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Effects of 2,3-Diphosphoglycerate on the Oxygen Equilibria of the Half-Cyanmet Hybrid Hemoglobins†

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ABSTRACT: The effects of 2,3-diphosphoglycerate on the oxygen equilibria of the half-cyanmet hybrid hemoglobins, $\alpha_2^+(CN)\beta_2(O_2)$ and $\alpha_2(O_2)\beta_2^+(CN)$, have been studied. Although the phosphate decreases the oxygen affinity of both the derivatives, the effects are several times larger for $\alpha_2^+(CN)\beta_2(O_2)$ than for $\alpha_2(O_2)\beta_2^+(CN)$. The oxygen binding of the stripped hybrids are essentially noncooperative. On the addition of 2,3-diphosphoglycerate, the oxygen binding to $\alpha_2^+(CN)\beta_2$ becomes cooperative, while the phosphate has no effect on the cooperativity in $\alpha_2(O_2)\beta_2^+(CN)$. The oxygen affinity of $\alpha_2^+(CN)\beta_2(O_2)$ decreases more gradually with DPG concentration than that of normal hemoglobin. The response of $\alpha_2(O_2)\beta_2^+(CN)$ to DPG suggests that the cyanmet β subunits change their conformation when the partner α subunits bind the oxygen molecules, *i.e.*, there exists a propagation of conformational changes from the α subunits to the β subunits. The present results do not follow both the original simple allosteric models of Monod, Wyman, and Changeux and of Koshland, Némethy, and Filmer and lead to some modifications of both the models.

Many studies with regard to the interaction of an unique allosteric effector, 2,3-diphosphoglycerate (DPG[1]) with hemoglobin have elucidated that it binds specifically to deoxyhemoglobin in a mole for mole ratio at physiological pH and salt concentration and profoundly lowers the oxygen affinity of hemoglobin (Benesch and Benesch, 1969). X-Ray studies (Perutz, 1970) and biochemical data (Benesch and Benesch,

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[1] Abbreviations used are: DPG, 2,3-diphosphoglycerate; bis-tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane. The MWC and KNF models are those presented by Monod, Wyman, and Changeux and by Koshland, Némethy, and Filmer, respectively. Molecular species of hemoglobin are represented by the formulae such as $\alpha_2^+(CN)\beta_2(O_2)$, $\alpha_2\beta_2^+(CN)$, *etc.*, where $\alpha(O_2)$ and $\beta(O_2)$, α and β, and $\alpha_2^+(CN)$ and $\beta_2^+(CN)$ indicate the oxygenated, deoxygenated, and cyanmet subunits, respectively.

