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THE INDUCTION OF DNA STRAND BREAKS AND FORMATION OF SEMIQUINONE RADICALS BY METABOLITES OF 2,4,5-TRICHLOROPHENOL

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The industrial pollutant 2,4,5-trichlorophenol (2,4,5-TCP) was metabolized with postmitochondrial liver fraction from Aroclor-1254 induced rats. The generated metabolites induced single strand breaks in PM2 DNA. Among the metabolites produced are the 3,4,6-trichlorocatechol (TCC) and the 2,5-dichlorohydroquinone (DCH), whereby the induction of DNA scission by DCH was approximately one hundred times greater than that of TCC. In the 2,4,5-TCP metabolization mixture radicals were observed by ESR. They were identified as the semiquinones of TCC and DCH. ESR studies confirmed that both TCC and DCH autoxidize in aqueous solution to their semiquinone radicals. The involvement of reactive oxygen species in the DNA strand scission was demonstrated by using DMSO, SOD, and catalase as scavengers. Inhibition of strand breaks with the scavenger enzymes did not give homogeneous results for DCH and TCC. This indicated that the directly damaging species might be different for DCH and TCC.

KEY WORDS: 2,4,5-Trichlorophenol, metabolic activation, 3,4,6-trichlorocatechol, 2,5-dichlorohydroquinone, semiquinone radicals, DNA strand breaks.

INTRODUCTION

2,4,5-Trichlorophenol (2,4,5-TCP) is an industrial product, which is mainly used as an intermediate in the manufacture of the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and its esters, and it remains as a major contaminant in these products. Small quantities of 2,4,5-TCP were also used in the past as a fungicide. Two case-control studies conducted in different parts of Sweden have pointed to a connection between soft-tissue sarcomas[1] or malignant lymphomas[2] and exposure to chlorophenols or 2,4,5-T. However, 2,4,5-TCP and 2,4,5-T used to be heavily contaminated with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), an experimental mutagen and carcinogen.[3] Animal studies using the pure 2,4,5-TCP have not been published. Therefore, a definitive conclusion about the carcinogenicity of 2,4,5-TCP is still lacking.

Mutagenicity testing of the purified 2,4,5-TCP gave negative results in the *Salmonella* test system (Ames test) which included metabolizing S-9 fractions.[4] However, many chlorinated chemicals have given negative responses in this test, including the structurally related experimental carcinogen 2,4,6-TCP.[4,5]

Previously we have shown, that 2,4,5-TCP forms seven *in vitro* metabolites.[6] Besides five dimerization products two were hydroxylated monomeric phenolic compounds.

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Of these, one was identified as the 3,4,6-trichlorocatechol (TCC), and a second one which had not been completely identified.[6] Identification of the latter compound is described here. The structurally related compounds pentachlorophenol and 2,4,6-trichlorophenol also form hydroxylated metabolites, either chlorinated catechols and/or chlorinated hydroquinones.[7,8,9] In the presence of oxygen these compounds spontaneously oxidize to quinones and form the semiquinone radicals as intermediates.[8,9] We have shown that during this process of autoxidation of the hydroquinones reactive oxygen species (ROS) were produced of which the hydroxyl radical (·OH) was found to be the causative agent of the observed DNA strand scission.[9,10]

In the present study we examined soluble fractions from metabolic activations of 2,4,5-TCP for induction of DNA scission in PM2 DNA. This DNA damage was compared to the DNA strand breaks produced by the synthesized monomeric phenolic metabolites. The formation of ROS and their involvement in the DNA strand breaking activity was investigated. Semiquinone radicals of 2,4,5-TCP metabolites were observed by ESR spectroscopy.

MATERIALS AND METHODS

Chemicals

2,4,5-TCP was purchased from EGA Chemie, F.R.G., and recrystallized from hexane. It was gas chromatographically free of chlorinated biphenyls, or diphenylethers, and dioxins. 3,4,6-TCC was synthesized from 2,4,5-TCP after Duff and Dakin[11] by Dr. J. Metzger of the Dept. of Chemistry, Universität Oldenburg. The structure of the synthesized compound was verified by ^{1}H-NMR and gas chromatography-mass spectrometry. 2,5-Dichlorohydroquinone (DCH) was purchased from Kodak Laboratories, Rochester, N.Y.. Catalase (from beef liver, spec. act. 6500 U/mg), glucose-6- phosphate, and PM2 DNA were obtained from Boehringer, Mannheim, F.R.G., NADP and superoxide dismutase (SOD) from bovine erythrocytes (spec. activity 3430 U/mg) were from Sigma.

