Metabolism of Xenobiotics in the Incubated Hen's Egg: Investigations with Ethyl 4-hydroxybenzoate

Lutz Kiep
D-Brunow

Summary
It is well known that in vitro preparations of the chick embryo can functionalise and conjugate selected model substances. Based on this biochemical knowledge an ex vivo model was developed to study xenobiotic metabolism in the incubated hen's egg. The xenobiotic is injected into the yolk sac, i.e. the nutritional compartment, on day 6 and metabolites are identified in the excretion medium of the embryonic kidneys (allantoic fluid) on day 11. During this developmental period the embryo lacks or has very limited sensitivity. Thus the model is in accordance with the 3R concept.
In the present investigation ethyl 4-hydroxybenzoate was chosen as a test substance. Concentrations of this paraben up to 24 mg/egg did not affect embryo viability. After inoculation of 6 mg/egg, 4-hydroxybenzoic acid and 4,4'-dihydroxy-L-ornithuric acid were identified in their free form. The 4-hydroxybenzoic acid was also eliminated in its conjugated form (glucuronide and/or sulphate). No unchanged paraben was excreted. 4,4'-dihydroxy-L-ornithuric acid [2,5-bis-(4-hydroxybenzoylamino)pentanoic acid] is a new metabolite. The structure of this amino acid conjugate was elucidated by synthesis and spectral methods (MS, ^{1}H and ^{13}C NMR).

Zusammenfassung: Metabolismus von Xenobiotica im bebrüteten Hühnerei - Untersuchungen mit Ethyl-4-hydroxybenzoat
Es ist seit langem bekannt, dass ausgewählte Modellsubstanzen mit in vitro Präparationen des Hühnerembryos funktionalisiert und konjugiert werden können. Auf der Grundlage dieser biochemischen Kenntnis wurde ein Modell zur Untersuchung des Metabolismus von Xenobiotica am bebrüteten Hühnerei entwickelt. Das Xenobioticum wird am 6. Tag in ein ernährendes Kompartiment des Bruteies (Dottersack) injiziert, und die Metaboliten werden am 11. Tag im Exkretionsmedium der embryonalen Nieren (Allantoisflüssigkeit) identifiziert. Innerhalb dieser Entwicklungsperiode ist von einer fehlenden oder eingeschränkten Sensitivität des Embryos auszugehen, wodurch sich das Modell in Übereinstimmung mit dem 3R Konzept befindet.
Für die vorliegende Untersuchung wurde Ethyl-4-hydroxybenzoat als Testsubstanz gewählt. Bis zu einer Konzentration von 24 mg/Ei beeinträchtigte das Paraben die Lebensfähigkeit der Embryonen nicht. Nach Inokulation von 6 mg/Ei wurden die Metaboliten 4-Hydroxybenzoesäure und 4,4'-Dihydroxy-L-ornithursäure in freier Form identifiziert. Die 4-Hydroxybenzoesäure wurde auch in konjugierter Form (Glucuronid und/oder Sulfat) eliminiert. Eine Exkretion unveränderter Ausgangssubstanz erfolgte nicht.
Die 4,4'-Dihydroxy-L-ornithursäure [2,5-Bis-(4-hydroxybenzoylamino)pentansäure] ist bisher nicht beschrieben worden. Die Struktur dieses Aminosäure-Konjugates wurde durch Synthese und spektrale Methoden (MS, ^{1}H und ^{13}C NMR) aufgeklärt.

Keywords: 3R, hen's egg, chick embryo, xenobiotic metabolism, ethyl 4-hydroxybenzoate

1 Introduction

Xenobiotic metabolism studies are performed routinely in laboratory animals. Numerous *in vitro* systems have been established for this purpose in the past few years (Worth and Balls, 2002; Gruber and Hartung, 2004). These include: (i) isolated and cultured hepatocytes; (ii) precision-cut liver slices; (iii) subcellular liver fractions; and (iv) heterologous expression systems. The avian embryo appears to represent another possible tool. *In vitro* preparations of the chick embryo can functionalise and conjugate selected model substances. The incubated hen's egg has previously been examined for its ability to ornithine conjugation and N-acetylation (Tab. 1). Species differences appear to be quantitative rather than qualitative except for the amino acid conjugation. Based on this biochemical knowledge an *ex vivo* model of the incubated hen's egg was developed to study xenobiotic metabolism (Kiep and Bekemeier, 1986). The xenobiotic is injected into the yolk sac, i.e. the nutritional compartment, on day 6 (d6) and metabolites are identified in the excretion medium of the embryonic kidneys (allan-

Received 20 April 2005; received in final form and accepted for publication 26 May 2005

toic fluid, AF) on day 11. Depending on the stage of development, the incubated hen's egg can be used both conservatively – in the sense of an animal experiment – and alternatively as a complementary method. Up to the end of the first half of the incubation period the embryo lacks or has very limited sensitivity (Peters et al., 1956). Investigations should be restricted to this period to avoid damage to the embryo (Rosenbruch, 1997). Consequently, this refinement is also in accordance with the 3R concept (Russel and Burch, 1959). A schematic overview of the model to study xenobiotic metabolism is presented in Figure 1.

