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DETOXIFICATION OF THE ESTERTIN STABILIZER BIS-(β-CARBOBUTOXYETHYL)TIN DICHLORIDE IN RATS BY HYDROLYSIS OF THE ESTER BOND

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SUMMARY

In a previous comparative toxicity study with alkyltin and estertin stabilizers, it was recognized that estertin compounds displayed in vitro lymphocytotoxic effects comparable to the dialkyltin compounds, but did not induce lymphoid atrophy when administered in vivo to rats as was found for the dialkyltin compounds. This discrepancy between the in vitro and in vivo toxicity of estertin compounds prompted us to study the metabolism of the estertin compound bis-(β-carbobutoxyethyl)tin dichloride (CBETC) in rats.

The hydrolysis product bis-(β-carboxyethyl)tin dichloride (CETC) was the only metabolite detectable using TLC. After daily intravenous administration of 20 mg CBETC/kg body weight CETC was detected in urine only, whereas no faecal excretion of organotin was found. Intravenous administration of relatively large amounts of 20 mg CETC/kg body weight indicated that this compound is not metabolized but rapidly excreted in urine, probably because of its hydrophilic nature.

Daily gavage of 15 mg CBETC/kg body weight resulted in the excretion of appreciable amounts of CETC in urine, but CETC was also found in faeces together with the parent compound. In the gastrointestinal tract

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Abbreviations: CMETC, bis-(β-carbomethoxyethyl)tin dichloride; CBETC, bis-(β-carbobutoxyethyl)tin dichloride; CETC, bis-(β-carboxyethyl)tin dichloride; DOTC, di-*n*-octyltin dichloride; DBTC, di-*n*-butyltin dichloride; HPTLC, high performance thin-layer chromatography; PCV, pyrocatechol violet; PVC, polyvinyl chloride; TLC, thin-layer chromatography.

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CETC would be formed locally probably by acid hydrolysis of CBETC as was shown also in vitro in acidified water. Esterases in the gastrointestinal tract, tissues and blood might also be responsible for the fast hydrolysis of CBETC. As shown in our previous study the hydrolysis product CETC did not cause any lymphocytotoxic effect. Therefore we conclude that in the rat the estertin compound CBETC is effectively detoxified by hydrolysis of the ester bond.

Key words: Estertin compounds; Estertin detoxification; Estertin hydrolysis

INTRODUCTION

Estertin compounds represent a new class of organotin compounds relatively recently introduced as stabilizers for PVC plastics [1,2].

Comparative toxicity studies between the dialkyltin compounds, di-*n*-butyltin and di-*n*-octyltin dichloride, and the estertin compounds, bis-(β-carbobutoxyethyl)tin dichloride (CBETC) and bis-(β-carbomethoxyethyl)tin dichloride (CMETC), revealed that when administered in vivo to rats the estertin compounds were much less toxic than dialkyltin compounds [3]. In 2-week feeding studies with the estertin compounds CMETC and CBETC a slight reduction in body weight gain and in liver weight was observed at a dietary level of 1350 mg/kg but not with lower amounts. In addition, CBETC produced a slight reduction of weights of thymus and spleen at this feeding level. At a dietary level of 450 mg of the estertin compounds/kg, toxicological effects were not observed, whereas 2-week feeding of the dialkyltin compounds, di-*n*-butyltin dichloride (DBTC) and di-*n*-octyltin dichloride (DOTC), at levels of 150 mg/kg in diet already induced a significant growth retardation, increased liver weights and a pronounced lymphoid atrophy. Thymus involution [4] associated with suppression of the immunoreactivity [5—7] was found to be the most sensitive criterion for the toxicity of dialkyltin compounds, which already occurred at dietary levels of 5 mg/kg. This immunosuppressive effect of DBTC and DOTC, probably related to a selective lymphocytotoxic and/or lymphocytostatic action [7—10], was not induced by the estertin compounds. However, in in vitro studies with rat lymphocytes both dialkyltin and estertin compounds [3] revealed a similar behaviour.

