Cytochrome a,a_3 Reoxidation

EARLY INDICATOR OF METABOLIC RECOVERY FROM HEMORRHAGIC SHOCK IN RATS

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ABSTRACT To assess the metabolic recovery of mitochondria after injury, we have monitored, *in vivo* and noninvasively, changes in the redox state of cytochrome (cyt) a,a_3 in 35 rats after tissue hypoxia induced by rapid exsanguination to a mean arterial pressure of 30–35 mmHg. This level of mean arterial pressure was maintained for a shorter period of time in group I ($n = 17$) and a longer period of time in group II ($n = 18$), then the shed blood was returned by infusion. The surviving animals were observed for 2 more h before terminating the experiments. During exsanguination, reinfusion and recovery intervals brain tissue parameters of blood oxygenation, relative blood volume, and cyt a,a_3 redox state were monitored continuously by spectrophotometry through the closed skull and intact skin. Group I had a high survival rate while group II had a very low survival rate. In both groups, with the onset of hypotension, there was a prompt rapid shift, followed by a slow continued progressive shift, of cyt a,a_3 toward a more reduced state. The extent of recovery of cyt a,a_3 following reinfusion was different in each group. In group I there was a rapid reoxidation of cyt a,a_3 to a level above the base line ($16 \pm 12\%$, mean $\pm$ SEM). In contrast, the extent of reoxidation of cyt a,a_3 in group II was significantly lower and stayed $31 \pm 6\%$ below the base-line level. To further evaluate the mechanisms responsible for these observations, another related experiment was performed. 12 rats were subjected to shock and resuscitation as outlined for groups I and II. After death or killing of the animal, we measured, *in vitro*, oxygen

consumption of cerebral cortical slices. Oxygen consumption of cortical tissue slices in subgroup I was significantly higher than in subgroup II.

We conclude that, under these experimental conditions, the oxidative response of cyt a,a_3 correlates closely with survival or death in the two groups. If in group I animals the greater oxidation of cyt a,a_3 , *in vivo* after resuscitation, reflects greater oxygen utilization, as is suggested by the *in vitro* observations in subgroup I, then we may be observing a useful adaptive response to tissue injury leading to preserved organ function and enhanced survival. Therefore, noninvasively measured cyt a,a_3 redox state, reflecting intracellular metabolic activity, seems to indicate both the overall cerebral cellular response to injury and the likelihood of survival.

INTRODUCTION

The availability of adequate oxygen and substrate is critical for the maintenance of normal intracellular function. Predictably, in tissue hypoxia induced by ischemia and shock, oxidative metabolism is impaired and cellular energy metabolism is grossly disturbed. Associated changes, such as inhibition of mitochondrial activity, have been observed by many investigators *in vitro*, after death of the experimental animal (1–10). It is not clear, however, whether one can extrapolate from these *in vitro* studies to the conditions existing *in vivo*. Recently, noninvasive and nondestructive approaches to cellular bioenergetics have become possible utilizing optical (11) or nuclear magnetic resonance (12) methodologies. Both techniques have potential clinical application. Interference with oxygen delivery to tissue or malfunction of mitochondrial oxidative phosphorylation can be assessed, along with coupling of these bioenergetic processes to functional activity *in vivo* (12). The optical techniques, in par-

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ticular, have been widely tested in living animals. The methodology is based on the fact that an organ, illuminated with appropriate wavelengths, yields signals delineating hemoglobin (Hb)¹ oxygenation and blood content as well as metabolic function. Information about ongoing oxidative metabolism can be obtained by NADH and flavoprotein fluorometry (13) and cytochrome spectrophotometry (14). This information can be interpreted in terms of intracellular oxygen availability and consumption, since oxygen utilization is directly proportional to the rate of electron transfer from cytochrome *c* oxidase (cyt *a*,*a*₃) to molecular oxygen and it has been shown that cyt *a*,*a*₃ redox state responds appropriately to variations in the level of tissue oxygenation within the physiological range in situ (15). Thus, changes in the in vivo redox state of cyt *a*,*a*₃ have served to indicate intracellular oxygen sufficiency and bioenergetic utilization (14–21).

Significant data are currently available showing the biochemical changes associated with a well-established model of brain ischemia originally developed by Nordström, Rehncrona, and Siesjö (22). This model, using bilateral carotid artery occlusion, has been tested in this laboratory using optical techniques (17, 23). In the present experiments we have extended our observations to the hemorrhagic shock model, a condition very similar to clinically observed hypovolemic shock.

