1 W.H. Johns and Th.R. Bates, J. Pharm. Sci. *58,* 179 (1969).
2 S.A. Hashim and T.B. van Itallie, J. Am. med. Ass. *192,* 289 (1965).
3 D.U. Datta and S. Sherlock, Br. med. J. *1,* 216 (1963).
4 R. Nelson, G.M. Murphy, S. Edkins, S. Nutter and C.M. Andersen, Gut *15,* 825 (1974).
5 J.T. Garbutt and T.J. Kenney, J. clin. Invest. *51,* 2781 (1972).
6 J.B. Carey, Jr, J. Lab. clin. Med. *56,* 797 (1960).
7 D.D. Brown, R.P. Juhl and S.L. Warner, Circulation *58,* 164 (1978).
8 J.H. Caldwell and N.J. Greenberger, J. clin. Invest. *50,* 2626 (1971).
9 A.V. Tembo and T.R. Bates, J. Pharmac. exp. Ther. *191,* 53 (1974).
10 K.J. Hahn, W. Eiden, M. Schettle, M. Hahn, E. Walter and E. Weber, Eur. J. clin. Pharmac. *4,* 142 (1972).
11 H.A. Rosenberg and T.R. Bates, Proc. Soc. exp. Biol. Med. *145,* 93 (1974).
12 D.G. Gallo, K.R. Bailey and A.L. Sheffner, Proc. Soc. exp. Biol. Med. *120,* 60 (1965).
13 J.J. Boylan, J.L. Bgle and P.S. Guzelian, Science *199,* 893 (1978).
14 P.M. Leeson and P. Fourman, Am. J. Med. *43,* 620 (1967).
15 S. Jain, P.J. Scheuer, S. Samourian, J.O.D. McGee and S. Sherlock, Lancet *1,* 831 (1977).
16 T.B. Deering, E.R. Dickson, C.R. Fleming, M.G. Geall, J.T. McCall and A.H. Baggenstoss, Gastroenterology *72,* 1208 (1977).
17 A. Bremmelgaard and L. Pedersen, Scand. J. Gastroent. *11,* 161 (1976).
18 P.R. Pal, J. biol. Chem. *234,* 618 (1959).
19 L.M. Hagerman and D.L. Schneider, Proc. Soc. exp. Biol. Med. *143,* 93 (1973).
20 W. Johns and Th. Bates, J. Pharm. Sci. *61,* 730 (1972).
21 A. Hurwitz, Clin. Pharmacokin. *2,* 429 (1977).
22 D.W. Newton and R.B. Keura, Drug Intelligence clin. Pharm. *12,* 546 (1978).

Structure elucidation of mammalian TCDD-metabolites

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Summary. Thin layer and gas chromatographic examination of the bile of dogs which were given tritium-labelled TCDD revealed the presence of several polar biotransformation products. The structure of 5 phenolic metabolites was elucidated by combined gas chromatography-mass spectrometry. A metabolic breakdown scheme for TCDD in the dog is proposed.

Quite high rates of conversion of 2,3,7,8-tetrachlorodibenzo-p-dioxin have recently been found in mammals,[2-4] whereas the metabolism of this compound by microorganisms appears to be very slow[5]. The differences among species in susceptibility towards TCDD[6,7] could be explained, at least partially, by different rates of metabolism, because earlier studies have shown that the dog, which is less sensitive to TCDD[6] than the rat, also converts the compound at a higher rate[8]. In addition, this animal species was chosen in our investigation because metabolites are excreted into the bile and, in the dog, bile duct cannulation is an ethically acceptable technique.

Tritium labelled TCDD (sp. act. 40 Ci/mmole; source: A. Kende, Rochester, NY) was purified by preparative gas chromatography (2 m×2 mm ID, DC 560, 220 °C) and mixed with unlabelled TCDD (source: Dow Chemical, Midland, MI), which was previously recrystallized several times from hot anisole, to yield a product with a sp. act. of 26.3 mCi/mmole. GC-MS analysis revealed only the presence of a penta-CDD (1%) but of no other isomers of TCDD. The radiochemical purity was higher than 98.5% (GC).

For the metabolism experiment a Thomas cannula was implanted in a 1-year-old beagle dog, weighing about 14 kg; the animal was then left to recover for a few weeks. A total amount of 5.4 mg of TCDD was administered enterally by direct introduction into the duodenal lumen in 4 portions of 1-2 mg, leaving time intervals of 2-7 days between applications. Treatment with TCDD was accompanied by very severe toxic symptoms, such as vomiting, anorexia and cachexy which ultimately caused the death of the animal (17 days after the 1st dose).

