—研究ノート—
Sceintific Note

Carbohydrate Metabolism in Antarctic Birds' Erythrocytes: Levels of IP5 and 2,3-DPG and Their Effect on Chicken Hexokinase Activity*

Edson RODRIGUES[1], Rubens ROSA[2], Dario OCAMPOS[3], Claudete D. ROSA[4] and Metry BACILA[5]**

南極鳥類の赤血球における炭化水素代謝: IP5, 2,3-DPG 量とニワトリのヘキソキナーゼに対する効果*

Edson RODRIGUES[1], Rubens ROSA[2], Dario OCAMPOS[3], Claudete D. ROSA[4] and Metry BACILA[5]**

要旨: 本文は鳥類赤血球中のグルコース代謝における，有機燐酸代謝産物である，イノシトール5リン酸 (IP5), 2,3ジホスホグリセリン酸 (2,3-DPG) のもつ意味を知ることを目的とするものである．使用したヘキソキナーゼは，セファローズ-N-アミノヘキサノイルグルコサミンを用いたアフィニティクロマトグラフィを用いて精製した．IP5のヘキソキナーゼ抑制作用は，Mg^{2+} に対して競争的であるが，グルコース，$MgATP^{2-}$ に対しては競争的ではない．一方，ヘキソキナーゼの活性は ATP^{4-} によって抑制される．赤血球中の Mg^{2+} 値 (2.76 μmol/m*l*) は，赤血球標準懸濁液中の ATP, IP5 の総量に比べて低い．

Abstract: The aim of the present study is to show the possible implication of organic phosphate metabolites=namely inositol pentaphosphate (IP5) and 2,3-bisphosphoglycerate (2,3-DPG)=in the glucose metabolism displayed by birds erythrocytes. For this purpose, a preparation of hexokinase (HK) has been purified from chicken erythrocytes (RBC) by affinity chromatography in Sepharose-N-aminohexanoyl glucosamine and used for kinetic experiments. It has been shown that IP5 is a competitive inhibitor of HK in regard to Mg^{2+}, but

[1] Faculdade de Ciências Farmacêuticas, Universidade São Francisco, Avenida São Francisco de Assis, 218, 12900-000, Bragança Paulista, SP, Brasil.
[2] Departamento de Medicina, Pontifícia Universidade Católica do Paraná, Curitiba, PR, Brasil.
[3] Faculdade de Zoologia e Tecnologia de Alimentos, Universidade de São Paulo, Pirassununga, SP, Brasil.
[4] Faculdade de Odontologia, Universidade Estadual Paulista-UINESP, São José dos Campos, SP, Brasil.
[5] Laboratório de Piscicultura, Universidade Federal do Paraná, Rua dos Funcionários, 1540, CEP 80. 035-050, Curitiba, PR, Brasil.
* With a grant-in-aid from the Brazilian Antarctic Program (Brazilian Antarctic Program, Laboratório de Piscicultura, Universidade Federal do Paraná, Curitiba, PR, Brasil)-PROANTAR-CNPq.
** To whom all correspondence should be sent: Prof. Metry BACILA.

not in regard to glucose and $MgATP^{2-}$. On the other hand, this preparation of HK is also competitively inhibited by ATP^{4-}. It has been found that the total intracellular content of Mg^{2+} (2.76 μmol/m*l*) is lower than the total value for the content of ATP and IP5 found in a standard packed red blood cell suspension.

1. Introduction

Since the work of BARRON and HARROP (1928) it has been known that contrary to the characteristic of most mammalian erythrocytes (RBC), avian RBCs do not produce lactic acid when incubated with glucose. EHRLENBACH (1938) showed that birds possess high levels of blood sugar. BENDELLA (1971) showed that most of the blood glucose in chickens is partitioned at the plasma site and very little or none in the RBC. According to BELL (1971), L. MARTINDALE measured the renal threshold for glucose in four 14-month-old hens and found values as high as 260, 310, 330 and 360 mg of glucose per 100 m*l* of plasma.

Several papers from our laboratories dealt with the problem of the defective glucose metabolism displayed by bird RBCs when compared with the glucose metabolism displayed by most of the mammalian RBCs. Studying the behavior of the glycolytic pathway in chickens (*Gallus gallus domesticus*) RBC, ROSA (1977) and ROSA *et al.* (1983) established the fact that those cells are unable to metabolize glucose and fructose in either aerobic or anaerobic conditions. Furthermore, they also demonstrated that in spite of this fact, chicken erythrocyte is endowed with a full set of glycolytic enzymes as well as of enzymes in the pentose phosphate pathway. Levels of blood glucose and of glycolytic enzymes in RBC and somatic tissues of *Pygoscelis* penguins were investigated by ROSA *et al.* (1989). They confirmed the occurrence of high levels of blood glucose in these birds, most of this glucose partitioned at the plasma site. It has been found also that among the enzymes linked to the phosphate metabolism in RBC, HK is present in very low levels in erythrocytes from both chinstrap (*Pygoscelis antarctica*) and gentoo (*Pygoscelis papua*) penguins.

