Cloning and characterization of organic anion transport systems in the adrenal cortex and their role in steroid release

Dissertation

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Tag der mündlichen Prüfung:	

ABSTRACT

The adrenal steroid hormones have a central role in maintaining homeostasis, as they have influence on almost every physiological process. Their movement across the cell membrane is still poorly understood, although this is of great interest to basic biology and medicine. Previous studies have suggested transporter(s) may participate in this process. In this study the characteristic features of the previously demonstrated ROAT1-like exchange transport system in bovine adrenal cells were investigated with representative substrates. Corticotrophin (ACTH) stimulated ^{3}H-PAH uptake into bovine adrenocortical cells, which could be inhibited by probenecid. Cortisol, glutarate and PAH in the incubation medium also cis-inhibited ^{3}H-PAH uptake, and preincubation with PAH trans-stimulated ^{3}H-PAH uptake. Preliminary studies on human adrenocortical cells also provided evidence for the existence of a probenecid inhibitable PAH-transporter. These results support the concept of an organic anion/dicarboxylate exchanger involved in cortisol release and PAH uptake into adrenocortical cells. Additionally, a sodium-dependent succinate uptake was also demonstrated in bovine adrenocortical cells. The uptake was inhibited by lithium, glutarate, fumarate, α-ketoglutarate and maleate, but not by 2,3-dimethylsuccinate or cis-aconitate. The lack of inhibition by citrate on succinate uptake at pH 7.4 is different from the data reported for the dicarboxylate transporters of all other organs investigated. These data are the first evidence for the existence of a Na^{+}-dicarboxylate cotransporter in adrenocortical cells. The expression of two families of organic anion transporters in adrenal cells was also investigated. This study represents the first demonstration of the renal organic anion transporter (ROAT1) and the three members of the organic anion transporting polypeptide family (oatp1, oatp2, oatp3) by PCR from rat adrenal cDNA. The rat organic cation transporter 2 (OCT2) was also detected from the adrenal gland by in situ hybridization. In addition, in situ hybridization was performed to determine the localisation of the expression of the newly demonstrated transporters in the rat adrenal gland. In the case of ROAT1, intense signal was observed in the outer zona fasciculata, while oatp3 and OCT2 mRNAs were clearly shown to be expressed in the zona glomerulosa. The oatp1- and oatp2-specific probes produced signals in single cells or small groups of cells in the inner zona fasciculata and zona reticularis. For all transporters tested in the adrenal gland, only ROAT1 expression showed clear increase upon exposure to ACTH. The number of cells expressing ROAT1 mRNA was

increased, especially in the inner zones of the adrenal cortex, while without ACTH, the signal was no longer detectable even in the inner zona fasciculata.. In summary these experiments provide clear evidence for the existence of a variety of transport systems in the adrenal gland. The evidence presented here suggests that of these transporters, the adrenal ROAT1 seems to be involved in glucocorticoid release from adrenocortical cells.

LIST OF ABBREVIATIONS

bp	base pairs
°C	Celsius
cDNA	complementary DNA
cRNA	complementary RNA
DMSO	dimethyl sulfoxid
dNTP	deoxyribonucleotide phosphate
fNaDC-3	flounder sodium / dicarboxylate cotransporter 3
fROAT	flounder renal organic anion transporter
h	hour
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
hROAT1	human renal organic anion transporter 1
K_m	Michaelis Menten constant
LB	Luria Bertani broth
M	molar (moles per litre)
µM	micromolar
mM	millimolar
min	minute
ml	millilitre
mRNA	messenger RNA
oatp	organic anion transporting polypeptide
OCT	organic cation transporter
ORI	oocyte Ringer's solution
PAH	para-aminohippurate
ROAT1	rat renal organic anion transporter 1
rOCT1	rat organic cation transporter 1
rOCT2	rat organic cation transporter 2
PCR	polymerase chain reaction
rpm	revolution per minute
sec	second
U	unit
UTR	untranslated region

1 INTRODUCTION

1.1 THE ANATOMY OF THE ADRENAL GLAND

In most mammalian species, including the rat, dog and human, the adrenal glands are paired organs, located close to the cephalic pole of the kidneys. The adrenals comprise two endocrine tissues which are embryologically and functionally distinct. The adrenal cortex which derives from the mesodermal lining of the coelom, and the adrenal medullary chromaffin tissue which originates in the neural crest and migrates into the centre of the cortical tissue during foetal development. The division of the mammalian adrenal cortex into three distinct concentric zones was first described by Harley in 1858, and the terms zona glomerulosa, zona fasciculata and zona reticularis were introduced by Arnold in 1866 (Neville & O'Hare 1982).

Blood flows centripetally through the adrenal cortex into the large medullary sinusoids, which drain into the central vein. The arrangement of sinusoids within the gland is such that almost every cell of the adrenal cortex is in direct contact with a blood vessel. For many years it was the commonly held view that the nerve bundles passed through the cortex without branching. It is now widely accepted that the adrenal cortex receives a rich innervation, mainly in the region of the zona glomerulosa and the connective tissue capsule. A range of neurotransmitters has been identified in the adrenal cortex, including both catecholamines and neuropeptides (Kondo 1985, Vinson *et al*. 1994). Nerve terminals have been found in close contact with both blood vessels and the adrenocortical cells, and it has been shown that certain neurotransmitters are able to influence blood flow and steroid secretion in the adrenal gland (Vinson *et al*. 1994).

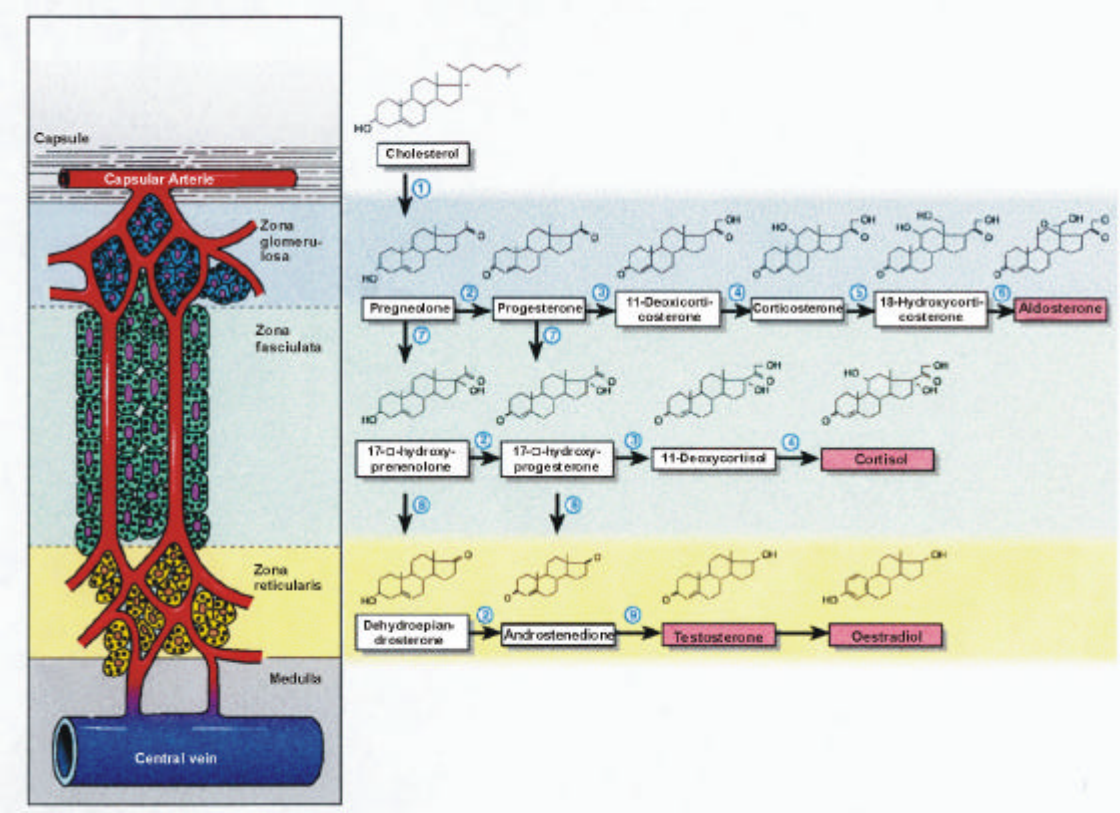
1.2 HORMONES OF THE ADRENAL GLAND

The principal secretory products of the adrenal medulla are the catecholamines, which are derivatives of the amino acid tyrosine, while the adrenal cortex secretes steroid hormones derived from cholesterol. The adrenal cortex is capable of producing about 50 different steroids with a wide range of activities. In most species, including the human, the most physiologically important of these corticosteroids are aldosterone, a mineralocorticoid, and cortisol, a glucocorticoid. The most abundant steroid produced

by the adrenal cortex, however, is an androgen, dehydroepiandrosterone sulphate (DHEAS). The adrenal cortex also produces estrogen, progesterone, and a wide range of precursors and metabolites of these steroids. In rats, which lack the 17α-hydroxylase activity necessary for cortisol and androgen production, the major glucocorticoid is corticosterone, and there is also a reduced androgen production. Recent evidence suggest that the mammalian adrenal cortex also produces an ouabain-like compound (Hinson *et al.* 1995).

1.2.1 Steroid hormone biosynthesis

The adrenal steroids are all synthesized from cholesterol, mainly by a series of hydroxylations involving the cytochrome P450 family enzymes. The major pathways of adrenal steroid biosynthesis are shown in Picture1.



Picture 1. Pathways of steroid biosynthesis and the vasculature of the mammalian adrenal gland. The outermost layer of the adrenal cortex, immediately below the capsule, is the zona glomerulosa which produces mineralocorticoids (aldosterone).The zona fasciculata comprises the greater part of the adrenal cortex and produces glucocorticoids (cortisol). The innermost zone of the adrenal cortex is the zona reticularis which produces sex steroids (testosterone, estradiol). Key to enzymes: **1** cytochrome $P450_{SCC}$ (cholesterol side-chain-cleavage), **2** 3β-hydroxysteroid dehydrogenase, **3** cytochrome $P450_{21}$ (21β-hydroxylase), **4** cytochrome $P450_{11\beta}$ (11β-hydroxylase), **5** cytochrome $P450_{18}$ (18-hydroxylase), **6** cytochrome $P450_{aldo}$ (aldosterone synthase), **7** cytochrome $P450_{17\alpha/17,20\ lyase}$ (17α-hydroxylase), **8** cytochrome $P450_{17\alpha/17,20\ lyase}$ (17,20-lyase), **9** 17-reductase

The cholesterol used in steroid synthesis is derived from two sources: *de novo* synthesis from acetate in the adrenals, or receptor-mediated uptake of plasma lipoproteins. Most cholesterol is stored in lipid droplets, in an esterified form, which is rapidly accessible in response to acute stimulation of steroidogenesis and is then replenished (Vinson *et al*. 1992). The endpoint of conversion of cholesterol to steroid hormones is zone-specific. In all mammalian species the zona glomerulosa is the only site of aldosterone synthesis. In rats the inner adrenocortical zones produce corticosterone as a major secretory product. In dog and human the inner zones favour the 17α-hydroxy pathway, with the zona fasciculata mainly producing cortisol and the zona reticularis mainly producing androgenes and sulphated steroids.

1.2.2 Regulation of adrenal function

Glucocorticoid secretion is regulated almost exclusively by corticotrophin (ACTH), a 39 amino acid peptide hormone released by the anterior pituitary gland. Corticotrophin has several distinct effects on the adrenal gland, which are evident at different time intervals after stimulation. Acutely, ACTH causes an increase in the rate of blood flow through the adrenal gland and an increase in the rate of steroid secretion. The long term effects include stimulation of the growth of the adrenal cortex and increased expression of the enzymes involved in steroid biosynthesis (Simpson & Waterman 1992). The adrenal gland requires a certain level of ACTH secretion to maintain its normal structure and function.

The regulation of aldosterone secretion is more complex, involving the interaction of several different systemic factors (for reviews see Müller 1988 and Vinson *et al*. 1992). Of these factors, the renin-angiotensin system is one of the most important, but very high concentrations of potassium ions can directly stimulate aldosterone secretion.

1.2.3 Transport of hormones in blood

The steroids secreted by the adrenal cortex, being hydrophobic in nature, are carried in the blood mostly bound to plasma proteins. Aldosterone is mostly carried by plasma albumin, while the glucocorticoids have a specific carrier protein, termed corticosteroid-binding globulin (CBG). There is a dynamic equilibrium between free and bound

glucocorticoid in plasma, and it seems to be that the free steroid is biologically active (Mendel 1989).

1.2.4 Actions of adrenal hormones

The glucocorticoids and the mineralocorticoids bind to intracellular receptors resulting in induction of mRNA and protein synthesis. Steroid binding induces conformational changes in the receptor, leading to activation and transport into the nucleus, where the complex binds to the promoter of a variety of different genes. Glucocorticoids were named for their effects on carbohydrate metabolism, but they have a wide range of action in many tissues as a result of activation of specific glucocorticoid receptors. They have effects on intermediary metabolism, immune function, fluid and electrolyte balance, bone and connective tissue function, mood and behaviour, and developmental processes. Glucocorticoids have an antagonistic action on insulin in intermediary metabolism as they increase hepatic glycogenesis by activating glycogen synthase and inactivating glycogen phosphorylase (Stalmans & Laloux 1979). They also increase hepatic gluconeogenesis by activation of glucose-6-phosphatase and pyruvate kinase and mobilisation of glucogenic substrates from peripherial tissues (Exton 1979). High glucocorticoid levels in the serum have prominent anti-inflammatory and immunosuppressive action, and thus glucocorticoids have an important role in the modulation and suppression of the acute stress response (Muck *et al*. 1984) and have pharmacological uses. However, their role in the normal modulation of immune processes is unclear. The effect on fluid and electrolyte balance is mediated via glucocorticoid receptors rather than an interaction with mineralocorticoid receptors (Gardner *et al*. 1986, Raff 1987). Glucocorticoids influence bone and mineral metabolism by reducing calcium absorption from the gut, resulting increased serum levels of parathyroid hormone, inhibiting osteoblast function and thus decreasing new bone formation (Hahn *et al*. 1979). In connective tissues, glucocorticoids inhibit the proliferation of fibroblasts and their production of collagen and glycosaminoglycans, resulting in impaired wound healing (Leibovich & Ross 1975). Glucocorticoids affect a diverse range of processes such as sleep patterns, cognition, and the reception of sensory input (McEwen 1979) and they have at least a maintaining role in depressive disorder (Bearn & Raven 1993), but the mechanism underlying these effects are poorly understood.

The main function of mineralocorticoids is in the regulation of water and electrolyte balance. Their principal effect is to increase the reabsorbtion of sodium in the kidney and in secretory epithelia.

Finally, there are groups of steroid hormones, namely the neural active steroids, which do not act through the classical intracellular, genomic mechanism described above. For example, pregnenolone sulphate and DHEAS are potent GABA antagonists and positive allosteric modulators at the N-methyl-D-aspartate (NMDA) receptor in the brain (Mellon 1994).

Under normal physiological conditions the corticosteroids have a central role in maintaining homeostasis and under pathophysiological stress work towards restoring it. Also, where the glucocorticoids are employed as pharmacological agents, a correct understanding of their way to reach their target cells and their manifold influences on cellular processes is essential if adverse reactions are to be avoided or minimised.

1.3 MOVEMENT OF STEROID HORMONES THROUGH THE PLASMA MEMBRANE

The different biosynthetic pathways and the regulation of steroid hormone synthesis, and also their action on their target organs and cells have been well characterised in recent years. In contrast, the release of steroids from the steroid-synthesizing cells into the blood and their entry into the target cells is poorly understood. It has long been assumed that this occurs via simple diffusion or exocytosis, based on the lipophilic structure of steroid hormones. However, in vitro studies demonstrated retention of steroids against a concentration gradient at the plasma membrane (Whitehouse *et al.* 1971, Inaba *et al.* 1974) and direct morphological evidence for exocytosis or any relevant storage of cortisol has never been demonstrated (Gemmell *et al.* 1977, Basset *et al*. 1980). Therefore, it is possible that a transport mechanism is also involved in steroid hormone release.

1.3.1 Transporter participation in cortisol uptake into hepatocytes

One of the first reports suggesting the transporter-mediated uptake of glucocorticoids was published by Rao *et al*. in 1976. They found that the uptake of cortisol into isolated liver cells was temperature dependent, showed saturation kinetics, was inhibited by

cortisone and corticosterone, and was significantly decreased by metabolic inhibitors and sulfhydryl reagents. The uptake was not dependent on sodium and was not affected by ouabain. The uptake from the external media into the liver cells was a rapid process, and showed characteristics of mediation by protein. Since the specific glucocorticoid binding proteins are localised in the cytoplasm in these cells, it suggests that these proteins are not directly involved in the uptake of cortisol. Their conclusion was that the transport of cortisol into the liver cells seemed to be in part a carrier-mediated action. However, a transport protein(s) mediating cortisol transport has yet to be identified.

1.3.2 The multispecific organic anion transport system (OAT)

One possible candidate for glucocorticoid transport is the organic anion transport system in the kidney. This multispecific system has been well characterised and has an important role in the excretion of potentially toxic organic anions, including endogenous compounds and their metabolites. The well characterised basolateral uptake step of this system involves exchange of an organic anion for an intracellular dicarboxylate, probably α-ketoglutarate, via the OAT1 transporter. This process is functionally coupled to sodium-dicarboxylate cotransport which recycles the exchanged dicarboxylates into the cell, and the sodium ions are pumped out via the Na^+-K^+-ATPase at the cost of one molecule of ATP (Fig. 1.). The model substrate for this system is para-aminohippurate, or PAH, a product of 4-aminobenzoate metabolism and its classical inhibitor is probenecid (Fig. 2.).

Although the functional characteristics of this organic anion system have been known for many years, the protein involved in the basolateral uptake of organic anions has only recently been identified, and is known as OAT1, from the rat and flounder kidney by functional expression in *Xenopus laevis* oocytes (Sekine *et al.*1997, Sweet *et al.*1997, Wolff *et al.* 1997). These proteins mediated PAH uptake, which was cis-inhibited by different kind of endogenous and exogenous organic anions and by dicarboxylates. From these observations it was suggested that these proteins moderate organic anion transport at the basolateral membrane of the proximal tubule of the kidney. During the present study, the human homologue was also cloned (Reid *et al.* 1998).

Inhibition studies on PAH uptake in the proximal tubule of the rat kidney were carried out by Ullrich and coworkers in order to determine the structural requirements of substrates of the system. This transport system has wide substrate specificity with a necessity of a negative or partial negative charge on a hydrophobic backbone, but the system also interacts with non-ionisable hydrophobic compounds. The saturable transport of cortisol at physiological concentration was also shown by these studies, and this process was inhibited by probenecid (Ullrich *et al*. 1991). Recently it was demonstrated that adrenocorticotroph hormone (ACTH)-stimulated cortisol release from primary bovine adrenocortical cells was inhibited by probenecid and trans-stimulated by PAH (Steffgen *et al*. 1996 and Rohrbach *et al*. 1997). Furthermore, oocytes expressing adrenocortical mRNA transport PAH in a probenecid-inhibitable manner, the existence of mRNA encoding a PAH transporter in the adrenocortical cells.

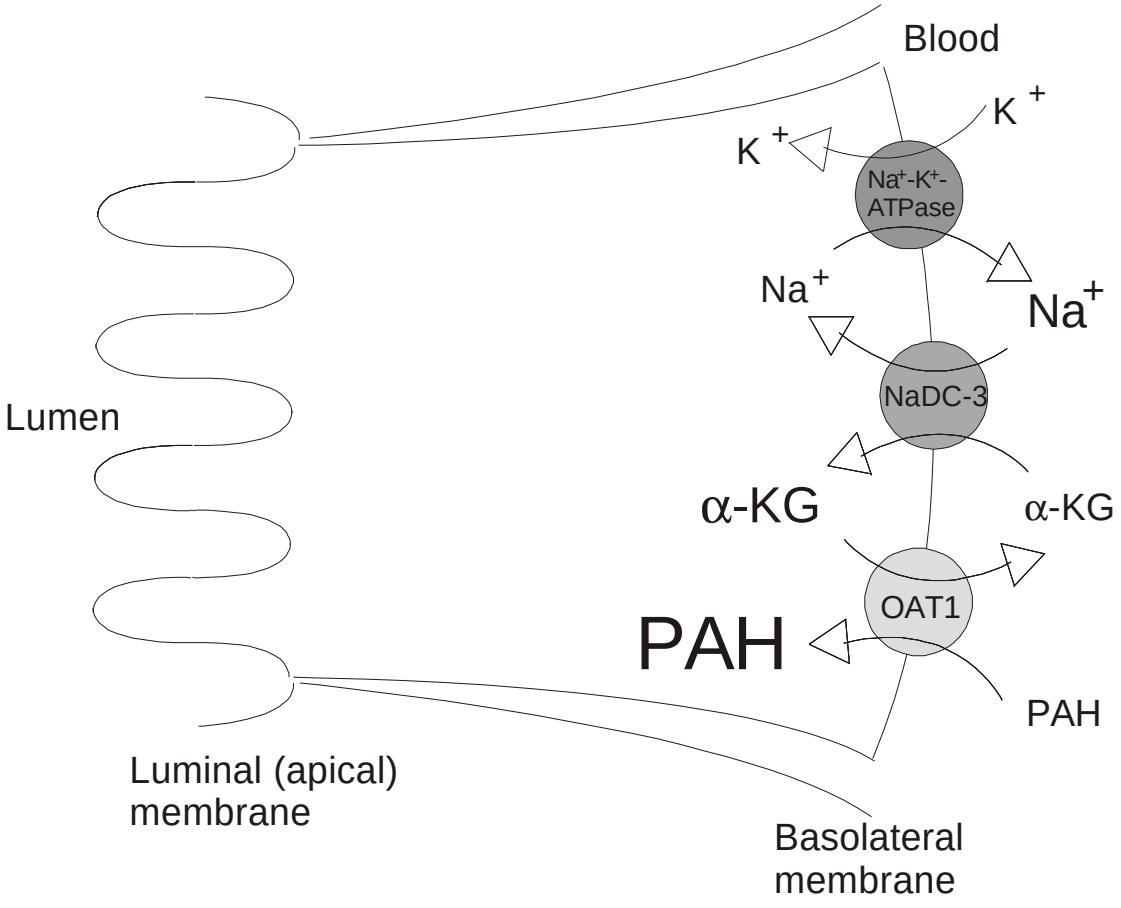


Figure 1. Model of basolateral organic anion uptake in proximal tubule cells of the kidney. The current model for basolateral organic anion uptake involves import of an organic anion, represented here by PAH, in exchange for an intracellular dicarboxylate (α-KG), by the multispecific organic anion transporting system (OAT1). The intracellular pool of α-ketoglutarate is maintained by metabolism and import by sodium-dicarboxylate cotransporter (NaDC-3), which returns α-ketoglutarate to the cell together with three sodium ions, in a process driven by the inwardly directed sodium gradient. The Na^+-K^+-ATPase pumps the sodium ions from the cell to maintain the sodium gradient.

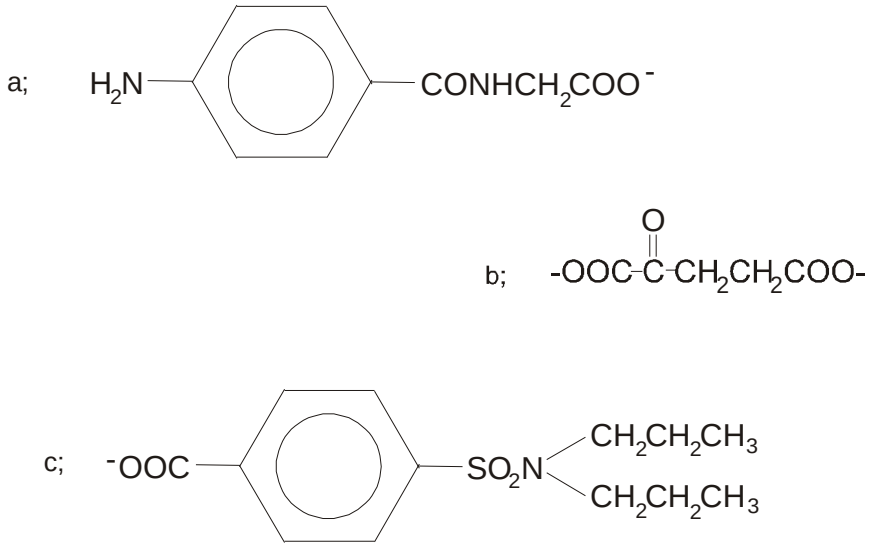


Figure 2. The chemical structure of the main compounds interacting with the organic anion system
a; para-aminohippurate, the model substrate of the multispecific organic anion transporting system
b; α-ketoglutarate, an intracellular exchange partner for organic anion uptake
c; probenecid, the classical inhibitor of the multispecific organic anion transporting system

1.3.3 The multispecific organic anion transporting polypeptide family (oatp)

Another candidate for steroid transport is the organic anion transporting polypeptide (oatp) family which represents a polyspecific transport system that can mediate charge-independent uptake of a wide variety of structurally unrelated amphipathic compounds (Fig. 3.).

Based on the expression studies of cloned members of the oatp family, it appears that steroid hormones are one of their main substrates. The first member of this family (oatp1) was cloned from rat liver (Jacquemin *et al*. 1994, Kullak-Ublick *et al.* 1994), but is also expressed in the proximal tubules of the kidney and in the brain. This transporter when transiently expressed in HeLa cells mediates sodium-independent uptake of conjugated (taurocholate) and unconjugated (cholate) bile acids, and conjugated steroids with a negative charge on the D ring (e.g. 17β-D-glucoronide, estrone-3-sulphate). Probenecid had a moderate inhibitory effect on the transporter, whereas PAH neither inhibited nor was transported (Kanai *et al*. 1996). The uptake of sulphobromophthalein (BSP), the model substrate of this transport system, was inhibited by corticosterone, but corticosterone was not transported by the transporter

(Kanai *et al*. 1996). In oocytes other steroid hormones such as cortisol and aldosterone were significantly transported via oatp1 (Bossuyt *et al*. 1996).

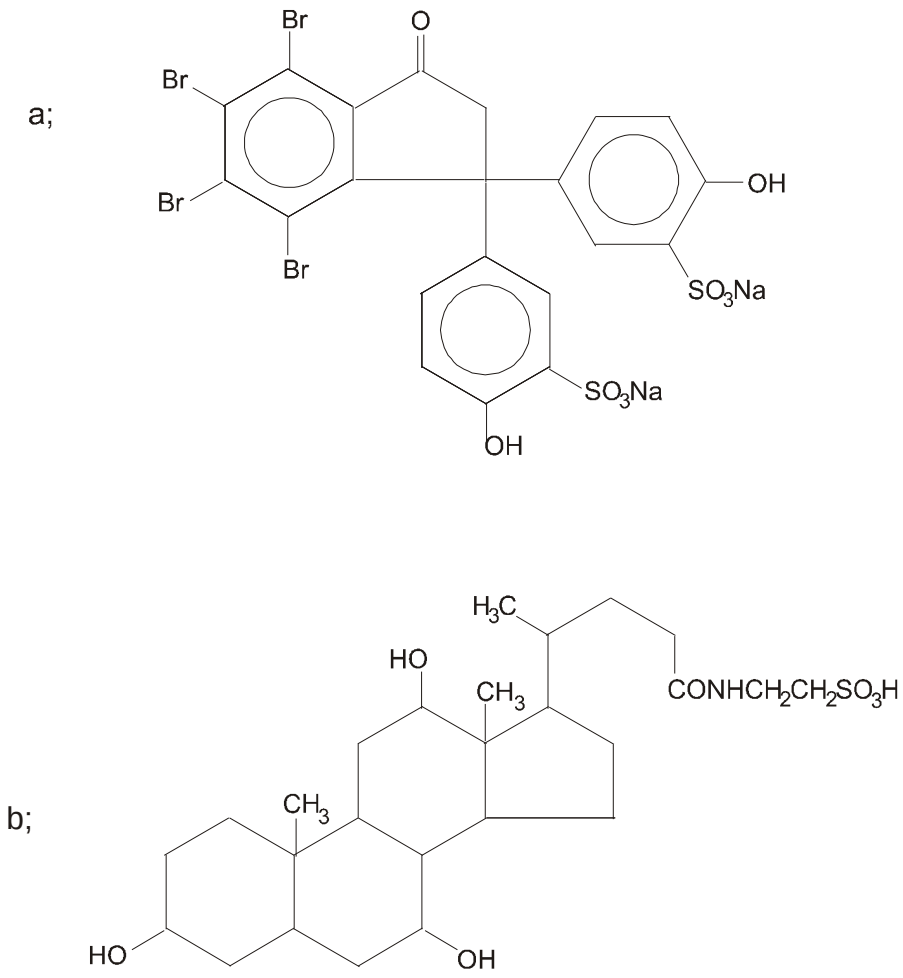


Fig. 3. The chemical structure of the main compounds interacting with the organic anion transporting polypeptide system.
a; sulfobromophthalein (BSP), the model substrate of the organic anion transporting polypeptide system
b; taurocholate, conjugated bile acid and model substrate of the organic anion transporting polypeptide system

The second member of this family, called oatp2 was isolated from rat brain (Noé *et al*. 1997), but is also expressed in the liver. This transporter also transports conjugated and unconjugated bile acids, conjugated steroids, as well as digoxin, thyroid hormones (triiodothyronine, thyroxine) and the cardiac glycoside ouabain. Dehydroepiandrosterone sulphate (DHEAS), which is produced by the adrenal cortex was significantly transported via oatp2. In contrast, unconjugated steroids such as aldosterone, estradiol and testosterone, and PAH were not transported.

