NAD/NADH: redox state changes on cat brain cortex during stimulation and hypercapnia

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GYULAI, LASZLO, EÖRS DORA, AND ARISZTID G. B. KOVACH. *NAD/NADH: redox state changes on cat brain cortex during stimulation and hypercapnia.* Am. J. Physiol. 243 (Heart Circ. Physiol. 12): H619–H627, 1982.—The redox state of the anterior suprasylvian gyrus of cats was measured during electrical stimulation and under hypercapnia on cats immobilized and artificially respirated. The state of the nicotinamide adenine dinucleotide-reduced nicotinamide adenine dinucleotide (NAD/NADH) redox system was monitored by in vivo fluorometry. Hypercapnia was produced by inhalation of 10, 15, and 30% CO_2, respectively. Hypercapnic acidosis led to NADH oxidation. The NADH oxidation under 30% CO_2 inhalation was significantly larger (−14.9 ± 2.9%) than that observed under 10% (−6.5 ± 1.9%) and 15% CO_2 (−7.0 ± 1.6%) inhalation. Under normocapnic conditions, stimulation induced NAD reduction to NADH (5.5 ± 0.8%). The magnitude of the NAD reductive response to stimulation was unaffected by 10% CO_2 inhalation, but it was decreased by 15 and 30% CO_2 inhalation. The increased concentration of NADH upon stimulation is interpreted as resulting from an increased rate of substrate mobilization. The cause of the oxidation of the NADH pool of the cell during hypercapnia is partly due to the direct inhibitory effect of CO_2 on the carbohydrate metabolism, but the role of other mechanisms cannot be neglected either.

in vivo fluorometry; energy metabolism of brain; glycolysis; cortical activation; mitochondria

ELECTRICALLY OR CHEMICALLY induced seizure results in a three- to fourfold increase in the rate of glycolysis and enhances oxygen consumption of the brain cortex (26, 30). Enzymatic fluorometric assays on rapidly frozen brain cortex have been used to map the changes in the concentration of substrates of carbohydrate metabolism and to calculate the nicotinamide adenine dinucleotide-reduced nicotinamide adenine dinucleotide (NAD/NADH) redox state of the tissue under these conditions (5, 15). It was found that the mitochondrial NAD/NADH ratio increased (5), whereas the cytosolic NAD/NADH ratio decreased during bicuculline-induced seizures (5, 15). The advantage of the enzymatic fluorometric techniques is that the substrate concentration on the tissue can be directly measured. However, this method is not suitable for continuous monitoring of the metabolic processes of a localized microregion.

The in vivo measurement of the characteristic band of NADH fluorescence made it possible to monitor directly the intracellular NADH level in a continuous nondestructive way (3, 17). This method is accepted as an effective tool to gain information on the energy metabolism of the brain cortex in living animals (6, 9, 17, 22, 27, 29). Some authors have suggested that in vivo NADH fluorometry is indicative exclusively of the intramitochondrial NADH changes (17, 22, 27, 29). They reported oxidation of NADH in the brain cortex during both electrical stimulation (22, 27, 29) and pentylenetetrazole-induced epilepsy (17). In vitro studies on isolated mitochondria showed that the rate of oxidative phosphorylation increased and the NADH pool became more oxidized when the respiration was stimulated by adenosine 5′-diphosphate (ADP) (4). The parallelism between the changes found in vitro and in vivo led these authors (17, 27, 29) to conclude that the measurement of the NADH fluorescence in vivo was a good indicator of the rate of oxidative phosphorylation in the intact brain.

Other studies revealed that the activation of brain slices (21) and isolated ganglia (1) resulted in an increase in intensity of NADH fluorescence; i.e., the concentration of NADH increased. Our preliminary studies also suggested that the direct electrical stimulation of the brain cortex in vivo reduced the cortical NAD to NADH (6, 7, 9). These conflicting results raise the question of whether in vivo NADH fluorometry is a suitable tool to follow the rate of oxidative phosphorylation.

This paper attempts to clarify this controversial problem. It is known that stimulation leads to substrate mobilization through the activation of phosphofructokinase (15, 30), which results in increased NADH production and its accumulation in the cytoplasm (5, 15). In contrast, hypercapnia inhibits phosphofructokinase and thus decreases substrate mobilization via glycolysis (30). It is therefore expected that under hypercapnic conditions the NADH production via glycolysis would decrease. We wanted to find out whether cytosolic changes in NADH production induced by hypercapnia and stimulation were reflected in the intensity of the NADH fluorescence. In addition, we studied the effect of hypercapnia on the cortical NADH fluorescence changes induced by direct electrical stimulation of the brain cortex.

In this investigation the hemodynamic artifacts (19) in NADH fluorescence measurements were eliminated by a correction procedure (8, 11, 19) in which changes in local vascular volume were measured by means of ultraviolet reflectance measurements. In this way not only the metabolic changes but also the vascular changes of the same part of the tissue were studied.

