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# An Analytical Method for Detecting TCDD (Dioxin): Levels of TCDD in Samples from Vietnam

by Robert Baughman\* and Matthew Meselson\*

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is an extraordinarily toxic substance that is produced as an unwanted side product in the industrial synthesis of 2,4,5-trichlorophenol, an intermediate in the manufacture of the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (1, 2). Because of its chemical stability and its lipophilic nature, the possibility exists that TCDD released into the environment could accumulate in food chains. A direct test of the possibility of biologically significant accumulation in animal tissues requires an analytical method able to detect TCDD at levels well below those known to be toxic. The lowest value known for the lethal dose of TCDD is that observed in the guinea pig, for which the single oral dose LD<sub>50</sub> is 600 parts per trillion (ppt) body weight (3). Allowing for sublethal toxic effects and providing for a conservative margin of safety, it seems desirable to have an analytical sensitivity of at least 1 ppt. For a 1-g sample this means the method must have a sensitivity of about 10<sup>-12</sup>g or 1 picogram (pg).

The most common method for analyzing chlorinated organic compounds in tissue samples is gas-liquid chromatography (GLC) with an electron capture detector. Its limit

of detection for TCDD, about 10<sup>-10</sup>g, is inadequate. This method is also susceptible to interference from other compounds and so is not very specific.

Mass spectrometry offers better possibilities. It is highly sensitive and in the high resolution mode of operation it is highly specific. We have previously described a time averaged mass spectroscopic method with an adequate limit of detection (4). However, full sensitivity could not be realized in most sample types because of interference from DDE (a major degradation product of DDT) and polychlorinated biphenyls (PCBs). In this paper we describe a clean-up procedure that overcomes this difficulty.

Homogenized samples are saponified in alcoholic potassium hydroxide and extracted with hexane. The extract is shaken with sulfuric acid and chromatographed on alumina. Elution with carbon tetrachloride-hexane removes most of the DDE and PCBs. Chlorinated dioxins are then eluted with dichloromethane-hexane. The TCDD containing fraction is further purified by preparative gas-liquid chromatography and analyzed by mass spectroscopy by use of a multichannel analyzer to average successive scans.

We also report the levels of TCDD found in a limited number of samples of fish and crustaceans from locations in South Vietnam near areas heavily exposed to 2,4,5-T.

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## Experimental

### Reagents and Apparatus

Hexane (pesticide grade, Fisher Scientific), dichloromethane (reagent grade, Eastman), carbon tetrachloride (reagent grade, Merck), 95–97% sulfuric acid (reagent grade, Dupont), sodium carbonate (powdered) (reagent grade, Mallinckrodt), and ethanol (pesticide grade, Matheson, Coleman and Bell) were used.

Activated alumina was Fisher A-540, activated at 130° C for 24 hr.

The gas chromatograph was a Bendix Model 2200 equipped with a thermal conductivity detector. The column was 5% SE-30 on 60/80 Chromosorb W, 2 m × 2 mm (id) stainless steel. The trap for preparative gas chromatography was a 150 mm × 1.5 mm (id) glass tube packed with 30 mm of glass wool.

An Associated Electrical Industries MS-9 double focusing mass spectrometer and a Varian 1024 time-averaging computer interfaced with the MS-9 as described earlier (4), were used.

### Cleanup Procedure for the Analysis of TCDD in Tissue Samples

(1) The sample was weighed and homogenized with 1.0–1.2 parts EtOH.

(2) This homogenate was transferred to a round-bottomed flask equipped with a reflux condenser (Teflon tape used on the ground glass joint). The sample was spiked with approximately 1000 ppt <sup>37</sup>Cl TCDD; 2 parts 40% aqueous KOH were added, and this mixture was refluxed for 2 hr. One part always refers to the original samples.

(3) The solution was partially cooled and 1 part hexane added.

(4) The solution was transferred to a separatory funnel, and the phases were separated. The aqueous phase was extracted with three more identical portions of hexane; the hexane extracts were combined and collected in the original round-bottomed flask.

(5) The hexane phase was transferred to the separatory funnel, the round-bottomed flask was rinsed twice with a few milliliters

of EtOH and then twice with a few milliliters of hexane; the solvent was refluxed each time; and the hexane was extracted with 1 part 1.0N NaOH.

