to reflect subtle differences in B_{12} metabolism. One might postulate that the greatest use of these compounds would be in clinical entities embodying highly deranged B_{12} metabolism, as in leukemia and liver disease(12,13,14).

Summary. The anilide, ethylamide, ethylamide monoacid, and di-(ethylamide) derivative of B_{12} inhibit cobalamin utilization by *O. malhamensis* and *E. coli.* The monobasic acid inhibits B_{12} utilization by *E. coli,* but not by *O. malhamensis:* the reverse is true for the dibasic acid. Methylamide and carbanilide derivatives are utilized as well as true B_{12}; all compounds are as active as true B_{12} for *E. gracilis* and *L. leichmannii.* In *E. coli,* suboptimal levels of methionine in presence of B_{12} amides and acids give increasing growth with increasing concentrations of B_{12} derivatives; neither methionine nor B_{12} acid and amide, when added alone, permit growth.

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Influence of ACTH upon Avian Species and Osteoporosis.* (25718)

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During previous investigations(1,2,3), osteoporosis was found in the culls of a flock of White Leghorn hens bred for heavy egg production. Endocrine interactions and role of pituitary, adrenals, and gonads in pathogenesis of this disorder are obscure. In the human, *species in which osteoporosis is common,* it is debatable whether exogenous ACTH is a causative agent or whether it only aggravates a pre-existing condition. Adrenal cortex increases in size and weight during reproduction in birds, due to hyperplasia and hypertrophy and occurs presumably through increased secretion of ACTH. In White Leghorn hens and roosters, the cortex represents 43% of weight of both adrenal glands(4). Bovine ACTH increases size of adrenal cortex, and prolongs survival of hypophysectomized birds; cortisone sustains the animal for indefinite period. Both ACTH and cortisone are powerful suppressors of bone growth in the chick(5). Pituitary - adrenocortical - gonadal hormone interactions have been demonstrated in birds(4,6,7) by the following experiments: (a) castration causes enlargement of adrenal cortex; (b) unilateral ovariectomy is followed by hypertrophy of opposite vestigial ovary if adrenals are intact but not if adrenal glands are also excised; (c) adrenal cortex may react to injections of small or large doses of estrogen or androgen in various ways, directly and indirectly through liberation or suppres-

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TABLE I. Reaction of Adrenal Glands, Ovaries, and Skeleton to ACTH.

No. of birds	Sex	Dose (mg lyophilized ACTH)	Days	Ovary wt. g/kilo	Adrenal wt. mg/kilo	Serum calcium	Blood sugar	Avg No. eggs/wk	Intramedullary bone†	Osteoporosis‡
7	♂		14		55.1 ± 5*	9.5 ± .5*	200. ± 21*		.0	1.5
7	♀		"	11.4 ± 2*	80.1 ± 3	20.3 ± 2.1	232. ± 28	6	1.	1.
4	♂	240	"		41.9 ± 5	8.8 ± .4	239.6 ± 22		.0	1.5
4	♀	"	"	10.1 ± 2	93.1 ± 3	34.5 ± 2.5	235.4 ± 19	4	.5	1.
21	♂	525	21	5.9 ± 1	91.1 ± 2	21.1 ± 1.9	230.1 ± 24	5	1.5	.7
21	♀	480	21		40.7 ± 3	10.6 ± 1.	208.1 ± 19			.0
21	♂	"	"	7.2 ± 2	101.2 ± 5	19.1 ± 1.	228. ± 18	5	1.5	.5
21	♀	770	42		58.1 ± 2	12.64 ± .6	200.5 ± 15		.0	.0
21	♂	"	"	3.4 ± 1	109.4 ± 5	54. ± 4.	239. ± 26	4	2.5	.2
21	♀	70	49	9.9 ± 2	81.5 ± 2	28.7 ± 2.1	221.4 ± 14	4	2.5	.2

* Mean ± stand. error. † Avg thickness of deposit in mm. ‡ Avg thickness of cortex in mm.

sion of ACTH by the pituitary, but in general estrogen increases and androgen decreases weight of adrenals; (d) enlargement of adrenal gland following castration is prevented by administration of androgens. Our object is to measure levels of plasma corticosterone and 17-OHCS (17-hydroxy-corticosteroid), weight of adrenal glands, and gonads, and to correlate such data with calcium and bone metabolism.

