The Validity of Using Plasma Corticosterone as a Measure of Stress in the Turkey.*† (26680)

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The present emphasis on stress in relation to disease has resulted in a tendency to classify as stress factors many of the unfavorable environmental and management factors of poultry, without first obtaining evidence of measured changes in the function of the adrenal glands.

Identification of adrenocortical steroids has been accomplished only recently in birds. The adrenal effluent blood of the capon has been reported to contain corticosterone, cortisone, and aldosterone(1), of which corticosterone was the principal component. Incubation of White Leghorn cockerel adrenals in Krebs-Ringer bicarbonate led to identification of corticosterone, aldosterone and tentative identification of small amounts of hydrocortisone(2). Corticosterone was the principal steroid obtained. Plasma levels of corticosterone in Gallinaceous birds have been reported. Adrenocorticotrophic hormone (8 I.U. ACTH intravenously) significantly increased corticosterone in adrenal effluent blood in pheasants and chickens 60 minutes after injection(3).

The present paper attempts to confirm that corticosterone is the principal corticosteroid found in turkey plasma. Data are also presented in support of the hypothesis that, *the concentration of corticosterone in peripheral plasma reflects the severity of stress conditions in the fowl.*

Materials and methods. Small White turkey poults were raised in batteries and were fed the Ohio Turkey Starter Ration(4) to 6 weeks of age for use in all experiments.

The procedure used to identify corticosterone was as follows: A 500 ml sample of plasma was extracted 3 times with 2 volumes of ethyl acetate, washed once with 0.1 volume of N NaOH and twice with water. The extract was dried by adding excess anhydrous Na_2SO_4 followed by filtration under vacuum through a cake of Na_2SO_4. This extract was then taken to dryness under vacuum in a water bath at 40°C. The residue was taken up in a small volume of ethyl acetate and layered onto a silica gel column(5). The column was first washed with a small amount of petroleum ether (B.P., 30-60°C) followed by 5% petroleum ether in benzene. The adrenal steroids were then eluted with ethyl acetate. The eluate was taken to dryness under vacuum in a water bath at 40°C. The residue was transferred in methanol to washed Whatman #1 filter paper and chromatographed with standards on a parallel lane in a system of ethylene glycol/toluene (6). The areas corresponding to the steroid standards were eluted in ethanol and rechromatographed in the E_2B system(7).

Identification of corticosterone was based on (1) mobility with reference to standards run on a parallel lane in the 2 solvent systems; (2) reaction with diphenyltetrazolium chloride and (3) sulfuric acid spectrum(8).

The level of free corticosterone in extracts of aliquots of peripheral (2 ml) plasma was determined by a fluorescence technic(9). Fluorescence was read in a Coleman Model 12 photofluorometer. Although this method measures hydrocorticosterone as well as corticosterone, the fluorescence attributable to the former compound is negligible since only trace amounts of it can be detected by chromatographic technics.

Results and discussion. Identification of corticosterone. A large number of chromatograms was used in development of the method. The results are summarized diagramatically in Fig. 1. The steroid present

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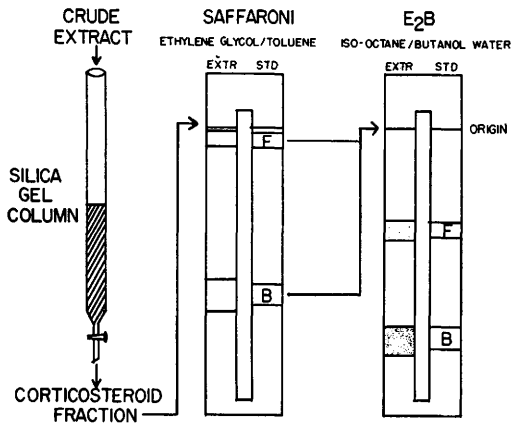

FIG. 1. Chromatographic separation of corticosterone (Compound B) and hydrocortisone (Compound F) from 200 ml equivalent of turkey plasma. Density of stippling indicates relative amounts of steroid.

in the largest quantity was identified as corticosterone. The material moved with a polarity of standard corticosterone run in parallel in both solvent systems, and reacted strongly with diphenyltetrazolium chloride. The sulfuric acid spectrum was substantially the same as that of pure crystalline corticosterone with the major peak at 285 mμ and smaller peaks at 333 mμ, 374 mμ, and 455 mμ. Ratio of corticosterone to hydrocortisone was approximately 20:1.

