on separate days for all samples. Food aliquots representing 25% or 50% of the weighed daily food intake, except the self-selected diets, were homogenized and assayed for riboflavin.

Results and discussion. The fecal riboflavin values observed during the control periods are consistent with other data from this laboratory for subjects ingesting essentially the same basal diet. Similar fecal riboflavin concentrations are also reported by Hathaway and Lobb[6] for subjects ingesting a natural diet containing 1.33 mg of riboflavin.

Upon supplementation of the diets of the subjects with 10 mg of pteroylglutamic acid daily for periods of 25 to 40 days, regular and consistent decreases were observed in fecal riboflavin. Values comparable to the lower range in this series have been noted in this laboratory only in one other instance, *i.e.*, when subjects were consuming a diet composed of milk with ascorbic acid and mineral supplements.[7]

Darby *et al.*[8] have noted that certain deficiency states due to decreases in gastrointestinal absorption may be relieved by the administration of pteroylglutamic acid. These observations suggest that in the present experiment, certain vitamin interrelationships involving the absorption of riboflavin might be concerned in the decrease in fecal riboflavin in the presence of long-continued daily dosage of 10 mg of pteroylglutamic acid. Another obvious possibility is that administration of pteroylglutamic acid measurably influences the character of the intestinal flora, resulting in a decrease in riboflavin synthesis, an increase in riboflavin destruction or both. The nature of this pronounced change in fecal riboflavin is being investigated; these results are being reported at this time because of their possible clinical implications.

Summary. As a part of long-time experiments in this laboratory on riboflavin metabolism in human subjects on weighed diets of known riboflavin content, it was noted that with the daily administration of 10 mg of synthetic pteroylglutamic acid for 25 to 40 days there was a striking decrease in fecal riboflavin values as compared to basal periods.

[6] Hathaway, M. L., and Lobb, D. E., *J. Nutrition*, 1946, **32**, 9.

[7] Gardner, J., Neal, A. L., Peterson, W. H., and Parsons, H. T., *J. Am. Dietet. Assn.*, 1943, **19**, 683.

[8] Darby, W. J., Jones, E., Warden, H. F., and Kaser, M. M., *J. Nutrition*, 1947, **34**, 645.

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Effect of Adrenal Cortical Extract on the Blood Picture and Serum Proteins of Fowl.

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Preliminary to studies on the effects of adrenal cortical extract (ACE) on embryonic development of the chick's blood, it became desirable to know how the blood elements of the adult fowl respond to cortical substances. We have been unable to find pertinent data for the fowl; furthermore the results obtained with various mammals are not consistent. Absolute peripheral lymphopenia has been observed in the normal mouse,[1] rat,[1,2,3] rabbit,[1] dog,[2] and in man[4,5] after injections of pituitary adrenotrophic hormone, and in the mouse,[1] rat,[1,6] rabbit,[1] and man[1,4,5] after

[1] Dougherty, T. F., and White, A., *Endocrinology*, 1944, **35**, 1.

[2] Reinhardt, W. O., Aron, H., and Li, C. H., PROC. SOC. EXP. BIOL. AND MED., 1940, **57**, 19.

[3] Yoffey, J. M., and Baxter, J. S., *J. Anat.*, 1946, **80**, 132.

[4] Forsham, P. H., Thorn, G. W., Prunty, F. T. G., and Hills, A. G., *J. Clin. Endocrinology*, 1948, **8**, 15.

adrenal cortical substances. However, others[3,7] have reported little or no change in the blood cell picture of the rabbit after therapy with ACE. The normal rat[8] and cat[9] have been shown to respond to pituitary adrenotrophic hormone therapy with a marked decline in number of thoracic duct lymphocytes. On the other hand Valentine *et al.*[10] observed no change in number of thoracic duct lymphocytes in cats injected with ACE.

