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Effects of furazolidone, PCB77, PCB126, Aroclor 1248, paraquat and *p*,*p*′-DDE on transketolase activity in embryonal chicken brain

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Abstract

The effect of in ovo exposure to PCBs, DDE and paraquat on transketolase activity was measured in 19-day-old chicken embryos. Furazolidone was used as a positive control for decreased activity of the enzyme. The potency of contaminants to interact with transketolase was also tested in an in vitro system, using control brain $7000\times g$ supernatants containing the enzyme. No effects were found on transketolase activity after in ovo or in vitro exposure to PCB126, Aroclor, DDE or paraquat. PCB77 decreased transketolase activity in vitro, but only at concentrations that, extrapolated to in ovo exposure, would be lethal to the embryo. Furazolidone decreased transketolase activity both in ovo and in vitro. For this contaminant, thiamine residues were analysed in the yolk sacs, but no differences were found between exposed and non-exposed eggs. Transketolase is dependent on thiamine pyrophosphate as a cofactor, and therefore, the decreased enzyme activity could be the result of an interaction between furazolidone and thiamine metabolism. Since thiamine residues were not affected by furazolidone and transketolase inhibition in vitro was similar to the inhibition after in ovo exposure, it was concluded that furazolidone interacted with transketolase on the enzymatic level rather than by a depletion of thiamine. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Transketolase; Chicken embryo; PCB; DDE; Paraquat

1. Introduction

The pentose phosphate pathway yields chemical energy (NADPH) and intermediates for assimilation (Stryer, 1988) and is as such crucial for the functioning of the organism. In this pathway, transketolase catalyses the transition of xylulose-5-phosphate and ribose-5-phosphate into seduheptulose-7-phosphate and glyceraldehyde-3-phosphate. These products can then be integrated into the glycolysis. Transketolase is dependent on thiamine pyrophosphate (Giguère and Butterworth, 1987; Schenk et al., 1998), which is formed

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from thiamine and ATP by the enzyme thiamine-pyrophosphotransferase (Basilico et al., 1979). Thiamine depletion has been suggested to be related to reproductive failures in salmon (Bylund and Lerche, 1995; Fitzsimons et al., 1999). A toxicological factor was suggested to be involved in the mechanism causing thiamine depletion, as high levels of hepatic cytochrome P450 enzyme activity have been shown in fish that produced offspring suffering from thiamine deficiency (Norrgren et al., 1993). It has been hypothesised that reproductive failures in birds occur via a similar mechanism (de Roode et al., 2000), i.e. reproduction failures may result from contaminant-induced thiamine deficiency. Since transketolase is directly dependent on the availability of thiamine at the enzymatic level, decreasing enzyme activities are associated with reduced thiamine levels and therefore, transketolase activity was suggested as a biomarker for thiamine status in fish (Masumoto et al., 1987; Amcoff et al., 2000) and in developing bird embryos (de Roode et al., 2000). In addition, contaminants may cause a reduction in transketolase activity by a molecular interaction with transketolase itself, thiamine pyrophosphate or thiamine-pyrophosphotransferase, which can be annotated as direct inhibition. In this case, transketolase cannot bind to thiamine pyrophosphate: the bioavailability of thiamine to transketolase is reduced. An example of a compound which has been suggested to affect transketolase in this manner is furazolidone (Ali and Bartlet, 1982). In this paper, this way of interaction will be annotated as 'interaction on the enzymatic level'. Indirect inhibition would be caused by the interaction between contaminants and intermediary metabolism, depleting thiamine levels as suggested for fish by Åkerman et al. (1998) and Balk et al. (1998). This would eventually lead to insufficient levels of thiamine pyrophosphate for transketolase to retain its activity. Examples of such interactions may be contaminant induced CYP450 synthesis and activity, or redox cycling, which are both energy requiring processes and increase the demand for NADPH. In rats an interference of PCBs and DDT with thiamine metabolism has been described after exposure to these contaminants via their food. Thiamine levels were found to decrease in liver (Pélissier et al., 1992), blood and sciatic nerve, and transketolase activity was lower than in control animals (Innami et al., 1977; Yagi et al., 1979). Paraquat is known to induce redox cycling in the cell (Grant et al., 1980; Brown and Seither, 1983; Melchiorri et al., 1998; Sanderson et al., 1999), and has been shown to result in effects similar to those of thiamine deficiency in birds, including skeletal aberrations and reduced righting reflex (Swank, 1940; Shaw and Phillips, 1945; Smalley, 1973; Classen et al., 1992). Based on the combined results it is hypothesised that the presence of high concentrations of PCBs, DDT and paraquat in bird eggs may affect the biochemical pathway in which thiamine and transketolase are involved in the production of metabolical energy, the pentose phosphate pathway.