Materials and methods. Fifteen adult Black Minorca roosters and 19 White Leghorn hens, purchased in open market and reared on standard commercial feed, were treated with lyophilized ACTH, 40 mg/injection at intervals and for periods of 14-49 days. Observations were made on various constituents of serum including proteins by paper electrophoresis, gross and microradiography, routine microscopic and special histochemical preparations, by methods described previously(2,3). Adrenal glands and ovaries were weighed wet before fixation in formalin for histological sections. Heparinized blood was collected from 14 untreated birds and 10 birds treated with intraperitoneal or intramuscular injections of either lyophilized or oxycel processed ACTH for 1 week. Level of 17-OHCS in plasma was measured by method of Nelson and Samuels(8); corticosterone was measured by method of Silber, *et al.*(9).

Results. Control roosters. Weight of 2 adrenal glands/kilo of control rooster was only 55.1 ± 5 mg (Table I). Level of blood sugar was approximately 200 mg%. There was no intramedullary new bone. Level of corticosterone was more than twice that of 17-hydroxy-corticosteroid (Table II).

Control hens. Weight of adrenal glands/kilo of control hen was 80.1 ± 3 mg. White Leghorn hens weighing an average of 1.6 kilo

TABLE II. Levels of Corticosterone and 17-Hydroxycorticosterone in Normal and ACTH-Treated Birds.

No. birds	Sex	Dose ACTH	Corticosterone Range in γ/100 cc plasma	17-OHCS Range in γ/100 cc plasma
7	♂		6.5 ± 2	2. ± 1
7	♀		8. ± 3	2.5 ± 1
5	♂	400 IU	12 ± 5	"
5	♀	"	15 ± 4	"

had adrenals weighing 60% more than Black Minorca roosters weighing an average of 3.5 kg. Level of blood sugar was as high as 232 mg %. Plasma level of both corticosterone and 17-hydroxy-corticosterone was also slightly higher in the laying hen than in the rooster. Deposits of intramedullary bone in the tibia varied from 0.5 to 2.5 mm in thickness from one phase to another in the egg-laying cycle.

Roosters injected with ACTH. Injections of mammalian ACTH produced slight increase in weight of avian adrenal gland. Level of corticosterone in plasma, however, increased approximately 2-fold (in both sexes); level of 17-hydroxy-corticosterone changed little if at all. Changes in chemical composition of other constituents of blood were hardly appreciable compared to effects observed previously in estrogen-treated birds(2). No change was found in serum total lipid, phospholipid, total nitrogen, albumin, globulin, A/G ratio, total phosphorus, inorganic phosphorus, or alkaline phosphatase. Blood sugar was elevated very slightly if at all. At conclusion of experiment in birds treated 6 weeks, there was a slight hypercalcemia. Bones showed slight, if any, thinning of cortex, and no intramedullary osteogenesis. There was no demonstrable evidence of osteoporosis.

