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A METHOD FOR STUDYING OXYGEN DIFFUSION BARRIER IN ERYTHROCYTES: EFFECTS OF HAEMOGLOBIN CONTENT AND MEMBRANE CHOLESTEROL

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SUMMARY

1. In order to study the kinetics of the oxygen egress from human red cells in the 50 sec–20 min time range, an apparatus for measuring the oxygen dissociation process was constructed, combining a spectrophotometer with an oxygen electrode of quick response.

2. Starting from air-saturated haemolysate or red cell suspensions, the velocity of oxygen dissociation from oxyhaemoglobin (V_{diss}) and of oxygen disappearance in the medium (V_{obs}) after addition of bakers' yeast (consuming the dissolved oxygen at the velocity of $V_{consump}$) were recorded. A parameter (r) was defined as the ratio of two velocities, V_{egress} (the velocity of oxygen egress into the medium) and V_{diss},

$$r \equiv V_{egress}/V_{diss} = (V_{consump} - V_{obs})/V_{diss}.$$

$V_{consump}$ could be calculated by the Michaelis–Menten equation as follows,

$$V_{consump} = V_{max} \cdot [O_2]/(K_m + [O_2]),$$

where V_{max} was the maximal velocity of oxygen consumption of bakers' yeast.

3. The r value was always 1·0 for the haemolysate, but it was less than 1·0 for the normal red cells. Further, the oxygen dissociation curve of red cells obtained at higher V_{max} was distorted, due to the non-equilibration between intra- and extracellular oxygen concentrations.

4. The r value was (i) independent of the amounts of the allosteric effectors (2,3-diphosphoglycerate and H^+) but (ii) dependent on the haemoglobin contents and (iii) dependent on the amounts of the membrane cholesterol. Therefore, the r value reflected only the process of the oxygen diffusion but not the 'chemical reaction' rate. The 'barrier' of the oxygen diffusion decreased at lower haemoglobin contents, but increased at higher cholesterol contents in the membrane.

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INTRODUCTION

During the capillary passage time of less than 1 sec, oxygen molecules must be transferred from red cells to the surroundings. There are two sorts of approach for estimating the *in vivo* oxygen egress from red cells. A popular consideration is based on the oxygen equilibrium curve, i.e., the relation between the oxygen tension of the medium and the oxygen saturation (%) of haemoglobin obtained under the slow equilibrium condition. Thus the measurement requires more than 20 min. However, this kind of analysis is not dynamic and may not be valid for the *in vivo* microcirculation.

On the other hand, the kinetic investigation, by means of a rapid mixing apparatus developed by Hartridge & Roughton (1923), observes faster phenomena (within ~ 500 msec) than those occurring *in vivo*. These studies (Gibson, Kreuzer, Meda & Roughton, 1955; Lawson, Holland & Forster, 1965; Lawson, 1966; Sirs, 1966*a*, *b*) have revealed that the rate of deoxygenation of red cells is much slower than that of haemoglobin solution; thus the diffusion process of oxygen must be taken into account. However, it is hard, without mathematical formulation on the basis of appropriate models (Roughton, 1932; Nicolson & Roughton, 1951; Mochizuki & Fukuoka, 1958; Strove, Colton & Smith, 1976), to analyse the rate-determining factors, such as (*a*) the oxygen dissociation process from oxyhaemoglobin ('chemical reaction' modulated by intracellular pH and organic phosphate contents) and (*b*) the oxygen diffusion process within the red cell interior and/or within the red cell membrane ('oxygen diffusion barrier').

There have been some disputes on weighing up these factors, e.g. whether or not the membrane itself is a main factor for the oxygen diffusion barrier (Roughton, 1932; Nicolson & Roughton, 1951; Forster, Roughton, Kreuzer & Briscoe, 1957; Roughton, Forster & Cander, 1957; Carlsen & Comroe, 1958; Kreuzer & Yahr, 1960; Thews, 1963; Forster, 1964; Kutchai & Staub, 1969; Sirs, 1969; Kutchai, 1975; Coin & Olson, 1979). In order to solve these problems experimentally, it seems to be worthwhile to obtain a kinetic parameter, which reflects the 'oxygen diffusion barrier' but is not influenced by the 'chemical reaction'. For this purpose, we try to deal with the oxygen transport kinetics by constructing an apparatus to monitor simultaneously the oxyhaemoglobin dissociation and the oxygen concentration in the medium, combining a spectrophotometer with an oxygen electrode of quick response. The apparatus allows us to analyse the oxygen egress from red cells in the 50 sec–20 min time range for the complete deoxygenation by using the oxygen consumption of bakers' yeast. A parameter, r, is defined operationally as the ratio of two velocities, the velocity of oxygen dissociation from oxyhaemoglobin and that of oxygen egress from red cells. The parameter actually represents the 'oxygen diffusion barrier', regardless of 'chemical reaction' rate.

This paper describes, thus, (i) the construction and characteristics of the apparatus, (ii) the independence of the parameter, r, of intracellular pH and 2,3-diphosphoglycerate which modulate the rates of the 'chemical reaction' and (iii) the influences of the decreased intracellular haemoglobin concentration (decreased internal viscosity) and of the increased membrane cholesterol (decreased membrane fluidity), as the extreme examples modifying the 'oxygen diffusion barrier'. Further, the factors affecting the 'oxygen diffusion barrier' are discussed.

METHODS

Materials

The fresh, heparinized blood was obtained from the healthy non-smoker (K.K.) and kept at 4 °C. Fresh pressed bakers' yeast was purchased from Kanebo Yeast Co. and kept at 4 °C. Adenosine triphosphate (ATP) and 2,3-diphosphoglycerate (2,3-DPG) were obtained from Sigma Chemical Co.; 2,3-DPG was further treated by Dowex 50W-X8 resin to remove cyclohexylammonium ion. The other chemicals were analytical grade.

Two kinds of isotonic buffered saline solution containing 0·1% glucose (as substrate for bakers' yeast) pH 7·4 at 37 °C were used: (*a*) in the absence of CO_2, the solution contained 0·8 mM-KH_2PO_4, 3·3 mM-K_2HPO_4, 7·2 mM-NaH_2PO_4, 36·8 mM-Na_2HPO_4 and 99·4 mM-NaCl (designated as buffer A) and (*b*) in the presence of 40 mmHg CO_2, 0·8 mM-KH_2PO_4, 3·2 mM-K_2HPO_4, 5·0 mM-NaH_2PO_4, 17·6 mM-Na_2HPO_4, 103·1 mM-NaCl and 24·0 mM-$NaHCO_3$ (designated as buffer B). The standard gas mixture, containing 5·55% CO_2 and 20·35% O_2 in pure N_2, was obtained from Seitetsu Kagaku Co.

Preparation of haemolysate

The red cells were washed three times with the buffer A after removing plasma and buffy coat, and resuspended in three volumes of the buffer A. After haemolysis by freezing and thawing, the clear supernatant was obtained by centrifugation (at 15,000 rev/min for 60 min at 4 °C).

Preparation of 2,3-DPG-increased red cells

The intracellular 2,3-DPG content was increased by the method of Deuticke, Duhm & Dierkesmann (1971), i.e. the red cells were washed with 0·9% NaCl and adjusted to haematocrit (Ht) $\simeq$ 45% with saline. The red cell suspension (1·3 ml.) was added to 3·7 ml. of an isotonic solution, containing 2·2 mM-KH_2PO_4, 9·4 mM-K_2HPO_4, 2·5 mM-NaH_2PO_4, 12·9 mM-Na_2HPO_4 94·8 mM-NaCl, 13·2 mM-inosine and 21·4 mM-sodium pyruvate, pH 7·3, and incubated at 37 °C. The 2,3-DPG content was varied by changing the incubation time.

Preparation of partially haemolysed–resealed red cells

Partially haemolysed red cells were prepared by giving hypo-osmotic shock and resealing, as follows. The packed red cells (2·5 ml.) were immersed into 2 mM-ATP solution (4 ml.) for 10 min at 15 °C, then 20 mM-2,3-DPG solution (adjusted to pH 7·4 with 1N-NaOH) (1·3 mM) was added. Three minutes later, the cells were resealed by addition of a hypertonic solution (5 ml.; consisting of 10 mM-2,3-DPG, 2 mM-ATP, 300 mM-KCl, 6 mM-$MgCl_2$, 10 mM-potassium phosphate buffer, pH 7·1), then incubated for 10 min at 37 °C. The resealed red cells were washed with the buffer A twice to remove the haemolysate. Since the resulting red cells were inhomogeneous with respect to the degree of haemolysis, the centrifugal fractionation was carried out in a narrow tube (inner diameter 2 mm, 10 cm long) at 1000 *g* for 10 min at 4 °C. Three layers were collected separately and resuspended into the original plasma. The degree of haemolysis was determined from the mean corpuscular haemoglobin concentration.

