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THE EFFECTS OF EXERCISE ON PHARMACOKINETICS AND PHARMACODYNAMICS OF PHYSOSTIGMINE IN RATS

FINAL REPORT

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This report deals with the effect of acute and trained exercise on pharmacodynamics (cholinesterase activity), pharmacokinetics and biochemical parameters in the rat after physostigmine (Phy) administration. Dose-response studies of Phy and ChE inhibition *in vivo* indicated that a dose of 70 μg/kg of Phy inhibited about 30% ChE activity in RBC. This dose was used throughout these exercise studies in consultation with USAMRDC. The oxygen consumption, respiratory exchange ratio (RER) and heat production showed a positive linear relationship in young and adult rats at different levels of exercise. However, significant differences were found in oxygen consumption and caloric expenditure in young vs. adult rats. Different levels of acute exercise affect the ChE activity in RBC and heart and alter the Phy-induced ChE activity in RBC and brain. Acute exercise (80% $VO_{2\ max}$ and Phy) decreased the half-time ($T_{½}$) of CnE recovery in RBC, brain and diaphragm whereas endurance training and Phy increased the $T_{½}$ in brain, diaphragm and heart compared to Phy alone. Phy followed by

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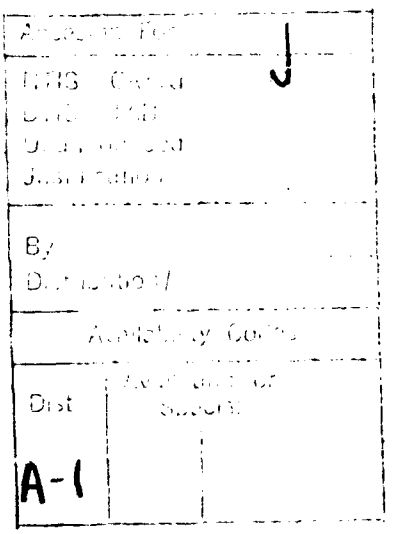
19. concurrent acute exercise increased the $T_{\frac{1}{2}}$ of ChE recovery in RBC but decreased in muscle and brain compared to acute exercise followed by Phy. The effect of endurance training on Phy pharmacokinetics resulted in the disappearance of absorption phase, C_{max} and T_{max} and an increase in AUC and $t_{\frac{1}{2}}$ and decrease in clearance. Phy caused metabolic stress in control rats by elevating the plasma lactate and pyruvate levels. Acute exercise and Phy showed a temporary metabolic stress. Endurance training reduced the metabolic stress of acute exercise as well as of Phy. Subacute Phy and/or trained exercise depress ChAT and/or AChE activities in brain regions differently and inconsistently, depending on the level of stress. The studies in this report would help in the development of a potential pretreatment agent and therapy regimen for the soldiers who need to maintain energetic fitness in the battlefield.



SUMMARY

Intense fitness is essential on the battlefield. Therefore, the effect of physical exercise (acute post, acute concurrent, trained post, or trained concurrent) on physostigmine (Phy)-induced cholinesterase (ChE) activity and biochemical parameters has been examined. The effect of subacute administration of Phy and trained exercise on choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) activities in brain regions were studied in order to determine the role of the neurotransmitter acetylcholine (ACh) in stressed conditions. The effect of trained exercise on pharmacokinetics of Phy was also examined. This study may be useful in the development of potential pretreatment agents and therapy regimen during exercise conditions. Pretreatment agents might also influence work performance. This summary has been written under separate headings, in order to point out the distinct studies, and reviews the research findings of the entire contract effort.

Dose-Response Studies of Phy: It was essential to conduct dose-response studies of Phy in order to determine the dose which would produce 30% ChE inhibition in red blood cells (RBC); this information was needed to carry out all the exercise experiments with this dose. This was done in consultation with the Commander at USAMRDC. Dose response of Phy was studied in rat using various doses (25-500 µg/kg, i.m.). Rats were sacrificed 15 min after Phy administration. RBC and tissues were analyzed for ChE activity by the radiometric method and for Phy concentration by high-performance liquid chromatography (HPLC). A comparison of ChE values in different tissues of rat indicated that ChE activity was highest in brain (7.11 mol/min/g) and lowest in diaphragm (0.67 mol/min/g). The enzyme activity was 11 times higher in brain than in diaphragm. From 50 to 200 µg/kg dose, Phy produced a dose-dependent inhibition of ChE in RBC (18-42%), brain (23-55%) and diaphragm (25-35%); then ChE inhibition plateaued at 200-500 µg/kg dose of Phy in these tissues. A dose-related ChE inhibition was seen in heart (16-50%) and thigh muscle (8-53%) at 50-500 µg/kg dose. Phy concentration increased linearly at 50-400 µg/kg dose in plasma, brain, heart, and thigh muscle. These results indicated that ChE inhibition was linear up to 200 µg/kg dose in RBC, up to 150 µg/kg dose in brain and up to 300 µg/kg dose in heart. This linearity was not consistent in other tissues.

Oxygen Consumption: As a first step in this exercise study, the oxygen consumption VO_{2max}, respiratory exchange ratio (RER) and heat production were compared in young and adult rats at different exercise levels. Open-circuit indirect calorimetry was used to determine metabolic variables in young (147 ± 2 g aged one month) and adult (332 ± 7 g aged four months) rats undergoing identical acute exercise regimens on a motor-driven treadmill with a computerized Oxyscan System. The animals were run within enclosed chambers with a positive air flow rate of 3650 ml/min using an incremental exercise protocol in order to compare oxygen consumption (VO_2), RER and heat production. The resting VO_2 was significantly higher in young (40.80 ml/kg/min) than in adult (29.23 ml/kg/min) rats. The young rats attained a higher VO_2 (81.55 ± 1.22) ml/kg/min) than did adult rats (68.97 ± 2.05 ml/kg/min) at a maximal level of exercise ($VO_{2\ max}$). Based on unit mass, the young rats had a higher heat capacity, both at resting (11.84 kcal/kg/hr) and at $VO_{2\ max}$ (24.11 kcal/kg/hr) than did adult rats. Adult rats became dependent on carbohydrates as the primary fuel source at 52% $VO_{2\ max}$ (Stage 1), whereas young rats did not resort to carbohydrate utilization as a

primary fuel source until reaching 87% $VO_{2\ max}$ (Stage 4). At peak exercise, adult animals burned only 15% less kcal/kg/hr than young rats, but adult rats burned 37% more carbohydrates than young animals. These data suggest that as exercise intensity increases, younger rats can more readily maintain a higher level of oxygen consumption accompanied by a more efficient use of fat as an energy source compared to adult rats. There was a positive linear relationship between VO_2, RER, and heat production at different levels of acute exercise in both groups of rats. These data showed that similar responses relative to VO_2, RER, and heat production occurred during acute exercise in young and adult rats, with the only difference between the 2 groups being a greater metabolic response in young than adult rats.

Phy and Intensities of Exercise: Since extreme fitness is required on the battlefield, it was necessary to evaluate how different intensities of exercise would influence Phy-induced ChE activity. If exercise contributes to alterations in ChE activity due to Phy, this in turn would affect work performance in the field. Therefore, the effects of 3 intensities of acute exercise (50%, 80%, and 100% $VO_{2\ max}$), administration of Phy (70 µg/kg, i.m.), and the combined effect of Phy and 3 intensities of acute exercise on ChE activity were investigated in RBC and various tissues. The endurance time in these rats was also determined. The ChE activity in RBC in exercised rats not exposed to Phy was significantly greater than that of unexercised controls (116%, 112%, and 108% of control, $p < 0.05$, at 50%, 80%, and 100% $VO_{2\ max}$, respectively), while in other tissues the ChE activity in general decreased slightly. In unexercised rats given Phy, the ChE activity ranges were 73-79%, 66-68%, 68-74%, 67-81%, and 57-61% of controls from 10 to 30 min in RBC, brain, heart, diaphragm, and thigh muscle, respectively. In exercised rats exposed to Phy, the ChE activity ranges were 54-51%, 58-50%, 77-73%, 71-83%, and 54-58% of controls from 10 to 30 min in RBC, brain, heart, diaphragm, and thigh muscle, respectively. These results suggest that different intensities of acute exercise (50%, 80%, and 100% $VO_{2\ max}$) showed a similar but significant inhibition of ChE activity in heart, without significantly affecting brain and thigh muscle. However, acute exercise produced a slight increase in ChE activity of RBC. Phy decreased ChE activity in RBC and tissues. The combined effect of Phy and acute exercise further decreased ChE activity in RBC and brain, without significantly affecting heart, diaphragm, and thigh muscle. Exercise potentiated the effect of Phy on ChE inhibition in RBC and brain, irrespective of the intensity of exercise. It seems that acute exercise affects ChE activity to a moderate degree in RBC and heart, and modifies the effect of Phy in RBC and brain. Exposure of rats to Phy (70 µg/kg, i.m.) increases endurance time in rats (160-200 g, weight [w]), possibly due to peripheral vasodilatation and lowering of core temperature.

Exercise is associated with production of lactic acid, metabolic acidosis and changes in hemoglobin (Hb), hematocrit, and plasma volume. These biochemical parameters were studied in this protocol. Phy administration followed by acute exercise at 80% and 100% $VO_{2\ max}$ significantly decreased plasma lactate concentration (144% and 179% of control), as compared to exercise alone (180% and 219% of control). These results indicate the interaction of Phy and acute exercise on lactate metabolism. Acute exercise at 80% and 100% $VO_{2\ max}$ increased plasma pyruvate concentration. At 100% $VO_{2\ max}$, Phy and acute exercise decreased plasma pyruvate content as compared to acute exercise alone. Different intensities of acute exercise slightly elevated Hb content. Phy administration

followed by acute exercise increased Hb content, compared to exercise alone. Significant change in hematocrit value was not observed.

Acute or Endurance Trained Exercise: It is likely that acute or endurance trained exercise modifies the effects of reversible ChE inhibitors by altering the time course of ChE activity in RBC and tissues of rat. Therefore the question was addressed whether the pharmacodynamics of Phy (rate of decarbamylation of ChE enzyme) is altered due to acute and/or trained treadmill exercise in RBC and various tissues of rat. The following iteration describes the interactive effects of acute exercise (AE), endurance training (ET), and Phy on time course of ChE activity in RBC and tissues of rat. Male Sprague-Dawley rats were divided into 5 groups (Gr) (each consisting of 4-8 rats): Gr I - sedentary control saline administration; Gr II - acute exercise (80% $VO_{2\ max}$); Gr III - endurance training; Gr IV - ^{3}H-Phy (70 µg/kg, i.m.); Gr V - acute exercise + ^{3}H-Phy; Gr VI - endurance training + ^{3}H-Phy. Rats from Gr III and Gr VI were endurance-trained for 6 weeks (wks) (5 days/wk) at progressively intensive levels on a 9-channel motor-driven treadmill (built in workshop of Southern IL University, School of Medicine). Rats from Gr II, III, V, and VI were given acute bout of exercise and were sacrificed at 2, 5, 10, 15, 30, 45, and 60 min. RBC and tissues (brain, heart, diaphragm, and thigh muscle) were analyzed for ChE activity by radiometric method. The results indicated that the % gain in body weight was similar in sedentary control (SC), as well as the ET Gr. It seems that AE + Phy increased, whereas ET + Phy decreased ChE activity in RBC and various tissues as compared to Phy alone. The % ChE inhibition was plotted on semilog graph to obtain the rate of decarbamylation (K_d min^{-1}) and half-time ($T_{½}$) of enzyme recovery. There was a slight increase (114% of control) in rate of decarbamylation of RBC-ChE in AE + Phy (0.024/min) as compared to Phy alone (0.021/min), with $T_{½}$ of enzyme recovery to be 29 and 33.5 min, respectively. The rate of decarbamylation of brain ChE was significantly increased (181% of control) by AE + Phy (0.025/min) and decreased (66% of control) by ET + Phy (0.009/min), as compared to Phy alone (0.014/min). The $T_{½}$ of recovery of enzyme was 50, 27.5, and 75 min in Phy, AE + Phy and ET + Phy Gr, respectively. The rate of decarbamylation of heart ChE was significantly decreased (44% of control) by ET + Phy (0.008/min), as compared to Phy alone (0.019/min). ET + Phy significantly increased the $T_{½}$ recovery of enzyme (85 min), as compared to Phy alone (37.5 min). The rate of decarbamylation of diaphragm ChE was significantly increased (384% of control) in AE + Phy (0.039/min) and decreased (80% of control) in ET + Phy (0.008/min), as compared to Phy alone (0.01/min). The $T_{½}$ of recovery of enzyme was 67.5, 17.5, and 84 min in Phy, AE + Phy and ET + Phy groups, respectively. The rate of decarbamylation of muscle ChE significantly decreased (67% of control) by AE + Phy (0.008/min), as compared to Phy alone (0.012/min). However, ET + Phy did not affect the rate of decarbamylation. The $T_{½}$ of enzyme recovery was 55, 83.5, and 60 min in Phy, AE + Phy and ET + Phy groups, respectively. These results suggested that AE and ET have opposite effects on the rate of recovery of ChE after Phy administration.

Phy is metabolized to eseroline, a phenolic compound. Phenolic drugs are known to alter mitochondrial function. When considering the potential effect of Phy on mitochondrial function, it is of interest to know what effect this may have on recovery from exercise performance. The level of blood lactate provides a fairly objective indication of the relative anaerobic demand of exercise. The effect of Phy on plasma lactate and pyruvate levels following an AE bout was

examined in untrained and trained rats. The Phy group (IV) elicited significantly higher plasma lactate (11.21 ± 0.75 mM) and pyruvate (0.25 ± 0.06 mM) levels ($p < 0.05$) than the SC group (I) (3.67 ± 0.52 and 0.13 ± 0.02 mM, respectively) at 2 min after injection. At 2 min after exercise, the AE + Phy group (V) had a significantly higher plasma lactate level (7.40 ± 0.72 mM) and pyruvate level (0.28 ± 0.04 mM) compared to the AE group (II) whose lactate and pyruvate levels were 4.18 ± 0.3 and 0.20 ± 0.01, respectively. From 5 to 30 min after exercise, lactate and pyruvate levels did not differ between these 2 acutely exercised groups. During exercise recovery, the ET + Phy group exhibited significantly lower levels of plasma lactate (5.89 ± 1.0 to 4.36 ± 0.29 mM) and pyruvate (0.18 ± 0.06 to 0.09 ± 0.02 mM) from 5 to 60 min after exercise, compared to ET (III) (6.50 ± 0.75 to 5.12 ± 0.61) for plasma lactate, and (0.19 ± 0.04 to 0.09 ± 0.01) for plasma pyruvate. These data show that the "additive" effect of Phy on after exercise plasma lactate and pyruvate levels can be attenuated by an enhanced fitness level in these rats.

The effects of various drugs on exercise in man and animal have been extensively reported, but there are very few reports with regard to the effects of exercise on disposition and pharmacokinetics of drugs. Phy is a flow-limited, poorly plasma-bound and highly extracted drug. We have therefore studied the effect of endurance-trained exercise on pharmacokinetics, as well as on the distribution of radioactivity (RA) in different tissues of rat. Male Sprague-Dawley rats (control) weighing 367 ± 9 g were administered [^{3}H]-Phy (70 µg/81.03 µCi/kg) and were sacrificed at 2, 5, 10, 15, 30, 45, and 60 min. Another group of rats (ET) were endurance-trained for 6 wk on a treadmill and were subjected to an acute bout of exercise (80% $VO_{2\ max}$) on the day of sacrifice. Immediately after exercise, rats weighing 320 ± 5 g were administered [^{3}H]-Phy (70 µg/81.03 µCi/kg) and were sacrificed at the same time points. Four to 6 rats were sacrificed at each time point. The % of weight gain in ET rats was similar to control rats during the training period. Blood, brain, heart, liver, lung, kidney, and muscle were collected. The tissues were oxidized in sample oxidizer, and the total RA was counted in a Beckman LS5800 liquid scintillation counter. Phy was determined in plasma by HPLC, collecting the fractions and counting the RA. The pharmacokinetic parameters in control vs. ET rats were computed using PC-NONLIN program of statistical consultants, and were found to be:

Area under the curve (AUC): (578.8 ± 88.7 vs. 834.2 ± 45.8 ng ml^{-1} min);
Half life ($t_{½}$): (8.8 ± 2.9 vs. 15.7 ± 1.6 min);
Clearance (Cl): (120.9 ± 18.5 vs. 83.9 ± 4.6 ml^{-1} min^{-1}/kg).

In control rats, C_{max} and t_{max} were 31.3 ng ml^{-1} and 4.9 min, respectively. The absorption phase, t_{max} and C_{max}, have disappeared in ET rats, indicating that increased blood flow due to exercise has caused this change compared to control rats. AUC and $t_{½}$ have also increased due to ET. At 2 min, the amount of RA in brain, heart, lung, kidney, liver, and muscle of trained rats showed 337%, 191%, 106%, 385%, 126%, and 80% over control rat, respectively. Kidney, liver, and muscle RA remained higher up to 10 min after exercise, whereas brain and heart RA decreased below control level within 5 min. These results indicate that training altered the distribution of [^{3}H]-Phy in tissues of rat.

Phy and Concurrent Exercise: Whether the rate of decarbamylation (K_d min^{-1}) of ChE in RBC and tissues of Phy-dosed rats is influenced by concurrent acute

exercise (CE) has been examined. Male Sprague-Dawley rats were divided into 4 groups: Gr I - sedentary control; Gr II - acute exercise (80% $VO_{2\ max}$); Gr III - Phy (70 µg/kg, i.m.) to rats sacrificed from 20 to 50 min; Gr IV - Phy + CE. Rats were sacrificed at various time intervals for CE (20-50 min) after Phy administration or from start of exercise. ChE activity in RBC and tissues were determined by radiometric method. The % ChE inhibition was plotted on semilog graph to obtain the K_d (min^{-1}) of ChE. Phy + CE increased the K_d (min^{-1}) compared to Phy alone in RBC (from 0.0165 to 0.0258); brain (from 0.0231 to 0.0385); heart (from 0.0252 to 0.0602), but decreased the K_d (min^{-1}) in diaphragm (from 0.038 to 0.0067) and muscle (from 0.0308 to 0.0135). These studies suggest that Phy followed by CE increased the $T_{½}$ of ChE recovery in RBC, but decreased $T_{½}$ in muscle and brain.

The effect of Phy, AE, and Phy + AE on time course of lactate, pyruvate, and L/P ratio in plasma, muscle, and brain was also studied. Phy administration prior to AE prolonged the conversion of pyruvate to lactate up to 50 min (30 min after exercise). The effect of Phy was more pronounced on muscle than brain during Phy + AE. This may be attributed to increased Phy concentration in muscle due to an increased blood flow in this organ. Phy might be creating an O_2 tension by increasing the formation of lactate after exercise.

Subacute Phy/Trained Exercise and ChAT/AChE in Brain Regions: The changes in pharmacokinetics of Phy due to trained exercise have been shown. However, changes in central neurotransmitter system due to physical exercise and ChE inhibitors (chemical stressors) have not received much attention. Therefore, this study sought to determine whether the biosynthetic (choline acetyltransferase (ChAT) and degradative (acetylcholinesterase [AChE]) enzymes for ACh in brain were affected in a regionally selective manner by the following chemical and physical stressors: (1) subacute administration of Phy, a prototypical ChE inhibitor; (2) subacute exercise; and (3) the combination of these 2 stressors. Male Sprague-Dawley rats (150-175 g) were divided into 5 groups: Gr 1 - sedentary control; Gr II - trained exercise for 2 wk using an incremental exercise program; Gr III - subacute Phy (70 µg/kg i.m., twice daily) for 2 wk; Gr IV - subacute Phy and a single bout of exercise (100% $VO_{2\ max}$); Gr V - subacute Phy + trained exercise. The rats were sacrificed in 20 min or 24 hr after the last dose of Phy and/or exercise. The ChAT activity decreased significantly ($p < 0.05$) in corpus striatum (Gr III, IV, and V) both in 20 min and 24 hr. AChE activity was depressed in all groups, but was significantly depressed in Gr IV up to 24 hr. ChAT activity decreased in cerebral cortex in all groups, but was significantly ($p < 0.05$) decreased in Gr V (79% of control), indicating that only combination of subacute Phy + trained exercise affected this region. Phy alone or exercise alone did not significantly affect ChAT. AChE activities in cerebral cortex decreased significantly in Gr IV (72%) and Gr V (75% of control) in 20 min, but recovered to control levels in all groups within 24 hr. ChAT activity significantly decreased ($p < 0.05$) in brainstem in all groups, at both 20 min and 24 hr. AChE activity in brainstem was depressed in all groups, but significantly ($p < -0.05$) in Gr II (81% of control) and Gr III (61% and 82% of control). AChE in brainstem decreased significantly ($p < 0.05$) in rats sacrificed after 20 min in Gr IV (79%) and Gr V (72% of control). The ChAT activity decreased significantly in hippocampus ($p < 0.05$) to 72% and 73% of control in Gr IV and V, respectively. However, Gr II and Gr III also showed decreased ChAT activity. AChE activity recovered to control level in all groups

by 24 hr. Brain regions involved with control of motor, autonomic, and cognitive functions were affected by subacute Phy and exercise in a regionally selective pattern that appears to depend on the type and interaction of these 2 stressors. These data are consistent with the hypothesis that the responsiveness of these brain regions to these different stressors is a function of the level of ongoing cholinergic transmission and that elevations in ACh levels due to AChE inhibition may have long-term effects on ChAT and AChE activities through a feedback mechanism.

The above studies were expanded to determine whether subacute Phy and trained exercise, or the combination of the two, regulate the biosynthetic and degradative enzymes of ACh in EDL (extensor digitorum longus) - fast muscle and soleus - slow muscle - of rat. Trained exercise (Gr II) decreased ChAT activity significantly ($p < 0.05$) to 68% of control in EDL and increased 124% of control in soleus. Trained exercise decreased ACh activity to 57% and 55% of control ($p < 0.05$) in both muscles at 20 min and remained depressed at 68% and 67% of control, even after 24 hr. Subacute Phy (Gr III) decreased ChAT activity to 89% and 94% of control in EDL and soleus at 20 min, but increased to 110% and 116% of control by 24 hr. In this Gr (III), AChE activity decreased to 56% and 57% of control in EDL and soleus at 20 min, and remained depressed to 68% and 67% of control, even after 24 hrs. Subacute Phy + single acute exercise decreased AChE activity significantly in EDL and soleus to 63% and 32% of control at 20 min; these ACh values recovered to 88% and 50% of control in soleus at 24 hr. Subacute Phy + trained exercise (Gr V) decreased ChAT activity significantly to 65% of control in EDL and remained depressed after 24 hr. ChAT activity in soleus did not change. AChE activity decreased significantly to 42% and 29% of control in EDL and soleus at 20 min, respectively. AChE activity in this group remained depressed (62% and 65% of control) in EDL and soleus muscle, even after 24 hr, respectively. These results showed constant decrease in AChE activity in both muscles in all groups at 20 min, which did not recover, even after 24 hr. ChAT activity showed a transient decrease at 20 min, in both muscles, and recovered to control level by 24 hr, except in the subacute Phy + trained exercise group. Phy + concurrent exercise seems to have a transient effect on ChAT but has a profound influence on AChE activity. Subacute Phy + trained exercise seems to regulate AChE, but not ChAT.

Endurance Training and Chronic Phy Infusion: Rats were endurance-trained for 5.5 wk, then a continuous infusion osmotic pump was inserted either with saline or Phy (34.5 µg/kg/hr). These rats were exercised daily and sacrificed on various days up to the 13th day to observe the steady-state level of ChE activity in RBC and also to compare the endurance time of rats between these 2 groups. It seems that Phy-induced ChE inhibition in RBC decreased due to endurance training, compared to Phy alone. Endurance time decreased by 13% in Phy + ET rat, compared to saline-administered + endurance-trained rats. The amount of physical activity decreased and the sleep increased due to stress and fatigue in endurance-trained rats, but more so in Phy-dosed + endurance-trained rats.

In conclusion, exercise modifies the ChE activity in the presence of ChE inhibitors and also alters the pharmacokinetics. Therefore, these studies would be useful in the development of an appropriate therapy regimen and pretreatment agent against organophosphate intoxication.

FOREWORD

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INTRODUCTION

Physostigmine (Phy), a centrally acting anticholinesterase drug, is considered to be a potential pretreatment drug for protection against organofluorophosphate agents (7,9,15,16,155,156,147,158). The rates of its absorption, distribution, metabolism, and excretion are most important in determining the protective action of Phy, as is its ability to inhibit cholinesterase (ChE) activity (1,2,3). Recently Somani et al. (61) have reviewed the effects of exercise on disposition and pharmacokinetics of drugs. Any alterations in the metabolism and pharmacokinetics of Phy due to exercise would also influence the pharmacodynamic effect of Phy, i.e., ChE activity, in RBC and different tissues. Phy, a flow-limited, poorly plasma-bound and highly extracted drug, is more likely to be affected by exercise. The pharmacodynamics of Phy (ChE activity) are likely to be altered by exercise due to altered blood flow rates to liver and pH of muscle. Physical exercise has broad effects on the body; it can evoke a number of enzymatic changes in muscles and liver (37,38,39). The changes in enzymatic activity are directly related to the intensity of physical exercise (40). Serum ChE activity has been reported to be increased after exercise (41,42). McMaster and Foster (43) have demonstrated that acute exercise increases behavioral sensitivity to Phy. The combined effects of physical exercise and chemical stressor, such as Phy, on the cholinergic system have not received attention. There seems to be an interaction between Phy, exercise and the neurotransmitter acetylcholine (ACh). This interaction can be monitored by one of the important biochemical markers of the cholinergic system, degradative enzyme of ACh, ChE.

Physical training improves efficiency when an individual subsequently engages in physical exertion (159-162). This is the end to which much physical preparation for military personnel is directed during basic and supplemental training (163-165). This notwithstanding, physiological stress is still to be expected, causing a redistribution of blood flow to serve the demands of active muscle cells (166,167), as well as to meet the needs of temperature regulation. This is especially true during prolonged submaximal (155,168,169) exercise. In addition, a considerable production of metabolic acidosis from substrate catabolism will lead to a marked reduction of intracellular pH (170-172). Exercise is associated with the production of lactic acid (173), metabolic acidosis (174), and changes in hemoglobin (Hb), hematocrit, and plasma volume (46,175,176). Since the time course of a drug may be influenced by exercise dynamics (177-178), it is important to know how exercise interacts with a drug which would potentially be administered under combat field conditions. This information would be of special consequence for a drug such as Phy, a tertiary amine, administered as a pretreatment drug in chemical defense (179). It is the intent of this research to simulate military conditions, using an animal model.