Hens injected with ACTH. Adrenal glands in hens were enlarged and increased in weight. Level of corticosterone in plasma increased significantly while 17-OHCS remained unchanged. Ovaries became progressively smaller at 2, 4, and 6 weeks after treatment. The reduction was apparently proportional to dose of ACTH and duration of treatment. In hens previously in heavy lay, egg production was gradually reduced from 6-7 to 4 eggs/week. Level of serum calcium in hens observed after 2 and 4 weeks treatment varied (as in control hens) from 10 to 35 mg%. In 2 hens observed after 6 weeks, total calcium was even higher. ACTH did not inhibit synthesis of phosphoprotein by the liver, but some calcium added to serum may not have been protein bound. Paper electrophoresis showed X_1-X_2 bands but they were not as dense as in control hens. Serum lipid and phospholipid and other components normally found in blood of laying hens were the same in treated birds and control birds. Level of blood sugar was not elevated. The cortex of long bones was thinner in hens than in roosters and showed increased number and size of vascular spaces; bone resorption appeared to occur without osteoclasts, and osteoporosis was extreme in hens treated with ACTH for 6 weeks. The marrow cavities were filled, however, with poorly calcified or uncalcified bone. The intramedullary deposit was not detached from the cortex as occurs in hyperparathyroidism or Vit. D and calcium deficient hens(3).

Discussion. The adrenal vein blood of the capon has been observed by Phillips and Jones (11) to contain 312 γ of corticosterone and 3 γ % of hydrocortisone. Our analyses of peripheral blood plasma of intact roosters and laying hens revealed 3 to 6 times higher levels of corticosterone than 17-OHCS. However, we found approximately the same concentration of 17-OHCS, only 2.5 γ %, in systemic blood as Phillips and Jones measured in adrenal vein blood. Using a small dose of ACTH, they were not able to stimulate secretion of corticosterone in adrenal vein blood; using 400 I.U. of ACTH, ACTH did raise the level of corticosterone in plasma, as well as increase size and weight of adrenal glands. The effect on the skeleton of pharmacodynamic doses of ACTH was to accentuate osteoporosis to which the White Leghorn female, but not the male, is predisposed. Resistance of rooster to osteoporosis may be related to metabolism of (precursors of) androgens; it is accordingly necessary to measure the output of these and related substances in blood or excreta.

Body weight. Doses of ACTH in above experiments produced gradual loss of body weight in hens in 6 weeks. Roosters were less affected in this respect. Loss of body weight is generally attributed to widespread catabolism of the labile fraction of body protein, especially muscle, similar to that which occurs in inanition. The bones of the hen were hard but became lighter and more brittle after ACTH. The osteons on the endosteal side of cortical bone were removed and replaced by large vascular spaces, fibrous tissue, and spongy bone. This occurred without formation of osteoclasts.

Level of the blood sugar normally varies from 161 to 280 mg % in birds. The range in hens and roosters treated with ACTH was 200.5 to 239.6, indicating no effect on blood sugar. In this respect, birds differ from mammals in which ACTH and adrenal corticoids have diabetogenic effects(10). Prolactin may elicit this response in oviparous animals.

The large dose of ACTH used in the foregoing experiments diminished ovarian weight and egg production steadily for 6 weeks. These changes could be attributed to suppression of production of gonadotropin by the anterior pituitary or secretion of estrogen by the ovary, or both, by corticoid hormones.

Adrenal cortex. The literature reviewed by Sturkie(10), reveals that ACTH extracted from the pituitary of a lower order can produce a response from an animal of a higher order, as well as *vice versa.* Riddle and his associates(12-14) observed a 40% enlargement of adrenal cortex and a 20% increase in level of blood sugar in doves and pigeons during reproductive cycle. Because egg production ceased in adrenalectomized birds, Riddle, *et al.*(14) observed that the adrenal cortex is essential for reproduction. It is reasonable to suppose that while mammalian ACTH affects avian adrenal cortex as evidenced by depletion of adrenal ascorbic acid (15), and(2) production of corticosterone (as shown above), avian ACTH might produce a quantitatively greater response than injections of heterogenous hormone.

Calcium protein complexes. A hypercalcemia which occurs in the laying hen is associated with formation of a calcium phosphoproteinate complex, and deposition of egg yolk, and this occurs under control of estrogen(1). In small doses the process is not altered by ACTH and adrenal cortical hormone. A different form of hypercalcemia, not associated with formation of phosphoprotein (as shown by chromatographs of their serum) occurs following prolonged treatment with large doses of ACTH. This hypercalcemia was relatively slight and could have been due to suppression of new bone formation by excess production of corticosterone. These observations require further investigation by studies of urinary and fecal excretion of calcium.

