In Vivo Comparison of Cerebral Tissue P_{O_2} and Cytochrome aa_3 Reduction-Oxidation State in Cats during Hemorrhagic Shock

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ABSTRACT To assess the adequacy of oxygen availability and utilization within the cerebral cortex in vivo, we have measured the partial pressure of oxygen in tissue (P_tO_2), as well as the reduction-oxidation state of cytochrome *c* oxidase (cyt aa_3) during shock induced by slow or rapid hemorrhage in anesthetized cats. P_tO_2 was measured with pyrene-butyric acid-generated fluorescence in cerebral cortical cells. Cyt aa_3 redox state was measured by the absorption of monochromatic light at 605 nm absorption peak of the enzyme reflected from the same cortical field. The P_tO_2 remained within the normal range until either 30±1.5 ml blood/kg was removed or the mean arterial pressure fell by 70±5% of base line. Beyond either point, the P_tO_2 fell rapidly to a low value approximating zero. By contrast, the reduction of cyt aa_3 began early when as little as 5 ml blood/kg was removed. Thereafter, the shift toward reduction was progressive and continuous with a slow rate at first and a rapid rate later. This accelerated rate of cyt aa_3 reduction preceded the rapid fall of P_tO_2. We conclude that, under these experimental conditions, cyt aa_3 reduction is a much earlier and more sensitive indicator of perturbed intracellular aerobic metabolism due to hemorrhage that is P_tO_2.

INTRODUCTION

Shock is associated with a generalized inadequacy of blood flow, to the extent that the tissue metabolism is deranged because of poor delivery of oxygen and substrate. In the absence of a continuously available supply of oxygen molecules, aerobic intracellular metabolism stops, cellular functions cease, and irreversible tissue damage rapidly results (1).

Evidence supporting a generalized inhibition of mitochondrial activity by shock and ischemia has been accumulated (2–9). Alterations such as uncoupling of phosphorylative activity from respiration, inhibition of ATPase enzyme activity, and defective ion transport are evident (3, 4). There is a progressive conversion of the pyridine nucleotides to the reduced form in the mitochondria of the livers in rats during shock (10). Structurally, the mitochondria swell, broken membranes are demonstrable, and in the final stages complete disappearance of the inner mitochondrial structure is apparent (11). The significance of these structural changes in the pathophysiology of shock is not fully elucidated, however, because this sequence occurs in a setting of prolonged shock and is observed, typically, after death. Thus, the importance of in vivo monitoring of the ongoing mitochondrial oxygen sufficiency and utilization in tissue ischemia induced by shock is apparent.

Because mitochondria use most of the oxygen available (12), and because cytochrome *c* oxidase (cyt aa_3)[1] is the most immediate reductant of oxygen in the electron transport system, we chose to monitor the changes of this cytochrome as an index of oxygen use by the mitochondria. Monitoring of the redox state of cyt aa_3 in the living brain is now possible by dual-beam reflection spectrophotometry (13, 14). In addition to monitoring changes in oxygen use, we also monitored oxygen availability by measurement of partial pressure of oxygen in the same tissue (P_tO_2) by optical technique. This was possible by applying the described tech-

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[1] *Abbreviations used in this paper*: cyt aa_3, cytochrome *c* oxidase; F, fluorescence emission; PBA, pyrene butyric acid; P_tO_2, partial pressure of oxygen in tissue; R, reflected light.

niques using pyrene butyric acid (PBA) as an in vivo fluorometric indicator for P_tO_2 (15, 16). The optical arrangement for noninvasive determination of regional P_tO_2 and cyt aa_3 redox state in the same cortical field enables us to compare the dynamics of both of these parameters in response to blood loss.

Our reasons for these experiments were to answer the following questions: (*a*) At what point of poor tissue perfusion due to hemorrhagic hypotension and shock does derangement in the mitochondrial oxygen use occur? (*b*) What are the relationships between tissue oxygen availability and use in hemorrhagic hypotension and shock?

We found that in shock induced by progressive hemorrhage the redox state of cyt aa_3 was a more sensitive indicator of cerebral tissue hypoxia than was P_tO_2.