Our present study aims to test this hypothesis, i.e. do contaminants influence the biological availability of thiamine, and, if the bioavailability is reduced, is this caused by a depletion of thiamine or by the direct interaction between contaminant and transketolase or its cofactor, thiamine pyrophosphate? The selected chemicals, furazolidone, PCB77, PCB126, Aroclor 1248, paraquat and *p,p′*-DDE, were tested in ovo and in vitro. In this way, it was possible to distinguish between thiamine depletion and interaction on the enzymatic level. Furazolidone was used as a positive control as it was found to decrease transketolase activity in bird embryos (de Roode et al., 2000). The in ovo experiments were designed to expose embryos throughout development and then measure transketolase activity in the brains. For contaminants with capacity to induce cytochrome P450 activity, concentrations used were high enough to induce EROD activity (Bosveld et al., 1992; de Roode and Bosveld, in preparation), which, according to the hypothesis, could lead to a depletion of thiamine. As hypothesised, a decreased transketolase activity in ovo may result from either a depletion of thiamine or a direct interaction between contaminant and enzyme or cofactor. Therefore, for the compound that had an effect on transketolase in ovo, thiamine residue analysis was performed on yolk sacs, in order to

establish the role of thiamine depletion in the observed decreased transketolase activity. In order to assess the capacity of the contaminants to interact directly with transketolase or its cofactor, in vitro experiments were conducted. This was also performed for the contaminants that did not decrease transketolase activity in ovo; as much higher concentrations could be used than in the in ovo experiments, this would give insight into the actual capacity of contaminants to interact with transketolase. These capacities may be apparent at contaminant concentrations close to lethal concentrations, and therefore hard to detect in 'in ovo' experiments. The in vitro experiments consisted of exposure of control brain $7000 \times g$ supernatants, containing transketolase and cofactors (available from in ovo experiments), to different concentrations of the test compounds, and measuring of transketolase activity.

2. Materials and methods

Fertile Lohmann Brown chicken eggs were kindly provided by a local hatchery (Verbeek poultry farm, Lunteren). Propylene glycol (99%) was purchased from Boom, The Netherlands. Trichloric acid (TCA), ethylacetate, hexane, potassium hexacyanoferrate, Tris–HCl, $MgCl_2 \cdot 6H_2O$, sucrose, NaOH and tetrasodiumphosphate were obtained from Merck. Dimethylformamide and acetonitrile were from Rathburn. Thiamine hydrochloride, thiamine monophosphate, thiamine diphosphate, paraquat (99%), DDE (99%), furazolidone (*N*-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidone), triosephosphate isomerase, α-glycerophosphate dehydrogenase, xylulose-5-phosphate, ribose-5-phosphate, NADH, NADPH, fluorescamine and bovine serum albumin (BSA) were from Sigma. PCB77 (purity 99.9%), PCB126 (purity 99.1%) and Aroclor 1248 were from Promochem.