Preparation of cholesterol-depleted and -loaded red cells

The washed red cells were suspended in the buffer A, and incubated with phospholipid vesicles, containing various amount of cholesterol, in the presence of heated plasma for 16 hr at 37 °C, according to Cooper, Leslie, Fischkoff, Shinitzky & Shattil (1978). The vesicles were composed of synthetic dipalmitoyl-phosphatidylcholine (Sigma) and cholesterol (Sigma), in which the cholesterol/phospholipid molar ratio was varied from 0·46 to 2·28. After incubation, the red cells were washed with the buffer three times to remove the vesicles.

Quantitative methods

The haemoglobin concentration was determined by the CN-methaemoglobin method (van Kampen & Zijlstra, 1970), 2,3-DPG by the method of Maeda, Chang, Benesch & Benesch (1971), adenylates by liquid chromatography (using a Hitachi model 634 A, with an anion exchange

resin, Hitachi no. 2634). The amount of membrane lipids was determined for the $CHCl_3$:methanol (2:1 by vol.) extracts (Ways & Hanahan, 1964) from red cells washed with isotonic Tris-saline buffer (pH 7·4) three times; cholesterol was determined by gas chromatography (using a Hitachi model 163, with a 2 m long OV-17 glass column and flame ionization detector) (Ikegawa, Matsui & Sato, 1971), and phospholipids were quantified by phosphate assay (Bartlett, 1959).

The intracellular pH was measured by a Radiometer PHM-64 with a capillary electrode, after haemolysing the packed red cells by freezing and thawing (Enoki, Tomita, Maeda, Kawase & Okuda, 1972). The red cell count, the mean corpuscular volume (m.c.v.) and the mean corpuscular haemoglobin (m.c.h.) were calculated by the standard method (Wintrobe, 1967).

The solubility of oxygen in the buffer A at 37 °C was determined by the method of Robinson & Cooper (1970), and the Bunsen coefficient was 0·023 ml./ml. per atm. The oxygen concentrations were calculated using the value throughout.

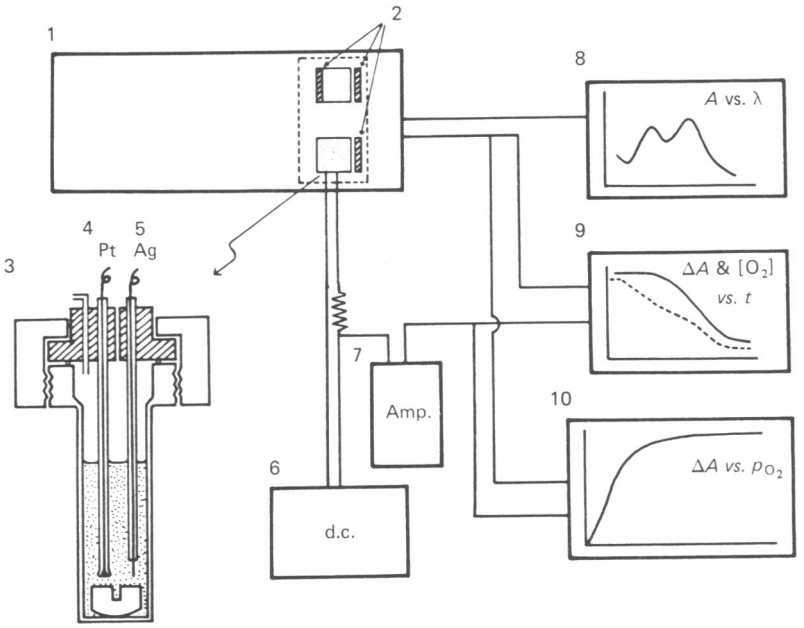

Fig. 1. Block diagram of the apparatus. 1, double beam spectrophotometer; 2, opal glass plates; 3, airtight cell; 4, Pt electrode (cathode); 5, Ag electrode (anode); 6 d.c. power supply; 7, amplifier for electrode current; 8, X–Y recorder for absorption spectrum; 9, two-pen recorder for measuring the time courses of electrode current and absorption changes; 10, X–Y recorder for oxygen dissociation curve.

Construction of apparatus and experimental procedures

Apparatus. The block diagram of the apparatus for recording the oxygen dissociation process of haemoglobin is shown in Fig. 1. The design was essentially similar to one described by Imai, Morimoto, Kotani, Watari, Hirata & Kuroda (1970), except for (*a*) the use of a home-made oxygen electrode of quick response, instead of a Clark-type electrode, (*b*) the use of opal glass for recording the deoxygenation process of the red cell suspensions and (*c*) the use of bakers' yeast as an oxygen consuming reagent.

(i) A double-beam spectrophotometer (Union Giken, SM-401) was used for monitoring the change of absorbance. An opal glass plate was placed as shown in Fig. 1, in order to cancel the considerable light scattering effect due to red cells and bakers' yeast (Shibata, 1959), and furthermore the reference beam was appropriately reduced by inserting an opal glass plate.

(ii) A home-made oxygen electrode (a set of Pt and Ag electrodes) was mounted to the airtight screw cap on the cuvette (1 × 1 cm). A polarizing voltage of −0·7 V was supplied by a

mercury battery and the polarographic current was amplified by an amplifier (Ohkura Electrical Co., AM-1001) as a DC voltage.

(iii) The reaction mixture in the cuvette was stirred at a constant rate by a spin bar (Cell Stirrer, F-37,150, Bel Art Co.), and a magnetic stirrer (Acrobat Stirrer, M.S. Co.) was placed under the thermostatically controlled cell holder. The temperature was maintained at 37 °C by circulating water from a thermostatically controlled bath.

Further details are described in the Appendix.

Procedures. The red cells were diluted with 3 ml. of the pre-warmed buffer A or B (37 °C) in the cuvette so as to give a haem concentration of about 0·1 mM. The red cell suspension in the cuvette was incubated for 5 min at 37 °C for temperature equilibration. An appropriate amount

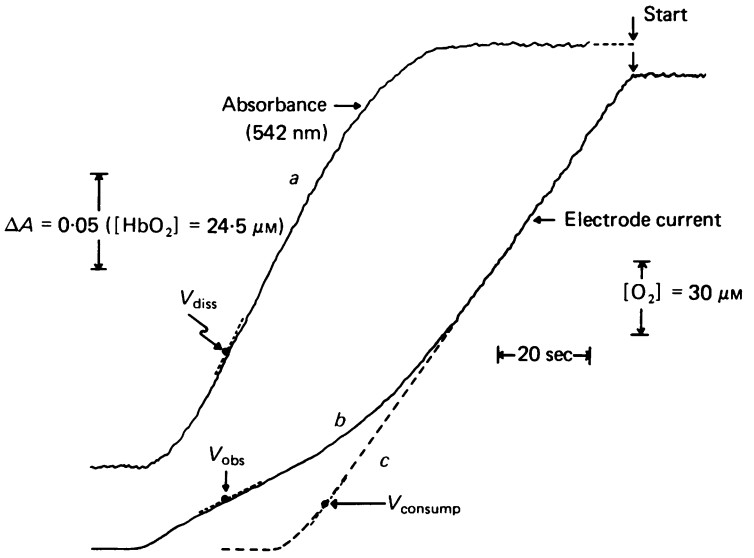

Fig. 2. Time courses of electrode current and absorption (at 542 nm) changes of red cell suspensions. The arrows marked under 'Start' indicate the time when yeast was added. Trace *a*, the absorption change of haemoglobin; trace *b*, the electrode current in the presence of oxyhaemoglobin (either in red cells or haemolysate); trace *c*, the calculated time course of electrode current in the absence of haemoglobin.

of bakers' yeast suspension was added by a microsyringe, through a small hole in the centre of the screw cap. After addition of bakers' yeast, the absorption change of haemoglobin at 542 nm and the current change of the oxygen electrode were recorded by a two-pen recorder (Watanabe Sokki Co., SR-652Z) for measuring the time courses and by a X–Y recorder (Watanabe Sokki Co., WX-440) for drawing the dissociation curve, simultaneously. In addition, the absorption spectrum could be drawn on another X–Y recorder (Watanabe Sokki Co., WX-442) before each run for the determination of the haemoglobin concentration.

