Japanese Journal of Physiology, **41**, 49–61, 1991

Optical Measurement of Perfused Rat Hindlimb Muscle with Relation of the Oxygen Metabolism

Akitoshi SEIYAMA,† Nobuji MAEDA, and Takeshi SHIGA*

Department of Physiology, School of Medicine, Ehime University, Onsen-gun, Ehime, 791-02 Japan
** Department of Physiology, School of Medicine, Osaka University, Suita, Osaka, 565 Japan*

Abstract Optical measurements of myoglobin (Mb) and cytochromes (Cyts) in the isolated rat hindlimb muscle perfused with cell-free medium were performed at 35 and 15°C under various oxygen supply, in the relation to the oxygen metabolism. Molar ratio of Cyt $a+a_3$, Cyt b, Cyt $c+c_1$, and Mb, oxygen affinity of Mb (P_{50}), and the thermodynamic parameter ($\Delta H°$) of Mb oxygenation in cyanide-perfused muscle were similar to those reported. The "apparent P_{50}" of Mb in cyanide-free muscle was almost two orders larger in magnitude than P_{50} in the presence of cyanide. O_2 uptake by the perfused hindlimb muscle was constant above O_2 supply of 0.73 μmol/(min·g muscle) (under a flow rate of 1.0 ml/(min·g muscle) at 35°C). Below the value of O_2 supply, the O_2 uptake decreased and lactate/pyruvate ratio increased. The critical mean oxygen tension in tissue (estimated by Mb oxygenation) for O_2 uptake at 35°C was ca. 10 mmHg. It was found that oxidation level (%) of Cyt $a+a_3$ was equivalent to oxygenation level (%) of Mb at 35°C, while the oxidation level of Cyt $a+a_3$ was higher than the oxygenation level of Mb at 15°C. Based on the results, the uneven distribution of O_2 in the muscle tissue and the intracellular O_2 gradient were discussed.

Key words: tissue oxygenation, perfused rat hindlimb muscle, myoglobin, cytochrome $a+a_3$, spectrophotometry.

Natural pigments contained in the tissue are useful indicators for monitoring of metabolic activities: hemoglobin (Hb) and myoglobin (Mb) for the oxygenation in the tissue (e.g., TAMURA *et al.*, 1978); members of respiratory chains for the oxidative activities (CHANCE and WILLIAMS, 1955a); and NADH, which also reflects the activity of the glycolytic pathway under certain circumstances (e.g., CHANCE *et al.*, 1965).

Received for publication October 1, 1990

† To whom all correspondence should be addressed.

In early works, JÖBSIS (1963a, b) has reported spectrophotometric observation of mitochondrial respiratory chain of intact skeletal muscle. Although the system, in which the isolated muscle is held in the flowing buffer, is useful for investigation of the nature of respiratory chains in the intact muscle, the function of the circulatory system, such as supply of O_2 and nutrients and removal of metabolites via capillaries, is lost.

Isolated perfused organ is a valuable preparation for physiological and biological studies, since it retains most of the functions in the intact organ *in vivo*. Isolated perfused rat hindlimb has been used for the study of metabolic state in skeletal muscle such as O_2 uptake, glucose uptake, lactate release, lactate/pyruvate ratio, creatine content, ATP/ADP ratio and so on (e.g., RUDERMAN *et al.*, 1971, 1980; SHIOTA and SUGANO 1986). Such metabolic property and function of the perfused rat hindlimb, however, have not been investigated in detail in the relation of tissue oxygenation or intracellular O_2 concentration.

In the present paper, we describe the optical measurement of isolated rat hindlimb perfused with cell-free medium and the oxygen metabolism of the hindlimb muscle. Using this technique, we show 1) molar ratio of Mb and cytochromes (Cyts) in the quadriceps muscle, 2) oxygen affinity of Mb (P_{50}) in the muscle at 35, 25, and 15°C, and 3) changes in oxygenation of Mb and oxidation of Cyt $a+a_3$ in the relation of oxygen metabolism. Based on the results, the uneven distribution of O_2 in the muscle and the intracellular O_2 gradient are discussed.

MATERIALS AND METHODS

Perfusion of rat hindlimb. Male Wistar rats (250 to 300 g in body weight) fed on a commercial diet were used. Rats were anesthetized with sodium pentobarbital (30 mg/kg body wt., i.p.). Preparation of the isolated rat hindlimb and the perfusion system were essentially the same as those of SHIOTA and SUGANO (1986). Experimental setup is shown in Fig. 1. Krebs-bicarbonate buffer (NaCl, 115 mM; KCl, 5.9 mM; $MgCl_2$, 1.2 mM; NaH_2PO_4, 1.2 mM; Na_2SO_4, 1.2 mM; $NaHCO_3$, 25 mM; $CaCl_2$, 2.5 mM; glucose, 10 mM, pH 7.4) containing 4% (w/v) polyvinylpyrrolidone (PVP-40T; purchased from Sigma Chem.: avg. mol. wt., 40,000) was perfused at 35, 25, and 15°C from abdominal aorta in a flow-through mode at 1 ml/(min·g muscle). The effluent was collected from inferior vena cava in order to measure the rate of lactate and pyruvate releases (see below). Before measurements, the rat hindlimb was perfused with the buffer equilibrated with 95% O_2 + 5% CO_2 for 30 min. Then, the O_2 concentration in the perfusate was varied stepwise by mixing buffer equilibrated with 95% O_2 + 5% CO_2 and that equilibrated with 95% N_2 + 5% CO_2, and the measurements were started. Perfusion pressure in the tubing leading to aorta catheter was monitored with a pressure transducer (SCK-580, Nihon Kohden Kogyo, Tokyo, Japan) connected to a carrier amplifier (AP-610G, Nihon Kohden Kogyo, Tokyo, Japan). During the measurements, perfusion pressure was maintained constant (e.g. 60–65 mmHg at 35°C). As required, antimycin A,

