Journal of Bioenergetics and Biomembranes, Vol. 22, No. 1, 1990

Influence of Cerebral Ischemia and Post-Ischemic Reperfusion on Mitochondrial Oxidative Phosphorylation

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Received June 24, 1989

Abstract

Unilateral ischemia in the right cerebral hemisphere of the rat was induced by ligation of the right common carotid artery coupled with controlled hemorrhage to produce hypotension (25 ± 8 mm/Hg). Where indicated after 30 min of ischemia, the withdrawn blood was reinfused to restore arterial pressure to normal. Mitochondria isolated from the ipsilateral hemisphere after 30 min of ischemia showed significantly lower respiratory rates than the organelles isolated from the contralateral side. Oxidation of NAD^+-linked substrates was more sensitive to inhibition in ischemia (30%) than was of ferrocytochrome *c* (12%), succinate oxidation being intermediate. The activities of membrane-bound dehydrogenases (both NADH and succinate-linked) were also significantly lowered. Ischemia did not affect the cytochrome content of mitochondria. Respiratory activity (NAD^+-linked) of mitochondria isolated from the ipsilateral hemisphere was twice as sensitive to inhibition by fatty acid as was of preparations from the contralateral side. Mitochondria isolated from cerebral cortex after 90 min of post-ischemic reperfusion showed no significant improvement in the rate of substrate oxidation. Adenine nucleotide translocase activity and energy-dependent Ca^{2+} uptake, both of which decreased significantly in mitochondria isolated from the ischemic brain, showed little recovery, on reperfusion. These observations suggested the strong possibility that the deleterious effects of ischemia on mitochondrial respiratory function might be mediated by free fatty acids that are known to accumulate in large amounts in ischemic tissues. The pattern of inhibition of ATPase activity was consistent with this view.

Key Words: Ischemia; brain mitochondria; respiration; calcium transport; adenine nucleotide translocase.

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0145-479X/90/0200-0061$06.00/0

Introduction

The incessant demands of energy for neuronal function require uninterrupted blood flow to the brain for the supply of glucose and oxygen. Negligible endogenous energy reserves and poor ability to oxidize free fatty acids because of low activity of the rate-limiting enzyme 3-ketoacylthiolase (Yang *et al.*, 1987) render the organ prone to rapid irreversible injury in ischemia. The maximum duration of experimental ischemia compatible with functional recovery appears to be not more than one hour (Hossman *et al.*, 1976; White *et al.*, 1984). Histopathologic examinations have revealed that alterations in mitochondrial ultrastructure and volume are the earliest signs of damage detectable in the ischemic nerve cell (Brown and Brierley, 1973). Swelling and structural damage to the inner mitochondrial membrane have been designated as marker for the identification of the "point of no return" (Trump *et al.*, 1982), at which an ischemic cell is condemned to irreversible injury and death.

Severe depletion of cerebral "adenylate energy charge" (Atkinson, 1968) within a few minutes of the onset of ischemia has been recorded in a number of laboratories (Kurup, 1988), indicating functional impairment of mitochondria *in vivo*. While the recovery of energy status was almost total on resumption of blood flow after complete ischemia of short duration, it was partial after incomplete ischemia (> 30 min) even on prolonged reperfusion (Nordstrom *et al.*, 1978; Welsh *et al.*, 1982).

The effect of ischemia on mitochondrial oxidative phosphorylation (Mela, 1979a,b, 1982; Hillered, 1984) seems to be influenced by a variety of factors, which include the animal model adopted, the method of induction and duration of ischemia employed, the anesthetic used, and the procedure devised for the isolation of mitochondria. These variations not withstanding, there is clear indication in the literature that reversible ischemia of long duration (30 min) caused significant decrease (30–50%) in oxidation rates (Schutz *et al.*, 1973; Rehncrona *et al.*, 1979; Hillered *et al.*, 1984). On reperfusion, partial recovery (Hillered *et al.*, 1984) and further deterioration (Rehncrona *et al.*, 1979; Mela, 1979a) have both been observed. Based on these premises, mitochondrial respiratory dysfunction has been designated as an important factor in cellular pathogenesis in cerebral ischemia (Fiskum, 1983; Hillered, 1984; Kariman, 1985).

Thus far, little effort has been made to study mitochondrial respiratory activity in a manner that would identify the electron transport sequence particularly sensitive to ischemic insults. Such information might prove to be of use in understanding the mechanism of mitochondrial damage, which was the aim of our experiments. The results presented in this paper indicate that free fatty acids, large quantities of which are known to accumulate in ischemic

brain (Bazan, 1970; Kuwashima *et al.*, 1976; Gardiner *et al.*, 1981; Rehncrona *et al.*, 1982; Yoshida *et al.*, 1982), may play a significant role in causing functional damage to the organelles.

Materials and Methods

Ischemia Model

Male Sprague-Dawley rats (Charles River Farms) weighing 440 ± 59 g and fed *ad libitum* on commercial chow pellets and water were anesthetized with halothane–air (3.5% v/v). After cannulation of the left femoral and right common carotid arteries, the latter was ligated to prevent blood flow to the right cerebral hemisphere. The dose of halothane was decreased to 1% (v/v) and blood was withdrawn from the femoral artery until the arterial blood pressure was lowered to 25 ± 8 mm/Hg and maintained for 30 min. This procedure, which is a combination of the techniques of Levine (1960) and Smith *et al.* (1984), was used to induce unilateral ischemia in the right cerebral hemisphere. Similar procedures have been used before to induce ischemia preferentially in one hemisphere of the brain (Brown and Brierley, 1968; Clendenon *et al.*, 1971). Measurements using the [^{14}C]iodoantipyrine method as adapted in this laboratory (Hallenbeck *et al.*, 1982) indicated that the average blood flow into the cortex of the ipsilateral hemisphere was 3.8% of the flow into that of the contralateral hemisphere (0.59 ml/g per min). Animals were kept warm on a heating pad or with a lamp.

After 30 min of ischemia, the animal was killed by decapitation (ischemic group), or was slowly (3 min) perfused with the withdrawn (warmed and heparinized) blood *via* the jugular vein (reperfused group). Reperfusion effectively raised the blood pressure to the original level (90–120 mm/Hg). The cannulae were removed, the wounds closed, and the animal allowed to recover from anesthesia. At the time of decapitation, the average blood flow to the right cerebral hemisphere was 21.4% of that to the left (control).

Isolation of Mitochondria

Mitochondria from the two cerebral hemispheres were isolated according to the procedure of Bernard and Cockrell (1979) with some modifications. After removal of white matter, the cortex was weighed, placed in cold homogenizing medium (300 mM mannitol, 5 mM glycylglycine buffer, pH 7.4, 100 μM EDTA, 2 mg BSA/ml), and minced. The fluid was drained off and the tissue was homogenized with fresh medium in a Potter–Elvehjem homogenizer (400 rpm) until the tissue was no longer discernible (6 strokes). The homogenate (6 ml) was centrifuged at 1500 × g for 8 min. The sediment

was suspended in 6 ml of medium (2 strokes) and centrifuged as before. The combined supernatant fractions were centrifuged at 10,000 × *g* for 10 min to sediment mitochondria. To avoid the danger of the leaching of cytochrome *c* (Jacobs and Sanadi, 1960), the hypotonic salt wash step in the procedure of Bernard and Cockrell (1979) and not adopted. Instead, the pellet was suspended in the homogenizing medium and synaptosomes were removed by two successive centrifugations, each of 2 min duration at 1500 × *g*. Mitochondria were then sedimented by centrifugation of the second supernatant fraction at 8,000 × *g* for 10 min and washed with 0.25 M sucrose (6 ml). The sediment was suspended in 0.25 M sucrose and used for the assay of oxidative phosphorylation with minimum delay (unless stated otherwise). All homogenizations except the first two were performed manually. Centrifugations were done in an IEC B-20A refrigerated centrifuge. The sucrose solution (0.25 M) contained 500 μM Tris-Cl buffer, pH 7.4.

