DUCK RED CELL 2, 3 DIPHOSPHOGLYCERATE: ITS PRESENCE IN THE EMBRYO AND ITS DISAPPEARANCE IN THE ADULT.

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SUMMARY

Erythrocytes drawn from embryos of the white Peking duck (Anas domesticus) contain appreciable amounts of 2,3 diphosphoglycerate and negligible amounts of inositol hexaphosphate. In the adult, inositol hexaphosphate becomes the major phosphorylated intermediate while 2,3 diphosphoglycerate is no longer detectable by ion-exchange column chromatographic separation and colorimetric assay procedures. This indicates that the metabolic pathways for the biosynthesis of 2,3 diphosphoglycerate and inositol hexaphosphate are influenced differentially during avian development. To our knowledge, this is the first report on the presence of 2,3 diphosphoglycerate in avian erythrocytes.

The erythrocytes of the bird are biochemically unique among nucleated and non-nucleated red blood cells in that they contain appreciable amounts of inositol hexaphosphate (IHP) and no measurable amounts of 2,3 diphosphoglycerate (1). We now report, for the first time, that erythrocytes from the duck embryo contain considerable amounts of 2,3 diphosphoglycerate (2,3-DPG) and negligible amounts of IHP. 2,3-DPG is absent in the erythrocytes of the adult duck. This observation may have implications for a switch mechanism whereby 2,3-DPG probably functions as the allosteric regulator of oxygen binding to hemoglobin during embryonic life while some other modifier assumes this role during the period of post-hatching development.

The *in vitro* binding of 2,3-DPG to mammalian hemoglobins was first reported by Sugita and Chanutin (2). The significance of the reciprocal binding of oxygen and 2,3-DPG to hemoglobin was elaborated by Benesch and Benesch (3) and Chanutin and Churnish (4). Although 2,3-DPG and ATP can both function, *in vitro*, as allosteric regulators of oxygen binding to hemoglobin, causing a

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shift in the oxygen dissociation curve to the right, 2,3-DPG is presumed to be the *in vivo* regulator. Support for this view is based on the observation that 2,3-DPG and hemoglobin are present in the human red cell in approximately equimolar concentrations and that *in vitro* binding occurs in the ratio of one mole of 2,3-DPG per mole of hemoglobin (3,5,6). A similar physiological role was proposed for IHP in the bird red cell based on its ability to decrease oxygen affinity when bound to both mammalian and avian hemoglobins (5,6,7). Our finding that embryo erythrocytes have nearly 15 times as much 2,3-DPG as IHP may have a direct bearing on the mechanism of oxygen delivery during avian development.

METHODS

Blood was obtained from the jugular vein of adult ducks and one day old ducklings. Embryonic erythrocytes were obtained by cutting the blood vessels covering the yolk sac, removing the blood with a Pasteur pipet and transferring the contents to a flask of cold isotonic saline to which heparin was added. Care was taken to minimize, as much as possible, the amount of yolk material transferred during this process. The blood was filtered twice through two layers of gauze to remove yolk material and small clots. The cells were washed 4 times with isotonic saline and the white cell layer removed by suction. Approximately 12-13 ml of cells can be obtained from 3 dozen 22 day old embryos. The normal incubation period of the fertilized egg is 28 days. After washing, the packed cells were resuspended in saline. The hematocrit was measured and a trichloracetic acid (TCA) extract was prepared as described by Bartlett (8). The neutralized TCA extract is adsorbed on 1 x 18.5 cm anion exchange columns in the formate form and the phosphorylated intermediates eluted with linear gradient of 0-5N formate buffer pH 3.0. The isolated intermediates were identified on the basis of their elution position and confirmed by the appropriate assay procedures for inorganic and total phosphorus, ADP, ATP and 2,3 diphosphoglycerate as described by Bartlett (8). Inositol hexaphosphate was identified only by its elution position. However, the quantitative values obtained for this intermediate in the present column experiments were in excellent agreement with values previously obtained in our laboratory (9) using Oshima's extraction and iron precipitation procedure for IHP (10).

RESULTS AND DISCUSSION

Figures 1A and 1B show the typical elution profiles for the normal one day old duckling and the adult duck. In both cases the major phosphorylated intermediates are inorganic phosphate, ATP and IHP. Adenosine diphosphate (ADP) which was present in all age groups studied constitutes a relatively minor intermediate. The results of column chromatography of 22 day old embryo red

