Influence of Allosteric Effectors and Temperature on Oxygen Binding Properties and Bohr Effect of Bovine Hemoglobin

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Summary

The oxygen (O_2) binding properties of bovine Hemoglobin (Hb) were examined and compared with those of human Hb. Bovine Hb had intrinsically lower oxygen affinity than human Hb, and the affinity was lowered further with increasing concentrations of chloride ion (Cl^-) and 2,3-diphosphoglycerate (DPG). The influence of Cl^- on P_{50} was greater in bovine Hb than in human Hb at the physiological concentrations of Cl^- and DPG and at 37℃. n_{max} was reduced at high Cl^- concentrations, whereas it was little effected by DPG. An increase in Cl^- concentration enhanced the Bohr effect, the magnitude of which reached a maximum at 0.1 M Cl^- and 20℃. This concentration was nearly equal to that at the highest slope of the log P_{50} vs. log [Cl^-] plot, and was equal to the physiological Cl^- concentration (0.1 M) in bovine blood. The influence of Cl^- on the Bohr effect in bovine Hb was similar to that in human Hb, and was independent of temperature. On the other hand, in the absence of Cl^-, bovine Hb was sensitive to DPG. An increase in DPG concentration enhanced the Bohr effect, which reached a maximum at 3 mM DPG and 20℃. This concentration was nearly equal to that at the highest slope of the log P_{50} vs. log [DPG] plot. The influence of DPG on the Bohr effect in bovine Hb was similar to that in human Hb. At low DPG concentrations, the influence of DPG on the Bohr effect became small with increasing temperature, whereas at high DPG concentrations, the DPG effect was insensitive to temperature changes. At the physiological

DPG concentration of 0.5 mM, increases in both Cl^- concentration and temperature diminished the DPG effect. At the physiological concentrations of Cl^- and DPG, the Bohr effect was –0.36 at 37℃. These results indicate that Cl^- and temperature are important determinants of O_2 affinity and the magnitude of the Bohr shift of bovine Hb. In the case of human Hb, the influence of DPG on the Bohr effect is greater than that of Cl^-, and at 37℃, in the presence of the physiological concentration of DPG, the influence of Cl^- on the Bohr effect is abolished.

The $\triangle H$ value of bovine Hb, which was measured at the physiological concentrations of Cl^- and DPG to be approximately –5.8 kcal/mol, is markedly lower than that of human Hb (approximately –8.1 kcal/mol). Low-temperature sensitivity contributes to O_2 unloading in cooled peripheral tissues. At the physiological concentration of DPG, the effect of Cl^- on P_{50} in bovine Hb at 37℃ is markedly higher than that in human Hb. This results seem to indicate that the Cl^- effect on P_{50} in bovine Hb contributes to the unloading of O_2 from Hb with marked influence of Cl^- during the Cl^- shift in bovine red blood cell.

Abbreviations

O_2, oxygen

Hb, hemoglobin

OEC, oxygen equilibrium curve

S, fractional saturation of Hb with O_2

PO_2 (p), partial pressure of O_2

P_{50}, partial pressure of O_2 at 50% O_2 saturation

DPG, 2,3-diphosphoglycerate

ΔH, overall heat of oxygenation

K_T, K_R and L, MWC parameters

Cl^-, chloride ion

Tris, tris(hydroxymethyl)aminomethane

L, length of light-path of sample cell

d.c., direct current

C_j, absorbance signal at a given PO_2

C_d, C_o, absorbance signal of the fully oxygenated sample and the fully deoxygenated sample, respectively

HEPES, 2-hydroxyethylpiperazine- N'-2-ethanesulfonic acid

MES, 2-morpholinoethanesulfonic acid

Met-Hb, methemoglobin

K_1, K_2, K_3, K_4, Adair constants

n_{max}, highest slope of Hill plot

[Eff], concentration of allosteric effectors

Bohr coefficient, slope of log P_{50} vs. pH plot (dlog P_{50}/dpH)

R, gas constant

T, absolute temperature

ΔH_1, ΔH_2, ΔH_3, ΔH_4, overall heat of oxygenation at individual step

f_0, f_1, f_2, f_3, f_4, fractional population in different oxygenation stages of Hb (Hb, $Hb(O_2)$, $Hb(O_2)_2$, $Hb(O_2)_3$, $Hb(O_2)_4$)

ΔS, arterio-venous difference in O_2 saturation

Contents

1. Introduction

The oxygen (O_2) binding property of hemoglobin (Hb) is characterized by position and sigmoidal shape of the oxygen equilibrium curve (OEC, O_2 saturation (S) vs. partial pressure of O_2 (P)). The position is expressed by the partial pressure of O_2 at 50% Hb saturation with O_2 (P_{50}). Mammalian Hbs can be broadly divided into two groups: low and high O_2 affinity Hbs. The Hbs of ruminant animals, such as bovine, goat and sheep, have intrinsically low O_2 affinity and are insensitive to 2,3-diphosphoglycerate (DPG) (Bunn, 1971; Perutz and Imai, 1980). The red blood cells of these animals have very low concentrations of DPG. In contrast, the Hbs of other mammalian species, such as human, horse and dog, have intrinsically high O_2 affinity and high sensitivity to DPG (Bunn, 1971, 1980; Perutz *et al.*, 1993; Perutz and Imai, 1980). The red blood cells of these animals have high concentrations of DPG.

The O_2 binding to Hb is generally an exothermic process: a decrease in temperature increases O_2 affinity, and the increased O_2 affinity suppresses O_2 release from Hb in peripheral tissues. Therefore, temperature is a critical determinant of O_2 transport by Hb. The temperature sensitivity of Hb is expressed as the overall heat of oxygenation, $\triangle H$, including the intrinsic heat of O_2 binding to the hemes (exothermic process) and the heats of all the processes associated to oxygenation, namely heats of proton and anion release which are of endothermic process. The $\triangle H$ value of human Hb has

been reported to vary from −9 to −14 under physiological conditions (Rossi-Fanelli *et al.*, 1964). Recently, an exceptionally low △H values were found in arctic ruminant animals, such as reindeer and musk ox: their △H values are approximately 1/3 of that of human Hb (Brix *et al.*, 1989-a; Condo *et al.*, 1988; Di Prisco and Giardina, 2000). The physiological significance of this characteristic low sensitivity to temperature has been interpreted from the animal's adaptation to low habitat temperature, i.e., the low sensitivity to temperature can allow for the supply of an adequate amount of O_2 to cooled peripheral tissues (such as skin and legs, which may be 10℃ cooler than lungs) (Brix *et al.*, 1989-a, 1989-b; Giardina *et al.*, 1989).

Domesticated ruminants, such as bovine, pig, and deer, also have relatively low △H values (approximately 2/3 of that of human) (Clerbaux *et al.*, 1993; Giardina *et al.*, 1990; Willford and Hill, 1986). Therefore, these ruminant animals are known to be cold-resistant. Investigations for the characteristic O_2 binding properties of bovine Hb have indicated that the low O_2 affinity of bovine Hb is not only an intrinsic property but also a result of the influence of allosteric effectors (Bunn, 1971, 1980; Perutz *et al.*, 1993; Perutz and Imai, 1980). The molecular basis of the low O_2 affinity of bovine Hb was explained by Perutz and Imai (1980), who pointed out that compared with human Hb, bovine Hb has the same K_R value, but a low K_T value (i.e., the intrinsic low O_2 affinity of the T state) and a high L value (i.e., stabilization of the T state), where K_R, K_T and L are the MWC parameters

(Monod *et al.*, 1965).

Recently, it has been suggested that the chloride (Cl^-) shift which is the movement of Cl^- from the plasma into red blood cells has important role to modulate O_2 delivery from Hb (Westen and Prange, 2003). It was also shown that the binding of extra Cl^- to bovine Hb contributed to lowering $\triangle H$ (De Rosa *et al.*, 2004).

From the viewpoint of the physiological significance of O_2 transport, the alkaline Bohr effect is also important to facilitate O_2 release at the peripheral tissues. In bovine adult Hb, the magnitude of the alkaline Bohr effect in the absence of organic phosphates is –0.53 (Smith *et al.*, 1979), and the Bohr effect is enhanced by the addition of Cl^- and DPG (Fronticelli *et al.*, 1984; Marta *et al.*, 1998; Perutz *et al.*, 1993). Furthermore, Clementi *et al.* (1996) showed that there is no difference in the log P_{50} vs. pH plot between the presence and absence of DPG at physiological Cl^- concentration (0.1 M).

To date, much attention has been focused on the structural basis for the low sensitivity to temperature of bovine Hb (Baudin-Creuz *et al.*, 2002; Fronticelli *et al.*, 1995; Marta *et al.*, 1998; Perutz *et al.*, 1993; De Rosa *et al.*, 2004), whereas the combined effects of temperature and allosteric effectors on the alkaline Bohr effect have received relatively little attention. It is important to examine the influence of a wide range of temperatures on the Bohr effect. Therefore, in this study, the O_2 affinity, cooperativity and the alkaline Bohr effect of bovine and human Hbs were investigated in detail

over the temperature range of 20 to 37℃, with focus on the influence of individual allosteric effectors and the combined effect of the effectors and temperature, and results were compared with human Hb.

2. Materials and Methods

Preparation of Hb solution

Bovine adult blood samples were purchased from Nippon Biological Material Center, and human adult blood samples were obtained from healthy nonsmoking individual by venipuncture. The red blood cells were washed four times by centrifugation at 3,000 rpm for 15 min with isotonic saline solution and lysed with distilled water. Stroma was removed by centrifugation at 15,000 rpm for 20 min. The red supernatant was stripped of organic phosphates with a 54 × 2 cm Sephadex G-25 column equilibrated with 0.05 M Tris buffer, pH 7.4. All preparative procedures were carried out at 0 – 5℃.

Oxygen equilibrium curve measurement and data analysis

Oxygen equilibrium data are measured with an automatic oxygenation apparatus developed by Imai and Yonetani (1977) and Imai (1981). Figure 1 shows the schematic diagram of the apparatus. A specially designed cell is mounted on the sample cell of a dual wavelength spectrophotometer (Shimadzu model UV-3000). A compact magnetic stirrer is affixed underneath the sample cell. The oxygen electrode is operated by an oxygen meter. The temperature of the sample cell is regulated by circulating water from a water bath and is monitored by a thermistor thermometer. Figure 2 is

a photograph of the apparatus.

The detailed structure of the sample cell is shown in Fig. 3. Monochromatic light is beamed through a pair of movable windows. The light-path length, which is indicated by L in the figure, is adjusted in the range of 0 to 16 mm by changing the distance between the two windows. The appropriate volume of the sample depends on L, and is 1.5 – 2.0 ml. At L = 13 mm, a 1.6 ml volume of 70 μM Hb solution is used. The stirring bar is made from a glass-covered wire. The oxygen electrode used is a Clark-type O_2 electrode (YSI model 5331).