(6) The hexane was extracted four times (or until acid phase was colorless) with 2 parts 95–97% H<sub>2</sub>SO<sub>4</sub>. Emulsions were broken with a few drops of saturated Na<sub>2</sub>CO<sub>3</sub> solution.

(7) The hexane was extracted with 1 part water, and several grams of Na<sub>2</sub>CO<sub>3</sub> were added to the hexane.

(8) The hexane was filtered through a column of Na<sub>2</sub>CO<sub>3</sub> (100 mm × 10 mm id for 300 ml hexane), the Na<sub>2</sub>CO<sub>3</sub> first being prewashed with several milliliters of hexane.

(9) The hexane was concentrated to 3–4 ml (Snyder column).

(10) The hexane residue was chromatographed on a column of activated Al<sub>2</sub>O<sub>3</sub> (50 mm in a 5 mm disposable pipet). The column should not be prewashed. Elution was with 12 ml of 20% CCl<sub>4</sub> in hexane, then 1 ml of hexane, and finally 4 ml of 20% CH<sub>2</sub>Cl<sub>2</sub> in hexane.

(11) The 20% CH<sub>2</sub>Cl<sub>2</sub> fraction was concentrated carefully to about 50 μl, 100–200 μl benzene added, and concentration repeated to 20 μl.

(12) A few micrograms of *m*-terphenyl in benzene were added to the residue and the mixture subjected to preparative chromatography. The retention time of *m*-terphenyl relative to that of TCDD was determined beforehand and used to make certain that the TCDD collection was carried out at the right retention time.

(13) The GLC trap containing TCDD was eluted with 60 μl followed by 10 μl of benzene. The total amount of eluant collected was measured, and the fraction size for the planned number of fractions (typically ten) calculated.

(14) The fractions for TCDD analysis were prepared in the sample tubes described previously (4). A known amount of TCDD was added to three or more fractions for quantitation of any TCDD observed. The amount of TCDD added per fraction for

quantitation should be approximately three or four times the amount expected to be present.

(15) The fractions were analyzed with the MS-9 instrument. Typical conditions were: source 220°C, resolution 10,000 (based on a 10% valley between peaks), trap current 1.0 mA (rhenium filament), electron multiplier 700, ionizing voltage 70 eV, time averaging at four scans per second.

(16) Peak heights were measured at  $m/e$  321.894. The quantity of TCDD (picograms), present in the fractions to which TCDD has not been added was computed from the ratio of their mean peak heights to the mean peak heights found with added TCDD.

(17) Steps (14)–(16) were repeated, but  $^{37}\text{Cl}$  TCDD was added and peak heights were measured at  $m/e$  327.885 in order to compute the amount of  $^{37}\text{Cl}$  TCDD recovered. The recovery through the complete cleanup procedure was then calculated based on the amount of  $^{37}\text{Cl}$  TCDD added to the sample at the beginning of the cleanup.

(18) The quantity of TCDD computed in step (15) was corrected by the recovery factor obtained in step (16) to give the final result.

### Sample Collection

Freshly caught fish and crustaceans were collected in South Vietnam in August and September 1970 from local fishermen. The samples were homogenized with a meat grinder, placed in acetone-rinsed glass bottles with aluminum foil-lined caps, and immediately frozen in solid  $\text{CO}_2$ . Later on the same day, samples were placed in a Linde LR-35 liquid nitrogen refrigerator where they remained until analysis. Water blanks were present in the liquid nitrogen refrigerator throughout the storage period and were analyzed with the samples. Fresh Cape Cod butterfish (*Poronotus tricanthus*, family *Stromateidae*) were obtained from a local market, homogenized, and kept at  $-20^\circ\text{C}$  until analysis. Domestic beef livers were obtained and treated similarly.

