Prediction of Hydroxylated Metabolites in Polychlorodibenzo-*p*-dioxins and Polychlorodibenzofurans by Hückel Molecular Orbital Calculations

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Hydroxylation either by mono- or di-oxygenases is the most common initial step in the metabolism of aromatic xenobiotic compounds by mammals and micro-organisms. From the observed metabolic patterns for polychlorodibenzo-*p*-dioxins (PCDD) and polychlorodibenzofurans (PCDF) it was shown that HMO calculations can predict the hydroxylated products derived from these compounds. The resistance of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and similarly substituted PCDDs towards enzymatic attack can be explained by the predicted lack of reactivity at positions 1, 4, 6, and 9 of the dibenzo-*p*-dioxin ring system. Influence of variations in the carbon skeleton and change in chlorine substitution on the level of HOMO energies are correlated with measured absorption of charge transfer complexes. Charge transfer properties of PCDD and PCDF probably play no role in difference of toxicities of various isomers.

Regioselectivity in the Oxidation of Aromatic Systems calculated by the PMO Approach.—In various fields of organic chemistry perturbation of molecular orbitals (PMO) methods have been applied with success in explaining stereo- and regio-selectivity. In particular the frontier molecular orbital (FMO) approach was successful in the study of $2\pi + 4\pi$ cycloadditions [1] and in a number of other fields. Higher organisms (*e.g.* mammals) epoxidize aromatic hydrocarbons by a mono-oxygenase enzyme. This intermediate epoxide gives rise to polar metabolites which are more rapidly excreted than the original compound. Hydroxylation *via* epoxides can also be the first step in the microbial breakdown of aromatic compounds (mainly fungi), though hydroxylation *via* enzymes called dioxygenases is the common mechanism for bacteria. This enzyme incorporates both oxygen atoms of one O_2 molecule into the substrate. Both reactions and mechanisms are shown schematically in Figures 1 and 2. This suggests that the ease and regioselectivity of the oxidation of aromatic compounds by mammals and micro-organisms should be predictable by the PMO approach. In the electrophilic attack of oxygen, only the occupied orbitals of the aromatic system or even only the highest occupied molecular orbital (HOMO; FMO approach) have to be considered. However, because of the possibility of a reaction being controlled by the underlying (subjacent) orbitals, it seems more justified to include these subjacent orbitals and to use the complete set of occupied orbitals. For this reason all occupied orbitals were considered in this study.