An explanation for the discrepancy between in vivo and in vitro effects may be an effective detoxification of the estertin compounds in vivo. Hydrolysis of the ester group was hypothesized to be one of the possible mechanisms for such a detoxification [3]. This possibility was suggested by the finding that bis-(β-carboxyethyl)tin dichloride (CETC), the hydrolysis product of CBETC, was inactive in both in vivo and in vitro test systems.

In this report the in vivo metabolism of the estertin compound CBETC was studied. The results obtained support the hypothesis of the hydrolysis

of the ester group, since after both oral and intravenous administration of CBETC the only detectable metabolite found was CETC.

MATERIALS AND METHODS

Materials

The estertin compound bis-(β-carbobutoxyethyl)tin dichloride (CBETC)-$[CH_3(CH_2)_3OOC(CH_2)_2]_2SnCl_2$ and its hydrolysis product bis-(β-carboxyethyl)tin dichloride (CETC)-$[HOOC(CH_2)_2]_2SnCl_2$ were provided by Dr. R.S. Talbot, AKZO Chemie UK, Ltd, Littleborough, England.

Animals

Specific pathogen-free Wistar-derived rats (WU-CPB) from the Central Institute for Breeding of Laboratory Animals, TNO, Zeist, were maintained at room temperature of 23 ± 2°C and in a relative humidity of 50—60% with a 12-h light/dark cycle. They were housed in individual metabolism cages allowing for separate collection of urine and faeces. Diet (Muracon, from Trouw and Co, Putten) and tap water were constantly available.

Treatment of animals

Oral treatment studies. CBETC and CETC were given by gavage in a vehicle consisting of a mixture of absolute ethanol and corn oil (1:9, by vol.). The test compounds were first dissolved in ethanol and while swirled on a whorl mixer, the corn oil was added slowly.

Groups of 3 male rats, each weighing 160—180 g, were daily administered 0 or 15 mg of CBETC or CETC/kg body weight in a vol. of 0.5 ml for 4 days. The daily urine and faeces production was recorded and separate samples from the individual animals were collected. Faeces samples were dried at 50°C. The urine samples were stored at 4°C. The rats were killed by decapitation 6 h after the last gavage.

The body weights and the weights of thymus, liver and kidney were recorded. The contents of the gastroinstetinal tract parts, i.e. stomach, ileum and caecum of each rat, were sampled (dried) and processed for identification of the organotin compounds and their metabolites, together with the urine and faeces samples.

Intravenous treatment studies. The vehicle used for the i.v. administration of the organotin compounds was a mixture of absolute ethanol/polysorbate 80/phosphate buffered saline (5:2:93, by vol.). The test compounds were first dissolved in ethanol and then mixed with polysorbate 80; finally, while the mixture was swirled on a whorl mixer, the saline solution was added slowly. Groups of 3 male rats, each weighing 140—150 g, were daily administered i.v. 0 or 20 mg of CBETC or CETC/kg body weight, in a vol. of 0.2 ml for 2 days. Urine and faeces of each rat were collected daily and processed for identification of estertin compounds and their metabolites. Rats were killed by decapitation 24 h after the last injection. The body weights and the weights of thymus, liver and kidney were recorded.

Sample preparation

Urine samples. Five milliliters of the urine, daily collected from each individual rat, was evaporated to dryness in a rotor vaporizer at 35°C, and the residue was resuspended in 1 ml of a solution of water/ethanol/acetic acid (5 : 4 : 1, by vol.). After centrifugation for 5 min in a micro-hematocrit centrifuge (Heraeus-Christ GmbH, Osterode/Harz, Germany), 2.5 μl of the supernatant was used for thin-layer chromatography (TLC) analysis. To evaluate the possible degradation of the test compounds during processing, CBETC and CETC were added to control urine samples and processed identically for TLC analysis (internal controls).

