INFLUENCE OF STRESS ON THE SECRETION OF CORTICOSTERONE IN THE DUCK (ANAS PLATYRHYNCHOS)

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SUMMARY

The influence of stress on the secretion of corticosterone has been investigated by radioimmunoassay in domestic ducks (Anas platyrhynchos) of 6–8 weeks of age. Intravenous and i.m. injections of synthetic ACTH (Synacthen, 25 i.u./bird and Synacthen Depot, 100 i.u./bird respectively) markedly increased (2.7- to 10-fold) basal concentrations of corticosterone in plasma (3–6 ng/ml) in comparison with those in birds injected with 0.9% saline (1 ml/bird). Maximum levels of 30–40 ng/ml plasma were reached after 30 min and 5 h respectively. Increased levels of corticosterone in plasma were also seen within 5 min of i.v. administration of saline and remained as high as those in birds treated with Synacthen for at least 20 min after injection. Blood sampling (from the brachial vein) was, by itself, sufficient to increase levels of corticosterone in plasma; a large (twofold) rise being observed as soon as 1 min after initial handling and bleeding, with concentrations of 30–40 ng/ml being found in birds bled 15 times during a period of 14 min. However, in a flock of birds, the alarm created in unhandled birds while others were being bled had no effect on concentrations of corticosterone in plasma. Deprivation of food or water for 24 h also enhanced levels of corticosterone in plasma, as did 24 h of adaptation to 0.2 m-NaCl drinking water. These results have demonstrated the lability of the secretion of corticosterone in response to stress and demonstrated its usefulness as a physiological indicator of stress in ducks.

INTRODUCTION

Activation of the pituitary-adrenal axis of birds in response to stress is well established (Siegel, 1971; Freeman, 1976; Holmes & Phillips, 1976) and is generally reflected by increased concentrations of corticosterone in plasma of the peripheral circulation. In ducks, although the influence of some stresses, including adrenocorticotropic hormone (ACTH; Macchi, Phillips & Brown, 1967; Bradley & Holmes, 1972), cold (Boissin, 1967; Ensor, Phillips & O'Halloran, 1976; Holmes & Slikker, 1976; Holmes, 1978) and osmotic stresses (Macchi et al. 1967; Allen, Abel & Takemoto, 1975) in enhancing secretion of corticosterone has been determined, the fluorometric and competitive protein-binding assays used in these studies are known to over-estimate levels of corticosterone in plasma (Culbert & Wells, 1975; Holmes & Phillips, 1976), as well as giving inconsistent results for birds (Simensen, Olson, Vanjonack, Johnson & Ryan, 1978). In view of the concern about stress in the poultry industry (Ministry of Agriculture, Fisheries and Food, 1965; Sorensen, 1978), the possible influence of stress on the secretion of corticosterone in the duck has been further investigated in the present study by using a sensitive radioimmunoassay for corticosterone.

MATERIALS AND METHODS

The birds used in these studies were obtained commercially (Cherry Valley Farms, Caistor, Lincolnshire) at 1 day of age and reared on deep litter under a photoperiod of 14 h.
light: 10 h darkness, and with food and fresh water or saline (0.1 m-NaCl until 3 weeks of age, 0.2 m-NaCl thereafter) freely available. When they were between 6 and 8 weeks of age (2–3 kg body weight), groups of birds (five to ten birds/group) were deprived of food or water for 24 h or were given hyperosmotic 0.2 m-NaCl drinking water for 24 h. Blood samples were taken from each bird from the brachial vein before and after treatment. Six other groups of birds were also bled in a known but random manner, to determine whether the order of bleeding affected levels of corticosterone in plasma. Serial blood samples (0.5–1 ml) were also taken from untreated birds which had been reared singly in cages of 60 cm × 60 cm × 60 cm from 4 weeks of age to determine the effect of bleeding itself on secretion of corticosterone. Serial blood samples were also taken from caged birds before and at intervals after injection of synthetic ACTH (Synacthen B (1–24) corticotrophin; Ciba Laboratories, Horsham, Sussex; 25 i.u./bird, i.v.; and Synacthen Depot; Ciba Laboratories; 100 i.u./bird, i.m.) or 0.9% saline (1 ml/bird i.v. or i.m.). All blood samples were collected in heparinized tubes, centrifuged and the plasma was separated and stored at −20 °C before assay. On each occasion that a bird was bled, the blood sample was obtained within 30–60 s of initial handling of the bird and each experiment was initiated between 09.00 h and 12.00 h.