2.1. In ovo exposure

Eggs were injected on day 0 of incubation and incubated according to de Roode et al., 2000. Compounds were dissolved in yolk lipids and transketolase activity was measured in brain. Injected doses were 0, 0.00115, 0.0115, and 0.115 μg per egg for PCB126 ($n = 15$); 0, 0.015, 0.15, 0.3 μg per egg for PCB77 ($n = 10$); 0, 1, 10, 100, 1000, 10 000 μg per egg for Aroclor 1248 ($n = 10$); 0, 11.9, 119, and 1190 μg per egg for *p,p′*-DDE ($n = 15$); 0, 3.3, 33, and 330 μg per egg for paraquat ($n = 15$) and 0 and 1000 μg per egg for furazolidone ($n = 15$; positive control; data reported in de Roode et al., 2000). On days 12 and 15 of incubation, eggs were candled for viability and infertile eggs and dead embryos were discarded. After 19 days of incubation, eggs were opened and embryos were decapitated. Brains were dissected, frozen in liquid nitrogen and kept at −80 °C for enzyme measurements. An extra experiment was performed injecting furazolidone (the compound that caused a decrease in transketolase activity) using filter sterilised propylene glycol as a carrier, resulting in exposure doses of 0, 10, 100 and 1000 μg per egg, for thiamine residue analysis in yolk sacs.

2.2. In vitro experiment

$7000 \times g$ brain supernatants were incubated with contaminants in the transketolase assay as described below, in absence of ribose-5-phosphate and xylulose-5-phosphate. Concentrations ranged from 1 nM to 1 μM and were increased stepwise by a factor 10. After 20 min, the substrates were added and activity was measured for 5 min.

2.3. Transketolase activity measurements

Homogenisation of brains was performed using a glass potter tube and teflon plunger (Braun, Germany) at 1200 rpm. The homogenate was centrifuged at $7000 \times g$ for 12 min after which the supernatant was frozen in liquid nitrogen and stored at −80 °C until analysis. Transketolase activity was determined spectrophotometrically at 340 nm from the change in NADH in a coupled reaction assay as described by Tate and Nixon (1987) and adapted for use in a multiwell platereader by de Roode et al. (2000).

2.4. Protein determinations

Protein content in the supernatants was determined fluorimetrically against a BSA standard (Lorenzen and Kennedy, 1993).

2.5. Thiamine analysis

Yolk sacs were freeze dried overnight and homogenised using morter and pistle. Thiamine and its phosphate ester thiamine pyrophosphate (TPP) were determined according to Brown et al. (1998) with some modifications. Approximately 500 mg of sample was homogenised with 2.1 ml of ice cold 2% TCA. After boiling in a water bath and cooling in ice, homogenisation was repeated with 3.0 ml of ice cold 10% TCA. The homogenate was quantitatively transferred into a centrifuge tube and centrifuged at $14\,000 \times g$. The supernatant was washed four times with 8.8 ml of ethyl acetate:hexane (3:2, v:v). Thiochromes were prepared and filtrated to HPLC. The HPLC apparatus consisted of two Waters 510 pumps, a Waters 717 Plus Autosampler equipped with a cooling unit, a thermostated column oven, a Waters Scanning Fluorescence HPLC Detector 474 and Millennium 32 software. The column was Hamilton PRP-1 (150×4.1 mm^2, particle size, 5 μm). The injection volume was 20 μl and the total flow was 1.0 ml/min. The column temperature was adjusted to 35 °C. The analysing time was 27 min using the gradient flow from eluents A to B:1–4 min 100% A, 4–14 min linear increase in eluent B to 100% and 14–27 min 100% A. Eluent A was 0.5% acetonitrile and 99.5% 25 mM phosphate buffer, pH 8.4 and eluent B 25% dimethylformamide and 75% 25 mM phosphate buffer, pH 8.4.

Blank samples were made by substituting NaOH with water. Standards were subjected to the same procedure as the tissue samples. With each sample set a sample from an egg homogenate was run as control. The recoveries of the determination were 82.8% for TPP and 106.1% for free thiamine, and the coefficients of variation from 39 determinations of the egg homogenate were 94.6% for TPP and 8.9% for free thiamine. High coefficients of variation for TPP are due to very low concentrations of this compound in the egg homogenate, its proportion of the total thiamine was less than 1%.