Following this research, BACILA *et al.* (1990) carried out a survey on the content of phosphorylated metabolic intermediates in RBCs from penguins and skuas at different stages of development. It has been found that on the 30th day of incubation-about 2 or 3 days before hatching-penguin embryos possess an erythrocyte in which both 2,3-DPG and IP5 are present, the concentration of 2,3-DPG being much higher than that of the IP5. In turn, IP5 is completely absent in the RBC of the 25th day embryos. However, the concentration of 2,3-DPG decreases around the hatching time, becoming almost completely absent in the 2-day-old chick. Coincidentally, IP5 concentration begins to rise around the 30th day of the egg incubation, increasing exponentially from the 28-day-old chick up to the adult penguin.

According to ROSA *et al.* (1983, 1989), chicken, penguin and skua erythrocytes seem to display similar behavior regarding glucose metabolism. In spite of the fact that those

---

Abbreviations used: IP5, inositol pentaphosphate; 2,3-DPG, 2,3-diphosphoglyceric acid; ATP, adenosine 5′-triphosphate, HK, hexokinase; RBC, red blood cells.

RBCs are endowed with a full set of glycolytic enzymes as well as of enzymes of the pentose phosphate pathway, they possess a defective glucose metabolism. As has been found already for chickens by BENDELLA (1971), ROSA *et al.* (1989) established that in penguin blood, most of the glucose is compartmentalized at the plasma site with very little permeating to the RBC.

The possible implication of the chelation of Mg^{2+} by 2,3-DPG and by IP5 on the impaired glucose metabolism displayed by bird RBC was first studied by RODRIGUES (1987). On the other hand, ROSA *et al.* (1988) found that IP5 is a competitive inhibitor for the activity of chicken erythrocyte phosphofructokinase. ROSA *et al.* (1983) also demonstrated that chicken RBC possesses the full set of glycolytic enzymes, glycolysis being defective at the hexosemonophosphate level but not at the level of triosephosphate metabolism.

In order to obtain deeper insight into this very interesting problem, a kinetic study on the effect of IP5 and 2,3-DPG on the activity of HK purified from chicken RBC was carried out. Consideration was also given to the regulatory aspects of glucose metabolism in birds in regard to the effects of IP5 and 2,3-DPG. This paper also presents data on the levels of hemoglobin modulators IP5 and 2,3-DPG in RBCs from penguin embryos and adult skuas.

2. Materials and Methods

For the analysis of RBC organic phosphate compounds from Antarctic birds, embryo penguins (*Pygoscelis adeliae*) and adult skuas (*Chataracta maccormicki*) were used. In order to collect blood from penguin embryos the following methodology described by BACILA *et al.* (1990) was used. Eggs previously marked were removed from the nests and carefully transported to the laboratory. Eggs on the 25th and 30th days of incubation were placed in a Petri dish and opened at the blunt pole. The embryos were removed and the whole blood collected by means of a Pasteur's pipette and transferred to a test tube containing EDTA. To collect blood from skuas, the birds were captured from their natural habitat and the blood collected by means of a puncture at the cubital vein with a vacuntainer tube containing heparin. The birds were set free immediately after this operation. The collected samples were pooled and the blood was spun down for 10 min at $480 \times g$ at room temperature. The packed RBCs were washed three times with cold saline and used for phosphate analysis. For this purpose, a protein free supernatant was prepared by adding two volumes of 1N $HClO_4$ to 2 volumes of packed RBC. The mixture, dipped in an ice bath, was homogenized by means of a glass rod and then spun down for 30 min at $12000 \times g$ in a refrigerated centrifuge. The supernatant was then collected and the pH adjusted to 3.5 with 5N K_2CO_3. This mixture remained dipped in the ice bath for 30 min and then it was spun down for 30 min at $12000 \times g$ for sedimentation of the $KClO_4$. The supernatant of this preparation was then collected and used for the assay of phosphorylated metabolic intermediates (organic phosphates) and Mg^{2+} ions.

Assays of organic phosphates were carried out by means of Dowex AG1×8 formate ion exchange chromatography according to BARTLETT (1968). For this purpose, a linear ammonium formate, formic acid (40 : 60) pH 3.45 gradient, was used for quantification of the eluted organic phosphates according to the method of ISAACKS *et al.* (1979). Inorganic

and organic phosphates were assayed according to the method of HESS and DERR (1974).

