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Some Factors Affecting Blood Formation in Turtles. (24382)

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The fundamental factors which influence blood formation in cold-blooded animals are unknown. Hypoxia, generally considered the universal stimulus for erythropoiesis, has no effect in Pisces, Amphibia, and in Reptilia (1,2). Cobalt readily produces polycythemia when administered to birds and mammals, but in frogs an increase in erythrocyte count was produced only by a highly toxic dose(3). Liver extracts, saponin, and even experimental hemorrhage, did not stimulate production of new blood cells in the hypoplastic frog marrow(4,5). It is quite unusual that factors which normally stimulate blood formation in warm-blooded animals fail to do so in cold blooded forms. Further investigation on the physiology of blood formation in cold-blooded animals is needed. Since most of the previous work has been on amphibians, it seemed desirable to study another class such as the reptiles. Experiments have been conducted on the effects of cobalt, cobalt with iron, copper and manganese supplements, crude liver extract, a folic acid antagonist, aminopterin, and of repeated bleeding on blood formation in box turtles.

Materials and methods. Adult box turtles (*Terrapene carolina carolina*) of both sexes were housed indoors with room temperature between 20 and 23°C. They were fed horsemeat, greens, bread and tomatoes. Large pans of water were kept in the cages. Experiments were conducted between August and March, a period of sexual rest. Cobalt chloride ($6H_2O$), iron ammonium citrate, copper sulphate, and manganese sulphate were administered in aqueous solution in amounts indicated. The liver extract (crude) was obtained from Lederle Laboratories. Each cc contained Vit. B_{12} activity equivalent to 2 μg of cyanocobalamin. The folic acid antagonist, aminopterin,* was dissolved by adding 0.4 cc of 1 M KOH/100 mg aminopterin in 100 cc H_2O. In the bleeding experiment blood samples were obtained by cardiac puncture using a 2-inch 20 gauge needle inserted near the forelimb. Repeated removal of 1 to 2 cc of blood proved adequate to stimulate blood formation. In other experiments each turtle was used for only one blood sample obtained by decapitation. Erythrocyte and leucocyte counts were made by using a diluting fluid previously described(6), except that 0.1% toluidin blue O replaced Wright's stain. Reticulocyte counts were made from smears stained with new methylene blue N following reticulocyte formula described earlier(2). A Klett-Summerson photoelectric colorimeter was used for hemoglobin determinations. Hemolysis was obtained by use of 0.3 M urea or 0.1% Na_2CO_3. Tissues were fixed in 10% buffered solution (pH 7.0) of formalin. Bones were decalcified by 5% formic acid. Paraffin sections were stained routinely with hematoxylin azure eosin.

Results. Control blood. Mean hematocrit values, hemoglobin concentration and erythrocyte counts of male turtles are significantly higher than in females (Table I). Leucocyte counts showed no significant sex difference. Thrombocytes were included in the leucocyte counts since they were difficult to distinguish in suspensions from lymphocytes and showed little tendency to clump. Differential analysis of leucocytes in blood smears was made. Mean control values for 13 turtles (both

* Aminopterin kindly supplied by Dr. J. M. Ruegsegger, Lederle Laboratories, Pearl River, N. Y.

TABLE I. Effects of Aminopterin on Blood of 13 Box Turtles.

Sex	No. inj.	No. days	Hemato-crit	Hb, g/100 cc	Erythrocyte count, mm³ × 100,000	White blood count, mm³ × 1000	Reticulo-cyte, %	Body wt, g
♀	8	10	28	5.0	7.0	13.5	.0	−13
♀	22	30	25.2	5.2	8.3	16.5	.0	−63
♀	22	30	29	8.2	7.6	25.0	1.0	−65
♀	22	30	15.5	4.2	4.7	5.0	.0	−37
♀	22	30	20	3.9	5.5	13.5	.0	− 7
♂	33	34*	18					−19
♀	34	35	23	5.1	6.5	12.0	1.0	−26
♀	40	42	25	2.1	1.6	2.0	.0	− 9
♂	40	42	31	7.5	7.8	3.5	1.0	−30
♂	40	42*						−16
♀	49	51	20	3.8	4.1	1.5	.0	−24
♀	49	51	19	3.1	2.8	2.0	2.0	−43
♂	49	51	16	3.0	3.4	2.5	.0	−22
♂	Control†		26.9 ± .5	6.2 ± .3	7.0 ± .2	21.4 ± 1.0	1.3 ± .4	
♀	" ‡		22.4 ± .7	5.4 ± .3	6.1 ± .2	25.1 ± 1.6	1.7 ± .3	

