



## Review Article

# FREE RADICAL GENERATION BY REDOX CYCLING OF ESTROGENS

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**Abstract**—Natural and synthetic estrogens elicit normal hormonal responses in concentrations in a clearly defined yet low range. At elevated doses, metabolic reactions of the phenolic moiety, while harmless at low levels, may become the predominant biochemical activity and may exert deleterious effects. These metabolic pathways, such as i) oxidation of estrogens to catechol estrogens and further to their respective quinones, and ii) free radical generation by redox cycling between catechol estrogens or diethylstilbestrol and their quinones, are investigated for their influence in physiological or pathophysiological processes. In this review, the *in vitro* capacity of various enzymes to oxidize estrogen hydroquinones to quinones or to reduce corresponding quinones to hydroquinones is evaluated. The *in vivo* activities of enzymes supporting redox cycling of estrogens and free radical generation is correlated with induction of kidney tumors in Syrian hamsters. Concomitant changes in activities in quinone reductase and other detoxifying enzymes in kidneys of hamsters treated with estrogen support a role of free radicals in the induction of tumors by estrogen. Free radical damage to protein and possibly to DNA in kidneys of estrogen-treated hamsters may be used as markers of free radical action *in vivo*.

**Keywords**—Estrogen, Redox cycling, Quinones, Catechol estrogens

## INTRODUCTION

Estrogenic activity is shared by a large number of substances varying in chemical structure. The major natural estrogens produced by the ovary are estradiol ( $E_2$ ) (Fig. 1) and estrone. Synthetic estrogens may be steroidal, such as  $17\alpha$ -ethinylestradiol, the estrogenic component of many contraceptive medications, or non-steroidal, such as DES or hexestrol. In addition, many naturally occurring or synthetic phenols possess some estrogenic activity.<sup>1,2</sup> The hormonal effects of this large number of substances are defined by their affinity for the estrogen receptor or by biological responses such as stimulation of uterine growth. Normal physiological responses are elicited by  $E_2$  and the more potent synthetic estrogens in concentrations in the low picogram range. However, at elevated doses, estrogens induce tumors in laboratory animals, for example, kidney tumors in Syrian hamsters and uterine tumors in mice.<sup>3,4</sup> Likewise, administration of high doses of the

synthetic estrogen DES has been clearly identified as carcinogenic.<sup>5</sup> Genital tract cancer incidence increased significantly in daughters of women treated with large doses of DES for the stabilization of pregnancies.<sup>6,7</sup> Thus, a maximal physiological response may be achieved with a defined yet low concentration of estrogen. At elevated concentrations, metabolic reactions of estrogens may become the predominant biochemical activity overshadowing their hormonal effects. Since estrogens are phenols, the metabolism of their phenolic moiety, while harmless at low levels, may exert deleterious biochemical effects at high concentrations. These metabolic pathways, such as CE formation, redox cycling of CE, and free radical generation, are investigated in our laboratory and elsewhere in attempts to elucidate the mechanistic details of estrogen-induced carcinogenesis. This review evaluates the current understanding of free radical generation by redox cycling of estrogens and its role in physiological and pathophysiological processes.

## OXIDATION OF DES OR CE TO QUINONES

The synthetic estrogen DES (structure shown in Fig. 1) may be viewed as a bridged hydroquinone, which

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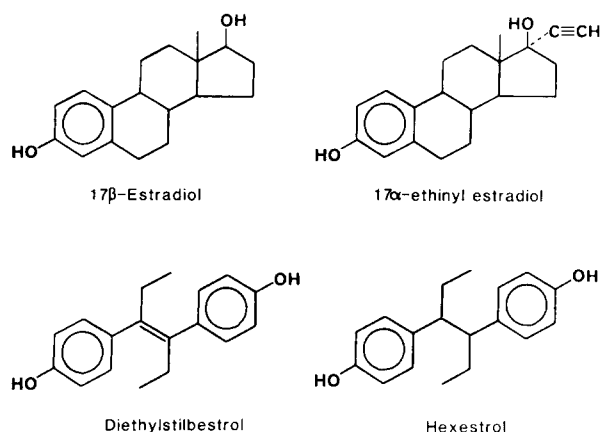


Fig. 1. Structures of 17β-estradiol ( $E_2$ ), the natural female hormone, and of the major synthetic estrogens 17α-ethinyl estradiol, diethylstilbestrol (DES), and hexestrol.

is oxidizable to a quinone (Fig. 2). Steroidal estrogens are phenols (Fig. 1) and cannot participate in redox cycling without prior conversion to CE (which is discussed below). 2-OH- $E_2$  or 4-OH- $E_2$  are hydroquinones and are readily oxidized to *ortho*-quinones (Fig. 3). Nonenzymatically, oxidations are carried out with silver oxide,<sup>8</sup> activated manganese dioxide,<sup>9</sup> or other metal oxides.

