

Use of a Rapid Brain-sampling Technique in a Physiologic Preparation:

Effects of Morphine, Ketamine, and Halothane on Tissue Energy Intermediates

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A new method of rapid sampling of brain tissue, "freeze-blowing," has been used to compare the neurochemistry of the brain during anesthesia with that in the awake state. The method avoids anoxia associated with the sampling process. Physiologic variables, including body temperature, blood-gas tensions and blood pressure, were carefully monitored and controlled in the experimental animals. None of the agents tested (halothane, morphine, and ketamine) reduced the brain tissue high-energy phosphate reserves. All three drugs doubled glucose levels. Morphine lowered both lactate and the lactate/pyruvate ratio. Uniformly, the three anesthetic agents led to twofold increases of brain cyclic 3'-5' adenosine monophosphate concentrations. These changes suggest a possible role for cyclic nucleotides in central neurotransmission. (Key words: Anesthetics, intravenous, ketamine; Analgesics, narcotic, morphine; Anesthetics, volatile, halothane; Brain, metabolism, ketamine; Brain, metabolism, morphine; Brain, metabolism, halothane; Metabolism, glycolysis; Metabolism, citric acid cycle; Metabolism, high-energy phosphate compounds; Metabolism, cyclic 3'-5' adenosine monophosphate.)

THE ENERGY STATE of the brain and its relationship to the mechanism of anesthesia

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have been of interest since the observation by Quastel and various co-workers¹⁻⁴ that anesthetics could block the oxidation of certain substrates at the NADH dehydrogenase step. Their studies, done with slices or minces of brain *in vitro*, have been expanded greatly, with localization of the block to one step in the electron-transport chain in the mitochondrion (see Cohen's recent review⁵).

However, most of the corresponding studies of the biochemical state of the brain *in vivo* have been limited either by anoxia associated with the sampling process itself⁶ or by anesthesia and surgery needed to obtain brain samples. Indirect methods, such as decapitation and freezing or whole-animal freezing, require some large finite interval for propagation of the thermal front through the tissue, allowing continued metabolism after the circulation has been stopped.⁷ The methods that avoided this artifact by exposing the brain or dura and directly applying liquid nitrogen or other coolant to the surface layers^{8,9} introduced the effects of the surgery and requisite anesthesia.¹⁰ Veech *et al.*¹¹ have developed a new method, called "freeze-blowing," that allows elimination of all of these artifacts, giving direct access to the supratentorial portion of the rat brain and providing for rapid freezing to the temperature of liquid nitrogen in less than one second. Their method has the added advantage that it can be used to obtain tissue from normal, unanesthetized animals.

An additional area of difficulty with the *in-vivo* studies has often been the lack of control of arterial carbon dioxide tension and temperature, both of which are well known to alter cerebral tissue perfusion and metabolism.¹² Therefore, we have developed a method to control physiologic variables in

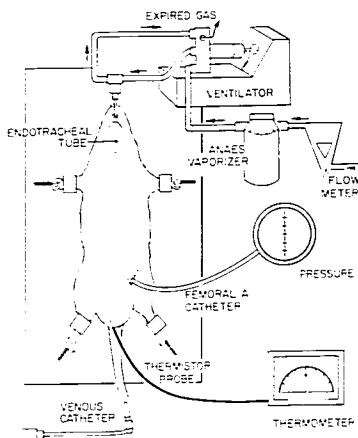


FIG. 1. Diagram of procedure for monitoring animals placed in the stereotaxic "freeze-blowing" apparatus.

experimental anesthetized animals specially adapted to the constraints imposed by the stereotaxic freeze-blowing apparatus, and have investigated the effects of anesthesia on cerebral tissue metabolism in this stable setting. The effects of three chemically diverse agents—halothane, morphine, and ketamine—on the concentrations of brain energy metabolites were measured and compared with corresponding values in awake, normal controls following freeze-blowing with liquid nitrogen, in the absence of anesthesiologically induced ventilatory depression or hypothermia. Representative compounds from the pathways of glycolysis, the citric acid cycle, and high-energy phosphate metabolism were measured to reflect any alterations associated with anesthesia.