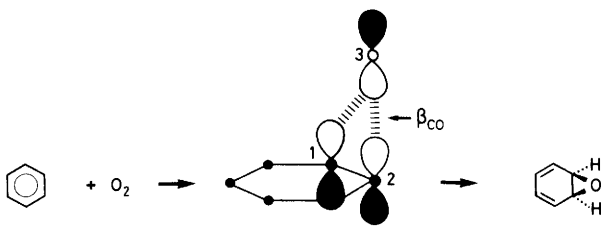

Figure 1. Orbital interactions in the mono-oxygenase reaction

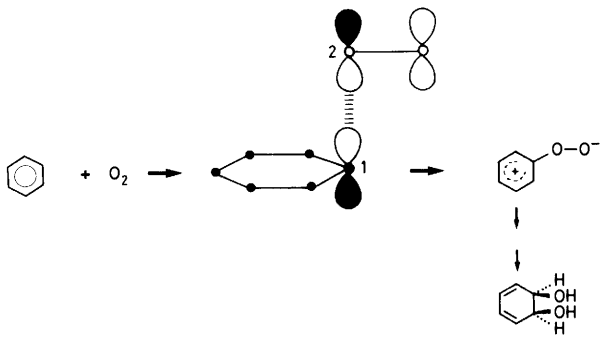

Figure 2. Orbital interactions in the dioxygenase reaction

Mono-oxygenases.—The formation of an epoxide (Figure 1) is a cheletropic cycloaddition and therefore the MO treatment of this reaction is basically the same as the treatment of all cycloadditions.[2] The epoxide is unstable and yields several products.[3] The occurrence of the *trans*-dihydrodiol and mercapturic acids in urine or culture medium is strong evidence for an intermediate aryl epoxide [3] while the catechols and phenols do not clearly indicate whether the initial attack had taken place by a mono- or a di-oxygenase, except when the formation of a phenol occurs simultaneously with an NIH shift. The ring opening appears to be under electronic control. The work of Kaubish *et al.*[4] led to a simple rule for predicting the ring opening in unsymmetrical epoxides. It was stated that of the two possible intermediates the one having the greatest number of tertiary carbenium ions in all mesomeric structures will predominantly be formed. Thus both 1,2- and 2,3-epoxytoluene will yield *o*-cresol while from 3,4-epoxytoluene only *p*-cresol will be formed (Figure 3).

Dioxygenases.—In lower organisms a second mode of attacking the π-system of aromatic compounds was described by Gibson *et al.*[5] Here, the responsible enzyme, a dioxygenase, incorporates both atoms of an O_2 molecule into the substrate yielding a *cis*-dihydrodiol. Several authors have proposed the mechanism involving a dioxetan intermediate. According to the Woodward–Hoffman rules, however, a $2\pi + 2\pi$ cycloaddition is thermally not allowed. A multistep mechanism is therefore more acceptable. Reineke and Knackmuss [6] have proposed a two-step mechanism of which the first step is shown in Figure 4. Presently, there is not enough evidence to distinguish between the zwitterion and the biradical intermediate and some data indicate that both mechanisms

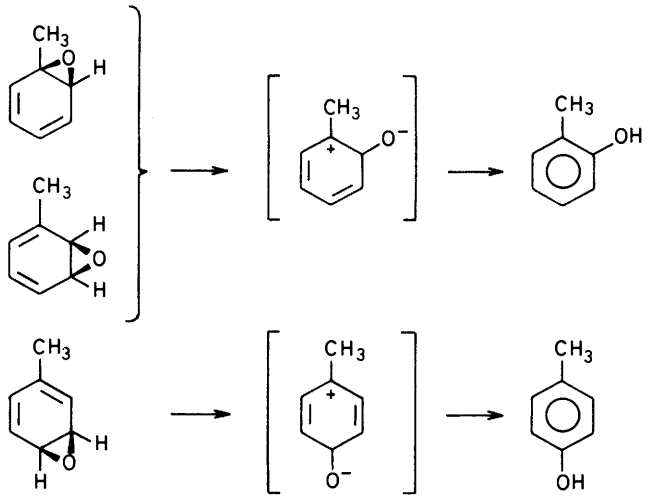

Figure 3. Prefential epoxide ring cleavage

may be operating. During the hydroxylation of benzoic acids by *Pseudomonas* and *Alcaligens* species for instance the application of the Hammett equation suggests the occurrence of a biradical,[6] while in the degradation of catechols, a charged intermediate seems more likely.[7] In both intermediates the π-system is separated from the terminal O-atom. It seems appropriate, therefore, to treat the subsequent reactions either as radical-radical, or anion-cation reactions.[5] In both cases the orbital-controlled part of the reaction is dominated by the same set of orbitals (The SOMO in the first case is the same orbital as the LUMO in the latter case.) In the heteropolar mechanism, the occupied orbital of oxygen interacts with the LUMO of the π-system while in the biradical mechanism the SOMOs of both the O-atom and the π-system give the main interaction. For the zwitterion the charge of both adjacent carbon atoms should also be taken into account since the reaction can be charge-controlled. Therefore, our theoretical approach to the dioxygenation of an aromatic system is considered in two steps, (1) the perturbation energy originating from the interaction of one carbon atom with one oxygen atom; (2) the subsequent interaction of the terminal oxygen atom with one of the adjacent carbon atoms.

Charge-transfer Complexing.—Measurements of the λ_{CT} of charge-transfer complexes will give information about the relative values of the energy levels of the interacting orbitals. This provides an independent check on the reliability of the calculated values of the energy values used in the perturbation expression (1). In predicting the ability of forming a charge-transfer complex of this type of compound with either electron donors or acceptors the energy levels of the HOMO and LUMO must be considered in comparison with those of electron donors and acceptors instead of taking the total valence shell interaction in account.[8,9] Charge-transfer complexing properties have also been used to explain cytosol binding capacity of PCDDs and PCDFs.[9,10] In these studies PCDD and PCDF were assumed to be electron acceptors which is rather unlikely as shown in our results.

