Hemoglobin Function in the Horse: The Role of 2,3-Diphosphoglycerate in Modifying the Oxygen Affinity of Maternal and Fetal Blood

By H. Franklin Bunn and Hyram Kitchen

The blood of the newborn horse was found to have a higher affinity for oxygen than that of the mother. This difference was due to the fact that the red cells of newborn foals contained 36% lower 2,3-diphosphoglycerate (2,3-DPG) than red cells from their respective mares. The ATP levels of foal and maternal red cells did not differ significantly. Following birth, a prompt rise in the foal’s red cell 2,3-DPG occurred, approaching normal (maternal) levels within 5 days. Unlike many other species, the hemoglobins of the newborn and adult horse have been shown to be structurally identical. Furthermore, phosphate-free solutions of newborn and maternal hemoglobins had identical oxygen saturation curves in the absence and presence of added 2,3-DPG. This study demonstrates that, in contrast to other species, the increased oxygen affinity of horse fetal red cells is due to a lower level of the cofactor 2,3-DPG rather than to the presence of fetal hemoglobin.

The oxygen saturation curve of the blood of newborn humans is shifted to the left of maternal blood, signifying increased affinity for oxygen. This phenomenon has also been demonstrated in a variety of other mammalian species including monkeys, rabbits, pigs, elephants, camelidae, and ruminants. The higher oxygen affinity of fetal blood has been considered physiologically advantageous in facilitating oxygen transport across the placenta. In many species including man, hemoglobins of fetal blood differ structurally from those of the adult.

The following footnote is for material that appears on p. 472:

*The structure of horse hemoglobin has been examined by several approaches in a variety of breeds. Thus far, structural differences have been limited to the alpha chains. Alpha chain duplication with subsequent mutation has been proposed by Kilmartin and Clegg. Their proposal considers variations at alpha 24 and alpha 60. No variation in the structure of beta chains have been reported. Electrophoresis and peptide mapping of hemoglobins from horses in this study identified hemoglobins with the expected physicochemical characteristics with the exception of one pony (Florida). The hemoglobin from this horse has a predominant electrophoretically fast component. Peptide mapping characteristics of this fast component was identical to other fast horse hemoglobin components studied previously.
turally from those found in the adult. In contrast, the hemoglobins of the fetal and adult horse have been shown to be structurally identical. We have compared the oxygen affinities of the blood of newborn foals and their mares. In this species, the higher oxygen affinity of the fetus appears to be due to decreased levels of red cell 2,3-diphosphoglycerate (2,3-DPG) rather than to structural or functional differences in fetal hemoglobin.

**MATERIALS AND METHODS**

Blood specimens were collected in heparin from mares and their newborn foals during the first few hours following delivery. Several breeds of horse were studied, including purebred and half-bred Arabs and Shetland ponies.* Additional blood specimens were drawn intermittently during the first 2 wk after birth. Specimens were obtained on three 5-mo-old fetuses by placing a catheter in the umbilical artery.† Within ½ hr following blood collection, hematocrit and hemoglobin measurements were made and neutralized protein-free extracts were prepared from each blood specimen.§ frozen, and air-shipped in dry ice. Duplicate measurements of 2,3-DPG were made on each extract by the method of Nygaard and Rørth,⁵ which is a modification of the method of Krimski.⁶ The assay is based on the requirement of 2,3-DPG as a cofactor in the monophosphoglycerate mutase reaction. The rate of conversion of 3-phosphoglyceric acid to lactate is reflected in the rate of oxidation of NADH in the lactic acid dehydrogenase reaction. In addition 2,3-DPG of ten of these extracts was also measured by the method of Keitt,⁵ in which 2,3-DPG serves as substrate and is converted stoichiometrically to glyceraldehyde-3-phosphate with the oxidation of an equivalent amount of NADH. A regression line plotted from these data showed very good agreement between the two methods with a slope of 1.0 and an intercept at zero. There was no significant difference between the two sets of data according to the paired t test. In comparison to human extracts, horse extracts contained an inhibitor which delayed the completion of the reaction sequence in the Keitt assay⁵ and made the end points less well defined. This probably accounts for the somewhat better (95%) reproducibility of determination of replicate samples using the method of Nygaard and Rørth.⁶ The 2,3-DPG content of these specimens did not change during storage at -20°C for several months. ATP was measured enzymatically by the coupled oxidation of NADH in the presence of 3-phosphoglycerate, phosphoglycerate kinase, and glyceraldehyde-3 phosphate dehydrogenase.

