Differences in the Interaction of 2,3-Diphosphoglycerate with Certain Mammalian Hemoglobins

Abstract. *The hemoglobins of man, horse, dog, rabbit, guinea pig, and rat all have relatively high (nonphysiologic) oxygen affinity when stripped of organic phosphates, and a strong reactivity with 2,3-diphosphoglycerate (2,3-DPG). Appropriately, their red cells contain high levels of 2,3-DPG. In contrast, the sheep, goat, cow, and cat have low oxygen affinity hemoglobins which interact weakly with 2,3-DPG, and low concentrations of red cell 2,3-DPG. These hemoglobins have structural differences at the NH_2-terminus of the β chain, a site where 2,3-DPG is thought to bind.*

2,3-Diphosphoglycerate (2,3-DPG) is the most abundant organic phosphate in the red cells of man and many other mammals (*1*). Benesch and Benesch and Chanutin and Curnish demonstrated that this compound is a potent modifier of hemoglobin function, due to its ability to lower oxygen affinity (*2*). Subsequently, a number of clinical and experimental studies have shown that 2,3-DPG can serve as a sensitive *control* in adaptation to hypoxia. The effect of 2,3-DPG on the oxygenation of various mammalian hemoglobins is relevant to both comparative physiology and biochemistry. Since the primary structure of many mammalian hemoglobins is known, a difference in the reactivity to 2,3-DPG among these hemoglobins should lead to a further understanding of the mechanism by which 2,3-DPG binds to hemoglobin.

Blood was drawn from the following animals: normal adult man, Arabian horse, Holstein cow, Dorset sheep, Alpine goat, New Zealand rabbit, Sprague-Dawley rat, mongrel dog, and domestic breeds of cat and guinea pig. Specimens were collected in heparinized tubes. Hemolysates were "stripped" of organic and inorganic phosphates by dialysis in a stretched cellulose membrane against 0.1*M* NaCl (*3*). Analysis of protein-free extracts of these hemolysates showed that at least 95 percent of the phosphates had been removed. Because of the heterogeneity of animal hemoglobins, the components of each hemolysate were analyzed by electrophoresis on cellulose acetate, *p*H 8.6, and, in most cases, isoelectric focusing on polyacrylamide gel (*4*). In this way the phenotypes of the sheep, goat, cow, and cat hemoglobins were readily determined. A and B hemoglobins from a heterozygous sheep were separated by chromatography on diethylaminoethyl cellulose (*5*). Cat A and B hemoglobins were separated on carboxymethyl cellulose (*6*). In each case the hemoglobin-rich fractions were pooled, concentrated by ultrafiltration, and dialyzed against 0.1*M* NaCl. Because the latter two hemoglobins autoxidized more readily than those of other species, experiments were completed within 48 hours after preparation of hemolysates.

2,3-Diphospho-D-glyceric acid was obtained from Calbiochem. The pentacyclohexylammonium cation was removed as described by Benesch *et al.* (*3*). The 2,3-DPG concentration of the reagent solution used in these studies was assayed by titration and phosphate determination and also by an enzymatic analysis specific for 2,3-DPG (*7*), all of which gave excellent agreement. We also measured 2,3-DPG enzymatically in neutral protein-free extracts prepared from fresh samples of animal blood.

Oxygen equilibrium curves were determined by a method adapted from Allen *et al.* and Riggs (*8*) and described in detail elsewhere (*9*). All experiments were done at 20°C with solutions consisting of 0.05 m*M* hemoglobin (tetramer) in 0.1*M* NaCl, 0.05*M* bis tris buffer, *p*H 7.2, and in the absence and presence of 2,3-DPG.

