A microbial metabolite of TCDD

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Summary. In the present study we describe the occurrence of a metabolite of TCDD, which arose in several microbial cultures after long term incubation. The polar metabolite amounted approximately 1% of the input material, and was found to be a hydroxylated derivative of TCDD.

The toxicity and persistence of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in the environment have caused concern, and studies of its metabolism in animals[2,3], soil[4-7] or aquatic systems[8,9] and by microbial populations[10-12] have been carried out. Whereas the metabolism of TCDD in rats appears to be considerable[2,13,14], microbial metabolism was found to be slow. Evidence for the occurrence of metabolism has either been based on the disappearance of the parent compound from the material tested, or on the appearance of polar fractions in thin layer chromatograms (TLC). In no case, however, has a microbial metabolite yet been characterized and identified. We present in this study the results of experiments on TCDD biodegradation by different microbial cultures under laboratory conditions.

Experiments with mixed microbial populations or single pure cultures were performed in closed systems to permit the determination of a total balance for input ^{14}C-TCDD. The cultures were incubated in different nutrient media for several weeks or months under aerobic or anaerobic conditions, usually at 28 °C[11,12]. Samples of the cultures were analyzed directly by radio-gas chromatography (GC)[11] and by TLC, and the evolution of $^{14}CO_2$ was also monitored. Less than 1‰ $^{14}CO_2$ evolved from the cultures and no metabolites could be detected in the radio-GC system (detection limit 0.04% of input material), as has previously been described[11,12]. But in several cultures polar bands appeared in TLC[11,12]. In most cases a band running approximately 1 cm from the start in the TLC system used (fraction B in fig. 1) was the most prominent one. The radioactive material contained in this band amounted to 1% or 1.5% at most of the input radioactive material[11,12]. Other polar fractions appearing were in the range of 0.3–0.4% at most, and usually less than 0.1%, and were not followed further.

Before analyzing the newly-arisen major polar fraction in detail it was necessary to establish the relative purity of the ^{14}C-TCDD used and to analyze the impurities contained in it. ^{14}C-TCDD was purchased from Kor Isotopes, Cambridge MA, USA, and had a specific activity of 2.6 or 4.7×10^9 Bq/mmole (different preparations). The material contained about 6.5% impurities, mostly 2,3,7-trichlorodibenzo-p-dioxin (TriCDD; 4%) and the isomers of 2,3,7-trichloro-8-(methoxyphenyl) dibenzo-p-dioxin (1.5%) which were presumably formed by radiolysis of TCDD in the standard anisole solution. The remaining 1% impurities consisted of several unidentified components, none of which amounted to more than 0.2% of the total. For our study it was of special interest to verify that no TCDD isomers other than the 2,3,7,8-TCDD were present in the ^{14}C-TCDD. This was indicated by the supplier, but additional tests were performed using combined gas chromatography-mass spectrometry (GC-MS) in the selected ion monitoring mode. The analysis was carried out on different glass capillary columns both in our laboratory (columns OV-73 and Carbowax 20 M) and in an independent laboratory (H.-R. Buser, Swiss Federal Research Station, Wädenswil[15]) (column Silar 10 C). The data obtained unequivocally demonstrated that the ^{14}C-TCDD used did not contain any detectable amounts of TCDD-isomers other than 2,3,7,8-TCDD at detection limits of 0.05% to 0.1%.

In order to characterize further the polar fraction in the 1-cm band (fraction B in fig. 1) by GC-MS, several extracts of culture 26 containing that component were combined (yielding a total activity of 1.2×10^5 Bq), and separated by TLC, and the corresponding band was extracted from the silicagel by dioxane. This purification step was performed by using 4 plates and very carefully purified solvents (triple distillation shortly before use). The entire dioxane extract was again centrifuged to remove the silica gel and then concentrated down to 1–2 µl. These were injected into the column by means of on-column injection[16,17] (for ex-