Recently, we have demonstrated that in hemorrhagic shock cyt *a*,*a*₃ reduction is a much earlier and more sensitive indicator of perturbed intracellular aerobic metabolism than is tissue PO₂ in cat cerebral cortex in vivo (24). The present experiments were performed in order to answer the following questions: (a) in hemorrhagic shock, to what extent is the reduction of cyt *a*,*a*₃ a reversible process? (b) what is the relationship between the reversibility of reduced cyt *a*,*a*₃ and recovery of the animal?

METHODS

In vivo experiments

Animal preparation. 35 adult male Wistar rats (350–400 g) were anesthetized with intraperitoneal injection of sodium pentobarbital (30 mg/kg). After insertion of an endotracheal tube the animal was ventilated with a gas mixture containing 30% oxygen (balance nitrogen) by means of a rodent respirator (model 607, Harvard Apparatus Co., Inc., S. Natick, MA). Cannulas were inserted into one femoral vein and both femoral arteries, and then heparin sodium (140 U/100 g) was administered intravenously. Arterial blood samples were analyzed for PO₂, PCO₂, and pH values in an Instrumentation Laboratory unit (IL-113-01-UM, Instrumentation Laboratory, Inc., Boston, MA). The tidal volume of the ven-

tilator was adjusted to maintain arterial PCO₂ between 35–40 mmHg and arterial PO₂ > 100 mmHg. Arterial blood pressure was constantly monitored via a Statham P23 Db pressure transducer (Statham Medical Instruments, Inc., Oxnard, CA). A constant body temperature, ~37°C, was maintained by a heating blanket.

Spectrophotometric measurements. Hair was removed from both sides of the head, using a depilatory cream. The head of the animal was positioned between an optical fiber bundle on one side and a photomultiplier detector on the other side, thus permitting transillumination directly through the head in the region immediately posterior to the eyes. Black cloth was then draped over the preparation in order to exclude extraneous light.

The techniques for monitoring the redox state of cyt *a*,*a*₃, using a differential spectrophotometer have been described previously (19, 20, 25). Briefly, cyt *a*,*a*₃ contains two copper atoms with low and high redox potentials designated CU_L and CU_H, respectively. When oxidized, the copper atoms have largely overlapping absorption bands in the near infrared (NIR) region; the reduced forms lack this absorption. Because of the depth of NIR penetration, these two copper atoms can be monitored quantitatively in this region of light spectrum without a need for craniotomy, thus permitting continuous measurement of the redox state of cyt *a*,*a*₃. When oxidized, a broad absorption band exists in the 800–900 nm region of the NIR, with a maximum ~845 nm. When oxygen becomes scarce, due to hypoxia or oligemia, a shift occurs in the total population of cyt *a*,*a*₃ molecules to a more reduced state. Spectrophotometrically, this is signaled by a decrease in optical density at 845 nm regions.

Since hypoxia is associated with changes in the oxyhemoglobin (HbO₂) content of arterial blood and cerebral hemodynamic parameters it is necessary to compensate for changes in blood PO₂ and in total organ blood volume. This can be accomplished by a differential technique described below. At 883 and 784 nm, respectively, the Hb-HbO₂ difference spectrum has equal optical density changes but of opposite sign. When these signals are averaged [$0.5 \times (883 + 784)\text{nm}$] they indicate the relative change in blood volume. The optical density change, which reflects relative blood volume changes, is subtracted from the 845-nm signal, yielding a nonambiguous value for cyt *a*,*a*₃ redox state. Since oxidized cyt *a*,*a*₃ does produce some light attenuation (absorption) at these two wavelengths the enzyme signal obtained in this way is an underestimate of the true cyt *a*,*a*₃ redox changes. No claims concerning quantitatively absolute concentration changes are made in the present article. The signal derived from 883 minus 784 nm reflects oxygenation changes of the total blood in the optically surveyed tissue. The three signals were displayed simultaneously and independently on a strip chart recorder.

