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The Effects of Xanthine Anologs on Neonatal Rat and Adult Mouse Respiration

(TITLE)

BY

Susan M. Reynolds

THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

Master of Science

IN THE GRADUATE SCHOOL, EASTERN ILLINOIS UNIVERSITY
CHARLESTON, ILLINOIS

1987
YEAR

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ABSTRACT

Apnea, a prolonged cessation of breathing, is commonly seen in premature infants. This condition, in its severest form, can be lethal and is a suspected cause of sudden infant death syndrome (SIDS). Sometimes referred to as apnea of prematurity, recurrent apnea is presently being treated with respiratory stimulants known as methylxanthines (MX) such as theophylline and caffeine. The benefits of MXs are accompanied by central nervous system stimulation and cardiostimulation which may be detrimental to a premature infant. Theophylline (1,3-diMX) has been shown to produce some of its effects by antagonizing endogenous adenosine. Another xanthine, enprofylline (3-propylxanthine), is presently being investigated as an antiasthmatic drug due to its bronchodilating effects. Unlike theophylline it has no CNS toxicity nor does it antagonize adenosine, except in high concentrations. Due to its lack of antagonism one would deduce that enprofylline would not stimulate respiration. The present study focused on the effects of these two xanthines on the respiration of adult mice and newborn rats. Respiratory studies were conducted on newborn rats (4- to 7-days old) and adult mice (20-30 g), using the volume displacement body plethysmograph. Subcutaneous injections of aminophylline (the ethylenediamine salt of theophylline) or enprofylline were administered and the effects compared to baseline respiration. Aminophylline

(20 mg/kg) significantly increased V_E by 30% in adult mice with a peak effect at 20 min. This increase was due to significant increases in both V_T (13%) and f (18%). Aminophylline significantly increased V_E by 44% in neonatal rats with a peak effect at 20 min. The increase was due to increases in both V_T (30%) and f (9%). Only f was significantly greater than saline controls. Enprofylline (20 mg/kg) showed no consistent pattern of effects nor were its effects significantly different from saline for adult mice or neonatal rats. There were no significant differences in V_E between doses (10, 20 and 40 mg/kg) of aminophylline in mice. Enprofylline (20, 40 and 80 mg/kg) also showed no significant differences in V_E between doses for mice. However, doses of enprofylline were significantly different from one another in neonatal rats. The trend shown by enprofylline (40 mg/kg) was a consistent respiratory depression whereas the 80 mg/kg dose showed a latent stimulation in respiration. During these experiments 7 of 8 pups died after injection of 80 mg/kg enprofylline. In summary aminophylline stimulated respiration in both adult mice and neonatal rat pups, while enprofylline did not stimulate respiration at any dose tested, except at a dose which was lethal to neonatal rats. These data support the hypothesis that theophylline stimulates respiration by adenosine antagonism.

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INTRODUCTION

Neonatal apnea is a respiratory condition whose effects claim thousands of lives each year. Neonatal apnea is described as prolonged cessation of breathing (greater than 20 sec) (Rigatto and Brady, 1972). It is observed in 25% of neonates weighing less than 2500 g at birth and in as many as 85% of neonates weighing less than 1000 g at birth (Aranda and Turmen, 1979). Apnea is usually reversed spontaneously but often requires vigorous resuscitative efforts. Infants requiring such treatment are found to be at increased risk of sudden infant death syndrome (SIDS) (Kelley et al., 1978). SIDS is defined as the unexpected, sudden death of a seemingly healthy infant for whom a routine autopsy fails to identify the cause of death. In the U.S. it kills 7,000 infants a year or about one in 500 babies born, making it the most frequent cause of death between the ages of one month and one year (Naeye, 1980). One of the strategies towards prevention of neonatal apnea is the use of respiratory stimulant drugs.

The respiratory stimulant effects of the chemical group known as the methylxanthines (MX) have been known for over a century (Leven, 1868). At one time MX's such as theophylline (1,3-diMX) (fig. 1) and caffeine (1,3,7-triMX) were used as diuretics, however they were eventually replaced with more potent diuretics having fewer side effects (Rall, 1985). The effectiveness of aminophylline,

the ethylenediamine salt of theophylline, in the treatment of adults with Cheyne-Stokes respiration was demonstrated in 1927 by Vogl. The primary clinical use of theophylline today is in the treatment of asthma in adults and children (Hendeles and Weinberger, 1983). Theophylline's bronchodilating effect relieves constriction of the bronchiolar smooth muscle brought on during an asthmatic attack.

Kuzemko and Paala (1973), found that aminophylline, rectally administered, decreased the incidence of apnea in premature infants. Since that time many researchers have confirmed these observations in addition to finding that caffeine is also effective in the treatment of neonatal apnea (Aranda and Turmen, 1979). Recurrent apnea of prematurity is now routinely treated with theophylline or caffeine. Effects of MXs which are beneficial in the treatment of a premature infant include an increase in minute ventilation (V_E), a decrease in the incidence of apnea and improvement of the arterial blood gases. These responses are apparently mediated at the level of the medulla (Eldridge et al., 1983). Another possible benefit could be an increased force of contraction of the diaphragm, a principle muscle of respiration (Aubier et al., 1981). Methylxanthines have also been shown to reduce fatigue of this muscle. It is pertinent to clarify that accompanying these beneficial effects of the MXs there also exist effects undesirable to a premature infant. The

leading side effect caused by some MXs is a stimulation of the central nervous system (CNS). Concentrations of theophylline 50% greater than the accepted therapeutic range can result in effects such as nervousness, restlessness, insomnia, tremors and a sensation of pain when pressure is applied to the skin. At even higher doses it can produce focal and generalized convulsions (Rall, 1985). Tachycardia above 180 beats per minute is also a common complication of MX treatment (Davi et al., 1978). Gastric irritation is also a concern. Due to the vomiting which may accompany MX treatment the gastric lining becomes irritated as well as a resulting loss of weight and water, which can be fatal in an already underweight, undernourished infant.

The mechanism(s) by which MXs have these effects is not yet known. There have been a few hypotheses however. One idea involves the inhibition of the enzyme cyclic nucleotide phosphodiesterase. Phosphodiesterase is responsible for catalyzing the reaction which breaks down cyclic adenosine-3',5'-monophosphate (cAMP) to its inactive form 5'-adenosine monophosphate (AMP) (Butcher and Sutherland, 1962). This process would decrease all actions fueled by cAMP. An in vitro study with theophylline resulted in delayed breakdown of cAMP but only at concentrations ten times greater than those concentrations that produce bronchodilation (Rall, 1985).

Another possible mechanism of MX action is the translocation of intracellular calcium (Rall, 1985). Methylxanthines cause an increase in the concentration of intracellular calcium as a result of increased permeability of the sarcoplasmic reticulum. This mechanism may explain the increased force of contraction sometimes caused by MXs however, it is an unlikely hypothesis since concentrations of theophylline and caffeine required to elicit such effects are greater than the maximum treatment for therapeutic use.

A third, more widely accepted hypothesis involves adenosine antagonism. Adenosine is a purine which is present nearly everywhere within the body. Adenosine causes contraction of tracheal smooth muscle (Fredholm et al., 1979) and bronchoconstriction in asthmatics (Cushley et al., 1983). Stable adenosine analogs have sedative and anti-convulsant effects on mice and rats (Dunwiddie and Worth, 1982).

Lagercrantz and colleagues, in 1984, studied the effects of adenosine on respiration using unanesthetized and anesthetized rabbit pups. Pups given intraperitoneal (ip) injections of increasing doses of L-PIA (0.1 to 5.0 umol/kg) showed depressed ventilation. Doses as high as 5.0 umol resulted in irreversible apnea and death. The effect of adenosine was completely reversed or prevented with administration of 20 mg/kg theophylline (Lagercrantz et al., 1984). In 1985 Hedner and colleagues, using the

occluded breath technique on anesthetized preterm rabbits, found that with ip administration of adenosine analogs such as phenylisopropyl adenosine (PIA) and 5'-N-ethylcarboxamidoadenosine (NECA) there were significant decreases in frequency (f), tidal volume (V_T) and V_E. Ten minutes postinjection V_E had decreased to 35% of the control. Treatment with theophylline 10 min after PIA injection reversed the PIA effect nearly completely by increasing both V_T and f towards control (Hedner et al., 1985). Watt and Rutledge (1985) did not, however, support these findings showing that respiration was dose-dependently stimulated by administration of adenosine in human subjects. This stimulation was primarily due to an increase in V_T. Runold (1986) used the barometric method of measuring respiration on unanesthetized 1- to 8-day-old rabbit pups. Ventilation was depressed with ip catheterized administration of 1.0 umol R-PIA. This depression was primarily due to a decrease in f. Those pups given pretreatment or subsequent treatment with a 10 mg/kg dose of theophylline showed prevention of or nearly complete recovery from the effects caused by the adenosine analog. R-PIA produced a greater effect in 1- to 3-day-old rabbits, which was accompanied by a higher affinity of R-PIA for adenosine receptors in the newborn rabbit brain.

Adenosine interacts with two types of cell-surface receptors designated as A_1 and A_2 to produce some of its effects (Daly, 1982). In general, A_1 receptors mediate an

inhibition of adenylate cyclase activity and A_2 receptors stimulate adenylate cyclase activity (Schwabe et al., 1985; Fredholm and Persson, 1982). The respective roles of A_1 and A_2 receptors in respiratory control are unclear.

The most accepted cellular mechanism of MX action at this time is adenosine antagonism. In terms of respiratory control, xanthines may have effects on peripheral chemoreceptors and the respiratory center.

There are two types of receptors responsible for detecting the blood gas status of the animal, known as the central and peripheral chemoreceptors. The major chemical determinant of respiratory activity is partial pressure of carbon dioxide (Pco_2) or pH. Ventilatory stimulation is probably not caused directly by CO_2 but instead is due to the ultimate increase in hydrogen ion concentration of the cerebrospinal fluid caused by the reaction of CO_2 with water, according to the following equilibrium equation:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

The increase in pH is then detected by central chemoreceptors located near the ventral surface of the medulla, and stimulation of breathing is initiated. This increase in ventilation results in CO_2 levels being lowered and pH being raised toward the normal. The peripheral chemoreceptors are located in the aortic bodies, within the arch of the aorta, and the carotid bodies, near the bifurcation of the carotid arteries. A decrease in partial pressure of blood oxygen (Po_2) is detected by these bodies

and nerve impulses sent along the vagus and glossopharyngeal nerves to the respiratory center in the medulla, stimulating ventilation. Davi and researchers (1978), using newborn infants showed that administration of theophylline resulted in an increased sensitivity of the brainstem to CO_2. In addition to this the CO_2-response curve was shifted to the left, suggesting an action on central chemoreceptors. Lundberg et al. (1981) supported these findings in a similar study using anesthetized adult rats. Eldridge and others (1983) supported Davi's and Lundberg's research showing that aminophylline shifted the CO_2-response curve to the left. However, this study was taken a step further in order to show that direct administration of aminophylline into the third ventricle resulted in no effect on the central chemoreceptors. They also determined that aminophylline could not penetrate the medulla to the deep neurons necessary for respiratory stimulation. Eldridge and colleagues did show that intravenous (iv) administration of aminophylline could reach the deep neurons having a stimulatory effect on respiration at the level of the medulla.

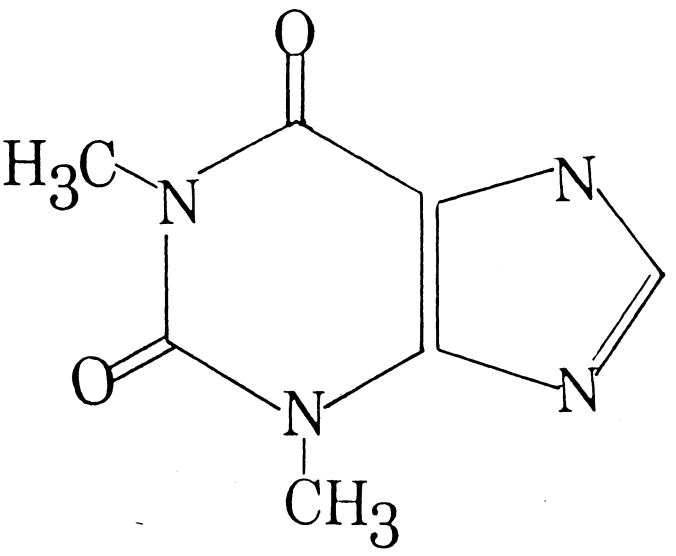
The systematic study of the structure activity relationships (SAR) of theophylline's core molecule will enable researchers to predict more desirable compounds for the treatment of premature apnea. SAR are the study of correlations between the structure of a molecule and its biological activity. For example, substitution at a

certain position may be responsible for the side effects of a drug and the size of the substituent at this position may dictate the potency or toxicity. Another group may be responsible for a desired action, for example, respiratory stimulation. Theophylline contains methyl groups on positions 1 and 3 of the xanthine nucleus as shown in figure 1. According to the work of Persson and others (1982b) the 1-position is necessary for adenosine antagonism, but not necessary for bronchodilation. The 3-position is necessary for bronchodilation and is responsible for some aspects of toxicity such as cardiostimulation (fig. 2). In general SAR studies have shown that larger, nonpolar substituent groups on positions 1 and 3 or additional groups on position 8 of the xanthine nucleus usually display enhancement of adenosine antagonism and bronchodilation as a result of these positions. Molecules lacking substituent groups at position 1 usually have reduced adenosine antagonism. Those having substituent groups on positions 7 and 9 show a general loss of potency. The addition of an aromatic ring at the 8 position increases potency considerably as an adenosine antagonist. Enprofylline (3-propylxanthine) (fig. 1) is currently undergoing clinical trials for its bronchodilating effect in the treatment of asthma. It is similar to theophylline in that it contains the same core molecule, xanthine, however enprofylline has a propyl group on position 3 and lacks a substituent group at position 1.

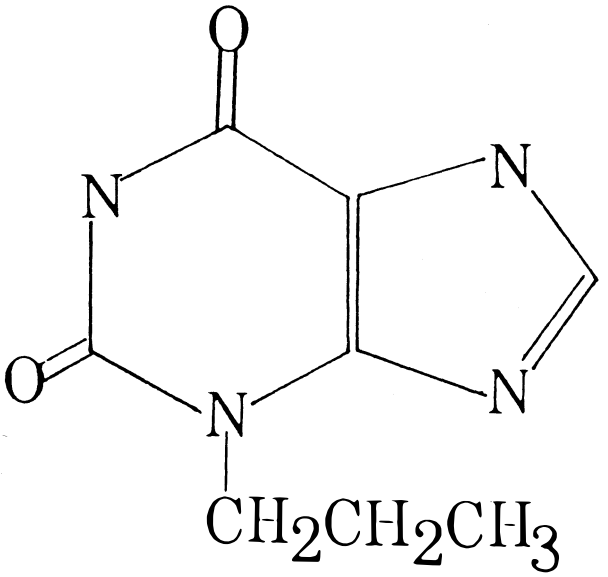
Enprofylline is five times more potent than theophylline as a bronchodilator and lacks the CNS stimulation exhibited by theophylline (Persson and Erjefalt, 1982; Persson et al., 1982a). Enprofylline does stimulate cardiac tissue as does theophylline (Trippenbach et al., 1980). Bronchodilation is brought about by direct relaxation or dilation of the smooth muscle of the bronchioles. Until recently it was proposed that enprofylline produced its effects without antagonizing adenosine receptors (Persson et al., 1982b). This was the reason for the lack of the CNS toxicity. In 1985 Wessberg and others, using the occluded breath technique on anesthetized rats, found that with intracerebroventricular (icv) and ip administration of adenosine analogs there was a dose-dependent depression of respiration. Frequency and V_T were depressed by as much as 25% of control and V_E depressed by 39% of control. Administration of theophylline prior to administration of an adenosine analogs resulted in complete antagonism of the respiratory depression. Pretreatment with enprofylline followed by adenosine analogs resulted in no antagonism, suggesting that enprofylline is not an antagonist of adenosine. However recent papers provide data supporting adenosine antagonism of both A_1 and A_2 receptor sites with high concentrations of enprofylline (Ukena et al., 1985; Schwabe et al., 1985). Although this antagonism may be minute it must be taken into account.

In summary, MXs such as theophylline and caffeine are being used in the treatment of apnea of prematurity. Theophylline, possessing substituent groups on positions 1 and 3 of the xanthine molecule, stimulates respiration in an already depressed system. Theophylline has been shown to antagonize adenosine, while enprofylline, lacking the 1-position substituent, has not been satisfactorally shown to antagonize adenosine. For these reasons it would be beneficial to study their effects on respiration in order to determine if the 3-position is important for respiratory stimulation. Subsequently it is hoped that this study will assist in the search for a more effective treatment of apnea of prematurity without the detrimental side effects. The hypothesis to be tested is that theophylline stimulates respiration by adenosine antagonism.

Figure 1. Structures of theophylline and enprofylline.