The effect of 2,3-DPG on the oxygenation of various mammalian hemoglobins is shown in Fig. 1. These animals fell into two distinct groups. When "stripped" of red cell phosphates, the hemolysates of man, horse, dog, rabbit, guinea pig, and rat had relatively high oxygen affinities. In the presence of 0.2 m*M* 2,3-DPG, the P_{50}'s increased 55 to 95 percent, indicating a substantial lowering of oxygen affinity. Thus, these animals have hemoglobins that are strongly reactive to 2,3-DPG. Furthermore, their red cells contained high concentrations of 2,3-DPG, in good agreement with values reported previously (*1*). These animals probably require such high levels of red cell 2,3-DPG in order to enable physiologic oxygen unloading. In contrast, the "stripped" hemolysates of ruminants (cow A, sheep AB, and B, and goat A) and cat had intrinsically *low* oxygen affinities and were weakly reactive to 2,3-DPG. Only a 6 to 8 percent rise in P_{50} was observed in the presence of 0.2 m*M* 2,3-DPG. Similarly, sheep A hemoglobin isolated from AB hemolysate increased 8 percent. As others have found (*1*), these animals had much lower levels of red cell 2,3-DPG than those in the first group (Fig. 1). It is unlikely that the red cells of these animals contain factors other than 2,3-DPG which significantly lower intracellular oxygen affinity. Benesch, Benesch, and Yu found that the P_{50} of sheep hemolysate was unchanged after stripping it of dialyzable substances (*2*). This hemolysate had a lower reactivity to 2,3-DPG than human hemolysate.

From these results, it appears that animals have high red cell 2,3-DPG only if they need it, that is, as a cofactor in lowering oxygen affinity sufficient for physiologic oxygen unloading. Such economy is appropriate, since

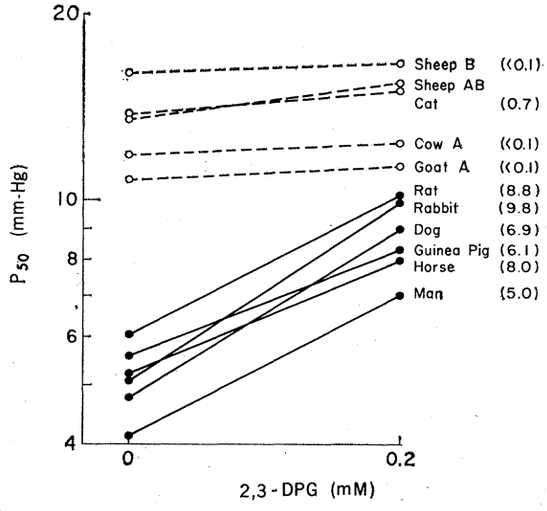

Fig. 1. The effect of 0.2 m*M* 2,3-DPG on the oxygen affinity of animal hemoglobins; P_{50} is the partial pressure of oxygen at which hemoglobin was half saturated. Oxygen equilibriums were done on 0.05 m*M* hemoglobin (tetramer) in 0.1*M* NaCl, 0.05*M* bis tris, *p*H 7.2, 20°C. The concentration of erythrocyte 2,3-DPG (in millimoles per liter of cells) is shown in parentheses beside each species tested.

Table 1. Effect of 2,3-DPG on oxygen affinity of cat hemoglobins.

2,3-DPG (mM)	Hemoglobin A (P_{50}, mm-Hg)	Hemoglobin B (P_{50}, mm-Hg)
0	10.6	15.0
0.2	11.4	15.0
2.0	14.6	15.8

the maintenance of a high concentration of 2,3-DPG is energetically expensive. Its synthesis involves bypassing the phosphoglycerate kinase reaction, a step resulting in the synthesis of adenosine triphosphate.