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METHODS

Experiments were carried out on cats of both sexes weighing 2.5–3.5 kg. The animals were anesthetized with 50–60 mg/kg α-chloralose, immobilized by 2–4 mg/kg gallamine triethiodide (Flaxedil), and artificially respired by a Harvard respiratory pump (no. 680). Respiration was adjusted to keep the arterial oxygen and carbon dioxide tensions (P_{O_2} and P_{CO_2}) around 100 and 32–35 mmHg, respectively. To maintain the arterial pH above 7.35, sodium bicarbonate solution (4.2%) was infused intravenously. Siliconized cannulas were introduced into both femoral arteries, the left femoral vein and the left lingual artery. After this, the head of the animal was fixed in a stereotaxic stand. For optical recording, a craniotomy was performed. A hole of 12 mm in diameter was drilled in the left parietal bone, and the dura overlying the suprasylvian gyrus was excised. Bleeding from the bone and the dura was stopped by bone wax (Bonewax, Ethicon) and electrocoagulation. A 150-μm-thick Corning glass coverslip fixed in a plastic frame was implanted and cemented by dental acrylic into the hole (Fig. 1). The operative procedure from the beginning of the anesthesia required approximately 2 h. Special care was taken to minimize surgical trauma to the brain. Only those animals were used for experimental observation in which there was no sign of edema or any visible damage of the brain cortex and in which no hyperemia could be seen during the preparation. Electrical stimulation of the brain surface was performed through two gold plates (10–20 μm thick) fixed into the frame of the window. The electrodes were just touching the pial surface. Electrical stimulation in the form of square-wave pulses had the following parameters: 4-8 V, 1.4 ms, 16 Hz. Hypercapnia was produced by respiring the animals with gas mixtures containing 21% O_2 with 10, 15, or 30% CO_2 in N_2. The gas mixtures were made with three rotameter tubes (Air Products). Blood acid-base status was measured with a Radiometer ABL-1 using arterial blood samples (0.7 ml) taken anaerobically. Blood gas measurements were performed within 1 min after sampling. Local blood volume and NADH fluorescence of the cortex were monitored by fluororeflectometry (4, 17, 19) whereby the tissue is illuminated with a light of 366 nm wavelength that penetrates to a depth of approximately 1 mm (4). The ultraviolet light is reflected diffusely by the tissue and is scattered by the stroma of the red blood cells but is absorbed by the hemoglobin in the blood (19). The intensity of the reflected light is inversely related to the red blood cell content of the illuminated tissue volume (11).

Ultraviolet light of 366 nm excites the NADH molecules, which fluoresce with a peak wavelength of 450 nm. The intensity of the fluorescent light is dampened by the red blood cells, and this produces an apparent change in NADH concentration. To eliminate the spurious change in NADH fluorescence due to the variation of the cortical blood content, a correction procedure was applied (8, 11, 19) in which 0.1–0.3 ml of dextran solution was injected via the lingual artery into the cerebral vascular bed. The dextran solution dilutes the blood but does not cause any change in the NADH level of the cortex (1, 19), resulting

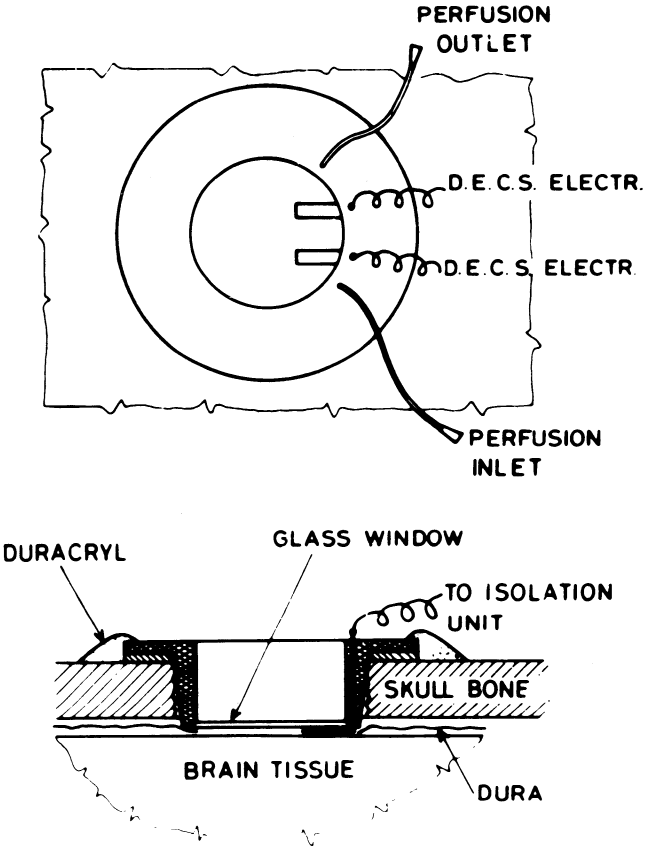

FIG. 1. Schematic diagram of window inserted into skull. DECS ELECTR, surface electrodes for direct electrical stimulation of brain cortex.

in an increase in both reflectance and fluorescence due to decreased absorption by hemoglobin. Dividing the maximum height of the fluorescence curve by the maximum height of the reflectance curve obtained during hemodilution provides a correction factor (k).

The real changes in NADH under any conditions can then be calculated by the following equation

$$\Delta CF_a = \Delta F_a - k\,\Delta R_a$$

where ΔCF_a is the corrected fluorescence; i.e. real NADH fluorescence change, and ΔF_a and ΔR_a are the measured changes in fluorescence and reflectance, respectively.

