# Aminophylline Increases Cerebral Metabolic Rate and Decreases Anoxic Survival in Young Mice

Abstract. *In weanling mice treated with pharmacologic doses of aminophylline, the concentrations of adenosine 3',5'-monophosphate and guanosine 3',5'-monophosphate in the brain increased 44 and 36 percent, respectively, and the cerebral metabolic rate was three times that in controls. In neonatal mice, therapeutic doses of aminophylline greatly decreased the rate of anoxic survival in vivo and the duration of gasping of the isolated head. The findings suggest caution in the use of this drug and other methylxanthines in hypoxic human newborns.*

Irregular or periodic respirations are a normal finding in premature infants. However, in about 30 percent of those weighing less than 1750 g, this breathing pattern is associated with sudden, recurring attacks of apnea (*1*). When the apneic episodes are associated with bradycardia or cyanosis, there is risk of anoxic brain damage with permanent neurologic or psychologic sequelae. In the past, clinical management of these infants has been a serious and difficult problem. In 1973 Kuzemko and Paala (*2*) reported that aminophylline rapidly abolished apneic attacks in these infants. Since then, equally impressive results from the use of methylxanthines in apnea of prematurity have been reported in at least six other studies. Over 50 years ago it was known that even with such severe complications as heart failure, uremia, and intracranial hemorrhage, aminophylline had a prompt, dramatic effect in restoring Cheyne-Stokes respirations to normal in adults (*3*).

The mode of action of methylxanthines in restoring normal breathing patterns in these conditions is not known. They appear to stimulate directly the respiratory center in the medulla by altering the sensitivity to $CO_2$ (*4*). Despite increased cardiac output (*5*), cerebral circulation is not improved. Wechsler *et al.* (*6*) studied the effects of intravenous injections of aminophylline (250 mg) on cerebral circulation and metabolism in ten randomly selected hospitalized patients without "cerebral depression." Four patients had a statistically significant increase in cerebral $O_2$ consumption and six showed a statistically significant decrease. Since all patients had a decrease in cerebral blood flow and a reduced amount of $O_2$ in blood from the internal jugular vein, it was concluded that aminophylline caused anoxia of cerebral tissue. It is of further interest that severe clinical side effects were only seen in those patients who showed an increase in cerebral metabolic rate. Others have confirmed the effect of aminophylline in reducing cerebral blood flow (*7*).

We have investigated the effects of aminophylline on carbohydrate and energy metabolism in the brains of normal 17- to 23-day-old mice (mean weight of 22 animals, 12.0 ± 0.4 g) (*8*). At an arbitrarily chosen dose of 100 mg of aminophylline per kilogram, injected intraperitoneally, we observed no clinical side effects.

Twenty minutes after aminophylline administration the amount of glucose in the plasma increased 29 percent, the amount of β-hydroxybutyrate 91 percent, and plasma glycerol 141 percent (Table 1). Since methylxanthines inhibit 3',5'-cyclic nucleotide phosphodiesterase activity (*9*), these results were attributed to the well-known effects of increased levels of adenosine 3'-5'-monophosphate (cyclic AMP) in peripheral tissues (glycogenolysis in liver; lipolysis in fat depots). The findings are also compatible with increased secretion of epinephrine or glucagon, or both.

Not surprisingly, in the brains of these animals, cyclic AMP increased 44 percent and guanosine 3',5'-monophosphate (cyclic GMP) 36 percent (Table 2). The most striking finding was the 79 percent increase in glucose concentration. The concentration of glucose 6-phosphate also increased, but there were no differences in the other glycolytic intermediates (Table 2). The increase in brain glucose was much higher than could be expected from the observed increase in plasma glucose (*10*). At first we considered that the finding might be due to a sparing of glucose utilization in brain be-

Table 1. The effect of intraperitoneal injections of aminophylline (100 mg/kg) on selected metabolites in the plasma of young mice. Data are expressed in millimoles per liter of plasma, and the values are means ± standard error. After the mice were decapitated blood was collected from the severed neck vessels in heparinized capillary tubes. The capillary tubes were promptly centrifuged at 4°C and extracts of the plasma were prepared in perchloric acid (*12*). β-Hydroxybutyrate was measured by the method of Williamson *et al.* (*21*), and glycerol by a modification of the method of Burch *et al.* (*22*).

| Metabolite | Controls ($N$ = 7) | Injected with aminophylline ($N$ = 7) | $P$ value |
|---|---|---|---|
| Glucose | 9.13 ± 0.14 | 11.80 ± 0.48 | <.001 |
| β-Hydroxybutyrate | 0.179 ± 0.017 | 0.342 ± 0.047 | .007 |
| Glycerol | 0.239 ± 0.020 | 0.575 ± 0.037 | <.001 |

