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STUDIES ON THE PRODUCTION AND METABOLIC CLEARANCE RATES OF CORTISOL IN THE EUROPEAN EEL, *ANGUILLA ANGUILLA* (L.)

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SUMMARY

Aspects of cortisol metabolism have been studied in the eel, *Anguilla anguilla* L., adapted to fresh water and sea water environments. The effects of hypophysectomy and removal of the corpuscles of Stannius on cortisol dynamics were assessed.

High specific activity tritiated cortisol reached a steady state in plasma 7 h after beginning a constant intravenous infusion. Plasma cortisol concentrations were determined in hourly blood specimens during the steady state situation. Plasma cortisol concentration, its metabolic clearance and production rates were obtained for the various experimental groups. Plasma electrolytes were also determined.

Similar cortisol concentrations occur in fresh water and sea water eels. Sea water adapted eels had higher metabolic clearance and production rates than fresh water animals.

After hypophysectomy both fresh water and sea water adapted eels had reduced plasma cortisol concentrations and cortisol production rates, although the metabolic clearance rates were significantly elevated. These alterations in cortisol dynamics following hypophysectomy were returned to normal by infusion of mammalian ACTH in a group of sea water adapted eels.

In fresh water eels removal of the corpuscles of Stannius produced almost twofold increases in the metabolic clearance and production rates of cortisol without there being any change in plasma cortisol concentration. Removal of the corpuscles of Stannius from sea water adapted eels induced a slight decrement in plasma cortisol concentration.

INTRODUCTION

The adrenocorticosteroids have been implicated in many aspects of the physiology of teleost fishes, including body fluid homeostasis [1], intermediary metabolism [2] and reproduction [3, 4]. Exogenously administered homologous or heterologous corticosteroids have profound actions in both marine and fresh water teleosts [5], and surgical removal of the adrenocortical homologue, from those species in which such intervention is possible produces major derangements in fluid and electrolyte balance [6]. Furthermore, degrees of hypoadrenocorticism and hyperadrenocorticism seem to characterize certain phases of the life history in many species [7].

The general pattern of adrenocorticosteroidogenesis within this enormous vertebrate group has been elucidated in but a few species [1, 8]. Cortisol appears not only to be the major corticosteroid produced *in vitro* by teleost adrenocortical tissue preparations, but is also quantitatively the major adrenal steroid identified in blood [8, 9]. Although additional corticosteroids, including aldosterone, cortisone and 11-deoxycortisol have been shown to occur in certain teleosts [8, 10–12], the physiology of these products has been little investigated [1, 5].

A variety of experimental approaches indicate that cortisol is of vital relevance to body fluid homeostasis in both marine and fresh water teleost fishes [1, 5]. Bearing in mind that the osmoregulatory mechanisms of fishes living in these two environments are fundamentally different [13–16], it is of interest that major changes in blood cortisol concentrations have not been demonstrated to be associated with changes in environmental salinity [17, 18]. The point obviously arises from such data as to whether cortisol metabolism is in any way related to environmental salinity and hence osmoregulation. A subsidiary enquiry might also question the meaning of steroid concentration in blood with respect to secretory rates, turnover and hence usage of the hormone. Other studies in teleost fishes have indeed suggested that the circulating titre of a steroid may not be a reliable index of adrenocortical activity [8].

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It is with these broad concepts in mind that the present studies were initiated to determine the normal production rates of cortisol in eels, adapted to fresh water and to sea water environments. Attempts were also made to elucidate endogenous endocrine factors that alter cortisol dynamics; of these the pituitary gland secretions, in particular ACTH, have been studied since much information is already present in the literature to indicate a pituitary–adrenocortical axis [1, 5]. The corpuscles of Stannius have been suggested as forming part of another endocrine axis with the adrenocortical steroid output [19, 20] although definitive proof is still awaited. The effects of removal of the corpuscles of Stannius upon cortisol production rates have thus been examined.

EXPERIMENTAL

A. *Animals*

Yellow and silver eels, weighing between 200 and 1000 g were obtained from Billingsgate Fish Market, London. All eels were kept in tanks of tap water for at least 2 weeks before experimental use. Some animals were then transferred to full strength sea water (1 osM/l), whilst others remained in fresh water. These two broad experimental groups were then kept in their respective environments for a further 2 weeks at a temperature of 17 ± 1°C under natural photoperiods.

