Oxidation of Methionine Residues of Porcine and Bovine Pepsins†

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ABSTRACT: By treating porcine and bovine pepsins with H_2O_2 at pH 3.2, 2.5 of the 4 methionine residues of porcine pepsin and 1.6 of the 3 residues of bovine pepsin were oxidized to methionine sulfoxide. The effect of modification on activity varied with the substrate. There were no significant changes in catalytic constants in the hydrolysis of acetyl-L-phenylalanyl-L-tyrosine by both pepsins and in the hydrolysis of benzyloxycarbonyl-L-glutamyl-L-tyrosine by porcine pepsin. Hydrolysis of benzyloxycarbonyl-L-glutamyl-L-tyrosine by bovine pepsin was too slow to measure. With benzyloxycarbonyl-L-histidyl-L-phenylalanyl-L-tryptophan ethyl ester as substrate, the modification decreased the catalytic efficiency (k_{cat}/K_m) by two-thirds for porcine pepsin and by half for bovine pepsin. With hemoglobin substrate, digestion was significantly less than with native pepsin for modified porcine pepsin, and slightly less for modified bovine pepsin. The results are interpreted as indicating the presence of a methionine residue that participates in the binding of long substrates, but is not close enough to the active site to reach short substrates. Cleavage of the modified pepsins with cyanogen bromide identified the methionine nearest the carboxyl terminus of both pepsins as a residue that remained partially unmodified.

It is well established that the activity of pepsin depends on two carboxyl groups (reviewed by Fruton, 1971 and Hofmann, 1974). Other residues that participate in the catalysis or binding of substrates are not so well understood. Chemical modification studies have shown that alteration of tyrosine (Herriott, 1935; Perlmann, 1966; Lokshina and Orekhovich, 1966; Rimon and Perlmann, 1968; Kozlov *et al.*, 1969; Sokolovsky and Riordan, 1969; Mains *et al.*, 1973) or tryptophan (Previero *et al.*, 1964; Lokshina and Orekhovich, 1964; Dopheide and Jones, 1968) residues affects the activity quantitatively, but does not destroy it altogether. Nitrous acid (Hofmann, 1969) deaminates the amino-terminal isoleucine and the single lysine residue and reacts with two tryptophan residues; about 60% of the activity on casein substrate remains. An arginine residue located 12 residues from the carboxyl terminus can be modified specifically by biacetyl, resulting in 80–85% loss of activity on hemoglobin substrate (Huang and Tang, 1972). When phenylglyoxal hydrate was used as the reagent to modify arginine (Kitson and Knowles, 1971) a decrease in k_{cat} was apparent with no change in K_m when the modified pepsin was assayed on acetyl-L-phenylalanyl-L-phenylalanylglycine at low pH, but at pH 4.7 the activity was equal to pepsin. The phosphate groups can be removed from both porcine (Perlmann, 1958; Clement *et al.*, 1970) and bovine (Meitner and Kassell, 1971) pepsins without change in activity on several substrates. From these studies it is clear that a number of residues participate either in the catalytic action or in maintaining the conformation of the active site.

Previous studies on modification of the methionine residues of porcine pepsin have been concerned with alkylation. At pH 2.2, pepsin was not alkylated by iodoacetic acid or iodoacetamide (Dopheide and Jones, 1968); in the experiments of Lokshina and Orekhovich (1964) at pH 4.6, methionine was alkylated to some extent without loss of activity measured with hemoglobin as substrate. During reaction of an active carboxyl group with 1,2-epoxy-3-(*p*-nitrophenoxy)propane (Chen and Tang, 1972) resulting in inactivation, one methionine residue was also modified; this residue was located 38 residues from the carboxyl terminus of pepsin.

The present paper deals with oxidation of the methionine residues of porcine and bovine pepsins. The activity of native and modified enzymes has been compared on both protein and synthetic substrates. In the case of porcine pepsin, a partially unmodified residue has been located in the known sequence proposed by Tang *et al.* (1973), which differs only in minor aspects from the partial sequence proposed by Morávek and Kostka (1973).

Materials and Methods

Materials. Crystalline porcine pepsin (batch PM 71A), from Worthington Biochemical Corp., Freehold, N.J., was purified by gel filtration on Sephadex G-75. Bovine pepsin was prepared according to Nevaldine and Kassell (1971) from an abomasum extract, which was a gift from Dairyland Food Laboratories, Waukesha, Wis.[1] Catalase was purchased from Sigma Chemical Corp., St. Louis, Mo.