Table 2. The effect of intraperitoneal injections of aminophylline (100 mg/kg) on selected metabolites in the brains of young mice. Heads were amputated directly into liquid nitrogen. Frozen brains were dissected at −35°C. Extracts of tissue were prepared in perchloric acid by the method of Lowry *et al.* (*12*). Glycogen was measured by the amyloglucosidase method of Passonneau and Lauderdale (*23*). Cyclic nucleotides were assayed by the procedure of Steiner *et al.* (*24*). All other metabolites were assayed by the specific enzymatic methods of Lowry and Passonneau (*25*). Data are expressed in millimoles per kilogram, and the values are means ± standard error; NS, not significant ($P$ > .05).

| Metabolite | Controls ($N$ = 7) | Injected with aminophylline ($N$ = 7) | $P$ value |
|---|---|---|---|
| Adenosine triphosphate | 2.55 ± 0.06 | 2.51 ± 0.02 | NS |
| Adenosine diphosphate | 0.617 ± 0.006 | 0.701 ± 0.012 | <.001 |
| Adenosine monophosphate | 0.079 ± 0.005 | 0.134 ± 0.013 | .002 |
| Phosphocreatine | 2.50 ± 0.07 | 2.34 ± 0.08 | NS |
| Glycogen | 1.43 ± 0.07 | 1.37 ± 0.04 | NS |
| Glucose | 0.85 ± 0.04 | 1.52 ± 0.11 | <.001 |
| Glucose 6-phosphate | 0.079 ± 0.001 | 0.110 ± 0.006 | <.001 |
| Fructose 1,6-diphosphate | 0.145 ± 0.007 | 0.141 ± 0.008 | NS |
| Pyruvate | 0.125 ± 0.005 | 0.126 ± 0.006 | NS |
| Lactate | 2.16 ± 0.16 | 2.33 ± 0.17 | NS |
| Lactate/pyruvate ratio | 17.4 ± 1.3 | 18.7 ± 1.6 | NS |
| Adenosine 3',5'-monophosphate | 2.88 ± 0.11 | 4.16 ± 0.17 | <.001 |
| Guanosine 3',5'-monophosphate | 0.053 ± 0.001† | 0.072 ± 0.004† | .010 |
| Glucose B/P ratio‡ | 0.093 ± 0.004 | 0.129 ± 0.008 | .002 |

*Measured in picomoles per milligram. †$N$ = 3. ‡The ratio of glucose concentration in the brain to that in the plasma, expressed in liters per kilogram.



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cause of the availability of ketone bodies as metabolic fuel, or that aminophylline might have decreased the cerebral metabolic rate. However, the latter possibility seemed unlikely since we have found that in mice of this age, increased brain glucose content secondary to reduced cerebral metabolic rate of diverse etiology was almost invariably associated with reduced lactate and increased phosphocreatine concentrations [sometimes there were increases in adenosine triphosphate (ATP) as well] (*11*). The amounts of these compounds were not changed by aminophylline. Furthermore, the concomitant increases in adenosine diphosphate (ADP) and adenosine monophosphate (AMP) 14 and 70 percent, respectively (Table 2), suggested increased ATP breakdown and an increase rather than a decrease in cerebral metabolic rate.

To test this hypothesis, we determined the effect of aminophylline on cerebral metabolic rate in weanling mice by the closed system technique of Lowry *et al.* (*12*). Measurements of cerebral metabolic rate for whole brain obtained by this method compare well with those obtained by different techniques (*13*). The Lowry model is particularly useful and reliable in immature, small laboratory animals where repeated collection of blood is difficult and where rapid freezing of the small head limits cerebral glycolysis after decapitation. Since the Lowry technique reflects the use of ATP produced from fuels other than glucose alone, it may be the preferred method in immature suckling animals where ketone body oxidation normally provides a significant fraction of the cerebral energy supply (*14*). In our experiments no attempt was made to keep the weanling mice away from the high-carbohydrate maternal diet. Figure 1 shows that during ischemia the rate of decrease of phosphocreatine, ATP, glucose, and glycogen was faster and the accumulation of lactate was greater in the aminophylline-treated animals. The energy-use rate, that is, the cerebral metabolic rate, calculated in terms of high-energy phosphate equivalents (~P) (*15*) was increased almost threefold by aminophylline: 34 mmole of ~P per kilogram per minute as opposed to 12 mmole $kg^{-1}$ $min^{-1}$ in controls (Fig. 1). The data do not permit an evaluation of the contribution of ketone body oxidation in vivo to the levels of the high-energy phosphate-containing compounds. If glucose were the major source of energy for the brain in these young animals, then, on the basis of the assumptions made by Lowry *et al.* (*12*), the rate of glucose utilization

