Adrenal Bioactivation and Toxicity of 3-MeSO₂-DDE, o,p′-DDD and DMBA Investigated in Tissue Slice Culture

BY

ÖRJAN LINDHE
I developed a precision-cut adrenal slice culture procedure to investigate cytochrome P450 (CYP) catalysed irreversible binding and adrenocortical effects in human, rodent, and fish adrenal tissue, \textit{ex vivo}. Autoradiography and radioluminography of exposed tissue slices showed that the potent adrenal toxicant 3-methylsulphonyl-2,2'-bis(4-chlorophenyl)-1,1'-dichloroethene (MeSO$_2$-DDE) causes a selective metabolite binding in \textit{zona fasciculata} (ZF), which is diminished by the CYP11B1 inhibitor metyrapone. MeSO$_2$-DDE also reduces corticosterone secretion, increases 11-deoxycorticosterone secretion and causes mitochondrial degeneration in ZF cells in cultured mouse adrenal slices. ACTH treatment of mice induces CYP11B1 and increases irreversible MeSO$_2$-DDE binding and toxicity in ZF cells. Metyrapone-sensitive binding of MeSO$_2$-DDE is also observed in human \textit{zona fasciculata/reticularis} (ZF/ZR) and 11-deoxycortisol/corticosterone secretion increases in MeSO$_2$-DDE-exposed cultured human adrenal slices. The adrenocortical drug 2-(2-chlorophenyl)-2-(4-chlorophenyl)-1,1-dichlorethane (o,p'-DDD, Mitotane\textsuperscript{\textregistered}) is also bound in ZF/ZR but does not to impair hormone secretion in human adrenal slices at equimolar concentration. A targeted, presumably CYP1B1-catalysed irreversible binding of the adrenocortical carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) in ZF/ZR occurs in rat adrenal slices, whereas presumably CYP1A1-catalysed irreversible binding in endothelial cells is observed in CYP1-induced rats and mice. The rat-specific adrenocortical activity of DMBA may rely on two independent pathological processes resulting in cell death and haemorrhage in the adrenal cortex. In Atlantic cod, selective binding of o,p'-DDD is observed in interrenal cells in cultured anterior kidney slices.

In conclusion, precision-cut adrenal slice culture is a simple \textit{ex vivo} test system with which to investigate CYP-catalysed metabolite binding, altered steroid hormone secretion and target cell ultrastructure in human, experimental and wild animal tissue. The results imply that organisms under stress could be at increased risk of MeSO$_2$-DDE induced adrenal toxicity. MeSO$_2$-DDE is an expected human adrenal toxicant, which should be evaluated as a possible alternative in the therapy of adrenocortical hypersecretion and tumour growth.

\textit{Keywords:} adrenal cortex, 3-MeSO$_2$-DDE, o,p'-DDD, DMBA, bioactivation, binding, toxic

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List of separate publications

This thesis is based on the Papers listed below, which will be referred to by their roman numerals.

**Paper I**  

**Paper II**  
Lindhe Ö., M. Bergström, M. Breitholtz, B. Långström, Å. Bergman, and I. Brandt. ACTH enhances adrenal tissue binding and adrenocorticoalytic activity of 3-methylsulphonyl-DDE [in manuscript]

**Paper III**  
Lindhe Ö., B. Skogseid, and I. Brandt. Cytochrome P450-catalysed binding of 3-methylsulfonyl-DDE and o,p’-DDD in human adrenal zona fasciculata/reticularis [submitted for publ.]

**Paper IV**  
Lindhe Ö., L. Granberg, and I. Brandt. Target cells for cytochrome P450-catalysed irreversible binding of 7,12-dimethylbenz[a]anthracene (DMBA) in rodent adrenal [in manuscript]

**Paper V**  
Lindhe Ö., I. Brandt, J. Schou Christiansen, and K. Ingebrigtsen. Irreversible binding of o,p’-DDD in interrenal cells of Atlantic cod (*Gadus morhua*) [submitted for publ.]

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Abbreviations

MeSO₂-DDE  3-methylsulphonyl-2,2′-bis(4-chlorophenyl)-1,1′-dichloroethene
2-MeSO₂-DDE  2-methylsulphonyl-2,2′-bis(4-chlorophenyl)-1,1′-dichloroethene
o,p′-DDD  2-(2-chlorophenyl)-2-(4-chlorophenyl)-1,1-dichloroethane
DMBA  7,12-dimethylbenz[a]anthracene
p,p′-DDE  2,2′-bis(4-chlorophenyl)-1,1-dichloroethene
p,p′-DDT  2,2′-bis(4-chlorophenyl)-1,1,1-trichloroethane
PCB 126  3,3′,4,4′,5-pentachloro-biphenyl
ACTH  adrenocorticotropic hormone
BNF  β-naphthoflavone (5,6-benzoflavone)
ANF  α-naphtoflavone (7,8-benzoflavone)
1-EP  1-ethynylpyrene
1-PP  1-(1-propynyl)pyrene
AhR  aryl hydrocarbon receptor
CYP  cytochrome P450
CYP11A1  gene encoding for cholesterol side-chain cleavage enzyme
CYP11B1  gene encoding for 11β-hydroxylase
CYP11B2  gene encoding for aldosterone synthase
EDC  endocrine disrupting chemical
GR  glucocorticosteroid receptor
GSH  glutathione
HPA axis  hypothalamus-pituitary-adrenal axis
HPLC  high performance liquid chromatography
PAH  polycyclic aromatic hydrocarbon
PCB  polychlorinated biphenyl
PSL  photo-stimulated luminescence
TEM  transmission electron microscopy
TCDD  2,3,7,8-tetrachlorodibenzo-\textit{p}-dioxin
**Introduction**

**Background**

The chlorinated insecticide DDT is a persistent environmental pollutant, which can be spread by the atmosphere over vast distances, subsequently becoming biomagnified in food chains. Although its biological degradation in the environment is slow, DDT is transformed to a number of lipophilic metabolites that are present in human tissues and in wild mammals, birds and fish.

The persistent DDT metabolite 3-methylsulphonyl-2,2’-bis(4-chlorophenyl)-1,1’-dichloroethene (MeSO$_2$-DDE) was first found in blubber from Baltic grey seal, a population suffering from adrenocortical hyperplasia and a suite of pathological lesions resembling those seen in Cushing’s syndrome in human subjects (1-3). MeSO$_2$-DDE has subsequently been shown to be a potent adrenocorticolytic compound that specifically targets the parenchymal zona fasciculata cells in mice (4-6). Its toxicity appears to derive from a reactive metabolite formed in situ in the targets cells (4-7). There are however major species differences in the toxic potency of MeSO$_2$-DDE, mice being highly sensitive whereas rats are resistant to toxicity (4, 6, 8-10). MeSO$_2$-DDE has also been found in human breast milk and blood serum (11-14) as well as in tissues of harbour seal, harbour porpoise, striped dolphin and polar bear (15-19). The toxicity of MeSO$_2$-DDE in the adrenal cortex in these species is not known.

The objective of this work was to develop and apply a precision-cut tissue slice culture procedure to facilitate assessment of adrenocorticolytic activity of chemicals in human and wild animal tissue. Using this procedure, MeSO$_2$-DDE, the adrenocorticolytic drug o,p’-DDD, and also the rat-specific adrenal toxicant DMBA were investigated with regard to cytochrome P450 (CYP) dependent metabolic activation, irreversible binding, and adrenocorticolytic effects in adrenal tissue of humans, rodents and fish.

**The adrenal gland**

The mammalian adrenal consists of a cortex and a central medulla. It lies close to the cranial end of the kidney. The adrenal cortex originates from the coelomic epithelium (mesoderm) and the medulla from the neuroectoderm of the neural crest. The cells of the cortex secrete steroid hormones, while those of the medulla secrete peptide hormones (20-24). The cortex differentiates into several layers, named by Julius Arnold in 1866 (25). Three zones can be distinguished in the mouse adrenal cortex: zona glomerulosa, zona fasciculata and zona reticularis (Figure 1).
Zonation in the mouse adrenal cortex. Closest to the surrounding capsule (C) is zona glomerulosa (ZG). Zona fasciculata (ZF) is the thickest zone with cells in parallel cords. Zona reticularis (ZR) is the innermost zone bordering to the medulla (M).

Regeneration of adrenocortical cells occurs continuously throughout life. The complete cell-renewal cycle takes about 200 days in mice (26). According to the presently favoured centripetal migration theory, most cells proliferate in the region between zona glomerulosa and zona fasciculata and migrate bi-directionally toward the capsule and the medulla (27-31). When cells reach the outer part of zona glomerulosa or the inner part of zona reticularis, they are eliminated by apoptosis (27, 31). Arterial blood flows through the cortex via a capillary network where almost every parenchymal cell is in contact with an endothelial cell. The capillaries debouch into large sinusoids that drain into the central vein in the medulla (23).