S-9 Fraction

Noninduced S-9 fractions were prepared according to Ames[12] from livers of male Wistar rats, 8–10 weeks of age. Aroclor-1254 induced rat liver fractions were purchased from Organon Technika (Eppelheim, F.R.G.). Protein determinations were performed by the method of Lowry.

Metabolic Activation and Gas Chromatography

2,4,5-TCP was dissolved to a 1 mM solution in 0.1 M sodium phosphate buffer, pH 7.4, containing 8 mM $MgCl_2$ and 33 mM KCl. 4 mM NADP and 5 mM glucose-6-phosphate (final concentrations) were added as solids and dissolved. The final pH was 7.2. The incubations were started by adding S-9 fractions to a final protein concentration of 3 mg/ml, and by incubating at 37°C. The controls did not contain 2,4,5-TCP, or S-9 fraction. At several time intervals of incubation, samples of 0.5 ml were taken, and the protein precipitated with 2 vol. of icecold acetone and subsequent centrifugation at 10,000 × g. The supernatant was dried in a rotary evaporator. The residue

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was dissolved in 0.5 ml ethyl acetate. 0.2 ml of this solution were mixed with 50 μl of the pyrolytic ethylation reagent triethylsulfonium hydroxide (0.02 M) in methanol/water (70:30),[13] and products were gas chromatographically analyzed on a Varian 3700 GC instrument, equipped with an electron capture detector, and a 40 m × 0.25 mm SE 54 column (Macherey-Nagel, Dueren, F.R.G.). The GC oven program was: initial temperature 170°C, rate 5°C/min., final temperature 270°C, final time 10 min. Isothermic analysis at 150°C was used for quantitative determinations of DCH and TCC, because this gave better separation of these two peaks. Pentachlorophenol was used as an internal standard for gas chromatography.

Electron Spin Resonance Spectroscopy

ESR spectra were recorded at room temperature on a Bruker ER-200 D X-Band ESR spectrometer using a quartz flat cell in a TF_{102} standard resonator. Instrumental conditions are in the legend to Figure 1. The preparation of the samples was as follows: The metabolite containing residue after evaporation of supernatant was redissolved twice in ethyl acetate to assure complete removal of salt. 0.1 M sodium phosphate buffer, pH 7.8, was then added immediately prior to ESR observations. A 0.5 M stock solution of 3,4,6-TCC in methanol was diluted with 0.1 M sodium phosphate buffer, pH 7.2, and 7.8, to the desired concentrations. 1 M DCH was dissolved in DMSO/Cremophore EL® (1:1) and diluted with buffer, either pH 7.2, or pH 7.8.

Measurement of DNA Strand Breaks

After metabolic activation of 2,4,5-TCP the metabolite containing supernatant was prepared as before for ESR spectroscopy. For 1 ml supernatant fraction 400 μl 0.1 M sodium phosphate buffer, pH 7.2, was added to the residue after evaporation from ethyl acetate. Aliquots of 30 μl were immediately incubated with PM2 DNA (0.2 μg DNA per 40 μl sample).

1 mM TCC and 0.5 mM DCH were dissolved in 0.1 M sodium phosphate buffer, pH 7.2, diluted to the desired concentration, and incubated with PM2 DNA. To some samples catalase, SOD, or DMSO were added to give final concentrations as specified in results. All incubations with DNA proceeded at 37°C for 4 hours. The reactions were stopped on ice with a solution containing 20% DMSO, 0.25% bromophenol blue and 10% Ficoll in water.

Superhelical and nicked forms of PM2 DNA were separated by agarose gel electrophoresis (0.9% agarose, 80 V, 7 h). The DNA was made visible by staining with ethidium bromide (1 mg/l) and UV illumination. The gels were photographed with a Polaroid camera using an instant pack film No. 667. Photographs were densitometrically evaluated. Strand breaks N per PM2 DNA molecules were calculated from $N = -\ln \alpha$, where α is the fraction of superhelical DNA molecules.