The Second-order Perturbation Energy.—The second-order perturbation energy (ΔE) resulting from overlap between 1, 2, and 3 or 1 and 2 (Figures 1 and 2) of the π-orbitals of carbon and one *p*-orbital of oxygen in the early stage of the reaction is dominated by the interaction between the occupied

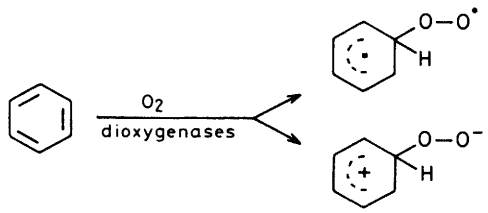

Figure 4. First step in dioxygenation

Table 1. Calculated energy levels of DD, DF, PCDD, and PCDF *

Compound	E_{HOMO}/β	E_{sHOMO}/β	E_{LUMO}/β	λ_{CT}/nm
DD	0.543	0.844	−1.013	635/423
1-MCDD	0.540	0.818	−1.016	608/425
2-MCDD	0.534	0.834	−1.020	620/416
2,3-DCDD	0.523	0.828	−1.028	585/400
2,7-DCDD	0.525	0.825	−1.028	590/405
1,2,4-tCDD	0.527	0.775	−1.025	555/420
1,2,3,4-TCDD	0.516	0.766	−1.033	578/—
OCDD	0.495	0.730	−1.053	n.d.
DF	0.699	0.783	−0.769	497
2-MCDF	0.693	0.766	−0.773	488
2,3-DCDF	0.673	0.762	−0.781	475
2,8-DCDF	0.686	0.752	−0.778	468

* DF = dibenzofuran, DD = dibenzo-*p*-dioxin, M = mono, D = di, t = tri, T = tetra, H = hexa, and O = octa. N.d. = not determined due to poor solubility.

orbitals of the aromatic system the lowest unoccupied orbital (LUMO) of oxygen, thus leading to expression (1) where β

$$E = \sum_n \frac{2\beta_n^2}{E_n - E_{LUMO}} \quad (1)$$

denotes the resonance integral between the LUMO of oxygen and the *n*th occupied MO of the substrate. In Figure 1 $\beta_n^2 = (C_{n,1} + C_{n,2})^2\ (1/2)^2\ \beta_{CO}^2$. In Figure 2 $\beta_n^2 = (C_n)^2\ (1/2)^2\ \beta_{CO}^2$. $C_{(n,j)}$ denotes the coefficient at atom *j* in the *n*th MO. These ΔE values are used to calculate the k_1/k_2 ratios for regioselective attack, *via* equation (2). k_n denotes the

$$k_1/k_2 = e^{-(\Delta E_1 - \Delta E_2)/RT} \quad (2)$$

relative rate constant for the attack on the *n*th position in a molecule. (For example, in Figure 5 there are three possible reactions.)

Results and Discussion

Charge-transfer Complexes.—The relative HOMO energies (and if possible of the subjacent orbital) of a series of ' related ' molecules can be determined by measuring the absorption maxima of their charge-transfer complexes with a single electron acceptor. In this study tetracyanoethylene (TCNE) was used as an electron acceptor. The results are summarized in Table 1. Although this simple HMO calculation method cannot predict the influence of the chlorine substitution pattern on the energy levels, this does not influence the validity of the perturbation energy calculations. The differences between the energy levels of the HOMO and the sHOMO on the one hand and the difference between dioxins and dibenzofurans on the other are of more importance in the calculations [*i.e.* E_n in equation (1)]. We define sHOMO as the first underlying occupied orbital under the HOMO.

