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ABSTRACT—The physiological significance of the position and shape of the oxygen equilibrium curve (OEC) of horse hemoglobin (Hb) is considered from the viewpoint of oxygen (O_2) transport efficiency and the effectiveness of the Bohr effect. In horse fetal and maternal bloods, their physiological O_2 affinities are nearly optimized with respect to the effectiveness of the Bohr shift occurring at the O_2 release site, when it is measured by the change in O_2 saturation per unit change in P_{50}. With relatively low cooperativity (n=2.69) of horse Hb under physiological conditions, the effectiveness of the Bohr shift for fetal blood at O_2 uptake site and maternal blood at O_2 release site is high. These facts imply that the position and the cooperativity of horse Hb OEC are optimized to receive maximal benefit from the double Bohr shift. Before exercise, the position of the OEC for adult mares is nearly optimized for the effectiveness of the Bohr shift occurring at the O_2 release site, whereas, at maximal exercise, the position of the OEC tends to become advantageous for O_2 transport efficiency.

Key words: cooperativity, effectiveness of the Bohr effect, hemoglobin, O_2-Hb equilibrium curve, horse

INTRODUCTION

An increase in partial pressure of CO_2 (PCO_2) or a decrease in pH lowers the O_2 affinity of Hb. Thus, they shift the OEC to the right, causing release of additional O_2 from Hb to the tissues. This shift caused by concomitant PCO_2 change and pH change is called the "classical Bohr effect" (Bohr *et al.*, 1904), while the shift of OEC only by pH change called the "Bohr effect". In the case of fetal blood, the Bohr shift is considered to be of particular importance. The diffusion of CO_2 from fetal blood into maternal blood increases pH of fetal blood and shifts the fetal OEC to the left, the simultaneous decrease in pH of maternal blood shifts its OEC to the right (MaCarthy, 1943). This phenomenon is called the "double Bohr effect". It has been considered that the enhanced difference in P_{50} between fetal and maternal bloods by the double Bohr effect promotes the mother-fetus transfer of O_2. P_{50} is partial pressure of O_2 at half saturation.

The magnitude of the Bohr effect is quantitatively measured by the change in log P_{50} per unit change in pH ($d\log P_{50}/dpH$), which is called the "Bohr coefficient". The

additional O_2 released from Hb by the Bohr shift without any change in venous PO_2 is dependent on not only the magnitude of the Bohr coefficient but also the steepness of the OEC. In our previous study, we found that the position of the OEC of human adult Hb at rest is optimal for the effectiveness of the Bohr shift occurring at O_2 release sites (venous PO_2(PvO_2)=40 torr) (Kobayashi *et al.*, 1996, Itoh *et al.*, 2001).

The cooperativity of O_2 binding is expressed in terms of the highest slope of the Hill plot (log($S/(1–S)$) vs. log P plot), n (Hill, 1910). The functional significance of the sigmoid shape of the OEC has been explained by efficient O_2 transport: the higher the cooperativity, the more O_2 is transported to the tissues. However, mammalian tetrameric Hb usually has relatively low cooperativity (n value of approximately 2.8). The physiological significance of this low cooperativity has not been explained so far. Recently, using the human OEC data, which was described by Adair's stepwise O_2 binding equation, it was reported that the relatively low cooperativity of the OEC of human tetrameric Hbs is designed to receive a maximal benefit from the double Bohr shift (Zhang *et al.*, 2003). In this study, the above-mentioned approach was applied to confirm the functional significance of the relatively low cooperativity of other mammalian tet-

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rameric Hbs.

In each many species of mammalian species, Hb of the fetal blood differs structurally from that found in the adult blood. The subunit structure of human adult Hb is $\alpha_2\beta_2$, and that of fetal Hb is $\alpha_2\gamma_2$. The amino acid sequences of the β-chain of adult Hb and the γ-chain of fetal Hb differ at 31 of 146 residues (Schroeder *et al.*, 1963). In contrast, the Hbs of the fetal and adult horse bloods have been shown to be structurally identical with each other (Stockell *et al.*, 1961; Comline and Silver, 1974). However, fetal blood has higher O_2 affinity than maternal blood because of the low concentration of 2,3-diphosphoglycerate (2,3-DPG) in fetal red blood cells compared to that in maternal red blood cells (Bunn and Kitchen, 1973). There are literature values for not only the Bohr coefficient of horse blood ($d\log P_{50}/dpH=-0.47$, Bunn and Kitchen, 1973; Fenger *et al.*, 2000), but also PO_2 for the arterial and venous blood and P_{50} for both fetal and maternal bloods (Comline and Silver, 1974; Comline and Silver, 1975). In addition, the OEC of horse adult blood, for which the four Adair constants (see below) were determined, is also available in the literature (Clerbaux *et al.*, 1993). To our knowledge, horse fetal blood OEC data, for which the four Adair constants have been determined, are not available in the literature. Then, we measured OECs of horse Hb at various 2,3-DPG concentrations and found that cooperativity is not sensitive to 2,3-DPG concentration. It is known that cooperativity is also insensitive to pH. These facts give the basis for generating an OEC for either adult or fetal Hb under given 2,3-DPG and pH conditions from the Adair constants for horse adult Hb. Using these calculated OECs, the physiological significance of the position and the cooperativity of horse Hb can theoretically be examined from the viewpoint of O_2 transport efficiency and the effectiveness of the Bohr shift.