In experiments designed to test the effect of gradient centrifugation (Clark and Nicklas, 1970) on ischemic mitochondria, the organelles sedimented at 10,000 × *g* were suspended in 1 ml of medium [255 mM mannitol, 75 mM sucrose, 100 μM EDTA, 2 mg/ml BSA,[5] and 3% (w/v) Ficoll-70], layered over 6 ml of 6% Ficoll in minnitol sucrose EDTA–BSA (Ginsberg *et al.*, 1977), and centrifuged at 10,500 × *g* for 30 min in a Sorvall OTD75B refrigerated ultracentrifuge. The pellet was washed with 0.25 M sucrose (6 ml) and used for the assay of oxidative phosphorylation.

Enzyme Assays

Polarographic determination of oxygen uptake was carried out at 30°C using a YSI Model 5300 biological oxygen monitor fitted with a Clark-type oxygen microelectrode (System 600 B, INSTECH Laboratories, Horsham, Pennsylvannia). The reaction system (total volume 650 μl) contained 50 mM Tris-Cl buffer, pH 7.4; 50 mM potassium phosphate buffer, pH 7.4; 112.5 mM sucrose; 5 mM $MgCl_2$; 1 mM EDTA; 2 mg/ml BSA (unless stated otherwise); and about 300 μg (protein) of freshly prepared mitochondria. After the addition of a substrate or 4 mM ascorbate + 100 μM TMPD), the rate of state 3 oxidation (Chance and Williams, 1955) was measured by stirring in 100–300 μM ADP. When succinate or ascorbate + TMPD was used as an electron donor, the reaction system contained 2.5 μM rotenone.

Phosphorylation coupled to Sites I and II of the respiratory chain was determined according to Lee *et al.* (1967). The reaction system contained 50 mM Tris-Cl buffer, pH 7.4; 12.5 mM potassium phosphate buffer, pH 7.4;

[5]Abbreviations: TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; BSA, bovine serum albumin.

5 mM $MgCl_2$; 2.5 mM ATP; 1.5 mM KCN; 1 mM EDTA; 50 mM glucose; 1 μCi $^{32}P_i$; 20 units of hexokinase; 45 mM sucrose, 2 mg/ml BSA; 4 mM pyruvate or 4 mM glutamate + 4 mM malate; or 8 mM malate; or 8 mM succinate; and 500–600 mg protein of freshly prepared mitochondria in a total reaction vol of 0.6 ml. After incubation at 30°C for 6 min, the reaction was initiated by the addition of 5 mM $K_3[Fe(CN)_6]$ and was stopped after 6 min by the addition of 0.6 ml of perchloric acid (16% v/v). The concentration of ferricyanide was determined by absorbance at 420 nm. After removal of P_i by extraction (Pullman, 1967), the radioactivity associated with glucose-6-P was determined with the addition of biofluor (Dupont) in a Beckman LS9000 liquid scintillation counter.

The reaction system for the uptake of Ca^{2+} under "limited membrane loading" (Carafoli, 1982) conditions contained 50 mM Tris-Cl buffer, pH 7.4, 15 mM succinate, 100 mM sucrose, 1 mg of mitochondrial protein, and 2 mg BSA/ml in a total reaction volume of 650 μl. After incubation at 30°C for 4 min, the reaction was started by the addition of 300 μM ^{45}Ca, Cl_2 (specific activity 100 nCi/μmol). After 1 min, the reaction was stopped by the addition of 15 μM ruthenium red. Samples in which ruthenium red was added at zero time served as blanks.

Adenine nucleotide transport was assayed according to Chan and Barbour (1979). The reaction system (0.5 ml) contained 25 mM Tris-Cl buffer, pH 7.4, 110 mM KCl, 1 mM EDTA, 5 mM $MgCl_2$, 3 mM KCN, 25 mM sucrose, 2 mg BSA/ml, and 1 mg mitochondrial protein. After equilibration at 0°C, the reaction was started by the addition of 90 μM [^{14}C]-ADP (1 μCi/μmol) and stopped after 30 sec by the addition of 400 μM atractyloside. Samples in which the inhibitor was added at zero time served as blank.

In both assays, the reaction mixture was centifuged quickly in a microfuge, the supernatant fraction was drained off, and any fluid adhering to the sides of the tube was wiped carefully with a filter paper wick. The sediment was taken up in 100 μl of Triton X-100 (0.5% v/v) and the radioactivity measured in a Beckman LS900 liquid scintillation counter after the addition of Filtron-X (National Diagnostics).

The activities of succinate and α-glycerophosphate dehydrogenase were assayed using 200 μM phenazine methosulfate + 100 μM 2,6-dichlorophenolindophenol (Nair and Kurup, 1986), and of NADH dehydrogenase using 0.8 mM $K_3[Fe(CN)_6]$ as electron acceptor (King and Howard, 1967). Mitochondrial ATPase activity was determined as described by Veldsema-Currie and Slater (1968). The time of incubation (3–6 min) and concentration of protein (300–600 μg) were adjusted to ensure linearity. Succinate and NADH-cytochrome *c* reductase activities were determined by the absorbance increase at 550 nm, the reaction medium containing 25 μM cytochrome *c* and 0.05% (w/v) deoxychloate. Cytochrome oxidase activity was measured by the

oxidation of ferrocytochrome *c*. The reaction system for all enzyme assays contained 2 mg/ml BSA.

The lysosomal marker enzyme *N*-acetyl-β-D-glucosaminidase was assayed using *p*-nitrophenyl-2-acetamido-2-deoxy-β-glucoside as substrate (Findlay *et al.*, 1958). Lactate dehydrogenase activity was determined by the rate of NADH oxidation at 340 nm in a medium containing 1 mM pyruvate, 3 mM KCN, 100 μM NADH and 0.1% Triton-X100 (Clark and Nicklas, 1970).

Other Estimations

The cytochrome content of mitochondria was estimated from difference (reduced minus oxidized) spectra as described by Gazzotti *et al.* (1980), with $Na_2S_2O_4$ and ascorbate + TMPD being used as electron donors. Protein was determined by the method of Ohnishi and Barr (1978); deoxycholate was used for solubilization.

Chemicals

All biochemicals were obtained from Sigma Chemical Co., St. Louis, Missouri. Other chemicals used were of the purest grades available. Solutions were prepared in deionized water and adjusted to pH 7.4 with KOH, where possible. Arachidonic acid was dissolved in oxygen-free water by adjustment of pH to 8 with KOH. The solution was kept frozen (−20°C) until use. Solutions of ferricyanide, KCN, ascorbate, phenazine methosulfate, sucrose, and homogenizing media were prepared fresh daily. Statistical analysis was done by the paired "t" test or the "t" test.

