NTP pattern of avian embryonic red cells: role of RNA degradation and AMP deaminase/5'-nucleotidase activity

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Baumann, Rosemarie, Robert Götz, and Stefanie Dragon. NTP pattern of avian embryonic red cells: role of RNA degradation and AMP deaminase/5'-nucleotidase activity. Am J Physiol Regul Integr Comp Physiol 284: R771–R779, 2003. First published November 21, 2002; 10.1152/ajpregu.00461.2002.—During terminal erythroid differentiation, degradation of RNA is a potential source for nucleotide triphosphates (NTPs) that act as allosteric effectors of hemoglobin. In this investigation, we assessed the developmental profile of RNA and purine/pyrimidine trinucleotides in circulating embryonic chick red blood cells (RBC). Extensive changes of the NTP pattern are observed which differ significantly from what is observed for adult RBC. The biochemical mechanisms have not been identified yet. Therefore, we studied the role of AMP deaminase and IMP/GMP 5'-nucleotidase, which are key enzymes for the regulation of the purine nucleotide pool. Finally, we tested the effect of major NTPs on the oxygen affinity of embryonic/adult hemoglobin. The results are as follows. 1) Together with ATP, UTP and CTP serve as allosteric effectors of hemoglobin. 2) Degradation of erythroid RNA is apparently a major source for NTPs. 3) Developmental changes of nucleotide content depend on the activities of key enzymes (AMP deaminase, IMP/GMP 5'-nucleotidase, and pyrimidine 5'-nucleotidase). 4) Oxygen-dependent hormonal regulation of AMP deaminase adjusts the red cell ATP concentration and therefore the hemoglobin oxygen affinity.

RED BLOOD CELLS (RBC) are of vital importance for embryonic and fetal development of higher vertebrates, and defective erythropoiesis/RBC function is a leading cause of embryonic/fetal death (17). Nevertheless, little is known about the biochemical mechanisms that regulate embryonic RBC function in vivo. During most of embryonic development, the circulating RBC population consists of nucleate immature erythroid cells, which terminate their differentiation inside the circulation. It is unknown if features of the normal erythroid differentiation process contribute to adaptive strategies specific for the embryo.

The oxygen transport function of embryonic RBC has been extensively studied in avian embryos, notably the chick embryo (7, 30, 43). Avian embryos continuously adjust the oxygen affinity to the changing conditions for gas transport during development (42). A key element of this process is the oxygen pressure-dependent control of the RBC organic phosphate pattern and concentration (6, 9). Erythrocytes from early and midterm chick embryos contain very high concentrations of ATP [up to ~15 mmol/l RBC (5, 8, 9)], and ATP is considered to be the dominant allosteric regulator of avian embryonic hemoglobin function during embryonic development (8, 29). In addition to ATP, the RBC from midterm embryos also contain large amounts of UTP and CTP [up to 9 mmol/l RBC (23)]. In the pre-hatching period when chick embryos become severely hypoxic (42), the ATP concentration decreases to less than 3 mM (UTP and CTP decline in parallel) and the erythrocytes transiently accumulate 2,3-bisphosphoglycerate (2,3-BPG) (23, 28), which is a weak allosteric effector of chick hemoglobin (8). The net result is a large increase of hemoglobin oxygen affinity (29, 30, 43). The biochemical mechanisms causing the complex changes of allosteric effector concentrations are not understood; in particular, it is not known by which metabolic mechanism the RBC of early and midterm embryos accumulate ATP, UTP, and CTP.

However, the major developmental signals that initiate the rapid decline of ATP and the switch to 2,3-BPG synthesis in late development have been identified: in vivo hypoxia causes a substantial reduction of ATP as well as an increase of 2,3-BPG and carbonic anhydrase II synthesis (6, 9) in chick embryos. Norepinephrine and adenosine, which activate the RBC cAMP signaling pathway, have been identified as mediators of the hypoxic effect under in vitro and in vivo conditions (21, 22, 24). The rapid degradation of UTP and CTP in late development is largely mediated by cAMP-dependent induction of pyrimidine 5'-nucleotidase (23), which is the key enzyme for metabolism of pyrimidine nucleotides.