Magnesium content in RBCs was assayed by the photometric method of BOHUON (1962) as a soluble red color complex formed with xylydil blue in alcoholic solution.

Inositol pentaphosphate (IP5) was purified as sodium salt from 1N $HClO_4$ chicken erythrocyte protein-free supernatant by ion exchange chromatography in Dowex AG1×8 resin. The IP5 sodium salt was then obtained by precipitation with a solution containing 10 mM $FeCl_3$ and 3.4 M NaCl.

Purification of HK from chicken (*Gallus gallus domesticus*) RBC was carried out first by DEAE-cellulose in order to remove hemoglobin from the preparation and then by salting out with ammonium sulfate followed by affinity chromatography in Sepharose-N-aminohexanoyl glucosamine. Homogeneity of the preparation, checked by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis, showed a single band of protein after the affinity chromatography.

HK activity was assayed spectrophotometrically by the method of UYEDA and RACKER (1965) following the reduction of $NADP^+$ ($\varepsilon = 6.22 \times 10$) at 340 nm. The assays were carried out at 30°C. Suitable concentrations of Mg^{2+} as $MgCl_2$ were used throughout the experiment. All auxiliary enzymes used in the kinetic assays were dialyzed 12 hours at 0–4°C, against 500 volumes of 125 mM glycylglycine buffer, pH 8.0, containing 0.1 mM EDTA and 3 mM β-mercaptoethanol.

3. Results and Discussion

The nature and the contents of organic phosphate metabolites and inorganic phosphate found in erythrocytes from 30-day-old Adélie penguin embryos and from adult skuas are shown in Figs. 1 and 2, respectively. During the biological development of both penguins and skuas, 2,3-DPG is replaced by IP5 (BACILA *et al.*, 1990) as a hemoglobin modulator. In the example shown in Fig. 2, IP5 accounts for 43.5% of the total phosphate found in the erythrocytes of the adult skua, the amount of 2,3-DPG being undetectable.

The inhibitory effect of 2,3-DPG in regard to the activity of chicken RBC hexokinase is shown in Fig. 3. It has been found that the inhibition displayed by this hemoglobin modulator is competitive in regard to the HK ligand site of $MgATP^{2-}$. A non-competitive effect of 2,3-DPG in regard to Mg^{2+} toward chicken HK is shown in Fig. 4, an indication that such inhibition is not due to the effect of ATP^{4-} generated in the system, the latter being a competitive inhibitor (see Fig. 5) toward the chicken HK.

The kinetic interrelationship between ATP^{4-} and $MgATP^{2-}$ in regard to the chicken RBC hexokinase activity is shown in Fig. 5. It can be seen that the effect is competitive and takes place in a monomolecular way.

When Mg^{2+} concentration was maintained at 5 mM above the total concentration of its natural chelating compounds, IP5 was unable to inhibit HK activity. However, when experiments were carried out in which different concentrations of Mg^{2+} ranging from 0.08 up to 1.2 mM were used in the presence of fixed concentrations of IP5 (0.0, 0.84, 2.11, 3.16 mM) a clear competitive effect of this organic phosphate metabolite toward Mg^{2+} was found in regard to HK activity (Fig. 6).

A significant competition toward the Mg^{2+} from the cell pool (Fig. 7) was found

