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Studies of the metabolism of tri- and hexachlorodiphenyl in the rat.

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Introduction.

In order to clarify the question whether and to what extent an accumulation of trichlorodiphenyl (TCD) and of hexachlorodiphenyl (HCD) occurred in the organism, investigations by residue were carried out in certain tissues of organs and in fats of rats, after a single oral administration.

We also investigated the segregation of unchanged TCD in the faeces and, in order to obtain an indication about the degradation in the organism, the metabolites which were present in the urine.

Method.

Compounds.

Mixtures of isomers of pure TCD and HCD were used for investigations.

Animal material.

Male and female SPF rats, CF_1 stock.

Apparatus.

Bühler homogeniser

Gas chromatograph 5750 G, a Hewlett-Packard product with E.C. detector (Ni^{63})

Rotary vacuum evaporator

Solvents.

Lutrol 9

n-hexane (Uvasol)

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n.p. chloroform

n.p. benzene

n.p. conc. ammonia (25%)

Experimental arrangement.

A. T.C.D.

1. Investigation for the detection of TCD in the tissue, following a single oral administration.

Using a pharyngeal probe, 5 rats each obtained per dose one single oral dose of 250, 125, 10 and 1 mg of TCD/kg in Lutrol 9 (1,0 ml solution/kg). They were killed 96 hours after the start of the experiment. Each dose was matched by 2 controls.

In animals of the 250 and 125 mg/kg dose, the hexane extracts of fat, liver, kidney, spleen, heart, stomach and the content of stomach were subjected to gas chromatographic investigation ; in animals of the 10 mg and 1 mg/kg dose, only the fat extracts were investigated by gas chromatography.

a) Method of extraction.

For the extraction of TCD, organs and adipose tissues were homogenised in " Bühler" with 7,0 ml (fat and liver) or 5,0 ml of hexane (kidney, spleen, heart, stomach). After 10 minutes centrifugation at 4000 rpm, the supernatant solution was siphoned off and was again extracted with 1 x 7,0 ml and 3 x 10,0 ml of hexane (or with 1 x 4,0 and 2 x 5,0 ml). The extracts were combined in a 50 ml or 25 ml measuring flask (extracts with small content of active substance were concentrated to 10,0 ml in a rotary vacuum evaporator).

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b) Gas chromatography.

This was governed by the following conditions:

Column: steel, 1/8 inch, 6 ft, 60 - 80 mesh, 10% UCCW 982 on Chromosorb W (AW - DMCS)

Injection block temperature	265°C
Detector temperature	290°C
Column temperature	200°C
Chart speed	0,5 inch/min.

1,0 - 2,0 µl of the extracts were injected into the gas chromatograph. For comparison, 1,0 µl of a standard was subsequently injected; the standard was so selected that the peak areas of the standard and analysis solution were of an approximately identical magnitude. The evaluation was carried out by calculating the product from the height and width at half of maximum intensity of the peaks. The peak area of the sample used for analysis was compared with that of the standard. The range of detection extended over 4 - 10 ng of TCD/µl.

2. Determination of TCD separated out in the faeces unchanged, after a single oral administration.

From 10 male rats, 5 received 250 mg of TCD/kg each and 5 were given 125 mg of TCD/kg each in Lutrol 9 using a pharyngeal probe. Each test included 2 control animals. The faeces of animals placed on metabolism cages was collected during 72 hours.

a) Method of extraction.

The total amount of faeces from each animal separated out daily was weighed, frozen at - 15°C and, before processing, dried for 24 hours in a vaccum desiccator over phosphorus pentoxide. Subsequently, homogenisation was carried out in a Bühler apparatus with 15 ml of

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hexane, it was centrifuged for 4 minutes at 4500 rpm, the supernatant solution was siphoned off and the residue was again extracted with 4 x 20 ml of hexane. The extracts were transferred into a 100 ml measuring flask and the TCD concentration was determined by gas chromatography (conditions given earlier). If the concentration of the active substance was slight, the extracts were concentrated to 50 or 25 ml in a rotray vacuum evaporator.