For the optical measurements a modified Chance fluororeflectometer was used. A 900-W alternating-current mercury arc lamp was used as a light source. The excitation light illuminated the brain cortex through a Leitz-microscope and an Ultropak X11 objective. Reflected and fluorescent light intensity were measured by end-window photomultipliers (EMI type 9524B) (Fig. 2).

The output of the photomultipliers was set to 1 V by adjusting the high voltage of the photomultiplier tubes. The changes in reflectance, fluorescence, and corrected fluorescence was expressed in percentage values of the control level. A 100% increase in these parameters means that the new value was twice the control.

The blood pressure was measured by a Statham pressure transducer. The temperature of the animals was kept between 37.5 and 38.5°C. The electrocorticogram (ECoG) was recorded by bifrontal leads using two stain-

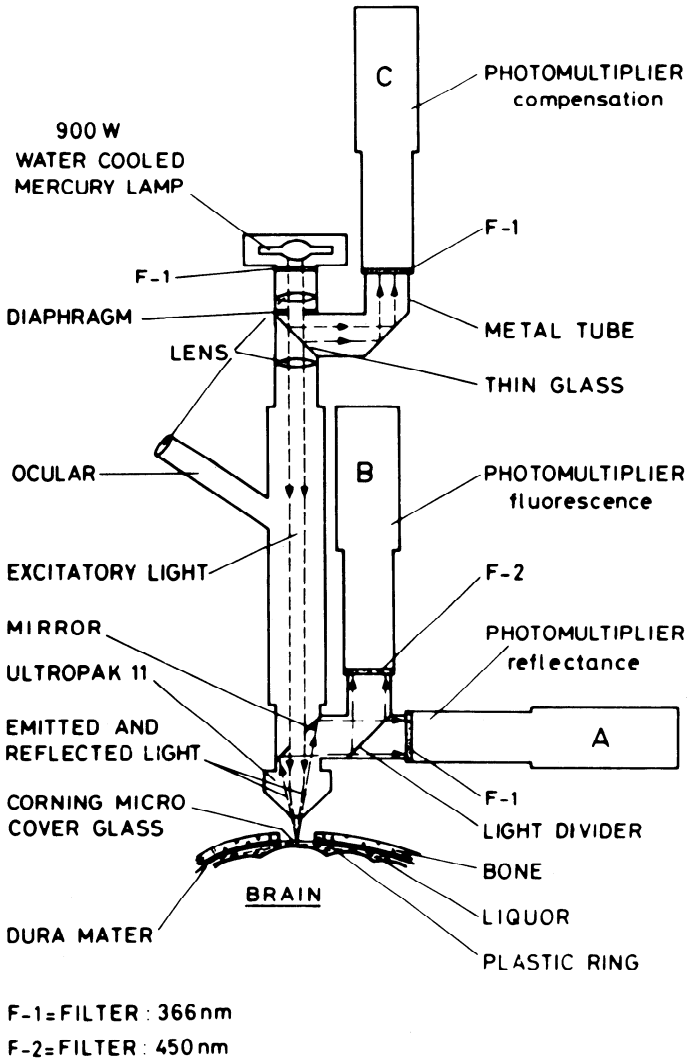

FIG. 2. Schematic diagram of fluororeflectometer. For details see METHODS.

less steel screws placed into the frontal bone.

Arterial blood pressure, ECoG, reflectance, and fluorescence were recorded on an eight-channel Galileo polygraph.

Experimental Procedure

For stimulation of the brain cortex, a voltage-inducing maximum vascular response in the control period was used. The intensity of stimulation was 2–2.5 times the threshold voltage, which was generally 2–3 V (9). The cortex was stimulated for 45–90 s before, during, and after each hypercapnic period. More than 10 min elapsed between stimulation and the start of the CO_2 inhalation.

Each CO_2 inhalation lasted for approximately 15 min, and the number of inhalations varied from experiment to experiment. More than 30 min elapsed between CO_2 inhalations.

The determination of the correction factor was carried out during steady states both before and during stimulation and hypercapnia.

Blood samples were taken before, during, and after CO_2 inhalations.

Data Processing

The fluorescence and reflectance signals were processed on a Hewlett-Packard digitizer using a sampling time of either 2.4 or 0.24 s, depending on requirements. Calculations from these data were made on Hewlett-Packard 9830A computer connected to a Hewlett-Packard *X-Y* plotter.[1]

Statistical analysis was performed by using nonparametric statistical tests. Nonparametric statistics were used because it seemed that the data to be analyzed did not follow normal distribution.

RESULTS

Stimulation

In a present experimental series the reactivity of the cerebrocortical energy metabolism to direct electrical stimulation at normocapnia was tested in 55 cases. In 51 cases the redox state of the NAD/NADH system of the cortex shifted toward reduction, whereas NADH oxidation was obtained only in 4 of 55 stimulations. The increase in NADH fluorescence of the tissue varied between +0.5% and +21.0%. The mean ± SE of the reductive response was +5.5 ± 0.8%. In some experiments the NAD reduction to NADH was preceded by a transient NADH oxidation (Fig. 3). The return to the control NADH level took about 3–6 min after the termination of the stimulation (Fig. 3).

Stimulation induced an increase in local vascular volume (−14.4 ± 1.1% reflectance decrease), although the mean arterial blood pressure did not change during stimulation.