The relationships of hypercortico-adrenal activity to serum protein levels in mammals is likewise controversial. Dougherty and White[11] report such activity to be associated with elevation in total serum protein in mice and rats. In the rabbit there is an increase in serum globulin due to rise in beta and gamma globulin.[12] In rats a decrease in gamma, but an increase in alpha and beta globulin, after ACE therapy has been reported.[13] Li and Reinhardt[14] found no effect of pituitary adrenotrophic hormone on plasma globulins in the normal rat, and Eisen *et al.*[15] detected no change in gamma globulin concentration after repeated injections of ACE, although a single injection did raise the serum antibody nitrogen in immunized rabbits.

It is our purpose in the present paper to report changes in the blood picture of 2 breeds of fowl at various periods after a single injection of ACE. Some observations on serum protein levels are included.

Method. Adult Single Comb White Leghorn laying hens and New Hampshire Red non-laying pullets were used. The Leghorns were 7 months of age at the outset of the experiment and weighed 1800 to 2200 g. The pullets were 3½ months old and weighed 1800 to 2200 g. The animals were housed outdoors in wire bottom cages protected from wind, dampness, and rain, and were maintained on commercial diets.

Blood was obtained from the ulnar vein and the smears were usually stained within 24 hours using Wright's and Giemsa, the latter diluted tenfold. The differential white blood cell determinations were made by counting 300 leucocytes, and occasionally 400, in representative regions of each slide.

Blood for both total leucocyte and erythrocyte counts was diluted 100 times[16] in a certified red blood cell diluting pipette with a solution[17] composed of 25 mg of neutral red added to 100 ml of 0.9% saline, and the cells were identified in a Neubauer Brightline Counting Chamber.

Total protein of the sera was estimated with the biuret reagent of Weichselbaum.[18] Optical densities of the sera were determined at 555 mu on a model 11A Coleman spectrophotometer by subtracting the optical density readings of serum-containing cuvettes from those with saline prepared at the same time. The density readings are thus simply measures of the relative total protein content rather than of absolute amounts.

Precipitation of total serum globulin was carried out with the ammonium sulfate method of Fine.[19] Nine and a half ml of 27.79% ammonium sulfate was added to 0.5 ml of serum placed in a 15 ml conical centrifuge tube, giving a final concentration of 2M ammonium sulfate. The tubes were refrigerated overnight and centrifuged the next

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7 Fox, C. A., and Whitehead, R. W., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 756.

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11 Dougherty, T. F., and White, A., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 26.

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19 Fine, J., *Biochem. J.,* 1935, **29**, 799.

TABLE I. Effect of a Single Injection of 2 ml of ACE* on Blood Cell Levels in 11 White Leghorn Laying Hens.

	Hr	RBC × 1000	Cells per cu. mm WBC	Polymorph	% Lymphocyte	Monocyte	Eosinophil	Basophil	Heterophil
Mean	Pre-O†	2,714	27,118	4,068	77.1	7.9	1.5	1.6	11.9
S.D.‡		243	4,288	1,792	4.8	2.1	0.6	0.8	5.0
Mean	0	2,738	30,727	5,715	76.0	5.4	0.8	1.1	16.7
S.D.		325	5,656	2,794	8.4	2.0	0.7	0.9	7.0
Mean	1	2,448	23,383	4,887	73.8	5.3	0.3	1.4	19.2
S.D.		274	6,862	3,132	7.9	2.4	0.3	0.9	7.6
Mean	3	2,367	18,378	5,440	64.3	6.1	0.4	1.5	27.7
S.D.		256	7,061	3,173	11.4	2.4	0.5	1.1	11.3
Mean	6	2,492	26,879	7,768	66.2	4.9	0.4	1.2	27.3
S.D.		238	6,967	2,858	10.0	2.5	0.4	0.9	9.1
Mean	9	2,588	30,107	7,165	72.2	4.0	0.5	0.8	22.5
S.D.		300	6,163	3,235	11.3	1.5	0.5	0.6	9.4
Mean	24	2,624	29,573	4,968	78.7	4.5	0.7	1.6	14.5
S.D.		447	5,875	3,247	10.4	2.2	0.6	1.2	8.4

* Wilson's product injected into 9 hens and Upjohn's into 2 hens.
† Derived from 40 counts made on the same 11 hens during the 4 weeks preceding injection.
‡ Standard deviation of the mean; S.D. $= \sqrt{\frac{\Sigma d^2}{n-1}}$

morning at 2500 r.p.m. for ½ hour. The supernatant, containing mainly albumin and ammonium sulfate, was separated from the packed precipitate by decantation, and the tubes were inverted for 1½ hours on filter paper to remove excess liquid. The globulin was dissolved in 0.3 ml of 0.85% saline. To 1/10 ml of this protein solution in a cuvette were added 4.9 ml of 0.85% saline and 5.0 ml of biuret reagent. Optical density readings were made at 555 mu on the spectrophotometer. Each serum sample was precipitated and tested in duplicate.