RESULTS

ESR Spectroscopy

Figure 1a shows the ESR spectrum of a mixture of metabolites in phosphate buffer, pH 7.8, from an incubation of 2,4,5-TCP with Aroclor induced S-9 fraction. This

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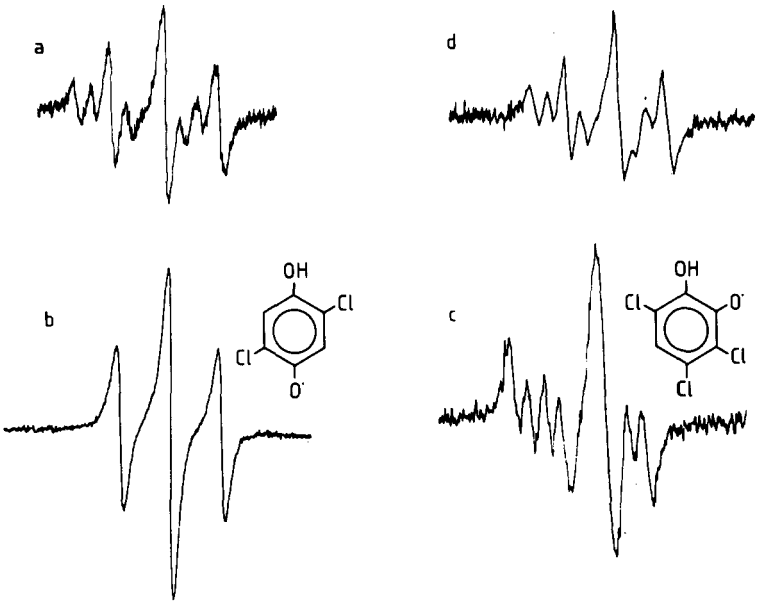

FIGURE 1 ESR spectrum of (a) a mixture of metabolites in phosphate buffer, pH 7.8, from an incubation of 2,4,5-TCP with Aroclor induced S-9 fraction; (b) DCH semiquinone radical; (c) TCC semiquinone radical; (d) a mixture of semiquinone radicals of DCH and TCC (concentration ratio: 10:1). All compounds were dissolved in 0.1 M phosphate buffer pH 7.8 and the ESR spectra were recorded with an X-band ESR spectrometer (Bruker ER 200 D) at $\nu = 9.5$ GHz and room temperature. Field sweep was 20 gauss and the sweep time 5 min.

spectrum is a superposition of two independent signals: a more intense three-line signal with an intensity ratio of 1:2:1 (hyperfine splitting $a_H = 1.9$ gauss), and a less intense eight-line signal with a splitting of 0.7 gauss and intensity ratios of approximately 3:2:2:3:3:2:2:3. The three-line signal is typical of two equivalent protons on a benzene ring.

In a previous report[6] a metabolite of 2,4,5-TCP was identified by GC-MS to be a dihydroxydichlorobenzene, but the positions of the two chlorine atoms and the OH-groups were not determined. We investigated by ESR spectroscopy, if the 2,5-dichlorobenzohydroquinone (DCH) would give a spectrum identical with that of the prominent three-line signal of the metabolite mix from the 2,4,5-TCP metabolization reaction. It was found, that the DCH semiquinone radical had a spectrum with three distinct lines of 1:2:1 intensities, a line width of 0.3 gauss and a hyperfine coupling constant of $a = 1.93$ gauss (Figure 1b). These data show very close agreement with the prominent signal pattern of Figure 1a, and with much confidence we can conclude that the metabolite is the DCH. The form of the spectrum of the dichlorobenzosemiquinone (DC semiquinone) and the splitting is not influenced by changing the pH value of the medium, whereas the intensity increases with increasing OH^- concentration from pH 7 to 8. The presence of 3,4,6-trichlorocatechol (TCC) in the mixture of metabolites was previously established. In a solution of TCC in 0.1 M phosphate buffer, pH 7.8, its semiquinone radical is formed. The ESR spectrum (Figure 1c) is a superposition of two independent signals: a one-line signal with a line width of $\leqslant 0.5$ gauss, whose intensity increases during the experiment, is superimposed asymmetrically to an eight-line signal, which itself consists of two groups of

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four lines having intensities of 3:2:2:3. The coupling constant within the group of four lines is 0.65 gauss. The centers of the two groups are 5 gauss apart. This signal was not observed at pH 7.2, at otherwise the same conditions. The 3,4,6-trichlorobenzosemiquinone (TC semiquinone) radical is similar to the less intense signal of the metabolic mixture.