N-Acetyl-L-phenylalanyl-L-tyrosine and Cbz-L-histidyl-L-phenylalanyl-L-tryptophan ethyl ester[2] were products of Cyclo Chemical Corp., Los Angeles, Calif. Cbz-L-glutamyl-L-tyrosine was purchased from Sigma Chemical Corp., St. Louis, Mo. These peptides were reported by the manufacturers to be chromatographically pure. Crystalline bovine hemoglobin was purchased from Pentex, Miles Labo-

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[1] We are grateful to Dr. John H. Nelson for arranging for us to receive the extract.

[2] Abbreviations used are: acetyl-Phe-Tyr, *N*-acetyl-L-phenylalanyl-L-tyrosine; Cbz-His-Phe-Trp-OEt, benzyloxycarbonyl-L-histidyl-L-phenylalanyl-L-tryptophan ethyl ester; Cbz-Glu-Tyr, benzyloxycarbonyl-L-glutamyl-L-tyrosine.

ratories, Kankakee, Ill. Fluorescamine (Fluoram®) was from Hoffmann-La Roche, Nutley, N.J.[3] Cyanogen bromide was from Aldrich Chemical Co., Milwaukee, Wis. Other chemicals were the best grades available.

Enzyme Assay. The enzyme concentration was determined spectrophotometrically at 280 nm using a factor of 676 μg of protein/A_{280} unit for both porcine pepsin (Blumenfeld *et al.*, 1960) and bovine pepsin (Lang and Kassell, 1971). Molecular weights of 34,644 for porcine (Tang *et al.*, 1973) and 33,367 for bovine pepsin (Lang and Kassell, 1971) were used to calculate molar concentrations. Each substrate was used at its previously determined pH optimum: for porcine pepsin according to references cited for each substrate, for bovine pepsin according to Lang and Kassell (1971).

The proteinase activity of pepsin was determined by a modification of the hemoglobin method of Anson (1939), as described previously (Chow and Kassell, 1968).

For the hydrolysis of synthetic substrates, free amino groups liberated were determined with fluorescamine (Udenfriend *et al.*, 1972). In each case, the incubation mixture at 37.6° contained 0.8 ml of substrate and 0.2 ml of enzyme (enzyme and substrate concentrations are given below). The reactions were stopped by addition of 0.5 ml of 0.2 M sodium borate buffer (pH 9.0). An aliquot of the solution was diluted to 1.5 ml with the borate buffer; 0.5 ml of fluorescamine solution in acetone (15 mg/ml) was added with rapid stirring on a Vortex mixer. The fluorescence was measured in an Aminco-Keirs spectrophosphorimeter equipped with a fluorescence attachment, with excitation at 390 nm and emission at 480 nm. Readings were corrected for fluorescamine-reactive material present at the start of the reaction by subtracting the transmission of a solution containing the same ingredients, but in which enzyme was added after the addition of 0.2 M borate buffer (pH 9.0). Standard curves were prepared, using the appropriate hydrolysis products. The kinetic parameters, K_m, V_{max}, and k_{cat} (V_{max}/[E]), were obtained from plots of [S]/v *vs.* [S] (Dixon and Webb, 1958; Wilkinson, 1961). Each experiment was carried out in triplicate, with duplicate assays on each sample. Lines were calculated by the method of least squares.

Acetyl-Phe-Tyr was initially dissolved in 2 equiv of warm NaOH solution (Jackson *et al.*, 1965) and adjusted to pH 2.0 with 1 N HCl. The solution was diluted with HCl of pH 2.0 containing 3% (v/v) methyl alcohol to give final concentrations of 0.6–2.4 mM in the incubation mixtures. The final enzyme concentration was 5.7×10^{-6} M for porcine and $17.4\text{–}23.2 \times 10^{-6}$ mM for bovine pepsin. The initial velocities were calculated from 8-min incubations, corresponding to hydrolysis of not over 7.5%, an amount not exceeding a linear reaction (Jackson *et al.*, 1965; Lang and Kassell, 1971).