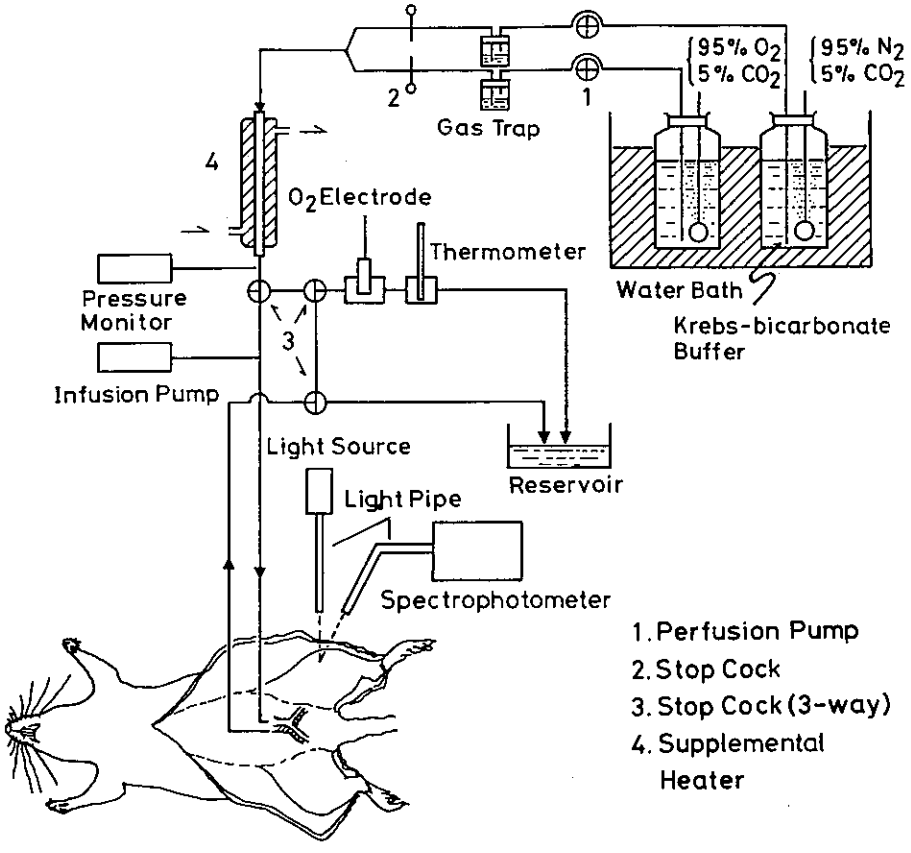

Fig. 1. Experimental setup for the perfusion of rat hindlimb and the optical measurement of thigh muscle. The optical measurement was devised for both reflectance and/or transmission mode. 1, perfusion pump; 2, stop cock; 3, three-way stop cock; 4, circulating water jacket connected to water bath.

potassium cyanide or sodium dithionite was infused into the tubing by an injection pump (Micro Feeder JP-W, Furue Science, Tokyo, Japan).

Spectrophotometric measurement. A computer-controlled rapid scanning spectrophotometer (USP-501, Unisoku, Osaka, Japan) was used for the analysis of Mb and Cyts in reflectance and/or transmission mode. Light was focused on the thigh (quadriceps) muscle through a light guide of 5 mm diameter. The transmitted light or the reflected light was conducted to the spectrophotometer through a light guide of 5 mm diameter. Spectra were recorded when constant O_2 uptake in the hindlimb muscle was attained after changing O_2 concentration in the buffer (and/or adding chemicals such as antimycin A). Simultaneously, the measurements of O_2 tension of perfusate (of both influent and effluent) and various metabolites were performed, as described below.

Analytical methods. O_2 tension in the perfusates was monitored with an oxygen electrode (GU-BMS; Iijima Products M.F.G. Co., Ltd., Aichi, Japan). O_2 concentration in the perfusate was calculated, based on oxygen solubility to the buffer, 1.5 μmol/(l·mmHg) at 35°C. The rate of O_2 uptake was calculated from the flow rate and the difference of O_2 concentration between influent and effluent. Concentrations of lactate and pyruvate in the effluent were determined enzymatically according to GUTMANN and WAHLEFELD (1974) and LAMPRECHT and HEINZ (1984), respectively.

Materials. Lactate dehydrogenase (LDH; EC 1.1.1.27), reduced and oxidized nicotinamide-adenine dinucleotide (β-NADH and β-NAD, respectively) for analysis of pyruvate and/or lactate were purchased from Boehringer Mannheim GmbH (Mannheim, West Germany). Antimycin A was obtained from Sigma Chem. (St. Louis, MO). All other chemicals were of analytical reagent grade.

RESULTS

Optical measurement of the perfused rat hindlimb

Absorption spectra of quadriceps muscle in rat hindlimb were recorded by perfusing the buffer of various O_2 concentration. Figure 2 shows changes of difference spectra (in the range of 500–650 nm) between aerobic condition (taken as a baseline) and the conditions under reduced O_2 supply. Upward peaks were observed at 520, 554, and 605 nm, and downward peaks, at 540 and 582 nm.