The technique used in this study achieves three important objectives. First, a true comparison between neurochemistry in the awake state and that in the anesthetized state may be made. Second, cerebral anoxic changes resulting from delays inherent in the sampling method are eliminated. Third, the secondary effects of anesthesiologically induced

hypercarbia and hypothermia on brain metabolism are excluded.

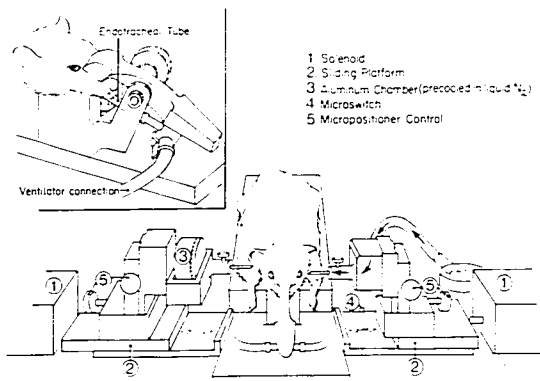
Materials and Methods

Male 200-g Wistar strain rats (Charles River Breeding Laboratories, fed *ad libitum* with Purina Rat Chow (Ralston Purina Company) and allowed free access to water, held in wire-bottomed cages at 22°C in a 12-hour-light (08:00–20:00)–12-hour-dark environment, were used for all studies. After percutaneous placement of a 22-gauge catheter (Argyle Medicut, Aloe Medical) in a tail vein and attachment of a "T" connector (Item 4612, Abbott Laboratories) filled with heparinized saline solution, experimental animals received a uniform one-hour course of anesthetic. Following induction of anesthesia, the trachea was intubated orally under direct visualization with a modified Miller Number 1 laryngoscope blade, adapted from the technique of Hey and Pleuvry,¹³ using a 15-gauge intravenous catheter (Jelco Laboratories) as the endotracheal tube. This allowed the control of ventilation with a volume-cycled, nonbreathing device (Model 680 Rodent Respirator, Harvard Apparatus), with supplemental oxygen. Next, a femoral artery was cannulated, distal to the origin of the profunda femoris, using a 22-gauge catheter (Bardic Cutdown Catheter, C. R. Bard, Inc.) with the tip beveled. The catheter was connected through a sampling stopcock to a strain-gauge pressure transducer (P23Db, Statham, Hato Rey, Puerto Rico), and the blood pressure displayed on an oscilloscope (ORM-2, Electronics for Medicine), previously calibrated against a mercury column.

A thermistor probe was placed in the rectum for monitoring with a telethermometer (43TA, Yellow Springs Instruments Company). Animals were warmed with either a heating coil under the abdomen or a warming lamp over the dorsum. Rectal temperature was held between 36.5 and 37.5°C. When all monitoring equipment was in place (fig. 1), the animal was transferred to the stereotaxic freeze-blowing apparatus (Precision Medical Instruments, Inc.) and its probes carefully aligned.

Using a 150- μ l microcuvette-electrode

FIG. 2. Schematic view of stereotaxic "freeze-blowing" device. Inset shows the arrangement of endotracheal tube and ventilator connections as they pass about the head restraint.



blood-gas device (BMS-3 Mk 2 Blood Micro System, with PHM-72 Mk 2 Digital Acid-Base Analyser with PHA 934 P_{O_2} module, Radiometer Copenhagen, The London Company), calibrated with analyzed gases (also The London Company), the ventilator and the supplemental oxygen were manipulated to maintain P_{aO_2} above 100 torr and P_{aCO_2} 35–45 torr. No more than three arterial blood samples of 0.30 ml each were removed from any rat, and the volume taken was always returned at least twofold with balanced salt solution given intravenously. Systolic arterial pressure was always 90 torr or more at the time of brain sampling.