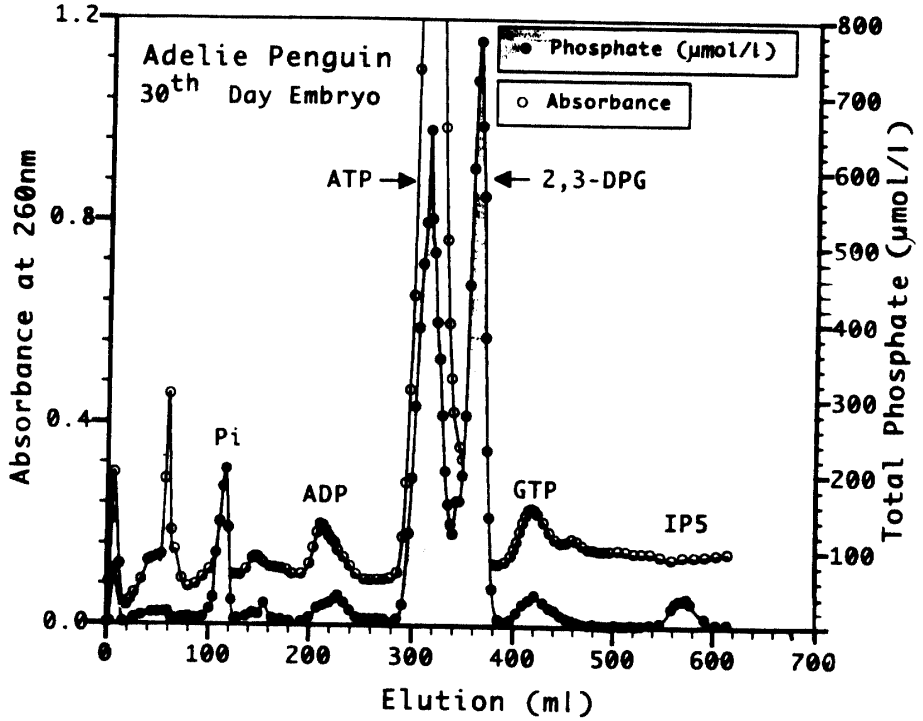

Fig. 1. Organic phosphate metabolites and inorganic phosphate content of erythrocytes from a 30-day-old penguin (Pygoscelis adeliae) embryo. Organic phosphates were separated by means of ion exchange chromatography in a Dowex AG-1×8 and assays of phosphate were carried out by a colorimetric technique using green malachite. Pi, inorganic phosphate; ADP, adenosine-5'-diphosphate; ATP, adenosine-5' triphosphate; 2,3-DPG, 2,3-bisphosphoglycerate; IP5, inositol pentaphosphate; GTP, guanosine-5'-triphosphate.

between ATP and IP5 due to the higher affinity of both organic phosphate compounds toward that divalent cation activator.

From the results of these experiments, it was possible to show that chicken RBC hexokinase is competitively inhibited by ATP^{4-} ($K_{I\,ATP^{4-}} = 800 \pm 40\ \mu M$). On the other hand, 2,3-DPG acts upon HK activity as a competitive inhibitor of $MgATP^{2-}$ ($K_{I\,2,3\text{-}DPG} = 1200 \pm 50\ \mu M$). However, 2,3-DPG acts as a non-competitive inhibitor of HK in regard to Mg^{2+}. In turn, IP5 is not an HK inhibitor in regard to glucose and $MgATP^{2-}$ but acts as a competitive inhibitor of HK in regard to Mg^{2+} ($K_{I\,IP5} = 2120 \pm 104\ \mu M$). Concentration of intracellular Mg^{2+} was found to be equal to 2.7 μmol/m*l* of a standard packed red blood cell suspension. This value is lower than the value for the total content of ATP and IP5 found in the same RBC suspension.

In spite of lacking organelles, including nuclei and mitochondria, most of the mammalian RBC operate a suitable mechanism that maintains adequate intracellular levels of NADPH and 2,3-DPG. Bird RBCs, on the other hand, in spite of being endowed with organelles, are defective cells in regard to glucose metabolism. Rosa *et al.* (1989) showed that penguin RBCs fall into this category of cells by possessing a defective glucose metabolism. Furthermore, the very low or almost non-existent glucose metabolism, peculiar to nucleated RBCs of birds and reptiles, impairs membrane integrity because of

