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# Corticosteroid Regulation of Colonic Ion Transport during Postnatal Development: Methods for Corticosteroid Analysis

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## Summary

Many mammalian species including human are immature at birth and undergo major developmental changes during suckling and weaning period. This problem is also conspicuous for the gastrointestinal tract that undergoes abrupt transitions coinciding with birth and weaning. This review deals with the maturation of ion transport functions in colon, the intestinal segment that plays an important role in sodium and potassium absorption and secretion. The purpose of the present review is to summarize the mechanism of sodium and potassium transport pathways and show how these transport processes change postnatally and how hormones, particularly corticosteroids, modify the pattern of development. Finally we describe some of the ways, how to analyze corticosteroid metabolism in target tissue.

**Key words**

Colon • Rat • Corticosterone • Aldosterone • 11β-hydroxysteroid dehydrogenase

## Ontogeny of intestinal ion transport

Physiological functions of mammals are not fully matured after birth. The survival of newborns depends, therefore, on their ability to adapt from intra- to extrauterine life as nutrition, respiration and regulation of body fluids are no longer maintained by placenta. The lung, kidney and the gastrointestinal tract are responsible for the maintenance of body homeostasis and growth after birth. The achievement of positive ion, water and nutrients balance is therefore one of the most important task of the gut and kidney during early postnatal development, although the structure and functions of these organs are immature. Maturation of renal and gastrointestinal functions is, therefore, one of the critical factors for survival and further development of the organism.

Ontogenetic development of the gastrointestinal tract represents topologically and temporally a highly organized process (Henning *et al*. 1994, Kedinger 1994) that can be divided into five phases: (1) morphogenesis, (2) cytodifferentiation and fetal development including preparation of the epithelium for absorption of colostrum and milk, (3) birth and the shift from the intra- to extrauterine environment, (4) suckling period, and (5) weaning of the offsprings from mother's milk to a solid diet. Although the development of intestinal functions is similar in all mammals, the developmental patterns show

considerable differences relatively to the ”ontogenetic time” in various species. The variations in timing and the extent of intestinal maturation reflect the duration of the gestational period. The altricial species such as mouse and rat, which are born after a short gestation, possess a very immature gastrointestinal tract that achieves fully developed digestive and absorptive functions after weaning. In contrast, the precocial species that have a long gestational period (guinea pig etc.) have the gastrointestinal tract much more matured at the time of parturition (Henning *et al*. 1994, Pácha 2000).

Tissue growth is associated with the accumulation of proteins, ions and water. Such accumulation is also obvious in the case of $Na^+$ and $K^+$ that represent the pivotal electrolytes of the extra- and intracellular space accumulated in bone and extracellular fluid and in muscles, respectively. In addition, it is well known that the concentration of $K^+$ in peripheral tissues decreases during postnatal development (Aperia and Celsi 1992, Aizman *et al*. 1998, Pácha 2000). It is therefore obvious that the epithelial intestinal and renal transport must operate efficiently even if the transport functions of both organs are not fully matured.

Concentration of $Na^+$ and $K^+$ in breast milk of rat decreases during suckling, but the $Na^+/K^+$ ratio is relatively constant and reaches the value 0.4-0.6. The $Na^+$ intake is 4.1-10.6 μmol $Na^+$. (g body weight . d)$^{-1}$ in 4-day-old rats and 5 μmol $Na^+$. (g body weight . d)$^{-1}$ in 14-day-old animals (Gouldsborough and Ashton 1998). Using the published data on average body weight gain and the percentage of extracellular fluid (Jelínek 1961, Babický *et al*. 1970, Redman and Sweney 1976) we can calculate the minimal $Na^+$ intake necessary for the normal development of the suckling rats. If we assume that the extracellular fluid volume represents 36 % of body weight and the average weight gain is usually 2.3 g daily, this means that the suckling rats expand their extracellular fluid volume by 0.83 ml a day, i.e. by 125 μmol $Na^+$ daily. If we consider the body weights 9 and 32 g in 4- and 14-day-old pups, the minimal $Na^+$ intake is 13 and 4 μmol $Na^+$.(g body weight . d)$^{-1}$, respectively. If this reasoning is true, the sucklings are threatened by relative $Na^+$ deficiency and the pups have to activate effective $Na^+$ transport pathways to absorb the bulk of dietary $Na^+$ and prevent renal $Na^+$ loss. This is in full agreement with premature human neonates of a gestational age of less than 32 weeks. They demonstrate high renal and intestinal losses of $Na^+$, very high plasma aldosterone and high rectal $Na^+$ absorption (Verma *et al*. 1989, Jenkins *et al*. 1990).

The tendency of the newborns or young animals to retain $Na^+$ and $K^+$ depends on the potential of renal and intestinal epithelium to absorb or reabsorb these ions. It has been shown that immature kidney is able to retain inappropriate amounts of $Na^+$ and $K^+$ not only following chronic administration of large amounts of ions but also when a load of ions is administered acutely. It was postulated that one of the main factors responsible for the retention of ions is the increased $Na^+$ reabsorption and decreased $K^+$ secretion in distal nephrons (Aizman *et al*. 1998, Satlin and Schwartz 1992, Spitzer and Chevalier 1992). In addition, the *in situ* perfusion experiments demonstrated a decrease of intestinal $Na^+$ and $K^+$ absorption during postnatal development (Finkel *et al*. 1985, 1988, 1994, Meneely and Ghishan 1982, Younoszai 1979). $Na^+$ and $K^+$ are absorbed both in the small and large intestine, however, the role of these two segments in maintenance of ion homeostasis changes during development. In particular, colon seems to play a much more important role in early postnatal development than in adulthood (Wilkinson and McCance 1971, Bentley and Smith 1975, Aizman *et al*. 1998).

The survival of newborns and the maintenance of homeostasis require regulation of the intestinal mucosa operating as a functional unit. This unit is composed of several cell types from which only some have transport functions and others serve predominantly regulatory or immunological role. Studies of the adult intestine have revealed that the regulation of intestinal transport depends not only on the intrinsic properties of enterocytes but also on systemic and local hormones, growth factors, neurotransmitters and complex interactions between enteric nerves and immune cells in the submucosa. The transport capacity of the intestinal epithelium depends, therefore, on a number of synergic and antagonistic signals of genetic, nervous, humoral and nutritional origin. These signals and their role have been partly deciphered in matured intestine, however, much less attention has been paid to the signals that trigger and/or modulate the developmental changes of intestinal transport (Pácha 2000).

## $Na^+$ transport in immature and mature colon

$Na^+$ absorption in the colon of sucklings and weanlings is higher than in adulthood (Finkel *et al.* 1985, 1988, 1994, Meneely and Ghishan 1982, Younoszai

1979). The $Na^+$ pathway that plays the key role in $Na^+$ absorption in immature rat colon is the electrogenic $Na^+$ transport *via* amiloride-sensitive $Na^+$ channels (Fig. 1). The transport capacity of this pathway increases during the suckling period, reaches its maximum during weaning, disappears later and is replaced by electroneutral $Na^+/H^+$ exchange in the adult colon (Pácha *et al*. 1987b, 1995, Finkel *et al*. 1988, Binder and Sandle 1994). Consequently it is obvious that the electrogenic $Na^+$ transport represents the dominant absorptive mechanism of $Na^+$ absorption in immature colon whereas the electroneutral $Na^+$ transport is characteristic for fully matured colonic epithelium. The developmental profile of $Na^+/H^+$ and the mechanisms of the replacement of electrogenic $Na^+$ absorption by the electroneutral pathway are unknown.