Since the principal aim of the experiment was the isolation of TCDD metabolites for structure elucidation, only a few parameters concerning the pharmacokinetics of the compound were investigated. Excretion of radioactivity in the bile reached a maximum on day 1 or 2 following administration, then decreased quite rapidly. This is an evident contrast to the elimination characteristics in the rat, in which the concentration of radioactivity in the bile remained constant over several days. The amount of radioactivity in the bile (expressed as a percentage of the amount administered) was 11 and 8% within 3 days after application of doses 3 and 4 on days 7 and 14, respectively.

GC-MS data of TCDD and methylated metabolites

Compound	Fraction	Elution temperature (°C)	MS-data M^+	No. of Cl atoms	Fragmentation
I (TCDD)		217.3	320	4	M^+-63; M^+-126
II	C1, C2	231	350	4	M^+-15; M^+-43
III	C1	223.9	316	3	M^+-15; M^+-43
IVa	C2	227	346	3	M^+-15; M^+-43
IVb	C2	228.8	346	3	M^+-15; M^+-43
V	B2	231.8	366	4	M^+-50
VI	B1	134.5	206	2	M^+-15; M^+-43

Finnigan 4000 quadrupole GC-MS instrument, EI 70 eV 240 °C; 24 m SP 2100 fused silica capillary; column conditions: 50 °C (2 min isothermal), 20 °C/min to 160 °C, 5 °C/min to 240 °C. Mass-spectra (m/z 100-450, 1.7 sec/scan) recorded using Finnigan 6115 data system.

This was significantly higher than the 2% excreted after doses 1 and 2 on days 0 and 2, respectively, and suggests a stimulation of drug-metabolizing enzymes in the liver by TCDD. In previous experiments we observed that, at the mg-dose level, intestinal absorption of TCDD is rather incomplete and does not exceed 50% of the administered amount, which implies that at least 15–20% of the TCDD absorbed from doses 3 and 4 was metabolized.

Isolation of metabolites from bile was carried out after dose 4 on a vacuum-dried sample of bile which was extracted twice with cold ethanol. The resulting extract contained about 50% of the total radioactivity. Extractability, using dichloromethane as extractant, was not increased by treatment of the bile with glucuronidase/arylsulfatase (Boehringer), which indicates that conjugation of the metabolites with glucuronic acid or sulfate does not occur in the liver of the dog. The extract was methylated (CH_3I in presence of K_2CO_3, 40–50 °C) to improve the volatility and chromatographic behavior of the compounds. The distribution of radioactivity on TLC after this procedure is shown in figure 1. 3 main radioactive zones, designated A, B and C were located. Zones B and C were separated and extracted with hexane or ethyl acetate. TLC-zone A was not investigated further. Most probably, the non-methylated radioactetrachloro-1-methoxydibenzo-p-dioxin as the most probable pyrolysis product with the CH_3O-group in a peri-(1-, 4-, 6- or 9-) position.

Compound **III** was identified as 2,7,8-trichloro-3-methoxydibenzo-p-dioxin. It was also formed by air oxidation of tive material remained at the starting zone, together with the majority of interfering material. TLC of methylated TCDD-derived material obtained from the bile of rats yielded a different distribution pattern; about 18% was located at the starting zone and the rest at r_f 0.2–0.4, but no radioactivity was detected at r_f 0.6–0.9. Both extracts from dog bile were concentrated and further purified by preparative GC (2 m × 2 mm SE 30, 200 and 220 °C). 2 radioactive fractions were trapped from each TLC-extract (designated B1, B2 and C1, C2, respectively) and, after reduction to a small volume, examined by GC-MS.

The table summarizes the GC-MS data for chlorinated compounds detected in the above fractions, and for TCDD. The amounts of the different compounds, as estimated from the GC-MS data were in accordance with the amounts expected from the radioactivity measured in the samples and were between 20 and 60 ng. None of the compounds identified was detected in the bile of non-treated dogs. The mass-spectrum (fig. 2) of the major compound **II** with a molecular ion at m/z 350 and 4 chlorine atoms shows a characteristic fragmentation to M^+-15 (M^+-CH_3) and M^+-43 (M^+-CH_3-CO), which identifies this compound as a methyl ether of a hydroxylated TCDD. The strong M^+-15 ion is indicative of a CH_3O-group in a lateral (2-, 3-, 7- or 8-) position[9] which would then identify this compound as 1,3,7,8-tetrachloro-2-methoxydibenzo-p-dioxin. This hydroxylated species can only be formed from TCDD in a rearrangement reaction involving migration of a chlorine substituent. This could arise via an NIH-shift subsequent to enzymatic formation of an arene oxide, precedents for which already exist in the case of other chlorinated aromatic compounds.[10] The assignment of the CH_3O-group to a lateral position in compound **II** is supported by the fact that high-temperature air oxidation of 2,3,7,8-TCDD (8 µg in sealed quartz ampoule, 620 °C), followed by methylation with diazomethane led to the formation of a small quantity (0.1%) of an isomer of compound **II** with an elution temperature of 232.5 °C and fragmentation to M^+-43. The absence of a strong M^+-15 in the mass-spectrum of this compound suggests 2,3,7,8-