#### Heme peroxidases

The *in vitro* enzymatic oxidation of DES to Z,Z-dienestrol presumably via DES Q by horseradish peroxidase has first been demonstrated by Liao and Williams-Ashman.<sup>10</sup> The initial formation of DES Q and subsequent spontaneous rearrangements of the quinone to Z,Z-dienestrol in this oxidation system has been confirmed by comparison of the ultraviolet absorption characteristics of DES Q formed by enzymatic oxidation with that of synthetic reference standard ( $\lambda_{\max}$ : 312 nm, 340 nm (shoulder)).<sup>8</sup> Horseradish peroxidase also catalyzes the oxidation of catechol estrogens. The formation of hexestrol 3',4'-quinone from 3'-hydroxy-hexestrol, the major catechol metabolite of the synthetic estrogen hexestrol, by this enzyme has been established by comparison of ultraviolet and mass spectra of the *in vitro* product with those of a synthetic standard<sup>11</sup> (Table 1). Peroxidases are known to oxidize substrates to free radicals by one-electron elimination.<sup>12,13</sup> The intermediacy of a semiquinone free radical in horseradish peroxidase-catalyzed oxidations was demonstrated by ESR analyses. ESR spectra of  $E_2$  2,3-semiquinone and  $E_2$  3,4-semiquinone could be readily recorded after stabilization of the semiquinone by  $Mg^{2+}$  ions.<sup>14-16</sup> Ross et al. also provided indirect ESR evidence for a DES semiquinone intermediate during oxidation of DES to DES Q.<sup>17</sup> Rat or mouse uterine

peroxidases also oxidize DES or catechol estrogens such as 3'-hydroxyhexestrol to their corresponding quinones.<sup>11,18</sup>

#### Tyrosinase

$E_2$  and 2-OH- $E_2$  are oxidized to a semiquinone free radical in the presence of mushroom tyrosinase,<sup>19</sup> presumably by two sequential one-electron oxidations (Table 1). Tyrosinase-catalyzed reactions may be a useful tool for ESR studies, but their biological significance remains to be explored.

#### Prostaglandin H-synthase

In the presence of hydrogen peroxide and prostaglandin H-synthase, DES and the synthetic analog indenestrol A are oxidized to DES Q and indenestrol quinone.<sup>20,21</sup> Both quinones have been identified by matching their ultraviolet spectra with those of synthetic standards. This enzyme catalyzes quinone formation by sequential 1-electron transfer reactions.<sup>13</sup> The semiquinone free radical intermediates in this oxidation have been demonstrated by ESR analysis.<sup>17</sup>

#### Cytochrome P-450 oxidase

2-OH- $E_2$  or DES are oxidized to their respective quinones presumably by two sequential one-electron oxidations<sup>22,23</sup> in the presence of phenobarbital-induced rat liver microsomes and cumene hydroperoxide<sup>24</sup> (Figs. 2 and 3 and Table 1). Purified cytochrome P-450 isozymes with hydrogen peroxide or cumene hydroperoxide as cofactor also oxidize DES or CE to their respective quinones. Both reactions follow first-order kinetics (D. Roy & J.G. Liehr, unpublished data). In these reactions,  $E_2$  2,3-quinone and DES Q were identified by time-dependent increases in absorption at 302 and 421 nm or 312 and 340 nm, respectively, which matched those of quinone standards. The oxidations are inhibited by the cytochrome P-450 inhibitor SKF 525A.

For the demonstration of redox cycling of estrogens by cytochrome P-450 enzymes, the formation of DES Q from DES was characterized further. Cytochrome P-450-mediated quinone formation may be quantified by determining DES Q concentrations using a high pressure liquid chromatography procedure.<sup>25</sup> Alternatively, DES Q may be allowed to rearrange quantitatively to Z,Z-dienestrol which may be quantified by high pressure liquid chromatography and/or identified by spectroscopic methods.<sup>24,25</sup> Thus, Z,Z-dienestrol was utilized as a marker of oxidation of DES to its quinone. Using both methods of analysis, DES Q formation