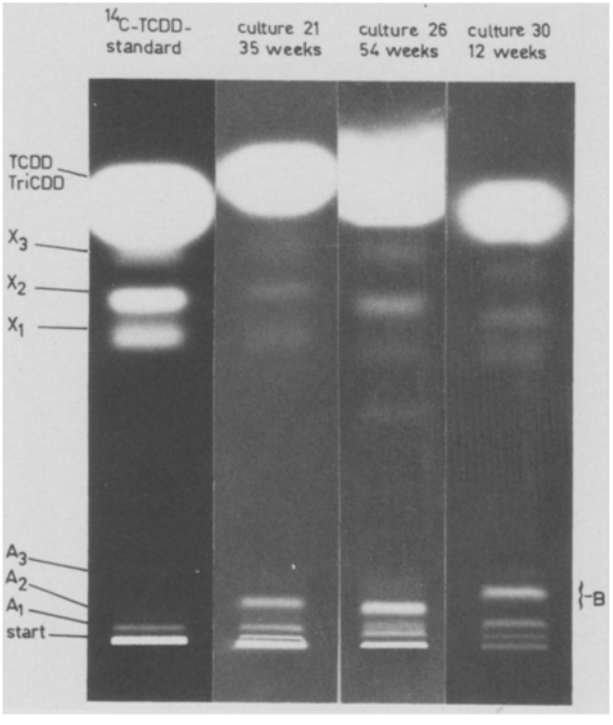

Figure 1. Autoradiography of thin layer chromatograms of extracts from microbial cultures incubated with ^{14}C-TCDD. Cultures of *Pseudomonas testosteroni* strain G 1036 (culture 21), of an unidentified bacterium isolated from Seveso soil (culture 26) and of a mixture of 6 unidentified bacterial strains isolated from Seveso soil[10] (culture 30) were incubated aerobically in 30 ml of complex nutrient media with ^{14}C-TCDD. Culture 21 contained 0.32 µg ^{14}C-TCDD per ml (total 199.2×10^3 Bq), culture 26 0.03 µg ^{14}C-TCDD per ml (total 298.7×10^3 Bq), and culture 30 0.067 µg ^{14}C-TCDD per ml (total 42.6×10^3 Bq). Samples were analyzed after 35 weeks (culture 21), 54 weeks (culture 26) or 12 weeks (culture 30) of incubation. They were extracted with isooctane (3×) and aliquots of the extracts containing about 1000 Bq were chromatographed on silicagel 60 plates (Merck, Darmstadt, FRG) with hexane/benzene (1:1). After chromatography the plates were dried and autoradiography was performed using Kodak No-screen film NS-2T. X_1 and X_2 represent anisole aduct isomers of TriCDD, X_3 possibly unchlorinated dibenzo-*p*-dioxin[11]. A_1–A_3 are polar impurities present in the ^{14}C-TCDD preparation. Fraction B designates the microbial metabolite discussed in this paper.