Cbz-His-Phe-Trp-OEt (Inouye *et al.*, 1966) was initially dissolved in warm ethyl alcohol and diluted with 10 mM sodium citrate buffer (pH 4.0) containing 2% (v/v) ethyl alcohol to give final concentrations of 0.1–0.4 mM in the incubation mixtures. The enzyme concentrations were 1.14×10^{-6} M for porcine and 3.48×10^{-6} M for bovine pepsin. The time of hydrolysis was 5 min for porcine and 70 min for bovine pepsin (per cent hydrolysis is discussed in Results).

Cbz-Glu-Tyr (Fruton *et al.*, 1939) was dissolved in 5 equiv of warm HCl solution and adjusted to pH 4.0 with 0.5 M NaOH. The solution was diluted with 10 mM sodium citrate (pH 4.0) to give a final concentration of 2 mM in the incubation mixtures. Incubation continued for 20 hr, with 1.4–2.4% hydrolysis.

Amino Acid Analysis. Analyses were carried out in a Beckman-Spinco analyzer, Model 120B, modified according to Eick *et al.* (1974), essentially by the method of Moore and Stein (1963). For total composition, samples were hydrolyzed in sealed evacuated tubes with 5.7 M HCl or with 3 M toluenesulfonic acid containing 0.2% tryptamine (Liu and Chang, 1971) for 24 hr at 110°. For determination of methionine sulfoxide, samples were hydrolyzed for 16 hr at 110° in 15% NaOH (Neumann *et al.*, 1962) in a small plastic tube inside a vacuum hydrolysis apparatus similar to that described by Bello (1963). Hydrolysates were adjusted to pH 2.2 before application to the analyzer.

Oxidation with Hydrogen Peroxide. The procedure was similar to that of Neumann *et al.* (1962). A 1% solution of pepsin, adjusted to pH 3.2 with 1% perchloric acid, was made 0.3 M in H_2O_2 and incubated for 70 min at 30°. The reaction was stopped by addition of sodium citrate (pH 6.0) to a final concentration of 0.1 M, followed at once by 2-μl portions of 2.5% catalase at 5-min intervals until bubbling stopped. The solution was dialyzed against water at pH 6.0 and lyophilized.

Cyanogen Bromide Cleavage. Before cleavage, a small amount of low molecular weight material, presumably resulting from self-digestion during oxidation, was removed from the modified pepsins by passage through a column of Sephadex G-75 (1.2 × 200 cm). The oxidized pepsins were then denatured by adjustment to pH 10.5 with 6 M ammonium hydroxide and incubation for 1 hr at 37°.

A 6% solution of each modified pepsin in 70% trifluoroacetic acid was treated with a 50-fold molar excess (based on methionine content) of cyanogen bromide for 24 hr at 37°, similar to the procedure of Tang *et al.* (1973). The solution was diluted tenfold with water and lyophilized. Fragments were separated on Sephadex G-75 in a column 1.2 × 200 cm, equilibrated with 50 mM ammonium bicarbonate (pH 8.0) containing 4% methanol.

Results

Amino Acid Analysis. Table I shows the methionine sulfoxide content of the native and modified proteins. Purified native porcine pepsin contained a detectable but small amount of methionine sulfoxide. Exposure of the pepsin to the experimental conditions (pH 3.2, 30°, 70 min) without peroxide caused oxidation of an additional half residue of methionine; this preparation is designated "control." Peroxide treatment resulted in the oxidation of about 2.5 methionine residues. With bovine pepsin, the native pepsin showed only a trace of the sulfoxide, and this was not altered by exposure to the experimental conditions without peroxide. About 1.6 methionine residues were oxidized by treatment with peroxide.

Parallel determinations of total compositions including tryptophan after 24-hr hydrolysis with *p*-toluenesulfonic acid (Liu and Chang, 1971) showed no significant variations in other amino acids between native and modified pepsins. No methionine sulfone was detected.

Effect of Oxidation on Activity. With hemoglobin as substrate, porcine pepsin showed a significant (18%) lowering of the activity in the course of 60 min of treatment with

[3] We thank Dr. Sidney Udenfriend and Mr. Thomas Cekoric, Jr., for gifts of fluorescamine before it was available commercially.

Table I: Methionine and Methionine Sulfoxide Content of the Pepsin Preparations[a] before and after Oxidation with H_2O_2.