* Died. † Mean ± SEM of 25 male turtles. ‡ Mean ± SEM of 29 female turtles.

sexes) are as follows: eosinophiles (spindle granules) 29.5 ± S.E.M. 5, eosinophiles (spherical granules) 15.5 ± 2.4, basophiles 10 ± 2.7, monocytes 5.2 ± 1.2, lymphocytes 40 ± 4, and blast cells 0. Both carapace and plastron contained marrow, but the relatively small number of marrow cells present made preparation of smears impractical. Smears prepared from limb bones were most useful in demonstrating blood cells in various stages of development. Histologic study of shell and limb marrow showed that eosinophiles with spherical granules were abundant, while lymphocytes and reticulum cells were less numerous and much more difficult to identify.

Cobalt injections. Three dosage levels were used. Eleven turtles were given 0.5 mg cobalt/100 g body weight subcutaneously 5 times weekly. One died after 14 injections in 19 days and the remainder were killed at intervals of 29 to 147 days. There was no significant alteration in numbers of erythrocytes, reticulocytes and leucocytes, in hemoglobin concentration, or in hematocrit values in turtles when compared with controls. Six turtles were given 2 mg cobalt/100 g body weight intraperitoneally. One turtle died and 3 were killed after 8 injections in 14 days, and the others were killed after 12 injections on day 16. Erythropoiesis was not increased in any of these turtles. Six turtles were given 5 mg/100 g body weight intraperitoneally. This dose proved to be toxic; 4 turtles died after 5 injections. The 2 others were killed after 5 injections in 6 days. Blood studies showed no evidence of stimulation of blood formation.

Eight turtles were given subcutaneous injections of 0.5 mg cobalt/100 g body weight and an intramuscular injection containing 0.05 mg copper sulphate, 0.08 mg manganese sulphate and 1 mg iron ammonium citrate/100 g body weight. This dose proved to be toxic, killing 3 turtles after 5 injections in 7 days. The remainder were sacrificed after receiving 6 injections in 10 days. Except for 1 turtle with a reticulocyte percent of 5.6/1000 erythrocytes, there was no evidence of any stimulation of blood formation.

Crude liver extract. Twenty-one turtles were given intramuscular injections of 0.1 cc of crude liver extract 5 times weekly from 1 to 4 weeks. One turtle had a reticulocyte % of 6.7/1000 erythrocytes after receiving 14 injections. The other turtles were examined at various intervals throughout the 4 weeks and none gave evidence of stimulation of blood formation.

Effects of repeated bleedings. The reticulocyte counts of turtles bled at various intervals are given in Table II. There was a marked increase in reticulocyte number after third and fourth bleeding. High reticulocyte counts persisted for at least 21 days and as observed in turtles 8 and 9 for as long as 50 days. The mean erythrocyte count of 9 turtles studied was reduced from 591,000 to 313,000/cu mm following 4th bleeding. Normal blood values were not restored until 4 months

TABLE II. Effects of Repeated Bleeding* on Reticulocyte Count of 9 Turtles.

Reticulocyte %					
Days of bleeding					
0	3	10	17	38	67
1.5		0	20		
1	3	3	20		
0	1	1	4		
4.5	2	0	16		
0	0	15	32	34	
1	3	1	4	36	
2	2	6	16	61	
.5	0	2	6		10.5
2.5	2	16	14		15.5

* 1 to 2 cc blood removed by cardiac puncture.

after last bleeding.

Aminopterin injections. Thirteen turtles (Table I) were given intraperitoneal injections containing 0.05 mg aminopterin/100 g body weight 5 times weekly from 10 to 51 days. All lost weight and 2 died. There was a consistent reduction in erythrocyte count, in hemoglobin concentration, and in hematocrit values after 6 weeks of treatment with aminopterin. A reduction in leucocyte counts was noted as early as second week. Only 1 turtle receiving aminopterin showed a normal leucocyte count. Severe leucopenia developed in all turtles after 6 weeks treatment. Analysis of blood smears showed that eosinophiles were completely eliminated after 30 days treatment. Within 6 weeks 4 of 6 turtles showed a marked reduction in lymphocytes to values as low as 4%. The numbers of basophiles and monocytes appeared to be unaffected by the drug. After 30 days treatment blasts cells appeared in peripheral blood occupying from 1 to 5% of total cells. One exceptional turtle had 18% blast cells in a smear examined following 51 days of aminopterin. Study of bone marrow smears also showed absence of eosinophiles after 30 days. The most conspicuous change in marrow was the great increase in number and size of blast cells. The blast cell in marrow of a normal turtle measures approximately 18 μ, after 5 weeks treatment with aminopterin many increased to as much as 24 to 30 μ. Large, irregularly shaped reticuloendothelial cells, observed only rarely in controls, were numerous in marrow and in spleen smears of treated turtles.