METHODS

Horse blood sample was purchased from Nippon biological material center. Hemoglobin was stripped of organic phosphates according to the method of Condo *et al.* (1992), and its OECs were measured with an automatic oxygenation apparatus developed by Imai and Yonetani (1977) and Imai (1981) at an Hb concentration of 60 μM on a heme basis and 25°C. Oxygen saturation of Hb was calculated from the change in absorbance at 576 nm measured with a Shimadzu spectrophotometer (model UV 2000). The concentration of O_2 in the sample cell was decreased by replacing air with pure N_2 gas and its change was monitored with a Clark-type O_2 electrode. The buffer solution used for OEC measurements was 0.05 M Tris-HCl (pH 7.4). Methemoglobin (Met-Hb) formed by auto-oxidation was reduced using an enzymatic reducing system as described by Hayashi *et al.* (1973). The Met-Hb concentration at the end of OEC measurement as determined as described by Evelyn *et al.* (1938), did not exceed 5% of total Hb. The 2,3-DPG concentrations were measured by the enzymatic procedure of Ericson and Verdier (1972). The experimentally obtained OEC data were analyzed by curve fitting method described by Imai (1981) to estimate the Adair constants. Using these Adair constants, P_{50} and n values were calculated.

The Adair equation (Adair, 1925) is given by

$$S=(A_1P+2A_2P^2+3A_3P^3+4A_4P^4)/4(1+A_1P+A_2P^2+A_3P^3+A_4P^4)$$

(1)

where S is the fractional saturation of Hb with O_2, $A_i(i=1\sim4)$ is the Adair constants and p is the partial pressure of O_2.

The literature A_i values for horse adult whole blood at pH 7.4, PCO_2 of 40 torr and 37°C are: $A_1=3.103\cdot10^{-2}$ torr^{-1}, $A_2=8.451\cdot10^{-4}$ torr^{-2}, $A_3=1.447\cdot10^{-5}$ torr^{-3} and $A_4=3.961\cdot10^{-6}$ torr^{-4} (Clerbaux *et al.*, 1993). The values of P_{50} and n are 23.8 torr and 2.69, respectively. Using Adair constants, theoretical OECs with various P_{50} values were generated by multiplying each PO_2 value by a common factor. In this multiplication, the position of the OEC was shifted freely without changing the shape.

In order to investigate the effect of cooperativity on the effectiveness of the Bohr shift, Hill's empirical equation (Hill, 1910) and OEC data of horse Hb solutions measured under various experimental conditions (Imai, 1983) were used.

The O_2 transport efficiency and the effectiveness of the Bohr shift of horse fetal and maternal bloods at rest were calculated using the following literature values of blood PO_2 and P_{50} (Comline and Silver, 1974). Fetal umbilical venous PO_2(arterialized fetal blood), fetal umbilical arterial PO_2 ("venous blood" coming from the fetus to the placenta), and physiological P_{50} under normal conditions were assumed to be 49, 33, and 27 torr, respectively. In the placental circulation, "arterial blood" and "venous blood" meant the blood flowing through the umbilical vein and the umbilical artery, respectively. Maternal arterial PO_2, uterine venous PO_2 and physiological P_{50} values were assumed to be 95, 50 and 31 torr, respectively.

The O_2 transport efficiency and the effectiveness of the Bohr shift during incremental exercise of horse adult were calculated using the PO_2, P_{50} and n values reported by Fenger *et al.* (2000). The arterial PO_2 (PaO_2), venous PO_2 (PvO_2), P_{50} and n values before exercise were 106, 38, 22.9 torr and 2.649, respectively, and these at maximal exercise were 86, 20, 31.8 torr and 2.707, respectively.

All computations were performed on a personal computer (model PC-9821 AP2; Nippon Electric Co., Tokyo) using MS-FOR-TRAN.

RESULTS AND DISCUSSIN

Effect of 2,3-DPG on cooperativity of horse Hb

Fig. 1 shows the effect of 2,3-DPG concentration on the O_2 affinity and cooperativity of horse Hb. These data demonstrate a strong influence of 2,3-DPG on the O_2 affinity, but little effect on cooperativity. Hill's coefficient (n) value was nearly constant. Therefore, in this study, the OEC with an n value of 2.69 of adult blood measured under standard conditions pH 7.4, PCO_2 40 mmHg, 37°C (Clerbaux *et al.*, 1993) was used to generate OECs for horse fetal and maternal bloods under various conditions.