2.6. Statistics

Statistical analyses were performed under Genstat. Differences in enzyme activities and thiamine contents were tested using a one-way ANOVA followed by a multiple range test, as well as using regression analysis with exposure dose as description variable. Regression analysis of maximum likelihood was performed on the in vitro data in order to find a dose-response. Multiple regression analysis was performed on the thiamine data to find out which form(s) governed the activity transketolase. The significance level was stated at 5%.

3. Results

In ovo transketolase activity ranged from 3.5 to 7.5 nmol/mg per min in controls. In ovo exposure to furazolidone decreased transketolase activity in embryonal brain by approximately 27% (Fig. 1). None of the other tested contaminants affected transketolase activity in bird embryos significantly in a dose dependent way (Fig. 1).

All compounds were tested in vitro as well to investigate if higher concentrations would affect transketolase activity by interaction on the enzymatic level rather than by depletion of thiamine. For furazolidone, the in vitro measurements revealed a significant dose dependent decrease in transketolase activity ($P < 0.001$; Table 1). PCB77 also decreased transketolase activity significantly; this was mainly due to the inhibition at the highest concentration ($P = 0.024$; Table 1). Aroclor, paraquat, p,p'-DDE and PCB126 did not influence transketolase activity at all ($P > 0.05$; Table 1).

Furazolidone was the only compound for which an in ovo, as well as an in vitro effect was observed, suggesting that the interaction was of enzymatic nature rather than one involving thiamine depletion. To validate this, thiamine levels were measured in yolk sacs. Thiamine pyrophosphate, free thiamine nor total thiamine levels were

affected by furazolidone ($P > 0.05$; Table 2). Neither a relation was found between transketolase activity and thiamine residues in the yolk sac ($P > 0.05$; Fig. 2).

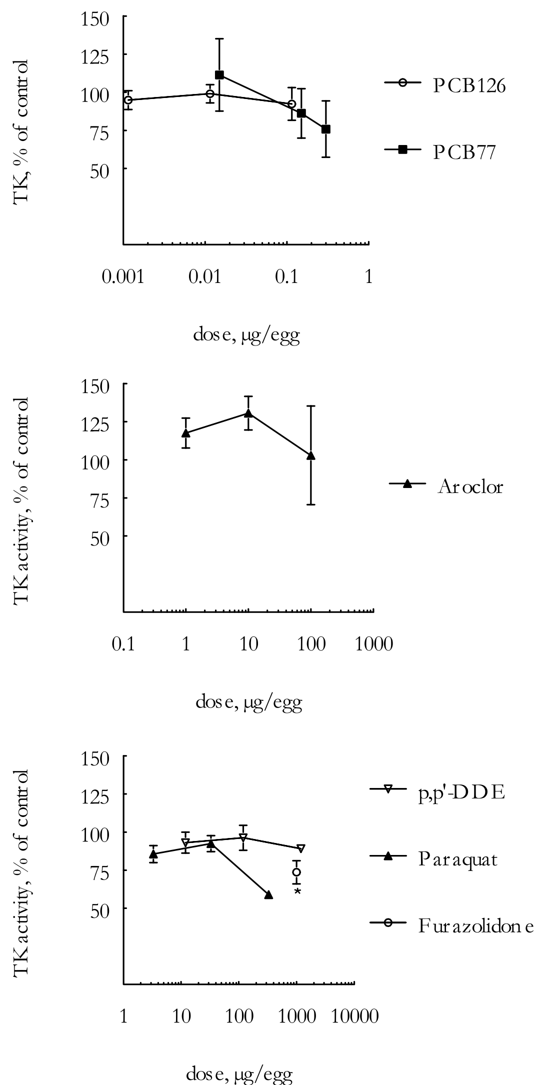

Fig. 1. Transketolase activity in brains of 19-day-old embryos exposed in ovo to different doses of contaminants, expressed as % of control values. Values presented are mean ± S.E. PCB126, $n = 11$, 13 and 2; PCB77, $n = 5$, 3 and 3; Aroclor, $n = 6$, 7 and 4; DDE, $n = 10$, 10 and 9, and paraquat, $n = 10$, 5, and 1 for increasing doses, respectively, and furazolidone, $n = 12$.

