Biochemical Pharmacology, Vol. 30, pp. 319–324.

0006-2952/81/040319-06 $02.00/0

INFLUENCE OF OXYGEN ON THE INHIBITION OF LIVER MICROSOMAL ACTIVATION OF CARBON TETRACHLORIDE BY THE CATECHOL 2-HYDROXYESTRADIOL-17β

HEINZ KIECZKA and HERBERT REMMER
Institute of Toxicology, University of Tübingen, Germany
and
HERMANN KAPPUS*
Section on Pharmacology, Med. Institute of Environmental Hygiene, University of Düsseldorf, Germany

(*Received* 26 *June* 1980; *accepted* 3 *September* 1980)

Abstract—In NADPH-supplied rat liver microsomes irreversible protein and lipid binding of ^{14}C-CCl_4-metabolites continuously increase with decreasing oxygen concentrations. In aerobic suspensions of rat liver microsomes and NADPH the catechol steroid 2-hydroxyestradiol-17β effectively inhibits not only CCl_4-initiated lipid peroxidation but also protein and lipid binding of ^{14}C-CCl_4-metabolites. With decreasing oxygen concentrations the inhibitory potency of 2-hydroxyestradiol-17β on all three parameters connected with activation of CCl_4 is reduced. In order to obtain the same inhibitory effect more catechol compound is needed under low than under high oxygen tensions. Anacrobically, 2-hydroxyestradiol-17β was found not to inhibit irreversible protein and lipid binding, and lipid peroxidation was not detectable in absence of oxygen. An oxidized catechol metabolite rather than the catechol molecule itself may be responsible for the enzymatic inhibition of the activation step of CCl_4 or may interfere with reactions of the $\cdot CCl_3$-radicals formed.

Several compounds protect the organism from the deleterious action of toxic chemicals like carbon tetrachloride (CCl_4; for review see [1, 2]). It has been suggested [1–3] that some of these protective agents act by inhibiting the metabolic activation step of CCl_4 by the cytochrome P-450 enzyme system of the endoplasmic reticulum of cells, especially hepatocytes, because an interrelationship between metabolism and toxicity of CCl_4 has already been established [1–4]. However, the so called antioxidants are presumably additionally interfering with the CCl_4-induced lipid peroxidation reaction which could be the real mechanism responsible for CCl_4-toxicity.

Catechol compounds belong to this 'antioxidant' group and are potent inhibitors of CCl_4-induced lipid peroxidation reactions *in vitro* and *in vivo* [1, 2, 5–7]. But the real molecular mechanism by which these compounds prevent toxicity is unclear. Catechols are substrates of a NADPH-dependent enzyme system of the endoplasmic reticulum of the liver, presumably the cytochrome P-450 reductase [8–15]. Their oxidation by hepatic microsomes results in the formation of semiquinones or quinones and hydrogen peroxide [15], probably due to superoxide anion radicals [11–15]. In order to find out how catechol compounds could prevent CCl_4-induced hepatotoxicity we investigated whether their oxidation occurring in hepatic microsomes in the presence of NADPH and oxygen is responsible for their inhibitory effect on the metabolic activation of CCl_4.

* To whom correspondence and reprint requests should be sent: Institute of Environmental Hygiene, Gurlittstraße 53, D 4000 Düsseldorf 1, Germany.

We selected the irreversible protein and lipid binding of CCl_4-metabolites as well as the lipid peroxidation as parameters of CCl_4-activation. We used several oxygen concentrations during incubation. 2-Hydroxyestradiol-17β was selected as a model catechol compound, because it has been demonstrated in our previous work that it is a very potent inhibitor of hepatic lipid peroxidation induced by CCl_4 or $CBrCl_3$ [5, 6], its efficiency probably being due to the lipophilicity of the steroid ring [5].

We found that the inhibitory potency of 2-hydroxyestradiol-17β on irreversible protein and lipid binding as well as on lipid peroxidation depends on the actual concentration of oxygen in liver microsomes. This indicates that the oxygen concentration in the hepatocytes could influence the 'antioxidant' activity of catechol compounds and of other hydroquinone and quinone molecules.

