obtained from G. D. Searle and Co., Chicago, Ill., through the courtesy of Dr. Frank Colton. The latter steroid was contaminated with a small amount of 17α-ethynyl-10β,17β-dihydroxy-4-estren-3-one (17α-ethynyl-10β-hydroxy-19-nortestosterone). Reference 17α-ethynyl-10β-hydroxy-19-nortestosterone was prepared and purified (Kirdani and Layne, 1964) in this laboratory. Randomly tritiated norethynodrel (20 mg) described previously (Arai *et al.*, 1962) was chromatographed on thin-layer silica gel G in ethyl acetate–cyclohexane, 1 : 1. The steroid was located by spraying the edges of the plate with dinitrophenylhydrazine reagent and was eluted with methanol. The product (0.02 μc/mg) was pure as judged by chromatography (Golab and Layne, 1962).

Experimental Procedure and Results

Enzymatic Reaction. The reaction medium was that described by Jellink and Irwin (1962): 0.02 ml 10^{-5} M $MnCl_2$, 0.02 ml 2×10^{-5} M H_2O_2, 20 μg peroxidase and 0.2 ml methanol or steroid solution in methanol, and 0.1 M *p*H 7.4 potassium phosphate buffer to 10 ml. When catalase was used the amount was the same as that of the peroxidase (20 μg). Measurements of reaction rate were made with a Cary Model 14 spectrophotometer. The base line was adjusted using medium containing no steroid. Various concentrations of the $\Delta^{5(10)}$-3-ketosteroid in methanol were added at $t = 0$ to the incubation mixture at 37° with rapid stirring. To minimize scattering the tip of the pipet was immersed in the medium during the addition of steroid. The prewarmed cell in the thermostated (37°) sample compartment of the instrument was filled with this solution and the recorder was started at $t = 1$ minute. A plot was obtained of optical density at 242 mμ versus time. The reaction velocity was calculated in optical density (OD) units/minute from the slope of the straight line obtained in the first 5–10 minutes.

The oxidation of norethynodrel as measured by the appearance of products absorbing at 242 mμ is shown in Figure 1 (curve A). The reaction gradually became slower after 15 minutes, and the optical density never reached the theoretical value calculated for the quantity of steroid added. The slope was 7.3×10^{-2} OD units/minute. The reaction is inhibited by catalase (curve B) and by the omission of $MnCl_2$ from the medium (curve C). Curve D shows the reaction rate in absence of enzyme. The reaction was also inhibited by ascorbate, but cyanide had little effect at a concentration of 10^{-3} M.

Effect of Concentration of Steroid on Reaction Velocity. Figure 2 shows the effect of varying the concentration of 17α-ethynyl-17β-hydroxy-5(10)-estren-3-one on the rate of reaction. By extrapolating the plot of $1/V$ against $1/S$ and obtaining the reciprocal, the Michaelis constant K_m was found to be equal to 6.26 M^{-1}.

*Effect of p*H *of the Medium.* The velocity of the reaction was determined at different *p*H values using

the same concentration of norethynodrel (3.35 μmoles/10 ml incubation medium). Table I shows that the reaction became faster as the *p*H of the medium was increased.

TABLE I: Effect of *p*H of Medium on the Rate of Oxidation of Norethynodrel by Horseradish Peroxidase.

*p*H	Velocity (OD units/min × 10^{-3})
6.0	4.8
6.5	9.6
7.0	11.0
7.4	15.8
8.0	17.0
8.5	19.0

Effect of Different 17α-Substituents of the Steroid. Little effect of the 17α-substituent was observed on the rate of oxidation of the $\Delta^{5(10)}$-3-keto-19-norsteroid molecule. The rates for 17β-hydroxy-5(10)-estren-3-one and for equimolar concentrations of its 17α-ethynyl- and 17α-ethyl- analogs were 3.45, 3.25, and 2.90 OD units/minute, respectively.