Faeces, stomach, ileum and caecum samples. They were dried to constant weight at 50°C and ground to a fine powder with a mortar and pestle. Each 1 g of dried faeces and stomach content, and 300 mg and 200 mg of ileum- and caecum contents, respectively, were suspended in 5 ml ethanol acidified to pH 1—2 by addition of 25 μl of HCl. After treating these suspensions for 15 min in an ultrasonic bath (Bransonic 221, Branson Europe, Soest, The Netherlands), they were centrifuged for 5 min at 3000 rev./min. The supernatants were evaporated to dryness in a rotor vaporizer at 35°C and the residues of faeces and stomach contents were resuspended in 1 ml and of ileum and caecum contents in respectively 300 and 200 μl ethanol/acetic acid (9 : 1, by vol.). After centrifugation for 5 min in a micro-hematocrit centrifuge 2.5 μl (faeces) or 5 μl (stomach, ileum and caecum) or the supernatants were used for TLC analysis. To evaluate degradation of the test compounds during processing, CBETC and CETC were added to dried control samples and processed identically for TLC analysis (internal controls).

Identification of organotin compounds by thin layer chromatography

Silica gel 60 HPTLC plates (0.25 mm layer thickness, without fluorescent indicator, No. 5547, Merck AG, Darmstadt, F.R.G.) of 20 × 20 cm were used for analyzing the test compounds and their metabolites. The solvent systems used by Petrowitz [11] and Kimmel et al. [12], to analyze mixtures of alkyltin compounds by TLC, as well as more polar solvent systems consiting of various mixtures of water, ethanol and acetic acid were tested to separate the estertin compound CBETC and its hydrolysis product CETC. Together with samples prepared from the CBETC or CETC exposed rats, also samples of internal controls (see above) and pure CBETC and CETC, dissolved in absolute ethanol, were spotted on each TLC plate.

To detect the tin compounds the following chromogenic reagents were used: (a) A 0.1% (w/v) solution of pyrocatechol violet (PCV) (J.T. Baker, Philipsburg, N.Y.) in 95% ethanol which yields blue-violet spots on a yellow background with mono- and dialkyl organotin compounds and inorganic tin compounds [11,12]; and (b) a 0.1% (w/v) solution of dithizone (Merck AG, Darmstadt, F.R.G.) in chloroform which yields salmon spots in a violet background with the same compounds as coloured with PCV [11,12].

Before spraying the chromogenic reagent, the TLC plates were exposed

to bromine vapor for 20 min which increased the stability of the coloured spots [13], since the CBETC spot fades rapidly. The plates were always photographed immediately after the spraying.

RESULTS

Separation of CBETC and CETC by thin-layer chromatography

The solvent systems used by Petrowitz [11] and Kimmel et al. [12] to analyze mixtures of alkyltin compounds by TLC were not successful to separate sufficiently the estertin compound CBETC and its hydrolysis product CETC. A much better separation was achieved by a solvent system consisting of a mixture of water/ethanol/acetic acid (5:4:1, by vol.). The non-polar compound CBETC was found near the origin, whereas the more polar compound CETC was located near the front, with R_f values of about 0.2 and 0.8, respectively (Fig. 1A, track C). In urine samples the R_f value for CETC was somewhat lower, maybe due to the interfering influence of urine components, which appeared as a yellow coloured rounded spot