MATERIALS AND METHODS

Materials. All chemical compounds including non-radioactive CCl_4 were of analytical grade and were obtained from Merck, Darmstadt, Germany. All biochemicals were of the purest grade available and were from Boehringer, Mannheim, Germany. ^{14}C-CCl_4 was purchased from Amersham Buchler, Braunschweig, Germany. Its specific radioactivity was 6.94 mCi/mmole, the purity being higher than 98 per cent. Unlabelled CCl_4 was mixed with the radioactive one to appropriate specific radioactivities.

2-Hydroxyestradiol-17β (Estra-1,3,5(10)-trien-2,3,17β-triol) was a gift from Schering, Berlin, Germany.

Microsomal incubations. Hepatic microsomes were freshly isolated according to standard procedures [5] from male Wistar rats of 180–220 g body weight which had free access to food (Altromin®) and tap water until the experiment. Microsomes (2 mg protein/ml) were incubated in KCl–Tris buffer with ^{14}C-CCl_4 (1 μl/ml; approx. 10 μmoles/ml) and with a NADPH-regenerating system in presence or absence of 2-hydroxyestradiol-17β as already described [5]. The special gas-tight incubation flask used during the present experiments which was comprised of 5 ml incubation fluid and 15 ml gas volume is described elsewhere in detail [16]. The methods of the incubations under different oxygen concentrations at 37° as well as the procedures to initiate and terminate the microsomal reactions are also given in this recent report [16].

Assays. Some samples withdrawn from the microsomal incubation were precipitated in ethanol (final concentration 70%), rehomogenized and washed with 70% ethanol four times (once with boiling 70% ethanol), similarly as described for the irreversible protein binding of imipramine [17]. The washed proteins were dissolved as described previously [17], and the irreversibly protein-bound radioactivity was counted by scintillation spectrophotometry [17].

The determination of irreversible binding of CCl_4-metabolites to lipids was identical to that described by Uehleke and co-workers [18, 19].

Malondialdehyde was estimated by the thiobarbituric acid method in the supernatant obtained after precipitation of the microsomal protein by trichloroacetic acid as described elsewhere [5].

Incubations were followed for 50 min. For every parameter measured at least 5 samples were withdrawn from the incubation mixture at different times. Control incubations were performed without NADPH. The rates of irreversible binding of CCl_4-metabolites or of MDA-formation were calculated from the linear phases of the time curves and expressed as nmoles CCl_4-metabolite irreversibly bound to protein and lipid or nmoles MDA formed per min. When experiments in the presence of the inhibitor 2-hydroxyestradiol-17β were performed, the different rates observed with different inhibitor concentrations were transformed into a Dixon-diagram as described previously [5]. From the Dixon-diagram obtained the inhibitor concentration (I_{50}) was calculated which inhibited the reaction to 50 per cent. The term I_{50} is used instead of the K_i-value which is not applicable to heterogenous enzyme systems [5].

RESULTS

Figure 1 shows how irreversible lipid and protein binding of CCl_4-metabolites formed by the action of hepatic microsomal enzymes depends on oxygen supply. As already pointed out [16] our system allows the examination of CCl_4-metabolism under defined conditions. The experimental design guarantees that constant amounts of CCl_4 are present in the microsomal suspensions as well as in the gaseous phases above [16]. No considerable changes of the oxygen concentrations present in the liquid phases have been observed, although during incubation some oxygen is used up by the microsomal activity [16]. With all oxygen concentrations applied the irreversible binding of CCl_4-metabolites both to proteins and lipids