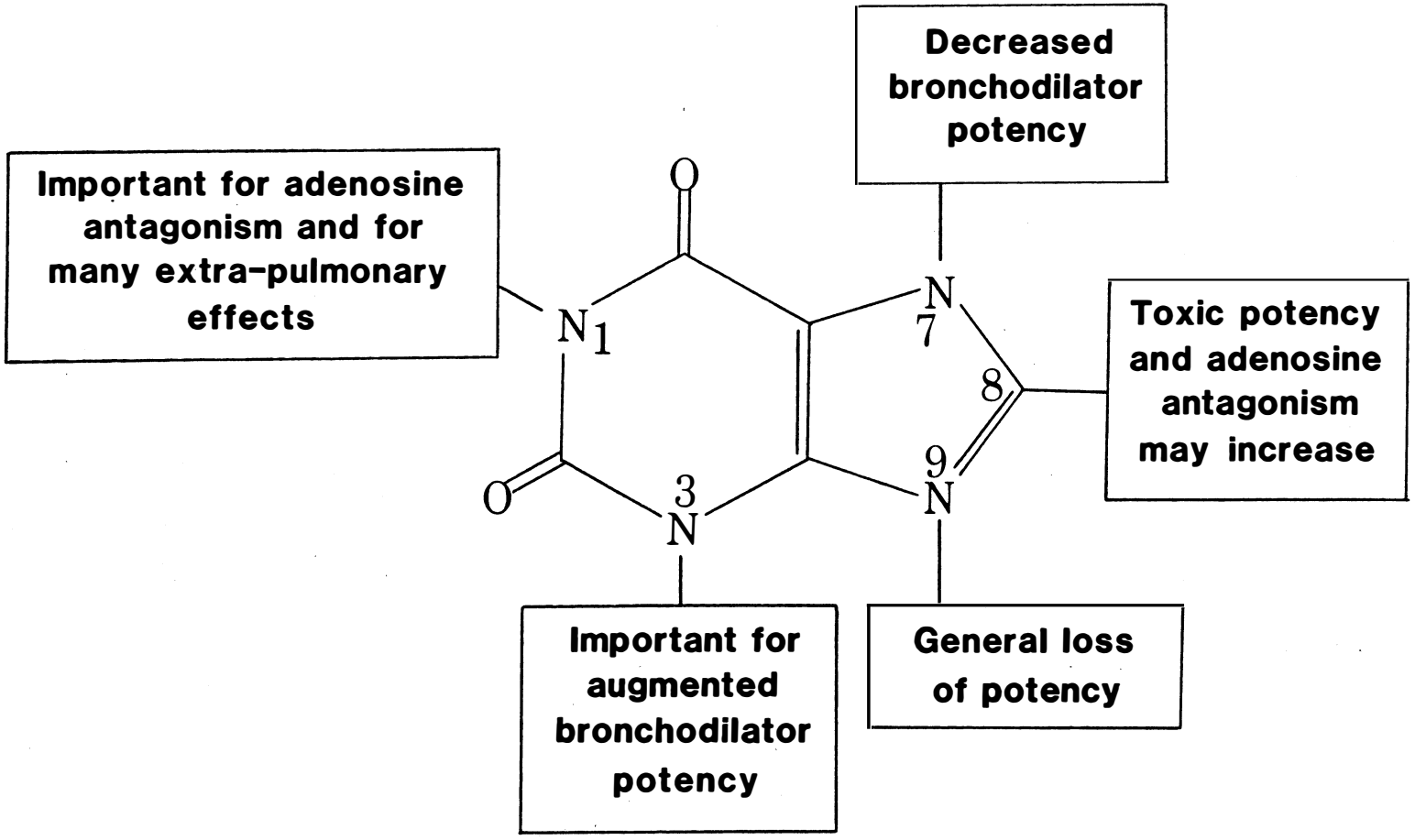


THEOPHYLLINE



ENPROFYLLINE

Figure 2. Structure of xanthine molecule displaying characteristics of substitutions at various positions.


Decreased bronchodilator potency
Important for adenosine antagonism and for many extra-pulmonary effects
Toxic potency and adenosine antagonism may increase
Important for augmented bronchodilator potency
General loss of potency
O
N1
N
7
8
9
N
3
N
O

METHODS

Animals: Experiments were performed on adult mice and newborn rats. Male mice, weighing 20-30 g, were obtained from a mixed strain colony housed in the Zoology Department's facilities. Newborn Sprague-Dawley rats were reared in the Zoology Department's animal care unit and kept with their mothers when not being tested. Only 4- to 7-day-old rats weighing 10-15 g were tested. Following each procedure the animals were returned to the animal colony or destroyed humanely.

Drugs: Aminophylline was purchased from Sigma Chemical Co., St. Louis, Mo. and enprofylline was a gift of A. B. Draco, Lund, Sweden.

Ventilatory Measurements: Ventilation was measured in unanesthetized adult mice and newborn rats with the use of a volume displacement body plethysmograph modified from the method of Alarie (1966). An open system was utilized for all experiments (Mead, 1960). The plethysmograph is composed of a plastic cylinder, 2.1 cm in diameter, covered at one end with a thin rubber sheath (fig. 3). The mouse or rat was maneuvered into the cylinder so that the head projected through a 9 mm diameter hole in the rubber sheath. A water-soluble lubricant sealed the area around the neck. The opposite end of the cylinder was sealed with a one-hole rubber stopper. Through the hole projected a piece of tubing connecting the plethysmograph to a #000

Fleisch Flow Transducer. Contraction and expansion of the chest wall resulted in a flow of air into and out of the cylinder and through the transducer. Air flow was measured and integrated by a Validyne CD 19A Carrier Demodulator and recorded as flow, volume and cumulative volume tracings on a Narco MK III-S Physiograph (fig. 4). Minute ventilation (V_E) and frequency (f) were determined from 10- to 30-sec segments of the polygraph recordings and tidal volume (V_T) was calculated ($V_T = V_E/f$). In mouse studies, the apparatus was cooled by placing crushed ice on the surface of the plethysmograph. This seemed to reduce the amount of struggling without altering baseline ventilation. Body temperature of newborn rats was maintained at 35°C by a heated water jacket surrounding the larger chamber.

Comparison of the ventilatory effects of aminophylline and enprofylline: Mice or rats were divided into three groups with each group consisting of eight animals. Respiration was measured in each animal for a minimum of 10 min in order to obtain a baseline (BL) control reading for each rodent. Each mouse or rat then received a subcutaneous (s.c.) injection in the scalp of 0.9% NaCl, 20 mg/kg aminophylline (AM) or 20 mg/kg enprofylline (ENP). This was accomplished without removing the animal from the plethysmograph. Respiration was then measured for 60 min postinjection with readings taken at 5 min intervals. This was done in order to determine if and at what time there

may be an effect for each drug. Once an effect was found for aminophylline, dose-response studies were attempted. This was accomplished by taking respiratory measurements every 5 min, following the injection of three consecutive doses of drug, up to and including that time of peak effect (25 min). An effect for enprofylline was never discovered during the primary experiments. This made it necessary to duplicate the tests at 40 and 80 mg/kg in order to determine if any dose had an effect. Once V_E, V_T and f were collected they were expressed as a percentage of the individual BL control values. Percent increases in V_E, V_T or f were plotted against time for the various doses tested. Theophylline at doses of 10, 20 and 40 mg/kg were compared to one another while doses of enprofylline, 20, 40 and 80 mg/kg, were compared.

Statistics: Data were analyzed by two-way analysis of variance (ANOVA) with repeated measures (Winer, 1962). These computations were performed using NWA Statpack software on an IBM PC/XT. Simple means analysis (Winer, 1962) was used to determine significance at each time interval and Tukey's multi-comparison test (Steel and Torrie, 1980) was then utilized to compare individual means. These computations, based on the results of the ANOVA, were performed by hand calculator. Differences with a probability level of less than 0.05 were considered statistically significant.

Figure 3. Diagram of mouse in body plethysmograph.

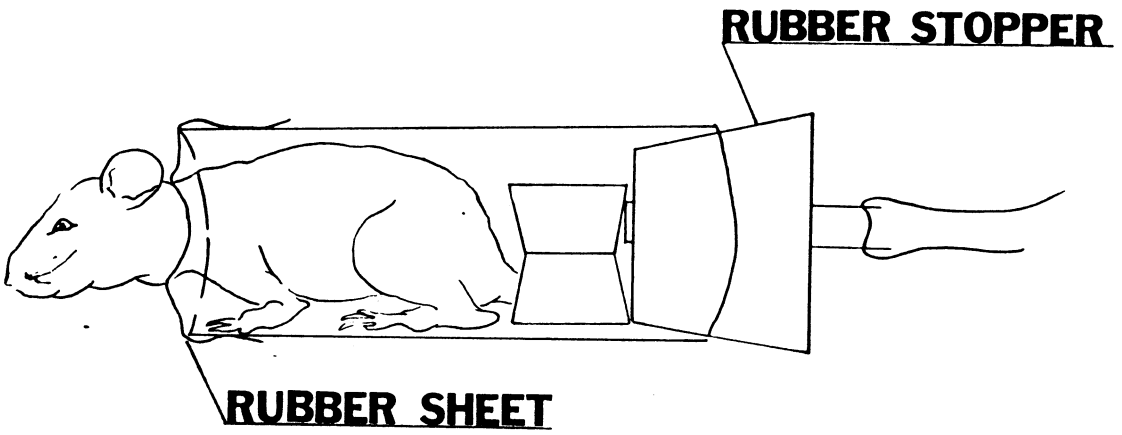
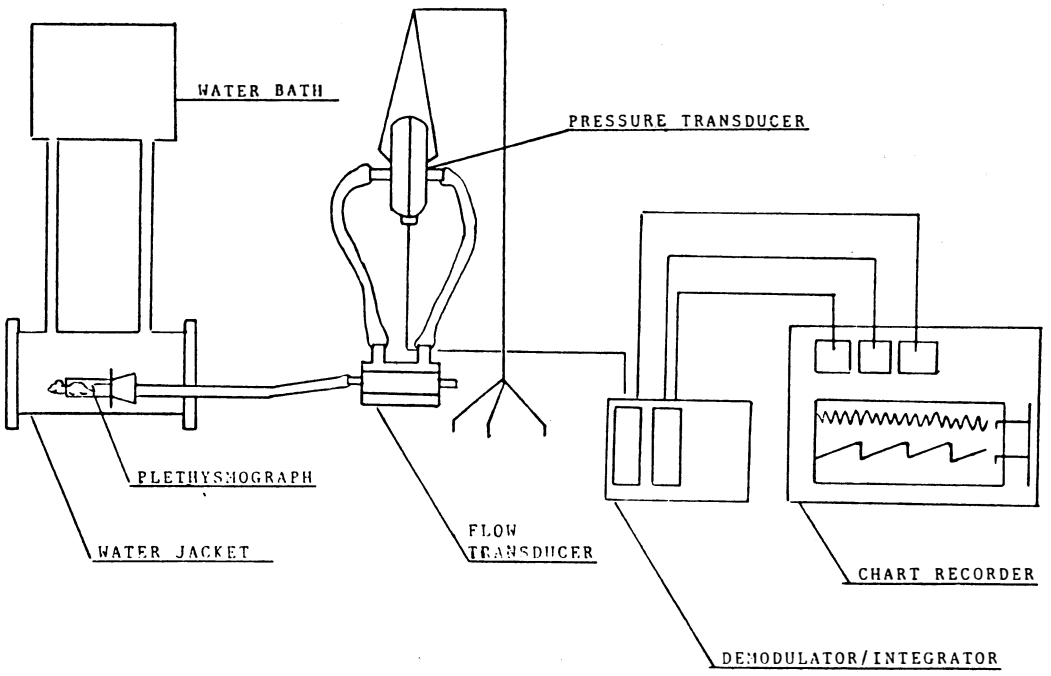

RUBBER STOPPER
RUBBER SHEET

Figure 4. Diagram of body plethysmograph and equipment used for measurement of ventilation.



PLETHYSMOGRAPH-PNEUMOTACHOGRAPH

RESULTS

Time Course of Saline, Aminophylline and Enprofylline in Adult Mice

Figure 4a represents a tracing of an adult mouse before and after administration of aminophylline (20 mg/kg). The saline treated mice had mean baseline (BL) controls for V_E, V_T and f of 14.9 ml/min, 0.068 ml and 217 breaths/min, respectively (see appendix I for individual values). For those animals that received aminophylline (20 mg/kg s.c.) BL means were 22.8 ml/min for V_E, 0.090 ml for V_T and 257 breaths/min for f. Mean BL V_E for the enprofylline group was 35.3 ml/min while BL V_T and f were 0.136 ml and 262 breaths/min, respectively.

Statistical analysis (repeated measures) showed a significant difference in V_E between treatments and over time ($F= 6.00$, $p < 0.01$ and $F= 4.58$, $p < 0.01$) (table 1). Aminophylline increased V_E in adult mice by 30% above BL at a peak time of 20 min, postinjection (fig. 5) (see appendix III for sample calculations of simple main effects and Tukey's test). V_E declined steadily after 20 min. The increase in V_E produced by aminophylline was due to increases in both f (18%, $p < 0.05$) and V_T (10%, $p < 0.05$) (figs. 6 and 7). The increase in f was significantly different from control at 20-35 min and 50 min while V_T was significantly different from control at 10 and 15 min (figs. 6 and 7).

V_E never increased more than 5% above BL after 20 mg/kg enprofylline (fig. 5). No enprofylline values for V_E were significantly different from saline controls. Similarly V_T and f were never significantly different from control (figs. 6 and 7).

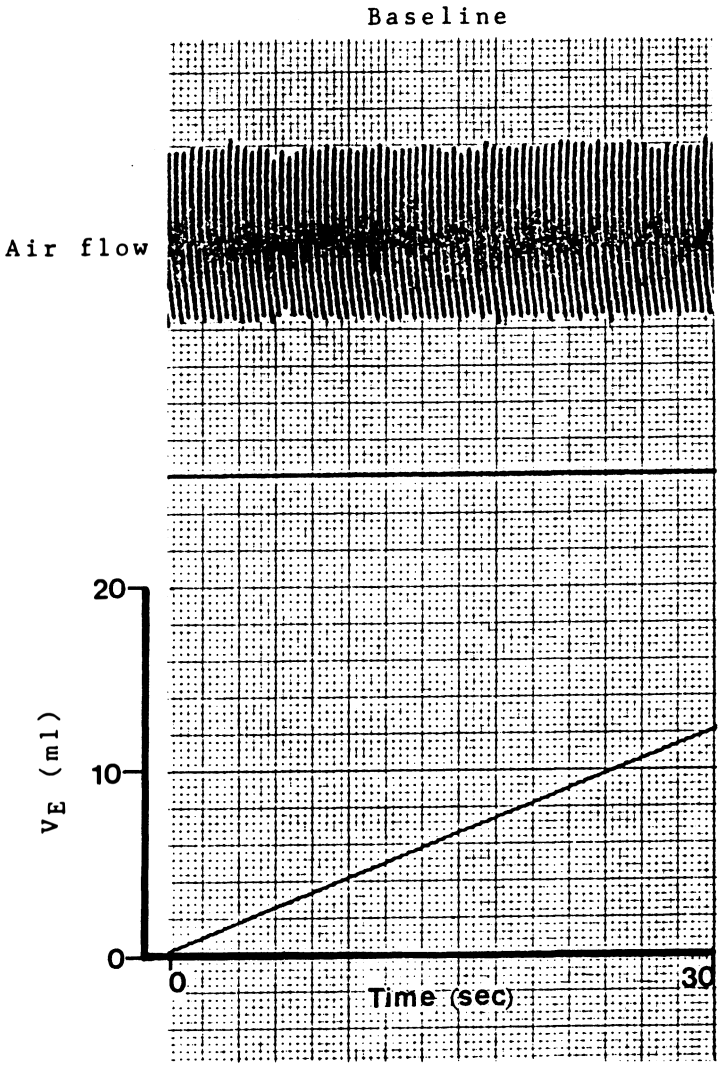
Dose Response of Aminophylline and Enprofylline in Adult Mice

Mean BL value for V_E of the aminophylline groups (10, 20 and 40 mg/kg, respectively) were 29.4, 28.6 and 28.9 ml/min, respectively. The BL values for V_T at doses 10, 20 and 40 mg/kg were 0.112, 0.122 and 0.115 ml while frequency BL values were 265, 237 and 247 breaths/min, respectively.

V_E, following administration of aminophylline (10 mg/kg), increased by 42% above BL at a peak time of 20 min (fig. 8). Doses of 20 and 40 mg/kg aminophylline increased V_E by more than 30% above BL at peak effects of 20 and 15 min respectively (fig. 8). There was no statistically significant difference between doses ($F= 0.13$, $p= 0.88$) (table 2). The increase in V_E was due to increases in both V_T (> 15%) and f (> 10%) (figs. 9 and 10). The peak effect for frequency of all doses (10, 20 and 40 mg/kg) was 15 min whereas the peak effect for V_T was 20 min for 10 and 20 mg/kg and 15 min for 40 mg/kg (figs. 9 and 10).