In the present study, we tried to assess the contributions of several different factors to the developmental regulation of the RBC trinucleotide pattern and concentration. 1) We determined if RNA degradation of maturing embryonic RBC correlates with the accumulation of NTPs. It is long known that more than 90% of cellular RNA are degraded during terminal differentiation of RBC (cf.37), causing a constant release of
nucleoside monophosphates (UMP, AMP, CMP, GMP), which can be rapidly converted to NTPs by the action of nucleotide kinases. Therefore, RNA degradation in maturing embryonic RBC may serve as an important source for NTPs that act as allosteric effectors of hemoglobin.

2) We previously demonstrated that pyrimidine 5’-nucleotidase plays a major role in the control of the UTP/CTP level of embryonic RBC in late development. To assess if similar mechanisms control the purine nucleotide pool, we determined the developmental profile of AMP deaminase (AMPD) and IMP/GMP 5’-nucleotidase. In adult erythrocytes, AMPD is the key enzyme for AMP degradation: it deaminates AMP to IMP (10, 35, 36). IMP and GMP are the preferred substrates for cytosolic purine 5’-nucleotidase while the affinity for AMP is substantially lower (11). In adult RBC, the AMPD activity is tightly controlled by negative (e.g., Pi and 2,3-BPG) and positive (e.g., ATP) allosteric effectors (45). Low-AMPD activity increases the erythrocyte ATP concentration (18). Red cell organic phosphates also control IMP/GMP 5’-nucleotidase activity: both 2,3-BPG and ATP are potent activators of the enzyme (11).

3) In mammalian RBC, the ATP content is stabilized by adenosine salvage from the extracellular space and subsequent phosphorylation via adenosine kinase. The net yield of the salvage pathway is critically affected by the activity of adenosine deaminase (ADA); a high ADA activity impairs adenosine salvage and causes a reduction of cellular ATP (16, 34). Therefore, we also determined the developmental profile of the ADA activity of embryonic RBC.

Finally, because UTP and CTP are transiently present in concentrations that are equimolar to hemoglobin, we tested if UTP and CTP are allosteric effectors of avian embryonic and adult hemoglobin.

The results of the present study suggest that RNA degradation in embryonic RBC is a major source for provision of allosteric effectors (ATP, UTP, CTP) to hemoglobin. AMPD and IMP/GMP 5’-nucleotidase activity show distinct changes during development that have a decisive influence on red cell trinucleotide pattern and concentration. In late development, AMPD is under oxygen-dependent control that is, in part, mediated by the AMP signaling pathway.

**MATERIALS AND METHODS**

Fertilized eggs from white leghorn chickens were incubated at 37.5°C and 60% relative humidity in a commercial incubator with automatic rotation. In some experiments, eggs were incubated under hypoxic (14% oxygen) or hyperoxic (60% oxygen) conditions in an incubator with automatic control of oxygen pressure (Heraeus, Nürnbberg, Germany). All experiments were carried out in observance of the “Guiding Principles for Research Involving Animals and Human Beings” (2).

Preparation of hemoglobin solutions. Blood was collected from chick embryos after 6 and 11 days of incubation, respectively. The erythrocytes were hemolyzed by freeze-thaw cycles and the lysates were subsequently centrifuged for 15 min at 16,000 g. The hemoglobin solutions were stripped from cofactors with a 1.5 × 50-cm Sephadex G-25 column equilibrated with 20 mM HEPES buffer (pH 7.4 at RT) and 100 mM KCl. The phosphate-free hemoglobin solution was dialyzed against measuring buffer and concentrated in an Amicon UF Cell with PM10 membrane. All procedures were carried out in the cold. Hemoglobin concentration was measured with the cyannemethoglobin method using the millimolar extinction coefficient of 44.0 and a molecular mass of 68,000 Da.

Determination of hemoglobin oxygen affinity. The hemoglobin concentration of the sample was adjusted to 50 g/l with measuring buffer [20 mM HEPES (adjusted to different pH values) and 100 mM KCl]. UTP, CTP, and ATP were added from buffered stock solutions to the desired final concentration. The samples of hemoglobin solutions were equilibrated at 37°C at 100% water vapor saturation in a Radiometer microinometer unit with different gas mixtures of known composition provided by Wösthoff gas mixing pumps. Oxygen saturation of the equilibrated samples was measured in duplicate to triplicate with the Radiometer OSM2. The pH of the samples was measured with an AVL blood gas analyzer.