Previous investigations (Kiep and Bekemeier, 1986; Kiep, 1997; Kiep et al., 2002) on this model were carried out with the slightly water-soluble substances sodium salicylate (metabolic reactions: aromatic hydroxylation, O-glucuronidation) and metamizol-sodium (metabolic reactions: aliphatic hydroxylation, deamination, N-acetylation, O-glucuronidation, O-sulphation). The method is evidently well suited to the study of these hydrophilic compounds. Also the main metabolic pathways these model substances take in the egg correspond to those taken in animals and humans. The fundamental suitability of this model was confirmed by investigations with (+)-methamphetamine (Neugebauer, 1995), 7-ethoxycoumarin (Clement and Mladek, 1995), p-nitrophenol (Clement and Mladek, 1996) and SFZ-47 (prodrug of an anti-inflammatory and analgesic agent) (Dong et al., 2003).

As the biokinetics of xenobiotics partially depend on their physico-chemical properties, the poorly water-soluble ethyl 4-hydroxybenzoate was chosen as a test substance for the following studies. This paraben is widely used as an antimicrobial preservative in pharmaceutical preparations, food products and cosmetics. Studies in rats (Derache and Gourdon, 1963; Kiwada et al., 1979), rabbits (Tsukamoto and Terada, 1964), cats (Phillips et al., 1978), dogs (Jones et al., 1956) and humans (Heim et al., 1957) have shown that the substance is absorbed rapidly, metabolised and eliminated. Corresponding results were found in humans with the substrate propyl 4-hydroxybenzoate (Sabalitschka and Neufeld-Crzellitzer, 1954). Urinary excretion of unchanged ethyl 4-hydroxybenzoate is very low, usually less than 1% of the administered dose. After ester hydrolysis, the main metabolites are the 4-hydroxybenzoic acid and 4-hydroxyhippuric acid in their free form. 4-hydroxybenzoic acid is also eliminated in its conjugated form (ester glucuronide, ether glucuronide, ether sulphate). The diglucuronic acid conjugate of 4-hydroxybenzoic acid was identified in dog urine (Quick, 1932). Protocatechuic acid (3,4-dihydroxybenzoic acid) was found in rabbit urine after application of 4-hydroxybenzoic acid (Bray et al., 1950). In rats and rabbits, the protocatechuic acid is partly methylated to vanillic acid (4-hydroxy-3-methoxybenzoic acid) (DeEds et al., 1957). The possible metabolic pathways of ethyl 4-hydroxybenzoate in animals and humans are summarised in Figure 2.

2 Materials and methods

2.1 Instruments and chemicals

The ESI (electrospray ionisation) mass spectrum (MS) was obtained with a Micromass LCT spectrometer. NMR (nuclear magnetic resonance) spectra were recorded in CD_3OD on a Bruker AC 300

Tab. 1: Biotransformation of selected model substances in the chick embryo

Reaction	Substrate	Source	Reference
Phase I			
Aromatic hydroxylation	Aniline	Liver microsomes	Powis et al., 1976
Aromatic N-heterocyclic hydroxylation	Antipyrine	Liver microsomes	Rifkind et al., 1982
Epoxidation	Benzo[a]pyrene	Culture of hepatocytes	Topp and van Bladeren, 1986
N-dealkylation	Aminopyrine	Culture of hepatocytes	Poland and Kappas, 1971
O-dealkylation	o-Nitroanisole	Liver microsomes	Strittmatter and Umberger, 1969
Oxidative deamination	Tryptamine	Liver homogenate	Ignarro and Shideman, 1968
S-oxidation	Chlorpromazine	Liver microsomes	Brodie and Maickel, 1962
Phase II			
O-glucuronidation	o-Aminophenol	Liver slices and liver homogenates	Dutton and Ko, 1966
O-sulfation	4-Methylumbelliferone	Embryo homogenate	Collett and Ungkitchanukit, 1979
N-acetylation	o-, m- and p-Aminobenzoic acid	Incubated hen's egg	Wolfe and Huang, 1959
O-methylation	Epinephrine	Liver cytosol	Ignarro and Shideman, 1968
Amino acid conjugation (ornithine)	Benzoic acid	Incubated hen's egg	Takahashi, 1928
Glutathione conjugation	Bromosulfophthalein	Liver homogenate	Brauer et al., 1963

spectrometer at 300.13 MHz (^{1}H) and 75.47 MHz (^{13}C); solvent signals were used as internal standards (^{1}H: 3.30, ^{13}C: 49.0). Ethyl 4-hydroxybenzoate (pharmaceutical grade, purity 99.6%, Lot-Nr. 0211A153) was purchased from Synopharm (Barsbüttel, Germany). The ethyl 4-hydroxybenzoate was dissolved in 1,2-propanediole to evaluate the substrate dose and for metabolism studies. Protocatechuic acid (3,4-dihydroxybenzoic acid) and vanillic acid (4-hydroxy-3-methoxybenzoic acid) were obtained from Serva (Heidelberg, Germany). 4-Hydroxybenzoic acid, 1,2-propanediole, β-glucuronidase/arylsulphatase, Extrelut® NT3-prepacked glass columns and PLC plates (silica gel 60 F_{254}/1mm with concentrating zone) were from Merck (Darmstadt, Germany). 4,4'-Diamino-L-ornithuric acid was synthesised according to a known methodology (Smith, 1958). All other chemicals were obtained from commercial suppliers and used without further purification. The following abbreviations are used: CD_3OD: methanol-d_4; Et_2O: diethyl ether; EtOAc: ethyl acetate; EtMeCO: 2-butanone; HOAc: acetic acid; MeOH: methanol.