This means, we could identify two radicals in the soluble fraction of a 2,4,5-TCP metabolization reaction, a relatively prominent signal of the semiquinone of DCH, and a less intense one of the TC semiquinone. Definite identification of the signals was accomplished by combining various concentrations of the two standard compounds. A ratio of 10:1 for DCH and TCC gave an almost identical signal to the one detected in the incubations (Figure 1d). The only difference is the asymmetrically superimposed line which was also observed in the ESR spectrum of the TC semiquinone. The 'artefact' is probably due to the formation of dimeric and higher polymeric radicals.[14]

The described signal in the aqueous solution of the metabolite mixture was observed at 30 minutes after starting the incubation of 2,4,5-TCP with Aroclor induced S-9 fraction, and up to 24 hours (final testing). The signal intensity increased over this time period. Incubations of 2,4,5-TCP with noninduced S-9 only yielded a faint signal after 4 to 5 hours of incubation, but the spectrum had identical characteristics as the one described for incubations with Aroclor induced S-9.

DNA Strand Breaks

The incubation of the metabolite mixture dissolved in 0.1 M sodium phosphate buffer, pH 7.2, with PM2 DNA resulted in single strand scission. Figure 2 shows that the

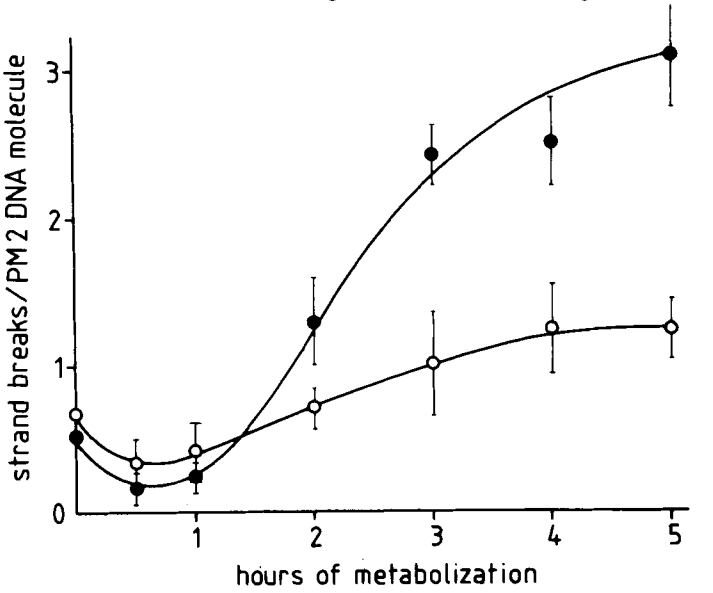

FIGURE 2 Single strand breaks in PM2 DNA as a function of metabolic activation time of 2,4,5-TCP. Incubation with DNA was 4 hr. Results are from two different experiments using the same Aroclor induced S-9 fraction (n = 3 to 8 data points in total).
● 2,4,5-TCP with Aroclor induced S-9 fraction
○ Aroclor induced S-9 fraction (control)

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number of breaks per PM2 DNA molecule increases with the time of metabolic activation. This corresponds with the increase of metabolites over the time period described. S-9 fractions (Figure 2, control) also produced single strand breaks (SSB's). These were inhibited by catalase, DMSO, and ethanol (unpublished results), and were most likely due to the production of ROS by some residual unknown factor of the S-9 fraction, which was soluble in ethyl ether, methanol, and phosphate buffer.

When S-9 fractions from noninduced rats were used for metabolic activation of 2,4,5-TCP, and subsequently incubated with PM2 DNA no strand scission was detected. This suggested that an enzyme inducible by Aroclor would be responsible for the production of a DNA damaging metabolite.