The marked differences in 2,3-DPG reactivity among these animal hemoglobins should be considered in light of the known structural differences among these proteins. Such a structural-functional approach may provide additional information as to the site of 2,3-DPG binding. Earlier data of Benesch *et al.* (*2*) indicated that 2,3-DPG binds in a one-to-one molar ratio with the β chains of deoxyhemoglobin tetramer, probably somewhere in the central cavity along the diad axis of symmetry. From a comparison of 2,3-DPG reactivity among different major and minor components of human hemoglobin, Bunn and Briehl concluded that the α amino groups of the β chains were involved in 2,3-DPG binding (*9*). Benesch *et al.* also came to this conclusion from "affinity labeling" studies on hemoglobin in which the same amino group was covalently linked to pyridoxal phosphate (*10*). Recently Perutz has fitted 2,3-DPG to an atomic model of human deoxyhemoglobin built from a Fourier synthesis at 3.5-Å resolution (*11*). One molecule of 2,3-DPG was placed into the internal cavity in such a way that the phosphates could form salt bonds with the two α amino groups of the β chains and the imidazoles of the β-H21 histidines, while the carboxyl was within bonding distance of the ε amino of one of the β-EF6 lysines. Among the hemoglobins used in this study, the primary sequences of man, horse, sheep A and B, goat A, cow A, and rabbit have been reported (*12*). These hemoglobins all contain β-H21 histidine and β-EF6 lysine. Perutz has recently pointed out that the ruminant hemoglobins contain a deletion at the NH_2-terminal end of the β chains (*11*). Thus the distance between α amino groups would be about 6 Å greater than in the hemoglobins having the usual 146 residues per chain. Because of this, one molecule of 2,3-DPG would not be expected to bind to both α amino groups, and thus the strength of binding at this proposed site would be considerably attenuated.

The structure of cat A and B hemoglobins has been partially worked out by Lessard (*13*). Among differences in their β chains, the NH_2-terminus of hemoglobin B is acetylated. In 0.2 mM 2,3-DPG neither of these hemoglobins interacts appreciably with 2,3-DPG (Table 1). However, in the presence of a high concentration of 2,3-DPG (2 mM), the oxygen affinity of cat A hemoglobin decreased considerably while that of cat B, hardly at all. Cat B resembles human F_1 and A_{IC}. All three hemoglobins have a blocked α amino group on the β chain and decreased reactivity to 2,3-DPG (*9*). Recently Taketa, Lessard, and Mauk have found a similar difference in the oxygenation of the two cat hemoglobins in the presence of a high concentration of 2,3-DPG (*14*).

H. Franklin Bunn
Thorndike Memorial Laboratory,
Boston City Hospital,
Boston, Massachusetts 02118

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Lymph Node Cells: Their Differential Capacity to Induce Tolerance of Heart and Skin Homografts in Rats

Abstract. *Rats of the BN strain, inoculated at birth with (Lewis × BN)F_1 hybrid lymph node cells are not tolerant of Lewis skin grafts but do display high degrees of tolerance of Lewis hearts.*

In rats high degrees of tolerance of Ag-B incompatible skin grafts, that is, skin grafts that differ from their host at the major histocompatibility locus of the species (*1*), can be induced effectively by inoculating neonatal recipients only with bone marrow cells (*2*). By comparison, splenic and lymph node cells are poor in inducing tolerance in respect to skin grafts, and thymocytes are ineffective (*3*). Nevertheless, when injected into neonatal hosts Ag-B incompatible lymphocytes are highly effective in inducing tolerance in respect to themselves (*4*). Thus blood lymphocytes from BN rats injected at birth with BN/Lewis F_1 hybrid lymph node cells display a significantly diminished reactivity when exposed to BN/Lewis lymphocytes in vitro in the mixed lymphocyte interaction, regardless of whether the inoculated animals are tolerant of Lewis skin grafts. On the other hand, BN rats treated in similar fashion with BN/Lewis F_1 hybrid thymocytes are neither tolerant of Lewis skin grafts nor do their lymphocytes have a diminished level of responsiveness when confronted with BN/Lewis lymphocytes in vitro (*4*). Inasmuch as tolerance of skin homografts is easily obtained with either lymphoid cells or thymocytes in Ag-B *compatible* strain combinations (*2, 5*), one explanation for these findings is that in rats skin and bone marrow cells may possess some Ag-B specificities that are absent or poorly expressed on lymphoid cells and thymocytes, and that the latter, in turn, are deficient in some Ag-B determinant or determinants present on lymph node and splenic cells.

To establish whether this inferior ability of lymphoid cells to induce tolerance of skin homografts applies to