2.2 Synthesis of 4,4'-dihydroxy-L-ornithuric acid

3 mL of 4 M HCl were chilled to 0° C in an ice/NaCl bath. Under stirring, a solution of 185 mg (0.5 mM) 4,4'-diamino-L-ornithuric acid and 70 mg (1 mM) sodium nitrite in 0.5 M NaOH (1 mL) was added dropwise at a temperature below 5° C. Five minutes after the last addition the reaction mixture was checked for free nitrous acid (iodine-starch paper) and the surplus was removed with a small amount of urea. Then the mixture was heated in a boiling water bath until nitrogen formation stopped. After cooling to room temperature the mixture was adjusted with 2 M NaOH to pH 2-3. Solid NaCl (1.5 g) was added to the mixture and then extracted exhaustively with Et_2O/EtOAc (1:1). The organic layer was dried with Na_2SO_4 and after that the solvent was distilled off *in vacuo*. The residue was dissolved in MeOH (500 µL) and purified with the solvent system $CHCl_3$/MeOH/HOAc (40:10:0.5; by vol.) by preparative TLC (thin layer chromatography).

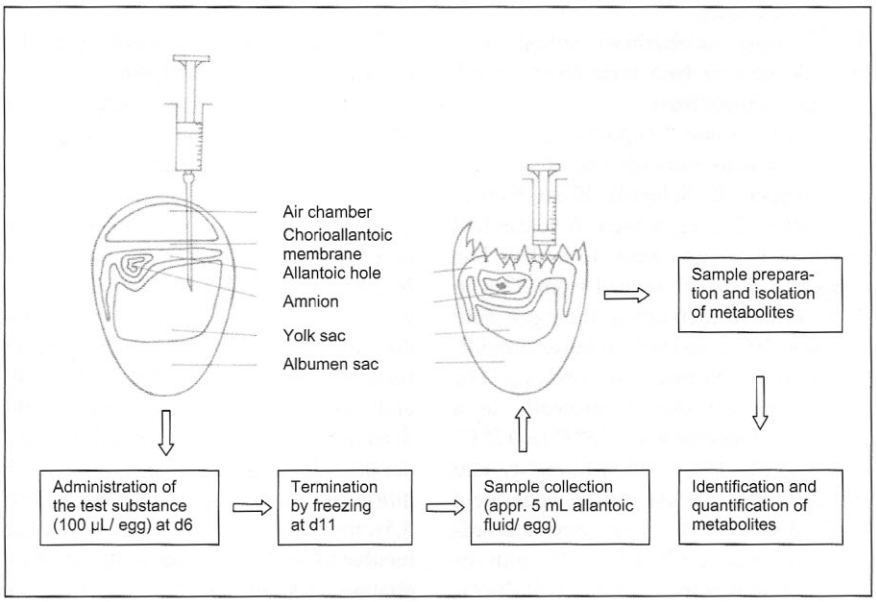

Fig. 1: Schematic overview of the model for studying xenobiotic metabolism in the incubated hen's egg

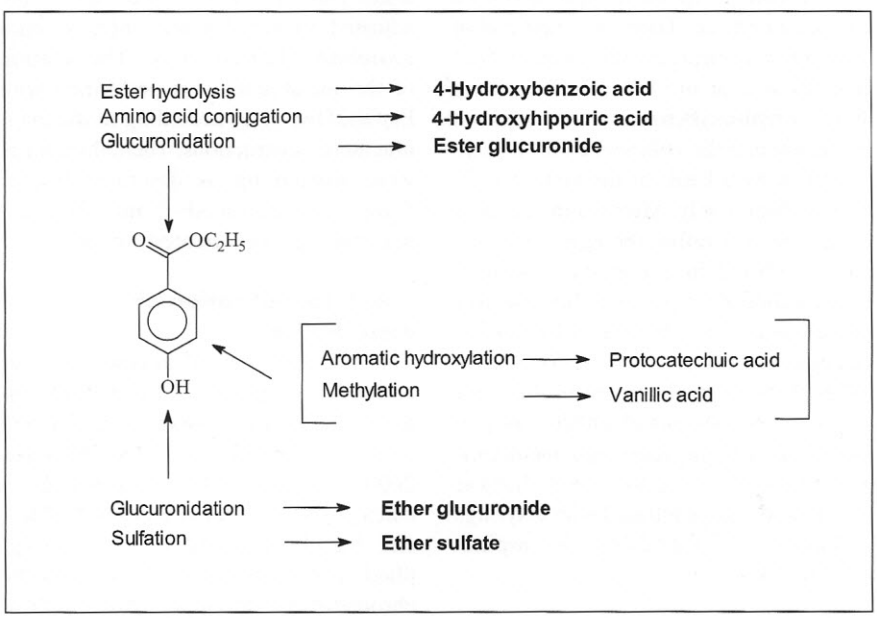

Fig. 2: Proposed metabolic pathways of ethyl 4-hydroxybenzoate in animals and humans