The third member of this family (oatp3) was isolated from the rat retina, and is also present in the kidney (Abe *et al.* 1998). This transporter is less well characterised, but in common with the other members of this family it mediates sodium-independent uptake of conjugated and unconjugated bile acids, and thyroid hormones.

1.4 THE AIM OF THE PRESENT STUDY

The goal of this study was to test the hypothesis that steroid release from adrenocortical cells is, at least in part, carrier mediated. Three lines of investigation were chosen to achieve this aim. Firstly, the classical renal organic anion transport system, previously shown to be present in bovine adrenal glands, was to be further characterised in bovine adrenocortical cell culture. To demonstrate the putative expression of organic anion transporters in the adrenal cortex, primers were designed for PCR-based homology cloning using the sequence data of previously cloned transporters (OAT1 and oatp1, oatp2, oatp3). These amplified sequences were then to be used for in situ hybridisation experiments, to determine where these genes are expressed in the adrenal gland. Finally, a human cell model was established for future characterisation of glucocorticoid release from the human adrenal gland.

2 MATERIALS

2.1 CHEMICALS

All chemicals used in this study were obtained from Sigma, Merk, Applichem, Serva, Roth, Bio Rad, Fluca, Amerscham, Gibco or Boehringer, unless otherwise stated in the text.

2.2 ENZYMES

Collagenase CLS II (Biochrom KG, Berlin, Germany)

Pfu DNA polymerase (Stratagene La Jolla, CA, USA)

PowerScript DNA polymerase (PAN Biotech GmbH)

Superscript (Gibco BRL Life Technologies)

Taq DNA polymerase (Promega GmbH)

T3 RNA polymerase (Promega GmbH)

T7 RNA polymerase (Promega GmbH)

Rnase H (Promega GmbH)

Trypsine (Gibco BRL)

Proteinase K (Boehringer Mannheim GmbH)

Hind III, *Xba*I, *Dnp*I, *Not*I (MBI Fermentas Vilnius, Lithuania)

2.3 BUFFERS

Blocking reagent 10%
(10 g Blocking-reagent powder (Boehringer Mainnheim GmbH) in 100 ml malic acid buffer (Boehringer Mannheim GmbH)

DEPC-water
(0.1% (v/v) diethylpyrocarbonate into demanded amount of distilled water)

EDTA 0.5M (pH 8.0)

$MgCl_2$ 1M

NaCl 5M

SDS 10%

SOC medium (Gibco BRL Life Technologies)

SSC buffer (Fluka Biochemika)

Tween-20 10%

TBST 10x
(125 ml 1M Tris-HCl (pH 7.5), 45 g NaCl, 500 µl Tween-20 (10%) DEPC-water was added to 500 ml)

Tris-HCl 1M (pH 7.0, 8.0, 9.5)

Tris-Nacl-PVA buffer
(10 ml Tris-HCl 1M (pH 9.5), 2 ml NaCl 5M, 10 g 70-100 KD polyvinyl alcohol and DEPC-water was added to 100 ml)

TEN-buffer
(500 µl 1M Tris-HCl pH 8.0, 100 µl 0.5M EDTA pH 8.0, 1000 µl 5M NaCl and DEPC-water was added to 50 ml

Wash buffer I
7.5 ml 20x SSC buffer, 1.5 ml 10% SDS and DEPC-water was added to 150 ml

Wash buffer II
1 ml 20x SSC buffer, 1 ml 10% SDS and DEPC-water was added to 100 ml

Blocking buffer
5 ml FCS, 5 ml 10% Blocking reagent, 40 ml 1x TBST buffer

Antibody solution
5 µl FCS, 50 µl 10% Blocking reagent, 1 µl Anti-DIG antibody, 444 µl 1x TBST buffer

NTM buffer
2 ml 5M NaCl, 10 ml 1M Tris-HCl (pH 9.5), 5 ml 1M $MgCl_2$ DEPC-water was added to 100 ml

Painting solution
1 ml Tris-Nacl-PVA buffer, 5 µl 1M $MgCl_2$ 18,6 µl NBT/BCIP mixture (Boehringer)

In situ grade hybridisation buffer (Amersham Pharmacia Biotech Europe GmbH)
PBS buffer
(0.144 g/L KH_2PO_4, 9 g NaCl, 0.795 Na_2HPO_4–$7H_2O$)

2.4 OLIGONUCLEOTIDES

General PCR reactions for screening and sequencing of clones and incorporation of restriction sites were carried out with sequence-specific primers from NAPS (NAPS Göttingen GmbH) or INTERACTIVA (INTERACTIVA Biotechnologie GmbH).
All primers used are listed in Results.

2.5 KITS

Nucleic acid purification:

QIAGEN RNA-DNA Maxi Kit (QIAGEN, Hilden, Germany)

Oligotex mRNA Mini Kit (QIAGEN)

QIAprep Spin Miniprep Kit (QIAGEN)

QIAquick™ Spin Miniprep Kit (QIAGEN)

PCR purification Kit (QIAGEN)

Nucleotrap™ Extraction Kit (Macherey-Nagel, Düren, Germany)

NucleoSpin™ Extract 2 in 1 (Macherey-Nagel)

PCR cloning kits:

TOPO™ TA Cloning Kit (Invitrogen, Carlsbad, CA, USA)

ZeroBlunt™ TOPO™ Cloning Kit (Invitrogen)

PCR-Script™ Amp Cloning Kit (Stratagene, La Jolla, CA, USA)

Mutagenesis:

QuickChange Site-directed Mutagenesis Kit (Stratagene)

cRNA synthesis:

T7 mMessage mMachine™ (Ambion, Austin, TX, USA)

2.6 BACTERIA

One Shot™ competent cell, TOP10F (Invitrogene Carlsbad, CA, USA)
(F1{lacI^q Tn10(Tet^R)}, mcrA, Δ(mrr-hsdRMS-mcrBC), ϕ80lacZΔM15ΔlacX74, deoR, recA1, araD139, Δara-leu)7697, galU, galK, rpsL(Str^R), endA1, nupG)

2.7 PLASMID VECTORS

pPCR-Script vector (Strategene, La Jolla, CA, USA)
(CoIE1 origin, ampicillin resistance, l*acZ* reporter, T7 and T3 RNA polymerase promoters)

pSPORT vector (Gibco BRL Life Technologies)
(pUC origin, ampicillin resistance, *lacZ* reporter and *lacI* repressor, T7 and SP6 RNA polymerase promoters)

2.8 RADICHEMICALS

Aminohippuric acid, P-[Glycyl-2-^{3}H]; ^{3}H–PAH
spec. activity: 5.0 Ci/mmol, conc.: 1 mCi/ml (NEN Life Science, Boston, MA, USA)

Corticosterone, [1,2,6,7–^{3}H(N)]–; ^{3}H-corticosterone
spec.activity: 83 Ci/mmol, conc.: 1 mCi/ml (NEN Life Science**)**

Succinic acid, [1,4–^{14}C]–,^{14}C-succinate
spec.activity: 58.4 mCi/mmol, conc.: 0.1 mCi/ml (NEN Life Science**)**

Taurocholic acid, [24-^{14}C]–,^{14}C-Taurocholate
spec. activity: 45.5 mCi/mol, conc.: 0.02 mCi/ml (NEN Life Science)

2.9 CELL LINES

HCI–H295R (American *Type Culture* Collection, Manessas, VA, USA)

2.10 CELL CULTURE MEDIA

Dulbecco's modified eagle's medium (DMEM)

Dulbecco's modified eagle's medium nutrient mixture F-12 HAM (SIGMA)
(with L-glutamine and 15 mM HEPES, without phenol red and sodium bicarbonate)

2.11 CELL CULTURE MEDIA SUPPLEMENTS

ITS+^{TM} Premix (Becton Dickinson Labware)

Nu-Serum (Becton Dickinson Labware)

Foetal calf serum

2.12 SEQUENCE ANALYSIS SOFTWARES AND ONLINE SEQUENCE

GCG for various sequence analyses (Wisconsin)

Gene Runner for primer design

Chromas for sequence reading

MAP multiple aligment (http://genome.cs.mtu.edu/map.html)

Webcutter (http://www.medkem.gu.se/cutter/)

Blast (http://www.ncbi.nlm.nih.gov/BLAST/)

Entrez Pubmed (http://www.ncbi.nlm.nih.gov/Entrez/)

2.13 EQUIPMENTS

Automated DNA sequencer, ABI Prism, Applied Biosystems (Laguna Beach, CA, USA)

Balance, Sartorius (Göttingen, Germany)

Centrifuges: Biofuge fresco, Heraus (Ostrode, Germany)

5417R, Eppendorf (Hamburg, Germany)

1394, Hettich

C-1200, National Labnet Co

RC-5B, Sorvall (Newtown, CT,USA)

Circulating water bath, RCB 300, Hoefer

Dissection microscope, Stemi 1000, Zeiss (Jena, Germany)

Gel Chambers: Midi, MWG-Biotech (Ebersberg, Germany)

VEU 2001, Pharmacia (Uppsala, Sweden)

Gel documentation, Gel Print 2000 I, Biophotonics (Ann Arbor, MI, USA)

Heated magnetic stirrer, Privileg

Microwave, 8017, 8521, Privileg

Nanoliter injector, World Precision Instrument (Sarasota, FL, USA)

pH meter, pH-Meter 611, Orion Research Inc (Beverly, MA, USA)

Refrigerated aspirator, Unijet II, UniEquip (Martinsried, Germany)

Scintillation counter 1500 Tri-Carb, Packard

Speed vac concentrator, SVC 100E, Savant (Holbrook, NY, USA)

Spectrophotometer, GeneQuant II, Pharmacia (Uppsala, Sweden)

Thermocyclers: 2400, Perkin Elmer

Omn-E HBTRE, Hybaid Ltd (Teddington, England)

PTC-200, MJ Research (Watertown, MI, USA)

UV transilluminator, TM40, UVP Inc (Upland, CA, USA)

Vortexer, REAX Top, Heidolph

3 METHODS

3.1 ISOLATION OF TOTAL RNA FROM RAT ADRENAL GLANDS

Reagents:

rat adrenal glands	
Qiagen Total RNA Midi Kit	
β-mercaptoethanol	
isopropanol	
RNase-free water	
formaldehyde–1.2% agarose gel	52 ml DEPC-water, 13.9 ml 5x MOPS buffer, 0.83 g agar

Total RNA from male Wistar rat adrenal glands was prepared with Qiagen RNA-DNA Maxi kit according to the manufacturer's protocol. The adrenal glands were excised from 250 g Wistar rats. The fresh tissue (4 adrenals) was immediately taken into a 15 ml polypropylene centrifuge tube containing 2 ml QRL1 buffer (10 µl β-mercaptoethanol per 1 ml lysis buffer was added before use). Homogenisation was performed instantly, to avoid RNA degradation, using rotor-stator homogenizer until the tissue was fully disrupted. To precipitate most of the proteins the lysate was mixed with 2 ml QRV1 buffer and eliminated by centrifugation for 20 min at 15.000 x g at 4°C. The supernatant was carefully aspirated into a new 15 ml tube and 3.2 ml ice-cold isopropanol were added and incubated 5 min on ice. To pellet the nucleic acids it was centrifuged for 30 min at 15.000 x g at 4°C. During centrifugation the QIAGEN-tip was equilibrated with 3 ml QRE buffer by allowing the column to empty by gravity flow. The pellet was resuspended and dissolved in 1 ml QRL1 buffer (β-mercaptoethanol was added). To create optimal conditions for binding RNA to QIAGEN resin the sample was diluted with 9 ml QRV2 buffer and applied to the QIAGEN- column. It was allowed to enter the resin by gravity flow. Contaminants (proteins, polysaccharides, carbohydrates …etc.) were washed away with 12 ml QRW buffer. RNA was then specifically eluted with 6 ml pre-warmed (45°C) QRU buffer into new 15 ml centrifuge tube, while DNA remained bound. To precipitate the RNA 1 volume of ice-cold isopropanol was added, and after 10 min incubation on ice it was centrifuged for 30 min at 15.000 x g at 4°C. To wash the RNA pellet 5 ml 70% ethanol

was added and centrifuged for 15 min at 15.000 x g at 4 °C. The supernatant was removed with care and the washing step was repeated. The RNA pellet was air dried for approximately 10 min and resuspended in 200 µl Rnase-free water. The amount of RNA was measured by photometer and visualised on formaldehyde–1.2% agarose gel. The gel was warmed up in a microwave until the agar was dissolved and it was then let to cool down and 3.7 ml formaldehyde was added. The probe and the ladder RNAs were prepared as follows: 1 ng RNA, 2 µl 5x MOPS, 3.3 µl formaldehyde and 10 µl formamide. The reaction mix was then incubated for 15 min at 55°C, then 5 µl probe buffer and 1 µl (1 µg/µl) ethidium bromide were added. The probes were run in 350 ml 1x MOPS buffer at 60 V for approximately 3 hours.

3.2 ISOLATION OF MESSENGER RNA FROM TOTAL ADRENAL RNA

Reagents:

mRNA isolation mini kit

Principle, the poly(A) tail of the mRNA binds with hybridisation to oligo(dT) molecule linked to latex particle while the other RNAs are washed away.

The total RNA solution (0-250 µg) was made up to 360 µl with DEPC-water, and 15 µl pre-warmed (37°C) oligotex suspension and 75 µl 6x binding buffer were added to it. To disrupt the secondary structure of the RNA molecules the mixture was incubated 3 min at 65°C. The hybridisation between the oligo$(dT)_{30}$ molecules and the poly(A) parts of the mRNAs was occurred at room temperature (10 min). Afterwards the solution was centrifuged at maximum speed and the supernatant was carefully aspirated. The pellet (the mRNA molecules bound to the latex particles) was then resuspended in 400 µl wash buffer and applied to a spin column. The column was centrifuged for 1 min at 1200 rpm, and the mRNA was washed again with 400 µl wash buffer. The mRNA was eluated from the column in two steps (20 µl and 30 µl) with preheated (80°C) elution buffer. The concentration was determined with photometer.

3.3 cDNA SYNTHESIS FROM ADRENAL mRNA

The isolated mRNA was used as a template to generate first strand cDNA using Superscript II enzyme.

Reagents:

Oligo $(dT)_{12-18}$ primer (500 µg/ml)	
Superscript II enzyme	
5x first stand buffer	250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$
DTT	0.1 mM
dNTP	500 µM each dATP, dTTP, dGTP, dCTP
DEPC-water	

The following components were assembled into a nuclease free PCR tube: 1µl Oligo$(dT)_{12-18}$ primer, 50-500 ng mRNA and DEPC-water to the volume 12 µl. The mixture was heated for 10 min at 70°C and chilled immediately on ice. Then 4 µl 5x first strand buffer, 2 µl 0.1 M DTT, 1 µl 10 mM dNTP mix, 1 µl (200 U) SuperScript II enyzme were added to the reaction. The reaction mix was incubated for 50 min at 42°C and the enzyme was then made inactive by heating for 15 min at 70°C. To remove RNA complementary to the cDNA 1 µl (2 U) Rnase H was added and incubated for 20 min at 37°C.

3.4 POLYMERASE CHAIN REACTION

The polymerase chain reaction (PCR) is a quick and sensitive process for in vitro enzymatic amplification of a definite segment of DNA for which flanking sequence information is available. The reaction mix contains a fragment of double-stranded template DNA and two single-stranded oligonucleotide primers designed with homology to the 5' and 3' ends of the target sequence, deoxyribonucleoside triphosphates (dNTPs), a buffer and a thermostable DNA polymerase. The mixture is cycled many times (about 30) through temperatures that permit denaturation, primer hybridisation to the template, and synthesis to exponentially amplify a product of definite size and sequence. This results in approximately million-fold amplification of

the defined DNA segment in the ideal case. The PCR products are then visualised on an adequate gel and examined for yield and specificity.

3.4.1 Degenerate PCR

Reagents:

template
degenerate 5' primer
degenerate 3' primer
dNTPs
10x PCR buffer
$MgCl_2$
Polymerase enzyme

To clone OAT and the oatps from rat adrenal cDNA a homology cloning strategy was used. Primers were designed based on the nucleic acid sequence alignment of previously cloned members of the related OAT (fROAT, OAT1, hROAT1) and oatp (oatp1, oatp2, oatp3) families (Table 6. and 7.). The PCR mixture consisted of the following mixture: 5x PCR buffer, additional 2 mM $MgCl_2$, 20 pmol of each primer, 10 mM dNTP mixture, 200 ng cDNA and 5 U Taq polymerase and nuclease free water to a volume of 50 µl. Amplifications were for 34 cycles of 94°C for 30 sec, 56°C for 45 sec and 72° C for 1-2 min; followed by a final extension step of 72°C for 10 min.

3.4.2 High fidelity PCR

The sequence of the examined clone for protein expression system has to be as close as possible to the sequence of the naturally expressed mRNA. Most of the polymerases used for PCR amplification have high productivity, but low fidelity. To maximise fidelity PCR was carried out with *PowerScript*, a proofreading polymerase. *PowerScript* has high polymerase fidelity and is less sensitive to changes in the concentration of cations, template and primers in the PCR mix.

Reagents:

template
sequence-specific 5' primer
sequence-specific 3' primer
dNTPs
10x PCR buffer
5x Optizymed enhancer
$MgCl_2$
PowerScript DNA polymerase

The amplification with *PowerScript* was carried out as per the manufacturer's instructions (PAN system), with 25 cycles of the following amplification parameters: 94°C for 20 sec, 55-60°C for 20 sec and 68°C for 5 min; followed by a final extension period of 10 min at 70 °C. The full-length cDNA was cloned into pSPORT vector, and the fidelity of the polymerase enzyme was confirmed by sequencing.

3.5 SITE DIRECTED MUTAGENESIS

In vitro mutation of targeted nucleotides allows correction of errors introduced by PCR amplification, amino acid exchanges and generation or disruption of restriction enzyme recognition sites. The Quick-Change™ Site-Directed Mutagenesis Kit is based on PCR technique as the *Pfu* polymerase copies the entire plasmid clone from two complementary primers, both of which contain the mutation. The template plasmids are removed by digestion with a methylation-dependent endonuclease, *Dpn*I, which reacts only with the methylated plasmids produced by bacteria (the target sequence is 5'-G^{m6} ATC-3'). The mutant plasmids with nicks can be transformed into *E.coli*, where the nicks are repaired.

Reagents:

Quick-Change™ Site-Directed Mutagenesis Kit
Oligonucleotide primers
*Dpn*I restriction enzyme
Competent cells
SOC medium

The primer pairs for site-directed mutagenesis were designed to anneal to the same sequence on opposite strands of the template plasmid containing the desired mismatch(s). The mutation was positioned possibly to the middle of the primer, with at least 12 bases of perfectly annealing sequence on both sides. The annealing temperature of the primers was about 75-80°C, each primer had GC content of at least 40%, and terminated in G or C bases. The reaction mix consisted of 50 ng template plasmid, 5 µl of 10x reaction buffer, 1 µl of the dNTP mix, 125 ng of each primer, 1 µl of *Pfu* DNA polymerase (2.5 U) and nuclease free water to 49 µl. Cycling parameters were: 95°C for 30 sec, 55°C for 1 min and 68°C for 2 min/kb of plasmid length for 12 (for point mutation) or 16-18 (for two or more mutations) cycles. The reaction mix was incubated with 1 µl *Dpn*I enzyme for 2 hours at 37°C to remove the template plasmid. The efficiency of the extension was checked on agarose gel. The mutant plasmid was transformed into One Shot competent cells as described in 3.12.2.

3.6 RESTRICTION DIGESTION

The restriction enzymes are bacterial enzymes that recognise specific 4-8 base pair nucleotide sequences called restriction sites, and then cleave both DNA strands at this site, generating a reproducible set of fragments.

To restrict the template DNA with the adequate restriction enzyme 100 ng – 5 µg DNA, 1–5 U/µg DNA restriction enzyme and 10% v/v enzyme specific restriction buffer was mixed and incubated 1-3 hours, depending on degree of digestion required.

3.7 LIGATION

Restriction enzymes generating fragments that have a single-stranded “tail” called sticky-end, at both ends. The tails on the fragments generated at a given restriction site are complementary to those on all other fragments generated by the same restriction enzyme. DNA ligase can insert DNA restriction fragments into replicating DNA molecules producing recombinant DNA.

Sticky-end ligation of restricted pSPORT vector and oatp3 clone was carried out using T4 DNA ligase. The standard insert to vector ratio was 3:1 and the reaction consisted of

1 U ligase/µg DNA and 10 µl 1x ligation buffer (66 mM Tris-HCl, 5 mM $MgCl_2$, 1mM dithioerythritol, 1 mM ATP, pH 7.5). Following at least 16 hours incubation at 4°C the reaction mix was used to transform One Shot competent cells.

3.8 AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis was used to visualise and isolate DNA molecules following PCR amplification or restriction digestion. Agarose (0.8–2 %) was dissolved in TBE buffer (45 mM Tris, 45 mM borate, 1 mM EDTA) by heating in a microwave. After cooling 2 µl of a 10 mg/ml ethidium bromide solution was added per 100 ml gel and the gel was poured. Gels were routinely run at 80-100 V for 1-2 hours, depending on the size of the examined DNA fragment or on the degree of band separation required.

3.9 GEL EXTRACTION

The DNA fragment was excised from the agarose gel and 3 volumes of binding and solubilization buffer (QB) was added to 1 volume of gel. It was incubated at 50°C until the gel slice was completely dissolved. To bind DNA, the solution was added to the QIAquick column and centrifuged at 13000 rpm for 1 min. During the DNA absorption step, useless primers, enzymes, nucleotides, agarose and ethidium bromide were not bound to the silica membrane. For direct sequencing and in vitro transcription the column was washed again with QB buffer. Salts were washed away by the ethanol-containing PE buffer. If the DNA was used for salt sensitive application, such as blunt-end ligation and direct sequencing, the column was incubated for 5 min with PE buffer, before centrifuging. Any residual PE buffer was removed by an additional centrifugation step. The DNA was eluted by centrifuging the column for 1 min at maximum speed with 50 µl EB buffer (10 mM Tris-Cl, pH 8.5) or H_2O (pH 7.0–8.5).

3.10 PCR PURIFICATION

To 1 volume of the PCR reaction 5 volumes of binding buffer (PB) were added and applied to the QIAquick column and centrifuged at 13000 rpm for 1 min. During the DNA absorption step, unimportant primers, enzymes, nucleotides, and ethidium

bromide were not bound to the silica membrane. Salts were washed away by centrifuging with ethanol containing PE buffer, and the remains of the wash buffer was cleared away by another centrifugation step. The DNA was eluted by centrifuging the column for 1 min at maximum speed with 50 µl EB buffer (10 mM Tris-Cl, pH 8.5) or H_2O (pH 7.0–8.5).

3.11 NON-RADIOACTIVE TERMINAL CYCLE SEQUENCING OF DNA (SANGER 1977)

Terminal cycle sequencing is a method of dideoxy sequencing in which the template DNA molecules are repetitively utilized to produce a sequencing ladder. The sequencing reaction mixture (consisting of template, specific primer, dNTP, fluorescence labeled 2',3'-dideoxynucleoside triphosphates (ddNTPs), and a termostable DNA polymerase) is subjected to repeated rounds of denaturation, annealing, and synthesis steps, similar to PCR. This method utilizes the ability of the DNA polymerase to apply ddNTPs as substrate. This 2',3'-dideoxynucleoside analog which lacks a 3' hydroxyl group, incorporated at the 3'end of the growing chain terminates the elongation. The amplified fragments were separated with electrophoresis and from the multiband pattern a computer program can assemble the sequence.

Reagents:

Premix	Tris/HCl, phosphatase, *Taq* DNA polymerase, $MgCl_2$, dNTPs, ddNTPs(fluorescence marked)
DNA template	ssDNA (0.1 µg), dsDNA (0.2–0.5 µg), PCR product(10–200 ng)
primer	4 pmol
dH_2O	to 20 µl
sequence loading buffe	3 µl formamid / 25 mM EDTA pH 8.0 (5:1)

The reaction consisted of 4 µl premix, 4 pmol sequence specific primer, DNA template and nuclease free water to 20 µl. The following PCR program was used: 94°C for 30 sec, 50°C for 10 sec, and 60°C for 4 min. After 25 cycles, the amplified DNA was precipitated with an ethanol containing mixture (250 µl 100% EtOH (RT), 10 µl 3M

NaAc pH 4.6, 80 µl H_2O) by centrifugation at 15.000 rpm for 15 min. The pelleted DNA was vacuum dried and diluted with sequence loading buffer. The separation of the DNA fragments was made by electrophoresis in a 0.4 mm thick 5% polyacrilamide gel. The sequence was assembled and analyzed with various software packages and online providers, as listed in 2.12.

3.12 CLONING OF AMPLIFIED PRODUCTS

Depending on the polymerase used for amplification, different vectors and methods were used to clone PCR products. Bacteria transformed with vectors containing the *lac*Z reporter gene were screened by blue-white selection. When no insert is present, a functional α-peptide is produced that complements the gene product of *lac*Z to produce a functional β-galactosidase protein. When plated on indicator plates containing IPTG and X-gal, the colonies are blue. When a cloned insert interrupts the *lac*Z gene, no complementation occurs and colonies appear white.

3.12.1 TOPO TA Cloning

Taq polymerase has a terminal transferase activity, which adds a single deoxyadenosine (A) to the 3' end of PCR products. TOPO Cloning™ exploits the ligation activity of topoisomerase by providing an activated, linearized vector, which has a single, overhanging 3' deoxythimidine (T) residue. Ligation occurs spontaneously within 5 min at room temperature. The pCR 2.1 TOPO vector allows blue-white selection of transformants.

Reagents:

pCR 2.1 TOPO vector	10 ng/µl in 50% glycerol, 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM DTT, 0.1% Triton X-100, 100 µg/ml BSA, phenol red
SOC medium	2% Tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4, 20 mM glucose, 0.5% Yeast extract

One Shot™ competent cells

In general, 0.5 to 2 µl of PCR sample (10 ng\µl) provides the proper insert to vector ratio for TOPO Cloning™. The ligation reaction was carried out in 5 µl volume with 0.5-2µl PCR product, nuclease free water to 4 µl and 1 µl pCR 2.1 TOPO vector. The reaction mixture was mixed gently and after incubation for 5 minutes at room temperature (~25°C) it was placed immediately on ice. To a 50 µl vial of competent cells 2 µl ligation reaction was added for transformation.