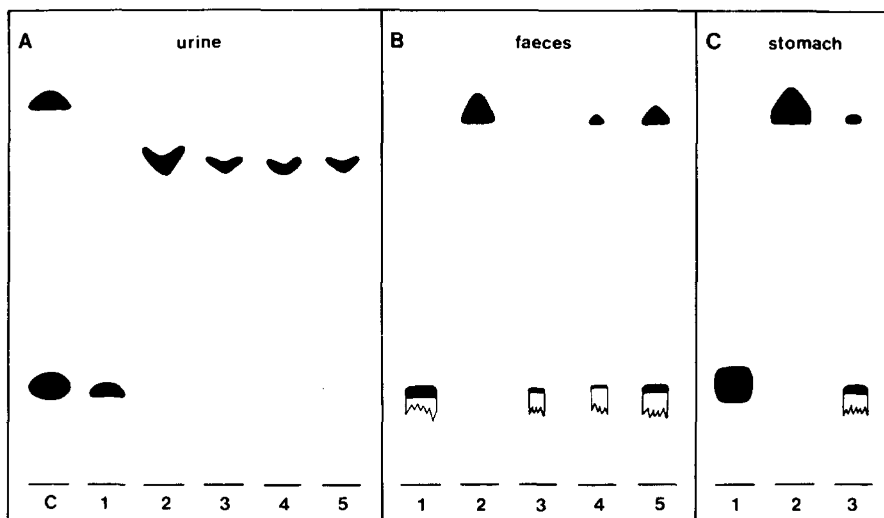

Fig. 1. TLC analysis of urine, faeces and stomach content from rats daily administered 15 mg CBETC/kg body weight by gavage for 4 days. Urine and faeces of the individual rats were sampled separately during day 1, 2 and 3, prepared for TLC analysis, and spotted in a vol. of 2.5 μl. (A) 3—5 urine samples and (B) 3—5 faeces samples from day 1, 2 and 3, respectively. Six hours after the last gavage stomach content was sampled, extracted, concentrated and spotted in a vol. of 5 μl (C, 3). As controls a sample of 10 μg CBETC and 10 μg CETC in ethanol (c), and samples of urine, faeces and stomach content containing 10 μg CBETC (A1, B1, C1) or 10 μg CETC (A2, B2, C2) were spotted in a vol. of 2.5 μl.

near the front and may also be responsible for the crescent shape of the CETC spot (Fig. 1A, track 2).

CBETC in faeces extracts did not show a clear concentrated spot but gave a more diffused elongated spot. In extracts of the gastrointestinal tract content similar shaped spots of CBETC were observed. The R_f values of CBETC and CETC were not changed. The detection limits of CBETC and CETC, added to control urine samples, were 0.6 μg/spot for CBETC and 0.3 μg/spot for CETC. In faeces extracts two times higher detection limits of 1.25 μg CBETC/spot and 0.6 μg CETC/spot were established. These data are based on colouring of the CBETC spot with dithizone and the CETC spot with the PCV-reagent.

Stability of CBETC and CETC during sample preparation and processing for TLC

Since it was observed that the estertin compound CBETC is easily hydrolyzed in water acidified with HCl to pH 2, the stability of CBETC and CETC during processing was tested by TLC of samples that were taken 2, 24 and 48 h after the addition of the compounds to the solvents used for faeces and urine extractions. In acidified ethanol, CBETC was not converted to CETC or any other detectable degradation product. CETC also appeared to be stable in the acidified ethanol. In the TLC solvent system (water/ethanol/acetic acid) only a slight conversion of CBETC to CETC was observed after a 48-h incubation at room temperature. Up to a 24-h incubation CBETC appeared to be stable in this solvent system. From CETC no degradation products could be detected. In the dried faeces samples no degradation of the test compounds was observed. However, in urine samples CBETC was unstable. Two hours after addition of CBETC to urine samples minor amounts of CETC were detectable. Degradation of CETC in urine did not occur.

Addition of CBETC or CETC to urine, faeces and gastrointestinal content, just before processing for TLC analysis, did not result in detectable amounts of degradation products (Figs. 1A,B,C; track 1 and 2).

Oral treatment studies

CBETC. Body weights and relative organ weights of thymus, liver and kidney as well as the urine and faeces production were not affected after a daily gavage of 15 mg CBETC/kg body weight for 4 days.

Thin-layer chromatography of the urine extract of the CBETC-exposed rats revealed that CETC was the only detectable metabolite (Fig. 1A). CETC was already detected in the urine sample on the first day. The parent compound CBETC was never detected in urine samples.

In faeces extracts the parent compound was present from the first day after administration (Fig. 1B). CETC was not detectable in the first-day faeces sample but appeared in increasing amounts after successive dosing (Fig. 1B). TLC analysis of the gastrointestinal content, 6 h after the last dose, showed that CBETC is already partly converted to CETC in the stom-

ach (Fig. 1C). In extracts of ileum and caecum content only the parent compound was present in detectable amounts. Any other degradation product than the hydrolysis product CETC was not found in faeces or gastrointestinal content.