## Results

### Methodology

The mass spectra of natural and  $^{37}\text{Cl}$  TCDD are shown in Figure 1. The most intense signal for natural TCDD occurs at  $m/e$  321.894 (nominal  $m/e$  322), corresponding to the isotopic isomer with one atom of  $^{37}\text{Cl}$  and three atoms of  $^{35}\text{Cl}$ . The natural abundances of the Cl isotopes are 75.53 and 24.47%, respectively. The observed spectrum for the synthetic  $^{37}\text{Cl}$  TCDD corresponds to an isotopic purity of 95.5%  $^{37}\text{Cl}$ , the same as the value claimed by the manufacturer (Oak Ridge National Laboratory) of the NaCl used in the synthesis of the labeled TCDD. The synthetic  $^{37}\text{Cl}$  TCDD contributes only 0.042% as much to the peak at  $m/e$  322 as it contributes to its most intense signal at  $m/e$  328. The contribution at  $m/e$  320 is even lower, by a factor of nearly 100. This allows an excess of  $^{37}\text{Cl}$  TCDD to be added to each sample before cleanup without interfering

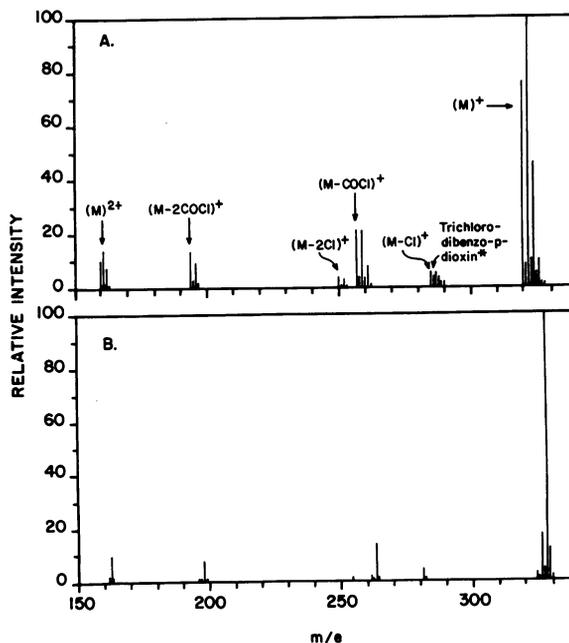


FIGURE 1. Mass spectra of (A) TCDD and (B)  $^{37}\text{Cl}$ -labeled TCDD. The isotopic purity of the  $^{37}\text{Cl}$  is 95.5%. The asterisk denotes an impurity. The multiplicity of lines associated with each major molecular species results from the presence of various isotopes of Cl and C.

