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Intraerythrocytic Organic Phosphates and Hemoglobins of Skua - *Catharacta maccormicki (Stercoraridae*) - at Two Different Stages of the Year in Relation to Antartic Migration

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ABSTRACT

Catharacta maccormicki *blood samples were collected in the winter (October) and in the summer (February) in order to study the intraerythrocytic organic phosphates, hemoglobin (Hb) electrophoretic patterns, oxygen blood equilibrium and stripped Hbs, as well as the effect of 2,3-biphosphoglycerate (BPG) and inositol hexaphosphate (IHP) on oxygen affinity. All the samples (five from the winter and five from the summer) showed the same electrophoretic pattern: one minor fast component and one major slow one. No differences in oxygen affinity and Bohr effect in the samples collected in the winter and in the summer were found. Oxygen affinity was higher in the stripped Hb than in the blood. BPG seemed to have no effect on the functional properties of skua Hb while IHP does. No BPG was found in any sample. Both inositol pentaphosphate (IP5) and IHP were found in all the samples. The IP5/IHP ratio in the winter samples was 3.0 while in summer 3.5. Adenosine diphosphate (ADP) was found in samples from both the seasons. Adenosine monophosphate (AMP) and adenosine triphosphate (ATP) were present only in the summer samples while guanosine triphosphate (GTP) was found in the winter samples. Since IP5 and IHP are very powerful HB allosteric effectors, ATP and GTP might function as other protein modulators.*

Key words: birds, blood, electrophoresis, cold, affinity, oxygen

INTRODUCTION

Antarctic birds are subjected to many seasonal changes of activity due to behavioral and environmental changes. Therefore, it would be of considerable interest to have information on the blood O_2 transport under natural conditions. In order to face the extreme life conditions, suitable mechanisms of temperature and migratory adaptations have been developed, involving both physiological and biochemical processes (Tamburrini et al. 2000; Lutfulllah et al. 2005).

Skuas (*Catharacta maccormicki*, Stercoraridae) are an example of migratory birds. This species inhabits Antarctica and does a seasonal migration to South America and even to the north of the Equator in the winter and comes back in the spring (Leotta et al. 2002), thus proving to be extremely resistant during the migration and to live in completely adverse conditions. All Antarctic birds, including the skua, are mostly well adapted to water and to extreme cold, possessing a body morphology particularly suited to a cold medium (Tamburrini et al. 1999; Jones et al. 2002). In this context, the study of hemoglobins (Hb) becomes

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extremely important, because Hb belongs to a protein family that represents a direct link between the environmental conditions and physiological demands. In this regard, Hb has been widely used as a subject for vertebrate evolution and molecular adaptation studies (Bordin et al. 1997).

The Hb oxygenation process is cooperative and the oxygen-binding properties are modulated by the interactions of specific amino acid residues with heterotropic allosteric effectors (Yuan et al. 2002). Allosteric effectors, such as 2,3-biphosphoglycerate (BPG) and inositol hexaphosphate (IHP), are known to bind at the β-chain, thereby markedly reducing the Hb oxygen affinity (Villar et al. 2002; Peng et al. 2003). In adult birds, inositol pentaphosphate (IPP) is the main allosteric effectors in the red cell, instead of BPG, which is found in human Hb (Zhang et al. 1996). However, bird embryos also present 2,3-BPG, which disappears at the moment of hatching (Isaacks et al. 1976a; Isaacks et al. 1976b; Isaacks et al. 1976c; Bartlett 1980a; Bartlett 1980b; Borgese Lampert 1975; Isaacks Harckness 1975). Since migration of skuas is a tremendous resistance test, an alteration of Hb functional properties due to different intraerythrocytic organic phosphates content in samples collected at different seasons (after migration and after a period of rest) could be expected.

The present work studied the Hb electrophoretic patterns, oxygen blood equilibrium and chromatographic patterns of intraerythrocytic organic phosphates obtained in the samples collected in the winter and summer, as well as the functional properties of stripped hemoglobin and the effect of BPG and IHP on these properties.