Pepsin	Residues/mole		
	Methionine	Methionine Sulfoxide[a]	Total
Porcine (4 residues[b])			
Native	3.85	0.13	3.98
Control[c]	3.30	0.67	3.97
Modified[d]	1.53	2.44	3.97
Bovine (3 residues[e])			
Native	2.88	Trace	2.88
Modified[d]	1.14	1.58	2.72

[a] Hydrolysis with 15% NaOH at 110° for 16 hr *in vacuo*. Methionine sulfoxide values are corrected 10% for destruction during hydrolysis (Neumann *et al.*, 1962). [b] Rajagopalan *et al.* (1966). [c] Sample kept under the conditions of footnote *d* without H_2O_2. [d] Oxidized according to Neumann *et al.* (1962): 1% protein, pH 3.2, 70 min, 30°, 0.3 M H_2O_2. [e] Lang and Kassell (1971).

Table II: Hydrolysis of Cbz-L-histidyl-L-phenylalanyl-L-tryptophan Ethyl Ester by Native and Modified Porcine Pepsin.[a]

Pepsin	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
Native	0.26	23	88
Control	0.47	20	43
Modified	0.54	15	28

[a] Experiments were conducted at 37.6° in 10 mM sodium citrate buffer (pH 4.0) containing 2% ethanol. [S] = 0.1–0.4 mM. [E] = 1.16×10^{-3} mM. The time of reaction was 5 min.

H_2O_2; with bovine pepsin, the change was small (6%) and hardly significant.

Oxidation of methionine residues had no effect within experimental error on K_m or k_{cat} for the action of either porcine or bovine pepsin on the substrate acetyl-Phe-Tyr.

In contrast, oxidation of methionine residues markedly affected the hydrolysis of Cbz-His-Phe-Trp-OEt. The graphic representation (Figure 1) shows that the differences between the native and modified pepsins were well beyond the standard deviations of each line. For modified porcine pepsin, K_m was increased and k_{cat} was decreased (Table II), so that the catalytic efficiency indicated by the ratio k_{cat}/K_m was only about one-third of the native enzyme. The control sample, in which only about half a residue of methionine was oxidized, fell between the native and modified pepsins in its hydrolysis of this substrate. For bovine pepsin, this peptide is a poor substrate (Lang and Kassell, 1966). To obtain significant hydrolysis, the enzyme concentration was increased, resulting also in higher blank values, and making it necessary to increase the time of hydrolysis. The percentage hydrolysis was then 28% at the highest substrate concentration and 68% at the lowest concentration. Because the time curve was not linear to this extent (Inouye

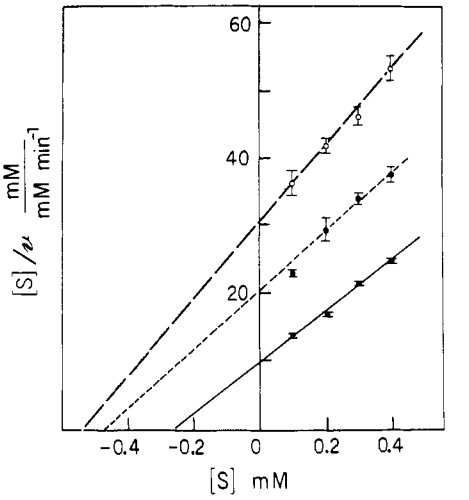

FIGURE 1: Determination of the Michaelis constant, K_m, the maximum velocity, V_{max}, and the molecular activity coefficient $k_{cat}(V_{max}/[E])$ for hydrolysis of Cbz-His-Phe-Trp-OEt by porcine pepsin. The enzyme concentration was 1.16×10^{-3} mM. The pH was 4.0. (■—■) native, (● - - - - ●) control, (○ - - ○) modified. Vertical bars indicate standard deviation.

et al., 1966; Lang and Kassell, 1971), kinetic constants could not be calculated. However, modification had a significant effect on the hydrolysis. At the highest substrate concentration, the modified pepsin gave 22% hydrolysis as compared to 28% for native. The reaction is nearly linear within this range.

Cbz-Glu-Tyr (Fruton and Bergmann, 1941) was chosen as a substrate slowly hydrolyzed by porcine pepsin. Methionine modification had no effect on the hydrolysis of this substrate. The bovine pepsins, both native and modified, gave almost no hydrolysis.