Measurement of corticosterone in normal and hypophysectomized turkeys subjected to ACTH injections and various stressors. The establishment of corticosterone as the predominant free corticosteroid found in turkey plasma suggests that concentration of this compound should reflect the severity of stress conditions in the fowl. Before this can be accepted as a working hypothesis, however, it must be shown: (1) that exogenous adrenocorticotrophic hormone (ACTH) will significantly increase the level of free corticosterone in peripheral plasma; (2) that various stressors will elevate plasma corticosterone and (3) that hypophysectomy will abolish this latter response to stress.

Effect of ACTH on plasma corticosterone. To test the effect of ACTH on free corticosterone in peripheral plasma, 6-week-old male Small White turkeys were injected intramuscularly (I.M.) with 8 I.U. ACTH (Upjohn, Depo ACTH) per bird per day for 3 days. One hour after final injection, heparinized blood was drawn from the wing vein and the plasma separated by centrifugation. The results are presented in Table I. The ACTH treatment elevated free corticosterone from 12.5 μg% in the controls to 28 μg% in the ACTH treated birds. Forty-eight hours after final injection the concentration had returned to 14.2 μg% which is at or near normal levels. Thus it was firmly established that exogenous ACTH will significantly incerase the level of free corticosterone in peripheral plasma.

Effect of stressors on intact and hypophysectomized turkeys. Hypophysectomies were performed on the birds used in these experiments at 6 weeks of age. One week after surgery the birds were subjected to the stressors of water deprivation or cold. At termination of each experiment the birds were sacrificed, their heads removed and assigned a number. The heads were then examined to determine completeness of surgery and thus assign each turkey to one of the following groups: (1) sham operated (all of the anterior pituitary remaining; (2) ap (anterior pituitary completely removed) and (3) ap + pp (complete hypophysectomy, *i.e.*, both anterior and posterior pituitary removed). Thus, there were 4 experimental groups; controls, sham operated, ap and ap + pp on each experiment.

The results obtained with water deprivation are presented in Table II. Water was withheld from all groups for 96 hours. At the end of this period the surviving birds were again allowed free access to water.

TABLE I. Plasma Corticosterone Concentration in 6-Week-Old Small White Turkeys Injected with ACTH (10 Birds per Treatment).

	Controls, 0.1 ml corn oil I.M./day (3 days)	8 U ACTH I.M./bird/day (3 days)
	μg %	
1st sample, 1 hr after last inj.	12.5	28.7
2nd sample, 48 hr after last inj.	11.9	14.2

I.M. = intramuscular. LSD (<.01) = 10.2.

TABLE II. Plasma Corticosterone Concentration and Hematocrits in 7-Week-Old Small White Tom Turkeys Subjected to Water Deprivation. (Operation performed at 6 wk of age.)

Treatments		0 hr	48 hr	96 hr*	168 hr (3 days after water was ret.)
		----------	μg %	----------	
Controls	Corticosterone	11.3	26.1	30.1	15.1
	Hematocrit	33.0	46.1	49.2	34.6
		(11.3)	(18.8)	(20.2)	(14.4)
Sham-operated	Corticosterone	11.7	19.5	25.8	14.2
	Hematocrit	33.2	45.4	48.0	33.9
		(11.6)	(16.4)	(17.7)	(13.8)
ap	Corticosterone	11.2	11.1		
	Hematocrit	33.9	42.4		
		(10.9)	(9.8)		
ap + pp	Corticosterone	12.4	16.3		
	Hematocrit	35.2	52.0		
		(11.6)	(10.3)		

( ) μg % corticosterone adjusted to a normal hematocrit – LSD ($<.01\%$) = 5.6.
* Birds were again allowed free access to water.