METHODS

Biological preparations and surgical procedures. 18 adult cats of both sexes were anesthetized with intraperitoneal injections of pentobarbital sodium (30 mg/kg). A tracheal tube was inserted, and after the intravenous administration of 5 mg/kg of gallamine triethiodide (Flaxedil; Davis & Geck, Pearl River, N. Y.), the animal was put on a mechanical ventilator (model 607, Harvard Apparatus Co. Inc., S. Natick, Mass.). Arterial blood samples were analyzed for P_{O_2}, P_{CO_2}, and pH values in an Instrumentation Laboratory Unit (IL 113-01-UM, Instrumentation Laboratory, Inc., Boston, Mass.). The tidal volume of the ventilator was adjusted to obtain a mean (±SE) arterial P_{CO_2} of 30.6±0.7 mm Hg and an arterial P_{O_2} between 100 and 120 mm Hg. Cannulas were inserted into one femoral vein and both femoral arteries. The brain was exposed through limited bilateral craniotomies, ~15 × 20 mm. The dura was removed, and a saline-soaked polyethylene film was used to eliminate the possible contamination of P_tO_2 by atmospheric oxygen. Arterial blood pressure was constantly monitored via a Statham P23 Db pressure transducer (Statham Medical Instruments, Inc., Oxnard, Calif.). Constant normal body temperature was maintained by a heating blanket. An electrocorticogram was obtained by a silver electrode, touching the pia, contralateral to the optical field. This electrode was referenced against the neck musculature.

Sodium PBA was prepared from pyrenebutyric acid (Eastman Kodak Co., Rochester, N. Y.) as described by Mitnick and Jöbsis (15).

Measurement of P_tO_2. The fluorometer used to monitor PBA fluorescence has been described (15, 17). Briefly, this unit consists of a water-cooled mercury arc lamp, appropriate optical filters, and photomultipliers, all incorporated into a 4320-Ernst Leitz microscope, (E. Leitz, Inc. Rockleigh, N. J.). A 340-nm optical filter was used for PBA excitation, and the fluorescence emission was monitored at 397 nm. The microscope was focused on a 3.8-mm wide cortical field along the midsuprasylvian gyrus.

Sodium PBA (25 mg/kg) was dissolved in 20 ml distilled water and injected intravenously over a 3–5-min period. Fluorescence from the probe increased rapidly and finally stabilized within 5 min at a level averaging 8.5 times the autofluorescence of the brain before injection (Fig. 1). The signal remained stable for at least 7 h, the maximal period of observation. Blood taken from animals after 5 min showed no PBA fluorescence, indicating cellular uptake, and also that blood PBA does not contribute to fluorescence signal. Both PBA fluorescence emission (F) and excitation light reflected (R) from the brain were monitored. Changes in reflected excitation light are primarily caused by changes in the volume of blood in the optical field (18). Correction for hemoglobin absorption in the optical field was made by an electronic circuit which subtracted the reflected excitation light signal from the emitted fluorescence signal which is also affected by blood volume changes. The reflected light subtracted from the emission signal (F − R) produced fluorescence values corrected for hemoglobin changes (17, 19). Methodology and rationale for deriving the corrected fluorescence signal essentially involves injecting a bolus of saline into a catheter positioned in the vena cava near the heart, causing hemodilution, and adjusting the amplification ratio of the two signals until there is no net change in the (F − R) signal as a result of the bolus (15, 17, 18).

In a series of control experiments when animals were ventilated with a low concentration of oxygen (15% O_2 + 85% N_2), the PBA fluorescence signal increased. It reached its maximum level when complete anoxia was induced by ventilating the animal with 100% nitrogen. A stepwise increase of the inspired concentration of oxygen resulted in a decrease in the PBA fluorescence signal and reached its lowest level when 100% oxygen was inspired by the animal.

For instrument calibration and expression of the results, a scale of 0–100% was used. The 0% fluorescence was that with no fluorescence-reflected light reaching the photomultiplier tube, and the 100% fluorescence signal was the light level measured from the surface of the brain in its resting or base-line state. Optical changes with anoxia or hemorrhage are then expressed as a fraction of this 0–100% scale. The time constant for signal acquisition was commonly 2 s, i.e., 2 s for reaching 63% deflection after a step change in optical input.