Analysis of red cell nucleotides. Whole blood from chick embryos was collected at various stages of development under stereomicroscopic control using a cooled glass capillary mounted onto a Leitz micromanipulator. The nucleotide pattern of embryonic RBC was analyzed by HPLC (41).

Measurement of total cellular RNA. Total RNA was prepared from RBC collected from 3- to 17-day-old chick embryos following the method of Chomczynski and Sacchi (15). To assess the quantitative recovery, we determined RNA from test samples containing known amounts of RNA. The results from several control experiments showed that there was a consistent recovery of 75% of the initial RNA, which was considered sufficient for our experimental purpose.

Preparation of dialyzed samples for enzyme activity measurements. Packed red cells were diluted with 5 vol of distilled water and hemolyzed by freeze-thaw cycles. Cell debris was removed by centrifugation (20 min, 4°C, 9,000 g), and the clear supernatant was subsequently dialyzed in Visking tubing against repeated changes of assay buffer.

**AMP deaminase activity.** The enzyme activity in dialyzed hemolysates was measured using the HPLC method of Smolen et al. (40). Assays were carried out at 37°C in a buffer containing 10 mM HEPES (pH 7.2), 100 mM KCl, 5 mM MgCl₂, and 10 mM AMP. The reaction was started by adding 20 µl of dialyzed hemolysate (concentration 20 g/l) and terminated after 10 min with perchloric acid (6% wt/vol). As AMP accounted for more than 97% of all AMP degradation products, calculation of AMP deaminase activity was based on AMP production only.

**5’-Nucleotidase and ADA activity.** 5’-Nucleotidase activity was measured by HPLC analysis (3). Assays of dialyzed samples were carried out in a medium with 50 mM HEPES, 10 mM MgCl₂, 1 mM DTT, pH 7.3 at RT using 1 mM GMP or 1 mM IMP as substrate and 5 mM 2,3-BPG as allosteric activator (10). The incubation was carried out for up to 60 min and samples for HPLC analysis were taken at various intervals. ADA activity was measured with the photometric method of Agarwal et al. (1). Assays were carried out in 1-mL medium containing 50 mM KH₂PO₄, pH 7.5 at 30°C, 0.05 mM adenosine and 300 mM hemolysate. The change in absorbance at 265 nm was followed for 30 min in a Beckman DU-64 spectrophotometer.

To assess the effect of in vivo hyperoxia or hypoxia on red cell function, chick embryos were either incubated for 12 days in air and 5 days in 60% oxygen (hyperoxia) or for 10 days in...
air and 24 h in 14% oxygen (hypoxia). Controls were kept in air.

**In vivo blockade of β-adrenergic and adenosine A2 receptors.** To test the effect of red cell adenosine receptor and β-adrenergic receptor blockade on AMPD activity of late embryos, the following procedure was adopted. Chick embryos were incubated for 11 days in air. At day 11, a small hole with a diameter of 1 mm was drilled into the blunt end of the egg and sealed with wax. At days 12 and 13, the seal was removed and at each time 6.5 nM propranolol (β-blocker) and 0.65 μM 3,7-dimethyl-1-propargylxanthine (DMPX; A2 receptor blocker) in 200 μl NaCl (0.9 g%) were injected onto the inner shell membrane. Controls were injected with 200 μl vehicle. At day 15, blood was sampled for determination of AMPD activity.

**Statistics.** Mean values were tested for significance difference using the *t*-test for unpaired samples.

**RESULTS**

Changes of red cell RNA content and nucleotide pattern between days 3 and 17 of incubation. Figure 1 shows the marked reduction of red cell RNA content between days 3 and 17 of incubation. A drastic decrease of RNA is observed between day 3 (239 μg RNA/mg Hb) and day 8 (23 μg RNA/mg Hb), after day 11 RNA levels change only to a minor extent. To assess to which extent nucleotides released by RNA degradation may have contributed to the expansion of the purine and pyrimidine nucleotide pool, we estimated total RNA nucleotide content (as mmol/l RBC) from the data in Fig. 1 [assuming an average weight per nucleotide of 350 Da, a RNA recovery rate of 75% and using published data (38) for red cell mean cellular hemoglobin concentration (MCHC)]. Calculated RNA nucleotide content declines from ~52 mmol/l RBC at day 3 to 15 mmol/l RBC at day 10 and 7 mmol/l RBC at day 17. The results are compatible with the idea that nucleotides released by RNA degradation account for the high red cell trinucleotide content as shown below (Fig. 2).