The general trend in the evolution of the adrenal gland can be simplified as an aggregation of steroid and peptide hormone secreting cells into a separate organ, which is well supplied by blood and is in contact with the nervous system (22, 32). Differences in the cellular organization and relative numbers of cells in the adrenal zones occur between mammalian species and between sexes (22, 23, 33). Non-mammalian vertebrates such as birds and fish lack the organization into a cortex and a medulla. The cortical cells are referred to as interrenal, adrenocortical or steroidogenic cells in these vertebrate classes, whereas the cells corresponding to the medulla are referred to as chromaffin cells. In fish, the steroidogenic cells are found in the anterior kidney.

**Steroid hormone synthesis**

The adrenal cortex secretes several steroid hormones necessary to maintain homeostasis. They can be divided into two groups: glucocorticosteroids (e.g. corticosterone and cortisol synthesized in the zona fasciculata/reticularis) and mineralocorticosteroids (e.g. aldosterone synthesized in zona glomerulosa). The
glucocorticosteroid synthesis is regulated by the adrenocorticotrophic hormone (ACTH) secreted by the anterior pituitary. Release of ACTH is controlled by the corticotrophin-releasing hormone (CRH) and vasopressin produced in the hypothalamus. Secretion may also be regulated by glucocorticosteroids via a negative feedback loop as illustrated in Figure 2. This chain of events is called the hypothalamus-pituitary-adrenal (HPA) axis (23). Following acute exposure, ACTH mediates an increased blood flow and increased glucocorticosteroid secretion by binding to the corticotrophin receptor on the cell surface (34, 35). Following prolonged exposure, ACTH induces adrenal hypertrophy/hyperplasia and hypersecretion (36, 37). In the absence of ACTH stimulation, the adrenal cortex rapidly becomes atrophic. The mineralocorticosteroid synthesis is stimulated by angiotensionogen II (regulated by renin) and extracellular K⁺.

The adrenal steroids are synthesized from cholesterol originating from two sources, viz. from receptor-mediated uptake of plasma low-density lipoproteins (LDL) and from synthesis in the adrenal cortex from acetate. Cholesterol fatty acid esters are stored in lipid droplets (23). There are species differences in the main steroid products secreted from the adrenal (38). Primates, dog, hamster, and fish secrete mainly cortisol, whereas corticosterone is the major glucocorticosteroid in mouse, rat, rabbit, birds, reptiles, and amphibians (38). Zona reticularis synthesizes various sex hormones and is the major site of cortisol synthesis in humans (39).

**Abnormal secretion of glucocorticosteroids**

Cushing’s syndrome (40), a disease caused by excessive glucocorticosteroid secretion, can give rise to obesity, hypertension, osteoporosis, debility, impotence, menstrual disorders, skin changes, hirsutism, suppression of the immune system, and/or psychological disturbances (41). Addison’s disease is caused by deficient glucocorticosteroid secretion, which can result in debility, fatigue, anorexia, weight loss, hyperpigmentation, hypotension, and/or gastrointestinal disturbances. Deficiency in glucocorticoid synthesis due to diminished ACTH secretion will lead to atrophy of zona fasciculata/reticularis and reduced glucocorticosteroid secretion (41).
Figure 2 The hypothalamus-pituitary-adrenal axis (HPA axis). Corticotrophin-releasing hormone (CRH) is released from the hypothalamus following stimulation by stress, exercise, hypoglycaemia or serotonin. The adrenocorticotropic hormone (ACTH) is secreted by the anterior pituitary into the blood. When adrenocortical cells are stimulated, glucocorticosteroids are synthesized and secreted. Release of CRH is inhibited by ACTH, γ-aminobutyric acid (GABA), noradrenalin, and glucocorticosteroids via the glucocorticosteroid receptor (GR) in the hippocampus. Glucocorticosteroids can also inhibit ACTH release by directly inhibiting CRH and ACTH secretion.

Figure 3 Steroid hormone synthesis in the adrenal cortex. The enzymes of the adrenal CYP11 family are located in the inner membrane of the mitochondria. Other adrenal CYP steroidogenic enzymes are located in the smooth endoplasmatic reticulum (SER). The enzymatic activities of the enzymes are: CYP11A1, 20,22-hydroxylase: 20,22-desmolase activity; 3β-HSD, 3-hydroxysteroid dehydrogenase: 5-ene-3-β-hydroxysteroid oxoreductase: 5-ene-3-oxosteroid-4,5-isomerase activity; CYP17A, 17α-hydroxylase and
Toxicity in the adrenal cortex

The adrenal cortex is a target for many xenobiotics and pharmaceutical agents (42-45) and has been estimated to account for more than 70% of all reported toxicological findings in the endocrine system (46). The adrenal cortex has a marked ability to accumulate lipophilic chemicals. Many xenobiotics are lipophilic and will consequently be associated with lipoproteins in the blood. Receptor mediated uptake of lipoproteins can cause a selective uptake of lipophilic xenobiotics by the adrenal cortex (23). On a weight basis, the adrenal has one of the most profuse blood supplies in the body, which therefore facilitates uptake of xenobiotics by the adrenal before storage in fat begins (42).

CYP enzymes are often involved in metabolic activation of toxic chemicals (42, 47-50). However, to my knowledge, only one steroidogenic CYP enzyme, CYP11B1, has been convincingly linked to the bioactivation of an environmental pollutant (MeSO₂-DDE) (5, 6). Metabolism of xenobiotics can cause the formation of reactive intermediates, which become irreversibly (covalently) bound to cellular macromolecules. Binding of reactive metabolites to proteins necessary for cell viability can lead to degeneration and cell death (51, 52). To protect cells from reactive intermediates, several detoxification processes are available in the adrenal cortex (53). One such is glutathione (GSH) conjugation. GSH conjugates to electrophilic centres in the molecule, rendering it more water-soluble. The conjugate and its degradation products are readily excreted (54).

Adrenocorticoletic substances examined

In this work, three substances that cause structural and/or functional changes in the adrenal cortex have been examined, viz. MeSO₂-DDE, o,p’-DDD, and DMBA. Their chemical structures are shown in Figure 4.

![Chemical structures of the test substances](image)
The toxic effects of these substances in the adrenal cortex have been described previously. They all appear to have an effect in the mitochondria. A brief summary of the toxicity of these chemicals is given below.

**MeSO₂-DDE**

Methylsulphones form by metabolism of organochlorines such as DDE, chlorinated benzenes and PCBs, during entero-hepatic circulation in the mercapturic acid pathway (55-57). MeSO₂-[¹⁴C]DDE gives rise to a highly specific irreversible binding in *zona fasciculata* of the adrenal cortex in mice. No corresponding binding has been reported in rats (4). The binding of MeSO₂-[¹⁴C]DDE is catalysed by CYP11B1, an enzyme present only in *zona fasciculata* of the mouse adrenal cortex (5, 58). This binding causes mitochondrial degeneration (disruption and loss of cristae), and loss of mitochondria following a single dose as low as 3 mg MeSO₂-DDE/kg b.w. in mice. Following a dose of 12.5 mg reduced corticosterone plasma concentration and cell death ensue in *zona fasciculata* (4, 6). As demonstrated by studies in lactating mice, there is an efficient transport of MeSO₂-DDE via the milk to suckling pups, which attain 2–4-fold higher tissue concentrations of MeSO₂-DDE than in the nursing dam (59). Consequently, MeSO₂-DDE causes adrenocortical cell death and reduces plasma corticosterone content also in suckling mouse pups (60, 61).

CYP11B1 activity in the mouse adrenocortical cancer Y1 cell line is reduced by MeSO₂-DDE, with an apparent Kᵢ (inhibition constant) of 1.6 µM (62). This indicates that MeSO₂-DDE competes for the binding site of CYP11B1 and is able to disturb corticosterone secretion also by enzyme inhibition.

**o,p´-DDD**

The adrenocorticoalytic activity of o,p´-DDD in dogs was first described in 1949 by Nelson & Woodard (63). o,p´-DDD inflicts mitochondrial damage in *zona fasciculata/reticularis* progressing through swelling, dissolution and eventual rupture of mitochondrial membranes, but with a minimal effect in *zona glomerulosa* (47). As proposed by Martz & Straw, o,p´-DDD metabolizes to a reactive metabolite via dechlorination at the C-2 position (64). It was later suggested that o,p´-DDD binds irreversibly to adrenal proteins following metabolism to a reactive acyl chloride (65). As with MeSO₂-DDE, o,p´-DDD shows major species differences in bioactivation and toxicity (64, 65). In mice, adrenal toxicity does not follow a single dose of o,p´-DDD (100 mg/kg) (4). In rats, 39 daily injections of 200 mg/kg failed to harm the adrenal cortex (21).
DMBA

Polycyclic aromatic hydrocarbons (PAHs) are environmental pollutants that form during incomplete combustion of organic materials. Humans are exposed to PAHs in the atmosphere, especially by inhaling tobacco smoke. PAHs are also present in food (66). 7,12-Dimethylbenz[a]anthracene (DMBA) is a potent model carcinogen, producing tumours in various tissues including the lungs, mammary glands and lymphoid tissue (67, 68).