The importance of the determination of the correction factor is shown in Fig. 3. As can be seen in Fig. 3, when a correction factor of 1 was used, a decrease in corrected fluorescence was obtained (4th curve in Fig. 3). With appropriate correction (made using either of the correction factors that were determined before or during cortical stimulation, 2.20 and 2.27, respectively) an increase in corrected fluorescence was obtained as a final change (2nd and 3rd curves in Fig. 3). The mean value of the correction factors determined in the control period before stimulation was 2.28 ± 0.13 and during stimulation 2.47 ± 0.18. The difference was statistically significant (Table 1).

Hypercapnia

Hypercapnia shifted the NAD/NADH redox state of the unstimulated cortex toward oxidation. The means of maximal changes in the NADH fluorescence were −6.5 ± 1.9, −7.0 ± 1.6, and −14.9 ± 2.9% during 10, 15, and 30% CO_2 inhalation, respectively (Fig. 4). The decrease in NADH fluorescence (NADH oxidation) induced by 30% CO_2 inhalation was significantly larger than that induced by 10 and 15% CO_2, whereas the amplitudes of the decrease in NADH fluorescence under 10 and 15%

[1] Biomedical Computer Programs P Series BMDP 77, University of California, Health Science Computing Facility, UCLA. W. J. Dixon and M. B. Brow.

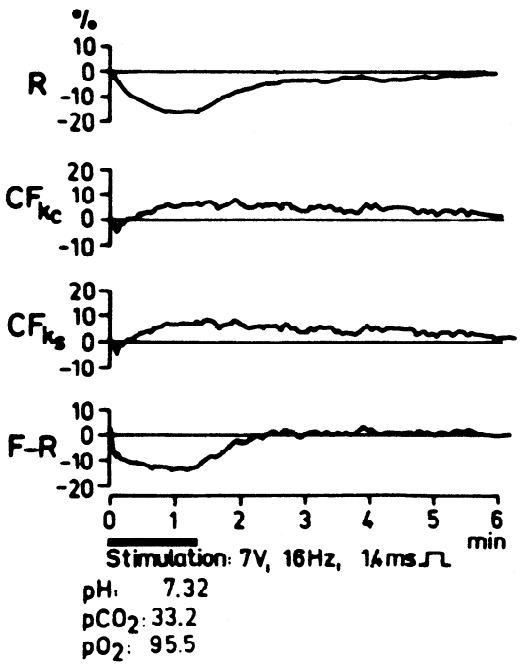

FIG. 3. Corrected fluorescence and reflectance changes induced by direct electrical stimulation of cortex in a single expt. R, reflectance; CF_{kc}, corrected fluorescence calculated by correction factor (k) determined prior to stimulation; CF_{ks}, corrected fluorescence calculated by correction factor determined during stimulation; F-R, subtraction of reflectance from fluorescence by a k factor of 1. Upward deflection in corrected fluorescence indicates NAD reduction. Downward deflection in reflectance indicates increase in local vascular volume. Graphs were plotted by a Hewlett-Packard 9830A computer and X-Y plotter after digitilization of original record using a sampling time of 2.4 s. pH, P_{CO_2}, and P_{O_2} are the values of arterial blood. Reflectance decreased (i.e., local vascular volume increased) during each stimulation at normocapnia (mean ± SE: −14.4 ± 1.1%).

CO_2 inhalation did not differ significantly from each other (Fig. 4).

The local vascular volume increased during 10, 15, and 30% CO_2 inhalation in the majority of the cases (−8.1 ± 1.4, −12.2 ± 2.8, −11.6 ± 3.0% reflectance decrease, respectively).

The value of the correction factor decreased during 15 and 30% CO_2 inhalation [from 2.19 ± 0.21 to 1.83 ± 0.20 and 2.54 ± 0.31 to 2.10 ± 0.26, respectively (Table 2)].

The dynamics of the change in NADH fluorescence during CO_2 inhalation were analyzed utilizing corrected fluorescence curves, which were obtained using three different correction factors. These three factors were determined before and during hypercapnia or were taken to be one (Fig. 5).

The corrected flourescence started to drop within 1–2 min after the start of the CO_2 inhalation, and the steady state was reached within 3–6 min. After the cessation of the 10 and 15% CO_2 inhalation, the corrected fluorescence usually returned to the control level in 5–25 min. Mainly after 30% CO_2 inhalation, but in a few cases after 15 and 10% CO_2 inhalation, the corrected fluorescence did not recover completely even in 30–40 min. Figure 5 shows the effect of 30% CO_2 inhalation on the corrected fluorescence calculated by the three different correction factors. In this case, the corrected fluorescence started to decrease about 80 s after the start of CO_2 inhalation and reached a steady state in approximately 5 min in all three curves. It remained at this level after termination of CO_2 inhalation.

The electrical activity of the brain cortex decreased during 30% CO_2 inhalation, but this change was reversible (Fig. 5). Direct electrical stimulation during 10% CO_2 inhalation produced the same extent of increase in NADH fluorescence as in normocapnia. However, during 15 and 30% inhalation, cortical stimulation elicited significantly smaller increase in NADH fluorescence (Figs. 5 and 6).