Figure 2, A and B, summarizes the developmental changes (concentration given as mol nucleotide/mol Hb) for major nucleotide compounds of the embryonic red cell (ATP, ADP, GTP, CTP, UTP). The time course for developmental changes of ATP and UTP/CTP differs slightly: whereas the ATP/Hb ratio declines after day 5, maximum values for UTP/CTP are established around day 10. Apparently, the high pyrimidine nucleotidase activity present in early development (Fig. 2C) initially limits accumulation of UTP/CTP.

In contrast to ATP/UTP/CTP, the red cell AMP, ADP, and GTP content changed only moderately during development (Fig. 2B). To account for the rapid increase of the MCHC during the first half of development, we recalculated the data of Fig. 2A to yield total NTPs in millimoles per liter of RBC together with the respective ATP and UTP/CTP concentrations (Fig. 2D). Maximum total NTP concentrations (ATP + UTP + CTP) are 21.5 mmol/l RBC at day 8 until day 10, which declined to 2.9

**Fig. 1.** Decrease of RNA content of circulating embryonic red blood cells (RBC; μg/mg Hb) as a function of developmental age. Data are means ± SD, n values are in parentheses.

**Fig. 2.** Embryonic red cell nucleotide concentrations Hb (A, B, D) and pyrimidine nucleotidase activity (Pyr. 5’-N; C) as a function of developmental age. Data are means ± SD with n = 10 except day 14 (n = 11), day 15 (n = 14), and day 16 (n = 7). Data for pyrimidine nucleotidase are taken from Ref. 22. C: data from A were recalculated as millimoles per liter of RBC with published values for mean cellular hemoglobin concentration (MCHC) from Ref. 38 (broken line) to cancel out the changes of red cell hemoglobin concentrations during development. NTP, nucleotide triphosphate.
mmol/l RBC at day 17. ATP decreases from a maximum of \(-14\) mmol/l RBC at day 6 to 8 to 2.2 mmol/l RBC at day 17. The pyrimidine nucleotides UTP/CTP have their maximum at day 10/11 with 8.9 mmol/l RBC and fall to 0.7 mmol/l RBC at day 17.

Previous experiments showed that experimental hypoxia causes a rapid decrease of red cell ATP (9). UTP and CTP concentrations respond in the same way. When day 10 embryos were submitted to short-term hypoxia (24 h at 14% oxygen), UTP and CTP (as well as ATP) were significantly decreased compared with the control (Fig. 3). In the hypoxic embryos, ATP fell by 0.54 mol/mol Hb (\(-2.4\) mmol/l RBC) and UTP + CTP together by 0.95 mol/mol Hb (\(-4.3\) mmol/l RBC).

Developmental changes of erythrocyte AMPD activity. Data presented in Figs. 1 and 2 demonstrate that accumulation of embryonic red cell ATP/UTP/CTP is correlated in time with RNA decrease. The combined NTP content reaches a maximum around day 8 to day 10 before it starts to decrease. Red cell ATP declines earlier than the pyrimidine trinucleotides, suggesting differences in the activities of the respective degradation pathways. As AMPD activity is a major point of control for the adenine nucleotide pool size (10, 35), we determined the AMPD activity of embryonic red cells between days 4 and 17. Figure 4 shows the developmental profile for basal AMPD activity measured at saturating substrate concentration (10 mM AMP). AMPD activity is very low at days 4 and 6 but increases continuously between days 6 and 11 from 3.6 to 24 U/g Hb. Between days 13 and 15, we measured AMPD activity at 12-h intervals and observed a large transient increase of AMPD activity (with a huge individual scatter) to a maximum value of 40 U/g Hb at day 14.5, followed by a rapid decline to a mean value of 15 U/g Hb at day 17.