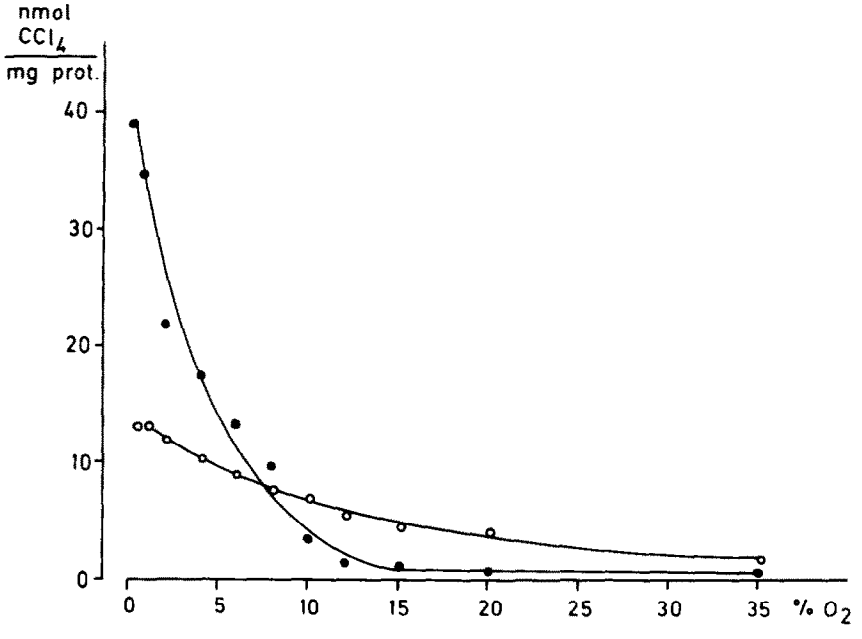

Fig. 1. Oxygen dependence of irreversible protein (○——○) and lipid (●——●) binding of CCl_4-metabolites after 50 min incubation with rat liver microsomes in the presence of NADPH. Irreversible binding to the total lipid isolated from microsomes containing 1 mg protein is given.

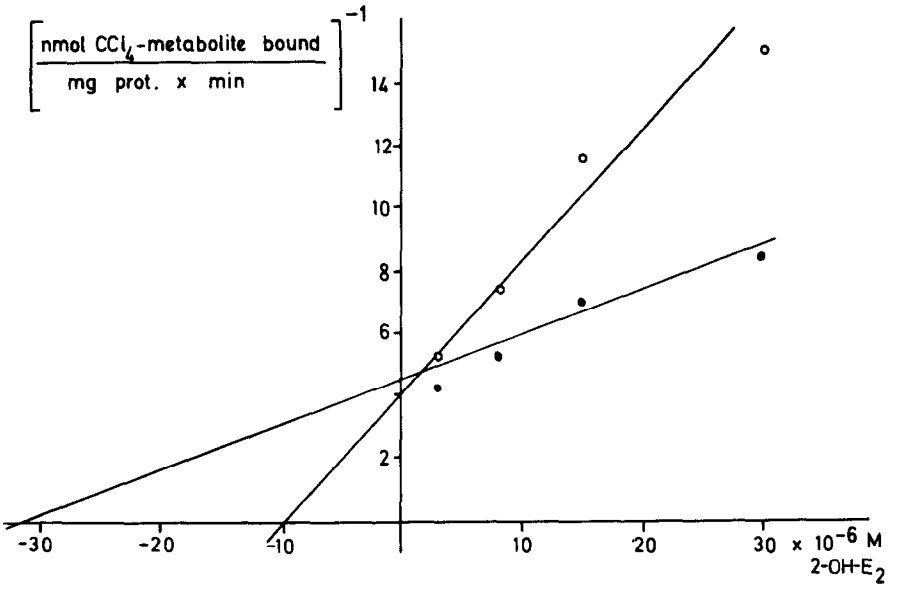

Fig. 2. Dixon-diagram of the inhibitory effect of 2-hydroxyestradiol-17β (2-OH-E_2) on irreversible binding of CCl_4-metabolites to proteins (●——●) and lipids (○——○) after incubation with rat liver microsomes in the presence of NADPH. Each point representing the rate of binding was obtained from the linear phase of a time curve with at least 5 points. Irreversible binding to the total lipid isolated from microsomes containing 1 mg protein is given. The diagram shows results from incubations in the presence of 4% oxygen and is representative for all other oxygen concentrations.

strongly depended on NADPH as evidenced by the lack of binding during incubations without NADPH (data not shown). This indicates that only metabolites of CCl_4 are irreversibly bound as already shown by others [19–21].

