BINDING OF 2,3-DIPHOSPHOGLYCERATE TO OXYHEMOGLOBIN

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Summary: Extensive binding of 2,3 diphosphoglycerate to oxyhemoglobin is demonstrated both by diafiltration and by Sephadex gel filtration. The average concentration of both 2,3 DPGA and hemoglobin in human erythrocytes is about 5 mM; a portion of the 2,3 DPGA remains bound to oxyhemoglobin and cannot be removed by Sephadex gel filtration.

2,3-diphosphoglycerate, discovered by Greenwald[1], was long suspected to be related to red cell viability since it was noted by many workers that its disappearance paralleled the decreased survival of stored human erythrocytes. The exciting findings of Chanutin[2] showing binding of 2,3-diphosphoglycerate (2,3 DPGA) to hemoglobin and of Benesch et al.[3] demonstrating a shift in the O_2 saturation curve of hemoglobin by 2,3 DPGA have awakened much interest. Until then the only molecular role known for 2,3 DPGA was its stimulating effect on 2,3 DPGA dependent phosphoglyceromutases[4,5].

On the basis of information in the literature and on the extensive studies on phosphoglycerates carried on for the last 15 years in this laboratory, it was suggested that there were other roles for 2,3 DPGA including that of affecting conformation of hemoglobin[6,7,8]. Moreover, as an extension of studies on binding of 2,3 DPGA to phosphoglyceromutases, we tried without success, to find marked changes in physical parameters[9] such as by optical rotatory dispersion or by ultracentrifugation accompanying binding of 2,3 DPGA to hemoglobin. However, as shown in this paper, 2,3 DPGA binds extensively to oxyhemoglobin. This is in disagreement with the results of Benesch et al.[3,10] who find binding of 2,3 DPGA to reduced hemoglobin but not to oxyhemoglobin.

Hemoglobin solutions were prepared from saline washed human red cells by hemolysis with H_2O and freezing, destromatization with toluene, followed by centrifugation. The hemoglobin was then "stripped" of 2,3 DPGA by gel filtration through Sephadex G-25 column equilibrated with 0.1 M NaCl as described by Benesch et al.[3] In some 16 experiments the hemoglobin solutions retained nearly 10% of their original 2,3 DPGA, i.e. showed molar ratios of 2,3 DPGA/hemoglobin of 0.1 to 0.15. The solutions were brought to the desired protein concentration by diafiltration through a Diaflo PM-30 membrane (M.W. cut off ≈ 30,000).

Fig. 1 illustrates binding of 2,3 DPGA to hemoglobin as measured by filtration dialysis. The experiments presented in Fig. 1 and Table 1 were carried out using the "wash-in" technique with a fixed-volume diafiltration assembly as described by Blatt et al.[11] Ten ml of the "stripped" oxyhemoglobin solution in 0.1 M NaCl was added to the filtration cell containing a Diaflo PM-30 membrane. The 2,3 DPGA solutions at the indicated concentrations were also prepared in 0.1 M NaCl and placed in the reservoir. The

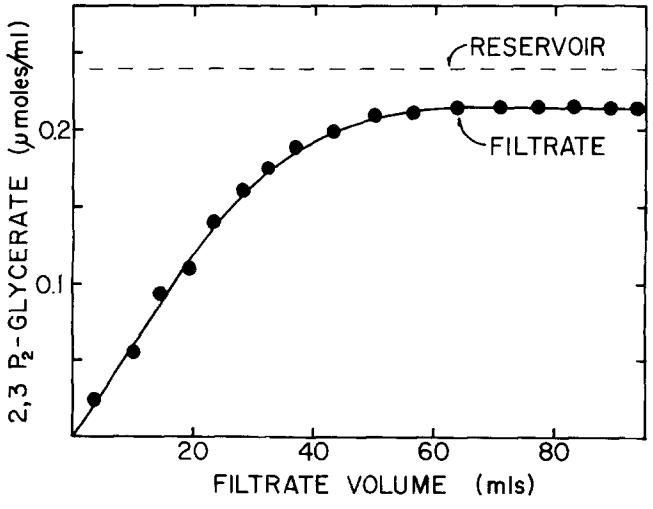

Figure 1. Binding equilibrium of 2,3 DPGA to oxyhemoglobin as measured by the filtration dialysis technique. The concentration of 2,3 DPGA in the reservoir remains constant. The values represented in this figure correspond to data from experiment 2 in table 1.