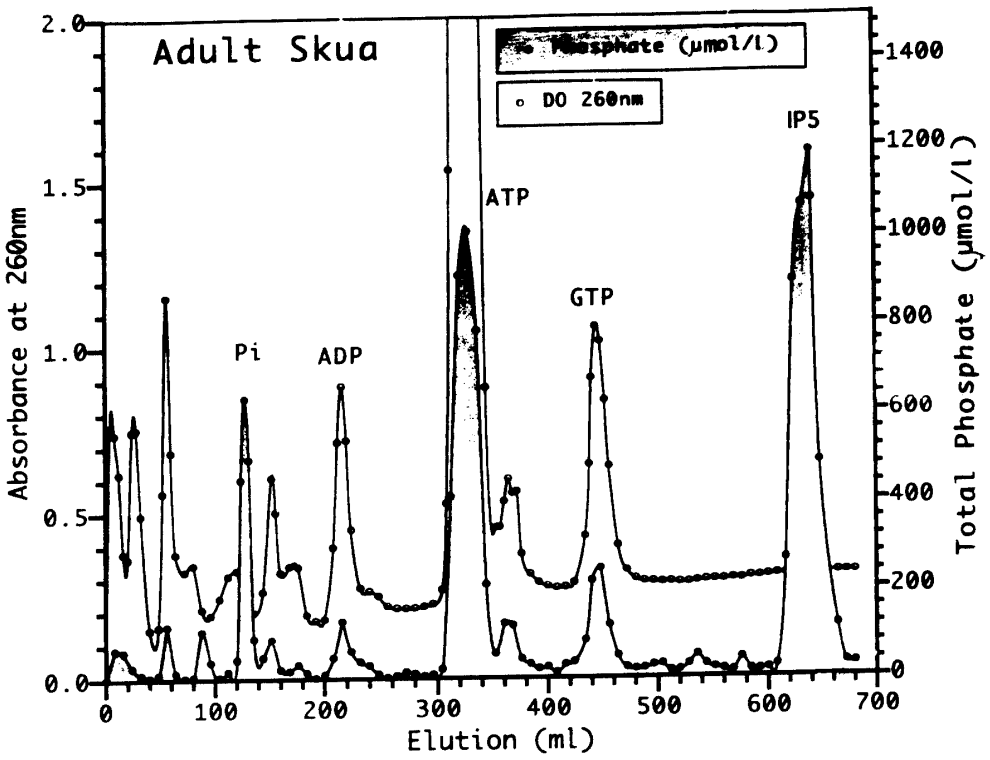

Fig. 2. Same as Fig. 1 but for erythrocytes from adult skuas (Chataracta maccormicki).

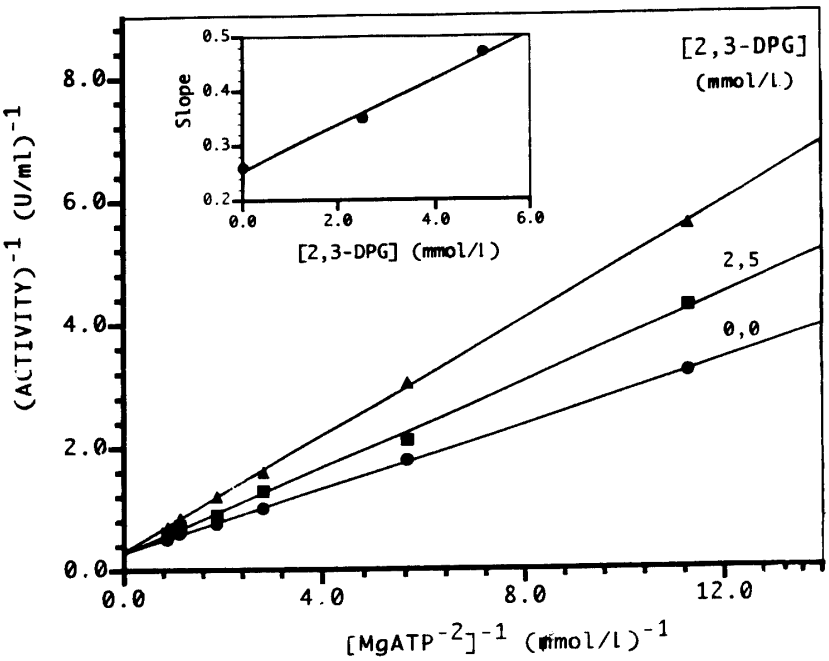

Fig. 3. Plot of the competitive inhibitory effect of 2,3-bisphosphoglycerate (2,3-DPG) ($K_{I\ 2,3\text{-DPG}} = 1200 \pm 50\ \mu M$) in regard to $MgATP^{2-}$ on the activity of hexokinase purified from chicken erythrocytes.

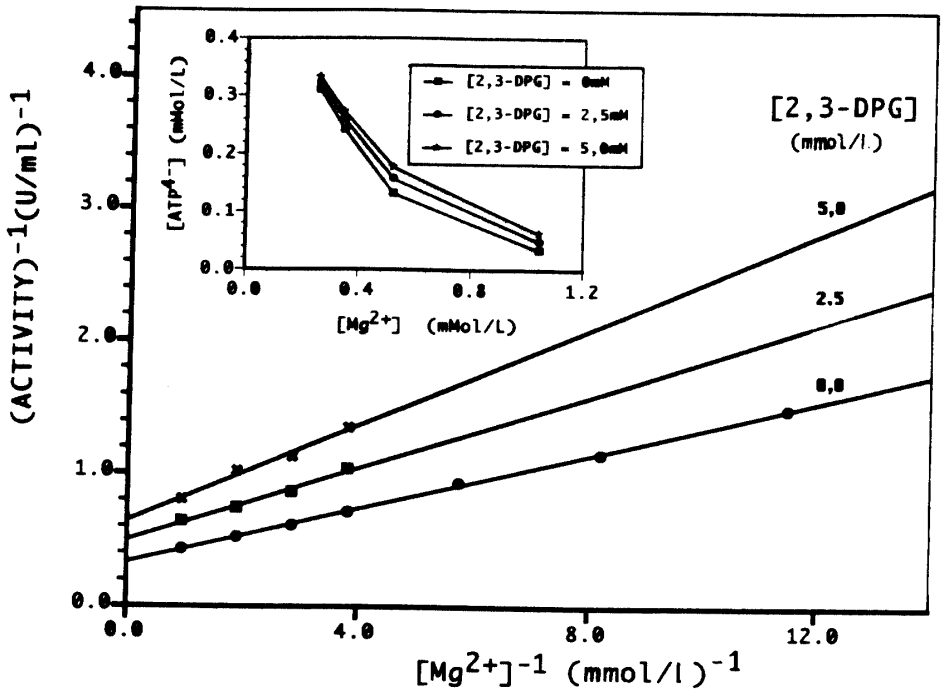

Fig. 4. Plot of the non-competitive inhibitory effect of 2,3-bisphosphoglycerate on the activity of hexokinase purified from chicken erythrocytes in regard to Mg^{2+}. The inserted plotting shows the correlation between 2,3-DPG, Mg^{2+} and the generation of ATP^{4-}.