3.12.2 pPCR-Script™ Amp cloning

Blunt-end PCR products generated by proof-reading polymerases can be ligated in pPCR-Script vector, using the pPCR-Script™ Amp cloning kit. The kit increase the efficiency of blunt-end ligation by inclusion of *Srf*I restriction enzyme during the ligation step as in the case of intramolecular vector ligation, the end of the vector form the restriction site for *Srf*I enzyme. The pPCR Script vector allows blue-white selection of transformants.

Reagents:

pPCR Script vector	10 ng/µl
pPCR Script reaction buffer	composition not provided by manufacturer
*Srf*I restriction enzyme	5 U/µl
rATP	10 mM
T4 DNA ligase	4 U/µl
One Shot™ competent cells	
SOC medium	

The molar ratio of insert to vector for successful ligation was calculated using the following formula:

$$\text{ng of insert} = \frac{(\text{size of the insert in bp}) \times (\text{ng of pPCR Script vector}) \times \text{molar ratio}}{\text{size of pPCR Script vector (2961 bp)}}$$

The manufacturers recommend an insert to vector ratio of between 40:1 and 100:1. The ligation reaction consisted of the following components: 0.5 µl of pPCR Script vector, 1µl pPCR Script 10x reaction buffer, 0.5 µl rATP, 4-6.4 µl insert DNA, 0.8 µl *Srf*I restriction enzyme, 0.8 µl T4 DNA ligase and nuclease free water to 10 µl. The reaction mix was incubated for 1 hour at room temperature, then heated for 10 min at

65°C to inactivate the enzymes, and stored on ice. For transformation 2 µl ligation reaction was added to a 50 µl vial of competent cells.

3.12.3 One Shot™ Transformation Reaction

After the One Shot cells were dissolved on ice, 2 µl of the ligation reaction was added into the vial and incubated on ice for 30 minutes. The cells were exposed to heat shock at a 42°C water bath for 30 seconds without shaking and then transferred immediately to ice. After 2 minutes incubation on ice, 250 µl room temperature SOC medium was added to the cells. The tube was shaken horizontally at 37°C for at least 1 hour for ampicillin\kanamycin selection, then placed on ice. From the transformation 100 µl was spread on a pre-warmed LB agar plate and was incubated overnight at 37°C.

3.12.3.1 Analysis of Positive Clones with PCR

A PCR cocktail consisting of PCR buffer, dNTPs, primers (M13 F and M 13 R) and *Taq* polymerase was prepared. White colonies were picked and analysed individually with a 25 cycles PCR (94°C for 45 sec, 54°C for 30 sec and 72°C for 1 min with a final incubation for 10 min at 72°C). The PCR products were visualized on a 2% agarose gel with ethidium bromide. The positive colonies were cultured overnight in LB medium containing 50 µg/ml ampicillin or kanamycin.

3.12.3.2 Plasmid isolation

Plasmid DNA was isolated from the overnight cultures according to the manufacturer′s instructions (Qiagen). Bacteria were lysed under alkaline conditions with P2 buffer, and the lysate was neutralized and adjusted to high-salt binding condition with N3 buffer. Afterwards the solution was centrifuged at maximum speed for 10 minutes, till a compact white pellet was formed. The supernatant was applied for purification to the QIAprep silica-gel membrane. Salts were wash away by centrifuging with ethanol containing PE buffer, and the remains of the wash buffer was cleared away by another centrifugation step. The DNA was eluted by centrifuging the column for 1 min at maximum speed with 50 µl EB buffer (10 mM Tris-Cl, pH 8.5) or H_2O (pH 7.0–8.5).

3.12.3.3 LB Medium and Plates

The LB medium consisted of 10 g (1.0%) Tryptone, 5 g (0.5%) yeast extract, 10 g (1.0%) NaCl and H_2O to 950 ml. The pH of the solution was adjusted to 7.0 with NaOH and the volume was brought up to 1 litre, and autoclaved on liquid cycle for 20 minutes at 15 psi. It was then stored in the fridge.

For LB agar plates 15 g/L agar was added to LB medium before autoclaving. After autoclaving, it was cooled down to about 55°C, and antibiotic (50 μg/ml of either ampicillin or kanamycin) was added before pouring into 10 cm plates. The plates were then stored in the fridge in the dark.

Before use for blue-white selection, the plates were warmed up to 37°C and 15 μl 4% X-GAL, 4 μl 100 mM IPTG in 181 μl sterile water was pipetted onto the plate, and spread with a sterile spreader. The solution was allowed to diffuse into the plate by incubating at 37°C for a half an hour.

3.13 NON-RADIOACTIVE IN SITU HYBRIDIZATION ON PARAFFIN EMBEDDED MATERIAL

3.13.1 Generation of riboprobes

RNA probes of the different transporters were produced by a modified protocol using PCR generated templates for in vitro transcription. Briefly, PCR products were cloned into pPCR-Script vectors, which were used as a template for another PCR using oligonucleotide primers specific for the T3- and T7 promoter regions of the plasmids. The PCR reaction mixture (50 μl) contained 200 ng plasmid DNA, 20 pmol of each primer, 1 μl dNTP mixture (10 mM each), 5 μl *Taq* buffer and 2.5 U *Taq* polymerase. After an initial 94°C denaturation step (2 min.), 30 cycles were carried out at 94°C (45 sec), 55°C (30 sec) and 72°C (1 min), followed by a final extension step of 72°C (10 min). The PCR products were visualised on a 1% agarose gel with ethidium bromide, excised and purified using QIAquick Gel Extraction kit. Digoxigenin-11-uridine-triphosphate (DIG-UTP) labelled sense and antisense probes were generated by in vitro transcription. Purified T3 or T7 PCR product was used as a template using either T3- or T7-polymerase, both according to the manufacturer's instructions. For the digoxigenin marking the following reaction mix was assembled: 200 ng sample with DEPC water

added to the final volume 9 µl, 2 µl DigRNA–label-mix, 4 µl transcription buffer, 2 µl DDT, 1 µl RNase inhibitor and 2 µl polymerase. After 2 hours incubation at 37°C the RNA was precipitated with 2.5 µl (4M) LiCl and 75 µl (100%) EtOH overnight at –20°C. On the next day the reaction mix was centrifuged for 30 minutes at full speed (1300 rpm) and after washing with 70% EtOH it was suspended in 20 µl DEPC water. The amount of transcripts was monitored on 1% agarose gel.

3.13.2 Non-radioactive in situ hybridisation

Reagents:

Proteinase K	14 mg/ml
acetic anhydride	
deionised formamide	
5-bromo-4-chloro-3-indolyl phosphate (BCIP)	50 mg ml
nitroblue tetrazolium salt (NTB)	75 mg/ml
The buffers are listed in 2.3	

Adrenals and kidney were taken from Wistar rats, fixed in 4% paraformaldehyde, and embedded in paraffin. In situ hybridisation was performed according to the method described by Breitschopf *et al.* 1992. To describe briefly, tissue sections were deparaffinised with Roticlear (Carl Roth GmbH), hydrated in serial dilutions of ethanol (100%, 90% and 70%) and postfixed in 4% TBS buffered paraformaldehyde. Samples were permeabilised using proteinase K (10 µg/ml) for 30 min at 37°C. Digestion was stopped by washing the samples in PBS (pH 7.4). To block the endogenous alkaline phosphatase slides were incubated with 0.25% acetic anhydride and dehydrated in serial dilutions of ethanol (70%, 90% and 100%). Digoxigenin labelled riboprobes were diluted 1:100 in hybridisation buffer. After application of sense and antisense probes the slides were covered with sterile cover slips and placed on a hot plate for 5 min at 85°C to denature the ribonucleotids. Hybridisation was performed overnight at 55–58°C in a sealed humidified chamber containing 50% formamide. Non-specific bound or unbound probes were removed by the following post-hybridisation washes: 1x SSC/0.1% SDS at room temperature (2 x 5min) and 0.2x SSC/0.1% SDS at hybridisation temperature (2 x 10 min). Finally, the sections were washed in TBS containing 0.1% Tween–20. DIG-labelled RNA probes were detected, after

hybridisation to target nucleic acid, by enzyme linked immunoassay using an antibody-conjugate (anti-digoxigenin alkaline phosphatase). A subsequent enzyme catalysed colour reaction with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium salt (NTB) were produced an insoluble blue precipitate, which visualised hybrid molecules.

3.14 cRNA synthesis

The T7 mMESSAGE mMACHINE kit enables the *in vitro* synthesis of large amount of cRNA from linear cDNA template, by incorporation of a 7-methyl guanosine cap analogue (m7G(5')ppp(5')G) during polymerisation.

Reagents:

10x Transcription buffer	composition not provided by manufacturer
2x Ribonucleotide mix	10 mM ATP,CTP, UTP, 2 mM GTP and 8 mM cap analogue
10x Enzyme mix	bacteriophage T7 RNA polymerase, ribonuclease inhibitor and other unlisted components
template DNA	*Not*I-cutted
*DNase*I	*RNnase*I–free in 50% glycerol buffer
precipitation solution	7.5 M LiCl, 75 mM EDTA

Template cDNA (5 µg) was digested with *Not*I (5 U/µg DNA) for 3 hour at 37°C, then purified using the PCR-purification kit from QIAGEN. For cRNA synthesis, the reaction mixture consisted of 2 µl 10x transcription buffer, 10 µl 2x ribonucleotide mix, approximately 1 µg *Not*I-cut template DNA, 2 µl enzyme mix and nuclease free water to 20 µl. The reaction mix was incubated at 37°C for 2 hours, then the template DNA was removed by *DNase*I digestion at 37°C for 15 min. The reaction was stopped by adding 30 µl nuclease free water and 25 µl precipitation solution and the samples were incubated at –20°C for 1-2 hours. Afterwards the RNA was collected by centrifugation at maximum speed for 15 min at 4°C and washed with 70% ethanol. The cRNA was resuspended in 10 µl nuclease free water and the concentration was determined. The samples were stored at –80°C.

3.15 BOVINE ADRENOCORTICAL PRIMARY CELL CULTIVATION

Reagents:

Freshly slaughtered bovine adrenal glands

Phosphate Buffered Salt buffer	0.144 g/L KH_2PO_4, 9 g/L NaCl, 0.795 g/L Na_2HPO_4–$7H_2O$, 100 U penicillin/ml, 100 µg streptomycin/ml
Wash media (300 ml)	3.6 g Dulbecco's modified Eagle's media, 3 ml Hepes solution (23.83 g Hepes/100 ml water), 12 ml penicillin (100 U/ml) treptomycine (100µg/ml) solution, 0.73 g $NaHCO_3$, 285 ml distilled water
Digestion media	See wash media plus 2.5% trypsine
Culture media (500 ml)	6 g Dulbecco's modified Eagle's media, 5 ml Hepes, 10 ml penicillin–streptomycine solution, 1.219 g $NaHCO_3$, 50 ml foetal calf serum (FCS)

Tris-ammonium-chloride buffer
Trypanblue solution 0.2% (w/v) in PBS buffer

3.15.1 Transport from the slaughter house

The bovine adrenals were transported in ice-cold PBS, which contained 100 U penicillin/ml and 100 µg streptomycin/ml.

3.15.1.1 Cell preparation

The adrenals were washed in 70% ethanol and cut longitudinally into two pieces. The marrow and the capsule were removed and the cortex was cut into very small pieces, which were put immediately into pre-warmed wash media. The pieces were washed three times for 20 minutes on 37°C shaker by replacing the wash-media through sterile sieves, and the filtered solutions were discarded. Sterile trypsin (2,5%) was put into the digestion-media and the pieces were digested three times—each time not longer than 20 minutes— in digestion media by replacing the medium through sterile sieves. Sterile PBS solution was put into the filtered solution to stop digestion Thereafter it was filtered through sterile Nylon-gauzes. The cell suspension was centrifuged at 1200 rpm for 8 minutes. To remove the erythrocytes 1 ml Tris-ammonium-chlorid buffer added to the sediment. It was incubated for 10 minutes in room temperature, then the cells were

washed and centrifuged two times with the pre-warmed culture media. The supernatant was removed and the cells were dispersed in pre-warmed culture media.

3.15.1.2 Viable cell counts using trypan blue solution

The reactivity of trypan blue is based on the fact that the chromopore is negatively charged and does not interact with the cell unless the membrane is damaged. Cell suspension (20 µl) and 1:10 diluted trypan blue stain (20 µl) were mixed and the cells were counted in a hemocytometer (Neubauer chamber).

[Counted cell number / 4] X 2 X 10 000 X volume (in ml) = total cell number

The cells were plated into Falcon 6-well plates at a density of approximately 6 x 10^5 cells/well. The culture media was changed every day.

3.16 CULTIVATION OF HUMAN ADRENOCORTICAL CARCINOMA CELLS (NCI-H295R)

Reagents:

Culture media — Dulbecco's Modified Eagle's Media Nutrient Mixture F-12 Ham cointains: 15 mM HEPES, 1 ml ITS^+ Premix/100 ml media (0.00625 mg/ml insulin, 0.00625 mg/ml transferrin, 6.25 ng/ml selenium, 1.25 mg/ml bovine serum albumin, 0.00535 mg/ml linoleic acid), 1 ml penicillin – streptomycinesolution, 1 ml glutaric acid, 2.73 ml Nu-Serum/100 ml media

Trypsine-EDTA solution (0.25% trypsine, 0.03% EDTA)

DMSO solution

3.16.1 Cryopreservation

The cells were cryopreserved to avoid loss by contamination, and to minimise genetic change. The cells were detached from the substrate with dissociation agents (trypsine), and afterwards resuspended in a complete growth medium, and established the viable cell count. The cells were aliquoted in a concentration between 1–5 x 10^6 cells/ml into freeze media (95% culture media and 5% DMSO). The cryogenic storage vials placed

on ice, and within 5 min in an insulated box in a −80 °C freezer for overnight. Finally, the vials were stored in liquid nitrogen.

3.16.1.1 Thawing of cryopreserved cells

Cryopreserved cells are fragile and require gentle handling. The cells were quickly thawed in a 37°C water bath after being removed from liquid nitrogen. Then the cells were plated directly into complete culture media and centrifuged at 1000 rpm for 5 min to remove cryopreservative (DMSO). The cells were then resuspended in complete culture media and plated in culture vessel.

3.16.1.2 Dissociation of cells from culture vessel

The following method is a gentle treatment to remove cells from the substratum while maintaining cellular integrity.

First the spent culture media was removed, and the cells were washed using balanced salt solution without calcium and magnesium. The dissociation solution (0.25% trypsine, 0.03% EDTA solution) was added to the side of the flask opposite to the cells (2.5 ml/25 cm^2). The flask was incubated for about 5 min at 37°C, then it was rocked gently and with adding culture media the reaction was stopped. The solution was centrifuged at 1000 rpm for 8 min and the cells were dispersed in complete media. The cells were counted and subcultured. The media were changed 2 times weekly.

3.17 RADIOCTIVE TRANSPORT INTO THE CELLS

Reagents:

PBS buffer

Transport media	Dulbecco's Modified Eagles's Medium Nutrient Mixture F-12 HAM with L-Glutamine and 15 mM HEPES, without phenol red and sodium bicarbonate
Ringer solution	5.4 mmol/L KCl, 10 mmol/L Tris, 2.8 mmol/L $CaCl_2$, 137 mmol/L NaCl 1.2 mmol/L $MgSO_4$, pH 7.4

ACTH (Synacten) 10^{-7} M
Forskolin
Para-aminohippurate solution (5 μM)
1N HCl and 1N NaOH

Confluent cell cultures (which were at least three days old) were taken for radioactive uptake experiments. After washing the cells with pre-warmed PBS buffer, 3 ml pre-warmed transport media or Ringer solution with or without substrate was added onto the cells. To stimulate cortisol synthesis 30 µl (10^{-7}) ACTH was added into the medium and incubated for 3 hours in the incubator. The medium was changed as required for the actual experiment and the cells were incubated with the test substrate for the indicated time period. Radioactive PAH (5.5 µM) was given into the medium (not directly onto the cells) and which was then mixed well. It was incubated for the indicated incubation time – normally 10 minutes – at 37°C. After incubation the plates were placed on ice, and 10 µl from each well was taken for measuring the radioactivity in the medium. For measurements of ^{3}H-PAH uptake, the cells were washed three times with ice-cold PBS and then lysed in 1 ml 1N NaOH. After neutralisation with 1N HCl, 4 ml of scintillation cocktail (Ultima Gold, Packard, Meriden, Conn., USA) was added, and radioactivity taken up by the cells was measured by liquid scintillation counting.

3.18 RADIOIMMUNOASSAY (RIA)

The concentrations of cortisol in the culture supernatants were determined by a radioimmunassay. The antiserum employed was raised against corticosterone, but showed 100% cross-reactivity with cortisol. The cross-reactivity with other steroids like aldosterone, progesterone, androstendion or testosterone was less than 0.1%. The antibody was diluted 1:2000 with PBS buffer containing 0.1% gelatin. The ^{3}H-corticosterone tracer was purchased from NEN (Bad Homburg, Germany), while unlabelled corticosterone used as a standard was supplied by Sigma. The sample volume was either 10 or 25 µl culture supernatant. Bound and free tracers were separated by dextran-coated charcoal. The limit of detection of this RIA was 1 ng/ml. Inter- and intra-assay coefficiens of variation, as determined by multiple duplicate determination of a pool of culture supernatants were 15.6% and 7.5%, respectively.

3.19 *XENOPUS LAEVIS* OOCYTES

To determine the function of oatp3 cloned from the rat adrenal gland, and to demonstrate corticosterone transport mediated by the ROAT1 clone, *Xenopus laevis*

oocytes were injected with cRNA derived from the previously mentioned genes and uptake assays were carried out.

3.19.1 Preparation of oocytes

Reagents:

Barth's medium	88 mM NaCl, 1 mM KCl, 0.3 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 15 mM HEPES, 10 mg/ml Gentamicin, pH 7.6
Collagenase	

Oocytes were separated by collagenase treatment involved overnight incubation of several ovarian lobes in 20 ml Barth's medium containing 5 mg/ml collagenase. On the following day the oocytes were washed several times with Barth's solution, and after sorting were then incubated for 10 min in Ca^+ free medium. Oocytes were stored in Barth's medium at 18°C.

3.19.2 Injection of cRNA

Oocytes were injected with approximately 25 ng of cRNA to be tested in a 23-46 nl volume, or the equivalent volume of nanopure water as a control, using Nanolitre injector.

3.19.3 Transport assay with *Xenopus* oocytes

Reagents:

ORI	90 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.6

On the second or third day after injection, surviving oocytes were sorted and transferred into ORI. After equilibration in ORI, oocytes were moved to ORI uptake medium containing the investigated radiolabelled and nonradiolabelled substrates. Uptake took

place from 10 min to 1 hour at room temperature. Adding ice-cold ORI medium stopped the uptake, and then the oocytes were washed three times with ice-cold ORI solution. After that individual oocytes were transferred to 5 ml scintillation vials containing 200 µl 1N NaOH to dissolve the oocytes. After incubation overnight, the solution was neutralised by addition of 200 µl 1N HCl, and finally 2 ml scintillation fluid was added. The mixture was vortexed thoroughly and the radioactivity taken by the oocytes was measured in a scintillation counter.

4 RESULTS

4.1 ^{3}H-PAH UPTAKE INTO BOVINE ADRENOCORTICAL PRIMARY CELLS

Glucocorticoids synthesized in the adrenocortical cells are released into the blood in response to ACTH. It has long been assumed that this occurs via exocytosis or simple diffusion, although the exact mechanism is still unknown. Recently, the existence of a probenecid-inhibitable exchange transporter system involved in cortisol release has been demonstrated in primary bovine adrenocortical cells (Steffgen *et al*. 1996). This transporter showed properties similar to the renal *p*-aminohippurate/dicarboxylate exchanger. To further characterize the attributes of the putative transporter, the uptake of radioactive *p*-aminohippurate (^{3}H–PAH) into bovine adrenocortical primary cell cultures under different conditions was measured. In parallel, the release of cortisol from adrenocortical cells was also tested (Steffgen *et al.* 1999).

Trans-stimulation means in an exchange transport system that the cells were incubated for the indicated time with a (possible) substrate of the investigated transporter. Cells should accumulate this substance, then the uptake of the radioactive material should be increased.

Cis-inhibition means that non-radioactive (possible) substrate was added at a high concentration besides the radioactive substance to the transport media. If the non-radioactive substance is also transported, the uptake of radioactivity should be decreased due to competition for the same transporter.

4.1.1 ACTH stimulation of bovine adrenocortical cells

Under physiological conditions ACTH increases the production of cortisol. The confluent monolayer cell culture was incubated with 1 nM ACTH to stimulate cortisol release from the cells. This treatment also stimulated ^{3}H–PAH uptake into the

adrenocortical cells (mean stimulation in 9 independent experiments was 1.98 fold, p< 0.05, figure 4.).

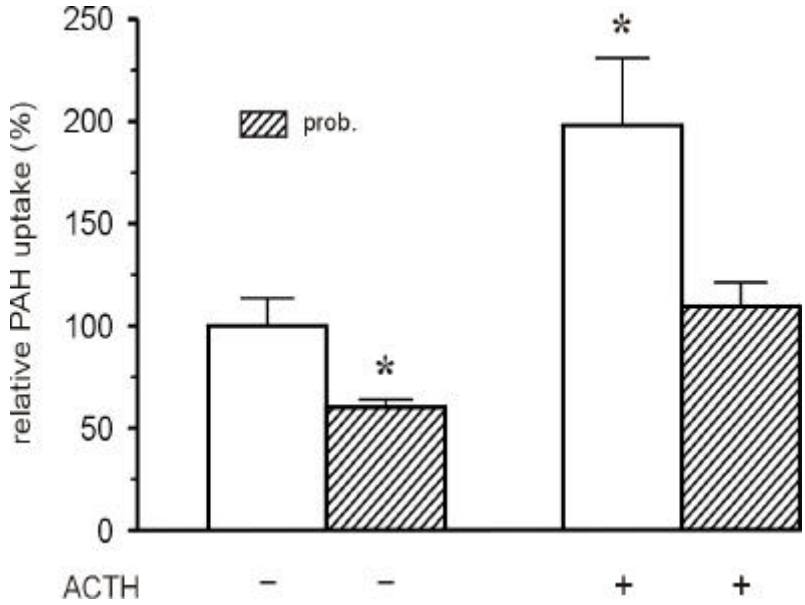


Figure 4. Stimulation by ACTH and inhibition by probenecid of uptake of ^{3}H-PAH into bovine adrenocortical cells. Cells were incubated with 5.5 µM ^{3}H-PAH for 10 min in the absence (open column) and in the presence (hatched column) of probenecid. The PAH uptake in the absence of ACTH and probenecid was set 100 %. Data represent means ± SEM of six independent experiments with 3-6 wells per condition. * $p < 0.05$

The stimulation of cortisol release and radioactive PAH uptake could be explained by an exchange transporter shared by both substrates. It follows that the increased synthesis and release of cortisol by ACTH trans-stimulated PAH uptake into the cells.

4.1.2 Inhibition of ^{3}H-PAH uptake into bovine adrenocortical cells by probenecid

In the presence of 5 mM probenecid (the classical inhibitor of the renal organic anion transporter), cortisol release from the cells and radioactive PAH uptake into the cells were decreased. Mean inhibition of probenecid of ACTH-stimulated radioactive PAH uptake was 45.7 ± 5.9 % in 10 independent experiments (Figure 4.). There was no significant difference in the relative inhibitory effect of probenecid on radioactive PAH

uptake between ACTH treated cells (45.7 ± 5.9 % inhibition) and non-treated cells (40.8 ± 3.7 % inhibition).

The culture media of the cells contained phenol red as pH indicator, which is known to interact with the presumed transporter (Shannon *et al.* 1938). To exclude this interaction probenecid inhibition on radioactive PAH transport was tested in phosphate-buffered saline (PBS) and Ringer's solution. There was no significant statistical difference between these three incubation solutions on probenecid inhibition of radioactive PAH uptake into the cells (Table 1.).

Incubation solution	Relative inhibition of radioactive PAH uptake by probenecid, %	Experiments/wells
Culture media	45.7 ± 5.9	10/48
PBS	39.9 ± 5.1	12/59
Ringer's solution	35.8 ± 3.3	4/15

Table 1. Relative inhibition of radioactive PAH uptake by probenecid in different incubation solutions. Data are mean ± SEM of the indicated number of experiments. Statistical analysis was done by Student's t test in comparison to culture media.

4.1.3 Trans-stimulation of ^{3}H-PAH uptake into bovine adrenocortical cells with 1 mM non-radioactive PAH

After preincubation of the cells for 1 hour with 1 mM non-radioactive PAH the cortisol release decreased about 33%. This might be interpreted as competition of the intracellularly accumulated non-radioactive PAH with the cortisol for the common transporter. On the other hand radioactive PAH uptake into the cells was increased by about 30% after 1 hour preincubation with 1 mM nonradioactive PAH (Figure 5.). This increase was probably due to more exchange substrate on the trans side of the shared transporter.

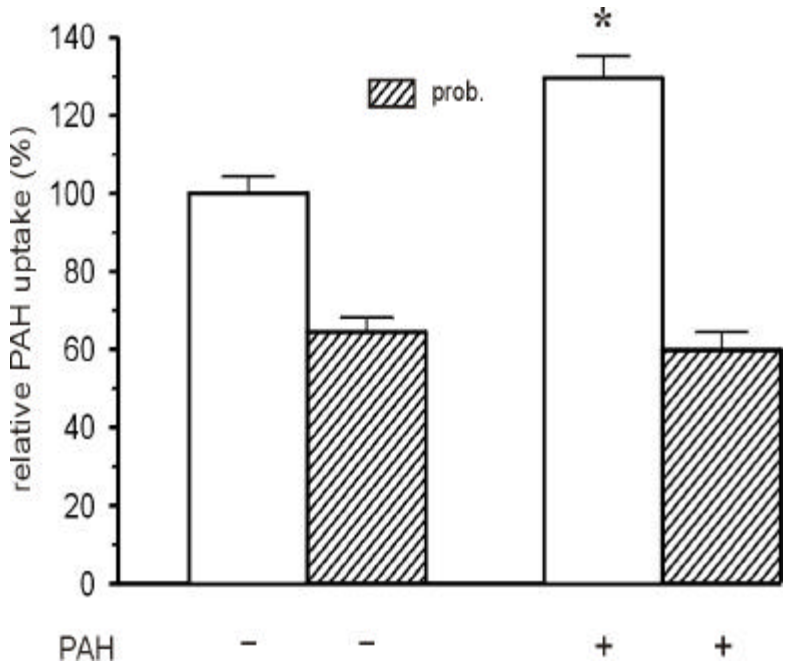


Figure 5. Influence of preincubation with 1 mM PAH on ^{3}H-PAH uptake into bovine adrenocortical cells. Cells were preincubated with cell culture medium (-) or with medium supplemented with 1 mM PAH (+) for 1 hour, washed, and incubated 10 min with 5.5 µM ^{3}H-PAH in the absence (open column) or presence (hatched column) of probenecid. Radioactive PAH uptake in the absence of probenecid and without preincubation was set to 100 %. Data represent means ± SEM of five independent experiments with 3-6 wells per condition. * $p < 0.05$

4.1.4 Cis-inhibition of ^{3}H-PAH uptake into bovine adrenocortical cells with non-radioactive PAH

As preincubation with 1 mM PAH increased radioactive PAH uptake into the cells, the effect of high concentrations of non-radioactive PAH in the incubation media was also tested. The expected outcome was cis-inhibition of radioactive PAH uptake as a result of competition. In comparison to inhibition by 5 mM probenecid, the mean inhibition of 1 mM nonradioactive PAH on radioactive PAH uptake into the cells was 40 ± 9.9% in 4 independent experiments, $p<0.01$ (Figure 5.). Increasing concentrations of non-radioactive PAH in the incubation media caused concentration-dependent inhibition of radioactive PAH uptake (Figure 6 A.). The inhibitory effect saturated around 1-5 mM non-radioactive PAH.