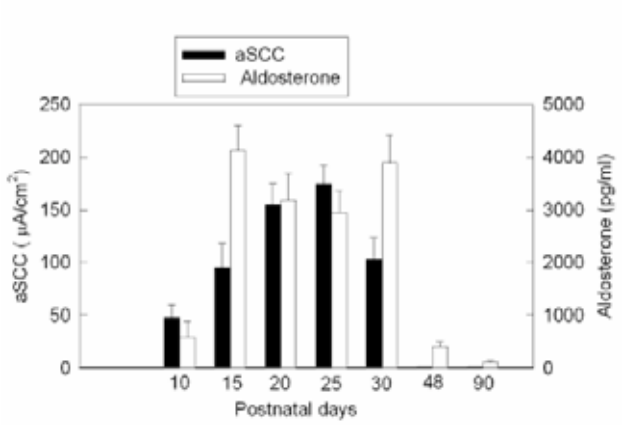


**Fig. 1.** Developmental profile of colonic electrogenic amiloride-sensitive $Na^+$ transport (aSCC) and plasma levels of aldosterone in rats fed a standard diet and housed with their dams until 30 days of life. According to Pácha et al. (1995).

In adult rats the electrogenic $Na^+$ pathway can only be induced by high pharmacological doses of corticosteroids (Pácha *et al*. 1987a) or by dramatic decrease of dietary $Na^+$ intake that is not adequate to the omnivorous character of rat (Pácha and Pohlová 1995**)**. In association with these changes in the distal colon the rat proximal colon increases electroneutral $Na^+$ transport (Vaněčková *et al*. 2001). The electrogenic $Na^+$ transport is induced only if dietary $Na^+$ intake decreases below 100 µmol $Na^+$. (100g body weight . d)$^{-1}$. If we consider that the $Na^+$ requirement of the rat for growth and reproduction is 0.5 g/kg diet (Warner and Breuer 1978) and the daily feed consumption corresponds to approximately 5 % of its body mass it can be calculated that the basal dietary $Na^+$ intake is approximately 100 µmol $Na^+$. (100g body weight . d)$^{-1}$ (Pácha and Pohlová 1995, Pácha *et al*. 1995**)**. It means that the electrogenic $Na^+$ transport is activated only if the animals are threatened by a relative $Na^+$ deficiency. This transport pathway is induced by aldosterone the plasma concentration of which increases with decreasing $Na^+$ intake (Fig. 2). The threshold plasma concentration of aldosterone for the induction of electrogenic $Na^+$ transport is 250-300 pg/ml in adult rats (Pácha *et al*. 1995).

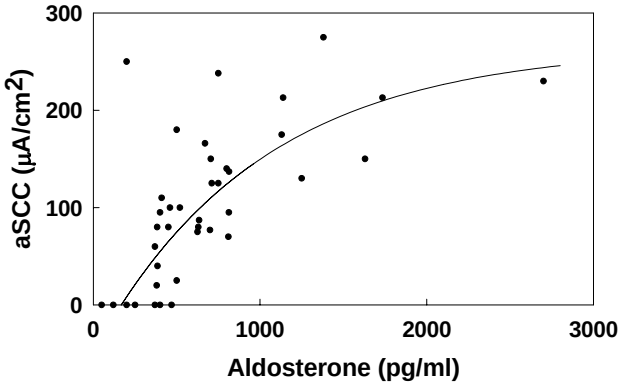


**Fig. 2.** Relationship between colonic electrogenic amiloride-sensitive $Na^+$ transport (aSCC) and plasma aldosterone level in adult rats with different dietary $Na^+$ intake. The curve was plotted by nonlinear regression analysis according to the equation $Y=A*(1-e^{-b(X-c)})$, where $A$ represents maximum transport capacity (265 µA/cm$^2$) and $c$ threshold plasma level of aldosterone for the induction of electrogenic $Na^+$ transport (170 pg/ml). According to Pácha *et al.* (1995).

The findings that electrogenic $Na^+$ transport is induced if dietary $Na^+$ intake decreases below 100 µmol $Na^+$. (100g body weight . d)$^{-1}$ and plasma concentration of aldosterone is higher than 300 pg/ml indicate that this transport system does not have any physiological meaning in most mammals with the exception of herbivorous animals. In adult animals the dietary $Na^+$ intake is higher and plasma aldosterone level lower that the threshold values. However, the above mentioned experiments demonstrate unequivocally the physiological importance of electrogenic $Na^+$ pathway in the early postnatal ontogenesis of rats. Similarly, this pathway was described in the distal colon of human newborns (Jenkins *et al*. 1990) even though electroneutral $Na^+$ transport was found in adulthood (Wills *et al*. 1984, Sandle *et al*. 1986, Sellin and Desoignie 1987). The herbivorous animals are threatened by $Na^+$ deficiency throughout the whole life and therefore the electrogenic $Na^+$ transport operates in their gut both in immature and mature large intestine (O'Loughlin *et al*. 1990, Binder and Sandle 1994).

## Regulation of electrogenic $Na^+$ transport in immature distal colon by corticosteroids

The developmental changes of electrogenic $Na^+$ transport correlate well with the increased plasma concentration of aldosterone in both animals (O'Loughlin *et al*. 1990, Pácha *et al*. 1995) and humans (Jenkins *et al.* 1990). Despite the generally accepted hypothesis that aldosterone regulates electrogenic $Na^+$ transport, some evidence has raised the question whether other factors might control this pathway (Fig. 3). First, the effect of adrenalectomy to inhibit colonic electrogenic $Na^+$ pathway is weaker in immature intestine (Pácha *et al*. 1987b). Second, premature weaning and hypothyroidism are accompanied by inhibition of this pathway without adequate decrease of plasma concentration of aldosterone (Pácha *et al*. 1995, 1996). Third, the dose of aldosterone that is able to restore electrogenic $Na^+$ transport in immature colon is smaller than the dose that is able to induce it in adult colon (Pácha *et al*. 1988).

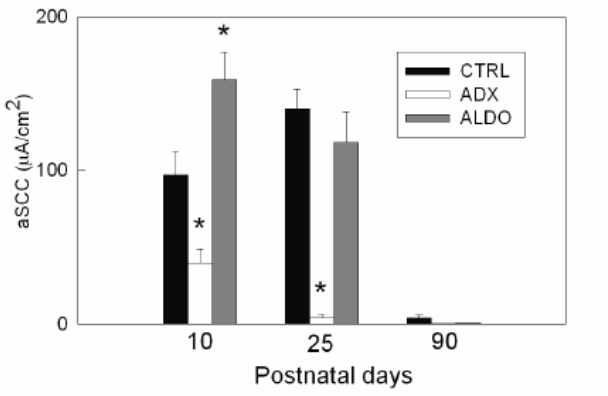


**Fig. 3.** Effect of adrenalectony (ADX) and aldosterone treatment (ALDO; 1.25 μg . (kg body weight . 4 h)$^{-1}$ for 24 h) to adrenalectomized rats on colonic amiloride-sensitive electrogenic $Na^+$ transport (aSCC) during postnatal development. *Significantly different from control rats.

These data indicate that in addition to aldosterone there are other factors that have permissive effect on the regulation of electrogenic $Na^+$ transport by mineralocorticoids. For example, aldosterone has been shown to increase the abundance of β- and γ-subunit but not the α-subunit of epithelial $Na^+$ channels (Lingueglia *et al*. 1994, Renard *et al*. 1995) in mature rat colon, whereas in immature colon the abundance of all three subunits increases during early postnatal development (Watanabe *et al*. 1998).

As the developmental profile of aldosterone is very similar to corticosterone or thyroid hormones (Henning 1978, Pácha *et al*. 1995, 1996) and the developing colon has an effective system of peripheral metabolism of glucocorticoids (Pácha and Mikšík 1996), we studied the role of thyroid hormones (Pácha *et al*. 1996). The inhibition of the developmental increase of thyroxine and triiodothyronine in hypothyroid pups was followed by the suppression of electrogenic $Na^+$ transport, while aldosterone concentrations remained elevated. Replacement therapy of hypothyroid pups with triiodothyronine restored this transport pathway, i.e. the regulatory role of aldosterone required the permissive effect of thyroid hormones. The mechanism of interaction between aldosterone and thyroid hormones is unknown. Nevertheless, this interaction cannot be localized at the level of the sodium pump of the basolateral membrane but it might occur at the level of apical epithelial $Na^+$ channels (Clauss *et al*. 1993, Pácha *et al*. 1996, Mrnka and Pácha 2000).