In all experiments the optical conditions in a strictly controlled physiological state [Nembutal anesthesia, partial arterial oxygen pressure (P_aO₂) > 100 torr, partial arterial carbon dioxide pressure (P_aCO₂) = 37.5 (±2.5) torr] was interpreted to reflect base-line normoxia. For instrument calibration the monochromator output signals were trimmed to 1.00 V using the monochromator slit height attenuators. At the end of each experiment the animal was ventilated with 100% nitrogen. After 10 min both reduction of cyt *a*,*a*₃ as well as the maximum Hb desaturation from the base line were recorded and given the value of 100. All experimental observations in reduction of cyt *a*,*a*₃ and desaturation of Hb were then expressed as a fraction of the signal change between base line (0%) and anoxia (100%). When oxidation of cyt *a*,*a*₃ and Hb occurred to a level above the base line these

¹ Abbreviations used in this paper: cyt *a*,*a*₃, cytochrome *c* oxidase; Hb, hemoglobin; NIR region, near infrared region.

changes were simply expressed in equal percentage terms. No attempt was made to determine in this animal model whether the base-line state of cyt a,a_3 reduction corresponded quantitatively to 85% reduction observed in another in situ experiment in which boundaries of redox changes were probed with hyperbaric hyperoxia as well as anoxia (21).

Shock protocol. After monitoring base-line conditions for 10 min, tissue hypoxia was induced by exsanguination, via an automatic pump, over a 6-min period to a mean arterial pressure of 30–35 mmHg according to a modification of Wigger's technique (26). The mean arterial pressure was regulated by intermittent minimal removal of blood over an interval of 40–50 min until the desired level of hypotension became stable with no further requirement for withdrawal of blood. All the blood withdrawn was stored in a syringe containing 0.15 ml heparin sodium. In 17 rats (group I), all the shed blood was reinfused immediately after the stabilization of blood pressure. In the remaining 18 animals (group II) this level of hypotension was maintained for a longer interval until the blood pressure began to fall progressively below the predetermined level. Then all the shed blood was reinfused over a 6-min period. The duration of shock was defined as extending from the end of rapid exsanguination to the beginning of blood reinfusion in each animal. Four animals died before reinfusion of blood and were excluded from the study.

Following reinfusion of shed blood, each surviving animal was monitored closely for 2 h, unless death supervened. Reversibility of shock was considered to have occurred after reinfusion if the blood pressure increased to the base-line (prehemorrhage) level or higher and remained so for 2 h. Shock was considered irreversible if, after reinfusion, the restoration of a base-line pressure proved temporary, with a subsequent progressive decline in mean arterial pressure to 60 mmHg or less. In each group, those animals that died or developed irreversible shock within the 2 h post reinfusion period were included in the overall mortality.

During the entire experimental period the redox state of cyt a,a_3 , Hb oxygenation in tissue, and the relative volume of blood in the optically surveyed tissue were continuously monitored. For analysis the continuous measurements were summed at 10-min intervals and tabulated. These computations and points recorded at key times in the experiment (the end of acute hemorrhage, before and immediately after the reinfusion) form the basis of all calculations and figures.

In vitro measurement of oxygen consumption of cerebral cortex

To elucidate further the in vivo observations, we measured cerebral oxygen consumption in vitro after parallel experiments. We chose to use intact slices obtained from cerebral cortex as the closest model to our in vivo experiments. This decision was based on the recognition that after extensive processing and purification, isolated mitochondria behave differently when compared with the in vivo model (14).

Animal preparations. In 12 rats, subdivided into two equal groups (subgroup I and subgroup II), direct in vitro measurements of cerebral cortical oxygen consumption were made after identical treatment with respect to preparation, hypovolemic shock, and resuscitation as described in the in vivo experiments. Immediately after death or killing the skull was opened, then the brain was removed and incubated in the ice-cold Krebs-Ringers phosphate buffer.

Tissue preparation. The cortex was separated and uni-

form slices of 0.35-mm thickness, oriented parallel to the outer surface, were rapidly cut from each lateral hemisphere using a McIlwain tissue chopper (Brinkmann Instruments, Westbury, NY). This 0.35-mm slice thickness was chosen after a series of pilot experiments performed on normal control rats. We found, as has been described elsewhere (27), that diffusion of oxygen through brain slices of this thickness provides optimal respiratory characteristics.