Whole blood oxygen saturation curves were determined as described previously¹³ within 3 hr after blood specimens were drawn from 2 foal-mare pairs. It is unlikely that the oxygen affinity of horse blood changes significantly during this 3-hr transit time. We observed no change in the PaO₂ of heparinized human blood during 5 hr storage on ice. Aliquots of blood were equilibrated at 37°C with hydrated gases containing known amounts of oxygen, 5% CO₂, balance nitrogen. On each equilibrated sample, oxygen saturation was determined in duplicate with a CO.Oximeter,§ and pH was measured anaerobically at 37°C. The equilibrium points were corrected to pH 7.40 according to the following equation:

\[
\frac{\Delta \log P_{O_2}}{\Delta \text{pH}} = -0.48
\]

This correction was always small since the ΔpH never exceeded 0.1.

Solutions of phosphate-free hemoglobin and standard solutions of 2,3-DPG were prepared and analyzed as described previously.¹⁴ Spectrophotometric oxygen saturation curves were done at 20°C on dilute solutions (0.1 mM tetramer) in 0.05 M bis Tris buffer, 0.1 M chloride, pH 7.2.¹⁴

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*See footnote on previous page.
† The normal gestation period of a horse is 11 mo.
‡ Cal Biochem Inc.: 2,3-DPG Stat Pack.
Recent x-ray diffraction studies of Arnone indicate that β2 (NA2) histidine is one of the specific residues of human hemoglobin which binds electrostatically to 2,3-DPG. Horse hemoglobin has glutamine at this site. This residue may serve equally well as a binding site for 2,3-DPG.

**RESULTS**

Whole blood oxygen saturation curves of two newborn foals and their respective mares are shown in Fig. 1. In both cases, the foal's blood had significantly higher oxygen affinity than that of its mare, as shown by P50 values that were lower by 2.0 mm Hg. The P50 values in these four blood specimens correlated directly with 2,3-DPG levels (Fig. 2). However, from the small number of measurements presented here, it appears that the increment of P50 with increasing red cell 2,3-DPG was less than that for human blood (Fig. 2). In order to investigate this difference, oxygen saturation curves were done on dilute solutions of phosphate-free hemoglobin from maternal and foal blood specimens. In the absence of 2,3-DPG, the maternal and fetal hemoglobins had nearly identical oxygen affinities (Fig. 3). In the presence of 0.2 mM and 0.5 mM 2,3-DPG, an equal reduction in oxygen affinity was observed. The reactivity of horse hemoglobin (foal, mare, and stallion) to 2,3-DPG (P50 DPG/P50 no DPG) was nearly identical to that of man (Fig. 3).* Thus, we cannot explain why intracellular oxygen affinity appears to be less responsive to changes in red cell 2,3-DPG in the horse, compared to man. More whole blood oxygen affinity measurements on horses with varying levels of 2,3-DPG are required in order to establish this point.

*Recent x-ray diffraction studies of Arnone indicate that β2 (NA2) histidine is one of the specific residues of human hemoglobin which binds electrostatically to 2,3-DPG. Horse hemoglobin has glutamine at this site. This residue may serve equally well as a binding site for 2,3-DPG.
These results indicate that red cell 2,3-DPG is a primary determinant of the relative oxygen affinity of the mare and her newborn foal. 2,3-DPG levels were measured on blood specimens drawn simultaneously from 14 mare-foal pairs (Fig. 4). The mean 2,3-DPG for newborn foals was $4.36 \pm 0.98^* \text{ m moles/l red cells}$ compared to a mean of $6.80 \pm 0.89^* \text{ m moles/l red cells}$ for their mares. Each foal had lower red cell 2,3-DPG than its respective mare. In a comparison by the paired $t$ test, these differences were highly significant ($p < 0.001$). Furthermore, the levels of 2,3-DPG found in three 5-mo-old fetuses were 4.96, 4.10, and 3.35 m moles/liter red cells. Thus, the red cell 2,3-DPG of the fetus did not appear to change significantly during the latter half of gestation. Figure 5 shows serial red cell 2,3-DPG levels of 3 foal mare pairs during the first 10 days postpartum. A prompt rise in the foals' 2,3-DPG was found during the first several days after birth, approaching maternal levels. In contrast, 2,3-DPG of their mares did not change appreciably during the last quarter of gestation and postpartum. We observed a similar prompt rise in the 2,3-DPG of 5 other foals fol-

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*Mean ± 1 SD.
Fig. 4. Red cell 2,3-DPG in newborn foals and their mares.