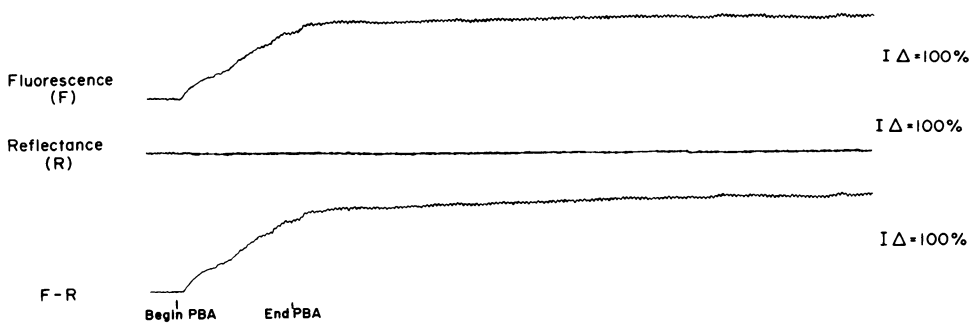

FIGURE 1 Increase in PBA fluorescence from brain following the intravenous injection of PBA during the interval indicated; (increase upward).

The Stern-Volmer equation was used to compute the P_tO_2 in relation to PBA fluorescence using Longmuir and Knopp's (20, 21) derivation for quenching constant. The details of this computation for in vivo monitoring of P_tO_2 in cat brain have been described (15, 22).

The base-line drifts were <1% over an 8-h period. The noise levels in the present experiments produced an uncertainty equivalent to ±5.9 mm Hg of P_tO_2.

Measurement of cyt aa_3 redox changes. Changes in the redox state of cyt aa_3 were measured by dual wavelength reflectance spectrophotometry as described by Jöbsis et al. (13). Briefly, two monochromatic light beams are delivered alternately to the cortex. One of the beams, "sample," is set near the absorption maximum of cyt aa_3 (605 nm), and the other, "reference," beam is set at a wavelength (590 nm) at which an equal optical density change occurs for hemoglobin oxygenation/deoxygenation as at the sample wavelength. The difference between the sample and reference signals thus compensates for changes in both blood volume and hemoglobin oxygenation states. In addition, changes in the reference wavelength at 590 nm provide an approximate indicator of the volume of blood in the optical field. For instrument calibration a scale of 0–100% for 605-590 nm signal was used. The 0% was assigned to no reflected light reaching the photomultiplier, and 100% was assigned to the light level measured from the surface of the brain in its resting or base-line (normoxic) state. Optical changes with anoxia or hemorrhage were then expressed as a fraction of this 0–100% scale. Progressive lowering of the inspired oxygen resulted in general reduction of cyt aa_3, and it reached a maximum level of reduction when the animal was ventilated with 100% nitrogen. As oxygen was provided in a stepwise fashion, cyt aa_3 became oxidized and reached a maximum level of oxidation when the animal was ventilated with 100% oxygen.

The instrument noise is <0.5%, and the biologically caused fluctations in the signal are ±1.0%. There is a 1.2% base-line drift over a 6-h period.

Experimental protocol. 1 h after the injection of the PBA, PBA fluorescence and the redox state of cyt aa_3 were measured simultaneously before and at the end of a 1–2-min ventilation with 100% nitrogen. Shock was induced either by slow hemorrhage from the femoral artery at a rate of 1 ml/kg per min in the first group (13 animals) and continued until the animal's death, or, by rapid bleeding of the cat, within 2–3 min, to a mean arterial pressure of 30–35 mm Hg in the second group (8 animals). The latter level of mean arterial pressure was then sustained until the animal's death. In both groups of animals, P_tO_2 and redox state of cyt aa_3 were monitored simultaneously during all the experimental manipulations. The experiment was terminated by ventilating the animal on 100% nitrogen and observing the maximal reduction of cyt aa_3 together with a decrease in P_tO_2 measured by PBA fluorescence.

Statistical analysis. Data are expressed as the mean ±SE. Means were compared by Student's *t* tests (paired or unpaired) and $P < 0.01$ was taken as a significant difference.