The rat as an animal model has been previously used to study the activity of neurotransmitters during exercise (180,181), as well as under other stressful conditions (182,183,184). The effects of drugs combined with other stressors on exercise performance have also been studied using the rat as the experimental animal (185,186). In a more recent study, Francesconi et al. (187) studied the effects of Phy on the ability of rats to work in the heat. In this study, the drug was found to reduce endurance time (ET) and to increase rectal and skin temperatures.

The fact that intense fitness is required in the battlefield and the question of how the different intensities of physical exercise and single acute exercise, trained exercise and concurrent exercise would influence pharmacokinetics of Phy-induced ChE activity and biochemical parameters need to be considered during the development of a potential pretreatment agent and therapy regimen. If exercise contributes to alterations in ChE activity due to Phy, this in turn would affect work performance in the field. We have attempted to answer some of these questions in this report. This report has been written under distinct headings, and a detailed introduction is given under each heading.

It was essential to determine the dose response of Phy in order to find out the dose required to inhibit about 30% ChE inhibition in red blood cells (RBC). It seems that 10-40% inhibition of acetylcholinesterase (AChE) by carbamates in RBC is a good measure of protective effects of carbamates against organophosphate intoxication. It was necessary to obtain information on significant differences in oxygen consumption and caloric expenditure in relation to the ages of rats and also to compare oxygen consumption, respiratory exchange ratio (RER) and heat production in young and adult rats at different exercise intensities. The rate of decarbamylation would indicate the extent of regeneration of ChE enzyme. Exercise is likely to influence this rate of decarbamylation; therefore, this study was undertaken to elicit the effects of acute postexercise, trained postexercise, and concurrent exercise on the regeneration of carbamate-inhibited ChE enzymes in RBC, brain, muscle, diaphragm, and heart.

Phy is a potential pretreatment drug which readily crosses the blood-brain barrier and enters the brain. The equilibration by brain and plasma seems to be immediately after i.v. injection of Phy, because at 2 min plasma concentration is much lower than the brain concentration. Phy gives rise to eseroline as a metabolite. The phenolic metabolite might disturb mitochondrial function because phenolic drugs are known to disturb the mitochondrial enzyme activities. Exercise and blood lactate concentration provide a good indication of strenuous work performance or exercise. Therefore, it would be of interest to know what potential effect Phy has on mitochondrial function during recovery from exercise performance and also during exercise.

Alterations in ChE activity in RBC, brain and muscle occur due to Phy administration, as well as to exercise and the combination of these 2 stressors. Choline acetyltransferase (ChAT) activity changes due to Phy and exercise, and whether there is a relationship between this biosynthetic enzyme ChAT and the degradative enzyme AChE due to treatment with Phy and exercise in different brain regions of rat needs to be answered. To answer these questions we have reported that the adaptive changes occur in these 2 enzymes due to exercise, and these changes are differentially expressed in different regions of the brain.

This report will provide the reader an understanding of the effects of acute postexercise, trained postexercise, concurrent exercise on: (1) time course of ChE activity in RBC, brain, muscle, diaphragm, and heart; (2) time course of biochemical parameters such as pyruvate and lactate in plasma and muscle; and (3) pharmacokinetics of Phy. This report will also provide information on: (4) kinetics of dose response of Phy *in vivo*; (5) comparison of oxygen consumption rate in young and adult rats at different intensities of exercise; and (63) effects of trained exercise and Phy on ChAT and AChE activities in different brain regions.

I. IN VIVO DOSE-RESPONSE RELATIONSHIP BETWEEN PHYSOSTIGMINE AND CHOLINESTERASE ACTIVITY IN RBC AND TISSUES OF RAT

Introduction

The disposition of Phy in rat after i.m., i.v., and oral administration was studied by Somani and Khalique (1,2) and Somani (3). These investigators have also determined the time course of ChE inhibition in plasma, brain and muscle and correlated with Phy concentration after i.m., i.v., and oral routes of administration. ChE inhibition in 6 different rat brain regions was also shown after i.m. and i.v. administration of Phy (4,5). ChE activity is an important parameter to monitor the efficacy of Phy. ChE inhibition in blood has been studied in mice (6), rabbit (7,8), rat (8,9,10), and guinea pig (8). These authors have estimated only the whole blood ChE enzyme for determining the dose response of Phy. There is a paucity of data on ChE inhibition in different tissues and any relationship between ChE inhibition and Phy concentration. The present study examines the effects of different doses of Phy on ChE activity in RBC, brain, heart, diaphragm, and thigh muscle and correlates Phy concentration in these tissues to the ChE activity.

Materials and Methods

Phy free base was obtained from Sigma Chemical Co. (St. Louis, MO). [^{3}H]-Phy (13 Ci/mmol) was custom-synthesized by Amersham Corp. (Chicago, IL). Methanol (HPLC grade) was obtained from Burdick and Jackson Laboratories, Inc. (Muskegan, MI). Ready-Solv EP was obtained from Beckman Instruments, Inc. (Fullerton, CA) and Monophase-40 plus was from Packard Instrument Co. (Downers Grove, IL). All other chemicals were analytical-grade and were obtained from the usual commercial sources.

Preparation of [^{3}H]-Phy Solution: Phy solution was prepared in 0.9% (w/v) saline to which 10 µl of hydrochloric acid was added to keep the Phy solution acidic (pH 3.5). Phy appears to be more stable in acidic solution. [^{3}H]-Phy (200 µCi/100 µl of 13 Ci/mmol) was added to unlabeled Phy (free base) to obtain the final concentration of 50, 200, 300, and 400 µg/ml. Radioactivity in each dose was determined by liquid scintillation counter and was found to be 50 µg/ml/84.4 µCi, 200 µg/ml/149 µCi, 300 µg/ml/123.5 µCi, and 400 µg/ml/145.5 µCi.

Dosing and Sacrifice: Male Sprague-Dawley rats (Harlan Industries, Indianapolis, IN) weighing 175-250 g were used. A group of 6-8 rats was administered various doses of Phy (25 µg/kg to 500 µg/kg) intramuscularly in the right thigh (dorsal surface). Control rats (10-12) were given saline. The animals were sacrificed by decapitation at 15 min after dosing. Blood was collected in heparinized tubes. Brain, diaphragm, heart and thigh muscle (left side) were removed, rinsed with ice-cold saline, and blotted dry. All samples were stored at -70°C until analysis.

Preparation of Blood Samples: Blood plasma was separated by centrifugation at 2000 rpm for 10 min at 4°C in Sorvall RC-5 centrifuge (DuPont Instruments Co.) and stored at -70°C until analysis. Immediately after collecting the blood, the erythrocytes were separated by pouring blood (0.5 ml) slowly drop by drop in a 15 ml glass tube containing 10 ml ice-cold saline, centrifuging at 2000 rpm for

10 min at 4°C and aspirating off the supernatant as much as possible. The suspension of erythrocytes was prepared by adding 2 ml of 0.1 M phosphate buffer (containing 1% Triton X-100 and 0.9% NaCl) and shaking the contents on vortex mixer for 15 sec.

Preparation of Tissue Homogenates: Tissues were weighed, minced and homogenized in buffer (0.1 M phosphate buffer, pH 7.2, 1% Triton X-100, 0.9% NaCl) using Polytron homogenizer (Switzerland), cooling the tubes as needed. A homogenate of 10% for brain, diaphragm, and thigh muscle and 5% for heart was prepared. The homogenates were centrifuged at 10,000 rpm for 10 min at 4°C. The clear supernatant was used for ChE determination.

Determination of the ChE Activity: The ChE enzyme estimation was carried out according to a modification of the radiometric method of Johnson and Russell (11). In this procedure, [^{3}H]-ACh was used as the substrate. This method measured the radioactivity (RA) due to [^{3}H]-acetate formed by the enzymatic hydrolysis of [^{3}H]-ACh. The substrate was prepared daily fresh by mixing 0.5 M Tris buffer (0.25 M Trizma base, 0.25 M Tris-HCl, 1.2 M NaCl, pH 7.4), AChCl (0.1 mmol for RBC, diaphragm, heart, and thigh; 1 mmol for brain) and [^{3}H]-AChI (1 mCi/0.0100 mmol).

Fifty-µl aliquots of RBC suspension and 50 µl of phosphate buffer were put in glass scintillation vials, and then 50 µl of freshly prepared [^{3}H]-ACh solution (2 µmol/1 µCi) was quickly added to each vial. The total volume of reaction mixture was 150 µl. For diaphragm, heart, and thigh muscle, an aliquot of 50 µl of homogenate and 30 µl phosphate buffer were taken and then added 20 µl of [^{3}H]-ACh solution (2 µmol/1 µCi), the total volume of reaction mixture being 100 µl. Brain ChE activity was determined by taking 50 µl of brain homogenate, 50 µl of [^{3}H]-ACh solution (1 µmol/1 µCi) and 50 µl of phosphate buffer, the total volume of reaction mixture was 150 µl. All the estimations were carried out in triplicate. Blanks representing the nonenzymatic hydrolysis of ACh were also prepared in triplicate with each sample run and subtracted off as background. The reaction mixture was incubated at 37°C for 15 min in Thermolyn waterbath (Syborn Corp., MI), the contents were immediately cooled on ice and stop solution (100 µl) was added to check the enzymatic hydrolysis. Then 4 ml of toluene scintillation cocktail (0.51% PPO, 0.03% POPOP and 10% isoamyl alcohol) was added to each sample. Ready-Solv (Beckman, Fullerton, CA) was used for measuring specific activity of the substrate. The samples were mixed on a vortex and the RA was counted in a Beckman LS 5800 Liquid Scintillation spectrometer.

The AChE values of RBC are expressed as µmol of ACh hydrolyzed/min/g of Hb content, whereas the tissue ChE values are expressed as µmol of ACh hydrolyzed/min/g of wet weight of tissue.

Determination of Hemoglobin: The Hb content of blood was determined by Sigma diagnostic kit using a Beckman Spectrophotometer at 540 nm.

Determination of Phy in Plasma and Tissues: Phy in plasma, brain, heart, and thigh muscle was determined by HPLC following the method of Somani and Khalique (1,12).

Statistical Analysis: ChE activity and Phy concentration were analyzed using Student's t-test to find out the significant differences in the individual experiments. A significance level of $p < 0.05$ was used.

Results

A comparison of the ChE values in different tissues in rats indicates that the ChE enzyme activity was highest in brain and least in diaphragm (Table 1). The enzyme activity was 11 times more in brain as compared to diaphragm. The ChE enzyme was found to be in the decreasing order of magnitude in different tissues as brain > heart > thigh > diaphragm. Phy concentration in plasma, brain, muscle and heart was determined by high-performance liquid chromatography (HPLC) and radiometric method. Brain concentrations were highest followed by plasma, muscle and heart for all dosages.

Table 1: Effect of various doses of physostigmine on cholinesterase activity in RBC and tissues of rats.

IM Phy Dose (µg/kg)	RBC ChE µmole/min/g of Hb	Tissue ChE µmole/min/g			
		BRAIN	HEART	DIAPHRAGM	THIGH MUSCLE
-	2.103±0.141	7.110±0.256	1.706±0.134	0.672±0.045	0.820±0.036
25	1.944±0.121	7.248±0.212	1.986±0.070	0.550±0.018	0.790±0.037
50	1.716±0.084[a]	5.489±0.369[b]	1.434±0.136	0.507±0.036[b]	0.751±0.029
100	1.451±0.048[c]	2.758±0.276[c]	1.238±0.109[a]	0.387±0.021[c]	0.495±0.042[c]
150	1.324±0.039[c]	2.401±0.248[c]	1.182±0.031[b]	0.402±0.017[c]	0.478±0.023[c]
200	1.223±0.075[c]	3.209±0.114[c,e]	1.045±0.037[c]	0.435±0.032[c]	0.475±0.033[c]
300	1.248±0.069[c]	3.619±0.120[c,d]	0.908±0.040[c]	0.468±0.050[b]	0.459±0.025[c]
400	1.374±0.103[c]	3.597±0.191[c]	0.875±0.040[c]	0.418±0.018[c]	0.469±0.034[c]
500	1.341±0.117[c]	3.718±0.149[c]	0.853±0.118[c]	0.374±0.016[c]	0.387±0.033[c]

ChE activity determined 15 min after Phy administration
[a]=$p < 0.05$; [b]=$p < 0.01$; [c]=$p < 0.001$, as compared to control group.
[d]=$p < 0.05$, as compared to 200 µg/kg of Phy.
[e]=$p < 0.01$, as compared to 150 µg/kg of Phy.

RBC: Phy produced a dose-dependent reduction of RBC-ChE activity in the dosages from 25 to 200 µg/kg (Fig. 1). The ChE inhibition produced was to the extent of 8%, 18%, 31%, 37% and 42% with 25, 50, 100, 150 and 200 µg/kg of Phy, respectively. However, the inhibition with 25 µg/kg was statistically insignificant as compared to control values. The percentage inhibition was plateaued at higher dosages of Phy (300, 400 and 500 µg/kg). The ChE inhibition was found to be 39%, 35% and 36% with 300, 400 and 500 µg/kg of Phy, respective-

ly. At these higher doses (300-500 µg/kg) ChE inhibition did not increase with increasing dose and the average level of inhibition was less than the maximal inhibition observed at a relatively lower dose (200 µg kg). Concentration of Phy in the plasma, on the other hand, showed a linear increase with increase in dose; the plasma concentrations of Phy at 50, 200, 300, and 400 µg/kg dose were 12.69, 47.02, 70.74, and 105.84 ng/ml, respectively.

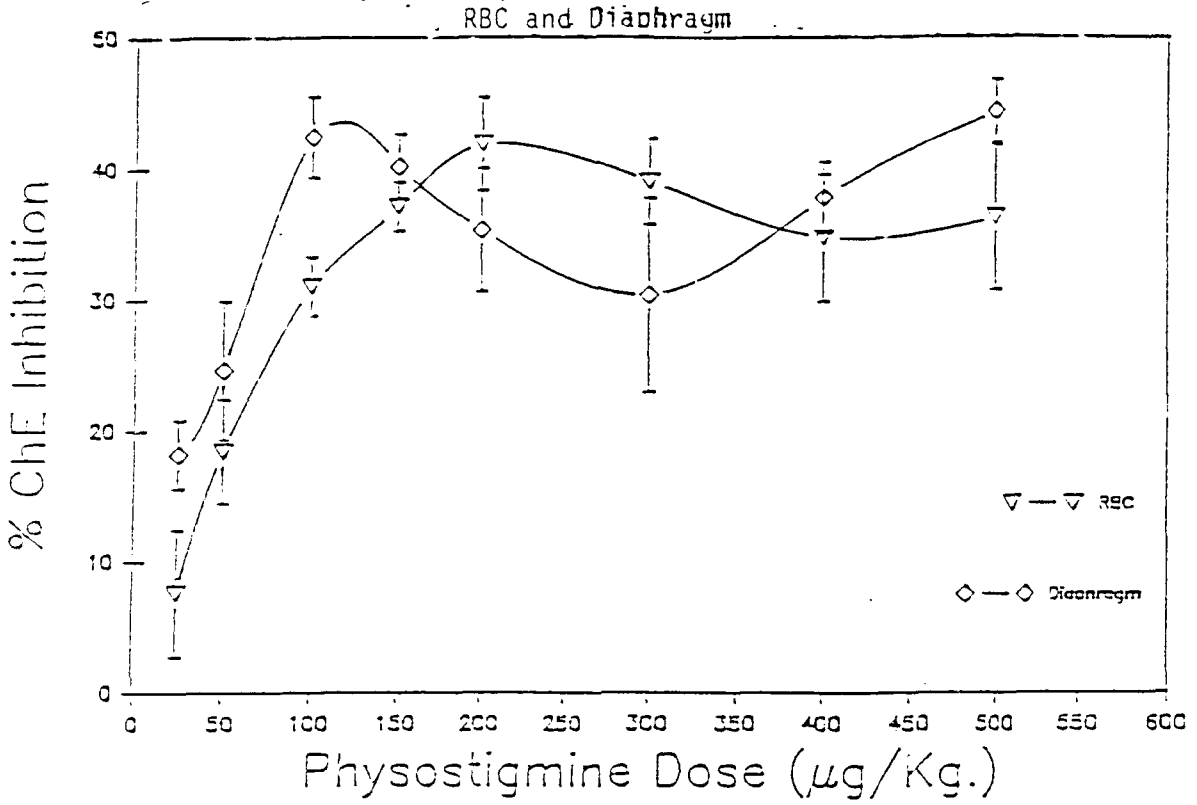


Fig. 1. Effect of i.m. administration of physostigmine (25-500 µg/kg) on % ChE inhibition in RBC and diaphragm of rats. Each point is the mean ± S.E.M. of 6-8 rats.

Diaphragm: Phy produced a dose-dependent inhibition of diaphragm ChE from 25 to 100 µg/kg, the inhibition being 18%, 25%, and 42% with 25, 50, and 100 µg/kg of Phy (Fig. 1). However, an increase in dose from 150 to 400 µg/kg of Phy produced a slight decrease in % ChE inhibition, as compared with 100 µg/kg. This decrease appeared to be statistically insignificant. Phy in dose of 500 µg/kg produced increased ChE inhibition (44%), which was similar to 100 µg/kg dose. It is difficult to explain these swings in ChE inhibition with different dosages.

Brain: A dose of 25 µg/kg of Phy did not produce any significant change in ChE activity of brain. A dose-dependent inhibition of ChE activity was observed with 50 to 150 µg/kg of Phy (Fig. 2). The percentage ChE inhibition was 23%, 61%, and 66% with 50, 100, and 150 µg/kg of Phy, respectively. Higher dosages of Phy did not increase the % inhibition of ChE; however, a decrease in % ChE inhibition was observed with increase in dosage (Fig. 2). The % ChE inhibition was 55%, 49%, 50%, and 48% with 200, 300, 400, and 500 µg/kg of Phy,

respectively. Even though there was constant increase in Phy concentration with increase in dose (Fig. 2), ChE inhibition plateaued between 200 to 500 µg/kg.

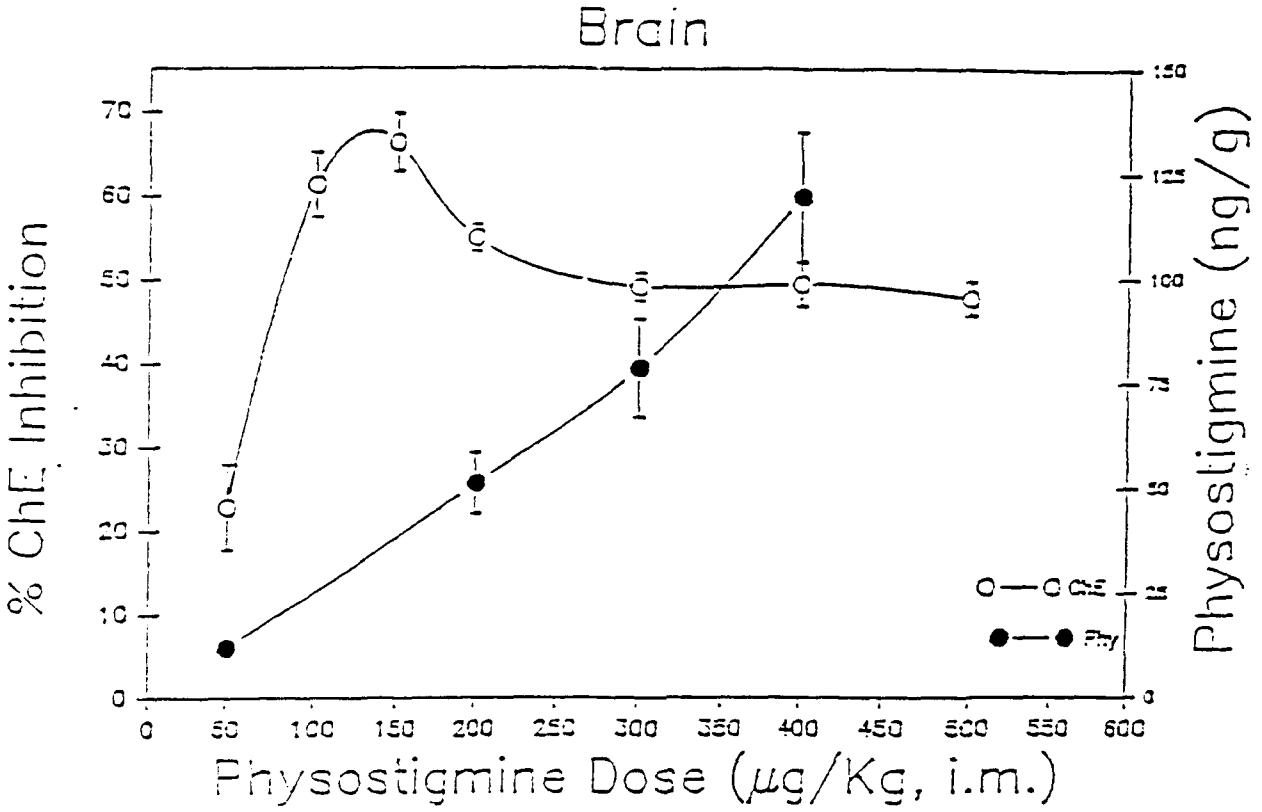


Fig. 2: Relation between physostigmine concentration (●——●) and % ChE inhibition (○——○) in brain of rats administered 25-500 µg/kg of physostigmine i.m. Each point is the mean ± S.E.M. of 6-8 rats.

Heart: Phy in a dose of 25 µg/kg produced a slight increase (16%) in heart ChE activity, which was statistically insignificant. Phy produced a dose-related inhibition of heart ChE, the inhibition was 16%, 27%, 31%, 38%, 47%, 49%, and 50% with 50, 100, 150, 200, 300, 400, and 500 µg/kg of Phy, respectively (Fig. 3). Even though there was constant increase in Phy concentration with increase in dose (Fig. 3), ChE inhibition plateaued between 200 and 500 µg/kg.

Thigh Muscle: Thigh muscle ChE inhibition was 4%, 9%, 40%, 42%, 42%, 44%, 43% and 53% with 25, 50, 100, 150, 200, 300, 400 and 500 µg/kg Phy. However, the inhibition produced with 25 and 50 µg/kg of Phy was statistically insignificant. The inhibition of ChE in muscle seems to be similar from 40% to 53% from 100 µg/kg to 500 µg/kg dose (Fig. 4). In spite of the constant increase in the concentration of Phy with increase in the dose (Fig. 4), the ChE inhibition remained plateaued at 150 to 400 µg/kg and again increased at 500 µg/kg.

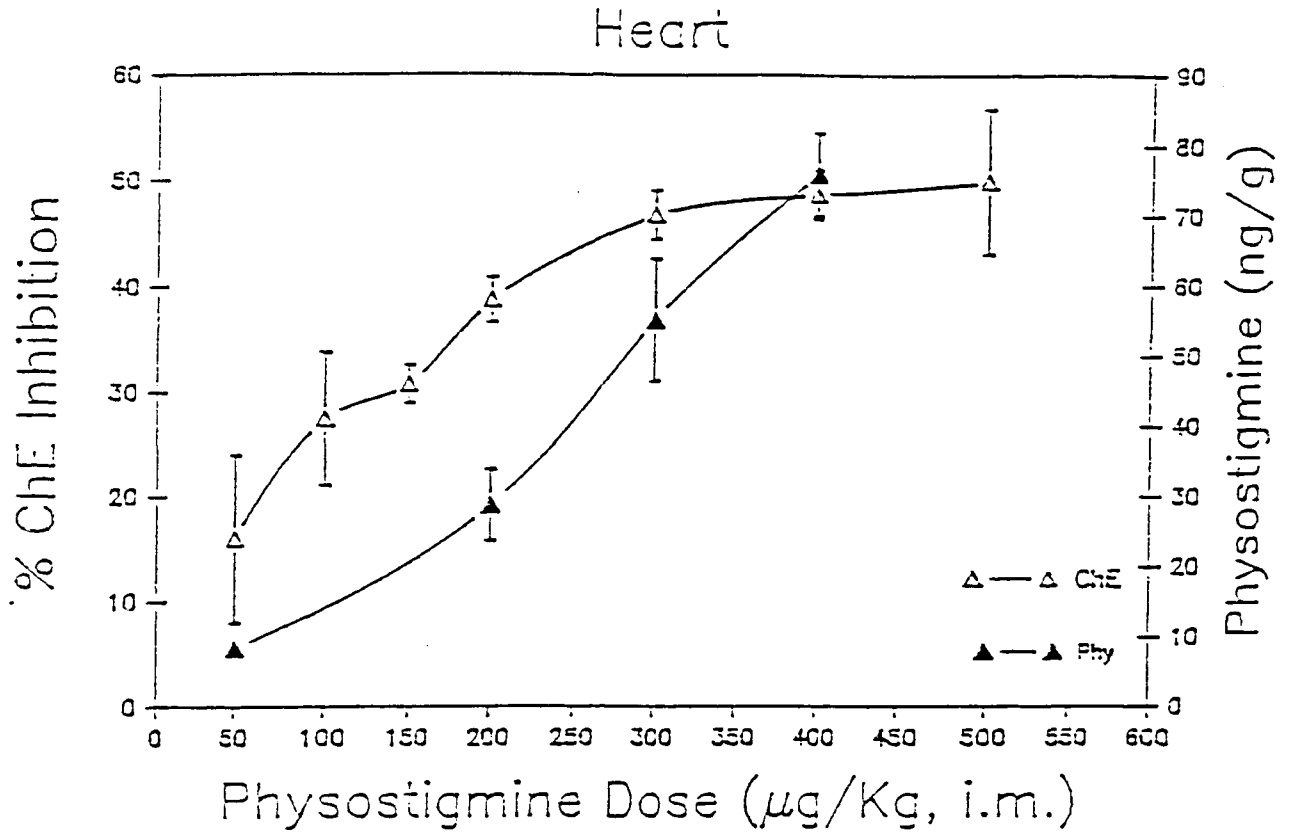


Figure 3: Relation between physostigmine concentration (▲——▲) and % ChE inhibition (△——△) in heart of rats administered with 25-500 µg/kg of physostigmine i.m. Each point is the mean ± S.E.M. of 6-8 rats.

Discussion

ChE inhibition is an important parameter in monitoring the efficacy of Phy in organophosphate intoxication or in Alzheimer patients. Phy has a narrow therapeutic range and the toxic dose of Phy appears to be very close to its optimum therapeutic dose of 0.25 - 1 mg i.v. infusion for 30 min (13), or 0.125 - 0.5 mg i.v. infusion for 30 min (14). A slight increase in dose of Phy can lead to adverse effects such as nausea, vomiting, hypersalivation and diarrhea. An excessive dose of Phy may produce severe toxic cholinergic manifestations leading to central and peripheral respiratory paralysis.