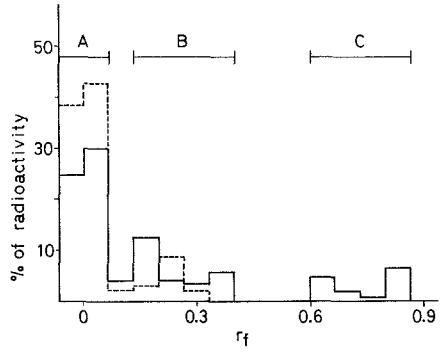

Figure 1. Radio-TLC of an extract from bile, before (dotted line) and after methylation (straight line) with methyliodide. Precoated silica gel 60 sheets (Merck) with a mixture of hexane and ethyl acetate (9:1) as developing solvent, were used. Zones designated B and C were extracted and used as described in the text.

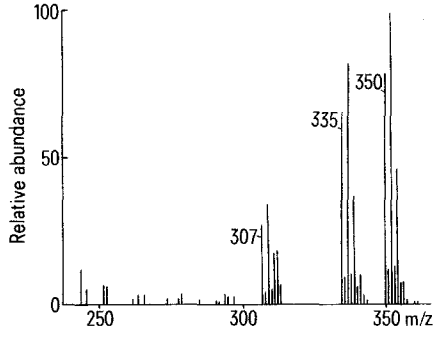

Figure 2. Electron impact (EI) mass-spectrum of major metabolite II. Experimental conditions see table.

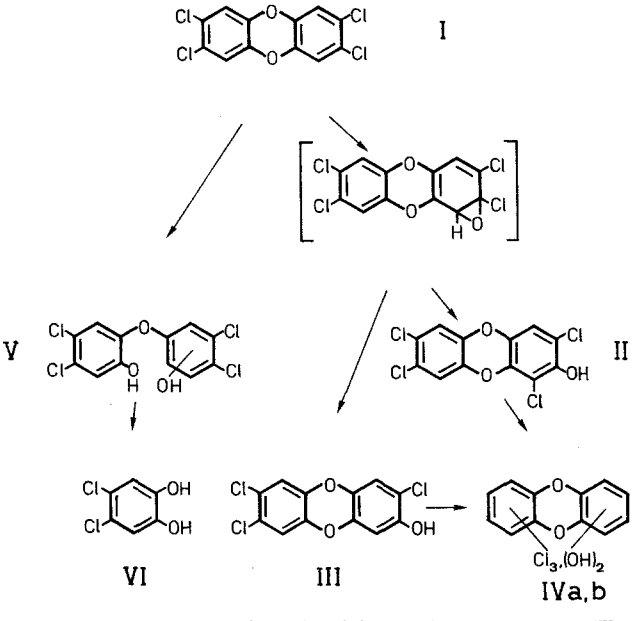

Figure 3. Proposed metabolic breakdown scheme of TCDD. The Roman numbers (**II–VI**) refer to the compounds after methylation at OH.

TCDD. 2 additional chlorinated compounds (**IVa** and **b**), a major and a minor, were identified as trichloro-dimethoxydibenzo-p-dioxins. The exact position of the substituents in these 2 metabolites remains unknown. Hydroxylation could take place at position 1, by direct chlorine replacement or via NIH-shift. In another metabolic study on various chlorinated dibenzo-p-dioxins different hydroxylated compounds were found but it was not possible to decide whether hydroxylation at position 1 does occur.[11] The mass-spectrum of compound **V** did not show the characteristic fragments of laterally methoxylated dioxins (M^+-15, M^+-43). Exact mass-measurements using high-resolution MS (Varian MAT 212 instrument, resolution 4000) indicated a composition of $C_{14}H_{10}O_3Cl_4$ (experimental mass 365.9352, exact mass 365.9385, courtesy J. Schmid, Givaudan Research Company Ltd, Dübendorf, Switzerland), suggesting a tetrachloro-dimethoxydiphenylether. The fragment M^+-50 (CH_3Cl) has been found with other chlorinated 2-methoxydiphenyl ethers[12]. The formation of this compound would have to involve cleavage of one of the ethereal bridges in the dioxin molecule, perhaps after epoxide formation at the angular carbon atom. Compound **VI** was identified as 1,2-dichloro-4,5-dimethoxybenzene based on its mass-spectrum and its co-elution with a synthetic reference sample (courtesy C. Rappe, University of Umea, Umea, Sweden). This metabolite must have been formed in a reaction involving cleavage of both ethereal bridges in the dioxin system.