Huggins and Morii found that in rats DMBA cause adrenal apoplexy (haemorrhage) and massive necrosis in zona fasciculata/reticularis, though zona glomerulosa and the medulla remained undamaged. The adrenal destruction was neither complete nor permanent, as regeneration invariably occurred (69). In a subsequent study, it was found that DMBA did not induce adrenal necrosis in immature rats or hypophysectomized mature rats (70). Pretreatment with ACTH rendered these rats susceptible to DMBA toxicity, suggesting involvement of a hormone-regulated mechanism (70). The first sign of adrenal injury was a selective destruction of steroid hormone-secreting cells, while adrenal capillary endothelium appeared undamaged (71). This was later confirmed with electron microscopy, where the first pathologic changes were seen in the mitochondria of zona fasciculata/reticularis (72, 73).

Horváth and co-workers proposed that DMBA-induced necrosis in the adrenal cortex is due to ischaemia caused primarily by injury to the capillary endothelial cells (74). Cefis and Goodall suggested the existence of two pathological processes (75), a direct cytotoxic effect and a vascular damaging action. According to another proposed mechanism of action, DMBA (or its liver metabolite 7-hydroxymethyl-12-MBA) may function as a pseudosubstrate for adrenal CYP enzymes. Pseudosubstrate binding results in uncoupling of the enzyme, release of reactive oxygen species, loss of enzyme activity, accumulation of peroxidation products and ultimately cell death (44, 76).

Despite these early and more recent attempts to explain DMBA-induced adrenal toxicity, its mode of action still remains incompletely understood.
Objectives of the study

The overall purpose of the present study was to develop and apply a precision-cut adrenal tissue-slice culture procedure to facilitate assessment of the adrenocorticolytic effects of xenobiotics in tissues of both humans and wild animals.

Another aim was to ascertain if simulated stress increases CYP11B1 mRNA and protein levels, irreversible metabolite binding and adrenocorticolytic activity of MeSO₂-DDE in mice.

The specific objectives were:

- to develop and apply precision-cut adrenal tissue slice culture (Paper I);
- to determine \textit{ex vivo} - in vivo correlations for adrenal MeSO₂-DDE, o,p'-DDD and DMBA metabolite binding and toxicity (Papers I, II, IV and V);
- to apply radioluminography to quantify irreversible metabolite binding in the adrenal cortex (Papers I, II, and III);
- to correlate irreversible binding of MeSO₂-DDE with localization and levels of CYP11B1 mRNA (Paper II);
- to examine the influence of ACTH and glucocorticoids on CYP11B1 protein levels, irreversible MeSO₂-DDE binding and toxicity, both \textit{ex vivo} and \textit{in vivo} (Paper II);
- to investigate irreversible binding and toxicity of MeSO₂-DDE and o,p'-DDD in human adrenal tissue (Paper III).
Comments on Material & Methods

Chemicals

3-methylsulphonyl-2,2′-bis(4-chloro-[14C]phenyl)-1,1′-dichloroethene (MeSO2-[14C]DDE; 495 MBq/mmol), unlabelled MeSO2-DDE, 2-methylsulphonyl-2,2′-bis(4-chloro-[14C]phenyl)-1,1′-dichloroethene (2-MeSO2-[14C]DDE; 495 MBq/mmol) and 2-(2-chlorophenyl)-2-(4-chloro-[14C]phenyl)-1,1-dichlorethane (o,p′-[14C]DDD; 414 MBq/mmol) were prepared as previously described and kindly provided by Dr Åke Bergman’s group at the Department of Environmental Chemistry, Wallenberg Laboratory, Stockholm University, Sweden (77, 78). The radiochemical purity of all compounds was >98%. [O-Methyl-11C]metomidate (373 MBq/µmol, radiochemical purity >95%, t½ 20.4 min) was prepared as described previously (79).

[G-3H]7,12-Dimethylbenz(a)anthracene ([3H]DMBA, 0.8–2.9 TBq/mmol) was purchased from Amersham Pharmacia Biotech Limited (Amersham, Bucks, England). 1-Ethynylpyrene (1-EP) and 1-(1-propynyl)pyrene (1-PP) were a kind gift from Dr William Alworth, Department of Chemistry, Bioorganic Chemistry Laboratory, Tulane University, New Orleans, USA. 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126) was a kind gift from Dr Bergman. All other chemicals were of commercial origin.

Animals and human tissue

Female C57Bl/6J mice were obtained from Charles River (Uppsala) or B & K Universal AB (Sollentuna, Sweden). Female Sprague Dawley rats were from Charles River (Uppsala). Juvenile Atlantic cod (Gadus morhua) of cultivated origin were obtained from Sea Farm A/S. (Bergen, Norway). Mature Atlantic cod was caught off Fiskebäckskil, Sweden. Specimens of human adrenal tissue were obtained from patients operated on for: an ipsilateral pheochromocytoma, a lymphnode metastasis of an aldosterone producing adrenocortical carcinoma, and a bilateral non-functioning adrenocortical hyperplasia. All three patients gave informed consent.

Preparation and incubation of tissue slices

Animals were killed by cervical dislocation or by CO2 exposure. The adrenals were rapidly excised and kept in ice-cold buffer until embedded in 3% agarose. During embedding, particular care was taken to orientate the mouse adrenals with the craniocaudal axis perpendicular to the sectioning plane. Precision-cut slices (200 µm) were prepared in a Krumdieck tissue slicer (Alabama Research and Development, Munford, Alabama, USA) in ice-cold phosphate-saline buffer. Slices of equal size were placed on titanium screen holders. The holders were transferred to standard six-well plates containing “Fully supplemented
Dulbecco’s Modified Eagle Medium” (2.5 ml). Slices were placed in culture within 1 h of excision. The plates were kept rotating (1 rpm) on an inclined plane in an incubator (95% air/5% CO₂ at 37.5°C), thus allowing the slices to alternate between medium and incubator atmosphere. Incubation time ranged from 3 to 96 h. The labelled test substances were added to the medium (typically 3.7 MBq/ml) dissolved in dimethyl sulfoxide and cultured for 3-24 h for localization of enzyme-catalysed irreversible metabolite binding. The ACTH analogue tetracosactide (henceforth referred to as ACTH) was added to the medium to stimulate steroid hormone secretion.

**Protein synthesis**

Incorporation of [³H]leucine into protein was measured in slices for up to 96 h to study protein synthesis. After sampling, slices were washed and homogenized in distilled water. Protein content was determined using a standard curve prepared with BSA (80, 81). Radioactivity incorporated into protein was measured in a Tri-Carb 1900CA liquid scintillator (Packard, Downers Grove, IL, USA).

**Hormone analysis**

Concentrations of cortisol, 11-deoxycortisol, corticosterone, 11-deoxycorticosterone, aldosterone, androstenedione and 17-OH-progesterone in the medium were measured with HPLC (Lichrosorb RP 18 column, 20 cm, 5 µm particle size) using UV detection (241 nm). The steroid products were separated using a linear gradient of acetonitrile (1 ml/min) according to Johansson et al. (62, 82).

**Histology and ultrastructure**

Following incubation or in vivo treatment, adrenal tissue was fixed in formaldehyde, dehydrated in an ethanol series (70%, 95%, and 100%) and embedded in methacrylate. Particular care was taken to orient the slices with the sliced plane parallel to the sectioning plane. This orientation was achieved by using a two-step embedding procedure whereby the sections were fixed in the correct position with a minimal amount of methacrylate and then mounted on a plastic holder with the remaining methacrylate. The embedded tissue was sectioned (2 µm) in a rotating microtome (HM 360, Mikrom Laborgeräte GmbH, Germany), stained with haematoxylin/eosin and examined by light microscopy. For reference purposes, some adrenal tissue was fixed directly. For transmission electron microscopy, slices were fixed overnight in glutaraldehyde. Examination was performed in a Philips EM 420 transmission electron microscope at 60 keV.
**Computer-aided histometry**

A Windows-based program, Leica QWIN (version 2.3, Leica, UK), was used to measure cell and nuclei parameters in the digital images obtained above. After manual thresholding of colour, the program selectively marked the pixel elements in the nuclei, thus separating them from the cytosol. The program measured by manual interaction both the thickness of the adrenal cortex and the area of zona fasciculata ($\mu m^2$). Only nuclei present in zona fasciculata and nuclei within a specified size range were accepted for further measurements. The area ($\mu m^2$) of the accepted nuclei and of cells in zona fasciculata was measured. Number of accepted nuclei per $\mu m^2$ in zona fasciculata was calculated. By allocating the pixel elements in the cytosol to their closest accepted nuclei, an estimated cell area ($\mu m^2$) was obtained. Before analysis, measurements were performed in a training set (one randomly selected image). The coefficients of variation for these measurements (three repeated measurements in the same image) were less than 4% for all measured variables.

