Blood Cell Changes Following ACTH Injection in the Chick.* (23554)

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Criteria of pituitary-adrenocortical function in the mammal in response to application of stressors or ACTH injection, in general have been found not to be adequate following similar treatments in birds. Adrenal hypertrophy has been reported not to occur in the chick following administration of various noxious agents(1) and to occur following chronic administration of ACTH (extract) or epinephrine(1,2). ACTH, epinephrine or cold did not produce an acute decrease (within 1-4 hours) in adrenal ascorbic acid content in the chick, duck or quail(2-5). Slover(6) demonstrated a decrease in adrenal ascorbic acid 12 hours following injection of ACTH in White Leghorn cockerels. Stamler *et al.*(7) failed to obtain an eosinopenia in the chick following chronic ACTH treatment. Elton and Zarrow(5) reported no depletion of adrenal cholesterol following ACTH injection; Slover (6) found a decrease in adrenal cholesterol 6 hours after ACTH injection in both male and female chicks, however repetition failed to substantiate this unpublished result.

Lack of suitable physiologic indicators of stress in the bird has prevented evaluation of the effects of stressors in the bird. The purposes of this investigation were: (a) to study the changes in blood cell counts of the chick following a single injection of ACTH and (b) to assess the possibility of using any resulting changes as possible indicators of stress (assuming that endogenous liberation of ACTH is a requisite event in the response of the bird to stressors).

Procedure. White Leghorn cockerels were obtained from a commercial hatchery on the day of hatching and maintained on Purina *Startena* and water *ad lib.* in brooder batteries. At 1-2 weeks of age, birds were injected with ACTH (ACTHAR, lyophilized, Armour) intraperitoneally; uninjected birds or water-injected (0.1 ml) birds of like age served as controls since in preliminary work it was found that neither water (0.1 ml) nor a dry needle puncture produced any significant changes in blood cell counts. At a specific time following injection of the hormone, intracardial samples of blood (1 ml or more) were drawn in a heparinized syringe from both treated and control birds. *Hematocrits* were obtained by centrifuging blood samples in Van Allen pipettes until constant readings were obtained. Direct acidophil counts were made in a standard hemocytometer at least 24 hours following dilution (200X) with Wiseman's staining solution(9); each blood sample is represented in the calculations by the mean of 4 counts. Differential white cell counts were made from smears stained with Wright's stain; a total of 200 cells was counted from each smear. Total white cell counts and absolute numbers of each kind of leucocyte were obtained by appropriate calculation from the direct acidophil and differential counts.

Results. Results of determinations on blood from cockerels 3, 6, 12 and 24 hours following intraperitoneal injections of ACTH are shown in Table I. Injection of ACTH was followed by an increase in total leucocytes at the 4 time periods following treatment. This leucocytosis was greatest during the first 6 hours and decreased in extent at the 12 and 24 hour intervals.

Changes in absolute numbers of eosinophils, basophils and monocytes were neither great in extent in most instances nor consistent, perhaps due to the small numbers of cells involved and therefore the relatively greater error involved in calculation. Furthermore, the changes in absolute numbers of these 3 types of cells in the treated birds were not beyond the range of variation found in the control groups. A marked heterophilia (neutrophilia) was obtained at all periods sampled except for one trial (24 hours). The increase in heterophils was greater at 3 and 6 hours than at 12 and 24 hours. A slight lymphocy-

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TABLE I. Blood Cell Determinations on Control and ACTH-Treated (1 IU) Birds.

Group	Treatment	No. birds	Acidophil count†	Total WBC‡	Basophils	Eosinophils	Heterophils	Lymphocytes	Monocytes	RBC§	Hematocrit
1	None	13	8.67 ± 5.38	11,665	210	315	1,610	9,355	175	2.064	33.7
	ACTH + 3 hr	14	19.10 ± 9.59	15,475	186	309	3,931	10,832	217	2.244	31.3
2	None	15	9.25 ± 5.16	14,260	242	171	1,882	11,751	214	2.078	27.4
	ACTH + 3 hr	14	24.34 ±17.67	18,568	278	56	5,348	12,663	223	2.009	25.9
3	None	16	5.84 ± 3.55	6,970	153	98	1,199	5,416	104	1.986	
	ACTH + 6 hr	15	19.67 ±10.38	14,270	242	143	4,224	9,390	271	1.894	
4	None	15	9.36 ± 4.72	11,806	248	59	2,019	9,362	118	2.149	31.2
	ACTH + 6 hr	13	36.69 ±23.87	18,201	182	27	8,118	9,774	100	1.969	28.0
5	None*										
	ACTH + 12 hr	15	13.88 ± 8.26	12,131	170	194	2,887	8,638	242	1.989	
6	None	25	5.82 ± 3.22	10,157	179	329	963	8,552	134		
	ACTH + 12 hr	19	10.92 ± 5.29	13,125	228	525	1,899	10,204	269		
7	None*										
	ACTH + 24 hr	16	9.39 ± 9.74	10,233	187	89	1,995	7,757	205	2.033	
8	None	16	10.73 ± 5.35	14,178	354	241	2,141	11,144	298	1.928	
	ACTH + 24 hr	15	7.02 ± 2.75	11,717	117	105	1,453	9,749	293	1.899	
9	None	12	11.38 ± 4.56								
	Needle	11	12.98 ± 5.44								
	H_2O + 6 hr	12	10.68 ± 4.82								
	H_2O + 12 hr	12	8.54 ± 4.37								
	H_2O + 12 hr	12	13.85 ± 7.69								