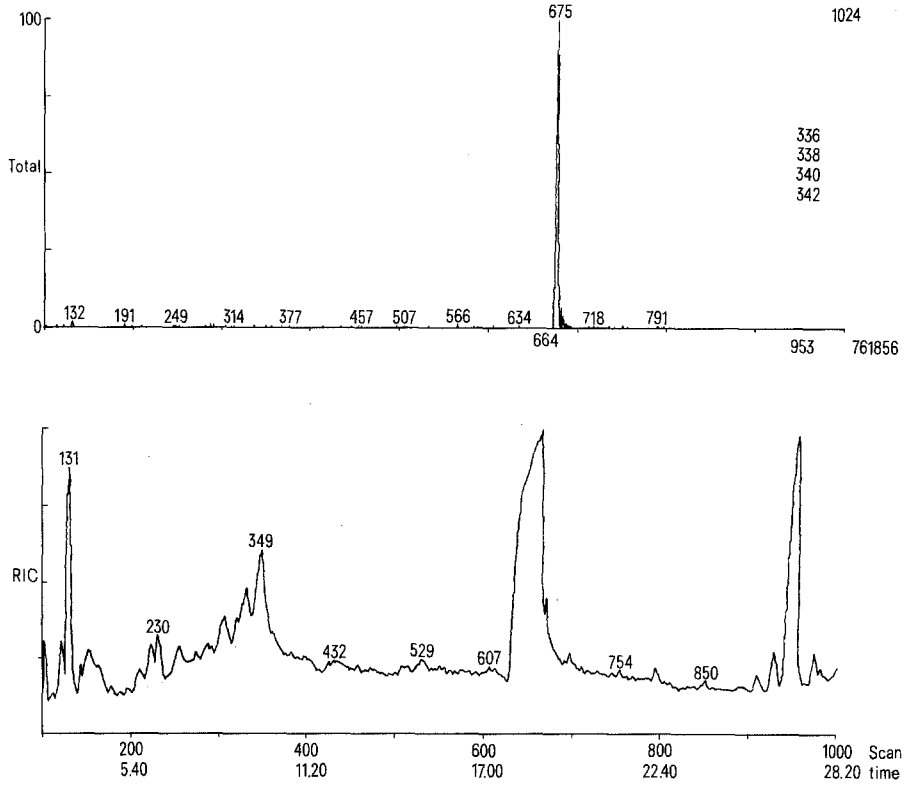

Figure 2. GC-MS analysis of fraction B from culture 26 (TLC, fig. 1) The lower trace shows the total ion current of the gas chromatogram; the peaks generally contain substances of no significance (e.g. hydrocarbons, phthalates, phosphates, antioxidants). The upper trace selectively picks out highly chlorinated compounds of mol. wt 336 by looking at 0.2-amu mass windows centered at m/e 335.87, 337.87, 339.87 and 341.87; the ion intensities found in these mass windows are added up to yield 1 single GC-trace. This selective procedure reveals, against a rather abundant background, the presence of a clear peak containing the metabolite of interest (mass spectrum, see fig. 3). For the analysis a glass capillary column[18,19] (SE-52, length 18 m, 0.31 mm i.d., programmed at 5 °C/min from 180 °C to 300 °C) and a Varian MAT212/Finnigan Incos GC-MS/Data System were employed.