ESI-MS: m/z (%) = 417 $[M-H+2Na]^+$ (100), 395 $[M+Na]^+$ (40), 373 $[M+H]^+$ (3); ^{1}H NMR: δ (ppm) = 7.74 (d, J = 8.7 Hz, H-2'/H-6'[a]), 7.70 (d, J = 8.7 Hz, H-2"/H-6"[a]), 6.83 (d, J = 8.7 Hz, H-3'/H-5'[b]), 6.81 (d, J = 8.7 Hz, H-3"/H-5"[b]), 4.49 (m, H-2), 3.40 (m, 2H-5); ^{13}C NMR: δ (ppm) = 179.6 (C-1), 170.0 (C-7'[c]), 169.1 (C-7"[c]), 162.1 (C-4'[d]), 161.9 (C-4"[d]), 130.2 (C-2'/C-6', C-2"/C-6"), 126.6 (C-1'[e]), 126.5 (C-1"[e]), 116.0 (C-3'/C-5', C-3"/C-5"), 56.2 (C-2), 40.6 (C-5), 31.6 (C-3), 26.9 (C-4); [a-e] signals are exchangeable; numbering according to Fischer's projection. The following abbreviations are used: M: molecular ion; m/z: mass-to-charge ratio; δ: chemical shift; J: coupling constant; d: doublet; m: multiplet.

2.3 Bioassay

2.3.1 Egg incubation, administration of the test substance and sample collection

Fertilised "White Leghorn" eggs from free-range hens were obtained from a local supplier (E. Schmoll, Klein Krams, Germany). The eggs were first candled and defective eggs were discarded. The weight of the eggs ranged from 52 g to 60 g. Prior to incubation, the eggs were stored at 10° C and 90% relative humidity for 24 h with their large ends up. The eggs were incubated horizontally in a commercial incubator at 37.8° C (± 0.2° C) and 65% (± 2%) relative humidity and turned every 6 h. At d6 the eggs were candled and infertile eggs were discarded. Using a dentist's drill, a 1-2 mm diameter hole was made in the shell directly over the air cell, without damaging the subjacent shell membrane. Two hours before injection, the eggs were stored in the incubator in an upright position with the large ends up. Then, the blunt end of each egg was cleaned with ethanol (70%) and 0.1 mL of the test solution (6 mg ethyl 4-hydroxybenzoate) was injected vertically into the yolk sac; controls were treated with 0.1 mL of the vehicle (1,2-propanediole) only. After sealing the hole with melted paraffin, the eggs were further incubated in a horizontal position and candled daily for viability. On d11 the bioassay was terminated by placing the eggs in a freezer at -18° C for 30 min. After thawing to room temperature, the eggshell over the air chamber was prepared off and the inner egg membrane was removed. Then, the chorioallantoic membrane was penetrated with a syringe and the AF (5.5-7.2 mL/egg) was aspirated (Fig. 1).

2.3.2 Evaluation of the substrate dose

Embryonated eggs (d6) were divided into five experimental groups and one control group, with five eggs in each group. The eggs in the experimental groups were injected with 0.1 mL of ethyl 4-hydroxybenzoate solutions containing 1.5 mg, 3 mg, 6 mg, 12 mg and 24 mg, respectively. The control group was inoculated with the vehicle (1,2-propanediole) only. After examination (d11) the toxicological test was ended by freezing the eggs.

2.4 Bioanalysis

2.4.1 Sample preparation and isolation of metabolites

For the extraction of metabolites, the AF of 5 eggs was combined. Five millilitres of the pooled AF were adjusted to pH 2-3 with 1 M HCl and extracted exhaustively with Et_2O/EtOAc (1:1). The combined organic layers were dried with Na_2SO_4 and evaporated to dryness *in vacuo*. For TLC (2.4.2) the residue was dissolved in 100 µL MeOH. The aqueous layer was neutralised with 1 M NaOH and hydrolysis was performed for the detection of conjugated metabolites under the following conditions: (i) After addition of 500 µL of acetate buffer (pH 5.5), the solution (5 mL) was stirred and incubated with β-glucuronidase/arylsulphatase (100 µL) at 37.5- 38.5° C for 24 h. (ii) After addition of 500 µL of HCl (37%), the solution (5 mL) was stirred and heated for 1 h in a boiling water bath. The hydrolysed fluids were adjusted to pH 2-3 and injected onto Extrelut® NT3-columns. The elution of the metabolites was performed with Et_2O/EtOAc (1:1) according to the manufacturer's instructions. Then, the eluates were worked up as described before. Samples of control AF (5 mL) from uninjected eggs were treated the same.