Radioimmunoassay of plasma corticosterone

[1,2,6,7-3H]corticosterone (New England Nuclear, Boston, Massachusetts, U.S.A; 1000 counts/min) was added to duplicate 75 μl portions of each plasma sample before extraction to determine efficiency of recovery of corticosterone. The samples were twice extracted with 1 ml redistilled ethanol and following centrifugation the combined supernatant fractions were evaporated under a stream of air in 15 ml conical glass centrifuge tubes at room temperature. The residue was concentrated in 200–300 μl redistilled ethanol and evaporated to dryness again. The residue was redissolved in 200 μl isooctane: methanol: benzene (8 : 1 : 1, by vol.), applied to a Sephadex LH-20 column (3.5 × 0.8 cm) and eluted with the same solvent. The elution profile of corticosterone on these columns (Fig. 1) was characterized using pure corticosterone, which did not cross-react in the assay (<0.09 ng/ml), in charcoal-stripped plasma (blank plasma) from adrenalectomized/gonadectomized animals. The first 12 ml of the eluate were discarded and the next 12 ml collected. The elution patterns of pure [1α,2α-3H]11-deoxy cortisol (DOC; The Radiochemical Centre, Amersham, Bucks.; 1·3–2·2 TBq/mm), [1,2,6,7-3H]cortisol (The Radiochemical Centre; 3–3·9 TBq/mm), [4-14C]cholesterol (The Radiochemical Centre; >1·85 GBq/mm), [1,2-3H]aldosterone (The Radiochemical Centre; 1·5–2·2 TBq/mm) and [1,2,6,7-3H]progesterone (3–4·1 TBq/mm) added to and extracted from blank plasma did not significantly overlap that of corticosterone under these conditions (Fig. 1). The corticosterone-containing eluate was then evaporated to dryness, redissolved in the eluting solvent, dried and dissolved in 500 μl phosphate-buffered saline (PBS; 0·05 m-sodium phosphate, 0·15 m-sodium chloride, 0·1% gelatin, 0·1% sodium azide, pH 7·6). The tubes were then vortexed, sealed with Parafilm (Gallenkamp, London) and allowed to stand at room temperature for 48 h. A portion (150 μl) of each sample was then counted in a Packard Tri-Carb scintillation counter (Packard Instruments, Illinois, U.S.A.) in 7 ml scintillation fluid (Packard Emulsifier Scintillator 299, Illinois, U.S.A.), to determine the efficiency of recovery, which ranged from 40 to 60%.

Antiserum to corticosterone was raised in New Zealand White rabbits immunized with 11β,21-dihydroxy-4-pregnene-3,20-dione hemisuccinate : bovine serum albumin conjugate (Steroids Inc., Wilton, U.S.A.) and at the final dilution used in the assay (1 : 18 000) gave a bound : free ratio of 0·30. Cross-reaction of other steroid hormones with the antiserum, determined by titrating the antibody with doses of 10, 102, 103, 104, 105 and 106 pg of each steroid, is shown in Table 1. Although DOC and progesterone showed good cross-reactivity their concentration in plasma from immature birds would be expected to be very low (Williams & Sharp, 1977; Sandor, Fazekas & Robinson, 1976; Simensen et al. 1978) and...
Fig. 1. Chromatographic profiles of pure labelled steroids: [\(^{14}\)C]cholesterol (O—O); \([\text{H}]\text{progesterone (●—●)}; \([\text{H}]\text{21-deoxycorticosterone (O - - O); [\text{H}]corticosterone (● - ●); [\text{H}]\text{aldosterone (x---x); [\text{H}]cortisol (x--x) on Sephadex LH-20 columns (3.5 × 0.8 cm) eluted with iso-octane : benzene : methanol (8 : 1 : 1, by vol).}