Nature of the Oxidation Products. To identify the products of the reaction, [^{3}H]norethynodrel was incubated as described. After 30 minutes the medium was extracted three times with ether, the combined ether extracts were dried over sodium sulfate, and the solvent was evaporated *in vacuo* below 30°. The extraction and evaporation of solvents were done under mild conditions in order to prevent any alteration in the composition of the product mixture. The residue was dissolved in 0.5 ml of methanol-benzene, 1:1, applied to a silica gel G plate, and chromatographed in ethyl acetate–cyclohexane, 1:1. Standards of [^{3}H]norethynodrel, 17α-ethynyl-19-nortestosterone, 17α-ethynyl-10β-hydroperoxy-19-nortestosterone, and 17α-ethynyl-10β-hydroxy-19-nortestosterone were run concurrently. The plate was heated in an air oven at 100° and sprayed with a saturated solution of $SbCl_3$ in chloroform (Golab and Layne, 1962). Ten areas in each of the two radioactive channels were scraped into liquid scintillation vials, eluted with methanol, and examined for radioactivity as described by Arai *et al.* (1962). The radioactivity of a blank area of silica gel of similar size was also counted. The radioactive products were found in five areas on the chromatogram of the extract. These were numbered starting from the point of application as: (I) polar compound(s) at the origin, (II) 17α-ethynyl-10β-hydroxy-19-nortestosterone, (III) 17α-ethynyl-10β-hydroperoxy-19-nortestosterone, (IV) 17α-ethynyl-19-nortestosterone, and (V) 17α-ethynyl-17β-hydroxy-5(10)-estren-3-one.

The expected compounds (V) and (IV) were the starting material and the product of isomerization, respectively. They were identified by coincidence of the spots with standards of the authentic compounds and similar color reactions with $SbCl_3$ in visible and ultraviolet light (Golab and Layne, 1962).

In order to establish the identities of compounds II and III, a large-scale experiment was carried out in which 9 mg of compound V was incubated for 30 minutes with fifteen times the medium used before. After extraction and chromatography, the material eluted from the area of the plate corresponding to standard compound II had a radioactivity of 5885 cpm. This was mixed with 9.81 mg of authentic compound II and crystallized from methanol-water. A specific activity of 630 cpm/mg was thus attained and did not change significantly after two further crystallizations. The eluate from the spot corresponding to compound III was mixed with a reference sample of the compound, the mixture was chromatographed as before on silica gel G, and the plate was stained with antimony trichloride. The radioactivity was found to be coincident with the spot corresponding to compound III.

Pathways of Product Formation. Three incubations were carried out in *p*H 7.4 phosphate buffer for 3 hours at 37°, in which [^{3}H]norethynodrel was used: (a) alone as control, (b) with enzyme but without hydrogen peroxide, and (c) with hydrogen peroxide but without enzyme. The incubates were either extracted immediately with ether or kept frozen for not more than 24 hours. Extremely variable total recoveries of the radioactivity ranging from 40 to 90% were obtained, and this was thought to be owing to the formation of very polar degradation products of the steroid. To facilitate comparison and eliminate the effect of different recoveries in the individual extractions, the results for compounds I, II, III, and IV are presented in Table II as percentages of the amount of starting material (V) recovered.

Column A of Table II shows that although the [^{3}H]norethynodrel used had previously been purified, after chromatography it contained approximately 3% of compound III and traces of II. Incubation of compound V in buffer (column B, Table II) caused an increase in air oxidation as evidenced by the higher percentage of compounds II and III. Slow isomerization to compound IV also occurred. There was a marked effect of hydrogen peroxide (column C, Table II) on the quantity of compound III, which increased to 40% of the recovered starting material. A simultaneous increase in compound II was also noted. With enzyme in the absence of hydrogen peroxide (column D, Table II), there was no increase in formation of compound II or of III, but an increase in isomerization was observed. This indicates that the peroxidase preparation may have had a small amount of an isomerase associated with it.

Time Study of the Enzymatic Reaction. To determine the formation of the various products as a function of time, 25-ml aliquots of an incubation of compound V in the complete system were extracted 5, 10, and 15 minutes after addition of steroid. The results obtained

TABLE II: Relative Amounts of Some of the Products Extracted from the Medium after the Incubation of [^{3}H]Norethynodrel with Peroxidase and with Hemoglobin at 37° in Phosphate Buffer at *p*H 7.4.