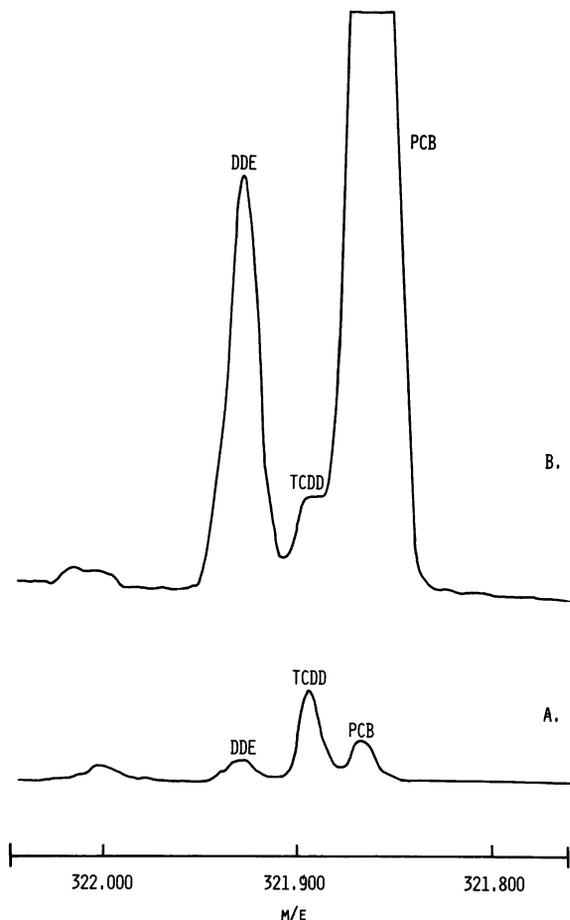


FIGURE 2. Mass spectra showing reduction of DDE and PCB levels in fish residue by means of alumina chromatography. Following the sulfuric acid cleanup step, the residue in hexane is added to a column of activated alumina: (A) Trace from the material eluted by 20%  $\text{CH}_2\text{Cl}_2$  in hexane after the column was first eluted with 20%  $\text{CCl}_4$  in hexane; (B) trace obtained from a similar 20%  $\text{CH}_2\text{Cl}_2$ -in-hexane elution after the column was first eluted with 1%  $\text{CH}_2\text{Cl}_2$  in hexane. Elution with 1%  $\text{CH}_2\text{Cl}_2$  in hexane was reported to be effective in reducing the amount of PCB residues (5). Elution with 20%  $\text{CCl}_4$  is clearly even more effective and was routinely used in obtaining the results reported here.

with analysis of natural TCDD at  $m/e$  322 and 320. The addition of  $^{37}\text{Cl}$  TCDD provides a carrier and makes possible the calculation of absolute recoveries.

An alumina chromatography step has been developed which, when combined with the cleanup steps described previously, (4)

makes possible the measurement of picogram quantities of TCDD in samples initially containing more than a millionfold excess of DDE and PCBs. Figure 2 shows the effectiveness of this procedure.

The calculation of TCDD levels described in steps (14)–(16) of the experimental section assumes a linear relationship between peak height and amount of TCDD present in any given sample. Figure 3 demonstrates that the response is indeed linear over the full range of TCDD amounts introduced into the MS-9 in the course of the analyses reported here.

The reproducibility and overall recovery of the complete analytical procedure is illustrated in Table 1. A sample of beef liver was homogenized and divided into three portions each of which was then spiked with 20 ppt TCDD and 1000 ppt  $^{37}\text{Cl}$  TCDD. The three samples were independently put through the cleanup procedure up to the GLC step. Each sample was then split into three portions before preparative GLC and mass spectrometric analysis, giving rise to a total of nine separate values for the recovery of both TCDD and  $^{37}\text{Cl}$  TCDD. The average recovery was  $34 \pm 7\%$  for TCDD and  $27 \pm 5\%$  for  $^{37}\text{Cl}$  TCDD. When the slight background signal at  $m/e$  322 in an unspiked

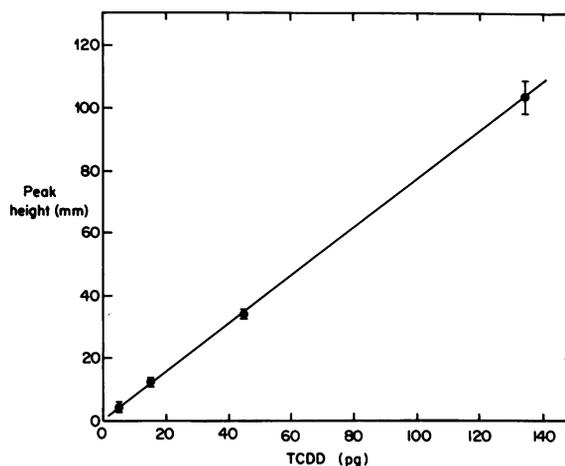


FIGURE 3. Linearity of response for TCDD in the presence of beef liver residue. The TCDD values are the amounts introduced into individual runs on the MS-9.

**Table 1. Recoveries of TCDD (added at 20 ppt) and <sup>14</sup>C TCDD (added at 1000 ppt) from beef liver.**

Sample	Recovery, %	
	TCDD	<sup>14</sup> C TCDD
Sample A		
GLC 1	47	24
GLC 2	36	30
GLC 3	36	25
Sample B		
GLC 1	28	35
GLC 2	31	29
GLC 3	24	20
Sample C		
GLC 1	29	27
GLC 2	40	32
GLC 3	37	21
Mean recovery for A, B, and C	34 ± 7.2	27 ± 5.0

sample of the same liver is taken into account, the calculated recoveries from the spiked samples become even more nearly equal. Experiments performed separately with each individual cleanup step established that the step with lowest recovery is preparative gas-liquid chromatography.