The typical recorder charts are shown in Fig. 2. In the absence of haemoglobin, oxygen dissolved in the buffer mixture was consumed by bakers' yeast (dotted line *c*). However, in the presence of oxyhaemoglobin (either in red cells or haemolysate), oxygen dissociated from oxyhaemoglobin (trace *a*) egressed into the medium, as the oxygen concentration in the medium decreased. Therefore, the electrode current (trace *b*) became higher than that in the absence of haemoglobin (dotted line *c*).

Analytical procedures and calculation of the parameter, r

The parameter, r, was defined as the ratio of two velocities at time t, i.e. the velocity of oxygen dissociation from oxyhaemoglobin (V_{diss}) and that of the net egress of oxygen from red cells (or from oxyhaemoglobin in haemolysate) to the medium (V_{egress}). These velocities were

computed by a digital computer (Hitachi, HITAC 10-II). The recorder readings (the absorbance at 542 nm and the electrode current) at every 2·5 sec were fed into a computer and converted to the degree of haemoglobin oxygenation (S_t, $0 \leqq S_t \leqq 1$) and to the oxygen concentration in the medium ($[O_2]_t$), respectively. Then these values were fitted to the third order curve by regression of seven points and velocities were calculated (*vide infra*).

(i) The apparent absorbance at 542 nm at time t, A_t, was converted to S_t, as follows. From A_{deoxy} (absorbance at $pO_2 = 0$, reached by yeast; i.e. $S = 0$) and A_{oxy} (absorbance at $pO_2 =$ 1 atm; see Appendix),

$$S_t = (A_t - A_{deoxy})/(A_{oxy} - A_{deoxy}).$$

The velocity of oxygen dissociation (V_{diss}, in μM/sec) could be computed as

$$V_{diss} = [Hb_4]_{total} \cdot (dS/dt).$$

where $[Hb_4]_{total}$ was the total haem concentration in the cuvette.

(ii) The velocity of the net egress of oxygen (V_{egress}) was calculated from changes of oxygen concentrations in the presence and absence of oxyhaemoglobin (either in red cells or haemolysate), as follows:

(*a*) The electrode current at time t, I_t, was converted to the oxygen concentration in the medium, $[O_2]_t$ by the equation

$$[O_2]_t = [O_2]_{initial} \cdot (I_t - I_{final})/(I_{initial} - I_{final}),$$

where $I_{initial}$ and I_{final} were the electrode currents at the initial (in the air-saturated condition) and final stages ($pO_2 = 0$), respectively, and $[O_2]_{initial}$ was the oxygen concentration of air-saturated buffer medium. V_{obs} could be computed as $V_{obs} = d[O_2]/dt$.

(*b*) V_{egress} (μM/sec) could be expressed by the difference between two velocities, i.e. the decrease in velocity of oxygen concentration in the presence of oxyhaemoglobin (either in red cells or haemolysate), V_{obs} (μM/sec) and that in the absence of haemoglobin, $V_{consump}$ (μM/sec) (*vide infra*), at the same oxygen concentration:

$$V_{egress} = V_{consump} - V_{obs}.$$

V_{egress} should be equal to V_{diss} for haemolysate, but was not necessarily equal to V_{diss} for red cell suspensions because of the 'oxygen diffusion barrier'.

(*c*) $V_{consump}$ could be determined in a separate experiment without haemoglobin, by adding the same amount of bakers' yeast into the same volume of the buffer medium. It was, however, for the purposes of the present study, calculated by the Michaelis–Menten equation, i.e.,

$$V_{consump} = V_{max} \cdot [O_2]/(K_m + [O_2]),$$

where V_{max} was maximal velocity of the oxygen consumption by bakers' yeast and K_m was the Michaelis constant for oxygen, 1·1 μM in average. V_{max} could be practically approximated by the velocity of oxygen consumption for each run, at higher oxygen concentration (e.g. at $[O_2] \geqslant$ 120 μM, which was more than 100 times of K_m) because of no detectable oxygen egress from red cells (or oxyhaemoglobin in haemolysate) during the initial stage. Actually, this operational estimate of V_{max} did not give an error of more than 1 %.

(iii) Finally, the parameter, r, was operationally defined as

$$r = V_{egress}/V_{diss}.$$

Here, the amount of dissolved oxygen in the red cell could be neglected, because it was about 1 % of that of haemoglobin-bound oxygen at 37 °C in the air-saturated condition (Singer, 1971). So, the parameter, r, represented the molar ratio of oxygen released from red cells into the medium to oxygen dissociated from oxyhaemoglobin during infinitesimal time.

RESULTS

Measurement of the oxygen dissociation curve

(i) *Haemolysate*

A typical oxygen dissociation curve of the haemolysate, obtained under the slow oxygen consumption velocity (V_{max} = 0·556 μM/sec) at pH 7·4 at 18 °C (pCO_2 = 0 mmHg), agreed perfectly with that measured by the standard spectrophotometric method (pCO_2 = 0 mmHg) using a tonometer as described by Allen, Guthe & Wyman (1950).

TABLE 1. The oxygen dissociation curve of the red cell suspension

Method ...	Present method[a]				
pCO_2 (mmHg) ...	0			40	
V_{max} (μM/sec)[c] ...	0·29	1·07	2·48	0·28	Reported
Number of experiments ...	9	4	7	8	value[b]
Oxygen tension (mmHg)[d]					
at 2·5% oxygenation	4·1 ± 0·3	3·7 ± 0·4	2·7 ± 0·2	—	—
5%	6·5 ± 0·3	5·9 ± 0·2	5·0 ± 0·4	—	—
10%	9·7 ± 0·3	8·7 ± 0·4	8·1 ± 0·5	10·7 ± 0·4	10·3
20%	14·3 ± 0·3	14·2 ± 0·3	12·8 ± 0·4	15·6 ± 0·5	15·4
30%	18·1 ± 0·5	17·9 ± 0·3	16·4 ± 0·7	19·4 ± 0·6	19·6
40%	21·4 ± 0·5	21·1 ± 0·3	20·0 ± 0·7	22·7 ± 0·6	22·8
50%	25·0 ± 0·4	24·8 ± 0·4	23·8 ± 0·4	26·6 ± 0·5	26·6
60%	28·9 ± 0·6	29·2 ± 0·3	27·9 ± 0·7	30·9 ± 0·7	31·3
70%	34·0 ± 0·8	34·0 ± 1·5	33·1 ± 0·8	36·3 ± 0·6	36·9
80%	40·8 ± 1·4	41·2 ± 0·5	40·8 ± 1·3	43·6 ± 0·7	44·5
90%	54·1 ± 1·0	53·7 ± 0·8	53·8 ± 0·7	57·5 ± 0·7	57·8
Hill's coefficient[d]	2·77 ± 0·02	2·77 ± 0·01[e]	2·74 ± 0·02[e]	2·78 ± 0·02	2·77

[a] In the absence of CO_2, buffer A was used, and in the presence of CO_2, buffer B was used.
[b] From Severinghaus (1966).
[c] The maximum velocity of oxygen consumption induced by bakers' yeast.
[d] Mean ± S.D.
[e] Hill's coefficient was the apparent value, since the oxygen dissociation curve was slightly distorted.

(ii) *Fresh red cells*

The oxygen dissociation curves of the fresh red cell suspensions obtained in the various conditions at pH 7·4 at 37 °C are summarized in Table 1. V_{max} and hence the time required for the complete deoxygenation was easily controlled by changing the amount of bakers' yeast added to the red cell suspensions. The curve obtained at higher V_{max} (e.g. V_{max} 2·5 μM/sec) was distorted, in particular at lower degrees of oxygenation (< 50%), as compared with that obtained at lower V_{max}. At 40 mmHg CO_2, the curve obtained at lower V_{max} agreed with that described by Severinghaus (1966), with respect both to the oxygen tensions at various haemoglobin oxygenations and to Hill's coefficient.