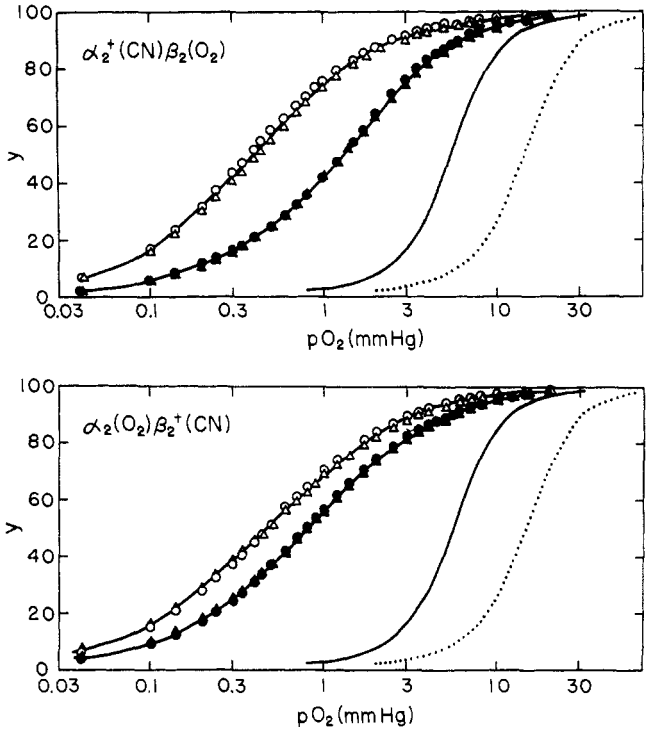

FIGURE 1: Effects of DPG on the oxygenation curves of $\alpha_2^+(CN)\beta_2(O_2)$ and $\alpha_2(O_2)\beta_2^+(CN)$. Hemoglobin concentration, 5×10^{-5} M (heme equivalent); in 0.05 M bis-tris buffer (pH 7.4), 2.5×10^{-4} M KCN, and 0.1 M NaCl; temperature, 25°. (a) $\alpha_2^+(CN)\beta_2(O_2)$, (b) $\alpha_2(O_2)\beta_2^+(CN)$. Open symbols, stripped; filled symbols, in 2 mM DPG. The curves for $\alpha_2(O_2)\beta_2(O_2)$ are given for comparison. Solid line, stripped; dotted line, in 2 mM DPG.

1969; Benesch *et al.*, 1969; Bunn and Briehl, 1970) have suggested that it cross-links the amino acids involving valines NA 1 (1), lysines EF 6 (82), and histidines H 21 (143) of the β subunits across the dyad axis and stabilizes the deoxy quaternary structure of hemoglobin.

In the present study we have investigated the effects of DPG on the oxygen equilibria of the half-cyanmet hybrid hemoglobins, $\alpha_2^+(CN)\beta_2(O_2)$ and $\alpha_2(O_2)\beta_2^+(CN)$, as a model system of partially oxygenated hemoglobins with a hope that further information on the regulatory function of DPG and the mechanism of cooperative effects in hemoglobin might be obtained.

Materials and Methods

Preparation of Half-Cyanmet Hybrid Hemoglobins. Human adult hemoglobin subunits were prepared as previously described (Maeda and Ohnishi, 1970) except that mercurated α chains were separated by the method of Bucci and Fronticelli (1965). The completeness of the regeneration of sulfhydryl groups from the mercurated subunits was confirmed by means of a disc electrophoresis using pH 8.3 buffer system. The isolated chains were oxidized to the cyanmet form at 30° for 15 min in the presence of 1.3-equiv amounts of potassium ferricyanide and 10-equiv amounts of potassium cyanide. After the complete oxidation of the chains had been spectrophotometrically confirmed, the excess potassium ferricyanide was removed through Sephadex G-25 column equilibrated with 0.05 M bis-tris buffer, pH 7.4 at 4°. The half-cyanmet hybrid hemoglobin was obtained by mixing the cyanmet chains with equivalent amounts of the partner oxy chains. The disc electrophoregrams of the reconstituted $\alpha_2^+(CN)\beta_2(O_2)$ and $\alpha_2(O_2)\beta_2^+(CN)$ showed that the reconstitutions were complete, no bands for the isolated chains being recognizable.

Measurements of Oxygen Equilibrium Curves. The oxygen equilibrium curves of hemoglobin were measured on 5×10^{-5} M (heme equivalent) hybrid hemoglobin in 0.05 M bis-tris buffer (pH 7.4), containing 2.5×10^{-4} M potassium cyanide, either in the absence or presence of 0.1 M NaCl at 25° by the automatic recording method of Imai *et al.* (1970). The measurements also performed on human adult hemoglobin at the same experimental conditions except without potassium cyanide. The measurement of a single curve was completed within 40–60 min. The exchange between cyanmet and ferrous hemes can be considered insignificant during that period because Bunn and Jandle (1966, 1968) showed that heme exchange in cyanmethemoglobins, deoxyhemoglobins, or oxyhemoglobins is negligible for that period at 25°. The content of the cyanmet chains in the hybrid hemoglobins, determined after measurements of oxygenation curves, was from 55 to 65%.

The oxygen equilibrium curves were expressed as conventional y *vs.* log p plot (y, percentage oxygen saturation; p, oxygen pressure) and further analyzed by the Hill plots (log $[Y/(1 - Y)]$ *vs.* log p plot, where Y is fractional oxygen saturation). The oxygen affinity and the extent of cooperativity in oxygen binding are expressed by the oxygen pressure at y = 50, P_{50}, and the slope of the Hill plots, n, respectively.