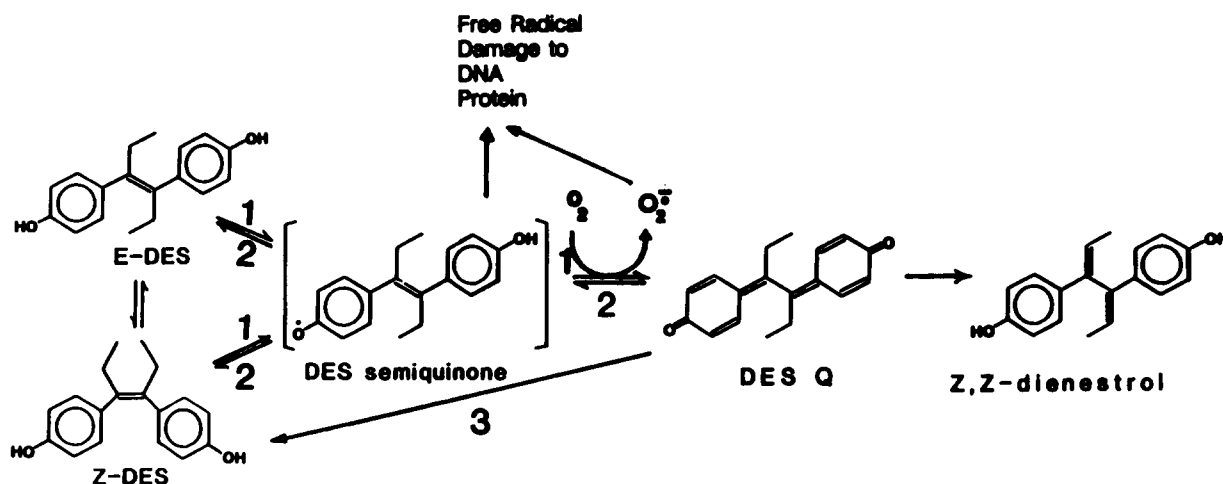


Fig. 2. Redox cycling between DES and DES Q and free radical generation. The intermediacy of a semiquinone in this process has been postulated<sup>24</sup> but not demonstrated. For this reason its structure is shown in brackets. Both the *E*- and *Z*-forms of DES are bridged hydroquinones and are capable of redox cycling without initial catechol formation. Both isomers of DES are oxidized by cytochrome P-450 oxidase (1) to the quinone.<sup>24</sup> The DES Q and semiquinone forms are reduced by NADPH-dependent cytochrome P-450 reductase<sup>24</sup> (2). Reductions catalyzed by 2 or by quinone reductase (3) preferentially form the *Z*-isomer of DES, which may equilibrate with the more stable *E*-form.<sup>27</sup> DES Q spontaneously rearranges to *Z,Z*-dienestrol.<sup>8</sup> The *Z*-form of DES and *Z,Z*-dienestrol have been taken as markers of reduction and oxidation, respectively.<sup>24</sup> The DES semiquinone may react with molecular oxygen and form the quinone and a superoxide radical.<sup>27</sup> Free radical-mediated damage to protein<sup>53</sup> and DNA<sup>40</sup> (and J. G. Liehr, D. Roy, & R. A. Floyd, unpublished data) has been observed. The nature of the active radical(s) is still being investigated.

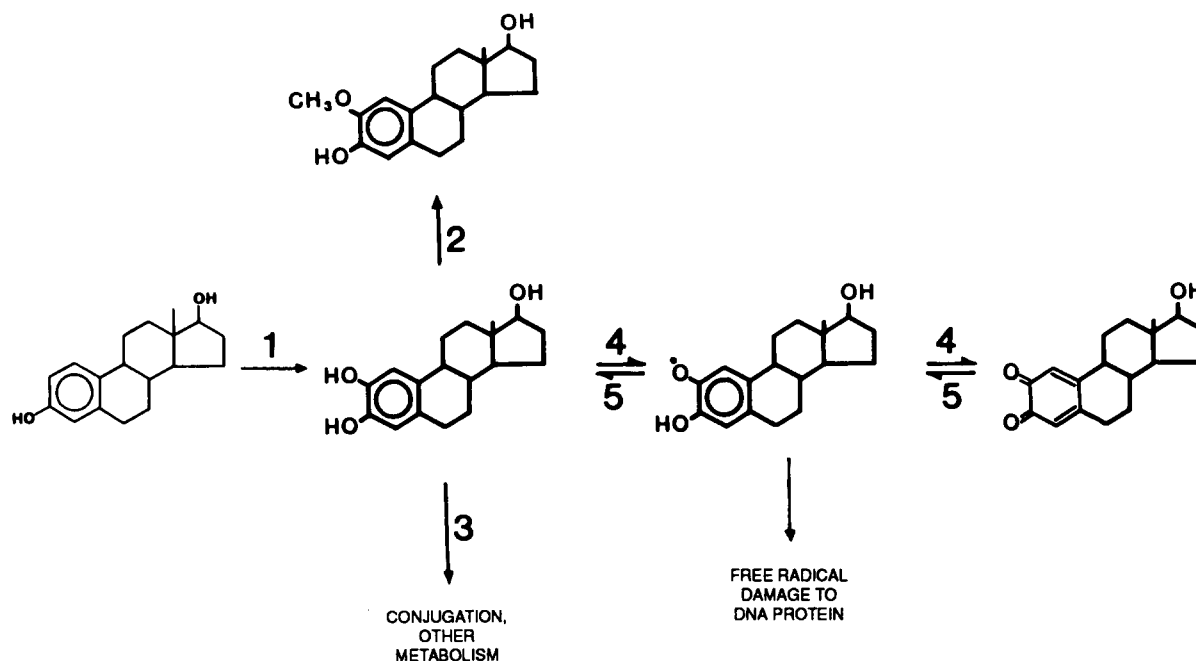
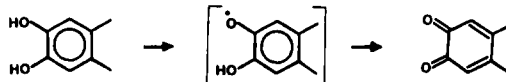
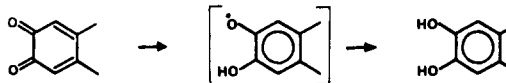