The oxygen meter is composed of two parts: a stabilized d.c. power supply and a high-sensitivity d.c. amplifier. Constant voltage (0.8 V) is applied to the oxygen electrode. The electrode current is converted into a voltage signal as the potential difference along a resistor and is amplified by a d.c. amplifier (5325 isolation amplifier; NF Electronic Instruments).

The ventilation system delivers air, nitrogen, or oxygen to the sample cell and monitors gas flow returning from the sample cell. It is an assembly of three three-way cocks, three small gas-washing bottles, and a gas flow monitor. Each gas is humidified by passing through water in each gas-washing bottle (capacity, 25 ml). The appropriate flow rate is 20 – 30 ml min^{-1}. Nitrogen in the cylinder must be of high purity (more than 99.999%).

To obtain the oxygen equilibrium curve, PO_2 and absorbance values are read from a set of points that are approximately picked from the

deoxygenation curve, and oxygen saturation values are calculated from the absorbance values at 575 nm using equation (1).

$$S = \frac{C_j - C_d}{C_o - C_d} \qquad (j = 1\text{-}N) \qquad (1)$$

Here, C_d and C_o are the absorbance signals of the fully oxygenated sample and the fully deoxygenated sample, respectively. C_j is the absorbance signal at a given P_{O_2}. N is the number of data collected. C_d value (deoxygenated sample) is obtained by extrapolating the bottom portion of the deoxygenation curve in the absorbance signal vs. P_{O_2} plot (Fig. 4). On the other hand, C_o value (oxygenated sample) is obtained by extrapolating the top portion of the deoxygenation curve in the absorbance signal vs. $1/P_{O_2}$ plot. In the graphical extrapolation, the quadratic function, absorbance signal = $Ax^2 + Bx + C$ is fitted and the best-fit C value is obtained.

Hepes buffer solutions (over the pH range of 6.8 to 8.1) and Mes buffer solutions (over the pH range of 5.9 to 6.4) were used for OEC measurements. The pH value was measured for each air-equilibrated oxygenated sample, and it was adjusted at the same temperature as that for OEC measurement. Met-hemoglobin (Met-Hb) formed by auto-oxidation was reduced with an enzymatic reducing system as described by Hayashi *et al.* (1973). The Met-Hb concentration at the end of OEC measurement, as described by Evelyn and Malloy (1938), was less than 5% of the total Hb concentration. The concentration of DPG was measured according to the enzymatic

procedure of Ericson and De Verdier (1972).

The experimentally obtained oxygen equilibrium data were analyzed by the curve fitting method described by Imai (1981), to estimate the four Adair constants (K_i, i = 1 – 4, equation (2)).

$$S = \frac{K_1 p + 3K_1K_2p^2 + 3K_1K_2K_3p^3 + K_1K_2K_3K_4p^4}{1 + 4K_1p + 6K_1K_2p^2 + 4K_1K_2K_3p^3 + K_1K_2K_3K_4p^4} \qquad (2)$$

Using Adair constants, OEC was generated. The sigmoid shape of OEC is expressed by Hill's coefficient (n_{max}) as the highest slope of Hill plot (log [S/(1-S)] vs. log P plot) (Hill, 1910), and enables large quantities of O_2 to be unloading in tissue upon small decreases in P.

The log P_{50} vs. log [Eff] plot was described by fitting experimental data to the Wyman equation (Wyman, 1964), where [Eff] is the concentration of allosteric effectors, and the slope of the log P_{50} vs. log [Eff] plot was calculated.

The magnitude of the Bohr effect was calculated from the slope of the log P_{50} vs. pH plot (dlog P_{50}/dpH, the Bohr coefficient) in the pH range of 7.2 to 7.6.

The △H value was calculated from the slope of the van't Hoff plot (log P_{50} vs. 1/T plot) (Wyman, 1964), that is,

$$\frac{\Delta H}{R} = \frac{d \ln P_{50}}{d(1/T)} \qquad (3)$$

Here, R is the gas constant and T is the absolute temperature. The △H

values obtained were corrected for the heat of O_2 solubilization (–3.0 kcal/mol).

3. Results and Discussion

Effect of allosteric effectors on O_2 affinity

Figure 5A shows the change in log P_{50} and n_{max} of bovine Hb induced by increasing Cl^- concentration at 20℃ and 37℃. The increases in Cl^- concentration and temperature decreased the O_2 affinity, and the total Cl^--induced changes in log P_{50} values were 0.56 and 0.42 at 20℃ and 37℃, respectively. The P_{50} value of stripped Hb (in the absence of both Cl^- and DPG, log P_{50} vs. log [Eff] = $-\infty$) was slightly higher compared with previous study (log P_{50} = 0.62 torr; De Rosa *et al.*, 2004). As judged from the position of the slope of log P_{50} vs. log [Cl^-] plot, the log P_{50} vs. log [Cl^-] plot at 37℃ was slightly shifted rightward along the log [Cl^-] axis compared with that at 20℃, indicating the reduction of sensitivity to Cl^- with increasing temperature.

In the case of DPG (Fig. 5B), the total changes in log P_{50} values were 0.52 and 0.32 at 20℃ and 37℃, respectively. A marked reduction of the sensitivity to DPG with increasing temperature was observed. At 20℃, Cl^- and DPG produced nearly equal total changes in log P_{50} value.

In the case of Cl^-, n_{max} was decreased with increasing Cl^- concentration and temperature. In contrast, the shape of OEC was invariant with change in DPG concentration.

Results shown in Figures 5A and 5B imply that the physiological concentration of Cl^- is not sufficient to saturate Hb at 37℃, therefore, Cl^-

plays important role in the regulation of O_2 transport *in vivo*. On the other hand, at 37℃ the physiological concentration of DPG (0.5 mM) is too low to regulate O_2 binding of bovine Hb.

Figure 6A shows the effect of increasing Cl^- concentration on log P_{50} and n_{max} of bovine Hb in the absence of DPG, in the presence of 0.5 mM DPG, and in the presence of 5 mM DPG at pH 7.4 and 20℃. At low Cl^- concentrations, DPG strongly reduced O_2 affinity. Increase in Cl^- concentration led to an increase in P_{50}, and the highest P_{50} value was reached at 1 M Cl^-. Further increases in the Cl^- concentration rather reduced P_{50} and caused drastic decreases in cooperativity, suggesting formation of Hb dimers due to extremely high salt concentrations.

Figure 6B shows the effect of increasing DPG concentrations at the physiological concentration of Cl^- on log P_{50} and n_{max} values. The effect in the absence of Cl^-, which was taken from Fig. 5, is also illustrated for comparison. In the physiological concentration of Cl^-, the log P_{50} value at the highest DPG concentration was higher than that attained by DPG only. In this connection, De Rosa *et al.* (2004) have reported that in the physiological concentration of Cl^-, the increase in DPG concentration enhanced log P_{50}, and the total change in log P_{50} was approximately 0.12 higher than that obtained by DPG only. The synergistic effects of Cl^- and DPG have explained this characteristic phenomenon. This phenomenon was noted in bear Hb as well (Colleta *et al.*, 1994). In the case of human adult Hb, such synergistic

effect was not observed, which is explained by the loss of additional Cl^- binding sites (De Rosa *et al.*, 2004).

Influence of allosteric effectors concentration on log P_{50} and n_{max} at three pH values

Figure 7A shows the effect of increasing Cl^- concentration on log P_{50} and n_{max} of bovine and human Hbs at 20℃ in the absence of DPG and at three pH values (7.2, 7.4 and 7.6). The log P_{50} vs. log [Cl^-] plot shifted rightward with increasing pH, and the shape of curves at the three pH values was almost identical. The intrinsically low O_2 affinity of bovine Hb compared with human Hb is clearly shown.

The effect of increasing DPG concentration in the absence of Cl^- on log P_{50} and n_{max} under the same experimental conditions as those for Cl^- is shown in Fig. 7B. The curve shifted rightward with increasing pH. The shape of the three curves was almost identical.

Figure 7C shows the effect of increasing Cl^- concentration in the presence of physiological concentration of DPG on log P_{50} and n_{max} under the same experimental conditions as Fig. 7A. In low Cl^- concentration, DPG enhanced log P_{50}. In bovine Hb, increasing Cl^- concentration decreased the DPG effect, and at 0.1 M Cl^-, the log P_{50} value was nearly equal to that of the DPG free medium (Fig. 7A). This apparent disappearance of the DPG effect is in agreement with the results of a previous study (Clementi *et al.*, 1996).

The reduction of n_{max} at high Cl^- concentrations was observed in Figs. 7A and 7C. In contrast to Cl^-, DPG had little effect on the n_{max} (Fig. 7B). Using the results shown in Fig. 7, the magnitude of the Bohr coefficient (dlog P_{50}/dpH) was estimated.

log P_{50} vs. log [Eff] plot and Bohr effect

Figures 8A and 8B show the relationship between the log P_{50} vs. log [Eff] plots at pH 7.4, and the magnitude of the Bohr coefficient of bovine and human Hbs. As shown in Fig. 7, the log P_{50} vs. log [Eff] plots showed nearly the same sigmoidal shape, it may be expected that the maximum difference in log P_{50} (i.e., the highest Bohr coefficient) occurred at the highest slope of the curve. As expected, the data in the figure showed that the Cl^- concentration that gave the highest slope was nearly equal to the Cl^- concentration at which the Bohr coefficient is maximized (Fig. 8A). Interestingly, at 37℃, this Cl^- concentration was nearly equal to the physiological Cl^- concentration.

In the case of DPG (Fig. 8B), the highest Bohr coefficient of bovine and human Hbs were also observed at the highest slope of the log P_{50} vs. log [DPG] plot. The DPG concentration that gave the highest Bohr coefficient at 37℃ (about 10 mM) was higher than the physiological DPG concentration of bovine blood (0.5 mM). The slope of the log P_{50} vs. log [Eff] plots of human Hb was higher than that of bovine Hb.

In the presence of 0.5 mM DPG (Fig. 8C), the Bohr coefficient was the largest at low Cl^- concentration, and was strongly reduced with increasing Cl^- concentration. Therefore, the Bohr coefficient vs. log [Cl^-] plot (Fig. 8C) was different from those of change in concentration of Cl^- or DPG alone (Figs. 8A, 8B).

At the physiological concentration of DPG, large differences in the effect of Cl^- on P_{50} were observed between bovine and human Hbs at 37℃. The large effect of Cl^- on P_{50} in bovine Hb at the physiological concentration of DPG seems to be important for O_2 unloading in bovine venous blood where Cl^- concentration is increased due to the Cl^- shift.

Influence of temperature on magnitude of Bohr effect

Figure 9A shows the influence of temperature on the magnitude of the alkaline Bohr effect of bovine and human Hbs at four Cl^- concentrations in the absence of DPG. Increase in Cl^- concentration enhanced the Bohr coefficient of bovine Hb, which reached a maximum at 0.1 M Cl^- and 20℃. However, further increases in Cl^- concentration rather decreased the Bohr coefficient. Very similar results are observed on human Hb. The influence of Cl^- on the Bohr effect is also same in bovine and human Hbs. These results are in accord with the proposal of Perutz *et al.* (1993). The change in temperature had little effect on the Cl^- dependent Bohr coefficient. The temperature insensitive alkaline Bohr effect has been reported in musk ox

(Brix *et al.*, 1989-a) and pig Hbs (Sinet *et al.*, 1982).