B. *Experimental groups*

Cortisol dynamics were examined in the following types of eel:

(i) Intact fresh water
(ii) Intact sea water
(iii) Hypophysectomized fresh water
(iv) Hypophysectomized sea water
(v) Fresh water eels after removal of the corpuscles of Stannius
(vi) Sea water eels after removal of the corpuscles of Stannius
(vii) Hypophysectomized sea water eels during infusion of porcine ACTH (Corticotrophin, Organon).

The surgical procedures for the removal of the pituitary gland and the corpuscles of Stannius have been described previously [6]. Eels in which such surgery was performed were left for some 7–10 days before measurement of cortisol dynamics (see below).

C. *Experimental protocols*

Under MS222 (Tricaine–methanosulfonate, Sandoz Ltd.) anaesthesia, a median ventral incision was made in the abdominal wall. Cannulae (PE50, Intramedic catheter, Clay Adams, New York) were implanted into the pneumogastric artery and into a branch of the left posterior cardinal vein. The body wall was sutured and the fish were placed in individual tanks of well aerated water. The animals were left undisturbed for at least 2 days before further manipulation, with the cannulae filled with heparinized saline (250 I.U./ml).

On the basis of several pilot experiments to determine equilibrium volumes for the constant infusion technique (see D, below and Fig. 1) all animals were treated according to the following protocol: at time zero a constant intravenous infusion of tritiated cortisol was begun, and 9 h later an initial arterial blood specimen was withdrawn. At hourly intervals thereafter further arterial blood samples were taken until the infusion was terminated some 14 to 16 h after time zero.

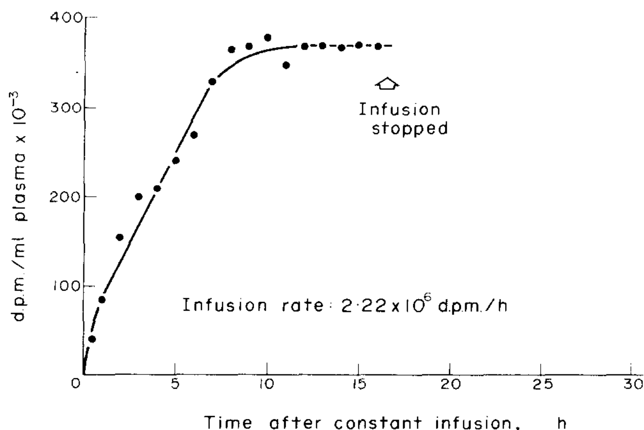

Fig. 1. Determination of the steady state during a constant infusion of tritiated cortisol in a typical experiment. Intravenous infusion of labelled cortisol was begun at time zero and continued for 16 h. Blood samples were taken every hour.

0·4 ml blood samples were drawn into disposable plastic syringes, and the arterial cannula was flushed such that minimal amounts of heparinized saline were introduced into the vascular system to avoid dilution of the isotope, and to minimize intravascular volume expansion.

D. *Constant infusion technique*

Two days after the surgical preparation of the eels, a constant infusion of [^{3}H]-cortisol (40 Ci/mmol, or approximately 10 μCi/0·1 μg; Radiochemical Centre, Amersham) was begun using a multichannel (Harvard Instruments, Milis, Mass.) infusion pump. Usually four eels were infused simultaneously. The rate of infusion was 0·240 ml/h (4 μl/min), and this rate applied to all eels regardless of the body weights. The amount of radioactivity infused was thus approximately 1 μCi/h. The time taken for this infusion rate to result in constant plasma level, that is to say the situation when the rate of removal of radioactivity exactly balanced the rate of infusion, was found to be 8 h (Fig. 1).

E. *Analytical*

Plasma sodium and potassium concentrations were determined by flame photometry and the plasma calcium and magnesium concentrations by atomic absorption spectrometry (Pye Unicam SP 90).