Fig. 3. Redox cycling of CE.  $E_2$  is oxidized to CE, that is, both 2-OH- $E_2$  and 4-OH- $E_2$  catalyzed by NADPH-dependent estrogen 2- or 4-hydroxylases<sup>42</sup> or by organic hydroperoxide-dependent estrogen 2/4-hydroxylase<sup>50</sup> (1). The oxidation of CE to their respective quinones is catalyzed by cytochrome P-450 oxidase<sup>24</sup> (4). Semiquinones are postulated intermediates in this oxidation. The reduction of estrogen quinones to their respective CE presumably via semiquinones is catalyzed by NADPH-dependent cytochrome P-450 reductase<sup>24</sup> (5). The semiquinone radicals are postulated to generate the protein damage observed in  $E_2$ -treated hamsters.<sup>53</sup> Defense mechanisms of the cell include removal of CE by methylation catalyzed by catechol-*O*-methyltransferase (2)<sup>41</sup> or by other conjugation reactions, such as glutathione, glucuronide, or sulfate formation (3).

Table 1. Metabolic Oxidation and Reduction Reactions of Estrogens

Reactions	Conditions	References
1. Oxidation 	A. Horseradish peroxidase, H <sub>2</sub> O <sub>2</sub> B. Tyrosinase, NADH/NADPH C. Cytochrome P450 oxidase, cumene hydroperoxide/H <sub>2</sub> O <sub>2</sub> or NADPH	11, 14-16 19 24, Roy and Liehr (unpublished)
2. Reduction 	D. Cytochrome P450 reductase, NADPH E. Cytochrome b <sub>5</sub> reductase, NADH* F. Quinone reductase, NADH/NADPH*	24 Roy and Liehr (unpublished) 27

\*This reaction has been demonstrated only for DES Q.

catalyzed by microsomal cytochrome P-450 oxidase was directly proportional to incubation time, concentrations of substrate, cumene hydroperoxide, and microsomal protein.<sup>25</sup> This reaction was inhibited by *N,N,N',N'*-tetramethyl-*p*-phenylenediamine, potassium cyanide, 2(3)-*t*-butyl-4-hydroxyanisole, or *n*-octylamine. These observations demonstrate that estrogens, that is, DES or CE metabolically formed from primary estrogens, may be oxidized to their respective quinones by various oxidizing enzymes. Some of these enzymes such as the cytochrome P-450 family are ubiquitous.

#### REDUCTION OF ESTROGEN QUINONES

For the operation of a complete redox cycle, the quinones formed by metabolic oxidation of DES or CE must be reduced to their respective hydroquinones. Quinones are reduced by several enzymes and also nonenzymatically by endogenous reducing agents.