Results

Mitochondria

The technique of induction of unilateral ischemia employed in the present study enjoys the distinct advantage of minimizing interanimal variation by maintaining one-half of the brain in the normal condition while keeping the other half ischemic. No significant difference in the fresh weight (0.57 ± 0.05 g) of the two hemispheres was seen in our experiments. The protein content (108 ± 5 mg/g) also showed no significant difference.

The recovery of mitochondria (7.0 ± 1.1 mg protein/g brain) in the experimental group showed a small (15%) but significant ($P < 0.025$) decrease from that in unanesthetized animals (8.3 ± 0.8 mg/g). Ischemia, however, had no effect. Increases in the content of protein recovered in

cerebral mitochondrial fractions have been observed in urethane anesthesia (Ginsberg *et al.*, 1977), as well as in ischemia (Hillered *et al.*, 1984). In contrast, Rehncrona *et al.* (1979) observed a decrease in mitochondrial recovery from the ischemic brain. Purification procedures generally result in serious loss of mitochondria, the final yield (6–8 mg protein/g brain; Clark and Nicklas, 1970) being only a fraction of the total population. An estimate of the content of mitochondria in the brain was therefore made by the enzymatic method. To eliminate errors due to possible intramitochondrial variations in specific activities, three enzymes located differentially in the inner mitochondrial membrane (succinate/dehydrogenase, α-glycerophosphate dehydrogenase, and cytochrome oxidase) were assayed in the homogenate and mitochondria. The values of mitochondrial protein calculated from these assays indicated that rat cerebral cortex contained 23 ± 4 mg mitochondrial protein/g (data not shown). Assuming the average brain weight to be 1.5 g, a protein yield of 28 mg/g may be calculated from the data of Clark and Nicklas (1970).

The assay of marker enzymes revealed that our preparation carried with it 43.6 ± 1.9% of the lysosomal and 13.0 ± 4.5% of the synaptosomal vesicles present in brain homogenate. The degree of contamination was unaffected by ischemia. Synaptosomal contamination in the preparation of Clarke and Nicklas (1970) may be calculated from their data as 9.3%. Leakage of hydrolases from lysosomes has been suggested as a forerunner of ischemia-associated membrane dissolution and cell death (Trump *et al.*, 1982). Consistent with this, a decrease in the hydrolase activity of hepatic lysosomes in endotoxemia and shock has been observed (Mela *et al.*, 1973). In our experiments, the "free" (detergent absent) activity of glucosaminidase in the ischemic tissue (2.2 ± 0.6 nmol *p*-nitrophenol released/min/mg protein) was not significantly higher than that in the control tissue (2.0 ± 0.5 nmol). Clendenon *et al.* (1971), who used an ischemic model similar to ours, also obtained similar results and concluded that the lysosomal enzyme released may not be a decisive factor in the pathogenesis of cell injury in ischemic brain.

Oxidative Phosphorylation

It was of interest to see how ischemia and reperfusion affected the oxidative activity of mitochondria with substrates that activate different regions of the respiratory chain. It is clear from the data presented in Table I that our ischemic model had effectively influenced mitochondrial electron transport function. Inhibition of state 3 (ADP present) oxidation was largest with NAD^+-linked substrates. The decrease in cytochrome oxidase activity (oxidation of ascorbate + TMPD), even though statistically significant, was

Table I. Effect of Ischemia on Oxidative Phosphorylation by Rat Brain Mitochondria[a]

Substrate	Function	Normal	Control	Ischemic
Pyruvate + malate	State 3 oxidation	215 ± 15	229 ± 37	162 ± 35* (29 ± 7)
	RCI	4.5 ± 0.2	5.3 ± 1.1	3.9 ± 1.3
	ADP/O	2.4 ± 0.1	2.7 ± 0.2	2.6 ± 0.4
Glutamate + malate	State 3 oxidation	218 ± 25	235 ± 46	164 ± 35* (30 ± 8)
	RCI	4.3 ± 0.4	4.3 ± 0.5	3.9 ± 1.7
	ADP/O	2.3 ± 0.2	2.5 ± 0.2	2.6 ± 0.3
Succinate	State 3 oxidation	172 ± 15	160 ± 31	121 ± 26* (24 ± 7)
	RCI	3.0 ± 0.3	2.8 ± 0.9	2.2 ± 1.0
	ADP/O	1.4 ± 0.4	1.7 ± 0.3	1.8 ± 0.2
Ascorbate + TMPD	State 3 oxidation	261 ± 47	254 ± 56	220 ± 60* (13 ± 4)
	RCI	1.7 ± 0.2	2.0 ± 0.3	1.8 ± 0.3
	ADP/O	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.1

[a]The rates of substrate oxidation (ng atom O/min per mg protein) given by brain mitochondria isolated from normal (unanesthetized) animals as well as from control (left) and ischemic (right; 30 min duration) hemispheres, when measured under phosphorylating conditions, are given. The ratio of respiratory rate in state 3 (ADP present) to that in state 4 (ADP exhausted; Chance and Williams, 1955) is given as respiratory control index (RCI). The values are the mean ± SD of six normal and 10 ischemic animals. The figures in parentheses represent the decrease (percent) in activity in ischemia from control. *Significant $P < 0.005$ control vs. ischemic.

minimal (13%). Inhibition of succinate oxidation appeared to be intermediate. Rehncrona *et al.* (1979) observed that the oxidation of glutamate + malate was inhibited more than that of succinate, while Hillered *et al.* (1984) observed the opposite.

The effects of post-ischemic reperfusion on mitochondrial oxidative phosphorylation are summarized in Table II. The data show that even 90 min after the readmission of blood and restoration of blood pressure, mitochondrial respiratory activity remained inhibited more or less to the same extent as observed after 30 min of ischemia (Table I).

It may be noted that neither ischemia nor reperfusion affected ADP/O, indicating that the phosphorylation system remained undisturbed. This is in agreement with previous observations (Hillered, 1984). The rate of state 4 (ADP exhausted) oxidation was not increased. The lower values of the respiratory control index (RCI) in the treated group (Tables I and II) reflected only the decrease in the rate of state 3 oxidation.