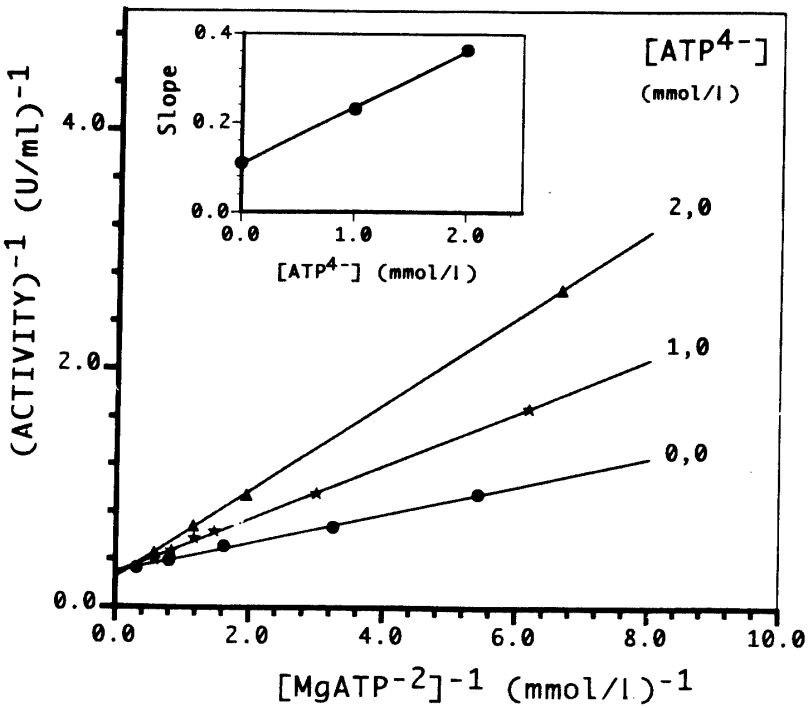

Fig. 5. The kinetics of the competitive inhibition displayed by ATP^{4-} ($K_{I\,ATP^{4-}} = 800 \pm 40$ μM) in regard to $MgATP^{2-}$ toward chicken erythrocyte hexokinase.

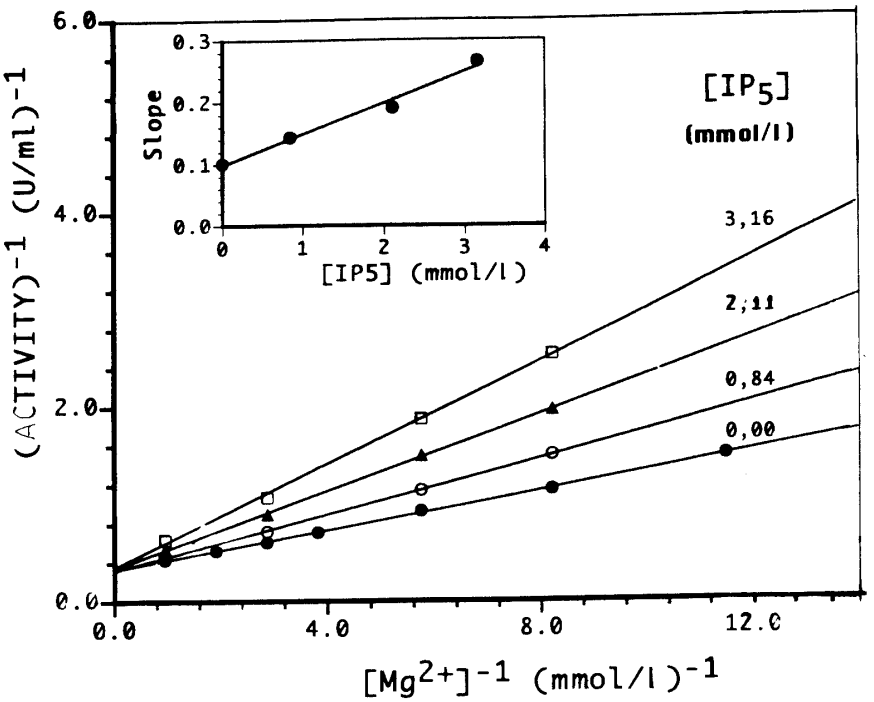

Fig. 6. The kinetics of the competitive inhibition in regard to Mg^{2+} displayed by inositol pentaphosphate ($K_{I\,IP5} = 2120 \pm 104\ \mu M$) on the activity of chicken erythrocyte hexokinase. The insert plot shows the linearity of the IP5 concentration and the inhibitory effect due to the chelating properties of this organic phosphate metabolite toward Mg^{2+}.