The adrenal cortical extracts (aqueous)* and sterile 0.85% saline were injected subcutaneously in the pectoral region. Immediately prior to the injections blood counts were taken from each animal and are designated in the tables as "0 hour" counts.

Experiments and Results. (1) *White Leghorn laying hens.* A single injection of 2 ml of ACE (Table I) was followed by a marked leucopenia, pronounced absolute lymphopenia, decrease in number of erythrocytes and an absolute polymorphonuclear leucocytosis. The severe lymphopenia, indicated by a post-injection mean of ½ the pre-injection mean at 0 hour accounts for the leucopenia. Differential counts revealed a 11.7% decrease in lymphocytes and a concomitant increase of 11% in polymorphonuclear cells 3 hours following injection. The latter change was apparently due mainly to heterophils. All changes were maximal at 3 hours after injection except for the absolute granulocyte alteration, which was maximal at 6 hours. The blood picture returned to normal by the end of 24 hours. The absolute and differential alterations are statistically significant; furthermore all animals showed the absolute changes, and only one hen failed to reveal the percentage deviations.

The effects of saline injections, of a 24-hour fast (with water *ad libitum*), and of fasting plus saline are shown in Table II. These procedures caused no appreciable alteration in the blood picture. The effect of fasting

* We are indebted to Dr. David Klein of the Wilson Laboratories and to Dr. W. F. Wenner of the Upjohn Company for supplying us with their adrenal cortical products.

TABLE II.

Effects of Saline, of Fasting, of Saline After Fasting, and of ACE* After Fasting on the Blood Picture of White Leghorn Laying Hens.

	No. of hens	Hr.	Cells per mm³ RBC × 1000	WBC	Lymphocyte	Polymorph	% Lymphocyte	Monocyte	Eosinophil	Basophil	Heterophil
Inj. of 2 ml of saline											
Mean	3	0	2,507	26,917	20,457	5,087	76.0	5.1	2.1	1.3	15.5
S.D.			249	4,653	3,253	1,274	3.0	1.0	0.9	0.8	3.9
Mean		3	2,703	24,917	19,012	4,460	76.3	5.8	1.7	1.9	14.3
S.D.			255	4,404	2,244	1,630	4.2	1.1	1.2	0.9	2.3
Mean		6	2,497	26,667	20,240	4,987	75.9	5.4	1.3	1.1	16.3
S.D.			255	5,125	3,778	1,708	4.7	0.8	0.6	0.6	4.4
Mean		9	2,635	25,167	18,322	5,863	72.8	3.9	1.7	1.2	20.4
S.D.			320	1,258	702	1,784	6.3	0.8	2.5	0.6	3.3
After 24-hr fast											
Mean	10	0	2,651	26,000	18,850	5,330	72.5	7.0	1.1	1.6	17.8
S.D.			320	5,678	4,286	2,833	10.8	2.9	0.5	0.8	9.0
Mean		24	2,730	25,500	17,570	5,916	68.9	7.9	1.2	2.0	20.0
S.D.			369	5,793	4,521	3,246	12.7	4.6	0.6	1.6	9.6
Inj. of 3 ml of saline after fasting											
Mean	3	0	2,670	23,583	16,720	5,613	70.9	5.3	1.6	2.0	20.2
S.D.			255	1,808	2,234	573	4.3	1.9	1.4	1.2	4.4
Mean		3	2,730	22,750	15,971	5,642	70.2	5.0	1.1	2.1	21.6
S.D.			152	2,750	3,783	1,785	9.9	1.8	1.1	0.2	6.3
Inj. of 3 ml of ACE after fasting											
Mean	10	0	2,334	23,000	17,204	4,623	74.8	5.1	0.9	1.7	17.5
S.D.			326	4,452	4,393	1,396	7.4	1.8	0.7	1.3	6.0
Mean		3	2,270	10,450	5,706	4,096	54.6	6.2	0.2	1.1	37.9
S.D.			263	2,020	1,704	1,075	11.3	2.7	0.3	0.8	10.3