It has been reported that stress conditions like starvation and heat exposure caused significant decreases in mitochondrial respiratory rates, which could be corrected by the exogenous addition of cytochrome *c* to the reaction system (Rasheed *et al.* 1980; Puranam *et al.* 1984). Addition of cytochrome *c* (25 μM) failed to stimulate the oxidation of NAD^+-linked

Table II. Effect of Post-Ischemic Reperfusion on Oxidative Phosphorylation by Rat Brain Mitochondria[a]

Substrate	Function	Control	Treated
Pyruvate + malate	State 3 oxidation	228 ± 28	159 ± 26* (30)
	RCI	4.4 ± 0.8	3.1 ± 0.4*
	ADP/O	2.6 ± 0.2	2.5 ± 0.3
Glutamate + malate	State 3 oxidation	266 ± 34	179 ± 30* (21)
	RCI	4.2 ± 0.7	3.1 ± 0.6*
	ADP/O	2.6 ± 0.3	2.6 ± 0.2*
Succinate	State 3 oxidation	167 ± 23	121 ± 26* (28)
	RCI	2.0 ± 0.4	1.7 ± 0.2
	ADP/O	1.6 ± 0.2	1.5 ± 0.3
Ascorbate + TMPD	State 3 oxidation	254 ± 53	229 ± 47 (10)
	ADP/O	0.6 ± 0.1	0.6 ± 0.2

[a] Unilateral ischemia was induced in the right cerebral hemisphere by artery ligation coupled with hypotension. After 30 min, the blood pressure was restored by reperfusion and the animal killed 90 min later. The rates of substrate oxidation (ng atom O/min per mg protein) shown by brain mitochondria isolated from the left (control) and right (treated) hemispheres, when measured under phosphorylating conditions, are given. The values are the mean ± SD of eight animals. The figures in parentheses represent the decrease (percent) in activity in the treatment. *Significant $p < 0.005$ control vs. treated.

substrates or of succinate by any of the mitochondrial preparation in our experiments. This would indicate that ischemia did not cause dissociation of the cytochrome from the inner mitochondrial membrane. Similarly, addition of the uncoupling agent, 2,4-dinitrophenol (150 μM), exerted no stimulatory influence on the rate of state 3 oxidation.

In our experiments, rats were kept under halothane (1% v/v) anesthesia throughout the duration of ischemia. There are reports that high concentrations of this anesthetic inhibited electron transport in mitochondria *in vitro* (Snodgrass and Piras, 1966; Cohen, 1973; Grist and Baum, 1974) and decreased blood flow and oxygen metabolism *in vitro* (Gjedde and Hindfelt, 1975). The data on the oxidative properties of mitochondria isolated from the brains of normal (unanesthetized) animals given in Table I make it clear that, under our experimental conditions, halothane anethesia caused no deleterious effects on mitochondrial respiration. Hillered *et al.* (1984) also observed that halothane anesthesia did not affect the oxidative functions of cerebral mitochondria.

Oxidation coupled specifically to the first and second coupling sites was measured using $K_3[Fe(CN)_6]$ as an electron acceptor. Under phosphorylating

Table III. Effect of Ischemia on Ferricyanide Reduction by Rat Brain Mitochondria[a]

Substrate	Reaction	Control	Ischemic
Pyruvate + malate	$K_3[Fe(CN)_6]$ reduction (nmol/min per mg protein)	662 ± 112	497 ± 116* (27 ± 10)
	$P/2\bar{e}$	1.6 ± 0.3	1.6 ± 0.4
Succinate	$K_3[Fe(CN)_6]$ reduction (nmol/min per mg protein)	258 ± 56	212 ± 59* (18 ± 8)
	$P/2\bar{e}$	0.6 ± 0.1	0.6 ± 0.1

[a]Experimental details are given in the Materials and Methods section. Other relevant details are given in the legend for Table IV. The values are the mean ± SD of 10 animals. The figures in parentheses denote decrease (%) from control. *Significant $P < 0.005$ control vs. ischemic.

conditions, ferricyanide interacts with a respiratory component (probably FeS center) between the second phosphorylation site and cytochrome *c* (Rasheed *et al.*, 1980). The data presented in Table III confirm the inhibitory effect of ischemia on electron transport activity in this region of the respiratory chain. The greater vulnerability of NAD^+-linked electron transport to inhibition by ischemia is clearly seen in this reaction as well.

The effect of ischemia on the activity of mitochondrial oxidoreductases is depicted in Table IV. Mitochondria isolated from the ischemic side of the brain yielded significantly lower activities when compared to the organelles obtained from the control side. Membrane-bound dehydrogenases (both NADH- and succinate-dependent) showed a 25% decrease in activity. However, the differential sensitivity shown in O_2 uptake rates was not apparent in this case. The disrupted state of the membrane and the artificial nature

Table IV. Effect of Ischemia on Respiratory Enzymes in Rat Liver Mitochondria[a]

Enzyme	Control	Ischemic
Succinate-cyt *c* reductase	66 ± 11	51 ± 7* (24 ± 8)
NADH-cyt *c* reductase	158 ± 27	122 ± 21* (23 ± 9)
α-Gly-P-dehydrogenase	54 ± 13	47 ± 5
Succinate dehydrogenase	103 ± 28	77 ± 17* (25 ± 9)
NADH dehydrogenase	939 ± 157	690 ± 87* (26 ± 8)
Cytochrome oxidase	867 ± 217	809 ± 138

[a]Enzyme activities were determined in mitochondrial samples kept frozen (−70°C) overnight. The reaction system contained 2 mg BSA/ml. Deoxycholate (0.05% w/v) was added to reaction systems containing cytochrome *c*. The values represent nmol ferricytochrome *c*, 2,6-dichlorophenolindophenol, or $K_3[Fe(CN)_6]$ reduced or ferricytochrome *c* oxidized/min per mg mitochondrial protein and are the mean ± SD of seven animals. The figures in parentheses represent decrease (%) from control. *$P < 0.005$ control vs. ischemic.

of the assay might have contributed to this. Exposure of animals to low oxygen tension has been reported to decrease succinate dehydrogenase activity in brain and increase it in the liver (Aithal and Ramasarma, 1969; Sivaramakrishnan and Ramasarma, 1975; Purushotham and Ghosh, 1975; Mela, 1979a). The insensitivity of cytochrome oxidase under nonphosphorylating conditions to inhibition in ischemia is noteworthy. The activity of α-glycerophosphate dehydrogenase also appeared to be unaffected by ischemia (Table IV).

The values for the cytochrome content of brain mitochondria differ widely in the literature. The concentrations in our experiments (aa_3 398 ± 82; *b*, 339 ± 86; and *c*, 305 ± 82 pmol/mg protein) were between those reported by Bernard and Cockrell (1979) and Clark and Nicklas (1970). Ischemia appeared to exert little influence on the cytochrome content of brain mitochondria. The data of Rehncrona *et al.* (1979) and of Hillered *et al.* (1984) show that the content of cytochrome aa_3 (200–250 pmol/mg protein) was not decreased in ischemia. Ischemic mitochondria retained the full complement of cytochrome *c*. This is consistent with the inability of exogenous cytochrome *c* to stimulate oxygen uptake.

The ATPase activity of mitochondria isolated from control and ischemic hemispheres of the brain indicated that ischemia did not cause stimulation of the spontaneous activity of the enzyme (12 ± 4 nmol P_i liberated/min per mg protein. This is consistent with the general observation that ischemia did not affect ADP/O or state 4 respiratory rates (Table I). In the absence of exogenous Mg^{2+} ions, the ability of 2,4-dinitrophenol (300 μM) to stimulate ATPase was significantly ($P < 0.025$) lower with mitochondria obtained from the ischemic hemisphere (39 ± 12 nmol P_i liberated) in comparison with control (51 ± 11 nmol P_i). This was corrected to a large extent on the addition of Mg^{2+} ions (196 ± 67 nmol P_i/min per mg protein), in agreement with previous reports (Ozawa *et al.*, 1967; Lazarewics *et al.*, 1972). Compression ischemia in rabbits caused a significant stimulation of the spontaneous (Mg^{2+} present) ATPase activity of cerebral mitochondria, while the uncoupler-stimulated activity (Mg^{2+} present) remained unchanged (Schutz *et al.*, 1973).