As expected from previous anaerobic studies [21–23] the irreversible binding increases continuously with decreasing oxygen concentrations (Fig. 1). This is not self-evident, because we recently found that microsomal CCl_4-induced malondialdehyde formation which was taken as a measure for lipid peroxidation showed an atypical oxygen dependency with a maximum at about 7% O_2 and a minimum at about 15% O_2 [16]. Binding of CCl_4-metabolites to the total lipid present in microsomes containing 1 mg protein is higher at low oxygen concentrations and slightly lower at high oxygen concentrations than irreversible binding to protein (Fig. 1). Because the protein/lipid ratio of our microsomes varied from 0.8 to 1.2, we related irreversible lipid binding to a certain amount of microsomal protein (Figs 1 and 2, Table 1). This seems reasonable as lipid binding of CCl_4 depends on its metabolism by enzymes which are present in definite amounts in the microsomal protein fraction.

Table 1 shows the results of irreversible protein and lipid binding of CCl_4-metabolites and of lipid peroxidation arising from microsomal incubations of CCl_4 with 2-hydroxyestradiol-17β under 0% and 10% oxygen. Binding to proteins and lipids and lipid

Table 1. Inhibitory effect of 2-hydroxyestradiol-17β (2-OH-E_2) on irreversible binding of CCl_4-metabolites to proteins and lipids and on MDA-formation after incubation with rat liver microsomes in the presence of NADPH, as influenced by the presence of oxygen*

	nmoles/mg microsomal protein/50 min			
	0% O_2		10% O_2	
	without	with 2-OH-E_2 (30 μM)	without	with 2-OH-E_2 (30 μM)
Irreversible binding of CCl_4-metabolites:				
To protein	13.1	12.9	7.5	3.7
To lipid†	38.9	39.4	3.4	i.a.
Lipid peroxidation:				
MDA-formation	i.a.	i.a.	28.0	i.a.

* The data are obtained from time curves with at least 5 points.

† Irreversible binding to the total lipid isolated from microsomes containing 1 mg protein is given. MDA = malondialdehyde. i.a. = insignificant amounts.

peroxidation are inhibited to 50–100 per cent by this catechol steroid in presence of 10% oxygen (Table 1) and also with higher oxygen concentrations (data not shown in Table 1), whereas at least binding is not influenced in absence of oxygen (Table 1, 0% O_2). Because lipid peroxidation is very low at 0% O_2 (Table 1), an inhibitory effect of 2-hydroxyestradiol-17β is not measurable.

To further evaluate the effect of oxygen on the inhibitory effect of 2-hydroxyestradiol-17β we carried out incubations with different oxygen and catechol steroid concentrations. The data obtained with 4% oxygen are shown in Fig. 2 which is a representative Dixon-diagram of irreversible binding of CCl_4-metabolites to proteins and lipids measured after incubation in the presence of different concentrations of the inhibitor 2-hydroxyestradiol-17β. These data clearly demonstrate that binding is inhibited by this catechol steroid and that it inhibits irreversible lipid binding to a higher extent than irreversible protein binding (Fig. 2, I_{50}-values of 10×10^{-6} M) compared to 32×10^{-6} M.

With 4% O_2 the potency of 2-hydroxyestradiol-17β on inhibition of lipid peroxidation as measured by malondialdehyde formation is in the range of the value obtained for irreversible lipid binding of CCl_4-metabolites (Fig. 3). Furthermore, Figure 3 demonstrates that in the presence of higher oxygen concentrations 2-hydroxyestradiol-17β is a good inhibitor of malondialdehyde formation and irreversible lipid binding of CCl_4-metabolites (I_{50}-values of 1–10×10^{-6} M), whereas it shows a weaker effect on irreversible protein binding. Concerning microsomal malondialdehyde formation induced by CCl_4 we have previously observed an I_{50}-value of about 0.9×10^{-6} M for 2-hydroxyestradiol-17β in the presence of 20% oxygen [5]. However, the exact inhibitory effect of 2-hydroxyestradiol-17β on irreversible protein and lipid binding could not be calculated with oxygen concentrations higher than 10 per cent (Fig. 3), because already the absolute amount of CCl_4-metabolites irreversibly bound during the uninhibited reaction was relatively low (see Fig. 1).