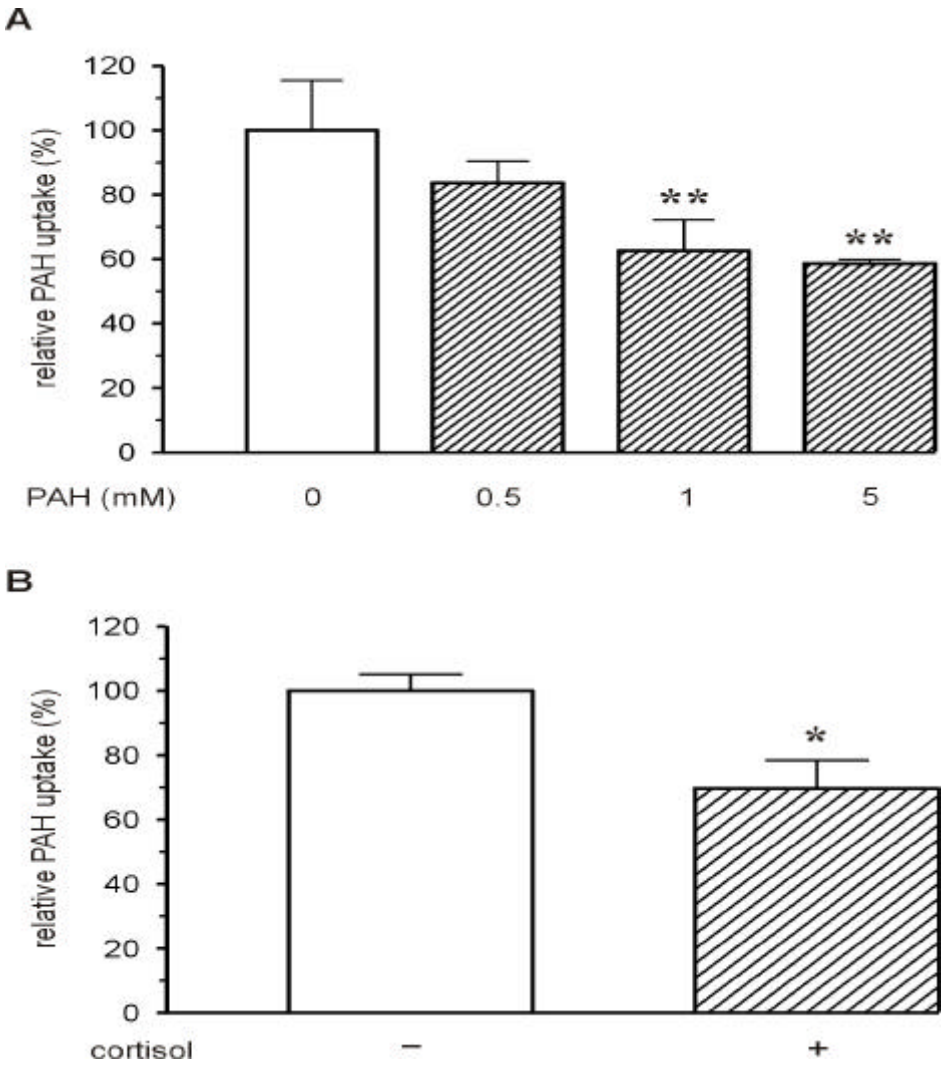


Figure 6. Influence of PAH and cortisol in the incubation medium on ^{3}H-PAH uptake into adrenocortical cells. A., Cells were incubated with 5.5 µM 3H-PAH in the absence or presence of different concentrations of non-radioactive PAH. **B.**, Cells were incubated for 10 min with 5.5 µM ^{3}H-PAH in the presence of 1 mM cortisol. The uptake of radioactive PAH in the absence of unlabelled PAH was set at 100 %. Data represent means ± SEM of five independent experiments with 3-6 wells per condition.

4.1.5 Cis-inhibition of ^{3}H-PAH uptake into bovine adrenocortical cells with 1mM cortisol

In a model of an exchange transporter, a high concentration of cortisol on the outside should cis-inhibit radioactive PAH uptake, since preincubation with PAH inhibited cortisol release from the cells. In the presence of 1 mM cortisol in the transport medium

31 ± 8.8 % inhibition was detected (Figure 6 B.), compared to 45.7 ± 5.9 % by probenecid. This inhibitory effect was indicative of competition between cortisol and PAH for the same transporter.

4.1.6 Cis-inhibition of ^{3}H-PAH into bovine adrenocortical cells with 1 mM glutarate

As glutarate – a model substrate of the renal organic anion transporter that is not metabolized – stimulated cortisol release from the cells about 2 fold, the effect of glutarate on radioactive PAH uptake was tested. Mean inhibition of radioactive PAH uptake by glutarate into the cells was 53% ($p<0.01$) in 4 independent experiments (Figure 7.). A higher concentration of glutarate (10 mM) in the incubation medium did not increase the inhibitory effect on radioactive PAH uptake.

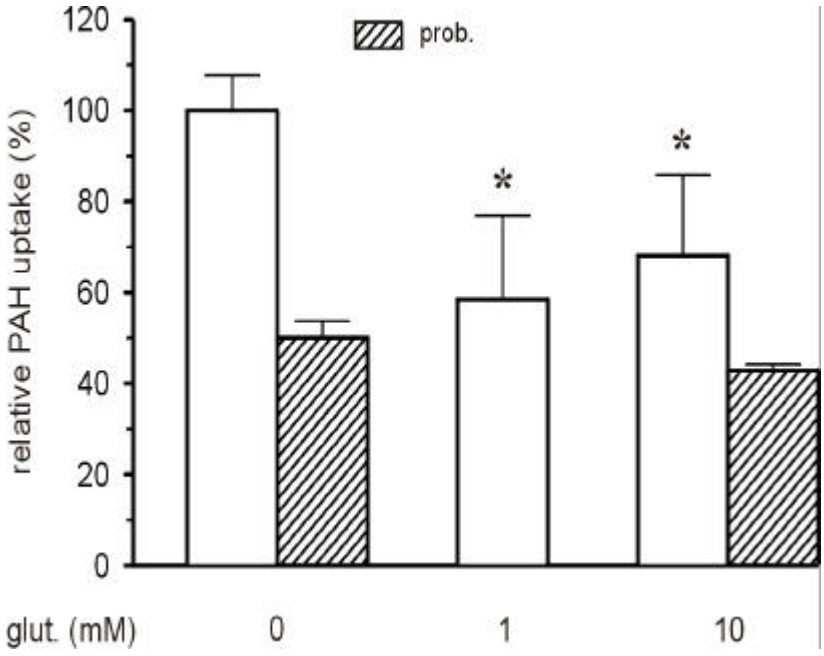


Figure 7. Influence of glutarate in the incubation medium on ^{3}H-PAH uptake into bovine adrenocortical cells. Cells were incubated with or without 1 or 10 mM glutarate (glut.) in the absence (open column) or presence (hatched column) of 5 mM probenecid for 10 min. Uptake of radioactive PAH in the absence of glutarate and probenecid was set at 100 %. Data represent means ± SEM of five independent experiments with 3-6 wells per condition.

4.2 CIS-INHIBITION OF ROAT1-MEDIATED PAH UPTAKE BY CORTICOSTERONE

Cis-inhibition studie was carried out with corticosterone, the main glucocorticoid in rats, in order to determine if corticosterone is a possible substrate of the rat renal organic anion transporter (Fig. 8.). Corticosterone strongly inhibited PAH uptake in ROAT1-injected oocytes by more than 70% (the expression clone of ROAT1 was a gift of Dr.Andrew Bahn, Department of Vegetative Physiology and Pathophysiology, Georg-August Göttingen, Germany).

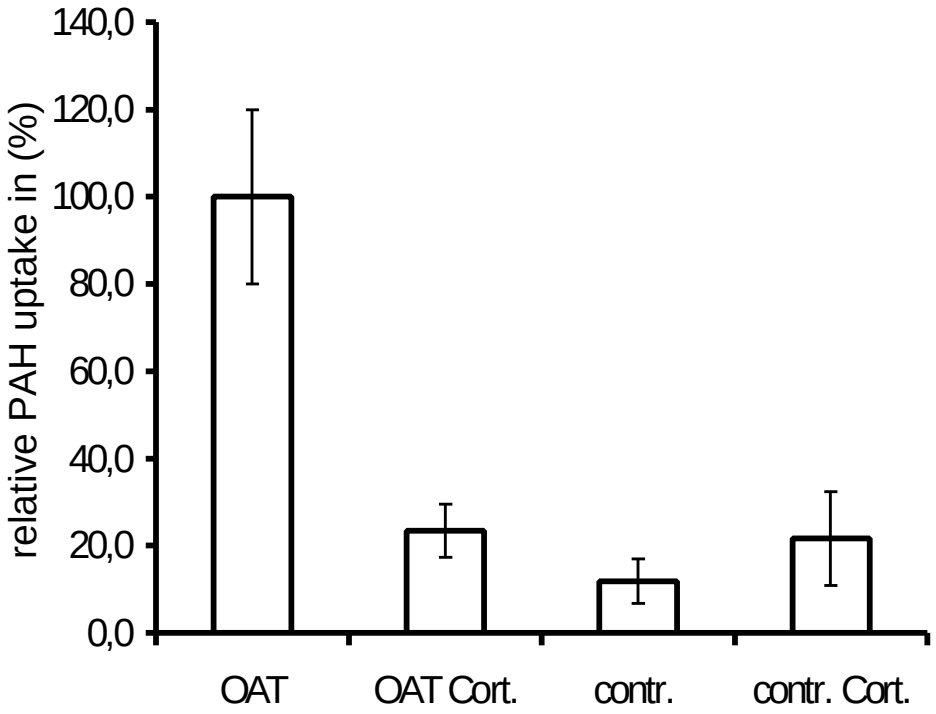


Figure 8. Cis-inhibition of ROAT1-mediated PAH uptake by corticosterone. The effect of corticosterone (Cort.) on control level of ROAT1-mediated PAH uptake is shown. Corticosterone was present in the uptake medium at a concentration of 500 µM. Uptake of radioactive PAH in the absence of corticosterone was set at 100 %. Data represent means ± SEM of 3 independent experiments with 7-12 oocytes per group.

4.3 DEMONSTRATION OF A Na^+-DICARBOXYLATE COTRANSPORTER IN BOVINE ADRENOCORTICAL CELLS

4.3.1 Characterization of ^{14}C-succinate uptake into bovine adrenocortical cells

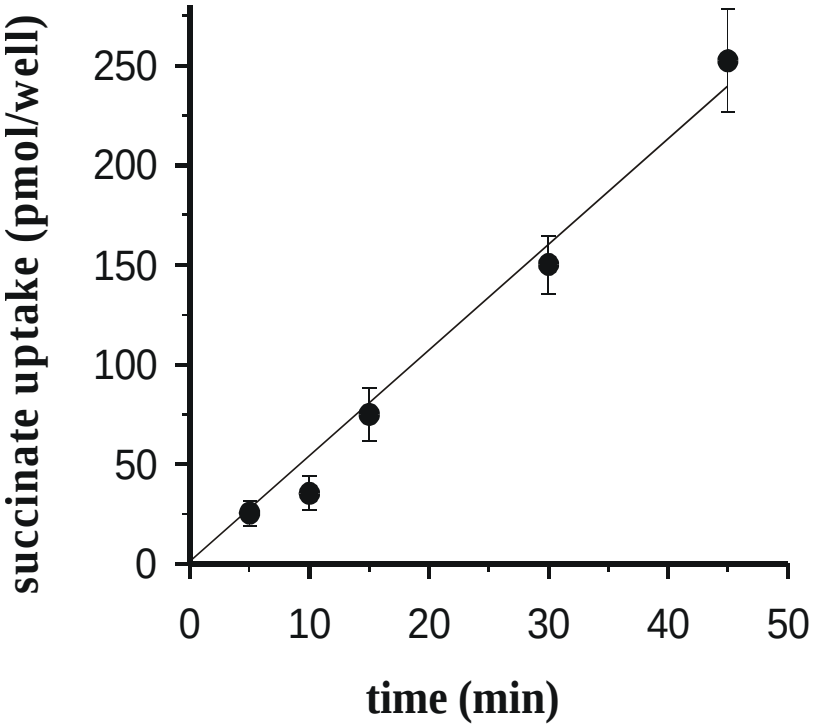


Figure 9. Time-dependency of ^{14}C-succinate (58 µmol/l) uptake into bovine adrenocortical cells. Data represent means ± SEM of five independent experiments with 3-6 wells per condition.

As shown in Figure 9., uptake of ^{14}C-succinate, the model substrate of the Na^+-dicarboxylate cotransporter, into bovine adrenocortical cells increased linearly with time for at least 45 min (r = 0.99). As calculated from the slope, the uptake rate amounted to 0.557 pmol succinate per min per well. For all further experiments, an incubation period of 30 min was chosen. Inhibition of dicarboxylate uptake by lithium has been reported for Na^+-dicarboxylate cotransporters in all mammalian tissues studied so far. Succinate uptake into bovine adrenocortical cells could be inhibited by 10 mM lithium by 75% ± 6.8% in three independent experiments (Fig. 10 A.).

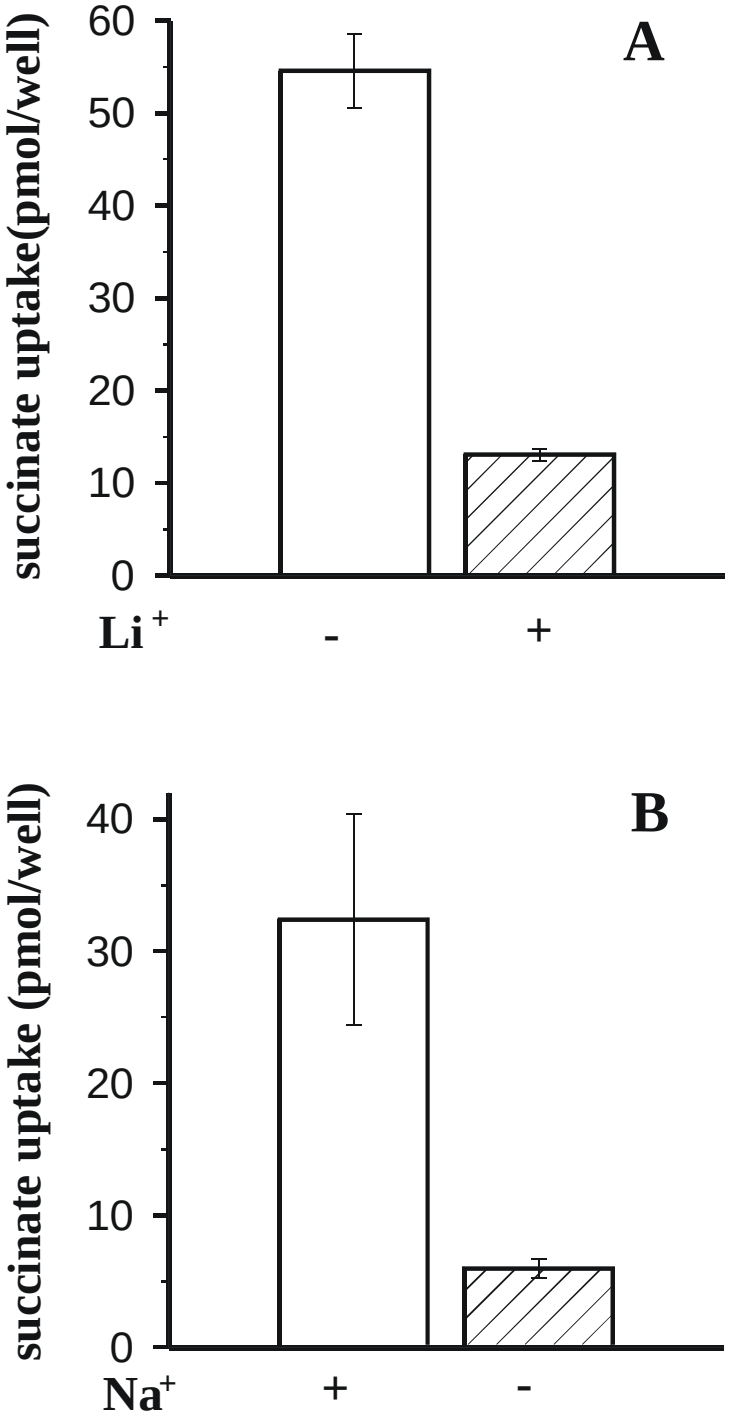


Figure 10. Inhibition of succinate uptake (30 min) by 10 mmol/l lithium in the presence of sodium in culture medium (A) and dependence of succinate uptake on sodium in Ringer's solution (B). To test sodium dependency, Na^+ was replaced by tetramethylammonium. Succinate concentration was 58 µmol/l in each experiment. Data represent means ± SEM of five independent experiments with 3-6 wells per condition

To test sodium-dependence of succinate uptake, experiments were performed in a Ringer's solution in the presence and absence of sodium (Fig. 10 B). Succinate uptake

into bovine adrenocortical cells incubated in the presence of sodium was about 7 times higher than in the absence of sodium, obviously proving sodium-dependence.

The succinate uptake was saturable with calculated apparent K_m value of 146.2 ± 5.1µM succinate (data taken from Steffgen *et al.* 1999).

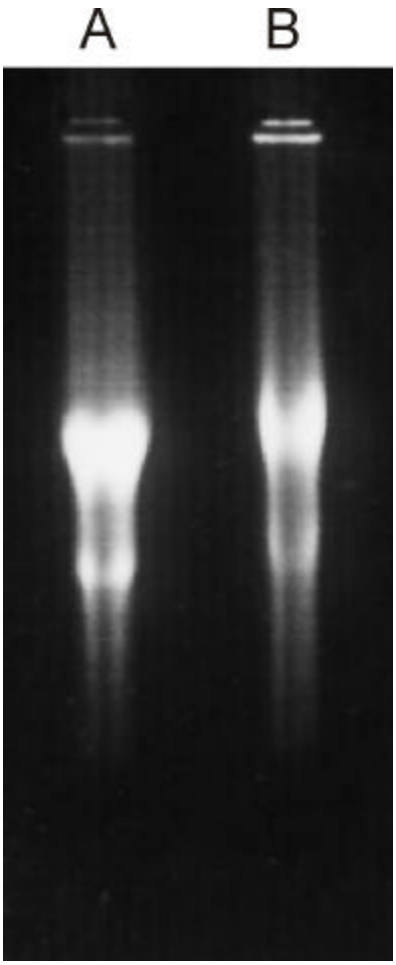
Substrate	Relative uptake (%)
None	100
Cis-aconitate	100 ± 12
Citrate	93 ± 12
Fumarate	30 ± 5
Glutarate	26 ± 4
Isocitrate	112 ± 13
α-Ketoglutarate	32 ± 4
Malate	125 ± 13
Maleate	77 ± 15
Malonate	80 ± 14
Succinate	14 ± 2

Table 2. Effect of 1 mmol/l of various potential substrates on ^{14}C-succinate (58 µmol/l) uptake into bovine adrenocortical cells. Uptake in the absence of nonradioactive substrate was set to 100%. Data represent means ± SEM from three to five independent experiments with three to six wells.

The specificity of the succinate transporter was also tested with several di- and tricarboxylates (Table 2.). Inhibition induced by 1 mM of potentional substrates ranked in the following order: succinate > glutarate > fumarate = α-ketoglutarate > maleate. There was only a slight inhibition by malonate and no inhibition by citrate, malate and isocitrate. The lack of inhibition by citrate on succinate uptake at pH 7.4 is different from the data reported for the sodium-dicarboxylate transporters in all other investigated organs.

4.4 PCR CLONING OF ORGANIC ANION TRANSPORTERS FROM THE ADRENAL GLAND

To support the hypothesis of the existence of organic anion transporter system(s) in the adrenal gland, a homology cloning PCR technique was employed, using the sequence data of the cloned flounder, rat and human transporters. As template, bovine and rat adrenal cDNA was used, prepared as described in 3.3.



Picture 2. Bovine and rat adrenal total RNA separated in a 1.2 % formamid-formaldehyd gel. Total RNA was isolated from rat and bovine adrenal glands with Qiagen Total RNA Maxi Kit. A: rat mRNA, B: bovine mRNA

PCR parameters (annealing temperature, template and $MgCl_2$ concentrations and adjuvants) for each sample were experimentally determined. The primers were based on conserved parts of the nucleic acid sequence of the cloned organic anion transporters.

Transporter clone	Appearance in GenBank
ROAT1	AF110022, AF008221
fROAT	Z97028
oatp1	L19031
oatp2	U95011
oatp3	AF041105
OCT1	NM012697
OCT2	D83044

Table 4. The GenBank EMBL accession numbers of transporter sequences used in this study.

4.4.1 Cloning of an OAT-like organic anion transporter

It was suggested from the uptake (this study) and the radioimmuno assay (Steffgen *et al.* 1999) that an anion exchanger is present in the bovine adrenocortical cells. This transporter was at least partly involved in cortisol release and showed properties similar to the renal para-aminohippurate/dicarboxylate exchanger. To clone this transporter from the adrenals a homology cloning PCR strategy was employed. Initially, bovine adrenal cDNA was used as a template for PCR using degenerate primers based on the sequence of the cloned rat, mouse and flounder organic anion transporters, a related transporter isolated from rat liver (NLT) and two rat organic cation transporters (OCT1 and OCT2) and obtained from Dr. Natasha A. Wolff, Department of Vegetative Physiology and Pathophysiologie, Georg-August University Göttingen, Germany .

Primer	Nucleotide sequence	Primers in pool	Degeneracy factor
5.1	$^{5'}$TIATGGCNWSNCAYAAY$^{3'}$	256	1024
5.2	$^{5'}$GGIACITGYGCNGCNTWY$^{3'}$	128	2048
5.3	$^{5'}$ARNCCRTARTANGCRAA$^{3'}$	256	256
5.4	$^{5'}$NCCRAADATNACYTGDAT$^{3'}$	576	576

Table 5. Degenerate primers used in homology screening PCR with bovine adrenal cDNA. Detailed description of the primers found in Reid *et al.* 1999. I = inosine, N = A/C/G/T, W = A/T, S = C/G, Y = C/T, R = A/G, D = A/G/T. The number of primers in each pool equals the total number of different primers; the degeneracy factor represents the total number of sequences with which each primer might theoretically bind.

Taking the species differences into consideration, the PCR parameters were chosen to render amplification possible even if the primers had no complete matching to the template. First of all, the annealing temperature was optimized, testing a range of temperatures setting out from the calculated melting temperature (about 40°C). As an alternative, a touchdown protocol was performed, where the annealing temperature was initially set high (50°C), but decreased by 0.4°C with each subsequent annealing step for 25 cycles. The amplified products were sequenced but they proved to be artifacts, mostly house-keeping genes. Moreover, in order to increase effectiveness, several concentrations of $MgCl_2$ (2.5–5 mM), primers (10–30 pmol) and template (0.1–1 µg) and adjuvants (0.01% gelatine, 4% DMSO) were also tried. Despite this fact, it proved to be unsuccessful. Afterwards rat adrenal cDNA was used as a template in PCR with less degenerate primers to minimize the problems associated with species-dependent sequence differences.

Rat adrenal cDNA was screened with degenerate primers based on similar nucleic acid sequence parts of rat (r), flounder (f) and human (h) clones (Table 6.). The fr–OAT forward and reverse primer were expected to yield PCR products of 241 bases, respectively. The PCR reaction was optimized for annealing temperature (55°C) and time (30 sec), $MgCl_2$ (3 mM), primer (20 pmol) and template (0.2 µg rat adrenal cDNA) concentrations and 4% DMSO was used to increase effectiveness. A product of the predicted size was sequenced and sequence homology searches revealed the product to have 98% homology to the cloned rat renal OAT1. That was the first molecular evidence for the presence of the renal organic anion transporter in the adrenal gland. This finding is consistent with the previous bovine transport results, as an organic transport-like system exists in the adrenal gland. Later, a 1700 base-pair product, amplified with OAT1U and rhOAT1R primers and rat adrenal cDNA as the template, proved to be the rat renal organic anion transporter, which confirmed the presence of this transporter in the adrenal gland.

Primer	Nucleotide sequence
frOAT1F	$^{5'}$CCACYAGCTTYGCTTYGCCTACTA$^{3'}$
frOAT1R	$^{5'}$TTCGCACCTSYCTGGCTGT$^{3'}$
OAT1F	$^{5'}$GATGTCGACCTATGGCCTTCAACG$^{3'}$
rhOAT1R	$^{5'}$CAGGCCTCARCACAAGAGAAG$^{3'}$

Table 6. Primers used to amplify ROAT1 from adrenal cDNA. The small letters of the primer names refer to the clones taken as a basis; f means flounder, r means rat, h means human. S = C/G, Y = C/T, R = A/G, F = forward, R = reverse

4.4.2 Cloning of organic anion transporting polypeptide transporters (oatp)-like transporters

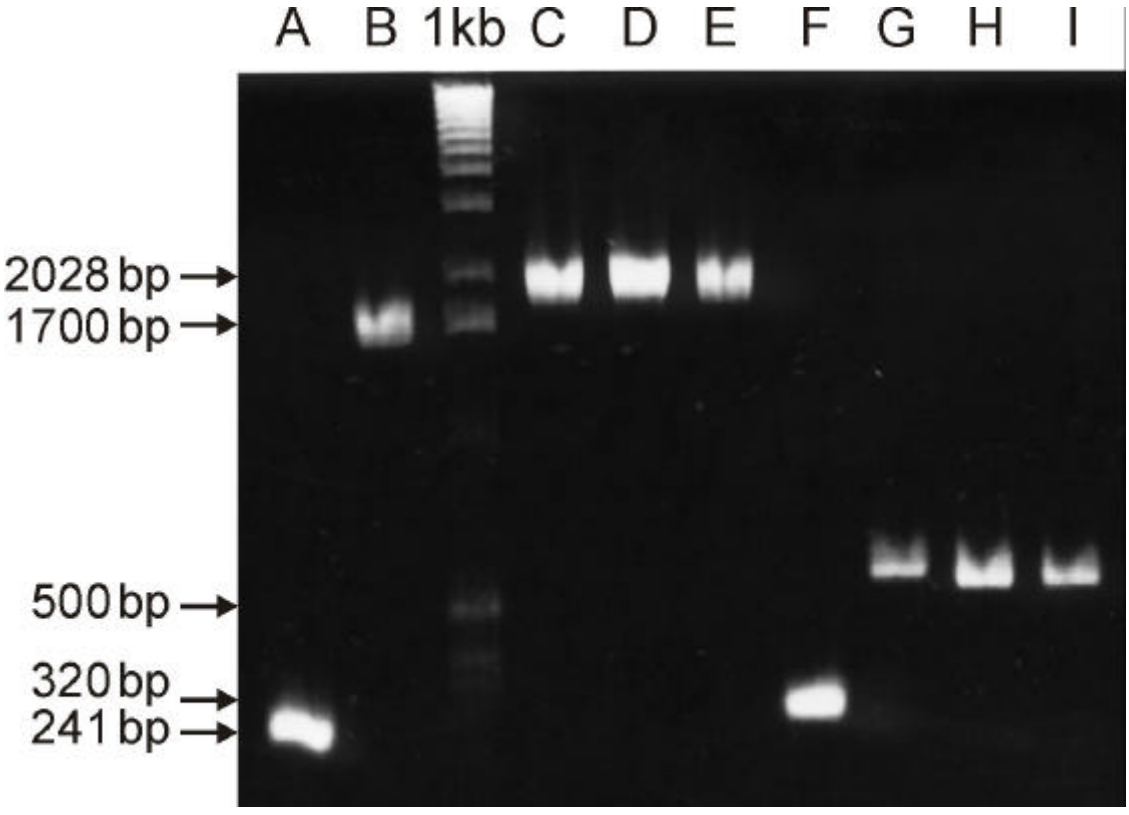
As described in the literature, the organic anion transporting polypeptide family (oatp) interacts with different kinds of steroid hormones (see 1.3.3). Thus, their existence in the rat adrenal cDNA was examined. The three known members of the family share about 86 % identity to each other. The primers were designed as to recognize and to be complementary to all members of the oatp family to screen whether they are expressed in the adrenal gland (Table 7.).