For the assay of plasma cortisol concentrations blood specimens were treated as follows: after centrifugation duplicate 20 μl aliquots of plasma were counted, and duplicate 100 μl fractions were assayed for cortisol. The actual assay procedure was that of Murphy[21] as modified for use in the eel [17, 22]. In the present studies, however, the amount of tritiated cortisol in the plasma samples (resulting from the infusion) varied from animal to animal and could not be ignored. Thus the percentage of counts that were bound was calculated from the combined amounts of tritiated cortisol in the binding mixture and those originally present in the plasma. It was judged necessary to test the validity of this procedure and a wide range of amounts of tritiated cortisol was added to 100 μl aliquots of an eel plasma pool, and the cortisol content determined. A rise in the apparent determined cortisol concentrations was observed with increasing amounts of tritiated cortisol; this was probably due to a change in the binding characteristics with increasing amounts of tracer. By plotting the increased apparent concentration of cortisol against increased tracer, a linear correction curve was established. This was then used to adjust for the cortisol concentrations in the unknown samples.

F. *Calculation of cortisol production rate*

The production rate of cortisol was calculated from the observed metabolic clearance rate (MCR) and the measured concentration of the steroid in the plasma. The MCR was determined by the constant infusion technique outlined by Owen and Idler[23]. Thus:

$$MCR = r/x_c,$$

in which r is the rate of infusion of tritiated cortisol (d.p.m./h) and x_c is the steady isotopic level of the steroid in plasma (d.p.m./ml). From this the production rate, (R), in μg/h adjusted for body weight, was assessed as R = MCR (ml/h/kg body weight) × cortisol concentration (μg/ml). In summary the metabolic clearance and production rates of cortisol were determined as follows: after establishing a steady isotopic state in the plasma, blood samples were withdrawn and the contained radioactivities and cortisol levels determined. The mean cortisol concentration of nine samples was used in the computation of the production rate.

G. *Possibility that cortisol was metabolized during the infusion*

In these studies it was obviously important to know whether the constantly infused tritiated cortisol was significantly metabolized within the circulation during the course of the experiment, since the method depends on maintaining a constant plasma level of tritiated *cortisol*. To this end, a large blood sample was taken from an eel at the end of the 16 h infusion period. Known amounts of [^{14}C]-cortisol and [^{14}C]-cortisone (Radiochemical Centre Amersham; specific activities 52 and 54 mCi/mmol respectively) and marker "cold" cortisol and cortisone were added to the separated plasma. After extraction with dichloromethane [17], the dried extract was run in a two dimensional thin layer chromatographic system: chloroform and chloroform–ethyl acetate (1:50 v/v). The combined U.V. absorbing spot, corresponding to cortisol and cortisone, was eluted and run in the Bush B5 paper system for 6 h. The cortisol and cortisone separated and were subsequently treated separately.

The *cortisol* fraction was acetylated with pyridine and acetic anhydride, and after evaporation of the reagents, the acetate was chromatographed in the paper partition system, cyclohexane–dioxane–methanol–water (4:4:2:1). After elution, 150 mg cortisol acetate was added and the mixture recrystallized to constant ^{3}H:^{14}C ratio. At each of the above stages, aliquots were taken for counting and the ^{3}H:^{14}C ratios determined. This ratio was found to be constant from acetylation through three crystallizations.

The procedures for the *cortisone* fraction were similar. After its separation in the Bush B5 system, the cortisone eluate was acetylated and run in the paper partition system, cyclohexane–benzene–methanol–water (100:70:100:25). After elution and addition of 150 mg cortisone acetate, the sample was recrystallized as before to constant $^3H:^{14}C$ ratio.

RESULTS

A. *Steady state characteristics in eels infused with tritiated cortisol*

Between 5 and 10 h after beginning the constant infusion of tritiated cortisol a steady state prevailed, and providing that the infusion continued the plasma concentration of radioactivity remained constant. Figure 1 shows a typical example: the plasma concentration of radioactivity (in d.p.m.) is plotted against time of infusion. In this instance a steady state, with stable levels of concentrations of tritium in the plasma was obtained about 7·5 h after beginning the infusion; this plateau was maintained until the infusion was stopped. Fifteen hours after stopping the infusion the plasma concentration of radioactivity had declined to less than 10% of the steady state value. Blood specimens used for the assessment of cortisol production rates were taken between 8 and 15 h after beginning the constant infusion. During the course of the infusions the measured plasma cortisol concentrations were not found to change. Thus in a typical example the plasma cortisol concentration before beginning the infusion was 2·1 μg/100 ml plasma and after 7 h, when the isotopic steady state had been reached a value of 1·8 μg/100 ml was observed. Subsequent hourly specimens gave values of 1·6, 2·1, 1·8, 3·1, 2·0, 3·0, 2·9 and 2·0 μg/100 ml. The mean and standard error was thus 2·2 ± 0·2 μg/100 ml, and this value was used to calculate the production rate of cortisol.