Intramedullary osteogenesis, like a secondary sex characteristic, is under control of estrogen and its function is to store and turn over a supply of calcium for calcification of the egg shell(3). Osteoporosis is a disorder consisting of increased porosity and decreased thickness of the cortex; the pathogenesis is not known.

Injections of ACTH for 4 weeks caused retention of intramedullary bone. At beginning of period of treatment while the hen was in heavy lay, overproduction of adrenal corticoids did not prevent synthesis of the proteinaceous matrix of intramedullary bone. Later, when total body weight was reduced and the cortex showed marked osteoporosis, egg production came to a halt and this relieved the skeleton of the demand for calcium for egg shells. Treatment with cortisone, as will be described(1), similarly produced retention of intramedullary bone and severe osteoporosis in laying hens. Storey investigated the action of ACTH, reviewed the literature on effect of adrenal steroids on bone in mammals and concluded that ACTH suppressed growth but did not produce osteoporosis in young rabbits(16). The circumstances in an adult animal are entirely different especially in the modern laying hen, now apparently under great stress. The modern laying hen in heavy lay has been observed to come off her legs as a result of a disorder known as "Cage Layer Fatigue"(17). CLF in birds, shortly after onset of lay, is associated with "decalcification of bones" without any change in serum calcium, phosphorus, or alkaline phosphatase(18). After a long period of laying when the pullet becomes an adult, the bones of a bird with CLF may show the characteristics of osteoporosis as described above.

Summary. Osteoporosis, occurring spontaneously in White Leghorn hens, was aggravated by injections of mammalian ACTH; the disorder did not develop in the male. It is suggested that there may be a causal relationship between ACTH, osteoporosis and the modern poultry disorder known as "Cage Layer Fatigue."

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Erythropoietic Activity of Proteolytic Enzymes. (25719)

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During some studies on effect of certain enzymes on activity of erythropoietin preparations, we found that samples of papain and bromelin could stimulate erythropoiesis. Consequently, a number of other proteolytic enzymes were assayed.

Methods and materials. A modification of the methods of Fried *et al.*(1) and Rambach *et al.*(2) was used to measure erythropoiesis. Each sample was injected subcutaneously daily for 4 days into 6 female Holtzman rats. Food was withheld throughout experiment but water was given *ad lib.* On fourth day, 0.5 μc of Fe^{59}, as ferric citrate, was injected into the tail vein. Twenty-four hours later amount of radioactive iron in an aliquot of whole blood was measured. In calculating % of Fe^{59} uptake by total red cell mass, it was assumed that blood volume was 5.18% of body weight at time of sacrifice. *Enzymes* were obtained from commercial companies (Table I). Sialic acid was determined by direct Ehrlich reaction described by Werner and Odin(3).

TABLE I. Erythropoietic Activity of Proteolytic Enzymes.

Enzyme	Purity	Source	Dose, mg	Fe^{59} uptake, avg %
Pepsin	Crystalline	Worthington	.5	7.4 ± .9
Trypsin	"	"	.1	11.1 ± 1.6
Chymotrypsin	"	"	.1	11.2 ± .2
B. subtilis proteinase	Crude	Novo	.5	9.2 ± 1.1
Bromelin	"	Takamine	.5	13.6 ± 1.1
Ficin	"	Lilly	.5	9.1 ± 1.4
Control				10.3 ± .9
Papain	Crude extract	Penick	5.0	14.3 ± 1.2
Control				7.8 ± 1.0
Papain	Partially purified	Lilly	.2	13.1 ± 1.3
Control				10.1 ± 1.5
Papain	Crystalline	Worthington	.1	13.4 ± 1.5
Control				10.3 ± .9