Primer	Nucleotide sequence
oatp5F	$^{5'}$GGCAACTTGTCCTCAAAC$^{3'}$
oatp6F	$^{5'}$GTGAATACAGATGACCTGACC$^{3'}$
oatp8R	$^{5'}$GCATGTAAATCGGATTGC$^{3'}$
oatp1F	$^{5'}$CCATGGAAGAAACAGAGAAAAAG$^{3'}$
oatp2F	$^{5'}$GAACACCATGGAAGAAAACAGAG$^{3'}$
oatp3F	$^{5'}$ATGGGAGAAACAGAGAAAAGGGTTGC$^{3'}$
oatp1-2-3R	$^{5'}$TTACAGCCTCGTTTTCAGTTCTCCG$^{3'}$

Table 7. Primers used to amplify the three members (oatp1, oatp2 and oatp3) of the organic anion polypeptide transporter family. The numbers 1 and 2 and 3 refer to the name of the respective transporter, number 5, 6 and 8 are serial numbers of the given primer. F = forward, R = reverse

The oatp–6 forward and oatp–8 reverse primers and the oatp–5 forward and oatp–8 reverse primers were expected to yield PCR products of 320 and 590 bases, respectively. As positive control rat liver cDNA was used. The PCR reaction was optimized for annealing temperature (55°C) and time (15 sec), $MgCl_2$ (3.5 mM), primer (20 pmol) and template (0.2 µg rat adrenal cDNA) concentrations and 4% DMSO was used to increase effectiveness. After successful amplification, the products of the

predicted size were sequenced. The sequence data were used to search for homologous clones in Blast searches. The 322 base pairs product was found to be oatp3 (98% identity) and the 590 base pairs product proved to be oatp2 (96% identity). The PCR-screen of rat adrenal cDNA indicated the presence of more members of the oatp family. To verify the first PCR results and demonstrate that more organic anion transporting polypeptide transporters are truly expressed in the rat adrenal gland, specific primers were designed for each member of the oatp family (Table 7.). The primers specifically fit to the ends of the open reading frame of the respective transporter and the PCR parameters were optimized to provide high stringency conditions (annealing temperature: 60°C). The whole open reading frame of oatp2 and oatp3 was successfully amplified with proof-reading polymerase (3.4.2), but the amplification of oatp1 ran into difficultes. The amount of the amplified end-product in the case of oatp1 was very low, therefore purification and reamplification of the first PCR product were required. On the basis of the sequence data of the PCR products it was shown that the oatp family with three members is also represented in the rat adrenal gland.



Picture 3. Amplification of OAT1 and oatp1, oatp2 and oatp3 from rat adrenal cDNA by degenerate PCR. A: ROAT1 amplified with frOAT1F-R primers, also template for in situ hybridization, B: ROAT1 amplified with OAT1F-rhOAT1R, C-E: oatp3, oatp2 and oatp1 open reading frames, F: oatp3 amplified with oatp6F-oatp8R, G: oatp2 DNA template for in situ hybridization, H: oatp1 DNA template for in situ hybridization, I: oatp2 amplified with oatp5F-oatp8R primers, Sizes of relevant bands from the 1 kb standard are shown.

4.5 NON-RADIOACTIVE IN SITU HYBRIDIZATION

PCR revealed that the organic anion transporter (ROAT1) and three members of the organic anion polypeptide family (oatp1, oatp2, and oatp3) are expressed in the rat adrenal gland. As the adrenal gland physiologically is divided into two major parts (cortex and medulla), and the cortex itself has three histologically and functionally separated zones, it was important to identify the localization of the transporters to show relation to the assumed substrates, i.e. steroids. The in situ hybridization method is able to visualize the expression of various genes within the intact cell, in their original context. Part of the respective sequence is used as a template for *in vitro* digoxigenin-labelled RNA amplification for non-radioactive in situ hybridization. This riboprobe, which hybridizes to its complementary sequence under suitable conditions, is applied to the prepared tissue sections. The hybrid molecules are visualised as an insoluble precipitate with the microscope.

The sequence parts used as DNA templates for in situ hybridization were chosen so as to have low identity to the related transporters. In the case of ROAT1, the sequence part amplified with frOAT1 forward and reverse primers was chosen, while it had low identity to the related organic anion and no identity to the organic cation transporters. In the case of organic anion polypeptide family, care had to be devoted to select the proper part of each transporter, as the members share high identity with each other (about 86%) in their open reading frame. Since the 3' untranslated regions of oatp1 and oatp2 show higher divergence (about 60 % identity), the probes were amplified from these regions. As the 3' untranslated region of oatp3 was not known, only the open reading frame was available for choosing a suitable part for in situ hybridization. As the fragment amplified with oatp6 forward and oatp8 reverse primers was used as probe for oatp3, to obtain specific results high stringency conditions and controls were used performing the in situ hybridization. Since the probes and the paraffin-embedded adrenal sections were self-made, furthermore the protocol was not applied to adrenal gland, therefore the method had to be optimised in every respect.

4.5.1 The empirical arts of in situ hybridization

It is important to optimize empirically the various experimental parameters of in situ hybridization such as proteinase K digestion, probe size and concentration, hybridization temperature, stringency of washing and method of detection to the specific application.

4.5.1.1 Proteinase K digestion

The main hitch of the in situ hybridization technique lies in the fact that the nucleic acids are bound inside a complex matrix of cellular structures, so that the target sequences are not readily accessible to the labelled probes. Therefore, the target RNA molecules were releaved by proteinase K digestion, which is the most critical step in the whole in situ hybridization procedure. The time and the temperature of the incubation and the amount of the enzyme have to be optimised for each tissue section type. With too little digestion, the cytoplasmatic membranes will not be sufficiently permeable to probes. With too much digestion, the membranes and protein structures will lose integrity and the template RNA or the hybrid complex may leak out of the cells and make surrounding cells falsely positive. Other adverse consequences can include high background and poor morphology. As determined experimentally, 1-2 μm thick, paraffin-embedded adrenal tissue requires 14 μg/ml proteinase K for 30 min at 37°C.

4.5.1.2 Probes

The choice of length depends on the intended target. When there are low levels of target (as is usually the case), long riboprobes are usually used as they are more sensitive. Because of problems with probe penetration when using intact cells or tissue, shorter probes give more rapid hybridization in situ, as they can easily gain access to the targets. The length of the probes is also dependent on the chosen specific part of the respective sequence. Probes of 201−668 base pairs in length were used in this study.

Transporter	Position and length of riboprobe		Hybridization temperature	Concentration
OAT1	1318 – 1559	241 base pairs	55°C	10 µg/ml
OCT1	1630 – 1831	201 base pairs	45-50°C	10-20 µg/ml
OCT2	1701 – 2009	298 base pairs	53°C	14 µg/ml
oatp1	2074 – 2682	608 base pairs	50°C	8 µg/ml
oatp2	2096 – 2764	668 base pairs	50°C	8 µg/ml
oatp3	678 – 958	320 base pairs	55°C	10 µg/ml

Table 8. The size and concentration of the riboprobes used for in situ hybridization. Numbers denote position of the first and last nucleic acid of the sequence and the length. The optimal concentrations determined experimentally are listed in this table.

Molecules used to label the probe for detection can further compromise penetration of the probe into the cells. These molecules can increase the rigidity of the probe, increase the steric hindrance, and adversely affect charge density and the distribution of the probe. The proper probe concentration must be also determined to give the best signal to background ratio (Table 8).

The chosen part of the of the respective sequence was amplified by PCR with proof-reading polymerase from rat adrenal cDNA (see 3.4.2). The amplified DNA fragment was cloned into into pPCR-Script vector containing T3 and T7 RNA polymerase promoters. To generate labelled RNA probes, in vitro transcription was performed using the previously prepared DNA template, DIG-RNA Labelling Mix (Roche) and T3 or T7 RNA polymerases (see 3.13.1).

4.5.1.3 Hybridization temperature and time

Stringent hybridization conditions were used to achieve high specificity and low background. This means high annealing temperature (between 50–55°C). Low salt concentration was used in the hybridization mixture, which means high stringency at any temperature. Long incubation time (overnight) was used with the experimentally determined probe concentration.

4.5.1.4 Washing

For washing after hybridization also stringent conditions were favoured. To remove probes bound to fatty acids, lipids and proteins, washing buffers with low salt concentration and soap basis were used (see 2.3). To increase stringency, the second washing step was carried out at the hybridization temperature.

4.5.1.5 Detection

All tissues have a certain level of endogenous enzyme activity (peroxidase, phosphatase etc.), and this can increase non-specific background levels. To avoid this, it is needed to choose an adequate enzyme-based detection system and/or pre-treat the tissue to remove or inhibit the endogenous enzymes. In the case of the adrenal gland, a kidney alkaline phosphatase-based detection system and pre-treatment with acetic anhydride were chosen (see 3.13.2).

4.5.1.6 Controls for in situ hybridization

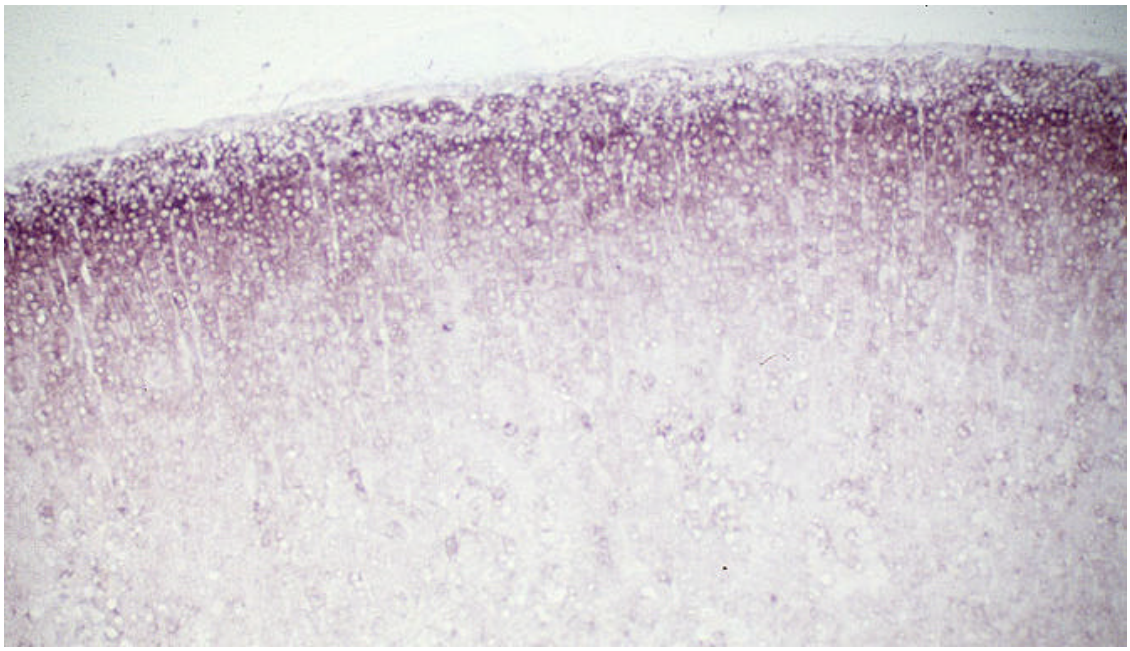
Controls should be included to monitor the hybridization reaction for probe specificity and to assess the level of background. In this study, sense probes were used as controls which were generated from the same DNA sequence labelled with DIG-dUTP and also the hybridization conditions were the same as for the respective antisense probes.

4.5.2 Expression pattern of the newly demonstrated organic anion transporters in the rat adrenal

The homology cloning PCR technique disclosed the presence of ROAT1, oatp1, oatp2 and oatp3 in rat adrenal cDNA. This means the presence of two organic transporter families with similar function but divergent substrate specificity and of which the oatp family is present with more members. This raises the questions of occurrence, functional role or rather the division of labour. Then non-radioactive in situ

hybridization method was chosen to detect the regions of expression of the investigated transporters. On the basis of the above detailed description, the method was optimized for every single probe to find out the optimal conditions for the most specific hybridization (Table 8.).

A



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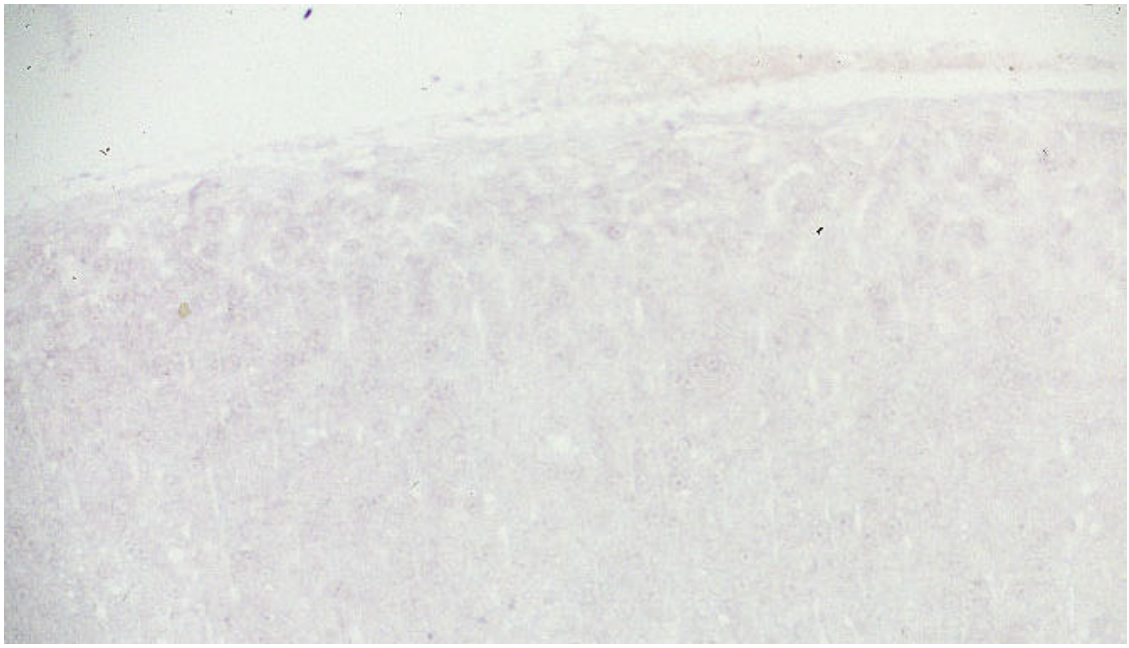
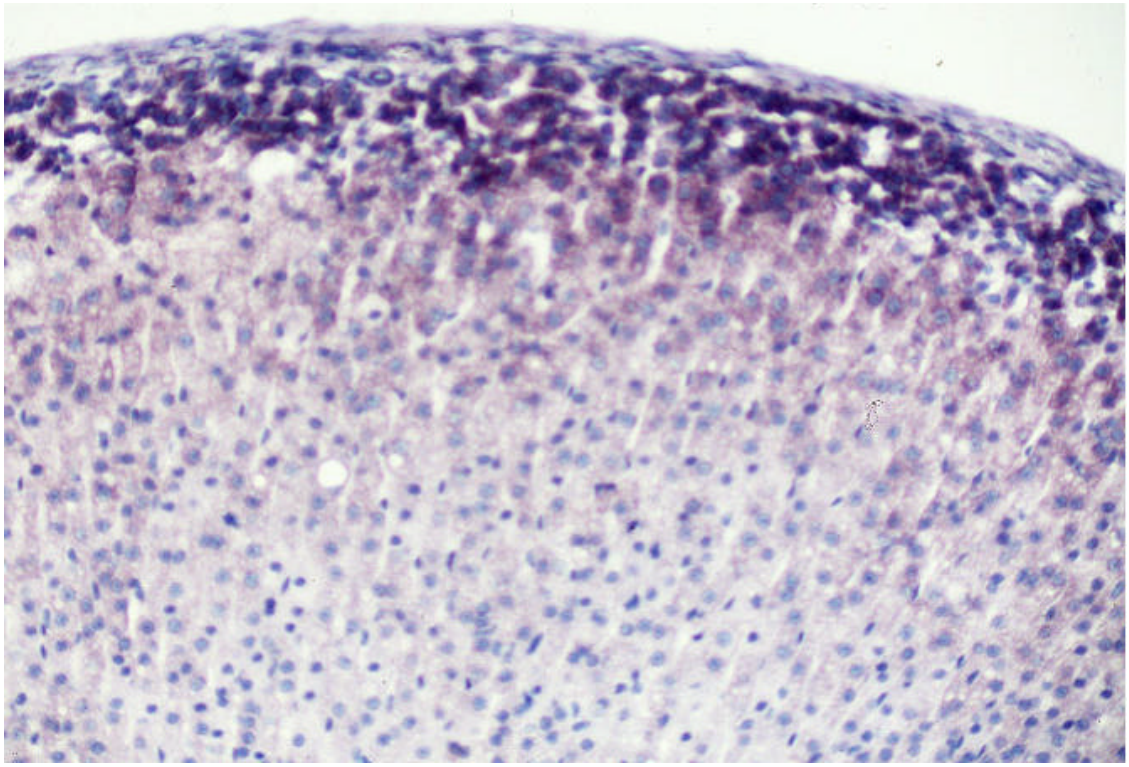


Picture 4. In situ hybridization of rat adrenal sections with digoxigenin (DIG)-labelled rat ROAT1 probe from control animals. ROAT1 mRNA was expressed in the outer zona fasciculata. A., antisense B., sense

A



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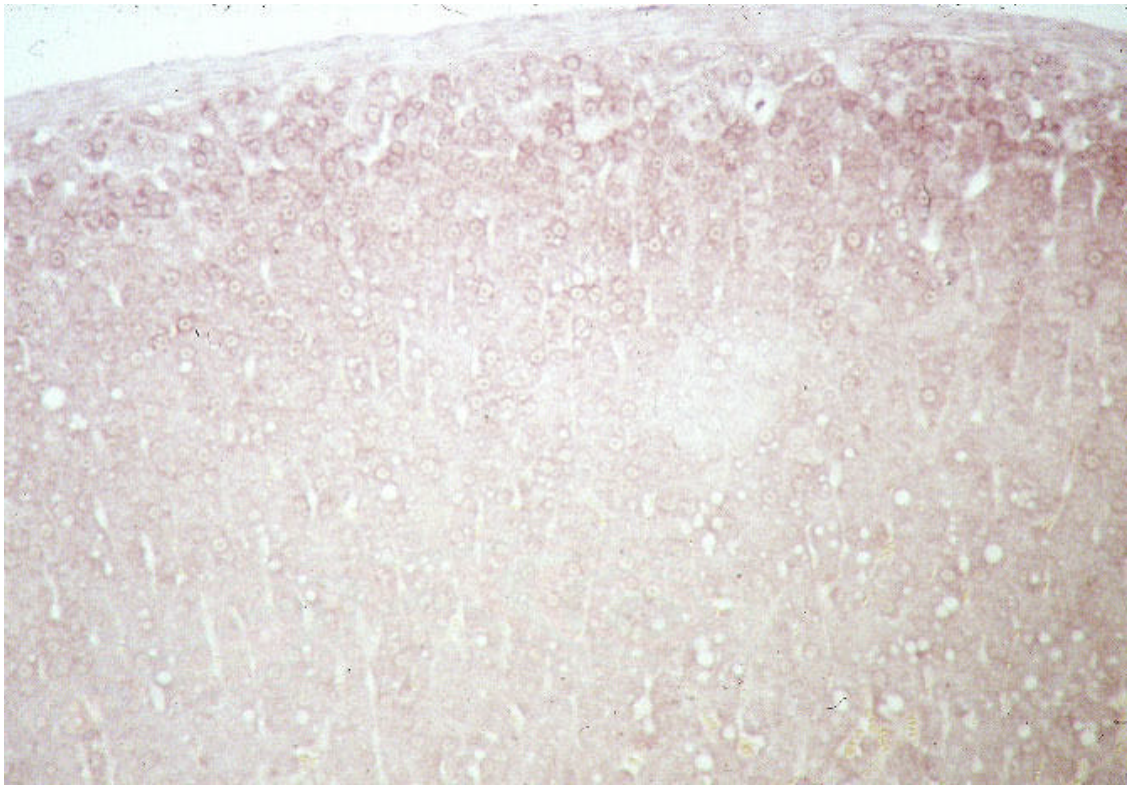
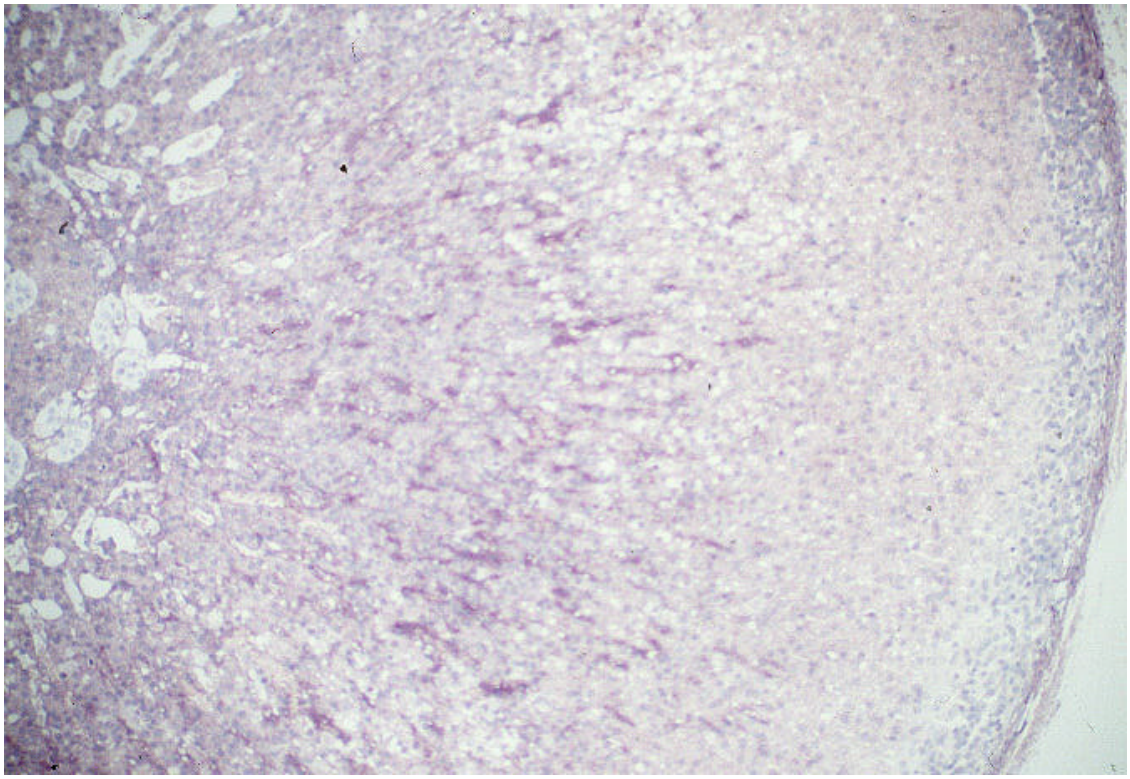


Picture 5. In situ hybridization of rat adrenal sections with DIG-labelled rat oatp3 probe from control animals. The expression of oatp3 mRNA was observed in the zona glomerulosa. A., antisense B., sense

A



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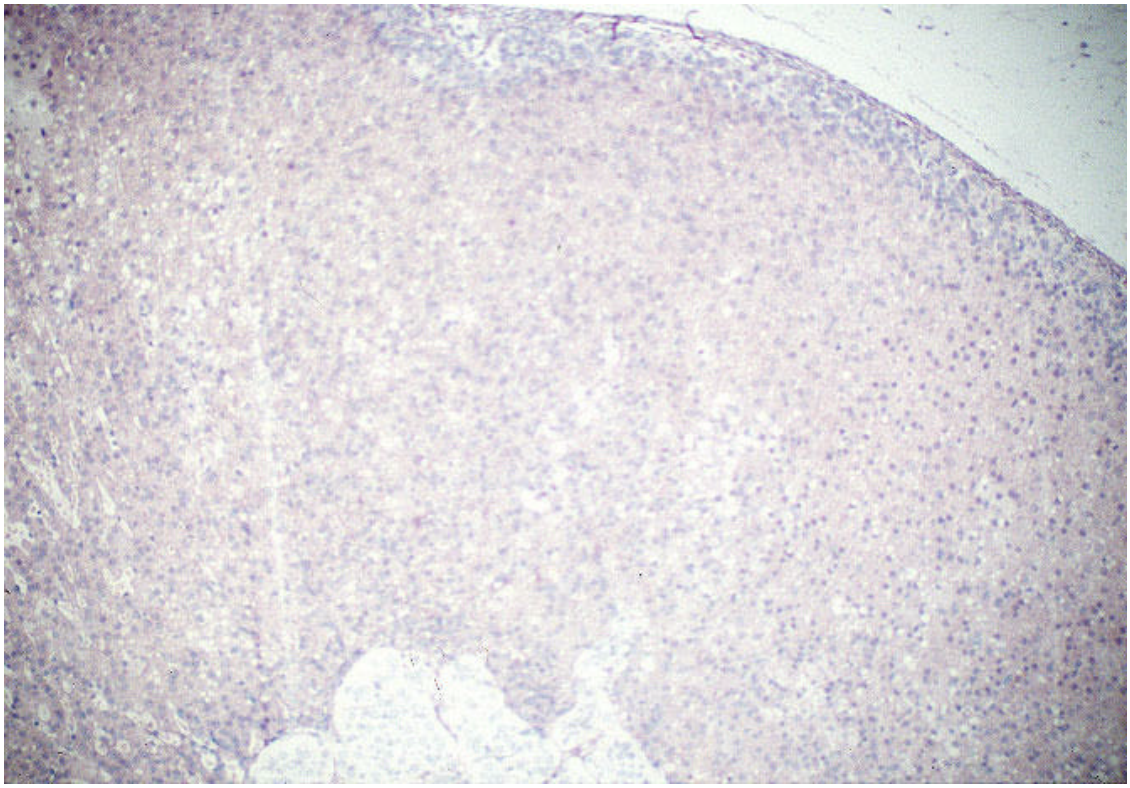
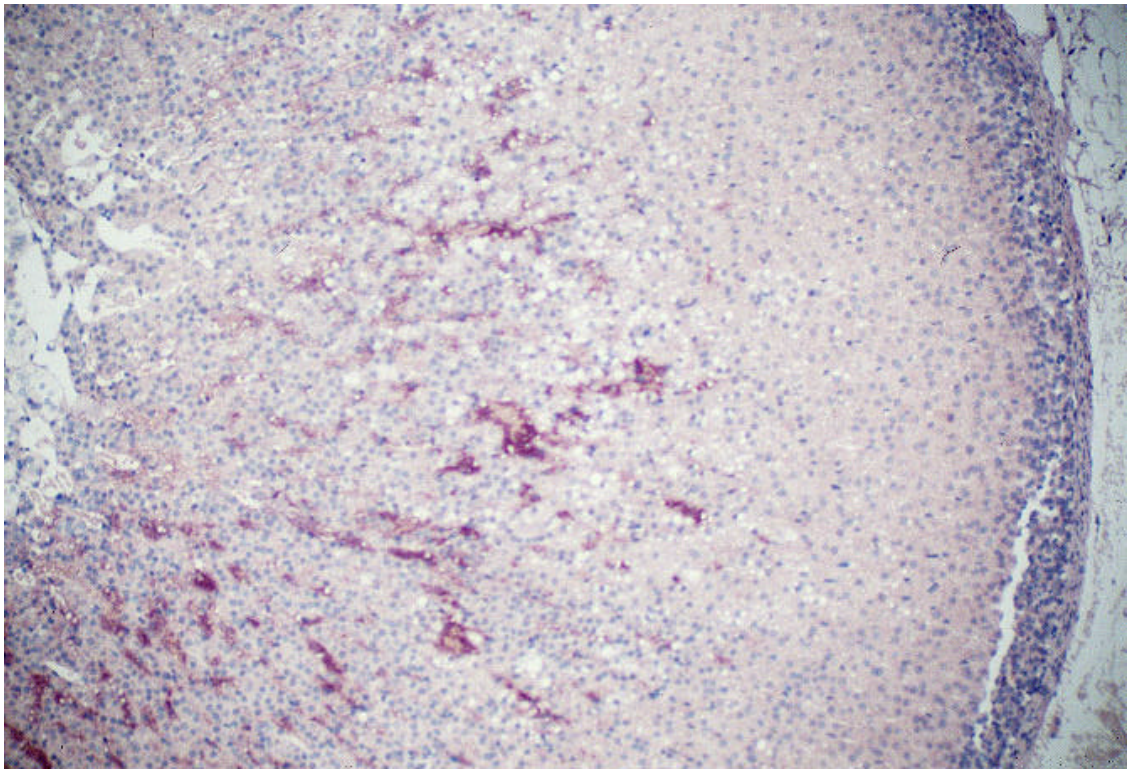