It can be seen from Fig. 3 that the inhibitory potency of 2-hydroxyestradiol-17β on malondialdehyde formation as well as on irreversible lipid and protein binding decreases continuously with decreasing oxygen concentrations. With oxygen concentrations lower than one per cent the I_{50}-values increased very much (Fig. 3) and could not be calculated from the data obtained during incubations with less than 0.2% oxygen. In the absence of oxygen the reactions were not inhibited even in the presence of relatively high concentrations of 2-hydroxyestradiol-17β (see Table 1).

DISCUSSION

Our experimental design guaranteed saturation of the microsomal enzymes with CCl_4 during the whole incubation [16]. Furthermore, the NADPH in the regenerating system used cannot become a rate-limiting factor because of the excess of this pyridine nucleotide. This is important because Slater and Sawyer had recently shown that the metabolic activation of CCl_4 can cause a loss of NADPH [24].

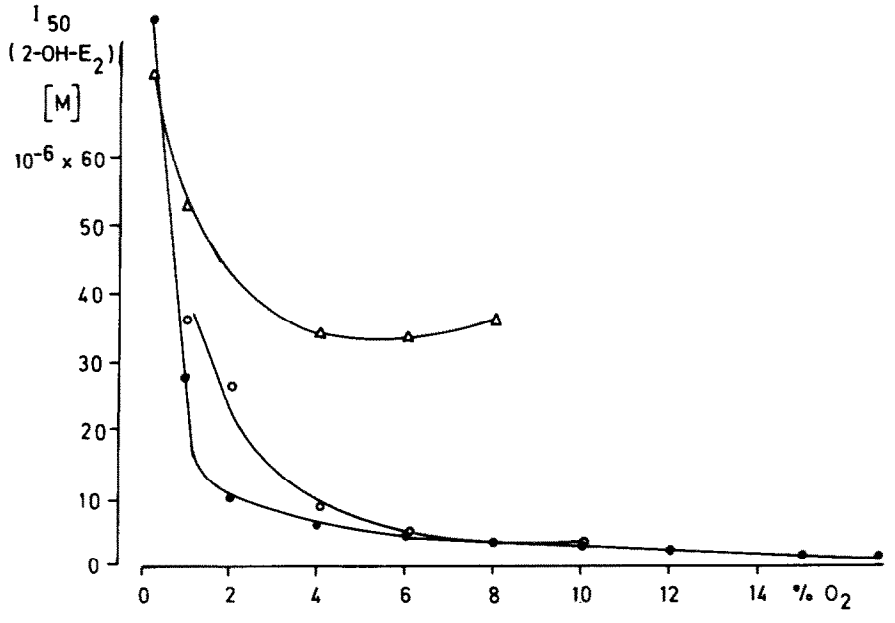

Fig. 3. Oxygen dependence of the inhibitory effect of 2-hydroxyestradiol-17β (2-OH-E_2) on irreversible binding of CCl_4-metabolites to proteins (△——△) and lipids (○——○) and on malondialdehyde formation (●——●) in rat liver microsomes incubated in the presence of NADPH. Each point representing the corresponding I_{50}-value was calculated from a separate Dixon-diagram with at least 4 concentrations of the inhibitor (2-OH-E_2) as described in Fig. 2 for the rate of irreversible binding or as described by Kappus *et al.* [5] for the rate of malondialdehyde formation. I_{50} (2-OH-E_2) implies the concentration of 2-hydroxyestradiol-17β which is necessary to inhibit the reaction to 50% under the present conditions.

probably due to irreversible binding of reactive CCl_4-metabolites to this molecule [40]. In addition, we were able to adjust the exact oxygen concentration in the microsomal mixture as controlled by an oxygen electrode [16]. With this system we measured the irreversible binding of CCl_4-metabolites to proteins and lipids over a wide concentration range of oxygen.

Although many other studies examined the irreversible binding in aerobic and anaerobic hepatic microsomes [4, 18–23], we report here for the first time a continuous increase of CCl_4-metabolites irreversibly bound to proteins and lipids with decreasing oxygen concentrations (Fig. 1). The curves obtained indicate that binding to lipids increases much more in presence of 10% oxygen and less, than binding to proteins.

