Rough Draft

Translation

INDUCTION OF MICROSOMAL LIVER ENZYMES AFTER POLYCHLORINATED BIPHENYLS (PCB) AND FOLLOWING STRESS

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ABSTRACT

One i.p. injection of PCB induces a high stimulation of rat liver microsomal drug oxidizing enzymes; equimolar doses of tetrachlorobiphenyls are distinctly more effective than dichlorobiphenyls. The effect can still be demonstrated distinctly four weeks after application. During this time the liver concentration of tetrachlorobiphenyls decreased to 7µg/g tissue whereas the concentration of adipose tissue rose to 252 µg/g. By stress tetrachlorobiphenyls are mobilized along with fat, causing an increase of liver concentration to 32 µg/g. Simultaneously, a new stimulation of microsomal activity can be seen, nearly equal to the maximum stimulation 4 days after i.p. application of 500 mg/kg b.w. Parallel to microsomal stimulation there is an increase of relative liver weight

Stress induced activation of microsomal activity caused by PCB redistribution is important for the evaluation of PCB in environmental toxicology, in view of rising PCB concentrations in human adipose tissue.

Besides DDT and its metabolites, polychlorinated biphenyls (PCB's) in an increasing extent are found in marine food chains, in feeds, birds, in human adipose tissue,

and in human milk (Koemann et al., 1969; Holmes et al., 1967; Risebrough et al., 1969; Biros et al., 1970). As far as the few qualitative analyses show, it is a matter of higher chlorinated PCB's (penta- to hepta-chlorobiphenyls) (Duke et al., 1971; Bagley et al., 1970). Experiments on animals with these compounds showed that a strong induction of microsomal enzymes could be induced (Risebrough et al., 1969). Since at present attempts have been made to replace the higher chlorinated PCB's by lower chlorinated compounds, the toxicological properties of these substances (di- to tetrachlorobiphenyls) are of a particular interest. In doing this, two exposure forms should be distinguished: the chronical absorption in the organism via a general environmental pollution with these compounds and an accute resorption of relatively large quantities through the commercial application of PCB's, among others as insulation, heat exchange, and hydraulic fluids.

Having described the distribution of the commercial PCB mixture Pydraul A 200^{R} (Monsanto) in the organisms of rats (Benthe et al., 1972) and having established an acute microsomal enzyme induction (Nissen, 1970), now we should investigate the transient duration of the enzyme induction and the possible mobilization of tetrachlorobiphenyl Aroclor 1248^{R} (Monsanto) deposited in the adipose tissue.

METHODS

Male Wistar rats (200 to 230 g) obtained a single intraperitoneal injection of tetrachlorobiphenyl (Aroclor 1248) and equimolar doses of dichlorobiphenyl (Aroclor 1232), respectively, (in the following abbreviated as TCB and DCB, respectively), in 1 + 1 mixture with olive oil, and until their killing obtained food (Altromin-R) and water in unlimited quantities. Rats of the same strain which obtained intraperitoneal injections of olive oil only serve as controls. The test animals were killed after the indicated periods of time (between 3 PM and 6 PM) by a blow in the neck and decapitation.

The conditions of stress consisted of withdrawal of food and of a surrounding temperature of 3°C.

Microsome Preparation

Immediately after killing the animals, the livers were removed and freed from blood in an ice-cold 0.1 m phosphate buffer of pH 7.4, were weighed, were chopped into small pieces in the ice-cold phosphate buffer of pH 7.4, were rinsed, and then were homogenized (Potter-Elvehjem) in 8 volumes of the fresh buffer. The homogenate was centrifuged for 10 minutes at 3000 xg and 20 minutes at 17,000 xg, the clear supernatant was siphoned off and was centrifuged 60 minutes at 105,000 xg (Rotor 30, Spinco L2). The deposited microsomes, after decanting the supernatant and after rinsing the loosely packed upper layers with a fresh phosphate buffer while leaving the glycogen sediment, were rehomogenized and centrifuged again during 60 minutes at 105,000 xg. The microsomes washed in this manner were taken up in the fresh buffer in such a way that about 40 mg protein/ml were suspended.

The oxidative O-demethylation of p-nitroanisole, measured according to the Netter (1963) method, served as a measure of the activity of the microsomal enzymes. In these tests, 2 ml incubation batch contained 200 µmol phosphate buffer of pH 7.4, 5.5 µmol glucose-6-phosphate (Boehringer), 12.5 µmol nicotinic acid amide, 30 µmol $MgCl_2 \cdot 6\ H_2O$, 0.3 µmol p-nitroanisole, 2 mg microsomal protein, 800 mU glucose-6-phosphate dehydrogenase. The incubation batch was saturated with O_2 and was filled into a 1 cm cell.