The results obtained from the same experiments performed as those above as a function of five DPG concentrations in the absence of Cl^- are illustrated in Fig. 9B. In bovine Hb, the highest Bohr coefficient was observed at 3 mM DPG and 20℃. In contrast to Cl^-, at low DPG concentrations (0.1 – 3 mM), the DPG effects were significantly reduced with increasing temperature, whereas at high DPG concentrations (10 mM), the DPG effect was insensitive to temperature changes. The influence of DPG on the Bohr effect in bovine Hb is very similar to that in human Hb. In both bovine and human Hbs, it is interesting to note that the influence of Cl^- and DPG alone on P_{50} resembles the influence of Cl^- alone on the Bohr effect.

Figure 9C shows the influence of temperature on the Bohr coefficient at four Cl^- concentrations in the presence of physiological concentration of DPG. In bovine Hb, the highest Bohr coefficient at 0.1 M Cl^- is similar to the literature values reported by Clementi *et al.* (1996). The Bohr coefficient was progressively decreased with increasing Cl^- concentration and temperature, and drastically diminished at 37℃ (–0.36).

By comparing the effect of temperature on the Bohr coefficient at the physiological concentration of Cl^-, which is shown in Fig. 9A (×), it is evident that DPG influences the Bohr coefficient at 20℃, whereas, its effect drastically disappears at high temperature (37℃). Taken together, these results confirm that Cl^- plays an important role in regulating the *in vivo*

function of bovine Hb. The DPG effect in human Hb is almost the same as that in bovine Hb at low temperatures, whereas the disappearance of the DPG effect at high temperatures is not as high as that observed in bovine Hb.

Correlation between P_{50} and magnitude of Bohr effect

The Bohr coefficient is useful for comparing interspecies difference in the magnitude of the Bohr coefficient. However, it does not directly indicate the Bohr effect-induced additional amount of O_2 released from Hb at the peripheral tissues and loading to Hb at respiratory organs. The additional amount of O_2 released due to the Bohr shift at O_2 release site depends on not only the magnitude of the Bohr shift but also the position and shape of the OEC and the O_2 pressure at the O_2 release site. In the previous study, we estimated the effectiveness of the Bohr shift in terms of the change in S at O_2 pressure per unit change in P_{50} ($dS_{(PO2)}/dP_{50}$), and it has been pointed out that the physiological P_{50} of human and horse Hbs is nearly optimized in order to receive the maximum benefits from the Bohr shift at O_2 release site (Itoh *et al.*, 2001; Zhang *et al.*, 2003-a, b). In this respect, it is also important to consider the magnitude of the Bohr effect in relation to P_{50}. Using the data shown in Fig. 9, the magnitude of the Bohr coefficient is plotted against log P_{50} (Fig. 10). The results showed that the influence of 0.1 M Cl^- alone and the combined influence of the physiological concentration of Cl^- and DPG on

the Bohr coefficient of bovine Hb at 37℃ was almost the same, meaning that Cl^- is a major determinant of the magnitude of the Bohr coefficient and P_{50}. However, it is important to note that the relatively low O_2 affinity (the physiological P_{50} of bovine Hb is 31.0 torr) is achieved at high temperatures. At physiological concentrations of Cl^- and DPG, the decrease in P_{50} with decreasing temperature slightly enhances the Bohr coefficient. This may be due to the presence of 0.5 mM DPG. The magnitude of the Bohr coefficient of bovine Hb is significantly lower than that of human Hb at physiological concentrations of Cl^- and DPG. The influence of DPG alone on the Bohr coefficient of human Hb at 37℃ is almost equal to combined influence of the physiological concentrations of Cl^- and DPG. This clearly indicates that DPG is the dominant effecter of the magnitude of the Bohr coefficient and P_{50}.

Van't Hoff plot at three pH values

Figure 11 shows the van't Hoff plots of bovine Hb at three pH values. In the absence of both Cl^- and DPG (Fig. 11A), no difference is observed among the slopes of the three plots at pH 7.2, 7.4 and 7.6. This means that the Bohr coefficient is independent of temperature and $\triangle H$ (temperature sensitivity) is independent of pH. The same trend is observed at the physiological concentrations of Cl^- (Fig. 11B).

At the physiological concentration of DPG (Fig. 11C), the slope of the plot at pH 7.6 is higher than that at pH 7.2. This indicates that the Bohr

coefficient is decreased as temperature is increased, and △H is increased at high pH values. Almost the same trend is observed at the physiological concentrations of Cl^- and DPG (Fig. 11D). The difference in △H values between neutral pH and alkaline pH is attributed to the heat generated by Bohr proton release upon oxygenation (Wyman, 1936).

Correlation between the △H and pH

For the same experimental conditions as Fig. 10, △H of bovine and human Hbs are illustrated as a function of pH (Fig. 12). The △H value markedly depends on pH and the presence of Cl^- and DPG. In bovine Hb, at the presence of 0.1 M Cl^- and in the absence and presence of 0.5 mM DPG, the △H values are nearly equal. The lowest values of –3.5 kcal/mol (absolute value) are observed in the presence of 0.5 mM DPG at pH 6.8. At the physiological concentrations of Cl^- and DPG and at pH 7.4, △H is –5.8 kcal/mol, and is significantly lower than that of human Hb (–8.1 kcal/mol). The △H value observed in this study is in good agreement with the previously reported value (△H = –9.6 kcal/mol; Imai and Yonetani, 1975).

The dependence on pH of △H and gradual decrease of △H with increasing concentrations of Cl^- and DPG are attributed to the heat generated by the oxygen-linked release of Bohr-proton (Wyman, 1936), Cl^- and DPG (Benesch *et al.*, 1969).

Heat of oxygenation at individual steps of bovine and human Hbs

The $\triangle H$ values at individual steps ($\triangle H_1$, $\triangle H_2$, $\triangle H_3$, $\triangle H_4$) are calculated from the Arrhenius plot of estimated Adair constants. As shown in Fig. 13, the $\triangle H$ values of bovine Hb at the physiological concentrations of Cl^- and DPG are minimal at the second step, whereas the $\triangle H$ value of human Hb is minimal at the third step. These results are probably due to the release of Cl^- from bovine Hb at the second step and the release of DPG from human Hb at the third step.

Population of intermediate molecular species

The fractional population of different oxygenated species (Hb (f_0), HbO_2 (f_4), $Hb(O_2)_2$ (f_2), $Hb(O_2)_3$ (f_3), $Hb(O_2)_4$ (f_4)) is calculated from the estimated Adair constants (Imai, 1982), and the results obtained in the absence and presence of physiological concentrations of Cl^- and DPG are illustrated in Fig. 14. Minimal values of f_2 and f_3 are observed for bovine and human Hbs, respectively. The low f_2 value of bovine Hb in the presence of Cl^- seems to indicate that two and three oxygen molecules combine with Hb simultaneously. In the case of human Hb, DPG has a depressing effect on f_3, and it seems to imply that in the presence of DPG, three and four oxygen molecules combine with Hb simultaneously.

Physiological significance of low $\triangle H$ on O_2 loading and chloride shift on O_2

unloading of bovine Hb

Figure 15 illustrates the theoretical OEC which clarifies the physiological significance of the low $\triangle H$ of bovine Hb on O_2 delivery to peripheral tissues, where the temperature is assumed to be 5℃ lower than that of the lungs (37℃). The OEC in (a) (P_{50} = 31.0 torr; Faber and Thornburg, 1983) is the standard OEC of bovine blood measured under physiological concentration of Cl^- and DPG and at pH 7.4 and 37℃. OECs in (b) (P_{50} = 24.5 torr) and (c) (P_{50} = 23.1 torr), respectively, were calculated for be those of bovine Hb ($\triangle H$ = –5.8 kcal/mol, cold-resistant Hb) and human Hb ($\triangle H$ = –8.1 kcal/mol, non cold-resistant Hb) under physiological solvent conditions and 32℃. The arrows ($\triangle S_{(a)}$, $\triangle S_{(b)}$ and $\triangle S_{(c)}$) indicate arterio-venous saturation difference, which express the amount of O_2 delivered to the peripheral tissues by OEC (a), (b, cold-resistant Hb) and (c, non cold-resistant Hb), respectively. The O_2 pressure (PO_2) of arterial (108 torr) and venous (42 torr) bloods were taken from Taylor *et al.* (1987). When there was no decrease in temperature of the peripheral tissues, the amount of O_2 delivered to the tissues is given by $\triangle S_{(a)}$. On the other hand, when the temperature was 5℃ lower, bovine Hb ($\triangle S_{(b)}$) could deliver O_2 about 6/5 of that delivered by human Hb ($\triangle S_{(c)}$). This difference seems to show the contribution of low temperature sensitivity of Hb on O_2 delivery at cooled peripheral tissues. On the contrary, human have non cold-resistant Hbs. At body temperature increased with hard exercise, these Hbs seem to have a

lower O_2 affinity than cold-resistant Hb, and are advantage for O_2 release.

It has been thought that the role of the chloride shift is to replace the lost negative charge of the outward moving HCO_3^- (Fig. 16). One recently discovered role of the chloride shift is that it modulates O_2 loading/unloading from Hb. Brix *et al.* (1990) suggested that the large influx of Cl^- during the chloride shift in brown bear contributes to O_2 unloading via modulation of Hb-O_2 affinity. In this regard, the large Cl^- effect in bovine Hb compared with that in human Hb may be due to O_2 unloading. Influxing Cl^- is considered to be an important contributor to the acid-base balance in blood and to O_2 unloading in bovine blood.