inadequate NADPH synthesis, essential to recovery from reduced glutathione levels, as a consequence of the low levels of glucose-6-phosphate produced. Other facts which corroborate the assumption of birds possessing a defective glucose metabolism peculiar to their physiological behavior include the compartmentalization of blood glucose mainly in the plasma; the low rate of glucose metabolism by chicken RBCs as assayed by the glycolytic flux; the lack of lactic acid production upon incubation with glucose; and the life span of the bird RBCs (around 30 days).

In RBCs of adult birds, there is a pool of Mg^{2+} that may be at the disposal of several organophosphate metabolites, mainly ATP and IP5, among others. Considering now that the total intracellular concentration of Mg^{2+} is much lower than those of ATP and IP5, it may be expected that part of the ATP will not be found as a complex in the cell. This means that ATP may inhibit hexokinase activity. This assumption is corroborated by the observation that IP5 is a competitive inhibitor of HK in regard to Mg^{2+}. A similar fact has been found (ROSA *et al.*, 1988) in regard to phosphofructokinase, which is also inhibited by competitive inhibition by IP5. Both 2,3-DPG and IP5 display a similar effect as hemoglobin modulators, 2,3-DPG for mammalian RBCs and IP5 for bird RBCs. As to their effect as inhibitors of the HK, however, 2,3-DPG is a competitive inhibitor in regard to the complex $MgATP^{2+}$, while IP5 is a competitive inhibitor in regard to the free Mg^{2+} from the cell pool.

According to BISWAS *et al.* (1984), RBC myo-inositol poliphosphates, as IP5, are the

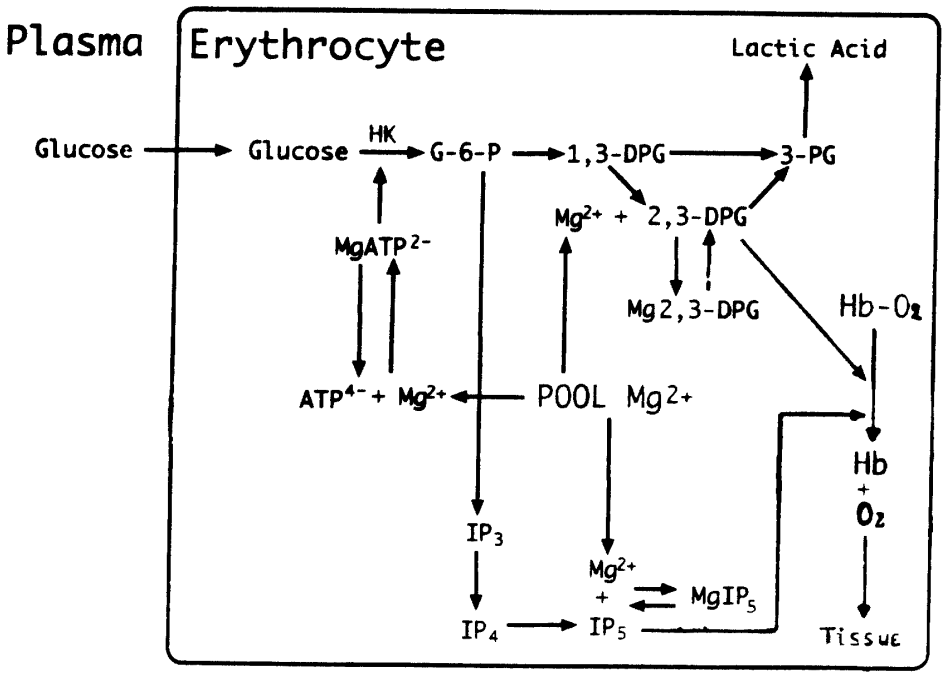

Fig. 7. The metabolic interrelationships in the kinetics of chicken erythrocyte hexokinase in regard to the Mg^{2+} cell pool. The Mg^{2+} from the RBC cell pool of adult birds, as in the case of adult penguins and skuas, as well as chickens, is chelated by the main organic phosphate metabolites as IP5, ATP and ADP as well as by other organic phosphate compounds suches GTP and UTP, among others. In mammalian RBC, 2, 3-DPG acts as a hemoglobin modulator, a property shared by IP5 that is the hemoglobin modulator for bird RBCs hemoglobin.

result of a biosynthetic pathway involving glucose-6-phosphate as the starting metabolite. This fact conflicts with the assumption that bird RBCs possess a defective glucose metabolism. It is possible, however, to suppose that glucose-6-phosphate may accumulate in the erythrocyte and inhibit hexokinase, building a suitable and adequate environment to feed myo-inositol synthesis and, from it, the synthesis of IP5 because of its fundamental property as a hemoglobin modulator.