Although the receptors of the corticosteroid and thyroid hormones belong to the same superfamily of transcriptional factors, the interaction between aldosterone and thyroid hormones is localized beyond the transcriptional step of $Na^+$ channel regulation (Mrnka and Pácha 2000). Both aldosterone and thyroid hormones exert a pleiotropic effect so that numerous genes are under their positive or negative control and some of them might concern proteins involved in the regulation of $Na^+$ channels. Recent studies have demonstrated that aldosterone can stimulate the expression and posttranslational targeting of the $Na^+$ channel regulatory G-proteins (Rokaw *et al*. 1996) and that G-protein expression is controlled by thyroid hormones in various tissues (Michel-Reher *et al.* 1993). Moreover, Komwatana *et al.* (1996) suggested that the G-protein α-subunit plays a role in the feedback regulation of electrogenic $Na^+$ transport. In addition, several lines of evidence suggest an association between $Na^+$ channels and the cytoskeletal actin (Cantiello *et al*. 1991, Berdiev *et al*. 1996) whose polymerization state is regulated by thyroid hormones (Farwell and Leonard 1992, Safran *et al*. 1993). Further studies will be necessary to ascertain the mechanism of post-transcriptional interaction of aldosterone and thyroid hormones.

The quantity of the $Na^+$ flux across the apical cell membrane (apical $Na^+$ permeability) is determined not only by the quantity of open $Na^+$ channels but also by the channel activity. This has been suggested by several studies, unfortunately all of them were done with non-intestinal epithelia (Garty and Palmer 1997). In cortical

collecting tubules that have similar transport properties to distal colon aldosterone increases the number of open apical $Na^+$ channels, i.e. increases primarily the density of channels at a constant open probability (Pácha *et al*. 1993).

## $Na^+,K^+$-ATPase and development of electrogenic $Na^+$ transport

Sodium ions enter the colonocyte across the apical membrane *via* passive transport pathways and are extruded across the basolateral membrane by sodium pump ($Na^+,K^+$-ATPase). $Na^+$ transport and the activity of $Na^+,K^+$-ATPase is roughly proportional in the epithelia inclusive adult colon (Rossier and Palmer 1992). In contrast, developmental studies have demonstrated a temporal dissociation between the developmental patterns of $Na^+$ transport and $Na^+,K^+$-ATPase activity (Finkel *et al*. 1985, Pácha *et al*. 1987b, 1991). $Na^+$ absorption decreases during postnatal development (Younoszai 1979, Finkel *et al*. 1985, 1988) together with an increase of the activity of basolateral $Na^+,K^+$-ATPase and mRNA expression for α- and β-subunits of this protein (Fuller and Verity 1990, Pácha *et al*. 1991, Zemanová and Pácha 1998). Although the activity of $Na^+,K^+$-ATPase [measured as the hydrolysis of ATP under maximum velocity ($V_{max}$) conditions] and the number of pump molecules increase during development, the maximum pumping activity of $Na^+,K^+$-ATPase remains constant (Pácha *et al*. 1991). There are two possibilities to explain this discrepancy. First, there is a considerable latent pool of individual pumps that increases during development. Such a pool of sodium pump molecules was demonstrated convincingly by Barlet-Bas *et al*. (1990) in renal collecting duct. Second, the turnover rate *per* a single pump molecule is higher during suckling and weaning period than in adulthood. In rat colon, the $Na^+$ turnover rate *per* a single sodium pump was found to be approximately ten times higher in immature than in mature colon (Pácha *et al*. 1991). Similar differences in sodium pump turnover rate were found in the rabbit colon that was or was not exposed to aldosterone (Roden and Turnheim 1988). It is therefore possible that the higher pump turnover rate in immature colon reflects the action of aldosterone. Is not clear in which way aldosterone influences the pump. One possibility might be that aldosterone modulates the composition of fatty acids in membrane phospholipids (Mrnka *et al*. 2000a,b, Jindřichová *et al*. 2003) as was shown for various membrane-bound transporters.

Detailed histochemical analysis of $Na^+,K^+$-ATPase activity (ouabain-sensitive $K^+$-dependent *p*-nitrophenylphosphatase activity) proved that the developmental increase of the enzyme is regulated by adrenal hormones, particularly by aldosterone (Zemanová and Pácha 1996, 1998). Adrenalectomy or high dietary salt intake decreased colonic $Na^+,K^+$-ATPase activity both in suckling and weaning period. This activity was restored by a mineralocorticoid, deoxycorticosterone acetate (DOCA), whose effect was blocked by the mineralocorticoid antagonist, spironolactone. Both adrenalectomy and DOCA modulated enzyme activity along the whole colonic crypt axis, i.e. not only in the surface colonocytes that are considered to be the place of the electrogenic $Na^+$ transport and expression of epithelial $Na^+$ channels (Lomax *et al*. 1994, Renard *et al*. 1995).

The hypothesis that the sodium pump that is expressed on the surface colonocytes represents the pump that participates in the $Na^+$ absorption also in the immature colon is supported by two findings. First, the decrease of dietary salt intake or administration of corticosteroids to adrenalectomized pups stimulates $Na^+,K^+$-ATPase activity predominantly in the upper part of the colonic crypts (Zemanová and Pácha 1998). Second, the increased salt intake during weaning prevents the developmental increase of basolateral membrane surface density (Vagnerová *et al*. 1997). In adult rats the increase of basolateral membranes has been found in colonocytes of rats with hyperaldosteronism (Kashgarian *et al*. 1980).

## $K^+$ transport in immature and mature colon

In mammals the colon is an organ that plays an important role not only in $Na^+$ but also in $K^+$ homeostasis. Colon is a place of significant bi-directional transport of $K^+$, its absorption and secretion driven by $K^+$-dependent adenosine triphosphatases (Kunzelmann and Mall 2002). The perfusion studies performed in situ showed that the net $K^+$ absorption is increased in immature colon (Aizman *et al*. 1996) and the renal excretion following intragastric KCl load is significantly lower in weanlings than in adult animals (Lorenz *et al*. 1986). These data indicate that the regulation of $K^+$ homeostasis during infancy is, owing to growth and development, different from later life.

In agreement with the well-known observations that in renal collecting ducts aldosterone stimulates not only the electrogenic $Na^+$ reabsorption but also $K^+$ secretion (Rossier and Palmer 1992), we found an increased $K^+$ secretion in immature colon that diminished during postnatal life (Fig. 4). Using various blockers of $K^+$ transporting proteins we were able to characterize the mechanism. This secretory pathway is electrogenic and appears to be mediated by $Na^+$,$K^+$-ATPase as well as by furosemide-sensitive $Na^+/2Cl^+/K^+$ cotransport on the basolateral side and by $Ba^{2+}$-sensitive $K^+$ channels on the mucosal side. The capacity of $K^+$ secretion correlates positively with the capacity of the electrogenic $Na^+$ transport. Nevertheless, the developmental changes of the driving force secondary to $Na^+$ absorption that result in the depolarization of apical membrane are not essential for the increased $K^+$ secretion in immature colon (Pácha *et al*. 1987c). Similar independence of electrogenic $Na^+$ absorption and $K^+$ secretion was also found in the guinea pig colon, i.e. in a herbivorous species with active colonic electrogenic $Na^+$ transport throughout the whole life (Rechkemmer and Halm 1989).

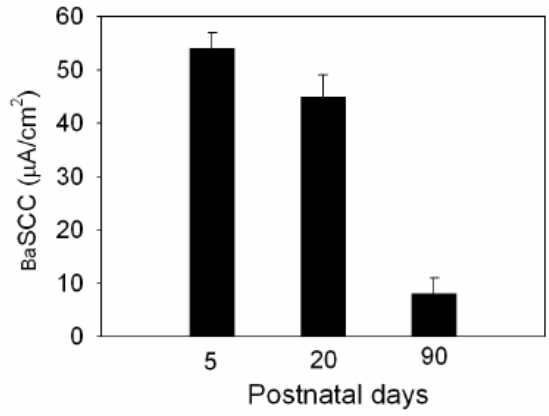


**Fig. 4.** Developmental pattern of electrogenic barium-sensitive $K^+$ secretion (BaSCC) in rat colon.

The regulatory factor that is responsible for the stimulated $K^+$ secretion in early postnatal life is aldosterone because the decreased dietary $Na^+$ intake increases, whereas adrenalectomy or increased dietary $Na^+$ intake diminishes $K^+$ secretion in weanling animals. In addition, the administration of aldosterone to adrenalectomized pups restores $K^+$ secretion (Pácha *et al*. 1987c, 1988).