2.4.2 Identification of metabolites

The identification of metabolites occurred by comparative TLC with the authentic reference substances in different solvent systems (Tyman, 1996; Wiltshire, 2000). TLC analyses were carried out on silica gel 60 F_{254} aluminium sheets (Merck). 1-5 µL of sample (2.4.1) were applied to a length of 5-15 mm and the chromatogram developed over a path of 10 cm in a paper lined chamber, previously left to equilibrate for at least 30 min at room temperature. The spots were visualised by UV absorption at 254 nm.

3 Results

Inoculation of ethyl 4-hydroxybenzoate in concentrations up to 24 mg/egg did not affect the viability of the embryos. A dose of 6 mg/egg was chosen for the metabolism studies and two compounds in their free form were detected in the AF. Excretion of unchanged paraben was not detected. The metabolites could be identified unambiguously as 4-hydroxybenzoic acid (major) and 4,4'-dihydroxy-L-ornithuric acid (minor) by comparative TLC with the authentic reference substances in three different solvent systems (Tab. 2). Regarding the results of enzymatic hydrolysis with β-glucuronidase/arylsulphatase, the 4-hydroxybenzoic acid is also eliminated in its conjugated form (glucuronide and/or sulphate). There were no indications for the presence of protocatechuic acid and vanillic acid. The metabolic profile of ethyl 4-hydroxybenzoate in the chick embryo is shown in Figure 3.

The 4,4'-dihydroxy-L-ornithuric acid [2,5-bis-(4-hydroxybenzoylamino)pentanoic acid] is a new metabolite. Departing from 4,4'-diamino-L-ornithuric acid (Smith, 1958), the synthesis was performed by diazotation of the primary aromatic amino groups and subsequent transformation of the diazonium salt to dihydroxyornithuric acid. The structure was confirmed by mass spectrometry and NMR spectroscopy. The ESI-MS shows a $[M-H+2Na]^+$ ion at m/z 417. The ^1H and ^{13}C NMR data are in agreement with the structure of 4,4'-dihydroxy-L-ornithuric acid (2.2).

4 Discussion

The urinary excretion of unchanged ethyl 4-hydroxybenzoate is very low in animals and humans since the ester linkage is hydrolysed readily. This is also the case in the incubated hen's egg, in which unchanged paraben was not detected. The metabolic profile of ethyl 4-hydroxybenzoate in the chick embryo corresponded to that in animals and humans (Fig. 2 and Fig. 3). Consequently, the model seems to be suitable for the investigation of the metabolism of the lipophilic and poorly water-soluble test substance. However, species differences exist in the conjugation of aromatic carboxylic acids with endogenous amino acids (Williams, 1967).

In mammals, the favoured conjugation partner is glycine (Steventon and Hutt, 2002). Most birds on the other hand con-

jugate aromatic carboxylic acids with ornithine (Jaffe, 1877; McGilvery and Cohen, 1950; Baldwin et al., 1960; Marshall and Koeppe, 1964; Bridges et al., 1970; Igarashi et al., 1992). This difference in the conjugation behaviour appears to be due to the difference in the major end product of nitrogen metabolism in mammals, i.e. urea, and birds, i.e. uric acid (Williams, 1967; Jondorf, 1981; Steventon and Hutt, 2002). Uric acid is characteristically produced by terrestrial species that develop within a shell in which nitrogenous waste products must be stored in an insoluble form. Since the biosynthesis of uric acid involves glycine as a precursor, it is conceivable that the non-proteinic amino acid ornithine serves as an alternative conjugation partner. Furthermore, the connection between the form of the N-excretion and the deamination of the proteolytic amino acids is striking. Mammals form ammonia by transdeamination, birds by oxidative deamination. The deamination of amino acids and the biosynthesis of uric acid during the ontogenesis of the chick embryo in the incubated hen's egg are well described (Drel, 1965; Emmanuel and Gilanpour, 1978).

The conjugation of benzoic acid (Takahashi, 1928) and of the aminobenzoic acid isomers (Wolfe and Huang, 1959) with ornithine is already possible in the chick embryo. Ornithine conjugation was detected on d6, just one day after uric acid excretion begins (Wolfe and Huang, 1959). This amino acid conjugation with a hydroxybenzoic acid isomer has not been described previously in the chick embryo. Recently, 2,2'-dihydroxyornithuric acid was identified in the excreta of broiler chickens after application of 2-hydroxybenzoic acid (Hillaert et al., 2003). The occurrence of this ornithuric acid in the AF after inoculation of salicylic acid into the incubated hen's egg has been discussed previously (Kiep and Bekemeier, 1986). Taken together, next to glucuronidation the conjugation of aromatic carboxylic acids with an amino acid is an important phase II reaction of xenobiotic metabolism in the chick embryo. Also, the further phase II reactions (Tab. 1) indicate that enzyme activities already appear in the first half of the incubation period. Moreover, the ability of the chick embryo to perform oxidative biotransformation prematurely is evident (Hamilton et al., 1983; Brunström, 1986; Heinrich-Hirsch et al. 1990).