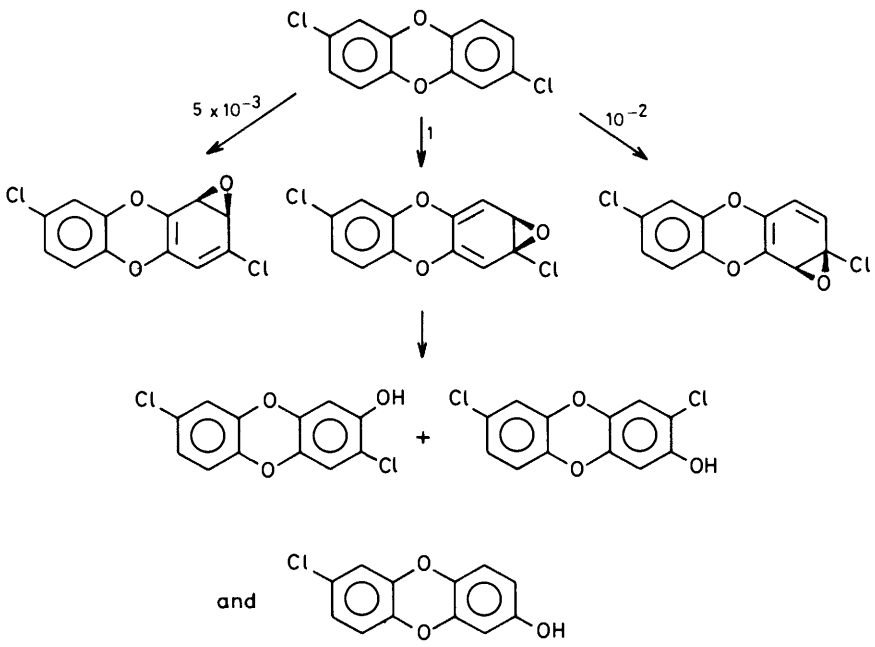

Figure 5. Metabolites of 2,7-DCDD. Relative rate based on β_{CO} 23 and β_{CC} 78 kcal mol^{-1}

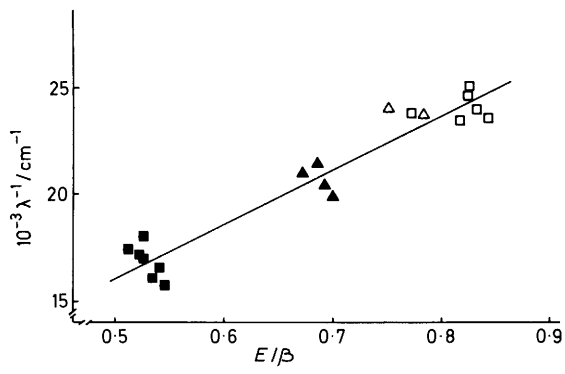

Figure 6. Correlation between E_{HOMO} (β) and $1/\lambda$: ■ HOMO of dioxins; ▲ HOMO of dibenzofurans; □ sHOMO of dioxins; △ sHOMO of dibenzofurans

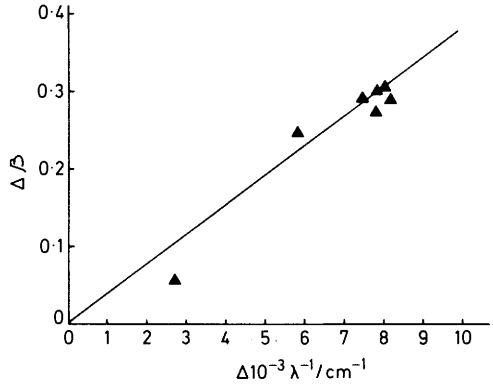

Figure 7. Correlation between Δβ and $\Delta(1/\lambda)$ for a number of dioxins and dibenzofurans

Figure 6 shows that these differences could be predicted rather well from the measured data. Attempts to synthesize charge-transfer complexes with a number of electron donors and DD or PCDD failed. This and the supporting evidence given by Shine *et al.*[11] (who succeeded in synthesizing $DD^{+}\cdot ClO_4^{-}$) can only lead to the conclusion that these types of compounds are, in fact, good electron donors, contrary to earlier statements.[8,9] We were also unable to establish a correlation between the π-electron densities and toxicological data of these compounds, thus making it likely that charge-transfer complexes do not play an important role in explaining the relative toxicity of either PCDD or PCDF.