4. Discussion

In this study, no effects on transketolase activity were found in embryos after in ovo exposure to PCB77, PCB126, Aroclor 1248, *p,p′*-DDE or paraquat. For these compounds, enzyme activities were always within the range of activities found for controls. Although there seemed to be a decrease in transketolase activity after exposure to the highest dose of paraquat, this was not significant, as it was only based on one surviving embryo. In repeated experiments, no embryos survived at this highest dose, and the decrease in activity could, therefore, not be reproduced. In vitro data confirm that paraquat, *p,p′*-DDE, Aroclor and PCB126 do not interact with the enzyme, even at concentrations well above those expected in brain tissue after in ovo exposure. PCB77 showed a decrease in enzyme activity in vitro, but only at a concentration much higher than the applied dose in the in ovo experiment, assuming that the exposure concentration in the enzyme assay was comparable to 361 μg per embryo. This assumed exposure concentration in the embryo was calculated from the exposure concentration in the enzyme assay, which was added to 4 mg of brain, and thus could be expressed as pmol/mg brain. Assuming that this concentration resembles the concentration in the whole embryo, with an average embryo weighing 30 g, the exposure dose in the embryo was calculated by multiplying the concentration by 30 000. With such high exposure doses in ovo, no survival can be expected; the LD50 after 3 days exposure starting at day 7 of incubation was found to be 0.5 μg per egg (Brunström and Andersson, 1988). Although PCB77 and PCB126 have a similar structure, a decrease in transketolase activity in vitro was not observed for PCB126. The reason for this difference is not clear, however, it should be noted that the observed decrease in transketolase activity for PCB77 was solely caused by the measurements at the highest concentration (statistical analysis excluding these three points yielded a value for P well above 0.05).

For furazolidone, the highest concentration used in vitro was comparable to the dose injected and resulted in approximately the same percent-

Table 1
Transketolase activity in vitro in brain $7000 \times g$ supernatants incubated with different contaminants

nM	Fz	Paraquat	PCB126	Aroclor	p,p'-DDE	PCB77
0.001	n.d.	n.d.	n.d.	n.d.	n.d.	8.90 ± 0.07
0.01	8.93 ± 0.03	8.66 ± 0.41	7.72 ± 2.0	7.67 ± 0.84	8.32 ± 0.59	9.11 ± 0.18
0.1	8.26 ± 0.3	8.05 ± 0.41	8.93 ± 0.84	8.03 ± 0.37	7.41 ± 0.62	8.29 ± 0.69
1	8.45 ± 1.16	5.31 ± 2.01	8.47 ± 0.56	7.57 ± 0.37	6.88 ± 1.12	8.39 ± 0.49
10	9.03 ± 0.21	8.77 ± 0.59	8.34 ± 0.90	8.18 ± 0.70	7.28 ± 0.53	9.17 ± 1.21
100	7.28 ± 0.26; $P < 0.001$	7.80 ± 0.27	8.79 ± 0.32	7.09 ± 0.28	7.71 ± 0.34	n.d.
1000	5.25 ± 0.90; $P < 0.001$	7.79 ± 0.66	7.42 ± 0.84	7.54 ± 0.31	7.56 ± 0.67	7.07 ± 0.78; $P = 0.024$

Values shown are mean ± standard deviation. P-values indicate level of significance in difference as compared with control; n.d., not determined.