There appears to be a controversy regarding the use of an appropriate dose and route of administration of Phy. Therefore, a dose response curve of Phy for ChE inhibition would be useful in designing the optimum dose and route of administration. Our results clearly show that Phy produced a dose-dependent inhibition of RBC ChE from 25 to 200 µg/kg doses. However, with the higher doses the percentage inhibition was plateaued, indicating the optimal dose of Phy for ChE inhibition in RBC to be about 150-200 µg/kg in rat at 15 min after its i.m. administration. Phy concentration at this dose in plasma was 42.05 ng/ml.

However, recently Maxwell et al. (8) showed 75% ChE inhibition in whole blood with 200 µg/kg of Phy after i.m. administration at 25 min. Harris et al. (9)

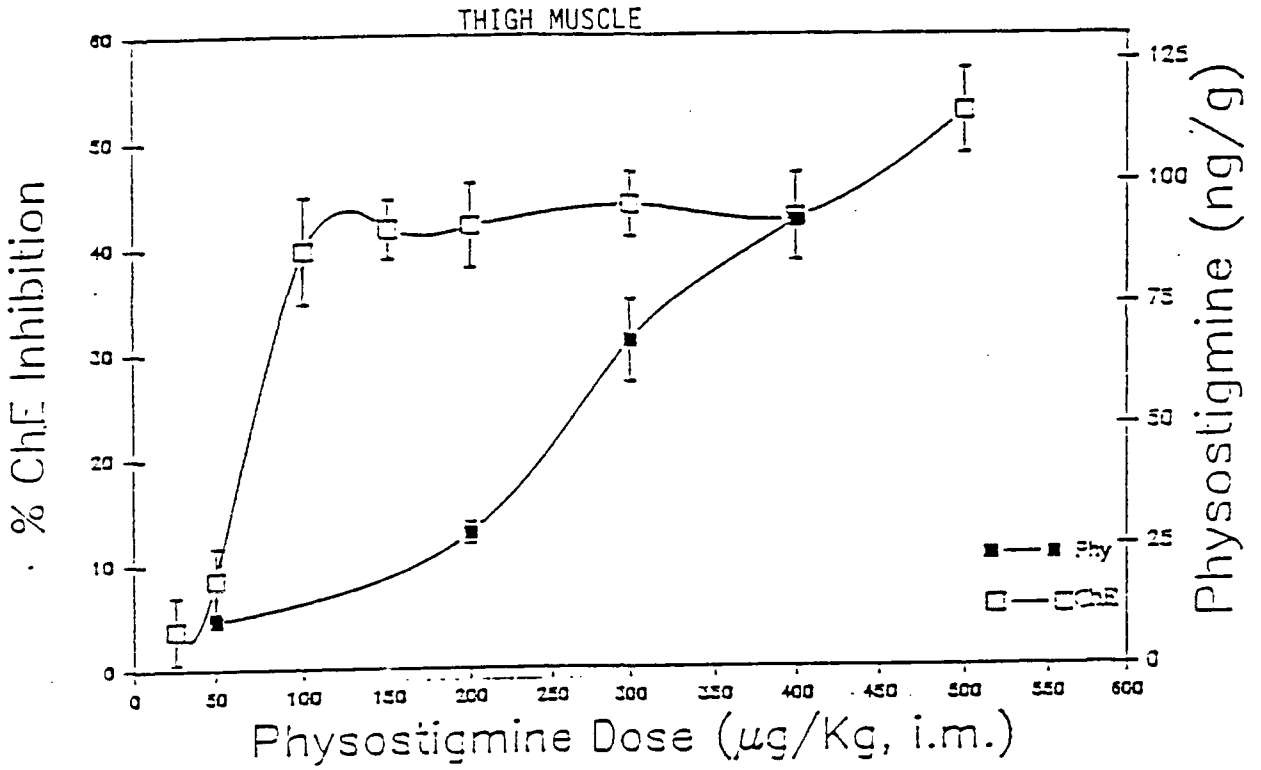


Fig. 4: Relation between physostigmine concentration (■——■) and % ChE inhibition (□——□) in thigh muscle of rats administered with 25-500 µg/kg of physostigmine i.m. Each point is the mean ± S.E.M. of 6-8 rats.

showed 58% ChE inhibition in whole blood at 15 min, with 70 µg/kg i.m. dose of Phy to rat. These authors have determined the total ChE present in the whole blood which comprises true ChE (AChE) and pseudocholinesterase (BuChE). It is understandable that our value of ChE inhibition is lower because we have estimated ChE values in RBC rather than whole blood. Heyl et al. (7) showed similar ChE inhibition (70%) at 15 min after 250 µg/kg of Phy i.m. to rats.

Our results show that the maximum ChE inhibition in brain and diaphragm was at about 200 µg/kg and then with an increase in dose there was a slight, but definite, decrease in inhibition of ChE. This slight decrease in inhibition of AChE in RBC, brain, and diaphragm may be related to the rates of carbamylation and decarbamylation of ChE of low- and high- molecular weights and the concentration of Phy in these tissues, which is dose-dependent. Somani and Khalique (2) have reported the rate of recovery of ChE activity at 0.027 min^{-1} in brain and .083 min^{-1} in muscle after 100 µg/kg i.v. administration of Phy. In our laboratory, we have shown that the rate of recovery of ChE in diaphragm is 0.05 min^{-1} after i.m. administration of Phy; however, after oral administration, the rate of recovery is biphasic (0.053 and 0.017 min^{-1}), indicating the different

rates of decarbamylation. The results in this study also show that the ChE inhibition can be predicted from Phy concentration up to 200 µg/kg dose, because there is a linear correlation between ChE inhibition and Phy concentration from 50 to 200 µg/kg in brain. However, in heart and thigh muscle, the linearity of ChE inhibition and Phy concentration is for all the doses studied.

Phy concentration is lowest in heart and highest in brain, followed by plasma and muscle. It seems that there is a rapid equilibration between blood and brain and Phy concentration proportionately increased in brain for all doses. The pKa of Phy is 7.9, and it is probably sequestered in the brain since it is a tertiary amine and a very lipid-soluble drug. Because of its use for ready penetration into the brain, the drug has a greater potential as a pretreatment agent against organophosphate intoxication, particularly in the central nervous system. Recently Deshpande et al. (10) have compared the efficacy of Phy (centrally acting tertiary amine) with those of pyridostigmine and neostigmine (peripherally acting quaternary amines). Pretreatment of rats with Phy 30 min prior to challenge of sarin reduced mortality to 28%. However, atropine, pyridostigmine and neostigmine injected alone did not protect rats against lethal effects of sarin. Deshpande et al. (10) have indicated that effective protection against lethality of sarin is due to protection of brain ChE by Phy. Our results (unpublished work) also show that the pretreatment of rat by Phy and then soman challenge protected the ChE enzyme from binding to soman.

Maximum ChE inhibition in brain was achieved by 150 µg/kg and then higher doses decreased ChE inhibition by indicating that there is a threshold of Phy in rat brain. Similar threshold for Phy may also be speculated in human, but no such study has been carried out in man. This study suggests that the higher doses in human might be ineffective in ChE inhibition and may produce many more side effects.

We have extended our studies and determined I_{max} and also ID_{50} dose corresponding to half maximal inhibition using the Lineweaver-Burke plots relating 1/inhibition to 1/dose or 1/concentration. These initial estimates were used in NONLIN program to generate the best fits to the nonlinear equation of the I_{max} model and to get better estimates of I_{max} and ID_{50}. ChE inhibition in brain increased up to 60% at 150 µg/kg. Above this dose, there was no increase in inhibition, rather the inhibition decreased to about 48% at higher doses of Phy (200-500 µg/kg). The estimated I_{max} was 58.47% and the ID_{50} was 25.15 µg/kg. From the ID_{50} value, it appears that saturation inhibition was attained faster (unpublished work).

Brain appears to have lowest ID_{50} and IC_{50}. It should have a greater clinical relevance that at lower dose the near maximal inhibition is achieved, especially in brain. Thus, there is no reason to give higher doses of Phy to patients with Alzheimer Syndrome.

The maximum sign-free dose of Phy has been determined (15,16) by observing the effects of graded doses of Phy on the occurrence of unmistakable signs of anticholinesterase poisoning (tremors, muscular fasciculations, unsteadiness, incoordination, or salivation). The maximum sign-free dose of Phy without atropine was 0.16 mg/kg in chicken and guinea pig: 0.1 mg/kg in mouse, rabbit and rat; and < 0.1 mg/kg in dog. However, the maximum-sign free dose of Phy with

atropine dosage (17.4 mg/kg) was 0.63, 0.3, 0.25, and < 0.1 mg/kg in guinea pig, rat, rabbit, and mouse, respectively (15). In the absence of atropine, the doses were very similar in all species, but in the presence of atropine there was greater variation for the maximum sign-free dose of Phy for the four species tested.

In conclusion, RBC, brain, and diaphragm showed dose-dependent ChE inhibition, whereas heart and muscle showed dose-related ChE inhibition at dosage studied. Phy concentration in plasma and tissues increased linearly with increase in dose.

II. RELATIONSHIP BETWEEN EXERCISE INTENSITY AND TYPE OF CALORIC EXPENDITURE IN YOUNG VS. ADULT RATS

Introduction

Several investigators have studied the respiratory capacity of rats using various methodological approaches (17,18,19,20,21). Using different approaches, relationships of oxygen consumption to different types of exercise and workloads have been identified in different species. None of these studies investigated the relationship of oxygen consumption to different exercise intensities in animals of different ages. However, recently Cartee and Farrar (22) have identified dissimilarities in oxygen consumption in trained young and old rats at different exercise workloads.

An important aspect which is lacking from the "above" investigations is the influence of exercise intensity on the type of foodstuff being used in young vs. older animals. Although an age-associated effect has been attributed to differences in $VO_{2\ max}$ (22), a difference in the type of caloric expenditure between different aged animals should not be discounted as a factor which limits $VO_{2\ max}$. Therefore, the present investigation was conducted to determine whether there is any difference in the type of caloric expenditure between young vs. adult rats in performing at progressive exercise intensities to $VO_{2\ max}$.

Materials and Methods

Animals: Two groups of male Sprague-Dawley rats (Harlan Industries, Indianapolis), young (w. 147 $\pm$ 2 g, aged 1 month) and adult (w 332 $\pm$ 7 g, aged 4 months) were studied for the determination of metabolic variables. Both groups of rats were fed ad libitum with Rodent Laboratory Chow (Ralston Purina Company, Indianapolis, IN). Feed consisted of protein (23.4%), fat (4.5%), and balanced with carbohydrate, fibers, vitamins, and minerals.

Description of Treadmill and Oxyscan System: The Oxyscan System and Omni-Pacer Treadmill (Omnitech Inc., Columbus, OH) were used for this study. The Oxyscan System consists of a multi-channel flow controller, thermal mass flow meter, Oxygen Analyzer (Zirconia Sensor), Carbon Dioxide Analyzer (NDIR Sensor), and an Oxyscan Analyzer/Computer.

The Flow Controller consists of 5 input ports (marked 0, 1, 2, 3, 4). Port 0 is the reference input, while ports 1 through 4 are connected to animal chamber 1 through 4, respectively. Associated with each port is a vacuum pump and a flow-control valve. The Flow Controller includes a mass flow meter with a digital flow indicator. Ambient air was drawn into each metabolic chamber through a Drierite column at an approximate rate of 3650 ml/min STPD. Good quality drierite (anhydrous $CaSO_4$, W.A. Hammond Drierite Co., Xenia, Ohio) was essential to remove moisture from the atmospheric air as well as from the expired air.

The oxygen and CO_2 sensors were calibrated using room air, 20.2% O_2/balance N_2, 99.99% N_2 and 0.5% CO_2/balance N_2 standard gases. Calibration of the sensors was carried out every day prior to exercising the rats.

The Omni-Pacer Treadmill is a compact, table-top model, with a continuous conveyor belt topped by a plastic housing, which is divided into 4 channels to allow exercise of four rats at a time. The treadmill includes speed and acceleration control electronics, grade control (-25° to +25°), a shock grid and an exercise duration timer.

Acclimatization Procedure: The rats were weighed and then placed in the treadmill chamber to determine the percent change in O_2 and CO_2 at 2.5 min intervals. At first, the animals were hyperactive in the treadmill, resulting in high RER values (above 1.2). Hence all rats were acclimatized to the treadmill by walking at 2 m/min followed by 10 m/min and then 2 m/min again for a period of 5 min at each of these speeds to obtain consistent metabolic values (oxygen consumption below 42 ml/kg/mm, RER below .85, and caloric expenditure below 15 kcal/kg/hr). After two steady-state values of the metabolic variables were recorded at rest, the rats were run using the incremental exercise protocol as given in Table 2. For each stage of the protocol, steady-state values were obtained prior to the next incremental stage.

Table 2: Protocol for exercising rats on treadmill, at different grades and speed for constant duration.

Stage	Grade/Degrees	Speed (m/min)	Duration (min)
1	0	8.2	5
2	5	15.2	5
3	10	19.3	5
4	10	26.8	5
5	12.5	26.8	5
6	12.5	30.3	5
Recovery	0	2	5

Measurements of maximal oxygen consumption ($VO_{2\ max}$) were considered valid only if the animal ran until it could no longer maintain pace with the treadmill. Oxygen consumption (VO_2, ml/kg/min), CO_2 production (VCO_2, ml/kg/min), RER (VCO_2/VO_2) and caloric expenditure (kcal/kg/hr) were continuously monitored and recorded at intervals of 2.5 min for each of 4 animals at different exercise levels. In order to determine the type of caloric expenditure utilized during the acute exercise bout, the percentage of total calories provided by carbohydrate and fat at each nonprotein RER was calculated by the method of Lusk (23).

Statistical Analysis: Statistical analyses were performed using a two-factor split-plot analysis of variance. The between subjects factor (group) consisted of two levels (young, adult). The within subjects factor was stage,

consisting of resting and stages 1-6. The linear increase over stage was evaluated by examining the linear components obtained from the split-plot analysis. Additional split-plot analyses were performed comparing resting with 10-min postexercise measures. Followup tests were conducted using the Games-Howell procedure (24). Independent t-tests were used to examine VO_2, RER, and caloric expenditure at their maximums. Statistical significance was set at the 0.05 level.

In an effort to examine the influence of decreasing sample size with increasing stage, split-plot analyses were performed three times for each variable (VO_2, RER, caloric expenditure). The first analysis utilized complete data (resting through stage 4) on all subjects. The next analysis contained a smaller sample of subjects including resting through stage 5. The third analysis contained the smallest sample (resting through stage 6). The results of these successive analyses yielded similar results across tests by variable. Therefore, the following split-plot significance tests are reported using results from the complete data set.

Results

Young rats had a significantly higher resting VO_2 than did the adult rats (Table 3). In addition, both the young and adult rats showed a linear increase between VO_2 and successive levels of exercise (stage 5). The VO_2, from resting to peak exercise, indicated a significant group effect, where young rats had larger mean values than adult rats (Table 3). The young rats attained a significantly higher $VO_{2\ max}$ (81.56 ml/kg/min) than the adult rats (69.98 ml/kg/min) despite reaching an identical level of exercise. However, VO_2 returned to resting values at 10 min after exercise for both groups.

Adult rats were unable to run at faster speeds and higher grades of exercise; only 13.33% of adult rats were able to run at stage 6. In contrast, 38.71% of the young rats were able to complete stage 6 of the exercise protocol.

Caloric expenditure, on a weight basis, was similar to the results for VO_2. That is, young rats had a significantly higher caloric expenditure than the adult rats (Fig. 5). Again, both young and adult rats showed a linear increase between caloric expenditure and successive levels of exercise. The young rats attained a significantly higher maximum caloric expenditure (24.11 kcal/kg/hr) than the adult rat (20.57 kcal/kg/hr) at maximal exercise. However, caloric cost returned to resting values at 10 min after exercise for both groups.

The results for RER are not as concise as those for VO_2 and caloric expenditure because the split-plot analysis indicated a significant group by stage interaction. This significant interaction effect indicates that the profiles for young and adult rats differ across stages, as shown in Fig. 6. Although both the young and adult rats exhibit an increase in RER with successive levels of exercise intensity, the increase is no longer linear (parallel). The Games-Howell followup tests indicate that adjacent means, especially in the adult group, fluctuate between increasing and decreasing trends. In addition, the followup tests also indicate that the adult group had significantly higher means than the young group at every stage (Fig. 6). At peak exercise, the adult rats had a higher mean RER than the young rats. However, at 10 min after exercise, adult rats had attained a lower mean RER than the young rats (Fig. 6).

Table 3: Changes in oxygen consumption (VO_2) during a progressive exercise in young and adult rats. Values are mean ± S.E.M..

Condition	Young Rat (VO_2) n	ml/kg/min	% of mean $VO_{2\ max}$	Adult Rat (VO_2) n	ml/kg/min	% of mean $VO_{2\ max}$
Resting 0 grade, 2 m/min	31	40.81 ± 0.47	50.04 ± 0.58	30	29.23* ± 0.73	42.38 ± 1.06
Stage 1 0 grade, 8.2 m/min	31	47.16 ± 0.56	57.83 ± 0.69	30	36.16* ± 1.02	52.43 ± 1.48
Stage 2 5 grade, 15.2 m/min	31	55.38 ± 0.81	67.91 ± 0.99	30	45.42* ± 1.34	65.85 ± 1.94
Stage 3 10 grade, 19.3 m/min	31	62.05 ± 0.87	76.09 ± 1.07	30	52.15* ± 1.33	75.61 ± 1.93
Stage 4 10 grade, 26.8 m/min	31	70.66 ± 0.83	86.65 ± 1.02	30	61.47* ± 2.01	89.13 ± 2.91
Stage 5 12.5 grade, 26.8 m/min	27	76.81 ± 1.31	94.19 ± 1.61	15	66.92* ± 2.79	97.03 ± 4.05
Stage 6 12.5 grade, 30.3 m/min	12	84.75 ± 2.11	103.92 ± 2.59	4	77.78 ± 4.04	112.77 ± 5.86
Mean $VO_{2\ max}$	31	81.55 ± 1.22	100	30	68.97* ± 2.05	100
10 min Post-Exercise 0 grade, 2 m/min	31	39.16 ± 0.77	48.02 ± 0.94	30	30.25* ± 0.61	43.86 ± 0.88

Based upon RER, both young and adult rats utilized fat as the primary fuel source at rest. The young rats did not resort to using carbohydrate as the primary fuel source during the acute exercise bout until 87% $VO_{2\ max}$, whereas the adult rats began utilizing carbohydrate more readily at 52% $VO_{2\ max}$ (Fig. 7). At peak exercise, the adult rats were utilizing 37% more carbohydrate than the young rats. By 10 min after exercise, both groups of rats were using a similar amount of fat as a primary fuel source.

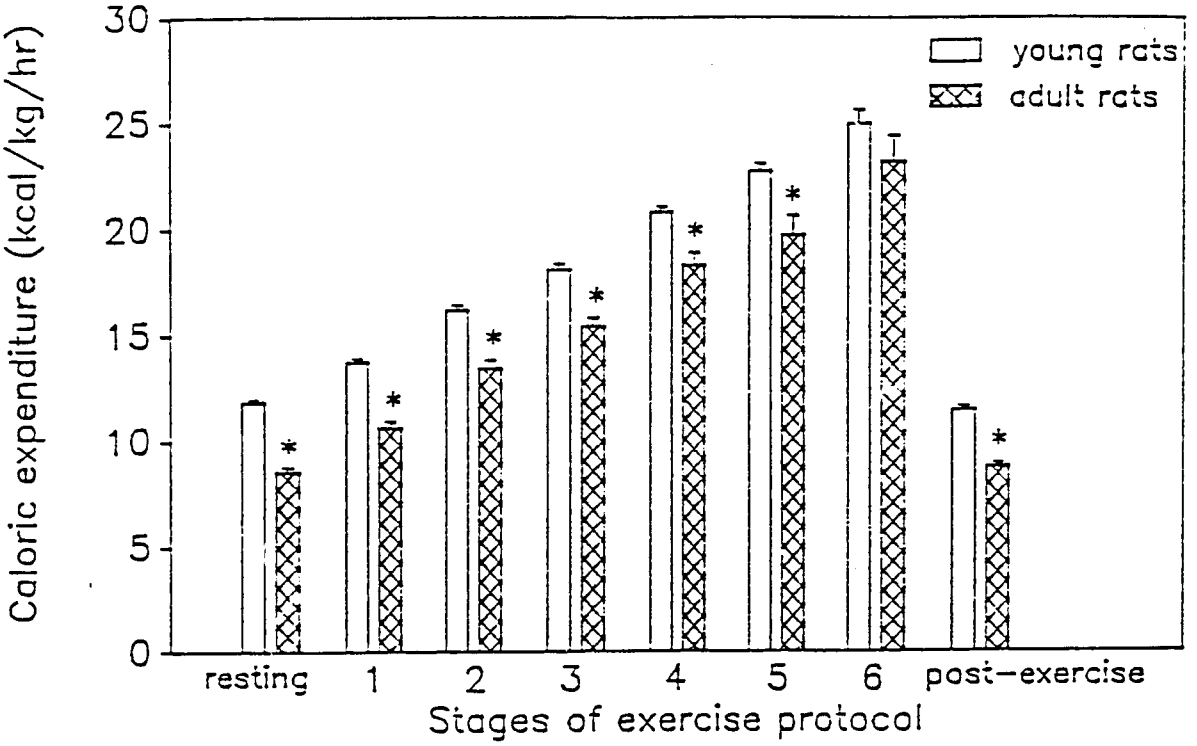


Figure 5. Effect of different levels of acute exercise on caloric expenditure in young vs. adult rats. The values are mean ± S.E.M.. *Significantly lower than young rats ($p < 0.001$).

Discussion

The importance of this investigation is that the age difference between 1- and 4-month old rats is significant enough to show that these animals cannot be grouped together relative to metabolic data. Although similar trends in VO_2 and caloric expenditure at different exercise intensities are observed in these two different age groups, the primary fuel utilized in performing an acute bout of exercise varies significantly between young vs. adult rats at different intensities. For the young rats, carbohydrate did not become the primary fuel source until 87% $VO_{2\ max}$ was reached, whereas in the adult rats carbohydrate was the primary fuel source from 52% $VO_{2\ max}$ to peak exercise. It would appear that even though there is a 3-month difference in these rats, a significant age-related change has occurred relative to efficiency in metabolizing energy stores. Although we have shown $VO_{2\ max}$ values similar to other investigators (17,18,19,20,21,22) in rats, no one has shown the relationship between age and type of fuel source utilized during a progressively intensive exercise.

The present investigation indicates that an age-related decline in VO_2 is evident at an early age in Sprague-Dawley rats (i.e., 1 month to 4 months of age).

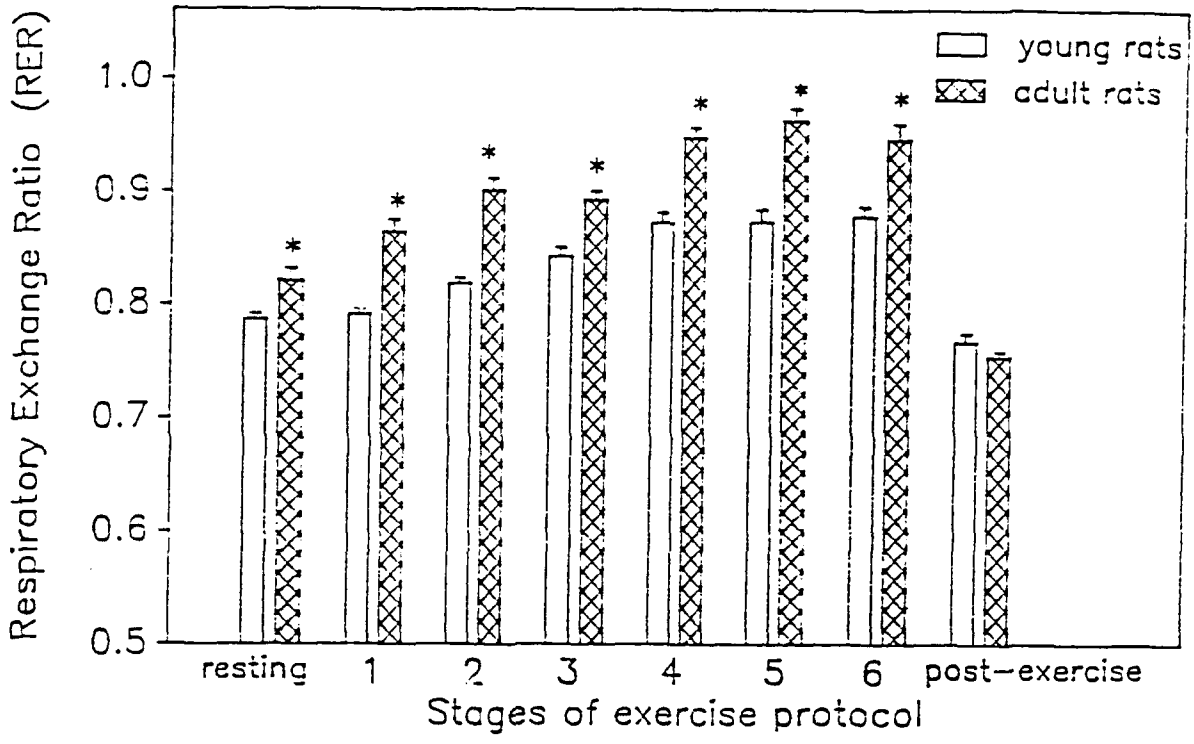


Figure 6. Effect of different levels of acute exercise on respiratory exchange ratio in young vs. adult rats. The values are mean ± S.E.M.. *Significantly higher than young rats ($p < 0.001$).