Results

Effects of DPG on the Oxygen Affinity. Effects of DPG on the oxygen equilibrium curves of $\alpha_2^+(CN)\beta_2(O_2)$ and $\alpha_2(O_2)\beta_2^+(CN)$ are shown in Figure 1. DPG evidently shifts the curves to the right-hand side in both the hybrids and the effect on $\alpha_2^+(CN)\beta_2(O_2)$ is larger than that on $\alpha_2(O_2)\beta_2^+(CN)$ in both the absence and presence of 0.1 M NaCl. Although the effect of DPG expressed by the ratio of P_{50} in the presence of 2 mM DPG to P_{50} for stripped hemoglobin is larger for normal hemoglobin $\alpha_2(O_2)\beta_2(O_2)$, than for both the hybrids in NaCl-free buffer, it is larger for $\alpha_2^+(CN)\beta_2(O_2)$ than for $\alpha_2(O_2)\beta_2(O_2)$ in the presence of 0.1 M NaCl as summarized in Table I, which is consistent with the result of Haber and Koshland (1971). Table I also includes the data of the isolated α and β chains for comparison (Tyuma *et al.*, 1971).

Since P_{50} of the stripped hybrids hardly changes by 0.1 M NaCl, that has large effects on the stripped normal hemoglobin, nonspecific salt effects on the hybrids seem to be small. Accordingly, the effects of DPG on the hybrids are considered to be due to specific interactions of the phosphate with the protein molecules.

Effects of DPG on the Cooperativity. All the Hill plots of oxygenation for the hybrids show a tendency to deviate slightly from the straight line at the very high range of Y as shown in Figure 2. The Hill plots for $\alpha_2^+(CN)\beta_2(O_2)$ given by Haber and Koshland (1971) also seem to have the same tendency. The origin of the deviation is still unknown. The Hill plot for $\alpha_2^+(CN)\beta_2(O_2)$ in the presence of DPG exhibits another deviation from the straight line at the lower part of the plot. This deviation may be ascribable to the Darling–Roughton effect due to the inevitable contamination of the cyanmet β chains formed from the oxy β chains in the hybrid, *i.e.*, an increase in oxygen affinity and a decrease in the Hill coefficient of hemoglobin caused by the partial oxidation (Darling and Roughton, 1942) or by the partial formation of cyanmet hemes (Benesch *et* al., 1965). Accordingly, we

TABLE I: Summary of the Effects of DPG on the Oxygen Equilibrium Functions of the Hybrid and Normal Hemoglobins and the Isolated Chains.

Hemoglobin Samples	Condition	P_{50} (mmHg)		P_{50}^{DPG}/P_{50}^{st} [a]	n	
		Stripped	In 2 mM DPG		Stripped	In 2 mM DPG
$\alpha_2(O_2)\beta_2(O_2)$	NaCl free	1.9	15.3	8.1	2.52	3.02
	In 0.1 M NaCl	5.6	15.1	2.7	2.95	2.93
$\alpha_2^+(CN)\beta_2(O_2)$	NaCl free	0.30	1.41	4.7	1.08	1.41
	In 0.1 M NaCl	0.40	1.38	3.5	1.17	1.52
$\alpha_2(O_2)\beta_2^+(CN)$	NaCl free	0.40	0.83	2.1	1.15	1.13
	In 0.1 M NaCl	0.47	0.79	1.7	1.10	1.14
α chain	In 0.1 M phosphate buffer	0.63			1.0	
β chain	In 0.1 M phosphate buffer	0.24			1.0	

[a] The ratio of P_{50} in 2 mM DPG to P_{50} of stripped hemoglobin. Experimental conditions are as described in Figure 1.

have confined the analysis and discussion to the main parts of the Hill plots giving a straight line.

The oxygen binding of the stripped hybrids is essentially noncooperative with the Hill coefficient, n, 1.08–1.17 as summarized in Table I. Even in the presence of DPG the oxygen binding of $\alpha_2(O_2)\beta_2^+(CN)$ is essentially noncooperative. However, the addition of DPG makes the oxygen binding of $\alpha_2^+(CN)\beta_2(O_2)$ significantly cooperative. These circumstances remain unchanged in the presence of 0.1 M NaCl.