The magnitude of the vascular response induced by

TABLE 1. *Effect of stimulation on the value of the correction factor*

	Normocapnia		Hypercapnia					
			10% CO_2		15% CO_2		30% CO_2	
	C	S	C	S	C	S	C	S
Mean	+2.28	+2.47	+1.87	+1.97	+1.83	+1.87	+2.10	+2.10
±SE	±0.13	±0.18	±0.18	±0.25	±0.20	±0.20	±0.26	±0.25
n	55	55	16	16	24	24	15	15
P		<0.05	0.46	0.30				0.33

10% CO_2, 15% CO_2, 30% CO_2, percent of CO_2 in inhaled gas mixture. C, control; S, stimulation.

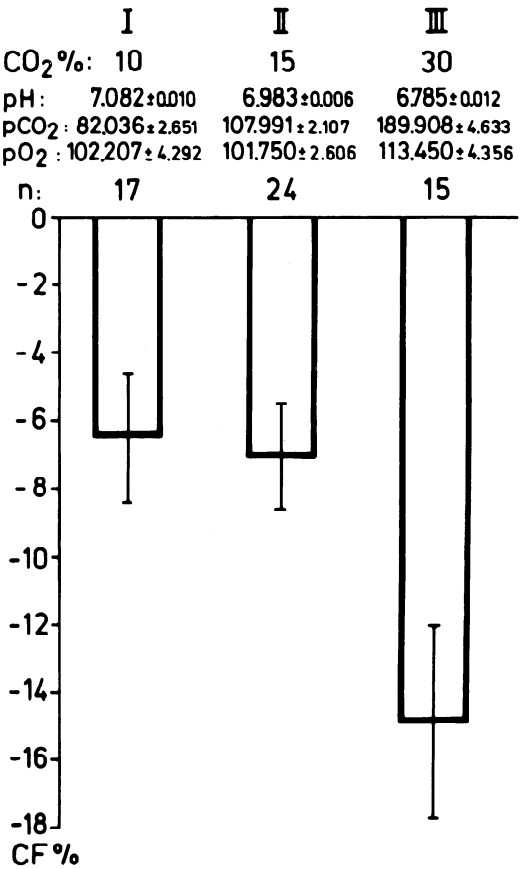

FIG. 4. Maximum changes in NADH fluorescence of cortex induced by hypercapnia. CO_2%, % CO_2 in inhaled gas mixture; CF, corrected fluorescence (NADH fluorescence). Negative values of CF means NADH oxidation. Data expressed as means ± SE. n, no. of cases averaged. pH, P_{CO_2}, and P_{O_2} are the values of arterial blood. Correction factor determined in steady state under hypercapnia was used to calculate corrected (NADH) fluorescence changes. Significant levels: I-II, nonsignificant; II-III, < 0.05; I-III, <0.05.

stimulation was as large during inhalation of 10% CO_2 as during the control. It decreased moderately during 15% CO_2 inhalation (from -15.4 ± 1.6 to -11.6 ± 1.2%) and decreased markedly during 30% CO_2 inhalation (from -11.8 ± 2.2 to -6.5 ± 1.5% reflectance decrease).

DISCUSSION

Methodological Aspects

The present study revealed that sustained direct electrical stimulation of the cortex resulted in a reduction of NAD to NADH. Most previous investigators found NADH oxidation under electrical stimulation of the cat brain cortex (20, 26, 28). The discrepancy between their results and ours can be explained as follows.

TABLE 2. *Effect of CO_2 inhalation on the value of the correction factor*

	CO_2 Inhalation					
	C	10% CO_2	C	15% CO_2	C	30% CO_2
Mean	+2.17	+1.89	+2.19	+1.83	+2.54	+2.10
± SE	±0.24	±0.46	±0.21	±0.20	±0.31	±0.26
n	17	17	24	24	15	15
P		0.096		<0.05		<0.01

10% CO_2, 15% CO_2, 30% CO_2, percent of CO_2 in inhaled gas mixture. C, control.

Methods of preparation. Traumas, even microtraumas of the brain, lead to disturbances in the vascular and redox responses induced by stimulation (9, 19). After long-lasting operation, heat trauma, or a hypotonic episode of 1 h, stimulation leads to a decreased vascular response, if any, and NADH oxidation (6, 19). Rosenthal and Jöbsis (27), Rosenthal and Somjen (29), and Lothman et al. (22) used intercollicular transection and generally could not observe consistent changes in local blood volume during stimulation, whereas the NAD/NADH system became more oxidized. The lack of vascular response during stimulation indicates a partial or complete paralysis of the cortical vessels (see explanation of NADH oxidation under these conditions on p. 16).

In our experiments special care was taken to minimize surgical traumas. The skull was closed by a window to restore the intracranial pressure and to maintain the physiological composition of the microenvironment of the cortical cells. Under such conditions we always observed increases in local blood volume during stimulation, showing that the brain circulation retained its normal responsive behavior.