Calculation of O_2 transport by fetal and maternal blood

Fig. 2 illustrates an example calculation of the O_2 transport by horse fetal and maternal bloods and the contribution of the Bohr shift occurring at the O_2 uptake and release sites.

The additional O_2 released from fetal blood due to the Bohr shift was estimated from the decrease in O_2 saturation at PO_2 of 33 torr, $\Delta S_{(33A-33B)}$. In the placenta, the additional O_2 uptake by fetal blood due to the Bohr shift was estimated from the increase in O_2 saturation at PO_2 of 49 torr,
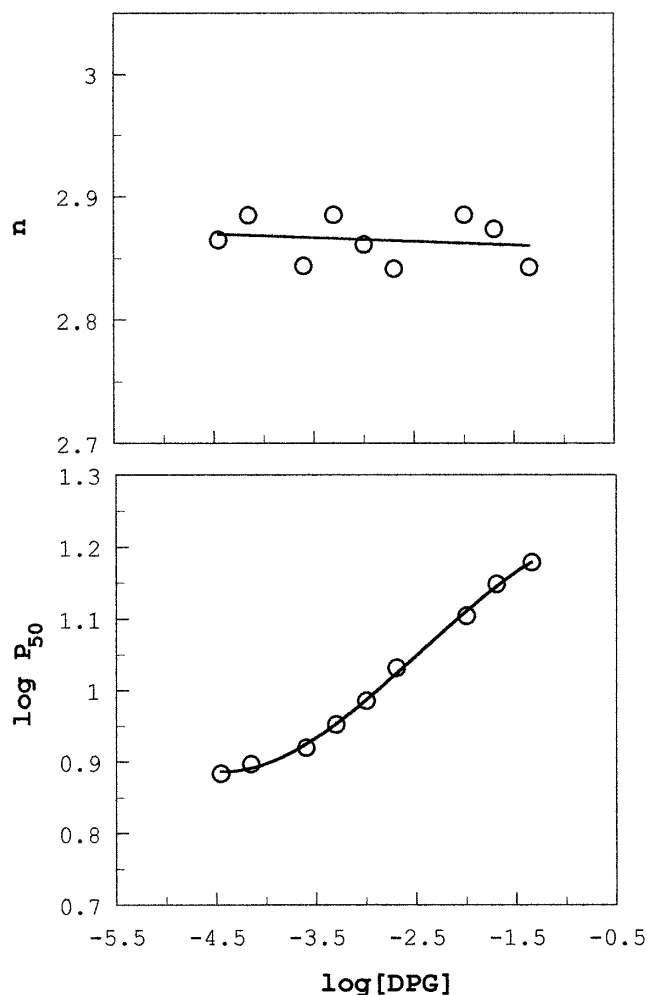
Fig. 1. Effect of 2,3-DPG on the O_2 affinity and cooperativity of horse adult Hb. Log P_{50} and n values are plotted against 2,3-DPG concentration (mol/liter). These parameter values were obtained from the OECs measured at pH 7.4 and 25°C.

$\Delta S_{(49A–49B)}$.

In maternal blood, the additional O_2 released *at PO_2 of 50 torr* due to the Bohr shift was represented by $\Delta S_{(50C–50D)}$, and the additional O_2 uptake in the lungs caused by the Bohr shift *at PO_2 of 95 torr* was represented by $\Delta S_{(95C–95D)}$.

The amount of O_2 transported to the tissues was estimated from the arterio-venous difference in O_2 saturation $\Delta S_{(PaO2–PvO2)}$.

O_2 transport efficiency of fetal and maternal blood

The slope of the OEC ($dS/dP=S'$) was quantified to represent the O_2 transport efficiency of Hb (Kobayashi *et al.*, 1994), and this measure was used to compare the fetal and maternal bloods. The S' vs. P plot of fetal blood exhibited a steep slope under physiological O_2 environment (at PO_2 ranging from 33 to 49 torr) (Fig. 3A), indicating high O_2 transport efficiency. In contrast, the slope of the OEC of maternal blood was rather flat under physiological O_2 environment (at PO_2 ranging from 50 to 95 torr), showing low O_2 transport efficiency (Fig. 3B).
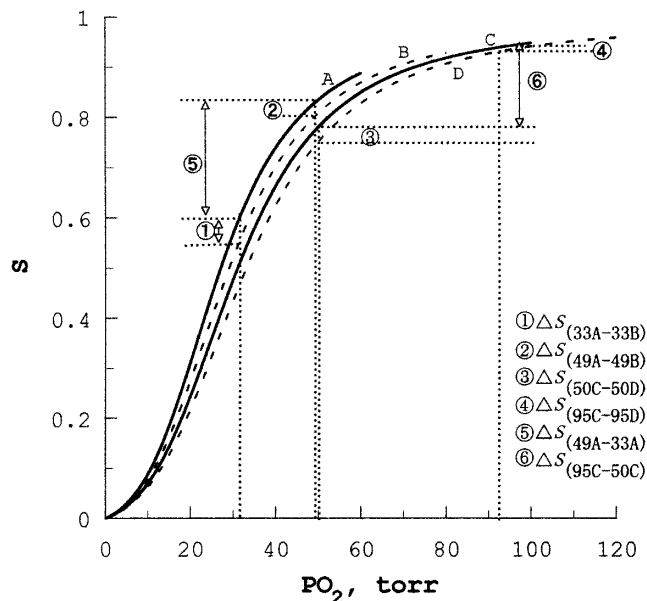