Inhibitory effect of hypoxia and adenosine/β-adrenergic receptor blockade on AMPD activity. In vivo hypoxia inhibits the fall of red cell ATP in late development of chick embryos (27). To test the effect of hyperoxia on AMPD activity, chick embryos were incubated for 12 days under normoxic conditions and subsequently under hyperoxic conditions (60% oxygen). Red cell AMPD activity and ATP were measured at daily intervals until day 17. The results (Fig. 5A) show that embryos incubated under hyperoxic conditions have a significantly lower red cell AMPD activity than the normoxic controls. At day 14, the controls had a nearly twofold higher AMPD activity (control 29.9 U/g Hb; hypoxia 16.1 U/g Hb). In turn, the hyperoxic embryos maintain their red cell ATP content at a high level (Fig. 5B). This suggests that the activity increase of AMPD in late development is also triggered by hypoxia and largely responsible for the rapid fall of the red cell ATP concentration.

Because the hypoxic effects are mediated via activation of red cell adenosine A2 and β-adrenergic receptors (22, 24), blockade of the adenosine A2 and β-adrenergic receptors attenuates the fall of red cell ATP in late development (21). Chick embryos received the β-adrenergic blocker propranolol and the A2 receptor blocker DMPX on days 12 and 13, and AMPD activity and NTPs were measured at day 15 (Fig. 6). The AMPD activity of the group treated with receptor blockers was significantly lower (16.3 U/g Hb (5.2 U/g Hb SD)) than that of the control (24.5 U/g Hb (3.6 U/g Hb SD)). Note that hyperoxic embryos had a RBC AMPD activity of 14.5 U/g Hb at day 15. As expected, the ATP and pyrimidine nucleotide (CTP/UTP) content was significantly increased in the group treated with receptor blockers. The data indicate that in late development norepinephrine and adenosine are involved in the control of AMPD activity.

**ADA activity of embryonic red cells.** Adenosine salvage is limited by ADA. The ADA activity of embryonic RBC between days 4 and 17 was maintained at a high level of \(-6\) U/g Hb (data not shown) throughout development, which is similar to values observed for rabbit erythroblasts (19). The ADA activity of embry-
onic RBC is about 12-fold higher than the ADA activity of adult human RBC under the same experimental conditions (0.5 U/g Hb). This suggests that adenosine salvage is less effective in embryonic RBC than in adult RBC.

**Developmental profile of RBC 5′-IMP/GMP nucleotidase activity.** The IMP/GMP 5′-nucleotidase that is the principal purine nucleotidase in embryonic RBC (we found no evidence for the presence of a specific AMP-nucleotidase) shows distinct activity changes during development. We used 2,3-BPG, which is equieffective to ATP as activator of IMP/GMP 5′-nucleotidase (11), to avoid ATP-dependent side reactions caused by the phosphotransferase activity of IMP/GMP 5′-nucleotidase (4). Figure 7 shows the developmental change of red cell IMP/GMP 5′-nucleotidase activity between days 4 and 15 that was assessed by measuring degradation of GMP (1 mM) during 60 min of incubation in the absence and presence of 2,3-BPG as activator. In view of the high concentration of ATP during early and midterm development, one can assume that the IMP/GMP 5′-nucleotidase is activated in this period. The results show that between days 4 and 6, the IMP/GMP 5′-nucleotidase activity is higher than at later stages (control 0.43 U/g Hb at day 4 vs. 0.06 U/g Hb at day 11; with 2,3-BPG 2.1 U/g Hb at day 4 vs. 0.41 U/g Hb at day 15). The decline of the nucleotidase activity is maintained when data are recalculated as units per liter of RBC (Fig. 7, inset).

**CTP and UTP decrease the oxygen affinity of embryonic and adult hemoglobin.** In the embryonic red cells, UTP and CTP are transiently present in concentrations equimolar to hemoglobin, and hypoxia rapidly decreases the UTP/CTP content; thus changes of red cell UTP/CTP could contribute to the normal control of embryonic blood oxygen affinity.