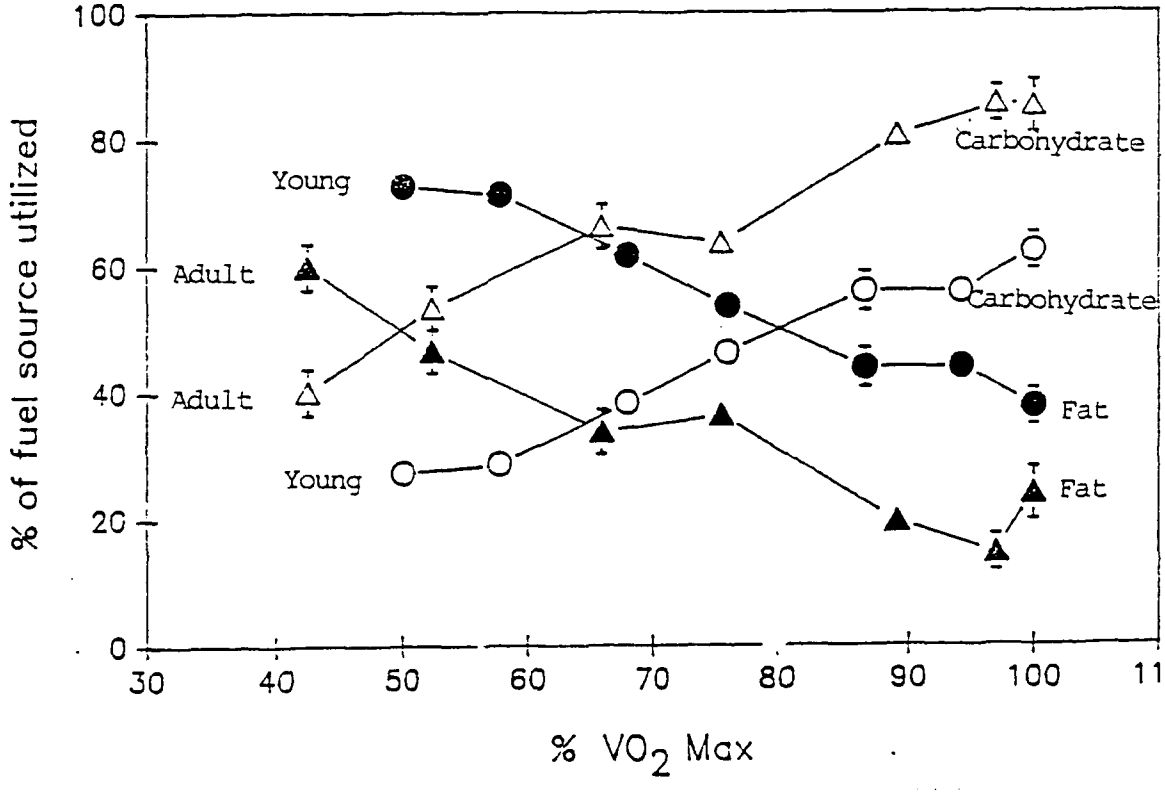


Figure 7. Relationship between exercise intensity and % of fuel source utilized in young vs. adult rats. Carbohydrates: young rat (○—○), adult rat (△—△); Fat: young rat (●—●), adult rat (▲—▲).

This occurrence is important to consider when attempting to identify other metabolic variables at optimal levels. An age-related difference in VO_2 has also been observed in 4-month old (young) vs. 18-month old (old) rats (22). Although Cartee and Farrar (22) investigated 4 month old rats, a direct comparison cannot be made with our studies, since the weight of rats was not mentioned by them.

The $VO_{2\ max}$ is considered to be the best single measure of aerobic capacity in man and animals and may be affected by a number of factors. Adult rats have shown a lower $VO_{2\ max}$ (68.97 ±2.05 ml/kg/min) than the younger rats (81.55 ± 1.22 ml/kg/min). Our findings are in agreement with other investigators (21,22) who have also reported a difference in $VO_{2\ max}$ of adult and older rats. However, there is a quantitative difference between their values and our values. This difference may be due to gender, strain, age, or weight of the animals. The lower $VO_{2\ max}$ found in adult rats may also be attributed to decreased physical activity (25). Old rats have a lower hindlimb muscle respiratory capacity than young rats (26,27). This difference, however, might not be the result of aging. It is likely that the documented decline in spontaneous physical activity in old rats (28,29,30,31,32) contributes to the age-related change. Recently, Musch et al. (33) have also shown a $VO_{2\ max}$ of 80 ml/kg/min in rats weighing 300 g; however, the method of measurement of $VO_{2\ max}$ was different from our method. The results indicate that adult rats had to consume a greater percentage of oxygen through the initial phase of the incremental exercise protocol compared to young rats, though both groups of rats were given identical exercise. It appears that this enhanced oxygen demand was in response to the switchover of fat to carbohydrate as the primary fuel source. RER values indicated that carbohydrates were being utilized more readily in adult than in younger rats toward the end of exercise. Young rats had significantly lower resting RER (0.787) compared to adult rats (0.821 ± 0.011). This indicates a greater dependence on fat metabolism in young compared to adult rats. We could not observe RER > 1.0 except in a few rats at $VO_{2\ max}$. Our data for RER is in agreement with other investigators (20,21). However, Cartee and Farrar (22) observed an RER of 1.24 in 4-month old rats and 1.12 in 18-month old rats at $VO_{2\ max}$. The difference in RER values observed at $VO_{2\ max}$ between our study and Cartee and Farrar (22) may be due to efficiency in fuel utilization. The strain of rat utilized might also contribute to this difference in fuel burning efficiency. Both our study and Bedford et al. (21) have reported higher $VO_{2\ max}$ values in young Sprague-Dawley rats compared to $VO_{2\ max}$ values reported in Fischer 344 rats by Cartee and Farrar (22).

In conclusion, the results have indicated significant differences in oxygen consumption and caloric expenditure in young vs. adult rats undergoing identical acute exercise. Young rats attained a higher $VO_{2\ max}$ (81.55 ml/kg/min) compared to adult rats (68.97 ml/kg/min). The younger rats possessed a higher resting caloric expenditure (11.84 kcal/kg/hr) compared to adult rats (8.53 kcal/kg/hr). Based upon these metabolic factors, it seems inappropriate to consider 1-month old and 4-month old rats as similar in their response to an incremental exercise, particularly since younger rats appear to utilize fats as a primary fuel source more readily through submaximal levels of exercise intensity compared to adult rats.

III. INTERACTION OF PHYSOSTIGMINE AND DIFFERENT INTENSITIES OF EXERCISE ON CHOLINESTERASE ACTIVITY IN RBC AND DIFFERENT TISSUES OF RAT

Introduction

Phy is a centrally acting anticholinesterase drug (1,2). Recently Somani and Dube (34) have reviewed Phy to be a potential pretreatment agent against organophosphate (OP) intoxication (9,16,35). Somani and Dube (36) have reported the *in vivo* kinetics of dose response of Phy and ChE activity in red blood cells (RBC) and tissues of rats.

Physical exercise has broad effects on the body; it can evoke a number of enzymatic changes in muscles and liver (37,38,39). The changes in enzymatic activity are directly related to the intensity of physical exercise (40). Serum ChE activity has been reported to be increased after exercise (41,42). McMaster and Foster (43) have demonstrated that acute exercise increases behavioral sensitivity to Phy. The combined effects of physical exercise and chemical stressor such as Phy on the cholinergic system have not received much attention.

There seems to be an interaction between exercise and the neurotransmitter acetylcholine (ACh). This interaction can be monitored by one of the important biochemical markers of the cholinergic system, degradative enzyme of acetylcholine, ChE). Since intense fitness is required in the battlefield, how the different level of physical exercise would influence the Phy-induced ChE activity needs to be considered during the development of potential treatment agent and therapy regimen. If exercise contributes to alterations in ChE activity due to Phy, this, in turn, would affect the work performance in the field. Therefore, this study investigates the effects of three intensities of acute treadmill exercise, Phy, the combined effect of Phy, and exercise on ChE activity in RBC and various tissues (brain, heart, diaphragm, and thigh muscle) and on endurance time in rats.

Materials and Methods

Chemicals: Phy free base was obtained from Sigma Chemical Co. (St. Louis, MO). Ready-Solv EP was procured from Beckman Instruments Inc (Fullerton, CA). Drierite (anhydrous $CaSO_4$), procured from W. A. Hammond Drierite Co. Xenia, Ohio, was used. Diagnostic kit was purchased from Sigma Chemical Co. (St. Louis, MO) for the determination of blood Hb. All other chemicals were analytical grade and were obtained from the usual commercial sources.

Treadmill and Oxyscan System: The detailed description of treadmill and oxyscan system is given in Section II. The Oxyscan System and Omnipacer Treadmill (Omnitech. Inc., Columbus, Ohio) were used. The Omnipacer treadmill is a compact table-top model, with a continuous conveyor belt divided into four channels to exercise four rats at a time. The treadmill includes speed and acceleration control electronics, grade control (-25° to +25°) shock grid and an exercise duration timer. The Oxyscan System consists of a multichannel flow controller, thermal mass flow meter, Oxygen analyzer (Zirconia Sensor), Carbon dioxide analyzer (NDIR sensor), and an Oxyscan analyzer/computer.

The Omnipacer treadmill and the Oxyscan System together form an integrated system for monitoring O_2 consumption (VO_2 = ml/kg/min), CO_2 production (VCO_2 = ml/kg/min), and caloric cost (cal/hr). Respiratory Exchange Ratio (RER = VCO_2/VO_2) is also calculated and presented. These parameters were continuously monitored and recorded at intervals of 2.5 min for up to 4 animals at different exercise levels.

Animals: Male Sprague Dawley rats (Harlan Industries, Indianapolis, In) weighing 160-200 g were used in this study.

Exercise and Phy Administration: The rats were exercised at different levels on treadmill described in Table 2 to obtain the $VO_{2\ max}$, RER, and caloric cost. After 3 days of determining the $VO_{2\ max}$ of each rat, the animals were divided into four groups. The following experimental protocol was carried out: (1) Rats were exercised at 50, 80 and 100% $VO_{2\ max}$, which corresponded to 10, 20 and 30 min, respectively, and soon after exercise, the rats were sacrificed. (2) Rats were administrated Phy (70 µg/kg, i.m.) and were sacrificed at 10, 20, and 30 min. (3) Rats were administered Phy (70 µg/kg, i.m.) and then immediately exercised at 50, 80 and 100% $VO_{2\ max}$. Immediately after exercise the rats were sacrificed. (4) In control group, rats were administered saline and sacrificed. All animals were sacrificed between 8:00 AM - 11:00 AM to minimize circadian cycle effects. Each group consisted of 4-12 rats. Soon after the decapitation, blood, brain, heart, diaphragm, and thigh muscle were collected. The blood was processed for the determination of RBC-ChE. The tissues were stored at -70°C until analysis for ChE determination.

Determination of ChE Activity: The ChE enzyme estimation was carried out according to a modification of the radiometric method of Jonnson and Russell (1975) (11). In this procedure, [^{3}H]-ACn is used as the substrate. This method measured the RA due to [^{3}H]-acetate formed by the enzymatic hydrolysis of [^{3}H]-ACh. The substrate is prepared daily by mixing 0.5 M Tris buffer (0.25 M Trizma base, 0.25 M Tris-HCl, 1.2 M NaCl, pH 7.4), AChCl (0.1 mmol for RBC, diaphragm, heart, and thigh muscle; 1 mmol for brain) and [^{3}H]-AChI (1 mCi/0.01 mmol).

We have previously reported the details of measurements of ChE activity in RBC, brain, heart, diaphragm, and thigh muscle (36). The Hb content of blood was determined by Sigma Diagnostic kit, using a Beckman Spectrophotometer at 540 nm.

The ChE values of RBC are expressed as µmol ACh hydrolyzed/min/g Hb content, whereas the tissue ChE values are expressed as µmol ACh hydrolyzed/min/g wet weight of tissue.

Statistical Analysis: The ChE values were subjected to one-way analysis or variance with 10 levels followed by Duncan's multiple range follow up tests. Significant differences were accepted at $p < 0.05$.

Results

Effect on Endurance Time: The values for endurance time for rats at different levels of exercise with or without Phy administration are given in Table 4. The end point for endurance time was that the rats were completely exhausted and they were not able to continue running at the given intensity of

exercise. Phy and acute exercise increased the endurance time of rats to 29-31%, as compared to the respective level of exercise alone. However, the increase in endurance time was statistically significant only at 100% $VO_{2\ max}$ ($p < 0.01$).

Table 4: Effect of physostigmine (70 µg/kg, i.m.) and/or different levels of acute exercise on endurance time in rats (n = 4-8)

Groups	Treatment	Endurance Time (min) Mean + S.E.
I	$VO_{2\ max}$ 50%	8.75 ± 2.61
II	Phy + $VO_{2\ max}$ 50%	11.25 ± 1.61 (29%)
III	$VO_{2\ max}$ 80%	16.25 ± 2.61
IV	Phy + $VO_{2\ max}$ 80%	21.25 ± 1.61 (31%)
V	$VO_{2\ max}$ 100%	23.13 ± 1.19
VI	Phy + $VO_{2\ max}$ 100%	30.10 + 1.02 *(30%)

* $p < 0.01$ as compared to $VO_{2\ max}$ 100%.

Values in parenthesis indicate the percentage increase in endurance time.

Effect on ChE Activity: Different intensities of acute exercise (50, 80, and 100% $VO_{2\ max}$) per se, Phy administration, and Phy and acute exercise did not show a significant effect on ChE activity in RBC and tissues over time (10, 20, and 30 min) corresponding to these three levels of exercise.

RBC: Exercise at 50, 80, and 100% $VO_{2\ max}$ produced a slight increase in ChE ($p < 0.05$) activity (116%-108% of control) (Fig. 8A). Phy administration inhibited ChE activity 73-79% of control from 10 to 30 min. Phy + acute exercise at different intensities of $VO_{2\ max}$ did not alter ChE activity significantly ($p < 0.05$). Phy + acute exercise further depressed the ChE activity in RBC (54-51% of control) as compared to Phy alone (73-79% of control). There is a significant difference in ChE activity between exercise alone ($p < 0.01$), Phy alone ($p < 0.01$), and Phy + exercise ($p < 0.01$) (Fig. 8A). ChE activity did not change due to different intensities of exercise in these three treatment groups.

Brain: Different intensities of acute exercise (50, 80, and 100% $VO_{2\ max}$) alone produced a slight but statistically in significant decrease in brain ChE activity (92-87% of control) (Fig. 8B). Phy significantly decreased ChE activity (66-68% of control; $p < 0.01$) in brain from 10 to 30 min (Fig. 8B). Phy and acute exercise further decreased the ChE activity (58-50% of control) ($p < 0.01$) at all three levels of exercise.

Heart: The acute exercise (50, 80 and 100% $VO_{2\ max}$) decreased the ChE activity significantly (82-87% of control, $p < 0.05$) (Fig. 8C), indicating that

the heart is the important organ to be affected by exercise. However, these intensities of exercise affected ChE activity to the same extent. Phy reduced ChE activity (68-74% of control) from 10 to 30 min. Phy and acute exercise showed ChE activity to be 77-73% of control at 50, 80, and 100% $VO_{2\ max}$ and did not show any significant difference as compared to Phy alone (Fig. 8C).

Diaphragm: Acute exercise did not produce a significant effect on ChE activity in diaphragm (Fig. 8D). Phy resulted in decline in ChE activity (67-81% of control) from 10 to 30 min. These results indicate that the recovery of ChE enzyme had started in the diaphragm, resulting in reduced ChE inhibition (81% of control) at 30 min. The effect of Phy + acute exercise on ChE inhibition was significantly greater compared to exercise alone. The ChE activity in Phy alone as well as Phy + exercise was almost the same from 10 to 30 min (Fig 8D). ChE activity was significantly higher (81 and 83% of control) at 100% $VO_{2\ max}$ in Phy and Phy + acute exercised rat, respectively, compared to 50 and 80% $VO_{2\ max}$.

Thigh muscle: Different intensities of acute exercise did not produce any significant effect on ChE activity (91-96% of control) of thigh muscle (Fig. 8E). Phy showed a significant decrease in ChE activity (57-61% of control) from 10 to 30 min ($p < 0.01$). The combined effect of Phy and acute exercise elicited significantly greater decrease in ChE activity ($p < 0.01$) at all three exercise levels when compared with exercise alone. ChE activity was 54, 58, and 57% of control at 10, 20, and 30 min, respectively, in the rats administered with Phy followed by acute exercise at 50, 80, and 100% $VO_{2\ max}$, respectively. However, there was no significant difference in ChE activity between Phy alone and Phy and acute exercise (Fig. 8E).

Discussion

This is the first report describing the combined effect of Phy (chemical stress) and different intensities of acute exercise (physical stress) on ChE activity in RBC and tissues. This investigation indicates that acute exercise alone, irrespective of the intensity, affects the ChE enzyme to a moderate degree in RBC and heart without affecting brain, diaphragm, and thigh muscle. The combined effect of Phy and different levels of exercise increased the ChE inhibition in RBC and brain without affecting other tissues. However, ChE activity was not affected by different intensity of exercise in RBC, brain, heart, and muscle. We have found (manuscripts in preparation) that the rate of decarbamylation of ChE increased in RBC, brain, and diaphragm and decreased in muscle due to acute exercise (80% $VO_{2\ max}$) and Phy compared to Phy alone. However, the endurance training has opposite effect to that of acute exercise. The rate of decarbamylation decreased significantly in all tissues due to endurance training and Phy compared to Phy alone. Phy (70 µg/kg, i.m.) inhibited the ChE activity in RBC (27-21%), brain (34-32%), heart (32-26%), diaphragm (33-19%), and thigh muscle (43-39%) from 10 to 30 min. This inhibition of ChE enzyme might have caused significant increase in endurance time in rats. Matthew et al. (44) have reported a decrease in endurance time of rats weighing 500 g by Phy salicylate (200 µg/kg, i.v.) and observed a rise in core temperature. However, Meeter and Wolthius (45) have reported that anticholinesterase agents induce peripheral vasodilation and lowers core temperature by increasing heat dissipation through the tail. It is possible that Phy in a dose of 70 µg/kg, i.m. may be reducing the core temperature of rats thereby resulting in an increase in endurance time.

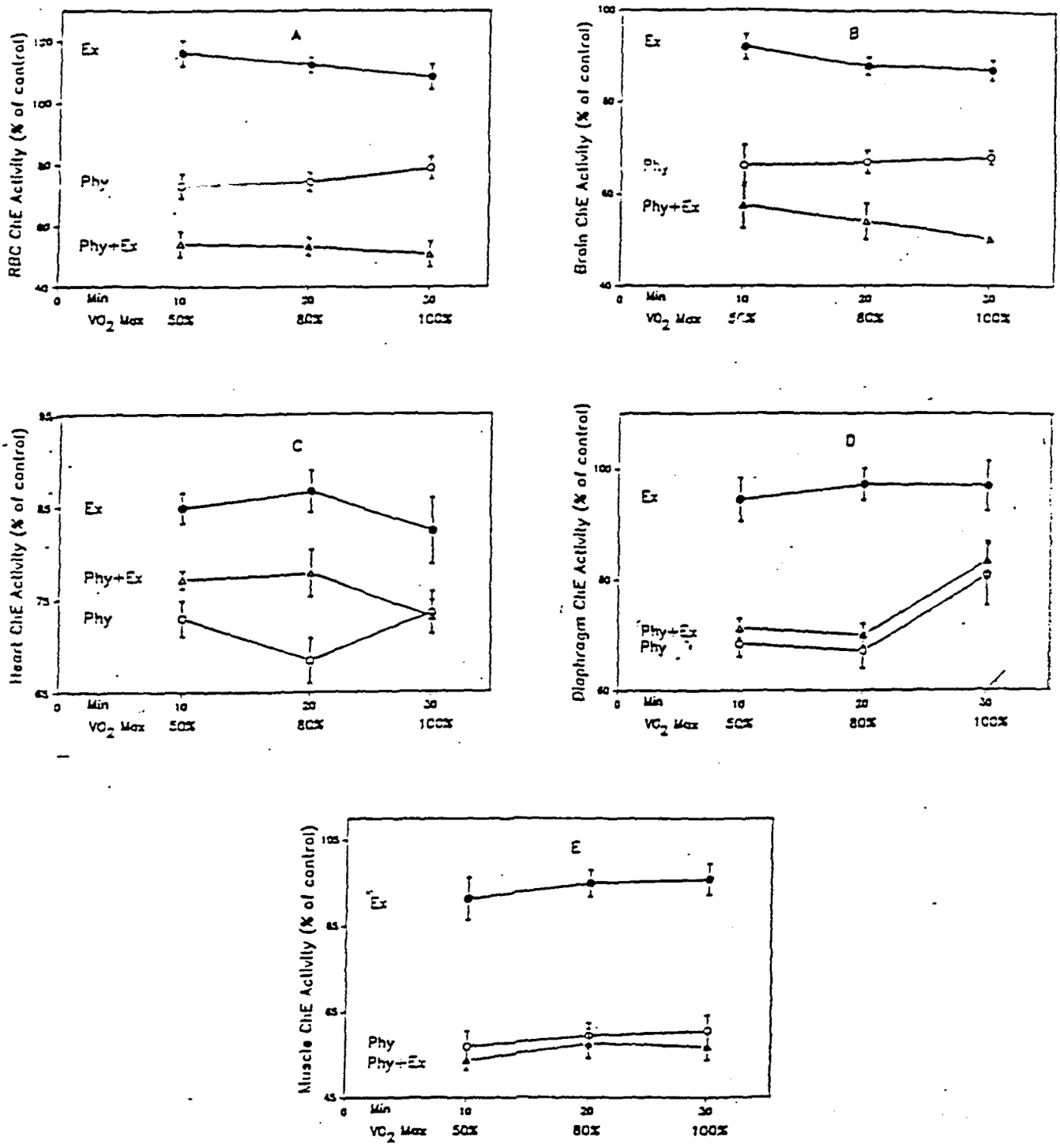


Fig. 8. Effects of physostigmine (70 µg/kg, i.m.) and different levels of treadmill acute exercise (Ex) on ChE activity in RBC (A), brain (B), heart (C), diaphragm (D) and thigh muscle (E) of rats. Values are mean ± S.E.M. (n=4-12). Control values of ChE in RBC 1.688 ± 0.144 µmol ACh hydrolysed/min/g Hb; tissues (brain 8.103 ± 0.609; heart 1.108 ± 0.124; diaphragm 0.723 ± 0.043; and thigh muscle 0.778 ± 0.045 µmol ACh hydrolysed/min/g wet tissue).

Acute exercise produced a slight elevation of RBC ChE activity. Changes in ChE activity observed in our experiment agree with the earlier work of Pawlowska et al. (41), who have also observed a significant increase in ChE activity in blood serum 1 and 2 hr after physical exercise (20 m/min for 30 min) in rats. The increase in RBC-ChE with acute exercise may be due to secondary effect of hypoxia (41) and hemoconcentration due to plasma shift (46) during exercise. Surprisingly, the increase in the intensity of exercise from 50% $VO_{2\ max}$ to 100% $VO_{2\ max}$ did not show corresponding increase in the ChE activity, rather we observed a slight decrease in ChE activity (108% of control) at 100% $VO_{2\ max}$ as compared to 116% of control at 50% $VO_{2\ max}$. This may be due to excitement, anxiety, and stress during initial period of exercise (50% $VO_{2\ max}$); after that, there may be a cholinergic acclimatization at higher intensity of exercise corresponding to treadmill exercise for 20 and 30 min, respectively.

Phy administration decreased ChE activity of RBC; however, Phy and acute exercise further decreased this ChE activity. This might be due to increased blood flow during exercise (47,48), resulting in the rapid transport of Phy from the site of injection. We have shown that the absorption phase, T_{max} and C_{max} have disappeared in acute exercise and endurance trained rats after i.m. Phy administration, indicating that increased blood flow due to exercise has caused change compared to control rats (manuscript in preparation).

Acute exercise produced a slight, but insignificant, decrease in brain ChE activity. This finding is in agreement with Ryhanen et al. (42) and is contrary to the finding of Pedzikiewicz et al. (49), who have shown a slight increase (3%) in brain ChE activity after single exercise. Holmstedt (50) has reported that physical exercise accelerates the nerve action in CNS resulting in the increased amount of ACh in the nerve endings, and hence increased ChE inhibition. Phy + acute exercise increased the ChE inhibition in brain, and this might be due to increased concentration of Phy in the brain during exercise. We have shown (manuscript in preparation) that the amount of RA in brain, heart, lung, kidney, liver, and muscle of trained exercised rats increased by 337, 191, 106, 385, 126, and 80% over control rat at 2 min, respectively, after the administration of [^{3}H]-Phy (70 µg) i.m. after exercise. Somani and Khalique (1,2) have reported that the concentration of Phy attains much higher level in brain than in plasma within 2 min of i.m. or i.v. injection of Phy.

Acute exercise produced a significant ($p < 0.05$) reduction of total ChE activity in heart. However, Tipton et al. (51) did not find any significant change in myocardial AChE and ChE activity after trained exercise. However, Herrlich et al. (52) have shown that atrial and ventricular ChE activity was significantly higher than AChE. Atria had higher AChE and ChE activity than the ventricles. We determined the total ChE activity in whole heart, and we did not quantitate the changes in ChE in atria or ventricle, nor did we differentiate the concentration of AChE and BuChE. Our results indicate that exercise significantly decreased ($p < 0.05$) ChE activity in the heart, irrespective of intensity of exercise. Phy produced a significant decrease in heart ChE activity, which was not modified by different intensities of exercise.

Acute exercise did not change the ChE activity in diaphragm, irrespective of the level of exercise. However, the subacute exercise for 5 days in a week increased in total ChE and BuChE activity in diaphragm (50). Phy reduced the ChE

activity in diaphragm. The combined effect of Phy and acute exercise did not alter the ChE activity in diaphragm, as compared to exercise alone.

In our studies, different intensities of acute exercise did not significantly affect the ChE activity in muscle. Contrary to our results, Pedzikiewicz et al. (49) reported an increase in muscle ChE activity (20%) with a very high intensity exercise protocol (48 m/min), which may be the cause of increase in ChE activity. The increase in ChE activity may be due to an increase in blood flow (25-fold) in skeletal muscles (47). In our protocol, the final speed reached 30 m/min for 5 min. This intensity might not be sufficient to observe changes in ChE activity in total muscle. Phy decreased ChE activity in muscle. The combined effect of Phy and different intensity of exercise showed the similar inhibition of ChE activity in muscle as that of Phy alone. Diaphragm and muscle contain mostly BuChE, and it appears that the exercise did not modify the effect of Phy on this enzyme. On the contrary, RBC and brain primarily contain AChE; Phy and exercise depressed this enzyme much more compared to Phy alone, indicating that the exercise modified the effect of Phy and AChE. Another explanation for the effect of exercise on Phy-induced inhibition of ChE in RBC and brain and not in diaphragm and muscle could be based on pharmacokinetics and distribution of Phy. We have previously shown that blood/brain equilibration of Phy is much more rapid. Brain concentration of Phy is higher than plasma concentration within 2 min of i.v. administration (2). However, it takes more time for Phy to reach peak concentration in muscle.

In conclusion, these results suggest that the different intensities of acute exercise (50, 80, and 100% $VO_{2\ max}$) showed a similar, but significant, inhibition of ChE activity in heart, without significantly affecting brain and thigh muscle. However, acute exercise produced a slight increase in ChE activity of RBC. Phy decreased the ChE activity in RBC and tissues. The combined effect of Phy and acute exercise further decreased the ChE activity in RBC and brain without significantly affecting heart, diaphragm, and thigh muscle. Exercise potentiated the effect of Phy on ChE inhibition in RBC and brain, irrespective of intensity of exercise. It seems that acute exercise affects the ChE activity to a moderate degree in RBC and heart, and modifies the effect of Phy in RBC and brain.