**Localization of CYP mRNA and metomidate binding**

*In situ* hybridization

*In situ* hybridization was performed to localize and semi-quantify mRNA, transcribed from the genes *cyp11b1, cyp11b2* and *cyp11a1*, in the mouse adrenal cortex. Plasmids inserted with double-stranded cDNA fragments (500-800 base-pairs) corresponding to these genes, were a kind gift from Prof. Keith L. Parker, Duke University Medical Center, North Carolina, USA. Anti-sense $^{35}$S-labelled UTP riboprobes homologous to the 3´-untranslated regions were prepared with prokaryote T3 RNA polymerase. Sections were hybridized over night with the probes at 56°C, then washed first at 50°C, then at room temperature, and dehydrated as above. Detection and quantification of mRNA–riboprobe complexes in the hybridized tissue sections was done as described below.

*[$^{11}$C]Metomidate binding*

To determine the level of the CYP11B1 enzyme, [$^{11}$C]metomidate was used as a biomarker for CYP11B1 protein. Adrenals from mice treated with saline, ACTH, and different doses of MeSO$_2$-DDE were frozen immediately after excision and stored at $-70^\circ$C. Adrenal freeze sections (20 $\mu m$) were incubated with [$^{11}$C]metomidate as described by Bergström et al. (79). Specific binding was determined by radioluminography as described below.
**Autoradiography**

**Light microscopy autoradiography**
Whole adrenals or tissue slices were fixed in buffered formaldehyde (4%). In order to remove the parent compound and unbound soluble metabolites, the tissues were extracted/dehydrated as above. Slides carrying the sections were dipped in NTB2 liquid emulsion diluted with distilled water. Autoradiograms were developed and sections were stained with toluidine blue and examined by light microscopy.

**Radioluminography**
Semi-quantification of tissue-bound radioactivity was performed by apposing histological sections or tape-sections (see below) to phosphor imaging plates (BAS-IP MP/MS 2040S Fuji, Japan), before subjecting them to autoradiography. The luminescence derived from radioactivity in the adrenal sections was detected by reading the imaging plate in a Phosphoimager (BAS 1500, Fuji, Japan) (83, 84). A computer-based bio-imaging analyser program was used for semi-quantification (MacBAS, version 2.2, Fujifilm, Japan or ImageGauge, version 3.46, Fujifilm, Japan). In order to correlate radioactivity and metabolically active regions, the labelled areas of the images were selectively marked at 1 pixel resolution (1 pixel =100 µm). Values obtained were expressed as photo-stimulated luminescence (PSL) minus background (BG) per mm² of tissue sections ((PSL-BG)/mm²). Radioluminography is referred to as phosphorautoradiography in Paper I.

**Tape-section autoradiography**
Groups of rats or mice were given an i.v. injection of MeSO₂-[¹⁴C]DDE or [³H]DMBA. One female mouse was injected with 2-MeSO₂-[¹⁴C]DDE. Atlantic cod were given o,p’-[¹⁴C]DDD intragastrically. Animals were killed and mounted in a gel of carboxy methylcellulose and water, frozen (−70°C) and subjected to tape section autoradiography according to Ullberg (85). Sagittal whole-body sections (20 µm) were collected onto tape (6890, 3M, USA) using a cryostat microtome (M-5160-C, PMV, Sweden) and freeze-dried. In order to prepare autoradiograms that show mainly irreversibly bound radioactivity, tape sections were extracted stepwise in a series of organic solvents. Following drying, tape sections were apposed to X-ray film (Structurix D-7, Agfa-Gevaert, Germany, or Hyperfilm-³H, Amersham, England) for exposure (4°C and −20°C).
Results & Discussion

Precision-cut tissue slice culture

The primary aim of this work was to develop a precision-cut slice culture procedure with which to examine bioactivation, binding and toxicity of MeSO$_2$-DDE in mouse adrenal tissue. The endpoints, protein synthesis, steroid secretion, irreversible binding and mitochondrial degeneration, were chosen to facilitate *ex vivo*–*in vivo* comparisons (Paper I).

Free-hand cut tissue slices have been used in pharmacological/toxicological research for a long time. A major disadvantage is the low reproducibility of slice thickness, as the surface to centre distance affects viability, nutrient transport, and oxygen supply, but most importantly exposure of cells to the test chemicals. In 1980, Krumdieck et al. described a fully automated precision-cutting tissue slicer (86). The ability of this machine to produce slices of standardized thickness at high speed gave slice culture a new start in toxicology. Since then, several studies have been published, applying this technique to different organs and endpoints (87-92).

To my knowledge, there are no reports of the use of precision-cut slice technique to examine the adrenal cortex. However, two studies used adrenal slices to examine release of catecholamine and acetylcholine from the adrenal medulla (93, 94).

Adrenal slice culture

I decided to use 200 µm thick adrenal slices to ensure sufficient oxygen and nutrient transport. Slice viability was ascertained by using parameters largely defined by the toxicity-related test variables to be examined. As determined in a series of experiments, ACTH-exposed slices maintained constant protein synthesis and steroid hormone secretion for at least 48 h (Paper I). Except for a slight swelling of *zona fasciculata* cells, no obvious histological changes could be observed after 24 h of culture, as compared with non-cultured slices fixed for histology immediately after slicing. The lack of apparent histological changes in the ACTH-exposed slices indicates good tissue viability. Ultrastructural examination, however, revealed reduced numbers of smooth endoplasmatic reticulum (SER) vesicles after 24 h in culture, showing that the functions of the SER could be affected at this time point. It is worth noting that the mitochondrial membrane structure was largely intact following 24 h in culture, as compared with tissue fixed immediately after slicing (Paper I). Previous studies with precision-cut liver, kidney, lung, and lymphoid tissue slices demonstrated maintained function up to 10 days in culture and that there are differences between organs and viability/test variables examined (90-92, 95, 96).
Induction and inhibition of steroid hormone secretion

Exposure of slices to ACTH (11 nM) for 24 h increased corticosterone secretion into the medium 8-fold \((p<0.05)\), compared with non-exposed control slices (Figure 5). Aldosterone secretion increased almost 4-fold \((p<0.01)\) under the same conditions (Paper I). Haidan et al. have shown that bovine adrenocortical cells co-cultured with medullary chromaffin cells produce 10-fold more cortisol than adrenocortical cells cultured alone. The secretory products of chromaffin cells were said to be responsible for stimulating the steroidogenesis in the co-culture \((97)\). This finding stresses the importance of studying glucocorticostroid-producing cells in their natural cellular environment.

![Figure 5](image_url)

**Figure 5** Corticosterone secretion to the medium from mouse adrenal slices. Curves represent secretion from slices exposed to ACTH (11 nM, \(r^2=0.99\)), to metyrapone (50 \(\mu\)M, \(r^2=0.89\)) and from non-exposed slices \((r^2=0.92)\). ACTH induced the rate of secretion 8-fold whereas metyrapone reduced secretion rate by 75%.

In slices exposed for 30 h to the CYP11B1 inhibitor metyrapone, the rate of corticosterone secretion was reduced by 75%, as compared with non-exposed slices (figure 5). Co-exposure of slices to metyrapone and ACTH for 24 h did not reduce corticosterone secretion significantly, compared with slices exposed to ACTH only. 11-deoxycorticosterone secretion from slices co-exposed to ACTH and metyrapone showed a 6-fold increase \((p<0.01)\), while aldosterone secretion was more than halved \((p<0.01)\) compared with slices exposed to ACTH only (Paper I). Metyrapone has also been reported to inhibit CYP11B2 activity \((98)\), but seems to have no or little effect on CYP21A and CYP17A activity \((82, 98, 99)\). The lack of effect by metyrapone in ACTH-stimulated slices can be explained by an overcapacity of the CYP11B1 enzyme in zona fasciculata. Although inhibition occurs, the increased levels of the
corticosterone precursor 11-deoxycorticosterone can explain the maintained corticosterone synthesis.

Several studies have shown that the activity of several CYP enzymes (CYP 1A2, 2A6, 2C9, 2C19, 2D6, 2E1, and 3A) is reduced in human liver slices after 4-8 h in culture. A similar loss of activity has been observed for phase II enzymes, e.g. 7-hydroxycoumarin glucoronyltransferase and 7-hydroxycoumarin sulfotransferase (100). In contrast to that study, the rates of CYP-dependent steroid hormone secretion in the present adrenal slice culture seem to be very stable.