CETC. Body weights and relative organ weights of thymus, liver and kidney, as well as the urine and faeces production, were not affected after the daily gavage of 15 mg CETC/kg body weight for 4 days. TLC-analysis of the urine extract showed only trace amounts of CETC (Fig. 2A). In faeces extracts clear CETC spots were observed during the experimental period (Fig. 2B). TLC analysis of the gastrointestinal content revealed clear spots of CETC as well (Figs. 2C,D,E). No degradation product was found in the samples from CETC exposed rats.

Intravenous treatment studies

After the i.v. application of a relatively high dosage of 20 mg CETC/kg body weight for 2 days no toxic effects were observed. The same dosage of CBETC induced a slight growth retardation (initial body weight was 145 ± 3 g, after 2 days the vehicle treated rats weighed 155 ± 1 g and the CBETC treated rats 147 ± 2 g) but did not affect the relative weights of liver, kidney or thymus. Urine production was remarkably increased by

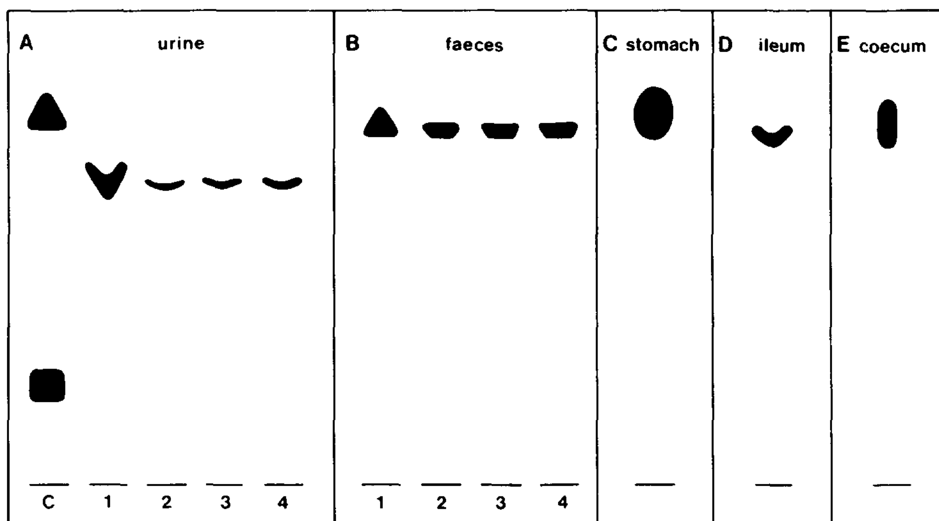

Fig. 2. TLC analysis of urine, faeces and gastrointestinal tract content from rats daily administered 15 mg CETC/kg body weight by gavage for 4 days. Urine and faeces of the individual rats were sampled separately during day 1, 2 and 3, prepared for TLC analysis and spotted in a volume of 2.5 μl. (A) 2—4 urine samples and (B) 2—4 faeces samples from day 1, 2 and 3, respectively. Six hours after the last gavage stomach- (C), ileum- (D) and caecum (E) content was sampled, extracted, concentrated and spotted in a vol. of 5 μl. As controls a sample of 10 μg CBETC and 10 μg CETC in ethanol (c), and samples of urine and faeces extracts containing 10 μg CETC (A1, B1) were spotted in a vol. of 2.5 μl.