Fig. 2. Example calculation of the O_2 transport and additional O_2 release and uptake caused by Bohr shift of horse blood. Solid line A represents the OEC of fetal "arterial" blood, and broken line B represents that of fetal "venous" blood. Solid line C represents the OEC of maternal arterial blood, and broken line D represents that of maternal uterine venous blood. $\Delta S_{(33A–33B)}$ (①) and $\Delta S_{(50C–50D)}$ (③) represent the O_2 released from fetal and maternal blood, respectively due to the Bohr shift. $\Delta S_{(49A–49B)}$ (②) and $\Delta S_{(95C–95D)}$ (④) represent the additional O_2 uptake by fetal and maternal blood, respectively due to the Bohr shift. $\Delta S_{(49A–33A)}$ (⑤) and $\Delta S_{(95C–95C)}$ (⑥) represent the amount of O_2 tansported to the tissues by fetal and maternal blood without the Bohr shift, respectively. Hypothetical OECs with physiological P_{50} values were constructed using the Adair constants of OEC for horse blood under standard conditions(Comline and Silver, 1974).

Relation to the optimal P_{50} for O_2 transport efficiency

In order to explain the significance of the position of the OEC of fetal blood, the arterio-venous difference in O_2 saturation ($\Delta S_{(PaO2–PvO2)}$) was calculated as a function of P_{50}. Fig. 4A shows the relationship between P_{50} and the fetal "venous blood" O_2 saturation ($S_{(33)}$), "arterial blood" O_2 saturation ($S_{(49)}$) and arterio-venous difference in O_2 saturation ($\Delta S_{(49–33)}$). The $\Delta S_{(49–33)}$ vs. P_{50} plot had one maximum value. The P_{50} that gave the highest O_2 transport efficiency was called the "optimal P_{50}" for O_2 transport. There are slight difference in the physiological P_{50} of horse blood and the optimal P_{50} values. The amount of O_2 transported at physiological P_{50} was slightly lower than that of the maximum value at optimal P_{50} value. In human fetal Hb, it is well known that the physiological P_{50} of human fetal Hb is close to the optimal P_{50} (Itoh *et al.*, 2001; Sold, 1982; Willford *et al.*, 1982). Therefore, the O_2 tranasport efficiency of horse fetal blood is not so high as that of human fetal blood.

In maternal blood, the amount of O_2 transported at physiological P_{50} (31 torr) was roughly one-half that of theoretically obtained maximum value (Fig. 4B). This indicates that maternal venous blood is a large O_2 reservoir and a rightward shift of the OEC enhances O_2 transport as
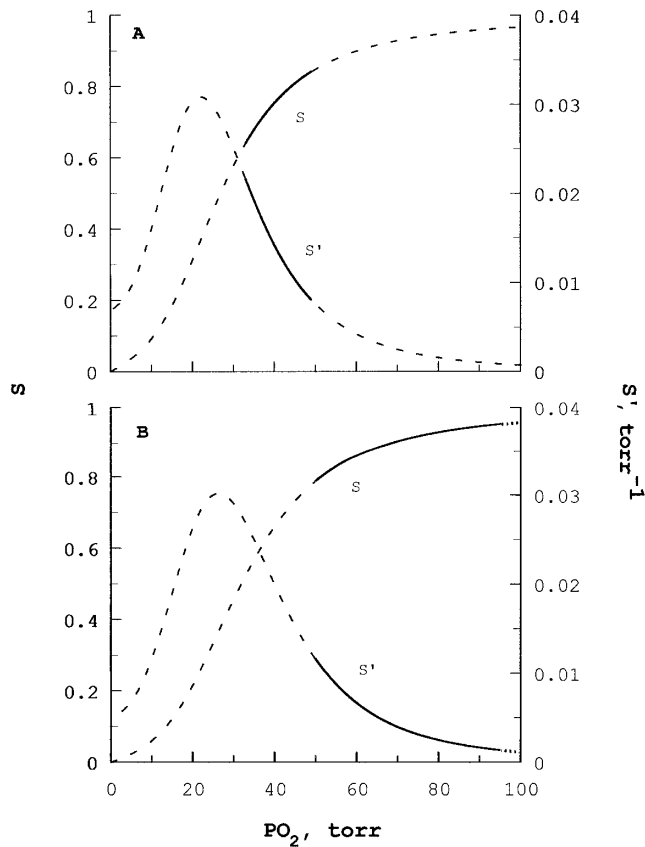
Fig. 3. OEC and *S'* vs. *P* plots of horse blood. **A:** Solid line indicate *S* and *S'* values calculated for the physiological PO_2 range of fetal blood. **B:** Solid line indicate S and *S'* values calculated for the physiological PO_2 range of maternal blood. These data were derived from hypothetical OECs with various P_{50} values that were constructed using the OECs shown in Fig. 2.

observed at hard exercise.