IV. EFFECT OF ACUTE AND TRAINED EXERCISE ON TIME COURSE OF CHOLINESTERASE ACTIVITY IN RBC AND TISSUES OF RAT AFTER PHYSOSTIGMINE ADMINISTRATION

Introduction

Physical exercise evokes a number of enzymatic changes in the body, especially in muscles and liver (37,39). Exercise is one of the important factors that alters ChE activity (49,42). The intensity of these changes depends upon the type and severity of exercise (41,40).

Phy, a reversible ChE inhibitor, is a centrally acting carbamate. Phy is a flow-limited, poorly plasma-bound, and highly extracted drug. Phy and its analog have been shown to improve the memory function (53) and was also used as a potential prophylactic against organophosphate intoxication (16,9,34,35). ChE inhibitors are likely to be used as prophylactic agents in battlefields or crop fields when a person is engaged in strenuous work. McMaster and Carney (54) have demonstrated that acute exercise increases the behavioral sensitivity to Phy. We have recently reported the *in vivo* kinetics of dose response of Phy and ChE activity in red blood cell and tissues of rats (36). Earlier Somani and Khalique (1,2,3) reported the pharmacokinetics and pharmacodynamics of Phy in rat after i.v., i.m., and oral administration.

The interaction of various drugs and exercise in man and animals has been reported widely (55,56). However, very few reports are available regarding the interaction of exercise on the disposition and pharmacodynamics of drugs. The different forms of physical exercise do not necessarily alter the processes of drug absorption to the same extent, resulting in the varied pharmacodynamic action of drugs. If exercise alters the ChE activity due to Phy, this, in turn, would interfere with the work performance in the fields when Phy is used as a pretreatment drug. Since that intense fitness is required in the battlefield, how the different types of physical exercise would influence Phy-induced ChE activity needs to be considered during development of potential pretreatment agent and therapy regimen. The combined effect of physical exercise and chemical stressor such as Phy on the cholinergic system has not received much attention.

Recently, we have shown that Phy or physical exercise or combination of two stressors depressed ChAT and/or AChE activities in different brain regions differently and inconsistently, depending on the level of stress. Physical exercise is one of the most important factors that alter ChE activity (49,42). The magnitude of the changes depends upon the type and severity of exercise. We have reported the rate of decarbamylation of BuChE in plasma, and ChE in brain to be 0.11 min^{-1} and 0.027 min^{-1}, respectively, after administration of [^{3}H]-Phy (100 µg/kg i.v.) (2). These rates were considerably higher than the elimination rate of Phy in plasma (0.046 min^{-1}) and in brain (0.063 min^{-1}), indicating a longer-lasting effect of Phy than is reflected by the drug level. It is likely that acute or trained exercise modifies the effects of reversible ChE inhibitors by altering the time course of ChE activity in RBC and tissues of rat. Therefore, this chapter addresses the question whether the pharmacodynamics of Phy (rate of decarbamylation of ChE enzyme) alters due to acute and/or trained treadmill exercise in RBC and various tissues of rat.

Methods

Animals: Male Sprague-Dawley rats (initial weight 160-200 g) were used. Rats were divided into 6 groups:

Gr I Sedentary control (SC), saline administration
Gr II Acute exercise (80% $VO_{2\ max}$) (AE)
Gr III Endurance trained + acute exercise (80% $VO_{2\ max}$) (ET)
Gr IV [^{3}H]-Phy (70 µg/kg, i.m.) (Phy)
Gr V Acute exercise (80% $VO_{2\ max}$) + [^{3}H]-Phy (70µg/kg, i.m.) (AE + Phy)
Gr VI Endurance trained + acute exercise (80% $VO_{2\ max}$) + [^{3}H]-Phy (70 µg/kg, i.m.) (ET + Phy)

Endurance Training of Rats: Rats from Gr III and Gr VI were acclimatized to treadmill in the beginning and were trained on a 9-channel motor-driven treadmill (built in our Southern Illinois University, School of Medicine workshop), utilizing an incremental exercise program 5 days a week for 6 weeks duration. During this program of exercise, the speed (meters/min), angle of inclination (degrees), and the duration (min) of exercise were varied to obtain different levels of exercise intensity as shown in Table 5. In the first 2 wk, conveyor belt speeds were 8.2, 15.2, and 19.3 m/min, and the angle of inclination was 6°. Exercise duration at each speed was 5 min the first week and 10 min the second week. In the 3rd and 4th weeks, the speeds were maintained at 19.3, 26.8, and 30.3 m/min. The duration of exercise at each speed was 10 min. The angle of inclination was 6° during the 3rd week and 9° in the 4th week. The final 2 wk of exercise involved sustaining speeds of 19.3, 26.8, and 30.3 m/min at a 9° angle of inclination for 10 min at each speed.

Table 5: Endurance training protocol for exercising rats.

Week	Belt Speed (m/min)	Angle of Inclination (Degrees)	Duration at Each Speed (min)
1	8.2, 15.2, 19.3	6	5
2	8.2, 15.2, 19.3	6	10
3	19.3, 26.8, 30.3	6	10
4	19.3, 26.8, 30.3	9	10
5	19.3, 26.8, 30.3	9	10
6	19.3, 26.8, 30.3	9	10

Determination of maximum oxygen consumption ($VO_{2\ max}$) was carried out in the beginning of the training protocol in order to determine the $VO_{2\ max}$ for each rat. Measurement of maximal oxygen consumption (100% $VO_{2\ max}$) was considered valid only if the animal ran until it could no longer maintain pace with the treadmill. During the training, the fifth day of every week, $VO_{2\ max}$ was determined for each rat.

Acute Bout of Exercise to Rats: The rats from Gr II, III, V, and VI were given an acute bout of exercise on the treadmill (Omnitech Electronics, Inc., Columbus, Ohio) at 80% $VO_{2\ max}$. The speed of the belt and angle of inclination were increased at different stages as shown in Table 2.

The oxygen consumption and heat production in individual rats undergoing different stages of exercise have been recorded once a week by Omnitech oxyscan analyzer. Body weights were recorded every day for all the groups.

Dosing and Sacrificing of Rats: On the day of the experiment, rats from Group I (SC) (8 rats) were administered saline and were sacrificed. The rats from Group II (AE) were subjected to an acute bout of exercise (80% of $VO_{2\ max}$) (see Table 2, Section II) and were sacrificed at 2, 5, 10, 15, and 30 min. The rats from Group III (ET) were endurance-trained and were subjected to acute bout of exercise (80% $VO_{2\ max}$) for 30 mins. using incremental exercise protocol, and they were decapitated at 5, 15, 30, and 60 mins. The rats from Group IV (Phy) were administered [^{3}H]-Phy (70 µg/kg i.m.) and were sacrificed at 2, 5, 10, 15, 30, 45, and 60 min. The rats from Group V (AE + Phy) were subjected to acute exercise at 80% $VO_{2\ max}$ and soon after exercise [^{3}H]-Phy was administered (70 µg/kg i.m.), and they were sacrificed at 2, 5, 10, 15, 30, 45, and 60 min. The rats from Group VI (ET + Phy) were endurance trained and were subjected to an acute bout of exercise for 30 min at 80% $VO_{2\ max}$ and then administered [^{3}H]-Phy (70 µg/kg i.m.) and were sacrificed at 2, 5, 10, 15, 30, 45, and 60 min. Four to 6 rats were sacrificed at each time point. Blood, brain, heart, diaphragm, and thigh muscle were collected for analysis of ChE activity.

Determination of ChE Activity: The ChE enzyme activity was determined by the radiometric method in RBC, brain, heart, diaphragm, and thigh muscle (11). The ChE values of RBC are expressed as µmol of ACh hydrolyzed/min/g of Hb content, whereas the tissue ChE values are expressed as µmol of ACh hydrolyzed/min/g of wet weight of tissue.

Determination of Hemoglobin: The Hb content of blood was determined by Sigma diagnostic kit, using a Beckman spectrophotometer at 540 nm.

Determination of Rate of Decarbamylation: The percent ChE inhibition was plotted on semilog graph to obtain a declining slope representing the rate of decarbamylation of the enzyme. The best fit lines were obtained by linear regression analysis and the correlation coefficient (r) were determined. However, the time points prior to maximum enzyme inhibition were excluded in estimating the best fit line since they do not represent the recovery phase of the enzyme. The half time of enzyme recovery is the time taken for the percent inhibition to be reduced to 50% of its maximum value.

Results and Discussion

Effect of Endurance Training on Metabolic Variables and Body Weight Gain: There was no significant effect of endurance training on metabolic variables, especially up to the third stage of exercise protocol. However O_2 consumption was decreased from the fourth stage to the last stage of exercise protocol in the trained rats, as compared to age-matched sedentary control rats. The initial $VO_{2\ max}$ was 77.1 ml/kg/min, which slightly decreased with the increase of training

period (68.7 ml/kg/min) on the 43rd day of training. The caloric expenditure increased with the increase in exercise duration as compared to rest. However, the RER was not affected. The percent gain in body weight was almost similar in sedentary control (73.22%) as well as trained rats (70.02%) during an observation period of 6 wk. The daily body weight gain was 3.72 and 3.27 g in sedentary control and endurance-trained rats, respectively.

Effect on ChE Activity and Rate of Decarbamylation: The interaction of acute exercise (AE), endurance-trained exercise (ET), and [^{3}H]-Phy (Phy, 70 µg/81.03 µCi/kg, i.m.) on time course of % control ChE activity in RBC and tissues are shown in Figs. 9-13. The rate of decarbamylation (K_d) of ChE activity and half-time ($T_{½}$) for recovery of enzyme in RBC and tissues are presented in Table 6.

Table 6: Effect of acute or trained exercise on rate of decarbamylation (K_d) ChE - in min^{-1} of ChE in RBC and Tissues of Rat.
r - is the correlation coefficient for % ChE inhibition vs. time for the declining curve.
$T_{½}$ - is the half-time in min for recovery of ChE enzyme.

GROUP		IV	V	VI
Treatment		Phy	AE + Phy	ET + Phy
RBC	K_d min^{-1}	0.021	0.024	-
	r	0.93	0.95	-
	$T_{½}$ min	33.5	29	-
Brain	K_d min^{-1}	0.014	0.0252	0.009
	r	0.97	0.90	0.91
	$T_{½}$ min	50.0	27.5	75.0
Heart	K_d min^{-1}	0.019	-	0.008
	r	0.95	-	0.89
	$T_{½}$ min	37.5	-	85.0
Diaphragm	K_d min^{-1}	0.01	0.039	.008
	r	0.97	0.99	0.98
	$T_{½}$ min	67.5	17.5	84.0
Muscle	K_d min^{-1}	0.012	0.008	0.012
	r	0.91	0.89	0.79
	$T_{½}$ min	55.0	83.5	60.0

RBC: Acute exercise showed a transient increase in ChE activity of RBC (114% of control) at 2 min, which returned back to almost control level after 10 min. Phy produced decrease in ChE activity with a peak effect at 10 min (67% of control) and recovered back to 79% of control at 60 min. Endurance training decreased ChE activity to 66% of control at 5 min and was almost maintained up to 60 min (76% of control). Endurance training + Phy further decreased ChE

There was a slight increase (114% of control) in rate of decarbamylation of RBC-ChE in AE + Phy (0.024/min), as compared to Phy alone (0.021/min), with $T_{½}$ of enzyme recovery to be 29 and 33.5 min, respectively (Table 6).

Brain: Acute exercise as well as endurance training produced a slight decrease in ChE activity of brain (91-97% of control) at various time points, which was not statistically significant ($p < 0.05$). Acute exercise + Phy showed an increase in ChE activity (70% of control) as compared to Phy alone (60% of control) at 15 min, which recovered to 99% of control at 60 min. Endurance training + Phy showed further decrease in ChE activity (48% of control) at 15 min, which recovered to 64% of control at 60 min (Fig. 10). It seems that acute exercise and endurance training delayed ChE recovery; however, there was almost complete recovery in acute exercise + Phy (99% of control) and slower recovery in endurance training + Phy (63% of control), as compared to Phy alone (78% of control) at 60 min.

The rate of decarbamylation was significantly increased (181% of control) by acute exercise (0.025/min) and decreased (66% of control) by endurance training (0.009/min) as compared to Phy alone (0.014/min) in brain (Table 6). The $T_{½}$ of recovery of enzyme was 50, 27.5, and 75 min in Phy, acute exercise + Phy and endurance training + Phy groups, respectively (Table 6) (Fig. 10-inset).

Heart: Acute exercise slightly decreased the ChE activity of heart (89-95% of control) at various time points. Acute exercise + Phy showed a faster recovery of ChE activity (100% of control) as compared to Phy alone (89% of control) at 60 min. Endurance training decreased ChE activity (85-75% of control) from 5 to 60 min. Endurance training + Phy produced further decrease in ChE activity (58% of control) as compared to Phy alone (69% of control) or training alone (80% of control) at 30 min (Fig. 11). Acute exercise completely recovered ChE activity (101% of control); however, training further depressed ChE activity to 73% of control as compared to Phy alone (89% of control) at 60 min.

The rate of decarbamylation was significantly decreased (44% of control) by endurance training (0.008/min) as compared to Phy alone (0.019/min). Endurance training significantly increased the $T_{½}$ of recovery of enzyme (85 min) as compared to Phy alone (37.5 min) (Fig. 11-inset).

Diaphragm: Acute exercise did not produce any significant effect on ChE activity of diaphragm (95-98% of control) at various time points. Acute exercise + Phy showed an increased ChE activity (73% of control) as compared to Phy alone (66% of control) at 15 min. Endurance training decreased ChE activity 85-78% of control from 5 to 60 min. Endurance training + Phy showed further decrease in ChE activity (50% of control) as compared to Phy alone at 15 min (Fig. 12). There was faster ChE inhibition as well as recovery by acute exercise (102% of control) as compared to Phy alone (82% of control) at 60 min. Endurance training delayed ChE inhibition as well as recovery of ChE activity.

The rate of decarbamylation of ChE activity was very significantly increased (384% of control) in acute exercise + Phy (0.039/min) and decreased (80% of control) in endurance training + Phy rats (0.008/min) as compared to Phy alone (0.01/min) (Fig. 12-inset). The $T_{½}$ of recovery of enzyme was 67.5, 17.5,

The rate of decarbamylation of ChE activity was very significantly increased (384% of control) in acute exercise + Phy (0.039/min) and decreased (80% of control) in endurance training + Phy rats (0.008/min) as compared to Phy alone (0.01/min) (Fig. 12-inset). The $T_{½}$ of recovery of enzyme was 67.5, 17.5, and 84 min in Phy, acute exercise + Phy and endurance training + Phy groups, respectively (Table 6).

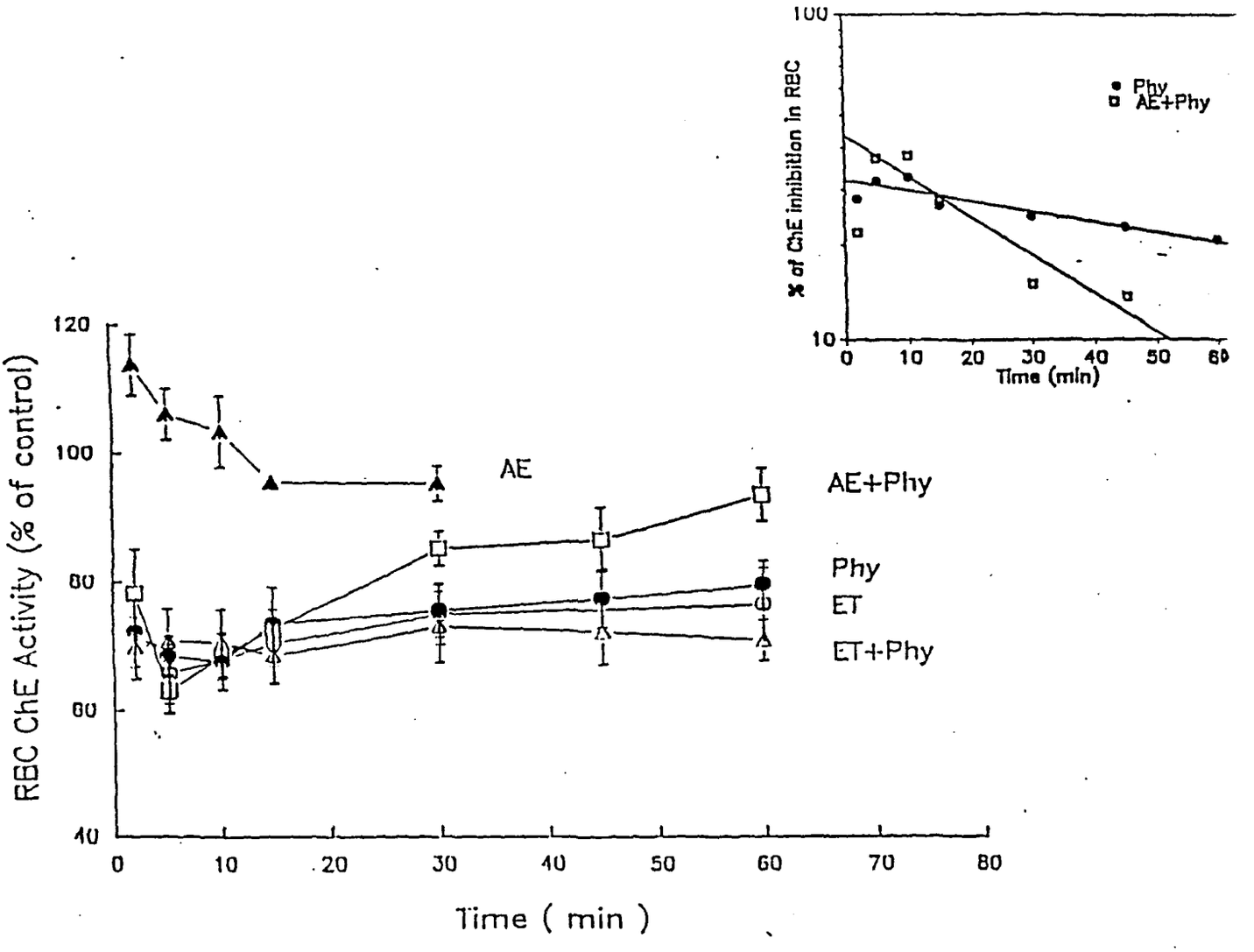


Fig. 9: Interaction of acute exercise (AE), endurance trained exercise(ET) and physostigmine (Phy) 70 µg/kg, i.m.) on time course of % control ChE activity in RBC of rats. Values are mean ± SEM. Inset figure shows the rate of decarbamylation of ChE in RBC.

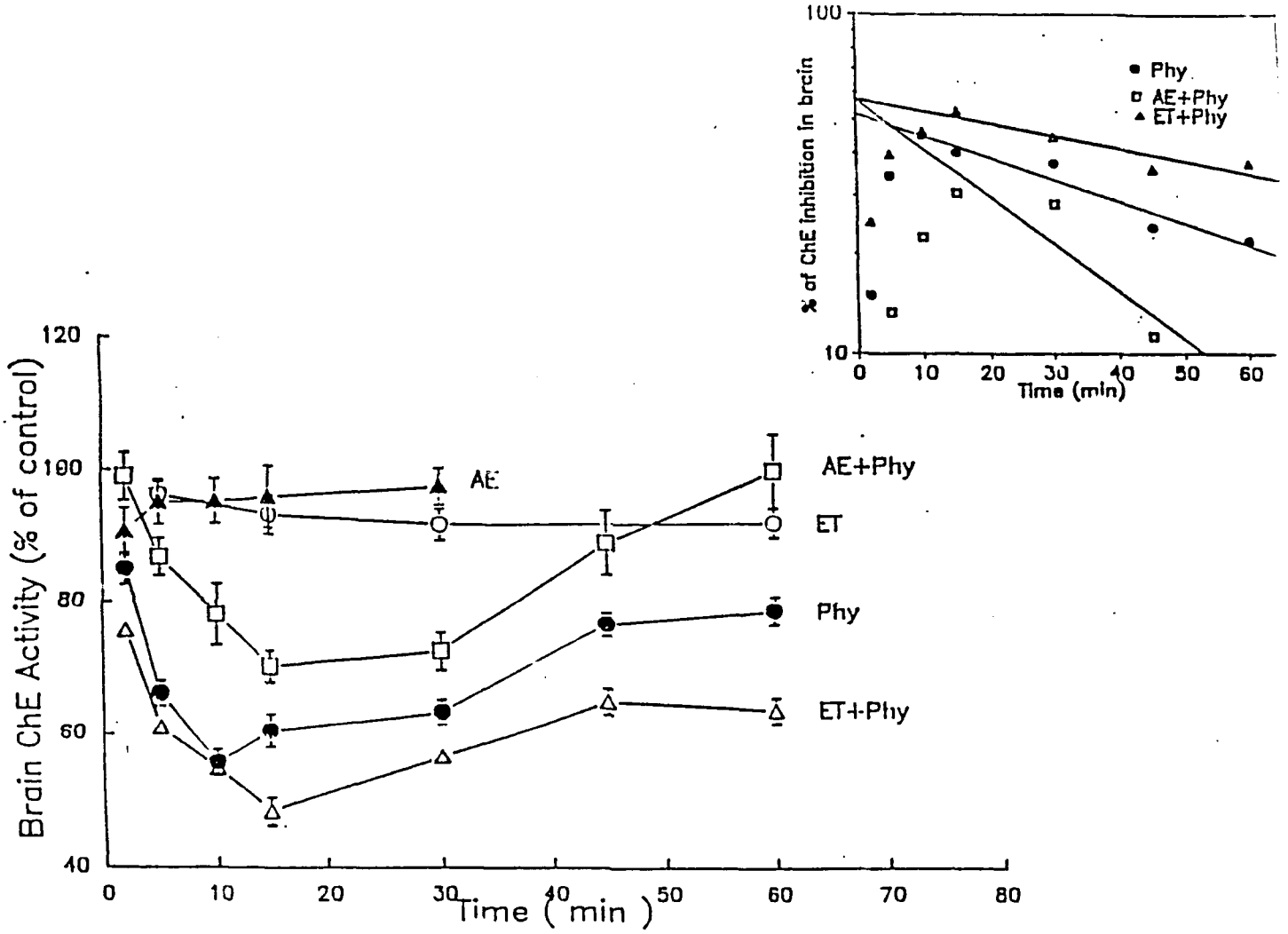


Fig 10: Interaction of acute exercise (AE), endurance trained exercise (ET) and physostigmine (Phy) 70 µg/kg, i.m.) on time course of % control ChE activity in brain of rats. Values are mean ± SEM. inset figure shows the rate of decarbamylation of ChE in brain.

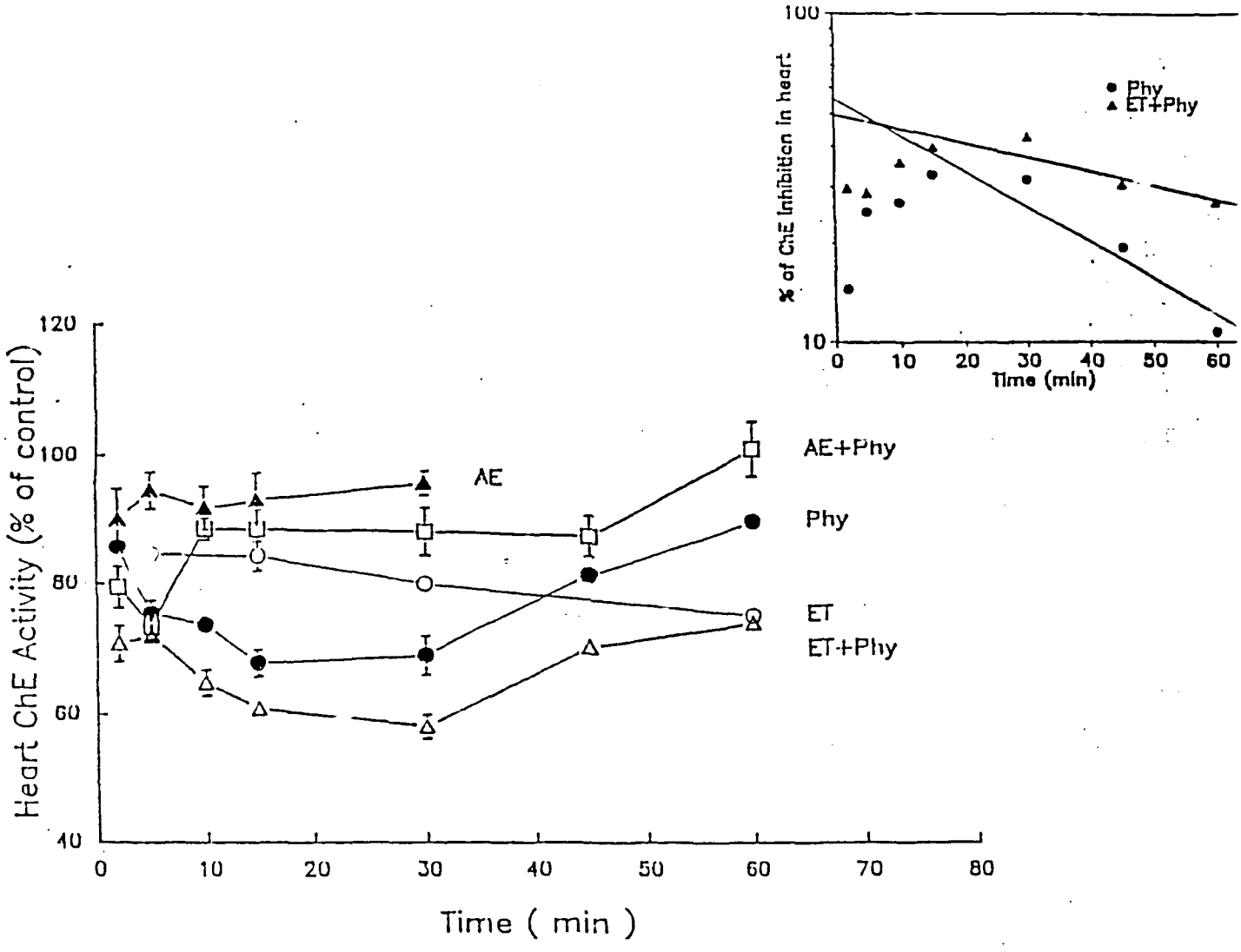


Fig. 11 Interaction of acute exercise (JAE), endurance trained exercise (ET and physostigmine (Phy) (70 µg/kg, i.m.) on time course of % control ChE activity in heart of rats. Values are mean ± SEM. Inset figure shows the rate of decarbamylation of ChE in heart.