##### Cytochrome P-450 reductase

NADPH-dependent cytochrome P-450 reductase catalyses the reduction by sequential one-electron transfer<sup>26</sup> of DES Q to both *Z*- and *E*-isomers of DES.<sup>24,27</sup> Although the *Z*-isomer is less stable than the *E*-form, *Z*-DES is the major product in this reduction. This stilbene may be used as marker product for the reduction of quinone to its respective hydroquinone. The reduction of DES Q catalyzed by NADPH-dependent cytochrome P-450 reductase, was dependent on time, concentrations of enzyme, and cofactor.<sup>24,27</sup> Cumene hydroperoxide can not be utilized by this enzyme as cofactor.<sup>27</sup> The reduction of DES Q was inhibited by adenosine 2'-monophosphate and by Cu(II) (3,5-diisopropylsalicylate)<sub>2</sub>, known inhibitors of this enzyme.<sup>28,29</sup>

##### NAD(P)H: quinone oxidoreductase

Unlike cytochrome P-450 reductase, NAD(P)H: quinone oxidoreductase (quinone reductase) is known to reduce quinones to their respective hydroquinones by two-electron transfer, thus bypassing formation of semiquinone free radicals.<sup>30</sup> In the presence of partially purified hamster liver quinone reductase and NADPH or NADH, DES Q is reduced to 65% *Z*-DES and 9% *E*-DES in a time-dependent fashion ( $K_m = 17.25 \mu\text{M}$ ).<sup>27</sup> The reduction is inhibited by dicumarol, a known specific inhibitor of this enzyme.<sup>31</sup>

##### Nonenzymatic reduction of quinones

The nonenzymatic reduction presumably via one electron transfer<sup>32</sup> of DES Q to both *E*- and *Z*-isomers of DES by vitamin C has been demonstrated under various conditions.<sup>33</sup> Uric acid, previously postulated as effective reductant in primates,<sup>34</sup> or vitamin E do not reduce this quinone.<sup>33,35</sup>

##### Biological significance of reduction

NADPH-dependent cytochrome P-450 reductase, quinone reductase, or vitamin C are major reducing systems for quinones but may only be part of a battery of enzymatic and nonenzymatic reducing capabilities of a cell. For instance, the reduction of DES Q to *Z*- and *E*-DES by hamster liver and kidney microsomes and NADH as a cofactor could only be inhibited partially by dicumarol, a specific inhibitor of quinone reductase. Since these microsomes have been shown to contain only very low quinone reductase activity, it has been postulated that other as yet unidentified enzymes are capable of reducing quinones to their respective hydroquinones. In other experiments, Roy and Liehr (unpublished data) have shown that cytochrome *b*<sub>5</sub> plus cytochrome *b*<sub>5</sub> reductase or xanthine and xan-

thine oxidase reduce DES Q to a mixture of *Z*- and *E*-DES. This great variety of reducing capabilities may afford ample protection to a cell against toxic effects of quinones. The covalent binding of CE to protein has been recognized for some time and has been postulated to proceed via metabolic oxidation of the CE to their respective quinones.<sup>36</sup> The genotoxicity of estrogen quinones has been detected only recently.<sup>37</sup> Covalent binding of estrogen quinones to protein and DNA is not discussed here, but is reviewed elsewhere.<sup>36,38</sup>

#### REDOX CYCLING OF DES OR CE

The redox cycling of estrogens has been demonstrated directly to occur between DES and DES Q, because marker products of oxidation and reduction had previously been identified for this stilbene estrogen<sup>24</sup> (Fig. 2). In the presence of hamster liver or kidney microsomes and NADPH, DES Q is reduced mainly to *Z*-DES by the cytochrome P-450 reductase component of this preparation.<sup>24,27</sup> Conversely, when *E*-DES is used as substrate and NADPH as cofactor, DES Q formation cannot be detected although *Z*-DES and *Z,Z*-dienestrol, the marker products of reduction and oxidation, respectively, accumulated in this preparation. It was concluded that cytochrome P-450 oxidase and NADPH-dependent cytochrome P-450 reductase maintain redox cycling between DES and DES Q. Some of the quinone spontaneously rearranges to *Z,Z*-dienestrol, which cannot be reduced by cytochrome P-450 reductase and thus accumulates with time. *Z,Z*-Dienestrol formation represents the exit from stilbene redox cycling.<sup>24</sup>

Redox cycling between 2-OH-E<sub>2</sub> and E<sub>2</sub> 2,3-quinone has also been demonstrated. Redox cycling of steroidal estrogens can only be monitored by spectrophotometric changes, since they do not appear to form marker products of oxidation or reduction.<sup>24</sup>

#### FORMATION OF FREE RADICALS

The formation of semiquinone free radicals by oxidation of CE has been demonstrated by ESR analysis<sup>14-16</sup> as discussed above. In addition, superoxide radicals are formed by redox cycling between DES Q and DES semiquinone catalyzed by hamster liver or kidney microsomes and NADPH.<sup>27</sup> Addition of DES Q to microsomal preparations and NADPH increased superoxide radical formation by 10 fold from base levels obtained without stilbene. The stimulation of superoxide radical formation was directly proportional to concentrations of DES Q, microsomal

protein, and NADPH, but was inhibited by addition of superoxide dismutase, quinone reductase, adenosine 2'-monophosphate, or Cu(II) (3,5-diisopropylsalicylate)<sub>2</sub>, inhibitors of cytochrome P-450 reductase.<sup>28,29</sup>