Polarographic measurement of oxygen consumption. Techniques for in vitro measurement of oxygen consumption of brain and other tissues have been well described (27–29) and were used here. Four 30–40-mg slices, obtained systematically from both hemispheres were used for measurement of oxygen consumption by one animal. Each sample was measured separately in a water-jacketed cell and the mean of the four measurements is reported here. The cell (Gibson Mechanical Electronics, Middleton, WI) contained 1.5 ml of Krebs-Ringer phosphate buffer incubation media, pH 7.4, and 5 mM glucose with a stirring rate of 2–3 spinbar. The polarographic cell was fitted with a Clark oxygen probe (model 4004, Yellow Springs Instrument Co., Yellow Springs, OH). Measurements of oxygen consumption were made within 1 h after the death or killing of the animal. For each sample oxygen consumption was determined at O_2 concentrations ranging from 0 to 90%. The electrode was calibrated by equilibration of the solution with selected gases containing O_2 and N_2 concentrations. The oxygen consumption was calculated from the slope of the polarographic output after the solution had been equilibrated with 100% O_2 and addition of specimens. Oxygen consumption was expressed as micromoles $O_2 \cdot h^{-1} \cdot g$ dry weight $^{-1}$. On a series of pilot experiments, using normal rats as controls, the base-line levels of cerebral cortical oxygen consumption at various oxygen concentrations were determined (Fig. 1). The rate of oxygen consumption obtained at 90% concentration ($401 \pm 44 \mu\text{mol } O_2 \cdot h^{-1} \cdot g$ dry wt $^{-1}$) was relatively close to values reported by Elliott (28) for rat cerebral cortex at 95% O_2 plus CO_2 ($500 \mu\text{mol } O_2 \cdot h^{-1} \cdot g$ dry wt $^{-1}$).

Statistical analysis. Data are expressed as the mean $\pm$ SEM. Means were compared either by Student's t tests (paired or unpaired), or Fisher's exact test. $P < 0.05$ was taken as a significant difference.

RESULTS

Fig. 2 is a typical example showing the effects of gas shift on cyt a,a_3 redox state, Hb oxygenation, and cerebral blood volume. When the animal was switched from 30% oxygen to room air some reduction of cyt a,a_3 occurred, Hb saturation was reduced and cerebral blood volume decreased. All of these parameters were reversed when the animal breathed 100% oxygen. Base-line values were restored when 30% oxygen was again used.

Figs. 3 and 4 demonstrate the response of these parameters to acute blood loss and reinfusion, respectively. The kinetics of reduction of cyt a,a_3 and desaturation of Hb were very characteristic. The changes in cyt a,a_3 redox state and Hb oxygenation in response to hemorrhage and shock can be observed in a typical case depicted in Fig. 3. There is rapid reduction of cyt a,a_3 and desaturation of Hb followed by continuous

FIGURE 1 Cerebral cortical oxygen consumption in normal control rats. 30–40-mg slices obtained from cerebral cortex were suspended in Krebs-Ringer phosphate buffer. The oxygen consumption was calculated from the slope of the polarographic output after the solution had been equilibrated with 100% oxygen. Mean $\pm$ SEM, $n = 5$.

slow decrements (Fig. 3). The rate of reduction of cyt a,a_3 was significantly faster than the shift of Hb to a less saturated state. When cyt a,a_3 was $50\pm 6\%$ reduced there was only $25\pm 3\%$ desaturation of Hb ($P < 0.05$). These changes slow to a plateau within 40–60 min and remain stable until the shed blood is reinfused. In gen-

eral, $>70\%$ of the observed cyt a,a_3 reduction and Hb unsaturation occurred within the first 20-min of exsanguination.

The mean duration of shock was 46 ± 5 min for group I and 63 ± 8 min for group II. There was no statistically significant difference between the two groups in the

FIGURE 2 Effects of ventilation with different concentrations of oxygen on cyt a,a_3 redox state, Hb oxygenation, and relative cerebral blood volume in rat brain. Note the reduction of cyt a,a_3 , deoxygenation of Hb, and decrease in cerebral blood volume when ventilation is switched from 30% O₂ to room air. All these parameters reverse with 100% O₂ and return to their base line with 30% O₂. Monitoring was done noninvasively by transillumination spectrophotometry through the closed skull and intact skin.