**MeSO₂-DDE toxicity ex vivo**

Exposure of slices to MeSO₂-DDE (50 µM) reduced the rate of corticosterone secretion by 90% after 24 h of incubation. In these slices, compared with non-exposed slices, the nuclei in the zona fasciculata cells were condensed (Paper I). Inhibition of corticosterone secretion (12% of control) has previously been observed in Y1 cells exposed to MeSO₂-DDE (20 µM) (62). Jönsson et al. reported that, following injection of MeSO₂-DDE (12.5 mg/kg i.p.), the nuclei of the zona fasciculata cells were pycnotic after 12 days (6). As determined by electron microscopy, vacuolated mitochondria were present in zona fasciculata of slices exposed to MeSO₂-DDE (50 µM) for 24 h, while the mitochondria of nearby zona glomerulosa cells (<5 cells away) remained largely intact (Paper I). The mitochondrial damage in mouse zona fasciculata appeared more extensive than that reported previously following a single injection of 3 mg MeSO₂-DDE/kg (6).

In conclusion, these findings show that all MeSO₂-DDE-induced effects previously reported in vivo can be reproduced in adrenal slice culture ex vivo. It can also be concluded that processes such as induction and inhibition of steroid hormone secretion can be examined in slice culture. Tissue slices therefore offer several advantages, compared with cultured adrenocortical cells and in vivo studies. The general strengths and weaknesses of in vivo, ex vivo, and in vitro systems have been reviewed elsewhere (89, 101-103).

** Autoradiography**

To facilitate distribution, cellular localization and quantification of tissue-bound radioactivity, autoradiography at different levels of resolution was employed. In the following sections, the different levels of resolution are exemplified.

**Tape-section autoradiography**

Qualitative examination of distribution of tissue-bound radioactivity in whole animals was done by tape-section autoradiography (Papers II, IV and V). In whole-body tape sections (20 µm) extracted with organic solvents, high levels of
MeSO₂-[¹⁴C]DDE binding were observed in the adrenal cortex (Figure 6). Non-extractable MeSO₂-[¹⁴C]DDE binding in the adrenal was about 18-fold higher than that in the livers of control mice. These results are in agreement with the original publication on irreversible tissue binding of MeSO₂-DDE in mice (4). Only weak 2-MeSO₂-[¹⁴C]DDE binding in the adrenal and a moderate binding in liver were observed in extracted tape sections (Figure 6). These results show that the structural change caused by the shift in the methyl sulphone moiety from the 3- to the 2-position was enough to dramatically change the pattern of irreversible binding.

Light-microscopy autoradiography
Cellular localization of tissue-bound radioactivity was determined by light-microscopy autoradiography (microautoradiography) of 2 µm thick adrenal methacrylate sections (Papers I, II, III, IV and V).

Mouse adrenal slices exposed to MeSO₂-[¹⁴C]DDE showed that intense irreversible binding of radioactivity was confined to zona fasciculata (Figure 7). In rat adrenal slices, only very faint labelling was observed in zona fasciculata. In mouse slices exposed to o,p′-[¹⁴C]DDD, very weak binding was observed.
Notably however, this binding was localized to both zona fasciculata and zona reticularis (Paper I). These discrepancies are noteworthy, as binding of MeSO$_2$-$[^14]$C]DDE in rat or o,p’-$[^14]$C]DDD in mouse adrenal cortex has not been reported previously. The level of o,p’-$[^14]$C]DDD binding was less than 5%, compared with MeSO$_2$-$[^14]$C]DDE binding in mouse adrenal cortex. The finding that o,p’-$[^14]$C]DDD was irreversibly bound also in zona reticularis indicates that it may also be activated by another enzyme than CYP11B1.

![Figure 7](image)

**Figure 7** Tissue binding profile in a MeSO$_2$-$[^14]$C]DDE-exposed mouse adrenal slice, cultured in a medium supplemented with ACTH (11 nM). MeSO$_2$-$[^14]$C]DDE binding is confined to zona fasciculata (ZF), as demonstrated by a bright-field (top) and the corresponding dark-field (bottom) image. Zona glomerulosa (ZG), zona reticularis (ZR), adrenal medulla (M).

**Radioluminography**

Quantification of tissue-bound radioactivity in cultured slices was determined by radioluminography (referred to as phosphorautoradiography in Paper I) of 2 µm thick adrenal sections. The images of tissue-bound MeSO$_2$-$[^14]$C]DDE obtained by radioluminography were found to closely match the images of the microautoradiograms of adrenal tissue sections (Papers I, II, and III; Figure 8).
There are several reports of radioluminography used to quantify radioactivity in histological sections. One such investigation quantified pro-opiomelanocortin A and B mRNA levels in the pituitary of rainbow trout (104). Thick (>20 µm) frozen tissue sections incubated with $^{11}$C-labelled substances have also been analysed by radioluminography (79, 105).

In the present work, human and mouse adrenal slices exposed to both MeSO$_2$-[14C]DDE and metyrapone showed a reduced level of binding (Papers I and III). Metyrapone also blocks irreversible MeSO$_2$-[14C]DDE protein binding in adrenal homogenate \textit{in vitro} (4, 106). These results are consistent with previous toxicity studies where metyrapone was reported to protect against MeSO$_2$-DDE-induced toxicity \textit{in vivo} (60).
Figure 9 Repeated exposures of $[^{14}\text{C}]$-labelled histological sections to an imaging plate showing a constant increase in luminescence over time, $r^2 > 0.98$ for both test substances. The time-dependent increase in detected luminescence is significantly different from the increase in the luminescence derived from background radiation ($p < 0.01$; mean and ± 95% confidence interval).

The values of radioactivity-derived luminescence (PSL/mm$^2$) obtained during exposure increased constantly with time (Figure 9). This result tallies with previous studies examining the system for linearity in radioactivity-derived luminescence ($83, 84, 107$). Variation in values between different exposures of the same MeSO$_2$-$[^{14}\text{C}]$DDE-exposed slice was less than 8%. Intra-exposure variation was less than 2% for three adjacent sections (Paper III).

Thus, radioluminography is a quick, sensitive method with which to quantify degrees of metabolite binding in the adrenal cortex. Combined with the cellular localization of binding obtained by microautoradiography, radioluminography seems to be an efficient means to semi-quantify the levels of [$^{11}\text{C}$]metomidate binding and MeSO$_2$-$[^{14}\text{C}]$DDE or o,p´-[$^{14}\text{C}]$DDD protein adducts in the target cells ex vivo.

**Cytochrome P450 mRNA localization**

To localize CYP mRNA expression in mouse adrenal, in situ hybridization with specific riboprobes was used. Effects of ACTH and metyrapone treatment in vivo on CYP11B1 mRNA levels were also examined (Paper II). The results show that CYP11B1 mRNA expression was confined to zona fasciculata, whereas CYP11B2 mRNA expression was observed only in zona glomerulosa. CYP11A1 mRNA was expressed throughout the adrenal cortex, with more intense labelling in the outer part of zona fasciculata and in zona reticularis. No expression of either cyp gene was observed in the adrenal medulla (Paper II;
These results are in agreement with a previous report on mouse CYP11B1 and CYP11B2 mRNA expression (58). I have found no previous report on the localization of CYP11A1 mRNA in the mouse adrenal cortex; in rat CYP11A1 mRNA expression is however found throughout the adrenal cortex (28). In rat CYP11B1 is expressed strongly in zona fasciculata, but weakly in zona reticularis. CYP11B2 is expressed only in zona glomerulosa (28, 29, 108-117). The level of radioactivity derived from the CYP11B1 riboprobe increased in the adrenal cortex of mice treated with ACTH (14 µg/kg/day x 5; p<0.05) and metyrapone (50 mg/kg/day x 5; p<0.05), compared with saline-treated control mice (Paper II). Repeated administration of ACTH in rats caused increased CYP11B1 mRNA expression, whereas CYP11B2 mRNA was reduced (112, 113). The increase in CYP11B1 mRNA in the mouse adrenal cortex following ACTH treatment is thus in agreement with previously reported data.

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Figure 10 In situ hybridization of CYP11B1, CYP11B2, and CYP11A1 in the adrenal cortex. In (A) a histological section, CYP11B1 mRNA expression (B) is confined to zona fasciculata (ZF), CYP11B2 mRNA (D) to zona glomerulosa (ZG), while CYP11A1 mRNA is expressed (C) throughout the adrenal cortex, labelling more intense in the outer part of zona fasciculata and in zona reticularis (ZR). No expression of either cyp gene is evident in adrenal medulla (M) (125-fold magnification, bar=125 µm).
The sites of MeSO₂-[¹⁴C]DDE binding and CYP11B1 mRNA localization were virtually identical in the mouse adrenal cortex. This result supports the contention that CYP11B1 catalyses activation of MeSO₂-DDE to a reactive metabolite in the adrenal zona fasciculata. It has also been shown that CYP11A1 and CYP11B2, transfected into COS cells, are not effective in metabolizing MeSO₂-DDE into a reactive metabolite (5). The differential localization of CYP11A1 and CYP11B2 mRNA supports the contention that these enzymes were not involved in MeSO₂-DDE binding and toxicity. On the other hand, the observation that the weak o,p′-DDD binding was partly localized to zona reticularis in mouse indicate that CYP11A1 can be the activating enzyme for this chemical (118).