Picture 6. In situ hybridization of rat adrenal sections with DIG-labelled rat oatp1 probe from control animal. The expression of oat1 mRNA was observed in the inner zona fasciculata and zona reticularis. A., antisense B., sense

A



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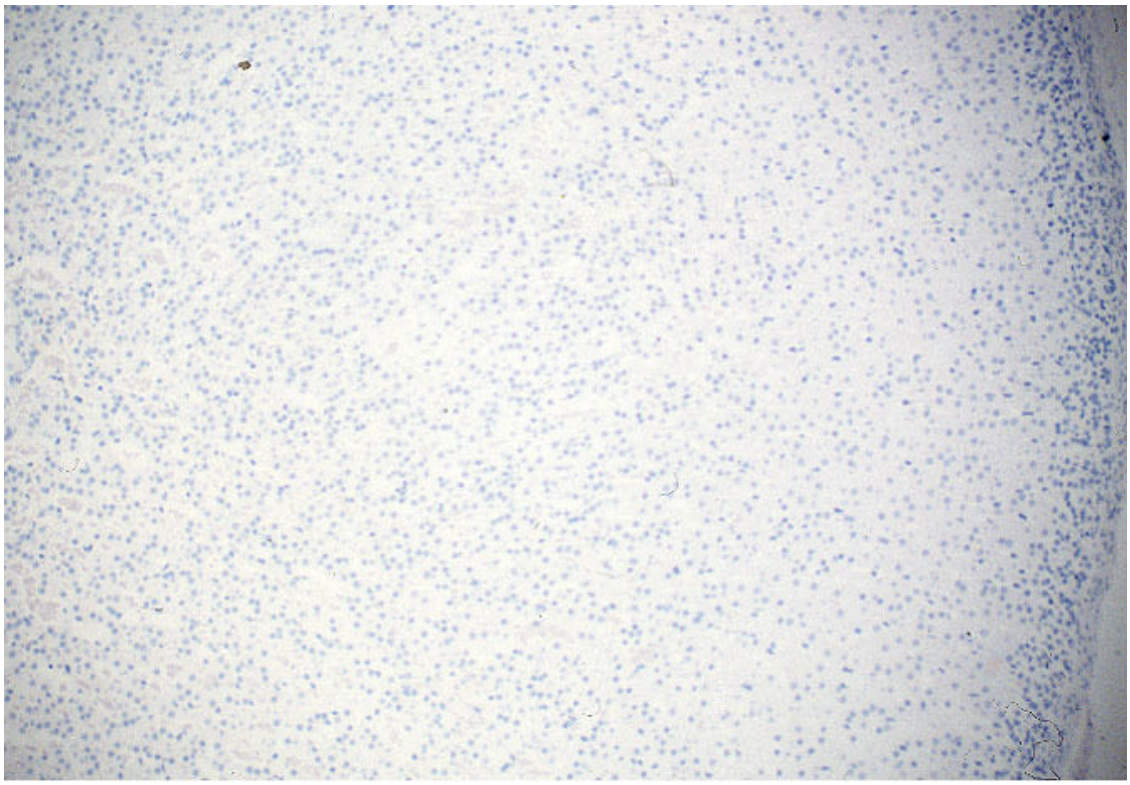
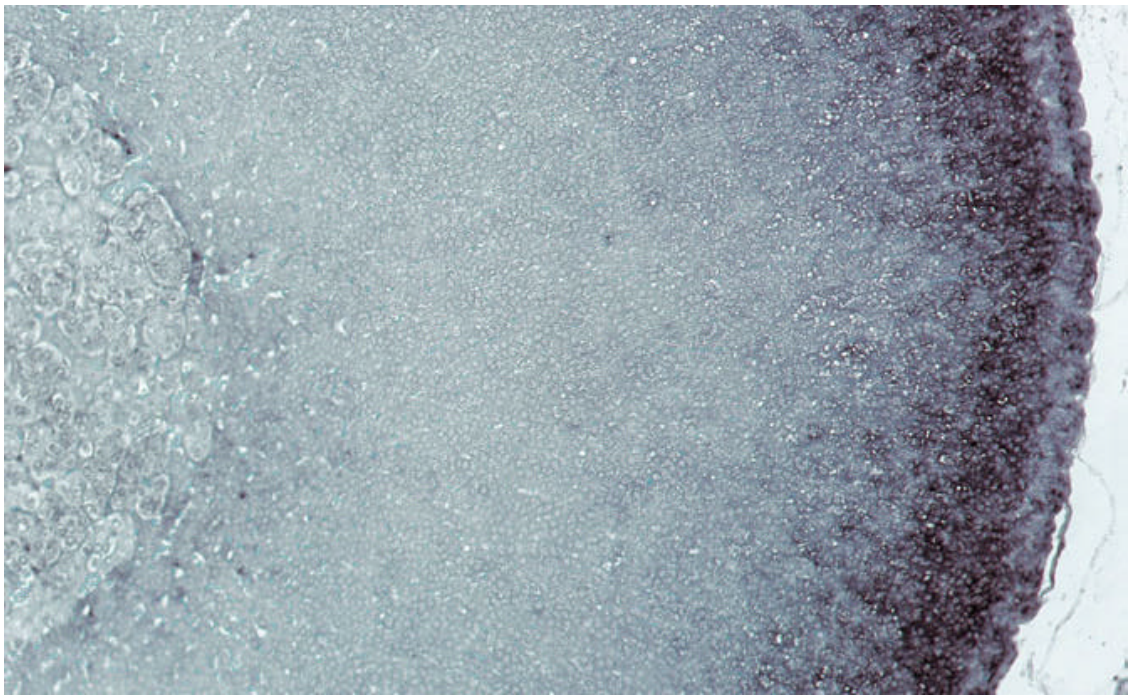


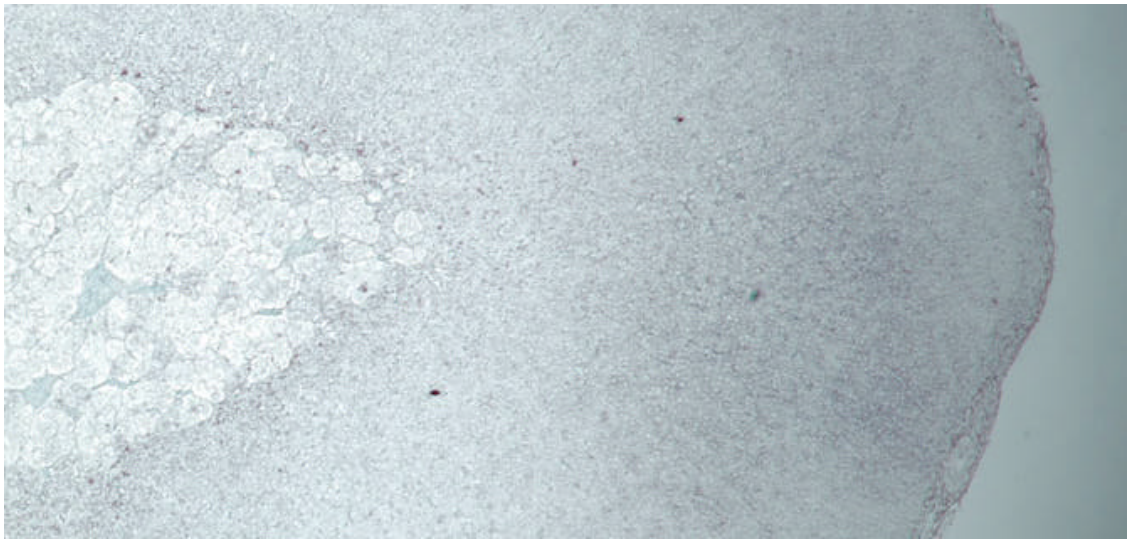
Picture 7. In situ hybridization of rat adrenal sections with DIG-labelled rat oatp2 probe from control animals. The expression oatp2 mRNA was observed in the inner zona fasciculata and zona reticularis. A., antisense B., sense

During the in situ hybridization studies, cis-inhibition transport assays were published with the recently cloned rat organic cation transporters 1 and 2. Among others, corticosterone decreased, in physiological concentration, organic cation transport via OCT2. In co-operation with Prof. Koepsell, organic cation transporter 1 (OCT1) and 2 (OCT2) probes which had already been used for in situ hybridization in kidney, were tested in rat adrenal gland for the presence of this cation transporters.

A



B



Picture 8. In situ hybridization of rat adrenal sections with DIG-labelled rat OCT2 probe from control animals. OCT2 mRNA was expressed in the zona glomerulosa and in the outer zona fasciculata. A., antisense B., sense

Transporter	Zona glomerulosa	Outer zona fasciculata	Inner zona fasciculata	Zona reticularis
OAT1	—	++	—	—
oatp1	—	—	+	+
oatp2	—	—	+	+
oatp3	++	—	—	—
OCT1	—	—	—	—
OCT2	++	++	—	—

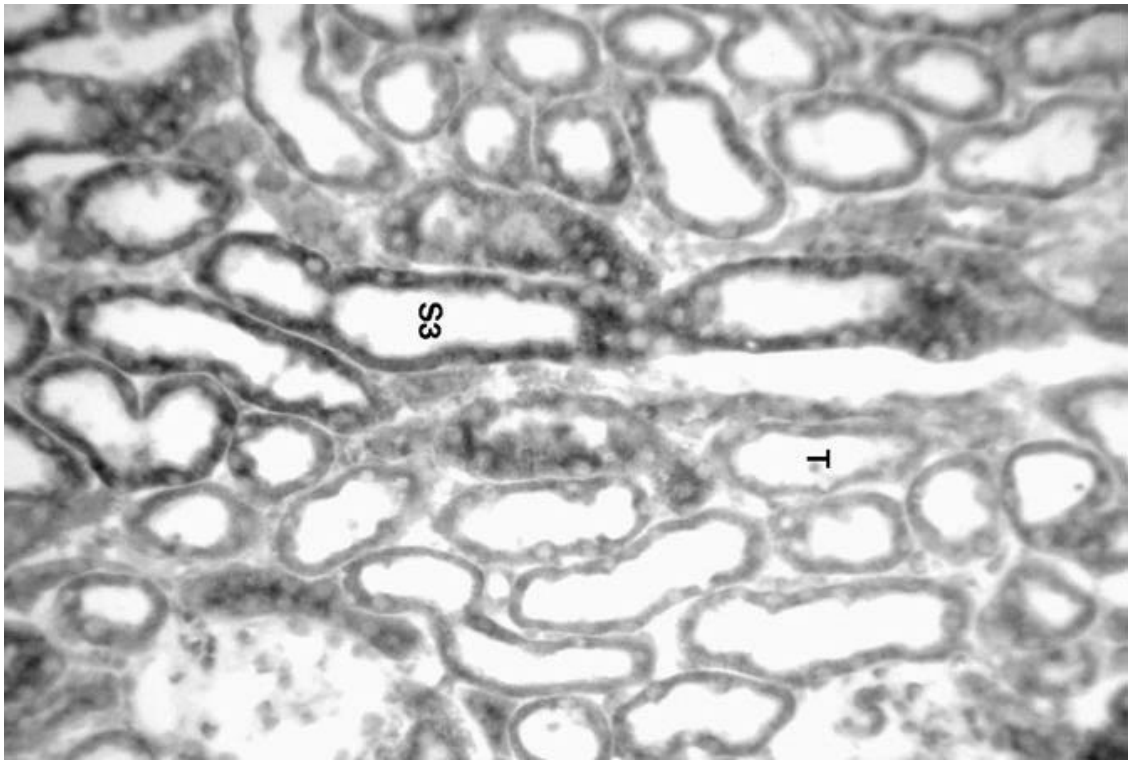
Table 9. Expression zonation of the investigated transporters in the adrenal gland. Dig-labelled RNA probes, typical of the studied transporter, were used in adjusted in situ hybridisation to detect the expression appearance and division of these transporters. —, means there was no signal +, means single cells were positive ++, means the whole zone was positive.

The results of the in situ hybridization confirmed that the investigated transporters were expressed separated and zone specific. Construing the in situ hybridization data and comparing them with the characterization of the transporters, a close relation is suggested in some cases between the product of the certain adrenal zone and the transporter expressed there. In detail, ROAT1 is expressed in the whole outer zona fasciculata, and corticosterone was produced without stimulation exclusively by the outer zona fasciculata according to the recent investigations. The zona glomerulosa produces aldosterone and ouabain and oatp3 and OCT2 was found in this zone. There are no experimental data whether there is any interaction between these steroids and transporters, but the common occurrence renders it probable. The point of interest is that OCT2 is also present in the outer zona fasciculata which produces corticosterone, a possible substrate of the transporter. The expression of the other two members of the organic anion transporter family (oatp1 and oatp2) turned out spotty in the inner zona fasciculata and zona reticularis. The positive cells were found mostly around vessels in one or two cell rows. These zones produce mainly conjugated steroids, sexual steroids and dehydroepiandrosterone(sulphate), which have been shown to interact with the above mentioned transporters. OCT1 gave no signal in the adrenal gland, not even when lower hybridization temperatures (45-50°C), higher template concentrations (10-20µg/ml) and increased proteinase K amount (up to 20µg/ml) were tried. However the negative result of the in situ hybridization was not confirmed with other methods.

4.5.3 Localisation of oatp3 in rat kidney

As oatp3 was a transporter from rat retina newly discovered during this study and only Northern blot analysis demonstrated its presence in the kidney, the localization of oatp3 expression in the kidney was a current issue. The previously used oatp3-RNA probes were employed on perfused rat kidney sections prepared by the group of Prof. Kaissling, Department of Anatomy, University of Zürich.

A



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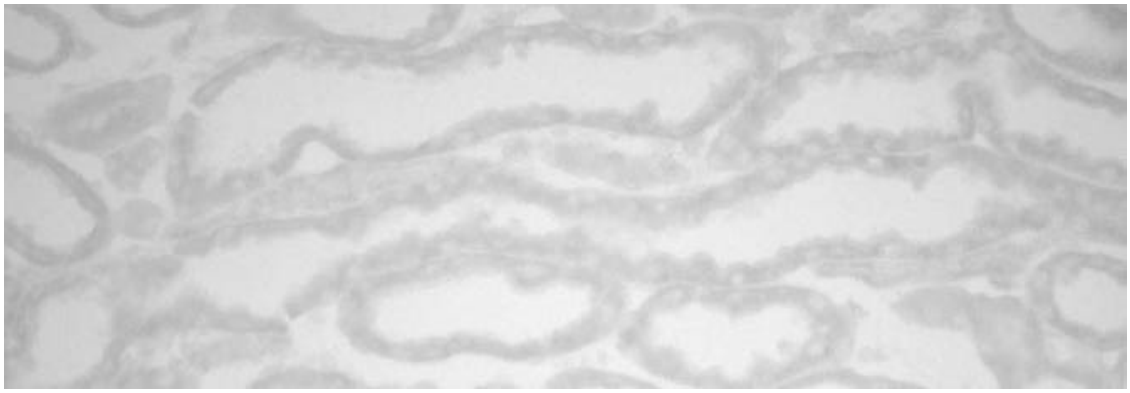


Picture 9. In situ hybridization of perfused rat kidney sections with DIG-labelled rat oatp3 probe from control animals. The expression of oatp3 was observed in the S3 segment of the proximal tubule. T: Thin ascending limb of Henle´s loop, A.,antisense B.; sense

This result was an additional proof of the existence of this transporter in the kidney, and the expression was restricted to the S3 segment of the proximal tubules.

4.5.4 Hormone regulation of the expressed transporters in the adrenal gland

The renal organic anion transporter (ROAT1) localizes exactly in the same zone as the 11-β hydroxylase, the end enzyme of corticosterone synthesis. Thus, the in situ hybridization result supports the previous assumption that this transporter might be involved in cortisol or corticosterone transport. It is known that ACTH controls corticosterone production and release. This effect is mediated by an increase in the number of 11-β hydroxylase enzyme molecules in the cells and in the whole adrenal gland. Under basal conditions, the enzyme is expressed only in the outer zona fasciculata, but under the influence of ACTH the inner zona fasciculata and zona reticularis cells also start to express the enzyme and in connection with it, the corticosterone production is also increased. Moreover, some glomerulosa cells change into fasciculata cells and they also start to express the hydroxylase enzyme and produce corticosterone. The idea was that if the transporter is involved in glucocorticoid transport, ACTH should have a regulatory effect on the expression of the transporter, similar to that on the enzyme. To find out whether ACTH has any effect on the expression of the transporter, rats were treated with ACTH (Synacten) for 5 days, other rats were hypophysectomized to investigate the effect of the lack of ACTH. All three groups were injected, including the control group, at the same time (the hypophysectomized and control rats were injected with 0.9% saline solution) to correct for errors caused by stress. This was very important as corticosterone is a so-called stress hormone, which is increased during stress. After treatment, the animals were killed and the adrenal glands were prepared for in situ hybridization and blood was taken to measure the corticosterone concentration with a radioimmunoassay (Figure 11.).

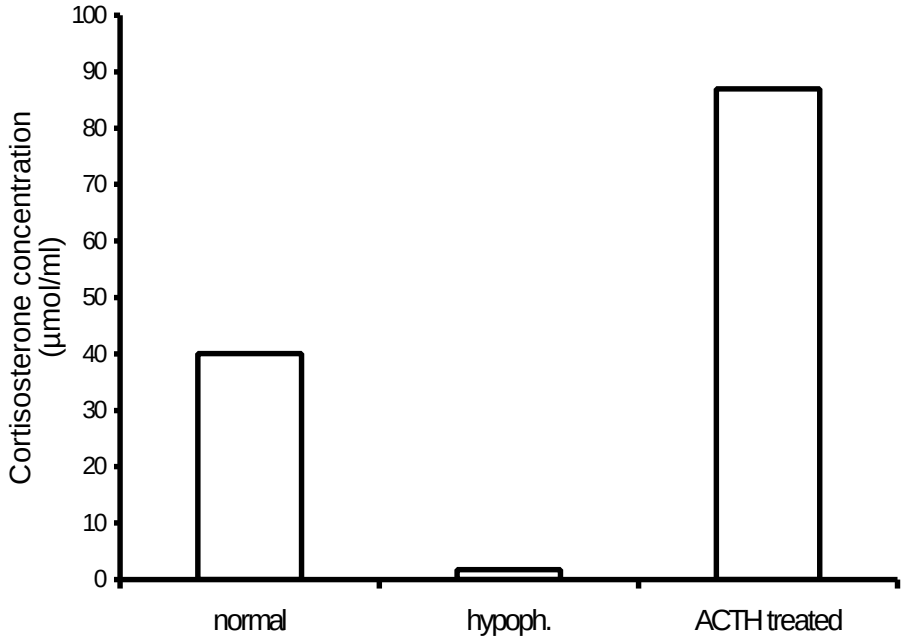
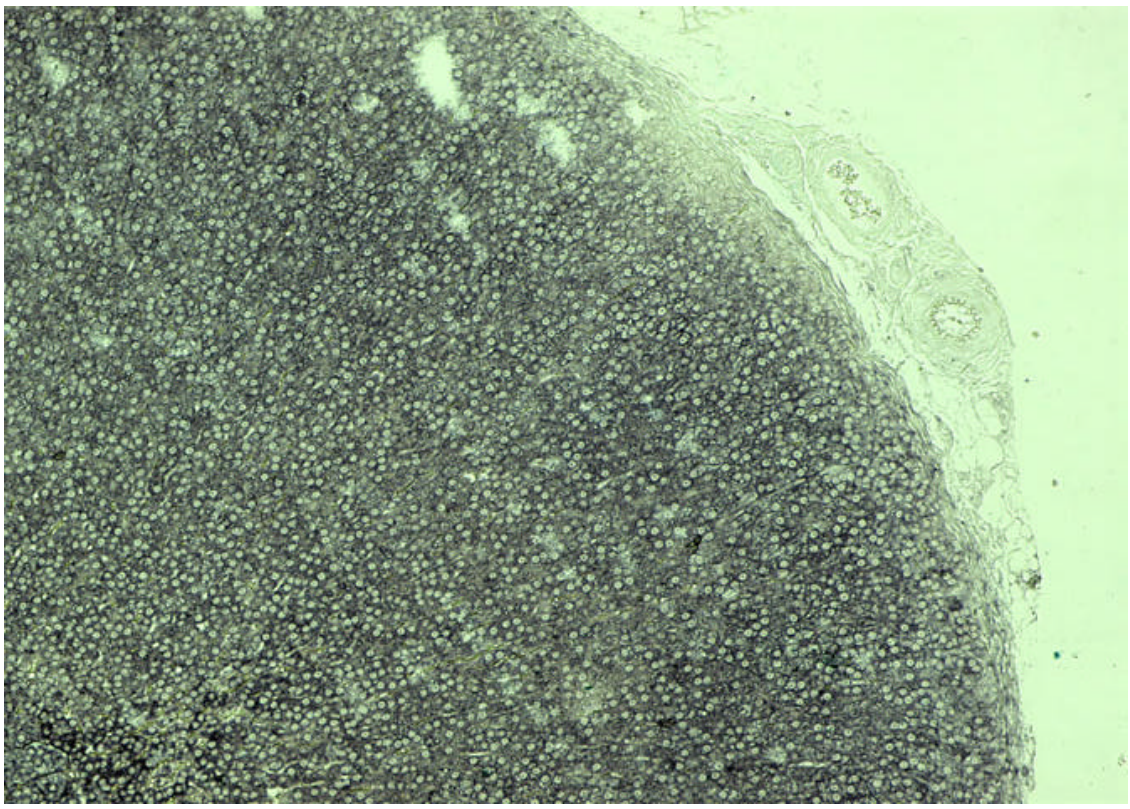


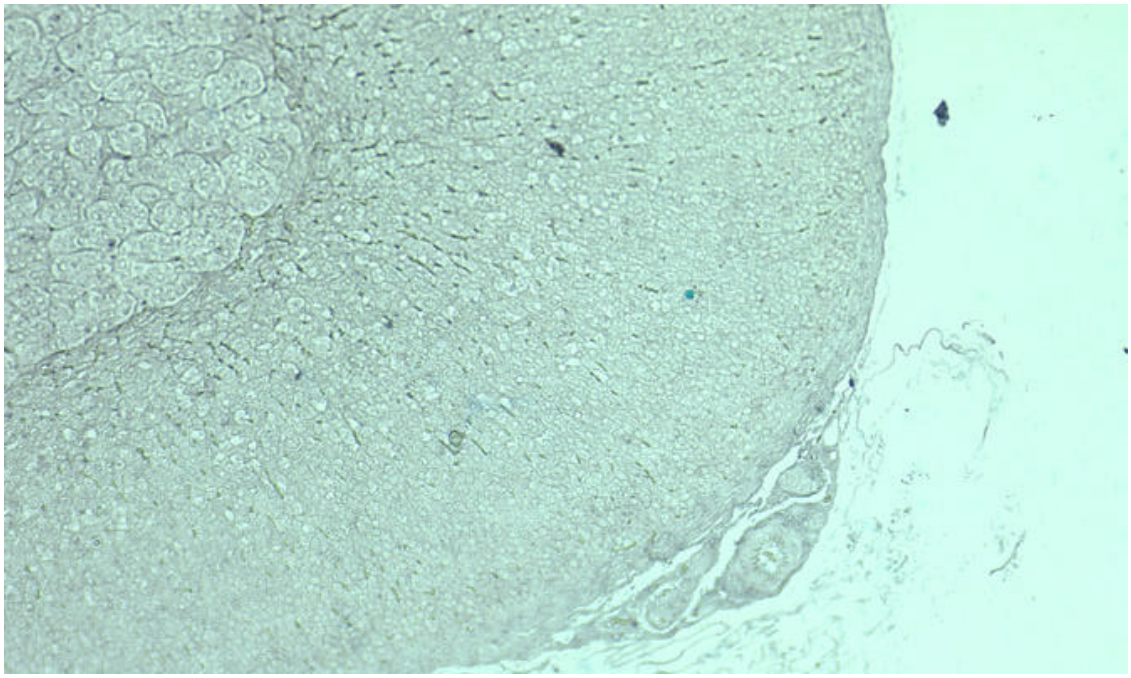
Figure 11. Corticosterone concentration in the blood of the treated rats. The rat was treated with ACTH (2.5 U Synacten) for 5 days, another rat was hypophysectomized to investigate the effect of the lack of ACTH. The hypophysectomized and control rats were injected with 0.9% saline solution at the same time as the ACTH treated ones. The concentration of corticosterone was determined by RIA.

4.5.4.1 ACTH regulation of ROAT1 expression in the adrenals

The regulation of ACTH on the rat "renal" organic anion transporter (ROAT1) was unambiguous. The effect was an obvious change in the expression pattern. In the hypophysectomized adrenal gland the expression of this transporter was no longer detectable (Picture 11.), while under normal, non-induced conditions the localization of the ROAT1 was limited to the outer zona fasciculata (Picture 4.). After ACTH treatment, modelling an induced state of corticonsterone synthesis, the localization was dramatically changed, spreading to other zones of the adrenal cortex. Strong signals were demonstrated in the inner zona fasciculata, zona reticularis and zona glomerulosa (Picture 10.).