	Compounds Incubated with Steroid in Buffer and Time of Incubation[a]							
Product[b]	A None No Incubation	B None 3 hr	C H_2O_2 3 hr	D Peroxidase 3 hr	E Hemoglobin, H_2O_2, $MnCl_2$ 3 hr	F Peroxidase, H_2O_2, $MnCl_2$ 5 min	G Peroxidase, H_2O_2, $MnCl_2$ 10 min	H Peroxidase, H_2O_2, $MnCl_2$ 15 min
17α-Ethynyl-19-nortestosterone (compound IV)	0.7	2.3	8.7	5.4	18.9	10.1	9.7	6.5
17α-Ethynyl-10β-hydroperoxy-19-nortestosterone (compound III)	3.0	5.9	40.4	4.2	20.9	14.7	11.5	8.0
17α-Ethynyl-10β-hydroxy-19-nortestosterone (compound II)	0.7	1.7	12.4	1.7	38.3	8.7	8.4	6.6
Origin of chromatogram (polar compounds I)	0.9	1.0	3.7	1.5	6.5	1.7	20.5	64.8
Ratio of II/III						0.59	0.73	0.83

[a] Amounts of radioactive steroid in 25 ml buffer were 3×10^{-3} μc in A, 8.6×10^{-3} μc in B, C, and E, and 2.45×10^{-2} μc in D. The concentration in F, G, and H was 6.15×10^{-2} μc in 75 ml buffer. H_2O_2 was 2×10^{-4} M and $MnCl_2$ was 10^{-5} M. Peroxidase was used at 2 μg/ml and hemoglobin at 20 μg/ml. [b] Values are expressed as radioactivity in product in per cent of the radioactivity in the recovered norethynodrel. See Discussion.

are given in columns F, G, and H of Table II. Compared to the controls (columns A, B, and C of Table II) in which there was negligible radioactivity remaining at the origin, formation of compound I after 15 minutes (column H, Table II) was extensive. This occurred at the expense of not only the starting material (V) but also of compounds II, III, and IV. It was also noted that the ratio of compound II/III increased with time, while the rate of decrease in the percentage of compound II was slower than that of III. This suggests that compound II was formed from III. A decrease with time in the percentage of compound IV was also observed.

Effect of Hemoglobin. Hemoglobin (grade A) was substituted for horseradish peroxidase in a 3-hour incubation in the complete system (Table II, column E). Compared to the similar incubations with peroxidase, there was very high conversion to compound II.

Discussion

In previous work in this laboratory it had been observed that when [^{3}H]norethynodrel is purified by column chromatography (Arai *et al.*, 1962), stored in solution in the cold for some time, and then chromatographed on silica gel G plates in ethyl acetate–cyclohexane, 1:1, contaminants invariably appeared as three spots with R_F values of 0, 0.32, and 0.5, respectively (Table II, column A). The possibility that these were oxidation products was suggested by analogy with the work of Fieser *et al.* (1955) who oxidized cholest-5-en-3-one in hexane with molecular oxygen at 25° to give 6β-hydroperoxy-cholest-4-en-3-one. Since norethynodrel is also a β,γ-unsaturated ketone, it seemed likely that air oxidation at position 10 with concerted migration of the double bond into conjugation with the ketone had taken place. Shapiro *et al.* (1964) have recently exposed norethynodrel at 45° to fluorescent light under an atmosphere of oxygen and produced 17α-ethynyl-10β-hydroperoxy-19-nortestosterone in 40% yield. Both groups (Fieser *et al.*, 1955; Shapiro *et al.*, 1964) reported the conversion of the hydroperoxides to the corresponding hydroxy derivatives by reduction with sodium iodide in acetic acid.