**Comparison of metomidate binding and MeSO₂-DDE binding/toxicity in mouse**

To establish whether simulated adrenal stress can cause increased susceptibility to toxicity in the adrenal cortex, the effect of repeated ACTH treatments on [¹¹C]metomidate binding (a marker for CYP11B1 protein), irreversible binding and adrenocortical activity of MeSO₂-DDE was investigated in mice. MeSO₂-[¹⁴C]DDE binding was examined *in vivo* and *ex vivo* following treatment with MeSO₂-DDE (1.56, 3.13, 6.25 and 12.5 mg/kg), with saline (0.1 ml x 3), ACTH (300 µg/kg x 3), metyrapone (100 mg/kg x 3), and dexamethasone (5 mg/kg x 3), (Paper II).

**[¹¹C]Metomidate binding**

The non-methylated metomidate analogue etomidate has a high affinity for the CYP11B1 protein (IC₅₀ of 15 nM) and inhibits corticosterone/cortisol secretion in rodents and humans (119-121). Etomidate has less affinity for the CYP11A1 protein (IC₅₀ = 400 nM) (119). The binding of [¹¹C]metomidate or [¹¹C]etomidate to rhesus monkey adrenal sections was selectively localized to the cortex (79). The degree of [¹¹C]metomidate binding in human adrenal tumour tissue is closely correlated with the level of CYP11B1 immunostaining (79), thus supporting a high affinity for the CYP11B1 protein. As observed in this study, intense [¹¹C]metomidate binding was confined to the mouse adrenal cortex. Repeated ACTH treatments increased [¹¹C]metomidate binding (*p*<0.01), compared with saline-treated controls (Paper II). These results substantiate that the degree of CYP11B1 protein was increased in the adrenal cortex following ACTH treatment.

**MeSO₂-[¹⁴C]DDE binding**

Repeated treatment with ACTH or metyrapone increased the degree of MeSO₂-[¹⁴C]DDE binding in mouse adrenal zona fasciculata (*p*<0.05), whereas dexamethasone treatment reduced the binding *in vivo* (*p*<0.05; Figure 11A)
(Paper II). As mentioned above, ACTH treatment increased CYP11B1 mRNA in mouse and rat (112, 113, 122). Metyrapone treatment has also been shown to increase the plasma ACTH concentration in rat (28). Thus the results seem to show that modulation of the HPA axis leads to predictable changes in MeSO₂-[¹⁴C]DDE binding and CYP11B1 mRNA/protein content in the adrenal cortex.

Figure 11 MeSO₂-[¹⁴C]DDE binding in vivo (A) and ex vivo (B) in mouse adrenal zona fasciculata. (A) MeSO₂-[¹⁴C]DDE binding increased in vivo following repeated treatments with ACTH (300 µg/kg) and metyrapone (100 mg/kg) whereas it decreased following repeated dexamethasone treatment (5 mg/kg). 100% in control represents 30.6 ± 1.8 (PSL-BG)/mm² (mean ± SEM; n=3). (B) MeSO₂-[¹⁴C]DDE binding ex vivo increased following a single dose of MeSO₂-DDE in vivo (3.13 and 6.25 mg/kg) given 24 h before dissection.
100% in control represents 32.9 ± 15.5 (PSL-BG)/mm² (mean ± SEM; n=5). * = p<0.05
Student’s t-test with Welch correction.

In mouse adrenal slices incubated with MeSO₂-[¹⁴C]DDE, increased binding of radioactivity was observed following ACTH treatment in vivo (p<0.05; 24 h), compared with slices obtained from saline-treated mice (Paper II). This increased binding tallies with the results obtained in vivo, showing that the effect of in vivo treatment can be examined ex vivo.

Increased MeSO₂-[¹⁴C]DDE binding was also found in cultured slices obtained from mice treated with a single dose of MeSO₂-DDE (3.13 and 6.25 mg/kg; p<0.05) 24 h before dissection, compared with slices from mice treated with corn oil (Figure 11B). No significant effect on [¹¹C]metomidate binding was observed following MeSO₂-DDE treatment in vivo (Paper II). This result indicates that MeSO₂-DDE did not affect CYP11B1 enzyme levels 24 h after dosing. It has been shown previously that addition of reduced GSH to mouse adrenal homogenates reduces irreversible MeSO₂-[¹⁴C]DDE binding and increases the content of water-soluble metabolites in the culture medium, suggesting inactivation of the putative reactive metabolite by GSH conjugation (4, 5). A reduced GSH level in the mitochondria and cytosol can thus contribute to greater irreversible binding to macromolecules in the zona fasciculata cells. The observed increase in MeSO₂-[¹⁴C]DDE binding ex vivo following MeSO₂-DDE treatment in vivo, can therefore be due to a local depletion of GSH, leading in turn to reduced GSH conjugation of the putative reactive intermediate in the zona fasciculata cells.

**MeSO₂-DDE toxicity**

To facilitate quantification of cellular changes in zona fasciculata, computer-aided analysis was used (Paper II). The program utilizes the colour information in digital images to distinguish between objects in the image (123). This selection was obtained through a stepwise user interaction.

The objective was to establish whether adrenal stress simulated by repeated ACTH treatments strengthens the toxicity of MeSO₂-DDE in mouse adrenal cortex. As expected, ACTH treatment increased the adrenal weight (p<0.05), nuclear area (p<0.05), and width of the adrenal cortex (p<0.001), compared with saline-injected mice (36, 112, 113, 122, 124, 125). Treatment of mice with ACTH followed by a single injection of MeSO₂-DDE (12.5 mg/kg i.p. dissolved in corn oil), reduced adrenal weight 48 h after dosing (p<0.05), compared with ACTH-treated mice given a single injection of corn oil only. No significant difference in adrenal weight was observed in mice treated with saline followed by a single injection of MeSO₂-DDE (Paper II). The decreased adrenal weight
observed in ACTH-treated mice suggests that adrenal toxicity was exacerbated by simulated stress.

An increase in nucleus and cell area as well as in width of the adrenal cortex was observed in the MeSO₂-DDE-treated mice (without ACTH exposure), compared with saline treated-controls. This response was similar to that resulting from ACTH treatment.

In conclusion, the combined results show that the toxicity of MeSO₂-DDE is dependent on the amount of the ACTH-regulated enzyme CYP11B1 expressed in the mouse adrenal cortex. The results imply that animals under stress can have a greater degree of MeSO₂-DDE bioactivation and therefore be at increased risk of adrenal toxicity.

**Human adrenal tissue slice culture**

The development of adrenal precision-cut slice culture (Paper I) provided the means to investigate irreversible binding, and of adrenocorticolytic activity of MeSO₂-DDE and o,p'-DDD in human adrenal tissue (Paper III).

**MeSO₂-[¹⁴C]DDE**

As demonstrated in Paper III, a marked irreversible binding of MeSO₂-[¹⁴C]DDE in tissue slice culture was found to be confined both to zona fasciculata and more intensely in zona reticularis in a normal human adrenal, leaving zona glomerulosa and the adrenal medulla devoid of binding. Selective binding of MeSO₂-[¹⁴C]DDE was also observed in an aldosterone producing adrenocortical carcinoma and in a non-functional adrenocortical hyperplasia (Paper III). CYP11B1 mRNA expression in the human adrenal has been observed to be moderately high in zona fasciculata and high in zona reticularis, whereas no expression has been reported in zona glomerulosa, the adrenal medulla, or the adrenal capsule (126, 127). This correlation of MeSO₂-[¹⁴C]DDE binding and CYP11B1 mRNA expression supports the conclusion that MeSO₂-DDE was activated also by the human CYP11B1 enzyme.

To compare the MeSO₂-[¹⁴C]DDE bioactivating capacity in human and mouse adrenal cortex, sections from slices exposed to MeSO₂-[¹⁴C]DDE (human 2.2 kBq µCi/ml and mouse 3.7 kBq/ml; 24 h) in culture were embedded in methacrylate. Tissue sections were apposed to an imaging plate. The results indicate that the normal human adrenal cortex had roughly >65% of the bioactivating capacity observed in the mouse adrenal (Figure 12).
Addition of the CYP11B1 inhibitor metyrapone (50 µM) to the culture medium reduced MeSO₂-[¹⁴C]DDE binding in human adrenal cortex to below the detection limit (Paper III). This finding is in agreement with the effect of metyrapone (10 µM) exposure observed in H295R cells, where reduced CYP11B1 activity (30% of control) was found (82). The previously reported inhibition of MeSO₂-[¹⁴C]DDE binding in human adrenal cortex homogenate by metyrapone (35% of control) also favours CYP11B1 as the activating enzyme in human zona fasciculata/reticularis (106). Binding to adrenal homogenate was also reduced by GSH (4).