Cyanogen Bromide Cleavage and Chromatography of Fragments. The methionine and methionine sulfoxide contents of the modified pepsins (Table I) show that in both cases somewhat over one residue of methionine remained unmodified. Since cyanogen bromide does not cleave at methionine sulfoxide, we attempted to locate the *unmodified* methionine by cleavage with this reagent.

The reaction was conducted with the modified pepsins as described under Materials and Methods. Separation of the fragments on Sephadex G-75 gave three peaks. In each case, the first peak was well separated and proved to be reasonably pure on amino acid analysis (Table III); it contained no significant amounts of homoserine or methionine, indicating carboxyl terminal peptides

For porcine pepsin, the composition of this peak corresponded to the known carboxyl terminal fragment (Kostka *et al.*, 1970; Tang *et al.*, 1973). The yield was 28%. For bovine pepsin, the composition of a carboxyl terminal fragment containing about 50 residues is significant (Table III); only the sequence of 20 amino acids of the carboxyl terminus has been published (Rasmussen and Foltmann, 1971). The composition found for peak 1 (Table III) includes these 20 amino acids. The yield was 45% which, allowing for some loss, indicates that more than half of the methionine residues about 50 residues from the carboxyl terminus was unmodified.

Fractions 2 and 3 for both pepsins were impure by amino acid analysis. Attempts to purify them further were unsuccessful. It is likely that the complexity of fractions 2 and 3 was caused in part by incomplete oxidation of most of the

Table III: Amino Acid Compositions of the Carboxyl-Terminal Cyanogen Bromide Fragments of Modified Porcine and Bovine Pepsins.

Amino Acid[a] (as Residues/Mole)	Porcine	Bovine
Tryptophan	0.6 (1)[b]	0.7 (1)[c]
Lysine	0.9 (1)	0.1
Histidine	0	0.1
Homoserine and lactone	0 (0)	0.1
Arginine	1.9 (2)	2.0 (2)
Aspartic Acid	5.0 (5)	7.0 (7)
Threonine	1.8 (2)	2.7 (3)
Serine	2.1 (2)	4.7 (5)
Glutamic Acid	2.2 (2)	3.3 (3)
Proline	1.9 (2)	1.6 (2)
Glycine	3.1 (3)	5.6 (5–6)
Alanine	2.9 (3)	2.6 (2–3)
Half-cystine	0 (0)	0.2
Valine	4.4 (5)	3.5 (4)
Methionine	0 (0)	Trace
Isoleucine	2.3 (2)	4.6 (5)
Leucine	3.0 (3)	3.9 (4)
Tyrosine	1.8 (2)	1.8 (2)
Phenylalanine	1.9 (2)	3.6 (3–4)
Total	(37)	(48–51)

[a] 24-hr hydrolysates. [b] Values in parentheses are taken from the known carboxyl-terminal sequence of 37 amino acids determined by several groups (*e.g.*, Kostka *et al.*, 1970). [c] Values in parentheses are estimated whole numbers and are *approximations;* the small amounts of lysine, histidine, homoserine, and cystine indicate that the peptide was not entirely pure.

methionine residues and in part by incomplete cleavage with CNBr. Tang *et al.* (1973) indicated that a Met-Thr bond of porcine pepsin was incompletely cleaved.

Discussion

The most interesting result of this investigation of conversion of methionine residues to the sulfoxide is the contrast in the effect on hydrolysis of different substrates. With porcine pepsin, the modification caused no significant change in the hydrolysis of acetyl-Phe-Tyr or Cbz-Glu-Tyr, decreased hemoglobin digestion moderately, and decreased hydrolysis of Cbz-His-Phe-Trp-OEt by about two-thirds (Table II). With bovine pepsin, already less active than porcine pepsin, the decreases in the rate of hydrolysis of the last substrate and of hemoglobin were smaller. The variation in the effects of modification are probably not related to the pH optima of the substrates, since both an unaffected substrate (Cbz-Glu-Tyr) and the most affected one (Cbz-His-Phe-Trp-OEt) were used at their optimum of pH 4. It seems more likely that the latter more extended substrate reaches more binding sites on the pepsin molecule (Fruton, 1971), one of them being a methionine residue, and that introduction of a larger group by oxidation of this methionine causes a change in the conformation of the binding site. This kind of modification can aid in mapping the distance of the methionine residue from the catalytic site.