There was no significant difference among the 4 groups at 0 time. The control corticosterone level increased significantly ($P<.01$) from 11.3 μg% at 0 time to 26.1 μg% at 48 hours and to 30.0 μg% at 96 hours. At 168 hours, 3 days after the surviving birds had been allowed free access to water, the corticosterone level in the control birds had returned to near normal levels. The sham operated birds also responded to the stress of water deprivation, but to a lesser extent than the intact controls. This may have been due to disturbance of the blood supply to and from the pituitary. There was a small increase in plasma corticosterone when both anterior and posterior pituitaries were removed, but no change when only the anterior pituitary was removed. It should be noted that hematocrits increased markedly in all groups at 48 hours of water deprivation, particularly in the ap + pp birds. When all corticosterone values were adjusted back to the levels that would have been attained if hematocrits had remained normal (*i.e.*, approx. 33%) (Table II) then both controls and sham operated birds showed significantly higher levels than did the ap or the ap + pp birds. Observation of these adjusted values indicates that hypophysectomy not only prevented elevation of plasma corticosterone, but also actually tended to reduce its levels from 11.3 μg% in controls to 9.8 μg% in the ap birds and to 10.3 μg% in the ap + pp birds.

All the ap + pp birds died almost immediately after the blood samples were drawn at 48 hours. The ap birds lived longer, but all died before the 96 hour collection. Observations indicated that the ap + pp birds passed tremendous quantities of fluid through their kidneys resulting in excessive water loss as urine. It is postulated that this would result in greater hemoconcentration, less efficient circulation and, therefore, less resistance to the stress of water deprivation than in the ap birds. Although the ap birds lived longer than the ap + pp birds, their early deaths indicate that they were unable to resist stress to the same degree as the control or the sham operated birds.

The effects of cold (35°-45°) on rectal temperatures and plasma corticosterone are shown in Table III. The plasma corticosterone in sham operated birds increased from 12.6 μg% at 0 time to 17.9 μg% at 24 hours. This response was prevented by hypophysectomy. As with water deprivation, the hypophysectomized birds subjected to cold stress tended to show lower than normal corticosterone levels 72 hours after the offending stressor was removed.

Although cold applied for 24 hours did not result in the death of the hypophysectomized birds, there was a significant ($P<.05$) drop

TABLE III. Effect of Cold (35°-45°F) on Plasma Corticosterone in 7-Week-Old Hypophysectomized Small White Turkeys. (Operation performed at 6 wk of age.)

Time (hr)	Sham-operated	ap	ap + pp
	μg %		
0	12.6 (41.1)	11.9 (40.6)	12.8 (39.9)
8	13.2 (41.0)	11.6 (40.1)	13.4 (38.9)
24	17.9 (40.8)	12.2 (39.8)	12.5 (36.0)
72 hr after returning to a 70°F room	12.6 (41.2)	9.9 (40.4)	10.3 (39.5)

( ) Rectal temperature in °C – LSD ($P < .01$) = 0.8°C.
LSD ($P < .05$) for corticosterone concentration = 5.4.

in rectal temperature of both ap and ap + pp birds. In another experiment the birds were placed in the cold and left until all the hypophysectomized birds died. Rectal temperatures were recorded every 24 hours, and the data are shown in Table IV. It is interesting to note that rectal temperatures of ap birds tended to be lower than normal and rectal temperature of the ap + pp birds was significantly ($P < .01$) lower than for the sham operated birds at 0 time. Rectal temperatures of the ap group dropped to 26.2°C at 96 hours and all birds in the group were dead at 120 hours. Rectal temperatures of the ap + pp birds dropped precipitously to 24.2°C at 72 hours and all birds in the groups were dead at 96 hours.

These data amply demonstrate that the normal turkey responds to the applied stressors of water deprivation and cold with an accelerated release and, consequently, an elevated plasma concentration of corticosterone. It has been also demonstrated that hypophysectomy prevents this response.

TABLE IV. Effect of Cold (35°-45°F) on Rectal Temperatures in 8-Week-Old Hypophysectomized Turkeys. (Operation performed at 6 wk of age.)

Time (hr)	Sham-operated	ap	ap + pp
0	41.2	40.8	39.8
24	41.1	39.4	37.2
48	41.0	36.7	33.3
72	41.2	32.0	24.2
96	41.1	26.7	all dead
120	41.2	all dead	

LSD ($P < .01$) = 0.9°C.

The effect of various replacement therapies on ability of hypophysectomized birds to resist stress conditions remains to be studied. The author recognizes that the thyroid may play an important role in resistance of hypophysectomized birds to stress. An attempt will also be made to study the influence of antidiuretic hormone on resistance of ap + pp birds to stress conditions. Thus the type of replacement therapies used will not only include ACTH and various adrenocorticosteroids, but also thyroxin and antidiuretic hormone preparations.