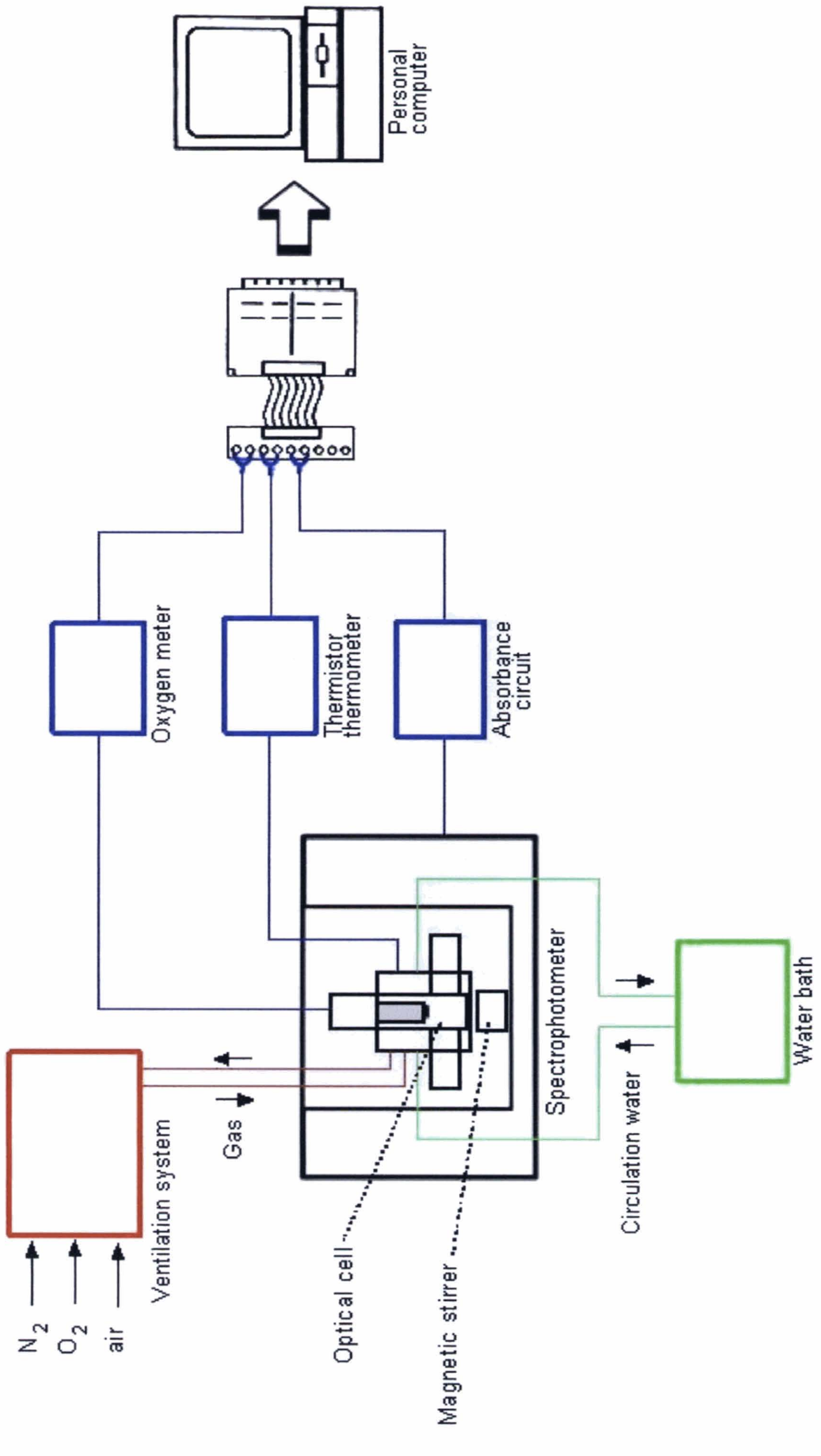

Fig. 1. Block diagram of the automatic oxygenation apparatus.

Fig. 2. The photograph of the automatic oxygenation apparatus.

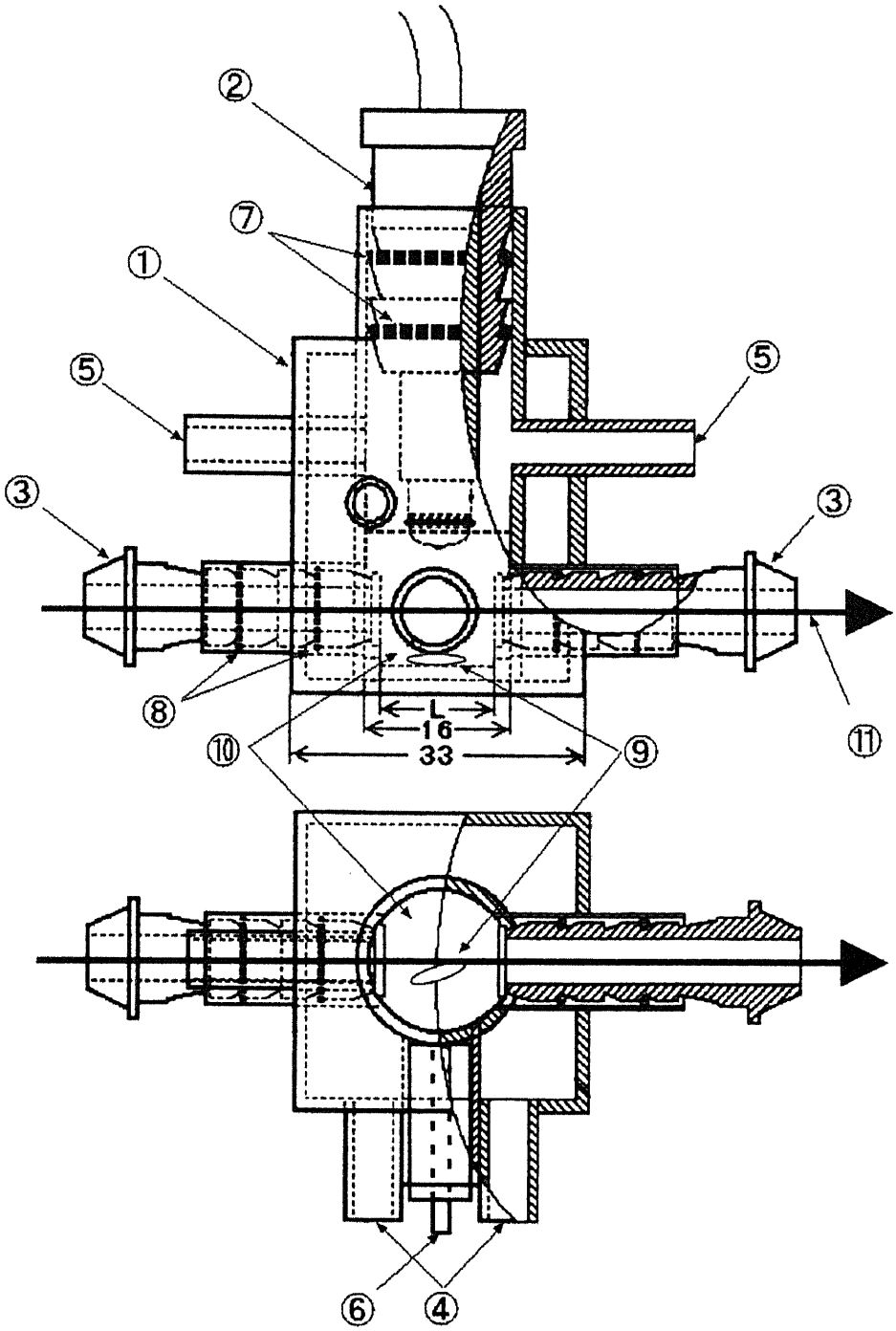

Fig. 3. The structure of optical cell. The upper and lower parts are side and top views, respectively. 1, main part of the cell; 2, oxygen electrode; 3, movable window; 4, inlet and outlet of circulating water from a water bath; 5, inlet and outlet of gases; 6, thermistor probe; 7, O-ring; 8, O-ring; 9, stirrer bar; 10, hemoglobin solution; 11, monochromatic light beam. L is length of light-path. Parts 1 and 3 are made of 18-8 stainless steel.

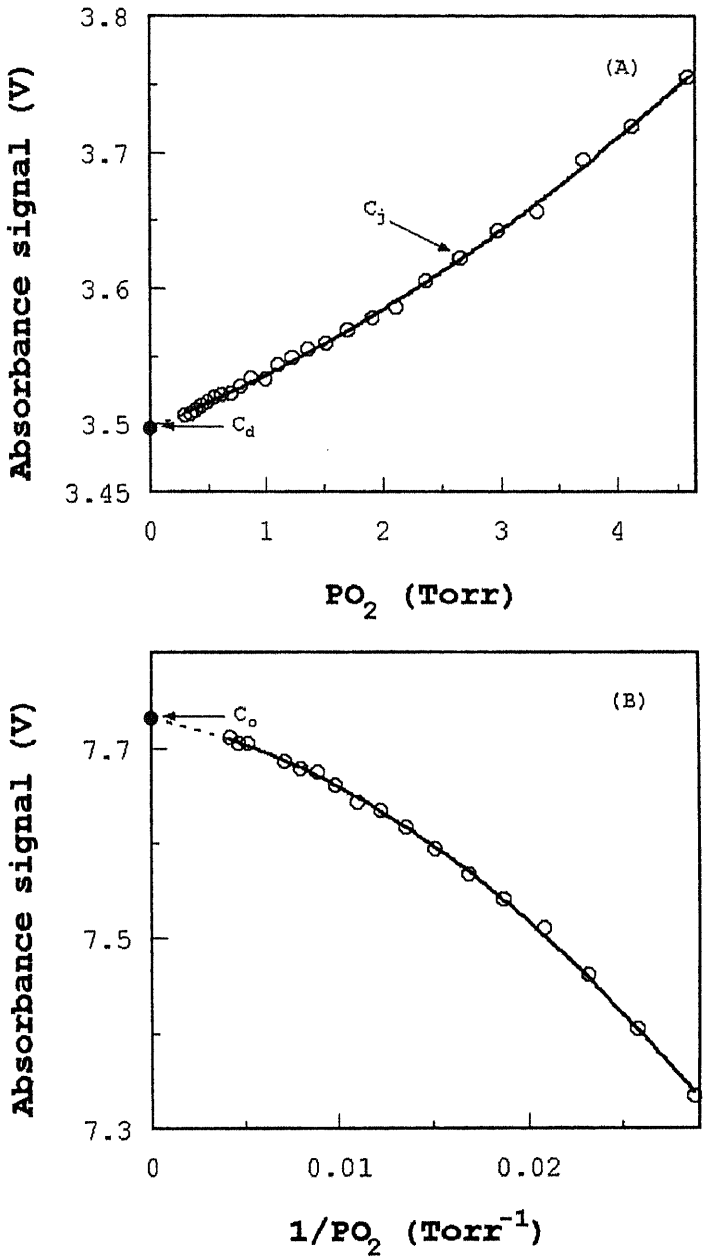

Fig. 4. Quadratic extrapolation of bottom (A) and top end (B) of oxygenation data. Lines were calculated by "Absorbance signal = $Ax^2 + Bx + C$". C_d and C_o are the absorbance signal of the fully oxygenated sample and the fully deoxygenated sample, respectively. C_j is the absorbance signal at given PO_2.

