Appearance of an Embryonic-Like Hemoglobin in the Methotrexate-Treated Adult White Peking Duck.* (30418)

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The adult duck has 2 hemoglobins(1,2), a major (Hb I) and a minor (Hb II) component. A third hemoglobin (Hb III) in the duck embryo bears a resemblance to human fetal hemoglobin in that it is present in the duckling but absent in the adult(3). The appearance of Hb III in the embryo and its disappearance by the 10th week of development after hatching provide a basis for the study of the epigenetic control of hemoglobin synthesis in this species. The present article reports the appearance of an embryonic-like hemoglobin (Hb "III") in the adult duck treated with the folic acid antagonist amethopterin (methotrexate).‡

Methods. Adult ducks weighing 3 to 3.5 kg were used. Daily intraperitoneal or subcutaneous injections of 150 mg of methotrexate were given. Hemoglobin was prepared for electrophoresis and chromatography from blood obtained from wing veins. The separated cells were washed 3 times with isotonic saline(4). After freezing and thawing once, lysis was completed by addition of 5 volumes of a hypotonic buffer pH 7.4(4). Centrifugation at 105,000 $\times$ *g* for 1 hour removed stroma, nuclei, and ribonucleoprotein. The hemoglobin was converted to cyanmethemoglobin and dialyzed 18 hours against 0.005 M phosphate buffer pH 8.6 to which KCN (100 mg/liter) was added(5).

Vertical-gel electrophoresis(6) in Tris-borate-EDTA buffer pH 9.2 was performed at 300 volts for 4 hours at 4°C, and the gel was stained with benzidine.

Chromatography on diethylaminoethyl cellulose (DEAE) was performed according to a modification of Huisman's procedure(5). Fifteen to 30 mg of hemoglobin, prepared as described, were adsorbed on 1 $\times$ 20 cm DEAE columns previously equilibrated with dialysis buffer. For elution (4°C) the mixing chamber held 500 ml of dialysis buffer, the reservoir held 0.006 M phosphate buffer pH 8.6 containing 0.05 M NaCl and 100 mg of KCN per liter; the flow rate was 1 ml per minute, and aliquots of 3.5 ml each were collected. Following the elution of Hb II the columns were "cleared" by direct application of 0.009 M phosphate buffer pH 6.0 containing 0.3 M NaCl and 100 mg of KCN per liter.

Cell content of the individual hemoglobins was determined by methods previously described(3).

Hemoglobin specific activity was determined as follows: 90 μc of Fe^{59}, as ferrous citrate, was administered by wing vein injection and the methotrexate treatment was discontinued. Four, 8, and 11 days later blood was drawn and the hemoglobins were separated by starch-block electrophoresis(7) in quadruplicate. Optical density measurements on the eluted samples were made at 415 mμ and 5 ml aliquots assayed for activity in a well-type scintillation counter.

Results. Examination of the bone marrow at the end of the 5th week of treatment with methotrexate provided evidence of megaloblastic changes. After the 6th week the red cell mass (hematocrit) had decreased from a control value of 45% to 9% and the dose of methotrexate was then reduced to variable levels which permitted the hematocrit to fluctuate between 8% and 18%. The methotrexate-induced anemia, during the first 6 weeks, was not associated with *in vivo* hemolysis but could be accounted for by the normal attrition of red cells due to *in vivo* aging and a severely depressed bone marrow. Chromium-51 red cell survival studies in a second