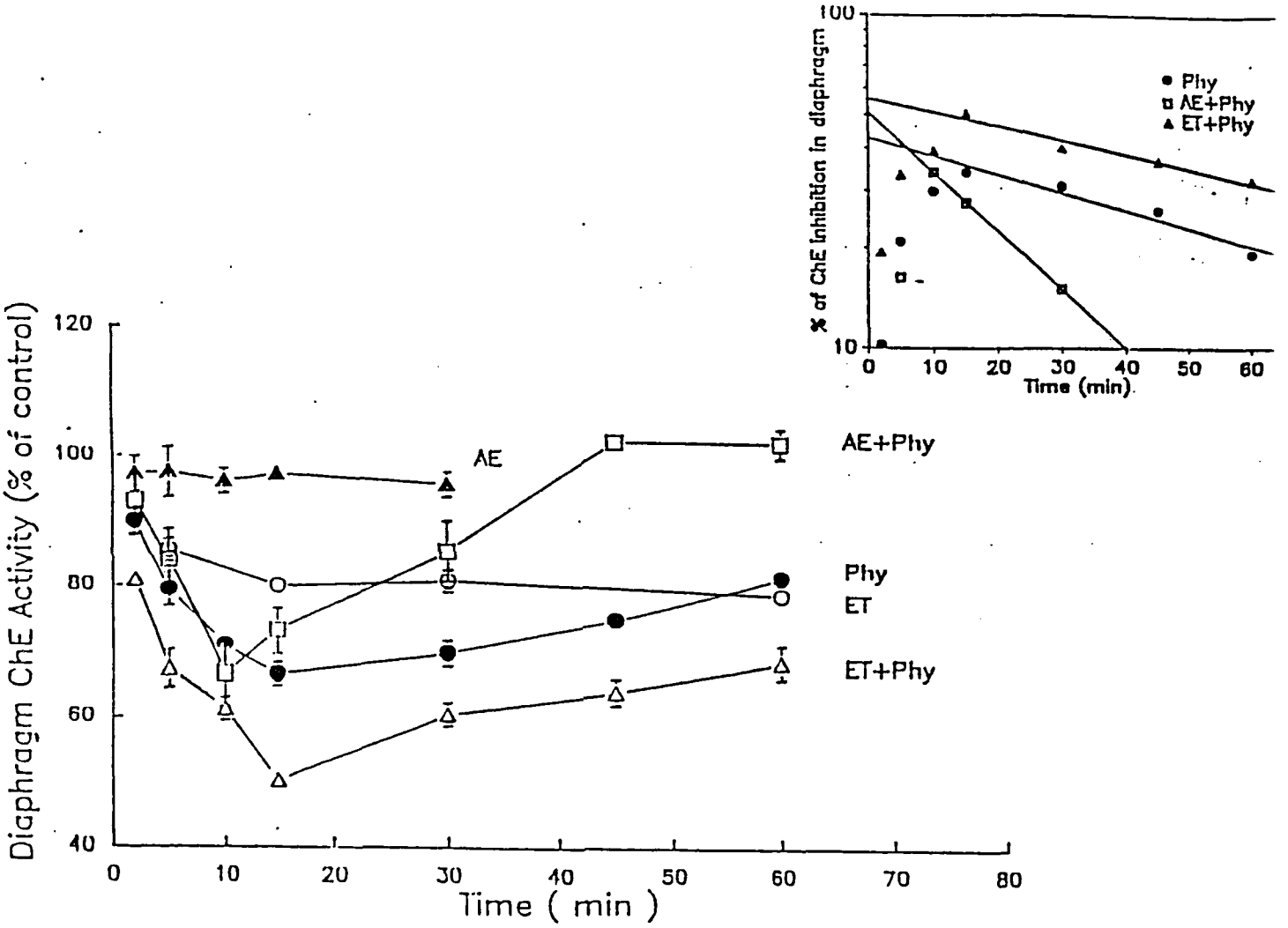


Fig. 12: Interaction of acute exercise (AE), endurance trained exercise (ET) and physostigmine (Phy) (70 µg/kg, i.m.) on time course of % control ChE activity in diaphragm of rats. Values are mean ± SEM. Inset figure show the rate of decarbamylation of ChE in diaphragm.

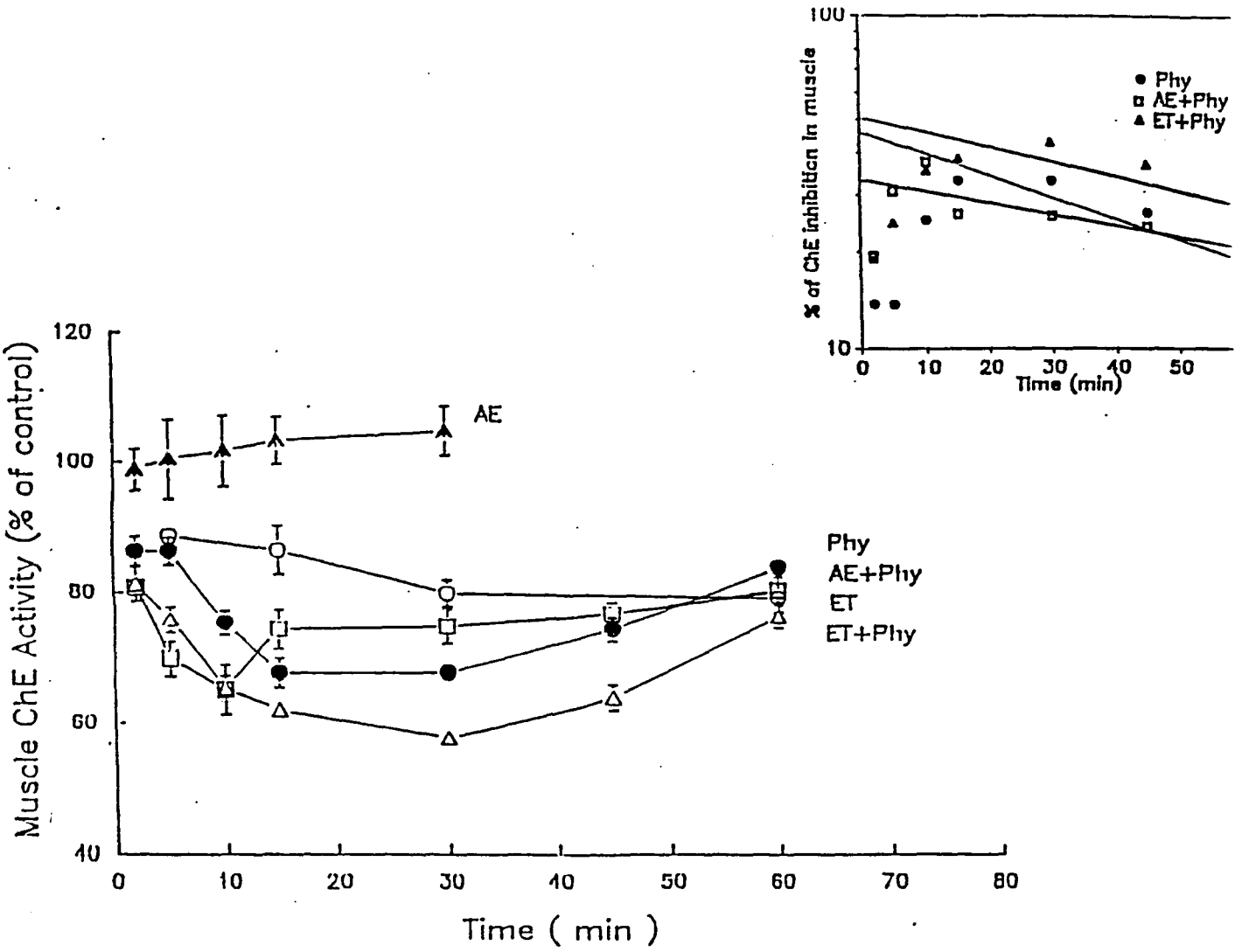


Fig. 13: Interaction of acute exercise (AE), endurance trained exercise (ET) and physostigmine (Phy) (70 µg/kg, i.m.) on time course of % control ChE activity in thigh muscle of rats. Values are mean ±SEM. Inset figure shows the rate of decarbamylation of ChE in muscle.

Muscle: Acute exercise did not produce any significant effect on ChE activity of muscle (99-105% of control) at various time points. Endurance training produced decrease in ChE activity (89-79% of control) from 5 to 60 min. Acute exercise + Phy as well as endurance training + Phy further depressed ChE activity up to 10 min, which was maintained up to 60 min in endurance training + Phy. However, acute exercise + Phy showed increased ChE activity from 15 to 45 min as compared to Phy alone (Fig. 13). Acute exercise enhanced the recovery, whereas training delayed the recovery of ChE activity.

The rate of decarbamylation of ChE activity significantly decreased (67% of control) by acute exercise (0.008/min) as compared to Phy alone (0.012/min). However, the endurance training did not affect the rate of decarbamylation (Fig. 13-inset). The $T_{½}$ of enzyme recovery was 55, 83.5, and 60 min in Phy, acute exercise + Phy, and endurance training + Phy groups, respectively.

Discussion

Effect on Metabolic Variables and Weight Gain: The prolonged exercise for 6 wk did not produce any significant effect on the metabolic variables, especially during the initial stages of an incremental exercise protocol. However, the O_2 consumption was slightly decreased due to endurance training of rats over a period of 6 wk as compared to age-matched sedentary control rats.

Effect on ChE Activity and Rate of Decarbamylation: The rate of decarbamylation of Phy-inhibited ChE varies in different tissues. The rate of decarbamylation is highest in RBC (0.021/min) and least in diaphragm (0.01/min) with intermediate rate in heart, brain, and muscle (0.019-0.012/min). The rate of decarbamylation in descending order is RBC > heart > brain > muscle > diaphragm. The $T_{½}$ of recovery of enzyme also follows the same pattern. Acute exercise (about 80% $VO_{2\ max}$) showed a transient increase in ChE activity of RBC (114% of control) at 2 min, which returned back to control level after 10 min. We have recently reported (57) that acute exercise (80% $VO_{2\ max}$) produced a slight increase in ChE activity of RBC (112% of control). This increase in ChE activity of RBC may be due to secondary effect of hypoxia, increased hemoconcentration and sequestration of RBC from spleen during initial time points to cope up with the increased demand of the body during exercise (41). Later on there may be a cholinergic acclimatization after 10 min. Endurance training followed by acute bout of exercise significantly decreased the ChE activity of RBC during an observation period of 5-60 min. The decrease in ChE activity may result in an increase in ACh level. There is a direct relationship between neurotransmitter ACh and its hydrolysing enzyme, ChE. The increased level of ACh may protect the body against the stressful situations of physical exercise (58).

The results show that Phy alone (70 µg/kg, i.m.) produced a significant decrease in ChE activity in RBC and various tissues. The maximum ChE inhibition varies 23-40% between 5 and 15 min. Phy-induced decrease in ChE activity is by its well-known anti-AChE property. Several workers have also shown the maximum effect of Phy to be within 15-30 min after its i.m. administration.

There appears to be controversy regarding the extent of ChE inhibition by Phy in various tissues of several animal species following different routes of administration (7,59). Harris et al. (9) have reported 58% ChE inhibition in

whole blood at 15 min with 70 µg/kg, i.m., dose of Phy to rat. Heyl et al. (7) have also shown similar ChE inhibition (70%) in whole blood at 15 min after 250 µg/kg of Phy, i.m., to rabbits. These authors have determined the total ChE present in whole blood, which comprises true cholinesterase (AChE) and pseudocholinesterase (BuChE). It is understandable that our value of ChE inhibition in RBC (27%) is lower because we have determined ChE enzyme in RBC and not in whole blood. The present findings on the extent of ChE inhibition are well corroborated with our earlier findings (36).

Acute exercise enhanced the recovery of Phy-induced ChE inhibition in RBC and various tissues. However, endurance training has potentiated the effect of Phy on ChE inhibition, thereby resulting in slower recovery of the enzyme. The modulatory effect of acute and endurance-trained exercise on pharmacodynamic effect of Phy may be due to several factors such as rate of decarbamylation, blood flow, rate of metabolism, $T_{½}$, etc.

Acute exercise has enhanced the rate of decarbamylation in RBC, brain, and diaphragm; however, there was a decrease in rate of decarbamylation in muscle by acute exercise. Endurance training decreased the rate of decarbamylation in brain, heart, diaphragm, and muscle. Different types of exercise have been reported to affect the blood flow in different ways (56). Change in blood flow will affect the amount of drug reaching the receptor site, thus affecting the pharmacodynamic activity. The main factor affecting drug absorption following i.m. administration is change in blood flow (60).

During exercise there is considerable increase in cardiac output with a redistribution of blood flow to different organs (56,61). The blood flow to other organs changes with exercise. Several studies have demonstrated the relationship between blood flow and exercise (55,56). Studies on the effect of different intensities of exercise in human beings have shown that moderate exercise increases the blood flow to 3-fold in heart and 10-fold in muscle, without affecting brain. The total blood flow increases to 3-fold by moderate exercise (56). In the present studies, the acute exercise (80% $VO_{2\ max}$) corresponds to moderate exercise. Thus, acute exercise has helped in quicker absorption as well as metabolism, resulting in faster recovery of ChE activity as compared to Phy alone. However, the prolonged exercise (endurance training for 6 wk) has delayed the metabolism of Phy, resulting in prolonged effect of Phy and thereby producing further decrease in ChE activity as compared to Phy alone. Ramos et al. (62) and Day et al. (63) have also shown that chronic exercise decreased the metabolism of hexobarbitone, resulting in increased sleeping time. It is possible that prolonged moderate exercise (endurance training) might be decreasing the hepatic blood flow, resulting in decreased clearance and rate of elimination of Phy, thereby potentiating the effect of Phy on ChE inhibition.

In the present studies, we have determined the ChE activity of thigh muscle in general and not in slow and fast muscle separately. Hence, we could not observe any significant change in ChE activity of muscle by acute exercise. It has been reported that muscle blood flow can vary as much as 19% (64) in different muscles, and there are large individual variations in drug absorption. Pedzikiewicz et al. (49) have reported an increase in muscle ChE activity (20%) after a short physical exercise. These authors reported the increase in ChE activity due to an increase in blood flow in skeletal muscles (47). However,

muscarinic cholinergic receptors do not play a significant role in elevating muscle blood flow in conscious rats, either during anticipatory phase or during slow locomotor exercise (48). Selective increase in G_4 AChE activity of adult male Fischer rats subjected to treadmill exercise has been reported by Fernandez and Donoso (65).

The present study also indicates that acute or endurance-trained exercise for 6 wk did not significantly alter the cholinergic functions of brain. This is in agreement with the commonly held belief that physical exercise does not improve the brain functions. However, acute exercise as well as training both modify the pharmacodynamic effect of Phy on ChE activity in brain. Acute and endurance trained exercise modulate both cholinesterases- AChE in RBC and brain and BuChE in heart, diaphragm, and muscle. It is possible that free drug concentration might be decreased during acute exercise and may be increased during endurance training on the brain, compared to Phy-administered sedentary control rat.

Our findings are in agreement with the reported pharmacokinetic observations (66). These authors have shown that training increased the area under the curve (AUC) from 579 to 834 ng/ml and $T_{½}$ from 8.8 to 15.7 min as compared to Phy alone. They have also reported the decrease in clearance from 121 to 84 ml/min/kg as well as rate of elimination from 0.08 to 0.04/mins as compared to Phy alone. Several authors have reported a decrease in clearance of many drugs by exercise (67,68).

In conclusion, acute exercise transiently increased ChE activity in RBC, which returned to normal within 10-15 min. Endurance training decreased ChE activity of RBC without affecting other tissues. Acute exercise enhances the rate of decarbamylation and decreases $T_{½}$ of recovery of enzyme as compared to Phy alone. However, endurance training potentiates ChE inhibition in RBC and various tissues due to decreased metabolism of Phy, possibly due to adaptation. Endurance training for 6 wk may be beneficial to prolong and potentiating the ChE inhibition by Phy. Endurance training may help in reducing the required dose of Phy and may be advantageous where Phy is used as a pretreatment drug against organophosphate intoxication.

V. EFFECT OF PHYSOSTIGMINE ON POST-EXERCISE PLASMA LACTATE AND PYRUVATE LEVELS OF UNTRAINED AND TRAINED RATS

Introduction

Phenolic drugs are known to alter mitochondrial function (69). A drug that changes the mitochondrial redox state may alter the balance between aerobic and anaerobic energy substrates within the cell. Phy, an anticholinesterase agent, was believed to be a potential pretreatment drug for organophosphate intoxication (34,36). Its treatment effects have significance relative to soldiers who may be exposed to chemical warfare. Phy has been a trial drug for the improvement of memory function in patients with Alzheimer's disease (70,71).

Phy is metabolized to eseroline, a phenolic compound, in plasma, muscle, brain, and liver (1,72). Recently Somani et al. (61), reported that eseroline causes neuronal cell death of mouse neuroblastoma (NLE 115), rat glioma (C_6), and neuroblastoma-glioma hybrid (NG 108-15) in *in vitro* studies. The mechanism of cell death seems to involve loss of cell ATP. King and Somani (73) studied the time course of an accumulation of [^{3}H]-Phy in brain subcellular fractions and reported that the mitochondrial RA increased continuously up to 60 min. The increase of Phy and its degradation products, eseroline and rubreserine, in mitochondria may interfere with normal physiological function of this organelle, resulting in altered redox potentials and subsequent changes in lactate and pyruvate concentrations. Changes in cellular concentrations of lactate and pyruvate will usually be reflected in plasma levels of lactate, since there is a concentration gradient and carrier-mediated "efflux" from the working muscle (74).

The level of blood lactate provides a fairly objective indication of the relative anaerobic demand of exercise (75). Upon its efflux from working skeletal muscle, lactate may be excreted in the urine or via sweat, it may be converted to glucose or protein, and it may be oxidized by various tissues in the body (76). A significant amount of lactate is oxidized and utilized as an energy source by working skeletal muscle. This process occurs not only during exercise but also during recovery from exercise. It is unknown if lactate would still be metabolized as readily during recovery if an individual was treated with Phy. For individuals exposed to chemical warfare, recovery from military exercises would be important in maintaining a state of "physical readiness."

The level of physical training may also have a significant effect on how individuals may be able to respond to chemical warfare. In endurance-trained individuals, the build-up of lactate concentrations in the blood is known to be attenuated when compared to untrained individuals when both of these groups are subjected to the same absolute workload. Donovan and Brooks (77) attribute this occurrence to an increase in plasma lactate clearance in endurance-trained subjects. However, Favier et al. (78) suggest that endurance training induces a slower production of lactate in contracting skeletal muscle. In either instance, it would seem that physically trained individuals would have a greater "recovery" potential from acute bouts of exercise. When considering the potential effect of Phy on mitochondrial function it is of interest to know what effect this may have on recovery from exercise performance, particularly in individuals in the armed forces exposed to anti-ChE agents. Therefore, this

study was designed to investigate the effect of Phy on postexercise recovery relative to lactate accumulation in the blood. Dependent upon the effect of Phy on mitochondrial function, it is possible that the metabolism of pyruvate may also be effected. Therefore, pyruvate levels in the blood were also examined.

Methods

Male Sprague-Dawley rats (initial weight 160-200 g) were used. Rats were divided into 6 groups: Gr I - sedentary control (SC), saline administration; Gr II - acute exercise (80% $VO_{2\ max}$) (AE); Gr III - endurance trained + acute bout of exercise (80% $VO_{2\ max}$) (ET); Gr IV - Phy (70 µg/kg i.m.) (Phy); Gr V - acute exercise (80% $VO_{2\ max}$) + Phy (70µg/kg i.m.) (AE + Phy); Gr VI - endurance trained + acute bout of exercise (80% $VO_{2\ max}$) + Phy (70 µg/kg i.m.) (ET + Phy).

Rats from Gr III and Gr VI were acclimatized and then trained on a 9-channel motor-driven treadmill (built in our SIU workshop) using an incremental exercise program. During this program of exercising, the speed (meters/min), angle of inclination (% grade), and the duration (min) of exercise were varied to obtain progressive levels of exercise intensity as shown in Table 5 (Section IV).

Rats from Gr I,II,IV, and V were not trained but were maintained under similar environmental conditions to those of the endurance trained rats. Each rat's weight was recorded daily before exercising the rats on the treadmill in order to determine the body weight changes during the entire period of training.

Determination of maximum oxygen consumption ($VO_{2\ max}$) was carried out prior to the beginning of the training protocol in order to determine the $VO_{2\ max}$ for each rat. Measurement of $VO_{2\ max}$ was considered valid only if the animal ran until it could no longer maintain pace with the treadmill. During the training, $VO_{2\ max}$ was determine for each rat on the fifth day of every week.

On the day of the experiment, rats from Gr I which served as sedentary control received saline and were sacrificed immediately. Rats from Gr IV were administered [^{3}H]-Phy (70 µg/kg, i.m.) and were sacrificed at 2, 5, 10, 15, 30, 45, and 60 min after injection. Rats from Gr II and V were exercised until they reached 80% $VO_{2\ max}$ and were subsequently removed from the treadmill. Rats from Gr V were then administered Phy (70 µg/kg i.m.), and were sacrificed at 2, 5, 10, 15, 30, 45, and 60 min after exercise in conjunction with rats from Gr II. The rats from Group III were endurance-trained and were subjected to acute bouts of exercise (80% $VO_{2\ max}$) for 20 min using incremental exercise protocol, and they were decapitated at 5, 15, 30, and 60 min. The rats from Group VI were endurance-trained and were subjected to an acute bout of exercise at 80% $VO_{2\ max}$ and then administered [^{3}H]-Phy (70 µg/kg, i.m.) and were sacrificed at 2, 5, 10, 15, 30, 45, and 60 min.

Blood was collected into precooled centrifuge tubes after decapitation. Plasma was separated from blood immediately at 4°C by centrifugation for 10 min at 5000 RPM (Jouan Inc., Winchester, Va) then deproteinized with 8% (w/v) perchloric acid. The supernatant was used for the estimation of lactate and pyruvate. Lactate and pyruvate were determined by the enzymatic method of Fleischer (1970) (79) (Sigma Diagnostics, St. Louis, MO) and expressed as mM.

Phy free base was obtained from Sigma Chemical Co. (St. Louis, MO). [^{3}H]-Phy (13 Ci/mmol) was custom-synthesized by Amersham Corporation (Chicago, IL). Diagnostic kits were purchased from Sigma Chemical Co. (St. Louis, MO) for the determination of lactate and pyruvate. All other chemicals were of analytical-grade and were obtained from the usual commercial sources.

Phy was labeled with tritium on both ortho positions to the carbamate chain on the aromatic ring of Phy. [^{3}H]-Phy was diluted with unlabeled Phy (162.07 μCi/140 μg/ml). The solution was prepared using physiological saline (0.9% w/v) in which 10 μl of hydrochloric acid was added to ensure that the solution was in an acidic pH range. The purity of Phy was assessed using high-performance liquid chromatography.

The data were subjected to a parametric two-way analysis of variance for unequal n's, using a general linear model approach. This approach tested the overall effect of experimental groups with time, both as independent factors. To compare experimental groups against the control group, a one-way analysis of variance was performed at each time point. In addition, each time point was compared in each group to evaluate the effect of time. Follow-up tests were performed using Duncan's multiple range test. Statistical significance was evaluated at the $p < .05$ level.

Results

The "Phy-dosed" (Gr IV) group elicited a significantly elevated plasma lactate from 2 min to 60 min after exercise (11.21 $\pm$ 0.76 to 4.86 $\pm$ 0.61 mM) (Fig. 14B) compared to sedentary "saline-dosed" (Gr I) rats (3.67 $\pm$ 0.52). The acute exercised + Phy (Gr V) had a significantly higher plasma lactate (7.40 $\pm$ 0.72) compared to the acutely exercised (Gr II) (4.18 $\pm$ 0.3) without Phy administration at 2 min after exercise. Thereafter, plasma lactate values did not differ between the acutely exercised groups during recovery. Endurance-trained rats treated with Phy (Gr VI) had significantly lower plasma lactate values from 5 to 60 min after exercise (5.89 $\pm$ 1.0 to 4.36 $\pm$ 0.29) (Fig. 14C), compared to the endurance-trained rats without Phy (Gr III) (6.50 $\pm$ 0.75 to 5.12 $\pm$ 0.61) (Fig. 14A) (Table 7).

Table 7: Effect of exercise (acute and endurance trained), physostigmine and exercise + physostigmine on time course of plasma lactate levels (mmol/L) in rats.

Time (min)	SED Control Group I	Acute Exercise Group II	Endurance Trained Group III	Phy Admin Group IV	Acute Exercise+Phy Group V	Endurance Trained+Phy Group VI
2	3.67±.52	4.18±0.30		11.22±0.76	7.40±0.72	7.40±0.86
5	3.67±.52	5.39±0.88	6.50±0.75	8.48±1.68	5.24±0.34	5.89±1.00
10	3.67±.52	4.51±0.37		5.12±0.39	4.92±0.69	5.31±0.27
15	3.67±.52	3.68±0.36	5.65±0.99	5.32±0.68	2.59±0.43	5.01±0.41
30	3.67±.52	3.78±0.56	5.48±0.61	4.88±0.66	4.29±1.45	5.12±1.45
45	3.67±.52			5.09±0.28	4.49±0.48	4.90±0.35
60	3.67±.52		5.12±0.61	4.86±0.61	3.75±0.57	4.36±0.29

Values are mean of 4 observations ± S.E.M.

Plasma pyruvate levels of the "Phy-dosed" (Gr IV) were significantly elevated above the "saline dosed" sedentary control (Gr I) for up to 30 min after injection (0.26 ± .07 to 0.12 ± .06 vs. 0.13 ± .02, respectively) (Fig. 14B). The acutely exercised + Phy (Gr V) elicited significantly higher plasma pyruvate levels at 2 min after exercise (0.28 ± .04) compared to the acutely exercised (Gr II) not receiving the drug (0.20 ± .01). This trend appeared to continue up through 30 min after exercise for the acutely exercised groups. With the endurance-trained groups, those rats receiving Phy (Gr VI), immediately after exercise had significantly lower plasma pyruvate levels from 5 to 30 min after exercise (0.18 ± .06 to 0.10 ± .097) (Fig. 14C) compared to their counterparts who didn't receive the drug (Gr III) (0.19 ± .05 to 0.13 ± .02) (Table 8) (Fig. 14A).

Table 8: Effect of exercise (acute and endurance-trained), physostigmine and exercise + physostigmine on time course of plasma pyruvate levels (mmol/L) in rats.