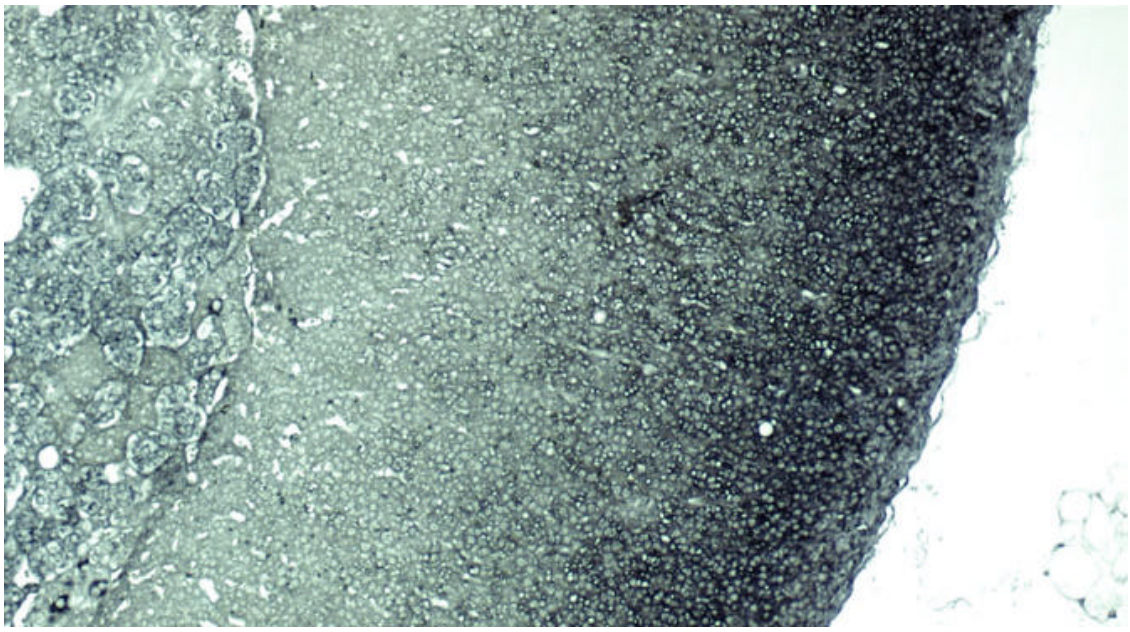
Picture 10. In situ hybridization of rat adrenal section with DIG-labelled ROAT1 probe from animals after ACTH treatment for 5 days. The expression of ROAT1 mRNA was enhanced particularly in the inner zona fasciculata and zona reticularis. Also zona fasciculata cells abutted directly against the connective tissue capsule



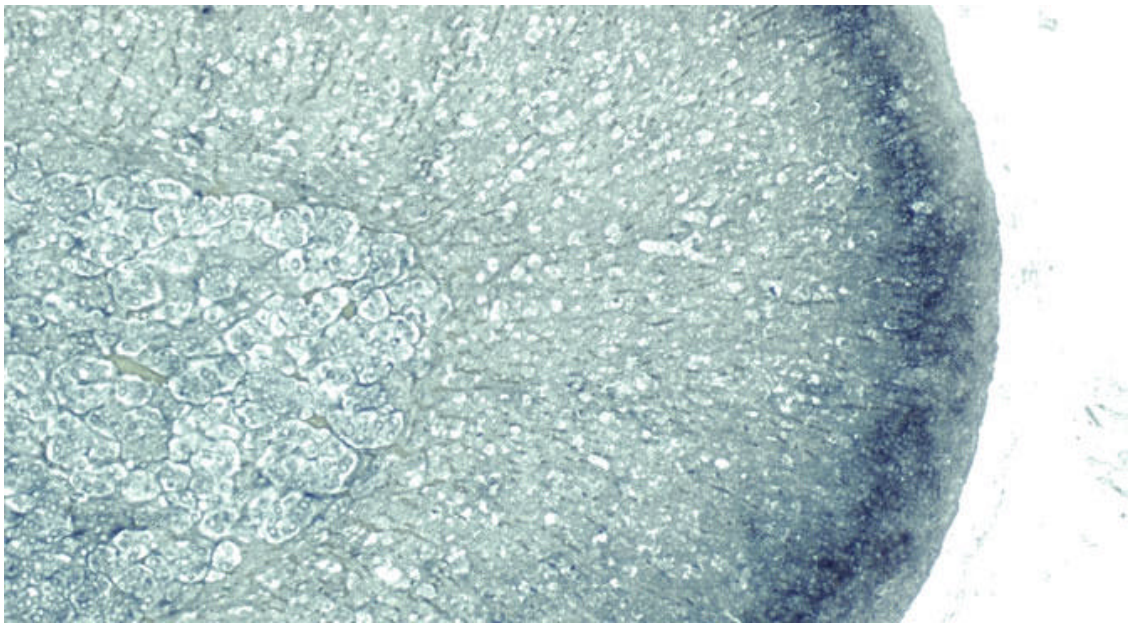
Picture 11. In situ hybridization of rat adrenal section with DIG-labelled ROAT1 probe from hypophysectomized animals. No detection of message was observed in the zona glomerulosa, zona fasciculata and zona reticularis.

4.5.4.2 ACTH regulation of oatp3 expression in the adrenals

Unlike the above transporter, ACTH had no clear effect on oatp3 expression since in comparison with normal conditions, in the hypophysectomized animal the signal was slightly weaker, but still detectable (Picture 13.). No significant change of localization similar to the one mentioned above was revealed. After the ACTH treatment, only the intensity of signals increased (Picture 12.)



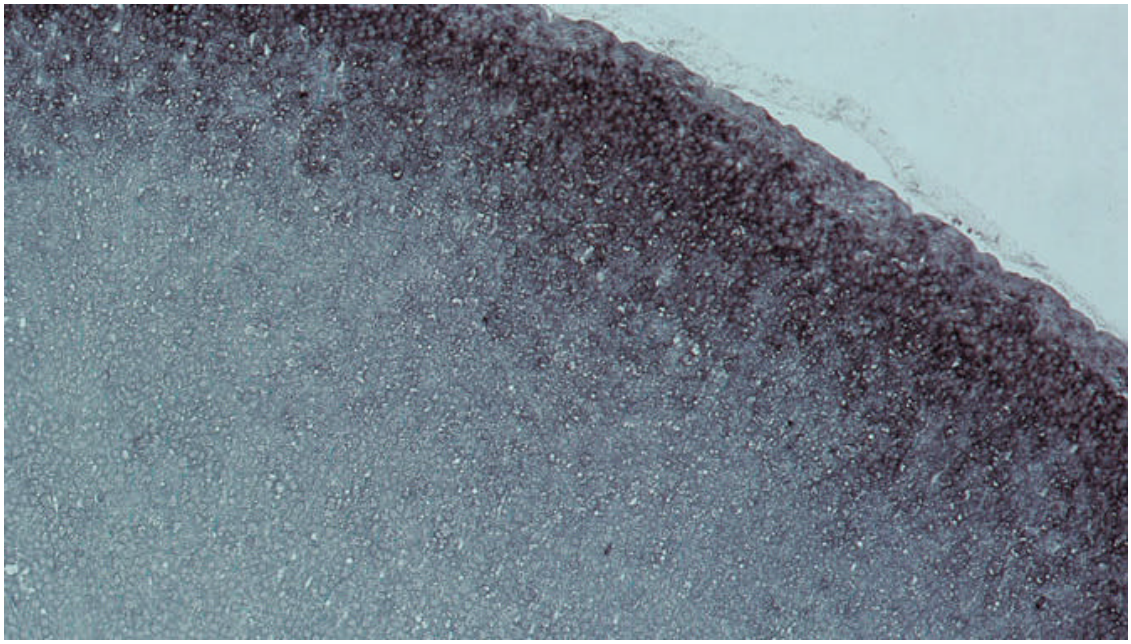
Picture 12. In situ hybridization of rat adrenal section with DIG-labelled oatp3 probe from animals treated with ACTH for 5 days.



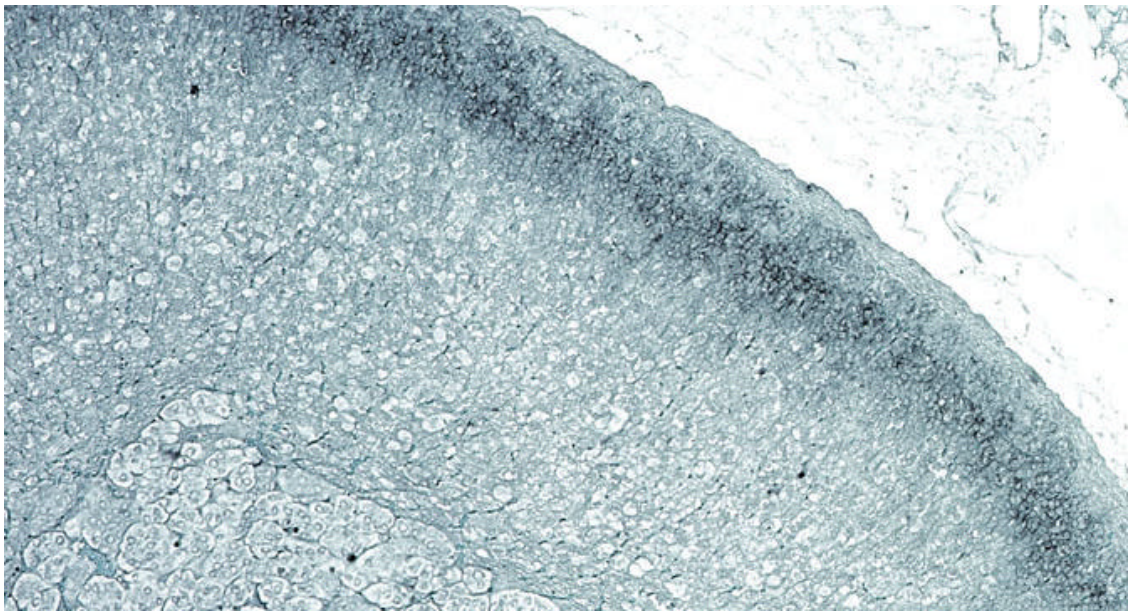
Picture 13. In situ hybridization of rat adrenal section with DIG-labelled oatp3 probe from hypophysectomised animals.

4.5.4.3 ACTH regulation of OCT2 expression in the adrenals

The effect of ACTH on the expression pattern of OCT2 was similar to oatp3. In hypophysectomized animals the signal became weaker (Picture 15.). The ACTH treatment resulted in small change of localisation and increased intensity (Picture 14.).



Picture 14. In situ hybridization of rat adrenal section with DIG-labelled OCT2 probe from animals after ACTH treatment for 5 days.



Picture 15. In situ hybridization of rat adrenal section with DIG-labelled OCT2 probe from hypophysectomized animals.

4.6 FUNCTIONAL CLONE OF OATP3

The organic anion transporting polypeptide 3 (oatp3) was originally cloned from a rat retinal cDNA library and Northern Blot analysis revealed it in kidney, too. In the present study, the transporter was also shown in the adrenal gland by a homology cloning PCR technique and in situ hybridization. The exclusive expressional appearance in the adrenal gland was in zona glomerulosa cells and in the kidney in the S3 segment of the proximal tubule. Since this transporter was newly discovered and poorly characterized, demand arose for further functional characterization. The whole open reading frame was amplified from rat adrenal cDNA by a proof reading polymerase. Performing the PCR reaction, the manufacturer's instructions (*PowerScript* DNA Polymerase, PAN Biotech GMBH, Germany) were followed accurately for the sake of high fidelity. The primers were designed to bind at a high annealing temperature (60°C), carrying *Xba*I and *Hind*III sites and used in 100 pmol quantity. The denaturation time was kept short (10 sec at 94°C) and 25 cycles were run. The PCR product was confirmed by sequencing and cloned into an expression vector, which was used for protein expression in *Xenopus laevis* oocytes. For expression, both 5' and 3' untranslated regions (UTRs) are necessary for cRNA translation, which, however, were not known for oatp3. That is the reason why the following strategy was used to construct functional clone with modification of a flounder sodium dicarboxylate transporter, fNaDC-3 (Steffgen *et al*. 1999), which is expressed at high levels in oocytes. A construct was generated which consisted of the oatp3 coding region flanked by the 5'and 3'UTRs of fNaDC-3. In detail, first the *Hind*III and *Xba*I sites of the vector were disrupted using site directed mutagenesis, then only the UTRs of fNaDC-3 and the complete vector were amplified by PCR. The primers included *Hind*III and *Xba*I restriction sites as close as was practical to the start and stop codons to enable subsequent subcloning. Both the amplification product of the vector and the oatp3 coding region were then sequentially digested with *Hind*III and *Xba*I and ligated together.

4.6.1 Functional characterization of oatp3

The function of oatp3 was investigated by injection of *Xenopus laevis* oocytes with cRNA derived from the expression clone described above. As model substrate, ^{14}C-taurocholate (20 µM) was given (10 min) to control its functional integrity, according to Abe *et al.* 1998, but this failed as there was no more uptake in the injected oocytes compared to the control (water injected) oocytes.

As a second attempt, the clone was subjected to control examinations, checking alignments based on the original sequence from the GenBank and from our labour. The comparison revealed three critical point mutations, which could influence the correct protein folding and could give some reason for the lack of function (App. 1.).

As a third attempt, these three point mutations were changed back to the original nucleotides by site directed mutagenesis. The reactions were carried out with the QuickChange^{TM} Site-Directed Mutagenesis Kit (Stratagene) following the manufacturer's instructions (see 3.5). The amplification products were verified by sequencing, whether the modification was successful. After the third mutation was corrected , this repaired clone was newly expressed again, but was still non-functional.

4.7 HUMAN ADRENOCORTICAL CELL LINE

The bovine and rat experimental results confirmed the original assumption that transporters are involved in the release of steroid hormones. In glucocorticoid release, the participation of the renal PAH transporter is strongly suggested, at least in bovine and rat adrenal. To find out whether the same model can be applied to humans, a steroid producing human adrenocortical carcinoma cell line (NCI-H295) was investigated under different conditions for cortisol release and ^{3}H-PAH uptake. The H295 cells represent a specific adrenocortical cell line which maintains the ability, under specified conditions, to produce all the adrenocortical steroids (ie., mineralocorticoids, glucocorticoids, and adrenal steroids).

Firstly, the ability of H295 cells to produce cortisol was evaluated. Cells were initially treated for 24 hours with forskolin (20 µM) and the medium content of cortisol was measured by RIA. The production of cortisol increased linearly with time and forskolin stimulation was unambiguous, about fifteen-fold after 24 hours (Figure 12.).

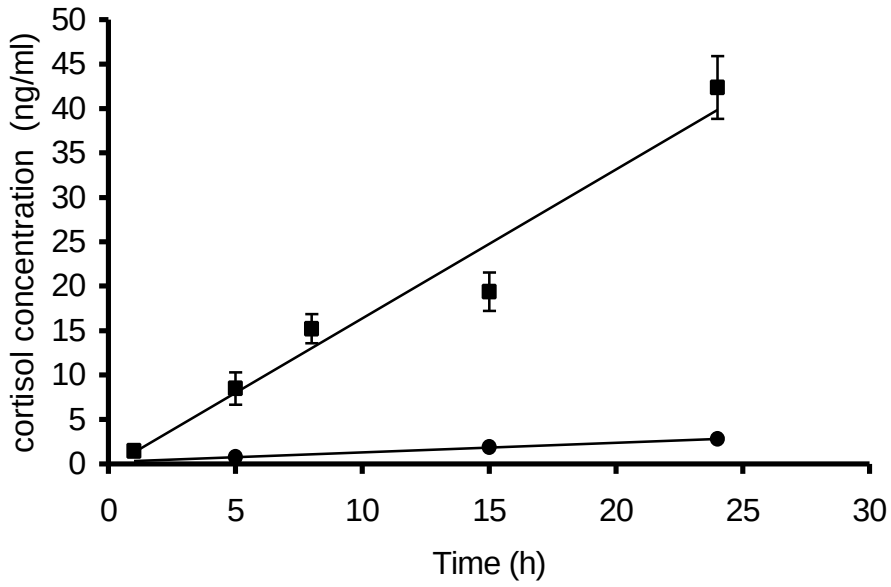


Figure 12. Time-dependency of the stimulation of cortisol with forskolin in H295 cells. Basal cortisol release (•) and incubation with 20 µM forskolin (▪). The medium content of cortisol was determined with RIA. Each point represents a mean ± SEM from three dishes of 1 representative experiment out of three independent experiments.

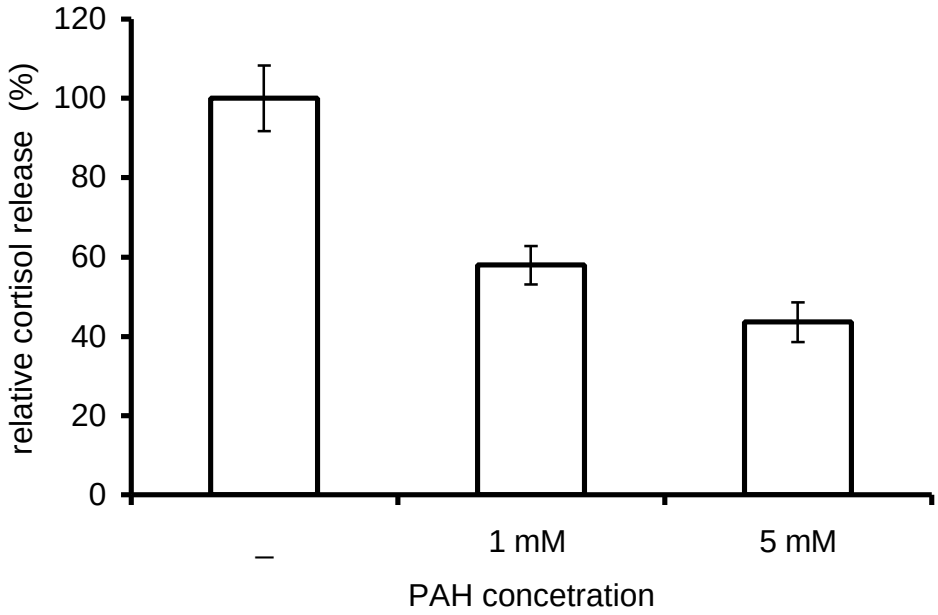


Figure 13. Influence of incubation with PAH on cortisol release from human adrenocortical carcinoma cell line. Cells were incubated with culture medium (-) or with medium supplemented with 1 mM or 5 mM PAH (+) for 24 hours. Forskolin (20µM) was added to both media. Cortisol release without PAH was set to 100 %. Data represent means ± SEM of two independent experiments with 3 wells per condition.

To repeat the previous experiments on human cells, the influence of para-aminohippurate in different concentrations was tested on cortisol release (Figure 13.) Cortisol release was inhibited in a concentration-dependent manner, suggesting intracellular accumulation of PAH, which was competing with cortisol for the same transporter.

Probenecid proved to be lethal at a 5 mM concentration or even at a tenfold dilution: the cells started to detach from the bottom after approximately 3 hours and after 24 hours all were dead.

Shorter incubation times (1/2 and 1 hour) were chosen to eliminate the side effects of the drug on other physiological functions of the cells, but they had to be long enough to measure cortisol in the supernatant of the cells. The cells were preincubated with forskolin in the further experiments for 24 hours so that cortisol became the major product of the cells.

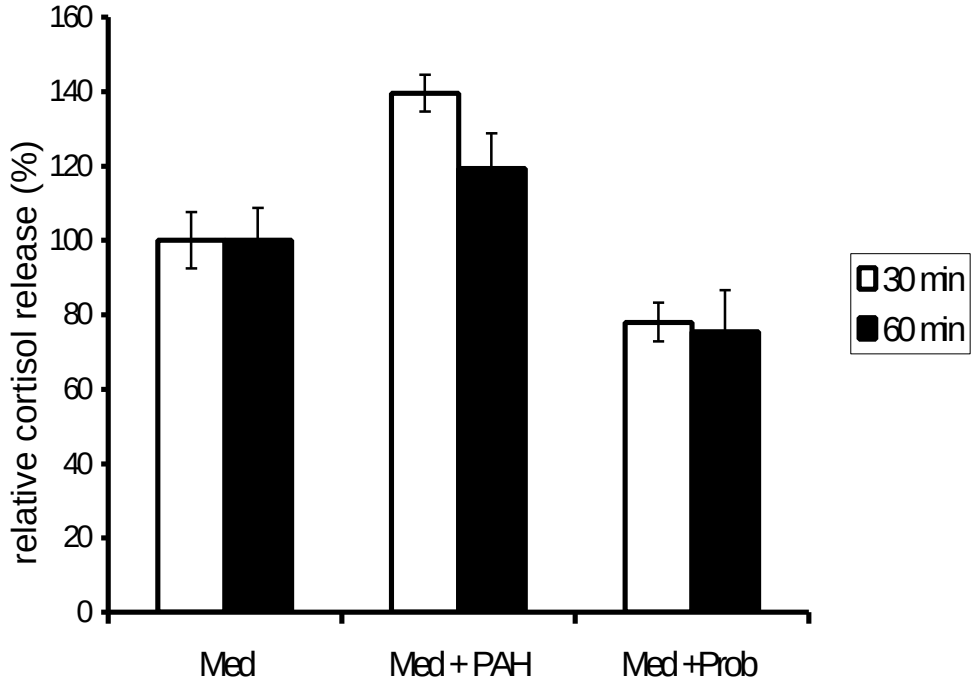


Figure 14. Influence of PAH and probenecid on cortisol release. After 24 hours preincubation with forskolin (20 µM) with 1 mM PAH or 5 mM probenecid (Prob) for 30 min (open column) or 60 min (filled column). Cortisol release in the absence of substrate was set to 100 %. Data represent means ± SEM of two independent experiments with three wells per condition.

After 1/2 hour incubation with 1mM PAH, cortisol release was increased about 40 % and after 1 hour about 20 % which indicated trans-stimulation (Fig.14.). Less stimulation of cortisol release by PAH with longer incubation time suggested that PAH was accumulated in the cells. Probenecid had a mild inhibitory effect on cortisol release under that conditions, about 25% (Fig.14.).

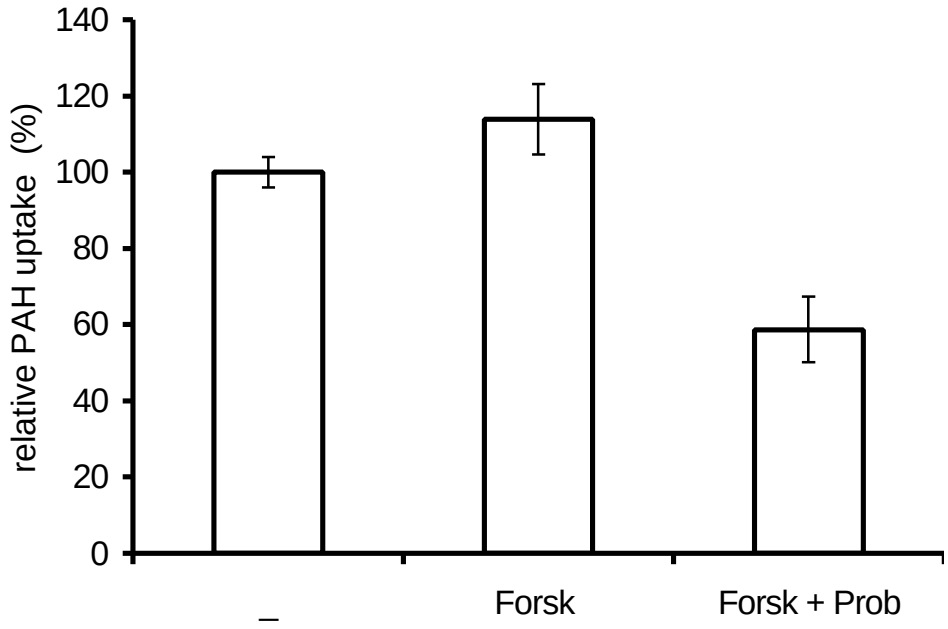


Figure 15. Effect of preincubation with forskolin and inhibition by probenecid on ^{3}H-PAH uptake into human adrenocortical carcinoma cell line. Cells were incubated with radioactive PAH (5.5 µM) for 10 min with or without preincubation with forskolin for 24 hours. Uptake of PAH was detected in the presence of 5 mM probenecid. PAH uptake in the absence of forskolin and probenecid was set at 100 %. Data represent means ± SEM of four independent experiments with three wells per each condition.

In contrast, 10 min uptake of radioactive para-aminohippurate was not significantly increased after forskolin treatment, but probenecid could inhibit about 40% of the uptake (Fig.15.). To further prove the concept of an exchanger, cis-inhibition and trans-stimulation with cortisol and PAH was tested on radioactive PAH uptake.

Preincubation with 1 mM PAH increased by about 35% radioactive PAH uptake while PAH in the transport medium inhibited uptake in a concentration dependent manner (two independent experiments Fig.16.).

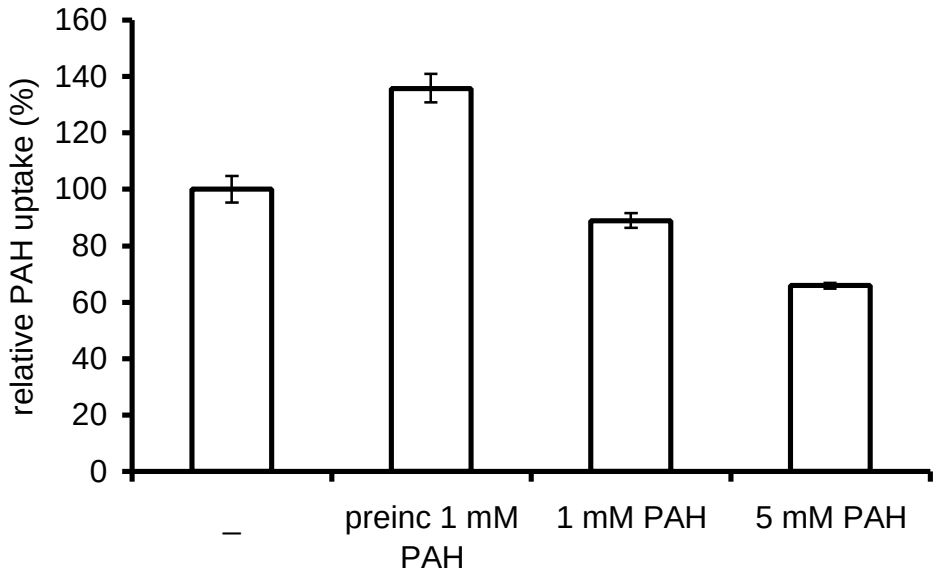


Figure 16. Influence of PAH on ^{3}H-PAH uptake into human adrenocortical carcinoma cell line. Cells were incubated with 5.5 µM ^{3}H-PAH for 10 min after 24 hours preincubation with 1 mM PAH or with 1 mM and 5 mM PAH in the incubation medium . Uptake of PAH in the absence of addition of nonradioactive PAH was set at 100 %. Data represent means ± SEM of two independent experiments with 3 wells per each condition.

5 DISCUSSION

5.1 STEROID HORMONE RELEASE FROM BOVINE ADRECORTICAL CELLS

The adrenal steroid hormones have a central, important role in the hormonal control, as they have influence on almost every physiological process. For this reason, the synthesis and action of adrenal steroids have been extensively investigated. In contrast, their movement through the plasma membrane is still poorly understood, although this is an important problem of basic biology and medicine. Due to their hydrophobic chemical structure, simple diffusion has been postulated as a release mechanism. However, in vitro studies demonstrated retention of steroids against a concentration gradient at the plasma membrane (Whitehouse *et al.* 1971, Inaba *et al.* 1974), whereas the interaction of steroids with carrier proteins in cell membranes has been shown in several studies (Rao *et al.* 1997, Raven *et al.* 1982, Thompson *et al.* 1995, Ullrich *et al*. 1991, Chen *et al*. 1996). Moreover, the recently cloned organic anion and organic cation transporter families proved to be able to transport different steroids (Burckhardt *et al.* 2000). Based on this evidence, it appears likely that transporter participation could be suggested in this process.

5.1.1 Demonstration and characterisation of a probenecid-inhibitable anion exchanger involved in cortisol release

It has recently been shown in bovine adrenal cells that basic and ACTH stimulated cortisol release was reduced by probenecid, a characteristic inhibitor of the renal basolateral PAH/dicarboxylate exchanger (Steffgen *et al.* 1996). Cortisol release was also trans-stimulated by para-aminohippurate itself, suggesting that the mechanism of release may involve a similar transporter. Further evidence for the existence of a renal basolateral PAH transporter-like system in the adrenocortical cells was provided by the expression of probenecid-inhibitable PAH transport after injection of adrenal mRNA into oocytes of *Xenopus laevis* (Steffgen *et al.* 1996).

In the present study, uptake of ^{3}H-PAH by bovine adrenocortical cells was examined under different conditions to obtain further evidence for such a transport system. Uptake of PAH into the cells could be stimulated with ACTH, consistent with stimulation of cortisol synthesis and subsequent trans-stimulation of PAH uptake by the increased intracellular cortisol concentration, of the exchange partner of PAH. Significant inhibition of PAH uptake into normal and ACTH-stimulated cells was also demonstrated. Nevertheless, the probenecid-inhibitable part of PAH uptake was somewhat lower (55%) than the probenecid-inhibitable part of cortisol release (63%). Therefore, it is possible that another transporter(s) is also involved in cortisol release from the adrenal cells. Experimental evidence for the involvement of the other transporter, however, has not been reported in the adrenals. Nor can it not be excluded that part of the inhibiting effect of probenecid on cortisol release might be due to inhibition of cortisol synthesis. However, intracellular cortisol rises in cells incubated with probenecid in comparison to cells which were not incubated with probenecid, indicating that there is no predominant inhibitory effect of probenecid on cortisol synthesis (Steffgen *et al.* 1996). There was always a residual part of PAH uptake and also cortisol release, which was not inhibited by probenecid and which might be due to simple diffusion.