The BL values for 20, 40 and 80 mg/kg doses of enprofylline were, 27.6, 28.2 and 27.9 ml/min for V_E, 0.11, 0.10 and 0.10 for V_T and 260, 284 and 272 breaths/min for f, respectively. Enprofylline showed no statistically

Figure 4a. Representative tracing of mouse respiration before and after administration of aminophylline.


Baseline
Air flow
20
10
0
V_E (ml)
0
Time (sec)
30

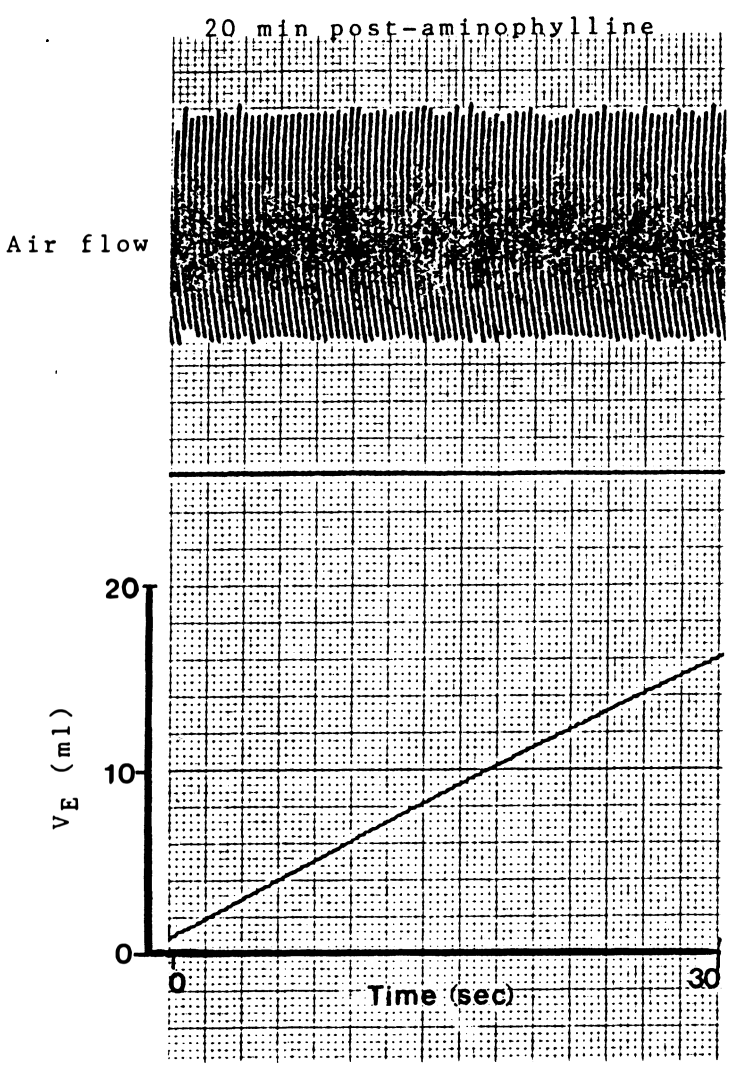

20 min post-aminophylline
Air flow
20
10
0
V_E (ml)
0
Time (sec)
30

Table 1. Two-way ANOVA, with repeated measures, of aminophylline and enprofylline time course experiments for adult mice, n= 8.

*** ANOVA TABLE ***

Source	DF	SS	MS	F	P
Treatment	2	41550.92	20775.46	6.00	<.01
Error	21	72660.75	3460.04		
Time	11	4772.59	433.87	4.58	<.01
Interaction	22	6602.33	300.11	3.17	<.01
Error	231	21897.46	94.79		

Figure 5. Effects of aminophylline and enprofylline on minute ventilation in adult mice. Bars indicate mean ± SEM. Asterisks indicate means significantly different from saline controls (* $p < 0.05$, ** $p < 0.01$; Tukey's test).

Effects of Aminophylline and Enprofylline on Minute Ventilation in Adult Mice

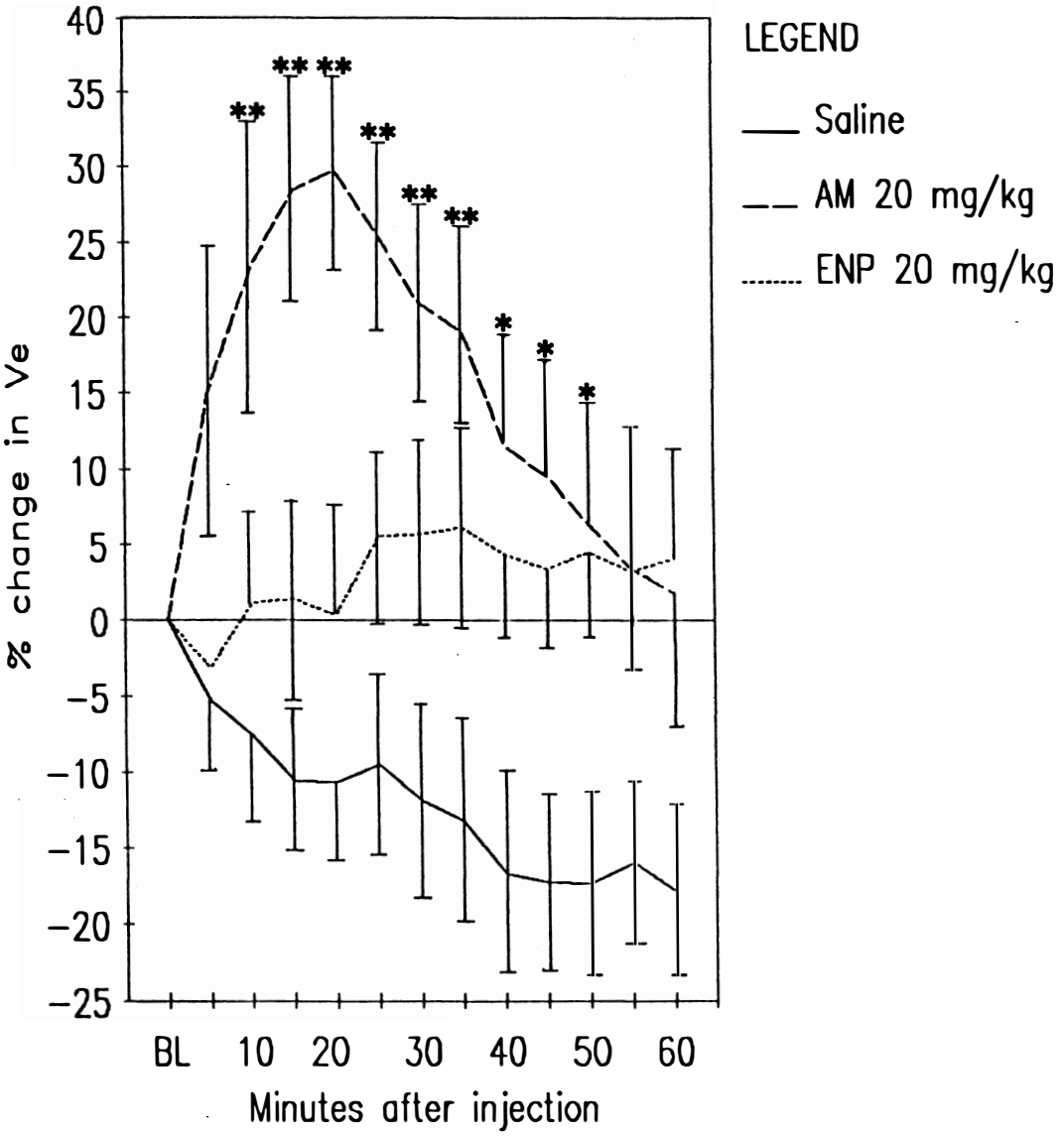


Figure 6. Effects of aminophylline and enprofylline on respiratory frequency in adult mice. Bars indicate mean ± SEM. Asterisks indicate means significantly different from saline controls (* $p < 0.05$, ** $p < 0.01$; Tukey's test).

Effects of Aminophylline and Enprofylline on Respiratory Frequency in Adult Mice

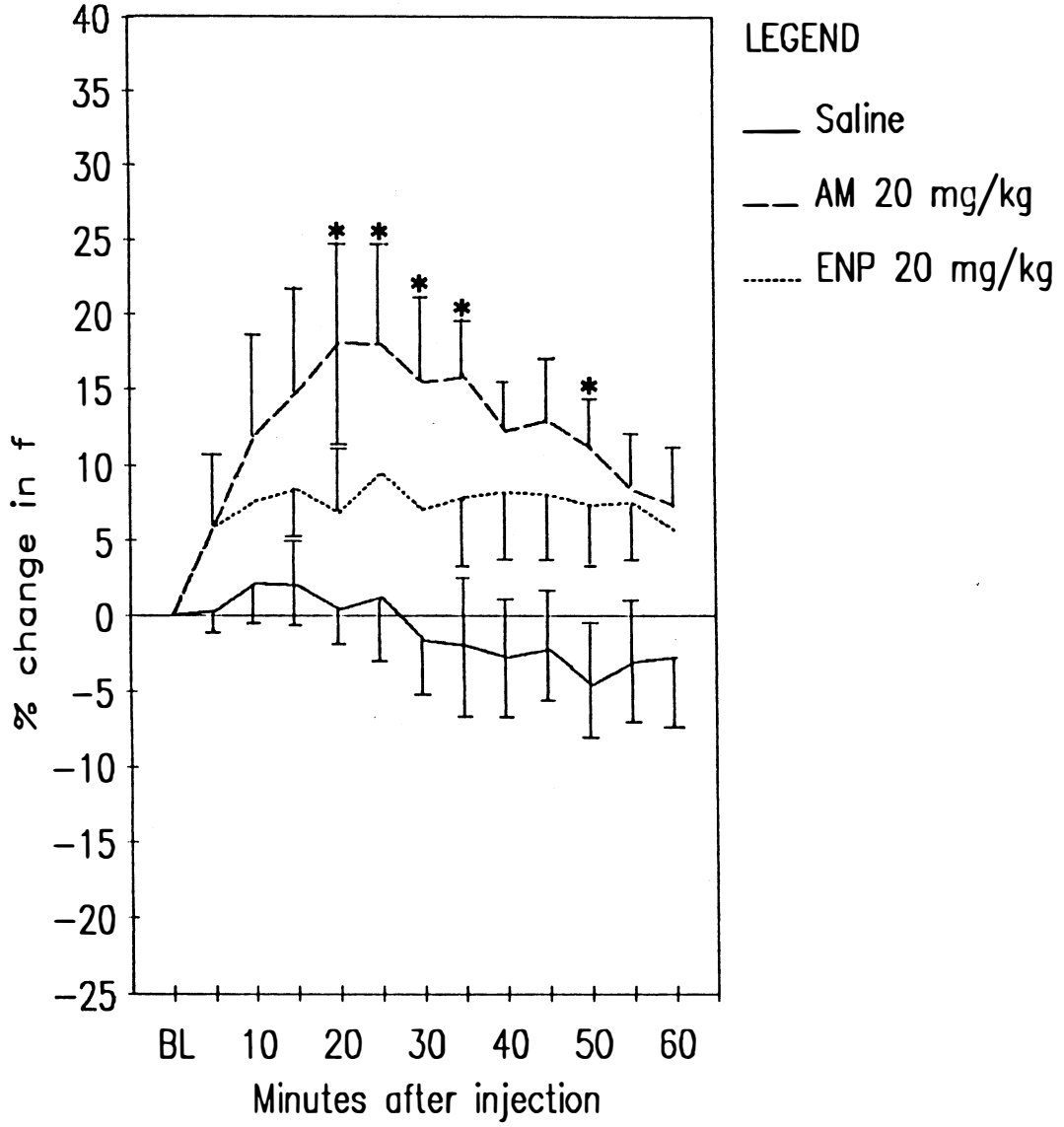


Figure 7. Effects of aminophylline and enprofylline on tidal volume in adult mice. Bars indicate mean ± SEM. Asterisks indicate means significantly different from saline controls (* $p < 0.05$, ** $p < 0.01$; Tukey's test).

Effects of Aminophylline and Enprofylline on Tidal Volume in Adult Mice

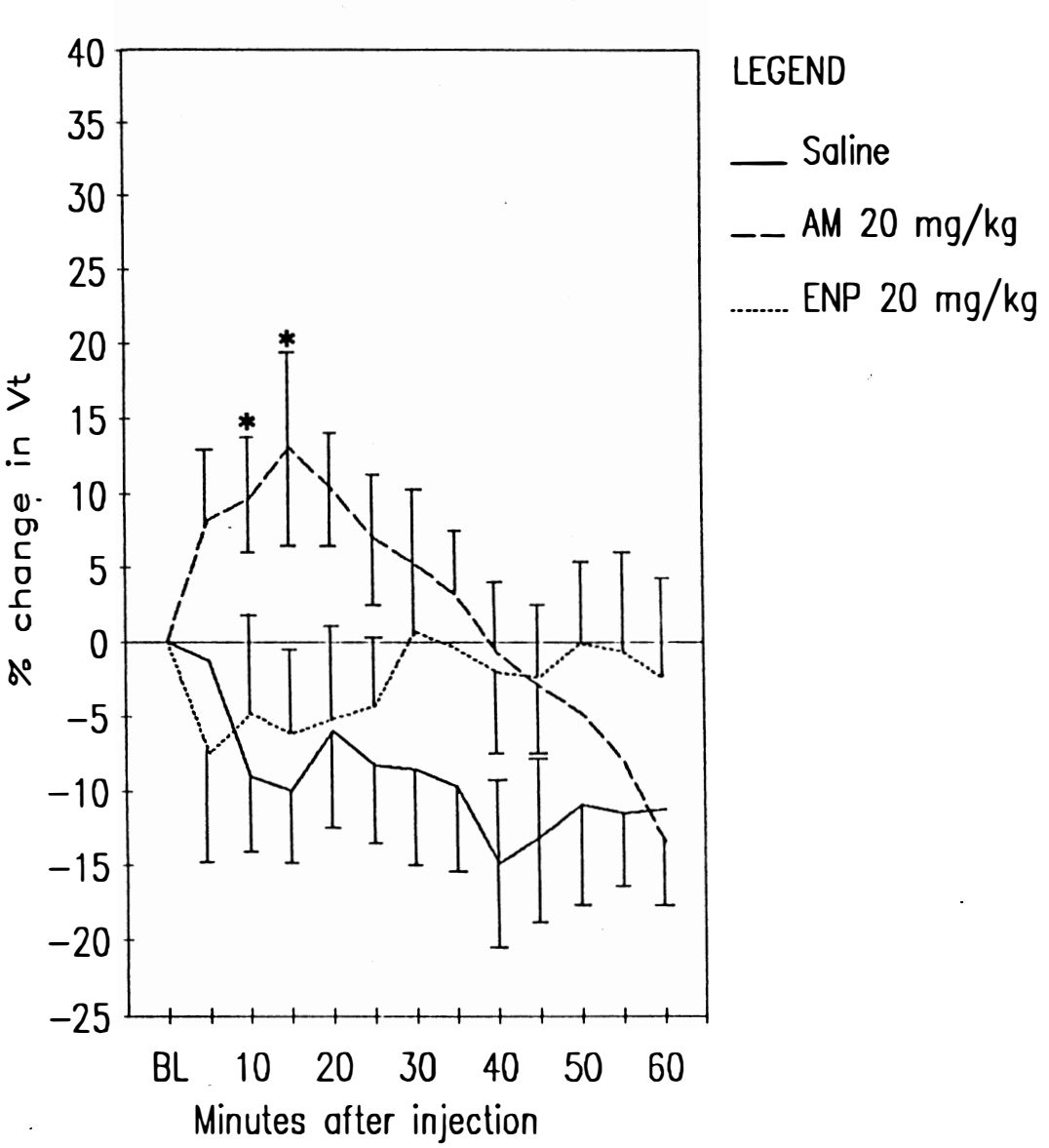


Figure 8. Effect of aminophylline on minute ventilation in adult mice. Bars indicate mean ± SEM. There were no significant differences between the tree doses of aminophylline ($p > 0.05$, two-way ANOVA with repeated measures).