RESULTS

When an animal is ventilated with 100% nitrogen for 1 min (Fig. 2), the PBA fluorescence (F − R) increases (indicating a decrease in P_tO_2), cyt aa_3 reduction occurs, the cerebral blood volume increases, and after a brief rise the arterial pressure drops. On returning the cat to room air breathing, there is a simultaneous increase in tissue P_O2 and a reoxidation of cyt aa_3. There is a modest decrease in cerebral blood volume, a sharp rise in arterial blood pressure, and a reappearance of electrical activity of the brain seen on electrocorticogram. The base-line P_tO_2 was 41±3 mm Hg. In response to 100% nitrogen ventilation, the reduction of cyt aa_3 and decrease in P_tO_2 were essentially parallel. However, the rate of reduction of cyt aa_3 was rapid and reached its maximum level earlier than P_tO_2.

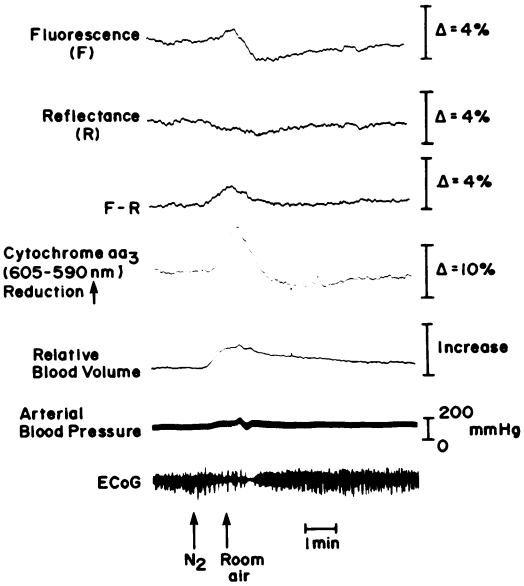

FIGURE 2 Effects of nitrogen anoxia on F, R, and corrected fluorescence (F − R) of PBA, and cyt aa_3 reduction, relative blood volume, blood pressure, and electrocorticogram. After 1 min nitrogen ventilation there is an increase in F − R signal (indicating a decrease in P_tO_2), an increase in cyt aa_3 signal (indicating reduction), and an increase in relative blood volume and blood pressure. Note the lag between the flattening of the electrocorticogram (ECoG) signal and other parameters.

In the first group of animals (slow hemorrhage), when arterial bleeding begins, the PBA fluorescence (F − R) remains essentially unchanged (minor, often fluctuating changes may occur probably due to regional adjustments in blood volume) until a certain point (closed arrowhead, Fig. 3), beyond which, it decreases rapidly and progressively to a point that P_tO_2 approaches zero. We have found this critical point to occur when 30±1.5 ml blood/kg was removed, and/or the mean arterial pressure fell by 70±5% of the base line. The mean arterial pressure at this point was 36±4 mm Hg. The findings for cyt aa_3 were somewhat different. Reduction of cyt aa_3 begins much earlier, when as little as 5 ml/kg is drawn, and then cyt aa_3 reduction increases steadily until it reaches a point beyond which there is a sudden further reduction of this enzyme. In other words, there are two rates of cyt aa_3 reduction after bleeding starts: slow initially and rapid later. In 9 of 13 animals in this group, the point when cyt aa_3 reduction rate begins to accelerate (open arrowhead in Fig. 3) occurred at an earlier time