On the other hand, it has been found that 2 mM DPG increases the Hill coefficient of normal hemoglobin from 2.5 to 3.0 only in the absence of NaCl (Tyuma *et al.*, 1971).

Dependence of the Oxygen Equilibrium Curves of $\alpha_2^+(CN)\beta_2(O_2)$ on the DPG Concentration. The oxygen affinity of $\alpha_2^+(CN)\beta_2(O_2)$ gradually decreases with increasing DPG concentration as shown in Figure 3. In NaCl-free buffer the effect of DPG in lowering the oxygen affinity, however, is weak for $\alpha_2^+(CN)\beta_2(O_2)$ compared to that for $\alpha_2(O_2)\beta_2(O_2)$. The concentration of DPG giving the half value of the maximum P_{50} shift is 2.3×10^{-4} M for $\alpha_2^+(CN)\beta_2(O_2)$ and 3×10^{-5} M for $\alpha_2(O_2)\beta_2(O_2)$. Moreover, the slope of the main part of the Hill plots gradually increases with DPG concentration (0.03–2 mM).

Discussion

Cooperativity in Oxygen Binding of the Half-Cyanmet Hybrid Hemoglobins. It has been found that 2 mM DPG increases the Hill coefficient of normal hemoglobin from 2.5 to 3.0 in the absence of NaCl (Tyuma *et al.*, 1971). The phosphate also increases the cooperativity in oxygen binding of $\alpha_2^+(CN)\beta_2(O_2)$. In $\alpha_2^+(CN)\beta_2(O_2)$ and $\alpha_2(O_2)\beta_2^+(CN)$ the oxygen binding is essentially noncooperative in the absence of DPG, while in the former cooperativity appears on the addition of 2 mM DPG. This cooperativity would be ascribable to the stabilization of the deoxy state through the cross-linkage between the β subunits by DPG. On the other hand, oxygen binding in $\alpha_2(O_2)\beta_2^+(CN)$ is essentially noncooperative even in the presence of DPG though it lowers the oxygen affinity of the hybrid. It may be due to weaker binding of DPG to $\alpha_2\beta_2^+(CN)$ than to $\alpha_2^+(CN)\beta_2$.

The present result that DPG is capable of interacting with the hybrid hemoglobins is consistent with the results of nmr studies by Ogawa and Shulman (1971), a study of the binding of a spin-labeled triphosphate with hemoglobin by Ogata and McConnell (1971) and a recent report of the effect of DPG on the oxygen equilibrium of $\alpha_2^+(CN)\beta_2(O_2)$ by Haber and Koshland (1971). Especially, the result obtained by Ogata and McConnell that a spin-labeled triphosphate binds more strongly to $\alpha_2^+(CN)\beta_2$ than to $\alpha_2\beta_2^+(CN)$ is in good agreement with the present result that DPG has larger effect on the oxygen affinity of $\alpha_2^+(CN)\beta_2(O_2)$ than on that of $\alpha_2(O_2)\beta_2^+(CN)$.

On the other hand, the observation of Haber and Koshland (1971) on the cooperativity in oxygen binding of $\alpha_2^+(CN)\beta_2(O_2)$ is in quite conflict with our results. They have found that DPG decreases the oxygen affinity of $\alpha_2^+(CN)\beta_2(O_2)$ without affecting the slope of the Hill plot ($n = 1.3$) in 0.2 M *N*-2-hydroxyethylpiperazine-*N'*-2-aminoethanesulfonic acid buffer (pH 7.2) containing 0.06 M NaCl and 5×10^{-4} M EDTA. The origin of this discrepancy in the conclusions on the cooperativity in $\alpha_2^+(CN)\beta_2(O_2)$ is unknown at present.

Mechamism of Oxygen Binding in Hemoglobin. Two different models have been presented for the molecular mechanism of the cooperative binding of substrate to allosteric proteins. In the one, the MWC model, the subunits of a protein molecule take two different states, arranged in a symmetrical fashion, and the protein molecule is assumed to maintain the symmetry during the conformational changes (Monod *et al.*, 1965). The KNF model, on the other hand, assumes progressive or sequential changes in the subunit conformation, only the subunit binding a ligand being able to transform its conformation (Koshland *et al.*, 1966). On the assumption that the oxidation of subunits to the cyanmet form is equivalent to the oxygenation, it is interesting to consider which model better fits to the present results.