In summary, the model of the incubated hen's egg is a highly organised test system for xenobiotic metabolism studies. The crucial advantage of the model exists in the consideration of biokinetic aspects. After inoculation of the xenobiotic into the nourishing compartment of the egg, absorption, distribution, metabolism and excretion (ADME) occur as in a mature organism. The model includes both phase I and phase II reactions of metabolism and only the bioavailable foreign compound is metabolised. In spite of interspecies differences, indications of the metabolic profile of a xenobiotic can be gained from the model. Consequently, this model could lead to the reduction of animal experiments in this area of xenobiochemistry.

References

Baldwin, B. C., Robinson, D. and Williams, R. T. (1960). Studies in detoxication - 82. The fate of benzoic

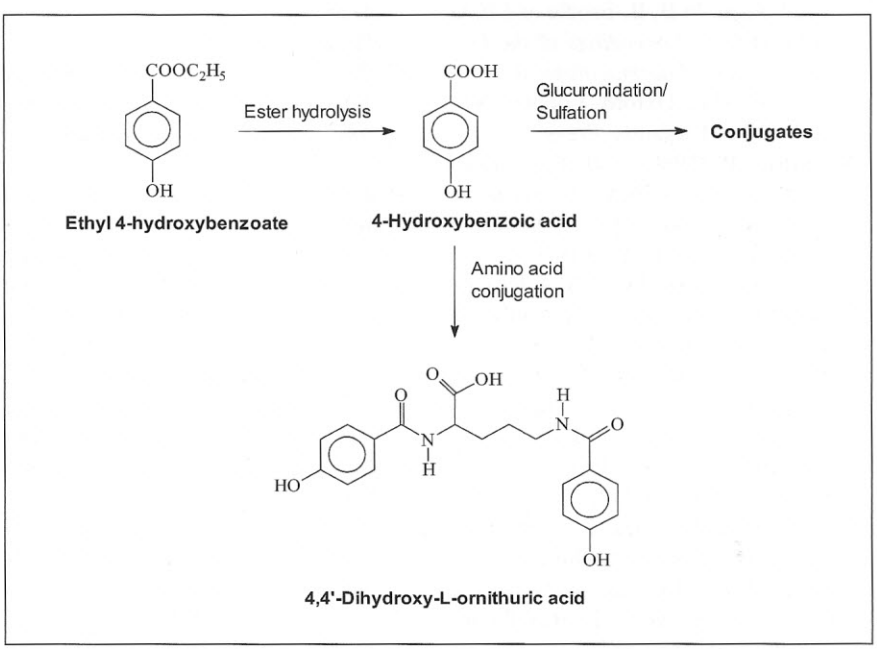

Fig. 3: Metabolic profile of ethyl 4-hydroxybenzoate in the chick embryo

Tab. 2: TLC data (RF values) of ethyl 4-hydroxybenzoate and its metabolites in different solvent systems (silica gel 60 F_{254}, visualisation by UV absorption)

	$CHCl_3$/EtOAc/ HOAc (10:8:2; by vol.)	**$CHCl_3$/MeOH/ HOAc** (16:4:0.2; by vol.)	**$CHCl_3$/EtMeCO/ HOAc/H_2O** (8:8:4:1; by vol.)
Ethyl 4-hydroxybenzoate	0.63	0.67	0.79
4-Hydroxybenzoic acid	0.53	0.56	0.68
4,4'-Dihydroxy-L- ornithuric acid	0.06	0.13	0.32

acid in some domestic and other birds. *Biochem. J. 76*, 595-600.

Brauer, R. W., Julian, L. M. and Krebs, J. S. (1963). Maturation of liver function in the chick embryo as explored with S^{35}-sulfobromophthalein: vascular factors, biliary secretion, and conjugation. *Ann. New York Acad. Sci. 111*, 136-156.

Bray, H. G., Thorpe, W. V. and White, K. (1950). The fate of certain organic acids and amides in the rabbit – 10. The application of paper chromatography to metabolic studies of hydroxybenzoic acids and amides. *Biochem. J. 46*, 271-275.

Bridges, J. W., French, M. R., Smith, R. L. and Williams, R. T. (1970). The fate of benzoic acid in various species. *Biochem. J. 118*, 47-51.

Brodie, B. B. and Maickel, R. P. (1962). Comparative biochemistry of drug metabolism. In B. B. Brodie and E. G. Erdös (Eds.), *Proceedings of the First International Pharmacological Meeting* (299-324). Oxford, London, New York, Paris: Pergamon Press.

Brunström, B. (1986). Activities in chick embryos of 7-ethoxycoumarin O-deethylase and aryl hydrocarbon (benzo[a]pyrene) hydroxylase and their induction by 3,3',4,4'-tetrachlorobiphenyl in early embryos. *Xenobiotica 16*, 865-872.

Clement, B. und Mladek, C. (1995). Biotransformationsuntersuchungen im Hühnerei – Teil 1: Metabolische Umwandlung von 7-Ethoxycumarin. *Pharmazie 50*, 207-210.

Clement, B. und Mladek, C. (1996). Biotransformationsuntersuchungen im Hühnerei – Teil 2: Metabolische Umwandlung von p-Nitrophenol. *Pharmazie 51*, 882-885.