Effectiveness of Bohr shift in fetal and maternal blood

The effectiveness of the Bohr shift at various P_{50} was estimated from the change in O_2 saturation per unit change in P_{50}, *i.e.* the slope of the $S_{(PO2)}$ vs. P_{50} plot. The slope $(dS_{(PO2)}/dP_{50})$ was usually a negative value because $S_{(PO2)}$ was decreased with an increase in P_{50}. Therefore, in this study, the magnitude of the effectiveness of the Bohr shift was expressed as $-dS_{(PO2)}/dP_{50}$.

Fig. 5A shows the effectiveness of the Bohr shift at the O_2 uptake and release sites (at PO_2s of 49 and 33 torr, respectively) for fetal blood. The theoretical highest effectiveness of the Bohr shift at O_2 release site was observed at P_{50} of 23 torr. Clearly the physiological P_{50} value is close to the optimal P_{50} value for the effectiveness of the Bohr shift. At O_2 uptake site, there are a slight difference in the physiological P_{50} and optimal P_{50} values.This implies that the position of the OEC of fetal blood is nearly optimal with respect to the effectiveness of the Bohr shift at O_2 release site.

In fetal blood, the highest value at the O_2 release site (PvO_2=50 torr) was observed at P_{50} of 38 torr (Fig. 5B). This
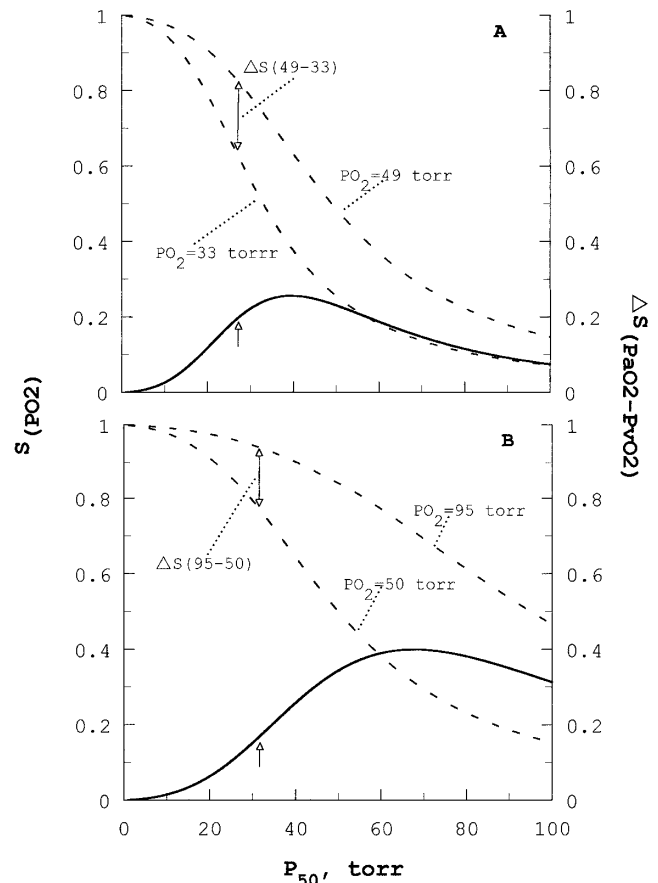

Fig. 4. O_2 saturation at arterial and venous PO_2 values and arterio-venous difference in O_2 saturation ($\Delta S_{(PaO2-PvO2)}$) of horse fetal and maternal bloods as a function of P_{50}. Hypothetical OECs with various P_{50} values were constructed using the OEC shown in Fig. 2. Open arrows are positioned at the physiological P_{50} for each blood. **A**: Broken lines represent the O_2 saturation of fetal "arterial" PO_2 of 49 torrand "venous" (PO_2 of 33 torr) blood. The solid line represents the fetal arterio-venous difference in O_2 saturation ($\Delta S_{(49-33)}$) of fetal blood. **B:** Broken lines represent the O_2 saturation of maternal arterial (PO_2 of 95 torr) and uterine venous (PO_2 of 50 torr) blood. Solid line represents the maternal arterio-venous difference in O_2 saturation ($\Delta S_{(95-50)}$) of maternal blood.

optimal P_{50} value is somewhat different from the physiological P_{50} value (31 torr). In contrast, at O_2 uptake site there was a large difference between the theoretical optimal P_{50} value (72 torr) and the physiological P_{50} value. Therefore, the position of the OEC of maternal blood is nearly optimal to maximize the effectiveness of the Bohr shift in venous blood. The effectiveness of the Bohr shift at O_2 uptake site of fetal blood is almost equal to that of maternal blood at O_2 release site with physiological P_{50}. This phenomenon is similar to that observed in human bloods (Zhang *et al.*, 2003)