The MWC model predicts that $\alpha_2^+(CN)\beta_2$ and $\alpha_2\beta_2^+(CN)$ are in the equivalent state and respond to DPG in the same manner. On the other hand, if we accept that DPG exclusively binds to the β subunits in the deoxy quaternary structure, the KNF model requires that DPG decreases only the oxygen affinity of $\alpha_2^+(CN)\beta_2$ and not that of $\alpha_2\beta^+{}_2(CN)$ since the β subunits in $\alpha_2^+(CN)\beta_2$ should be still in the deoxy conformation and those in $\alpha_2\beta_2^+(CN)$ should have already transformed into the oxy conformation. Neither prediction, however, accords with the present results since DPG decreases the oxygen affinity of both the hybrids and the magnitude of the effects is larger for $\alpha_2^+(CN)\beta_2(O_2)$ than for $\alpha_2(O_2)\beta_2^+(CN)$.

Here two explanations corresponding to the two models with some modifications will be possible. One of them is the

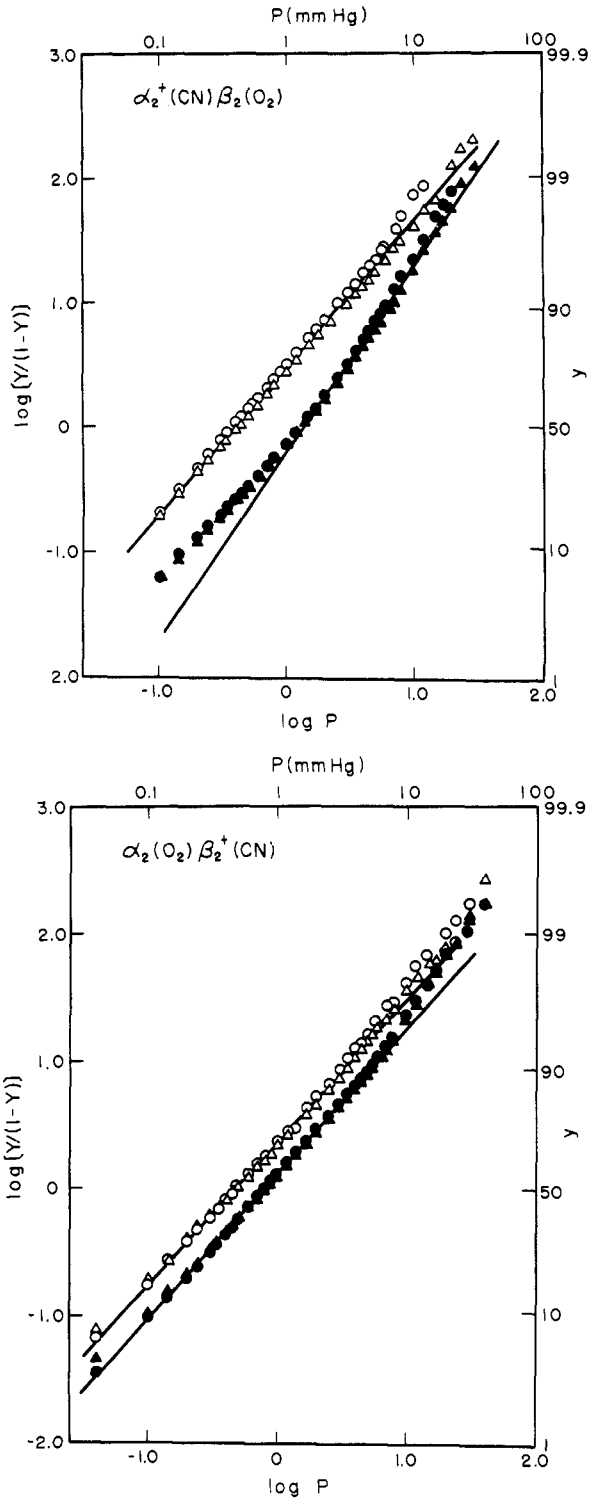

FIGURE 2: Hill plots of the oxygenation of $\alpha_2^+(CN)\beta_2(O_2)$ and $\alpha_2(O_2)\beta_2^+(CN)$ in the absence and presence of DPG. Experimental conditions and symbols are as described in Figure 1. (a) $\alpha_2^+(CN)\beta_2(O_2)$, (b) $\alpha_2(O_2)\beta_2^+(CN)$.