When assayed under condition of "massive loading" (permanent anion present), mitochondria isolated from ischemic brain showed decreased ability for energy-dependent calcium uptake (Ginsberg *et al.*, 1977; Mela, 1979b). In contrast, Rosenthal *et al.* (1987) found no change in the extent of calcium uptake in ischemia, but an increase on reperfusion. We have assessed the effect of ischemia and post-ischemic reperfusion on mitochondrial calcium uniporter under "limiting loading" conditions (permeant anion absent). The results presented in Table V show that the energy-dependent uptake of Ca^{2+} was inhibited in ischemia by about 40% and remained at the same level even after reperfusion. Measurements of calcium-stimulated respiration made

Table V. Effect of Ischemia and Post-Ischemic Reperfusion on Calcium Uptake and Adenine Nucleotide Translocation by Rat Cerebral Mitochondria[a]

Treatment	Function	Control	Treatment
Ischemia	Ca^{2+} uptake (ng ion/mg protein)	25.9 ± 6.6	16.3 ± 6.2* (37)
Reperfusion		22.0 ± 2.3	12.3 ± 4.7* (44)
Ischemia	Translocase (pmol ADP/mg protein)	499 ± 145	249 ± 88* (50)
Reperfusion		541 ± 118	301 ± 43* (44)

[a]Calcium uptake was measured by using [^{45}Ca]Cl_2. Adenine nucleotide binding was determined at 0°C for 30 sec using [^{14}C]-ADP. The values are the mean ± SD of eight animals for ischemia and four for reperfusion. The inhibition of uptake (%) is shown in parentheses. Other details are given in the Materials and Methods Section. *Significant $p < 0.005$ control vs. treated.

separately on the same samples under similar conditions indicated that the Ca : O ratio (3.1 ± 0.3) was not affected by ischemia (data not given).

The data presented in Table V also reveal that the capacity of cerebral mitochondria to bind and transport ADP was inhibited (50%) under ischemic conditions, and that reperfusion (90 min) did not release the organelles from inhibition.

Fatty Acid Efffect

A gradation in the sensitivity of electron transport associated with the three coupling sites to inhibition by ischemia was discernible in the data presented here. Oxidation reduction at the first coupling site was affected more than that at the second site, the terminal one (cytochrome oxidase) being relatively refractory. There are reports that lipophilic inhibitors of mitochondrial oxidative phosphorylation exhibit a similar gradation in potency (Wilson, 1978; Saikumar and Kurup, 1984a,b; Chaturvedi and Kurup, 1986). The first coupling site (Complex I), which contains up to 26 proteins, is the largest and most complicated part of the electron transport chain and is characterized by the presence of a large number of Fe–S clusters and a large, uncharacterized hydrophobic protein core (Castello and Frey, 1987). The greater affinity shown by lipophilic inhibitors to this site becomes understandable in this context. Unusual lipids (Natarajan *et al.*, 1986), free fatty acids (Bazan, 1970; Kuwashima *et al.*, 1976; Gardiner *et al.*, 1981; Rehncrona *et al.*, 1982; Yoshida *et al.*, 1982), and their oxidation products (Gaudet *et al.*, 1980) are known to accumulate in the ischemic brain. Free fatty acids and their CoA derivatives are potent inhibitors of mitochondrial oxidative phosphorylation (Wojtczak, 1976). In the light of these facts, it was thought to be of interest to see how mitochondria isolated from control and

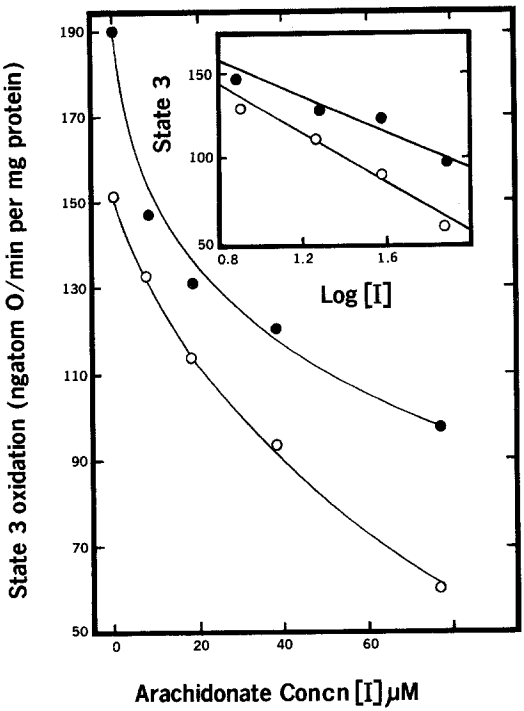

Fig. 1. Effect of cerebral ischemia on the inhibition of mitochondrial respiration by arachidonate. Mitochondria isolated from control (●) and ischemic (○) hemispheres of rat brain were incubated for 1 min with arachidonate (concentrations indicated) before the initiation of state 3 respiration by the addition of pyruvate + malate and ADP Regression lines fitted by the least-squares method are given in the inset. The correlation coefficients for control and ischemic samples were 0.96 and 0.97, respectively.

ischemic parts of the brain would react to the addition of free fatty acids. Although palmitic and stearic acids are present in higher amounts in the ischemic brain, the rate of increase of arachidonic acid is the fastest and the net increase highest. Therefore, we choose arachidonate for our study. Titration of mitochondria with the fatty acid revealed that the inhibition of oxidative phosphorylation was a linear function of the logarithm of arachidonate concentration (see Fig. 1). Concentrations of the fatty acid required to inhibit respiratory activity by 50% (I_{50}), calculated from titration curves, are presented in Table VI. A greater sensitivity of electron transport at coupling Site I to inhibition is clearly shown by the data. The concentration of arachidonate required for inhibition of succinate oxidation was 5–10 times that required to inhibit malate oxidation. An I_{50} value of 126 μM arachidonate may be calculated from the data of Kuwashima *et al.* (1976) for the inhibition of succinate oxidation. It may be mentioned in this context that

Table VI. Effect of Ischemia on the Sensitivity of Rat Brain Mitochondria to Inhibition by Arachidonate[a]

Substrate	I_{50} (μM)	
	Control	Ischemic
Pyruvate + malate	35.9 ± 13.4	14.1 ± 6.0* (63 ± 8)
Gutamate + malate	28.6 ± 6.5	16.4 ± 7.9* (45 ± 7)
Succinate	122.7 ± 45.8	146.7 ± 42.9

[a]The values of I_{50} [concentration of arachidonate (μM) required for 50% inhibition of state 3 oxidation] calculated from regression equations fitted as shown in Fig. 1 are the mean ± SD of five animals. The correlation coefficients ranged from 0.95 to 0.99. The numbers in parentheses represent percent decrease from control. The reaction system contained no BSA and the same amounts (400 μg protein) of mitochondria. *Significant $P < 0.005$ control vs. ischemic.

arachidonate concentrations as high as 615 μM exerted no inhibitory effect on the oxidation of ascorbate + TMPD, the rates of oxygen uptake (ngatom O/min/mg protein) being 211 ± 34 and 288 ± 43 (arachidonate present) for control, and 178 ± 32 and 197 ± 38 (arachidonate present) for ischemic mitochondria, respectively.