MATERIALS AND METHODS

Blood samples from ten skuas (five from the winter and five from the summer, in first of September 1994 and first of February 1994, respectively) were obtained in heparinized syringes from the main vein in the wings and divided into three parts in the field. The birds were captured from the King George Island and after this procedure, they were released. One part of the blood was immediately submitted to oxygen equilibrium experiments (Johansen et al. 1980). The second part was used to obtain phosphate extracts (Bartlett 1959), and the third was washed three times with 1% NaCl, and then tris-EDTA ($1.5x10^{-2}$ M pH 8.0) was added to the packed erythrocytes in a 1:1 ratio. Then the hemolysis was achieved by freezing and thawing the samples three times. To free the hemolisate of the cellular debris, the sample was centrifuged at 12,000g at 4^oC for 15 mins. The pellet ('ghost cells') was discarded and the Hb solution was frozen at -20^oC for further analysis. In order to check if some variation was present in the samples, electrophoresis of the individual hemolysates was carried out in starch gel (Smithies 1955; Smithies 1959; Val et al. 1980).

For the spectrophotometric oxygen equilibrium experiments, the supernatant was stripped of salt and organic phosphates by passing through a 1 x 30cm column, filled with Sephadex G-25 resin and equilibrated in 10mM Tris-HCl (pH 8.0). Methemoglobin, when present, was reduced with dithionite in a Sephadex G-25 column (Schwantes et al. 1976), with the following changes: the sample was applied only after no dithionite solution remained at the top of the resin. Due to the changes in the sample color, reduction and reoxygenation could be accompanied visually as the sample passed through the column.

The oxygen equilibrium of stripped Hb was carried out at 20^oC by the spectrophotometric method (Riggs Wolbach 1956). The effect of organic phosphates (IHP, 2,3-BPG) was measured by the addition of appropriate volumes of solutions of 2,3-BPG and IHP salts to the stripped Hb. The organic phosphates (OP) were tested in a molar ratio of 1:8 (Hb : OP). The P50, Bohr effect and n values (Hill plot) were interpolated from linearized logarithmic plots of O_2 equilibrium data.

The separation of the red blood cell organic phosphates (mainly inositols) was carried out by the liquid ionic exchange chromatography in Pharmacia columns (1 x 30 cm), which were filled with DOWEX 1x8, 200 MESH resins. The chromatograms were registered with a linear gradient of hydrochloric acid (Issacks et al. 1976a). After this, total phosphorus was quantified (Bartlett 1959). The separation and quantification of other organic phosphates (ATP, ADP, AMP and GTP) was carried out by the liquid ionic exchange chromatography in Pharmacia columns (1cm x 10cm) filled with Q-Sepharose Fast Flow resin. The buffers utilized were trietanolamine 20 mM plus KCl 1 M and a linear gradient was achieved from zero to 1 M KCl. The separation was carried out in an automated HPLC system. The chromatographic profile was obtained at 220 nm.

Main peaks of the eluted fractions were identified for comparison with the chromatograms of standards run in similar conditions to those of the samples and then compared with the chromatographic profiles. A T-student test was used for calculation.

RESULTS

Starch gel electrophoresis of the 10 specimens' (five from each season) hemolysates showed two components: one slow moving major one, and a fast moving minor one. This pattern was constant from one individual to another (Fig.1) and in the same figure is shown, in order to compare the pattern obtained with human Hb.

Oxygen equilibrium determined from the unfractionated blood samples of *C. maccormicki* and stripped Hb in the presence and absence of BPG and IHP (measured at 20°C in the pH range 6.2 - 9.75) are shown in Figures 2 and 3, respectively. In the unfractionated blood, no differences between the two seasons were seen, indicating that the migration did not change the blood O_2 affinities

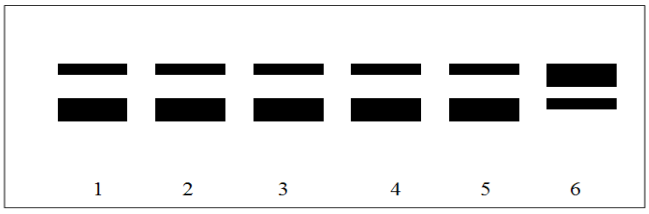

Figure 1 - Schematic electrophoretic pattern of hemolysates of *C. maccormicki* colleted in the winter and in the summer. 1, 2 and 3 - Winter; 4 and 5 - Summer; 6 - Human Hb (pattern). The support was starch gel 13% and the buffers were Tris-Borate-EDTA 0,036 M pH 8.6.