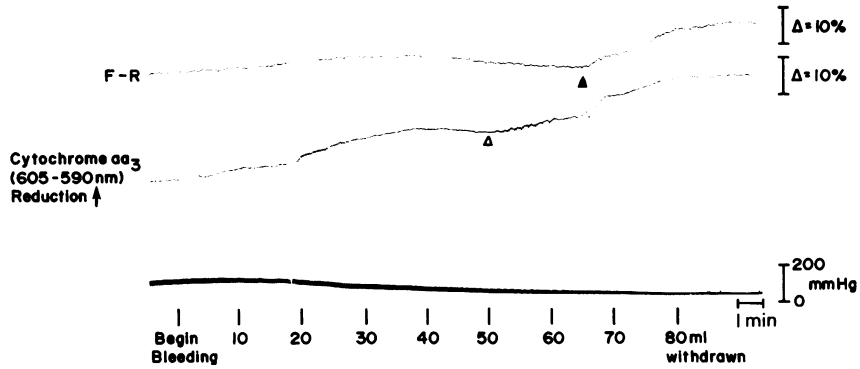

FIGURE 3 Effects of slowly induced hemorrhagic shock on P_tO_2 and the redox state of cyt aa_3. Only in the late stage of hemorrhage does the F − R signal exhibit a sustained marked change as indicated by the closed arrowhead. Beyond that point, a progressive increase in this signal, indicating a decrease in P_tO_2, occurs. Note the two rates of increase in cyt aa_3 reduction signal. The open arrowhead indicates the point of acceleration of cyt aa_3 reduction signal. The onset of this change precedes the marked shift in the F − R signal by several minutes (closed arrowhead).

of hemorrhage, or higher arterial pressure ($P < 0.01$), than the point when PBA fluorescence speeds up. In three animals there were no significant differences between these two points. In one animal of this group, although there was only minimal decrease in P_tO_2 in response to hemorrhage (even after the animal's death), cyt aa_3 reduction did occur. This animal's baseline P_tO_2 was low (17.3 mm Hg).

To provide comparisons between the P_tO_2 and cyt aa_3 reduction in relation to bleeding, Table I shows data for repetitive bleedings at 5 ml/kg increments as well as changes in mean arterial pressure. It can be seen that P_tO_2 remains essentially within the normal range until 25 ml/kg of hemorrhage. However, at 25–30 ml/kg exsanguination and above, P_tO_2 rapidly decreases. In contrast, a progressive increase in cyt aa_3 reduction is seen continuously, with a faster rate of reduction at 25–30 ml/kg, or greater, of exsanguination. The relationship between the percent decrease of P_tO_2 and the percent increase in cyt aa_3 reduction in response to hemorrhage can be seen in Fig. 4. Thus, in all experiments cyt aa_3 showed a consistent, monotonical increasing reduction well before a consistent drop in P_tO_2 was observed.

To determine the effects of rapidly induced hemorrhagic shock on P_tO_2 and cyt aa_3 reduction, we bled eight animals in the second group to a mean arterial pressure of 30–35 mm Hg, while observing both optical signals. The P_tO_2 begins to fall rapidly when critical mean arterial pressure is achieved (Fig. 5). At this point, the amount of exsanguination was 34±6 ml/kg and the P_tO_2 within the range of 10 to 16 Torr. Cyt

TABLE I

Comparison of the P_tO_2 (mm Hg) and cyt aa_3 Reduction (Percent Maximal) in Relation to Bleeding at 5-ml/kg Increments

Blood removed	P_tO_2 (Mean±SEM)	*P value* vs. base line	*cyt aa_3* reduction (Mean±SEM)	MAP* (Mean±SEM)
ml/kg	*mm Hg*		*Percent maximal*	*mm Hg*
0	41±3	—	0	121±8.5
5	36±5	NS	12±3	101±7
10	35±6	NS	18±3.5	87±5
15	34±6	NS	29±5.7	78±5
20	34±6	NS	36±7	65±4
25	29±6	NS	44±8	59±5
30	14±6	<0.001	59±8	38±1.2
35	8.5±5	<0.001	73±6	33±1.3

* Mean arterial pressure.

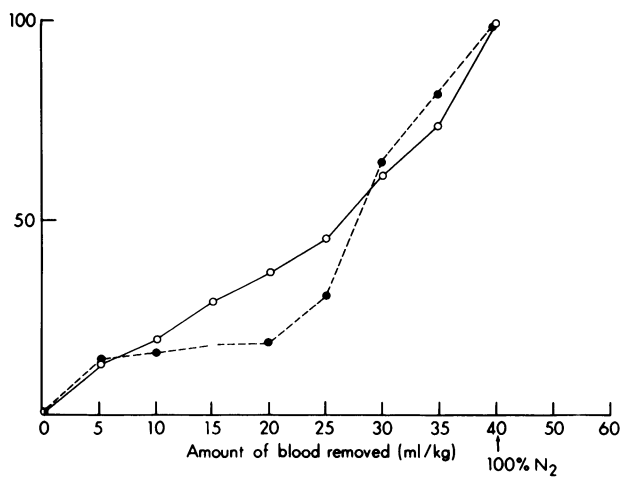

FIGURE 4 Relationship between the percent decrease of P_tO_2 (●) from the base line, and cyt aa_3 (○) reduction (percentage of maximal) in response to bleeding at 5-ml/kg increments. Note the acceleration of the rate of both values beyond 25 ml/kg of hemorrhage.

aa_3 reduction started much earlier and reached almost a maximum level before the P_tO_2 began to fall. Thus, an even greater disparity between cyt aa_3 and P_tO_2 changes was observed with this protocol.