We therefore tested the influence of UTP and CTP on 1) the oxygen affinity of purified hemoglobin solutions from day 11 chick embryos, when the definitive red cells with adult hemoglobin A and D are the predominant blood cells and 2) on hemoglobin solutions from day 6 embryos, when the blood contains ~90 to 95% primitive erythrocytes with embryonic hemoglobin (12). ATP and CTP were equally effective in decreasing oxygen affinity of hemolysates from day 6 embryos, whereas UTP was slightly less effective (Fig. 8A). When NTPs were added in a stoichiometric ratio of 2:1 to hemoglobin solutions, the oxygen half-saturation pressure (P_{50}) increased nearly fourfold with UTP and about fivefold with CTP and ATP compared with the control value of 4.7 mmHg. ATP and UTP together (3:1 ATP and 2:1 UTP) gave a P_{50} value of 30 mmHg compared with 30.7 mmHg for ATP (5:1). Data are mean values of two experiments in each case. These

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**Fig. 5.** Effect of hyperoxia on red cell AMPD activity (A) and ATP content (B). Embryos were incubated from day 12 to day 17 in air or in 60% oxygen, and AMPD and ATP were measured at daily intervals. Data are means ± SD, with n values in parentheses (n values are identical for A and B). *Significant difference of at least 2P < 0.05.

**Fig. 6.** Effect of in vivo β-adrenergic and adenosine A2 receptor blockade with propranolol (P) and DMPX (D) on red cell AMPD activity (A) and NTP pattern (B). Embryos were treated with receptor blockers on days 12 and 13 and measurements were made on day 15. Data are means ± SD. *Significant difference to control (*2P < 0.05; **2P < 0.005).
results show that the oxygen affinity of the embryonic hemoglobins is more dependent on the total concentration of purine and pyrimidine trinucleotides rather than on a specific trinucleotide compound. Qualitatively similar results were obtained, when we tested the effect of CTP and UTP on hemoglobin from day 11 chick embryos. At this stage of incubation, CTP and UTP account for nearly half of the red cell trinucleotide content. Figure 8 shows the results obtained in the absence and presence of UTP, ATP, or CTP, which were added at twofold excess over hemoglobin. The P50 values (mean values for n = 2 in each case) obtained at pH 7.2 increased by a factor of ~4 (UTP) to 5 (ATP/CTP) compared with the control (6.9 mmHg). In conclusion, the results indicate that the developmental changes of hemoglobin oxygen affinity in chick embryos are, in part, mediated by alterations of the UTP/CTP concentration.

DISCUSSION

The regulation of the red cell organic phosphate pattern and concentration during embryonic development is the major determinant for the oxygen affinity of avian embryonic blood (7, 29). The data of the present study show that regulation is complex and involves mechanisms that have not been described for adult RBC.

The present investigation contains three main findings: 1) mononucleotides derived from RNA degradation are apparently a prominent source for allosteric effectors of hemoglobin in embryonic RBC, 2) IMP/GMP nucleotidase activity and AMPD activity are important for the developmental control of embryonic red cell nucleotide pattern and markedly change their activities during embryonic development, and 3) in late development AMP-deaminase activity is oxygen dependent and controlled by cAMP-dependent pathways.

RNA degradation contributes to embryonic red cell purine and pyrimidine trinucleotide concentration. It has previously been estimated that RNA degradation during terminal erythroid differentiation may lead to liberation of up to 40–50 mmol/l RBC mononucleotides (37). The time course of RNA degradation between days 3 and 10 is closely correlated to the increase of total red cell NTP content. The calculated RNA nucleotide content (in mmol/l RBC) declines by more than 30 mmol/l RBC in this period, whereas the red cell NTP content increases between days 4 and 8 by ~100% to a maximum of ~22 mmol NTP/l RBC between days 8 and 10. This suggests that initially the RNA-derived mononucleotides are largely retained in the cell and subsequently phosphorylated to NTPs. To which extent nucleotides derived from RNA are retained is decided by the activity profile of the key metabolizing enzymes AMPD and 5'-nucleotidase. Clearly salvage of extracellular pyrimidine and purine nucleoside could also contribute to accumulation of NTPs in the embryonic RBC. However, several arguments argue against a major contribution from this side.