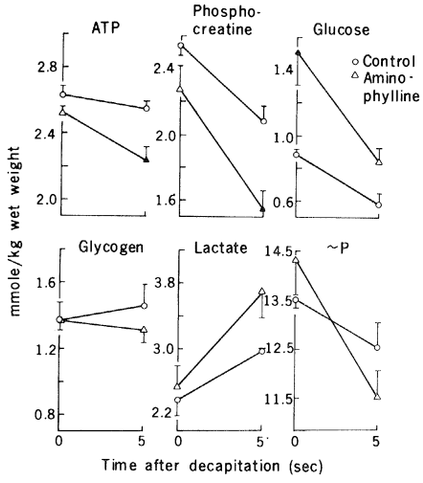


Fig. 1. Effect of aminophylline (100 mg/kg) on changes in selected metabolites and the anoxic energy reserve after decapitation. Each point represents the mean value from four mice. Vertical lines represent ± standard error of the mean. Closed symbols indicate a significant difference from control ($.011 \geq P \leq .022$). The method for calculating ~P is given in (*15*).

in those which received aminophylline was calculated to be 1.02 mmole $kg^{-1}$ $min^{-1}$ as opposed to 0.36 mmole $kg^{-1}$ $min^{-1}$ in controls.

Our finding of increased cerebral metabolic rates in mice that have received pharmacologic doses of aminophylline supports the observations of Wechsler *et al.* (*6*) who found increased rates in some patients given therapeutic doses of the drug. Although there was no evidence of cerebral anoxia as determined by the amounts of lactate and pyruvate in the brain and by the lactate-pyruvate concentration ratio (Table 2) (*16*), the increased concentrations of AMP and ADP suggest an imbalance between energy utilization and energy production in the aminophylline-treated animals (*16*). The reason for the increased energy-use rate in aminophylline-treated mice is not clear. There is evidence that neurotransmitters such as catecholamines and acetylcholine may exert some of their effects in the central nervous system by way of cyclic AMP- and cyclic GMP-mediated responses (*17*). Therefore, the increase in cerebral metabolic rate may be a reflection of increased neuronal activity caused by increased concentrations of brain cyclic nucleotides.

Provided that the supply of fuels for ATP production is commensurate with the demand, no ill effects of increased metabolic rate would be expected. In fact, the data suggest that the clinical effects of aminophylline in restoring normal respiratory rhythms in premature infants with apnea and in adults with Cheyne-Stokes respirations may be due to increased metabolic activity of neurons in the respiratory centers. However, if for any reason the supply of glucose or oxygen was limited, increased cerebral metabolic rate would be a distinct disadvantage. This suspicion was confirmed in a study of 3- to 9-day-old mice (4.9 ± 0.3 g, $N = 32$) in vivo. In these experiments, the dose of aminophylline was reduced to 7.5 mg/kg and was injected subcutaneously (again no clinical side effects were observed). This dose is at the upper limit of the amount of aminophylline used for the treatment of apnea in premature infants (*18*). Fifteen to 60 minutes after the aminophylline injection, one control and one aminophylline-treated littermate (carefully matched for weight) were exposed together to an atmosphere of $N_2$ at room temperature. Depending on the age of the animals, the interval of anoxia ranged from 2 to 28 minutes. The survival rate of 16 pairs of mice was 62 percent for controls and 0 percent for the aminophylline-treated mice. In young animals, gasping of the isolated head has been used to study the effect of drugs on survival of this reflex respiratory mechanism (*19*). Such a preparation excludes the influence of the circulation. Using this model we have confirmed the harmful effect of aminophylline on anoxic survival observed in vivo. In 32 mice (8 days old) injected subcutaneously with aminophylline (7.5 mg/kg) and decapitated 15 to 45 minutes later there was an earlier onset of gasping of the isolated head, and the last gasp (which signals death in the intact animal) occurred much earlier, at 3.5 ± 0.25 minutes ($N = 16$) as opposed to 5.08 ± 0.25 minutes ($N = 16$) in controls ($P < .001$). We have not studied the effect of aminophylline on cerebral metabolic rate in 3- to 9-day-old mice. However, since the duration of survival of neurons in the respiratory centers after decapitation appears to be determined by the balance between the anoxic energy reserve and the cerebral metabolic rate (*20*), and since the energy reserve is, if anything, a little larger in aminophylline-treated animals (Fig. 1), the results suggest that in younger mice, as in man, therapeutic doses of aminophylline may increase the cerebral metabolic rate.

Currently there is an intense interest in the use of aminophylline (and other methylxanthines) in the treatment of apnea of prematurity. Although findings in animals may not be applicable to the human situation, the deleterious effect of aminophylline in neonatal mice sub-

jected to acute anoxia indicates the need for caution in the use of these drugs in hypoxic human newborns.