Time (min)	SED Control Group I	Acute Exercise Group II	Endurance Trained Group III	Phy Admin Group IV	Acute Ex + Phy Group V	Endurance Trained+Phy Group VI
2	0.13±.02	0.20±0.01		0.26±0.07	0.28±0.04	0.18±0.04
5	0.13±.02	0.20±0.02	0.19±0.05	0.35±.10	0.18±0.04	0.18±0.06
10	0.13±.02	0.16±0.02		0.17±0.03	0.34±0.03	0.16±0.01
15	0.13±.02	0.16±0.02	0.15±0.01	0.16±.0.04	0.18±0.03	0.11±0.01
30	0.13±.02	0.14±0.02	0.13±0.02	0.12±0.06	0.17±0.04	0.10±0.09
45	0.13±.02			0.11±0.02	0.14±0.04	0.10±0.02
60	0.13±.02		0.09±0.01	0.09±0.03	0.14±0.03	0.09±0.02

Values are mean of 4 observations ± S.E.M.

The lactate/pyruvate ratio (L/P), sometimes used as an indicator of intracellular or intravascular changes was significantly greater throughout 60 min after injection for the Phy-dosed group (43.2 to 54.0, from 2 to 60 min after injection, respectively) compared to a L/P of ?8.2 in the saline-dosed sedentary control (Gr I). In the acutely exercised groups, there was only a slight increase in L/P for both groups from 2 to 30 min after exercise (20.9 to 27.0 for the AE (Gr II) vs. 22.9 to 25.2 for the AE+Phy (Gr V). L/P did not significantly differ through these time points for the acutely exercised groups. In the endurance-trained groups, the L/P was similar at 5 min after exercise for both groups (33.9 for ET (Gr III) vs. 33.4 for ET+Phy (Gr VI)). But by 60 min after exercise, there was an approximate 17% higher L/P in the endurance-trained group receiving Phy vs. the endurance-trained group not receiving Phy.

Discussion

The results of this investigation showed that when acute exercise precedes Phy dosing, the plasma levels of both lactate and pyruvate are diminished. Further, rats that are untrained and subjected to Phy following an acute bout of exercise are more likely to experience elevated lactate and pyruvate levels in the blood shortly after the cessation of exercise compared to those not receiving the drug. However, this effect does not appear to linger beyond 2 min after exercise. When these rats are endurance-trained, lower lactate and pyruvate levels appear in the blood when given Phy vs. when they don't receive the drug.

It is clear that Phy-dosing in sedentary rats raises lactate and pyruvate levels beyond that observed in rats receiving a similar dosing of saline. This suggests that Phy is interrupting the "normal" oxidizing reducing capacity of the mitochondria as suggested by King and Somani (73). If Phy and its metabolites

(quinone compounds) accumulate in muscle cell mitochondria, this circumstance may interfere with the cell's redox state. Olgin et al. (75) reported that changes in the muscle reflect changes in muscle mitochondrial redox state and concluded that an increase in L/P ratio could reflect the redox state of mitochondria during exercise. Phy and its metabolites may create a type of stress similar to exercise. An accumulation of quinone-type compounds in muscle mitochondria may alter the NADH/NAD ratio which is proportional to changes in cytosolic lactate/pyruvate ratio (80). As noted by Olgin et al. (75), these occurrences within the muscle would potentially be reflected in the blood due to the concentration gradient build up between these two intervascular areas. We observed a L/P ratio of (43.2 to 54.0, from 2 to 60 min after injection) in plasma of Phy-dosed rats compared to 28.2 of saline-dosed rats. This significant "quantitative" difference lends support to the possibility that muscle mitochondrial function has been altered by Phy. Yet other possibilities may exist which have not been experimentally explored, such as an enhancement of rate-limiting enzyme activities particularly within the process of glycolysis.

One factor which did alter the build-up of lactate and pyruvate in the plasma was the incorporation of exercise just prior to Phy-dosing. In untrained animals who were subjected to acute exercise up to 80% $VO_{2\ max}$, the level of plasma lactate and pyruvate at 2 min after exercise was 63% and 24% lower, respectively, compared to the sedentary group that was Phy-dosed. When Phy was administered to animals who were acutely exercised, plasma lactate and pyruvate were attenuated less than the animals who didn't receive the drug when compared to the sedentary, Phy-dosed group. In this case, plasma lactate and pyruvate levels were 34% lower and 7% higher at 2 min after exercise compared to the sedentary, Phy-dosed group. These results suggest that Phy only has a temporary effect on raising lactate levels in the blood in the early stage of recovery from a submaximal exercise effort at 80% $VO_{2\ max}$. It is likely that since blood flow is still elevated, above "resting" in the early stages of recovery that plasma lactate and pyruvate were more readily transported and metabolized at various target tissues. It may also be true that Phy was more readily metabolized during the early part of recovery, since the metabolic rate is still elevated.

Another factor which has been shown to effect plasma lactate (and potentially plasma pyruvate) levels in response to a single exercise bout is endurance training. Favier et al. (78) reported that there was a 28% less build-up of lactate in trained skeletal muscle compared to that in an untrained, sedentary animal in response to an acute exercise bout. However, contrary findings have been reported which show that submaximal exercise results in slightly greater lactate concentrations in skeletal muscle and blood of trained compared to untrained animals and humans (81,82,83,84). This latter finding would lend support to our study in that plasma lactate was slightly higher from 5 to 30 min after exercise in the trained groups compared to untrained, acutely exercised groups.

From this investigation, it appears that Phy dosing is associated with a metabolic stress in that elevated plasma lactate and pyruvate levels were observed in sedentary, untrained rats. Exposure to Phy, immediately after exercise in untrained animals, only results in a temporary metabolic stress. In the case of endurance-trained rats, the metabolic stress of both the acute exercise and Phy dosing is reduced during recovery from a submaximal exercise effort.

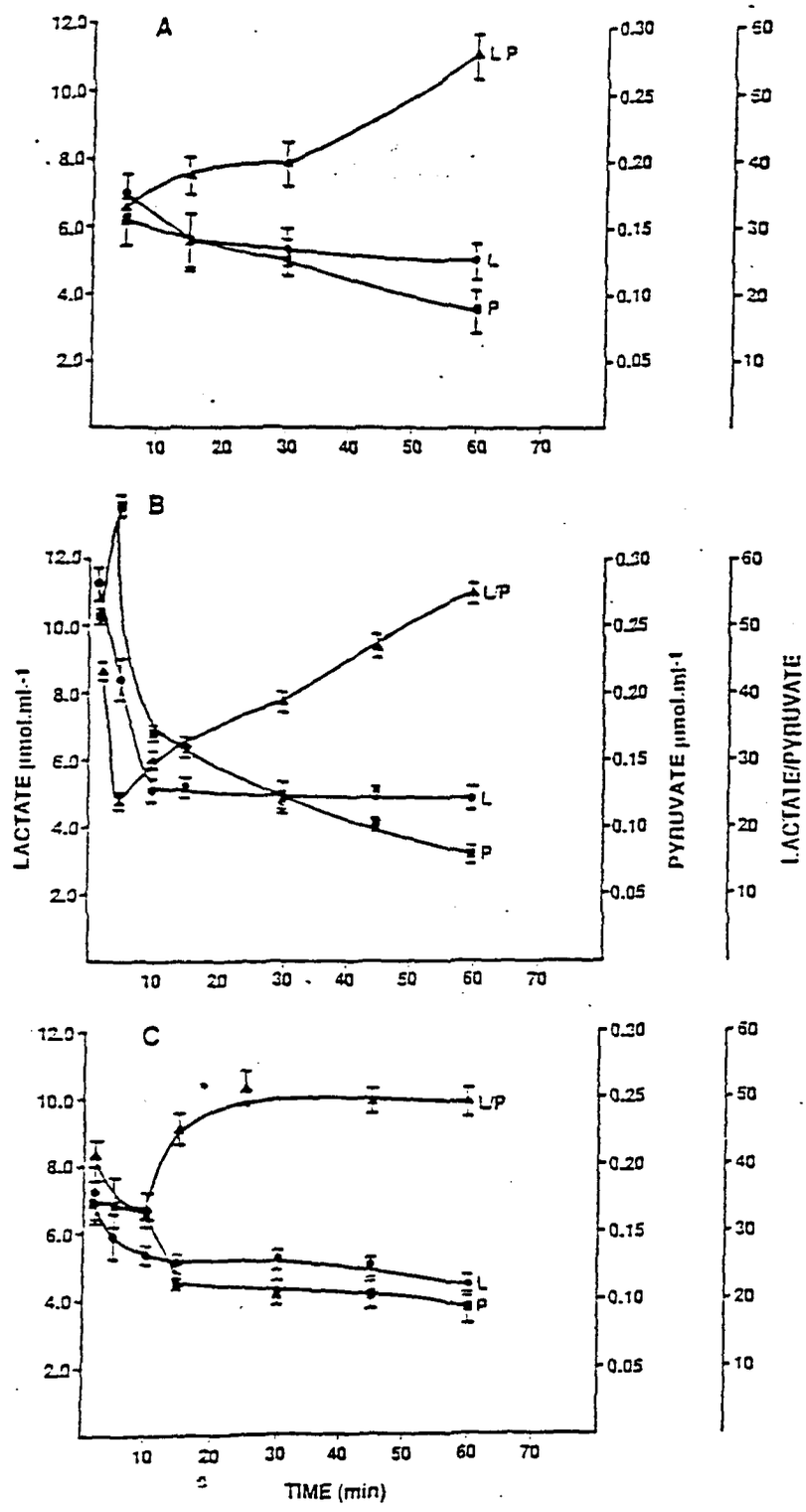


Fig. 14: Plasma lactate (L), pyruvate (P) and lactate-to-pyruvate ratio (L/P) during recovery period after endurance training (A), physostigmine (Phy) (70 µg/kg i.m.) administration (B), endurance training + Phy (70 µg/kg i.m.) administration (C) in rats. Values are mean ± SEM; n=4. Control lactate 5.05 ± 0.30 $\mu mol.ml^{-1}$

VI. EFFECT OF TRAINED EXERCISE ON DISTRIBUTION AND PHARMACOKINETICS OF PHYSOSTIGMINE

Introduction

The effect of various drugs on exercise in man and animals has been reported, but there are very few reports with regard to the effect of exercise on distribution and pharmacokinetics of drugs. Recently Somani et al. (61) have reviewed the effect of exercise on disposition and pharmacokinetics. Several mathematical models have been discussed relating the effect of altered blood flow to drug absorption, taking into account altered permeability (66,85,86,87,88), distribution (89,90), elimination (91,92,93,94,95,96,97,98,99), and general disposition (100). Although these models provide many interesting theories of the differential effects of blood flow on the pharmacokinetics of flow vs. capacity-limited drugs, one- vs. two-compartmental models, or permeable vs. impermeable drugs, they do not discuss the effect of exercise which may alter the drug pharmacokinetics.

Phy, an alkaloid, is a flow-limited, poorly plasma bound, and highly extracted drug. It was considered as a potential prophylactic agent against organophosphate poisoning (34). The pharmacokinetics and pharmacodynamics of Phy and the distribution of [^{3}H]-Phy and metabolites in various tissues of rat after i.v., i.m., and oral administration have been reported by Somani and Khalique (1,2,3). There is no information as to whether trained exercise affects the distribution and pharmacokinetics of Phy and metabolites.

During exercise, there is a considerable increase in cardiac output with a redistribution to various organs. The alterations in blood flow to the different organs will also affect the distribution of the drug in the body. Moreover, the changing blood flow will affect the amount of the drug reaching the receptor site, thus affecting the pharmacodynamic activity.

It seems that endurance-trained exercise is likely to alter the distribution and pharmacokinetics of flow-limited drugs, such as Phy. Therefore the purpose of this investigation is to study the effect of endurance-trained treadmill exercise on time course of distribution of RA in plasma, brain, liver, muscle, heart, kidney, and lung after intramuscular administration of [^{3}H]-Phy during the recovery phase after exercise. This investigation was also carried out to study the effects of endurance-trained exercise on pharmacokinetic parameters of Phy.

Materials and Methods

Phy (free base) was obtained from Sigma Chemical Co. (St. Louis, MO). [^{3}H]-Phy (13 Ci/mmol) was custom-synthesized by Amersham Corp. (Chicago, IL). Monophase 40 Plus was obtained from Packard Instruments (Downers Grove, IL). All other chemicals were analytical-grade and were obtained from the usual commercial sources.

Preparation of [^{3}H]-Phy Solution: Phy is labeled with tritium on both ortho positions to the carbamate chain on the aromatic ring of Phy. [^{3}H]-Phy was diluted with unlabeled Phy (167.07 μc/140 μg/ml). The solution was prepared using physiological saline (0.9% w/v) in which 10 μm of hydrochloric acid was

using physiological saline (0.9% w/v) in which 10 µm of hydrochloric acid was included to ensure that the solution was in an acidic pH range. The purity of Phy was assessed using high performance liquid chromatography (HPLC) using an ultraviolet detector and also by monitoring the [^{3}H]-Phy in the eluant. The solution used in all experiments was greater than 95% pure.

Animals: Male Sprague-Dawley rats (initial weight 160-200 g) were divided into 2 groups. Control-Phy: Rats were administered [^{3}H]-Phy (70 µg/kg) (specific activity 167.07 µCi/140 µg/ml). Trained exercise + Phy: Rats were endurance-trained as per Table 5; acute bout of exercise (80% $VO_{2\ max}$) was given as per Table 2 prior to [^{3}H]-Phy dose 70 µg/kg i.m. (specific activity 167.07 µCi/140 µg/ml) and sacrificed.

Endurance Exercise Training of Rats: Rats were trained on a 9-channel motor-drive treadmill (built in our Southern IL University School of Medicine workshop), utilizing an incremental exercise program 5 days a wk for 6 wk duration. During this program of exercise, the speed (meters/min), angle of inclination (degrees), and the duration (min) of exercise were varied to obtain different levels of exercise intensity as shown in Table 5.

In the first 2 wk, conveyor belt speeds were 8.2, 15.2, and 19.3 m/min, and the angle of inclination was 6°. Exercise duration was 5 min the first wk and 10 min the second wk. In the third and fourth wk the speeds were maintained at 19.3, 26.8, and 30.3 m/min. The duration of exercise was 10 min. The angle of inclination was 6° during the third wk and 9° in the fourth wk. The final 2 wk of exercise involved sustaining speeds of 19.3, 26.8, and 30.3 m/min at a 9° angle of inclination for 10 min.

Dosing and Sacrificing of Rats: On the day of the experiment, trained rats were subjected to an acute bout of exercise 80% $VO_{2\ max}$ on the treadmill (Omnitech Electronics, Inc., Columbus, OH) using incremental exercise protocol (Table 2). The rats were administered [^{3}H]-Phy (70 µg/kg i.m.; specific activity 167.07 µCi/140 µg/ml) and were sacrificed at time intervals of 2, 5, 10, 15, 30, 45, and 60 min. The control group was maintained similarly to the trained group, but was not exercised. On the day of experiment, rats from the control group were administered [^{3}H]-Phy (70 µg/kg i.m.; specific activity 167.07 µCi/140 µg/ml) and were sacrificed at the same time intervals as above. Plasma was separated from blood at 4°C immediately through centrifugation for 10 min at 5,000 rpm (Jouan, Inc., Winchester, Va.) and 50 µl of plasma was used for sample oxidizer. Brain, liver, kidney, heart, lung, and muscle were removed, rinsed with ice-cold saline, and blotted dry. The tissues were immersed in liquid nitrogen for 30 sec, wrapped in aluminum foil, and stored at -70°C until analysis.

Determination of [^{3}H]-Phy By HPLC: Phy was determined by radiometric method in plasma by HPLC by modifying the procedure of Somani and Khalique (2). The HPLC was performed using a Waters C_{18} µBondapak column (30 cm x 0.39 cm i.d.). The flow rate was adjusted to 2 ml min^{-1}. A 100-µl sample loop was used to load the column. The mobile phase consisted of 0.005 M octanesulfonic acid, 0.005 M sodium phosphate monobasic, and 1% v/v/ acetic acid in a mixture of methanol: water (40:60). The pH of the mobile phase was 3.1. Fractions from the HPLC eluant were collected at 0.5-min intervals for 15 min in scintillation vials. Scintillation fluid (Ready-Solv EP) was then added to each fraction and

the RA was counted. Radiochromatograms were graphed for disintegrations per min (dpm) in each fraction vs. retention time. The percentages of RA at the retention time of Phy and metabolites were calculated from the total RA in the HPLC fractions. The amount of Phy in plasma and brain was then calculated on a per milliliter or per gram basis from RA as per (2). The accuracy and precision of the method were ascertained by analyzing 5 different plasma and brain samples with the same amount of [[^{3}H]]Phy. The mean recovery from plasma was 97.9%, with a coefficient of variation of 6.4%. The recovery in brain was 99.9%, with a coefficient of variation of 4.6%.

Radioactivity Counting in Plasma and Tissues: Tissue samples (100-300 mg) of brain, liver, kidney, heart, lung, and muscle were brought to room temperature, and the RA was measured using a Packard Tri-Carb Model 306 sample oxidizer and a Beckman LS 5800 liquid scintillation counter. The efficacy of the sample oxidizer was found to be more than 95%.

Analysis of Data: The concentration of tissue RA, which constituted [^{3}H]-Phy + metabolites, was expressed as nCi g^{-1} wet weight of tissue. The total RA in plasma was expressed as nCi ml^{-1} of plasma. The concentration of RA vs. time was plotted on semilog graph and the regression line was obtained. The elimination rate constant (Ke) of Phy + metabolites was determined from the regression line and the t½ was calculated from Ke ($t_{½} = \frac{0.693}{Ke}$).

Pharmacokinetics of Phy: The Phy concentration of plasma was plotted as a function of time on semilogarithmic graph paper. The peak plasma concentration (C_{max}) represented the time at which the rate of absorption became equal to the rate of elimination. Initial estimates were obtained graphically by the method of residuals, and these values were used as initial values for NONLIN Program.

Pharmacokinetic Analysis Using PC-NONLIN Program: The plasma concentration-time curves for all data points were subjected to analysis utilizing a nonlinear modeling program (PC-NONLIN, Statistical Consultants, Inc., Lexington, KY) on a Zenith computer (Model 50) to derive pharmacokinetic constants. A one-compartment model with first-order input and first-order output and no lag-time was used. The absorption rate constant (k_a) and elimination rate constant (k_e) values were needed as initial values to compute k_a and k_e using the above PC-NONLIN modelling program. K_a is the rate constant at which the drug enters the central compartment from outside the system, and k_e is the rate constant at which the drug leaves the system from the single compartment.

Statistical Analysis: The data was analyzed by one-way analysis of variance to examine whether trained exercise affected the distribution of RA in different tissues. This analysis was conducted on plasma and each tissue separately. In addition, means at 2 to 60 min were compared using the Garnes-Howell test for multiple comparisons. This test indicated whether changes in drug + metabolite levels were significant from one time to the next. Statistical significance was evaluated at 0.05 level.

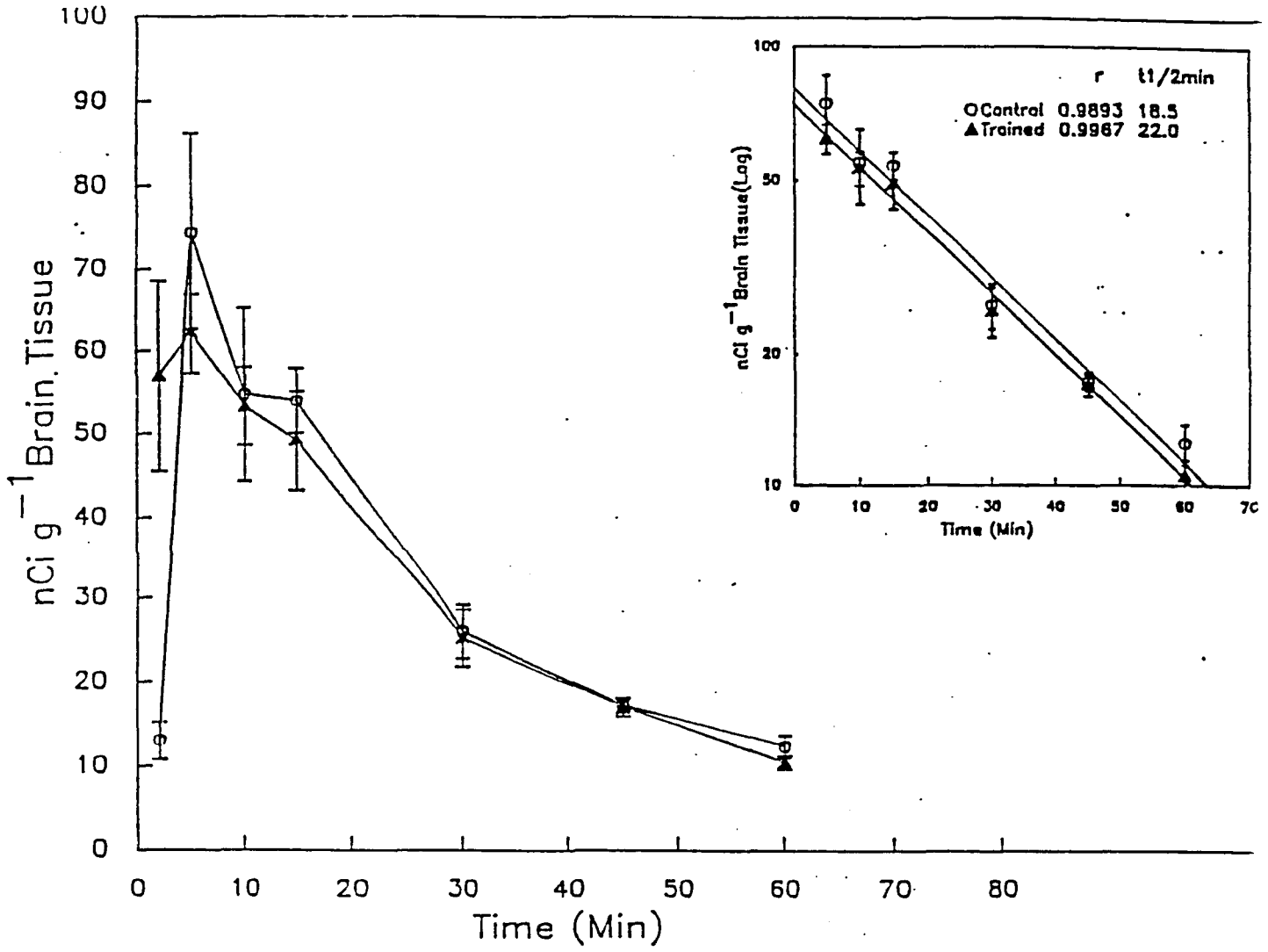


Fig. 15: Time course of radioactivity in brain of control (O - O) and trained (▲ - ▲) rats after [^{3}H]-physostigmine (70 g/kg, i.m.).

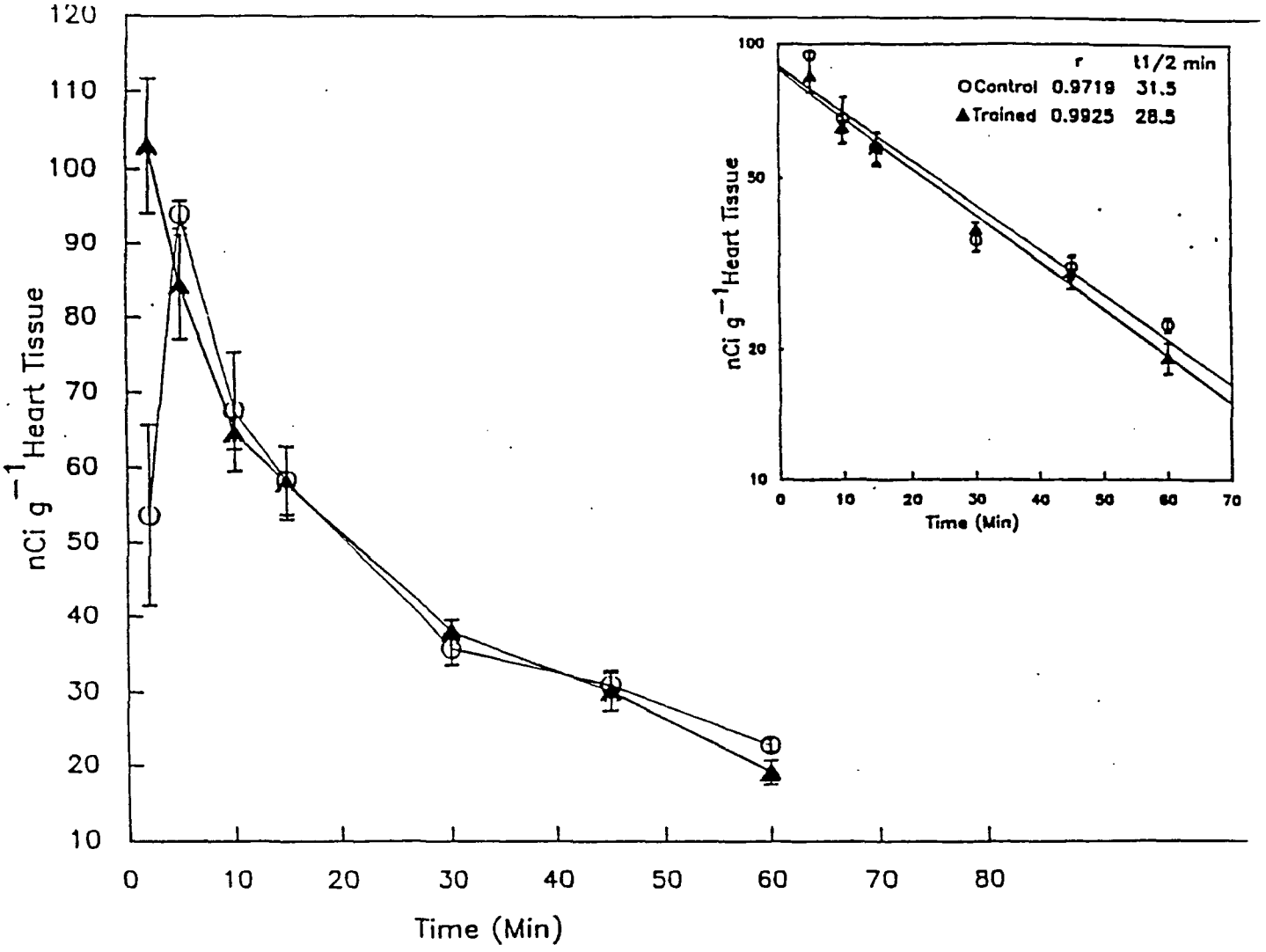


Fig. 16: Time course of radioactivity in heart of control, pyruvate 0.12 0.02 mol.ml^{-1}.