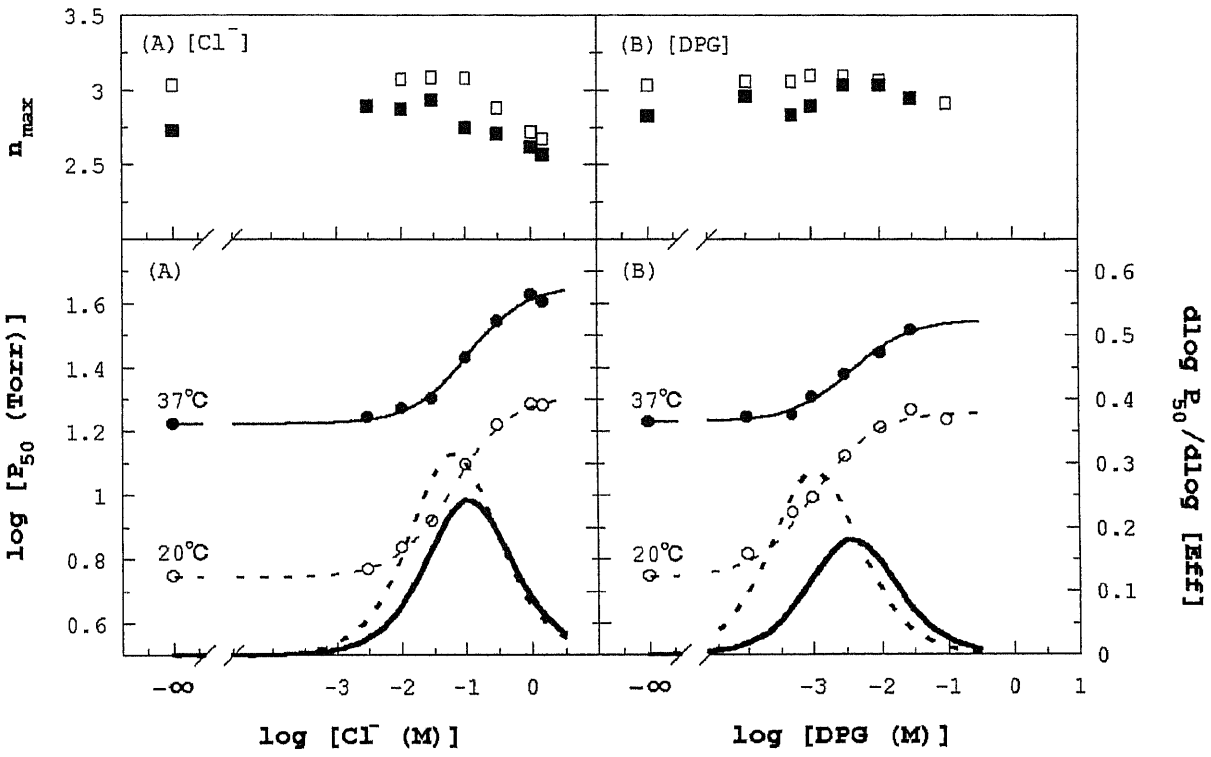

Fig. 5. Effect of Cl^- (A) and DPG (B) concentration on log P_{50} (○, ●) and n_{max} (□, ■) of bovine Hb in 0.1 M Hepes buffer at pH 7.4. Dashed and solid lines represent the slope of log P_{50} vs. log [Eff] plot. The temperature was 20℃ for open symbols and dashed line and 37℃ for closed symbols and solid line.

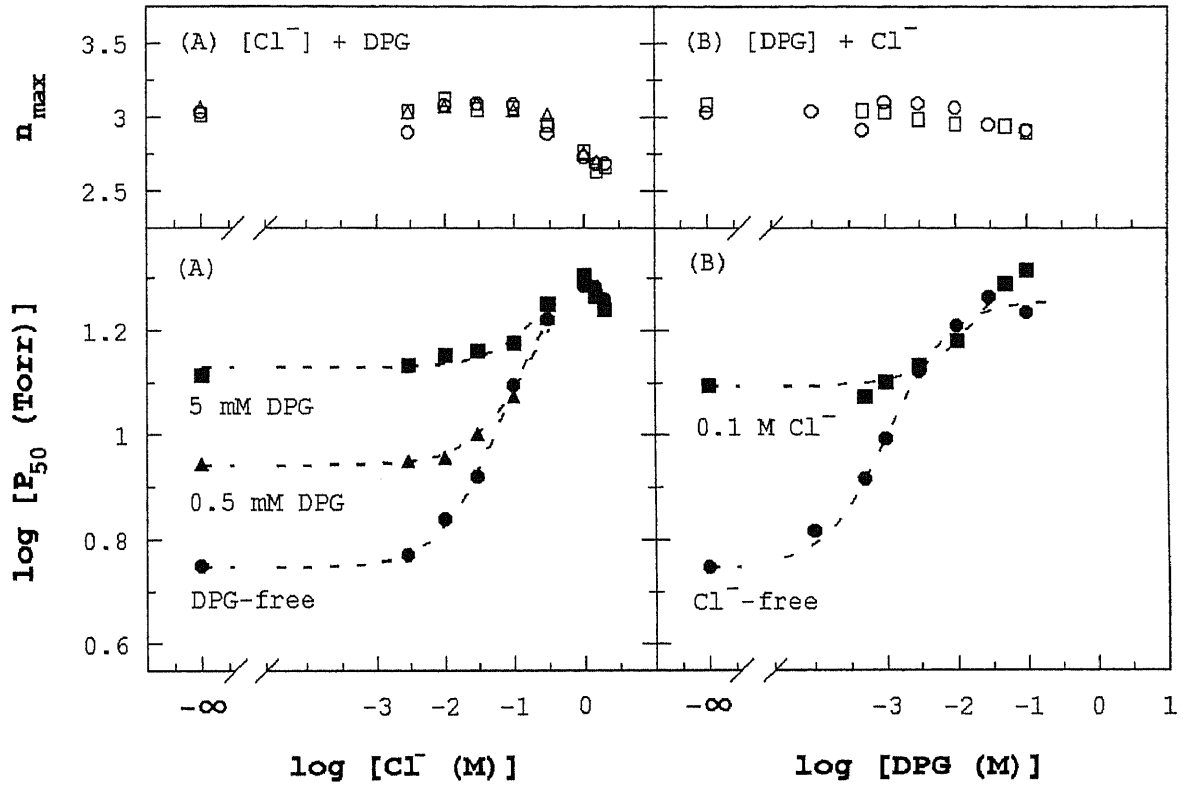

Fig. 6. Effect of Cl^- (A) and DPG (B) concentration on log P_{50} (closed symbols) and n_{max} (open symbols) of bovine Hb in 0.1 M Hepes buffer at pH 7.4 and 20℃.
(A) ●, ▲ and ■, were in the absence of DPG, in the presence of 0.5 mM and 5 mM DPG, respectively.
(B) ● and ■, were in the absence of Cl^- and in the presence of 0.1 M Cl^-, respectively. Data shown by ● was taken from Fig. 5.

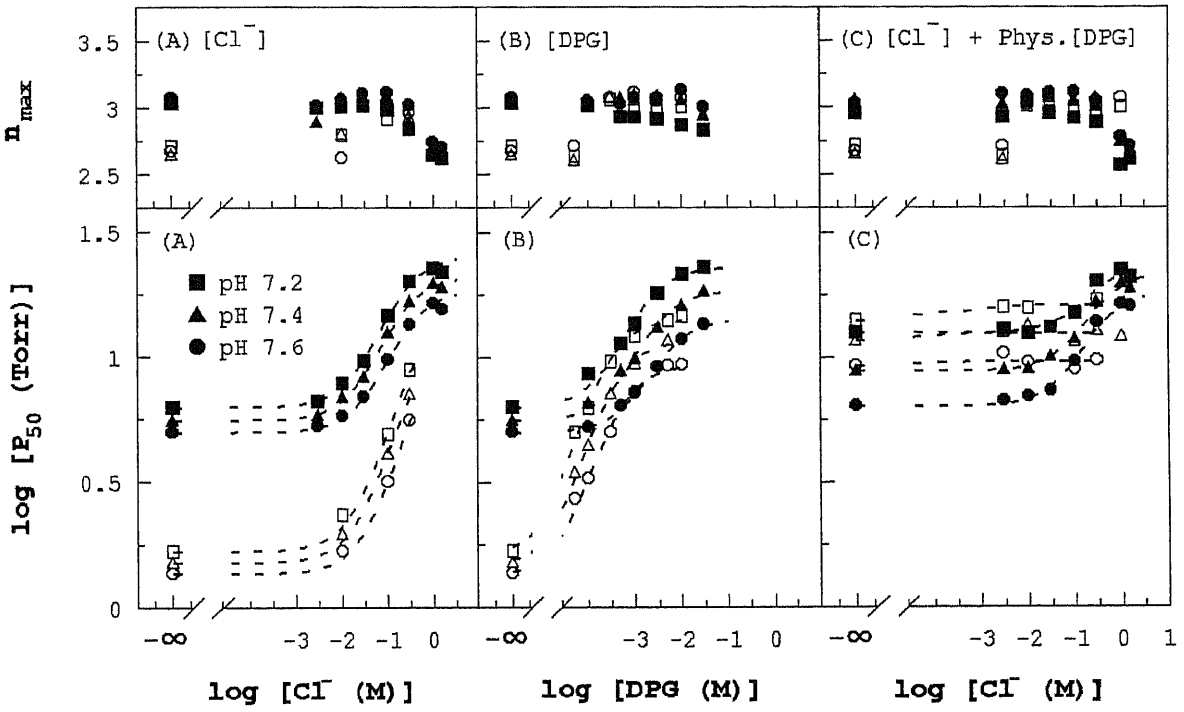

Fig. 7. Effect of Cl^- concentration in the absence of DPG (A), DPG concentration in the absence of Cl^- (B), and Cl^- concentration in the presence of physiological DPG concentration (C) on log P_{50} (lower) and n_{max} (upper) of bovine (closed symbols) and human Hbs (open symbols) in 0.1 M Hepes buffer at three pH values of 7.2 (■), 7.4 (▲) and 7.6 (●) and 20℃.