We conclude from these and other controls that the present analytical method provides the sensitivity and reproducibility required for biologically meaningful analyses of animal tissue samples. The method makes possible investigations of such samples at levels approximately 10<sup>-4</sup> times those reported heretofore (6).

## Observed TCDD Levels

Signals at *m/e* 320 and 322 were conspicuously present in each of the fish and crustacean samples from Vietnam. The calculated levels of TCDD, summarized in Table 2, range from 18 ppt to 814 ppt, based on total wet body weight.

No peak was observed at *m/e* 320 or 322 with Cape Cod butterfish. The background signal corresponded to a level of 3 ppt of TCDD. No peaks were observed in water blank samples present in the liquid nitrogen refrigerator throughout the sample collection and storage period.

Confirmation that peaks observed at *m/e* 320 and 322 are in fact produced by TCDD is routinely provided by the criteria outlined in part A of Table 3. All three of these criteria are met by the mass spectra from each of the Vietnamese samples.

The additional confirmatory procedures listed in part B of Table 3 were carried out on a sample of Vietnamese fish. This sample, carp from the Dong Nai River, exhibited a mean TCDD level of 540 ppt. The mass spectrum in the region *m/e* 322 is shown in Figure 4. The compound observed in this fish behaved identically to TCDD in each of the three additional confirmatory tests. We consider it extraordinarily unlikely that this compound is anything other than a tetrachlorodibenzo-*p*-dioxin. In contrast to the significant amounts of 2,3,7,8-tetrachlorodi-

**Table 2. TCDD levels in fish and crustaceans.**

Map site <sup>a</sup>			Level, ppt total wet body weight <sup>b</sup>			
			I	II	III	Mean
A	Dong Nai River (interior)	Carp (Cyprininae)	690	320	610	540
B	Dong Nai River (interior)	Catfish (Siluridae)	610	1020		810
B	Dong Nai River (interior)	Catfish (Tachysuridae)	510	530		520
C	Sai Gon River (interior)	Catfish (Schilbeidae)	52	89		70
C	Sai Gon River (interior)	River Prawn (Palaemonidae)	34	49		42
D	Can Gio Village (seacoast)	Croaker (Sciaenidae)	110	49		79
D	Cape Gio Village (seacoast)	Prawn (Peneidae)	23	14		18
	Cape Cod, Massachusetts	Butterfish (Stromateidae)				≤3

<sup>a</sup> Letters refer to sites on map in Figure 5.

<sup>b</sup> Roman numerals refer to independent cleanups of different portions of the same sample. All values are corrected for recovery.

Table 3. Confirmation Procedures

- A. Routine
1. Follows  $^{37}\text{Cl}$  TCDD through highly specific cleanup
  2. Has expected mass ( $\pm 2-3$  mmu) at  $m/e$  320 and 322
  3. Has expected ratio of isotopic isomers at  $m/e$  320 and 322
- B. Additional<sup>a</sup>
1.  $\text{M}^+-\text{COCl}$  fragmentation peak has expected mass and isotopic isomer ratio
  2. Percent recovery after partial photolytic decomposition equals that of  $^{37}\text{Cl}$  TCDD (7, 8)
  3. Partition coefficient between dichloromethane-hexane and acetonitrile equals that of  $^{37}\text{Cl}$  TCDD (7).

<sup>a</sup> Steps 2 and 3 of the additional procedures were carried out on the dichloromethane-hexane eluant from the alumina chromatography prior to preparative GLC.

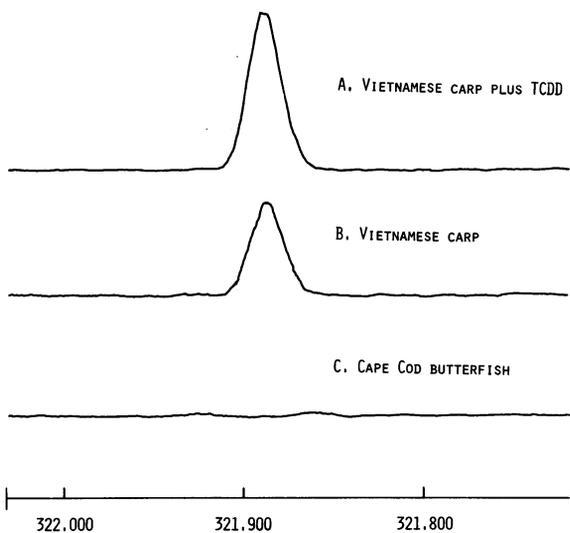


FIGURE 4. TCDD signals observed in fish samples: (A) Vietnamese carp plus 60 pg TCDD, (wet weight of fish 0.18 g); (B) Vietnamese carp, (wet weight of fish 0.18 g); (C) Cape Cod butterfish (wet weight of fish 0.16 g).