Effect of Aminophylline on Minute Ventilation in Adult Mice

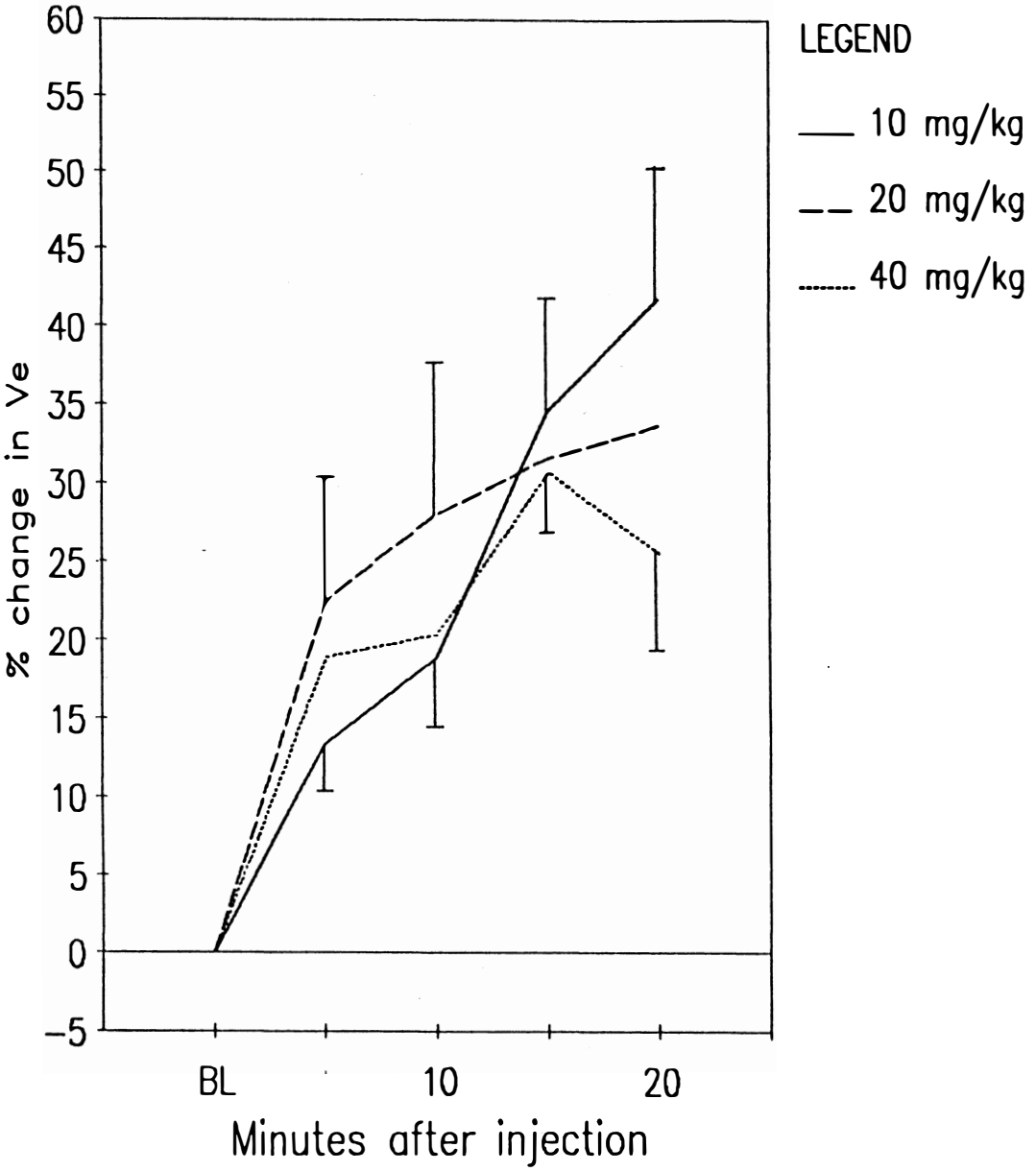


Table 2. Two-way ANOVA, with repeated measures, of aminophylline dose-response in adult mice, n= 6.

*** ANOVA TABLE ***

Source	DF	SS	MS	F	P
Treatment	2	303.84	151.92	.13	0.88
Error	16	17988.28	1199.22		
Time	3	3045.46	1015.15	12.40	<.01
Interaction	6	1073.20	178.87	2.19	0.06
Error	45	3683.02	81.84		

Figure 9. Effect of aminophylline on respiratory frequency in adult mice. Bars indicate mean ± SEM. There were no significant differences between the three doses of aminophylline ($p > 0.05$, two-way ANOVA with repeated measures).

Effect of Aminophylline on Respiratory Frequency in Adult Mice

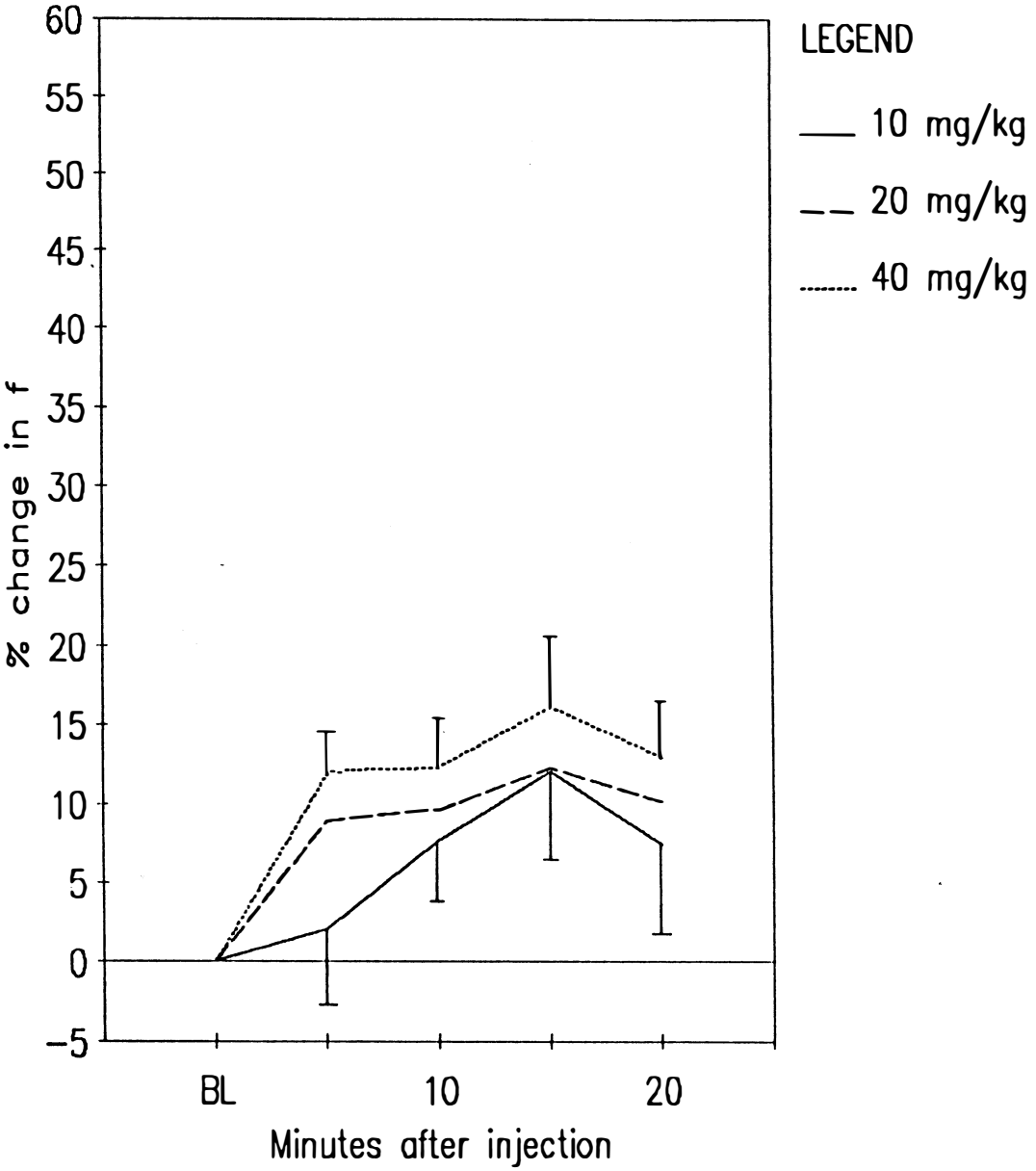


Figure 10. Effect of aminophylline on tidal volume in adult mice. Bars indicate mean ± SEM. There were no significant differences between the three doses of aminophylline ($p > 0.05$, two-wa ANOVA with repeated measures).

Effect of Aminophylline on Tidal Volume in Adult Mice

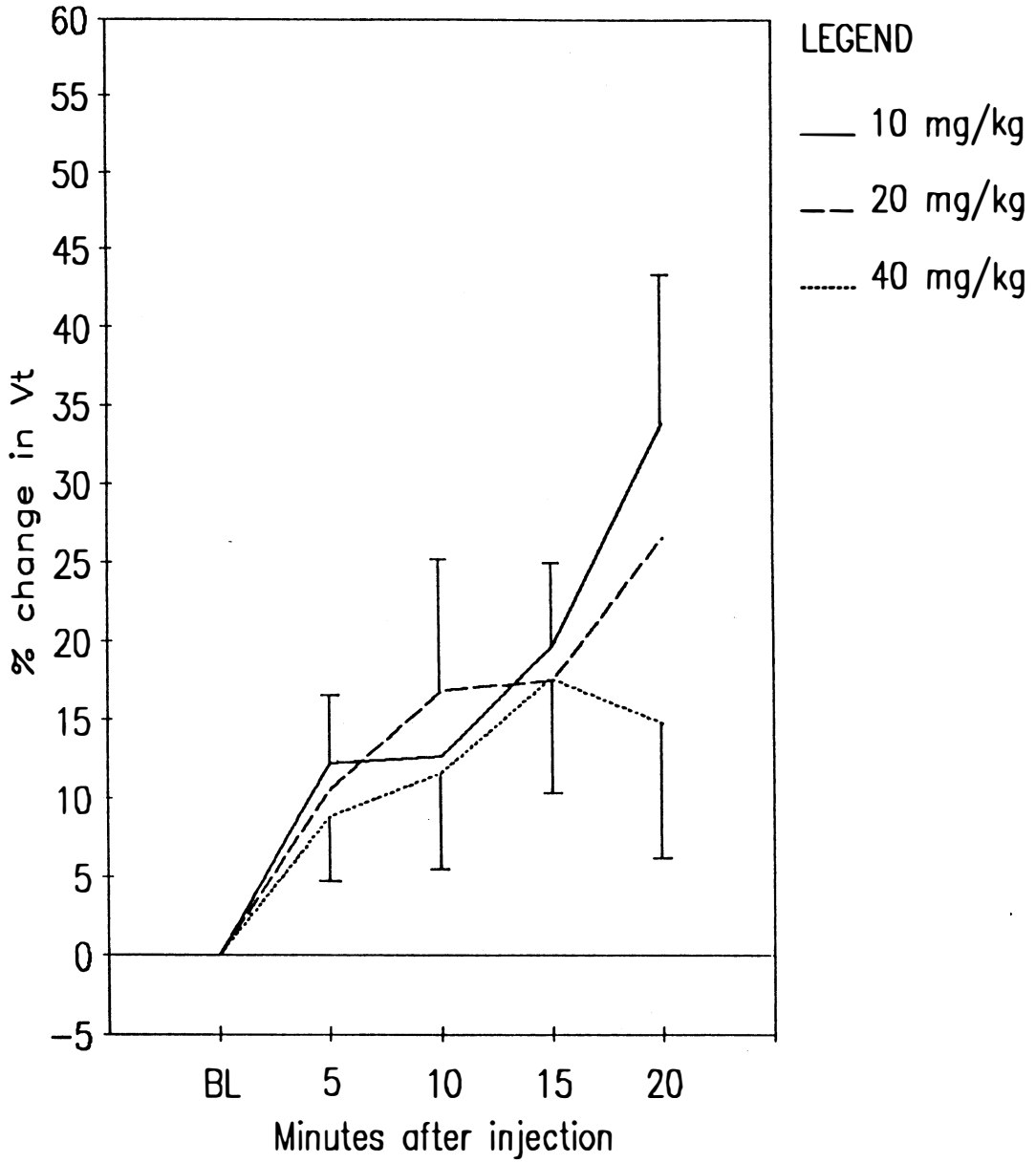


Table 3. Two-way ANOVA, with repeated measures, of enprofylline dose-response in adult mice, n= 6.

*** ANOVA TABLE ***

Source	DF	SS	MS	F	P
Treatment	2	4763.69	2381.85	.418	0.67
Error	15	85574.36	5704.96		
Time	11	857.60	77.96	.603	0.83
Interaction	22	1977.79	89.90	.695	0.84
Error	165	21343.09	129.35		

Figure 11. Effect of enprofylline on minute ventilation in adult mice. Bars indicate mean ± SEM. There were no significant differences between the three doses of enprofylline ($p > 0.05$, two-way ANOVA with repeated measures).

Effect of Enprofylline on Minute Ventilation in Adult Mice

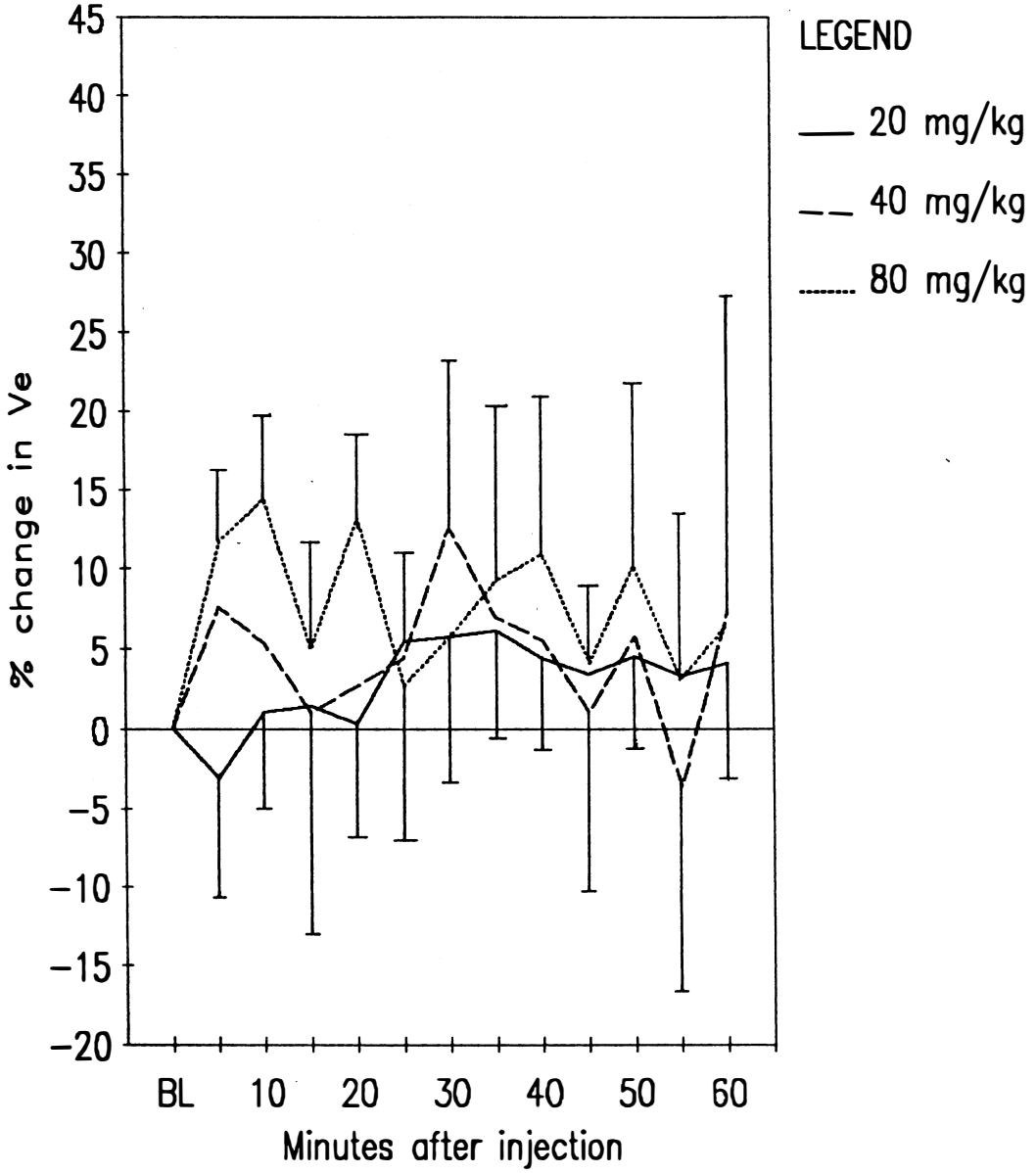


Figure 12. Effect of enprofylline on respiratory frequency in adult mice. Bars indicate mean ± SEM. There were no significant differences between the three doses enprofylline ($p > 0.05$, two-way ANOVA with repeated measures).