In order to identify the components contributing to these absorbance changes

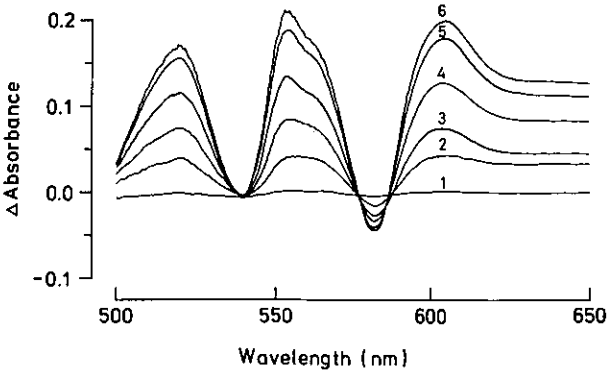

Fig. 2. Spectral change of the thigh (quadriceps) muscle accompanied with decrease of oxygen concentration in influent. Difference spectra were recorded in transmission mode at 35°C. Aerobic state, where O_2 supply was 1.02 μmol/(min·g muscle), was taken as a baseline (Trace 1). All difference spectra were obtained using 540 nm as reference wavelength. The isolated rat hindlimb was perfused at constant flow rate (1.02 ml/(min·g muscle)). The O_2 supply (μmol/(min·g muscle)) was stepwise decreased: 1.02 (Trace 1), 0.81 (Trace 2), 0.57 (Trace 3), 0.31 (Trace 4), 0.09 (Trace 5), 0 (Trace 6). Trace 6 was obtained by infusing the buffer containing 2 mM $Na_2S_2O_4$.

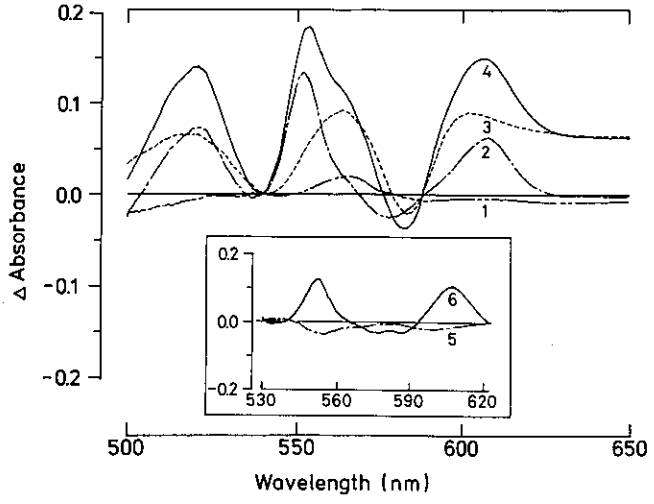

Fig. 3. Spectrophotometric separation of myoglobin and cytochromes in quadriceps muscle of perfused rat hindlimb. Difference spectra in the transmission mode at 15°C and those at 35°C (inserted figure) are shown. Traces 1 and 5, (aerobic +67 μM antimycin A) − (aerobic); Traces 2 and 6, (aerobic + 1.7 mM KCN) − (aerobic); Trace 3, (anaerobic) − (aerobic + 1.7 mM KCN); Trace 4, (anaerobic) − (aerobic).

and then to determine the appropriate wavelength pairs for measurements of oxygenation (or deoxygenation) of Mb and oxidation (or reduction) of Cyt $a+a_3$, difference spectra under various experimental conditions were measured, as follows (Fig. 3).

(a) Identification of Cyts and Mb. Difference spectra, (aerobic + antimycin A) − (aerobic), were measured to separate a spectrum of reduced Cyt b (Fig. 3, Trace 1 at 15°C and Trace 5 at 35°C). In this measurement, 40 to 80 μm antimycin A gave the same spectra. At 15°C, Mb was almost oxygenated and Cyts were almost oxidized, respectively, under the perfusion with 95% O_2 + 5% CO_2)-equilibrated buffer at a flow rate of 1.02 ml/(min · g muscle). Thus, only broad peak was observed at about 565 nm by reduction of Cyt b (Trace 1).

The spectrum of reduced state of respiratory chain components was obtained at 15°C by treatment with cyanide in aerobic condition, (aerobic + 1.7 mM KCN) − (aerobic), as shown in Trace 2 (1 to 4 mM KCN gave the same spectra). Various peaks in Trace 2 were identified as follows: β band of Cyt b and Cyt $c+c_1$ (as broad peak) at about 520 nm, α band of Cyt $c+c_1$ at 551 nm, α band of Cyt b (as shoulder) at 564 nm, and α band of cyanide complex of Cyt $a+a_3$ at 605 nm (cf., JÖBSIS, 1963a). The difference spectrum between deoxygenated and oxygenated state of Mb could be obtained by the subtraction of Trace 2 from Trace 4, i.e., (anaerobic) − (aerobic + 1.7 mM KCN), as shown in Trace 3. Abve 600 nm, a slight discrepancy from purified Mb spectrum was observed, probably due to a slight

Table 1. Molar ratio of myoglobin and cytochromes in rat quadriceps muscle.