Measurement of the parameter, r

(i) *Accuracy of the parameter, r*

It would be desirable to determine the r values at all oxygenation ranges of haemoglobin. However, V_{diss} and V_{egress} decreased at the higher and lower degrees of haemoglobin oxygenation, as shown in Fig. 3. At the higher degrees, both velocities were so small that differentiation between V_{obs} and $V_{consump}$ was very difficult. Also, at the lower degrees, the errors for the estimation of $V_{consump}$ became large, and V_{diss} and V_{obs} were very small. In the range of 40–60 % oxygenation, V_{diss} was the largest, thus the measurement of V_{diss} was more accurate and the deviation of

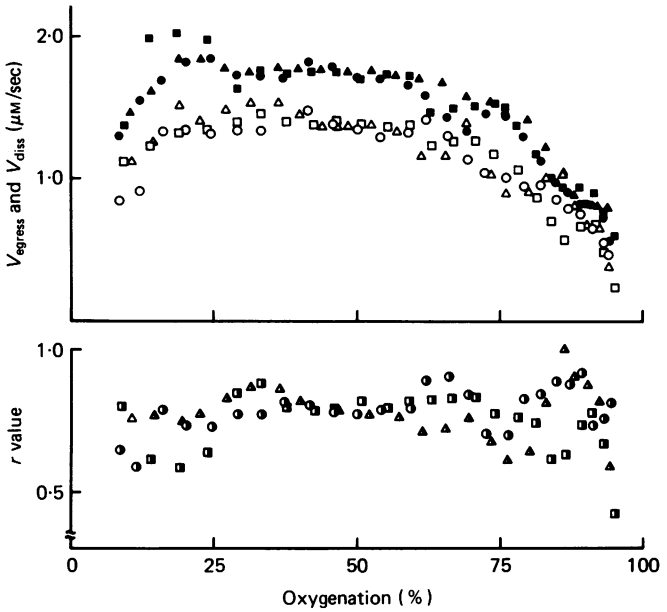

Fig. 3. Deviations of V_{diss}, V_{egress} and the r value. Top: V_{diss} (filled symbols) and V_{egress} (open symbols) *vs.* % oxygenation of haemoglobin. Bottom: The r value (V_{egress}/V_{diss}) *vs.* % oxygenation of haemoglobin. The results from three separate experiments, obtained in the same conditions in buffer A at 37 °C, are summarized by the different symbols. Haem concentration, 102 μM; V_{max}, 2·346 μM/sec.

the r value became minimal. In addition, the r values were constant in these regions. Therefore the r value obtained in the 40–60 % oxygenation range was shown throughout and the standard deviation of the r value, as well as V_{diss} and V_{egress}, was within 3 %.

(ii) *Comparison of the r value for red cells and haemolysate*

In order to verify the meaning of the r value, the two velocities, V_{diss} and V_{egress}, and the r values were compared for both haemolysate and red cells at various values of V_{max}. As shown in Fig. 4, V_{diss} and V_{egress} for haemolysate coincided regardless of V_{max}: thus the r values were always unity. On the other hand, V_{diss} for red cells was greater than V_{egress} except for those at lower V_{max}, and the r values

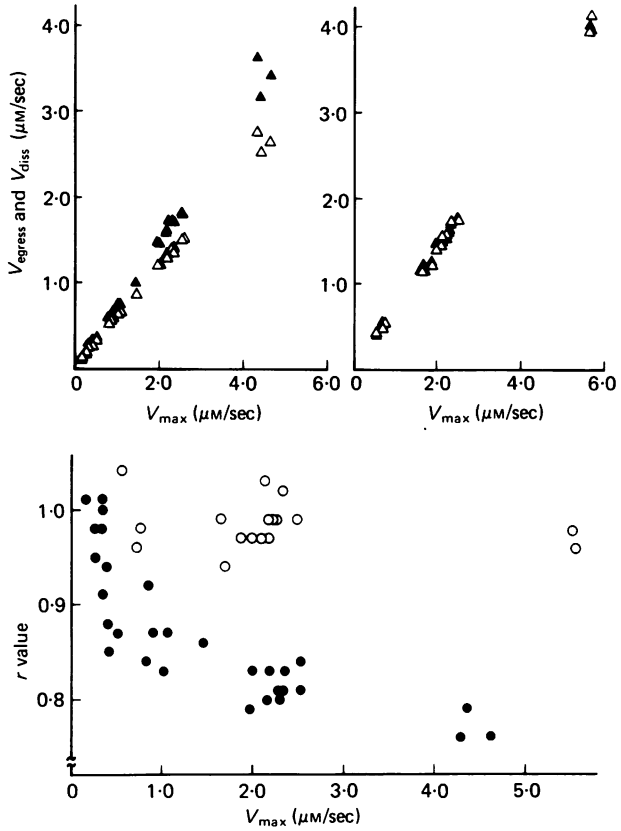

Fig. 4. Comparison of the r value and the constituent velocities obtained for red cells and haemolysate. Top: V_{diss} (▲) and V_{egress} (△) *vs.* V_{max}, obtained for red cells (left) and haemolysate (right). Bottom: the r value *vs.* V_{max}, obtained for red cells (●) and haemolysate (○). Velocity of oxygen consumption in the abscissa represents V_{max}. Measured in buffer A at 37 °C. P_{50} (mmHg) and the Hill's coefficient (n) were $24{\cdot}6 \pm 0{\cdot}7$ and $2{\cdot}75 \pm 0{\cdot}02$ for red cells, and were $19{\cdot}7 \pm 0{\cdot}5$ and $2{\cdot}68 \pm 0{\cdot}03$ for haemolysate, respectively. The haem concentrations for red cell experiments and for haemolysate were 98·9 ~ 105·3 μM and 97·3 μM, respectively.

were dependent on V_{max} ($r \simeq 0{\cdot}8$ at $V_{max} \simeq 2{\cdot}0$–$2{\cdot}5$ μM/sec). As V_{max} (dependent on amounts of bakers' yeast added) increased, the r values decreased correspondingly to the appearance of the distortion of the oxygen dissociation curve. However, when V_{max} was decreased, the r value approached unity.

(iii) *Effect of intracellular 2,3-DPG and pH*

As is well known, changes of 2,3-DPG content or intracellular pH affect the oxygen dissociation curve of red cells by modulating the on and off rate constants between haemoglobin and oxygen (Salhany, Eliot & Mizukami, 1970; Bauer, Klocke, Kamp & Forster, 1973). However, the parameter, r, was scarcely affected at higher V_{max} ($V_{max} \simeq 2{\cdot}0$–$2{\cdot}5$ μM/sec), in spite of remarkable shift of the oxygen dissociation curves (Table 2).

With increasing 2,3-DPG, P_{50} shifted from 26·8 mmHg to 37·2 mmHg, but the r values were unchanged ($r \simeq 0{\cdot}8$ at $V_{max} \simeq 2{\cdot}0$–$2{\cdot}5$ μM/sec), although V_{diss} and V_{egress} were decreased (V_{diss}: from 1·62 to 1·50 μM/sec, V_{egress}: from 1·35 to 1·20 μM/sec, at $V_{consump} = 2{\cdot}31$ μM/sec, $0{\cdot}4 < S < 0{\cdot}6$). Similarly, when pH was raised, P_{50} shifted from 23·1 to 16·5 mmHg, but r values were unchanged ($r \simeq 0{\cdot}8$ at $V_{max} \simeq 2{\cdot}0$–$2{\cdot}5$ μM/sec), in spite of increase in V_{diss} and V_{egress} (V_{diss}: from 1·75 to 1·85 μM/sec, V_{egress}: from 1·40 to 1·51 μM/sec, at $V_{consump} = 2{\cdot}31$ μM/sec, $0{\cdot}4 < S < 0{\cdot}6$).