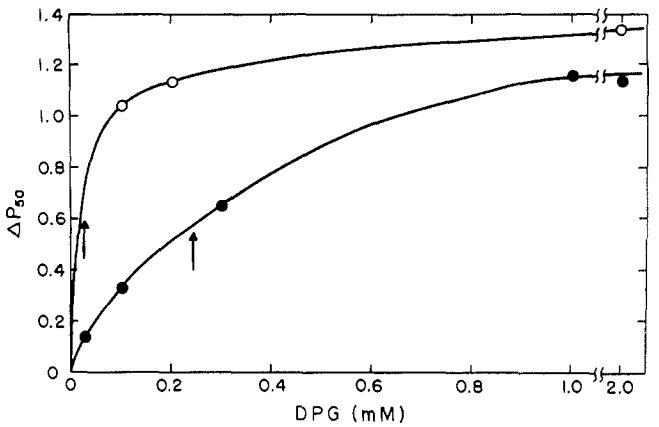

FIGURE 3: Dependence of the oxygen affinity of $\alpha_2(O_2)\beta_2(O_2)$ and $\alpha_2^+(CN)\beta_2(O_2)$ on the concentration of DPG. The ordinate is the shift of P_{50} from that of stripped hemoglobin and given by $\times$ 10 mmHg for $\alpha_2(O_2)\beta_2(O_2)$ and mmHg for $\alpha_2^+(CN)\beta_2(O_2)$. Open circles, $\alpha_2(O_2)\beta_2(O_2)$; filled circles, $\alpha_2^+(CN)\beta_2(O_2)$. Arrows show the half values of the maximum P_{50} shift. The concentration of DPG giving those values is 3×10^{-5} M for $\alpha_2(O_2)\beta_2(O_2)$ and 2.3×10^{-4} M for $\alpha_2^+(CN)\beta_2(O_2)$. Experimental conditions are as described in Figure 1, except in NaCl-free buffer.

generalized MWC model (Ogata and McConnell, 1971), in which the α and β subunits have different intrinsic affinities for ligand and the ligation of them affects the allosteric equilibrium between two states unequally. The introduction of the nonequivalence of the α and β subunits into the MWC model permits the oxygen equilibrium curves of $\alpha_2^+(CN)\beta_2(O_2)$ and $\alpha_2(O_2)\beta_2^+(CN)$ to be affected by DPG in different manners.

The alternative is the KNF model with a modification that the conformation of a subunit depends not only on the ligation of the subunit itself, but also partially on that of neighboring subunits (Haber and Koshland, 1967). According to this model the β subunits are able to take at least four different conformations corresponding to $\alpha_2\beta_2$, $\alpha_2(O_2)\beta_2$ (or $\alpha_2^+(CN)\beta_2$), $\alpha_2\beta_2(O_2)$ (or $\alpha_2\beta_2^+(CN)$), and $\alpha_2(O_2)\beta_2(O_2)$ (or $\alpha_2^+(CN)\beta_2(O_2)$ and $\alpha_2(O_2)\beta_2^+(CN)$). Assuming that $\alpha_2\beta_2(O_2)$ (or $\alpha_2\beta_2^+(CN)$) can bind DPG with a smaller binding constant than that for $\alpha_2(O_2)\beta_2$ (or $\alpha_2^+(CN)\beta_2$), the modified KNF model can also well account for the present results on the effects of DPG on the oxygen affinity of both the hybrids. Accordingly, although the present results fail to fit to the original simple MWC and KNF models, both the models with the above modifications can account for them equally.