The finding of increased $K^+$ absorption in immature colon indicates that the "secretory" serosa-to-mucosa $K^+$ flux is lower than the opposite "absorptive" flux from the mucosal to the serosal side (Aizman *et al*. 1996). This mucosa-to-serosa $K^+$ flux is mediated by two $K^+$-ATPases ($H^+$,$K^+$-ATPases), ouabain-sensitive and ouabain-insensitive pumps, that are independent of $Na^+$ (Kunzelmann and Mall 2002). Both of these ATPases are expressed in colonocytes and the ratio of their transport capacity in adulthood is 1.0:1.3 (Vaněčková *et al*. 2001). Similar ratio of both forms was ascertained by Aizman *et al*. (1996) using a different approach. Nevertheless, it is unknown whether this ratio is changing during early postnatal development.

In agreement with an increased transport of $Na^+$ and $K^+$ across the apical and basolateral membrane the colonocytes of immature colon have increased $Ba^{2+}$- and tetraethylammonium(TEA)-sensitive $K^+$ conductance. The $K^+$ conductance progressively decreases during ontogeny and this reduction is associated with a diminished expression of $Ba^{2+}$- and TEA-sensitive conductance in mature crypts (Beskid and Pácha 2003).

## Metabolism of glucocorticoids in developing intestine

Studies of the $Na^+$ and $K^+$ transport in the immature intestine have revealed that the developmental changes of this transport depend on aldosterone. However, its plasma concentration is lower by several orders of magnitude than that of corticosterone (Henning 1978, Pácha *et al*. 1995) and the affinity of aldosterone and corticosterone to mineralocorticoid receptor is very similar (Schulman *et al*. 1986). Considering the higher glucocorticoid concentration and promiscuity of mineralocorticoid receptors, corticosterone could mimic aldosterone regulation in the mineralocorticoid target tissue. The exclusive occupancy of mineralocorticoid receptors by aldosterone under the conditions of much higher circulating levels of corticosterone is attained by the enzyme 11β-hydroxysteroid dehydrogenase (11βHSD). This enzyme converts glucocorticoids (cortisol, corticosterone) to biologically less active 11-oxoderivatives (cortisone, 11-dehydrocorticosterone) that have low affinity to glucocorticoid and mineralocorticoid receptors (Stewart and Krozowski 1999).

We have shown that the activity of 11βHSD is expressed in the intestine but is not distributed homogeneously in the intestinal segments and shows a sexual dimorphism (Pácha and Mikšík 1994, Vylitová *et al*. 1998, Mazancová *et al*. 2003). The activity of 11βHSD is high in distal colon, medium in caecum and proximal colon, low in ileum and absent in jejunum and

duodenum, i.e. the distribution of 11βHSD correlates with the distribution of gut mineralocorticoid sensitivity. Also the developmental profile of 11βHSD is not identical in all intestinal segments (Pácha and Mikšík 1996, Pohlová *et al*. 1997). 11βHSD activity is high in the caecum and colon at birth and alters little until adulthood. In contrast, the activity in the ileum is low during the first two weeks of life and rises in the next 20 days to reach the value of adult animals. Very low activity in the ileum is limited to the period when glucocorticoids are able to modulate maturation of the small intestine.

The difference between the developmental profile of 11βHSD in the small and large intestine may facilitate the effect of glucocorticoids in the small intestine during maturation and the colonic regulation of the $Na^+$ transport by aldosterone. Several data support this hypothesis. Treatment of pups with carbenoxolone, an inhibitor of 11βHSD, increased colonic but not ileal $Na^+$,$K^+$-ATPase (Pohlová *et al*. 1997). The explanation of this discrepancy concerns carbenoxolone-inhibited colonic 11βHSD and the impaired corticosterone metabolism resulting in glucocorticoid induction of $Na^+$,$K^+$-ATPase *via* mineralocorticoid receptors (Sheppard 1998, Whorwood *et al*. 1994, Tsuganezawa *et al*. 1995). Similarly, the inhibition of corticosterone degradation by carbenoxolone increased plasma corticosterone concentration that resulted in a stronger stimulation of glucocorticoid receptors, intestinal growth and maturation of villus height and crypt depth (Pácha *et al*. 2003).

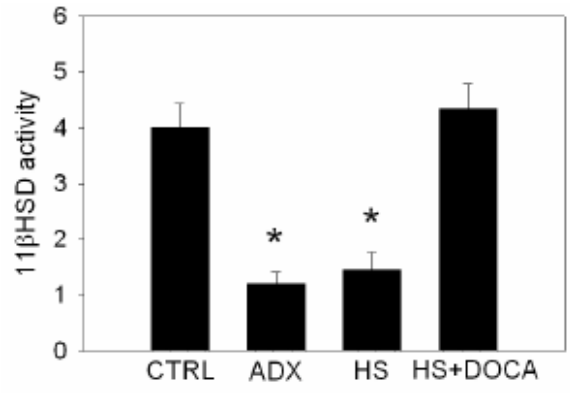


**Fig. 5.** Effect of adrenalectomy (ADX) and high dietary $Na^+$ intake (HS) without or with deoxycorticosterone acetate treatment (500 μg . (100 g body weight . day)$^{-1}$ from day 20 to day 25) on activity of 11β-hydroxysteroid dehydrogenase (11βHSD) in weanling rats. The activity of 11βHSD is expressed as the conversion of corticosterone to 11-dehydrocorticosterone per hour and gram of dry weight. *Significantly different from control sham-operated rats (CTRL).

11βHSD is regulated by several signals including corticosteroids (Fig. 5). Adrenalectomy and high salt diet decrease 11βHSD activity and their effect can be prevented by administration of dexamethasone or DOCA (Pácha and Mikšík 1996). This effect of corticosteroids represents a way of direct influencing of the intestinal tissue (Pácha *et al*. 1997). The second important factor in 11βHSD regulation is cytodifferentiation that upregulates this enzyme (Pácha *et al*. 2002).

## Separation methods for analysis of steroid metabolism

The qualitative and quantitative determination of steroids, including corticosteroids is not trivial. Their structures are closely similar and they contain several functional groups which make their separation difficult. As mentioned in previous chapter steroids can be metabolized not only in peripheral but also in target tissues to gain or to lose the biological activity. For example, 5α-reduction of androgens is an important step in the formation of biologically active androgens, or extraovarial aromatization of androgens to estrogens by aromatase in fat tissue is an important source of estrogens in post-menopausal women. Similarly, the reduction of the keto group at $C_{20}$ and oxidation of the hydroxy group at $C_{11}$ seem to play an important role in local regulation of progesterone, cortisol and corticosterone. The reduction of 11-oxo steroids cortisone and 11-dehydro-corticosterone may increase *vice versa* the local concentration of biologically active steroid in the target tissue *via* autocrinic or paracrinic effect.

The major metabolic pathways for inactivation of steroids is reduction of the A-ring giving tetrahydro derivatives and reduction at $C_{17}$ or $C_{20}$ keto group or oxidation at $C_{11}$ as mentioned above. Most of these reactions give rise to epimeric products. It means that for analyzing the impact of steroids on the organism, their metabolism and further conversion there is an urgent need of good analytical methods capable to determine the individual compounds of interest. These methods should be not only sensitive but also selective. This means that one needs not only to determine low concentrations of steroids in biological samples (which by itself could be a considerable problem), but also to separate (i) individual steroids of interest one from the other and (ii) to separate these steroids from other accompanying compounds of different chemical nature (Makin *et al*. 1995, Shimada *et al*. 2001, Shu *et al*. 2003).