Table 1. Cross-reactivity of steroids with antiserum to corticosterone

<table>
<thead>
<tr>
<th>Steroid</th>
<th>% Cross-reactivity*</th>
<th>Steroid</th>
<th>% Cross-reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corticosterone</td>
<td>100.0</td>
<td>Aldosterone</td>
<td>1.1</td>
</tr>
<tr>
<td>Deoxytocorticosterone</td>
<td>100.0</td>
<td>Cholesterol</td>
<td>0.2</td>
</tr>
<tr>
<td>Progesterone</td>
<td>63.6</td>
<td>Dehydroepiandrosterone</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Cortisol</td>
<td>11.0</td>
<td>Oestrone</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Testosterone</td>
<td>6.6</td>
<td>Oestriol</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Dihydrotosterone</td>
<td>2.6</td>
<td>Oestradiol-17β</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>17α-Progesterone</td>
<td>2.3</td>
<td>Oestradiol-17α</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

* (Dose of corticosterone causing a 50% fall in disintegrations/min bound)/(dose of other steroid causing a 50% fall in disintegrations bound).

would be removed from the plasma samples, together with plasma lipids, by chromatography with Sephadex LH-20.

Quadruplicate corticosterone standards (0.025, 0.05, 0.10, 0.20, 0.40, 0.60, 1.00, 2.00, 3.00 and 4.00 ng/tube) in 250 μl PBS or 250 μl of each sample were incubated with 500 μl antiserum to corticosterone for 30 min at room temperature. Tritiated corticosterone (10 000 counts/min) in 100 μl PBS was added to each of the tubes, which were vortexed and incubated at 37 °C for 1 h and then incubated at 4 °C for 24 h. Dextran-coated charcoal (0.05% dextran, 0.5% charcoal) was added to each tube in 200 μl ice-cold PBS, the tubes were capped, inverted ten times and incubated for 20 min at 4 °C. Following centrifugation (1540 g for 15 min at 4 °C), the supernatant fractions were decanted into scintillation vials and counted in 7 ml scintillation fluid.

Concentrations of corticosterone in the samples were determined from a logit–log transformation of the standard curve, using the computer program devised by Rodbard & Frazier (1972) and Rodbard, Faden, Hutt & Knisley (1975). In 13 assays the minimum detectable dose (two standard deviations from maximal binding in tubes containing no
competing corticosterone) was $11.9 \pm 1.0$ pg/tube. The intra-assay coefficient of variation for the concentration ($14.05 \pm 0.31$ (S.E.M.) ng/ml) of a pooled plasma sample assayed seven times in the same assay was 5.83% and the interassay coefficient of variation for its concentration ($16.88 \pm 0.52$ ng/ml) in nine assays was 9.33%. The recovery of triplicate doses of unlabelled corticosterone (1.00 and 0.5 ng/ml) added to and extracted from blank plasma was 98 ± 1 and 106 ± 6% respectively. The recovery of higher doses of unlabelled corticosterone (4, 8 and 16 ng/ml) added to and extracted in triplicate from a pooled plasma sample (mean concentration $8.81 \pm 0.24$ ng/ml, $n = 6$) was 102 ± 2, 105 ± 4 and 105 ± 3%. The estimated concentration of corticosterone in ten plasma samples assayed with this antiserum and with an antiserum to corticosterone generously donated by Dr H. A. Gross, Bethesda, Maryland, U.S.A. (Gross, Ruder, Brown & Lipsett, 1972) also showed a good correlation (correlation coefficient, $r = 0.93$, $P < 0.001$). Dose–response inhibition curves parallel with those of the standard curve were also obtained with different volumes (150, 75, 50 and 25 μl) of a pooled plasma sample and its concentration was independent of plasma volume (21.17, 22.34, 21.92 and 23.31 ng/ml respectively).