benzo-*p*-dioxin known to have been disseminated as a contaminant of 2,4,5-T (1), we know of no likely route by which other isomers of TCDD might have been introduced into the Vietnamese environment.

The locations from which the Vietnamese samples were obtained are designated in Figure 5. The letters correspond to those in Table 2. Areas heavily treated with 2,4,5-T before its use was ordered discontinued in

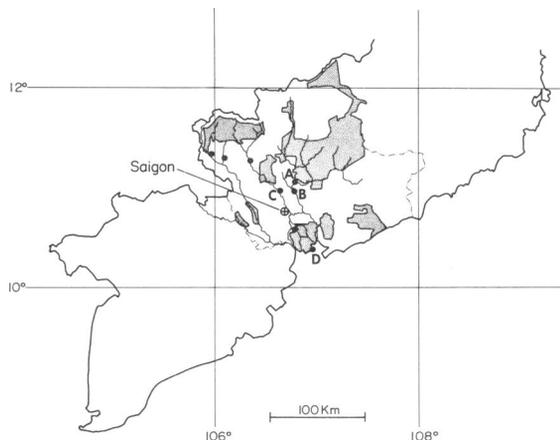


FIGURE 5. Map showing sampling sites in relation to rivers and principal sprayed areas. Sites A and B are located on the Dong Nai River, site C is on the Sai Gon River, and site D is on the coast at Can Gio. Sprayed areas are depicted only within the region bounded by the dashed lines (- · -).

April 1970 are shown as stipled. The number of samples is not adequate to permit reliable conclusions concerning the differences between various locations and species, although this certainly should be a subject of future studies.

## Discussion

Considering the limited number of samples we have analyzed and the fact that they were collected 2½ yr ago, it does not seem appropriate to attempt any detailed evaluation of the possible toxicological significance of our results. Such discussion is made even more difficult by the complexity and incompleteness of the existing toxicological data. However, in order to provide perspective for such discussion, a tabulation of some of the principal toxicity data on TCDD is presented in Table 4. It may be noted that guinea pigs consuming their weight of food contaminated with TCDD at a level of 600 ppt would have ingested a quantity corresponding to the lethal dose. In contrast, a far greater quantity of TCDD is required to reach the  $\text{LD}_{50}$  cited for rats. The table shows that teratogenesis in the rat occurs at doses substantially lower than those required to kill.

Table 4. Levels of TCDD giving various biological effects.

Effect	TCDD to obtain effect, ppt body weight	Reference
<b>Lethality</b>		
Female rat, single oral dose LD <sub>50</sub> (observations terminated at 44 days)	45,000	(3)
Male rat, single oral dose LD <sub>50</sub> (observations terminated at 44 days)	23,000	(3)
Male guinea pig, single oral dose LD <sub>50</sub> (observations terminated at 50 days)	600	(3)
<b>Teratogenicity</b>		
Cleft palate in 50% NMRI mice, daily oral dose, days 6-15	5,000	(9)
Intestinal hemorrhage and subcutaneous edema in 50% Sprague-Dawley rats, daily oral dose, days 6-15	125-500	(3)
Edema and death in chicken embryo, single injection	20	(10)
<b>Enzyme induction</b>		
Doubling of δ-aminolevulinic acid synthetase in chicken embryo, single injection	30	(11)
<b>Mitotic arrest</b>		
Lily endosperm, ambient concentration	<200	(12)