It has been postulated that the bird adrenal may be relatively autonomous and, therefore, function at a relatively high level in absence of the anterior pituitary(10). The data presented here tend to support this hypothesis in that non-stressed hypophysectomized birds exhibit plasma corticosterone levels not significantly different from normal controls. The ap birds live relatively well until subjected to severe stressors, then their inability to respond with an accelerated release of corticosterone appears to render them less resistant to the applied stressor. The relatively low level of corticosterone in ap birds which have been subjected to stress, indicates that there may be an accelerated utilization of corticosterone in response to the stressors applied.

Summary. Corticosterone was established as the principal free corticosteroid found in turkey plasma. A routine procedure based on the fluorescence of corticosterone was used for quantitation of free corticosterone in peripheral plasma of the turkey. Using this technic it was demonstrated that exogenous ACTH injected intramuscularly significantly increases the level of free corticosterone in peripheral plasma. Likewise, it was shown that the stressors of water deprivation and cold significantly elevate plasma corticosterone and that hypophysectomy abolishes this latter effect. It is suggested that these data indicate the validity of using plasma corticosterone concentration as a measure of severity of stress conditions in the birds. These data also support the postulate that the bird adrenal may be relatively autonomous and, therefore, functions

at a relatively high level in the absence of anterior pituitary function.

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Demonstration of Antigen-Reagin Interaction by Free Boundary Electrophoresis Using Buffer Contaminated with Specific Antigen.* (26681)

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In vitro experiments showing the specific interaction between human reaginic serum and homologous antigen have not been demonstrated. We assume that such specific activity exists and that it can be observed through alterations in molecular behavior of reaginic serum as can be demonstrated with free boundary electrophoresis. Efforts to discover a difference between reaginic and non-reaginic serum by electrophoresis have not been clearly successful(1). Interaction of antigen with specific antibody in free boundary electrophoresis was first demonstrated by Campbell, *et al.*(2).

TABLE I. Calculation of Mobility and Percentage Composition.

				α				β					
		Alb.		α_1		α_2		β_1		β_2		γ	
Patient	Conc.	$\times 10^{-5}$	%	$\times 10^{-5}$	%	$\times 10^{-5}$	%	$\times 10^{-5}$	%	$\times 10^{-5}$	%	$\times 10^{-5}$	%
	WSR*												
DR	.0	7.08	58.19	6.01	3.86	4.45	8.77	3.56	5.20	3.19	13.53	1.25	10.45
	.02	7.29	50.13	6.19	5.85	4.43	9.97	3.23	20.21	—	—	1.15	13.81
	.04	6.95	52.48	5.88	4.95	4.37	9.05	3.28	22.46	—	—	1.26	11.02
	.06	7.09	57.78	6.25	3.80	4.40	9.77	3.60	3.26	3.20	15.98	1.29	9.38
	.10	6.82	59.18	5.73	4.23	3.87	8.39	3.41	5.34	3.04	14.82	1.07	8.01
	WSK†												
DR	.0	7.08	58.19	6.01	3.86	4.45	8.77	3.56	5.20	3.19	13.53	1.25	10.45
	.01	6.77	54.90	5.72	4.08	4.20	10.70	3.22	6.23	2.90	13.25	1.05	10.83
	.02	7.19	55.26	6.09	4.22	4.55	9.99	3.56	7.33	3.23	11.98	1.29	11.23
	.04	6.74	54.79	5.74	5.92	4.28	9.11	3.71	3.58	3.20	15.32	1.30	11.30
	.06	6.69	56.69	5.31	3.58	3.97	11.97	3.02	17.12	—	—	1.06	10.63
	WSR*												
UN	.0	6.85	65.80	4.40	6.34	—	—	3.06	13.32	—	—	1.09	14.52
	.01	6.87	65.88	4.53	7.12	—	—	3.09	13.14	—	—	1.10	13.83
	.02	6.82	64.66	4.46	8.18	—	—	3.09	13.56	—	—	1.23	13.58
	.04	6.96	62.05	4.55	10.13	—	—	3.11	13.30	—	—	1.19	14.52
	.06	6.90	65.27	4.53	8.06	—	—	3.30	13.50	—	—	1.14	13.18

α_1 and β_1 represent total α and β unless shown in respective columns.
* WSR = Water soluble ragweed.
† WSK = " " Koeleria.

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