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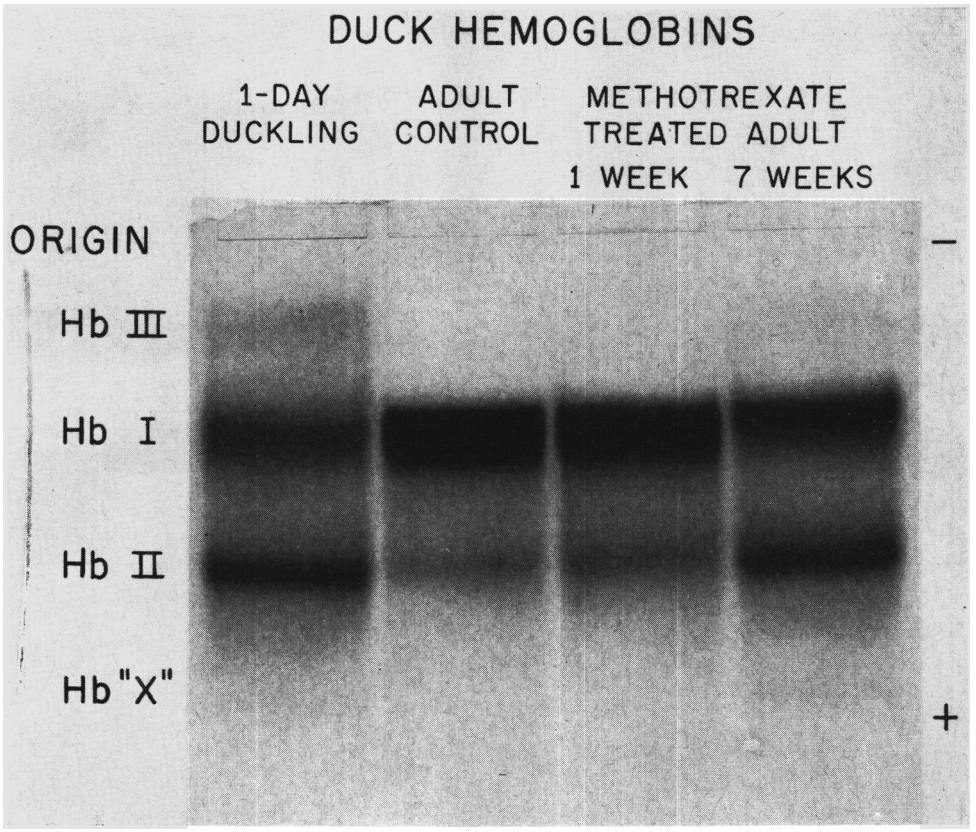

FIG. 1. Vertical-gel electrophoresis of hemoglobins from a normal duckling and adult compared with methotrexate-treated animal.

similarly treated adult duck did not suggest a shortened life span when compared with values previously reported in the literature (8,9).

Methotrexate was discontinued for 5 days when the animal's hematocrit had reached 9% (end of the 6th week of treatment) and a rise to 15% followed. The animal was bled at this time and the results of vertical-gel electrophoresis of hemoglobins from a normal duckling, adult, and the treated duck after 1 and 7 weeks of methotrexate are shown in Fig. 1. Hemoglobin III is the slowest moving anodal component in the duckling. Electrophoresis of hemoglobin from the normal adult duck and from the experimental animal after one week of treatment showed only the 2 adult hemoglobins but a third hemoglobin band has appeared, after 7 weeks of treatment, which corresponds in electrophoretic mobility to Hb III. A fast moving band, here labeled Hb "X," was inconstantly seen in the electrophoretic pattern of duckling hemoglobin but was consistently observed in the treated animal from the 7th to the 11th week of the experiment. The adult duck, which served as the control for these experiments, was subsequently treated with methotrexate and similar results were obtained.

Fig. 2 illustrates the DEAE cellulose elution pattern of hemoglobin from the 21-day duck embryo, the normal adult, and the methotrexate-treated animal. Hemoglobin "III" in the experimental animal is eluted at the position of Hb III in the embryo. No comparable hemoglobin is present in the normal adult duck. The peak following Hb II is the result of "clearing" the column (described in *Methods*), and has not been further identified.

Cell content of the individual hemoglobins

TABLE I. Cell Content of Individual Hemoglobins Before and After Treatment with Methotrexate.

Weeks treated with methotrexate	Hemoglobin/cell (pg)				
	Total Hb	Hb I	Hb II	Hb "III"	Hb "X"
0	42.5	34.2	8.3	Absent	Absent
10	50.3	32.4	14.7	1.8	1.7

in the adult duck before and after 10 weeks of treatment is shown in Table I. Hemoglobins "III" and "X," absent at start of the experiment, were evident at 10 weeks by electrophoresis on starch-block and vertical-gel. The folic acid antagonist also led to the preferential synthesis of Hb II relative to Hb I: the results indicate that Hb II content per cell increased approximately 70%. Analogous alterations in hemoglobin proportions, and increased fetal hemoglobin in the adult, have been noted in the human megaloblastic anemias resulting from vit. B_{12} and folic acid deficiency(10,11).

During the 11th week of treatment methotrexate was discontinued and hemoglobin was labeled *in vivo* with Fe^{59}. The specific activities of the individual hemoglobins are shown in Table II. Though Hbs "III" and "X" had slightly higher specific activities than Hbs I and II during this period no precursor-product relationship was apparent. Four weeks after methotrexate was discontinued only a trace amount of Hb "III" was detectable by starch-block electrophoresis.

Discussion. Several interpretations are possible regarding the appearance of Hb "III" in the methotrexate-treated adult duck. Hemoglobin "III" may be the product of the polymerization or dissociation of the adult hemoglobin chains. Hemoglobin "III" may also be the product of an altered genetic determinant which is expressed as a structurally different hemoglobin but with elec-

TABLE II. Hemoglobin Specific Activities Following Administration of Fe^{59} *in vivo* After Methotrexate had been Discontinued and the Animal Allowed to Recover.