In the present work when a solution of norethynodrel in *p*H 7.4 phosphate buffer was allowed to stand at 37° in air (Table II, column B), the optical density of the solution increased slowly. This increase is caused partly by isomerization of the $\Delta^{5(10)}$ bond to the corresponding Δ^4-3-ketone (λ_{max}^{aq} 248 mμ). The products with an oxygen function at position 10β absorb at 242 mμ. If the foregoing incubation was repeated in the presence of hydrogen peroxide (Table II, column C), the formation of 17α-ethynyl-10β-hydroperoxy-19-nortestosterone (III) took place in appreciable yield and a smaller amount of 17α-ethynyl-10β-hydroxy-19-nortestosterone (II) was produced. This reaction of hydrogen peroxide with norethynodrel was comparatively slow. The addition of Mn^{2+} and horseradish peroxidase to the system resulted in a much more rapid oxidation (Figure 1, curve A). The requirement for hydrogen peroxide and the inhibition of the enzyme catalysis by catalase and also by ascorbate, which is a competitive substrate for peroxidase, indicate that the reaction is truly peroxidative in nature. This conclusion is compatible with the increase with time of the ratio of compound II

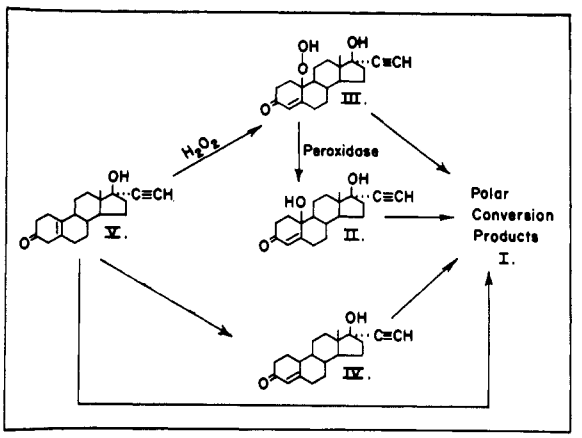

FIGURE 3: Postulated pathways for the formation of oxidation products of 17α-ethynyl-17β-hydroxy-5(10)-estren-3-one. See text and Table II.

to III (Table II, columns F, G, and H) in the reaction mixture. This increase can be interpreted as the formation of the 10-hydroxy product from the hydroperoxide.

A rapid increase in the percentage of polar compounds in the medium (Table II, columns F, G, and H) occurs as the reaction progresses and is accompanied by a decrease in the percentage of compounds II, III, and IV. This indicates that further oxidation of all three of these compounds occurs, although it does not exclude the oxidation of compound V by routes which do not involve these compounds as intermediates. The formation of polar products (I) from norethynodrel and from compounds II and III accounts for the failure of the optical density at 242 mμ to reach the theoretical value for the amount of steroid added. Following a rise which is still evident after 15 minutes' incubation (Table II, column H), the percentage of polar products has again decreased after 3 hours. This indicates that further degradation of these products occurs to compounds not extractable with ether. The poor total recoveries obtained in many of the experiments suggest that this degradation may be extensive, and the nature of the conversions is being investigated. In spite of these poor total recoveries, however, the data in Table II should provide valid comparisons of the relative amounts of compounds IV, III, and II in the conversion products of compound V, since all these steroids have a very high partition coefficient in favor of ether in the ether-water system and should therefore be extracted quantitatively from the incubation medium. It should be noted that the accuracy of the quantitative measurements, which involve elution from silica gel before radioactivity measurements, is probably only about $\pm 20\%$. However, this accuracy is sufficient to establish the general conclusions drawn in this work.

The results in Table II can be summarized in the scheme shown in Figure 3. This scheme does not attempt to depict the mechanism of the peroxidatic reaction. It is possible that there may be a one-electron oxidation involving the formation of a free radical, analogous to the mechanism proposed by Booth and Saunders (1956) for the peroxidatic hydroxylation of mesitol.

Hemoglobin is well known to possess a pseudoperoxidase activity (Paul, 1963). However, the results in Table II, column E, suggest that horseradish peroxidase and hemoglobin differ in their action on norethynodrel, since substitution of hemoglobin for peroxidase in the reaction medium leads to the formation of larger amounts of compound II in relation to the other products. The results are not sufficient to indicate whether the action of hemoglobin or similar substances may contribute to the extensive 10-hydroxylation of norethynodrel which occurs *in vivo* (Arai *et al.*, 1962, Layne *et al.*, 1963).

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