Calculation of β.—The linear correlation of E_{HOMO} (β units) and $1/\lambda_{CT}$ allows a quantitation of the value of β if it is assumed [12] that the slope of this correlation should be close to unity. This leads to a relative β_{CC} of 84 ± 10 kcal mol^{-1} (Figure 6). In addition it is possible to quantitate β from the measured differences in the charge-transfer bands for the HOMO and the sHOMO. In Figure 7 ΔE (in β units) *versus* $\Delta 1/\lambda_{CT}$ is plotted. A straight line was obtained *via* the least squares method. These data were obtained from measurements of the lower chlorinated dibenzo-*p*-dioxins. This leads to a relative β_{CC} of 78 ± 10 kcal mol^{-1}. In the calculation of the perturbation energy [equation (1)] β_{CC} is used. Evaluation of the carbon–oxygen resonance integral in the early stage of the reaction has been done with considerable success, in the reaction of singlet oxygen with mono- and di-methylnaphthalenes.[12] An optimum value of $\beta_{CO} = 0.29\beta_{CC}$, thus leading to β_{CO} of 23 kcal mol^{-1}.[12] This value was used in the calculation of the k_1/k_2 ratios given in Figures 5 and 8.

The Application of the PMO Approach to Mono-oxygenases.—Rat metabolism of DD and PCDD on the one hand and DF and PCDF on the other show some remarkable differences. For DD and PCDD [13] hydroxylation took place only in the 2- and 3-positions, whereas in the case of the DF and PCDF a variety of products was found.[14] In Figure 9 the structure and numbering of DD and DF are given. The preferential attack on both substrates can be calculated with the equation (1) for the perturbation energies. The results are given in Tables 2 and 3. The predicted attack for both DD

Table 2. Perturbation energy for mono-oxygenase reaction ($\Delta E/\beta^2_{CO}$) * of polychlorodibenzo-*p*-dioxins

Number of chlorines	Chlorine substitution pattern	C-1, C-2	C-2, C-3	C-3, C-4	C-6, C-7	C-7, C-8	C-8, C-9
0		1.778	2.157	1.778	1.778	2.157	1.778
1	1	1.857	2.129	1.807	1.776	2.121	1.779
	2	1.805	2.203	1.724	1.784	2.114	1.779
2	1,2	1.862	2.137	1.824	1.787	2.067	1.747
	1,3	1.876	2.185	1.774	1.736	2.070	1.789
	1,4	1.891	2.152	1.891	1.778	2.123	1.778
	2,3	1.818	2.309	1.818	1.621	2.133	1.621
	2,7	1.716	2.192	1.792	1.716	2.192	1.792
3	1,2,4	1.815	2.222	1.916	1.788	2.103	1.711
4	1,2,3,4	1.939	2.358	1.939	1.618	2.142	1.618
	1,2,3,7	1.911	2.312	1.857	1.692	2.223	1.774
	1,2,3,8	1.908	2.328	1.864	1.741	2.222	1.699
	1,3,6,9	1.877	2.226	1.741	1.768	2.152	1.902
	1,4,6,9	1.890	2.161	1.890	1.890	2.161	1.890
	2,3,7,8	1.834	2.319	1.834	1.834	2.319	1.834
6	1,2,3,4,6,7	1.958	2.322	1.824	1.884	2.171	1.843
8		1.964	2.382	1.964	1.964	2.382	1.964

* In units of $1/\beta_{CC}$. See equation (1).