TABLE 2. Effect of pH and 2,3-DPG on the r value

	pH varied[a]			2,3-DPG varied[b]		
Experiment no. ...	1	2	3	4	5	6
Haemoglobin($\times 10^{-3}$ mole/l..cell)	5·48	5·45	5·35	5·66	5·56	5·67
2,3-DPG($\times 10^{-3}$ mole/l..cell)	5·60	5·23	5·22	6·04	8·50	13·30
2,3-DPG/haemoglobin (molar ratio)	1·02	0·96	0·98	1·07	1·58	2·31
P_{50} (mmHg)[c]	16·5	19·7	23·9	26·8	33·0	38·0
Hill's coefficient	2·65	2·70	2·77	2·77	2·80	2·79
Number of experiments ...	5	7	5	4	5	4
r value ± S.D.[d]	0·83 ± 0·02	0·81 ± 0·02	0·79 ± 0·03	0·81 ± 0·02	0·81 ± 0·04	0·80 ± 0·03
Apparent P_{50} ± S.D.(mmHg)[d]	16·0 ± 0·3	19·3 ± 0·5	23·1 ± 0·3	26·5 ± 0·4	32·2 ± 0·6	37·3 ± 0·4
Apparent Hill's coefficient ± S.D.[d]	2·61 ± 0·02	2·67 ± 0·02	2·74 ± 0·03	2·75 ± 0·02	2·77 ± 0·02	2·77 ± 0·02
Extracellular pH[e]	7·81	7·65	7·44	7·38	7·37	7·37
Intracellular pH[f]	7·53	7·37	7·21	7·08	7·05	6·90

[a] The pH of the medium was varied by changing the pH of buffer A, and the suspension was incubated for 10 min at 37 °C before measurement.

[b] The 2,3-DPG content was varied by incubating the red cells in the inosine-pyruvate-phosphate medium for 25 min (expt. no. 4), 45 min (expt. no. 5) and 85 min (expt. no. 6) (see Methods). After incubation, the red cells were washed with buffer A three times.

[c] P_{50} (pO_2 at half-oxygenation of haemoglobin) was measured in the equilibrium condition ($V_{max} \simeq 0{\cdot}3$ μM/sec).

[d] Measured in buffer A. The r value, the apparent P_{50} and Hill's coefficient were determined at higher V_{max} ($V_{max} \simeq 2{\cdot}0$–$2{\cdot}5$ μM/sec); the oxygen dissociation curve was slightly distorted.

[e] pH of the buffer A used for the measurement at 37 °C.

[f] Measured by the method of Enoki *et al.* (1972), at 37 °C.

The relation between P_{50} and the observed velocities is shown in Fig. 5, in which V_{diss}/V_{obs} and V_{egress}/V_{obs} were plotted for normalization (see Discussion).

(iv) *Effect of intracellular haemoglobin content*

The results obtained with the cells partially haemolysed to different degrees are summarized in Table 3. The r values changed from 0·8 to 0·9 (at $V_{max} \simeq 2{\cdot}0$–$2{\cdot}5$ μM/sec), depending on the intracellular haemoglobin content. The plot of haemoglobin content *vs.* average r values clearly showed that the r value approached 1·0 as the intracellular haemoglobin content decreased at the same V_{max} range. The P_{50} of these samples varied slightly but not systematically, probably due to the changes

of the amounts of the allosteric effectors. The mean corpuscular volumes also differed from sample to sample, but no relation to the r values was observed within these variations. When haemoglobin content was low (e.g. the mean corpuscular haemoglobin was about 14 pg/cell), slight haemolysis (less than 4%) occurred during the measurement, but this did not affect the P_{50} and r values.

TABLE 3. Effect of intracellular haemoglobin content on the r value

Experiment no. ...	1	2	3	4	5
Haemoglobin($\times 10^{-3}$ mole/l..cell)	5·43	3·96	3·60	3·19	2·45
M.c.v. (μm^3)[a]	88·9	77·7	76·2	85·7	88·8
M.c.h. (pg/cell)[b]	31·2	19·9	17·7	17·6	14·1
	(100%)	(63·7%)	(56·7%)	(56·5%)	(45·1%)
2,3-DPG($\times 10^{-3}$ mole/l..cell)[c]	5·23	6·05	7·54	5·66	5·00
P_{50} (mmHg)[d]	27·2	26·8	26·9	24·8	24·0
Hill's coefficient	2·77	2·78	2·78	2·75	2·75
Number of experiments	9	5	7	5	10
r values ± S.D.[e]	0·81	0·81	0·87	0·84	0·92
	± 0·03	± 0·03	± 0·03	± 0·03	± 0·05
Apparent P_{50} ± S.D.(mmHg)[e]	25·6	26·5	26·5	23·9	23·2
	± 0·9	± 0·5	± 0·7	± 0·9	± 0·6
Apparent Hill's coefficient ± S.D.[e]	2·75	2·75	2·77	2·74	2·73
	± 0·02	± 0·02	± 0·02	± 0·01	± 0·02
Extracellular pH[f]	7·39	7·38	7·40	—	7·37
Intracellular pH[g]	7·15	7·11	7·11	—	7·10

[a] Mean corpuscular volume.

[b] Mean corpuscular haemoglobin.

[c] In addition, the concentration of ATP ranged from 0·96 to 1·42 mmoles/l..cell in this experiment.

[d] P_{50} was measured at the equilibrium condition ($V_{max} \simeq 0{\cdot}3$ μM/sec).

[e] Measured in the buffer A. The r value, the apparent P_{50} and Hill's coefficient were determined at higher V_{max} ($V_{max} \simeq 2{\cdot}0$–$2{\cdot}5$ μM/sec); the oxygen dissociation curve was slightly distorted.

[f] pH of the buffer A used for the measurement at 37 °C.

[g] Measured by the method of Enoki *et al.* (1972), at 37 °C.

(v) *Effect of membrane cholesterol content*

As summarized in Table 4, the r value was dependent on the cholesterol/phospholipid molar ratio of the red cell membrane. For the cholesterol-loaded cells, the r value decreased to *ca.* 0·6 (at $V_{max} \simeq 2{\cdot}0$–$2{\cdot}5$ μM/sec). Though the cell volume tended to increase during the preparation, the r values were independent of the mean corpuscular volume within these variations. Also, the small variations of the intracellular ATP and 2,3-DPG contents shifted P_{50} in some extent, but the r values should not be affected as described above.

TABLE 4. Effect of the membrane cholesterol on the r value

Series ...	Cholesterol-depleted or -loaded cells									Fresh cells
	A[a]						B[b]			
Experiment no. ...	1	2	3	4	5	6	7	8	9	10
Cholesterol($\times 10^{-16}$ mole/cell)[c]	2·70	2·90	3·15	4·84	6·96	7·47	2·49	3·00	5·84	3·26
Phospholipids($\times 10^{-16}$ mole/cell)[c]	4·06	3·50	3·14	3·73	3·86	3·76	3·18	3·34	3·07	3·81
Cholesterol/phospholipids (molar)	0·67	0·83	1·00	1·30	1·80	1·99	0·78	0·90	1·90	0·86
M.c.v. (μm^3)	97	114	101	110	97	101	112	104	120	104
2,3-DPG($\times 10^{-3}$ mole/l..cell)[d]	5·80	5·85	5·85	5·98	5·90	6·14	3·92	3·60	3·35	5·17
P_{50}(mmHg)[e]	31·2	29·3	31·5	30·3	30·3	31·4	26·5	25·8	25·4	25·2
Hill's coefficient	2·78	2·77	2·77	2·79	2·78	2·80	2·78	2·78	2·76	2·77
Number of experiments	8	6	3	7	9	5	6	4	8	14
r value[f]	0·76	0·74	0·76	0·68	0·67	0·62	0·80	0·78	0·67	0·79
(± S.D.)	(0·04)	(0·04)	(0·05)	(0·04)	(0·06)	(0·04)	(0·04)	(0·04)	(0·06)	(0·04)
Apparent P_{50} (mmHg)[f]	29·9	28·8	31·5	29·0	28·9	30·1	26·0	25·6	24·5	24·4
(± S.D.)	(0·4)	(0·3)	(0·6)	(0·6)	(0·8)	(0·4)	(0·4)	(0·9)	(0·6)	(0·6)
Apparent Hill's coefficient[f]	2·77	2·76	2·75	2·76	2·75	2·77	2·78	2·75	2·75	2·77
(± S.D.)	(0·02)	(0·02)	(0·03)	(0·01)	(0·02)	(0·03)	(0·03)	(0·03)	(0·01)	(0·01)
Extracellular pH[g]	7·37	7·38	7·37	7·38	7·38	7·38	—	—	—	7·37
Intracellular pH[h]	7·04	7·07	7·04	7·07	7·06	7·10	—	—	—	7·16

[a] Buffer A was used for the preparation (see Methods).