An interesting point that emerged from the data in Table VI was the differential sensitivity of NAD^+-linked electron transport to inhibition by arachidonate between mitochondria obtained from ischemic and control sides of the brain, the I_{50} value for the former (14–16 μM) being less than half of the value for the latter (29–36 μM). This difference between the two preparations was not seen readily in the inhibition of succinate oxidation, probably because of nonspecific binding arising from the large concentrations of inhibitor required.

Ficoll Gradient

Mitochondrial preparations made by the procedure of Clark and Nicklas (1970), which involves passage through a discontinuous gradient of Ficoll, have been reported to give low respiratory rates (Moore and Jobsis, 1970; Bernard and Cockrell, 1979). The rates reported by Hillered *et al.* (1984), who used the Clark and Nicklas procedure, were only about half those reported in this paper. The altered sensitivity of the preparation to inhibition by fatty acid indicated the possibility that ischemic conditions might alter the permeability characteristics of mitochondrial membranes. If this is taken into consideration, it is conceivable that Ficoll gradient centrifugation can affect mitochondrial preparations from the normal and ischemic brain differently, which might contribute to the greater damage of respiratory function in

ischemic mitochondria observed in our laboratories (Hillered *et al.*, 1984; Mela *et al.*, 1975, 1978). To clarify this point, our preparation was subjected to gradient centrifugation as described by Ginsberg *et al.* (1977). The treatment significantly decreased ($P < 0.05$) the recovery of mitochondria from ischemic tissue (4.3 ± 0.4 mg protein/g grain). However, the pattern of inhibition of state 3 respiration under ischemic conditions was not substantially altered by the treatment, the extent of inhibition with different substrates being the same as reported in Table I. Interestingly, the greater sensitivity of NAD^+-linked electron transport to inhibition by ischemia was seen in this experiment as well (data not given).

Discussion

Investigations on cerebral energy metabolism have led to the general conclusion that from the point of view of neuronal recovery, incomplete ischemia of long duration is more injurious than complete ischemia (Siesjo, 1981; White *et al.*, 1984). The observation made in many laboratories that mitochondria isolated from ischemic brain showed decreased rates of substrate oxidation (Hillered, 1984; Sims and Pulsinelli, 1987; this paper) is consistent with the rapid depletion of energy status suffered by the ischemic tissue. However, the correlation between the two became less consistent on reperfusion. Thus, after 30 min of incomplete ischemia induced by bilateral carotid artery occlusion coupled with hypotension, reperfusion (60–90 min) led to a 60% recovery of energy status (Nordstrom *et al.*, 1978), while mitochondrial respiratory rate deteriorated from the ischemic level by 45% (Rehncrona *et al.*, 1979). In contrast, under similar conditions of ischemia and reperfusion, Hillered *et al.* (1984) observed a 50% reversal of inhibition of mitochondrial function. The results presented in this paper reveal that after 90 min of reperfusion, no significant reversal of respiratory inhibition takes place. It has also been reported that in certain cerebral regions oxidative function recovered in 1 h of reperfusion, but badly deteriorated in 24 h (Sims and Pulsinelli, 1987). Apparently, post-ischemic resuscitation of cerebral energy metabolism is more complex than its deterioration in ischemia.

Three metabolic events, namely lactic acidosis, generation of oxygen radicals, and intracellular calcium overload, have been implicated as causes of ischemic cell injury and mitochondrial respiratory damage (Hillered, 1984; Siesjo, 1981; Fiskum, 1985). The addition of exogenous lactic acid, which lowered the pH of the reaction medium to 6.1, significantly decreased state 3 oxidation rates of isolated brain mitochondria (Hillered *et al.*, 1985a). The treatment decreased ADP/O as well. Moreover, these adverse effects were corrected when the pH was raised to 7.1. The present view is that lactic

acidosis may not be a critical cause of injury in ischemia (Siesjo, 1981; Haas, 1983; Hillered *et al.* 1985b).

It has been proposed that in incomplete ischemia, electron transport systems generate reactive oxygen radicals, which initiate extensive peroxidation of membrane phospholipids (Fridovich, 1979; Demopoulos *et al.*, 1982). Exposure of rat brain mitochondria to the radical generating the xanthine–xanthine oxidase system caused a significant decrease in the rate of oxidation of NAD^+-linked substrates (Hillered *et al.*, 1983). The treatment destroyed the state 3 to state 4 transition, which does not occur in ischemia. Xanthine oxidase activity of the brain is low, and direct evidence for radical formation in cerebral ischemia has not been forthcoming (Watson and Ginsberg, 1988).

The fact that the energy-dependent uptake of Ca^{2+}, whether under conditions of limited loading (this paper) or of massive loading (Ginsberg *et al.*, 1977; Mela, 1979a), was decreased in mitochondria isolated from ischemic tissue, could be a consequence of the inhibition of respiratory activity or of a partial preloading of matrix with Ca^{2+} ions. The latter would be favored by the postulates of calcium overload (Siesjo, 1988) and lipid peroxidation (Watson and Ginsberg, 1988). The predilection of mitochondrial phospholipids for peroxidation (Sharma, 1977), the adverse effect of lipid peroxidation on Ca^{2+} permeability (Vladimirov *et al.*, 1980), and the ability of hydroperoxides to liberate bound calcium (Richter and Frei, 1988) could all lead to accumulation of the metal ion in mitochondria. Hillered *et al.* (1984) observed no increase in the content of mitochondrial calcium in ischemia, but detected a significant increase in post-ischemic reperfusion. It would appear that chemical methods do not faithfully report the concentration of ionic calcium in mitochondria, which is low (< 3 ng ion/mg protein; Somlyo, 1985). Moreover, the physiological significance of mitochondrial accumulation of Ca^{2+} is in doubt (Carafoli, 1987). In light of this, uptake and deposition of calcium as a major cause of mitochondrial membrane damage (Fiskum, 1985) has to be discounted until support from valid measurements of calcium content *in situ* is adduced (Cheung *et al.*, 1986).

Strangely enough, despite reports on the massive accumulation of free fatty acids in the ischemic brain (Moskowitz *et al.*, 1984), their role in the mediation of mitochondrial respiratory damage has received scant attention. Our results point strongly toward free fatty acids as the primary agents of mitochondrial dysfunction in the ischemic brain. This interpretation is based on the following premises:

1. There is reasonable resemblance between the differential sensitivity shown by electron transport at the three coupling sites in the inhibition in ischemia and by exogenous fatty acids.

2. The oxidation of NAD^+-linked substrates by mitochondria prepared from the ischemic brain is more sensitive to inhibition by fatty acid than is the oxidation by preparations obtained from control tissue (Table VI). This could mean either that the preparation has already accumulated fatty acids or that the organelles have been sensitized to their action. The ability of fatty acids to partition readily into cell membranes (Pujra *et al.*, 1984) and perturb membrane structure and alter properties like fluidity and permeability is well recognized (Schullery *et al.*, 1981).