Open skull vs. window technique. The fact that the skull was open throughout the experiments of Rosenthal and Jöbsis (27), Rosenthal and Somjen (29), and Lothman et al. (22) could lead to different results from those

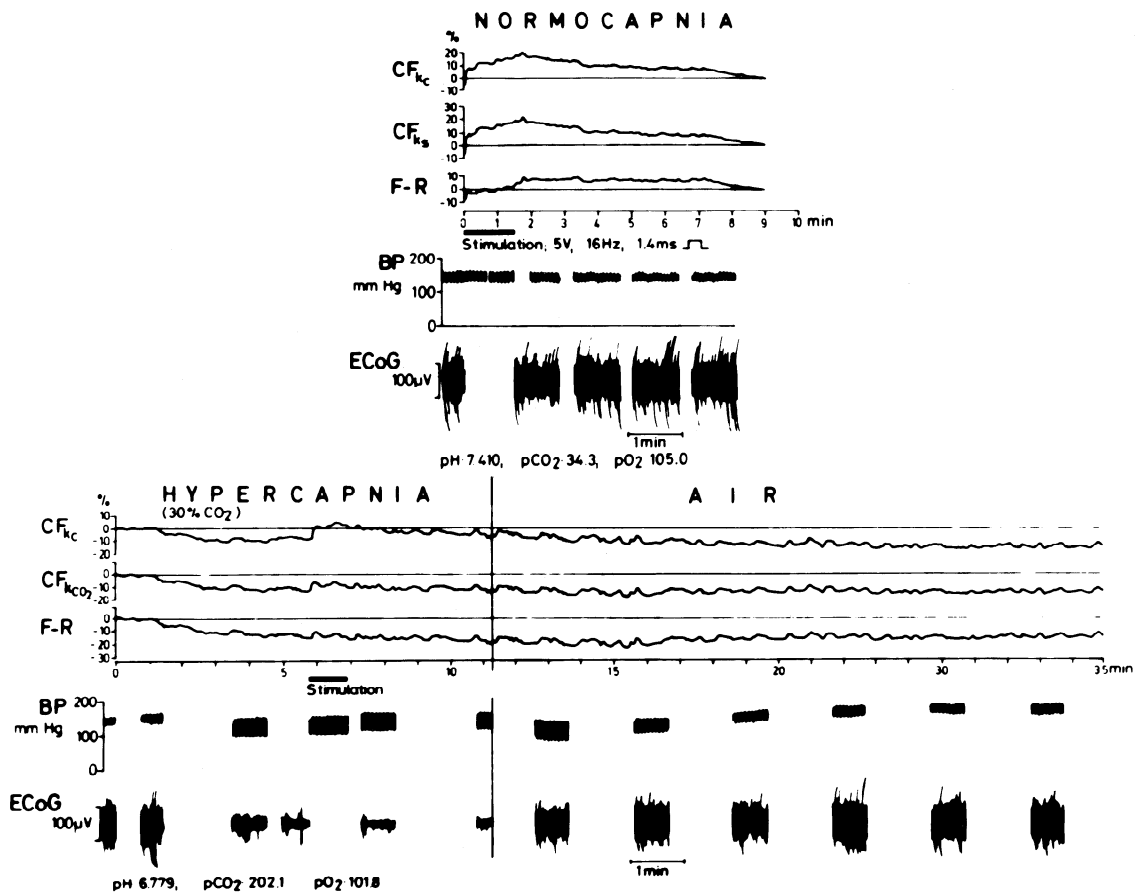

FIG. 5. Effect of 30% CO_2 inhalation on NADH fluorescence of cortex on electrocorticogram (ECoG) and on mean arterial blood pressure in a single experiment. CF_{kc}, corrected fluorescence calculated by correction factor (k) determined prior to stimulation; CF_{ks}, corrected fluorescence calculated by correction factor determined during stimulation; F-R, subtraction of reflectance from fluorescence by a k factor of 1; CF_{kCO_2}, corrected fluorescence calculated by correction factor determined under hypercapnia. pII, P_{CO_2}, and P_{O_2} are values of arterial blood. BP, blood pressure. Time from left to right.

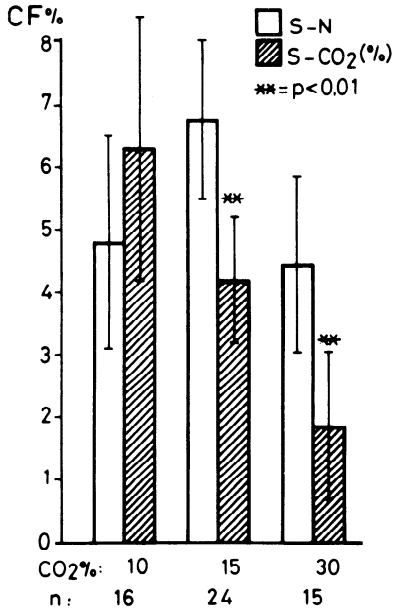

FIG. 6. Effect of hypercapnia on NADH fluorescence changes induced by direct electrical stimulation of cortex. S-N, stimulation under normocapnic conditions prior to hypercapnia; S-CO_2(%), stimulation under hypercapnia; CF, corrected fluorescence (NADH fluorescence). Positive values of CF mean NAD reduction. Data expressed as means ± S.E. *n*, no. of cases averaged.

described in this paper. One reason may be the loss of CO_2 from cerebrospinal fluid (CSF) into the air through either the oil or artificial liquor covering the cortical surface (24). However, we observed the increase in NADH upon stimulation to be similar during hypocapnia and normocapnia (unpublished observations), suggesting that the loss of CO_2 from CSF is not responsible for the disparate results.

Methods of stimulation. In our experiments field stimulation with surface electrodes was used. All the other authors who observed NADH oxidation to stimulation also used field stimulation with surface electrodes (22, 27, 29), so the difference in the way of stimulation cannot be responsible for the different results. Furthermore, Rosenthal and Jöbsis (27) compared fluorometric responses to stimulation on the surface and those evoked by stimulation 1 mm below the surface and found no significant differences to the different kinds of stimulation.