Component	λ_s–λ_r (nm)	ε ($mM^{-1} \cdot cm^{-1}$)	Molar ratio
Mb	561–587	5.6	4.69±0.15
Cyt a+a_3	605–630	24.0	1
Cyt b	564–575	20	0.54±0.03
Cyt c+c_1	550–540	19	1.78±0.07

Molar ratio was calculated from difference spectra measured at 15°C by using wavelength pair of λ_s–λ_r. Mb: anaerobic − (aerobic +1.7 mM KCN). Cytochromes: (aerobic +1.7 mM KCN) − aerobic. Extinction coefficient applied for Cyt a+a_3 was cited from VAN GELDER (1966) and those for Cyt b and Cyt c+c_1 from CHANCE and WILLIAMS (1955b). The values are means±S.E. from 4 animals.

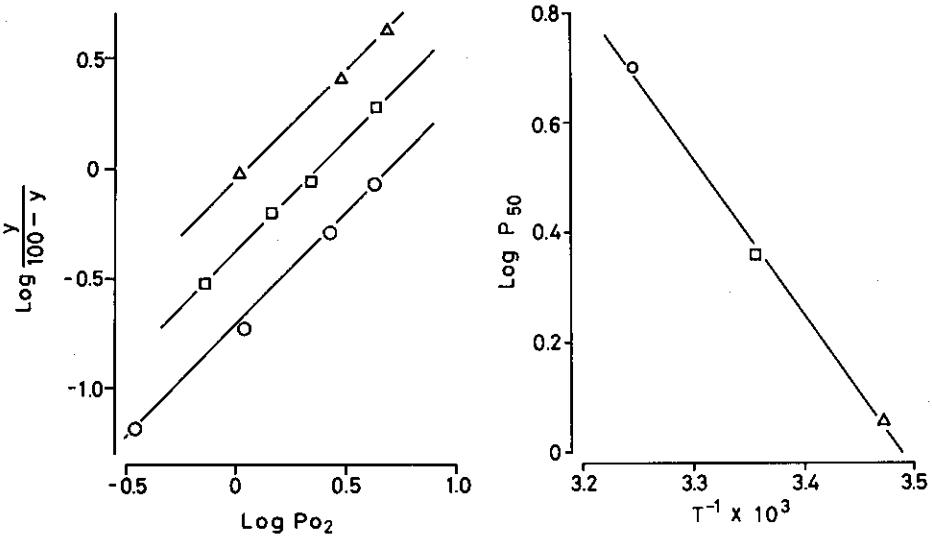

Fig. 4. Hill plot of oxygen equilibrium of myoglobin (left) and van't Hoff plot of P_{50} (right) in perfused hindlimb muscle. Tissue respiration was inhibited by addition of 2 mM KCN to the perfusate. ○, at 35°C; □, at 25°C; △, at 15°C. At the end of the oxygen titration, the buffer containing 2 mM $Na_2S_2O_4$ was infused to obtain complete anaerobic state. The value of P_{O_2} was in influent. Y represents % oxygenation of myoglobin calculated from the difference of absorbance at 561–582 nm. The results obtained from 3 animals are shown.

contribution of Cyt a+a_3 (i.e., spectral difference between reduced the state and the cyanide complex: VANNESTE, 1966; VAN BUUREN *et al.*, 1972; NICHOLLS *et al.*, 1976; NICHOLLS and HILDEBRANDT, 1978; WIKSTRÖM *et al.*, 1981).

At 35°C, however, complex spectrum (Trace 5) was observed under the perfusion with (95% O_2+5% CO_2)-equilibrated buffer. Moreover, the reduction

of Cyts by inhibition of respiration with cyanide was accompanied by the additional oxygenation of Mb (a small peak at 580 nm, as shown in Trace 6). Analyzing the spectra at 35°C, 10–15% of Mb and Cyt $a+a_3$ appeared to be deoxygenated and reduced, respectively.

(b) Determination of Cyts and Mb. Optimal wavelength pairs and extinction coefficients for the determination of molar ratio of Cyts and Mb are summarized in Table 1. Molar ratio of Cyt b, Cyt $c+c_1$, and Mb to Cyt $a+a_3$ estimated from the difference spectra at 15°C was 0.54, 1.78, and 4.69, respectively (Table 1).

Based on the results of Figs. 2 and 3, wavelength pairs of 587–630 and 605–630 nm were chosen for the measurements of oxygenation (or deoxygenation) of Mb and oxidation (or reduction) of Cyt $a+a_3$, respectively, where the interference of Cyts absorbance to Mb measurement was less than 10%, and that of Mb to Cyt $a+a_3$ measurement less than 12%, as estimated from their molar ratio and their extinction coefficients.

Oxygen affinity of myoglobin in perfused hindlimb muscle

Figure 4 shows the Hill plot of oxygenation of Mb obtained in cyanide-perfused hindlimb muscle (left) and the van't Hoff plot at 35, 25, and 15°C (right). In the presence of cyanide, O_2 uptake of hindlimb was completely inhibited, thus O_2 gradient in the tissue was neglected under steady state of perfusion. P_{50} of Mb at