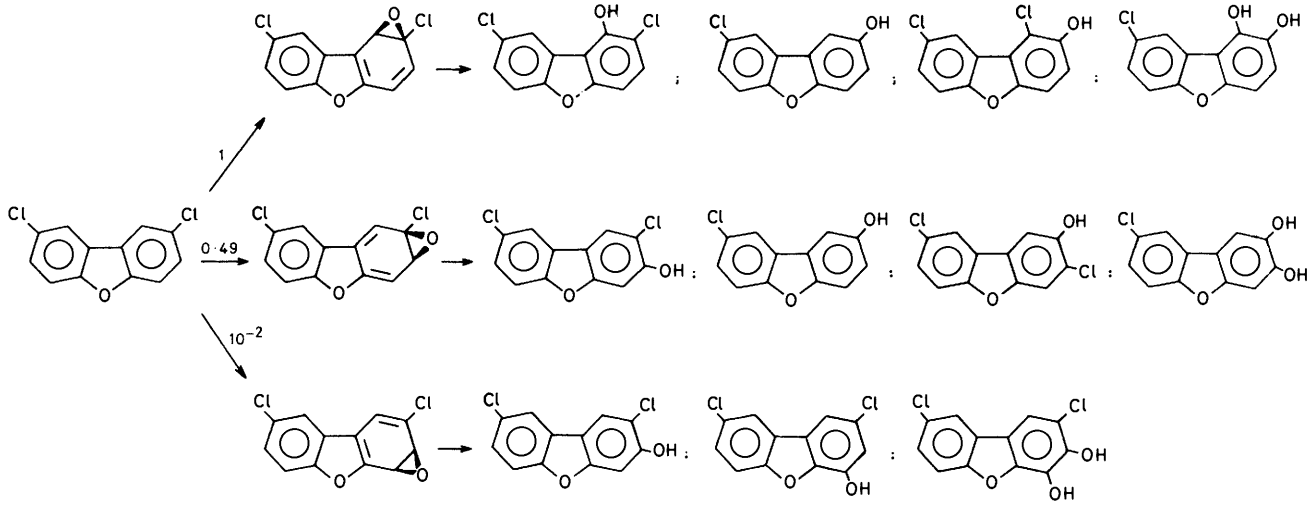

Figure 8. Possible metabolites of 2,8-DCDF

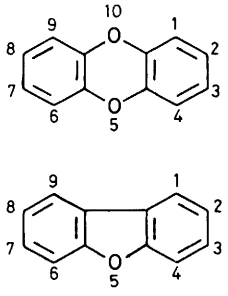

Figure 9. Structure and numbering of DD and DF

and PCDD are on the 2,3 position, leading to a 2,3-epoxide. This reaction appeared to be HOMO-controlled. This epoxide is leading to the formation of the 2- and 3-hydroxy metabolites. This is experimentally confirmed by the findings of Poiger and Schlatter [15] and Tulp and Hutzinger.[13] Figure 5 gives the possible epoxidation reactions for 2,7-dichlorodibenzo-*p*-dioxin and the relative *k* values for the formation of the different epoxides. The only metabolites found were 3-hydroxy-2,7-dichloro-, 2-hydroxy-3,7-dichloro- (due to NIH shift), and 2,3-dihydroxy-7-chloro-dibenzo-*p*-dioxin (due to dechlorination). Based on the calculated *k* values (Figure 5) the 2,3-epoxide is expected to be formed as a major product, which is in agreement with experimentally found metabolites already mentioned. For DF and PCDF the predicted products are, contrary to the DD and PCDD, more dependent on the chlorine substitution pattern (see Table 3). Figure 8 gives the possible epoxidation products and further reactions of 2,8-dichlorodibenzofuran and the relative calculated *k* values for the epoxidation. Compared with the metabolism of PCDD the differences in activity for PCDF are less pronounced, which should give rise to a less specific degradation pattern. The major metabolite from 2,8-PCDF for example was 2-chloro-8-hydroxydibenzofuran [14] originating from the epoxide in the 1,2- or 2,3-positions. However, five more monohydroxydichloro metabolites were found thus confirming the large diversion in the degradation pattern.