DISCUSSION

Tissue P_{O_2} observations. Techniques for determining tissue P_{O_2} have differing attributes and liabilities. We chose to use a modification of the method described by Mitnick and Jöbsis (15), using an intracellular oxygen indicator, PBA. PBA is nontoxic at levels needed for cellular applications, and since quenching is a physical reaction that does not consume oxygen, it neither interferes with oxygen consumption nor with physiological function (20, 21). Hempel (22) found a precise linear relationship between arterial P_{O_2} and the reciprocal of the fluorescence of PBA in the brain as well as a progressive increase in cerebral venous P_{O_2} when cats were ventilated with sequentially greater oxygen concentrations under hyperbaric pressures up to an atmospheric pressure of 4 atmospheres absolute (ATA). In our experience, the major limitation of PBA fluorometry has been the noise level that can produce an uncertainty equivalent to ±5.9 mm Hg of P_tO_2. Using this technique, Mitnick and Jöbsis (15) determined a resting tissue P_{O_2} of 42.8±8 (SD) mm Hg (cerveau isolé preparation), a value very close to our observation of 41±3 (SE) mm Hg.

We observed a decrease in P_tO_2 in response to hemorrhagic hypotension. The fall in brain tissue P_{O_2} during hemorrhagic shock has been reported by others (23–25) using the microelectrode technique. It is noteworthy that in all these experiments the level of hypotension was lower than in our experiments. Our findings are also consistent with the concept that cerebral blood flow remains relatively unchanged until the mean arterial pressure decreases to below a critical value. Below that point, the autoregulatory mechanisms preserving cerebral blood flow apparently fail (26).

Response of the respiratory chain. The shift toward reduction of cytochrome oxidase is entirely consistent with numerous observations of hypoxic injury in tissue subjected to similar experimental manipulations (13, 27–29). As mentioned earlier, cyt aa_3 reduction during slow hemorrhage has two rates: slow initially and rapid later. The observation of the reduction of cyt aa_3 during an earlier stage of hemorrhage, when brain P_tO_2 is still apparently adequate, is in support of previous reports that metabolic and functional abnormalities of the brain do occur even when mean arterial pressure is only mildly reduced. For instance, moderate increases in lactate content and lactate/pyruvate ratio (30, 31), as well as increases in NADH fluorescence (32) occur when mean arterial pressure is only mildly reduced, and the cerebral perfusion pressure is still much above the critical level. It is generally agreed that functional derangement occurs in two stages (33). Certain neurophysiological functions functions (e.g., evoked cortical responses) are lost first and at a relatively high cerebral perfusion pressure, whereas gross derangement of cerebral energy metabolism, depolarization of cells, and a massive efflux of K^+ ions (34) occur when the second stage is reached. The change from a slow to a rapid reduction of cytochrome oxidase during progressive hemorrhage and shock may reflect the beginning of this second stage, i.e., the point at which critically low perfusion begins.

What accounts for increase in cyt aa_3 reduction while P_tO_2 is adequate? This clearly is a new observation that may point toward a more complex interaction between the availability of oxygen molecules and mitochondrial metabolism at the cellular level than has been appreciated heretofore. The maintenance of base-line P_tO_2±5.9 mm Hg while cyt aa_3 is shifting toward a more reduced state is in general agreement with observations made on isolated mitochondria in vitro by Chance and Williams (35). The in vitro findings (36) indicate that over a fairly wide P_{O_2} range the rate of oxygen use by mitochondria remains constant. This relationship stems from the fact that the rate of oxygen consumption, being a bimolecular reaction between reduced cyto-

FIGURE 5 Effects of rapidly induced hemorrhagic hypotension on P_tO_2 and cyt aa_3 redox state. Note that the F − R signal beings to increase (indicating a decrease in P_tO_2) when cyt aa_3 is almost maximally reduced.