1) Compared with
phosphorylation of NMP derived from RNA breakdown, salvage of extracellular nucleoside is energetically more costly, because it includes one additional phosphorylation reaction. 2) Embryonic RBC have an ADA activity that is more than 10-fold higher than ADA activity of adult mammalian RBC, making it unlikely that adenosine salvage is as effective as in adult mammalian RBC. The finding of high ADA activity in immature embryonic RBC is similar to observations on rabbit erythroblasts, where ADA activity is about 10-fold higher than in the mature rabbit erythrocyte (19). 3) Uridine kinase, the key enzyme for the pyrimidine salvage reaction, is subject to strong competitive inhibition by UTP and CTP at submillimolar concentrations (14). This effective inhibition of uridine kinase precludes accumulation of UTP or CTP via the salvage pathway to the very high levels that we found (~6 mmol/l RBC UTP and ~3 mmol/l RBC CTP at day 10/11), which are about 20- to 30-fold higher than values for UTP and CTP found in normal tissues (44). Indeed, preliminary experiments with day 11 red cells that were incubated for 4 h in the absence and presence of uridine (0.5 mM) failed to detect an increase of either uridine or cytidine nucleotides in red cells that were incubated with uridine. Taken together, these arguments are not in favor of nucleoside salvage as major contributor to the expansion of the NTP pool of embryonic RBC between days 4 and 10 of development.

Function of embryonic red cell UTP and CTP. Our results show for the first time that UTP and CTP are potent allosteric regulators of embryonic and adult chick hemoglobin. At physiological pH, the less-abundant CTP is equieffective with ATP, and UTP is slightly less effective than ATP. It is not surprising that UTP and CTP are allosteric effectors of hemoglobin, because the hemoglobin organic phosphate binding site accommodates a large range of different substrates [e.g., 2,3-BPG, ATP, GTP, inositolpentaphosphate etc. (Ref. 13)]. It follows that during most of chick embryonic development, the oxygen affinity is determined by the total concentration of ATP, UTP, and CTP in the RBC (given the pronounced homology of development, the same might be inferred for other avian embryos). Like ATP, both UTP and CTP rapidly decline when the embryos are subjected to hypoxia, a result that is explained by cAMP-dependent induction of pyrimidine-5'-nucleotidase (23) by adenosine and norepinephrine.

The total concentration of red cell trinucleotides (ATP/UTP/CTP) decreases by ~18 mmol/l RBC between days 11 and 17. During this period, the growing embryo develops progressive hypoxia and hypercapnia due to increasing limitations for diffusive gas transfer, which is reflected in a fall of the oxygen pressure of the chorioallantoic vein of ~40 mmHg and a PCO2 increase of ~30 mmHg (42). The fall of the red cell NTP content causes an increase of the hemoglobin oxygen affinity due to decreased cofactor binding. In addition, the decrease of red cell NTPs should contribute to the large reduction of the pH difference across the red cell membrane in late development (7), because the falling concentration of impermeable anions affects the Donnan equilibrium. The reduction of the red cell NTP content helps to stabilize cell pH in the face of progressive respiratory acidosis. This is of advantage as a reduced cell pH and the corresponding fall of the oxygen affinity would compromise oxygen uptake.

The measurements of organic phosphate concentration (and RNA content) were made on whole blood RBC samples with different amounts of primitive and definitive RBC and one may ask to which extent these data are representative for the different RBC subpopulations. To assess functional heterogeneity, we previously fractionated embryonic RBC from day 7 and made measurements of the oxygen affinity of immature definitive RBC (containing only adult hemoglobin) and whole blood containing 70% embryonic hemoglobin (which is only found in primitive RBC). These measurements showed little difference between the oxygen binding curve of definitive cells and whole blood at day 7 (7). Likewise, fractionation of blood at later stages demonstrated only minor differences in ATP content and oxygen transport function between the various fractions (7). Furthermore, after day 6 of development, primitive and definitive RBC show the same response to experimental hypoxia, i.e., increased 2,3-BPG synthesis and reduction of NTPs (9, 32).

The high content of ATP and particularly UTP may also serve other purposes. Embryonic RBC could be a major source for release of UTP (ATP) to the extracellular space; release of ATP from mammalian RBC due to mechanical deformation is a well-established phenomenon (20). Of particular interest in the present context is the recent finding that UTP is a potent angiogenic factor for the chick chorioallantois, where it is as effective as VEGF (39). As the chick embryonic RBC contain the highest UTP concentrations of all cells analyzed so far and as peak concentrations are found at the time of maximum chorioallantoic growth, release of UTP from embryonic RBC may be an important local factor for regulation of chorioallantoic growth and development.