After about 3 minutes preincubation at 37°C, the reaction was started with the addition of 0.02 µmol NADP. The formation of p-nitrophenol was registered by the extinction increase at 420 nm in spectrophotometer PM Q_2 (Zeiss) against a reference cell (without NADP) in the time intervals of 1 minute over a period of 15 minutes. The reaction proceeded to about the 10th minute with a constant rate. For the microsomes of the untreated normal animals the p-nitroanisole conversion amounted to 6.72 nmol/mg microsomal protein/10 min. at 37°C. This value was verified in each series by means of 2 control animals, was set 100%, and the enzyme activity of the rats treated with tetrachlorodiphenyl was referred to it.

Analysis

The determination of the protein contents of the microsome preparations was carried out by the biuret reaction with bovine albumin as a standard substance. To determine the PCB contents in the liver, 1 g of the tissue was ground with anhydrous Na_2SO_4 to a dry powder, the powder was extracted twice with 40 ml $CHCl_3$ (analytical grade), the combined $CHCl_3$ extracts were evaporated in a rotary evaporator to dryness, and the residue (in the following designated as total lipide) was determined gravimetrically. The extraction of PCB from the total lipide was carried out according to Grant et al. (1971), and the quantitative determination was accomplished according to Benthe et al. (1972). By this method, the yield amounted to 85 $\pm$ 5.1%.

All the chemicals were of analytical grade, hexane and heptane were spectrally pure (Uvasol, Merck), G-6-P, G-6-PDH, NADP were obtained from Böhringer Co., p-nitroanisole, analytical grade, from Schuchardt. The donation of tetrachlorodiphenyl Aroclor^R 1248 and of dichlorobiphenyl Aroclor^R 1232 by Monsanto is acknowledged.

RESULTS

To find a suitable dose, PCB was administered to the rats in increasing quantities, and the microsomal activity increase was measured after 2 and 4 days, respectively (Table 1).

According to these tests the threshold dose is about 5 mg TCB/kg, while that for DCB is considerably higher. In further studies of TCB, to expect a definite microsomal activity increase, a dose of 500 mg TCB/kg was chosen. As Table 1 shows, an equimolar dose of DCB has a considerably weaker effect.

Table 1. Increase of the Oxidative Demethylation as a Function of Equimolar Quantities of TCB and DCB; Average Values from 4 Animals ± s

mg PCB/kg	Days After Injection	Microsomal p-Nitroanisole Demethylation % of Control
Control	2	100[a] ± 4.6
Control	4	100 ± 4.6
5 TCB	2	146 ± 65.0
5 TCB	4	108 ± 31.2
25 TCB	4	146 ± 35.8
50 TCB	4	208 ± 20.0
500 TCB	4	488 ± 14.0
38.2 DCB	4	146 ± 25.0
382 DCB	2	315 ± 34.4
382 DCB	4	266 ± 52.7

[a]100% = 6.72 ± 0.31 nmol p-nitrophenol/mg microsomal protein/10 min.

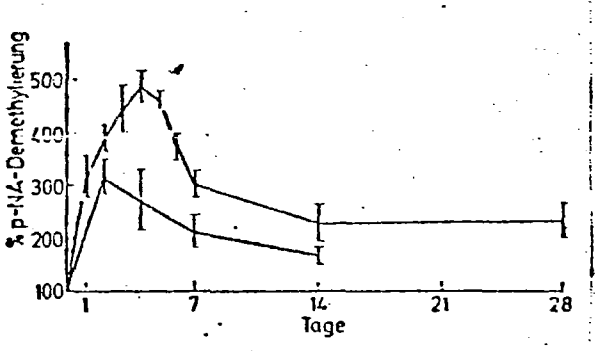

Figure 1. Demethylation of p-nitroanisole in percent of the control (100% = 6.72 nmol ± 0.31 nmol p-nitrophenol/mg microsomal protein/10 min) as a function of time after a single i.p. injection of 500 mg TCB (upper curve) and 382 mg DCB/kg KG (lower curve), respectively. Average values from 5 rats ± s.

A test of the transient course of the induction effect shows that the maximum is reached 3 to 4 days after 500 mg TCB/kg, up to the 7th day a rapid decrease of the activity takes place, and after that it hardly decreases and consequently, even 28 days after the TCB application, the microsomal activity exceeds the control value by more than two-fold. Individual measurements 8 and 13 weeks after application still

always gave increased values. On the other hand, relative to TCB, the extent of activation after an equimolar dose of DCB is considerably lower, and the maximum seems to be reached after 2 days. However, the effect is still clearly detectable after 14 days (Figure 1).