The difference between the effects of stimulation with different parameters cannot be excluded. In the present study stimulation with square waves of 1.4 ms duration of 16 Hz and 4–8.0 V were used. In the work of Rosenthal and Somjen (29) and Lothman et al. (22), when long-lasting stimulation was applied (30 s or more), the duration of square-wave pulses was 0.5 ms and the frequency was between 10 and 20 Hz. The difference in duration of square-wave pulses cannot be responsible for the differences, because Dóra and Kovách (7) observed NAD reduction to NADH and an increase in vascular volume when the cortex was stimulated for 40 s with square-wave pulses of 0.5 ms duration with a frequency of 15 Hz.

We tested whether the strength of the stimulation could be responsible for differences in fluorometric responses by stimulating the brain cortex with a voltage in the range other authors used (22, 27, 29). Furthermore, we tested the effect of stimulus strength on the changes in NADH level and cortical ultraviolet reflectance (9). In most of the cases stimulation caused an increase in NADH and always a decrease in ultraviolet reflectance of the cortex. We never were able to observe NAD/NADH changes without concomitant changes in the cortical ultraviolet reflectance. The authors referred to above always observed NADH oxidation during stimulation of any strength, whereas they never were able to see any consistent vascular response. These data suggest that the strength of the stimulation cannot be the factor that is responsible for the differences between our results and those of others. In contrast, the length of stimulation does seem to be an important factor. In our studies short-lasting stimulation [up to 0.5 s in our experiments (10)] resulted in NADH oxidation, whereas longer stimulation evoked NAD reduction to NADH, but the local vascular volume increased in each case. Rosenthal and Jöbsis (27) applied short stimulation and observed NADH oxidation but no consistent changes in the local blood volume to stimulation with any parameter. We feel that this lack of any consistent change in vascular volume to stimulation may be due to impaired integrity of the cortex, which can be responsible for the uniform NADH oxidation response.

Based on the above described considerations we suggest that the difference in parameters of the long-lasting stimulation should not be responsible for the different results of our experiments and those of others.

Methods of correction of hemodynamic artifacts. In our experiments the correction procedure of Harbig et al. (9) and Kovach et al. (19) was used. This proved to be a suitable method for eliminating the apparent NADH changes caused by the variations of the local blood content (11, 19). The mean value of the correction factor was around two, although there were great differences on various animals. This can be explained by the fact that the value of the correction factor is influenced by the optical arrangement (angle of illumination, illumination apparatus) and by the intensity of the background fluorescence (8). The correction factor as it is presented in Tables 1 and 2 was altered by electrical stimulation and CO_2 inhalation. Therefore, frequent determination of the correction factor is essential for the estimation of the real NADH changes.

Rosenthal and Jöbsis in their early paper used no correction at all for the contribution of local blood content changes to the fluorescence alterations during stimulation (27). Rosenthal and Somjen used a correction factor of one (29). In most cases in these studies no consistent or negligible changes in local blood volume were observed under stimulation. This means that these fluorometric transients were true measures of oxidative activity of the brain cortex because there was no need to correct for vascular changes. On those occasions when the cortical ultraviolet reflectance changes, mainly under seizures and spreading depression (29), the inappropriate correction could lead to erroneous estimations of the NADH changes in the cortex (see Fig. 3).

Metabolic Mechanisms

The NADH level of the cells is affected predominantly

by the following metabolic mechanisms. *1*) Production of NADH through glycolysis tends to increase the cytosolic NADH level. *2*) Production of NADH through Krebs-cycle and the oxidative decarboxylation of pyruvate tends to increase the mitochondrial NADH level. *3*) State 4-state 3 transition of mitochondrial oxidative phosphorylation tends to increase the NADH level of the cell, whereas state 3-state 4 and state 3-state 5 transitions leads to opposite changes. *4*) The overload of the capacity of carrier system transporting reducing equivalents from the cytosol to mitochondria tends to increase the cytosolic NADH level.

Increased neuronal activity leads to an increase in ADP and a decrease in adenosine 5′-triphosphate (ATP) concentration (30). This activates the mitochondrial oxidative phosphorylation (state 4-state 3 transition) (4), and, if sufficient amounts of oxygen are available, oxidation of the mitochondrial NADH pool takes place. There should be enough oxygen, because tissue Po_2 increases (20, 30) as does venous Po_2 (2, 14) during electrical stimulation of the cortex or during epileptic seizures. The local oxygen concentration at the mitochondria in situ should not be much lower than the extracellular oxygen concentration even at high respiratory activity (33). The venous Po_2 and Po_2 measured by microelectrodes do not necessarily correspond to the tissue level, leaving the question open as to whether the cortex is or is not hypoxic under seizure and electrical stimulation of the brain cortex. However, the following experiments on brain cortex show that during increased electrical activity NADH is oxidized to NAD even when the CBF is decreased: in hemorrhagic shock and when the blood pressure is lower than the autoregulatory level of the brain circulation, NADH oxidation also occurs as a result of direct cortical stimulation (7). Furthermore, cytochrome aa_3 becomes more oxidized during epileptic seizures in normotension and on low blood pressure (13). NADH oxidation takes place in the cortical tissue as a response to direct stimulation of the cortex under conditions when the cortical vessels are unresponsive (22, 29). These data strongly suggest that during increased neuronal activity the mitochondria do not become hypoxic. Hence, we have to assume that in our experiments NADH fluorescence increased because the total rate of NADH production throughout the cells exceeded the rate of mitochondrial NADH oxidation. Accordingly, Chapman et al. showed that the NAD/NADH ratio increased in the mitochondria but decreased in the cytosol (3) during bicuculline-induced epileptic seizures.