The experiments on the inhibition of irreversible binding of CCl_4-metabolites to proteins and lipids clearly demonstrate that the catechol compound used is not only able to inhibit lipid peroxidation as has been shown previously [5], but can also efficiently inhibit this binding reaction in presence of oxygen (Table 1, Fig. 2). However, whereas the inhibitory potency (I_{50}) of 2-hydroxyestradiol-17β on lipid binding is in the range of the values observed on lipid peroxidation, the effect on protein binding is smaller (Fig. 3).

A number of possibilities have been discussed in order to explain the irreversible protein and lipid binding of CCl_4-metabolites and the lipid peroxidation occurring in presence of CCl_4 metabolism. Most likely $\cdot CCl_3$-radicals [1–4, 18–27] or CCl_2-radicals [29] are involved, although there is still no real proof for their formation [28]. Other new theories deal with the formation and reaction of Cl_3COOH [30, 31] or phosgene [32–35]. But both compounds cannot be formed anaerobically and are therefore not responsible for the irreversible protein and lipid binding in absence of oxygen.

It has been suggested by several authors [1–3, 18–23] that chemical compounds inhibit metabolic activation of CCl_4 to $\cdot CCl_3$-radicals, thereby modifying irreversible protein and lipid binding as well as lipid peroxidation. This cannot be the case in our experiments, because anaerobically the catechol compound does not inhibit the parameters measured, although CCl_4 is activated maximally. However, Slater and co-workers [2] discriminate between unspecific inhibitors and typical free radical scavengers, the latter group being also represented by 2-hydroxyestradiol-17β [5]. They conclude from their results that for inhibition of the activation step of CCl_4 to $\cdot CCl_3$-radicals 10–100 times the amount of chemical is necessary compared to the amount needed for inhibition of lipid peroxidation. They further suggest that free radical scavengers react directly either with the $\cdot CCl_3$-radical itself or with lipid radicals or lipid peroxides formed by the action of $\cdot CCl_3$-radicals.

Our data give no evidence in favour of this hypothesis for the following reasons: From the theoretical standpoint it can be assumed that the more $\cdot CCl_3$-radicals are formed the more free radical scavengers are necessary to trap them. This fact could simply explain our data on oxygen dependency of the inhibitory potency of 2-hydroxyestradiol-17β. But the formation of free radicals originating from CCl_4 increases only tenfold (Fig. 1), whereas the inhibitory potency of 2-hydroxyestradiol-17β is lowered to a much greater extent resulting in a total lack of inhibition in absence of oxygen (Table 1) where the $\cdot CCl_3$-radical formation is maximal and should be decreased by the catechol compound. The same facts argue against the interaction of the catechol molecule with lipid radicals, their amount being fully dependent on the amount of $\cdot CCl_3$-radicals.

On the other hand, the hypothesis that catechols interfere with CCl_4-induced lipid peroxides, as already suggested [1, 2, 5], is not favoured by the present findings because very low oxygen concentrations should give minor amounts of lipid peroxides [16], which could be easily trapped by small amounts of this scavenger. In contrast, in the presence of low oxygen concentrations more catechol compound is needed to get the same inhibitory effect on CCl_4-induced lipid peroxidation than in the presence of higher oxygen concentrations (Fig. 3).