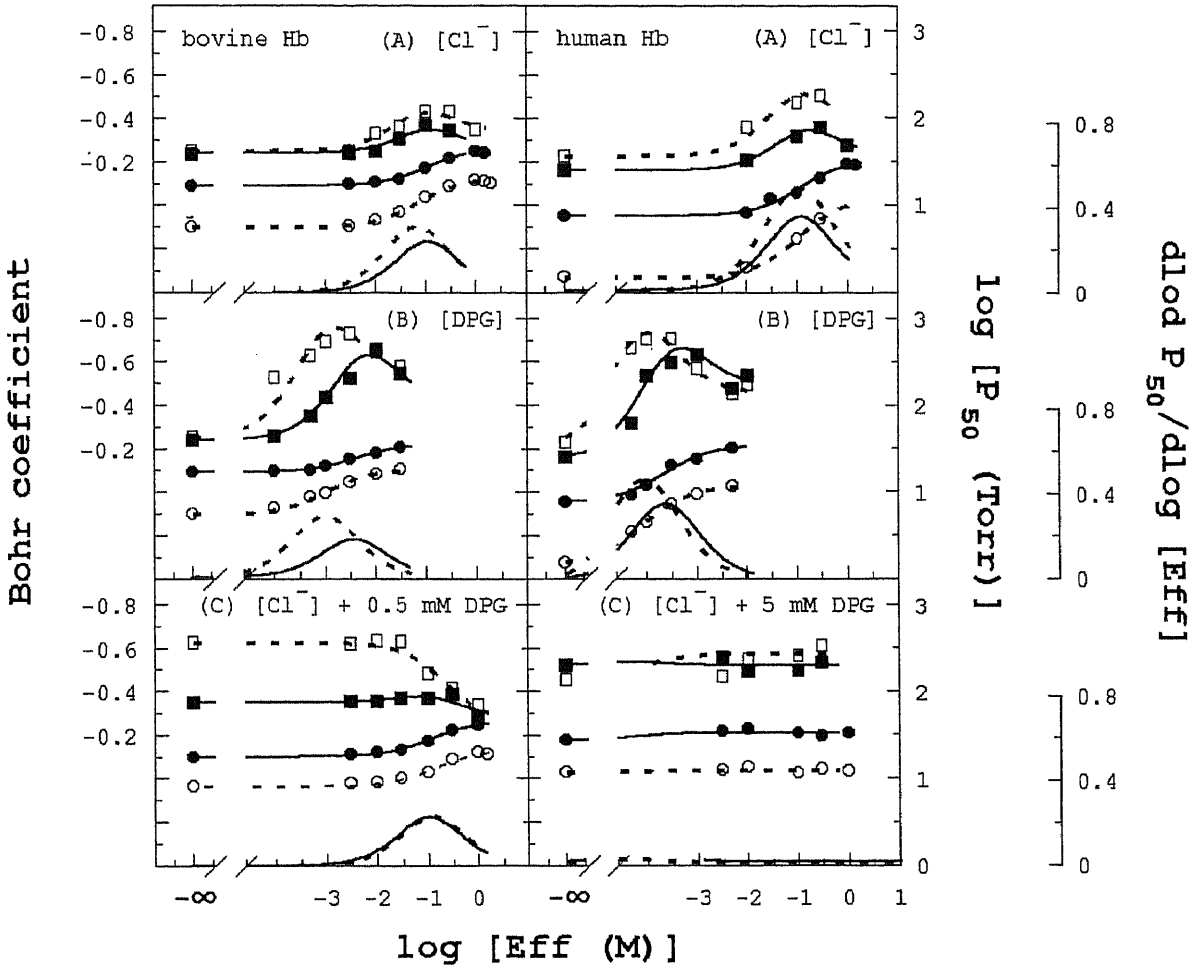

Fig. 8. Effect of Cl^- concentration (A), DPG concentration (B) and Cl^- concentration in the presence of physiological DPG concentration (C) on log P_{50} (○) and the magnitude of the alkaline Bohr effect (□) of bovine and human Hbs in 0.1 M Hepes buffer.
Dashed and solid lines represent the slope of the log P_{50} vs. log [Eff] plot at pH 7.4, and 20℃ and 37℃, respectively. Open and closed symbols represent at 20℃ and 37℃, respectively.

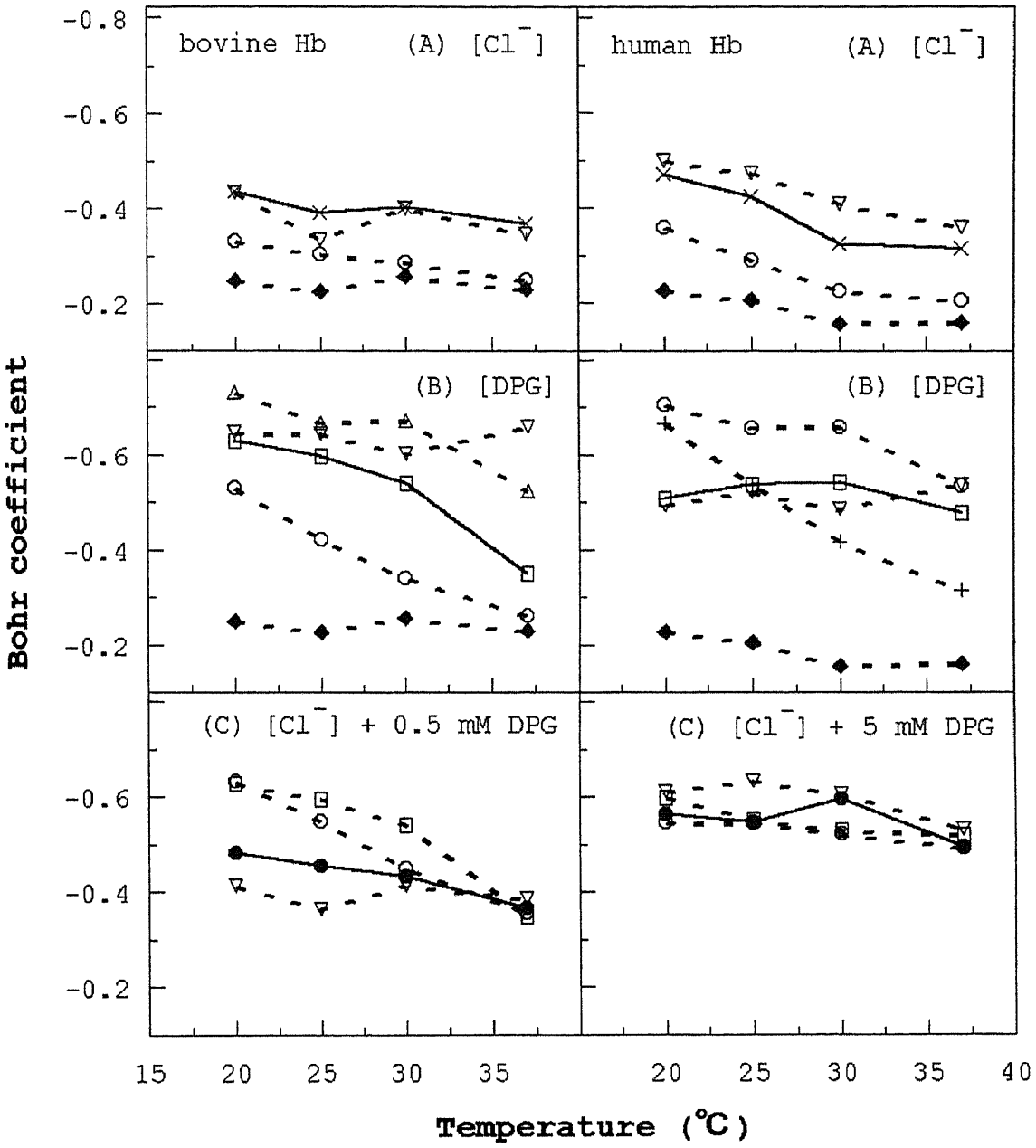

Fig. 9. Effect of temperature on the magnitude of the alkaline Bohr effect of bovine and human Hb in 0.1 M Hepes buffer. (A) in various concentration of Cl^-. (B) in various concentration of DPG. (C) in various Cl^- concentration in the presence of physiological concentration of DPG.
(A) (◆) Cl^--free; (○) 0.01 M Cl^-; (×) 0.1 M Cl^-; (▽) 0.3 M Cl^-.
(B, bovine Hb) (◆) DPG-free; (○) 0.1 mM DPG; (□) 0.5 mM DPG; (△) 3 mM DPG; (▽) 10 mM DPG.
(B, human Hb) (◆) DPG-free; (+) 0.05 mM DPG; (○) 0.1 mM DPG; (□) 5 mM DPG; (▽) 10 mM DPG.
(C) (□) Cl^--free + phys.[DPG]; (○) 0.01 M Cl^- + phys.[DPG]; (●) 0.1 M Cl^- + phys.[DPG]; (▽) 0.3 M Cl^- + phys.[DPG]. Physiological DPG concentrations of bovine and human bloods are 0.5 mM and 5 mM, respectively.

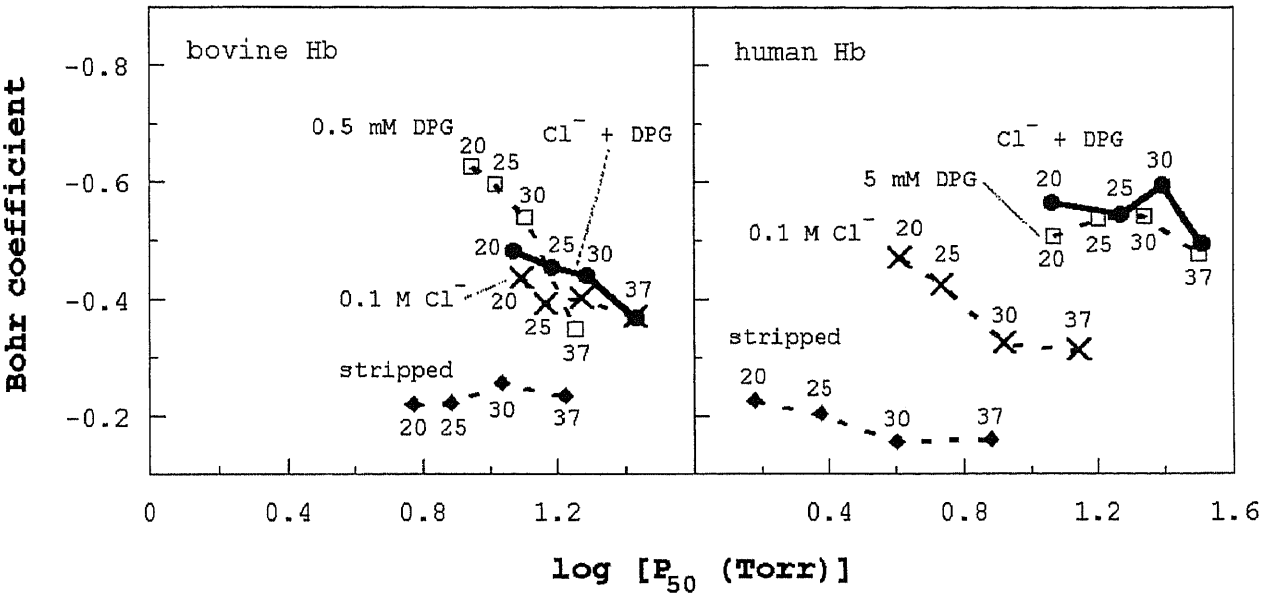

Fig. 10. Relationship between the magnitude of the alkaline Bohr effect and log P_{50} at pH 7.4 of bovine and human Hb. Conditions: 0.1 M Hepes buffer at four temperature (20, 25 30 and 37℃) in the absence of both Cl^- and DPG (stripped, ◆), in the presence of 0.1 M Cl^- (×), in the presence of physiological DPG concentration (□), and in the presence of physiological concentrations of Cl^- and DPG (●). Physiological DPG concentrations of bovine and human bloods are 0.5 mM and 5 mM, respectively. Number in the figure represents the temperature. All data were taken from Fig. 9.