Dicumarol, an inhibitor of quinone reductase,<sup>31</sup> increased superoxide radical formation.<sup>27</sup> Superoxide radical formation has also been reported as a result of DES metabolism by horseradish peroxidase.<sup>39</sup> Using this enzymatic activation of DES, formation of 8-hydroxydeoxyguanosine *in vitro* has also been observed, which indicates damage to DNA by hydroxyl radicals.<sup>40</sup> Hydroxyl radical formation has also been detected by ESR analysis of DES metabolism catalyzed by mushroom tyrosinase.<sup>16</sup>

#### FREE RADICALS IN ESTROGEN-INDUCED CANCER

The occurrence of free radicals *in vivo* cannot be detected with present technology. Therefore, only indirect evidence for free radical action can be obtained by using marker products or reactions. The largest body of evidence for free radicals generated by estrogen exists for the hamster kidney tumor model. In this species, DES or E<sub>2</sub> induce kidney tumors in nearly all animals treated with hormone.<sup>3</sup> Estrogen-induced free radicals have been postulated to play a tumor-initiating role in this model.<sup>27,39</sup>

#### *Influence of estrogen treatment on CE formation*

CE are the substrates for redox metabolism and free radical formation by estrogen and therefore their formation in kidney of estrogen-treated hamsters must be discussed. Under normal physiological conditions, CE are rapidly methylated. These reactions decrease the CE reactivity with DNA and protein, that is, their toxicity. 2-OH-E<sub>2</sub> has been shown to inhibit the methylation of 4-OH-E<sub>2</sub>.<sup>41</sup> This inhibition assumes physiological significance in organs which generate 2-OH-E<sub>2</sub> and 4-OH-E<sub>2</sub> in comparable amounts such as the hamster kidney,<sup>42</sup> the mouse uterus,<sup>43</sup> or the rat pituitary.<sup>44</sup> These three organ systems are established models for estrogen-induced cancer.<sup>3,4,5</sup> Chronic E<sub>2</sub> treatment of hamsters decreases activity of renal estrogen 4-hydroxylase by only 25%, that of 2-hydroxylase by 75% of control values.<sup>42</sup> Thus, 4-OH-E<sub>2</sub> remains available as a major substrate for redox cycling in kidneys of hamsters treated chronically with estrogen. Rates of CE formation decreased also in livers of E<sub>2</sub>-treated hamsters, but not differentially as observed in kidney.

### *Influence of estrogen treatment on redox cycling*

As outlined above, the oxidation of CE or DES to corresponding quinones is catalyzed by heme peroxidase or cytochrome P-450 oxidase.  $E_2$  treatment is known to stimulate peroxidase activity in uterus and mammary tumor tissue.<sup>45,46</sup> In the hamster kidney, a target organ of estrogen-induced carcinogenesis,  $E_2$  treatment did not affect cytochrome P-450 oxidase, this activity increased from 317 in controls to 1025 pmol/nmol cytochrome P-450 after 2 months of  $E_2$  treatment.<sup>42</sup>

The activity of renal cytochrome P-450 reductase, which catalyzes the reduction of estrogen quinones to their corresponding hydroquinones, is not altered by treatment of hamsters with  $E_2$  for 2 months. However, when expressed in relation to specific content of cytochrome P-450, this activity increases 2-3-fold under  $E_2$  treatment conditions.<sup>42</sup>

Any effects of the changes in activities of cytochrome P-450 reductase and of cytochrome P-450 oxidase on quinone concentrations in vivo are not known. Concentrations of DES Q have been measured after injection of 20 mg/kg DES and have been found to be 76 and 26 pmol/g liver and kidney, respectively, of adult, hamster.<sup>25</sup> In neonates and fetus, concentrations were even less (0.026 and 0.047% of adult levels in fetal liver and kidney, respectively). The effect of estrogen treatment on in vivo quinone concentrations remain to be analyzed. These observations demonstrate that quinones are formed in vivo in low albeit detectable concentrations. Moreover, estrogen treatment of hamsters modulates the activities of enzymes catalyzing redox cycling.