Statistical differences in the results were determined by Student’s $t$-test.

RESULTS

Long-acting ACTH (Synacthen Depot) greatly increased (>sixfold, $P < 0.001$) concentrations of corticosterone in the circulation between 1 and 5 h after i.m. administration (Fig. 2a), whereas the levels of corticosterone in plasma of the saline-injected control birds remained unaltered during the same periods. Figure 2b shows that 30 min after i.v. injection of Synacthen, the concentration of plasma corticosterone was markedly (ninefold, $P < 0.001$) increased and remained significantly ($P < 0.01$) higher than the pretreatment level after 60 and 120 min and higher ($P < 0.01$) than the level of corticosterone in the saline-injected birds after 30 and 60 min. In both groups of ACTH-injected birds the maximum levels of corticosterone were between 30 and 40 ng/ml. Figure 2b also shows that the concentrations of corticosterone in the control birds were significantly ($P < 0.05$) enhanced within 30 min of the initial blood sample being taken and the saline injection being given. This stressful effect of blood sampling and saline injection was confirmed and further investigated in another experiment in which six birds were serially bled over a period of 60 min after being injected i.v. with 0.9% saline. In each bird a very pronounced increase (1.4- to 6.2-fold) was observed within 5 min of the first sample being taken and this increase
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persisted for at least 20 min. The mean level of corticosterone in these birds 60 min after initial blood sampling and injection (8.48 ± 1.04 ng/ml) was not significantly different from the pretreatment level (5.96 ± 1.42 ng/ml). Figure 3 shows that levels of corticosterone in plasma were also greatly and progressively increased in birds bled every minute over 14 min (linear regression between corticosterone level and time, \( r = 0.569 \) \( n = 90 \), \( P < 0.001 \)). In these birds a significant \( (P < 0.05) \) increase in concentration of corticosterone was already discernible within 1 min of the first blood sample being taken, with levels of 30–40 ng/ml being found after 14 min. As these peak levels of corticosterone were as high as the maximum levels in birds bled less frequently but injected with ACTH (Fig. 2), birds were injected i.v. with ACTH (Synacthen) or saline and bled at intervals during the following 40 min, to determine whether administration of ACTH could further increase corticosterone levels during this period. Figure 4 shows that in both the saline- and ACTH-injected birds the levels of corticosterone were strikingly \( (P < 0.01) \) increased 5, 10 and 20 min after injection, although the concentrations of corticosterone in the ACTH-treated birds were not significantly different from those in birds injected with saline. Thirty minutes after injection of saline the corticosterone level fell \( (P < 0.01) \) but remained higher \( (P < 0.05) \) than the pretreatment level, as it was after 40 min. The concentration of corticosterone in the ACTH-treated birds was higher \( (P < 0.01) \) than that in the control birds after 30 and 40 min. The distress transmitted from birds being caught and undergoing venepuncture to unhandled birds awaiting to be bled had no consistent effect on levels of corticosterone in plasma (Fig. 5). The overall correlation coefficient between the corticosterone level and the order in which the birds were bled was \( r = 0.058 \) \( n = 60 \), \( P > 0.1 \).

Concentrations of corticosterone in plasma in two groups of 6-week-old ducks (6.95 ± 0.80 ng/ml \( n = 10 \) and 6.95 ± 1.41 ng/ml \( n = 10 \) respectively) were also increased \( (P < 0.01) \) by 24 h of food or water deprivation (by 11.68 ± 1.57 ng/ml and 10.67 ± 1.55 ng/ml respectively). Levels of corticosterone in plasma were also increased \( (P < 0.01) \) in birds given 0.2 M-NaCl drinking water for 24 h (Fig. 6). The basal concentration

Fig. 3. Concentrations of corticosterone in plasma of untreated 8-week-old ducks serially bled for 14 min. Values are means ± s.e.m. \( (n = 6) \).
Fig. 4. Concentrations of corticosterone in plasma of 8-week-old ducks following i.v. injection with synthetic ACTH (Synacthen; 25 i.u./bird; ●) or saline (1 ml/bird; ○). Values are means ± S.E.M. (n = 6).