[b] Isotonic Tris-saline (33 mM-Tris HCl, 130 mM-NaCl and 5 mM-glucose, pH 7·4 at 20 °C) was used for preparation of the red cells. In this series, the red cell sample was obtained from a different donor.

[c] For the quantitative analyses of cholesterol and phospholipids, the red cells were washed with isotonic Tris-saline buffer prior to the lipid extraction.

[d] In addition, the concentration of ATP ranged from 1·40 to 1·65 m-mole/l..cell in this experiment.

[e] P_{50} was measured in equilibrium ($V_{max} \simeq 0{\cdot}3$ μM/sec).

[f] Measured in buffer A. The r value, the apparent P_{50} and Hill's coefficient were determined at higher V_{max} ($V_{max} \simeq 2{\cdot}0$–$2{\cdot}5$ μM/sec); the oxygen dissociation curve was slightly distorted.

[g] pH of buffer A used for the measurement at 37 °C.

[h] Measured by the method of Enoki *et al.* (1972) at 37 °C.

DISCUSSION

Characteristics of the apparatus

The present apparatus was essentially similar to that of Imai *et al.* (1970), in the sense that the change of the haemoglobin absorbance and the current of the oxygen electrode were recorded simultaneously. In addition to the oxygen dissociation curve, the time courses of the phenomena were recorded simultaneously. However, the following points were modified in pursuance of our intention. (*a*) The essential feature of the platinum electrode, coated with cellulose acetate, has been reported recently by Hagiwara, Ishibashi, Sasaki & Kamigawara (1978). Our cathodic platinum electrode was designed to obtain quick response (time constant was less than 1·5 sec), though the noises became greater compared with those coated with a thick membrane or covered with a Teflon membrane. Therefore, it could follow the time course in 50 sec for the complete deoxygenation. (*b*) The light scattering effect due to red cells and bakers' yeast could be cancelled by means of opal glass plates. Although the absorbance/mole haemoglobin was inevitably reduced due to the flattering effect (Duysens, 1956), the linearity between the difference in absorbance and the haemoglobin concentration was assured. (*c*) The use of bakers' yeast, as a consuming agent of oxygen, was particularly beneficial in controlling the velocity of oxygen consumption by varying its amount. Beef heart muscle particles have been used by Colman & Longmuir (1963), but bakers' yeast seems to be superior, because of the simplicity of the preparation and of the reproducibility of the activity. The technical details of these points are described in the Appendix.

As a whole, the apparatus demonstrated reasonable accuracy as shown in Table 1, i.e. a perfect oxygen dissociation curve could be obtained in the condition of slow deoxygenation at pH 7·4 in the presence of CO_2, and coincided with those of Severinghaus (1966).

Rate processes

According to the rapid mixing experiments, it has been recognized that the half-time of oxygen dissociation from oxyhaemoglobin in the red cell ($\simeq$200 msec) is far slower than that of haemoglobin solution ($\simeq$16 msec) at about 20 °C. On the basis of the mathematical treatment (Roughton, 1932; Nicolson & Roughton, 1951), the 'diffusion barrier' for oxygen molecules in the red cell has been considered. Although the present technique required more than 50 sec for the complete deoxygenation, a difference of oxygen egress between red cells and haemolysate was clearly observed.

In the case of haemolysate, the velocity of oxygen dissociation from oxyhaemoglobin should be equal to that of increase of oxygen concentration in the medium, i.e. no diffusion barrier existed; thus the r value was unity. For the oxygen egress from red cells, however, the following processes should be taken into account: (*a*) the deoxygenation of oxyhaemoglobin in red cells, (*b*) the recombination of oxygen to deoxyhaemoglobin in red cells, (*c*) the diffusion of oxygen inside red cells and/or within the cell membrane, and probably (*d*) the molecular motion of haemoglobin itself in red cells. Basically, the present method is intermediate between the oxygen equilibrium measurement and the rapid mixing method, with respect to the decreasing velocity of oxygen in the medium. The experimental evidence suggested that the

r value was independent of the rate of the deoxygenation–reoxygenation reaction ('chemical reaction') but highly influenced by the diffusion processes.

(i) It is empirically known that the oxygen dissociation curve must be measured slowly, otherwise the curve may be distorted because of non-equilibration between the intra- and extracellular oxygen concentrations (*vide supra*, and Table 1). At lower V_{max}, the intra- and extracellular concentrations should approach equality, because the diffusion process of oxygen was not the rate-limiting step any more.

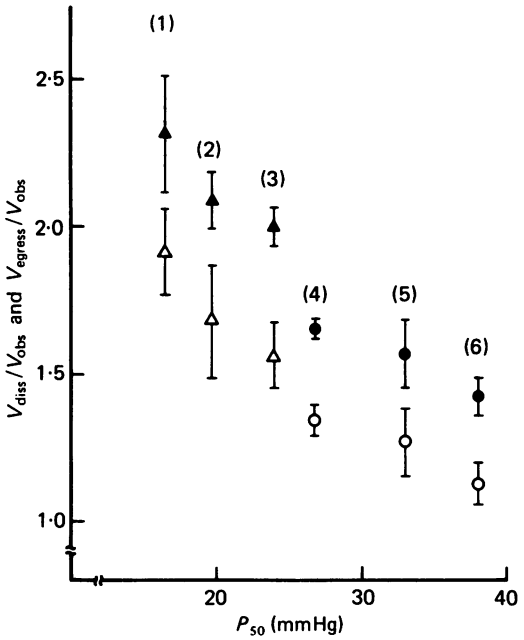

Fig. 5. The relationship between P_{50} and velocity ratios (V_{diss}/V_{obs} and V_{egress}/V_{obs}). The oxygen affinity was modified by changing pH of the medium (triangles) and by increasing 2,3-DPG content in red cells (circles). Filled and open symbols represent V_{diss}/V_{obs} and V_{egress}/V_{obs} respectively. The data were obtained from the same experiment as in Table 2. Numbers in parentheses correspond to the experiment number in Table 2.

Therefore, the r value approached unity (i.e. $V_{egress} = V_{diss}$, similarly to the haemolysate). On the other hand, when the amount of bakers' yeast was augmented, the oxygen dissociation curve of red cells was distorted so as to shift towards the left and the distortion became evident as the deoxygenation proceeded (Table 1). The r values were smaller than unity at higher V_{max} (i.e. $V_{egress} < V_{diss}$); thus one should consider whether the presence of the oxygen diffusion barrier resulted in the non-equilibration between the intra- and extracellular oxygen concentrations.

(ii) The r values for red cells, even at higher V_{max}, were independent of the concentrations of allosteric effectors (such as 2,3-DPG and H^+) in spite of alteration of P_{50}, V_{diss} and V_{egress} (Table 2). Therefore, the 'chemical reaction' rates (i.e. the rates of the deoxygenation and recombination reactions) were not reflected to the r values.

On the other hand, the rate of oxygen dissociation, observed by the rapid mixing

studies, is known to be highly dependent on 2,3-DPG and pH both in haemoglobin solution and in red cells (Salhany *et al.* 1970; Salhany, Keitt & Eliot, 1971). Accordingly, it was predicted that V_{diss} might increase at the same V_{obs} by increasing 2,3-DPG or by lowering intracellular pH, because the oxygen affinity decreased. However, the opposite results were obtained. As P_{50} increased, V_{diss} decreased (Fig. 5). This discrepancy resulted from the difference in experimental conditions, in particular from the different velocity for decreasing the oxygen concentration in the medium. In the rapid mixing method, the first order rate constant could be obtained (Dalziel & O'Brien, 1961; Salhany *et al.* 1971) in the absence of oxygen in the medium. However, in our experiment, the decrease of oxygen concentration in the medium was slower.

The ratio, V_{diss}/V_{obs}, should be proportional to $\Delta S/\Delta[O_2]$, which was actually the slope of the oxygen dissociation curve.

$$V_{obs} = d[O_2]/dt \quad ([O_2]: \text{oxygen concentration in the medium})$$
$$V_{diss} = [Hb_4]_{total} \cdot (dS/dt) \quad (\text{see Methods})$$

Thus,

$$V_{diss}/V_{obs} = [Hb_4]_{total} \cdot (dS/d[O_2])$$

Thus, when the oxygen dissociation curve shifted to the right, V_{diss}/V_{obs} decreased as shown in Fig. 5 at the same haem concentration.