Incubations of PM2 DNA with the synthesized compounds, TCC and DCH, both resulted in DNA single strand breaks (Figures 3 and 4). However, the strand breaking activity differed greatly. While a 1 mM TCC solution produced 0.3breaks/PM2 DNA molecule, DCH formed the same number of breaks at a concentration a 100 times lower. A metabolite mixture of a 4-hour incubation including Aroclor induced S-9 contained approximately 0.2 mM TCC and 0.03 mM DCH (GC analysis). These data also correspond well with the ESR observations, where such a concentration ratio of TCC and DCH gave a spectrum comparable to the one of the metabolite mix (Figure 1a,1d). Therefore, we conclude that the DNA strand breaks produced by the metabolite mixture are probably to a large extent due to the DCH metabolite, although TCC is involved, too. We cannot totally exclude more DNA strand breaking species among the dimeric phenolic metabolites of 2,4,5-TCP.[6]

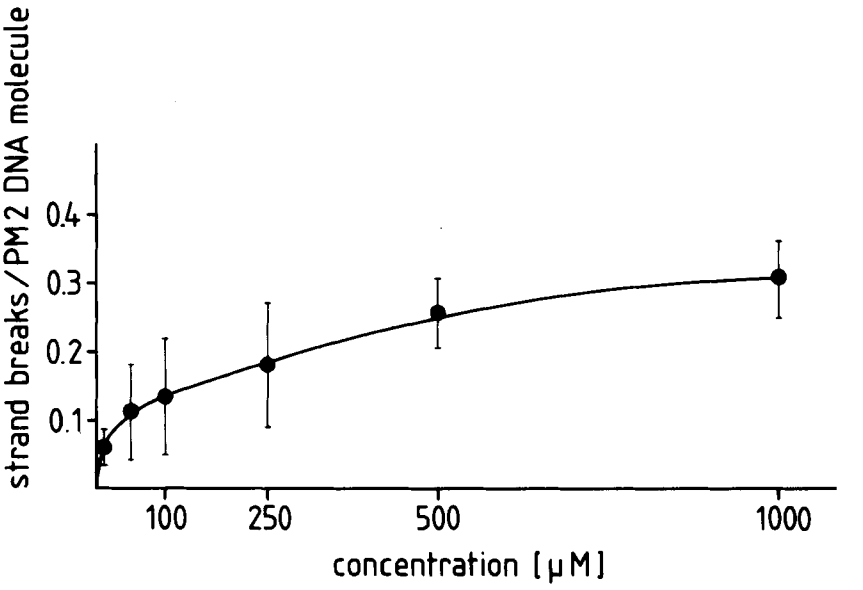

FIGURE 3 Single strand breaks induced in PM2 DNA as a function of 3,4,6-trichlorocatechol concentration. Incubation with DNA was 4 hr. Results indicate the mean ± SD of 3 to 5 experiments.

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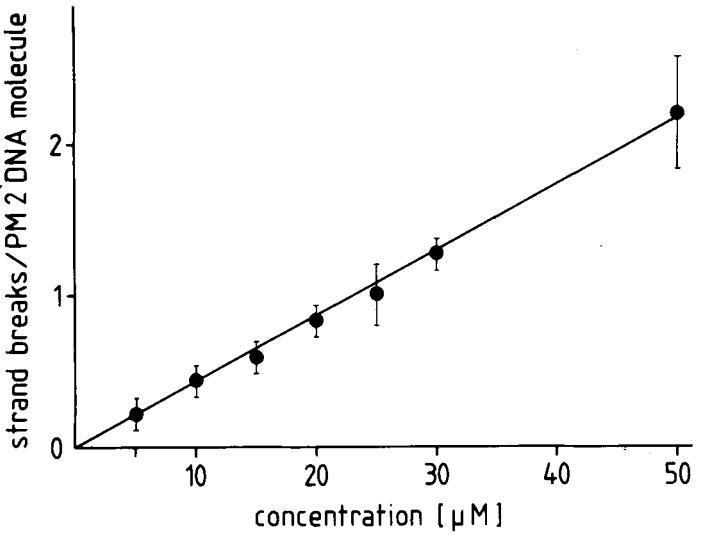

FIGURE 4 Single strand breaks induced in PM2 DNA as a function of 2,5-dichlorohydroquinone concentration. Incubation with DNA was 4 hr. Results indicate the mean ± SD of 3 experiments.