From our data we suggest that in hepatic microsomes metabolizing CCl_4 catechols neither react directly with $\cdot CCl_3$-radicals nor interfere with lipid radicals or with lipid peroxides. Three mechanisms could explain the results obtained: (a) Previous studies have shown that phenols and catechols interfere with the microsomal electron transport chain of cytochrome P-450 leading to a so-called uncoupling of mixed function oxidase reaction [10–12]. This might occur by the reaction of the catechol with superoxide anion (O_2^-) formed in microsomes. During this reaction step hydrogen peroxide (H_2O_2) and the semiquinone of the catechol are formed under the conditions described [8–15], the latter being reduced to the parent catechol compound. This 'uncoupling' would withdraw electrons from the electron transport chain of cytochrome P-450 which then are not available for the reduction of CCl_4 to $\cdot CCl_3$. The inhibition of microsomal cytochrome P-450 reduction by 2-hydroxyestradiol-17β [12] and also by catecholamines [11] has been observed previously and has been ascribed to the action of catechols on cytochrome P-450 reductase. (b) During microsomal oxidation of catechols, e.g. 2-hydroxyestradiol-17β, reactive intermediates, probably the semiquinone or quinone molecules, are formed which bind irreversibly to proteins to a high extent [9, 36–39]. They could compete with $\cdot CCl_3$-radicals at the protein or lipid binding site, although lipid binding of catechols has not yet been investigated. This competition could only occur aerobically, fully explaining all of the results obtained, because minor amounts of catechol-metabolites are formed in the absence of oxygen [36–39]. (c) If it were not the catechol itself but only the semiquinone molecule which reacts with $\cdot CCl_3$-radicals, oxygen must be present to form the semiquinone, whereas anaerobic conditions would favour the reduction to the parent catechol by NADPH-dependent reductases [11–15]. Chloroform ($CHCl_3$) and the quinone compound would result from this reaction. However, the effect of catechol compounds on $CHCl_3$ formation has not yet been studied under different oxygen concentrations. Such an oxidation reduction cycle is very efficient in trapping free oxygen radicals and is quite similar to the reaction mechanism involved during

inhibition of lipid peroxidation induced by iron ions in hepatic microsomes. It differs from the mechanism suggested by Slater and co-workers [2] only to the extent that it is not the catechol itself but its oxidized metabolite which is the real radical scavenger.

Regardless of the mechanism responsible, our results indicate that oxygen concentration is an important factor during the inhibitory activity of catechols on irreversible protein and lipid binding as well as on lipid peroxidation. Low oxygen tension in cells *in vivo*, as observed in the centrolobular area of the liver where CCl_4-induced necrosis starts [1–4], might be responsible for minor inhibitory effects of some catechol compounds and of other free radical scavengers on metabolic effects of CCl_4. If toxicity is related to these parameters and if α-tocopherol would behave similarly, low oxygen concentrations concomitant with a low activity of this radical scavenger could contribute to the CCl_4-induced hepatotoxicity *in vivo*.

Acknowledgements—We thank the Deutsche Forschungsgemeinschaft, Bonn-Bad Godesberg, Germany, for financial support.