References

BACILA, M., RODRIGUES, E., ROSA, R. and LUCCHIARI, P.H. (1990): Developmental biology of Antarctic birds in regard to the organic phosphate compounds of erythrocytes. Korean J. Polar Res., **2**, 37-42.

BARRON, E.S.G. and HARROP, G.A. (1928): Blood cell metabolism. II. The effect of methylene blue and other dyes upon the glycolysis and lactic acid formation of mammalian and avian erythrocytes. J. Biol. Chem., **79**, 65-87.

BARTLETT, G.R. (1968): Phosphorus compounds in the human erythrocytes. Biochem. Biophys. Acta, **156**, 221-230.

BELL, D.J. (1971): Plasma Glucose. Physiology and Biochemistry of Fowl, Vol. 2, ed. by D.J. BELL and B.M. FREEMAN. London, Academic Press, 913 p.

BENDELLA, A. DE (1971): Inaugural Dissertation: Tieraztlichen nochshule, Vienna. Physiology and Biochemistry of Domestic Fowl, Vol. 2, ed. by D.J. BELL and B.M. FREEMAN. London, Academic Press, 913 p.

BISWAS, B.B., GOSH, B. and MAJUMDER (1984): Myo-inositol poliphosphates and their role in cellular metabolism. A proposed cycle involving glucose-66-phosphate and myo-inositol phosphates. Subcellular Biochemistry, Vol. 10, ed. by D.B. RODYN. New York, Plenum Press, 237-273.

BOHUON, C. (1962): Microdosage du magnsésium dans divers millieux biologiques. Clin. Chim. Acta, **7**, 811-817.

EHRLENBACH, F. (1938): Experimental study of the blood sugar of birds. Z. Vgl. Physiol., **26**, 28.

HESS, H.H. and DERR, J.E. (1974): Assay of inorganic and organic phosphorus in the 0.1-5 nanomole range. Anal. Biochem., **63**, 607-613.

ISAACKS, R.E., HARKNESS, G.A., FROEMAN, P.H. and SUSSNUN, S.A. (1979): Studies on avian erythrocytes metabolism. I. Procedure for separation and quantification of the major phosphorylated metabolic intermediates by anion exchange chromatography. Comp. Biochem. Physiol., **53A**, 95-99.

RODRIGUES, E. (1987): Hexoquinase purificada de eritrócito de galinha (*Gallus gallus domesticus*): Propriedades cinéticas e físico-químicas relacionadas ao metabolismo glicídico. pH. D. Thesis. University of São Paulo, São Paulo, Brasil.

RODRIGUES, E., ROSA, R., OCAMPOS, D., ROSA, C.D. and BACILA, M. (1996): Carbohydrate metabolism in birds erythrocytes: Levels of IP5 and 2,3-DPG in Antarctic birds and their effect on chicken hexokinase activity. Abstracts of XIX Symposium on Polar Biology, December 5-6, 1996. Tokyo, Natl Inst. Polar Res., 81.

ROSA, C.D., FARIA, J.B., RODRIGUES, E. and ROSA, R. (1988): The effect of inositol pentaphosphate on the activity of phosphofructokinase from chicken erythrocytes. Arq. Biol. Tecnol., **31**, 489-494.

ROSA, R. (1977): Comportamento bioquímico do eritrócito de *Gallus gallus domesticus* com relação à operabilidade do sistema multienzimático da glicólise. Doctor's Thesis. University of São Paulo, São Paulo, Brasil.

ROSA, R., ROSA, C.D., OCAMPOS, D. and BACILA, M. (1983): The profile of the glycolytic system and the metabolic activity of chicken erythrocytes. Comp. Biochem. Physiol., **75B**, 141-145.

ROSA, R., RODRIGUES, E. and BACILA, M. (1989): Blood glucose partition and levels of glycolytic enzymes in erythrocytes and somatic tissues of penguins. Comp. Biochem. Physiol., **92B**, 307-311.

UYEDA, K. and RACKER, E. (1965): Regulatory mechanisms in carbohydrat metabolism. VI. Hexokinase and phosphofructokinase. J. Biol. Chem., **24**, 4682-4688.

(Received February 17, 1997; Revised manuscript accepted January 7, 1998)