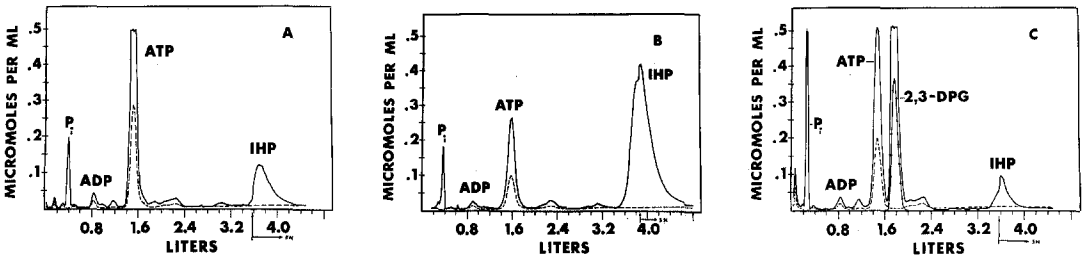

Figure 1. TCA extracts of duckling (A), adult duck (B) and embryo (C) erythrocytes were adsorbed on anion exchange resins (BioRad AG 1 x 8, 200-400 mesh) converted from the chloride to the formate form. After degassing, the slurry was poured into 1 x 30 cm columns to a height of 19 cm. The column was washed with distilled water until it gave a negative reaction with Nessler's reagent. The top portion of the column was removed until a final column height of 18.5 cm was produced. The neutralized extract was adsorbed onto the column and elution accomplished with 4 liters of a linear gradient of 0-5N formate buffer, pH 3.0. Fifteen ml fractions were collected and all tubes were analyzed for total phosphate and E_{260} absorption for nucleotides. Total phosphate peaks corresponding to adenine nucleotides or inorganic phosphate were analyzed for ribose by the orcinol procedure and inorganic phosphate respectively. Bartlett's chromotropic acid test for 2,3-DPG was used to identify this intermediate in the embryo extracts. In the duckling (A) the peak tube for ATP was not plotted on this profile but was calculated to be 0.81 u moles / ml eluate. Similarly, in the embryo (C), the peak tube for 2,3-DPG phosphorus was 0.83 u moles / ml eluate and that for inorganic phosphorus 0.72 u moles / ml eluate. The abscissa records the volume of buffer passing through the column. Abbreviations: Inorganic phosphorus (P_i), adenosine diphosphate (ADP), adenosine triphosphate (ATP), 2,3 diphosphoglycerate (2,3-DPG) and inositol hexaphosphate (IHP). Total phosphate (_____), E_{260} (--------) and chromotropic acid (▲▲▲).

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cell extracts are shown in Figure 1C. The most striking qualitative change, relative to the duckling and adult duck, is the phosphate peak appearing after ATP and eluting between 1.65 and 1.95 liters of buffer. This is the elution position expected for 2,3-DPG and its identity was confirmed, in replicate experiments by Bartlett's chromotropic acid (CTA) test (8).

The major quantitative changes associated with the period of post-hatching development involve reciprocal changes in the cell content of ATP and IHP. In replicate columns we have determined that the mean values for ATP and IHP in the one day old duckling are 5.45 and 1.74 u moles / ml cells respectively. The corresponding values in the normal adult duck are 3.15 and 4.54 u moles / ml cells. These results are shown in Table 1. The considerably

Table 1

Mean concentration of some major duck red cell phosphorylated intermediates

	u moles / ml cells				
	P_i	ADP	ATP	IHP	2,3-DPG
Embryo (22 Day old)	2.26	0.20	2.00	0.30	4.82
Duckling (1 Day old)	1.96	0.32	5.45	1.74	-
Adult Duck	1.71	0.39	3.15	4.54	-

lower values for IHP in the duckling led us to predict that it might be even more markedly depressed in the embryo. The value of 0.30 u moles / ml cells observed for the 22 day old embryo confirmed this prediction. However, the most striking and unexpected finding was the presence of 2,3-DPG. The concentration of this intermediate (4.82 u moles / ml cells) in the embryo may be compared with the concentration of IHP (4.54 u moles / ml cells) in the adult duck.

The presence of 2,3-DPG in the embryo and its disappearance in the adult duck suggested a possible relationship to the kinds of hemoglobin present during embryonic and post-hatching development. We have reported (11) that multiple hemoglobins exist in the embryo and adult duck. Hemoglobins III and IV (26% and 74% of the total hemoglobin) are the earliest hemoglobins we have been able to detect in the 6 day old embryo. These two hemoglobins are electrophoretically different from the two hemoglobins which are invariably present in the adult duck (HbsI and II). During embryonic development Hb IV disappears and Hbs I and II appear by day 14 of embryonic life. At this time,

hemoglobins I, II and III are present in the embryo and they account for 34%, 50% and 16% respectively of the total hemoglobin. At 21 days of embryonic life the comparable values are 45%, 45% and 10%. There is a reciprocal change in the concentration of Hbs I and II such that in the adult the former becomes the major fraction (85 %) while Hb II accounts for the remaining 15%. Hemoglobin III persists for approximately 70 days after hatching and is thereafter never normally detected in our animals; although treatment of adult ducks with methotrexate results in the appearance of a hemoglobin with electrophoretic and chromatographic properties indistinguishable from Hb III (12). The appearance of Hb III in the embryo, its presence after hatching and its disappearance in the adult is reminiscent of fetal hemoglobin in Man.

Since it is now generally accepted that ATP, 2,3-DPG and IHP can modify oxygen binding to hemoglobin, we believe it is plausible that allosteric modification of oxygen binding to hemoglobin is mediated in the duck embryo by 2,3-DPG and in the adult by some other regulator such as IHP and/or ATP. Information regarding the possible binding site of these intermediates to duck hemoglobins is presently unknown. Investigations on the structure and physiological properties of embryonic and adult duck hemoglobins are currently in progress.

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