Factors affecting the parameter, r

Nicolson & Roughton (1951) have pointed out that the oxygen diffusion barrier consists of the red cell interior and the cell membrane. Two extreme cases were studied in detail, in order to test the validity of the r value as an operational measure of the oxygen diffusion barrier.

(i) Partially haemolysed red cells, of which the haemoglobin contents were varied from 31·2 to 14·1 pg/cell, showed the dependence of r values on the intracellular haemoglobin concentration (Table 3). The r value increased with decreasing haemoglobin content. Although the 2,3-DPG/haemoglobin molar ratio inevitably varied during the preparation, the r values were independent of 2,3-DPG, as shown already.

Some of membrane components, as well as the intracellular components, might be lost during the preparation of the partially haemolysed red cells, but the viscosity of the cell interior depended mainly on the haemoglobin contents. Longmuir & Roughton (1952) actually showed that the diffusion constants of N_2 and CO in haemolysate decreased as haemoblobin concentration increased. Therefore, the decreased oxygen diffusion barrier may be due to the decreased viscosity inside red cells. Holland & Forster (1966) showed the relationship between the red cell diameter and oxygenation time, comparing many animal species. However, the mean corpuscular volume of our samples was not significantly altered and no relation between the mean corpuscular volume and the r values was noticed, thus the slight variation in the mean corpuscular volume was not reflected in the r value.

In short, the decreased viscosity in the red cell accelerated the diffusion rate of oxygen as well as the motion of haemoglobin molecules (Kreuzer, 1970), and increased the r value.

(ii) The cholesterol-loaded and -depleted red cells gave another series of examples. During the preparative procedures, the red cell constituents were kept fairly constant except for the amount of membrane cholesterol, though the slight changes of mean corpuscular volume and 2,3-DPG inevitably occurred (Table 4). As judged from the oxygen dissociation curve and the contents of various metabolites, the red cells were functionally intact. However, the increase of the cholesterol/phospholipid ratio decreased the r value. This demonstrated that change in the membrane organization affected the oxygen diffusion.

Cholesterol in the phospholipid bilayer is known to possess a condensing effect, immobilizing the fatty acid acyl chains, at the temperature studied here. (*a*) Fischkoff & Vanderkooi (1975) have shown by the fluorescent dye method that the diffusion rate of oxygen in the membrane was retarded by the presence of cholesterol. This could explain the decreased r value in the cholesterol-loaded cells. (*b*) Cooper *et al.* (1978) showed that the 'membrane viscosity' measured by the fluorescent dye decreased as the cholesterol/phospholipid ratio increased in the red cell ghosts. Also, we have shown decreased motion of the fatty acid spin labels in cholesterol-loaded red cells (which were obtained from the same donor and prepared in the same manner as in the present study) and demonstrated anti-parallelism between the spin label motion and cholesterol/phospholipid ratio of the red cells from twelve persons (Suda, Maeda, Sekiya, Mastuoka, Tokita & Shiga, 1978). Thus the decreased fluidity of the lipid portion might directly retard the diffusion rate of oxygen to some extent, and decrease the r value. (*c*) In addition, as the membrane became harder with cholesterol-loading, the 'tank-tread movement' of the red cell membrane (Schmid-Schönbein, 1976; Fischer, Stöhr-Liesen & Schmid-Schönbein, 1978) might be decreased (if any, in our experimental conditions); then the motion of haemoglobin in the cell interior could be indirectly reduced. Consequently, the diffusion rate of oxygen could be retarded. (*d*) Actually, the decreased red cell deformability was recognized for colesterol-loaded red cells (Shiga, Maeda, Suda, Kon, Sekiya & Oka, 1979). The above direct and indirect actions of cholesterol could explain the decreased r value, i.e. the increase of the oxygen diffusion barrier, observed for cholesterol-loaded cells.

At any rate, the parameter, r, could be operationally useful for the analysis of the determinant factors of the oxygen diffusion barrier, regardless of the chemical reaction rate, without mathematical treatment and assumption. It may be important that the oxygen diffusion barrier is operative, even in the relatively slower deoxygenation process compared with the process in the *in vivo* microcirculation.

APPENDIX

Characteristics of the oxygen electrode

The cathodic platinum electrode was made of a platinum wire (diameter 0·6 mm, length 9 cm, purity 99·99 %, purchased from Ishifuku Kinzoku Co.). After making a sphere of diameter 2 mm in the flame, the wire was covered with plastic tubing except the spherical portion. The spherical portion was coated with epoxy-resin and fixed to the tubing. The spherical portion was polished with fine emery paper

to expose a smooth surface of platinum with a diameter of about 2 mm, then immersed into 0·5 % cellulose acetate solution (in acetone) and dried in air. Cellulose acetate was obtained from Eastman Kodak Co. (39·8 % in acetyl, lot no. ASX). The fundamental properties of the cellulose acetate-coated electrode have been reported in detail by Hagiwara *et al.* (1978). However the thickness of coating was reduced in order to obtain a quick response.

The anodic silver electrode was made of a silver wire (diameter 0·8 mm, length 9 cm, purity 99·99 %), covered with plastic tubing and epoxy resin except the tip portion (*ca.* 1 cm).

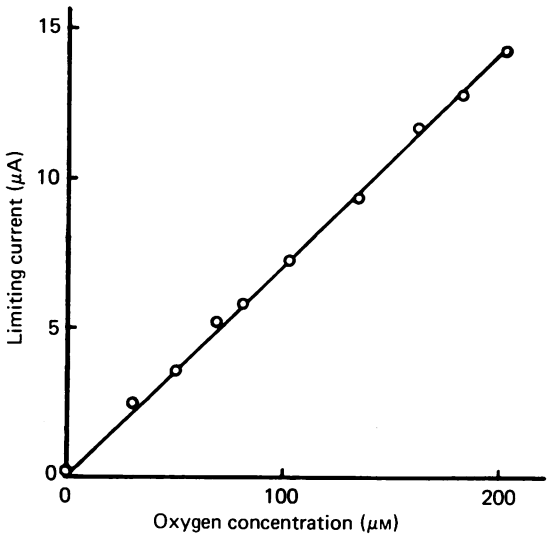

Fig. 6. Relationship between the oxygen concentration (μM) and the limiting current of the oxygen electrode. Measured in buffer A at 20 °C. Details are described in the text.

It was recommended that, in order to minimize the residual current, the electrode should be polarized (at −0·7 V) in the buffer for more than 2 hr prior to use.

The linearity between the oxygen concentration in the medium and the limiting current was tested as follows: two solutions (the buffer A saturated with either air or pure N_2) were volumetrically and anaerobically mixed in a gas-tight syringe, then transferred into a closed cuvette (till overflowing with solution) equipped with the electrode and the spin bar. Perfect linearity between the oxygen concentration and the current was obtained (Fig. 6).

The response time of the system was tested by a flow system, which could alternately switch two solutions (air-saturated and N_2-saturated buffers) by a three-way cock. The electrode was placed in the flow system. The time constant, as well as the limiting current, depended on the thickness of the cellulose acetate coating. When the thickness was adjusted to give a limiting current of 14–17 μA for air-saturated buffer A at 37 °C in the stirred cuvette, the time constant of the whole system was ascertained to be less than 1·5 sec (in where the time constant of the DC amplifier was *ca.* 1 sec and that of the recorder was *ca.* 0·8 sec).

The calibration of the oxygen concentration in the medium was carried out by

taking the final point, after reaction with an excess amount of bakers' yeast, to be zero, and the initial point, for the air-saturated buffer, to be the calculated value using the experimentally determined solubility coefficient of oxygen in the buffer used.

Reliability of the optical measurement

As the red cells and the bakers' yeast gave considerable light scattering, a set of opal glass plates was used throughout as shown in Fig. 1.