B. *Specificity of the cortisol determinations*

The constancy of the $^3H:^{14}C$ ratio from the acetylation stage through to the final recrystallization is taken as proof of the radiochemical purity of the cortisol and cortisone under investigation. Using the ^{14}C activity to assess the recovery, the amount of ^3H activity in the form of cortisol and cortisone in the original plasma was calculated from the isotope ratios to be 68 and 1·8% respectively. Thus about 30% of the radioactivity appears to be present as other metabolites, which were not identified. Since the competitive protein binding assay used to measure cortisol only measures cortisol in eel plasma [22], the figure of 68% was used in the calculation of the MCR of cortisol in all animals studied.

C. *Cortisol dynamics*

Tables 1 and 2 give the basic features of cortisol concentrations as well as its metabolic clearance and production rates in the various categories of eel investigated. In addition the plasma electrolyte concentrations are also presented.

The concentrations of cortisol in plasma of fresh water and sea water adapted eels are similar, being between 4 and 5 μg/100 ml plasma. There is however a marked and statistically significant difference in the metabolic clearance rate of the steroid in these types of fish. Thus in fresh water eels, an average of 20·6 ml of plasma are cleared of cortisol/h, whilst in sea water fish the average is 28·4 ($P < 0{\cdot}01$; Tables 1 and 2). Alongside and perhaps associated with the lower metabolic clearance in fresh water eels, the cortisol production rate is about half that of sea water adapted eels ($P < 0{\cdot}05$). The plasma electrolytes differed in their concentrations when fresh water and sea water eel values were compared confirming previous observations in this laboratory [6].

Table 1. Changes in cortisol dynamics and plasma electrolyte concentrations in fresh water adapted eels

Experimental group	Plasma cortisol concentration (μg/100 ml) plasma)	Cortisol metabolic clearance rate (ml/h/kg body weight)	Cortisol production rate (μg/h/kg body weight)	Plasma electrolyte concentrations (mmol/l) Na	K	Ca	Mg
Intact control ($n = 18$)	4·1 ± 0·8	20·6 ± 1·3	0·72 ± 0·08	143 ± 1	1·8 ± 0·15	2·3 ± 0·06	2·4 ± 0·2
Hypophysectomized ($n = 10$)	0·51 ± 0·08*	31·5 ± 3·3*	0·17 ± 0·04*	113 ± 2*	2·8 ± 0·1*	1·75 ± 0·2*	2·4 ± 0·3
Corpuscles of Stannius removed ($n = 10$)	4·2 ± 0·5	36·6 ± 2·7*	1·5 ± 0·17*	123 ± 4*	3·2 ± 0·3*	3·8 ± 0·27*	2·7 ± 0·4

Values are means ± standard error.
* Indicates values statistically different from control group ($P < 0{\cdot}001$).

Table 2. Changes in cortisol dynamics and plasma electrolyte concentrations in sea water adapted eels

Experimental group	Plasma cortisol concentration (μg/100 ml plasma)	Cortisol metabolic clearance rate (ml/h/kg body weight)	Cortisol production rate (μg/h/kg body weight)	Plasma electrolyte concentrations (mmol/l) Na	K	Ca	Mg
Intact control (n = 19)	4·8 ± 0·4	28·4 ± 2·5	1·29 ± 0·21	178 ± 3	3·5 ± 0·2	2·3 ± 0·05	3·7 ± 0·2
Hypophysectomized (n = 11)	0·43 ± 0·1***	45·57 ± 5·3**	0·18 ± 0·04***	189 ± 3*	2·6 ± 0·06***	3·2 ± 0·2***	4·1 ± 0·6
Corpuscles of Stannius removed (n = 8)	3·5 ± 0·37*	31·6 ± 3·7	1·07 ± 0·13*	192 ± 5*	4·3 ± 0·3*	4·2 ± 0·3***	8·6 ± 1·0*

Values are means ± standard error.
Values statistically different from control group are indicated as follows * $P < 0·05$; ** $P < 0·01$; *** $P < 0·001$.