* Product of Wilson Laboratories.

was studied since it was considered desirable to reduce the high lipid content of the sera[20] for electrophoretic analyses.

Two months after the above injection of 2 ml of ACE, the same group of birds was fasted for 24 hours and then given 3 ml of ACE (Wilson). Cell count alterations were similar to those obtained 3 hours after the first injection of 2 ml of ACE except that there was no significant decrease in number of erythrocytes (Table II). The lymphocyte mean was ⅓ the pre-injection mean. In one hen showing an absolute lymphopenia involving a drop from 22,400 to 5,490, an electrophoretic analysis (using veronal buffer)† of the serum showed no significant change in any protein fraction from levels found in the same animal's serum after saline injection 3 days earlier. However, the post-extract serum showed a 15% total serum protein increase.†

(2) *New Hampshire non-laying pullets.* A single injection of 3 ml of saline into 12 New Hampshire pullets did not alter the peripheral blood cell picture significantly (Table III). Ten days after the saline injection, each animal received a single injection of 3 ml of ACE. Three hours after injection there was an absolute lymphopenia (Table III) with a mean of 12,833 compared to the pre-injection mean of 20,466 and a slight drop in the number of erythrocytes. The total leucocyte level remained normal as a result of increase in the number of granulocytes. Differential leucocyte determinations indicate a mean decline of 27.9% in lymphocytes, an increase of 26.3% in polymorphonuclear cells, mainly due to heterophils, and a slight drop in eosinophils.

Although the aforementioned changes in blood cell values are statistically significant, data for total protein variation between post-saline and post-extract levels (Table III) yields a t value of 2.0 which is slightly below the criterion of significance (2.2). If the protein values for individual birds are considered

TABLE III. Effect of 3 ml of ACE (Wilson) on Blood Counts, Total Serum Protein, and Total Globulin Levels in 12 New Hampshire Red Non-laying Pullets (treated 10 days earlier with 3 ml of saline).

	Hr.	Cells per mm³ RBC × 1000	WBC	Lympho-cyte	Poly-morph	% Lympho-cyte	Mono-cyte	Eosino-phil	Baso-phil	Hetero-phil	Serum optical density reading Total protein	Total glob.
Pre-sal. Mean	0	2,432	30,208	20,511	7,884	67.9	6.0	1.8	3.0	21.3		
S.D		344	8,818	6,188	4,005	9.8	1.7	1.4	1.1	9.5		
Post-sal. Mean	3	2,398	28,854	18,669	8,397	64.7	6.2	1.4	3.2	24.5	.425	.200
S.D.		196	5,303	4,917	3,667	10.6	2.2	0.6	1.2	9.3	.091	.064
Pre-ACE Mean	0	2,679	27,000	20,466	5,184	75.8	5.0	1.2	2.3	15.7		
S.D.		390	4,979	4,749	1,357	5.7	2.1	1.0	1.2	4.5		
Post-ACE Mean	3	2,535	25,792	12,833	12,190	47.9	6.6	0.3	1.9	43.3	.456	.187
S.D.		313	5,405	3,395	3,631	8.9	2.2	0.4	1.4	8.5	.087	.048

Note: t value for protein change is 2.0, and for globulin 1.2; $t = \frac{x}{s}\sqrt{n-1}$, x = difference in means and $s = \sqrt{\frac{\Sigma(x_i - x)^2}{n}}$ where x_i = difference between the control and experimental value in the same animal.

[20] Herrmann, G. R., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 229.

† Performed through the courtesy of Dr. John Mehl, Professor of Biochemistry at the University of Southern California School of Medicine. Total protein determined by a biuret method.