3. Investigation of TCD decomposition products in urea.

10 female rats were given TCD by means of a pharyngeal probe in a dose of 1000 mg/kg and were subsequently placed, together with 5 control animals, over metabolism cages. The urea was collected during 72 hours and it was frozen up to the time of processing (-15°C).

a) Method of extraction.

In order to be also able to identify the decomposition products bound on glucuronic acid, half of the collected urea (24, 48 and 72 hour urea) from the treated animals and from the controls was adjusted to pH 4,5 (N CH_3COOH), it was mixed with β-glucuronidase (3000 Fishman units/ml) and incubated for 18 hours at 37°C. Subsequently, the enzymatically split and the untreated urea samples were adjusted to pH 9,0 (20% sodium carbonate solution) and shaken out four-times with the three-fold amount of hexane (centrifugation for 10 minutes at 4000 rpm). The hexane phases were combined and concentrated in a rotary vacuum evaporator at 45°C. The residue was absorbed in a small amount of hexane (1/100 of the original amount of urea).

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b) Thin-layer chromatography.

The urine extracts were separated on silica gel G with chloroform/benzene/ammonia (127:22:1) as eluent (2 x in the same direction of motion) . For rendering the individual fractions visible, spraying was carried out with Rodamin B -solution, Folin's and Gibb's reagent (Rodamin B: 0,05% in abs. ethanol ; Gibb's reagent: 1,0% solution of dichloroquinone chlorimide in 96% ethanol: Folin's reagent: conc. solution (Merck) to dilute with water in a ratio of 1:3).

For the isolation of the TCD decomposition products, the hexane extracts of the 24 and 48 hour urine treated with β-glucuronidase were applied onto a large number of plates (layer thickness 0,5 cm). For the localisation of the metabolites, on completion of the test, the border strips of chromatograms were sprayed with reagent solution. The silica gel layer at the height of individual metabolites was scraped out and was transferred to centrifugal tubes (100 ml). By repeated washing of the silica gel with hexane (3 x 80 ml, centrifugation for 10 minutes at 4000 rpm), it was possible to dissolve out the still strongly contaminated metabolites. The extracts were concentrated in the rotary evaporator to 0,5 - 1,0 ml, the concentrated solutions were again chromatographed (2 x in the same direction of motion), the fractions were once more eluted with hexane from the silica gel and subjected to investigation by gas chromatography.

B. H C D .

Investigations for the detection of HCD in the adipose tissue, after a single oral administration.

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By using a pharyngeal probe, 5 rats each were given orally 1,4 mg of HCB/kg in Lutrol 9 (1,0 ml of solution/kg). They were killed 96 hours after the begin of the experiment. Two untreated animals were used as controls.

a) Method of extraction.

The extraction of the adipose tissue was carried out according to the method given for TCD.

b) Gas chromatography.

The following conditions were valid for the gas chromatographic investigation:

Column: steel, 1/8 inch, 6 ft, 100 - 120 mesh, 10% SE 30, Chromosorb W , (AW-DMCS)

Injection block temperature	265°C
Detector temperature	290°C
Column temperature	235°C
Chart speed	0,5 inch/min.

It was possible to identify 4 - 10 ng/ul.

Results.

A. T C D .

1. The gas chromatogram of the pure TCD mixture of isomers used for our tests showed at least 8 peaks of which 3 could be identified by comparison with the corresponding pure substances. These were the following compounds :

2,4,4'-trichlorodiphenyl (2,4,4'-TCD),
3,4,4'-trichlorodiphenyl (3,4,4'-TCD) and
2,5,2'-trichlorodiphenyl (2,5,2'-TCD), see Fig. 1.

In the tissue of rats, from all the isomers, only 2,4,4'-TCD could be

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identified 96 hours after the administration of 250, 125, 10 and 1 mg of TCD/kg ; see Figures 2 and 3.

In the adipose tissue, 8 - 15% of 2,4,4'-TCD were accumulated, in the other organs only about 1% (related to that contained in the TCD mixture of isomers administered), see Tables 1 - 3 .