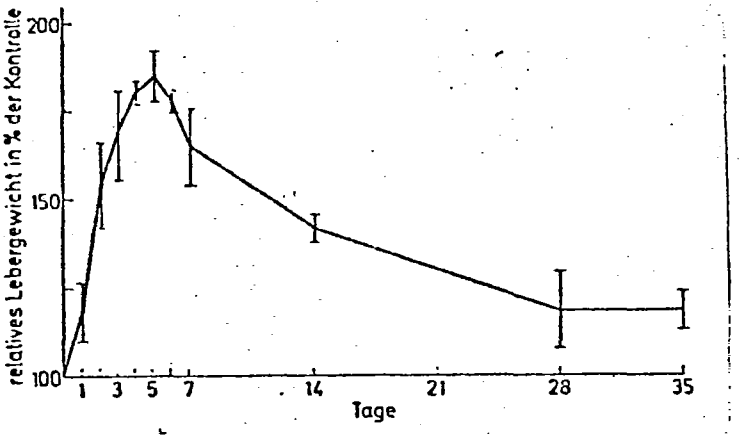

Figure 2. The relative liver weight (g/100 g body weight) in percent of the control as a function of time after a single i.p. injection of 500 mg TCB/kg body weight. Average values from 6 rats (the value after 35 days from 4 rats) ± s.

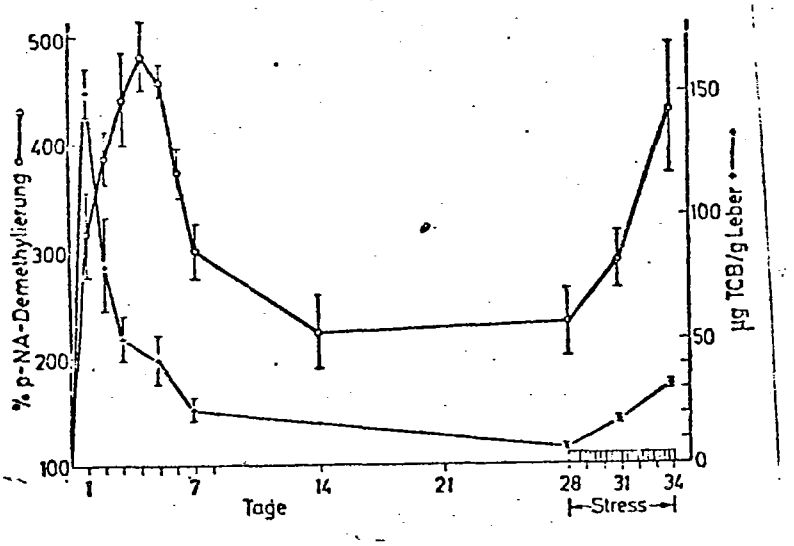

Figure 3. The TCB concentration of the liver and the microsomal p-nitroanisole demethylation (in % of the control) as a function of time after a single dose of 500 mg TCB/kg body weight (see Figure 1) and afterthe 28th day of the stress duration. For every 6 control animals 100% = 6.72 ± 1.5 (28th day), 13.2 ± 2.15 (31st day), 10.2 ± 1.2 (34th day) nmol p-nitrophenol/mg microsomal protein/10 min.

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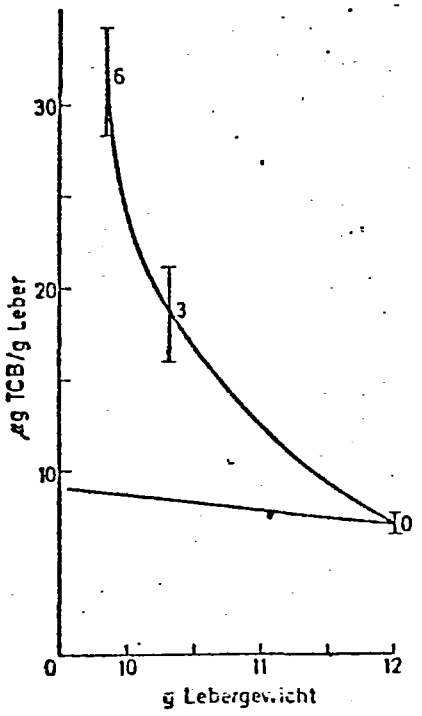
An increase of the liver weight is parallel to the activity increase (Figure 2). A steep weight increase within the first 5 days is followed by a slow decrease, and only after 4 weeks the liver weight approaches that of the controls.