3. The inhibition of mitochondrial adenine nucleotide translocase activity in ischemia and post-ischemic reperfusion (Table V) is noteworthy. Undoubtedly, the best documented effect of fatty acids and their CoA esters on mitochondria is the inhibition of adenine nucleotide translocase (Wojtczak, 1976). It may be mentioned in this context that both ADP concentration (Jacobus, 1985; Chance *et al.*, 1986) and adenine nucleotide translocation (Kholodenko *et al.*, 1987; Westerhoff *et al.*, 1987; Aprille, 1988) have been assigned key roles in the physiological regulation of mitochondrial function.

4. The inhibition of uncoupler-stimulated ATPase activity in the absence of Mg^{2+} ions observed in mitochondria obtained from ischemic tissues (this paper; Ozawa *et al.*, 1967; Lazarewicz *et al.*, 1972) is reminiscent of the action of fatty acids and other liphophilic membrane-perturbing agents (Panini and Kurup, 1975; Wojtczak, 1976; Saikumar and Kurup, 1984b, Chaturvedi and Kurup, 1984). It may be pointed out that the media used for the preparation and assay of mitochondria contained BSA (2 mg/ml). Functional impairment of such preparations would signify that free fatty acids are localized in the membrane at inaccessible regions like the hydrophobic interior of the bilayer. If so, on reperfusion of the ischemic tissue, functional recovery of mitochondria, which would depend on the removal of partitioned fatty acids predominantly by reincorporation into phospholipids, could become rate-limiting.

Acknowledgments

The excellent technical help rendered by Jessie R. deJesus in the surgical induction of ischemia and the rapid removal of brain is acknowledged.

The experiments reported herein were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals", Institute of Laboratory Animal Resources, National Research Council, DHHS, Publ. No. (NIH)86-23.

This work was supported by Naval Medical Research and Development Command Work Unit No. MR040101.1126.

References

Aithal, H. N., and Ramasarma, T. (1969). *Biochem. J.* **115**, 77–83.
Aprille, J. R. (1988). *FASEB J.* **2**, 2547–2556.
Atkinson, D. E. (1968). *Biochemistry* **7**, 4030–4045.
Bazan, N. G., Jr. (1970). *Biochim. Biophys. Acta* **218**, 1–10.
Bernard, P. A., and Cockrell, R. S. (1979). *Biochim. Biophys. Acta* **548**, 173–186.
Brown, A. W., and Brierley, J. B. (1968). *Br. J. Exp. Pathol.* **49**, 87–106.
Brown, A. W., and Brierley, J. B. (1973). *Acta Neuropathol.* **23**, 9–22.
Carafoli, E. (1987). *Annu. Rev. Biochem.* **56**, 395–433.
Carafoli, E. (1982). In *Physiology of Shock, Anoxia, and Ischemia* (Cowley, R. A., and Trump, B. F., eds.), Williams and Wilkins, Baltimore, pp. 92–112.
Castello, M. J., and Frey, T. G. (1987). In *Membraneous Structures* (Harris, J. R., and Horne, R. W., eds.), Academic Press, New York, pp. 377–444.
Chan, S. H. P., and Barbour, R. L. (1979). In *Membrane Bioenergetics* (Lee, C. P., Chatz, G., and Ernster, L., eds.), Addison-Wesley, Reading, Massachusetts, pp. 521–532.
Chance, B., and Williams, G. R. (1955). *J. Biol. Chem.* **217**, 407–427.
Chance, B., Leigh, J. S., Jr., Kent, J., McCully, K., Nioka, S., Clark, B. J., Maris, J. M., and Graham, T. (1986). *Proc. Natl. Acad. Sci. USA* **83**, 9458–9462.
Chaturvedi, V. K., and Kurup, C. K. R. (1986). *Indian J. Biochem. Biophys.* **23**, 156–161.
Cheung, J. Y., Bonventre, J. V., Malis, C. D., and Leaf, A. (1986). *New Engl. J. Med.* **314**, 1670–1676.
Clark, J. B., and Nicklas, W. J. (1970). *J. Biol. Chem.* **245**, 4724–4731.
Clendenon, N. R., Allen, N., Komatsu, T., Liss, L., Gordon, W. A., and Heimburger, K. (1971). *Arch. Neurol.* **25**, 432–448.
Cohen, P. J. (1973). *Anesthesiology* **39**, 153–164.
Demopoulos, H. B., Flamm, E., Seligman, M., and Pietronigro, D. D. (1982). In *Pathology of Oxygen* (Autor, A. P., ed.), Academic Press, New York, pp. 127–155.
Findlay, J., Levvy, G. A., and Marsh, C. A. (1958). *Biochem. J.* **69**, 467–476.
Fiskum, G. (1983). *Am. J. Emerg. Med.* **2**, 147–153.
Fiskum, G. (1985). *Ann. Emerg. Med.* **14**, 810–815.
Fridovich, I. (1979). *Adv. Neurol.* **26**, 255–259.
Gardiner, M., Nilsson, B., Rehncrona, S., and Siesjo, B. K. (1981). *J. Neurochem.* **36**, 1500–1505.
Gaudet, R. J., Alam, I., and Levine, L. (1980). *J. Neurochem.* **35**, 653–658.
Gazzotti, P., Malmstrom, K., and Crompton, M. (1980). In *Membrane Biochemistry* (Carafoli, E., and Semenza, G., eds.), Springer-Verlag, Berlin, pp. 62–76.
Ginsberg, M. D., Mela, L., Wrobel-Kuhl, K., and Reivich, M. (1977). *Ann. Neurol.* **1**, 519–527.
Gjedde, A., and Hindfelt, B. (1975). *Acta Anesthesiol. Scand.* **19**, 310–315.
Grist, E. M., and Baum, H. (1974). *FEBS Lett.* **48**, 41–44.
Hallenbeck, J. R., Leitch, D. R., Dutka, A. J., Greenbaum, D. J., Jr., and Mckee, A. E. (1982). *Ann. Neurol.* **12**, 124–156.
Hass, W. K. (1983). *Neurol. Clinics* **1**, 345–353.
Hillered, L. (1984). In *Mitochondrial Physiology and Pathology* (Fiskum, G., ed.), Van Nostrand Reinhold, New York, pp. 120–146.
Hillered, L., and Ernster, L. (1983). *J. Cereb. Blood Flow Metab.* **3**, 207–214.
Hillered, L., Siesjo, B. K., and Arfors, K. E. (1984). *J. Cereb. Blood Flow Metab.* **4**, 438–446.
Hillered, L., Ernster, L., and Siesjo, B. K. (1985a). *J. Cereb. Blood Flow Metab.* **4**, 430–437.
Hillered, L., Smith, M. L., and Siesjo, B. K. (1985b). *J. Cereb. Blood Flow Metab.* **5**, 259–266.
Hossmann, K. A., Sakaki, S., and Kimoto, K. (1976). *Stroke* **7**, 301–305.
Jacobs, E. E., and Sanadi, D. R. (1960). *J. Biol. Chem.* **235**, 531–534.
Jacobus, W. E. (1985). *Annu. Rev. Physiol.* **47**, 707–725.
Kariman, K. (1985). *Life Sci.* **37**, 71–73.
Kholodenko, B., Zilinskiene, V., Borutaite, V., Ivanoviene, L., Toleikis, A., and Praskevicus, A. (1987). *FEBS Lett.* **223**, 247–250.