Influence of cooperativity on effectiveness of the Bohr shift in fetal and maternal blood

Using physiological PaO_2, PvO_2 and P_{50} values, the influence of cooperativity on the effectiveness of the Bohr shift was investigated for a wide range of *n* values.
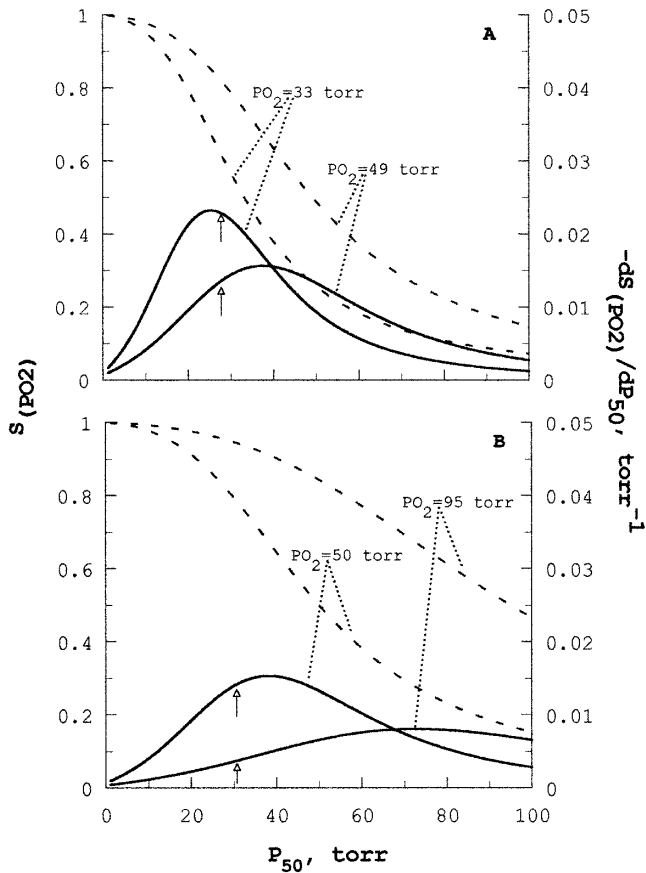
Fig. 5. Effectiveness of Bohr shift of arterial and venous horse blood as a function of P_{50}. Arrows indicate the physiological P_{50}. Hypothetical OECs with various P_{50} values were constructed using the OEC shown in Fig. 2. **A:** Dashed lines represent O_2 saturation of Hb ($S_{(PO2)}$) in fetal "arterial" (PO_2 of 49 torr) and fetal "venous" (PO_2 of 33 torr) blood. Solid lines represent the effectiveness of the Bohr shift at the O_2 uptake site in fetal "arterial" blood, $-dS_{(49)}/dP_{50}$, and that at O_2 release site in umbilical "venous" blood, $-dS_{(33)}/dP_{50}$. **B:** Dashed lines represent O_2 saturation of Hb ($S_{(PO2)}$) in maternal venous and arterial blood with PO_2 of 50 and 95 torr. Solid lines represent the effectiveness of the Bohr shift at the O_2 uptake site in arterial blood, $-dS_{(95)}/dP_{50}$, and that at the O_2 release site in uterine venous blood, $-dS_{(50)}/dP_{50}$.
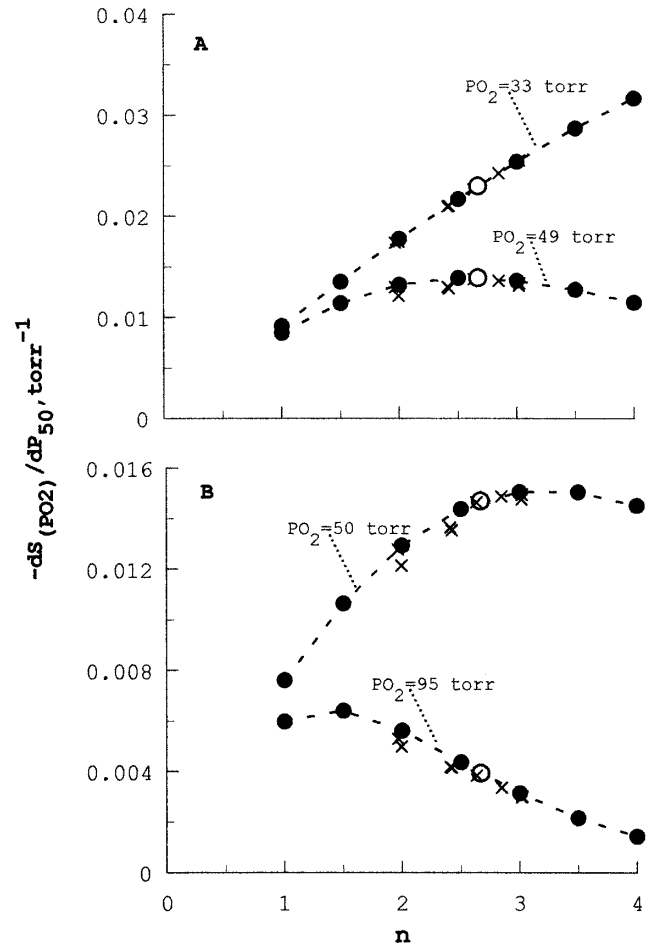