Scavenging of Oxygen Radicals

Semiquinone production in the presence of oxygen suggests the formation of superoxide and other reactive oxygen species (·OH, H_2O_2). Incubation of TCC, DCH, or the metabolite mixture with DNA in the presence of hydroxyl (·OH) radical quenchers, and ROS converting enzymes gave unexpected results (see Table 1). While the DNA single strand breaks due to DCH were totally abolished by catalase, this enzyme had only a slight effect on the inhibition of damage done by TCC. In contrast,

TABLE 1

Effect of DMSO, catalase and SOD on DNA strand breaks induced by metabolites of 2,4,5-TCP. PM2 DNA was treated for 4 hours in the presence of additives with the 'metabolite mix' TCC, or DCH in 0.1 M Na-phosphate buffer, pH 7.2, at 37°C. SSB's/PM 2 DNA molecule without additives: Metabolite mix: 1.96 ± 0.27 (6), TCC 1 mM: 0.31 ± 0.05 (18), DCH 0.03 mM: 1.72 ± 0.34 (8). Numbers indicate the mean ± SD; numbers in parentheses = n, nd = not determined.

Additive		Metabolite mix % Inhibition	TCC 1mM % Inhibition	DCH 0.03 mM % Inhibition of SSB's
DMSO	0.1%	82 ± 7 (4)	69 ± 13 (6)	93 ± 2 (6)
	1.0%	nd	91 ± 10 (6)	100 ± 2 (6)
	10.0%	100.5 ± 0.5 (5)	97 ± 3 (6)	99 ± 0.4 (6)
Catalase	50 μg/ml	nd	16 ± 6 (4)	99 ± 0.8 (6)
	100 μg/ml	85 ± 2 (4)	16 ± 10 (16)	103 ± 2 (6)
SOD	10 μg/ml	nd	74 ± 13 (6)	3.4 ± 0.8 (6)
	100 μg/ml	6 ± 11 (4)	94 ± 6 (6)	−3.6 ± 9.4 (6)

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TABLE 2
Effect of TCC on the catalase activity. Concentrations: 1 mM TCC, 0.05 mM DCH, 100 μg/ml catalase, 0.1 M sodium phosphate buffer, pH 7.2. Values are the mean ± SD; numbers in parentheses = *n*.

Incubation conditions	SSB's/PM 2 DNA molecule	% Inhibition of SSB's by catalase
TCC, DNA, 3 h	0.27 ± 0.02 (4)	
TCC, DNA, catalase, 3 hr	0.24 ± 0.01 (4)	12
DCH, DNA, 3 h	2.62 ± 0.37 (5)	
TCC, catalase, 1 hr, then DCH, DNA, 3 hr	0.42 ± 0.03 (11)	94[1]

[1]SSB's due to DCH

SOD was ineffective in reducing DCH strand scission, but was almost completely inhibitory for TCC damage. Incubation with 10% DMSO of TCC, DCH and the metabolite mix resulted in a complete inhibition of single strand breaks.

To test if the inability of catalase to inhibit TCC induced strand breaks was caused by an inhibitory effect of TCC on catalase, we incubated this compound with catalase for one hour, and subsequently added DCH and PM2 DNA for a three-hour incubation. The results are shown in Table 2. An 94% inhibition of strand breaks due to DCH indicates that the catalase is to a large extent intact after a pre-incubation with TCC.

DISCUSSION

Metabolites from incubations of 2,4,5-TCP with Aroclor induced S-9 fraction showed DNA single strand scission at physiological pH. Seven metabolites have previously been identified in the incubation mixture.[6] Of these, TCC and the here identified DCH were found to have strand breaking properties at pH 7.2. TCC is quantitatively the main metabolite in the incubation mixture, and its concentration is about ten times greater than that of the DCH. But because the concentration needed to initiate the same number of strand breaks is much lower for DCH, most of the strand scission by the metabolic mixture is presumably due to the DCH metabolite. This is substantiated by metabolic reactions of 2,4,5-TCP with noninduced S-9. Concentrates of these incubations do not show DNA strand breakage, although TCC is present at concentrations similar to those found in incubation mixes with Aroclor induced S-9. DCH, however, is only present in trace amounts after prolonged incubation times. These results could possibly explain, why no DNA damage was detected in liver and white blood cells from noninduced rats with the alkaline elution assay after oral dosage of animals with 2,4,5-TCP.[15] TCC has been detected as an *in vivo* metabolite of 2,4,5-TCP.[11] It was shown to be mutagenic in a mammalian cell (V79) mutagenicity assay.[16] But the damage produced by TCC might not be detectable by the relatively insensitive method of the alkaline elution assay. The semiquinone radicals of the TCC and the DCH were observed in aqueous solution in the presence of oxygen by ESR