JEAN HOLOWACH THURSTON
RICHARD E. HAUHART
JOHN A. DIRGO
*Edward Mallinckrodt Department of Pediatrics, Washington University School of Medicine,* and *Division of Neurology, St. Louis Children's Hospital, St. Louis, Missouri 63110*

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## Stimulated DNA Synthesis in Frog Nuclei by Cytoplasmic Extracts of Temperature-Sensitive Mammalian Cells

Abstract. *Cytoplasmic extracts of proliferating cells stimulate DNA synthesis in isolated nuclei of* Xenopus laevis *liver. When tested by the same assay, cytoplasmic extracts of resting cells are completely inactive. When cytoplasmic extracts are prepared from cell cycle–specific temperature-sensitive mutants arrested in the $G_1$ phase of the cell cycle by the nonpermissive temperature, they also fail to stimulate DNA synthesis in frog nuclei. The results indicate that, to stimulate DNA synthesis in isolated frog nuclei, essentially all information of $G_1$ cells must be present.*

Jazwinski *et al.* (*1*) reported that DNA synthesis in nuclei isolated from frog liver or spleen can be directly stimulated by the addition to the incubation mixture of cytoplasmic extracts prepared from proliferating cells. Electron microscopic analysis of the DNA molecules from the reaction mixture strongly indicated that the cytoplasmic extracts stimulated initiation of DNA replication in the chromatin of the normally resting frog nuclei. A comparison of the stimulatory activity in extracts from various mammalian and avian sources showed that the activity was present only in proliferating cells. In contrast, cytoplasmic extracts from normally resting tissues and cells had no stimulatory activity on DNA synthesis in isolated frog nuclei. In subsequent experiments, Jazwinski and Edelman (*2*) used the same cell-free system, consisting of isolated frog nuclei, to investigate the stimulatory effect of cytoplasmic extracts from temperature-sensitive (ts) mutants of *Saccharomyces cerevisiae*. The ts mutants used by Jazwinski and Edelman (*2*) were mutants of the cell division cycle isolated and described by Hartwell and collaborators (*3*). All of these mutants were deficient in events of the dependent pathway leading to the initiation of DNA synthesis in the yeast cell cycle. When the yeast cells were incubated for one generation at the nonpermissive temperature, 36°C, their extracts showed very low or no stimulatory activity on DNA synthesis in frog liver nuclei. On the other hand, cytoplasmic extracts from yeasts grown at the permissive temperature had stimulatory activity.

We have extended these studies to ts mutants of the mammalian cell cycle and, specifically, to ts mutants in the $G_1$ phase of the cell cycle. These are operationally defined as mutants that, at the nonpermissive temperature, are arrested in the $G_1$ phase of the cell cycle. Three such mutants have been studied in our laboratory: (i) AF8 cells, originally derived from BHK cells by Basilico and coworkers (*4*). The execution point of the ts defect in these cells has been located by shift-up experiments at 8.6 hours from the beginning of the S phase (*5*); (ii) K12 cells, a ts mutant from Chinese hamster cells originally isolated by Roscoe *et al.* (*6*) and characterized by Smith and Wigglesworth (*7*). This mutant is also arrested in $G_1$ at the nonpermissive temperature, and the execution point has been located by Ashihara *et al.* (*5*) at 1.6 hours before the S phase; and (iii) ts13 cells, another $G_1$ phase ts mutant from BHK cells originally isolated and partially characterized by Talavera and Basilico (*8*). Its execution point has been located (*9*) at 3.2 hours before the S phase.

In these experiments we asked a simple question, namely, Is the activity in the cytoplasmic extracts of proliferating cells that is responsible for the stimulation of DNA synthesis in frog nuclei present when the cells are prevented from reaching S by exposure to the nonpermissive temperature? The advantages over the previous experiments with yeast cells (*2*) are essentially two: (i) the work is extended to mammalian cells and (ii) more important, by appropriate manipulations the cells can be arrested in $G_1$ without their having to be exposed to the nonpermissive temperature for a whole generation.

Temperature-sensitive mutants were made quiescent by serum deprivation (36 to 48 hours in 0.5 percent serum) and were then stimulated by fresh medium plus 10 percent serum at either the permissive or the nonpermissive temperature. Under these conditions, at the permissive temperature, 85 to 90 percent of the cells enter DNA synthesis with median times of entry into S of 24, 11, and 24.5 hours for AF8, K12, and ts13, respectively. At the nonpermissive temperature, less than 5 percent of the cells are labeled by continuous exposure to [$^3$H]thymidine (*5, 10*). Cytoplasmic extracts were prepared as described by Jazwinski *et al.* (*1*) from quiescent cells,


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