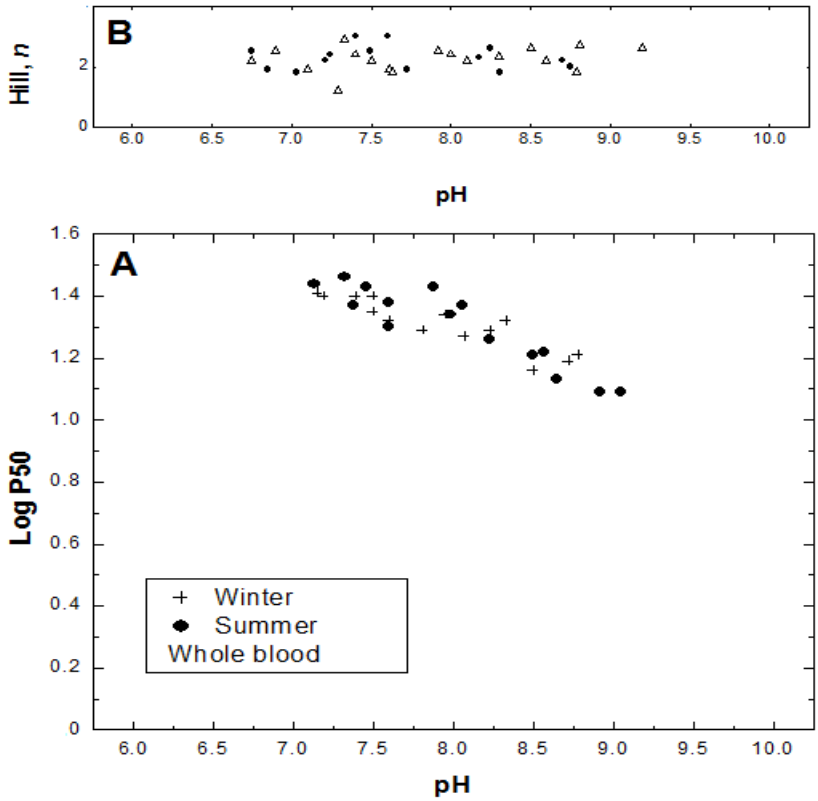

Figure 2 - (A) Oxygen affinity in unfractioned blood from *C. maccormicki* collected in both seasons, expressed as P_{50}, the partial pressure of oxygen required to saturate 50% of the hemes measured at 20°C. Crosses: specimens collected in winter; closed circles: specimens collected in summer. (B) Hill values (*n*) obtained from dissociation curves. Open triangles: summer; closed circles: winter.