The difference in the effects of modification on the hydrolysis of different substrates is reminiscent of the methionine to methionine sulfoxide conversion with chymotrypsin (Weiner *et al.* 1966), which produced even larger quantitative differences. With the pepsins and chymotrypsin, only decreases in rates of hydrolysis have been seen on modification, whereas with the zymogen, chymotrypsinogen, increased reactivity resulted from oxidation of methionine-192 (Gertler *et al.*, 1974).

The methionine residues of porcine pepsin were more reactive toward the smaller reagent, H_2O_2, than they were toward iodoacetic acid or iodoacetamide in the earlier experiments of Lokshina and Orekhovich (1964) and of Dopheide and Jones (1969). This was also the case with another protein, the pancreatic trypsin inhibitor (Kassell, 1964). Thus H_2O_2 is a more effective reagent for methionine modification.

In porcine pepsin, part of the methionine is sensitive to oxidation by air at pH 3.2, as judged by the increase in methionine sulfoxide in the "control" sample containing no H_2O_2 (Table I). Probably this is an exposed residue. Bovine pepsin did not undergo spontaneous oxidation and has one less methionine residue.

The unusual position of the small carboxyl terminal peptides of both modified pepsins preceding many larger peptides cannot be explained at present, but agrees with the results of Tang and coworkers (personal communication) obtained after cyanogen bromide cleavage of porcine pepsin.

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Conversion of an Androgen Epoxide into 17β-Estradiol by Human Placental Microsomes†

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ABSTRACT: Three androgen epoxides, 17β-hydroxy-4β,5-oxido-5β-androstan-3-one (II), 3β,19-dihydroxy-5,6β-oxido-5β-androstan-17-one 3-acetate (VIII), and 19-hydroxy-4β,5-oxido-5β-androstane-3,17-dione (V), were synthesized and subsequently evaluated as potential precursors in the biosynthesis of estrogens by incubation with human placental microsomes. One of these epoxides (V) was converted into 17β-estradiol, whereas the other two were metabolized to unidentified products. The possible intermediacy of an androgen epoxide in the biosynthesis of estrone and of 17β-estradiol is discussed and a mechanism is proposed for the aromatization process.

The placental aromatization of the A ring of androgens requires NADPH and oxygen (Ryan, 1958, 1959) and both 19-hydroxy-4-androstene-3,17-dione (XII, R = OH; R′ = H) (Meyer, 1955; Ryan, 1959; Longchampt *et al.*, 1960; Morato *et al.*, 1961; Wilcox and Engel, 1965) and 19-oxo-4-androstene-3,17-dione (XII, R, R′ = O) (Axelrod *et al.*, 1965; Akhtar and Skinner, 1968; Oh and Tamaoki, 1971) have been shown to be involved as intermediates. The same cofactors are required for the aromatization of 19-norandrogens (Townsley and Brodie, 1968) and androgens with an oxygen function at C-19 (Morato *et al.*, 1961) and there is evidence (Meigs and Ryan, 1971; Bellino and Osawa, 1974) that several mixed function oxidases may be involved in the aromatization process. After the introduction of the oxygen at C-19 there remain, in chemical terms, only two operations to be effected for estrogen formation. One is the removal of the hydrogen atoms at C-1 and C-2 and the other is the expulsion of the angular substituent at C-10. Although it is well established (Morato *et al.*, 1962; Brodie *et al.*, 1969; Fishman *et al.*, 1969; Osawa and Spaeth, 1971) that the C-1 and C-2 hydrogen atoms are stereospecifically lost from the β side of the molecule, the actual mechanism for the final stages of the aromatization of the A ring is still not clear. Morato *et al.* (1962) have suggested that aromatization of 19-oxoandrogens may involve a substituent at C-1 or concerted removal of the 1β-hydrogen atom with expulsion of the angular group at C-10. Experimental results with various androgen derivatives (Townsley and Brodie, 1968; Brodie *et al.*, 1969) have not confirmed these views.

Recently, Morand *et al.* (1974) proposed that epoxidation of the double bond in the A ring (XIII) or the B ring (XI) of androgens may play a role in the biological aromatization process. This paper describes the synthesis of some potential androgen epoxide precursors and the results of incubation of these compounds with human placental microsomes.

Experimental Procedure

Materials. Solvents used for extractions and chromatography were reagent grade. Testosterone, 17β-estradiol, es-

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