2. In the faeces, up to 72 hours after the end of the test, altogether about 10% of unchanged TCD mixture of isomers could be identified. The main amount was separated out after 48 hours. In addition to the TCD spectrum, gas chromatograms of the treated animals showed a number of further peaks which were absent in the controls and which could not be identified; see Fig. 4. It is possible that we deal with decomposition products of TCD separated out via the bile.

3. Unchanged TCD was not found in urine. After break-down with β-glucuronidase, the thin-layer chromatographic separation gave a greater number of decomposition products which showed a phenol-type reaction. 6 main metabolites were found, see Fig. 5.

It was possible to separate to a large extent metabolite 6 by repeated thin-layer chromatography from the accompanying substances and it only showed 1 peak in the gas chromatogram, see Fig. 6.

B. H C D .

Gas chromatographic analysis showed that, even at a concentration of 1,4 mg of HCD/kg, the spectrum of the total mixture of isomers could be identified in the fat extract, see Fig. 7. The accumulation in the adipose tissue amounted to about 15%. An exact evaluation was not possible.

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Discussion.

In the organism, the isomeric trichlorodiphenyls become hydroxylated to a certain percentage and are, bound on glucuronic acid, separated out in urine. Introduction of OH-groups into the molecule takes predominantly place in p- and o-positions. If these are partly occupied, as for instance in 2,4,4'-TCD, hydroxylation and, consequently, separation can only occur to a very limted extent, if at all. This is possibly the reason for the accumulation of this particular isomer in the adipose and organ tissue.

The concentration of the TCD mixture of isomers separated out unchanged in the faeces was much smaller than expected. On the other hand, peaks occurring additionally in the gas chromatograms indicated decomposition products of TCD. These may possibly separate out via the bile. But there is also the possibility of decomposition by intestinal bacteria.

In contrast to TCD, the total spectrum of isomers could be identified in the adipose tissue, after the administration of HCD. Unfortunately, no pure isomers were available, so as to determine how high was the percentage of the p and o-chlorinated compounds in the mixture of isomers.

Summary.

1. The single oral administration of 250, 125, 10 and 1 mg of TCD mixture of isomers/kg caused in rats an accumulation in the tissue by only one of the isomeric compounds identified as 2,4,4'-TCD.

In the adipose tissue, 8 - 15 %, in other organs about 1% of 2,4,4'-TCD

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could be identified.

In the faeces, up to 72 hours after oral administration, unchanged TCD was separated out. Beside the spectrum of the mixture of isomers, the gas chromatograms of the faeces extracts of treated animals showed some additional peaks which could not be identified.

In urine, unchanged TCD was not identified. The separation in thin-layer chromatography gave, after break-down with β-glucuronidase, a number of metabolites with phenolic OH-groups.

2. In contrast to the results obtained with TCD, the entire spectrum of isomers was identified in the fat extracts, after the single administration of 1,4 mg/kg of HCD. The accumulation in the adipose tissue amounted to about 15%.

Signatures of the two authors.