age of enzyme inhibition. This suggests that the effect of furazolidone on transketolase is the result of an interaction between furazolidone and either one of the enzymes or the cofactor involved. The assumption that furazolidone interacts with transketolase on an enzymatic level is confirmed by the results of the thiamine analysis. No effects were found on yolk sac residues of free thiamine, thiamine pyrophosphate or total thiamine, and there was no relation between yolk sac thiamine residues and transketolase activity. Although the relation between yolk sac residues and levels of thiamine in the cell is not exactly known, a decrease in yolk sac residues would be expected if transketolase activity was due to limited thiamine availability. Ali and Bartlet (1982) hypothesised that furazolidone inhibited thiamine-pyrophosphotransferase. However, no ATP is supplied to the system in the reaction assay, implying that the cofactor for transketolase (thiamine pyrophosphate) cannot be formed de novo during the reaction assay. Consequently, the inhibition of thiamine pyrophosphotransferase would not affect the availability of thiamine pyrophosphate in the in vitro test system. Therefore, the observed effects on transketolase activity in vitro is most likely due to a direct interaction between furazolidone and transketolase or thiamine pyrophosphate.

PCB126, Aroclor, DDE or paraquat did not affect transketolase activity in vitro or in ovo, whereas the observed effect of PCB77 on transketolase activity in vitro may be deceptive, as this was only observed at a concentration where no embryo would survive. Thus, the suggested toxicological factor involved in the mechanism of thiamine deficiency in fish (Norrgren et al., 1993), was not found to play a role in the developing chicken embryo as was tested in the present study.

Nevertheless, effects on transketolase activity have been described for mammals. PCBs and DDT decreased thiamine levels in mammals (Innami et al., 1977; Yagi et al., 1979; Pélissier et al., 1992), as well as transketolase activity (Yagi et al., 1979). If the mechanism behind these effects is based on an interaction between contaminants and thiamine bioavailability, the discrepancy between mammals and bird embryos may be explained by the different circumstances the animals live in. Thiamine is an essential vitamin, implying it should be present in the daily diet of adult mammals. However, bird embryos live in a closed egg with thiamine supplies stored in the yolk sac (Adiga and Ramana Murty, 1983). When present at sufficiently high levels, this may prevent the embryo from contaminant induced thiamine defi-

Table 2
Thiamine residues in yolk sacs of 19-day-old embryos exposed to different doses of furazolidone

μg per egg	TPP	Free thiamine	Total thiamine
0	0.44 ± 0.29	1.38 ± 1.49	2.23 ± 1.89
10	0.47 ± 0.25	1.07 ± 0.91	1.96 ± 1.15
100	0.54 ± 0.38	1.23 ± 0.39	2.14 ± 0.48
1000	0.23 ± 0.24	0.59 ± 0.48	1.20 ± 0.84

TPP, thiamine pyrophopshate; free thiamine, thiamine without phosphate groups.

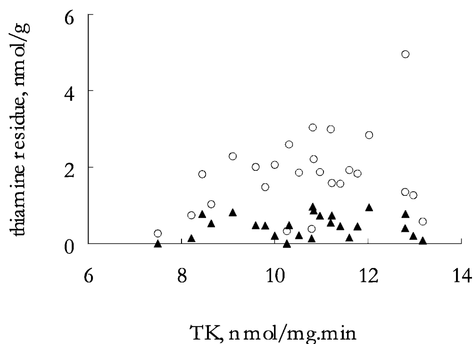

Fig. 2. Relation between yolk sac residues of thiamine (thiamine pyrophosphate, filled triangles; and total thiamine, open cirkels) and transketolase activity (TK) in livers of 19-day-old embryos exposed to different doses of furazolidone.

ciency and effects may only be observed when eggs receive reduced amounts of thiamine from the mother bird.

In conclusion, it was found that furazolidone inhibits transketolase activity, and that this effect as observed after in ovo exposure is due to an interaction between the compound and transketolase or its cofactor thiamine pyrophosphate. PCBs, paraquat and *p,p′*-DDE did not affect transketolase in the developing chicken embryo. It should be noted that up till now, no reports exist on the potency of other contaminants to interact with the pentose phosphate pathway in the developing bird embryo.

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