Exposure of human slices to MeSO₂-DDE (25 µM) increased the accumulation of 11-deoxycorticosterone ($p<0.05$), 11-deoxycortisol and androstenedione ($p<0.05$) in the culture medium. As in the mouse, no apparent histological changes were found in MeSO₂-DDE-exposed human slices (Paper III). In the human adrenocortical carcinoma cell line H295R, MeSO₂-DDE (10 µM) reduces CYP11B1-catalysed cortisol formation (66% ±10%) (82). Studies using human adrenal cortex homogenates have previously shown that irreversible binding of MeSO₂-[¹⁴C]DDE has an apparent $K_m$ of 1.4 µM, while in mouse Y1 cells there is an apparent $K_i$ of 1.6 µM (62, 106).

Combined with the zone-specific binding observed, the above findings support the contention that MeSO₂-DDE is metabolized by CYP11B1 and can exert adrenocorticolytic activity in the human adrenal cortex.
**o,p´-[\textsuperscript{14}C]DDD**

By virtue of its tissue-selective toxicity, o,p´-DDD is currently used as an adrenocorticolytic drug for treatment of adrenocortical carcinoma and Cushing’s syndrome (128, 129). However, only one-third of the patients respond to post-operative o,p´-DDD treatment (129). In addition to hypocortisolism, o,p´-DDD gives rise to dose-dependent side effects in the gastrointestinal tract (nausea, vomiting and diarrhoea) and CNS (dizziness and headaches). In a substantial proportion of patients, these side effects are intolerable at therapeutic doses and the drug has to be withdrawn. The effective dose for treatment of adrenocortical cancer gives a plasma concentration exceeding 14 µg/ml (44 µM), whereas plasma concentrations below 10 µg/ml (31 µM) are therapeutically insufficient (130-132).

o,p´-[\textsuperscript{14}C]DDD binding was observed in zona fasciculata/reticularis in a normal human adrenal, in an aldosterone producing adrenocortical carcinoma and in a non-functional adrenocortical hyperplasia. However, in contrast to MeSO\textsubscript{2}-[\textsuperscript{14}C]DDE binding, o,p´-[\textsuperscript{14}C]DDD binding was reduced by only 42% following metyrapone exposure (Paper III). This observation implies that o,p´-DDD was metabolized by more than one enzyme in the adrenal cortex. This enzyme could be another steroidogenic enzyme, such as CYP11A1 (118).

Unlike MeSO\textsubscript{2}-DDE, exposure to o,p´-DDD (25 µM) did not produce any visible effects on the steroid secretion. Nor were any histological changes found in o,p´-DDD-exposed slices (Paper III). Previous binding experiments using human adrenal homogenates have shown that o,p´-DDD has an apparent K\textsubscript{m} of 24 µM (106). Irreversible o,p´-DDD binding in this study was only partly reduced by exposure to metyrapone (49% of control), whereas addition of GSH reduced binding only slightly.

In conclusion, the similar localization of binding and the lower (1/17) K\textsubscript{m}-value for irreversible protein binding indicate that MeSO\textsubscript{2}-DDE could be toxic at concentrations below the therapeutically effective o,p´-DDD plasma concentration (130). Considering the low potency and the potentially severe side effects frequently observed following o,p´-DDD treatment, MeSO\textsubscript{2}-DDE should be evaluated as a possible alternative for the treatment of adrenocortical hypersecretion and tumour growth.

**Target cells for [\textsuperscript{3}H]DMBA in rat and mouse adrenals**

In order to make use of the tissue slice culture procedure to examine activation and irreversible binding of lipophilic substances, another classical adrenocorticolytic agent with an unclear mode of action was examined in rats and mice. The polycyclic aromatic hydrocarbon DMBA is a known potent adrenal toxicant in rat, but completely lacks toxicity in mouse. Therefore, a
comparative examination of cellular localization of irreversible binding *ex vivo* as well as binding and distribution *in vivo* was performed in rats and mice. Some animals were pre-treated with 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126) and β-naftoflavone (BNF) to induce Ah-receptor regulated enzymes such as CYP1A1 and 1B1 (Paper IV).

In rat adrenal slices, irreversible binding of [³H]DMBA was confined to *zona fasciculata* and *zona reticularis*. In slices from rats treated with PCB 126 *in vivo*, binding in *zona fasciculata/reticularis* was roughly doubled. Very weak [³H]DMBA binding was observed in mouse adrenal *zona fasciculata/reticularis*.

Intense binding to clusters of cells (henceforth called focal binding) was found at the border between *zona fasciculata/glomerulosa*, and more intensely in *zona reticularis* in the rat adrenal. [³H]DMBA binding was induced by PCB 126 but inhibited by ellipticine, ANF, 1-PP, and 1-EP, but not by metyrapone (Paper IV). Essentially all DMBA metabolism in rat adrenal is catalysed by CYP1B1 (133), while in mouse adrenal no CYP1B1 protein has been found (134-136). DMBA-induced apoptosis has been reported in pre-B cells co-cultured with bone marrow stromal cells from AhR-null mice, but not from CYP1B1-null mice, indicating a requirement for CYP1B1-catalysed metabolism (137). The ability of the CYP1 inhibitors ellipticine, ANF, 1-PP, and 1-EP to reduce DMBA binding (138-143) in cultured rat adrenal slices, supports the contention that parenchymal binding in *zona fasciculata/reticularis* was due to a local, presumably CYP1B1-catalysed, formation of a reactive DMBA metabolite.

A marked induction of [³H]DMBA binding was found in endothelial cells, in *zona glomerulosa* and in the capsule in PCB 126-treated rats and mice. This binding was completely abolished by ellipticine, 1-EP, and 1-PP (10 µM) but remained unaffected by metyrapone (Paper IV). CYP1A1 immunostaining and/or [³H]DMBA binding are reported to be inducible by BNF in endothelial cells of heart, lung, liver, skeletal muscle, and uterus in rats and mice, whereas neither immunostaining nor binding occurs in vehicle-treated animals (143, 144). No constitutive expression of CYP1A1 has been found in human vascular endothelial cells (145). TCDD induced CYP1A1, but not CYP1B1, in these cells (145). The inducible DMBA binding in endothelial cells may therefore be catalysed by CYP1A1.

Tape-section autoradiograms of vehicle-treated rats given [³H]DMBA revealed intense labelling in the adrenal cortex. In contrast, no apparent labelling was observed in the adrenal cortex of vehicle-treated mice. In PCB 126- and BNF-treated mice, non-extractable radioactivity was induced in the adrenal capsule, but not in vehicle-treated mice (Paper IV; Figure 13).
Figure 13 $[^3]H$DMBA labelling in autoradiograms from extracted tape sections. Comparison of a rat (left) and a mouse (right), pre-treated with PCB 126 (bottom) and vehicle (corn oil; top). Considerable DMBA labelling is evident in the control rat adrenal cortex. In the mouse treated with PCB 126, non-extractable labelling in the adrenal capsule is seen.

The results presented here suggest that the adrenocorticolytic activity of DMBA may be dependent on a dual mode of action. In agreement with the observations on cellular sites of DMBA-toxicity (69-71), intense, presumably CYP1B1-catalysed, DMBA binding was observed in zona fasciculata/reticularis in rat (but not mouse) adrenal cortex. The observed irreversible DMBA binding presumably catalysed by CYP1A1 in endothelial cells in the adrenal cortex also corroborates the suggestion that the endothelial cell is a primary site of toxicity (74). In support of the proposal by Cefis & Goodall (75), the results of this study favour the presence of two modes of action in the adrenal cortex. The adrenocorticolytic activity of DMBA may rely on two independent pathological processes resulting in cell death and haemorrhage.

**o,p'-[^14]C]DDD binding in Atlantic cod interrenal cells**

The tissue-slice culture procedure was also applied to determine the ability of interrenal cells to activate o,p'-[^14]C]DDD in Atlantic cod (Paper V). Previous studies have shown that o,p'-DDD can impair cortisol secretion in teleost fish (146-149). Whether this effect is mediated by *in situ* bioactivation of o,p'-DDD in the glucocorticoid-secreting interrenal cells (the equivalent of the mammalian adrenal cortex) has not been established. Microautoradiography of precision-cut tissue slices from the anterior kidney exposed to o,p'-[^14]C]DDD, revealed that radioactivity was bound selectively in the interrenal cells. Furthermore, autoradiography at different levels of resolution performed on Atlantic cod
given o,p′-[¹⁴C]DDD intragastrically confirmed this selective localization of non-extractable radioactivity (Paper V).