Effect of Enprofylline on Respiratory Frequency in Adult Mice

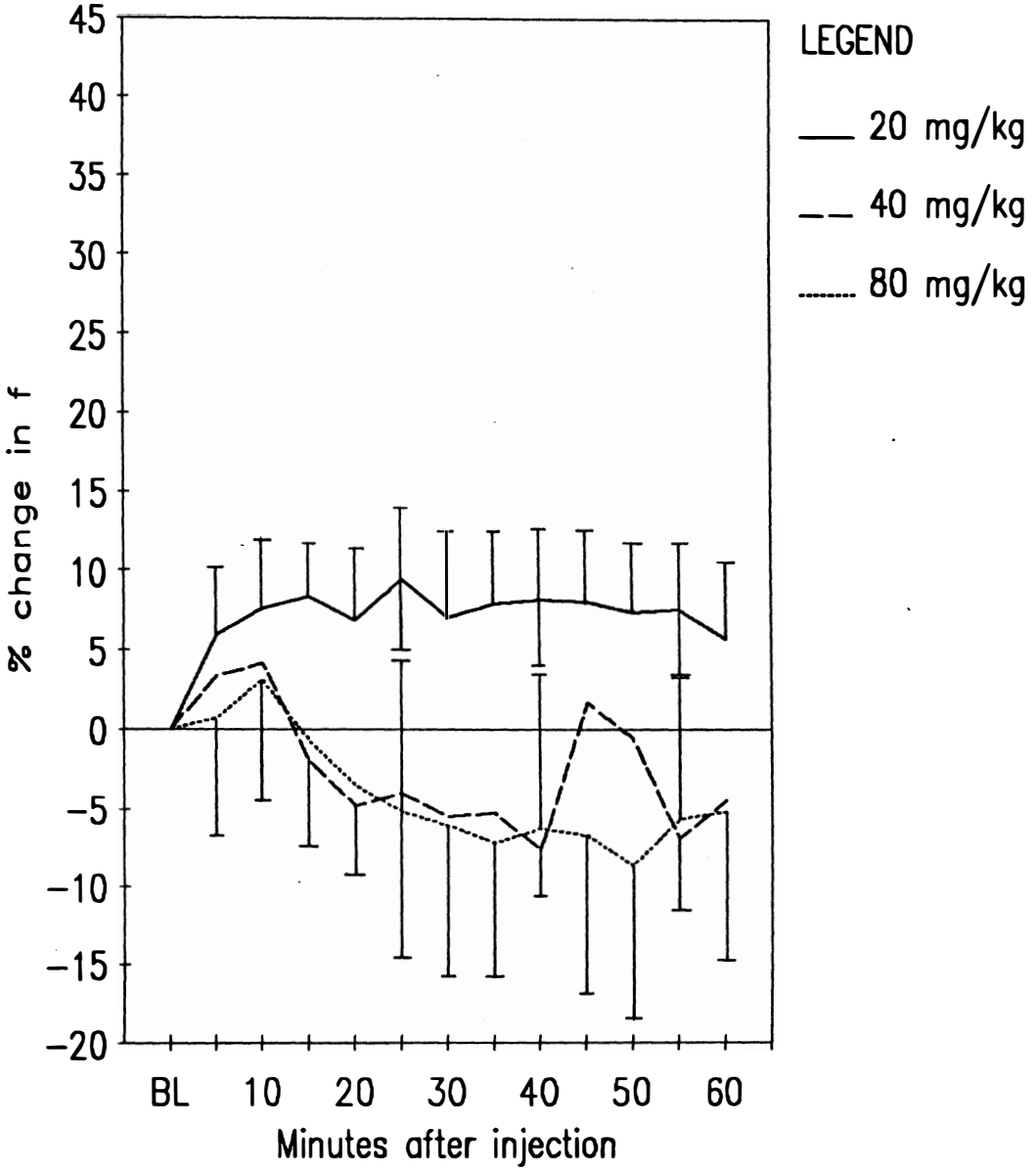
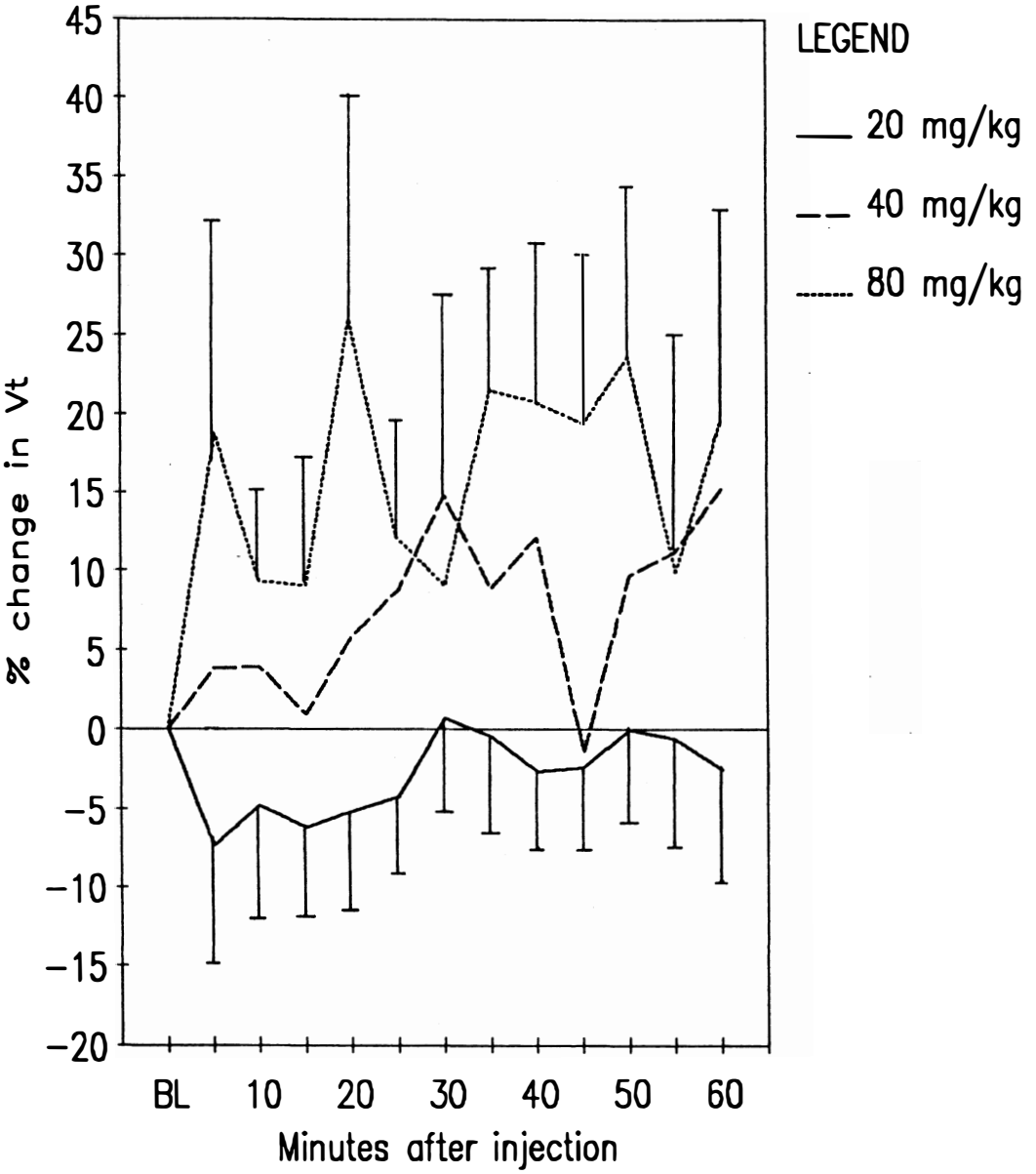


Figure 13. Effect of enprofylline on tidal volume in adult mice. Bars indicate mean ± SEM. There were no significant differences between the three doses of enprofylline ($p > 0.05$, two-way ANOVA with repeated measures).

Effect of Enprofylline on Tidal Volume in Adult Mice



significant differences between doses or times (F= 0.418, p= 0.67; F= 0.603, p= 0.83) (table 3). There was no consistent pattern in V_E, f or V_T throughout the experiments (fig. 11, 12 and 13).

Time Course of Saline, Aminophylline and Enprofylline in Newborn Rats

Figure 13a represents a tracing of newborn rat respiration before and after administration of aminophylline (20 mg/kg). The saline group had mean BL values for V_E, V_T and f of 16.5 ml/min, 0.124 ml and 134 breaths/min, respectively (see appendix II for individual values). BL means for those animals that received aminophylline (20 mg/kg s.c.) were 4.7 ml/min for V_E, 0.042 ml for V_T and 114 breaths/min for f. The enprofylline group (20 mg/kg s.c.) showed BL means of 6.6 ml/min for V_E, 0.047 ml for V_T and 143 breaths/min for f.

Statistical analysis (repeated measures design) showed a significant difference in V_E between times (F= 5.30, $p < 0.01$) and no significant drug effects (F= 1.85, p= 0.18) but a significant interaction of drugs and time (F= 2.03, $p < 0.01$) (table 4). Aminophylline increased V_E in 4- to 7-day-old rats by 44% above BL at a peak time of 20 min postinjection (fig. 14, $p < 0.01$). V_E declined steadily after 20 min. The increase in V_E produced by aminophylline was primarily due to a significant increase in f (> 5%, $p < 0.01$) and a 30% increase in V_T which was not significantly different from controls (figs. 15 and 16).

Statistical analysis showed no significant change in V_E at any time after injection of 20 mg/kg enprofylline (fig. 14). Enprofylline peaked at 5 min with an 18% increase above BL ($p > 0.05$). At other times V_E was elevated by less than 7%. None of these values were significantly different from control. V_T and f of enprofylline-treated rats were not significantly different from either saline or aminophylline (figs. 15 and 16).

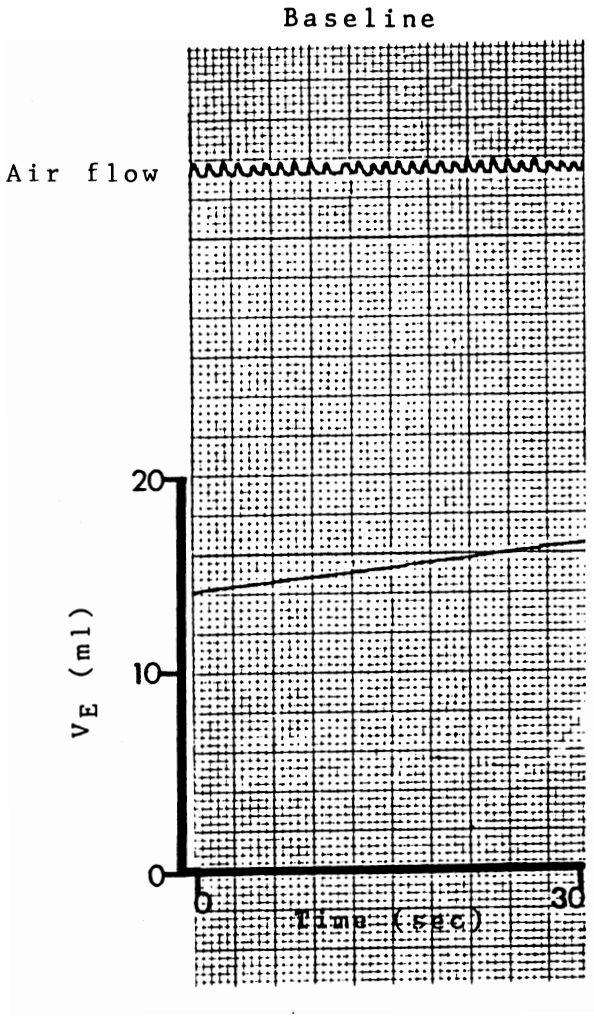
Dose Response of Enprofylline in Newborn Rats

The 40 mg/kg enprofylline group exhibited BL means of 9.9 ml/min for V_E, 0.067 ml for V_T and 152 breaths/min for f. BL values for the 80 mg/kg dose group were 9.6 ml/min, 0.064 ml and 153 breaths/min for V_E, V_T and f respectively. Statistical analysis resulted in significant differences between treatments ($F= 12.51$, $p < 0.01$) and interaction ($F= 2.99$, $p < 0.01$) (table 5), but not between times ($F= 0.86$, $p= 0.58$). Enprofylline (40 mg/kg) showed statistically significant decreases in V_E at 5, 15 and 40 min ($p < 0.05$) and 45 min ($p < 0.01$) when compared to the 20 mg/kg dose (fig. 17). There were no statistically significant changes in V_T or f (fig. 18 and 19). When compared to the 20 mg/kg dose, V_E for 80 mg/kg dose was significantly decreased at 5 min ($p < 0.01$) yet significantly increased at 50 and 60 min ($p < 0.01$) (fig. 17). Again, there were no statistically significant changes in V_T or f (fig. 18 and 19).

Lethal Dose Study

Seven of the eight pups given the 80 mg/kg dose of enprofylline died between 3 to 8 hrs after injection. Shortly before death a pale color to the pups was noted. To determine if death was caused by the dose of enprofylline (80 mg/kg) or the high pH of the solution (pH= 9.0), 8 pups were injected with a 0.9% NaCl solution with an adjusted pH of 9.0. Pups were injected with volumes coinciding to those given in the enprofylline tests. No pups died after receiving pH 9.0 NaCl and no changes in behavior were observed.

Figure 13a. Representative tracing of rat respiration before and after administration of aminophylline.


Baseline
Air flow
20
10
0
V_E (ml)
0
Time (sec)
30

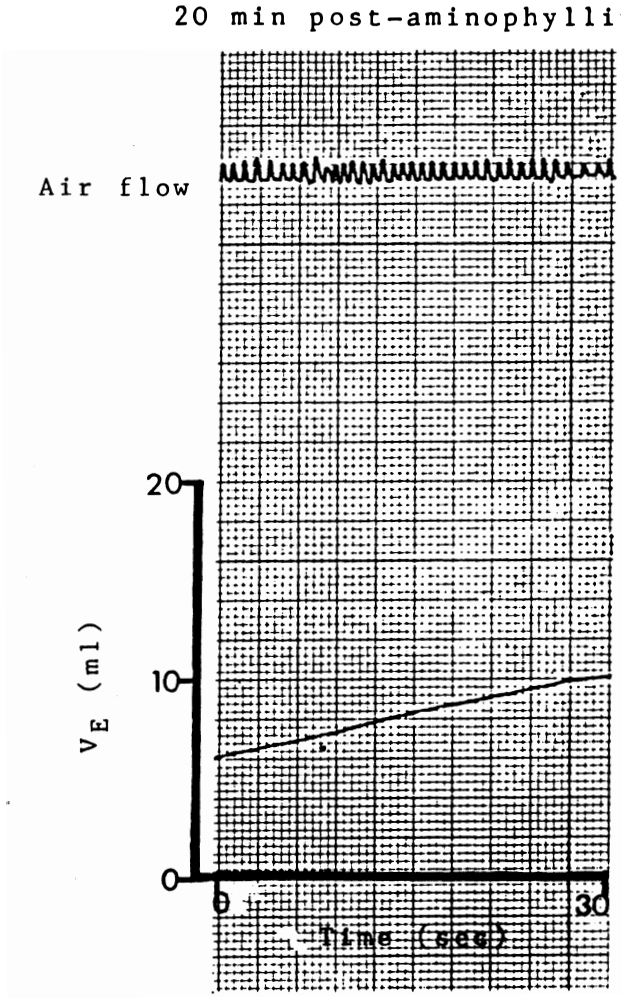

20 min post-aminophylline
Air flow
20
10
0
V_E (ml)
0
Time (sec)
30

Table 4. Two-way ANOVA, with repeated measures, of aminophylline and enprofylline time course experiments for newborn rats, n= 8.

*** ANOVA TABLE ***

Source	DF	SS	MS	F	P
Treatment	2	16903.74	8451.87	1.85	0.18
Error	21	96141.69	4578.18		
Time	11	10653.95	968.54	5.30	<.01
Interaction	22	8161.12	370.96	2.03	<.01
Error	231	42186.99	182.63		

Figure 14. Effect of aminophylline and enprofylline on minute ventilation in newborn rats. Bars indicate mean ± SEM. Asterisks indicate means significantly different from saline controls (* $p < 0.05$, ** $p < 0.01$, Tukey's test).