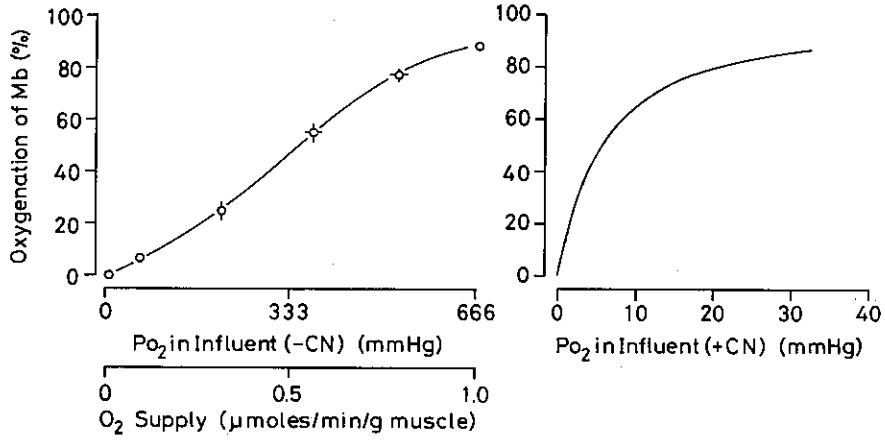

Fig. 5. Relationship of myoglobin oxygenation between cyanide-perfused hindlimb (right) and cyanide-free perfused hindlimb (left). The rat hindlimb was perfused at constant flow rate (1.02 ml/(min·g muscle)), and the oxygen concentration in the buffer was varied. Oxygenation of myoglobin in the cyanide-free perfused hindlimb was calculated from the absorbance difference at 587–630 nm. The value shows mean±S.E. from 6 animals at 35°C (the data include both transmission and reflectance measurements). The oxygen equilibrium curve of myoglobin in the cyanide-perfused hindlimb was drawn by using $P_{50}=5.0$ mmHg at 35°C in Fig. 4. The P_{O_2} in the abscissa was in the influent.

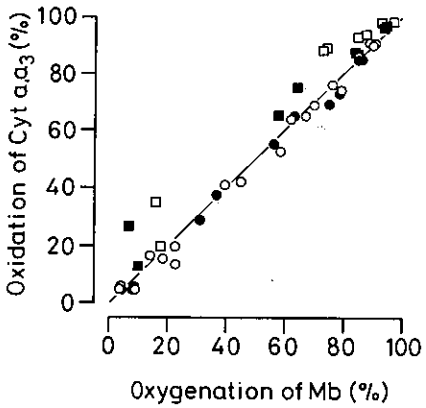

Fig. 6. Steady-state oxygen titration in the perfused rat hindlimb. The experiments were performed by reducing oxygen tension in influent from the aerobic to the anaerobic conditions stepwise. Oxidation level of Cyt $a+a_3$ measured at 605–630 nm was plotted against oxygenation level of Mb measured at 587–630 nm. ○, reflectance measurements (from 4 animals) and ●, transmission measurements (from 2 animals) at 35°C. □, reflectance (from 2 animals) and ■, transmission (from 2 animals) at 15°C.

35, 25, and 15°C was 5.0, 2.3, and 1.1 mmHg (in the perfusate), respectively, and the Hill's coefficient was unity. Thermodynamic parameter for the reaction ($\Delta H°$) was -13.0 kcal/mol.

Tissue oxygenation and oxidation in the perfused hindlimb muscle

Relationship between the oxygenation of Mb in the cyanide-perfused hindlimb and that without cyanide is shown in Fig. 5. P_{O_2} of the influent at half oxygenation of Mb in the cyanide-free muscle was ca. 360 mmHg at 35°C, whereas that in cyanide-perfused hindlimb was 5.0 mmHg.

Figure 6 shows the relationship between oxidation of Cyt $a+a_3$ and oxygenation of Mb measured under different O_2 supply. At 35°C, the oxidation (or reduction) level (%) of Cyt $a+a_3$ in the resting perfused hindlimb muscle was equivalent to the oxygenation (or deoxygenation) level (%) of Mb. However, at 15°C, the oxidation level of Cyt $a+a_3$ was higher than the oxygenation level of Mb.

Oxygen metabolism in perfused rat hindlimb

Figure 7 shows changes in lactate release, lactate/pyruvate (L/P) ratio and O_2 uptake, under different O_2 supply to the perfused hindlimb. O_2 uptake at 35°C was constant under O_2 supply above 0.73 μmol/(min·g muscle) ($P_{O_2}=480$ mmHg in the influent), and below the value, O_2 uptake decreased and L/P ratio increased.

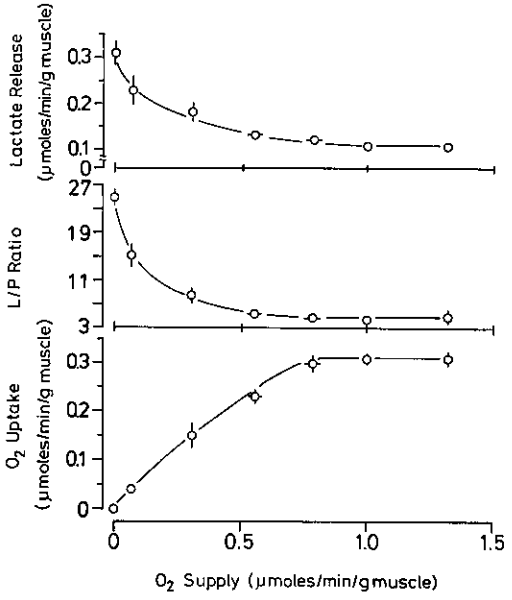

Fig. 7. Changes in oxygen uptake, lactate release and lactate/pyruvate (L/P) ratio with reducing the O_2 supply. The values are means ± S.E. (from 3 animals for the measurements of lactate release and L/P ratio, and from 6 animals for the measurements of O_2 uptake). The hindlimb was perfused at 1.02 ml/(min·g muscle) in a flow-through mode except for O_2 supply of 1.35 μmol/(min·g muscle) of which the flow rate was 1.35 ml/(min·g muscle). The 0 μmol/(min·g muscle) of O_2 supply was obtained by infusing 2 mM $Na_2S_2O_4$.