A site-specific irreversible binding of o,p′-[¹⁴C]DDD therefore seems to take place in glucocorticoid producing interrenal cells in Atlantic cod, both ex vivo and in vivo. Precision-cut tissue slice culture could become a useful laboratory screening tool for evaluation of potential wildlife effects of adrenocorticolytic environmental pollutants.

**Hypothesis on species differences in MeSO₂-DDE bioactivation**

As shown by previous work in our laboratory, there are major species differences in irreversible binding and toxicity of MeSO₂-DDE in glucocorticosteroid-producing cells (10, 150). The results presented in this thesis suggest that there might be a gradient from sensitive to insensitive species, as compiled in Table 1. Mouse and most likely human adrenal cortex has a good bioactivating capacity for MeSO₂-DDE, whereas rat and mink are poor bioactivators. For comparative reasons, data on o,p′-DDD are included in Table 1.

**Table 1** Species differences of irreversible MeSO₂-DDE and o,p′-DDD binding and toxicity in glucocorticoid-producing cells. ++++, very high; ++, high; +, yes; (+), low but present; −, no (at relevant doses); (−), unclear; ?, not examined. * Toxicity ex vivo with MeSO₂-DDE exposure was observed at a concentration where o,p′-DDD did not give rise to an effect in the same experiment. ♣ Slight binding ex vivo was observed.

<table>
<thead>
<tr>
<th>Species</th>
<th>MeSO₂-DDE Binding</th>
<th>MeSO₂-DDE Toxicity</th>
<th>o,p′-DDD Binding</th>
<th>o,p′-DDD Toxicity</th>
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<td>Human</td>
<td>++</td>
<td>(+)*</td>
<td>++</td>
<td>+++*</td>
<td>Paper III, (64, 106, 151)</td>
</tr>
<tr>
<td>Mouse</td>
<td>+++</td>
<td>+++</td>
<td>(−)*</td>
<td>−</td>
<td>Papers I and II, (4, 6, 60, 150)</td>
</tr>
<tr>
<td>Rat</td>
<td>(−)*</td>
<td>−</td>
<td>(−)</td>
<td>−</td>
<td>Paper I, (64)</td>
</tr>
<tr>
<td>Seal</td>
<td>+</td>
<td>?</td>
<td>+</td>
<td>?</td>
<td>(152)</td>
</tr>
<tr>
<td>Dog</td>
<td>?</td>
<td>?</td>
<td>+</td>
<td>+</td>
<td>(63, 64)</td>
</tr>
<tr>
<td>Otter</td>
<td>−</td>
<td>?</td>
<td>+</td>
<td>?</td>
<td>(8)</td>
</tr>
<tr>
<td>Mink</td>
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<td>?</td>
<td>+</td>
<td>+</td>
<td>(8)</td>
</tr>
<tr>
<td>Chicken</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(9)</td>
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<tr>
<td>Eider/Duck</td>
<td>+</td>
<td>?</td>
<td>+</td>
<td>?</td>
<td>(9)</td>
</tr>
<tr>
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<td>(+)</td>
<td>?</td>
<td>(+)</td>
<td>?</td>
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</tr>
<tr>
<td>Atlantic cod</td>
<td>(+)</td>
<td>?</td>
<td>+</td>
<td>+</td>
<td>Paper V, unpublished</td>
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<tr>
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<td>?</td>
<td>?</td>
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<tr>
<td>Rainbow trout</td>
<td>(−)</td>
<td>?</td>
<td>+</td>
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The enzymes in the CYP11 family are closely related and there is considerable inter-species amino acid homology in the CYP11B1 proteins. In mouse, the homology of amino acids in CYP11B1 and CYP11B2 is 80%, while in humans it is 93%. Despite this high degree of homology in mouse and human, no irreversible binding of MeSO₂-[¹⁴C]DDE was found in zona glomerulosa (only in zona fasciculata/reticularis) in these species.

No correlation between irreversible binding and CYP11B1 amino acid homology is apparent. A possible explanation for the observed species differences could be differences at single codons in the substrate-binding region of the CYP11B1 enzyme. 11β-Hydroxylase deficient human subjects display several point mutations in the CYP11B1 gene, leading to amino acid substitutions in the CYP11B1 protein (153-155). One such “hot spot” for mutation can be distinguished at codon T318 (154-156).

Amino acid alignments of human CYP11B1 and CYP11B2 with three prokaryote P450 genes with determined tertiary protein structure (P450CAM, P450Terp and P450BM-3) have been made (157-161). A region of amino acids, located at codons 295-335 in CYP11B1/2, was proposed to be analogous to the I-helix in the prokaryote enzymes and might be a determinant of substrate binding and specificity (160, 161). To ascertain the importance of individual amino acid substitutions and combinations thereof in this region, human CYP11B1 and CYP11B2 mutants have been constructed and transfected into COS-1 cells (160, 161). A CYP11B2 mutant with substitution in codon 320 resulted in increased 11β-hydroxylase activity and a reduction in aldosterone synthase activity, compared with the wild type CYP11B2 (160). CYP11B1 variants with substitution of the amino acids S288G and V320A convert CYP11B1 into an aldosterone-producing enzyme (162). A CYP11B1 mutant with substitution in codon 320 has a partly reduced 11β-hydroxylase activity compared with the wild-type CYP11B1 (161). The two apparent differences in the proposed substrate binding region in the CYP11B1 and CYP11B2 amino acid sequences in mouse and human are a shift from Serine/Valine to Glycine in codon 288 and Serine/Valine to Alanine in codon 320 (Table 2).

Based on these considerations, a hypothesis can be formulated as follows:

| The presence of a Serine/Valine in codon 320 of CYP11B1 predisposes to a high degree of MeSO₂-DDE bioactivation. |

According to this hypothesis the CYP11B1 enzyme of Cape baboon and hamster would have a good MeSO₂-DDE bioactivating capacity. Other species, such as pig and guinea pig, would be expected to have a poor MeSO₂-DDE-bioactivating capacity. These possibilities can be tested with the slice culture system. Another way to test this hypothesis is to make use of the mutant human
CYP11B1 and CYP11B2 genes (A320V & V320A variants) created by Böttner et al. to examine irreversible protein binding in transfected COS cells (160, 161).

Table 2 Comparison of the amino acid sequences between different species, in region 282-345 of CYP11B1 and 11B2 expressed in the adrenal cortex. Standard amino acid nomenclature is used. The mentioned amino acids are A, Alanine; S, Serine; G, Glycine; V, Valine. Alignment was calculated using ClustalW with default settings (163-165), http://www.ebi.ac.uk/clustalw. The accession numbers (obtained by searches with Swiss-PROT/trEMBL and ExPASy) for CYP11B1 are; mouse, Q9QW69; human, P15538; rat, P15393; sheep, P51663; pig, Q29552; bovine, P15150; frog, Q92104; hamster P97720; guinea pig, Q64408; rainbow trout Q9PT16; Cape baboon, Q29527; CYP11B2; mouse, P15539; human, P19099.

Studies regarding the three-dimensional structure of the substrate-binding region of CYP11B1 and the binding of MeSO₂-DDE isomers and structurally related molecules to the active site are warranted to extend our understanding of the interactions of environmental pollutants and pharmaceuticals with the CYP11B1 protein.
Conclusions

- Adrenal slice culture is a simple *ex vivo* test system with which to examine zone-specific irreversible metabolite binding, altered steroid hormone secretion, and target cell ultrastructure in various species. The slice culture procedure can be useful for evaluating the endocrine disrupting potential of chemicals and pharmaceuticals.

- Radioluminography is an efficient means with which to measure irreversibly bound adducts in restricted target cell populations.

- The adrenocortical activity of DMBA can rely on two pathological processes, resulting in necrosis and haemorrhage. Metabolic activation and irreversible binding of DMBA in rat adrenal parenchymal cells is presumably catalysed by CYP1B1, whereas in endothelial cells it seems to be catalysed by CYP1A1.

- The localization of MeSO$_2$-DDE binding is linked to the expression of CYP11B1.

- A close correlation exists between irreversible binding and adrenocortical activity of MeSO$_2$-DDE *ex vivo* and *in vivo* in the mouse adrenal zona fasciculata.

- Amount of irreversible binding and adrenocortical activity of MeSO$_2$-DDE are dependent on the content of the ACTH-regulated enzyme CYP11B1. Organisms under stress can have a greater degree of MeSO$_2$-DDE bioactivation and therefore be at increased risk of adrenal toxicity.

- Irreversible binding of MeSO$_2$-DDE and o,p´-DDD in human adrenal was observed in zona fasciculata/reticularis. MeSO$_2$-DDE should be evaluated as a suspected human adrenal toxicant, especially with regard to the risk posed to suckling infants in developing countries. MeSO$_2$-DDE should also be evaluated as a possible alternative to the presently used drug (o,p´-DDD) for the treatment of adrenocortical hypersecretion and tumour growth in human subjects.
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