In seven normal adult horses, who were neither pregnant nor postpartum, the mean red cell 2,3-DPG was 6.40 ± 0.85* mmoles/liter red cells, close to the values which we obtained for mares (both pregnant and postpartum) and foals 5 or more days postpartum.

The difference in 2,3-DPG levels of mares and their newborn foals still pertains when concentrations are expressed per gram hemoglobin. The average mean corpuscular hemoglobin concentration (MCHC) of mares' red cells was 36.5 g/100 ml, compared to a value of 34.8 g/100 ml for newborn foals. Furthermore, the MCHC of foals' red cells did not change significantly during the first 2 wk following birth.

ATP has been found to be nearly as potent as 2,3-DPG in lowering the oxy-

Fig. 5. Serial changes in red cell 2,3-DPG of three foal-mare pairs.

*Mean ± 1 SD.
gen affinity of human hemoglobin. We found no significant differences between ATP levels in six foal-mare pairs (mares: $0.15\pm0.05$ mM/l RBC; foals: $0.21\pm0.08$ mM/l RBC). These results confirm earlier reports that the horse has considerably lower levels of red cell ATP than most other mammals including man. Because of its low concentration and the likelihood that it is largely bound to magnesium ion, ATP is of no significance as a determinant of oxygen affinity in horse blood.

**DISCUSSION**

Increased oxygen affinity of fetal blood has been observed in a wide variety of mammalian species (Table 1). (The only exception reported to date is the cat.) It is tempting to conclude that this difference provides an adaptive advantage, perhaps in facilitating the transfer of oxygen across the placenta to the fetus. However, this phenomenon may be of limited significance in man. At least 12 kindreds have been reported in which hemoglobin variants of very high oxygen affinity were inherited as a codominant trait. There are a number of instances in which affected mothers, with a markedly left-shifted oxygen saturation curve ($P_50 = 15$ mm Hg) have borne entirely normal offspring, who did not inherit the mutant hemoglobin. In these cases the blood of the fetus presumably had oxygen affinity considerably lower than that of the mother. Furthermore, Novy et al. have observed that intrauterine transfusion of adult red cells into fetuses with erythroblastosis fetalis had no obvious adverse effect on tissue oxygenation.

Differences between maternal and fetal oxygen affinities vary widely among species (Table 1). The difference which we have found between the mare and the foal is small. Whether it is physiologically significant is open to question. The importance of the differential oxygen affinity of maternal and fetal blood

<table>
<thead>
<tr>
<th>Species</th>
<th>$P_{50}$-mm Hg</th>
<th>pH 7.40, 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Man</td>
<td>26</td>
<td>22</td>
</tr>
<tr>
<td>Rhesus monkey</td>
<td>32</td>
<td>19</td>
</tr>
<tr>
<td>Rabbit</td>
<td>31</td>
<td>27</td>
</tr>
<tr>
<td>Sheep</td>
<td>34</td>
<td>17</td>
</tr>
<tr>
<td>Goat</td>
<td>30</td>
<td>19</td>
</tr>
<tr>
<td>Cow</td>
<td>31</td>
<td>22</td>
</tr>
<tr>
<td>Pig</td>
<td>33</td>
<td>22</td>
</tr>
<tr>
<td>Elephant</td>
<td>24</td>
<td>21</td>
</tr>
<tr>
<td>Camel</td>
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<td>17</td>
</tr>
<tr>
<td>Llama</td>
<td>21</td>
<td>18</td>
</tr>
<tr>
<td>Cat</td>
<td>36</td>
<td>36</td>
</tr>
<tr>
<td>Dog</td>
<td>31</td>
<td>21</td>
</tr>
<tr>
<td>Rat</td>
<td>38</td>
<td>28</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>30</td>
<td>19</td>
</tr>
<tr>
<td>Seal</td>
<td>29</td>
<td>21</td>
</tr>
<tr>
<td>Horse</td>
<td>25</td>
<td>23</td>
</tr>
<tr>
<td>Horse</td>
<td>26</td>
<td>24</td>
</tr>
</tbody>
</table>

Except for the horse, these values were taken from the papers of Novy and Parer and Metcalfe, Dhindsa, and Novy.