Effect of Aminophylline and Enprofylline on Minute Ventilation in Newborn Rats

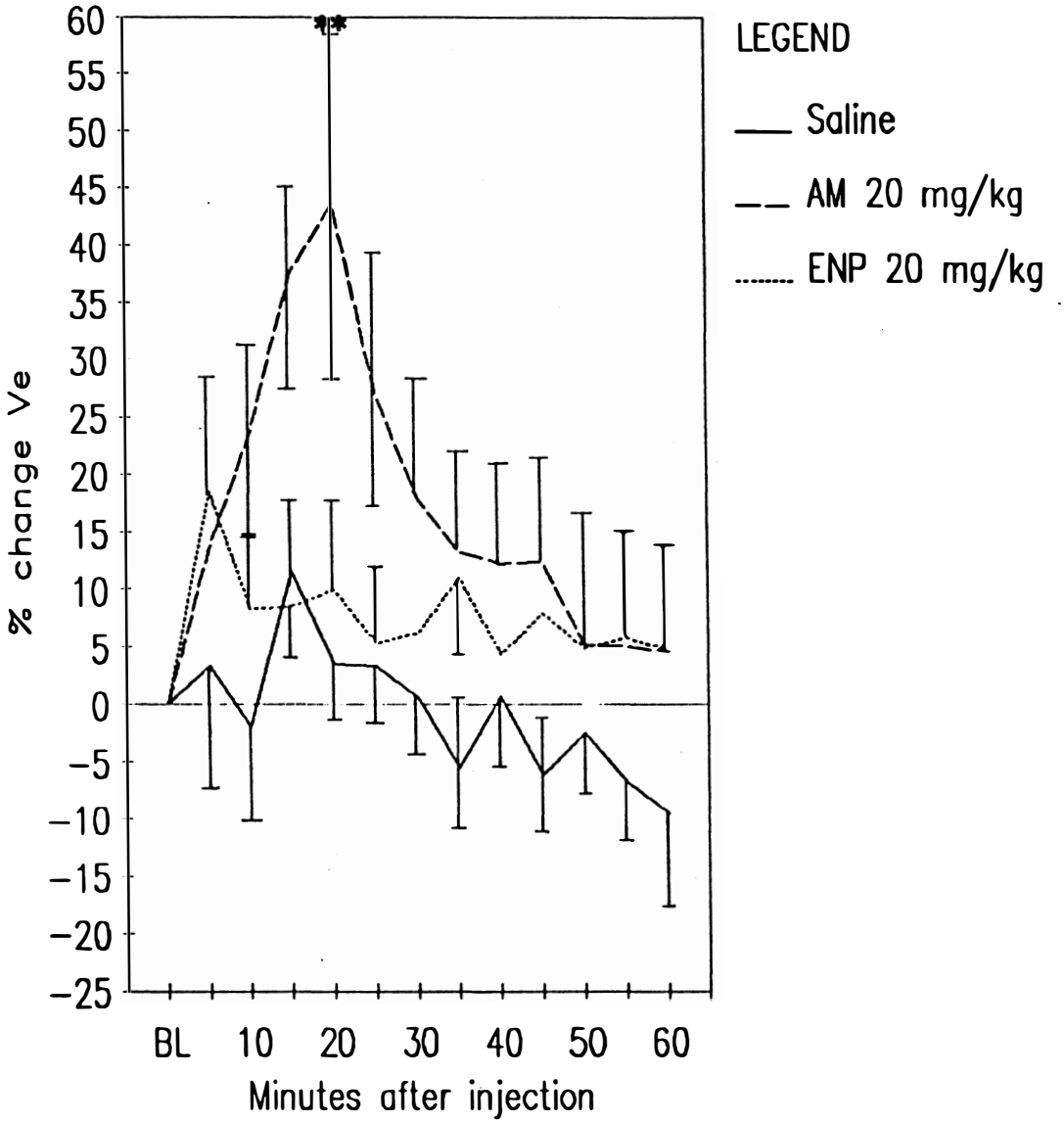


Figure 15. Effect of aminophylline and enprofylline on respiratory frequency in newborn rats. Bars indicate mean ± SEM. Asterisks indicate means significantly different from saline controls (* $p < 0.05$, ** $p < 0.01$; Tukey's test).

Effects of Aminophylline and Enprofylline on Respiratory Frequency In Newborn Rats

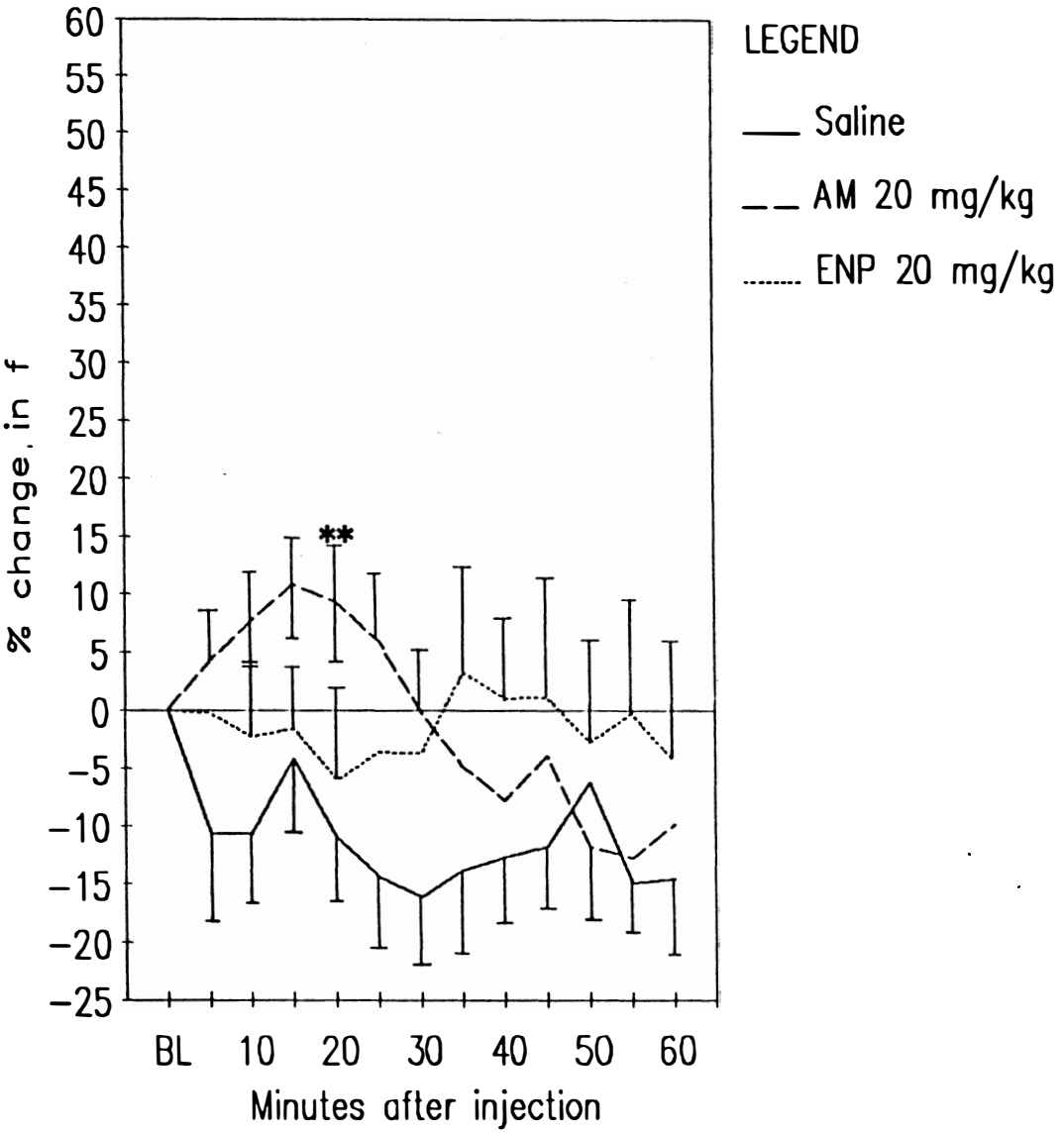


Figure 16. Effect of aminophylline and enprofylline on tidal volume in newborn rats. Bars indicate mean ± SEM. There were no significant differences with aminophylline nor enprofylline.
($p > 0.05$, two-way ANOVA with repeated measures).

Effect of Aminophylline and Enprofylline on Tidal Volume in Newborn Rats

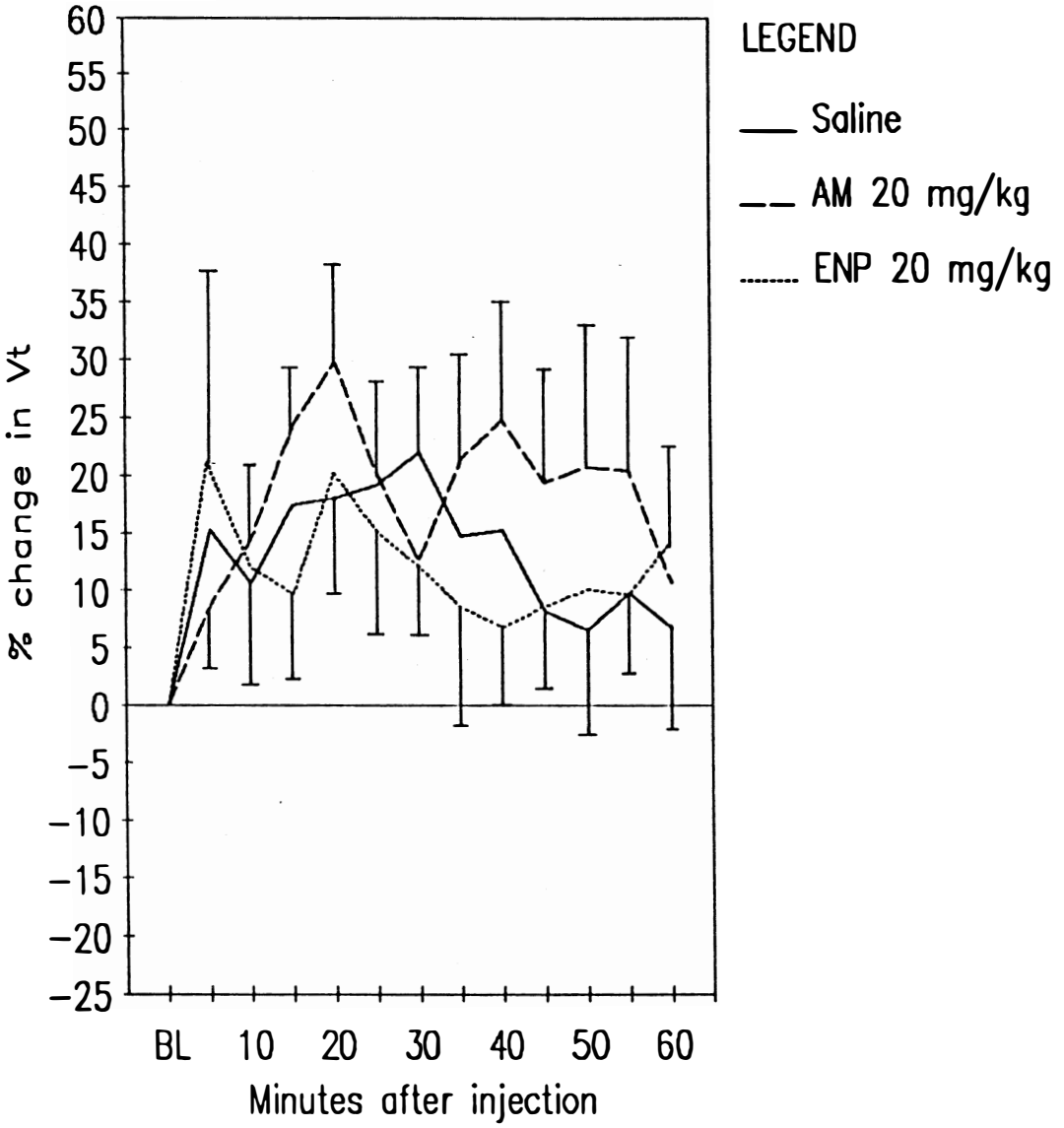


Table 5. Two-way ANOVA, with repeated measures,of enprofylline dose-response newborn rats, n= 6.

*** ANOVA TABLE ***

Source	DF	SS	MS	F	P
Treatment	2	41384.29	20692.15	12.51	<.01
Error	15	24804.17	1653.61		
Time	11	1512.43	137.49	.86	0.58
Interaction	22	10525.58	478.44	2.99	<.01
Error	165	26357.48	159.74		

Figure 17. Effect of enprofylline on minute ventilation in newborn rats. Bars indicate mean ± SEM. Asterisks indicate means significantly different from 20 mg/kg controls (* $p < 0.05$, ** $p < 0.01$; Tukey's test).

Effect of Enprofylline on Minute Ventilation in Newborn Rats

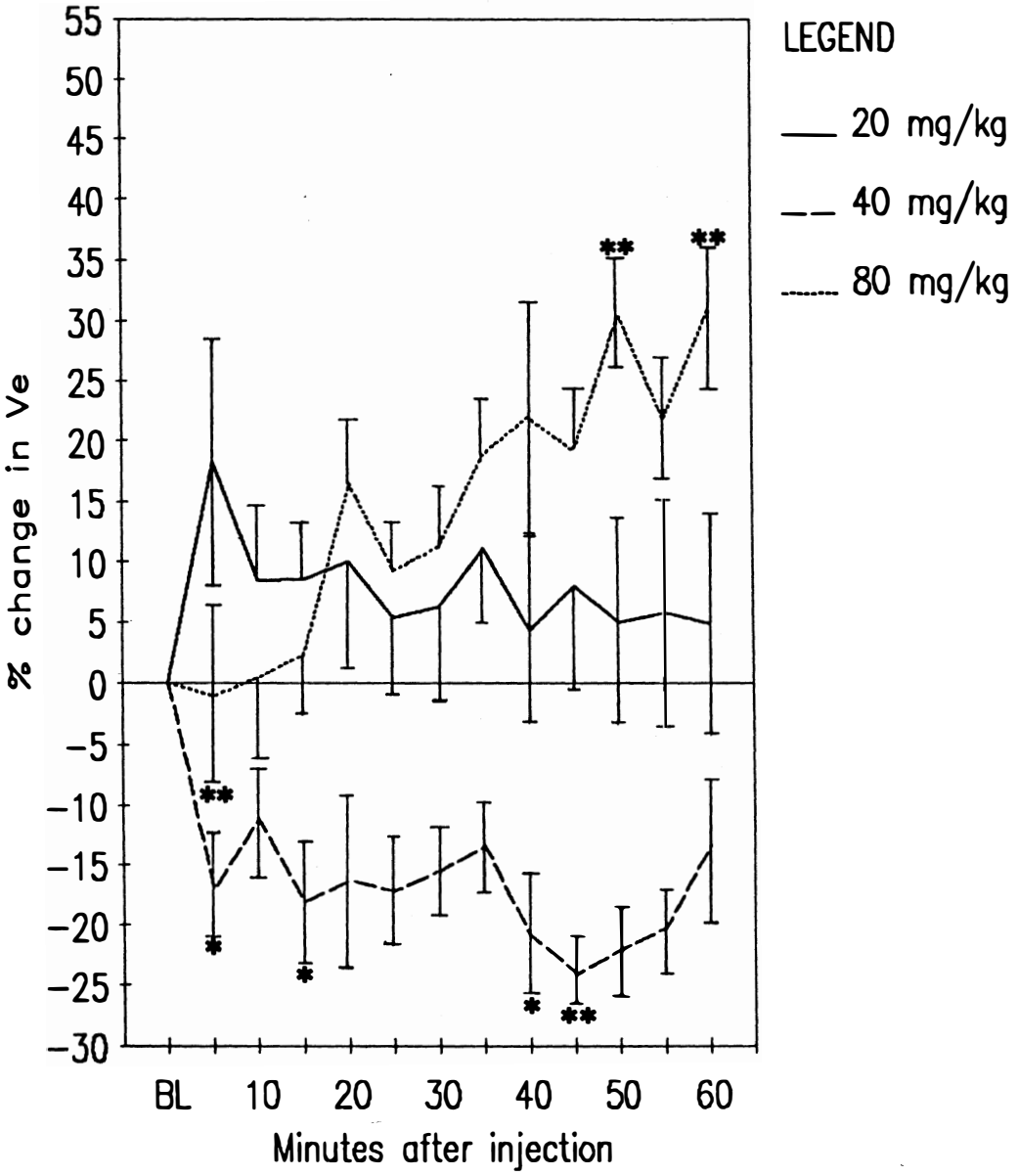


Figure 18. Effect of enprofylline on respiratory frequency in newborn rats. Bars indicate mean ± SEM. There were no significant differences between doses of enprofylline. ($p > 0.05$, two-way ANOVA with repeated measures).