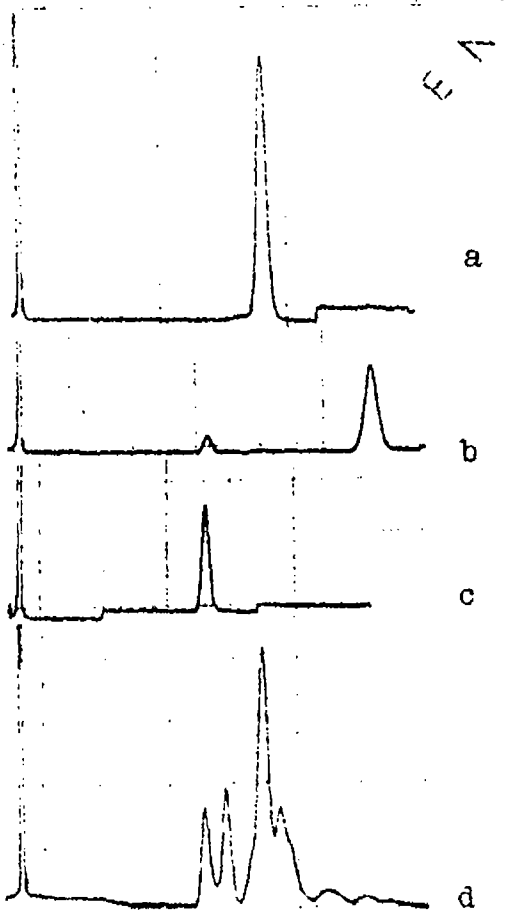


Figure 1

Gas chromatograms of

a. 2,4,4'-TCD, 4,2 ng/µl
b. 3,4,4'-TCD, 4,0 ng/µl
c. 2,5,2'-TCD, 2,2 ng/µl
d. TCD mixture of isomers, 11,9 ng/µl

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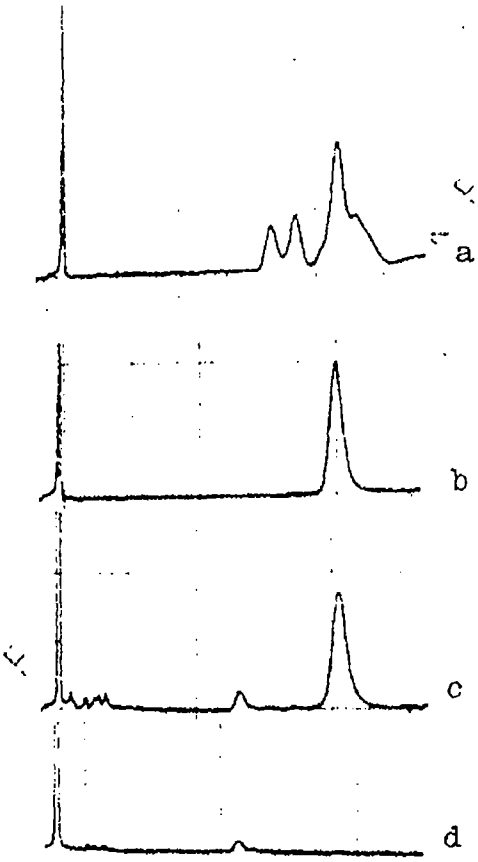


Figure 2.

Gas chromatogram of

a. TCD mixture of isomers, 6,08 ng/µl
b. 2,4,4'-TCD (pure), 2,28 ng/µl
c. fat extract TCD rat (10 mg TCD/kg)
d. fat extract (control rat).

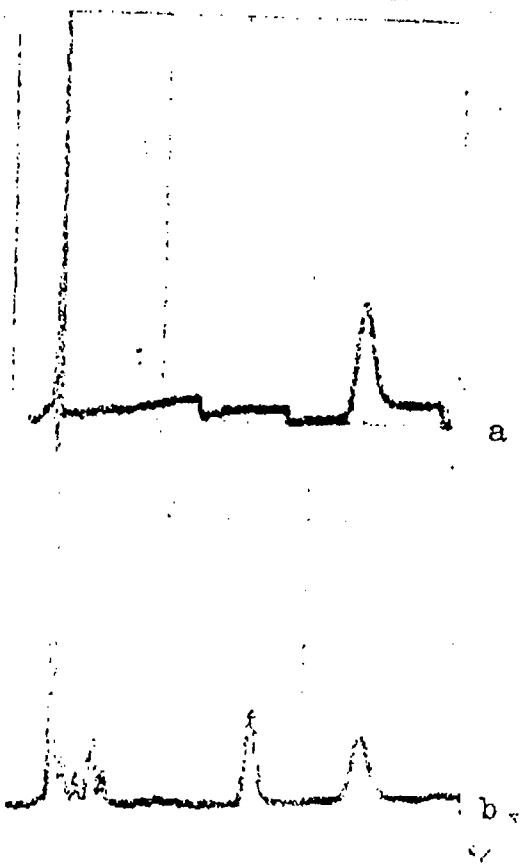


Figure 3.