FIGURE 3 Continuous recording of changes of relative cerebral blood volume, Hb saturation, and cyt a,a_3 redox state in response to acute hemorrhage and shock in rat brain. Note the rapid, followed by a slow progressive, decrease of relative blood volume, deoxygenation of Hb, and shift of cyt a,a_3 toward reduction. These parameters were monitored noninvasively by transillumination spectrophotometry through closed skull and intact skin.

volumes of blood withdrawn and reinfused. There was no statistically significant difference in the amount of cyt a,a_3 reduction and Hb deoxygenation at the end of shock period (just before blood reinfusion) between the two groups. No statistically significant difference was observed in base line and post reinfusion mean arterial pressure within each group (101 ± 4 vs. 101 ± 4 for group I and 89 ± 7 vs. 91 ± 10 mmHg for group II) as well as between the two groups. After reinfusion of blood, reoxidation of cyt a,a_3 occurred in both groups; maximal values were noted ~ 4 – 6 min after the replacement of shed blood. The degree of this reoxidation differed, however, between the two groups. In group I (Fig. 5) there was rapid reoxidation of cyt a,a_3 with a mean overshoot of $16 \pm 8\%$ above the base-line value ($P < 0.05$). A gradual decrease in overshoot was observed over the following 20 min, after which the level of oxidation of cyt a,a_3 reached a new steady state above the base-line and remained at this level until the termination of the experiments. In group II, on the other hand, the extent of reoxidation of cyt a,a_3

was significantly lower ($P < 0.001$) remaining $31 \pm 6\%$ below the base line (Fig. 6). Despite additional reoxidation of cyt a,a_3 during the ensuing 10 min the subnormal redox state persisted throughout the remainder of the experiment.

15 of 17 animals (88%) in group I were alive and had normal mean arterial pressure at the end of 2 h post reinfusion period while only 2 of 18 animals (11%) in group II survived and had a mean arterial pressure more than 60 mmHg ($P < 0.001$). Of the remaining 16 animals in the latter group, 8 of them died after exhibiting irreversible shock within the 2 h post reinfusion period. Only one of these eight animals died sooner than 40 min after reinfusion. Eight other rats in group II were alive at the end of the 2-h observation period but already had developed irreversible shock with a mean arterial pressure of 60 mmHg or less. The relationship of survival to the completeness of cyt a,a_3 reoxidation is summarized in Table I. Since there were no significant differences in the time course of cyt a,a_3 reoxidation within group II animals there is a reason-

FIGURE 4 Continuous recording of changes of relative cerebral blood volume, Hb saturation, and cyt a, a_3 redox state of rat brain in response to reinfusion following the induction of hemorrhagic shock. Note the rapid increase in blood volume and reoxygenation of Hb and cyt a, a_3 after reinfusion. These parameters were monitored noninvasively by transillumination spectrophotometry, through the closed skull and intact skin.

able presumption that the altered redox state relates in some direct manner to the mechanism leading to death.

Immediately after reinfusion of blood in group I, reoxygenation of Hb was incomplete (Fig. 5). The difference was statistically significant ($P < 0.05$) with a mean O_2 saturation remaining $12 \pm 8\%$ below base-line levels. Despite some later increase in Hb oxygenation this value stayed below the base line in group I, for the remaining period of experiment. The animals in group II demonstrated on the average reoxygenation of Hb, immediately after reinfusion, to a value that did not differ significantly from base-line levels ($P > 0.05$) (Fig. 6). Oxygenation of Hb gradually decreased to a new level below the base line and was associated with a simultaneous early increase in cyt a, a_3 oxidation, although the level remained below the base-line value throughout the experiment. At a later stage, when irreversible shock and circulatory collapse developed, all parameters fell.

Relative cerebral blood volume decreased rapidly

initially and then fell slowly after acute hemorrhage and before reinfusion of blood in both groups (Figs. 5 and 6). In neither group did relative cerebral blood volume return to the base-line level after reinfusion (Figs. 5 and 6). Immediately after reinfusion, cerebral blood volume was significantly greater in group I ($83 \pm 8\%$ vs. $57 \pm 6\%$ of base line in group II, $P < 0.01$). In group I, after a transient fall, relative blood volume remained constant for the remaining period of the experiment (Fig. 5). In group II, by contrast, a gradual further increase in relative blood volume was observed. Blood volume remained stable for a period of ~ 70 –80 min, then a rapid fall in all parameters occurred in association with overt circulatory collapse.

Among animals utilized for in vitro experiments the survival rates in the two subgroups were similar to groups I and II. Four of six animals (66%) in subgroup I were alive and had normal mean arterial pressure at the end of 2-h post reinfusion period while none of the six animals (0%) in subgroup II survived or had a mean arterial pressure > 60 mmHg. Four rats in the

FIGURE 5 Changes of relative cerebral blood volume, Hb saturation, and cyt a_3 redox state in response to hemorrhagic shock and resuscitation in group I rats with high survival rate. Note the hyperoxidation of cyt a_3 and incomplete reoxygenation of Hb after blood reinfusion. Note the lack of complete return of relative blood volume to base-line levels after reinfusion. Each point represents the mean $\pm$ SEM for $n = 17$.