The proposed metabolic breakdown scheme for TCDD is shown in figure 3. The biotransformation of TCDD in the dog appears to be a detoxification process, since it could be shown that the acute toxicity of the metabolites (using a crude extract from the bile of TCDD-treated dogs, which contained a mixture of all the compounds mentioned) in the guinea-pig is at least 100 times lower than that of the parent compound.[13] To our knowledge this is the 1st report on the identification of mammalian metabolites of TCDD and it demonstrates that this compound, in some organisms, is not as inert to metabolic attack as has hitherto been believed.

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2 H. Poiger and Ch. Schlatter, Nature *281*, 706 (1979).
3 J.R. Olson, T.A. Gasiewicz and R.A. Neal, Toxic. appl. Pharmac. *56*, 78 (1980).
4 J.C. Ramsey, J.G. Hefner, R.J. Karbowsky, W.H. Braun and P.J. Gehring, Toxic. appl. Pharmac. *48*, A126 (1979).
5 F. Matsumura and H.J. Benezet, Envir. Health Perspec. *5*, 253 (1973).
6 B.A. Schwetz, J.M. Norris, G.K. Sparschu, V.K. Rowe, P.J. Gehring, J.L. Emerson and C.G. Gerbig, Envir. Health Perspec. *5*, 87 (1973).
7 J.R. Olson, M.A. Holscher and R.A. Neal, Toxic. appl. Pharmac. *55*, 67 (1980).
8 H. Poiger, H. Weber and Ch. Schlatter, Pergamon Ser. Envir. Sci. *5*, 317 (1982).
9 M.Th.M. Tulp and O. Hutzinger, Biomed. Mass Spectrom. *5*, 224 (1978).
10 J.W. Daly, D.M. Jerina and B. Witkop, Experientia *28*, 1129 (1972).
11 M.Th.M. Tulp and O. Hutzinger, Chemosphere *7*, 761 (1978).
12 C.A. Nilsson, Doctorial Dissertation, University of Umea, Umea, Sweden (1977).
13 H. Weber, H. Poiger and Ch. Schlatter, submitted to Toxic. Lett. (1981).

Urinary catecholamine metabolites (vanylmandelic and homovanillic acids) in the rat after subchronic treatment with sodium nitrate or nitrite

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Summary. Sodium nitrate and nitrite in rat food (5 and 0.5% respectively) result in a decrease of both food intake and growth. These agents induce a specific increase in the urinary excretion of 2 catecholamine metabolites: vanylmandelic and homovanillic acids.

According to the studies of Goodall et al.[2-4] vanilmandelic acid (VMA), arising principally from the peripheral organs, is the main catabolite of adrenaline and noradrenaline after methylation and oxidative deamination. Homovanillic acid (HVA), generated through similar degradation pathways, is the main catabolite of circulating dopamine[5]. Both these acids are eliminated in their free form in urine and correspond to the most important forms of excretion for 3-O-methylated amines like metadrenaline, normetadrenaline and 3-O-methyldopamine. So, the measurement of these 2 urinary catabolites of catecholamines can provide additional information in this field[6].

The metabolism of catecholamines, and also their role in certain physiological functions (e.g. blood pressure regulation) have been the subject of many investigations. In the present report we consider these basic physiological data with respect to nutrition and food toxicology where nitrates and nitrites are involved. These compounds pose an important problem because of their high concentrations in foodstuffs[7,8], which is due to various factors: accumulation by plants, high levels in drinking water, use as preservatives in meat products. Their physiopathological effects have often been described[9-13] and it is known for example that nitrates and nitrites affect the vasomotricity of the blood pressure[14,15] and the blood supply to various organs[16-18]. Furthermore, in an epidemiological study, Malberg et al.[19] found a relationship between the frequency of hypertension in a population in the state of Colorado (USA) and the high level of nitrate in the drinking water (up to 125 ppm). Egashira et al.[20] reported that nitrates and nitrites disturbed monoamine oxidase activity in vitro in various tissues in the rat. We have investigated the *in vivo* impact of these compounds on cardiovascular activity by investigating their possible effects on the metabolism of catecholamines. It was with this aim that we studied the urinary excretion of the two major metabolites of dopamine and noradrenaline after subchronic administration of sodium nitrate or nitrite.

Materials and methods. 1. Experimental material: 49 Sprague-Dawley rats weighing 75 g at the beginning of experiment were maintained at 23 ± 1 °C in a thermostated