Fig. 6. Influence of cooperativity on effectiveness of the Bohr shift in horse blood. The effectiveness of the Bohr shift as a function of n value were calculated using OEC data of horse whole blood measured under standard condition ($\bigcirc$) (Comline and Silver, 1974), OEC data of horse Hb solutions measured under various experimental conditions ($\times$) (Imai,1983) and Hill's empirical equation ($\bullet$). **A:** Effectiveness of the Bohr shift in fetal "arterial" blood, $-dS_{(49)}/dP_{50}$, and that in "venous" blood, $-dS_{(33)}/dP_{50}$, are plotted against n. **B:** Effectiveness of the Bohr shift in maternal arterial blood, $-dS_{(95)}/dP_{50}$, and that in uterine venous blood, $-dS_{(50)}/dP_{50}$, are plotted against n.

In fetal blood, the effectiveness of the Bohr shift at the O_2 release site ($dS_{(33)}/dP_{50}$) was increased with an increase in n value (Fig. 6A), and reached the highest value at approximately $n=7$ (this point is out of range in Fig. 6A). Conversely, at the O_2 uptake site of fetal "arterial" blood, the highest effectiveness of the Bohr shift ($dS_{(49)}/dP_{50}$) was observed at $n=2.6$ (Fig. 6A).

In maternal blood, the highest value at the O_2 release site ($dS_{(50)}/dP_{50}$) was observed at $n=3.3$, and that at the O_2 uptake site ($dS_{(95)}/dP_{50}$) was observed at $n=1.5$ (Fig. 6B).

Correlation between effectiveness of the Bohr shift of fetal "arterial" blood and that of maternal uterine venous blood

The effectiveness of the Bohr shift at physiological P_{50} was compared between at the O_2 uptake site (fetal "arterial" blood) and that at O_2 release site (maternal uterine venous blood) to consider the gas exchange across the placental membrane. As shown in Fig. 7, the two $dS_{(PO2)}/dP_{50}$ values at PO_2 values of 49 and 50 torr were nearly equal at n values below 3.0. The highest values were observed at an n values ranged from 2.5 to 3.0. The n value of horse Hb under standard conditions was reported to be 2.69 (Clerbaux *et al.*, 1993). If there was large difference in effectiveness of the Bohr shift of both bloods, the increase or decrease in CO_2 and H^+ concentration in the fetal blood could not be prevented. The results obtained in this study seem to show that relatively low cooperativity is adequate for gas exchange across the placental membrane.

2,3-DPG has little effect on the Bohr coefficient of horse blood (Pellegrini *et al.*, 1996) and this seems to suggest that
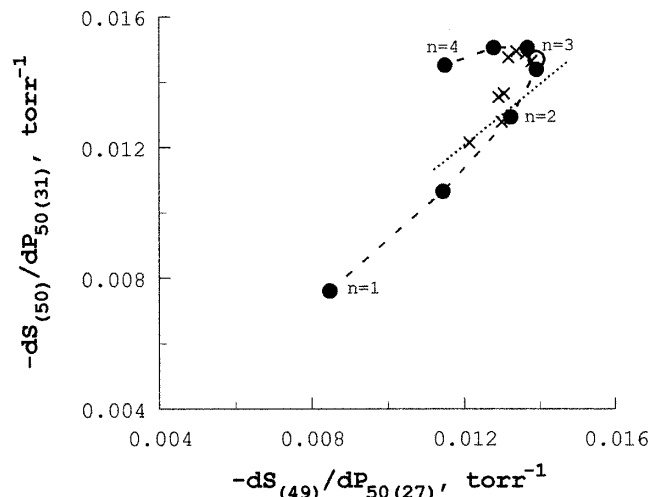
Fig. 7. Correlation between effectiveness of Bohr shift in horse fetal blood and that in horse maternal blood at various n values. The effectiveness of the Bohr shift at the O_2 uptake site in fetal "venous" blood ($-dS_{(49)}/dP_{50\,(27)}$) is plotted against that at the O_2 release site in maternal uterine venous blood ($-dS_{(50)}/dP_{50\,(31)}$). The straight dotted line represents the relation: $-dS_{(49)}/dP_{50}=-dS_{(50)}/dP_{50}$

fetal blood have the same Bohr coefficient as that of maternal blood . From these results, it is concluded that the physiological P_{50} values of horse fetal and maternal bloods are appropriate for the effectiveness of the Bohr shift occurring at the O_2 uptake and release sites. The relatively low cooperativity of fetal and maternal bloods is well suited for the efficient coupling of O_2 and H^+ transport through the placental membrane. These theoretically obtained results confirm the results on human Hbs reported in our previous paper (Zhang *et al.*, 2003)