FIGURE 6 Changes of relative blood volume, Hb saturation, and cyt a_3 redox state in response to hemorrhagic shock and resuscitation in group II rats with low survival rate. Note the lack of complete reoxidation of cyt a_3 . Complete reoxygenation of Hb is seen immediately after reinfusion. Note the lack of complete return of relative blood volume to base-line level after reinfusion. Each point represents the mean $\pm$ SEM for $n = 18$.

TABLE I
Comparison of the Extent of Reversibility of Cyt *a*, *a*₃
Following Blood Reinfusion and Survivorship of Animals

	Lived Cyt <i>a</i> , <i>a</i> ₃ recovery		Died Cyt <i>a</i> , <i>a</i> ₃ recovery	
	Complete	Incomplete	Complete	Incomplete
Group I (<i>n</i> = 17)	15	0	1	1
Group II (<i>n</i> = 18)	1	1	0	16

latter group were alive at the termination of the experiment, but already had developed irreversible shock with the mean arterial pressure of 60 mmHg or less. The remaining two died of progressive shock at ~70–80 min post reinfusion.

The rates of oxygen consumption in cortical tissue slices from the two subgroups of rats were different. In subgroup I oxygen consumption was significantly higher ($P < 0.05$) than in subgroup II, at essentially all concentrations of oxygen. The rate of oxygen consumption obtained in the two subgroups are shown in Table II and Fig. 7. Although the rate of oxygen consumption in subgroup II rats was somewhat higher than normal control rats, a statistically significant difference could not be demonstrated between the two populations.

DISCUSSION

Previous studies have indicated that an increased mortality can be anticipated if hypotension is allowed to persist to the point that the blood pressure becomes unstable and falls below desired levels. Replenishment of blood at this time rarely restores a stable base-line blood pressure (30). Under these experimental conditions cerebral anoxia and respiratory failure will be the most frequent cause of death (31). If reinfusion of blood takes place during the period when the blood pressure is still stable, however, full recovery ensues. The recovery of >80% of the group I rats in our experiment is consistent with 75% survivorship reported by Butcher and Braitberg (30) following induction of 60 min of shock. This prior demonstration of a time-related reversible phase of experimental hypovolemic shock led to our choice of a similar model to test the relationship of reversibility to mitochondrial bioenergetics.

We report the new observation that, after tissue injury induced by hemorrhagic shock, survival can be correlated directly with restoration of the base-line redox state of cyt *a*, *a*₃. In this experimental model full reoxidation was a reliable predictor of survival after

resuscitation. There was a strong correlation as well between incomplete reoxidation and death during the observation period in animals subjected to more prolonged hypotension.

The shift toward reduction of cyt *a*, *a*₃ and deoxygenation of Hb in response to hemorrhage are fully expected and have been observed previously during hypoxic injury in tissue subjected to similar manipulations (14, 32, 34, 36, 37). The kinetics of cyt *a*, *a*₃ reduction and Hb deoxygenation in response to tissue hypoxia in these experiments are entirely consistent with previously reported changes in brain tissue PO₂ in this laboratory (24), as well as observations by others (35). The decrease in relative cerebral blood volume after reinfusion may be due to the hemodilution commonly seen in shock (26). With the optical technique used here, we monitor the relative amount of Hb in the tissue and this value may not correlate directly with cerebral blood flow; during hemodilution cerebral blood flow may increase while the amount of Hb in the surveyed optical field decreases.

Blood O₂ saturation in surveyed cerebral cortex of group I animals (Fig. 5) immediately following reinfusion was lower than normal and lower than in group II (Fig. 6), although a statistically significant difference could not be demonstrated between the two groups. The observed desaturation for group I becomes physiologically more significant when correlated with concurrent larger relative blood volumes. This observation, after replenishment of shed blood, of a larger relative blood volume, a greater than base-line reoxidation for cyt *a*, *a*₃ (Fig. 5) and of persistent relative

TABLE II
Comparison of the Rate of Cerebral Cortical Oxygen
Consumption in the Two Subgroups of Rats

O ₂ concentration	Micromoles O ₂ consumed · h ⁻¹ · g dry weight ⁻¹		
	Subgroup I	Subgroup II	<i>P</i> *
%	mean ± SEM		
2	137 ± 13	143 ± 17	NS
10	348 ± 24	260 ± 24	<0.05
20	456 ± 76	338 ± 41	NS
30	561 ± 55	384 ± 51	<0.05
40	625 ± 60	408 ± 58	<0.05
50	643 ± 65	429 ± 65	<0.05
60	686 ± 73	454 ± 63	<0.05
70	740 ± 86	489 ± 69	<0.05
80	864 ± 86	532 ± 79	<0.02
90	897 ± 86	553 ± 63	<0.001

Oxygen consumption was measured polarographically in slices obtained from rat cerebral cortex immediately after the death or killing of the animal.