DISCUSSION

In this paper, oxygen metabolism of rat hindlimb muscle perfused with cell-free medium was studied by an optical method, and 1) molar ratio of Mb and Cyts, 2) oxygen affinity of Mb in the muscle, and 3) oxygenation of Mb and oxidation of Cyt $a+a_3$ were measured.

Molar ratio of Mb and Cyts. The molar ratio of Cyts b and $c+c_1$ to Cyt $a+a_3$ was similar to those determined in purified mitochondria of rat skeletal muscle (MAKINEN and LEE, 1968), and that of Mb to Cyt $a+a_3$ (4.7 : 1) is also similar to that reported by TAMURA *et al.* (1987), when the value was compared by recalculating on the basis of an extinction coefficient of Cyt $a+a_3$ reported by VAN GELDER (1966) (Table 1). The ratio of Mb to Cyt $a+a_3$ in the muscle obtained in the present experiment was much smaller than that in rat heart (8–10 : 1; TAMURA *et al.*, 1978; KANAIDE *et al.*, 1982; KENNEDY and JONES, 1986), since quadriceps muscle of rodents is composed of three types of muscle with different concentration of Mb and mitochondria; i.e., white, red and intermediate muscles (BALDWIN *et al.*, 1973).

Heterogeneous oxygenation of tissue. Oxygenation of Mb in the perfused

muscle was measured in the absence and the presence of 2 mM cyanide (Figs. 4 and 5). In the presence of cyanide, Hill plot of Mb showed the linear slope of unity (Fig. 4, left). P_{50} of Mb was 5.0 mmHg at 35°C, 2.3 mmHg at 25°C, and 1.1 mmHg at 15°C (Fig. 4, left), and the $\Delta H°$ was -13.0 kcal/mol (Fig. 4, right). These values were quite similar to those of purified Mb of rat skeletal muscle (STRICKLAND *et al.*, 1959; ANTONINI and BRUNORI, 1971). Thus, in the present preparation of perfused rat hindlimb, the interference of contaminated hemoglobin (Hb) in isolated hindlimb, if any, for the measurement of Mb and Cyts can be negligible.

In the absence of cyanide, oxygenation of Mb showed sigmoid saturation and the "apparent P_{50}" was almost two orders larger than P_{50} in the presence of cyanide (Fig. 5, left). This relation in the shape of Mb saturation curve and value of apparent P_{50} depends on the rate of O_2 uptake and/or temperature of the tissue (unpublished data). This relation may be due to the heterogeneous oxygenation of cyanide-free perfused muscle. Thus, mean tissue P_{O_2} ($\overline{tP_{O_2}}$) in cyanide-free perfused hindlimb muscle was estimated from the oxygenation of Mb (Fig. 5, left), based on the oxygenation curve of Mb in the cyanide-perfused muscle (Fig. 5, right). In O_2 supply below 0.73 μmol/(min·g muscle) (the influent $P_{O_2}=480$ mmHg in the present experimental condition), O_2 uptake of perfused hindlimb muscle started to decrease and L/P ratio to increase (Fig. 7). At this "critical" point of O_2 uptake, mean oxygenation of Mb in cyanide-free perfused muscle was ca. 70% (Fig. 5, left) and, thus, critical $\overline{tP_{O_2}}$ was estimated as ca. 10 mmHg. In several tissues, the *in vivo* critical $\overline{tP_{O_2}}$'s as high as 10 mmHg have been reported (CHANCE *et al.*, 1964; FABEL, 1968). These values are much higher than that reported for the respiration of isolated mitochondria (OSHINO *et al.*, 1974). As for the reason, uneven distribution of O_2 in the perfused organ due to the inhomogeneity of capillary function (HONIG *et al.*, 1971), and also P_{O_2} difference from extracellular space to mitochondrial inner membrane (KENNEDY and JONES, 1986) should be considered.

Oxygenation of Mb and oxidation of Cyt $a+a_3$ in the perfused hindlimb muscle. Coherent relation of oxygenation of Mb and oxidation of Cyt $a+a_3$ has been reported in the perfused rat heart (TAMURA *et al.*, 1978; KANAIDE *et al.*, 1982): the respiration state of mitochondria is expected to be near state 3 (SUGANO *et al.*, 1974) and the O_2 affinity of Cyt $a+a_3$ is about 10-fold higher than that of Mb (cf. OSHINO *et al.*, 1974). The interpretation of linear relationship between the oxygenation (or deoxygenation) of Mb and the oxidation (or reduction) of Cyt $a+a_3$ in the perfused heart is still disputed experimentally and theoretically as follows: (1) presence of oxygen gradient between cytosol and mitochondrion (KENNEDY and JONES, 1986; CHANCE and CHANCE, 1987; TAMURA *et al.*, 1989), termed as gradient coherence (TAMURA *et al.*, 1989; CHANCE, 1989); (2) heterogeneity of oxygen tension among cells (CASPARY *et al.*, 1985; CLARK *et al.*, 1987; WITTENBERG and WITTENBERG, 1989), termed as geometric coherence (TAMURA *et al.*, 1989) or pseudo-coherence (CHANCE, 1989); (3) difference in oxygen affinity of Cyt $a+a_3$ between *in vivo* and *in vitro* (i.e., O_2 affinity of Cyt $a+a_3$ *in vivo* may be much lower than that *in vitro*) (JÖBSIS, 1974).