Effect of Enprofylline on Respiratory Frequency in Newborn Rats

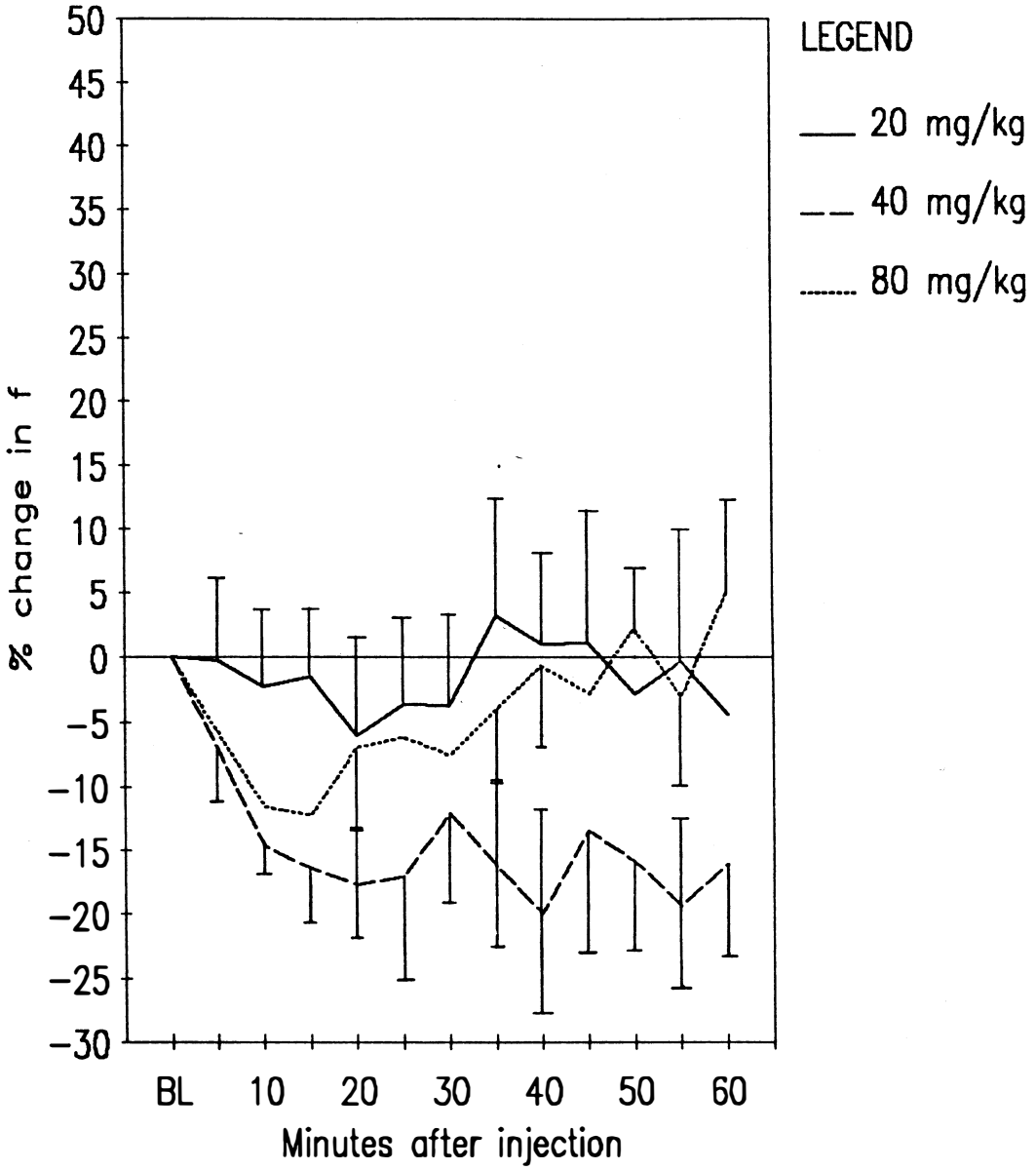
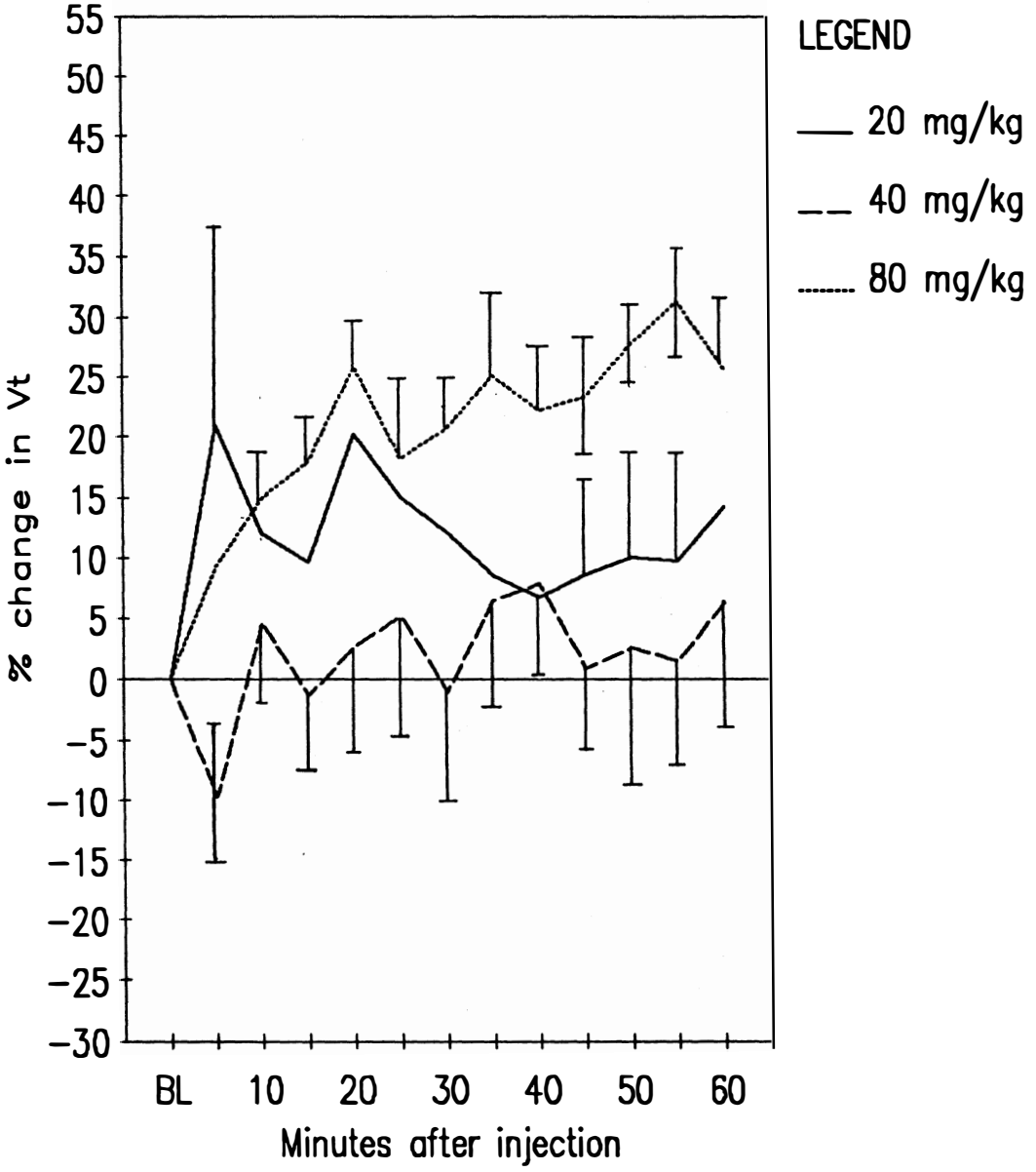


Figure 19. Effect of enprofylline on tidal volume in newborn rats. Bars indicate mean ± SEM. There were no significant differences between doses of enprofylline. ($p > 0.05$, two-way ANOVA with repeated measures).

Effect of Enprofylline on Tidal Volume in Newborn Rats



DISCUSSION

It has become increasingly important to determine the causes of neonatal apnea. Apnea of prematurity, a condition often associated with sudden infant death syndrome (SIDS), leads the causes of death among premature infants (Naeye, 1980). Of great necessity is the study of those drugs presently being used for the treatment of apnea and the continued search for a more effective treatment with fewer side effects.

As surmised from the literature, a widely accepted hypothesis behind the mechanism of apnea is the release of endogenous adenosine in the brain. Adenosine may be released in the brain during an hypoxic attack (Winn et al., 1981). Hypoxia, a deficiency of oxygen at the tissue level, may then cause extended interruption of the respiratory rhythm that has previously been referred to as apnea (Cross and Oppe, 1952; Rigatto et al., 1975).

Respiratory stimulants, such as theophylline and caffeine, are presently being used for treatment of recurrent neonatal apnea (Aranda and Turmen, 1979). The effects, as well as the toxicity, of the MXs depend upon substituent groups being attached to certain positions of the xanthine nucleus. SAR studies have shown that substitution on the 1-position is necessary for adenosine antagonism while the 3-position contributes to bronchodilation. Theophylline has methyl groups on both the 1- and 3-positions while enprofylline has a single

propyl group on position 3. Since enprofylline contains no substituent group on position 1 it has very little adenosine antagonistic capabilities. For these reasons enprofylline and theophylline were chosen for the present studies, theophylline representing a potent adenosine antagonist and enprofylline, a weak adenosine antagonist, if one at all.

The hypothesis which was tested in the present study was that theophylline stimulates respiration by adenosine antagonism. According to this hypothesis theophylline would stimulate respiration and enprofylline would not.

Enprofylline had not, until recently, been shown to antagonize adenosine receptors in any tissues. Fredholm and Persson (1982) did show that enprofylline selectively antagonized adenosine A_2 receptors in rat hippocampal slices, however at doses much greater than those used to produce bronchodilation. These results were supported by a pair of papers (Ukena et al., 1985; Schwabe et al., 1985) in which data showed that enprofylline (100 and 1000 uM) stimulated adenylate cyclase by competitively antagonizing adenosine receptors in human blood platelets, however enprofylline did not antagonize adenosine in platelet aggregation studies. Selectivity of enprofylline for either A_1 or A_2 receptors was disputed. However, Schwabe et al. (1985) showed that enprofylline was a weak antagonist of adenosine, being less potent than xanthine itself, at A_1 receptors of rat and bovine brain and rat fat

cells. In other pharmacological studies enprofylline has been shown not to antagonize adenosine receptors or stimulate the CNS (Persson et al., 1982a).

It is well-known that theophylline stimulates respiration (Aranda and Turmen, 1979; Persson and Fredholm, 1982; Persson et al., 1982a,b, 1983). If the respiratory stimulant effects of theophylline are indeed the result of adenosine antagonism then enprofylline should not stimulate respiration. The results of this thesis support this hypothesis.

There was considerable variability in BL means between the three groups of mice in the initial study, however the values for f were consistent with previous studies (McGilliard and Takemori, 1978). The BL means for V_E of enprofylline are much higher than aminophylline or controls. This can be attributed to poor random sampling from a diverse population. Mice used for the control experiments came from facilities where the temperature was kept fairly constant and the population closely monitored (Life Science room 323). Although these experiments took place during the months of February and March the building temperature averaged 25^{o}C. Once this population was exhausted a population housed in a separate building (the vivarium) was exploited. This facility was much colder and cages more crowded. Coincidentally, these mice were used primarily for enprofylline and a few aminophylline experiments. Although the respiratory studies were

conducted under constant conditions for all mice, the low ambient temperatures in which these animal were bred and raised may have resulted in a higher metabolic rate, hence higher respiratory rate. The overpopulation of cages may have also contributed to differences in BL through a stress mechanism. To reduce these problems all animals used in the dose-response studies were brought into the core facility and assigned to separate cages of 5 animals or less. The problem of randomization was not so easily reduced. To eliminate variability due to the diversity in BL between animals, drug effects were expressed as a percent change from the BL value of each animal.

Aminophylline, the ethylenediamine salt of theophylline, was chosen for these studies because of its improved solubility in water. After injection it dissociates resulting in theophylline and ethylenediamine. Aminophylline (20 mg/kg) stimulated respiration in both the adult mice (fig. 5, 6 and 7) and the neonatal rats (fig. 14, 15 and 16). Minute ventilation was increased by as much as 30% in mice (fig. 5) and more than 40% in rat pups (fig. 14). In 1986 Runold and colleagues published data from a study similar in some ways to the present studies. With unanesthetized rabbit pups they showed that theophylline (10 mg/kg) stimulated respiration by more than 20% of the control value. Theophylline treatment also resulted in nearly complete reversal of the respiratory depressant effects of R-PIA. Hedner et al. (1985) also

showed that theophylline (50 mg/kg) reversed the respiratory depressant effects of PIA. Since PIA has been shown to be a potent A_1 receptor agonist (Ukena et al., 1985), Runold's and Hedner's results suggest that theophylline had its effect by antagonism of the A_1 receptor site. These data, using newborn rabbits, support the present studies involving newborn rats.

Enprofylline is five times more potent than theophylline as a bronchodilator (Persson and Kjellin, 1981; Lunell et al., 1982) and cardiostimulant (Persson et al., 1983). When given at the same dose as aminophylline (20 mg/kg), enprofylline did not stimulate respiration in either adult mice (fig. 5, 6 and 7) or newborn rats (fig. 14, 15 and 16). According to the literature theophylline must have its respiratory effects by the mechanism of adenosine antagonism.

Even though no dose-response was determined for doses of aminophylline (10, 20 and 40 mg/kg) in adult mice, data duplicated that of the time course studies nicely (fig. 8, 9 and 10). Since differences were not significant it appeared that the 10 mg/kg dose may be the maximum dose at which an effect will be elicited for adult mice and doses greater than that produce no greater stimulation. Dose-response studies for aminophylline (10, 20 and 40 mg/kg) with neonatal rats were flawed. The respiratory effects of these doses appeared to be minimal. Upon analysis of data it was concluded that the drug solutions may no longer have

been effective. Enprofylline dose-response studies for the adult mice resulted in no significant differences between doses nor was there any effect on the respiration of these animals (fig. 11, 12 and 13). Dose-response experiments for the newborn rats were intriguing (fig. 17, 18 and 19). The 40 mg/kg dose showed a significant depression of V_E compared to the 20 mg/kg dose. The 80 mg/kg dose also depressed respiration slightly, but significantly compared to the 20 mg/kg dose at 5 min postinjection. However with time V_E was consistently stimulated to more than 35% above BL which was a significant increase. This effect is puzzling and possibilities endless. It is possible that enprofylline (40 mg/kg), providing it is a selective antagonist of A_2 adenosine receptor sites (Fredholm and Persson, 1982), may bind to the A_2 sites at this dose leaving only the A_1 sites available for adenosine binding. Since A_1 sites are responsible for decreasing synthesis of adenylate cyclase it may be possible that this decrease causes respiratory depression. Subsequently, if enprofylline (80 mg/kg) binds to A_1 as well as A_2 sites then adenosine has no available binding site resulting in net synthesis of adenylate cyclase and consequential stimulation in respiration.

Seven of the eight rat pups (88%) receiving 80 mg/kg enprofylline died between 3 to 8 hr after injection. After some consideration, a fatal pH effect or lethal drug effect were concluded to be possible causes. Because no pups were

killed when given a pH 9.0 NaCl solution it was determined that 80 mg/kg enprofylline is a lethal dose for neonatal rats. A pale color was noted just before death, but no unusual behavior was observed. Although cause of death is uncertain, since no convulsions were observed CNS toxicity was not considered as the cause of death. It is possible however, that convulsions took place between observations or that the toxicity of the enprofylline solution was such that the CNS system was severely depressed. In this case CNS toxicity could be a possibility without observing convulsions. The pale color of the skin, however, may indicate a cardiovascular complication. If enprofylline is indeed 5 times greater a cardiostimulant than theophylline (Persson et al., 1983) then these rat pups may have died due to an incredible insult to the cardiovascular system. No adult mice died from any of the enprofylline dose. The 80 mg/kg dose may have affected the rat pups while not affecting adult mice since many drugs are metabolized more slowly in newborns than in adults. It may also be possible that the immature cardiovascular system could be affected by this drug to a greater extent than the more mature cardiovascular system of adult mice. This complication warrants further study.

In summary it was found that theophylline stimulated respiration in adult mice and newborn rats while enprofylline did not. There were no significant differences between doses of aminophylline in the mice nor

were there any significant differences between enprofylline doses in mice. Enprofylline produced significant alterations of respiration in newborn rats only at lethal and nearly lethal doses. These data support the hypothesis that theophylline stimulates respiration by adenosine antagonism.

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Appendix I. Effect of aminophylline and enprofylline on V_E in adult mice with respect to % change from baseline.