* Unpaired *t* test with *n* = 12.

FIGURE 7 Comparison of the rate of cerebral cortical oxygen consumption in subgroup I with high survival rate and subgroup II rats with low survival rate. Note that the oxygen consumption in subgroup I ($n = 6$) was significantly higher than subgroup II rats ($n = 6$). Cerebral cortical oxygen consumption was measured as described in Fig. 1.

desaturation of Hb are consistent with enhanced oxygen utilization. Conversely the finding in group II animals, under otherwise comparable conditions, that cyt a,a_3 remained in a reduced state and blood O_2 saturation remained relatively normal (Fig. 6) is consistent with decreased utilization of oxygen by mitochondria. The observations in tissue slice in subgroups I and II are also consistent with this interpretation of in vivo events. It is noteworthy that Jöbsis et al. (36) showed that, in contrast to in vitro, under in vivo conditions the rate of electron transfer to oxygen molecules ($\dot{V}O_2$) from cyt a,a_3 correlates with the degree of oxidation for cyt a,a_3 . These investigators observed oxidation of this cytochrome during increased energy-utilizing cortical activity induced by seizure (36), which is known to be associated with increased oxygen utilization and simulates the state 4 to state 3 transition of isolated mitochondria in vitro. More extensive measurements, later on, on the excised toad brain confirmed these observations (37).

What accounts for the greater than base-line oxidation of cyt a,a_3 following blood reinfusion? One proposed explanation is greater O_2 availability accompanying reactive hyperemia due to increased cerebral blood flow after resuscitation. Although we did not measure cerebral blood flow in our experiments, previous observations suggest that increased perfusion is an unlikely mechanism. In related experiments the occurrence of reactive hyperemia required total interruption of cerebral blood flow; minimal residual perfusion prevented a hyperemic response after res-

toration of blood flow (39). In our experiments, cerebral perfusion was certainly decreased but not interrupted completely. Even more relevant, perhaps, is the recent observation by Frischka et al. (40) that cortical blood flow did not increase after reinfusion in a dog model of hemorrhagic shock. This observation is particularly pertinent because, in our experiments, the mitochondrial signal is obtained almost entirely from the cortex (33). Our in vitro observations in brain slices are also consistent with these observations, since the increased oxygen consumption occurred in a diffusion-limited system wherein perfusion played no role.

The initial overshoot of cyt a,a_3 reoxidation may be explained best on the basis of increased oxidative ATP production as a result of high energy demands of the tissue for restoration of lost ion gradients, cytosolic Ca^{++} , etc., leads to a higher O_2 consumption (33). This presumed transient requirement, however, is unlikely to account for the persistence of some hyperoxidation of cyt a,a_3 until the end of the 2-h observation period. The presumed in vivo differences in oxygen consumption by cyt a,a_3 in the two groups were observed unambiguously in vitro in slices obtained from cerebral cortex. In the latter experiments, oxygen consumption in tissue slices was significantly higher in subgroup I, when compared with normal control and subgroup II rats. The likely inference for the in vivo observation is that after the initial injury induced by shock cyt a,a_3 activity alters and continues to utilize more oxygen. These findings are in agreement with prior observations made by Mela et al. (41) in isolated mitochondria