It should be noted in the present result that coherent relation between oxidation (or reduction) of Cyt $a+a_3$ and oxygenation (or deoxygenation) of Mb was observed unexpectedly in the perfused hindlimb of rat at 35°C (Fig. 5), since mitochondrial respiration state in the resting skeletal muscle is expected to be close to the state 4 (CHANCE *et al.*, 1962), where the O_2 affinity of Cyt $a+a_3$ is much higher than that of Mb (cf. OSHINO *et al.*, 1974). Loss of the relationship and the increased oxidation level of Cyt $a+a_3$ were observed at 15°C (Fig. 6), wherein O_2 uptake at 15°C in the aerobic condition was about one-fourth of that at 35°C. This indicates that the relation between oxidation (or reduction) level of Cyt $a+a_3$ and oxygenation (or deoxygenation) level of Mb depends on the rate of O_2 uptake in tissue. This effect of metabolic rate on the relationship between oxygenation level of Mb and oxidation level of Cyt $a+a_3$ is characterized by gradient coherence (CHANCE, 1989). On the other hand, as discussed before, uneven distribution of O_2 and heterogeneous composition of three types of muscle with different concentrations of myoglobin and mitochondrion result in the heterogeneous oxygenation and metabolic state in tissue, which is characterized as geometric or pseudo-coherence (CHANCE, 1989). Thus, the coherent relation between oxidation level of Cyt $a+a_3$ and oxygenation level of Mb in the perfused hindlimb muscle may be caused by superposition of such heterogeneities in tissue on intracellular O_2 gradient.

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan, and a grant from the Ehime Health Foundation.

REFERENCES

ANTONINI, E. and BRUNORI, M. (1971) Specific aspects of the reactions of myoglobin with ligands. *In*: Hemoglobin and Myoglobin in Their Reactions with Ligands, ed. by NEUBERGER, A. and TATUM, E. L., North-Holland Publ. Co., Amsterdam, pp. 219–234.

BALDWIN, K. M., WINDER, W. W., TERJUNG, R. L., and HOLLOSZY, J. O. (1973) Glycolytic enzymes in different types of skeletal muscle: Adaptation to exercise. *Am. J. Physiol.*, **225**: 962–966.

CASPARY, L., AHMAD, H. R., HOFFMANN, Y., and LÜBBERS, D. W. (1985) Multicomponent analysis of reflection spectra from the guinea pig heart for measuring tissue oxygenation by quantitative determination of oxygen saturation, of a myoglobin, and of the redox states of cytochromes aa_3, b, and c. *Adv. Exp. Med. Biol.*, **191**: 263–270.

CHANCE, B. (1989) Metabolic heterogeneities in rapidly metabolizing tissues. *J. Appl. Cardiol.*, **4**: 207–221.

CHANCE, B., MAURIELLO, G., and AUBERT, M. (1962) ADP arrival at muscle mitochondria following a twitch. *In*: Muscle as a Tissue, ed. by RODAHL, K. and HORVATH, S., McGraw-Hill, New York, pp. 128–145.

CHANCE, B., SCHOENER, B., and SCHINDLER, F. (1964) The intracellular oxidation-reduction state. *In*: Oxygen in the Animal Organism, ed. by DICKENS, F. and NEIL, E., Pergamon, London, pp. 367–388.

CHANCE, B. and WILLIAMS, G. R. (1955a) Respiratory enzymes in oxidative phosphorylation. I. Kinetics of oxygen utilization. *J. Biol. Chem.*, **217**: 383–393.

CHANCE, B. and WILLIAMS, G. R. (1955b) Respiratory enzymes in oxidative phosphorylation. II. Difference spectra. *J. Biol. Chem.*, **217**: 395–407.

CHANCE, B., WILLIAMSON, J. R., JAMIESON, D., and SCHOENER, B. (1965) Properties and kinetics of reduced pyridine nucleotides fluorescence of isolated and *in vivo* rat heart. *Biochem. Z.*, **341**: 347–377.

CHANCE, E. M. and CHANCE, B. (1987) Oxygen delivery to tissue: Calculation of oxygen gradients in the cardiac cell. *Adv. Exp. Med. Biol.*, **222**: 69–75.

CLARK, A., Jr., CLARK, P. A. A., CONNET, R. J., GAYESKI, T. E. J., and HONIG, C. R. (1987) How large is the drop in P_{O_2} between cytosol and mitochondrion? *Am. J. Physiol.*, **252**: C583–C587.

FABEL, H. (1968) Normal and critical O_2-supply of the heart. *In*: Oxygen Transport in Blood and Tissue, ed. by LÜBBERS, D. W., LUFT, U. C., THEWS, G., and WITZLEB, E., Intercontinental Book Corp, New York, pp. 159–171.

GUTMANN, I. and WAHLEFELD, A. W. (1974) L(+) lactate. Determination with lactate dehydrogenase and NAD^+. *In*: Methods of Enzymatic Analysis, 2nd ed., ed. by BERGMEYER, H. U., Academic, New York, Vol. 3, pp. 1464–1468.