Seven to 10 days after *hypophysectomy*, the plasma cortisol concentrations were about one tenth of their normal values in both fresh water and sea water adapted eels ($P < 0·001$ in both instances). There was no significant statistical difference between the plasma cortisol concentration of hypophysectomized fresh water eels compared with that of hypophysectomized sea water eels. In both groups the reduced concentrations of cortisol occurred together with an obvious and very clear cut reduction in the production rates ($P < 0·001$ in both instances). This "basal" production rate in sea water eels was 0·18 μg/h/kg body weight, and in fresh water eels was 0·17 μg/h/kg body weight; these values do not differ statistically (Tables 1 and 2). In both hypophysectomized fresh water and sea water eels statistically significant increments in the metabolic clearance rates of cortisol were observed. Thus in sea water eels hypophysectomy resulted in a mean MCR of 45·6 ml/h/kg compared with the control average of 28·4 ml/h/kg ($P < 0·001$) and in fresh water eels the mean value of 31·5 ml/h/kg represents a 50% increase over the control ($P < 0·001$). The MCR of hypophysectomized sea water eels was higher ($P < 0·05$) than that of hypophysectomized fresh water fish, contrasting with the fact that the cortisol production rates were similar in these two experimental groups. Following hypophysectomy sea water eels were characterized by hypernatraemia, hypercalcaemia and hypokalaemia, whilst in fresh water eels hyponatraemia and hypocalcaemia were accompanied by hyperkalaemia.

To investigate further the curious and in many respects unexpected effect of hypophysectomy upon the MCR of cortisol, some additional experiments were performed on 4 sea water adapted eels which had been hypophysectomized for 10 days. Exactly the same protocol was used as for all other animals in this study, except that in this particular group 5 mU ACTH/h/kg was added to the tritiated cortisol infusion. Prior to the infusion, the cortisol concentration in plasma was 0·4 ± 0·1 μg/100 ml plasma, and 16 h after beginning the tritiated cortisol–ACTH infusion this had increased to 6·25 ± 0·4 μg/100 ml. The observed production rate in these fish was 2·05 ± 0·3 μg/h/kg body weight, compared with the hypophysectomized sea water eel value of 0·18 μg/h/kg (Table 2). The production rate of cortisol was thus returned towards the normal sea water value, and the changes in concentration of production rates were accompanied by a depression in the elevated MCR (Fig. 2).

Removal of the *corpuscles of Stannius* from fresh water adapted eels was without effect on plasma cortisol concentration, but there were significant increments in both MCR ($P < 0·001$) and production rate ($P < 0·001$). In sea water eels removal of the corpuscles of Stannius resulted in slightly reduced cortisol production rates ($P < 0·05$) and plasma concentrations

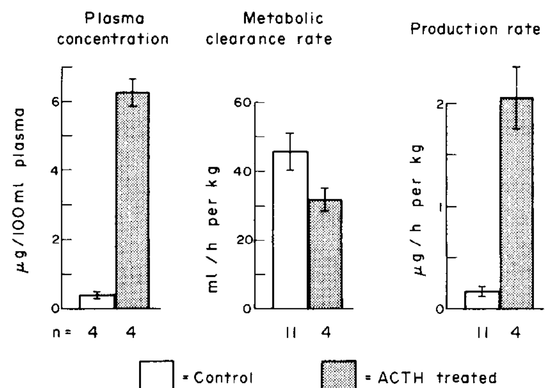

Fig. 2. Effects of ACTH on cortisol dynamics in hypophysectomized sea water adapted eels. Mammalian ACTH was infused with the tritiated cortisol (see text).

($P < 0.05$). The MCR was unaltered. In both fresh water and sea water eels removal of the corpuscles of Stannius had the typical effects of elevating plasma calcium and potassium concentrations; in fresh water animals hyponatraemia was present contrasting with the hypernatraemia of sea water eels without their corpuscles of Stannius.

DISCUSSION

The measurement of the rate of appearance of a corticosteroid in the adrenocortical venous effluent blood provides directly the steroid secretion rate. Such collections are not always possible because the morphology of the adrenocortical homologue and its vasculature precludes the necessary surgical approaches. An alternative method widely applied to mammals and to man in particular, determines the secretion rate of a particular steroid indirectly by observing the systemic or urinary dilution of the specific radioactivity of an administered isotopically labelled steroid by endogenously produced hormone [25, 26]. These isotope dilution methods not only determine the secretion rate of a steroid, but also its metabolic clearance rate, a relevant index of physiological usage of the hormone. Such methods have been applied to the measurement of corticosteroid dynamics in man [26–29], sheep [30, 31], rat [32] and guinea-pig [33] among the mammals. Of non-mammalian vertebrates, whose adrenocortical homologues are frequently unsuitable for the collection of genuine adrenal venous effluent blood, the duck [34], a few teleostean fish species [4, 23, 36 39], an elasmobranch fish [40], and the bullfrog [41] have been studied using such indirect methods.