Role of AMPD and IMP-GMP 5'-nucleotidase for regulation of the purine nucleotide pool in embryonic RBC. The AMP deaminase content, estimated by measuring activity at saturating substrate concentration, is initially (days 4 and 6) very low but increases about 30-fold between day 4 and day 14/15. That AMPD activity is low in the most active phase of RNA degradation allows accumulation of ATP to a peak level of ~14 mmol/l RBC at day 6 to day 8. Because little IMP is produced, GMP will be the preferred substrate for IMP/GMP 5'-nucleotidase. The IMP/GMP 5'-nucleotidase activity at days 4 and 6 is apparently high enough to process most of the GMP liberated from RNA, since we do not observe accumulation of GTP. The fall of red cell ATP after day 8 is largely explained by the increased AMPD activity and decreasing influx of AMP from RNA.

We do not yet know how AMPD synthesis and allosteric behavior are regulated during development. The present results suggest that control is complex and
unlikely to be tied to a single regulatory event. There may be different expression of erythrocyte AMPD isoforms during development, as several splice variants of erythrocyte AMPD exist (31), as well as phosphorylation-dependent change of function as has been demonstrated for heart muscle AMPD (26). In late embryonic development, AMP deaminase is oxygen regulated via the cAMP signaling pathway. Hyperoxia suppresses the transient increase of AMPD activity after day 13, and the same result is obtained by in vivo blockade of red cell β-adrenergic and adenosine A2 receptors. This explains previous findings showing that exposure to hyperoxia (27) or in vivo blockade of RBC β-adrenergic and adenosine A2 receptors attenuates the physiological fall of red cell ATP in late chick embryos (21). Thus, regulation of AMPD activity is an important target for oxygen pressure-dependent control of red cell ATP.

In conclusion, from the present data, we can identify the major factors controlling the RBC nucleotide pattern of the chick embryo during three different developmental phases: phase 1 (days 4–7), phase 2 (days 8–12), and phase 3 (days 13–17). Phase 1 sees accumulation of ATP, UTP, and CTP due to rapid degradation of RNA, low activity of AMPD, and decreasing activities of pyrimidine 5′-nucleotidase (23) and purine 5′-nucleotidase. In phase 2, RNA degradation rapidly declines, and AMPD activity increases, whereas pyrimidine nucleotidase remains at a low level (23); as a result, ATP levels begin to decrease, whereas UTP/CTP levels remain nearly constant. Phase 3 sees a rapid (but transient) cAMP-dependent induction of AMPD and 5′-pyrimidine nucleotidase (23) and low to absent influx of nucleotides from RNA degradation; in consequence ATP as well as UTP/CTP levels decline rapidly.

Thus, the developmental profile for red cell ATP as well as UTP/CTP content reflects the varying input from RNA degradation and the changes of AMPD activity as well as purine- and pyrimidine 5′-nucleotidase activity. Oxygen-dependent hormonal regulation of AMPD and pyrimidine 5′-nucleotidase allows rapid adaptation of the NTP pool size (and therefore oxygen affinity) to changing environmental and/or embryonic conditions (e.g., hypoxia) in midterm and late development.

It remains to be seen if similar phenomena occur during embryonic development of vertebrates from other classes. In early crocodile embryos, a similar decline of RBC NTPs from very high to low values has been described (25). Likewise, it is well established that the nucleate RBC of adult teleost fish respond to hypoxia by reduction of RBC NTPs; the mechanism is still unresolved (33). It would be interesting to see if in these species changes of nucleotide metabolizing enzyme activities and/or RNA degradation contribute to the regulation of the NTP pool size.

**Perspectives**

During erythroid differentiation, as documented for embryonic and adult vertebrates, RNA degradation and release of large amounts of mononucleotides that can be converted to NTPs occur at the same time as maximum synthesis of hemoglobin. This coincidence may have contributed to the selection of organic phosphates as allosteric cofactors for tetrameric vertebrate hemoglobin.

**REFERENCES**


