Oxygen Affinity in Red Cells: Inability to Show Membrane-Bound 2,3-Diphosphoglycerate

In 1971 and 1972, Pendleton *et al.* (*1*), in our laboratories, reported on the effect, on the hemoglobin-oxygen dissociation curve, of incubating washed erythrocytes with propranolol. The curve showed a greater than + 7-mm shift to the right at the P_{50} point [the partial pressure (mm-Hg) of O_2 at which hemoglobin is 50 percent saturated]; however, a concomitant increase in the concentration of 2,3-diphosphoglycerate (2,3-DPG) was not observed. More recently, Oski *et al.* (*2*) reported a release, induced by propranolol, of 2,3-DPG bound to inner membranes into the erythrocyte itself (both in vitro and in vivo), and postulated that the increase in 2,3-DPG not bound to membrane (unbound) might partially account for the shift of the curve to the right reported by Pendleton *et al.*

We repeated the in vitro experiments described by Oski *et al.*, with freshly drawn heparinized blood from nonsmoking volunteers of both sexes, but made two modifications to the procedure. The first modification was the determination of hemoglobin content on the blood sample taken for 2,3-DPG assay, and the second was the use of the stoichiometric 2,3-DPG assay method of Keitt (*3*) in place of the routine kinetic technique. With these modifications, we were unable to demonstrate any differences in the ratio of 2,3-DPG to hemoglobin (expressed as nanomoles of 2,3-DPG per milligram of hemoglobin) between whole blood, lysates, or supernatant solutions, whether or not the samples were treated with propranolol. Representative data are shown in Table 1.

Table 1. Ratios of 2,3-DPG in which the technique of Oski *et al.* (*2*) and the 2,3-DPG assay of Keitt (*3*) were used. Standard error of our 2,3-DPG assay was ± 7 percent. Concentration of propranolol, $6 \times 10^{-5} M$.

Treatment	Ratio of 2,3-DPG (nmole)/Hb (mg)		
	Whole blood	Lysate	Supernatant
	Donor 1		
None	15.68	14.62	15.27
None	16.06	15.75	14.82
Propranolol	15.66	15.83	15.57
Propranolol	16.06	15.68	15.49
	Donor 2		
None	15.78	14.45	14.61
None	15.13	13.85	14.40
Propranolol	15.80	15.72	15.96
Propranolol	15.58	15.33	15.80

Because of our inability to confirm the findings of Oski *et al.*, we redesigned the experimental procedure, and performed a series of experiments in which we used combinations of the following: high-speed centrifugation of lysate (50,000*g* to 100,000*g* for 25 to 60 minutes), precipitation of protein by perchloric acid prior to 2,3-DPG assay, the Schroter and Winter (*4*) kinetic assay for 2,3-DPG, and in all cases, only untreated blood. Again, we found no significant differences in the ratios of 2,3-DPG to hemoglobin in whole blood, lysate, and supernatant, even when six determinations were performed on the same blood sample.

In addition to the modifications above, we also altered the type of volunteer donors. Thus, instead of blood from nonsmokers, we drew blood from both light (< one pack per day) and heavy (> two packs per day) smokers of both sexes. The results were similar to those shown in Table 1.

In order to see if experimental technique could influence the results, we performed paired experiments, in which blood samples were divided after being lysed, with one set being treated as described by Oski *et al.*, and the other set subjected to high-speed centrifugation (> 50,000*g*). No significant differences were found between these lysates when the ratios of 2,3-DPG to hemoglobin were compared in lysates and in supernatant solutions.

As a final confirmation of our results, we lysed an untreated blood sample by the usual technique, and included a small amount (1 percent of total 2,3-DPG), of [^{32}P]2,3-DPG, free of [^{32}P]-inorganic phosphate, in the lysing solution. We divided the radioactive lysate into two parts and treated them as in the experiments reported above; the number of counts per minute and the amount of 2,3-DPG were determined (Table 2).

Table 2. Lysis of two blood samples in the presence of [^{32}P]2,3-DPG. SA, specific activity.

Fraction	Ratio of 2,3-DPG (nmole)/Hb (mg)	Radioactivity (count/min)	SA
	*Centrifugation: 75,000*g		
Lysate	16.72	10,136	606
Lysate	17.04	10,550	619
Supernatant	16.40	10,646	649
Supernatant	17.36	11,459	660
	Method of Oski et al. (2)		
Lysate	16.40	10,191	621
Lysate	16.40	10,357	631
Supernatant	17.04	11,610	680
Supernatant	16.72	11,368	680

The results in Table 2 indicate that under these conditions, no significant portion of the total 2,3-DPG pool is bound to membranes or to membranous particles, in agreement with the results of 14 other experiments performed on blood drawn from 10 volunteer donors.

E. G. BRANN
D. J. NEWMAN
Biochemistry Department,
Smith, Kline and French Laboratories,
Philadelphia, Pennsylvania 19101

References and Notes

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5. We thank H. Green for his advice and encouragement during these studies.

10 August 1972

I am disappointed that Brann and Newman were unable to duplicate our results. T. Huisman and G. Peskin have also informed me that they were unable to demonstrate any binding of 2,3-DPG to the red cell membrane. Brann and Newman fail to mention in their comment that they were able to confirm our results, on samples prepared in our laboratory, after they observed our procedures. The method of preparing the cell lysates is important in producing the results. The discrepancies might be accounted for if there was a variation in the *p*H of the deionized water used to hemolyze the cells. The binding of 2,3-DPG to the interior of the cell is presumably an electrostatic phenomenon, with the anion 2,3-DPG binding to the positively charged internal membrane. Changes in *p*H of the lysing medium would disturb this interaction.

The failure to observe the in vitro binding by propranolol leaves unexplained the effect of epinephrine on blocking the phenomenon we reported, and also leaves unexplained the observed in vivo alterations in P_{50}.

FRANK A. OSKI
Department of Pediatrics,
Upstate Medical Center,
Syracuse, New York 13210

14 November 1972