* All birds in this group were processed at the same time as group 3; those inj. with ACTH are to be compared with the controls of group 3.

† Count = No. of acidophils/0.045 mm^3 blood ± stand. dev.

‡ WBC and leucocytes expressed as cells/mm^3 blood.

§ RBC expressed as millions/mm^3 blood.

tosis was obtained in 6 of the 8 reported trials; the magnitude of this increase was greatest at 3 hours and decreased thereafter. From the above results it is evident that the observed leucocytosis was due primarily to the heterophilia and to a lesser extent to the lymphocytosis.

The above absolute numbers of leucocytes are based on acidophil counts which, in the chicken, are comprised of both eosinophils and heterophils (neutrophils). Injection of ACTH resulted in a statistically significant ($P = < .005$) increase in acidolphils throughout the first 12 hour period. This acidophilia is evidently due to the marked and consistent heterophilia rather than to an eosinophilia.

Examination of the diffential counts on which the absolute numbers of cells in Table I are based, indicates a consistent relative increase in heterophils and a decrease in lymphocytes following injection of ACTH.

Treatment with ACTH was followed in the 3 instances where determined by a lowered hematocrit of 5.5, 7.2 and 10.3% of the respective control values. The RBC counts indicate no great or consistent change in the treated birds, but were slightly lower in 4 of 6 determinations. This decrease is consistent with the lowered hematocrit.

Neither injection of distilled water (0.1 ml) nor puncture with a dry needle produced a significant change in acidophilia 6 or 12 hours following treatment (see group 9, Table I). These results indicate that the acidophilia obtained following injection of ACTH was not due merely to the mechanics of injection.

To determine if the degree of acidophilia was proportional to dose, graded doses of ACTH were administered to 2 series of birds. Dose-response curves based on data obtained 6 and 12 hours following injection are shown in Fig. 1. Statistical analysis indicated that there is a highly significant (beyond the 1% level of probability) linear relation between

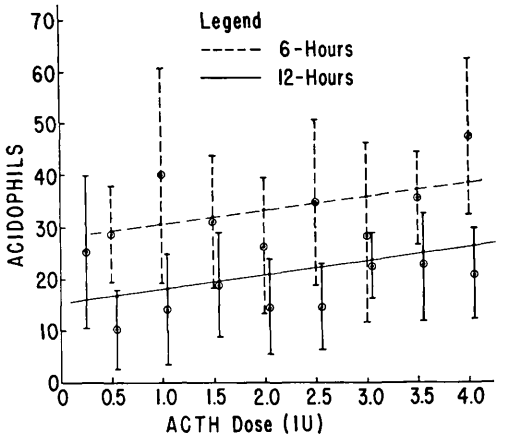

FIG. 1. Response of acidophils (number/.045 mm³ blood) to graded doses of ACTH (IU). Each point represents the mean of 12 birds; vertical lines represent ± stand. dev. of means.

dose and magnitude of response in each case. In the 6-hour trial, $Y = 27.85 + 2.65\ X$; in the 12-hour trial, $Y = 15.55 + 2.65\ X$. It will be noted that the acidophilia was uniformly greater at the 6-hour period than at the 12-hour period. Coefficients of variation of the acidophilia were: 44.67% for the 6-hour series and 52.23% for the 12-hour series; refinement of procedure could possibly reduce this high value if it could be shown that its sources were not predominantly attributable to the experimental subjects. By inspection of the data (Fig. 1) it appears that the acidophilia is not a very precise quantitative criterion for ACTH under the circumstances of the treatment described herein.