*Mean $\pm$ 1 SD.
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probably depends on several factors including uterine blood flow, placental anatomy, and fetal oxygen requirement. This subject is discussed thoroughly in recent reviews.\(^1\)\(^2\) Fetal and maternal horse hemoglobins appear to be structurally and functionally identical. (See footnote on page 471). They interact as strongly with 2,3-DPG as human hemoglobin. The higher oxygen affinity of blood from newborn foals could be accounted for by the subnormal levels of 2,3-DPG in their red cells. Following birth, 2,3-DPG increased promptly toward normal. Presumably, there was a corresponding decrease in oxygen affinity. A prompt right shift following birth has been observed in other species,\(^23\)\(^-\)\(^26\) and may be an important adjustment in the neonate, facilitating tissue oxygenation.

This increase in red cell 2,3-DPG of the newborn foal is so rapid that it cannot be attributed to a new population of red cells. Like many other tissues, erythrocytes probably undergo significant metabolic alterations following birth. The mechanism responsible for the increase in 2,3-DPG is not clear. Rapid alterations in the 2,3-DPG concentration of human red cells may follow changes in plasma pH or inorganic phosphate.\(^27\)

There appear to be three mechanisms by which fetal blood can have a higher oxygen affinity than maternal blood (Table 2):

1. The fetal hemoglobin may have an intrinsically higher oxygen affinity, independent of any intracellular cofactor. Examples include the sheep and the goat.\(^23\)\(^,\)\(^24\)

2. The fetal hemoglobin may have impaired interaction with a red cell cofactor. For example, the oxygen affinity of “stripped” human fetal hemoglobin is very similar to (adult) hemoglobin A. However, hemoglobin F binds less strongly to 2,3-DPG,\(^28\)\(^-\)\(^30\) and therefore in the presence of 2,3-DPG within the red cell, its oxygen affinity is correspondingly greater.

3. The content of 2,3-DPG may be significantly less in fetal red cells. This is true for the horse, as shown in this report, and also for the pig\(^25\)\(^,\)\(^31\) and the dog.\(^26\)

The first two mechanisms cited above require the presence of a fetal hemoglobin which differs structurally and functionally from the adult hemoglobin. In the third, the two hemoglobins may be identical, as appears to be the case for the horse, dog, and pig. However, structural identity of fetal and adult hemoglobins cannot be definitely established unless primary amino acid sequences have been determined.

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Species</th>
<th>Distinct Fetal Hemoglobin</th>
<th>Relative Oxygen Affinities in Absence of RBC Organic Phosphates</th>
<th>Red Cell 2.3-DPG mmole/liter RBC</th>
<th>Interaction of Hemoglobin With 2.3-DPG</th>
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</thead>
<tbody>
<tr>
<td>I. Sheep. goat</td>
<td>Yes</td>
<td>Fetal &gt; Adult</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>Weak(^24)</td>
</tr>
<tr>
<td>II. Man</td>
<td>Yes</td>
<td>Fetal = Adult</td>
<td>-</td>
<td>-</td>
<td>Weak(^28)(^,)(^29)</td>
</tr>
<tr>
<td>III. Horse</td>
<td>No</td>
<td>Fetal = Adult</td>
<td>4.5</td>
<td>6.8</td>
<td>Strong</td>
</tr>
<tr>
<td>Pig</td>
<td>No(^23)(^,)(^34)</td>
<td>Fetal = Adult(^31)</td>
<td>-4(^25)</td>
<td>-10(^25)</td>
<td>?</td>
</tr>
<tr>
<td>Dog</td>
<td>No(^26)</td>
<td>?</td>
<td>0.5(^26)</td>
<td>4.4(^26)</td>
<td>?</td>
</tr>
</tbody>
</table>

* Bunn HF, Seal US: unpublished observations.
ACKNOWLEDGMENT

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