

In Vitro Augmentation of Muscle Oxygen Consumption by Hemoglobin (35697)

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(Introduced by S. F. Marotta)

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It has been suggested by Nicolson and Roughton (1) that hemoglobin and related respiratory pigments, in addition to their traditional role as oxygen "carriers," also aid the diffusion of oxygen across short distances both intra- and extracellularly. Wittenberg (2, 3) and Scholander (4) showed that when a thin, aqueous film of hemoglobin separates an oxygen-rich atmosphere from one with low oxygen, oxygen diffuses across the film several times faster than can be accounted for by the P_{O_2} gradient alone. Films of intact erythrocytes exhibit the same property (5, 6). While hemoglobin unquestionably increases the availability of oxygen to respiring tissues, the exact manner in which hemoglobin influences the relationship between respiratory rate and P_{O_2} is vague. Interesting questions remain: is there a particular P_{O_2} at which the hemoglobin is most effective in facilitating tissue oxygen uptake; can hemoglobin depress oxygen consumption at low P_{O_2} by competing with tissues for available oxygen; in *in vitro* respiring systems does agitation completely replace the effects of hemoglobin in increasing oxygen availability to tissues? The experiments reported here were designed to determine the effects of the presence of hemoglobin on the respiratory rates of excised tissue exposed to different oxygen partial pressures.

Materials and Methods. Manometric measurements of oxygen consumption were carried out on upper leg muscles of large grass frogs. Muscles were dissected out into cold amphibian Ringer's solution, cleaned of all tendons and blood vessels, and teased apart with pointed forceps into pieces containing only a few fibers. Approximately 100 mg of muscle were transferred to each 15-ml War-

burg vessel equipped with a gassing cock. Media consisted of 5 ml of 1% glucose-amphibian Ringer buffered to pH 7.6 with Tris-HCl, with or without 1% bovine hemoglobin. Center wells contained 0.2 ml of 10% KOH to absorb CO_2 . Hemoglobin was prepared by lysing washed bovine red blood cells with an equal volume of distilled, deionized water and then removing whole cells and ghosts by centrifugation. The final stock solution, which contained 9.3% hemoglobin as determined by the acid hematin method (7), was frozen and stored at 0-4° in 10-ml aliquots for use as needed. Spectral analysis of the thawed material showed absorption peaks at 540 and 576 $m\mu$ indicating the presence of oxyhemoglobin; the absence of a peak at 630 $m\mu$ indicated no appreciable methemoglobin. The final media containing 1% hemoglobin was prepared by diluting the stock solution with Tris-glucose-Ringer.

Mixtures of oxygen and nitrogen containing 2.5, 5.0, 7.5, 10, and 15% oxygen were prepared using a double mixing flowmeter and a Beckman C2 oxygen analyzer. Reaction vessels containing tissues in either plain or hemoglobin media were prepared in triplicate and gassed for 15 min with one of the above mixtures or ambient air. An additional 3 vessels containing only hemoglobin media were included in each experiment to measure oxygen uptake or release by the pigment itself; and these values were then used to correct oxygen consumption measurements of tissues in hemoglobin media. Oxygen uptake was measured at 25° for 1-hr periods and expressed as Q_{O_2} (μ l of O_2 consumed/mg of dry tissue wt/hr). To test the effects of agitation, periods of shaking (60 cpm) were alternated with stationary periods. At each of

the six oxygen concentrations, tissues from the same four animals were used to measure Q_{O_2} under four conditions: shaking control; shaking hemoglobin; nonshaking-control; and nonshaking-hemoglobin.

To examine the possibility that other factors such as nonfunctional heme groups, general proteins, denatured hemoglobin or red blood cell salts might be responsible for the altered Q_{O_2} , experiments were also carried out in which methemoglobin, casein, or the centrifugate of boiled stock solution were substituted for hemoglobin at equivalent concentrations. Measurements were made using 5% oxygen in the gas phase.

Results and Discussion. Examination of the shaken and nonshaken control curves in Fig. 1 shows that in the P_{O_2} range studied (19–154 mm Hg), oxygen consumption increased with P_{O_2} in a hyperbolic fashion similar to that observed with many other tissues (8) and intact animals (9); that is,