Fig. 5. Basal concentrations of corticosterone in plasma in six groups of 6-week-old ducks in relation to the order (1–10) in which they were bled.
of corticosterone in birds chronically adapted to 0·2 M-NaCl (7·70 ± 1·30 ng/ml, n = 20) was not significantly higher than that in the birds maintained on fresh water (4·99 ± 0·73 ng/ml, n = 20).

DISCUSSION

Under the conditions used, the specificity, sensitivity, accuracy and precision of the radioimmunoassay procedure compare favourably with the same parameters in other corticosterone radioimmunoassays (Gross et al. 1972; Etches, 1976; Nishida, Matsumura, Horino, Oyama & Tenku, 1976; Schmelling & Nockels, 1978) and therefore the assay was considered to be suitable for the determination of corticosterone in ducks. The estimated basal concentration of corticosterone in plasma of ducks in this study was generally between 1 and 5 ng/ml, which was very similar to the level in Aylesbury and Pekin ducks estimated in some studies by fluorometry (Bradley & Holmes, 1971; Holmes & Kelly, 1976) or competitive protein-binding assay (Allen et al. 1975; Ensor et al. 1976), although much lower than in other studies (Macchi et al. 1967; Bouillé, Daniel, Boissin & Assenmacher, 1969).

The striking increase in the level of corticosterone in plasma following ACTH administration clearly demonstrates the role of ACTH in the secretion of corticosterone. The stimulatory effect of ACTH on corticosterone secretion in intact and adrenohypophysectomized birds is well established (reviewed by Wells & Wight, 1971; Holmes & Phillips, 1976; Holmes, 1978), despite some negative reports (Newcomer, 1959; Siegel & Siegel, 1966; Macchi et al. 1967). However, its effect on the levels of corticosterone in plasma of intact ducks has only once been followed in a single bird (Macchi et al. 1967), in which the maximal corticosterone increase after administration of 10 i.u. ACTH/kg i.v. was only 199% and occurred 90 min after injection. The size and time-course of the corticosterone response to ACTH in the present study were very similar to those seen in other avian studies (Holmes & Phillips, 1976), especially in studies of the chicken (Etches, 1976; Beuving & Vonder, 1978), although direct comparisons with other studies cannot be made because of differences in species, age and sex of the birds used, in the dose and preparation of ACTH and in assay methodology.

Since the half-life ($t_1/2$) of corticosterone in the duck is normally about 10 min (Baylé, Boissin, Daniel & Assenmacher, 1971; Helton & Holmes, 1973; Holmes, Broock & Devlin, 1974; Holmes & Kelly, 1976) and is even less in stressed birds (Daniel & Assenmacher, 1971), the high levels of corticosterone maintained after stress or ACTH administration may have
resulted from a greater release of the hormone from the adrenal cortex throughout these periods. However, the inability of exogenous, heterologous ACTH (which would probably have a \( t_4 \) of less than 10 min) to have any greater effect than saline during the first 20 min after its administration demonstrated that the release of corticosterone during this time must be maximal and that peak levels of corticosterone of 30–40 ng/ml plasma resulted only from ACTH-induced corticosteroidogenesis. The rapid stimulation of corticosterone secretion in response to stress may result partly from the semi-autonomous control of adrenal gland function in birds (Bradley & Holmes, 1971; Chan, Bradley & Holmes, 1972; Holmes & Phillips, 1976; Holmes, 1978), increases in level of corticosterone in plasma being observed within 1 min of initial handling and bleeding (Fig. 3).