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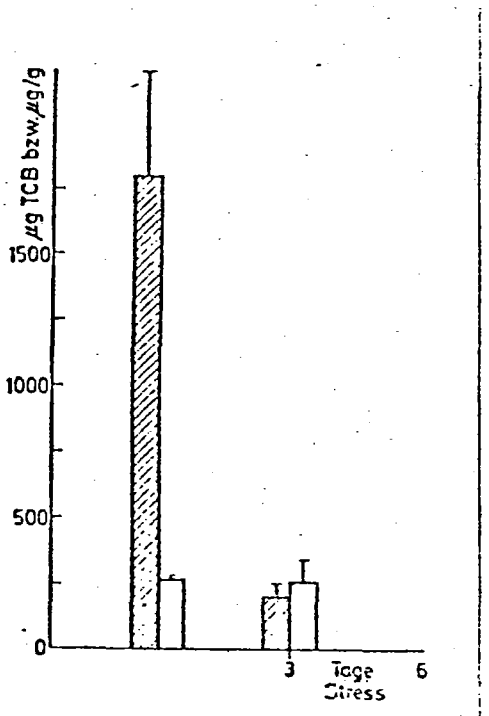

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Figure 4. The TCB accumulation in the liver under stress conditions. The upper curve: TCB concentration at the beginning and after 3 and 6 days stress plotted against the corresponding liver weights. The lower curve: conversion of the TCB concentration at the stress start (28 days after the TCB application) to the decreasing liver weights.

Figure 5. The TCB quantity (µg TCB) in the total retroperitoneal adipose tissue (shaded column) and the TCB concentration (µg/g adipose tissue) after a single injection of 500 mg TCB/kg body weight and after the following the 3 day long stress. Average values from 6 rats $\pm s_x$.

For the PCB concentration in the liver (Figure 3) a maximum value (150 µg TCB/g) is measured 24 hours after the application which then decreases within 7 days in about exponential course to a value of 22 µg/g, after which it still decreases only slightly.

A comparison of the TCB contents with the microsomal stimulation shows that the stimulation proceeds parallel to the PCB content in the liver with a time phase shift of 2 to 3 days.

It is known from previous distribution studies (Benthe et al., 1972) that PCB is redistributed in the adipose tissue. In the present investigation the PCB concentration amounted to 252 µg/g adipose tissue 28 days after application. Now the question arises whether under stress conditions the PCB stored in the adipose tissue is mobilized and triggers a renewed increase of the enzyme activity in the liver. For this purpose, 28 days after the PCB application the rats were exposed to a surrounding temperature of 3°C and they were deprived of food.

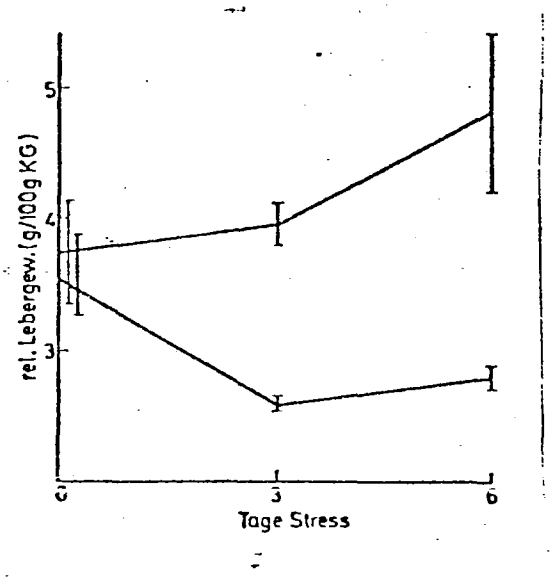

Figure 6. The course of the relative liver weight (g/100 g body weight) of the TCB treated rats (upper curve) and of the untreated control animals under the stress conditions. The starting value four weeks after a single i.p. injection of 500 mg TCB/kg body weight.

For two animal groups after 3 and 6 days the following values were determined: O-demethylation, TCB concentration of the liver and of the adipose tissue and the relative liver weight. As a control, we used under the same conditions the control animals of the same rat group which were treated with olive oil. As Figure 3 shows, the TCB concentration in the liver increased more than two-fold after 3 days stress (from 7.0 to 18.2 µg/g), and to 32 µg/g after 6 days stress. This concentration increase cannot be explained by the decrease in the absolute liver weight. When we plot the PCB starting concentration as a function of the liver weight, we obtain the lower curve of Figure 4. The actually measured concentrations correspond to the

upper curve and show an extremely steep increase. This increase is based on a mobilization of PCB from the adipose tissue. Figure 5 shows that under stress the TCB quantity in the entire adipose tissue decreases, but the TCB concentration (µg/g fat) remains constant due to lipolysis. After 6 days of stress, the entire retroperitoneal adipose tissue dissipated so that no measurement could be carried out anymore.