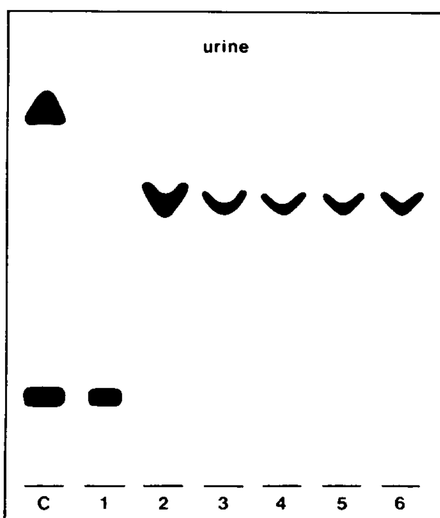

Fig. 3. TLC analysis of urine from rats daily administered 20 mg CBETC or CETC/kg body weight intravenously for 2 days. Urine sampled separately during days 1 and 2, respectively, from the CBETC (3,4) and CETC (5,6) exposed rats was extracted and spotted in a vol. of 2.5 μl. As controls a sample of 10 μg CBETC and 10 μg CETC in ethanol (c), and 10 μg CBETC (1) and 10 μg CETC (2) in urine was spotted in a vol. of 2.5 μl.

more than twice that of control animals. Chromatography of the urine extracts of the CBETC dosed rats revealed clearly detectable CETC spots on the first as well as the second day, whereas no other metabolite nor the parent compound CBETC could be detected (Fig. 3, spots 3,4). In faeces extracts no organotin compound was detected. After i.v. administration of CETC, this compound appeared to be only excreted in urine (Fig. 3, spots 5,6). No metabolite of CETC was detected.

DISCUSSION

From this study it appears that after both oral and intravenous administration, the estertin compound CBETC is effectively metabolized in rats to the dicarboxylic tin compound CETC. In rats intravenously dosed with 20 mg CBETC/kg body weight for 2 successive days, CETC excreted in urine was the only metabolite formed in a detectable amount. CBETC nor any metabolite other than CETC could be detected in urine or faeces extracts. Probably CBETC is readily converted into CETC by esterases, and because of its hydrophilic nature CETC is rapidly excreted by the kidney without further conversion. This is supported by netabolism studies with CETC. When this compound was given i.v. in relatively large amounts of 20 mg/kg body weight for 2 successive days it was the only compound excreted in urine.

After oral administration of 4 successive daily doses of 15 mg of CBETC/ kg body weight by gavage, CETC was found together with CBETC in faeces extracts. In urine CETC was the only metabolite present in detectable amounts. TLC analysis of the gastrointestinal tract content, 6 h after the last gavage, revealed that CBETC was already partly hydrolyzed to CETC in the stomach. This may be the result of an acid hydrolysis, since in vitro CBETC is converted to CETC in an acidified aqueous solution. Conversion in the gastrointestinal tract by esterase activity may further contribute to the presence of CETC in faeces. Once formed CETC will be poorly absorbed from the gastrointestinal tract. This is supported by oral treatment studies with CETC. After gavage of relatively large amounts of 15 mg CETC/ kg body weight for 4 successive days, only minor amounts of CETC were detected in urine.

Since CBETC is easily converted to CETC by acid hydrolysis, the possibility of a conversion during sample extraction and processing for TLC analysis was carefully examined. In urine hydrolysis of CBETC does occur. But it is very unlikely that CBETC will be present in urine, since this compound probably does not pass the kidney barrier, because of its lipophilic character. In other solvents used during the extraction and processing CBETC was found to be stable for a considerable time. Therefore we conclude that CETC is not produced artifactually during processing for TLC.

Although relatively high doses of CBETC or CETC were administered to rats, in order to get detectable amounts of metabolites, no severe signs of toxicity were observed. The intravenous administration of CBETC induced a remarkable diuresis. The total amount of urine produced was more than doubled. Also a minor growth retardation of these animals was observed. Relative organ weights were not affected and morphological organ changes were not observed. Oral administration of CBETC and CETC, and intravenous treatment with CETC as well, did not induce any sign of toxicity. These results are in agreement with those obtained from a comparative 2-week feeding study with diester and dialkyltin compounds [3]. In 2-week feeding studies the dialkyltin compounds DBTC and DOTC already induce lymphocyte depletion at dietary levels of 5 mg/kg [8,14], whereas CBETC did not effect the lymphoid system up to dietary levels of 1350 ppm [3]. However, in vitro a similar lymphocytotoxicity of CBETC and the dialkyltins was observed [3]. CETC was found to be inactive in both in vivo and in vitro test systems. Because of its 2 negatively charged carboxyl groups CETC is probably unable to penetrate the cell membrane. This may explain the minor absorption from the intestinal tract, the absence of general toxicity and in vitro cytotoxicity as well. Since CETC is the hydrolysis product of CBETC, it was hypothesized that the estertin compounds are effectively detoxified in vivo by hydrolysis. In this study it is demonstrated that in rats CBETC is easily converted to CETC, which is rapidly excreted in the urine.