chrome a_3 and oxygen, is set by the product of their concentrations. As the P_{O_2} falls and oxygen becomes scarce, reduced a_3 begins to accumulate and partially compensates for the low oxygen concentraion—the "cushioning effect." Correlation between the rate of oxygen uptake and the percentage of oxidized cytochrome a_3 in isolated mitochondria (36) indicates that only a few millimeters of mercury decrease in partial pressure of oxygen is associated with a drastic increase in reduced cytochrome a_3 and only a slight decrease in oxygen consumption. It is important to emphasize that the in vitro studies show that until very low P_{O_2} levels are reached (5 Torr), no increase in reduction of cytochrome can be seen, indicating a high affinity of cytochrome for oxygen. Unfortunately, the value depends on the rate of electron transport (i.e., oxygen consumption), and since mitochondria usually respire at a maximal rate in the in vitro condition, it would be difficult to extrapolate from these in vitro studies to the conditions existing in vivo (30). In fact, although controversial, it has been demonstrated in certain experimental models that the affinity of cyt aa_3 for oxygen may be remarkably low in the cerebral cortex in vivo (13, 37). Because of this more reduced state of cyt aa_3, in vivo, any decrease or increase in P_{O_2} around the normal value will cause reduction or oxidation of cyt aa_3, respectively (14). Our findings are also in agreement with those of Ji et al. (38) in isolated perfused rat liver. These investigators showed that the reduction of pyridine nucleotide induced by a "sudden" ischemia consists of at least two components: the oxygen-sensitive component and the flow-sensitive component. They also found that the reduction of pyridine nucleotide was approximately twice as great during "no flow" anoxia as in "normal flow" anoxia, underlying the previous observation (39) that no flow anoxia is much more detrimental to the preservation of ATP in the perfused liver than normal flow anoxia.

Based on the above in vitro and in vivo observations we hypothesize that: (*a*) As bleeding continues there is a gradual decrease in P_tO_2 resulting in a progressive accumulation of reduced cyt aa_3, but a small decrement in P_tO_2 cannot be reliably detected by our technique. As bleeding continues, however, there is a point when the fall in P_tO_2 >5.9 mm Hg and can be clearly observed on the PBA fluorescence signal. In fact, as shown in Table I, although minimal and not statistically significant, there is a progressive decrease in the mean P_tO_2 as blood is continuously withdrawn. In contrast, because of the marked sensitivity of cyt aa_3 to ischemia and hypoxia, even a minimal decrease in P_tO_2 is associated with significant reduction of this cytochrome which can be detected by this technique. (*b*) Small adjustments in cerebral blood flow or vascular tone in response to hemorrhage result in cyt aa_3 reduction before O_2 depletion becomes critical. This implies that in vivo mitochondrial redox state is maintained by mechanisms more sensitive to substrate supply or blood-borne compounds (other than oxygen) than to oxygen alone.

Other explanations are equally plausible. Inhomogeneity of perfusion during shock may occur (40). Although lateral inhomogeneity should not produce differences in the two measurements, the longer wavelengths used for cyt aa_3 monitoring could possibly contribute to a discrepancy if inhomogeneity develops vertically in the perfusion of the various cortical layers. Nevertheless, both optical signals derived predominantly from the outermost layers and the putative differential derivation of the signals would be relatively small. Clearly, further studies on this and other possibly interfering effects must and will be performed.

No classic pathophysiological correlates have been investigated so far, however, and it is not known whether these intracellular events foreshadow the establishment of irreversible shock. In fact, the preliminary observations in rat brain (41) indicate the presence of a close correlation between the rate of cyt aa_3 reduction and irreversibility of shock.

Recent noninvasive methods for studying some of these parameters are now available for clinical investigation (42, 43). It will be of interest and importance to determine whether these more comparable parameters can be useful in studying, monitoring, and treating shock as it occurs in human illness. Since cyt aa_3 becomes gradually more reduced as oxygen becomes less available to mitochondria, monitoring of this enzyme may prove to be a more sensitive means of determining when a tissue enters a hypoxic state at a stage before irreversible mitochondrial damage occurs.

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