With the increase of the TCB concentration in the liver, a renewed microsomal stimulation takes place, which after 6 days of stress amounts to 434% and, therewith, reaches again almost the maximum of the initial stimulation after 500 mg TCB/kg.

The weight increase of the liver found at the beginning is measurable again under the stress conditions (Figure 6): while the relative liver weight of the control group abruptly decreases in the first 3 days, it shows a clear tendency toward an increase in the animals treated with TCB.

DISCUSSION

In the investigations published to date on the induction of microsomal liver enzymes in rabbits (Villeneuve, 1971), rats (Street, cited from Peakall and Lincer, 1970) and falcons (Lincer and Peakall, 1970) with higher chlorinated biphenyls (penta- to hepta-chlorobiphenyls) singular observations were always made at the end of the chronical feeding tests. Nissen (1970) was, however, able to show that even a single, short-lasting exposure caused this induction.

As Table 1 and Figure 1 show, low chlorinated biphenyls after a single application lead also to a microsomal stimulation. Now it was of interest to ask how rapidly TCB is eliminated from the liver and how long, in relation to it, the increased activity of microsomal enzymes remains. While from the classical inducer phenobarbital, for example, the microsomal aminopyrine-demethylase is again found normal 4 days after the last injection (Orrenius and Ernster, 1964) the increased enzyme

activity after DDT lasts for several weeks (Ghazal et al., 1964). Consequently, the activity of TCB can be very well compared with that of DDT: 4 weeks after the TCB administration, the activity of p-nitroanisole demethylase is decreased to 47% of the maximum value and still remains exactly as high as after 2 weeks. Individual measurements after 8 to 13 weeks still did not give a normal value. The TCB contents of the liver decreases rapidly within the first 3 to 5 days, but after that only very slowly. After 4 weeks there are still 5% of the initial value present in the liver (Figure 3). This finding is in good agreement with the analyses of Grant et al. (1971), who 3 weeks after administration of 500 mg pentachlorobiphenyl (Aroclor 1254)/kg still found 13% of the concentration of the 2nd day. Likewise, the increase of the relative liver weight by Aroclor 1254 is also valid for TCB (Aroclor 1248). The transient course (Figure 2) is parallel with the microsomal activity and reaches a maximum, the double weight of the control, with 6.75% (of the body weight). Besides, it is questionable whether this enormmous increase can be explained only by the increase of the endoplasmic reticulum.

When we compare the transient course of the TCB contents of the liver with that of the adipose tissue, it becomes clear that a large part of TCB is redistributed from the liver into the adipose tissue, as we were also able to detect after the PCB application as aerosol (Benthe et al., 1972).

Now we raise the question: is the TCB deposited in the adipose tissue mobilizable when the adipose tissue is dissipated under lipolytic conditions? For DDT this redistribution from the adipose tissue was reported many times (Dale et al., 1962; Brown, 1970; Findlay and Freitag, 1971). Moreover, when 7 ug TCB/g liver are still sufficient for maintaining the microsomal activity on a higher level, then in the case of a redistribution a repeated increase of the induction could be expected under the condition that the TCB can act sufficiently long on the liver. Figures 4 and 5 show a confirmation of our hypothesis that during dissipation of the adipose tissue TCB is mobilized and the liver concentration increases again.

The attained concentration with 32 µg/g liver tissue corresponds to about the 7 day value after an i.p. injection. The increase of the O-demethylation after 3 days stress with nearly 300% corresponds to the 7 day value and after 6 days reaches again almost the maximum value. The increases refer to the untreated control animals of the same colony which were exposed to the same conditions. This is also therefore important since the conditions of fasting alone lead to an increase in the activity of the microsomal enzymes (Figure 3), as was already shown by Kato (1967) and Gram et al. (1970). The increase of the relative liver weight also agrees with these findings. In addition, it should be noted that the decrease of the absolute body weight in the groups showed no significant differences.

Therewith, it can be considered as proved that under the lipolysis conditions a concentration of 260 µg TCB/g adipose tissue is sufficient for inducing microsomal enzymes of the rat liver. Although this concentration is 40 times higher than in the adipose tissue of a normal person (6 ppm) (Quentin, 1972), but in view of the long retention in the adipose tissue in an occupational exposure, it can easily lead to an accumulation up to these concentration ranges.

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