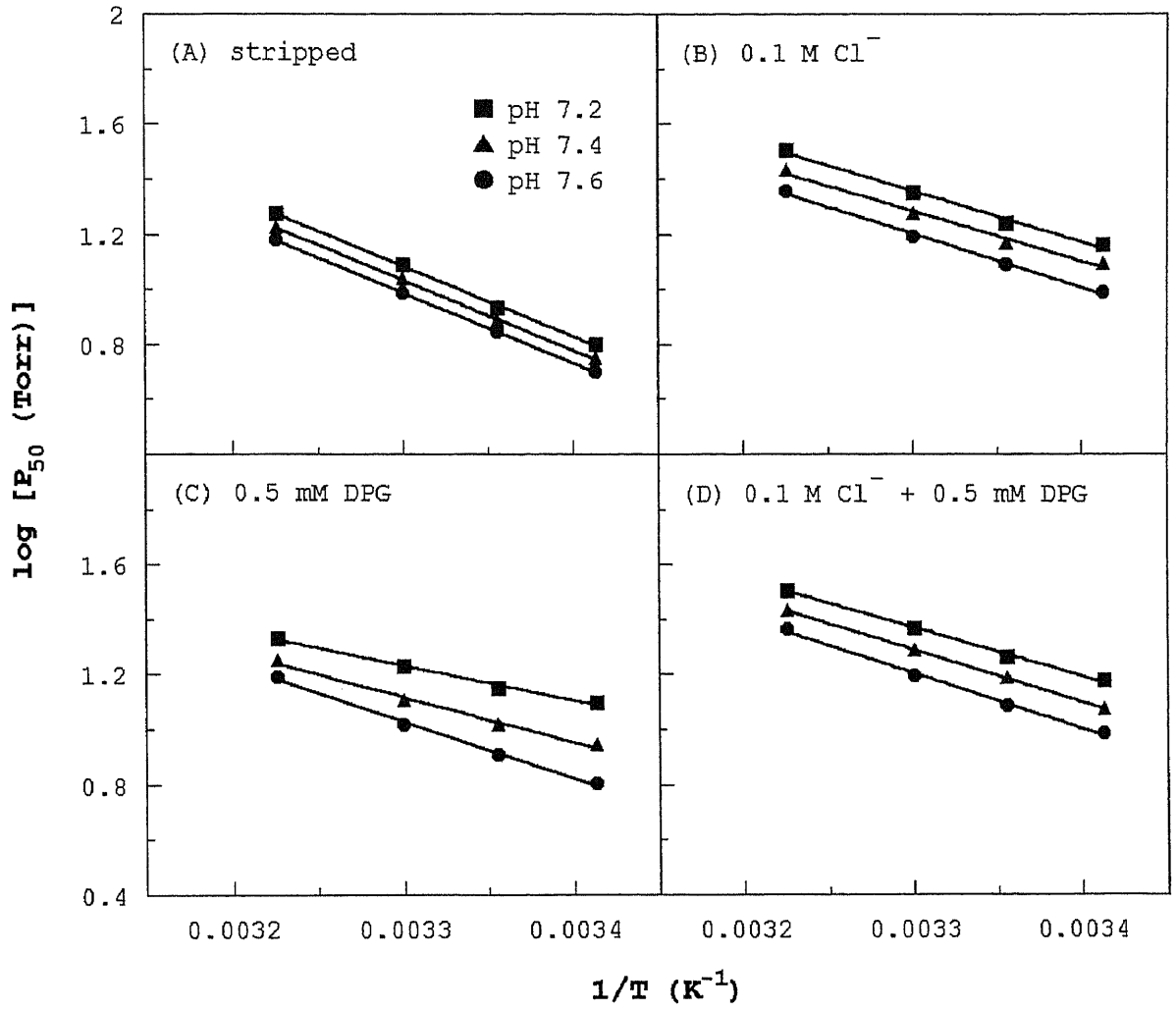

Fig. 11. Van't Hoff plots of the temperature dependence of log P_{50} values for bovine Hb in 0.1 M Hepes buffer at pH 7.2 (■), 7.4 (▲) and 7.6 (●). (A) in the absence of Cl^- and DPG (stripped Hb). (B) in the presence of 0.1 M Cl^-. (C) in the presence of 0.5 mM DPG. (D) in the presence of 0.1 M Cl^- and 0.5 mM DPG.

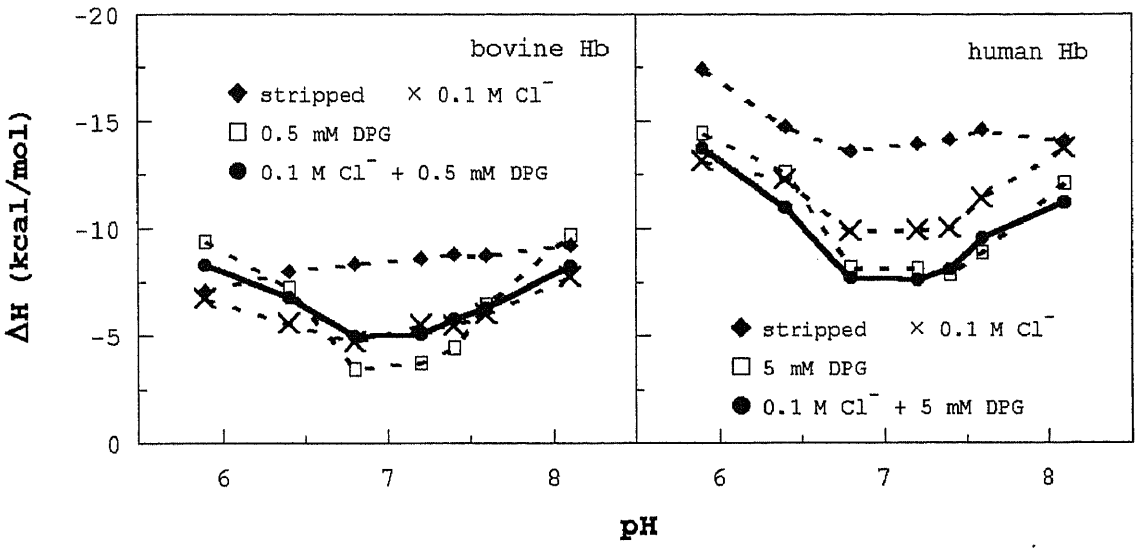

Fig. 12. Relationship between the ΔH of bovine and human Hbs and pH. The ΔH values were calculated from the integrated van't Hoff equation by using the OEC in 0.1 M Hepes buffer and 0.1 M Mes buffer in the absence of both Cl^- and DPG (stripped, ◆), in the presence of 0.1 M Cl^- (×), in the presence of physiological DPG concentration (□), and in the presence of physiological concentrations of Cl^- and DPG (●). Physiological DPG concentrations of bovine and human bloods are 0.5 mM and 5 mM, respectively. ΔH values are corrected for the heat contribution of O_2 in solution (–3.0 kcal/mol).

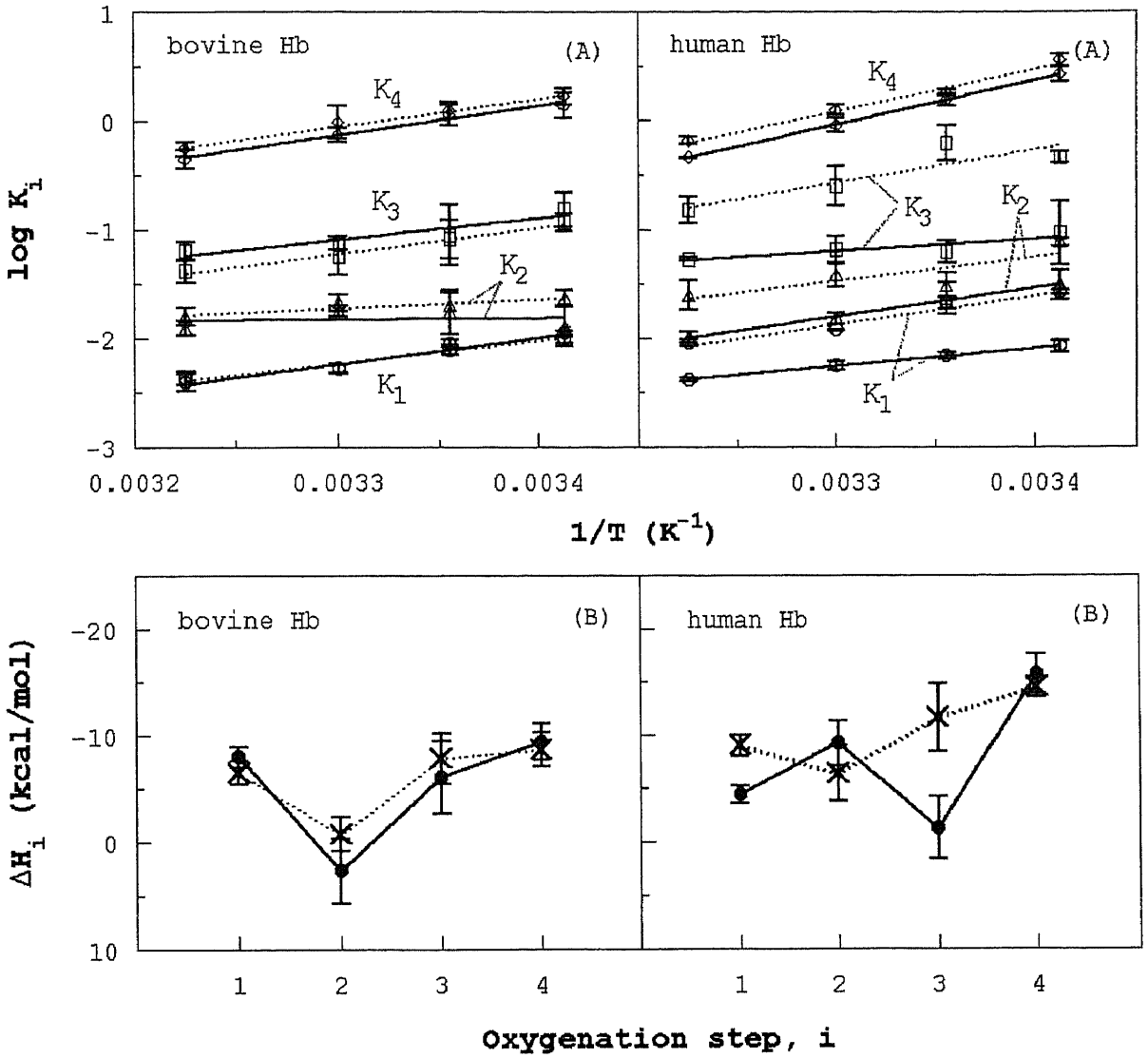

Fig. 13. Effect of temperature on Adair constants (K_i, i = 1 – 4; A) and heats of oxygenation at individual steps (B) of bovine and human Hbs. (A) ○, △, □ and ◇ represent K_1, K_2, K_3 and K_4, respectively. (B) The ΔH_i values were calculated from the Arrhenius plot of estimated Adair constants. Dotted and solid lines represent in the presence of 0.1 M Cl^- (×), and in the presence of physiological concentrations of Cl^- and DPG (●), respectively. Physiological DPG concentrations of bovine and human bloods are 0.5 mM and 5 mM, respectively.