Sample preparation is the first step in the analytical procedure. In the case of steroid analysis in biological samples (fluids or tissues) the traditional method originally was liquid-liquid extraction (for details see Makin *et al*. 1995). In the present day solid-phase extraction (SPE) has become the most common. This method is relatively simple and really powerful. In principle this approach is applicable to liquid samples; consequently the first step is homogenization of the sample (tissue etc.) in a suitable buffer followed by centrifugation. The sample is then applied to a cartridge packed by reversed-phase material (C18). In our works we used the Sep-Pak cartridges (from Waters; Milford, MA, USA) (Pohlová *et al*. 1997). After sample application the polar compounds were eluted by water; in the next step steroids were eluted by pure methanol. Then the cartridges were conditioned by water to be re-used with another sample. Collected methanol fractions were evaporated under nitrogen and then reconstituted in a suitable volume of methanol for injection to the analytical (HPLC) system.

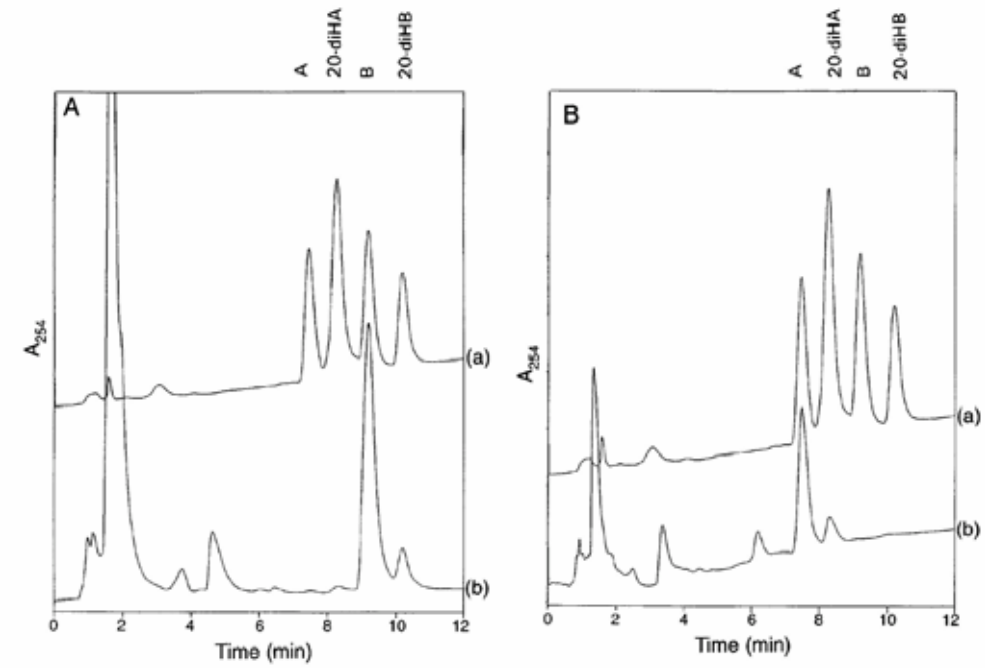


**Fig. 6.** HPLC chromatograms with UV detection applied for the analysis of corticosterone metabolite in hen jejunum. (**A**) Metabolism of corticosterone: (**a**) standards of steroids; (**b**) jejunum incubated with corticosterone (1.45 μM). (**B**) Metabolism of 11-dehydrocorticosterone: (**a**) standards of steroids; (**b**) jejunum incubated with 11-dehydrocorticosterone (1.45 μM). Conditions: Lichrospher 100 RP-18 (125 x 4 mm) column (Merck, Darmstadt, Germany) and eluted with a linear methanol-water gradient from 45:55 (v/v) to 65:35 (v/v) over 15 min followed by isocratic washing with 100 % methanol for another 10 min. The flow rate was 1.0 ml/min and the column temperature was held at 45 °C. Ultraviolet absorbance at 254 nm. Identification: corticosterone (B), 11-dehydrocorticosterone (A), 20β-dihydrocorticosterone (20-diHB), and 11-dehydro-20-dihydrocorticosterone (20-diHA) (Vylitová *et al*. 1998). Originally published by Elsevier.

## HPLC methods

*UV detection*

UV absorbance of steroids depends on their structure. Good UV absorbing properties possess steroids containing conjugated dienes and trienes, unsaturated ketones or aromatic chromophores. Corticosterone, cortisol or aldosterone represent typical examples of compounds with good spectral properties.

The very separation method is simple; it is based on reversed-phase chromatography and elution with a water-methanol gradient. An example of this method is shown in Figure 6. We used this analytical approach for studies of corticosterone metabolism in mammalian and avian intestinal tissue and found as the main metabolic products 11-dehydrocorticosterone in mammalian (rat, guinea pig) and 20-dihydrocorticosterone and 11-dehydro-20-dihydrocorticosterone in the avian intestine (Pácha and Mikšík 1994, 1996, Pohlová *et al*. 1997, Vylitová *et al*. 1998). These data indicate that the metabolism of glucocorticoids in peripheral tissue is not identical in all vertebrates and that not only

11β-hydroxysteroid but also 20β-hydroxysteroid dehydrogenase (20HSD) operates in the avian tissue. Our unpublished data show that 20HSD might also be involved in prereceptor protection of mineralocorticoid receptors against glucocorticoid excess in chicken mineralocorticoid target tissues.

Naturally, in addition to our method, it is possible to derivatize these steroids (both in the pre- or post-column mode) exploiting thus the non-specific UV absorption of the arising derivatives and improve their UV or fluorescence detectability (Blau and Halket 1993, Makin *et al*. 1995); hydrazones (for oxo compounds) or post-column detection with sulphuric acid (Sudo 1990, Blau and Halket 1993, Makin *et al*. 1995) represent typical examples.

*Radioactivity detection*

To obtain detailed information about the origin of the arising steroid compounds we use the substrates labeled with $^3$H. This approach gives us the possibility to trace the fate of the substrate in the target tissue. Everything what we will see by the radioactivity detector stems from our labeled compounds of interest. Another feature of this approach is a high sensitivity that allows to analyze low concentrations of compounds. When using the radioactivity detector we have in principle two possibilities for detection. We can use the solid cell or the flow cell. In the case of using solid, heterogeneous, cell (packed with fine grains of solid scintillating material), the price of the analysis is relatively low. A considerable disadvantage of this method is its low sensitivity (efficiency is around 7–10 %). In the method exploiting flow, homogeneous, cell the sensitivity is considerable increased (efficiency is above 50 %). On the other hand this latter approach is more expensive because it consumes the scintillation cocktail (typically the flow-rate is 3-times higher than the mobile-phase flow). An application of this method for the detection of products arising from conversion of corticosterone in chicken intestine is shown in Figure 7. It can be seen that corticosterone (20 nM) at concentration similar to $K_m$ value for 11βHSD type 2 (isoform that prevents mineralocorticoid receptors against glucocorticoids) was converted into three metabolites 11-dehydro-20-dihydro-corticosterone > 11-dehydrocorticosterone > 20-dihydro-corticosterone. In this experiment corticosterone metabolism showed a different pattern than in experiments using corticosterone concentration (1.45 μM) that was close to the $K_m$ value for 20HSD (Fig. 6). The question is what is the physiological role of 20HSD in chicken intestine. One of the explanations is that 20HSD protects the mineralocorticoid target tissue not only against corticosterone but also against progesterone (lacking hydroxyl group at $C_{11}$). Considering that plasma concentration of progesterone is much higher that that of aldosterone during egg laying period, progesterone secretion is not restrained by increased dietary $Na^+$ intake and corticosteroid receptors show relatively large affinity to various steroids including progesterone, this conclusion seems to be plausible.