Discussion. The results of this study differ in a number of respects from other reports on blood cell changes in birds following injection of ACTH or adrenocortical extracts or hormones. Shapiro and Schechtman(10) reported that injection of adrenocortical extracts into White Leghorn hens resulted in a "leucopenia, absolute and relative lymphopenia and absolute and relative increase in granulocytes attributable mainly to heterophils." Calculation of their printed data also indicates an eosinopenia.

The present results are similar to (although more extensive than) those reported by Wellar and Schechtman(11) who treated embryos with one injection of adrenocortical extract and sampled blood prior to and after hatching (up to 1 month). No lymphopenia was produced, however a marked "polymorphonuclear leukocytosis" occurred 24 hours after injection.

The present results also differ from studies on mammals in which lymphopenia was reported following injection of ACTH in mice, rats, rabbits and human beings(12). Lymphopenia was regarded as a specific response to ACTH since it did not occur in adrenalectomized animals; the polymorphonuclear leucocytosis was regarded as non-specific because it occurred in adrenalectomized as well as intact animals(13).

Details of the physiologic mechanism responsible for the results in this study are unknown. That the leucocytosis was not due to hemoconcentration is indicated by the facts that it occurred in spite of the slightly lowered hematocrit and that the differential counts were markedly changed also.

In spite of the high coefficient of variation and apparent low precision of acidophilia as a *quantitative* criterion of ACTH injection, under the described experimental conditions, results of the two "assay" trials do confirm the use of this criterion as a *qualitative* indicator of ACTH injection and presumably of intrinsic ACTH release.

Summary. Injection of ACTH into 7-14 day old White Leghorn cockerels was followed within 6 hours by a marked absolute leucocytosis and heterophilia and a slight lymphocytosis; no consistent change in numbers of eosinophils, basophils or monocytes was evident within 24 hours. A slight decrease in hematocrit was also produced. The magnitude and constancy of the acidophilia obtained following ACTH injection suggests its possible use as a qualitative criterion of ACTH and/or acute stress in the bird.

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Probable Polypeptidic Nature of Erythropoietin.* (23555)

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Previous work from this laboratory has been concerned with the erythropoietic stimulating effects of plasma extracts from anemic animals and human subjects(1-4) and from hypoxic hypoxic animals(5). The present communication describes certain chemical and physical properties of erythropoietic stimulating factor (EPF) from anemic rabbits. It is hoped that further study of this factor may reveal information relevant to the isolation and purification of the factor.

Materials and methods. Preparation of plasma extracts. New Zealand rabbits were made anemic by daily subcutaneous injections of 1 ml of neutralized (with NaOH to pH 7.0-7.5) (2.5%) aqueous solution of phenylhydrazine hydrochloride until the rabbits' hemoglobin was less than 5 g %. Plasma drawn in heparin (20 mg 100 ml blood) was pooled and frozen at −5°C until approximately 1 l was collected. It was then deproteinized by a modification of the method of Borsook *et al.*(6). The frozen plasma was melted and adjusted to pH 5.5 with 1 N HCl. It was then deproteinized in batches of 250 ml by placing in boiling water for 15 min. The precipitate, which was removed by filtration, was washed with 400 ml of boiling distilled water which was again placed in boiling water for 5 min. before filtration. The filtrates and washings were combined and concentrated *in vacuo* in a flash evaporator to ⅓ the original plasma volume (T < 55°C). Control plasma was obtained from normal untreated rabbits and deproteinized in the same manner. Extracts which received only this treatment are called "pH 5.5 extracts". Some extracts were treated further with trichloroacetic acid (TCA extracts) or by heating at pH 9 (pH 9 extracts). The preparation of the TCA extract was done essentially according to the method of Borsook *et al.*(6). To 1 volume of pH 5.5 extract at 5°C was added 0.1 volume of 50% TCA. After filtration the filtrate was extracted 3 times with equal volumes of ether to remove the TCA. Dissolved ether was removed in the flash evaporator. It was then dialyzed in 36/32" Nojax casing against running tap water for 48 hrs. (T = 9°C). An easier procedure which, however, removed less protein was afforded by heating at pH 9. Alkali (1 N NaOH) was added to the pH 5.5 extract until pH 9 was reached. The solution was then placed in a boiling water bath for 5 min. After the small amount of precipitate had been removed by centrifugation, it was dialyzed against running tap water. *Dialysis.* Dialysis was performed at 5°C against an equal volume of 0.16 M NaCl for 48 hrs. Plasma extract was contained in 20/32" Nojax cellulose casing. Both the dialysate and the dialysand (inside the casing) were tested.

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