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REFERENCES

1 R.E. Hutton and V. Oakes, Synthesis of novel substituted alkyltin halides, in J.J. Zuckermann (ed.), Organotin Compounds: New Chemistry and Applications. American Chemical Society, Washington, D.C., 1976, p. 123.
2 D. Lanigen and E.L. Weinberg, The use of estertin stabilizers in PVC, in J.J. Zuckermann (ed.), Organotin Compounds: New Chemistry and Applications. American Chemical Society, Washington, D.C., 1976, p. 134.
3 A.H. Penninks and W. Seinen, Comparative toxicity of alkyltin and estertin stabilizers. Food Chem. Toxicol., 20 (1982) 909.
4 W. Seinen and M.I. Willems, Toxicity of organotin compounds. I. Atrophy of thymus and thymus-dependent lymphoid tissue in rats fed di-*n*-butyltindichloride. Toxicol. Appl. Pharmacol., 35 (1976) 63.
5 W. Seinen, J.G. Vos, R. van Krieken, A.H. Penninks, R. Brands and H. Hooykaas, Toxicity of organotin compounds. III. Suppression of thymus-dependent immunity in rats by di-*n*-butyltin dichloride and di-*n*-octyltin dichloride. Toxicol. Appl. Pharmacol., 42 (1977) 213.
6 W. Seinen, J.G. Vos, R. Brands and H. Hooykaas, Lymphocytotoxicity and immunosuppression by organotin compounds. Suppression of graft-versus-host reactivity, blast transformation and E. rosette formation by di-*n*-butyltin dichloride and di-*n*-octyltin dichloride. Immunopharmacology, 1 (1979) 343.
7 W. Seinen and A.H. Penninks, Immune suppression as a consequence of a selective cytotoxic activity of certain organometallic compounds on thymus and thymus-dependent lymphocytes. Ann. N.Y. Acad. Sci., 320 (1979) 499.
8 W. Seinen, Immunotoxicity of alkyltin compounds, in R.P. Sharma (ed.), Immunological Considerations in Toxicology, Vol. 1, CRC Press, Palm Beach, FL, 1981, p. 103.
9 A.H. Penninks and W. Seinen, Toxicity of organotin compounds. IV. Impairment of energy metabolism of rat thymocytes by various dialkyltin compounds. Toxicol. Appl. Pharmacol., 56 (1980) 221.
10 A.H. Penninks and W. Seinen, The lymphocyte as target of toxicity: a biochemical approach to dialkyltin induced immunosuppression, in J.W. Hadden, L. Chedid, P. Dukor, F. Spreafico and D. Willoughby (Eds.), Advances in Immunopharmacology, Vol. 2, Pergamon Press, Oxford and London, 1983, p. 41.
11 H.J. Petrowitz, in E. Stahl (ed.), Thin-Layer Chromatography, 2nd edn., Springer Verlag, New York, 1969, p. 664.
12 E.C. Kimmel, R.H. Fish and J.E. Casida, Bio-organotin chemistry. Metabolism of organotin compounds in microsomal mono-oxygenase systems and in mammals. J. Agric. Food Chem., 25 (1977) 1.

13 R.R. Miller, Effects of dialkyltins in the thymus and thymocytes of rats. Thesis, University of Michigan, 1977, p. 47.
14 W. Seinen, J.G. Vos, I. van Spanje, M. Snoek, R. Brands and H. Hooykaas, Toxicity of organotin compounds. II. Comparative in vivo and in vitro studies with various organotin and organolead compounds in different animal species with special emphasis on lymphocyte cytotoxicity. Toxicol. Appl. Pharmacol., 42 (1977) 197.