King, T. E., and Howard, R. E. (1967). *Methods Enzymol.* **10**, 275–294.
Korup, C. K. R. (1988). *Ind. J. Biochem. Biophys.* **25**, 615–617.
Kuwashima, J., Fujitani, B., Nakamura, K., Kadokawa, T., Yoshida, K., and Shimizu, M. (1976). *Brain Res.* **110**, 547–557.
Lazarewics, J. W., Strosznajder, J., and Gromek, A. (1972). *Acad. Pol. Sci. Bull.* **20**, 599–606.
Lee, C. P., Sottocasa, G. L., and Ernster, L. (1967). *Methods Enzymol.* **10**, 33–37.
Levine, S. (1960). *Am. J. Pathol.* **36**, 1–17.
Mela, L. (1979a). *Neurol. Res.* **1**, 51–63.
Mela, L. (1979b). *Circ. Shock Suppl.* **1**, 61–67.
Mela, L. (1982). In *Pathophysiology of Shock, Anoxia, and Ischemia* (Cowley, R. A., and Trump, B. F., eds.), Williams and Wilkins, Baltimore, pp. 84–95.
Mela, L., Miller, L. D., Bacalzo, L. V., Jr., Olofsson, K., and White, R. R. (1973). *Ann. Surg.* **178**, 727–735.
Mela, L., Crowe, W., Harbig, K., Wrobel-Kuhl, K., and Kovach, A. (1975). *Surg. Forum* **26**, 51–53.
Mela, L., Crowe, W., Wrobel-Kuhl, K., and Miller, L. D. (1978). In *Cerebral Ischemia and Arterial Hypertension* (Mossakowski, M. J., Zelman, I. B., and Kroh, H., ed.), Polish Medical Publishers, Warsaw, pp. 96–98.
Moore, C. L., and Jobsis, F. F. (1970). *Arch. Biochem. Biophys.* 295–305.
Moskowitz, M. A., Kiwak, K. J., Heikimian, K., and Levin, L. (1984). *Science* **224**, 886–889.
Nair, N., and Kurup, C. K. R. (1986). *Indian J. Biochem. Biophys.* **23**, 76–79.
Natarajan, V., Schmid, P. C., and Schmid, H. H. O. (1986). *Biochim. Biophys. Acta* **878**, 32–41.
Nordstrom, C. H., Rehncrona, S., and Siesjo, B. K. (1978). *Stroke* **9**, 335–343.
Ohnishi, S. T., and Barr, J. K. (1978). *Anal. Biochem.* **86**, 193–200.
Ozawa, K., Seta, K., Araki, H., and Handa, H. (1967). *J. Biochem. (Tokyo)* **61**, 512–514.
Panini, S. R., and Kurup, C. K. R. (1975). *Arch. Biochem. Biophys.* **168**, 188–197.
Pujra, W. J., Klienfeld, A. M., and Karnovsky, M. J. (1984). *Biochemistry* **23**, 2039–2043.
Pullman, M. E. (1967). *Methods Enzymol.* **10**, 57–60.
Puranam, R. S., Shivaswamy, V., Kurup, C. K. R., and Ramasarma, T. (1984). *J. Bioenerg. Biomembr.* **16**, 421–431.
Purushottam, T., and Ghosh, N. C. (1975). *Environ. Physiol. Biochem.* **5**, 73–77.
Rasheed, B. K. A., Chhabra, S., and Kurup, C. K. R. (1980). *Biochem. J.* 191–198.
Rehncrona, S., Mela, L., and Siesjo, B. K. (1979). *Stroke* **10**, 437–446.
Rehncrona, S., Westerberg, E., Akesson, B., and Siesjo, B. K. (1982). *J. Neurochem.* **38**, 84–93.
Richter, C., and Frei, B. (1988). *Free Radical Biol. Med.* **4**, 365–375.
Rosenthal, R. E., Hamud, F., Fiskum, G., Varghese, P. J., and Sharpe, S. (1987). *J. Cereb. Blood Flow Metab.* **7**, 752–758.
Saikumar, P., and Kurup, C. K. R. (1984a). *Biochim. Biophys. Acta* **776**, 263–266.
Saikumar, P., and Kurup, C. K. R. (1984b). *Indian J. Biochem. Biophys.* **21**, 309–313.
Schullery, S. E., Seder, T. A., Weinstein, D. A., and Bryant, D. A. (1981). *Biochemistry* **20**, 6818–6824.
Schutz, H., Silverstein, P. R., Vapalahti, M., Bruce, D. A., Mela, L., and Langfitt, T. W. (1973). *Arch. Neurol.* **29**, 408–416.
Sharma, O. P. (1977). *J. Neurochem.* **28**, 1377–1379.
Siesjo, B. K. (1981). *J. Cereb. Blood Flow Metab.* **1**, 155–185.
Siesjo, B. K. (1988). *Ann. N.Y. Acad. Sci.* **522**, 638–661.
Sims, N. R., and Pulsinelli, W. A. (1987). *J. Neurochem.* **49**, 1367–1374.
Sivaramakrishnan, S., and Ramasarma, T. (1975). *Environ. Physiol. Biochem.* **5**, 189–200.
Smith, M. L., Bendek, G., Dahlgren, N., Rosen, I., Wieloch, T., and Siesjo, B. K. (1984). *Acta Neurol. Scand.* **69**, 385–401.
Snodgrass, P. J., and Piras, M. M. (1966). *Biochemistry* **5**, 1140–1149.
Somlyo, A. P. (1985). *Cell Calcium* **6**, 197–212.
Trump, B. F., Berezesky, I. K., and Cowley, R. A. (1982). In *Pathophysiology of Shock, Anoxia, and Ischemia* (Cowley, R. A., and Trump, B. F., eds.), Williams and Wilkins, Baltimore, pp. 6–46.
Veldsema-Currie, R. D., and Slater, E. G. (1968). *Biochim. Biophys. Acta* **162**, 310–319.

Vladimirov, Y. A., Olenov, V. A., Suslova, T. B., and Cheremisina, Z. P. (1980). *Adv. Lipid Res.* **17**, 173–249.

Watson, B. D., and Ginsberg, M. D. (1988). In *Oxygen Radicals and Tissue Injury* (Halliwell, B., ed.), FASEB, Bethesda, pp. 81–91.

Welsh, F. A., O'Connor, M. J., Marcy, V. R., Spatacco, A. J., and Johns, R. L. (1982). *Stroke* **13**, 234–242.

Westerhoff, H. V., Plomp, P. J. A. M., Groen, A. K., Wanders, R. J. A., Bode, J. A., and Van Dam, K. (1987). *Arch. Biochem. Biophys.* **257**, 154–169.

White, B. C., Wiegenstein, J. G., and Winegar, C. D. (1984). *J. Am. Med. Assoc.* **251**, 1586–1590.

Wilson, S. B. (1978). In *Plant Mitochondria* (Ducet, G., and Lance, C., eds.), Elsevier, Amsterdam, pp. 215–222.

Wojtczak, L. (1976). *J. Bioenerg. Biomembr.* **8**, 293–311.

Yang, S., He, X., and Schulz, H. (1987). *J. Biol. Chem.* **262**, 13027–13032.

Yoshida, S., Abe, K., Busto, R., Watson, B. D., Kogure, K., and Ginsberg, M. D. (1982). *Brain Res.* **245**, 307–316.