Feeding studies in monkeys show that dioxin poisoning is cumulative (13). Various levels of a toxic fat known to contain chlorodioxins were incorporated into the daily diet of *Macaca mulatta* monkeys. As pointed out by the investigators, the mean survival time depended inversely on the daily dose. A plot of their data (Fig. 6) conforms rather well to the relation  $T = K/D + K'$ , where  $T$  is mean survival time,  $D$  is daily dose, and  $K$

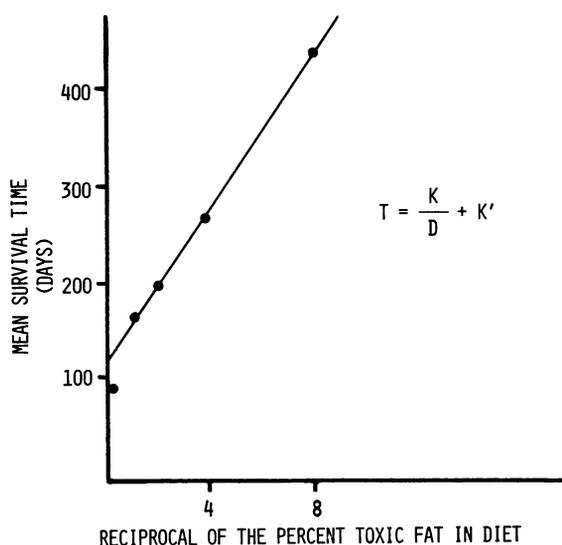


FIGURE 6. Mean survival time of monkeys fed toxic fat plotted against the reciprocal of the per cent of toxic fat present in the diet (13).

and  $K'$  are constants corresponding respectively, to the accumulated lethal dose and to the lag time between the accumulation of this dose and the time of death. No departure from this relation is seen even at the lowest level of toxic fat tested, where the mean survival time was 445 days. The importance of this result is that repeated intake of quantities of TCDD individually equal to only a small percentage of the single oral dose LD<sub>50</sub> may over time cause serious poisoning. Unfortunately, the LD<sub>50</sub> for TCDD in these primates cannot be computed since all the animals died (5/5), even at the lowest dose level, and the concentration of TCDD in the toxic fat has not been established.

In South Vietnam itself we have little information regarding the possible occurrence of toxic effects of TCDD in humans. Certainly, it should be pointed out that while we were in South Vietnam in 1970, the medical member of our group, Dr. John Constable, Professor of Surgery at Harvard Medical School, did not encounter evidence of any severe and widespread unusual illness in visiting Can Gio and several other villages or in discussions with officials of the South Vietnamese Ministry of Health. However, it was felt that certain indications in birth statistics ought to be investigated further

for possible connections with herbicide exposure (14). It is of obvious interest to survey appropriately chosen populations in South Vietnam more closely, especially if TCDD residues should be found in human tissue samples.

Finally, turning from questions of environmental toxicology to the biological mechanisms of action, we note that TCDD seems to be particularly toxic to proliferating tissues, as suggested by its effects on spermatogenesis and hematopoiesis and its apparent toxicity to the intestinal epithelium (13) and the thymus (15). These indications are consistent with the effects of a mitotic poison, such as TCDD is known to be in the African blood lily (12) and possibly in *Drosophila melanogaster* (16). We are led by these observations to speculate that TCDD may be able catalytically to disrupt microtubules, the subcellular elements of which spindle fibers are constructed and which are ubiquitous in their structural roles in cell extension and cell movement.

## Summary

A procedure has been developed for the reliable detection of TCDD in animal tissues down to levels approaching 1 ppt. It makes use of chemical cleanup, preparative gas-liquid chromatography, and analysis by time-averaged high resolution mass spectroscopy.

A limited number of fish and crustacean samples was collected in South Vietnam in 1970 near areas heavily exposed to the herbicide 2,4,5-T. TCDD was detected in these samples at levels ranging from 18 to 810 ppt. TCDD was not detected in a sample of Cape Cod butterfish used as a control.

These results suggest that TCDD may have accumulated to biologically significant levels in food chains in some areas of South Vietnam exposed to herbicide spraying.

**Note added in proof:** Overall recoveries have been increased to 60–80% by replacing the GLC step with an additional  $Al_2O_3$  column step. Details of this procedure will be described in a future publication.

## Acknowledgement

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