Although haemorrhagic shock may be responsible for some of the latter increase in corticosterone level in serially sampled birds (Fig. 3), the results of the present study demonstrated that merely handling and bleeding the birds enhanced corticosterone secretion. Very similar effects of restraint and blood sampling have also been observed in some studies in domestic fowl (Beuving & Vonder, 1978), although not in others (Culbert & Wells, 1975; Etches, 1976; Freeman & Manning, 1979). The reason for these discrepancies is unclear. In ducks concentrations of corticosterone in plasma of agitated birds 2–8 min after insertion of a wing-vein cannula are also higher than the level in restrained but undisturbed birds, 65 min after cannulation (Bradley & Holmes, 1971); after which time it is claimed that injection of test substances and frequent removal of blood samples has no effect on levels of corticosterone (Holmes et al. 1974; Holmes & Kelly, 1976). Thus, although handling and bleeding procedures might appear to be relatively mild stressors, under some circumstances they can markedly alter the levels of corticosterone in plasma of the peripheral circulation. Moreover, in other avian studies, these procedures have also been found greatly to increase heart rate (Howard, 1971) and blood volume (McFarland, 1963), to induce hyper-lipacidaemia (Freeman & Manning, 1976, 1977, 1979) and hypoglycaemia (Davison, 1975; Freeman & Manning, 1976, 1977) and to affect other endocrine systems profoundly (Wilson & Sharp, 1975; Zachariasen & Newcomer, 1974; Freeman & Manning, 1976; Jurani, Vyboh, Lamosova & Nvota, 1978; Davison, Scanes, Harvey & Flack, 1980). However, the present results (Fig. 5) also show that the concentrations of corticosterone in plasma of birds witnessing others being restrained and bled were unaffected by their distress. Similar results in domestic fowl have been reported (Culbert & Wells, 1975). In contrast, corticosterone levels in rat plasma are directly related to the order in which the animals are bled (Henning, 1978).

Although stress is difficult to quantify, deprivation of food or water is generally considered to be a relatively severe stress (Freeman, 1976; Beuving & Vonder, 1978) and has been found to stimulate secretion of corticosterone in chickens and turkeys (Brown, 1961; Constantin, Raszyk, Holub & Kotrabacek, 1977; Beuving & Vonder, 1978), while undernutrition results in adrenal hypertrophy in pigeons and domestic fowl (Vincent & Hollenberg, 1920; Breneman, 1942; Conner, 1959). Deprivation of food and water similarly increased levels of corticosterone in plasma in this study, possibly as a result of the need to increase gluconeogenesis and achieve glucose homeostasis (Holmes & Phillips, 1976; Veiga, Roselino & Migliorini, 1978). The fact that the \( t_4 \) of corticosterone is unchanged during fasting in the duck (F. Malaval & I. Assenmacher, unpublished observations cited by Assenmacher, 1973) suggests that the increased levels of corticosterone seen in plasma after 24 h of starvation in this study and after 17 days in others (F. Malaval & I. Assenmacher, unpublished observations cited by Assenmacher, 1973), represent a definite stimulation of adrenocortical function.

Adrenal glucocorticoids have profound effects on electrolyte balance in birds and are intimately involved in extrarenal excretion in marine birds (Phillips, Holmes & Butler, 1961; Holmes & Phillips, 1976; Holmes & Pearce, 1978) and as expected in the present study levels of corticosterone in plasma were increased in birds transferred from fresh drinking water to
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0.2 m-NaCl, although corticosterone levels were not enhanced in birds chronically adapted to saline drinking water (Fig. 6). Others have also found that acute osmotic challenges increase the rate of corticosteroid secretion in ducks (Donaldson & Holmes, 1965; Macchi et al. 1967; Allen et al. 1975), while basal levels of corticosteroid in serum are unchanged in birds chronically adapted to drinking salt water (Allen et al. 1975). These findings are therefore in agreement with Selye's classical concept of stress in that an initial period of alarm is followed by a period of adaptation (Selye, 1950).

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REFERENCES


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