The brain-sampling process followed the method of Veech and co-workers,¹¹ with the freeze-blower modified to allow passage of the endotracheal tube (fig. 2). Their arrangement used a set of two solenoid-driven hollow-needle probes, stereotaxically aligned, to sample the brain. One probe was connected to a high-pressure propellant gas supply when both had been driven into the skull, thereby displacing the tissue out of the cranial vault through the other hollow probe into an aluminum receiver. This set of aluminum plates is designed with appropriate geometry to force the brain specimen into a very thin layer. The plates are held in liquid nitrogen until immediately before sampling,

and their high heat capacity and good thermal conductivity ensure that the thin tissue layer will freeze in less than 1 second, arresting all biochemical activity. Most of the supratentorial brain except the most rostral tip of the olfactory bulbs is obtained. Control animals, fully awake, breathing room air spontaneously, were placed in the stereotaxic device in a special restraining cage provided with the machine.

In rats receiving halothane, anesthesia was induced with high flows of halothane (Fluothane, Ayerst Laboratories, Inc.) in oxygen, delivered through a calibrated vaporizer (Fluotec 3, Cyrane Ltd. through Fraser Sweatman, Inc.) via a nose cone, using 5 per cent v/v inspired for 5 minutes. The tracheas were then intubated as described and the animals placed on the ventilator with the inspired concentration reduced to 1.0–1.5 per cent for maintenance. For the last 5 minutes before sampling, the inspired concentration was always 1.5 per cent.

In rats anesthetized with morphine, anesthesia was induced with an intravenous dose of 20 mg/kg body weight, given as the sulfate. A second dose of 10 mg/kg was given at 30 minutes.

Animals anesthetized with ketamine could not be satisfactorily ventilated without a supplemental muscle relaxant; consequently,

they formed the only group to receive more than one drug. Ketamine HCl (Ketalar, Parke, Davis and Company) was given intravenously in 10-mg/kg body weight increments at induction, at 5 minutes and 10 minutes, then at 10-minute intervals through 50 minutes. A final dose was given 90 seconds prior to sampling. Pancuronium Br (Pavulon, Organon, Inc.) was given as a supplemental relaxant in two 0.1-mg/kg doses intravenously, the first immediately after intubation and the second at 32 minutes.

Biochemical assays were performed using enzymes and cofactors from Boehringer Mannheim Corporation and Sigma Chemical Company, with other chemicals being reagent-grade materials from Fischer Scientific Company, Mallinckrodt Chemical Works, and J. T. Baker Chemical Company. Specimens were stored under liquid nitrogen until a group of six was available to process as a set. Each brain was ground to a powder under liquid nitrogen with mortar and pestle, yielding around 0.8 to 1.0 g, then deproteinized and extracted at -20°C as described by Miller and co-workers.¹⁴ Most individual assays were performed as described by Bergmeyer,¹⁵ Lowry and Passonnan,¹⁶ Miller and co-workers,¹⁴ and Biebuyck and co-workers,¹⁷ using enzymatic coupling to pyridine nucleotides and following the characteristic spectral shift at 340 nm with the transition between oxidized and reduced forms, as seen on a spectrophotometer (PMQ II, Carl Zeiss Inc.). Concentrations of cyclic

3'-5' adenosine monophosphate, however, were kindly determined for us by the laboratory of Dr. Hans Bode by the procedure of Gilman,¹⁸ using displacement of labeled AMP from a binding protein (Cyclic AMP Assay Kit, Affitron Corporation).

Control groups for individual assays included 11 to 22 animals, and experimental groups included 6 to 14 individuals. Data were combined to yield group means and standard errors of the means. Tests for significant differences from the control values were based upon these group means and standard errors, using Student's *t* test,¹⁹ with $P < 0.05$ accepted as significant.

Results

Table 1 shows the physiologic state of the test animals after stabilization in the brain-blowing apparatus prior to brain sampling. Pa_{O_2} 's were above saturation for all three groups. The major variation noted between groups was that the systolic arterial pressures obtained in the animals anesthetized with halothane were lower than those obtained with morphine and ketamine.