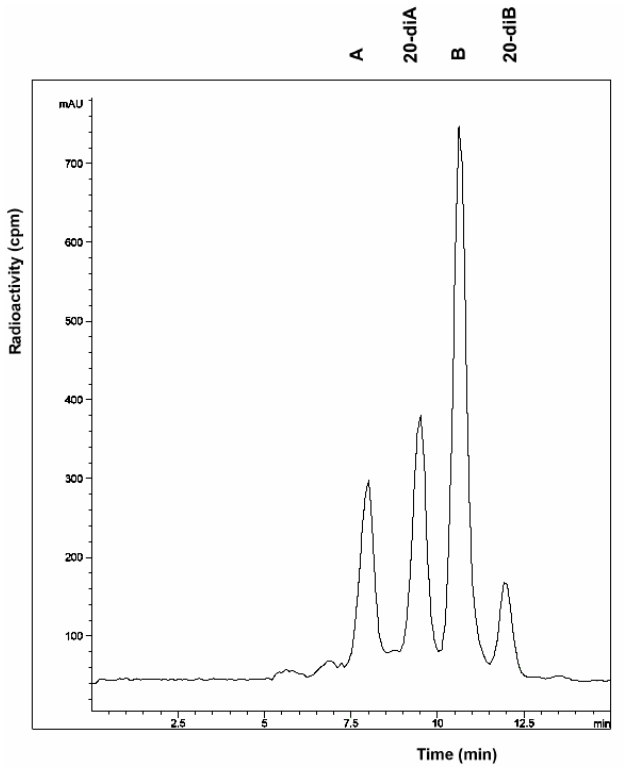


**Fig. 7.** HPLC chromatograms with radioactive detection applied for the analysis of corticosterone metabolite. Conditions: Lichrospher 100 RP-18 (125 x 4 mm) column (Merck, Darmstadt, Germany) and eluted using a linear methanol-water gradient from 42:58 (v/v) to 62:38 (v/v) at 15 min followed by isocratic washing with 100 % methanol for another 10 min. The flow rate was 1.0 ml/min and the column temperature was held at 45 °C. Radioactivity detector (Radiomatic 150TR; Canberra Packard) with a flow cell (flow rate of scintillation cocktail/mobile phase was 3:1 from 5 to 15 minutes). Identification: corticosterone (B), 11-dehydrocorticosterone (A), 20β-dihydrocorticosterone (20-diHB), and 11-dehydro-20-dihydrocorticosterone (20-diHA).

*HPLC/Mass Spectrometry*

Recently the HPLC/MS methodology gained popularity and became a relatively frequently used approach for steroid analysis. It can be exploited both for the analysis of free steroids and conjugates (sulphates or glucuronides) (Gaskell *et al.* 1987, Park *et al*. 1990, Steffenrud and Maylin 1992, Barrón *et al*. 1996, Bean and Henion 1997, Shibasaki *et al*. 1997, Fiori *et al*. 1998,

Polettini *et al*. 1998, Marwah *et al*. 2001). Regarding corticosterone, HPLC-thermospray mass spectrometry (Park *et al*. 1990, Steffenrud and Maylin 1992, Shibasaki *et al*. 1997), HPLC-atmospheric pressure chemical ionization mass spectrometry (Fiori *et al*. 1998) or HPLC-electrospray ionization (ESI) mass spectrometry (Marwah *et al*. 2001) methods can be found in the literature.

We developed two HPLC/MS methods (Mikšík *et al*. 1999, 2004) that are capable to separate overall 23 steroids that could participate in the corticosterone metabolism (Figs 8 and 9). These methods are relatively simple and exploit the separation by reversed-phase chromatography with the acidified water-methanol (1 % acetic acid) gradient. This approach allows to separate and to quantitate a much wider scale of compounds, not only those which posses suitable UV properties, as mentioned above (Fig. 8). The application of extracted ions method (that are specific for individual steroids) allows to detect (and to quantitate) all steroids, even those not separated by chromatography (Fig. 9). This analytical approach helped us with identification of various steroid products particularly with identification of 3α/3β and 20α/20β epimers. The practical applicability of the developed methods was proven by monitoring the activity 20β-hydroxysteroid dehydrogenase and 11β-hydroxysteroid dehydrogenase in avian intestine (Mikšík *et al*. 1999), 20β-hydroxysteroid dehydrogenase in chicken intestine (conversion of corticosterone and progesterone) and chicken oviduct and also the activity of 3-hydroxysteroid dehydrogenase in chicken oviduct (Mikšík *et al*. 2004). Using this approach we were able to characterize intestinal 20HSD as 20β-hydroxysteroid dehydrogenase (unpublished data). This method was further used for the characterization of an so far unknown metabolic product of corticosterone in colonic epithelial cell line Caco-2 (Pácha *et al*. 2002).

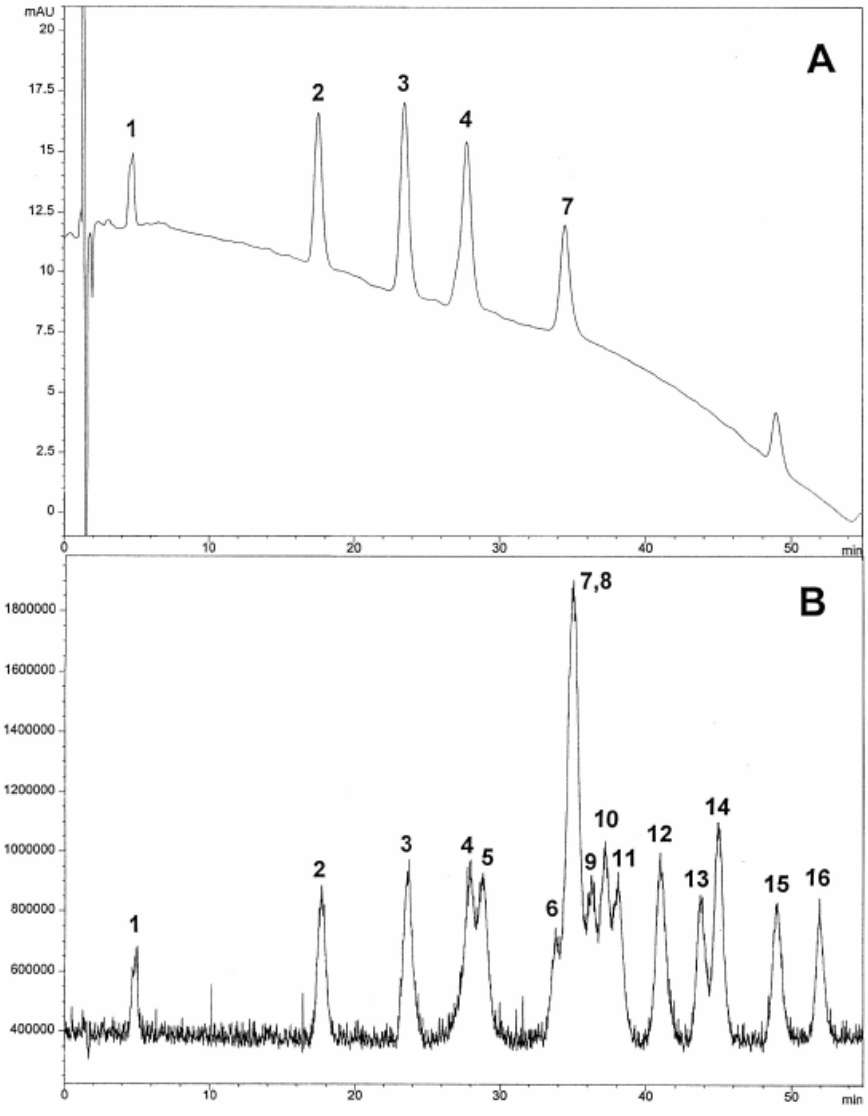


**Fig. 8.** HPLC separation of a standard mixture of corticosteroid metabolites monitored by (**A**) UV absorbance detection at 254 nm and (**B**) mass detection (TIC 200-500 *m/z*). Chromatographic conditions: Zorbax Eclipse XDB-C18 column (150x2.1 mm I.D., 5 µm, Rockland Technologies [Hewlett-Packard]). Elution was achieved by a linear gradient with a mobile phase A (methanol-water-acetic acid 40:60:1, v/v/v) and B (methanol-water-acetic acid 60:40:1, v/v/v). Gradient started from 10 % B to 40 % B at 30 min, followed by a 20 min gradient to 50 % B; then the column was eluted with 100 % B for 5 min. Equilibration before the next run was achieved by 10 min washing with buffer **A.** Flow-rate was 0.25 ml/min, column temperature was held at 25 °C. Mass detector LC/MSD (Hewlett-Packard, Palo Alto, CA, USA) with atmospheric pressure ionization-electrospray ionization (API-ESI), positive mode. Operating conditions: drying gas ($N_2$), 6 l/min; drying gas temperature, 350°C; nebulizator pressure, 20 psi (138 kPa); capillary voltage, 4 500 V; ions were observed at mass range *m/z* 200-500; fragmentor was set at 80 V. Reconstructed ion chromatogram for selected ion was set considering the whole mass range of the considered ion, e.g. for *m/z* 315, the mass range *m/z* is 315–316. Injection: 59 ng of each steroid. For peak identifications see Fig. 9 (Mikšík *et al*. 1999). Originally published by Elsevier.