Discussion. Lack of erythrocyte stimulation in box turtles when treated with cobalt of varying concentration or by cobalt with mineral supplement emphasizes how ineffective cobalt treatment is in cold blooded animals. A strong dose of cobalt (20 to 30 mg/100 g body weight) administered to frogs(3) produced a 2 to 4 fold increase in erythrocytes in a few days; however, this dose produced anemia after 1 week. It is not clear whether or not the increase in erythrocytes in frogs was due to the action of cobalt on erythropoietic tissue or to hemoconcentration resulting from toxicity of the drug. Reticulocyte studies were not reported. The failure of hypoxia(1,2) and of non toxic doses of cobalt to stimulate erythropoiesis in cold blooded animals suggests that there may be a fundamental difference in the mechanism of blood formation between cold and warm-blooded animals. According to current concepts(7) from research on mammals, reduced oxygen tension, cobalt injections and loss of blood stimulated red cell production through a humoral substance called erythropoietin. The presence of erythropoietin has not been established in cold-blooded animals.

In our study it has been clearly shown that loss of blood stimulated red cell production. Since 4 months were required for restoration of normal blood values following last bleeding, it is suggested that the turtle erythrocyte matures very slowly. It has previously been found(8) that longevity of turtle erythrocyte exceeds 11 months.

The absence of an hematopoietic effect by crude liver extracts in normal turtles and in frogs(5) is not unusual since there was no demonstrated liver deficiency. It is of interest that there was no stimulation of erythropoiesis in the anemic salted frog when treated with a purified liver extract containing an antipernicious anemia factor(9). Little is known of the nutritional requirements of poikilotherms.

Folic acid antagonists, such as aminopterin, interfere with maturation of erythrocytes and leucocytes and cause an increase in size of stem cells in birds and mammals(10). We have shown that a similar inhibition occurs in cold-blooded animals such as turtles. The sensitivity of the turtle to aminopterin lay be-

tween that of rat and guinea pig. For example, rats give 0.25 mg aminopterin daily (11) develop pancytopenia and maturation arrest at level of stem cell within 24 days. Turtles receiving 0.20 mg 5 times weekly develop anemia and leucopenia with enlarged stem cells within 5 to 7 weeks. Guinea pigs receiving 0.25 mg daily develop anemia and leucocytosis after 2 months(12). In this study it has been shown that folic acid is probably necessary in blood formation in the turtle, and in this respect blood formation of reptiles is similar to that of birds and mammals.

Summary. Sex differences in control blood values of box turtles are presented. Mild to toxic doses of cobalt, including mineral supplement and injections of crude liver extract failed to stimulate erythropoiesis. Marked reticulocytosis was induced by repeated bleeding. High reticulocyte counts persisted for 3 weeks following the last bleeding and 4 months were required for restoration of normal erythrocyte values. Blood formation was altered by injections of folic acid antagonist, aminopterin. Arrested maturation of blood cells was detected in form of enlarged marrow blast cells as early as 5 weeks and anemia and leucopenia followed after 6 weeks treatment.

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Antimycotic Effect of Spermine.* (24383)

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Spermine, a biologic polyamine, is widely distributed in animal tissues(1) and has also been found in yeasts(2) and bacteriophages (3). Shown to be a growth factor for some micro-organisms(4), it has, on the other hand, been found to inhibit the growth of various bacteria, mainly Gram positive(5). The present communication deals with the effect of spermine on growth of yeasts. Spermidine, a polyamine closely related to spermine was also examined.

Materials. Spermine tetrahydrochloride and spermidine phosphate (Hoffmann La Roche & Co., Ltd., Basle, Switzerland) were used. *Test organisms.* The yeasts examined were obtained from the collection of the Dept. of Bacteriology. *Saccharomyces cerevisiae* served as the main test organism. *Media.* The organisms were grown on the following medium: 0.5% Bacto peptone, 0.5% yeast extract (Difco), 2% glucose, 0.2% $CaCO_3$, 2% agar agar (Difco). The antimycotic effect of spermine was tested in Difco nutrient broth enriched with 0.5% glucose (w/v), pH 7.6.

Antimycotic spectrum of spermine. The antimycotic effect of spermine was tested by the double dilution method(6). Several species

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