Gas chromatogram of

a. 2,4,4'-TCD (pure) 1,4 ng/µl
b. fat extract TCD rat (1,0 mg/kg)

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Table 1: Detection of 2,4,4'-trichlorodiphenyl (2,4,4'-TCD) in the adipose tissue of the rat (♂) after a single oral administration of 250, 125, 10 and 1 mg of trichlorodiphenyl (TCD,mixture of isomers)/kg (mean values from 5 animals each)

Dose TCD mg/kg	Absolute dose		Accumulation of 2,4,4'-TCD in the fat	
	T C D mg	2,4,4'-TCD mg	mg	%
250	92,0	30,66	2,53	8,2
125	44,5	18,83	2,15	14,5
10	3,31	1,16	0,107	9,2
1	0,33	0,11	0,011	10,0

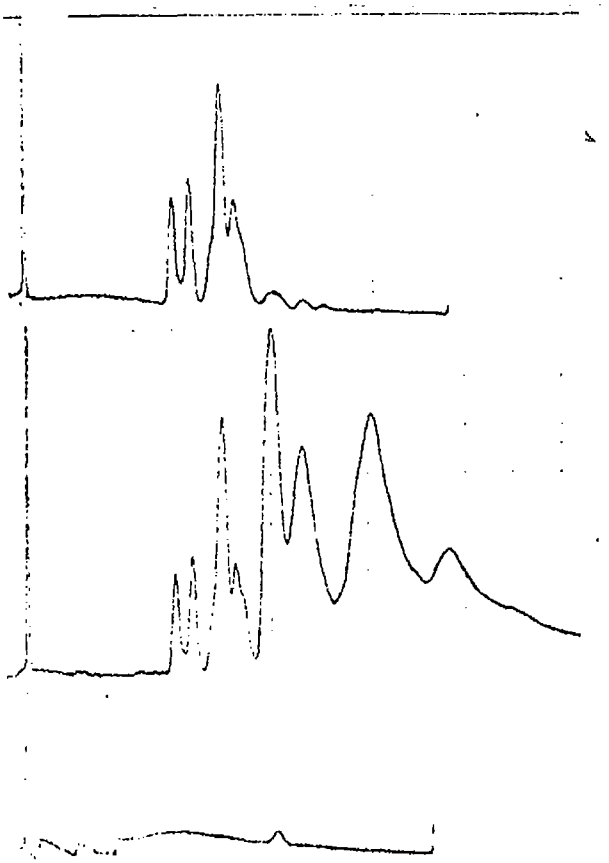


Figure 4.

Gas chromatogram of

a. pure TCD,mixture of isomers,23,856 ng/µl
b. faeces extract TCD rat (125 mg/kg)
c. faeces extract control rat

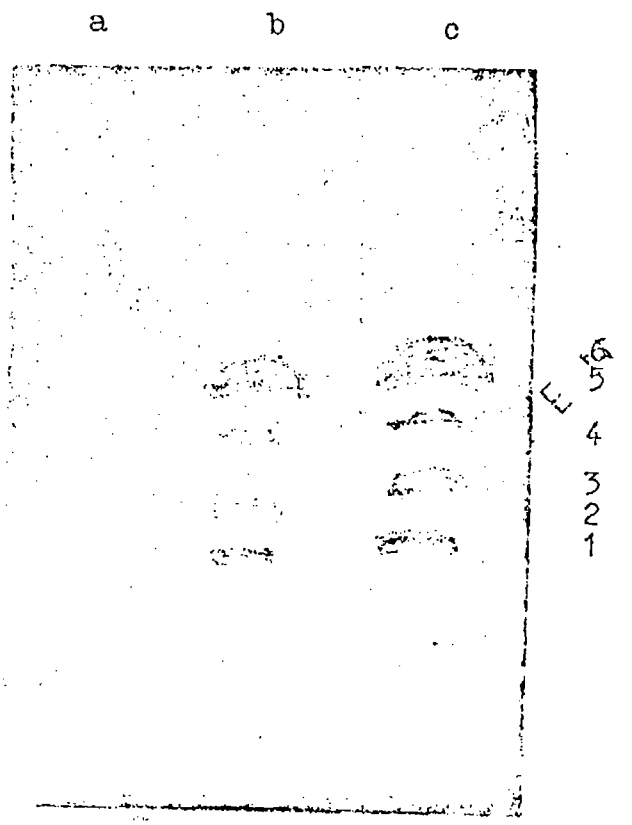


Figure 5.