The spectral changes in the visible region during the deoxygenation of red cells by bakers' yeast are shown in Fig. 7. In this Figure, the base line of the spectro-

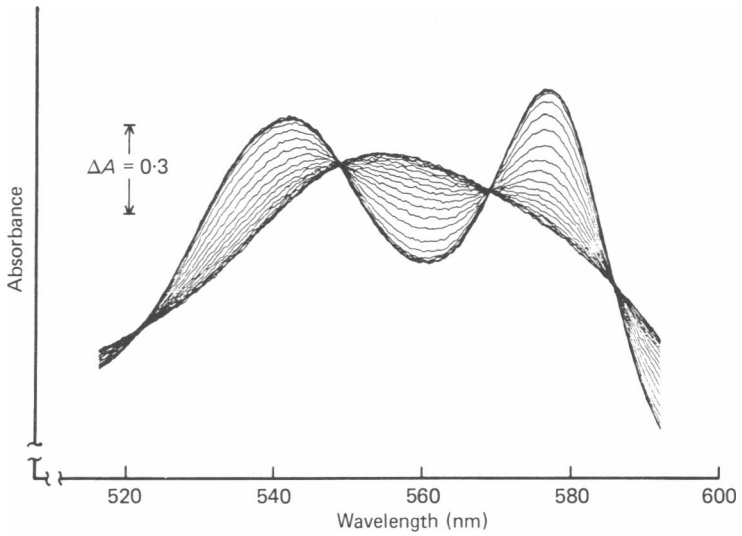

Fig. 7. Spectral changes of the oxygenated red cell suspensions after addition of bakers' yeast. Observed in buffer A at 20 °C. Haem concentration, 103·6 μM; Bakers' yeast added, 10 μl.

photometer output must be moved down by applying an appropriate DC voltage, in order to expand the spectral difference between oxy- and deoxy-haemoglobin. The isosbestic points appeared at 522, 548·5, 569 and 586 nm, and coincided with the reported wave-lengths for human haemoglobin solution (van Assendelft, 1970). However, the calculated absorbance/mole ('apparent molar extinction coefficient') in the visible region decreased, as compared with the clear haemoglobin solution, due to the flattering effect as demonstrated by Duysens (1960).

The linearity of the absorbance against concentration of haemoglobin in the oxy- and deoxy-forms, both in red cells and in solution in the presence of bakers' yeast, is shown in Fig. 8. The absorption difference between 542 nm (the peak of the oxy-form) and 522 nm (the isosbestic point), ΔA, was proportional to the haemoglobin concentration up to ΔA of 0·85. Correspondingly, the apparent absorbance/mole of red cell suspensions (in the presence of yeast) decreased to 84 % of that of haemoglobin solution in both the oxy- and deoxy-forms (without yeast). In addition, the cytochromes in bakers' yeast did not influence the spectra, i.e. the apparent spectrum

of bakers' yeast suspensions was flat and did not change during the deoxygenation in the present experimental conditions.

The degree of haemoglobin oxygenation in red cells was calibrated as follows: (i) for the deoxy-form, the absorbance at $pO_2 = 0$ (reached by the addition of yeast) was taken and (ii) for the 100 % oxy-form, the absorbance was approximated with the sample equilibrated with pure O_2 gas flow at 1 atm, after addition of KCN (20 mM) into the deoxygenated sample containing bakers' yeast.

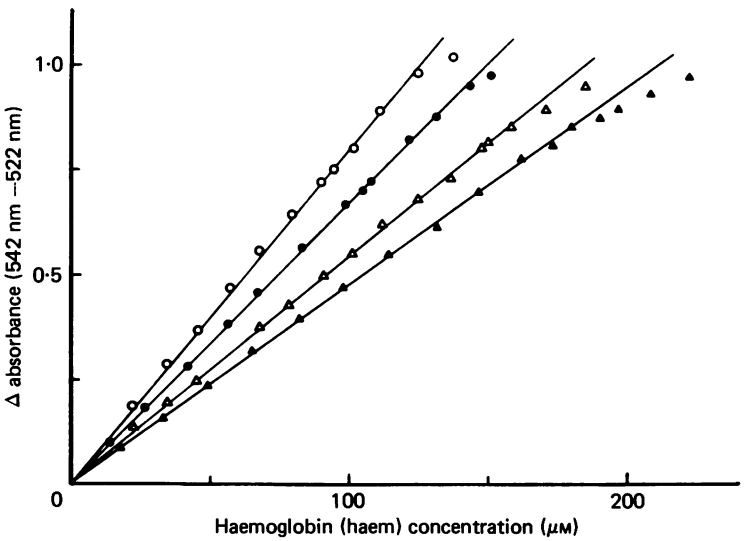

Fig. 8. Relationship between △ absorbance (542 nm − 522 nm) and the haem concentration. The spectra of the deoxygenated form were measured by adding 30 μl. bakers' yeast suspension, and those of the oxygenated form measured in the presence of the same quantity of bakers' yeast suspension and 20 mM-KCN. The oxygenated forms of red cells (●) and haemolysate (○), and the deoxygenated forms of red cells (▲) and haemolysate (△).

Properties of bakers' yeast as an oxygen consuming reagent

The bakers' yeast suspension was prepared freshly each day as follows: 9 g of pressed yeast were washed with 0·9 % NaCl solution three times and resuspended in 5 ml. buffer A, using a Teflon homogenizer, then kept at room temperature. When 30 μl. suspension was added to 3 ml. buffer A, the dissolved oxygen was consumed at the rate of *ca.* 5·4 μM/sec at 37 °C, and was completely used up within 50 sec. The oxygen concentration in the medium (in the presence of 0·1 % glucose, without haemoglobin) linearly decreased with time up to 30 μM. The velocity was proportional to the amount of added bakers' yeast. The Lineweaver–Burk plots, $1/V$ *vs.* $1/[O_2]$, is shown in Fig. 9. The K_m for oxygen was always 1·1 μM, similar to the value of Sargent & Tayler (1971) and the $V_{max}/[\text{yeast}]$ was 0·009 μM/sec per μl. (here, the bakers' yeast suspension of 1 μl. contained 83 μg protein, as measured by Lowry's method), at pH 7·4 in buffer A at 37 °C. However, when the cellulose acetate coating (of the Pt electrode) was too thick, erroneous K_m and V_{max} were obtained.

Bakers' yeast consumed oxygen and inevitably produced CO_2; thus the pH might

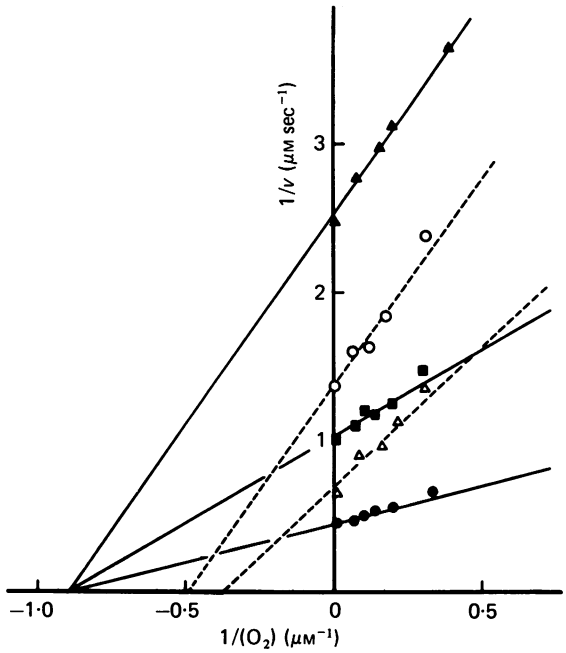

Fig. 9. Lineweaver–Burk plots of the oxygen consumption by bakers' yeast and influence of the thickness of coating of the electrode. Measured in buffer A at 37 °C. The amounts of bakers' yeast were varied: 30 μl. (●), 11 μl. (■), 5 μl. (▲), 30 μl. (△) and 11 μl. (○). The limiting currents of the electrode were 17 μA (filled symbol) and 5 μA (open symbol), which corresponded to thinner and thicker coating of cellulose acetate, respectively.

change during measurement and carbaminohaemoglobin might be produced. Actually, a slight decrease of pH (less than 0·02) was observed during deoxygenation, but this pH change did not affect the measurement (within our experimental error). Further, the production of carbaminohaemoglobin could be neglected, because (*a*) equilibrium of CO_2 and bicarbonate between the inside and outside of cells could be attained rapidly (Forster & Steen, 1968) and (*b*) the amount of CO_2 produced during the deoxygenation (= the amount of oxygen consumed by yeast, ~200 μM) corresponded to about 0·3 mmHg in CO_2 tension. Thus, the effect of CO_2 produced by bakers' yeast could be neglected.

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