Collett, R. A. and Ungkitchanukit, A. (1979). Phenol sulphotransferase in developing chick embryo. *Biochem. Soc. Trans. 7*, 132-134.

DeEds, F., Booth, A. N. and Jones, F. T. (1957). Methylation and dehydroxylation of phenolic compounds by rats and rabbits. *J. Biol. Chem. 225*, 615-622.

Derache, R. and Gourdon, J. (1963). Metabolism of a food preservative: parahydroxybenzoic acid and its esters. Food Cosmet. *Toxicol. 14*, 189-195.

Dong, Q. G., Gu, J. K., Zhong, D. F. et al. (2003). Metabolism of SFZ-47 in chicken embryo by liquid chromatography-electrospray ion trap mass spectrometry. *Acta Pharmacol. Sin. 24*, 576-580.

Drel, K. O. (1965). Deamination of some amino acids in embryo livers during transition from ureotelism to uricotelism. *Fed. Proc. Transl. Suppl. 24*, 39-42.

Dutton, G. J. and Ko, V. (1966). The synthesis of o-aminophenyl glucuronide in several tissues of the domestic fowl, Gallus gallus, during development. *Biochem. J. 99*, 550-556.

Emmanuel, B. and Gilanpour, H. (1978). Studies on enzymes of nitrogen metabolism, and nitrogen end products in the developing chick (Gallus domesticus) embryo. *Comp. Biochem. Physiol. B 61*, 287-289.

Gruber, F. P. and Hartung, T. (2004). Alternatives to animal experimentation in basic research. *ALTEX 21, Suppl. 1*, 3-31.

Hamilton, J. W., Denison, M. S. and Bloom, S. E. (1983). Development of basal and induced aryl hydrocarbon (benzo[a]pyrene) hydroxylase activity in the chicken embryo in ovo. *Proc. Natl. Acad. Sci. USA 80*, 3372-3376.

Heim, F., Leuschner, F. und Wunderlich, G. (1957). Über das intermediäre Verhalten des p-Oxybenzoesäureäthylesters. *Klin. Wochenschr. 35*, 823-825.

Heinrich-Hirsch, B., Hofmann, D., Webb, J. and Neubert, D. (1990). Activity of aldrinepoxidase, 7-ethoxycoumarin-O-deethylase and 7-ethoxyresorufin-O-deethylase during the development of chick embryos in ovo. *Arch. Toxicol. 64*, 128-134.

Hillaert, U., Baert, K., Rozenski, J. et al. (2003). 2,5-Bis-(2-hydroxybenzoylamino)pentanoic acid, a salicylic acid-metabolite isolated from chicken: characterization and independent synthesis. *Bioorg. Med. Chem. Lett. 13*, 335-337.

Igarashi, K., Suzuki, R., Kasuya, F. and Fukui, M. (1992). Determination of ornithine conjugates of some carboxylic acids in birds by high-performance liquid chromatography. *Chem. Pharm. Bull. 40*, 2196-2198.

Ignarro, L. J. and Shideman, F. E. (1968). Catechol-O-methyltransferase and monoamine oxidase activities in the heart and liver of the embryonic and developing chick. *J. Pharmacol. Exp. Ther. 159*, 29-37.

Jaffé, M. (1877). Über das Verhalten der Benzoesäure im Organismus der Vögel. *Ber. Dtsch. Chem. Ges. 10*, 1925-1930.

Jondorf, W. R. (1981). Drug metabolism and drug toxicity: some evolutionary considerations. In P. Jenner and B. Testa (Eds.), *Concepts in Drug Metabolism, Part B* (307-376). New York, Basel: Marcel Dekker.

Jones, P. S., Thigpen, D., Morrison, J. L. and Richardson, A. P. (1956). p-Hydroxybenzoic acid esters as preservatives - III. The physiological disposition of p-hydroxybenzoic acid and its esters. *J. Am. Pharm. Assoc. Sci. Ed. 45*, 268-273.

Kiep, L. (1997). Eignung früher Entwicklungsstadien des bebrüteten Hühnereies für eine Ersatz- und Ergänzungsmethode zur Untersuchung der Biotransformation von Xenobiotica. In H. Schöffl, H. Spielmann und H. A. Tritthart (Hrsg.), *Ersatz- und Ergänzungsmethoden zu Tierversuchen, Band IV* (428-429). Wien, New York: Springer-Verlag.

Kiep, L. und Bekemeier, H. (1986). Biotransformation und Toxizität von Xenobiotica im Kükenembryo. *Pharmazie 41*, 868-872.

Kiep, L., Maderner, S. and Seifert, K. (2002). Metabolism of metamizol in early stages of the incubated hen's egg. *Pharmazie 57*, 829-833.

Kiwada, H., Awazu, S. and Hanano, M. (1979). The study on the biological fate of paraben at the dose of practical usage in rat – I. The metabolism and excretion of ethyl p-hydroxybenzoate (ethyl paraben) and p-hydroxybenzoic acid. *J. Pharmacobiodyn. 2*, 356-364.