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spectroscopy. Radical formation most likely occurs during a single electron transfer from the hydroquinone or catechol to molecular oxygen, which is reduced to the superoxide radical (O_2^-). It is generally assumed that catechols do not autoxidize at physiological pH.[17] At pH 7.2 we did not detect TC-semiquinone radicals by ESR, but DNA strand scission occurred at that pH. If these strand breaks are dependent on the autoxidation process, then this would mean, that our method for measuring DNA damage is more sensitive than the detection of semiquinone radicals by ESR.

We tested if ·OH radicals may be initiators of the DNA damage by using scavengers of ROS. Superoxide (O_2^-), which is produced during autoxidation, dismutates to O_2 and H_2O_2, which will then produce ·OH radicals by the Fenton reaction. The strand scission observed during DNA incubation with DCH was inhibited by catalase and DMSO, but SOD was not protective. These results are in accordance with the assumption that DCH dependent strand breaks are directly induced by ·OH radicals. Catalase removes H_2O_2 from the incubation and interferes in this way with the production of ·OH radicals by the Fenton reaction, so that no strand breaks will be observed. DMSO, an efficient ·OH radical scavenger, also gave protection. The inability of SOD to protect against DCH induced strand scission can be explained by the well known function of this enzyme to convert O_2^- to H_2O_2. The net amounts of H_2O_2 produced from O_2^- should be the same in an incubation mixture with or without SOD, if autoxidation of the hydroquinone has run to completion. Time kinetics studies of DCH dependent strand breaks have shown that there is no further increase of damage after four hours of incubation (data not displayed). Autoxidation is no longer occurring at this time. These above results agree with the assumption, that ·OH radicals must be the final DNA damaging species. We have previously shown that chlorinated hydroquinones, the 2,3,5,6-tetrachlorohydroquinone and the 2,6-dichlorohydroquinone, metabolites of pentachlorophenol and 2,4,6-trichlorophenol, respectively, also produce single strand breaks induced by ·OH radicals.[9,10]

The results of TCC cannot be explained on the basis of the cited enzyme functions. While SOD inhibited most of the strand breaks, catalase gave very little protection. Cadenas *et al*[18] have recently suggested that SOD also functions as an O_2^- semiquinone oxidoreductase. In a sequence of events O_2^- would reduce SOD-Cu^{++} to SOD-Cu^+, which again would be oxidized by a semiquinone radical with the formation of the hydroquinone. We have no evidence for such a reaction sequence in the case of TCC, but this function of SOD would certainly explain the inhibitory effect of SOD on the DNA strand scission. The low degree of protection by catalase suggests, that H_2O_2 and the production of ·OH radicals via the Fenton reaction does not play a major role in the DNA strand scission due to TCC. Very recently van Maanen *et al.*[19] reported an inactivation of ssΦX174 DNA by the semiquinone radical of the catechol metabolite of the antitumor agent etoposide. The O_2^- seemed to be involved in the formation of the semiquinone radicals, but ·OH radicals were found to be of no importance. It is possible that in our case the catechol TCC also produces damage via the semiquinone. The strong inhibition of strand scission by SOD shows that O_2^- anions are also of major importance here. We conclude that DCH and TCC autoxidize at physiological pH and produce O_2^-, but the mechanism for the production of the damaging species, or the damaging species itself must be different for both compounds.

Semiquinone radicals are reactive intermediates, which have been suggested to react with and bind to cellular macromolecules.[8,20,21] ROS have been implicated in the initiation and progression of a variety of human diseases,[22] but also in the promotion-

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al process of carcinogenesis[22,23,24]. Boutwell *et al.*[25] found 2,4,5-TCP to be a promoter of skin carcinogenesis, but his experimentation did not include an evaluation on the TCDD content. In view of our results and the importance of 2,4,5-TCP as an industrial pollutant, a reevaluation of this compound as a promoter of carcinogenesis seems to be necessary.

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