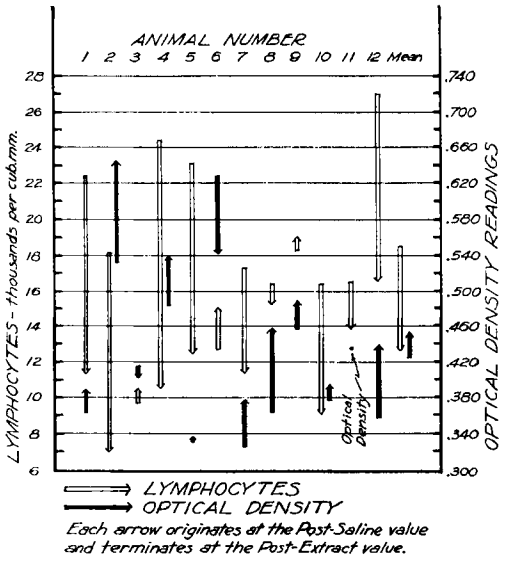

FIG. 1.
Effect of a single injection of 3 ml of ACE on lymphocyte and total serum protein levels in each of 12 New Hampshire Red non-laying pullets.

(Fig. 1), it is evident that 9 of the 12 pullets showed higher serum protein levels and one showed no change. Despite the borderline t value, this indicates a trend toward elevation in total serum protein.

It is evident from Fig. 1 that increase in serum protein levels is associated with lymphopenia. Of 9 animals revealing a lymphopenia, 8 also showed an elevation in serum protein, and one no change. Two pullets (No. 3 and 6, Fig. 1) deviated from this general picture, showing an apparent decrease in serum protein accompanied by an increase in lymphocytes. In one of these (No. 3) the changes observed are insignificant. While the results suggest a correlation between lymphopenia and serum protein increase in individual pullets, there appears to be no obvious quantitative relationship.

The t value for the difference between globulin readings was 1.2, 6 animals showing a decrease, 5 an increase, and 1 no change after extract injection. We have, therefore, no indication of a consistent globulin change as a result of ACE injection.

Discussion. The different degrees of lymphopenia obtained in the present experiments may be due to an inherent breed difference in reactivity, to differences in age since lymphoid tissue is less active in older birds,[21] or to differences in amounts of injected extract. The overall picture, however, indicates absolute and relative lymphopenia and absolute and relative heterophil leucocytosis occur consistently after a single injection of ACE into both breeds of fowl. Furthermore the maximal lymphopenic effect occurred at 3 hours in the present study on the fowl, which is in good agreement with the work on the rat and rabbit.[1]

The present results suggest an augmentative effect of ACE therapy on total protein levels in fowl. However, we can not be certain that the effect was due solely to the ACE injected inasmuch as 10 days elapsed between bleeding of animals for control and experimental serum protein determinations. The apparent increase in protein level may possibly have been due to 10 days of maturation, although no significant changes were found in the lymphocyte counts. The data of Herrmann[20] suggests that non-laying chickens of 11-22 weeks of age have a serum protein mean of 4.02 $\pm$ .5 (S.D.) as compared to 6.1 $\pm$ 1.3 for laying hens. Definite conclusions on the effects of ACE on serum protein levels must await more complete data on protein changes in the serum of the normal fowl during the 15th and 16th week of development.

Conclusions. 1. White Leghorn laying hens injected subcutaneously with aqueous adrenal cortical extract, showed leucopenia, absolute and relative lymphopenia and absolute and relative increase in granulocytes attributable mainly to heterophils. The effects were maximal at 3 hours and the blood picture returned to normal by the end of 24 hours.

2. New Hampshire Red non-laying pullets injected with ACE displayed similar changes with the exception of leucopenia which was masked by increase in polymorphonuclear cells.

3. ACE therapy produced no statistically significant increase in total serum protein or serum globulin levels. However, a trend toward increase in total serum protein, accompanied by lymphopenia, is indicated for the majority of the individual birds studied.

[21] Biester, H. E., and Devries, L., Diseases of Poultry, The Collegiate Press, Inc., 1945, ch. IV.