Thin-layer chromatogram of urine extracts obtained after break-down with β-glucuronidase (pH 9)

Eluent: chloroform/benzene/ammonia (127:22:1)
Spray: Gibb's reagent.
a. control urea b. 24 hour urea
c. 48 hour urea.

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Table 2: Identification of 2,4,4'-trichlorodiphenyl (2,4,4'-TCD) in the adipose tissue of the rat (♂) after a single oral administration of:

1. 250 mg TCD (mixture of isomers) / kg

Rat No.	Absolute dose		Adipose tissue in g	Accumulation of 2,4,4'-TCD in the fat	
	T C D mg	2,4,4'-TCD mg		mg	%
6	95,00	31,67	3,550	1,94	6,1
7	96,25	32,08	11,963	4,50	14,0
8	93,75	31,25	5,730	3,23	10,3
9	88,75	29,58	10,516	1,75	5,9
10	86,25	28,75	6,507	1,23	4,2

2. 125 mg TCD (mixture of isomers) / kg

Rat No.	Absolute dose		Adipose tissue in g	Accumulation of 2,4,4'-TCD in the fat	
	T C D mg	2,4,4'-TCD mg		mg	%
1	42,5	14,17	7,696	1,75	12,4
2	45,6	15,21	8,928	2,10	13,8
3	42,5	14,17	8,309	3,15	22,2
4	44,9	14,96	9,003	1,75	11,7
5	46,9	15,63	6,395	2,00	12,8

3. 10 mg TCD (mixture of isomers) / kg

Rat No.	Absolute dose		Adipose tissue in g	Accumulation of 2,4,4'-TCD in the fat	
	T C D mg	2,4,4'-TCD mg		mg	%
11	3,45	1,15	7,4	0,1066	9,3
12	3,40	1,13	11,9	0,1011	9,0
13	3,00	1,00	11,4	0,0873	8,7
14	3,60	1,20	16,5	0,1379	11,5
15	3,10	1,33	8,5	0,1011	7,6

4. 1 mg TCD (mixture of isomers) / kg

Rat No.	Absolute dose		Adipose tissue in g	Accumulation of 2,4,4'-TCD in the fat	
	T C D mg	2,4,4'-TCD mg		mg	%
16	0,34	0,11	13,4	0,0145	13,2
17	0,33	0,11	11,9	0,0121	11,0
18	0,32	0,11	14,5	0,0114	10,4
19	0,34	0,11	12,6	0,0079	7,2
20	0,31	0,10	9,9	0,0096	9,6

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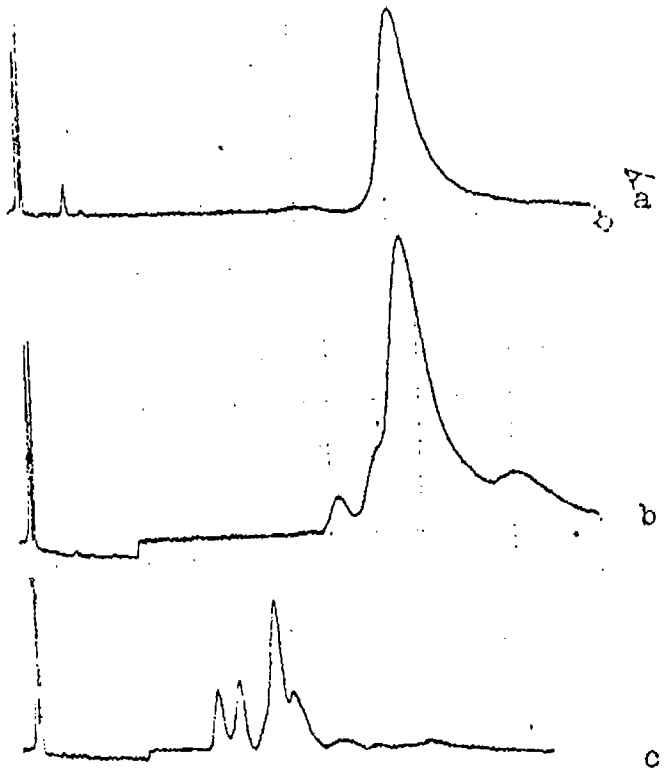


Fig. 6.