O_2 transport efficiency and effectiveness of the Bohr shift before exercise and at maximal exercise

Using literature values of *in vivo* O_2 environment and P_{50} during incremental exercise in horse (Fenger *et al.*, 2000), we calculated O_2 transport efficiency at O_2 release site and the effectiveness of the Bohr shift before exercise and at maximal exercise. The fetal umbilical arterial PO_2 and physiological P_{50} are different to the literature values used in Fig. 2 (Comline and Silver, 1974). The O_2 consumption rate at maximal exercise was approximately 20 times higher than that before exercise. Fig. 8 shows the relationship between the O_2 transport efficiency and the effectiveness of the Bohr shift at various P_{50} values with n fixed at physiological values. Before exercise, the physiological P_{50} (22.9 torr) was nearly optimal for the effectiveness of the Bohr shift. The O_2 transport efficiency is about 2/5 that of the maximal value (Fig. 8A). In contrast, at maximal exercise, the physiological P_{50} (31.8 torr) tended to be advantageous for O_2 transport efficiency (Fig. 8B). The effectiveness of the Bohr shift was low, approximately 3/4 of that before exercise. The decrease in O_2 affinity and the effectiveness of the Bohr shift at maximal exercise indicate both a decrease in
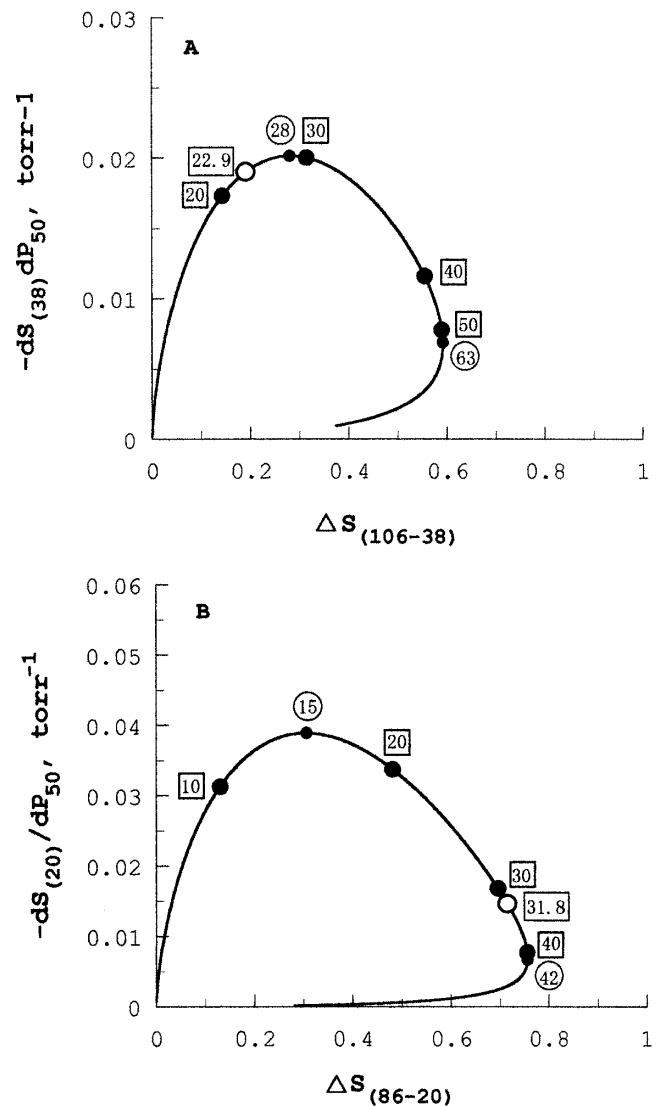

Fig. 8. Relationship between effectiveness of Bohr shift at venous PO_2 (the ordinate) and O_2 transport efficiency (the abscissa) of horse blood before exercise (A) and at maximal exercise (B) at physiological n and various P_{50} values. Open circles represent the $-dS_{(PvO2)}/dP_{50}$ and $\Delta S_{(PaO2-PvO2)}$ values obtained at physiological P_{50} values. The number in the squares attached to the line represent P_{50}. The numbers in the circles represent the P_{50} which gives the maximal effectiveness of the Bohr shift or O_2 transport efficiency.

pH and an increase in PCO_2 must occurr. In fact, $PvCO_2$ and pH values changed from 49.9 torr and 7.43 before exercise to 82.9 torr and 7.15 at maximal exercise (Fenger *et al.*, 2000).

The P_{50} of the OEC before exercise, which is lower than PvO_2, is nearly optimized with respect to the effectiveness of the Bohr shift. On the other hand, at maximal exercise, the physiological P_{50}, which is higher than PvO_2 but lower than PaO_2, tends to be advantageous for O_2 transport efficiency. These trends are basically similar to those of human Hb (Itoh *et al.*, 2001)

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