Fluorometric measurements on isolated rat ganglion and brain slices indicate that stimulation leads to transient NADH oxidation followed by a long NADH reduction to NADH. After addition of pyruvate to the ambient medium, the NAD reductive response decreased by 90% (21) and 100% (9), signifying that the fluorometrically measured increase in NADH level to stimulation can be attributed to at least 90% cytosolic, glycolytic NAD reduction to NADH.

On the basis of the above facts we tentatively suggest that the increased NADH fluorescence during stimulation of the brain cortex may arise predominantly from the increased cytosolic production of NADH.

We cannot exclude the possibility that the mitochondrial NADH level also increases, but we have mentioned above reasons for believing that there is sufficient oxygen to support oxidation of NADH to NAD. We presume that the H^+-transporting system from the cytosol to the mitochondria and, to a lesser extent, the mitochondrial oxidation of NADH may not match the increased NADH production. The occasional small NADH oxidation in the early phase of stimulation shows that in this phase the mitochondrial NADH oxidation exceeds the NADH production. In light of this, the question arises, what is the reason for the NADH oxidative response to stimulation on traumatized brain?

Traumas of the brain can lead to serious deterioration of the brain metabolism in a manner most similar to ischemic insults (23, 25, 32) and, at least in the early phase, resembling excitation of neurons (23, 25). The cerebral metabolic rate of oxygen ($CMRO_2$) decreases after concussion or contusion of the head, showing that the mitochondrial substrate oxidation is decreased (23). Excess lactate (23) and the lactate-to-pyruvate ratio (25) increases because the rate of glycolysis increases (32). Under these conditions additional stimulation of the brain cortex cannot increase the flux of reducing equivalents to the mitochondrial respiratory chain as much as under normal physiological conditions because glycolysis is already activated. Therefore, electrical stimulation during trauma cannot bring about the same extent of activation of glycolysis and NADH production as in nontraumatized cortex. As a result, the increased NADH oxidation in the mitochondria due to stimulation exceeds the extra NADH production. Consequently, a decrease in NADH fluorescence is measured by fluorometry. This interpretation is valid only if we assume that there is sufficient oxygen to match the increased demand of oxidative phosphorylation. As we pointed out above, there should be enough oxygen because, even during severe arterial hypotension, NADH (7) and cytochrome aa_3 oxidation occurs (13) as a response to cortical stimulation and epileptic seizure.

In the present study we demonstrated that inhalation of gas mixtures containing 10, 15, and 30% CO_2 induced NADH oxidation. At least four mechanisms can be responsible for this phenomenon.

First, NADH oxidation can be explained as the direct effect of CO_2 on the carbohydrate metabolism, i.e., as the inhibition of the phosphofructokinase (30). It can be assumed that the decreased rate of glycolysis brings about decreased production of NADH that is measured as a decrease in NADH fluorescence.

The second mechanism that could participate in the NADH oxidation is the increase in O_2 supply through vasogenic mechanisms in those parts of the brain that were nearly hypoxic when the blood gas values and blood flow were normal (12, 28). This might explain the marked oxidation of cytochrome aa_3 when hyperbaric and/or normobaric hyperoxia is combined with hypercapnia. However, decreased supply of substrate or reducing equivalents to mitochondria could also lead to cytochrome aa_3 oxidation in these studies (12, 18). Increased oxygen delivery to the mitochondria under hypercapnia can be the result of increased oxygen delivery from hemoglobin as a result of decrease in arterial pH (Bohr effect) as well.

Third, NADH oxidation can be a consequence of the uncoupling of the mitochondria produced by hypercapnia (30).

Fourth, if H^+ is rate limiting in the cytochrome aa_3 oxidation, as suggested by Jöbsis (16), and if we accept the near-equilibrium theory of the oxidative phosphorylation (33), then the increase in intramitochondrial H^+ concentration due to the increased diffusion of CO_2 through the mitochondrial membrane can lead to NADH oxidation.

There are conflicting results concerning how the $CMRO_2$ changes during hypercapnia (31). The combination of the above described mechanisms may lead to increased, decreased, or unchanged O_2 consumption depending on the magnitude of their changes. Our data are not sufficient to clarify the quantitative aspects of the metabolic changes under hypercapnia. But we can suggest that certain amounts of NADH oxidation during 15 and 30% CO_2 inhalation is a consequence of the inhibitory effect of CO_2 on the glycolysis, whereas this effect is negligible in 10% CO_2 inhalation. This can be concluded because direct cortical stimulation induced a smaller NAD reduction during 30 and 15% CO_2 inhalation than in the control stimulation. This shows that the glycolytic NADH production was decreased either by direct inhibition of the glycolysis or due to the depression of neuronal activity. The occurrence of NADH production induced by stimulation under severe hypercapnia may be indicating that the in vivo CO_2 inhibition of the phosphofructokinase can be overridden by other positive modulators.

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