HONIG, C. R., FRIERSON, J. L., and NELSON, C. N. (1971) O_2 transport and V_{O_2} in resting muscle: Significance for tissue-capillary exchange. *Am. J. Physiol.*, **220**: 357–363.

JÖBSIS, F. F. (1963a) Spectrophotometric studies on intact muscle. I. Components of the respiratory chain. *J. Gen. Physiol.*, **46**: 905–928.

JÖBSIS, F. F. (1963b) Spectrophotometric studies on intact muscle. II. Recovery from contractile activity. *J. Gen. Physiol.*, **46**: 929–969.

JÖBSIS, F. F. (1974) Intracellular metabolism of oxygen. *Am. Rev. Respir. Dis.* (Suppl), **110**: 58–63.

KANAIDE, H., YOSHIMURA, R., MAKINO, N., and NAKAMURA, M. (1982) Regional myocardial function and metabolism during acute coronary occlusion. *Am. J. Physiol.*, **242**: H980–H989.

KENNEDY, F. G. and JONES, D. P. (1986) Oxygen dependence of mitochondrial function in isolated rat cardiac myocytes. *Am. J. Physiol.*, **250**: C374–C383.

LAMPRECHT, W. and HEINZ, F. (1984) Pyruvate. *In*: Methods of Enzymatic Analysis, 3rd ed., ed. by BERGMEYER, H. U., Verlag Chemie, Weinheim Deerfield Florida Basel, Vol. 6, pp. 570–577.

MAKINEN, M. W. and LEE, C. P. (1968) Biochemical studies of skeletal muscle mitochondria. 1. Microanalysis of cytochrome content, oxidative and phosphorylative activities of mammalian skeletal muscle mitochondria. *Arch. Biochem. Biophys.*, **126**: 75–82.

NICHOLLS, P. and HILDEBRANDT, V. (1978) Binding of ligands and spectral shifts in cytochrome c oxidase. *Biochem. J.*, **173**: 65–72.

NICHOLLS, P., PETERSEN, L. C., MILLER, M., and HANSEN, F. B. (1976) Ligand-induced spectral changes in cytochrome c oxidase and their possible significance. *Biochim. Biophys. Acta*, **449**: 188–196.

OSHINO, N., SUGANO, T., OSHINO, R., and CHANCE, B. (1974) Mitochondrial function under hypoxic conditions: The steady states of cytochrome $a+a_3$ and their relation to mitochondrial energy states. *Biochim. Biophys. Acta*, **368**: 298–310.

RUDERMAN, N. B., HOUGHTON, C. R. S., and HEMS, R. (1971) Evaluation of the isolated perfused rat hindquarter for the study of muscle metabolism. *Biochem. J.*, **124**: 639–651.

RUDERMAN, N. B., KEMMER, F. W., GOODMAN, M. N., and BERGER, M. (1980) Oxygen consumption in perfused skeletal muscle. Effect of perfusion with aged, fresh, and

aged-rejuvenated erythrocytes on oxygen consumption, tissue metabolites and inhibition of glucose utilization by acetoacetate. *Biochem. J.*, **190**: 57–64.

SHIOTA, M. and SUGANO, T. (1986) Characteristics of rat hindlimbs perfused with erythrocyte- and albumin-free medium. *Am. J. Physiol.*, **251**: C78–C84.

STRICKLAND, E. H., ACKERMAN, E., and ANTHONY, A. (1959) Effects of altitude acclimatization on the equilibrium constant of rat oxymyoglobin. *Am. J. Physiol.*, **197**: 211–213.

SUGANO, T., OSHINO, N., and CHANCE, B. (1974) Mitochondrial functions under hypoxic conditions; The steady state of cytochrome c reduction and of energy metabolism. *Biochim. Biophys. Acta*, **347**: 340–358.

TAMURA, M., HAZEKI, O., NIOKA, S., and CHANCE, B. (1989) *In vivo* study of tissue oxygen metabolism using optical and nuclear magnetic resonance spectroscopies. *Annu. Rev. Physiol.*, **51**: 813–834.

TAMURA, M., OSHINO, N., CHANCE, B., and SILVER, I. A. (1978) Optical measurements of intracellular oxygen concentration of rat heart *in vitro*. *Arch. Biochem. Biophys.*, **191**: 8–22.

TAMURA, M., SEIYAMA, A., and HAZEKI, O. (1987) Spectroscopic characteristics of rat skeletal muscle and cardiac tissue in the visible and near-infrared region. *Adv. Exp. Med. Biol.*, **215**: 297–300.

VAN BUUREN, K. J. H., ZUURENDONK, P. F., VAN GELDER, B. F., and MUIJSERS, A. O. (1972) Biochemical and biophysical studies on cytochrome aa_3. V. Binding of cyanide to cytochrome aa_3. *Biochim. Biophys. Acta*, **256**: 243–257.

VAN GELDER, B. F. (1966) On cytochrome c oxidase. I. The extinction coefficients of cytochrome a and cytochrome a_3. *Biochim. Biophys. Acta*, **118**: 36–46.

VANNESTE, W. H. (1966) The stoichiometry and absorption spectra of components a and a_3 in cytochrome c oxidase. *Biochemistry*, **5**: 838–848.

WIKSTRÖM, M., KRAB, K., and SARASTE, M. (1981) Cytochrome Oxidase: A Synthesis, Academic Press, New York, pp. 198.

WITTENBERG, B. A. and WITTENBERG, J. B. (1989) Transport of oxygen in muscle. *Annu. Rev. Physiol.*, **51**: 857–878.