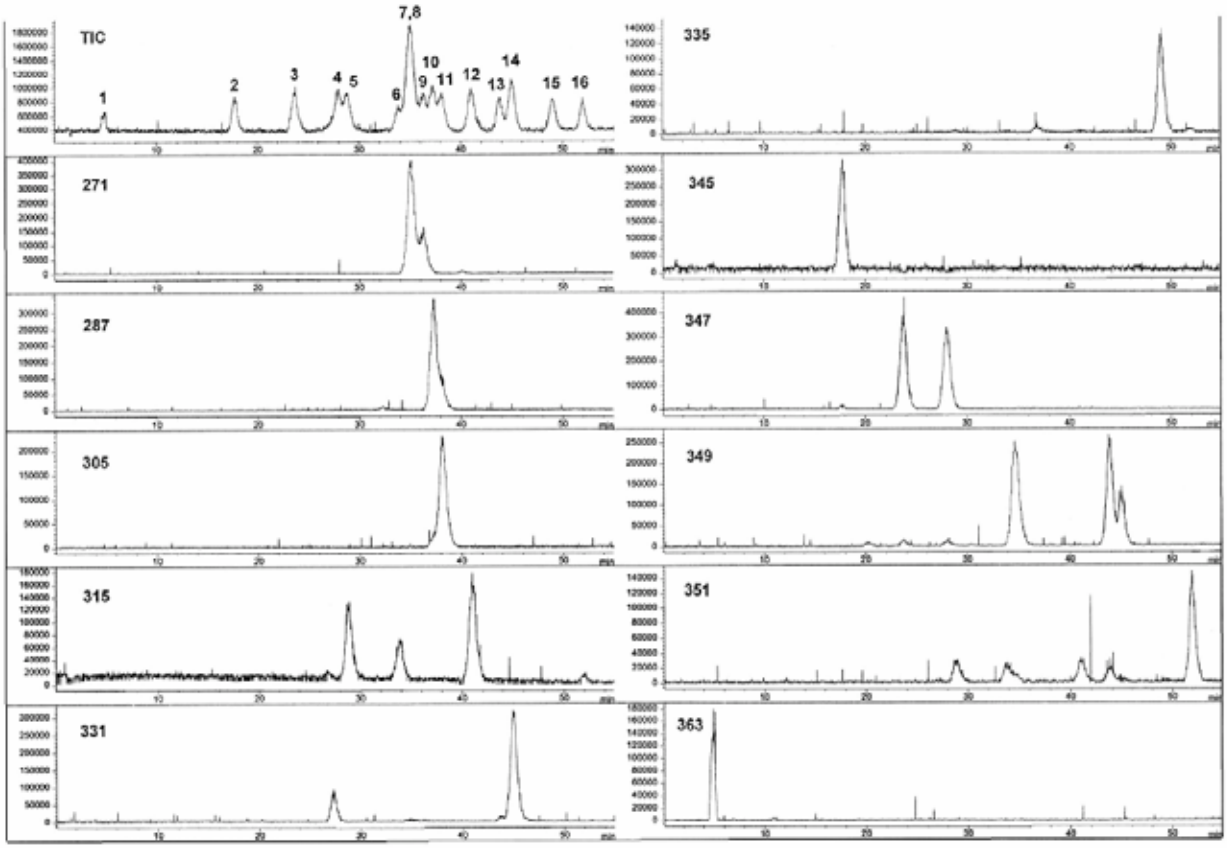


**Fig. 9.** The same separation of steroids as in Fig. 8, monitored by extracted ions. Specification of individual ions (*m/z*): **271**, 5β-androstan-3α,11β-diol-17-one (**8**) and 5α-androstan-3α,11β-diol-17-one (**9**); **287**, 5β-androstan-3α-ol-11,17-dione (**10**); **305**, 5α-androstan-3α-ol-11,17-dione (**11**); **315**, 5β-pregnan-3β,11β,21-triol-20-one (**5**), 5α-pregnan-3β,11β,21-triol-20-one (**6**) and 5α-pregnan-3α,11β,21-triol-20-one (allotetrahydrocorticosterone) (**12**); **331**, 5β-pregnan-3α,21-diol-11,20-dione (tetrahydro-11-dehydrocorticosterone) (**14**); **335**, 5β-pregnan-3α,11β,20β,21-tetrol (**15**); **345**, pregn-4-ene-21-ol-3,11,20-trione (11-dehydrocorticosterone) (**2**); **347**, pregn-4-ene-20β,21-diol-3,11-dione (**3**), pregn-4-ene-11β,21-diol-3,20-dione (corticosterone) (**4**); **349**, pregn-4-ene-11β,20β,21-triol-3-one (20β-dihydrocorticosterone) (**7**) and 5α-pregnan-3α,21-diol-11,20-dione (**13**); **351**, 5β-pregnan-3α,20β,21-triol-11-one (**16**); **363**, pregn-4-ene-6β,11β,21-triol-3,20-dione (6β–hydroxycorticosterone) (**1**) (Mikšík *et al.* 1999). Originally published by Elsevier.

## Capillary electrophoretic methods

Capillary electrophoresis is another method for the separation of steroids. Various modes as micellar electrokinetic chromatography (MEKC) or micro-emulsion electrokinetic chromatography (MEEKC) (Vomastová *et al.* 1996, Wiedmer *et al.* 1998, Wu *et al.* 2003) can be used for this purpose.

To confirm further the products of corticosteroid metabolism in the intestine identified originally by HPLC methods we developed capillary electrophoretic method (MEKC) with diode-array detection for the determination of steroids (Vylitová *et al.* 1998). The comparison of individual modes of capillary electrophoresis (MEKC, MEEKC) is presented in Figure 10. The best results were obtained with microemulsion electrokinetic chromatography in which higher aliphatic alcohols were used as microemulsion-forming modifiers. The system consisted from n-hexanol (0.81 %), SDS (3.31 %), n-butanol (6.61 %) in 20 mM phosphate buffer pH 10.0 (89.28 %, w/w) (Vomastová *et al.* 1996). Using this approach we were able to detect 20-dihydro and 11-dehydro derivatives of corticosterone in biological material (Fig. 11) obtained from chicken intestine (Vylitová *et al.* 1998). In combination of HPLC with UV and radioactivity detection the results obtained by this method were successfully applied for the confirmation of the presence of 20βHSD in avian intestine.

Capillary electrochromatography represents another possibility for steroid analysis. Two operational modes can be used, namely separation in columns (capillaries) packed with sol-gel bonded ODS/SCX (Tang and Lee 2000) or on functionalized macroporous polyacrylamide gels (Fujimoto *et al.* 1996, Que *et al.* 2000) (for review see Deyl and Mikšík 2001, Mikšík and Deyl 2003). Separation on Zorbax ODS packed capillaries is also possible (Huber *et al.* 1997).