Table 1

BINDING OF 2,3 DPGA TO OXYHEMOGLOBIN AS DETERMINED BY FILTRATION DIALYSIS

Experiment	Conditions		Binding ratio
	HbO_2	2,3 DPGA	2,3 DPGA/HbO_2
	mM	mM	
1	0.10	0.10	0.7
2	0.25	0.24	0.45
3	1	0.25	0.2
4	2	1	0.5
5	2.3	1.8	0.5

system was pressurized at 20 psi with O_2 and the hemoglobin in the filtration cell was continuously stirred with a magnetic stirrer. Aliquots of the ultrafiltrate were collected and assayed for 2,3 DPGA to determine the concentration at binding equilibrium. This value corresponds to the free 2,3 DPGA inside the cell. The 2,3 DPGA determined inside the filtration cell at the completion of the experiment represents the total 2,3 DPGA (bound plus free). The difference between these two values is the amount of 2,3 DPGA bound to the protein. All binding ratios were corrected for membrane retention, experimentally determined in "blank" experiments following diafiltration of hemoglobin solutions, with H_2O replacing the hemoglobin in the filtration cell. All experiments were conducted at room temperature. Table 1 summarizes a number of experiments by this technique demonstrating extensive binding of 2,3 DPGA to oxyhemoglobin at a wide range of concentrations, even approximating those which occurred in the red cell.

Fig. 2 typifies again the extensive binding of 2,3 DPGA to oxyhemoglobin demonstrated by the technique of Pfleiderer and Auricchio[12]. Varying amounts of 2,3 DPGA were added to known concentrations of "stripped" oxyhemoglobin

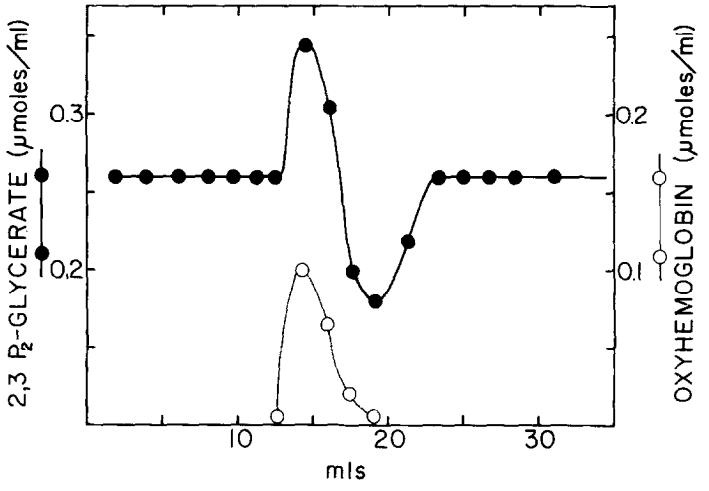

Figure 2. Elution profile of a representative binding experiment using a Sephadex G-25 fine column (1 x 25). The values represented in this figure correspond to data from experiment 9 in table 2.

Table 2

BINDING OF 2,3 DPGA TO OXYHEMOGLOBIN AS DETERMINED BY GEL FILTRATION

Experiment	Conditions		Binding ratio
	HbO_2	2,3 DPGA	2,3 DPGA/HbO_2
	mM	mM	
1	0.3	0.1	0.58
2	0.3	0.24	0.64
3	0.7	0.05	0.44
4	0.7	0.05	0.5
5	0.7	0.1	0.77
6	0.7	0.1	0.83
7	0.7	0.1	0.69
8	0.7	0.1	0.66
9	0.7	0.26	0.85
10	2.8	2.4	0.98
11	2.8	1.4	0.75

in 0.02 M NaCl and allowed to stand at room temperature for 1/2 hour. One-half to 1 ml samples of these solutions were then passed through a 1 x 25 cm column of Sephadex G-25 fine, previously equilibrated at room temperature with equimolar concentrations of 2,3 DPGA in 0.02 M NaCl. Two ml aliquots were collected and assayed for 2,3 DPGA and hemoglobin concentrations. Calculation of the molar binding ratio of 2,3 DPGA/hemoglobin for each column was based upon the amount of 2,3 DPGA in excess of the baseline level in the peak protein tube. Table 2 summarizes the results of a series of experiments at fairly wide concentrations of oxyhemoglobin and 2,3 DPGA, and as shown in all cases, there is extensive binding.

As reported above, there is a portion of 2,3 DPGA bound which cannot be removed by Sephadex as described by Benesch[3] and in addition there is in all cases extensive binding of 2,3 DPGA to oxyhemoglobin. Possibly the discrepancies may reflect the fact that we use the highly specific and sensitive enzymatic method for 2,3 DPGA first described in this laboratory[13] and which has recently been automatized[14].

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