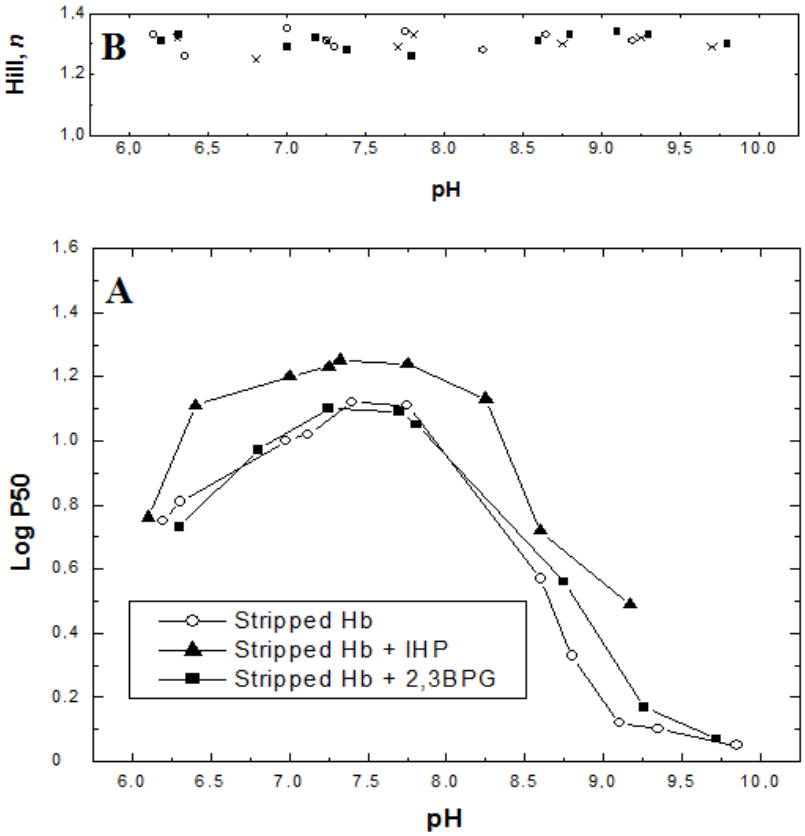

Figure 3 - (A) Oxygen affinity in stripped Hb of *C. maccormicki*, with or without addition of organic phosphates, IHP and BPG, expressed as P_{50} at 20°C. Open circles: stripped Hb; closed triangles: stripped Hb plus IHP; closed squares: stripped Hb plus 2,3BPG. All of them provided a molar ratio of 1:8 (Hb:OP). (B) Hill values (*n*) obtained from dissociation curves. Crosses: stripped Hb; open circles: stripped Hb plus IHP; closed squares: stripped Hb plus 2,3BPG.

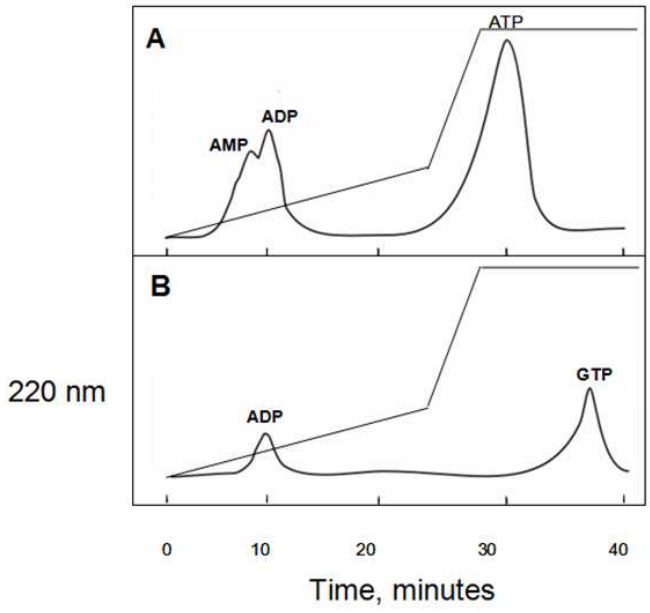

Figure 4 - Chromatographic profiles of phosphate compounds obtained of skuas collected in both seasons. (A) Summer; (B) Winter. The absorbance was 220 nm and the total elution time was 40 minutes. The buffer used was trietanolamine 20 mM pH 7.5 and the gradient was KCl (straight line) from 0 to 1 M.

Table 1 - Total values of organic phosphates of Skuas collected in winter and in summer, in mM phosphorus / mL RBC (Red blood cells). A) No inositols B) Inositols. A *T-student* test was used for calculation.

A	**Winter**	**Summer**
AMP	Not detectable	1.25 ± 0.3
ADP	0.41 ± 0.02	1.75 ± 0.25
ATP	Not detectable	0.36 ± 0.11
GTP	0.16 ± 0.01	Not detectable
2,3 BPG	Not detectable	Not detectable
B	**Winter**	**Summer**
IP5	2.01 ± 0.18	2.13 ± 0.25
IHP	0.67 ± 0.13	0.61 ± 0.1
IP5 / IHP	3.0	3.5
Σ Total inositols	2.68 ± 0.16	2.74 ± 0.27

The Bohr effect (pH 7 - 9.75) in the blood and stripped Hb experiments was normal. Blood oxygen affinity was lower than stripped Hb. The addition of IHP to stripped Hb increased the P50 in pH values between 6.7 and 9.2, but the addition of BPG had no similar effect.

DISCUSSION

Skuas are very aggressive and have rapacious habits. They have a rapid, sustained and powerful flight, enabling them to overtake many other birds (Tamburrini et al. 2000). They are effective predators, feeding on fish, crustaceans, and penguin eggs and chicks, but they also scavenge carcasses and feed at sewage outlets (Leotta et al. 2002). The electrophoretic Hb pattern of two bands was one major slow and one minor fast moving, each component showing functional and structural differences, as described by Tamburrini and co-workers (2000). This pattern is common in birds, similar to that described by (Hiebl et al. 1987a) (Hiebl et al. 1987b) (Hiebl et al. 1987c) in *Aegypies monachos*, *Accipiter gentilis* and *Chloephaga melanoptera*. The allosteric properties of the bird Hb depend on temperature and are modulated by the protons, chloride, and inositol polyphosphate, especially pentaphosphate (Vorger 1994). Besides this latter characteristic, bird Hbs are considered to be functionally similar to mammalian ones (Perutz 1983). The present results are in agreement with that found in the literature, where the influence of the IP5 in reducing the Hb-O2 affinity was more pronounced than that one of BPG. IP5, in the physiological concentrations, is the primary modulator in quail (Rieira et al. 1991).