From the abovementioned data it is obvious, that a sub-

Table 3. Perturbation energy for mono-oxygenase reaction ($\Delta E/\beta^2_{CO}$) * of polychlorodibenzofurans

Number of chlorines	Chlorine substitution pattern	C-1, C-2	C-2, C-3	C-3, C-4	C-6, C-7	C-7, C-8	C-8, C-9
0		1.833	1.600	1.742	1.742	1.600	1.833
1	1	1.789	1.814	1.652	1.760	1.704	1.866
	2	1.943	1.630	1.823	1.723	1.629	1.810
	3	1.844	1.655	1.816	1.749	1.611	1.843
	4	1.780	1.703	1.711	1.753	1.700	1.868
2	2,3	1.962	1.693	1.896	1.732	1.640	1.818
	2,8	1.925	1.861	1.557	1.557	1.861	1.925
3	2,3,8	1.941	1.923	1.621	1.574	1.868	1.927
4	1,2,3,4	1.867	1.961	1.892	1.709	1.786	1.889
	2.3.7.8	1.952	1.934	1.638	1.638	1.934	1.952
8		1.872	1.622	1.885	1.885	1.622	1.872

* In units of $1/\beta_{CC}$. See equation (1).

Table 4. Perturbation energies for the dioxygenase reaction ($\Delta E/\beta^2_{CO}$) * of polychlorodibenzo-*p*-dioxins

Number of chlorines	Chlorine substitution pattern	C-1	C-2	C-3	C-4	C-6	C-7	C-8	C-9
0		0.631	0.659	0.659	0.631				
4	1,2,3,4	0.691	0.736	0.736	0.691	0.631	0.665	0.665	0.631
4	2,3,7,8	0.654	0.709	0.709	0.654				

* In units of $1/\beta_{CC}$. See equation (1).

Table 5. Calculations on the intermediate after initial attack on C(1)

	C(0)	C(2)
C_{LUMO}^2	0.36	0.23
q	0.29	−0.01

Table 6. Calculations on the intermediate after initial attack on C(2)

	C(1)	C(3)
C_{LUMO}^2	0.31	0.26
q	0.27	−0.06

stitution pattern including the 2,3,7,8-positions of dibenzo-*p*-dioxin has a more pronounced influence on the metabolism than the same chlorine pattern has in dibenzofuran. In the latter case preferential attack will be on the 1,2-position.

The Application of the PMO Approach to Dioxygenases.—When a *Pseudomonas sp.* is grown on salicylate in the presence of dibenzo-*p*-dioxin,[16] the key metabolite formed is *cis*-1,2-dihydroxy-1,2-dihydrodibenzo-*p*-dioxin. According to the mechanism (see Introduction) the formation of a *cis*-dihydroxy metabolite can only occur by the action of a dioxygenase enzyme system. Thus the initial step can be treated as the sole interaction of one carbon atom and one oxygen atom. In Table 4 the calculated perturbation energies for the attack on DD, 1,2,3,4-TCDD, and 2,3,7,8-TCDD in the different positions are shown.

The relative k values for attack on C-1 and -2, calculated from the data in Table 4 are 0.7 and 1.0, respectively. Further reactions is either orbital controlled (radical and charged intermediate) on charge controlled (charged intermediate). To be able to predict ring closure, calculations were performed assuming a pentadienyl model for the intermediates. Tables 5 and 6 give the results for the second step after attack of O_2 on C-1 and -2. Attack on C-1 will, according to these calculations, not give a *cis*-dihydrodiol on C-1 and -2 but attack on the adjacent carbon atom (bridgehead). Initial attack on C_2 will yield *cis*-dihydrodiol on C-1 and -2 both in the charge controlled and orbital controlled reaction (Table 6).

Experimental

Hückel MO calculations were performed with a computer program developed by the Organic Chemistry Department of this University. This program was kindly supplied to us by Professor J. W. Verhoeven and was modified to enable us to use the ω-technique.[17] Throughout our calculations 1.4 was used for ω. We used the suggested parameters for heteroatoms given by Streitwieser.[17]

The charge-transfer complexes were made by adding the substrate to a saturated solution (2 ml) of TCNE in dichloromethane. The absorption was measured with an u.v. spectrometer (Cary 17D). DD, 1-MCDD, 2-MCDD, 2,3-DCDD, and 2,7-DCDD were purchased from Analabs. DF, 2-MCDF, and 2,3-DCDF were synthesised as described by Choudhry *et al.*[18] The synthesis of 1,2,4-TCDD, 1,2,3,4-TCDD, and OCDD was also performed as described before.[19,20]

Acknowledgement

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