REFERENCES

1. G. L. Plaa and H. Witschi, *Ann. Rev. Pharmac. Tox.* **16**, 125 (1976).
2. T. F. Slater, in *Biochemical Mechanisms of Liver Injury* (Ed. T. F. Slater), p. 745. Academic Press, London (1978).
3. T. F. Slater, in *Oxygen Free Radicals and Tissue Damage* (Ciba Foundation Symp. 65), p. 143. Excerpta Medica, Amsterdam (1979).
4. E. S. Reynolds and M. T. Moslen, in *Free Radicals in Biology* Vol. IV (Ed. W. A. Pryor), p. 49. Academic Press, London (1980).
5. H. Kappus, H. Kieczka, M. Scheulen and H. Remmer, *Naunyn-Schmiedeberg's Arch. Pharmac.* **300**, 179 (1977).
6. H. Kappus, D. Köster-Albrecht and H. Remmer, *Archs. Tox.* Suppl. **2**, 321 (1979).
7. D. Köster-Albrecht, U. Köster, H. Kappus and H. Remmer, *Tox. Lett.* **3**, 363 (1979).
8. F. Marks and E. Hecker, *Hoppe-Seyler's Z. Physiol. Chem.* **349**, 523 (1968).
9. F. Marks and E. Hecker, *Hoppe-Seyler's Z. Physiol. Chem.* **350**, 69 (1969).
10. N. Oshino and R. Sato, *J. Biochem.* **69**, 169 (1971).
11. D. McKillop and G. Powis, *Biochem. J.* **158**, 135 (1976).
12. P. Wollenberg, M. Scheulen, H. M. Bolt, H. Kappus and H. Remmer, *Hoppe-Seyler's Z. Physiol. Chem.* **357**, 351 (1976).
13. H. M. Bolt and H. Kappus, *J. Steroid Biochem.* **7**, 311 (1976).
14. T. Uemura, E. Chiesara and D. Cova, *Molec. Pharmac.* **13**, 196 (1977).
15. J. B. Schenkman, I. Jansson, G. Powis and H. Kappus, *Molec. Pharmac.* **15**, 428 (1979).
16. H. Kieczka and H. Kappus, *Tox. Lett.* **5**, 191 (1980).
17. H. Kappus and H. Remmer, *Biochem. Pharmac.* **24**, 1079 (1975).
18. H. Uehleke, *Proc. Europ. Soc. for the Study of Drug Toxicity* XV, 119 (1973).
19. H. Uehleke and Th. Werner, *Archs. Tox.* **34**, 289 (1975).
20. M. C. Villarruel, M. I. Diaz Gomez and J. A. Castro, *Toxic. appl. Pharmac.* **33**, 106 (1975).
21. I. G. Sipes, G. Krishna and J. R. Gillette, *Life Sci.* **20**, 1541 (1977).
22. H. Uehleke, K. H. Hellmer and S. Tabarelli, *Xenobiotica* **3**, 1 (1973).
23. E. A. Glende, A. M. Hruszkewycz and R. O. Recknagel, *Biochem. Pharmac.* **25**, 2163 (1976).
24. T. F. Slater and B. C. Sawyer, *Chem.-Biol. Interact.* **16**, 359 (1977).
25. J. L. Poyer, R. A. Floyd, P. B. McCay, E. G. Janzen and E. R. Davis, *Biochim. biophys. Acta* **539**, 402 (1978).
26. A. Ingall, K. A. K. Lott, T. F. Slater, S. Finch and A. Stier, *Biochem. Soc. Transact.* **6**, 962 (1978).
27. E. K. Lai, P. B. McCay, T. Noguchi and K. L. Fong, *Biochem. Pharmac.* **28**, 2231 (1979).
28. B. Kalyanaraman, R. P. Mason, E. Perez-Reyes, C. F. Chignell, C. R. Wolf and R. M. Philpot, *Biochem. biophys. Res. Commun.* **89**, 1065 (1979).
29. C. R. Wolf, D. Mansuy, W. Nastainczyk, G. Deutschmann and V. Ullrich, *Molec. Pharmac.* **13**, 698 (1977).
30. E. H. Cheong and W. R. Bidlack, *Proc. West. Pharmac. Soc.* **20**, 97 (1977).
31. J. E. Packer, T. F. Slater and R. L. Willson, *Life Sci.* **23**, 2617 (1978).
32. H. Shah, S. P. Hartman and S. Weinhouse, *Cancer Res.* **39**, 3942 (1979).
33. D. Mansuy, P. Beaune, T. Cresteil, M. Lange and J. P. Leroux, *Biochem. biophys. Res. Commun.* **79**, 513 (1977).
34. L. R. Pohl, B. Bhooshan, N. F. Whittaker and G. Krishna, *Biochem. biophys. Res. Commun.* **79**, 648 (1977).
35. J. L. Stevens and M. W. Anders, *Biochem. Pharmac.* **28**, 3189 (1979).
36. M. Scheulen, P. Wollenberg, H. M. Bolt, H. Kappus and H. Remmer, *Biochem. biophys. Res. Commun.* **66**, 1396 (1975).
37. E. Dybing, S. D. Nelson, J. R. Mitchell, H. A. Sasame and J. R. Gillette, *Molec. Pharmac.* **12**, 911 (1976).
38. S. D. Nelson, J. R. Mitchell, E. Dybing, H. A. Sasame, *Biochem. biophys. Res. Commun.* **70**, 1157 (1976).
39. M. Scheulen, H. Kappus and H. M. Bolt, in *Microsomes and Drug Oxidations* (Ed. V. Ullrich), p. 661. Pergamon Press, Oxford (1977).
40. I. Harders, W. Kunz, H. Uehleke and B. Werner, *Naunyn-Schmiedeberg's Arch. Pharmac.* Suppl. **293**, R 65 (1976).