Haber and Koshland regarded the demonstration of cooperativity in oxygen binding of $\alpha_2^+(CN)\beta_2(O)_2$ and the response of the hybrid to DPG as evidence that ligand binding to hemoglobin follows a mechanism of sequential conformational changes rather than a concerted pathway and analyzed the oxygen equilibrium curves of $\alpha_2^+(CN)\beta_2(O_2)$ in the absence and presence of DPG on the basis of the simple KNF model. However the cooperativity in oxygen binding of $\alpha_2^+(CN)\beta_2(O_2)$ and the response of the hybrid to DPG observed by them do not necessarily exclude the MWC model and moreover the simple KNF model is inadequate to account for the present results on the response of both the hybrids to DPG.

Recent studies on the binding of a spin-labeled triphosphate to hemoglobin by Ogata and McConnell (1971) have supported their generalized MWC model rather than the simple KNF model. They have shown that the electron resonance spectra of the spin label are only the superposition of the signals of free labels and those bound to hemoglobin and have sharp isosbestic points at the various stages of ligation. The results suggest that the spin label bound to hemoglobin experiences only one environment and there exists only one conformation in deoxyhemoglobin and partially liganded hemoglobins available for DPG binding as far as observed by the spin-label technique. Ogawa and Shulman (1971) and Cassoly *et al.* (1971) showed by nuclear magnetic resonance

studies that the cyanmet subunits in the deoxygenated hybrids apparently are in an equilibrium between different conformations and organic phosphates shift the equilibrium. Although this result also supports the MWC model, further studies are necessary to establish an unique model of cooperative oxygen binding in hemoglobin.

Finally, in $\alpha_2(O_2)\beta_2^+(CN)$ the response of the oxidized β subunits to DPG implies that the β subunits change the conformation with the oxygenation of the α subunits, *i.e.*, there is a propagation of conformational changes from the α subunits to the β subunits. Otherwise, the oxygen affinity of $\alpha_2(O_2)\beta_2^+(CN)$ would be insensitive to DPG. The conformational changes propagating to neighboring subunits over a subunit where ligation occurs, would contribute to the cooperativity in oxygen binding of hemoglobin. The conclusion is consistent with recent physicochemical studies of hemoglobin (Hayashi *et al.*, 1967; Maeda and Ohnishi, 1971; Ogawa and Shulman, 1971; Ogata and McConnell, 1971; Asakura and Drott, 1971).

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A Requirement for Cytochrome b_5 in Microsomal Stearyl Coenzyme A Desaturation†

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ABSTRACT: The stearyl coenzyme A desaturase activity of hen liver microsomes was resolved into two fractions by gel filtration in the presence of deoxycholate. Both the large molecular weight fraction (P_3) and the smaller molecular weight fraction (P_4G) were required for desaturase activity. This activity was stimulated by addition of NADH–cytochrome b_5 reductase and deoxycholate. The P_4G fraction contained lipid and cytochrome b_5. Removal of the lipid destroyed desaturase activity which could then be restored by addition of lipid dispersions. Partially purified cytochrome b_5 was prepared by the standard trypsin procedure (trypsin–cytochrome b_5) and by ion exchange and gel chromatography of a detergent extract of acetone-extracted microsomes (detergent–cytochrome b_5). Only detergent–cytochrome b_5 could replace P_4G in the desaturase assay. Maximal desaturase activity was obtained with P_3, NADH–cytochrome b_5 reductase, detergent–cytochrome b_5, lipid, and deoxycholate. The requirement for these components is further evidence for the involvement of the NADH-specific electron transport chain of microsomes in stearyl coenzyme A desaturation.

The conversion of stearic acid into oleic acid has long been known to be catalyzed by the microsomal fraction of a liver homogenate but the mechanism of the reaction remains to be elucidated. Morris (1970) has investigated the stereochemistry of this conversion in animals and has suggested that the two hydrogens are removed from stearic acid in a simultaneous concerted reaction. This would indicate that formal oxygenated intermediates are not involved in desaturation. The same conclusion had been reached earlier from experiments with hydroxystearic acids in animals (Elovson, 1964) and *Euglena gracilis* (Gurr and Bloch, 1966). Attempts have been made to solubilize and purify the desaturase but to date only a fivefold purification has been obtained and this partially

† From the Department of Biochemistry, University of Virginia School of Medicine, Charlottesville, Virginia 22901. *Received March 15, 1972.* This work was supported by Grants P-559 and BC-71A from the American Cancer Society.

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