Marshall, F. D. and Koeppe, O. J. (1964). Enzymatic synthesis of benzoylornithines. *Biochemistry 20*, 1692-1695.

McGilvery, R. W. and Cohen, P. P. (1950). Enzymatic synthesis of or-

nithuric acids. *J. Biol. Chem. 183*, 179-189.

Neugebauer, M. (1995). Biotransformation von (+)-Metamfetamin im bebrüteten Hühnerei. *Pharmazie 50*, 201-206.

Peters, J. J., Vonderahe, A. R. and Powers, T. H. (1956). The functional chronology in developing chick nervous system. *J. Exp. Zool. 133*, 505-518.

Phillips, J. C., Topp, C. S. and Gangolli, S. D. (1978). The metabolism of ethyl and n-propyl-p-hydroxybenzoate ("parabens") in male cats. *Toxicol. Lett. 2*, 237-242.

Poland, A. and Kappas, A. (1971). The metabolism of aminopyrine in chick embryo hepatic cell culture: effects of competitive substrates and carbon monoxide. *Mol. Pharmacol. 7*, 697-705.

Powis, G., Drummond, A. H., MacIntyre, D. E. and Jondorf, W. R. (1976). Development of liver microsomal oxidations in the chick. *Xenobiotica 6*, 69-81.

Quick, A. J. (1932). The relationship between chemical structure and physiological response – II. The conjugation of hydroxy- and methoxybenzoic acids. *J. Biol. Chem. 97*, 403-419.

Rifkind, A. B., Troeger, M. and Muschick, H. (1982). Kinetic evidence for heterogeneous responsiveness of mixed function oxidase isozymes to inhibition and induction by allylisopropylacetamide in chick embryo liver. *J. Biol. Chem. 257*, 11717-11727.

Rosenbruch, M. (1997). Zur Sensitivität des Embryos im bebrüteten Hühnerei. *ALTEX 14*, 111-113.

Russel, W. M. S. and Burch, R. L. (1959). *The principles of Humane Experimental Technique*. London: Methuen.

Sabalitschka, T. und Neufeld-Crzellitzer, R. (1954). Zum Verhalten der p-Oxybenzoesäureester im menschlichen Körper. *Arzneim. Forsch. 4*, 575-579.

Smith, J. N. (1958). Comparative detoxication – 5. Conjugation of aromatic acids in reptiles: formation of ornithuric acid, hippuric acid and glucuronides. *Biochem. J. 69*, 509-516.

Steventon, G. B. and Hutt, A. J. (2002). The amino acid conjugations. In C. Ioannides (Ed.), *Enzyme Systems that Metabolise Drugs and Other Xenobiotics* (501-520). Chichester: John Wiley & Sons.

Strittmatter, C. F. and Umberger, F. T. (1969). Oxidative enzyme components of avian liver microsomes: changes during embryonic development and the effects of phenobarbital administration. *Biochim. Biophys. Acta 180*, 18-27.

Takahashi, M. (1928). Über den Entgiftungsvorgang im Fötalorganismus - Synthese der Ornithursäure im bebrüteten Hühnerei bei Einspritzung von Benzoesäure. *Z. Physiol. Chem. 6*, 291-297.

Topp, R. J. and van Bladeren, P. J. (1986). Oxidative biotransformation in primary cultures of chick embryo hepatocytes: induction of cytochrome P-450 and the metabolism of benzo(a)pyrene. *Arch. Toxicol. 59*, 150-155.

Tsukamoto, H. and Terada, S. (1964). Metabolism of drugs - XLVII. Metabolic fate of p-hydroxybenzoic acid and its derivatives in rabbit. *Chem. Pharm. Bull. 12*, 765-769.

Tyman, J. H. P. (1996). Phenols, aromatic carboxylic acids, and indoles. In J. Sherma and B. Fried (Eds.), *Handbook of Thin-Layer Chromatography* (877-920). New York, Basel, Hong Kong: Marcel Dekker.

Williams, R. T. (1967). Comparative patterns of drug metabolism. *Fed. Proc. 26*, 1029-1039.

Wiltshire, H. (2000). Thin-layer chromatography. In R. F. Venn (Ed.), *Principles and Practice of Bioanalysis* (149-159). London, New York: Taylor & Francis.

Wolfe, H. J. and Huang, K. C. (1959). The conjugation of the aminobenzoic acid isomers in the adult and embryonic Gallus domesticus. *J. Cell. Comparat. Physiol. 54*, 243-249.

Worth, A. P. and Balls, M. (2002). Alternative (non-animal) methods for chemical testing: current status and future prospects. *ATLA 30, Suppl. 1*, 1-125.

Acknowledgement

The author is thankful to Prof. Dr. Karlheinz Seifert, Organic Chemistry, NW II, University of Bayreuth, Germany, for providing spectral data (MS, ^{1}H and ^{13}C NMR) of the 4,4'-dihydroxy-L-ornithuric acid and for helpful discussions.

Correspondence to

Dr. Lutz Kiep
Ringstr. 4
D-19372 Brunow/Mecklenburg
Germany
e-mail: lutz.kiep@t-online.de