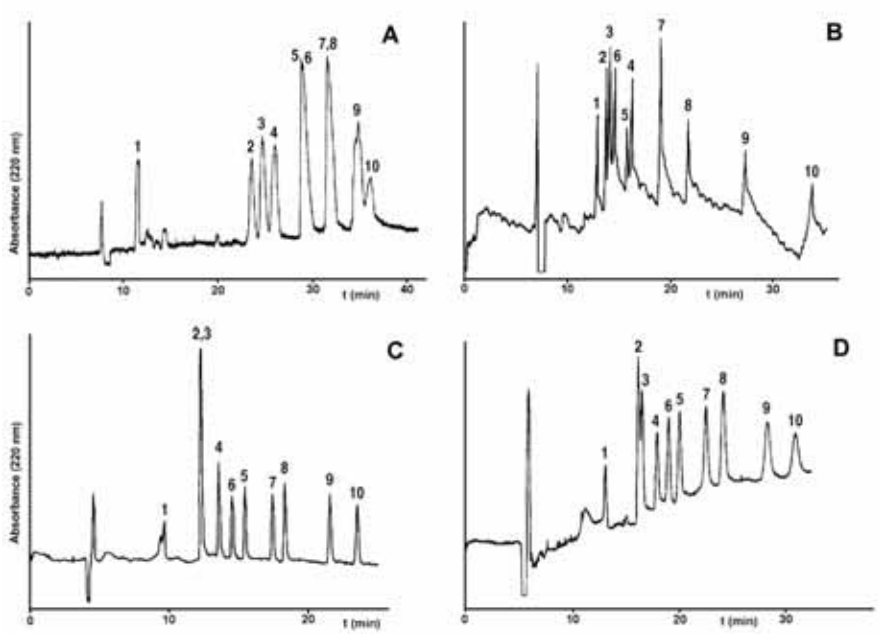


**Fig. 10.** Separation of the test mixture of steroids by capillary electrophoretic metods: **A**) micellar electrokinetic chromatography with SDS (25 m*M* SDS, 25 m*M* borate buffer pH 9.5); **B**) micellar electrokinetic chromatography with glycodeoxycholate (50 m*M* glycodeoxycholate, 25 m*M* borate buffer pH 6.5); **C**) microemulsion electrokinetic chromatography with hexane (n-hexane (0.81 %), SDS (3.31 %), n-butanol (6.61 %) with 20 mM phosphate buffer pH 10.0 (89.28 %, w/w)); **D**) microemulsion electrokinetic chromatography with hexanol (n-hexanol (0.81 %), SDS (3.31 %), n-butanol (6.61 %) with 20 m*M* phosphate buffer pH 10.0 (89.28 %, w/w)). Conditions: Fused-silica capillaries, 60 cm long (50 cm to the detector) x 50 µm I.D., mounted into a home made capillary electrophoresis. Separations were run at 15 kV and monitored at 220 nm at ambient temperature. Identification of peaks: 1 - triamcinolone, 2 - aldosterone, 3 - cortisone, 4 - cortisol, 5 - dexamethasone, 6 - 11-dehydrocorticosterone, 7 - corticosterone, 8 - cortexolone, 9 - deoxycorticosterone, 10 - deoxycorticosterone acetate (Vomastová *et al.* 1996). Originally published by Elsevier.

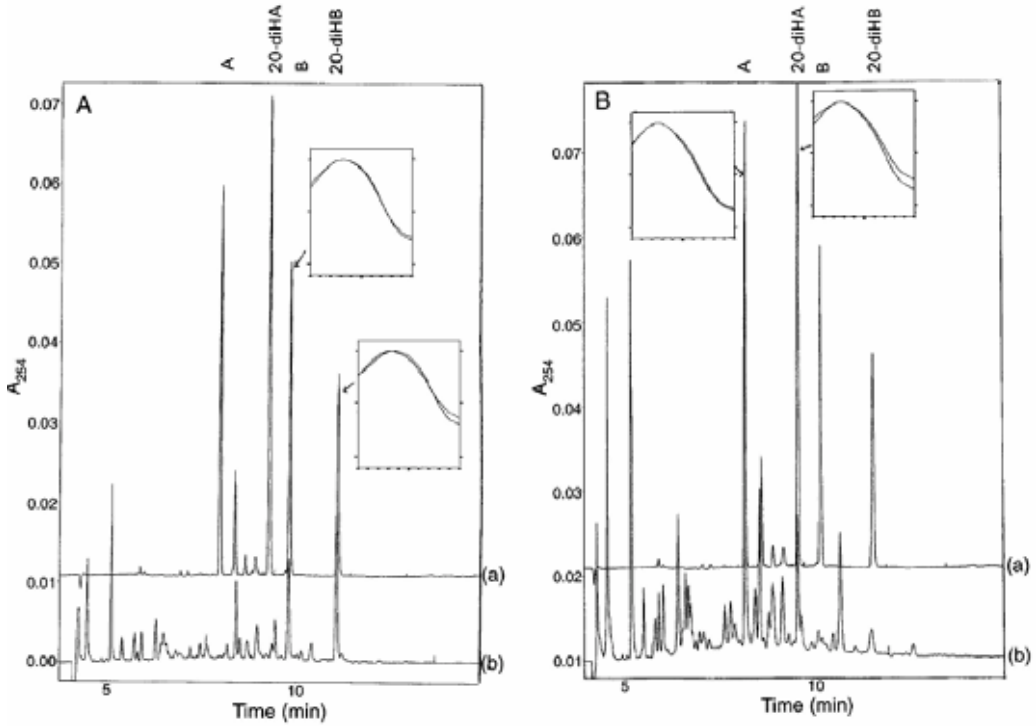


**Fig. 11.** Analysis of steroid metabolism in the hen jejunum by MEKC with diode-array detection. (**A**) Metabolism of corticosterone, (**a**) standards of steroids; (**b**) jejunum incubated with corticosterone (1.45 µM). (**B**) Metabolism of 11-dehydrocorticosterone; (**a**) standards of steroids; (**b**) jejunum incubated with 11-dehydrocorticosterone (1.45 µM). (Insets) Comparison of spectra of the steroid metabolites and the corresponding standards (similarity index: A, 0.9997; B, 0.9997; 20-diHA, 0.9978; 20-diHB, 0.9990); the x axis is 230–280 nm. MEKC was performed on a Beckman P/ACE 5500 Instrument. An unmodified fused-silica capillary 47 cm long (40 cm to the detector), 75 µm i.d. was used with a background buffer consisting of 50 mM sodium deoxycholate and 50 mM borate buffer adjusted to pH 10.0 with 1 M NaOH. Separations were run at 15 kV at 30°C and monitored at 250 nm. Peak identification: corticosterone (B), 11-dehydrocorticosterone (A), 20β–dihydrocorticosterone (20-diHB), and 11-dehydro-20-dihydrocorticosterone (20-diHA) (Vylitová *et al.* 1998). Originally published by Elsevier.

## Perspectives for future research

The aim of this paper was to draw attention to the development of colonic $Na^+$ and $K^+$ transport in mammals particularly in the rat and to show how analytical methods can be used for investigation of peripheral steroid metabolism. Much work has been done in studying intestinal transport functions in mature intestine, but less attention has been paid to the ontogenetic regulation of these processes. Our studies tried to contribute to filling these gaps. Nevertheless, a lot of questions are at the forefront of contemporary developmental biology and physiology of the gastrointestinal tract. Among the most important questions belong the increased sensitivity of immature intestine to infectious diarrhea and the enteric neuroimmune interactions in gastrointestinal hyper-sensitivity responses in babies.

The intestine is capable of transporting water, nutrients and salts that come by oral ingestion and by endogenous secretion from a variety of sources including the intestine, salivary glands, pancreas etc. There is a balance between intestinal absorption and secretion, but it can be easily reversed to net secretion. The excessive loss of water into the intestinal lumen induced by enterotoxigenic bacterial infection is a typical example of this imbalance. Despite our current knowledge about how the immature intestine absorbs water and electrolytes, it is not clear how the immature intestine responds to secretory stimuli and whether this response undergoes any developmental changes (Pácha 2000). This information is very important because infantile diarrhoea is one of the major causes of infant morbidity and mortality world-wide and the response to bacterial enterotoxins changes during development (Chu and Walker 1993). These practical problems stress how important is to understand the regulatory mechanisms of irreversible adaptations and maturation of regulatory systems operating in reversible adaptations of intestinal transport functions during perinatal period and during weaning. This knowledge will help to improve the treatment of pre-term and full-term newborns and infants, many of whom suffer from intestinal diseases.

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