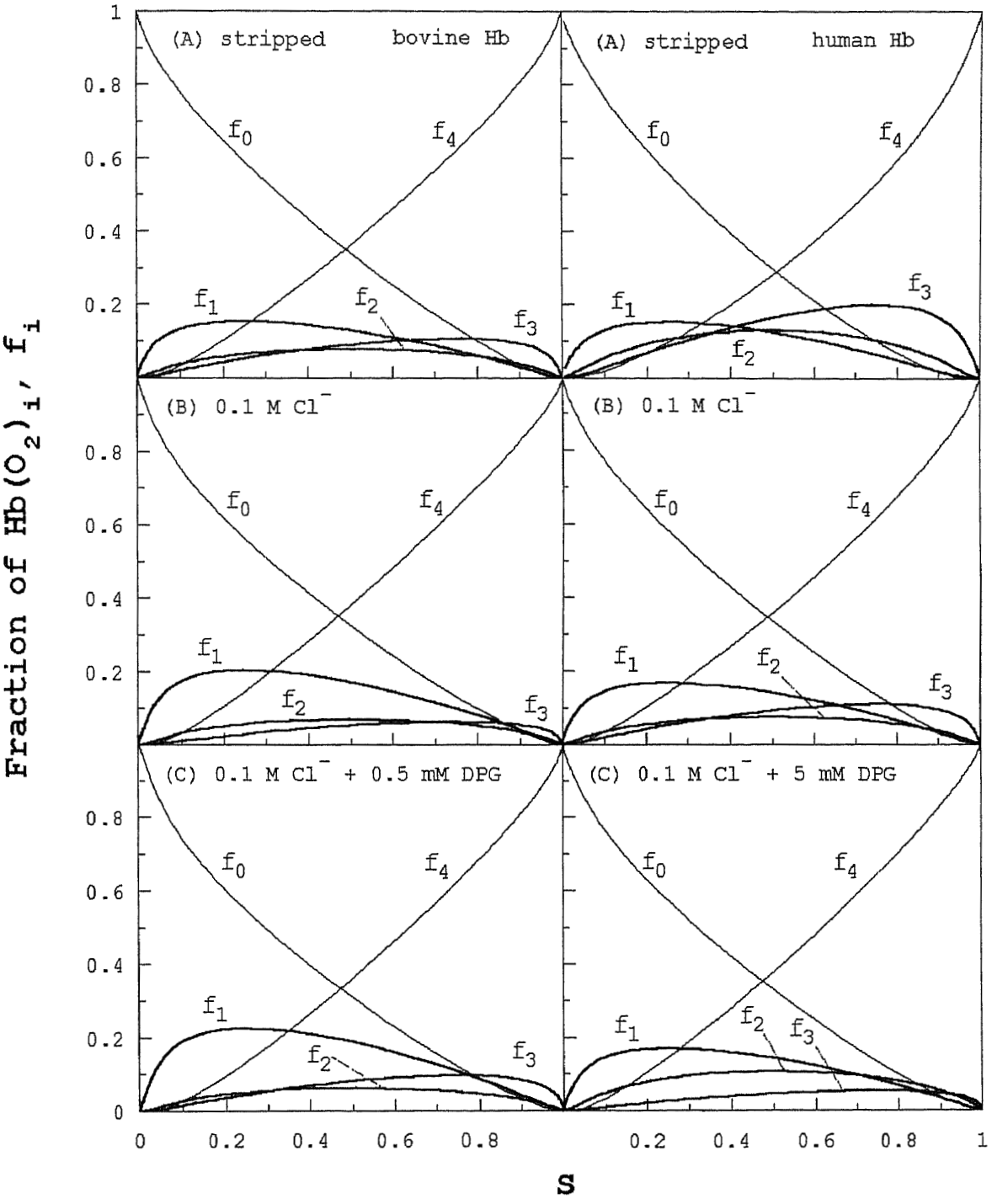

Fig. 14. Fraction population (f_i, i = 0 – 4) of molecules in different oxygenation stages of bovine and human Hbs in the absence of both Cl^- and DPG (stripped) (A), in the presence of 0.1 M Cl^- (B), and in the presence of physiological concentrations of Cl^- and DPG (C). Physiological DPG concentrations of bovine and human bloods are 0.5 mM and 5 mM, respectively.

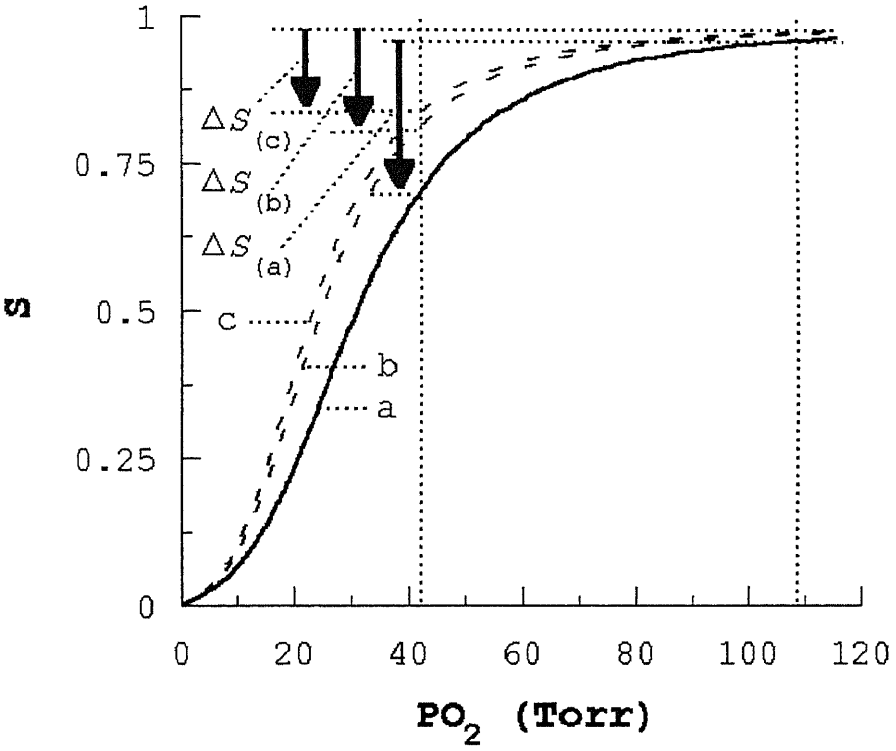

Fig. 15. Example data showing amount of O_2 delivered to the tissues by Hb with ΔH values of –5.8 and –8.1 kcal/mol at peripheral tissues at 32 and 37℃. The OEC data with P_{50} of 31 torr and n_{max} of 2.8 of adult bovine blood measured under standard condition (Faber and Thornburg, 1983) was used to generate OEC with various P_{50} values. O_2 delivered to the tissues was estimated using the theoretical OEC with P_{50} value of 31.0 (a, 37℃), 24.5 (b, 32℃) and 23.1 torr (c, 32℃). The arrows, $\Delta S_{(a)}$, $\Delta S_{(b)}$ and $\Delta S_{(c)}$, represent the arterio-venous O_2 saturation difference, O_2 delivered to the tissues, calculated using the OEC of (a) , (b) and (c), respectively. O_2 pressures of arterial and venous bloods were 108 and 42 torr, respectively (Taylor *et al.*, 1987).

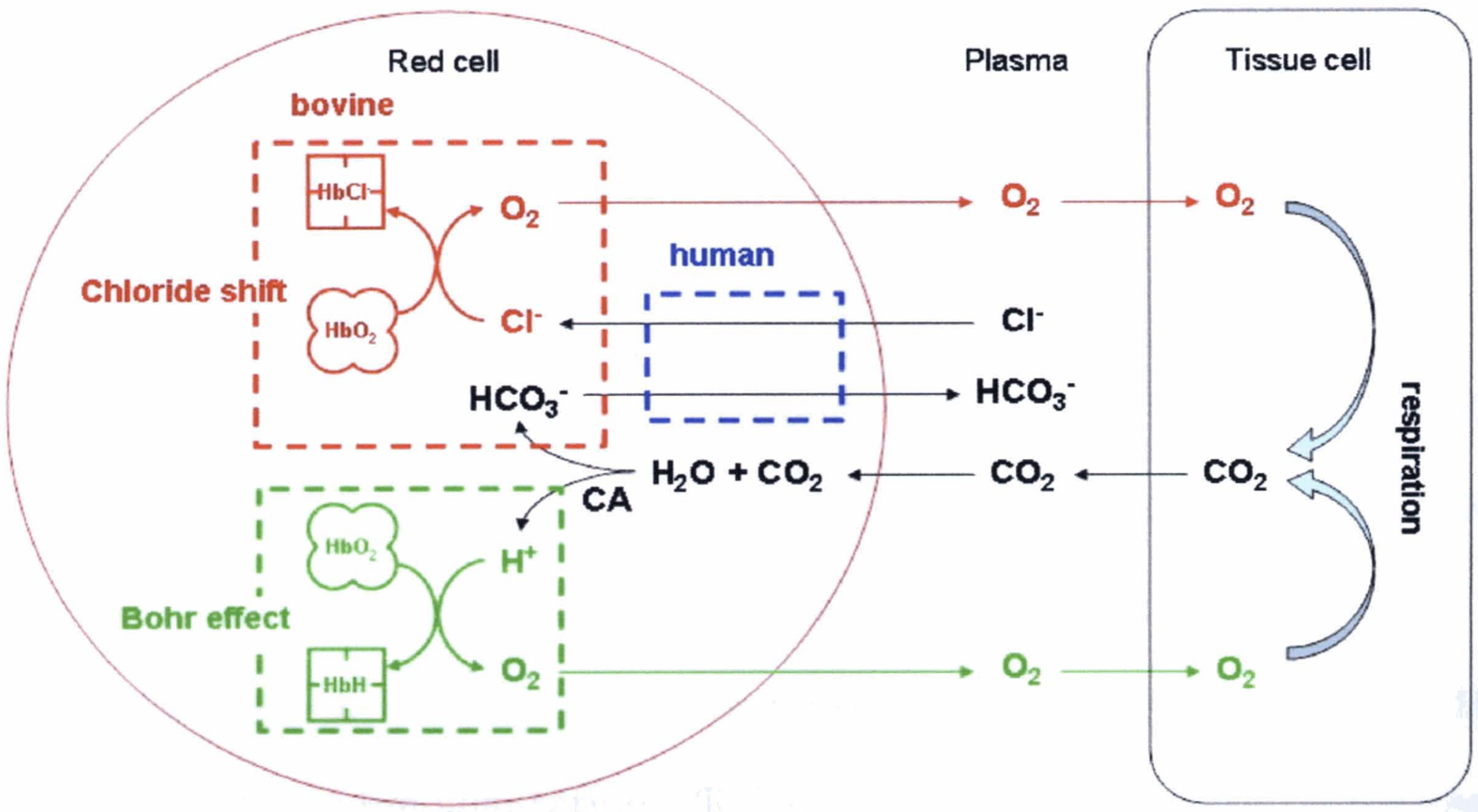

Fig. 16. The unloading of oxygen and the uptake of protons and Cl^- during the circulation of the red cell through tissues.

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