Gas chromatogram of

a. Metabolite 6, after three-fold thin-layer chromatography

b. Metabolite 6 after the 1. thin-layer chromatography

c. Spectrum of the pure TCD mixture of isomers.

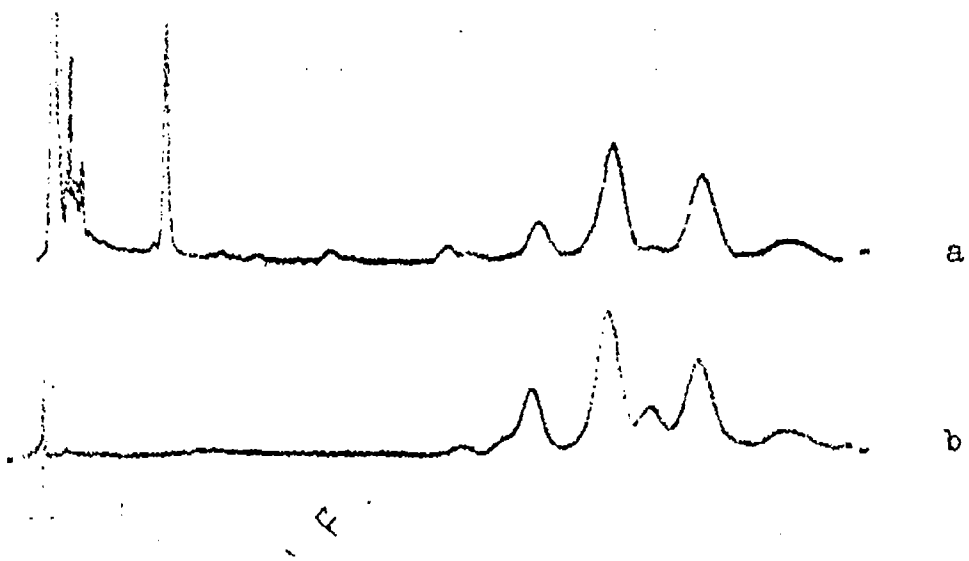


Figure 7. Gas chromatogram of

a. Fat extract HCD rat (1,4 mg/kg)

b. HCD mixture of isomers (pure), 6,64 ng/µl

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Table 3: Identification of 2,4,4'-TCD in the adipose tissue after a single oral administration of 250 and 125 mg trichlorodiphenyl (TCD, mixture of isomers)/kg

Mean values from 5 rats each (♂), killed 96 hours after the begin of the experiment

Dose mg TCD/kg	Absolute dose		FAT		LIVER		KIDNEY		SPLEEN		HEART		STOMACH		CONTENT OF STOMACH		TOTAL AMOUNT 2,4,4'-TCD	
	T C D	2,4,4'-TCD mg	mg	%	mg	%	mg	%	mg	%	mg	%	mg	%	mg	%	mg	%
250	92,0	30,66	2,53	8,2	0,171	0,6	0,023	0,07	0,006	0,02	0,0115	0,037	0,024	0,08	0,0013	0,004	2,7668	9,0
125	44,48	14,83	2,15	14,5	0,09	0,6	0,013	0,1	0,0014	0,01	0,0039	0,03	0,014	0,09	0,005	0,004	2,2813	15,3

Table 3: Selection of 2,4,4' – TCD in the rat tissue, in the single administration of 250 and 125 mg trichlorodiphenyl (TCD, mixture of isomers)/kg.
Mean values from 5 rats each (♂), killed 96 hours after the begin of the test

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