	BL	5 min		10 min		15 min		20 min		25 min		30 min		35 min		40 min		45 min		50 min		55 min		60 min	
	V_E	V_E	%	V_E	%	V_E	%	V_E	%	V_E	%	V_E	%	V_E	%	V_E	%	V_E	%	V_E	%	V_E	%	V_E	%
NaCl	16.0	13.6	-15.0	13.2	-17.5	13.8	-13.8	14.4	-10.0	14.0	-12.5	14.0	-12.5	13.8	-13.8	13.6	-15.0	13.6	-15.0	13.6	-15.0	13.2	-17.5	13.6	-15.0
	17.2	18.0	4.7	15.6	9.3	16.0	-7.0	16.8	-2.3	16.8	-2.3	16.4	-4.7	16.8	-2.3	15.6	-9.3	13.6	-20.9	14.4	-16.3	14.8	-14.0	13.2	-23.3
	16.0	10.4	-35.0	9.6	-40.0	9.6	-40.0	9.2	-42.5	9.2	-42.5	8.8	-45.0	8.8	-45.0	8.0	-50.0	8.6	-46.3	7.6	-52.5	10.0	-37.5	9.2	-42.5
	9.6	10.4	8.3	10.4	8.3	9.6	0.0	9.6	0.0	10.4	8.3	10.0	4.2	10.4	8.3	9.6	0.0	9.2	-4.2	9.2	-4.2	9.6	0.0	9.2	-4.2
	17.2	17.2	0.0	17.2	0.0	16.8	-2.3	16.8	-2.3	16.8	-2.3	17.2	0.0	16.8	-2.3	17.6	2.3	17.0	-1.2	16.8	-2.3	17.2	0.0	16.8	-2.3
	16.0	14.4	-10.0	14.4	-10.0	14.4	-10.0	12.8	-20.0	12.0	-25.0	10.0	-37.5	9.8	-38.8	9.2	-42.5	10.0	-37.5	10.4	-35.0	9.6	-40.0	9.6	-40.0
	12.0	12.0	0.0	10.0	-16.7	10.0	-16.7	11.0	-8.3	12.0	0.0	12.0	0.0	10.8	-10.0	10.8	-10.0	11.2	-6.7	11.2	-6.7	10.8	-10.0	11.2	-6.7
	17.6	18.4	4.6	18.6	5.7	18.4	4.6	17.6	0.0	17.6	0.0	17.6	0.0	17.2	-2.3	16.8	-9.6	16.4	-6.8	16.4	-6.8	16.0	-9.1	16.0	-9.1
Mean	14.9	14.3	-5.3	13.7	-7.6	13.7	-10.7	13.5	-10.7	13.6	-9.5	13.3	-11.9	13.1	-13.3	12.7	-16.7	12.5	-17.3	12.5	-17.3	12.7	-16.0	12.4	-17.9
SEM	1.1	1.2	5.1	1.2	6.0	1.2	4.9	1.2	5.1	1.1	5.9	1.3	6.7	1.2	6.7	1.3	6.7	1.1	5.9	1.2	6.2	1.1	5.4	1.1	5.6
AM	17.2	30.4	76.7	32.0	86.1	27.2	58.1	27.2	58.1	25.6	48.8	23.2	34.9	23.2	34.9	17.6	2.3	16.2	-5.8	15.2	-11.6	15.5	-9.9	15.2	-11.6
	22.4	22.4	0.0	24.8	10.7	25.6	14.3	27.6	23.2	26.0	16.1	24.4	8.9	24.0	9.1	23.2	3.6	21.2	-5.4	20.0	-10.7	19.6	-12.5	18.4	17.9
	18.4	19.2	4.4	20.4	10.9	23.2	26.1	23.2	26.1	23.2	26.1	21.6	17.4	20.4	10.9	20.4	10.9	21.6	17.4	19.6	6.5	18.0	-2.2	18.0	-2.2
	24.0	24.0	0.0	24.0	0.0	25.2	5.0	24.4	1.7	22.8	-5.0	22.4	-6.7	22.0	-8.3	19.2	-20.0	19.6	-18.3	19.2	-20.0	15.6	-35.0	15.2	-36.7
	24.8	31.2	25.8	34.4	38.7	39.2	58.1	35.6	43.6	33.6	35.5	33.6	35.5	32.4	30.7	32.0	29.0	31.6	27.4	30.8	24.2	30.4	22.6	30.4	22.6
	27.2	28.4	4.4	28.4	4.4	29.6	8.8	30.4	11.8	29.2	7.4	28.8	5.9	28.8	5.9	27.6	1.5	27.2	0.0	27.2	0.0	28.0	2.9	26.8	-1.5
	24.0	25.6	6.7	28.0	16.7	28.8	20.0	33.2	38.3	32.0	33.3	30.0	25.0	29.2	21.7	27.6	15.0	26.8	11.7	26.8	11.7	21.2	-11.7	21.2	-11.7
	24.0	24.8	3.3	28.4	18.3	32.8	36.7	32.4	35.0	34.0	41.7	35.2	46.7	36.0	50.0	36.0	50.0	36.0	50.0	36.0	50.0	36.0	50.0	36.0	50.0
Mean	22.8	25.8	15.2	27.6	23.2	29.0	28.4	29.3	29.7	28.3	25.5	27.4	20.9	27.0	19.1	25.5	11.5	25.0	9.6	24.4	6.3	23.0	3.5	22.7	1.8
SEM	1.2	1.4	9.3	1.6	9.9	1.8	7.4	1.5	6.4	1.6	6.4	1.9	6.3	1.9	6.7	2.3	7.4	2.3	7.7	2.5	8.0	2.7	9.0	2.7	9.4
ENP	36.4	37.6	3.3	37.6	3.3	36.0	-1.1	36.0	-1.1	35.2	-3.3	37.6	3.3	37.2	2.2	35.2	-3.3	33.6	-7.7	34.0	-6.6	33.2	-8.8	32.0	12.1
	28.0	22.8	-18.6	25.2	-10.0	25.6	-8.6	24.0	-14.3	26.0	-7.1	25.6	-8.6	26.4	-5.7	27.2	-3.9	29.6	5.7	29.2	4.3	28.4	1.4	27.6	-1.4
	32.0	29.2	-8.8	32.4	1.3	33.2	3.8	33.6	5.0	33.2	3.8	34.4	7.5	34.8	8.8	34.4	7.5	33.2	3.8	32.4	1.3	32.4	1.3	32.0	0.0
	28.8	34.8	20.8	38.4	33.3	39.2	36.4	38.4	33.3	38.4	33.3	39.6	37.5	40.4	40.3	36.0	25.0	37.2	29.2	36.8	27.8	40.0	28.9	39.2	36.1
	40.0	44.0	10.0	42.8	7.0	40.8	2.0	46.0	15.0	48.0	20.0	46.4	16.0	44.8	12.0	47.2	18.0	44.0	10.0	47.2	18.0	41.6	4.0	36.8	8.0
	35.2	35.2	0.0	34.8	-1.1	34.8	-1.1	34.8	-1.1	33.6	4.6	36.0	2.3	37.2	5.7	38.0	8.0	37.6	6.8	40.0	13.6	40.0	13.6	40.0	13.6
	42.4	22.8	-46.2	30.0	-29.3	29.6	-30.2	26.8	-36.8	34.8	-17.9	32.4	-23.6	32.0	-24.5	30.8	-27.4	32.0	-24.5	32.0	-24.5	31.2	-26.4	26.4	-37.7
	39.2	44.8	14.3	40.8	4.1	43.2	10.2	40.2	2.6	43.2	10.2	43.6	11.2	43.2	10.2	43.6	11.2	40.8	4.1	40.0	2.0	38.4	2.0	38.4	2.0
Mean	35.3	33.9	-3.1	35.3	1.1	35.3	1.4	35.0	0.3	36.6	5.4	37.0	5.7	37.0	6.1	36.6	4.4	36.0	3.4	36.5	4.5	35.7	3.2	34.1	4.1
SEM	1.9	3.0	7.6	2.1	6.2	2.1	6.6	2.5	7.2	2.4	5.7	2.3	6.3	2.1	6.4	2.3	5.7	1.7	5.4	2.1	5.7	1.7	6.6	1.9	7.3

%= % change in V_E from baseline

Appendix II. Effect of aminophylline and enprofylline on V_E in neonatal rats with respect to % change from baseline.

	BL	5 min		10 min		15 min		20 min		25 min		30 min		35 min		40 min		45 min		50 min		55 min		60 min	
	V_E	V_E	%	V_E	%	V_E	%	V_E	%	V_E	%	V_E	%	V_E	%	V_E	%	V_E	%	V_E	%	V_E	%	V_E	%
NaCl	31.5	31.8	1.0	28.5	-9.5	26.7	-15.2	25.5	-19.1	24.2	-23.2	24.0	-23.8	24.8	-21.2	24.0	-23.8	28.2	-10.5	28.5	-9.5	24.0	-23.8	26.3	-16.5
	32.0	21.0	-34.3	26.4	-17.5	32.1	0.3	32.4	1.3	33.6	5.0	30.0	-6.3	31.8	-0.6	30.0	-6.3	26.4	-17.5	24.0	-25.0	23.4	-26.9	23.7	-25.9
	18.0	25.0	38.9	20.0	11.1	21.0	16.7	17.4	-3.3	17.4	-3.3	18.0	0.0	18.6	3.3	20.0	11.1	18.0	0.0	17.0	-5.6	19.2	6.7	18.1	0.6
	24.0	12.6	-47.5	12.6	-47.5	31.8	32.5	27.3	13.8	28.2	17.5	26.4	10.0	18.4	-23.3	26.4	10.0	18.0	-25.0	22.0	-8.3	21.0	-12.5	21.5	-10.4
	6.6	6.9	4.6	6.0	-9.1	6.0	-9.1	6.3	-4.6	6.0	-9.1	6.0	9.1	6.0	-9.1	6.0	-9.1	5.7	-13.6	6.0	-9.1	6.0	-9.1	3.3	-50.0
	7.5	8.1	8.0	8.4	12.0	8.4	12.0	7.8	4.0	7.5	0.0	8.1	8.0	6.0	-20.0	6.6	-12.0	6.9	-8.0	8.1	8.0	7.5	0.0	7.8	4.0
	5.7	8.1	42.1	6.9	21.1	7.5	31.6	7.2	26.3	6.9	21.1	6.9	21.1	7.2	26.3	6.9	21.1	6.6	15.8	6.3	10.5	6.6	15.8	7.2	26.3
	6.6	7.5	13.6	8.1	22.7	8.1	22.7	7.2	9.1	7.8	18.2	6.9	4.6	6.6	0.0	7.5	13.6	7.2	9.1	7.8	18.2	6.3	-4.6	6.3	-4.6
Mean	16.5	15.1	3.3	14.6	-2.1	17.7	11.4	16.4	3.4	16.5	3.3	15.8	0.6	14.9	-5.6	15.9	0.6	14.6	-6.2	15.0	-2.6	14.3	-6.8	14.3	-9.6
SEM	4.0	13.4	11.1	3.2	8.4	4.0	6.4	3.8	4.8	3.9	5.4	3.5	4.8	3.5	5.9	3.6	5.5	3.3	4.8	3.2	4.9	2.9	5.1	3.2	8.0
AM	4.8	3.9	-18.8	3.9	-18.8	5.5	14.6	4.5	-6.3	4.5	-6.3	3.9	-18.8	3.9	-18.8	3.5	-27.1	4.0	-16.7	3.3	-31.3	4.2	-12.5	4.5	-6.3
	6.0	5.4	-10.0	6.6	10.0	6.9	15.0	6.3	5.0	6.3	5.0	6.0	0.0	6.0	0.0	6.0	0.0	6.0	0.0	5.4	-10.0	4.8	-20.0	4.5	-25.0
	3.6	4.8	33.3	5.7	58.3	6.9	91.7	8.1	125.0	6.3	75.0	5.4	50.0	5.1	41.7	5.1	41.7	5.1	41.7	4.8	33.3	4.8	33.3	4.8	33.3
	4.2	5.4	28.6	5.4	28.6	5.7	35.7	5.7	35.7	5.7	35.7	5.4	28.6	4.8	14.3	4.8	14.3	4.8	14.3	4.8	14.3	4.8	14.3	4.8	14.3
	6.3	6.6	4.8	6.6	4.8	7.8	23.8	7.5	19.0	5.4	14.3	5.4	14.3	5.4	14.3	5.7	9.5	5.1	9.0	3.6	42.9	3.0	52.4	3.0	54.4
	4.5	6.0	33.3	6.6	46.7	6.9	53.3	8.1	80.0	7.2	60.0	7.2	60.0	6.6	46.7	6.6	46.7	6.6	46.7	6.6	46.7	6.6	46.7	6.0	33.3
	5.4	6.9	27.8	6.9	27.8	6.9	27.8	8.1	50.0	6.6	22.2	6.3	16.7	6.3	16.2	6.0	11.1	6.0	11.1	6.0	11.1	6.0	11.1	6.0	11.1
	3.0	3.3	10.0	3.9	30.0	4.2	40.0	4.2	40.0	4.2	40.0	3.6	20.0	3.6	20.0	3.6	20.0	3.6	20.0	3.6	20.0	3.6	20.0	3.9	30.0
Mean	4.7	5.3	13.6	5.7	23.4	6.4	37.7	6.6	43.6	5.8	27.2	5.4	17.8	5.2	13.3	5.2	12.1	5.2	12.3	4.8	5.2	4.7	5.0	4.7	4.6
SEM	0.4	0.4	7.2	0.4	8.6	0.4	9.0	0.6	15.0	0.4	11.1	0.4	10.0	0.4	8.4	0.4	8.7	0.4	8.5	0.4	10.9	0.4	11.3	0.4	11.1
ENP	5.7	6.0	5.3	5.4	-5.3	5.4	-5.3	4.8	-15.8	5.1	-10.5	5.1	-10.5	6.0	5.3	4.8	-15.8	5.4	-5.3	5.7	0.0	5.7	0.0	5.1	-10.5
	8.7	7.8	10.3	7.8	10.3	7.8	10.3	6.9	-20.7	7.2	-17.2	6.9	-20.7	7.2	-17.2	7.2	-17.2	6.0	-20.7	5.4	-31.0	4.8	-44.8	4.8	-44.8
	6.6	12.0	81.8	9.0	36.4	8.0	21.2	8.4	27.3	7.5	13.6	5.4	-18.2	6.0	-9.1	8.4	27.2	6.6	0.0	6.0	-9.1	6.3	-4.6	6.3	-4.6
	7.8	7.5	-3.9	6.0	-23.1	7.2	-7.7	7.8	0.0	8.4	-7.7	7.8	0.0	6.6	15.4	7.2	-7.7	7.2	-7.7	6.3	-19.2	7.5	-3.9	6.6	-15.4
	6.6	8.1	27.7	7.8	18.2	9.2	9.1	8.1	22.7	7.2	9.1	7.8	18.2	8.4	27.3	9.8	18.2	8.7	31.8	8.1	22.7	7.8	18.2	8.4	27.3
	6.0	7.2	20.0	6.3	5.0	6.3	5.0	6.3	5.0	6.6	10.0	8.1	35.0	7.5	25.0	8.4	40.0	8.1	35.0	8.4	40.0	8.7	45.0	7.5	25.0
	5.7	5.1	10.5	6.0	5.3	6.0	5.3	6.3	10.5	6.0	5.3	6.6	15.8	6.6	15.8	5.1	-10.5	5.4	-5.3	6.6	15.8	6.6	15.8	6.3	10.5
	6.0	7.2	20.0	7.3	20.0	7.8	30.0	9.0	50.0	8.4	40.0	7.8	30.0	7.5	25.0	6.0	0.0	8.1	35.0	7.2	20.0	7.2	20.0	7.8	30.0
Mean	6.6	7.6	18.2	6.9	8.4	7.0	8.5	7.2	9.9	7.0	5.3	6.9	6.2	7.0	10.9	6.9	4.3	6.9	7.9	6.7	4.9	6.8	5.7	6.6	4.8
SEM	0.4	0.7	10.0	0.4	6.3	0.3	4.4	0.5	5.3	0.4	6.3	0.4	7.6	0.3	5.9	0.5	7.6	0.5	7.9	4.0	8.4	0.4	9.2	0.4	9.0

Appendix III

Sample computation of simple main effects (Winer, 1962):

Variation due to treatments at 20 min after injection.

$$SS_{A \text{ at } 20 \text{ min}} = (\text{Sum of 20 min NaCl})^2 + (\text{Sum of 20 min AM})^2 + (\text{Sum of 20 min ENP})^2$$
$$= -85.49^2 + 267.75^2 + 2.56^2$$
$$= 9875.6447 - 1423.2680$$
$$= 8452.3767$$

$$MS_{A \text{ at } 20 \text{ min}} = \frac{SS_{A \text{ at } 20 \text{ min}}}{r-1}$$
$$= \frac{8452.3767}{2}$$
$$= 4226.1884$$

$$SS_{\text{within cell}} = SS_{\text{error A}} + SS_{\text{error}}$$
$$= 72660.75 + 21897.46$$
$$= 94558.21$$

$$MS_{\text{within cell}} = \frac{SS_{w \text{ cell}}}{rc(n-1)}$$
$$= \frac{94558.21}{252}$$
$$= 375.2310$$

$$F = \frac{MS_{A \text{ at } 20 \text{ min}}}{MS_{\text{within cell}}}$$
$$= \frac{4226.1884}{375.2310}$$
$$= 11.2629$$

Compared to tabulated $F_{.01\ (2,\ 252)} = 4.8$

Conclusion: There is a significant difference between treatments at 20 min ($p < 0.01$)

Sample computation of Tukey's Multicomparison Test (Steel and Torrie, 1980):

Comparison of treatment means at 20 min

$$\text{Tukey's } w_{0.01} = q_{0.01\ (3,\ 252)} \times S_{\bar{Y}}$$

$$S_{\bar{Y}} = \frac{MS_{\text{within cell}}}{n}$$
$$= \frac{375.2310}{8}$$
$$= 6.8486$$

$$\text{Tukey's } w_{0.01} = 4.15 \times 6.8486 = 28.4217$$

$$\text{Tukey's } w_{0.05} = 3.33 \times 6.8486 = 22.8058$$

Differences between treatment means at 20 min:
SAL - AM = 33.4688 - (-10.6825) = 44.1513
SAL - ENP = 0.3200 - (-10.6825) = 11.0025

Conclusion: Aminophylline V_E at 20 min is significantly greater than saline ($p < 0.01$).
Enprofylline V_E is not significantly different from saline ($p > 0.05$).