### *Influence of estrogen treatment on detoxifying enzymes*

Quinone reductase (*DT*-diaphorase) activity, which reduces quinones to hydroquinones by two-electron transfer bypassing semiquinone free radicals,<sup>30,31</sup> is decreased by 80% from control levels in kidney of hamsters treated with  $E_2$  for 1 month.<sup>27</sup> Although this activity recovers upon prolonged treatment, a temporary loss of protection likely occurs from toxic effects of estrogen quinones and their redox cycling. The temporary decrease in quinone reductase activity is particularly pronounced in the kidney, the target of estrogen-induced carcinogenesis, but is less in liver, which is not a target for the disease.

Renal catalase activity in hamsters treated with  $E_2$  for 1 month decreased by 55% from controls.<sup>47</sup> After prolonged hormone treatment, this activity recovered to approximately 34% to 24% below control levels, but never reached the activity in untreated hamster

kidney. Estrogen treatment had no effect on superoxide dismutase activity. Glutathione metabolism is affected by chronic estrogen treatment of hamsters in two ways: i) The activity of glutathione peroxidase increased specifically in the kidney by 45–114% over control activities;<sup>47</sup> ii) total levels of reduced and oxidized glutathione were significantly increased over controls. These changes have been postulated to represent a part of the kidney cell defenses against chronic exposure to free radicals resulting from redox cycling of estrogens over prolonged time periods. Target organ-specific increases in glutathione peroxidatic activity and total glutathione levels in cardiac tissue of rats treated chronically with adriamycin have previously been taken as evidence for redox cycling of adriamycin and free radical toxicity specifically in this organ.<sup>48</sup>

### *Influence of estrogen treatment on lipid peroxidation*

Chronic  $E_2$  treatment of hamsters did not alter lipid peroxidation when assayed by reaction of thiobarbituric acid with malondialdehyde, a product of lipid peroxidation.<sup>47</sup> However, the more stable fluorescent products of lipid peroxidation increased 110% in kidney of hamsters treated with estrogen for 1 month over control values. The corresponding increase in liver, which is not a target of estrogen-induced cancer, was only 12% over controls. These results were taken as evidence for lipid peroxidation by free radicals generated specifically in kidneys of estrogen-treated hamsters. Failure to detect lipid peroxidation by thiobarbituric acid assay may have been due to difficulties inherent in this assay<sup>49</sup> or, more likely, to low steady-state concentrations of lipid peroxides in these kidneys. As shown in Figure 4, lipid peroxides are continually used as cofactors for cytochrome P-450-catalyzed oxidation of CE or DES to semiquinones and then to quinones.<sup>24</sup> Moreover, there are organic hydroperoxide-dependent estrogen 2/4-hydroxylases which catalyze the oxidation of estrogens to CE, the substrates for redox cycling.<sup>50</sup> As a result, redox cycling of estrogens is a self-maintaining process generating free radicals, which react to form lipid hydroperoxides,<sup>51</sup> which support redox cycling so long as there is a supply of steroid hormone.

### *Influence of estrogen treatment on protein and DNA*

There is a large number of reports on estrogens binding to DNA or proteins.<sup>38</sup> Free radicals may be involved in this binding. Since their participation has not been demonstrated unequivocally, such binding will not be reviewed here unless there is evidence of free radical action. Because of the high reactivity of

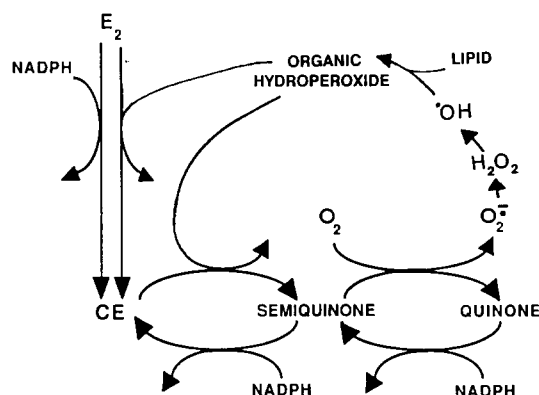


Fig. 4. Proposed maintenance of redox cycling of estrogens. Organic hydroperoxide (OHP) is required for the oxidation of  $E_2$  to CE catalyzed by the OHP-dependent estrogen 2/4-hydroxylase.<sup>50</sup> This process operates in addition to NADPH-dependent estrogen hydroxylation.<sup>42</sup> Moreover, CE is oxidized to their respective quinones catalyzed by cytochrome P-450 oxidase<sup>24</sup> (and D. Roy, H. W. Strobel & J. G. Liehr, unpublished data). A semiquinone is postulated to be an intermediate in these enzymatic oxidations. The estrogen quinones are reduced to hydroquinones by NADPH-dependent cytochrome P-450 reductase<sup>24</sup> presumably via a semiquinone.<sup>26</sup> Redox cycling is illustrated for CE, however, both DES and CE have been demonstrated to undergo this process.<sup>24</sup> Superoxide radicals formed by redox cycling of DES<sup>27</sup> and possibly of CE, react to form hydroxyl radicals, which start the lipid peroxidation process.<sup>51</sup> Estrogen treatment of hamsters has been shown to result in increased levels of lipid peroxidation.<sup>47</sup> These lipid peroxides are postulated to function as cofactors for enzyme dependent oxidations as outlined above.

the estrogen quinones, covalent estrogen-protein or estrogen-DNA addition products may be formed without involvement of free radical processes.