$$Q_{O_2} = \frac{P_{O_2}}{K_1 + K_2 \cdot P_{O_2}}, \quad (1)$$

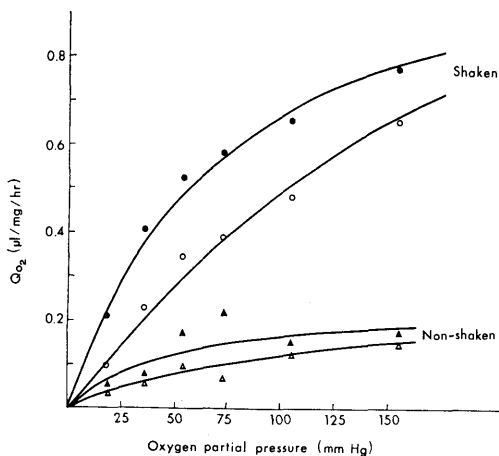


FIG. 1. Effects of shaking and hemoglobin on frog skeletal muscle Q_{O_2} . Treatments: shaking + the presence of 1% bovine hemoglobin (●); shaking controls (○); no shaking + hemoglobin (▲); and no-shaking controls (△). With shaking, hemoglobin mean values are all significantly higher than controls using the paired means t test ($p < .05$). Without shaking, hemoglobin mean values are always higher than controls, but the differences are not significant at the 5% level of confidence. Hyperbolic curves were fitted using linear regression analyses with $n = 24$.

where $1/K_1$ reflects the rate at which Q_{O_2} approaches a maximal value with increasing P_{O_2} and $1/K_2$ is the maximum Q_{O_2} at infinite oxygen concentration. Highest observed values occurred in air where the Q_{O_2} was 0.66 $\mu\text{l}/\text{mg}/\text{hr}$ in shaken vessels and 0.15 $\mu\text{l}/\text{mg}/\text{hr}$ in nonshaken vessels. Thus, though shaking increases oxygen consumption by decreasing diffusion distances (10), oxygen availability was a limiting factor both in shaken control vessels and also in the nonshaken, particularly at the lower P_{O_2} levels.

In the presence of 1% hemoglobin, muscle tissues consumed oxygen at a higher rate than did the controls. In the shaken vessels, at all six oxygen concentrations, the mean hemoglobin values were significantly higher than the means of their paired controls ($p < .05$), with the greatest absolute increase, 0.19 $\mu\text{l}/\text{mg}/\text{hr}$, occurring at 10% O_2 (72.5 mm Hg) as shown in Table I. In the nonshaken vessels, all of the hemoglobin means were again higher than the paired control means, but the differences were not significant. Maximum absolute increase, 0.15 $\mu\text{l}/\text{mg}/\text{hr}$, again occurred at 10% O_2 . Since, in the nonshaking experiments, at least three of the individual hemoglobin Q_{O_2} values at each P_{O_2} were higher than their paired controls, we suggest that hemoglobin facilitation of Q_{O_2} occurred in both the shaken and nonshaken vessels; however, in the latter, extremely low respiratory rates combined with

TABLE I. Influence of P_{O_2} on Hemoglobin Facilitation of Q_{O_2} Showing Peak Effect at 72.5 mm Hg (10% O_2).

P_{O_2} (% O_2) (mm Hg)	+ ΔQ_{O_2} with Hb ^a ($\mu\text{l}/\text{mg}/\text{hr}$)	
	Nonshaking	Shaking
18.4 (2.5%)	0.019 \pm 0.012	0.110 \pm 0.022 ^b
36.0 (5.0%)	0.024 \pm 0.019	0.178 \pm 0.036 ^b
53.7 (7.5%)	0.078 \pm 0.065	0.181 \pm 0.022 ^b
72.5 (10%)	0.154 \pm 0.073 ^b	0.191 \pm 0.052 ^b
104.4 (15%)	0.031 \pm 0.019	0.171 \pm 0.032 ^b
151.1 (air)	0.029 \pm 0.025	0.115 \pm 0.030 ^b

^a Difference between hemoglobin and control paired means \pm standard error of the difference.

^b Significant increases ($p < .05$) due to presence of hemoglobin using the paired means t test with $N = 4$ pairs.

TABLE II. Effects of Shaking and Hemoglobin on Rate of Q_{O_2} Increase ($1/K_1$) and Maximal Q_{O_2} at ∞O_2 ($1/K_2$) Derived by Fitting Eq. (1) to the Data.

Treatment	N	$\frac{1}{K_1}$	$\frac{1}{K_2}$
		$\left(\frac{\mu\text{l/mg/hr}}{\text{mm Hg}}\right)$	$(\mu\text{l/mg/hr})$
Nonshaking			
Hemoglobin	24	0.0048	0.246
Control	24	0.0021	0.276
Shaking			
Hemoglobin	24	0.0161	1.38
Control	24	0.0068	1.85

^a Not significant at the 5% level of confidence based on analysis of variance.

a normal variability to render the consistent, small increases in Q_{O_2} with hemoglobin insignificant.