None of the three drugs tested caused reduction in any of the high-energy phosphate compounds (see table 2). Halothane elevated phosphocreatine to 17 per cent above control; adenosine diphosphate levels were increased 10 per cent by ketamine. A significant change uniformly induced by all three drugs was a doubling of cyclic 3'-5' adenosine

TABLE 1. Physiologic Variables (Means \pm SEM)*

	Halothane	Morphine	Ketamine
P_{O_2} (torr)	213.9 \pm 21.7 n = 16	268.9 \pm 21.2 n = 17	324.9 \pm 32.5 n = 15
P_{CO_2} (torr)	37.27 \pm 1.37 n = 16	40.23 \pm 0.80 n = 17	37.79 \pm 0.66 n = 15
pH	7.438 \pm 0.018 n = 16	7.389 \pm 0.014 n = 17	7.367 \pm 0.012 n = 15
Systolic arterial pressure (SAP) (torr)	103.3 \pm 5.95 n = 9	156.3 \pm 5.51 n = 12	151.7 \pm 3.86 n = 15
Temperature (C)	37.66 \pm 0.185 n = 16	38.06 \pm 0.240 n = 17	37.32 \pm 0.293 n = 15

* Measured as described in Materials and Methods.

TABLE 2. Brain Tissue Concentrations of Phosphate Energy Reserves and of Cyclic 3'-5' Adenosine Monophosphate (cAMP) (Means \pm SEM)

	Control n = 16	Halothane n = 8	Morphine n = 8	Ketamine n = 8
Adenosine triphosphate (μ mol/g wet weight of tissue, uncorrected)	2.21 \pm .055	2.193 \pm .0683	2.065 \pm .070	2.13 \pm .0373
Phosphocreatine (μ mol/g wet weight of tissue, uncorrected)	3.21 \pm .046	3.753 \pm .1061**	3.24 \pm .158	3.325 \pm .157
Adenosine diphosphate (μ mol/g wet weight of tissue, uncorrected)	.434 \pm .0179	.4311 \pm .0168	.4336 \pm .0176	.479 \pm .0125*
Adenosine monophosphate (μ mol/g wet weight of tissue, uncorrected)	.041 \pm .0027	.0419 \pm .0012	.036 \pm .00362	.0393 \pm .0021
Cyclic 3'-5' adenosine monophosphate (nmol/g wet weight)	1.288 \pm .1008	2.50 \pm .130**	2.649 \pm .1544**	2.33 \pm .308**

** Significant difference from control, $P < 0.01$.

monophosphate concentrations. None of the three agents caused any decrease in the adenylate energy charge²⁰:

$$\frac{(\text{ATP}) + 0.5 (\text{ADP})}{(\text{ATP}) + (\text{ADP}) + (\text{AMP})}$$

felt to be an important intracellular control signal for energy metabolism.

Table 3 shows the levels of representative constituents of glycolytic and tricarboxylic acid cycle intermediates. Ketamine elevated pyruvate 25 per cent, while morphine caused both lactate and the lactate/pyruvate ratio to decrease significantly. All agents approximately doubled glucose levels, but only halothane altered 2-oxoglutarate to 130 per cent of control and glucose-6-phosphate to 83 per cent. Morphine depressed malate concentrations by about a third, but halothane increased them to about 20 per cent above control levels.

Discussion and Conclusions

In 1972, using the freeze-blowing technique, Biebuyck and Hawkins²¹ investigated changes in cerebral tissue metabolic intermediates that occur with halothane, pentobarbital, and ketamine. They found increases in ATP with halothane and pentobar-

bital, reductions in lactate by half with pentobarbital and by a third with halothane and an increase in tissue glucose with halothane. All three of the agents lowered glucose-6-phosphate, but only pentobarbital and halothane decreased 2-oxoglutarate while ketamine caused an increase.

The preparation they used was the spontaneously breathing rat. No method then existed for controlling the body temperature or for the administration of inhalation anesthetics or controlled gas mixtures once the animal had been positioned in the stereotaxic freeze-blowing apparatus, nor had the appropriate monitoring modalities been adapted to use with the apparatus. The difference in the present results may relate to these differences in the techniques, especially as regards the influences of various inhalation anesthetic concentrations, drug-induced respiratory depression, and hypothermia.