Free radical damage to proteins can be measured by sodium [ $^3H$ ]-borohydride reduction of carbonyl-containing amino acid residues of proteins.<sup>52</sup> Amino- and thiol-containing proteins are converted to carbonyl or thione moieties by free radical-mediated oxidation. This assay may be used as monitor of free radical generation *in vivo*, since it involves postlabeling methodology.<sup>53</sup> Protein isolated from kidneys of hamsters treated with  $E_2$  for 1 month incorporated 4 fold more  $^3H$  when reduced with sodium [ $^3H$ ]-borohydride than untreated controls. These results demonstrate that free radicals are generated in hamster kidney prior to estrogen-induced tumor development. Free radical damage appears to be greatest within the first 30 days of exposure to estrogen<sup>53</sup> in a time period when the enzymatic protection from free radical action is inhibited temporarily.

Free radical-mediated damage to DNA has also been postulated to occur specifically in kidney of hamsters treated with estrogen.<sup>54,55</sup> In this organ estrogen-induced indirect DNA modifications accumulate prior to malignancy.<sup>54,55</sup> These adducts do not contain estrogen but have been formed by addition to DNA of electro-

philic endogenous substances of as yet unknown structure. Because of their chromatographic mobilities, these estrogen-induced indirect adducts have been postulated to be formed by binding of lipids or lipid peroxides to DNA. An accumulation of 8-hydroxydeoxyguanosine, a marker of hydroxyl radical action, in DNA of hamsters treated with DES has also been observed (J. G. Liehr, D. Roy, & R. A. Floyd, unpublished data).

#### PHYSIOLOGICAL ROLE OF REDOX CYCLING OF ESTROGEN?

The oxidation of estrogens to CE and their participation in the formation of free radicals by redox cycling most likely is not a process unique to estrogen-induced carcinogenesis. As a matter of fact, almost all biochemical events occurring during carcinogenesis also function, at least at some time as necessary components of the normal physiological life cycle of a cell or organism. Unfortunately, much less is known about a physiological role of redox cycling of CE and free radical formation, because most investigators focus on disease states and their prevention.

One possible physiological function of redox cycling of CE and free radical generation is participation in induction of parturition. There is abundant evidence that prostaglandins are involved in parturition<sup>56</sup> and that hydrogen peroxide stimulates uterine contractions through a prostaglandin release mechanism.<sup>57</sup> This hydrogen peroxide induces chemiluminescence in uterine tissue.<sup>58</sup> Since hydrogen peroxide itself does not chemiluminesce, it has been concluded that other reactive oxygen species such as hydroxyl radical, are formed upon contact with the tissue and that they may mediate contractile activity via prostaglandin production.<sup>58</sup> These processes may be enhanced by stimulation of uterine prostaglandin synthase activity by CE.<sup>59,60</sup> In addition, the reactive oxygen species necessary for contractile activity may be generated by redox cycling of CE. Additional research is needed to clarify the physiological role of free radicals generated by estrogens.

#### CONCLUSION

The data demonstrate that  $E_2$  and the more potent synthetic estrogens exert their hormonal actions when present in low concentrations. At these levels, the metabolic conversions to CE or other intermediates do occur but may not be biologically deleterious. At elevated concentrations, formation of CE, their redox cycling and free radical generation may assume physiological or pathophysiological significance in organs containing elevated enzyme activity catalyzing CE for-

mation/activation coupled with decreased enzyme activity catalyzing CE detoxification. Such a spectrum of enzyme activities is induced in kidneys of hamsters treated with estrogen prior to the appearance of renal cancer. There appears to be a physiological role of free radicals by redox cycling of CE during parturition.

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#### ABBREVIATIONS

$E_2$ —17 $\beta$ -estradiol  
 DES—diethylstilbestrol  
 CE—catechol estrogen(s)  
 2-OH- $E_2$ —2-hydroxyestradiol  
 4-OH- $E_2$ —4-hydroxyestradiol  
 DES Q—diethylstilbestrol-4',4''-quinone