In the present studies, the constant infusion technique applied to the eel, indicates that this species not only has plasma cortisol concentrations below those of other teleosts studied [8], but also the secretory and metabolic clearance rates are considerably less than those observed in other teleosts and vertebrates [23, 30, 31]. The cortisol secretory rates have been calculated over the period during which the plasma radioactivity concentration was constant, and a steady state existed between the rate of infusion and the metabolic removal of the tritiated cortisol. The rate of infusion was chosen to avoid as far as possible any blood volume expansion effects which might alter cortisol dynamics. Thus the chosen rate of 4 μl/min was below the normal urine flows of both fresh water and sea water eels, which respectively produce 20 and 5 μl/min/kg body weight [6]. In addition blood samples were withdrawn at an overall rate of 400 μl/h. Taking all these factors into account even the smallest eels used were unlikely to be affected by the infused volumes. Furthermore the sequential measurement of plasma cortisol concentrations during the infusion period indicates that the circulating concentrations were stable.

A major and perhaps the most relevant finding of the present investigation is that in sea water, eels have higher metabolic clearance and secretory rates of cortisol than when they are adapted to fresh water. These elevated values are seen despite similar blood cortisol concentrations [17, 18]. In some recent preliminary experiments in this laboratory, it was found that cortisol concentrations in the cardinal veins (the effective venous output of the diffuse adrenocortical homologue of this species [42]) is considerably higher than in arterial blood in both fresh water and sea water eels. Taking into account the measured rate of cardinal vein blood flow, the production rates were: 0.39 ± 0.05 μg/h/kg in four fresh water adapted eels, and 1.4 ± 0.07 μg/h/kg in four sea water eels. The differing pattern of production rate shown in detail in the present study, has thus been confirmed in small experimental groups.

It is tempting to suggest that the increased cortisol production rates observed in sea water adapted eels are related to the increased rates of sodium turnover in this environment. This is dependent upon the adrenal cortical steroids and cortisol may be the active hormone in this respect [5]. More data are of course required as to volumes of distribution, metabolic fate and sites of action of cortisol before firm conclusions can be reached as to the physiological significance of the differing cortisol dynamics in fresh water and sea water adapted eels.

As in other teleostean species [43] the production rates of cortisol are dependent upon the presence of an intact pituitary in both marine and fresh water eels. A curious and as yet unexplained effect of hypophysectomy in the eel is the considerable increase in the cortisol metabolic clearance rate. The changes induced by hypophysectomy are all rectified by the infusion of mammalian ACTH. Explanations for the increased metabolic clearances in the absence of ACTH may include induced changes in binding proteins to give a greater percentage of "free" steroid in the circulation; hence degrading enzymes might have a relatively greater fraction of plasma cortisol on which to act. More data are required on hepatic metabolism of cortisol in both intact and hypophysectomized eels before such an explanation is accepted. Furthermore recently it has been suggested that the metabolic clearance of other corticosteroids may be increased in the absence of trophic agents as a result of an increased hepatic blood flow [44].

The corpuscles of Stannius appear to affect the cortisol dynamics especially in fresh water eels, in which this steroid's production rate was elevated after remo-

val of these glands. In addition the MCRs were increased by almost 90%. In sea water adapted eels the changes seen in cortisol concentrations and production were slight after removal of the corpuscles, although the direction of the change was opposite to that of fresh water animals. It is unlikely that the corpuscular principle(s) acts directly on the adrenal cortical homologue, unless it is postulated that the function of this gland is to inhibit the fresh water eel corticosteroid output.

The usual changes in electrolyte composition of sea water and fresh water adapted eels occurred following removal of the corpuscles of Stannius or the pituitary. It was not possible to relate these observed changes in sodium, potassium, calcium or magnesium to changes in cortisol metabolism, in such a way as to suggest a direct effect of these ions upon adrenocortical function.

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