Maximum stimulation at 10% O_2 suggested that the hemoglobin effect may be operational mainly at lower, more physiological P_{O_2} levels. To investigate this possibility we examined statistically the effects of hemoglobin on $1/K_1$ and $1/K_2$ in the fitter hyperbolic equations. Confidence limits for these constants were estimated from analysis of variance tables using data obtained from the linear regression analyses. The results, shown in Table II, indicate that hemoglobin alters the hyperbolic relationship between Q_{O_2} and P_{O_2} in a very specific manner. That is, hemoglobin approximately doubles the rate of rise ($1/K_1$) of Q_{O_2} toward its maximum, but has little effect on the maximum itself ($1/K_2$). This suggests that hemoglobin exerts its effect primarily by increasing the rate of oxygen diffusion from media to tissues, possibly in a manner similar to facilitation in hemoglobin films, rather than by directly stimulating the metabolic machinery of the cells or by increasing the oxygen content of the media as shaking does.

That these effects are due to oxyhemoglobin is shown in Fig. 2. When centrifugate of boiled hemoglobin media, casein, or methemoglobin are substituted for hemoglobin, there is no augmentation of Q_{O_2} . Spectral analyses showed that neither the methemoglobin nor the centrifugate possessed the twin absorption peaks of oxyhemoglobin; however, a methemoglobin peak at $630 m\mu$ was

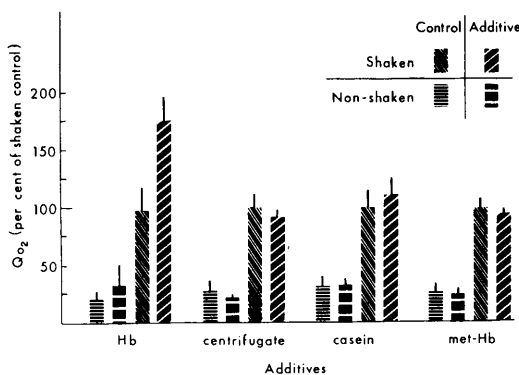


FIG. 2. Comparison of effects of hemoglobin, centrifugate of boiled hemoglobin, casein, and methemoglobin on frog skeletal muscle Q_{O_2} : gas phase = 5% O_2 . Each bar represents the mean of four animals plus one standard deviation, expressed as percentage of the mean Q_{O_2} for the shaken control values of that group. The only significant increase occurred with hemoglobin in the shaking experiment ($p < .02$).

present in both.

The greater effect of hemoglobin in the shaken vessels is interesting since it might be expected that agitation of the media would improve oxygen diffusion and thus mask a hemoglobin effect, while in the nonshaken vessels, hemoglobin facilitation should have a marked effect on the oxygen-starved cells. The results, however, are consistent with the model proposed by Zilversmit (11), and substantiated experimentally by Scholander (12) to describe hemoglobin facilitation of oxygen diffusion in thin films. In this model the two components of oxygen diffusion are: (a)

simple diffusion of oxygen along a P_{O_2} gradient in the media, which operates mainly at the oxygen-rich surface; and (b) diffusion of oxyhemoglobin along its concentration gradient with continuous release of oxygen at the low P_{O_2} surface. The two gradients interact in the film by reoxygenation of reduced hemoglobin. In this experiment, agitation of the tissue and media during shaking aided diffusion of both oxygen and oxyhemoglobin, and also increased the probability of interactions between oxygen and reduced hemoglobin. Hence, since all of its dependent reactions are accelerated, hemoglobin facilitation should operate more efficiently in the shaken vessels.

These results demonstrate that hemoglobin increases oxygen availability to *in vitro* respiring tissues in a manner quantitatively different from that produced by shaking, and that the hemoglobin effect is potentiated by shaking. They also suggest that hemoglobin could be used in tissue respiration studies to maximize Q_{O_2} , particularly when carried out at lower, more physiological P_{O_2} levels.

Summary. Frog skeletal muscle Q_{O_2} increased hyperbolically with increasing P_{O_2} when the latter was varied from 19 to 154 mm Hg. Shaking the reaction vessels at 60 cpm or adding 1% hemoglobin to the media significantly increased muscle respiration. However, while shaking alone increased both the rate of rise of Q_{O_2} with increasing P_{O_2} , as well as the calculated maximum Q_{O_2} at infinite oxygen supply, addition of hemoglobin significantly increased only the rate of

Q_{O_2} rise (maximal effect at $P_{O_2} = 72.5$ mm Hg) but not maximum Q_{O_2} . When the two treatments were tested together, shaking potentiated the hemoglobin effect. A variety of related substances tested did not augment muscle respiration. The results suggest that hemoglobin increases the availability of oxygen to tissues by increasing the rate of diffusion of oxygen from media to cells in a manner related to the facilitated diffusion observed in hemoglobin films.

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Received Jan. 19, 1971. P.S.E.B.M., 1971, Vol. 137.