In the species analyzed in this work, IHP reduced the Hb-O2 affinity in comparison with the stripped Hb, suggesting that this phosphate could negatively affect this affinity in agreement with the fact of the birds possess inositols as main modulators. The values obtained for the Hill plots usually were about 1.0, indicating the absence of cooperativeness. However, in some situations, the n values obtained were very high, reaching up to 5. These high n values could be due to polymerization of the hemoglobin molecules, or in function of the conformational state, or proportion of the two components. In the unfractionated blood in hen, the Hill coefficients reached up to 5, when 80 to 90% oxygenated Hb was present (Cobb et al. 1992). According to these authors, the D components, but not the A, self-associates to form octamers of Hbs. Since the components A (slow) and D (fast) had the β chain in common, tit was concluded that the contacts tetramer-tetramer should depend on residues (probably Lys-71, Gly-78 and Glu-82) on the surface of the α chain.

The data obtained in relation to inositol levels found in the erythrocytes of *C. maccormicki* was very interesting considering that this bird was migratory par excellence. In the winter as well as in the summer, IP5 and IP6 were in slightly different concentrations. However, with the added total amount of inositols, very close values were observed. Therefore, the migration did not alter the total levels of inositols. However, in the winter, IP5 was three times more than IP6, while in the summer it was 3.5 times more than IP6. Assuming that IP6 was more effective in the modulation of the Hb to the oxygen than IP5, it could be postulated that the migratory act required a larger amounts of IP6, or that the latter changed into IP5, because the obtained values for the sum of total

inositols in the winter and summer were very close. A second binding site for inositol polyphosphates, at the chains, should be important in *C. maccormicki* since its affinity for this polyphosphate was about one third of that for the main binding site (Tamburini et al. 2000; Riccio et al. 2001). There are several avian species with another inositol polyphosphate (generally hexa or tetra) besides pentaphosphate, but most of the avian species show only inositol pentaphosphate. If this second inositol binding site shows special affinity for these other inositols (hexa or/and tetra), these should be tested. It is also possible that, due to the physiological weakness of the bird in the return to Antarctica, the levels of IP6 remain the same with the purpose of facilitating the delivery of the oxygen to the muscles.

The analysis of the phosphate chromatograms obtained for samples showed differences among the profiles. In both the seasons ADP was at levels, similar to described by other authors in other species of birds (Issacks et al. 1976a; Issacks et al. 1976b; Issacks et al. 1976c; Bartlett 1982c). However, the most interesting was the presence of GTP in the winter and its absence in the summer. With ATP, there happened exactly the opposite- its presence in the summer and its absence in the winter.

To confirm the identity of ATP and GTP, other experiments were done. New samples were concentrated and chromatographed in order to compare with a pattern. There was exclusive presence of GTP in the winter samples and of ATP in the summer samples. There was no report of similar data in the recent literature. However, it was possible that GTP did not have the specific function of modulating Hb in the birds, because the concentration found was very low. It was possible that the GTP and/or ATP could act as modulators in other metabolic pathways, for instance, in the glicolysis cycle. This metabolic pathway is the major source of energy of most of the organism erythrocytes and occupies a central position in the metabolism, providing lots of energy when the metabolic demands suddenly increase. In this case, when *C. maccormicki* migrated from the Antarctica to South America, these phosphates could act as modulators of other important proteins. Hence, it would be necessary to study the Hb oxygen equilibrium, with the addition of ATP and GTP in the found concentrations to check if, in the concentration found, these nucleotides functioned as Hb effectors. The role of ATP and GTP as allosteric effectors of key enzymes of the erythrocytes glucose metabolism of skuas must be found out. The presence of GTP in the birds' erythrocytes was never described. The present results on the BPG were in agreement with the literature, where its presence in the erythrocytes of adult birds was not related. However, in the literature, the amount of data on birds' erythrocytic nucleotides is very limited.

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