Days after Fe^{59}	Specific activity, cpm/OD 415 mu			
	Hb I	Hb II	Hb "III"	Hb "X"
4	266	240	278	284
8	262	270	325	320
11	243	257	297	298

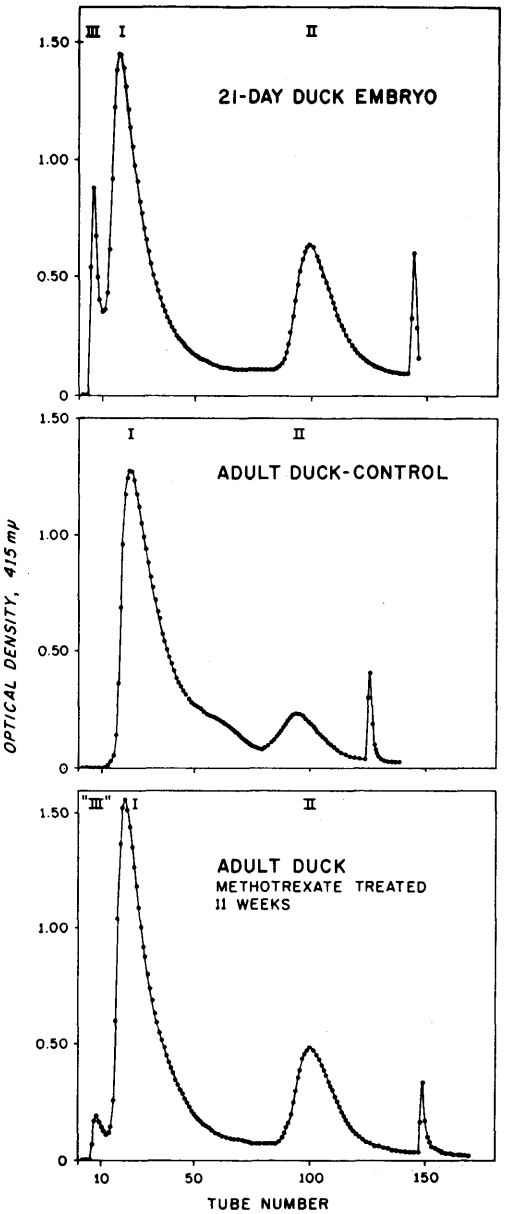

FIG. 2. DEAE cellulose chromatography of hemoglobins from 21-day duck embryo, normal adult, and methotrexate-treated adult duck.

trophoretic and chromatographic properties indistinguishable from Hb III. Finally, Hb "III" in the methotrexate-treated adult duck and Hb III in the embryo and duckling may be structurally identical. On the basis of current genetic concepts(12) the last possibility would imply that the folic acid antagonist has led to the recall of Hb III by derepressing the gene responsible for the synthesis of this protein.

Summary. Methotrexate-induced megaloblastic anemia led to altered proportions of Hbs I and II in the adult Peking duck and to the appearance of the 2 new hemoglobins which we have tentatively labeled Hbs "III" and "X." One of these, Hb "III," has electrophoretic and chromatographic properties indistinguishable from Hb III seen only in the embryo and duckling.

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Carbohydrate and Protein Metabolism in Rats During Chronic Administration of Glucagon and Epinephrine.* (30419)

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Both epinephrine and glucagon are effective against insulin hypoglycemia and when given to the normal animal usually produce a temporary rise in blood sugar. However, it is thought that their hyperglycemic and glycogenolytic actions involve different carbohydrate reserves. In the normal untreated animal, glucagon, which is secreted into the portal vein, acts almost exclusively on hepatic glycogen, whereas epinephrine, which is diluted and widely distributed by the blood, acts principally on muscle glycogen stores(1). Since glucagon reduces plasma amino acid concentration(2) and increases nitrogen excretion(3), it is almost certain that gluconeogenesis from protein also occurs, either as a direct effect or as a result of liver glycogen depletion(4). This may account in part, for the adverse effect of glucagon administration on growth(5).

Although there have been many studies of metabolic effects of epinephrine and glucagon, we have not seen data comparing the effects of these hormones on both carbohydrate and protein metabolism in the same animal. The purpose of the present experiments was to study changes in liver and muscle glycogen, blood glucose, and plasma amino acids in fed and fasted rats during chronic administration of epinephrine and/or glucagon. Changes in body weight were also recorded.

Materials and methods. Adult male Sprague-

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