and in *in vivo* studies reported from this laboratory (42). Mela et al. (41) found a significant increase in liver and heart mitochondrial respiratory activity of rats exposed to low-inspired oxygen for 5 min to 3 h. Respiratory activity in state 3 and the turnover of cytochrome oxidase increased after hypoxia and decreased in response to hyperoxic exposures. These observations are similar to our recent observations in another experiment comparing the kinetics of tissue PO_2 and cyt a,a_3 redox state in response to hypoxia induced by nitrogen breathing in cats (42). After brief anoxia, followed by restoration of oxygenation, a marked hyperoxidation of cyt a,a_3 occurred while tissue PO_2 remained near zero. Then cyt a,a_3 activity returned gradually toward the base line over a long interval of time. Analysis of these results was consistent with the hypothesis that after brief periods of anoxia the affinity of cyt a,a_3 for oxygen increased. Similarly, in this study, after reinfusion, the initial cyt a,a_3 overshoot was followed by both an increase in oxygenation of Hb, probably reflecting a greater availability of oxygen, and a gradual decrease in the extent of oxidation of cyt a,a_3 during the subsequent period of observation. Since higher oxidation of cyt a,a_3 following blood reinfusion was associated with higher survival rate in group I animals, and under comparable circumstances *in vitro* oxygen utilization increased, a reasonable hypothesis is that this phenomenon reflects an adaptive mechanism occurring in mitochondria after hypoxic injury. There may well be a prolonged requirement for increased oxygen consumption in order to repair the sequelae of injury, accounting for sustained hyperoxidation of cyt a,a_3 .

The mechanisms responsible for this "adaptation" are not known and can only be postulated on the basis of observations made by other investigators. Overoxidation of cyt a,a_3 after reinfusion may be the result of augmented respiration in uncoupled mitochondria. This phenomenon in mitochondria, after cerebral ischemia or hypoxia, has been reported from several laboratories under *in vitro* (43–47) as well as *in vivo* (17) conditions. Since free fatty acids inhibit oxidative phosphorylation due to uncoupling of ATP formation from oxygen consumption (48), the known increase of these substrates in both shock (49) and brain ischemia (50) might have triggered the uncoupling process. If uncoupling of the oxidative phosphorylation and increased O_2 utilization does occur, one biochemical link may be the presence of an energy-conservation site at cyt a,a_3 or between a_3 and O_2 as has been proposed already (21, 33). Loss of such a conservation site would result in an increased rate of O_2 uptake and an oxidation of cyt a,a_3 .

What accounts for the different mitochondrial ki-

netics and high mortality rate in the rats studied after delayed reinfusion in this experiment? We relate the incomplete reoxidation of cyt a,a_3 after delayed reinfusion to interstitial and mitochondrial edema. Because of increased capillary permeability in the late stage of shock (51) reinfusion of blood at this stage will result in a significant increase of interstitial edema. The resulting interstitial edema will not only decrease the total blood volume, a pronounced finding in this group, but would also compromise blood flow and oxygen delivery from capillaries to mitochondria. In fact, in subgroup II animals, when brain slices were exposed to adequate concentrations of oxygen *in vitro*, wherein diffusion alone limited aerobic metabolism, the O_2 uptake was normal, but not high as was the case for subgroup I. Since mitochondrial edema is frequently observed at this stage of shock (52, 53), this phenomenon may be responsible for the lack of overoxidation of cyt a,a_3 in group II as opposed to Group I. Alterations of mitochondrial function by mitochondrial edema have been well documented in intact cells as well as in isolated mitochondria (54, 55). Although "adaptive" phenomena may have occurred in group II rats, either interstitial edema or subsequent mitochondrial injury may have prevented observation of overshoot and subsequent recovery. Normal, rather than decreased levels of oxygen consumption in brain slices in this group is consistent with this hypothesis. Extrapolation of these intracellular changes from brain tissue to other vital organs may explain the increased mortality as well.

Siesjö et al. (56) and Rehncrona et al. (57) have speculated that free radicals may initiate irreversible damage after incomplete ischemia. Others have found that long-term ischemia was associated with increased free radical production in cerebral and other tissues (58, 59), and suggested that they may lead to tissue damage (59, 60).

The existing data, including our own, do not allow a choice between these various proposed mechanisms. We hypothesize, however, that in reversible shock as a result of initial injury induced by poor tissue perfusion, an adaptive mechanism develops in mitochondria leading to increased respiratory activity. This adaptation is characterized by increased oxygen utilization by mitochondria either *in vivo*, when tissue perfusion is restored, or *in vitro* when oxygen is provided to tissue, and is associated with a complete recovery of the animals studied in a parallel manner. This recovery can be detected spectrophotometrically and is characterized by hyperoxidation of cyt a,a_3 . On the other hand, in irreversible hemorrhagic shock, as a result of continuous decreased tissue perfusion, the apparently useful hyperoxidation of cyt a,a_3 does not

occur. At this stage, the failure of restored perfusion to induce full reoxidation of reduced cyt a,a_3 may account for the observed high mortality rate.

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