Using the freeze-blower with the same animal preparation, Miller and co-workers¹¹ investigated the effects of morphine. They found that many of the alterations observed in cerebral-tissue metabolite levels with morphine could be readily duplicated by either carbon dioxide inhalation or acetazolamide injection. These changes in

TABLE 3. Brain Tissue Concentrations ($\mu\text{mol/g}$ Wet Weight of Tissue, Uncorrected) of Glycolytic and Tricarboxylic Acid Cyclic Intermediates (Means \pm SEM)

	Control	Halothane	Morphine	Ketamine
Glucose	1.20 \pm .0317	2.66 \pm .198**	2.708 \pm .1897**	2.02 \pm .1461**
Glucose-6-phosphate	.1338 \pm .005	.1108 \pm .0038**	.1215 \pm .00599	.131 \pm .0068
Pyruvate	.0845 \pm .0066	.0889 \pm .0066	.0675 \pm .0056	.106 \pm .0069*
Lactate	1.073 \pm .02695	1.041 \pm .0714	.734 \pm .0800**	1.144 \pm .0771
[Lactate] [Pyruvate]	13.85 \pm .9097	11.94 \pm 1.117	11.03 \pm .898*	11.25 \pm .326
2-oxoglutarate	.1184 \pm .0092	.1544 \pm .0094*	.140 \pm .0101	.1138 \pm .0114
Malate	.238 \pm .062	.2844 \pm .0199*	.1415 \pm .0205**	.2202 \pm .0163

* Significant difference from control, $P < 0.05$.** Significant difference from control, $P < 0.01$.

cluded elevation of glucose and decreases of lactate, pyruvate, 2-oxoglutarate, malate, and creatine phosphate. They saw no change in tissue levels of adenine nucleotides. Their observations reaffirm the concept that P_{CO_2} must be rigidly controlled in neurochemical experiments.

With the elimination of these methodologic difficulties, our study shows that there is no depression of high-energy phosphate reserves, or of the adenylate energy charge,²⁰ upon exposure to three anesthetics of widely differing chemical natures. That the energy reserves of the brain are not decreased, and that brain lactate is normal or decreased, are indicative of adequate energy supply and the presence of adequate tissue oxygenation. The brain redox state, both cytoplasmic and mitochondrial, has also been shown to be unaltered during exposure to these agents.²¹ Derr and Zieve²⁵ suggest that the decreased cerebral oxygen uptake in coma (and anesthesia) may result from decreased oxidative phosphorylation, probably on the basis of decreased utilization of ATP.

The increase in brain-tissue glucose shown in these experiments in the absence of anoxia due to sampling delay is in agreement with the increase in glucose in mouse brain during halothane anesthesia demonstrated by Brunner *et al.*¹⁰ These authors suggested that the mechanism for transport of glucose into brain may be altered because of an elevated ratio of glucose in brain to that in blood. This

brain/blood glucose relationship is the experimental situation discussed in detail by Biebuyck *et al.*²⁴

The uniform elevation of brain cAMP during anesthesia in this study raises the question of the possible role of cyclic nucleotides in central neurotransmission. Iversen²⁶ suggested that the inhibiting effects of norepinephrine could be mediated indirectly by cAMP, produced and released by glial cells. Clearly, further work is necessary, not only in whole brain, but also in isolated brain compartments, before these changes can be fully understood.

The main purpose of the present communication is to indicate that by the use of a new technique of rapid sampling and freezing it is possible to compare, for the first time, the neurochemistry of the fully awake state and that of the anesthetized state. Lack of regional detail is a disadvantage of the present technique. In view of the extremely early changes in labile intermediates that occur post mortem, it is essential that the rapid sampling and freezing method be adapted to regional brain sampling also. This improvement, together with arteriovenous substrate differences across the brain and simultaneous CSF sampling, will further our understanding of the neurochemical basis of anesthesia.

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