As characteristic features of an exchange transporter system, trans-stimulation and cis-inhibition were tested with the representative substrates of the likely transporter. As the investigated transporter was believed to be an organic anion/dicarboxylate exchanger, glutarate was tested for the ability to inhibit PAH uptake into adrenocortical cells. Significant inhibition was observed with 1 mM glutarate. A higher concentration (10mM) did not increase the inhibitory effect, demonstrating saturation. This effect fits in with the expected model. Inhibition by cortisol was also investigated in order to ascertain – from the opposite side – whether cortisol can interact with the PAH transport system, to confirm the radioimmunoassay findings, showing that the release of cortisol could be influenced by PAH (Steffgen *et al.* 1996 and 1999). In the presence of 1 mM cortisol in the transport medium, the uptake of radioactive PAH was significantly inhibited. This can be explained with competition (cis-inhibition) for a common transporter. Similarly to glutarate 1 mM, nonradioactive PAH caused cis-inhibition of radioactive PAH uptake, as has been shown for the renal organic anion transporter system (Berner *et al.* 1976, Eveloff *et al.* 1979). Preincubation of the cells with 1 mM PAH for 1 hour trans-stimulated ^{3}H-PAH uptake into the cells indicating the presence of

transporter mediating PAH/PAH self-exchange. A similar effect has been shown for the renal basolateral PAH transport system (Shimada *et al.* 1987, Schmitt *et al.* 1993). The result confirms the concept of a carrier-mediated exchange of PAH for cortisol in the bovine adrenocortical cells. Taken together these findings suggest that the disclosed transporter involved in cortisol release in bovine adrenocortical cells is similar to the renal organic anion/dicarboxylate exchanger.

5.2 DEMONSTRATION OF A Na^+-DICARBOXYLATE COTRANSPORTER IN BOVINE ADRENOCORTICAL CELLS

Side-chain cleavage (SCC) of endogenous cholesterol in adrenal mitochondria isolated from ACTH-treated rats indicates that the size of the reactive cholesterol pool depends, via NADPH, on the reducing precursor (McNamara *et al.* 1990). McNamara and Jefcoat have elaborated the existence of two pools of reactive cholesterol, one being partially activated by succinate, the other one solely by isocitrate. It has been postulated that these pools are localized in a subpopulation of mitochondria or different pools of activity within mitochondria.

Serum contains a notable level of some Krebs cycle intermediates (e.g. 42 µM for succinate or 99 µM for citrate in human serum were detected by Krebs in 1950). Northern blots using the luminal dicarboxylate transporter cloned from rabbit kidney as a probe revealed that this transporter is transcribed in the adrenals (Pajor *et al.* 1995). Therefore, it could be suggested that energy requirement of these cholesterol pools can be activated by Krebs-cycle intermediates transported into the cells via a dicarboxylate transport system. Sodium-dependent dicarboxylate transporters have been well characterized in different organs, but not yet in adrenals. In renal proximal tubules, a Na^+-dicarboxylate cotransporter has been described in the basolateral and another one in the luminal membrane (Burckhardt 1989, Wright *et al.* 1987). Both systems differ with respect to their functional characteristics.

To determine whether these transporters are expressed in the adrenals, uptake of succinate by bovine adrenortical cells was tested. As in all other organs tested so far, the uptake displayed sodium dependence and was saturable with an apparent K_m value of approximately 150 µM. This value is between that reported for the high affinity transporter found in the rabbit basolateral membrane of the proximale tubule (12µM)

and that for the rabbit renal luminal transporter (610 µM) (Wright *et al.* 1987). Lithium inhibition of succinate transport into bovine adrenocortical cells was also demonstrated, similar to the inhibition of both basolateral and luminal dicarboxylate transporters by lithium (e.g., Burckhardt 1989, Wright *et al*. 1987). This inhibition is probably due to the replacement of one sodium ion by lithium.

A range of dicarboxylates were tested for their ability to cis-inhibit succinate transport in bovine adrenocortical cells. Strong inhibition was observed with fumarate, glutarate and α-ketoglutarate, as has been reported for renal basolateral and luminal dicarboxylate transporters. On the other hand, isocitrate and citrate did not affect succinate uptake at physiological pH in bovine adrenal cells. This differs from the inhibitory effect of citrate on succinate uptake shown for kidney (Wright *et al*. 1987), liver (Zimmerli *et al*. 1992), intestine (Wolframm *et al*. 1994) and placenta (Ogin *et al.* 1989). Whether another transporter is involved in the uptake of isocitrate and citrate requires further studies. Substrates with trans-configuration (fumarate) were more effective than those with cis-configuration (maleate), as has been reported previously for both the renal luminal and basolateral dicarboxylate transporters (Burckhardt *et al*. 1984, Ullrich *et al*. 1984, Wright *et al.* 1987). Further characterization of this transporter showed no inhibition of succinate uptake by 2,3-dimethylsuccinate or cis-aconitate (Steffgen *et al.* 1999). Both substrates have previously been shown to solely inhibit the basolateral and not – or only weakly – the luminal dicarboxylate transporter in the kidney (Ullrich *et al*. 1984). Moreover, succinate uptake into adrenocortical cells was higher at pH 6.0 than at pH 7.4 or 8.5 (Steffgen *et al*. 1999), which distinguishes this system from both the luminal as well as from the basolateral renal dicarboxylate transporters with respects to pH dependence.

This was the first demonstration of a dicarboxylate transporter in the adrenal gland, which showed some similarities to the renal luminal transporter, as well as differences from both luminal and basolateral transporters. This carrier might supply adrenocortical cells with reducing precursors for cholesterol side-chain cleavage.

5.3 CORTICOSTERONE TRANSPORT VIA THE CLONED RAT ORGANIC ANION TRANSPORTER (ROAT1)

In addition to the cellular expression, the previously cloned ROAT1 – reamplified from rat kidney – was tested for the ability to transport corticosterone, the main

glucocorticoid in rats, when expressed in *Xenopus laevis* oocytes. In contrast to PAH, corticosterone was not taken up specifically into ROAT-1 injected oocytes, as the radioactivity was approximately as high as in the water-injected control oocytes and also the variation of radioactivity within the groups was high. A possible explanation for this observation could be that the oocyte expression system is not suitable for investigating steroid uptake since the oocyte membrane is repleted with progesterone receptors, which are required for maturation, and corticosterone can bind to this receptor with high affinity (Liu *et al.* 1993). In addition to binding, the hydrophobicity of corticosterone may allow passive diffusion through the plasma membrane.

The attempt to inject the radioactive corticosterone into the oocytes to measure efflux also failed. One possible reason could be that the steroids were bound to intracellular receptors or to other cytoplasmic components (eg. intracellular lipids).

Finally, the effect of corticosterone of ROAT1-mediated PAH uptake was tested in a cis-inhibition study. Although corticosterone strongly cis-inhibited PAH uptake, this demonstrates only that the transporter interacts with corticosterone, but does so far not demonstrate transport of corticosterone.

5.4 PCR CLONING OF ORGANIC ANION TRANSPORTERS FROM ADRENAL cDNA

5.4.1 Rat renal organic anion transporter 1 (ROAT1)

Recently, the renal organic anion transporter was cloned from several species (flounder: Wolff *et al.* 1997; rat: Sweet *et al.* 1997; human: Reid *et al.* 1998) and the proteins expressed from the clones showed the same functional characteristics as in the cellular model (Uwai *et al.* 1998, Wolff *et al.* 1997, Hosoyamada *et al.* 1999). The in vitro transport assays with bovine adrenal cells revealed carrier-mediated cortisol release, which showed properties similar to the renal organic anion exchanger. To obtain molecular evidence for the presence of this transporter in the adrenal gland, a homology cloning technique was applied, using the nucleic sequences of the cloned flounder and rat OAT1 to design primers. Since this attempt failed in bovine cDNA, as an alternative rat adrenal cDNA was screened to eliminate the problems according to the species differences. Using a PCR-based cloning strategy, ROAT1 was successfully amplified from rat adrenal cDNA, confirming the assumption that ROAT1 is expressed in the

adrenal cortex according to the transport results. This was the first molecular evidence for the existence of a transporter, at all, in the adrenal gland.

5.4.2 Rat organic anion transporting polypeptide family (oatp)

The bovine transport data suggested the presence of another transporter in adrenals and the participation in cortisol release (Steffgen pers.comm.). Based on the literature, a likely candidate was the organic anion transporting polypeptide (oatp) which represents a polyspecific transporter that can mediate charge-independent uptake of a wide variety of structurally unrelated amphipathic compounds. This broad substrate spectum of oatp includes the organic anions (eg., sulfobromophtalein and bile salts) (Jacquemin *et al.* 1994, Kullack-Ublick *et al.* 1994), as well as estrogen conjugates (Bossuyt *et al.* 1996, Kanai *et al.* 1996), aldosterone and cortisol (Bossuyt *et al.* 1996).

In addition the corresponding parameter for cortisol uptake by oatp1 expressed in *Xenopus* oocytes (K_m = 13.0 µM by Bossuyt *et al.* 1996) was close to the K_m value for the low affinity transport component of cortisol uptake into isolated rat liver cells (Rao *et al.* 1976). In contrast to the data of Bossuyt and co-workers, in a transient expression study of oatp1 in HeLa cells, strong inhibition of oatp-mediated sulfobromophthalein uptake by corticosterone and hydrocortisone was found, whereas no transport of these substrates was detected (Kanai *et al.* 1996). However, conclusions are difficult to draw based on these studies as the two groups did not use the same substrate, because cortisol and corticosterone have very different chemical properties. Nevertheless, demonstrating oatp1 expression in the adrenals was the first step in addressing this question. In addition, besides oatp1, two other members of the rat oatp family had been cloned at the beginning of this study, namely oatp2 (Noé *et al.* 1997) and oatp3 (Abe *et al.* 1998), but their characterization was incomplete. To demonstrate oatp in adrenals, a PCR cloning strategy was employed using primers based on homologous parts of the known members of the oatp family. The PCR screening of rat adrenal cDNA disclosed all three oatp transporters. This was the first molecular demonstration of the respective transporters in rat adrenal gland.

5.4 LOCALISATION OF ROAT1 AND OATP1, OATP2 AND OATP3 IN RAT ADRENAL GLAND

In the next step, the transporters revealed by PCR in this study were localized in the different zones of the adrenal gland by non-radioactive in situ hybridization.

The previous results in the other organs showed that the expression of the various members of the investigated transporter family typically appear in a certain place of the organ. In the kidney, oatp1 is localised to the S3 segment of the proximal tubule in the outer medulla (Bergwerk *et al*. 1996), just as oatp3 (according to our data). In contrast, ROAT1 is expressed in the S2 segment (Tojo *et al*. 1999). In the liver, oatp1 is expressed in the apical membrane of all hepatocytes (Bergwerk *et al*. 1996), while oatp2 is found in the basolateral membrane of the hepatocytes around the central vein (Kakyo *et al*. 1999).

It was a very important issue to determine the localisation of the revealed transporters, as the adrenal gland divides into cortex and medulla, two functionally and originally different parts. The cortex is characterised by a unique anatomical zonation that supports functional diversities, as the three zones produce different steroid hormones. The early steps of the steroidogenesis are all common to all cortical zones, just the late steps are zone specific. In the zona fasciculata and reticularis, corticosterone synthesis depends on the expression of cytochrome P450 11-β hydroxylase (P45011β), which converts 11-deoxycorticosterone to corticosterone, which is the main glucocorticoid in rat, while it is cortisol in bovine and human. The zona reticularis also produces conjugated and sexual steroids. In the zona glomerulosa aldosterone synthesis depends on the expression of cytochrome P450 aldosterone synthase (P450aldo), which converts deoxycorticosterone to aldosterone. The exact zonation of the end enzymes was detected with in situ hybridisation and immunohistochemistry (Ogishima *et al*. 1992, Ho *et al*. 1993, Engeland *et al.* 1997 and Wotus *et al.* 1998).

A non-radioactive in situ hybridisation method, established and optimized for all probes and the adrenal gland, was adopted to disclose the expression pattern of the investigated transporters. The specific Dig-labelled riboprobes could recognize and detect all

transporters (ROAT1, oatp1, oatp2 and oatp3), which were previously demonstrated by PCR in the adrenal gland.

5.4.1 Renal organic anion transporter 1 (ROAT1)

In the case of ROAT1, the presence in the outer zona fasciculata is congruent whith the nonstimulated expression of P450 11-β hydroxylase in the outer zona fasciculata with faint signal in the inner zona fasciculata and zona reticularis, revealed by in situ hybridisation and immunohistochemistry. As the involvement of ROAT1 in corticosterone transport has been suggested from the uptake experiments, this is supported by the co-localized expression with the P45011β enzyme.

It is known that in response to ACTH the adrenal cortex increases the amount of cortisol or corticosterone in the blood. The Engeland and Ho group investigated whether the adrenal gland has the capacity to increase P450 11-β hydroxylase expression in response of adrenal activation. The results showed that one injection of high doses of ACTH increased P45011β mRNA within the initial 24 hours after injection and than repeated injections maintained the elevated expression. The response resulted primarily from an expansion of the area of hybridisation in the inner zones of the adrenal cortex, suggesting that ACTH acts to increase the number of cortical cells expressing P45011β mRNA, contributing to the increase of the total message expression in the adrenal cortex. Extension of fasciculata-like cells to the adrenal capsule after chronic ACTH treatment was observed by Pudney *et al*. in 1984 and the new in situ results showed that the expression of P45011β also appeared in the zona glomerulosa (Engeland *et al*.1997 and Ho *et al*.1993). The change of the mitochondrial cristae from the tubular to the vesicular form and the appearance of the P45011β enzyme in the glomerulosa cells suggest that ACTH induced a transformation of the zona glomerulosa cells to zona fasciculata cells (Hornsby *et al.* 1974, Bertholet 1980, Wolkersdörfer *et al*. 1998). The recruitment of steroidogenic cells may represent a novel mechanism for amplifying the steroid response to adrenal activation.

Similar gene regulation was assumed for ROAT1, if it takes part in corticosterone release. Therefore, the in situ hybridisation experiments were repeated with ACTH-treated, rats injected with Synacten for 5 days, hypophysectomized and control rats.

The control and hypophysectomized rats were also injected with neutral saline at the same time as the ACTH-treated rats with the aim of harmonizing the results, as stress has also been shown to increase corticosterone synthesis in the same manner as ACTH (Engeland *et al.* 1997). The corticosterone concentration in the blood was also determined with corticosterone radioimmunoassay to verify the efficiency of the treatments.

Activation of the adrenal cortex by ACTH increased the amount of ROAT1 mRNA in the same manner as in the case of P45011β. The change in hybridisation resulted from an increase in hybridisation area, as the signal was spread into the zona fasciculata and zona reticularis, and cells in the zona glomerulosa also became positive for the transporter. In addition, the effect of lack of ACTH (hypophysectomy) on the expression of the transporter was tested to consider the regulation from the other side. The signal was no longer detectable even in the outer zona fasciculata. These data clearly show that the physiological stimulus produced by ACTH is able to up-regulate the gene expression of ROAT1, as well as that ACTH is required for a basic transcription of ROAT1 gene. On the basis of these results the direct regulation of the transporter by ACTH is suggests link with corticosterone synthesis.

5.4.2 Organic anion transporting polypeptide 3 (oatp3)

The result of in situ hybridisation revealed oatp3 mRNA exclusively in the zona glomerulosa of the adrenal gland which produces ouabain, as was recently demonstrated (Beck *et al*. 1996, Foster *et al*. 1998) and aldosterone. It was not tested if these compounds are substrates of the transporter, although oatp3 was shown to transport thyroid hormone besides taurocholate (Abe *et al*. 1998). The transporter was also demonstrated to be expressed in the kidney by Northern Blot analysis. As the kidney is the main target of aldosterone action, this suggests a connection between the transporter and aldosterone. However, according to the data presented here, oatp3 expression was found in the S3 segment of the proximale tubule, but not in the collecting ducts which are the site of aldosterone action. Because it was not possible to detect taurocholate transport with the oatp3 cDNA cloned in this study, the two possible adrenal substrates, aldosterone and ouabain, could not be tested, leaving the question open for further examination.

To test whether ACTH has a regulatory effect on oatp3 expression, adrenals from ACTH-treated and hypophysectomized rats were used for detection of any changes in the expression pattern of oatp3. The ACTH-treatment yielded small changes in the intensity of the signal and in hybridisation area, but these were not at all comparable with the large ACTH-induced increases in ROAT1 expression. The decreased amount of oatp3 mRNA in response to the lack of ACTH could be due to the fact that the adrenal requires a certain level of ACTH secretion to maintain its normal structure and function.

5.4.3 Organic anion transporting polypeptide 1 and 2 (oatp1 and oatp2)

In contrast to the previously discussed transporters, in situ hybridisation revealed oatp1 and oatp 2 to be expressed only in single cells or small groups of cells in the inner zona fasciculata and zona reticularis, particularly around blood vessels. These were adrenocortical cells, not epithelial cells, based on their morphology. These adrenal cells produce mainly dehydroepiandrosterone(sulfate) (DHEAS) and also sexual steroids (estrogen, progesterone) and a wide range of precursors and metabolites of these steroids. The appearance of these transporters corresponds with the functional data obtained with oatp1 and oatp2 clones, expressed heterologously in *Xenopus* oocytes (Kullack-Ublick *et al*. 1998, Kakyo 1999) and HeLa cells (Kanai *et al.* 1996), which revealed transport of conjugated steroids, such as estradiol 17 β-D glucuronide and DHEAS. In the rat which among others, lacks the 17α-hydroxylase activity necessary for cortisol and androgen production there is a negligible androgen production, which could account for the rare and specified expression of the above transporters.

5.4.4 Organic cation transporter 1 and 2 (OCT1 and OCT2)

In the course of evaluating the results of the bovine cellular transport experiments the possibility of participation of another transporter in cortisol release arose. Although the first candidate, oatp1, was expressed in the rat adrenal, the localisation in the inner cortical zones indicated another function. In the mean time the organic cation transporter family had been further characterized and this came into consideration.

The kidneys efficiently excrete organic cations of diverse chemical structure. The structural requirements for substrates are a hydrophobic moiety, the ability to form hydrogen bonds and the presence of ionic or partial electrical charges (Ullrich *et al.* 1994 and 1997). Besides the kidneys, a wide tissue distribution was found for the organic cation transporter family (Burckhardt *et al*. 2000)

As the bovine cellular uptake results with primary adrenocortical cells suggested that another transporter participates in cortisol release, and since oatp1 proved not to be involved in this process in rat, the OCTs got into focus. The investigation of OCTs in the adrenal gland was carried out in collaboration with Prof. Koepsell, who showed cis-inhibition by corticosterone of heterologously expressed rat renal OCT1 and OCT2 transporters (Koepsell *et al*. 1999). The presence of the above family in the adrenal cortex could be also demonstrated with in situ hybridisation. In contrast to the results of the cis-inhibition studies, OCT1 seemed not to be expressed in adrenals, while OCT2 was expressed mainly in zona glomerulosa. Interestingly, the expression of OCT2 crossed the border between the zones, also appearing in the upper cell row of the zona fasciculata, a phenomenon only observed with this transporter. The question of function of OCT2 in adrenals is still open, as it could be involved in glucocorticoid release or in aldosterone or/and ouabain transport, which must be tested.

The effect of ACTH regulation on the expression of OCT2 was also tested as in the case of ROAT1 and oatp3. Direct regulation could not be unambiguously demonstrated, though the expression was diminished in the hypophysectomized individuals due to the reduced adrenal function. ACTH treatment did not influence the expression of OCT2, indicating no connection between the transporter and the glucocorticoid synthesis. Despite the inconclusive results regarding regulation of OCT2 expression in the adrenals, this is the first evidence for zone- and cell-specific expression of OCT2 in the adrenal gland.

For all transporters tested by us in the adrenal gland, only ROAT1 showed clear dependency on ACTH, which indicates indirectly a connection between ROAT1 and the glucocorticoids. In summary, it can be stated that ACTH could stimulate PAH uptake into bovine adrenal cells and could also increase the expression of ROAT1 mRNA in rat adrenals. Furthermore, corticosterone could cis-inhibit PAH uptake by heterologously expressed ROAT1 and the localization and regulation of ROAT1 and P45011β enzyme expression proved to be synchronised.

5.5 CLONING AND ANALYSIS OF RAT ADRENAL OATP3

The whole open reading frame of oatp3 was isolated from rat adrenal cDNA using a proof-reading polymerase enzyme under optimized conditions. As the 3' and 5' UTRs were not known for oatp3, which are required for functional expression in *Xenopus* protein expression system, the PCR product was placed between the untranslated regions of the flounder sodium dicarboxylate cotransporter, fNaDC-3 (Steffgen *et al.* 1999). The same strategy was employed for the human OAT1 (Reid pers.comm.) and a recently cloned rabbit OAT1 clone (Bahn *et al*. 2000). Referring to the characterisation of the above mentioned clones, the fNaDC-3 UTRs seemed to improve the expression of mammalian cDNAs in oocytes (unpublished data).

5.5.1 Expression of oatp3 in *Xenopus* oocytes

The construction of the functional oatp3 clone flanked by fNaDC-3 UTRs was verified with sequencing. The sequence of the adrenal clone proved to be 98 % identical with the original retinal oatp3. The insertion of the oatp3 clone between the fNaDC-3 UTRs occurred in frame and the START and STOP codons were intact. However, a transport assay in *Xenopu*s oocytes with taurocholate as substrate, carried out according to the protocol of Abe and coworkers, with the cloned adrenal oatp3 was unsuccesful. When the whole sequence of this clone was compared on the amino acid sequence level with the original retinal one and also with fragments which were amplified during the screening of the adrenal cDNA for oatps, three point mutations were revealed, suggesting alteration in protein folding. Twice, the original amino acids were replaced with prolins which can brake the secondary structure of the protein. The third divergence in the 11^{th} transmembrane domain, a change from phenylalanin to serin, can also have an influence on the correct folding on steric ground. These changes were reversed using site-directed mutagenesis. After sequencing, the clone was found to correspond to the published sequence. However, the repaired expression clone still did not mediate taurocholate uptake, when injected in *Xenopus* oocytes. Further attemps are intended after additional improvement of the adrenal oatp3 clone and the experimental conditions.

5.6 HUMAN ADRENOCORTICAL CELLS

The steroid release was investigated in the bovine and rat adrenal model, but naturally the most important was to become acquainted with the mode of glucocorticoid release from human adrenal cells. For this reason, a human adrenocortical carcinoma cell line (NCI-H295) was obtained, which can serve as an in vitro model for human adrenal cell function. Furthermore, the ability of H295 cell to be manipulated into a cell producing mineralocorticoids, glucocorticoids or C19-steroids provides a valuable model for elucidating the molecular mechanisms which give rise to each of the three distinct zones of the human adrenal cortex (Gazdar *et al.* 1990, Rainey *et al.* 1993 and 1994).

Under unstimulated conditions, the cells do not produce cortisol. Significant increases in cortisol synthesis could be seen after 5 hours of forskolin treatment, an agonist of the protein kinase A pathway. Forskolin was used for stimulation of cortisol production, because the previous experiments indicated the lack of response to ACTH treatment (Mountjoy *et al.* 1994). The reason of the insensitivity was not yet cleared up, but decreased expression of ACTH receptor was assumed (Rainey *et al.* 1993). Cortisol becomes the major steroid product after 24 hours of forskolin treatment (Rainey *et al.* 1994). Therefore, in the present study, the cells were pre-treated for 24 hours with forskolin.

On the basis of the bovine experiments, the human adrenocortical cells were tested for the existence of an OAT-like organic anion transporter involved in cortisol relese from the cells. The release of cortisol could be trans-stimulated with 1 mM PAH and probenecid had a mild inhibitory effect on the release, detected by RIA. In contrast, forskolin could not increase PAH uptake into the cells, but probenecid could inhibit about 40% of the uptake, which is comparable with the bovine results. The uptake could also be significantly inhibited with 5 mM PAH in the incubation medium, indicating low substrate affinity of the transporter.

Due to the technical difficulties as the cells did neither tolerated probenecid, even in low concentrations, for extended periods of time, nor high concentrations of the investigated substrates the results can only be regarded as preliminary. Those obtained are (in part) consistent with the proposed model of an exchanger mediating cortisol release, but further experiments are needed to characterize this system in human adrenal cells.

If cortisol is released in human by transport systems, treatment of hypercortisolism (Cushing) by inhibitors of the transporter would be possible and this would be a good alternative to operation, or at least for bridging the time until the patient is in a better condition for operation.

6 APPENDIX

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A.  MGETEKRVATHEVRCFSKIKMFLLALTWAYVSKPLSGIYMNTMLTQIERQFDIPTSIVGF   60
R.  MGETEKRVATHEVRCFSKIKMFLLALTWAYVSQSLSGIYMNTMLTQIERQFDIPISIVGF   60
                          I

A.  INGSFEIGNLLLIIFVSYFGTKLHRPIMIGVGCVIMGLGCFLMSLPHFLMGRYEYETTIS  120
R.  INGSFEIGNFLLIIFVSYFGTKLHRPIMIGVGCVIMGLGCFLMSLPHFLMGRYEYETTIS   20
       II                              III

A.  PTSNLSSNSFLCMENRSQTLKPTQDPAECIKEMKSLMWIYVLVGNIIRGIGETPIMPLGI  180
R.  PTSNLSSNSFLCMENRSQTLKPTQDPAECIKEMKSLMWIYVLVGNIIRGIGETPIMPLGI  180
                                       IV

A.  SYIEDFAKSENSPLYIGILETGKVFGPIVGLLLGSFCASIYVDTGSVNTDDLTITPTDTR  240
R.  SYIEDFAKSENSPLYIGILETGKVFGPIVGLLLGSFCASIYVDTGSVNTDDLTITPTDTR  240
                             V

A.  WVGAWWIGFLICAGVNILSSIPFFFFPKTLPKEGLQDDVDGTNNDKEEKHREKAKEENRG  300
R.  WVGAWWIGFLICAGVNILSSIPFFFFPKTLPKEGLQDDVDGTNNDKEEKHREKAKEENRG  300
                    VI

A.  ITKDFLPFMKSLSCNPIYMLLILTSVLQINAFINMFTFLPKYLEQQYGKSTAEVVLLIGV  360
R.  ITKDFLPFMKSLSCNPIYMLLILTSVLQINAFINMFTFLPKYLEQQYGKSTAEVVLLIGV   60
                                    VII

A.  YNLPPICIGYLLIGFIMKKFKITVKKAAYMAFCLSLFEYLLYFLHFMITCDNFPVAGLTA  420
R.  YNLPPICIGYLLIGFIMKKFKITVKKAAYMAFCLSLFEYLLYFLHFMITCDNFPVAGLTA  420
     VIII                            IX

A.  LYEGVHHPLYVENKVLADCNRGCSCSTNSWDPVCGDNGLAYMSACLAGCKKSVGTGPNMV  480
R.  LYEGVHHPLYVENKVLADCNRGCSCSTNSWDPVCGDNGLAYMSACLAGCKKSVGTGTNMV  480


A.  FQNCSCIRSSGNSSAVLGLCKKGPECANKLQYFLIMSVIGSFIYSITAIPGYMVLLRCIK  540
R.  FQNCSCIRSSGNSSAVLGLCKKGPECANKLQYFLIMSVIGSFIYSITAIPGYMVLLRCIK  540
                                       X

A.  PEEKSLGIGLHAFCTRVSAGIPAPIYFGALIDRTCLHWGTLKCGEPGACRMYNINNFRRI  600
R.  PEKKSLGIGLHAFCTRVFAGIPAPIYFGALIDRTCLHWGTLKCGEPGACRMYNINNFRRI  600
                       XI

A.  YLVLPAALRGSSYLPALFILILMRKFQFPGEIDSSETELAEMKITVKKSECTDVHGSPQV  660
R.  YLVLPAALRGSSYLPALFILILMRKFQFPGEIDSSETELAEMKITVKKSECTDVHGSPQV  660
              XII

A.  ENDGELKTRL  670
R.  ENDGELKTRL  670
```

Appendix 1. Amino acid comparison of the retinal and adrenal oatp3. Residues shaded grey are those different in the retinal and adrenal clone. The putative transmembrane regions are indicated by solid lines (data taken from Abe *et al*. 1998) A means adrenal, R means retinal.

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