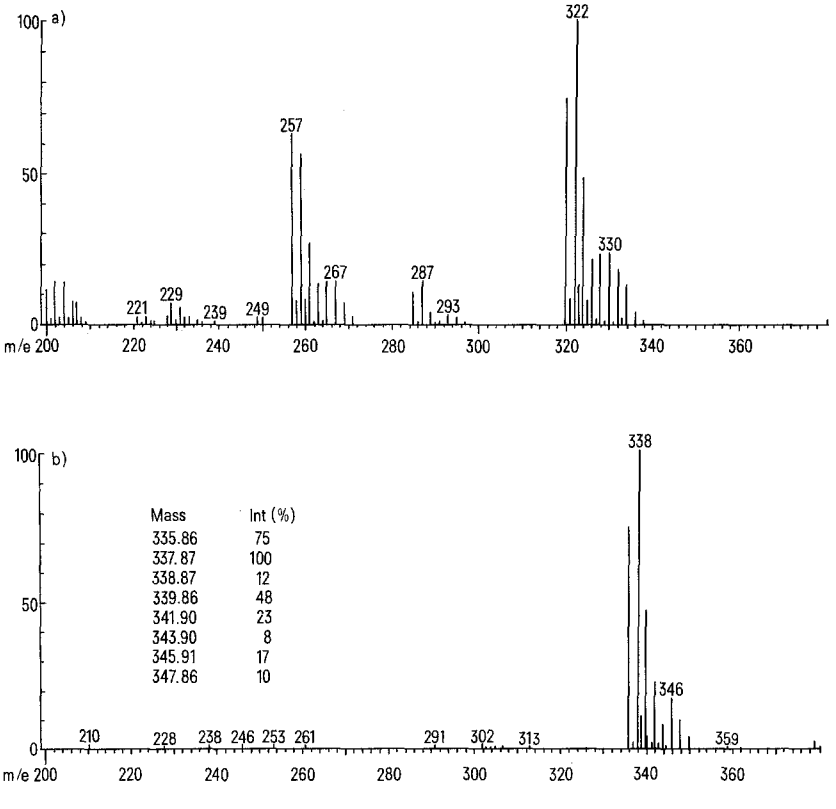

Figure 3. Mass spectra of ^{14}C-labelled TCDD-standard spectrum (a) and metabolite spectrum (b). The normal 4-Cl patterns (maxima at m/e 322 and 338, respectively) are superimposed by a second group of peaks (maxima at m/e 330 and 346, respectively) which reflect the presence of the ^{14}C-label. Spectrum b represents the sum of 4 scans during the GC-peak (fig. 2), calculated after accurate-mass subtraction of the well separated background peaks (mass spectrometer resolution of 800). The characteristics of spectrum b are in good agreement with the molecular formula $C_{12}H_4O_3Cl_4$ and the presence of ^{14}C-label.

perimental details see fig. 2). The GC-MS findings are summarized by the 2 gas chromatograms shown in figure 2 and by the mass spectrum of the new metabolite in comparison to the spectrum of the ^{14}C-TCDD-standard (fig. 3). These data clearly indicate the presence of a compound of the formula $C_{12}H_4O_3Cl_4$ which, according to the molecular ion pattern, is labelled with ^{14}C identically with the starting TCDD and is eluted, under the given experimental conditions, some 3–4 min after TCDD on a nonpolar glass capillary column.

In a separate experiment it was demonstrated that most of the radioactivity contained in the TLC-band B could be extracted into a basic water phase (2 moles/l sodium hydroxide) and re-extracted into isooctane after acidification (1 mole/l sulfuric acid). It was shown by TLC that the re-extracted material was identical to fraction B. This behavior indicated the phenolic nature of the metabolite. The data strongly support the conclusion that the polar metabolite is a hydroxylated derivative of TCDD itself, i.e. 1-hydroxy-2,3,7,8-tetrachlorodibenzo-p-dioxin, provided no rearrangement of the chlorine substituents has occurred. Unfortunately trials to prepare the compound synthetically in order to compare GC retention times have failed so far.

This is the first report of the identification of a microbial metabolite of TCDD. Structure identification of mammalian TCDD-metabolites has been reported[13]. Even if the amount of metabolite formed was low, it shows that TCDD, although highly persistent in the environment, is not totally inert to microbial attack. In the long range, even a low contribution from microbial metabolism may turn out to be important.

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General solution of the pseudo first-order rate equations for consecutive reactions with identical rate constants[1]

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Summary. The integrated rate equations for any number of consecutive pseudo first-order reactions $A \rightarrow B \rightarrow \ldots$ are given for the case in which the rate constants are all identical. The general solution coincides with the description of a Poisson process, and therefore may be more widely applicable to the kinetics of morphological alterations or other cellular processes than previously supposed.

The general solution of the differential rate equations for pseudo first-order series reactions is well-known[2]. However, the integrated equations contain a limitation in that they do not apply in the case where the rate constants are identical[3]. The anomaly arises because of the occurence of rate constant terms of the form $1/(k_i - k_j)$ which are undefined when the differences are zero. Although it is easy to derive the missing equations, it is apparently not generally recognized that the description coincides with the description of a Poisson process. This important class of reactions provides a basis for the analysis of the birth and growth of whole populations, for example, and might be useful in the analysis of other, smaller scale biological processes as well. The integrated equations which complete the pseudo first-order rate theory for any number of consecutive reactions connected by identical rate constants are found as follows.

Consider first the general reaction scheme

$$C_1 \xrightarrow{k_1} C_2 \xrightarrow{k_2} \cdots C_{N-1} \xrightarrow{k_{N-1}} C_N \qquad (1)$$

in which no restrictions are placed on the values of the rate constants k_n. If c_n is the concentration of the n'th species with time derivative dc_n/dt, the general differential rate equations for scheme (1) are

$$dc_n/dt = k_{n-1}c_{n-1} - k_n c_n \qquad 1 \leqslant n \leqslant N-1 \qquad (2)$$