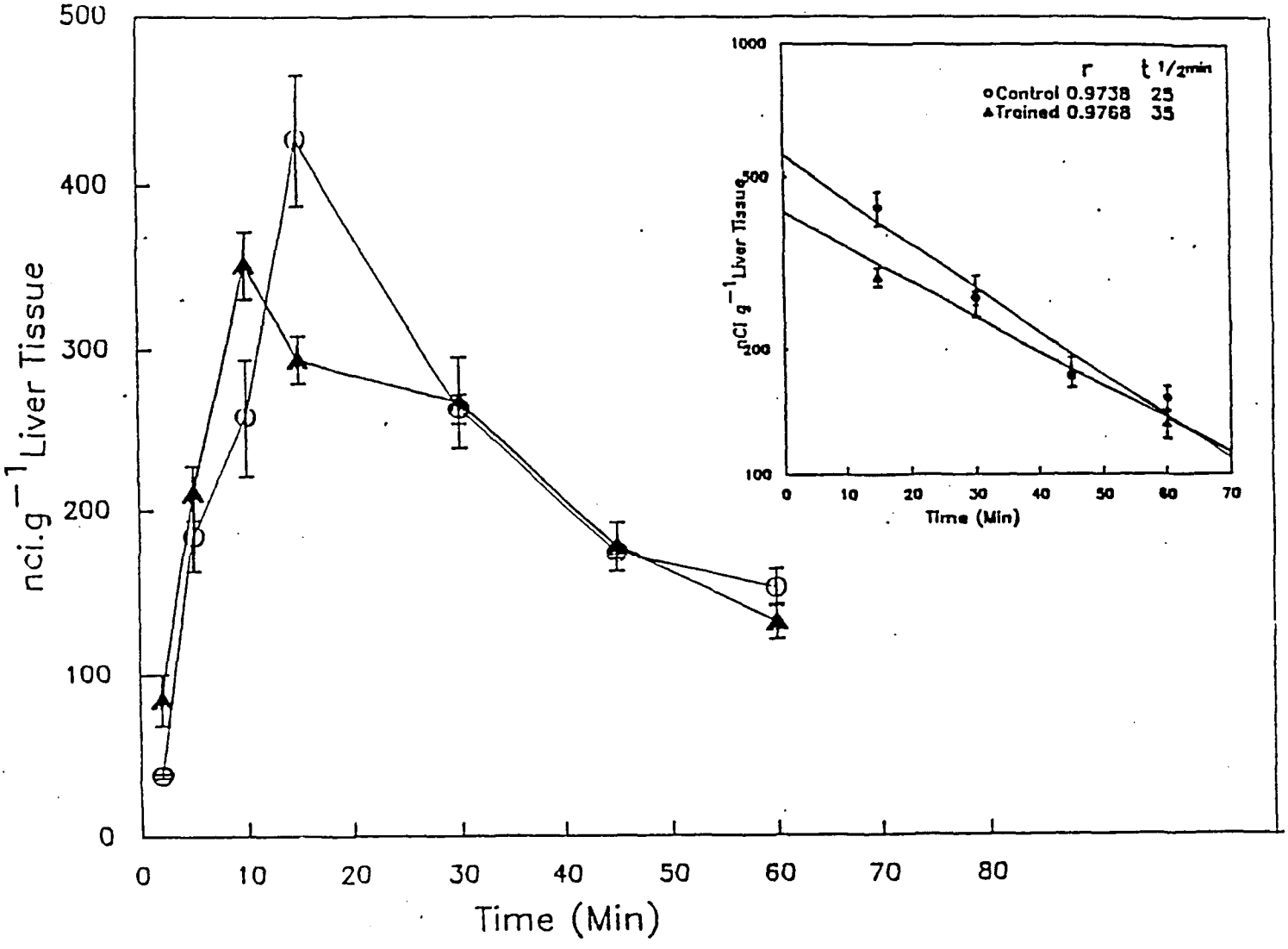


Fig. 17: Time course of radioactivity in liver of control (0 - 0) and trained (▲ - ▲) rats after [^{3}H]-physostigmine (70 g/kg, i.m.) administration.

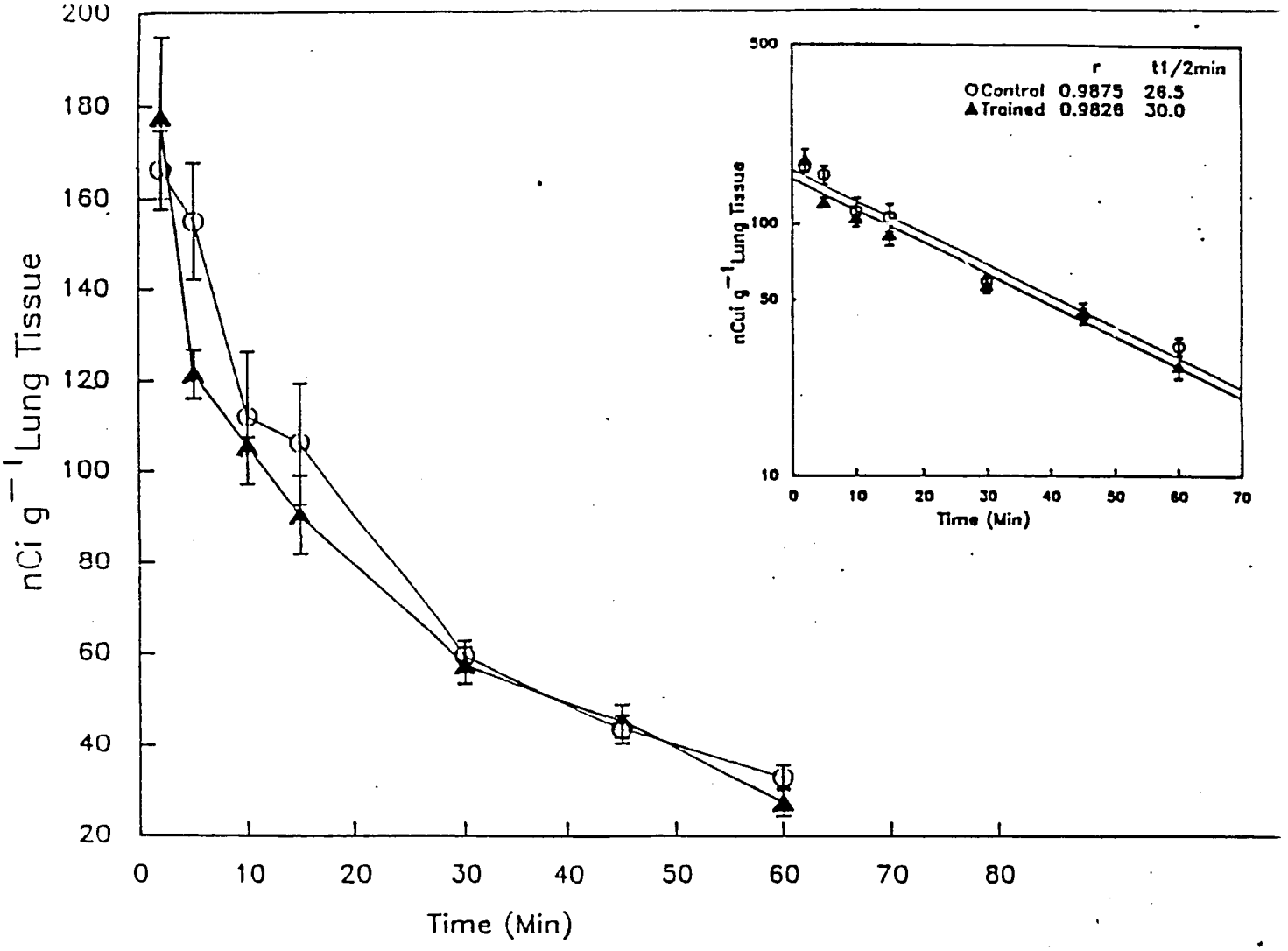


Fig. 18: Time course of radioactivity in lung of control (0 - 0) and trained (▲ - ▲) rats after [^{3}H]-physostigmine (70 g/kg, i.m.) adminsitration.

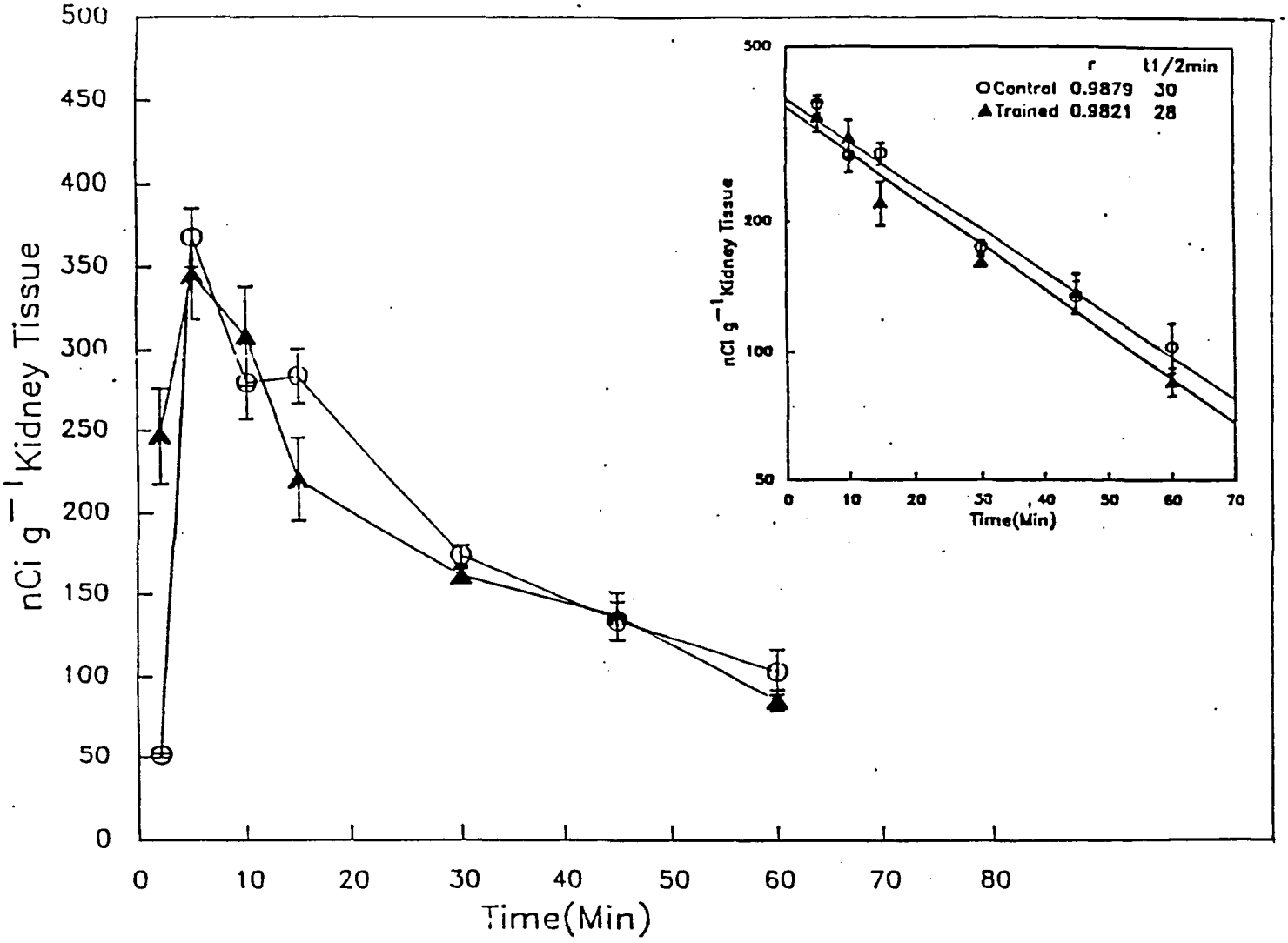


Fig. 19: Time course of radioactivity in kidney of control (0 - 0) and trained (▲ - ▲) RATS AFTER [^{3}H]-physostigmine (70 g/kg, i.m.) administration.

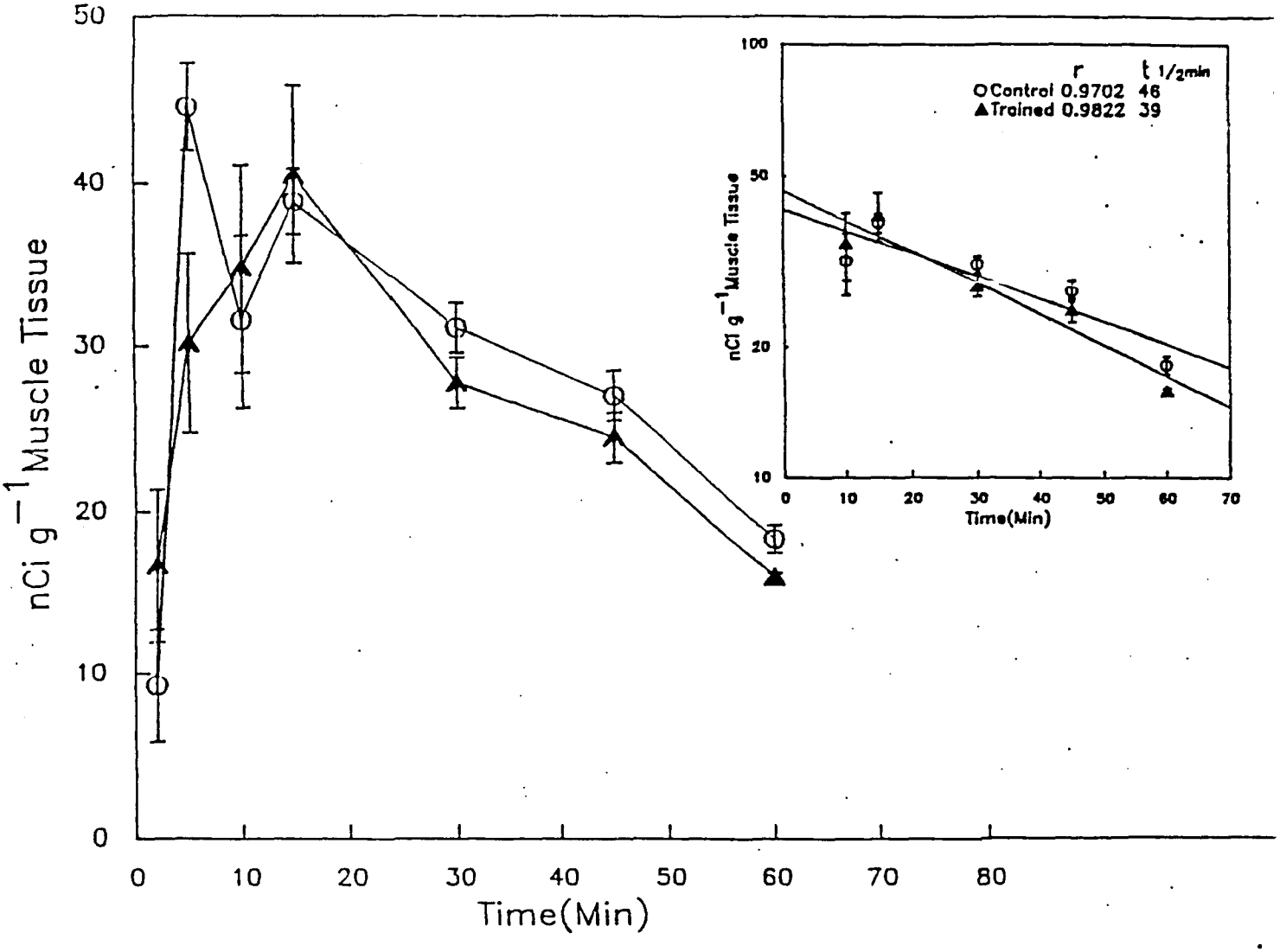


Fig. 20: Time course of radioactivity in muscle of control (0 - 0) and trained (▲ - ▲) rats after [^{3}H]-physostigmine (70 µg/kg, i.m.) administration.

Results

The results of effects of trained exercise on the distribution of RA (Phy + metabolites) are shown below. Fig. 15 shows that the RA in the brain of the control group was less (13.0 nCi g^{-1}) than in the trained group (57.0 nCi g^{-1}) at 2 min (Fig. 15). There was no significant difference in RA in both the groups from 10 to 60 min. In the control group, the t½ of Phy + metabolites in brain was significantly lower than in the trained group (Fig. 15-inset).

The RA in heart of the control group was significantly less (53.6 nCi g^{-1}) than in the trained group (102.9 nCi g^{-1}) (Fig. 16). There was no significant difference in RA in both the groups from 5 to 60 min. In the control group the t½ of Phy + metabolites was slightly higher than in the trained group (Fig. 16-inset).

The RA in the liver was highest on a per g basis compared to all other tissues (Fig. 17). The highest amount of RA (426.2 nCi g^{-1}) was observed at 15 min in the control group, whereas in the trained group the highest amount of RA (351.0 nCi g^{-1}) was observed at 10 min. The t½ of the control group was less than that of the trained group (Fig. 17-inset). These results suggest that trained exercise decreased the distribution of RA in the liver because exercise decreases the blood flow to the liver by about 20%.

The RA in the lung in both groups was almost similar except at 5 min, where it was slightly higher in the control group (155.3 nCi g^{-1}) (Fig. 18) than in the trained group (121.4 nCi g^{-1}) (Fig. 18). In the control group, the $t_{½}$ was slightly lower than in the trained group (Fig. 18-inset).

The RA in the kidney was significantly less ($p < 0.05$) in the control group (50.8 nCi g^{-1}) than in the trained group (246.9 nCi g^{-1}) at 2 min (Fig. 19). The maximum amount of RA (284.0 nCi g^{-1}) was observed at 15 min in the control group, whereas the maximum amount of RA (308.3 nCi g^{-1}) was observed at 10 min in the trained group. In the control group the t½ was slightly higher than in the trained group (Fig. 19-inset).

The RA in the muscle was less on a per g basis compared to all other tissues (Fig. 20). In the control group muscle RA was 9.2 nCi g^{-1}, whereas in the trained group RA increased to 16.6 nCi g^{-1} at 2 min. The control group RA was slightly higher from 30 to 60 min. In the control group, the t½ was slightly higher than in the trained group (Fig. 20-inset).

Table 9: Half-life of [^{3}H]-physostigmine + metabolites in tissues of control and trained rats

	Half-life (min)	
Tissue	Control	Trained
Brain	18.5	22
Heart	31.5	28.5
Liver	25	35
Lung	26.5	30
Kidney	30	28
Muscle	46	39

Table 9 summarizes the $t_{1/2}$ of RA in various tissues of control and trained rats. Liver showed significant increase in $t_{1/2}$, and muscle showed significant decrease in $t_{1/2}$ in trained rats compared to control.

Table 10: Effect of training on [^{3}H]$^+$physostigmine pharmacokinetics in the rat after intramuscular administration (70 µg/kg)

	Control	Trained
AUC (ng*min/ml)	578.8 ± 88.7	834.2 ± 45.8
$T_{1/2}$ (min)	8.8 ± 2.9	15.7 ± 1.6
K_e (min^{-1})	0.08 ± 0.03	0.04 ± 0.005
CL (ml/min/kg)	120.9 ± 18.5	83.9 ± 4.6
V_D (ml/kg)	1511	2097
C_{max} (ng/ml)	31.3	- - -*
T_{max} (min)	4.9	- - -*

$^+$Phy was determined by HPLC.
*C_{max} and T_{max} disappeared due to training exercise.

Pharmacokinetic parameters for Phy were determined using a PC-NONLIN program of Statistical Consultants. Table 10 clearly shows that the absorption phase, T_{max} and C_{max} have disappeared in endurance-trained rats. Trained exercise significantly increased the AUC from 579 to 834 ng/min/ml ($p < .05$) and $t_{1/2}$ from 8.8 to 15.7 min ($p < .05$) compared to control, whereas exercise significantly decreased clearance (Cl) from 121 to 84 ml min^{-1} kg^{-1} compared to control.

Discussion

This study has clearly shown that trained exercise altered the pharmacokinetic parameters of Phy, a flow-limited drug. Exercise increases cardiac output but diverts blood flow away from the liver (101,102) and could decrease the Cl of drugs, particularly those flow-limited drugs which are hepatic extractable, such as propranolol (103,104). Phy is highly extracted by liver and its Cl may be dependent on hepatic blood flow (61). A decrease in liver blood flow due to exercise will decrease the amount of Phy reaching the parenchymal cells, which, in turn, reduces the metabolism of Phy, thereby decreasing the Cl of Phy and increasing the AUC and t½.

The pharmacokinetics and disposition of flow-limited drugs are more likely to be affected by exercise, whereas the pharmacokinetics and disposition of capacity-limited drugs which are strongly bound and poorly extracted are less likely to be influenced by exercise (61).

Exercise causes the plasma shift (46) which results in a decrease in plasma volume and a change in the volume of distribution. It is not necessary to correct the plasma shift, since this is one of the effects of exercise, and no correction is justified (104).

This study shows that the time course of Phy distribution is different in tissues in trained exercise rats compared to control rats. Trained exercise altered the time taken by different tissues to peak concentration of the drug plus metabolites. These results showed that the RA of Phy + metabolites was higher as percent of control in brain (337%), liver (126%), heart (191%), kidney (385%), lung (106%), and muscle (180%) at 2 min after exercise in endurance-trained rats. However, RA in trained rats declined below control at 5 min after exercise in kidney, muscle, brain, heart, and lung, whereas in the liver RA declined below control at 15 min after exercise. The amount of total RA in the different tissues reveals the distribution of Phy and its metabolite's affinity to different tissues. The highest amount of RA accumulates in liver when compared to other tissues.

Peak concentrations of RA were observed in 2 min time in heart and lung—the organs of very high blood flow. It seems the distribution of RA was dependent on blood flow. Peak concentration of RA was observed in brain at the 5-min time point, and it decreased within 30 min. Muscle showed peak concentration of RA only after 15 min. The blood flow to different organs changes with intensity of exercise. During exercise the arterioles in muscle will inflate, and during cessation of exercise these arterioles will come to normal condition, which will not allow higher blood flow from arterioles, thereby helping to sequester the drug in muscle mass. In plasma, RA showed a decreasing trend from the beginning. The half-lives of RA in trained vs. control rats were for brain (18 vs. 20 min), liver (25 vs. 35 min), heart (31 vs. 26 min), kidney (30 vs. 28 min), lung (26.5 vs. 30 min), and muscle (45 vs. 31 min). There was no significant difference in t½ except muscle and liver. Exercise influences the profile of distribution of RA in all tissues and pharmacokinetics of Phy. It appears that these influences may be due to the flux of blood flow after the cessation of exercise, severity of exercise, pH changes due to lactic acid production, ionization of the drug, lipid solubility, and other undetermined factors.

VII. EFFECT OF CHOLINESTERASE INHIBITOR AND EXERCISE ON CHOLINE ACETYLTRANSFERASE AND ACETYLCHOLINESTERASE ACTIVITIES IN BRAIN REGIONS

Introduction

Alterations in central neurotransmitter systems due to physical exercise and/or chemical stressors has not received much attention. Of the few studies that have specifically examined biochemical markers of the brain cholinergic system, the two most frequently measured indices have been the biosynthetic enzyme of acetylcholine (ACh), choline acetyltransferase (ChAT), and the degradative enzyme AChE.

Apparent discrepancies in the literature regarding the effects of stressors on cholinergic function have been common. For example, Gottesfeld et al. (105) reported diminished ChAT activity in rat brain exposed to immobilization stress. Longoni et al. (106) reported increased ChAT activity in rat cerebral cortex after acute and repeated electroshock. Unchanged ChAT activity after exposure to immobilization for 2 hr was also reported in hypothalamic regions: brain stem, striatum, and hippocampus (105,107,108). None of these previous studies directly compared both cholinergic enzymes, and none directly compared the effects of physical exercise and chemical stressors such as Phy, a ChE inhibitor. Phy was considered to be a potential pretreatment agent against organophosphate poisoning (reviewed by Somani and Dube (34). There is even greater discrepancy concerning the measurement of AChE in the brain exposed to different drugs and stressors (109,110,111,112).

We have recently reported the effect of Phy administration and different levels of exercise on ChE activity in red blood cells (RBC) and various tissues of rat (57). We observed that exercise enhances the inhibition of ChE activity in RBC and whole brain elicited by Phy. In order to more clearly delineate these effects on central cholinergic function, this study sought to determine: Will trained exercise, subacute Phy administration, or the combination of these two treatments elicit adaptive changes in the biosynthetic or degradative enzymes for ACh? If so, are these changes differentially expressed within subregions of the brain?

Materials and Methods

Phy free base, acetyl-Co A, and acetyl choline chloride were obtained from Sigma Chemical Co. (St.Louis, MO). [^{3}H]-Acetyl-Co A and [^{3}H]acetyl choline iodide were obtained from Amersham Corp. (Chicago, IL). Ready-solve was procured from Beckman Instruments Inc. (Fullerton, CA). All other chemicals were analytical-grade and were obtained from the usual commercial sources.

Animals: Male Sprague-Dawley rats (w 150-175 g) were obtained from Harlan Industries, Indianapolis, IN. These rats weighed 250-300 g at the time of sacrifice. They were 14-15 wk old and were young adults. The rats were divided into five groups (Gr).

Gr I: received saline and served as sedentary controls. These rats were sacrificed on the day of the experiment.

Gr II: was trained for 2 wk as per the protocol (Table 11), and exercise was stopped 24 hr prior to sacrifice.

Gr III: received Phy (70 µg/kg, i.m.) twice daily for 2 wk. This group was divided into two subgroups. Gr IIIa was given Phy on the day of the experiment and sacrificed after 20 min; Gr IIIb was given Phy 24 hr prior to sacrifice.

Gr IV: Phy was administered (70 µg/kg i.m.) twice daily for 2 wk. Acute exercise (100% $VO_{2\ max}$) was given 24 hr before sacrifice. These rats were divided into two subgroups. Gr IVa was given Phy on the day of the experiment and sacrificed after 20 min; Gr IVb was given Phy 24 hr prior to sacrifice.

Gr V: Phy was administered (70 µg/kg, i.m.) twice daily for 2 wk and trained every morning for 30 min, as per protocol (Table 11). This group was also divided into two subgroups. Gr Va received Phy on the day of experiment and sacrificed after 20 min, Gr Vb received Phy 24 hr prior to sacrifice.

Training of Rats: Rats from Gr II and Gr V were acclimatized to treadmill in the beginning and were trained on a 9-channel motor driven treadmill (custom built at SIU), using an incremental exercise program. During this program of exercising, the speed (meters/min), angle of inclination (% grade), and the duration (min) of exercise were varied to obtain different levels of exercise intensity as shown in Table 11.

Table 11: Training protocol for exercising rats for 2 weeks.

Week	Belt Speed (m/min)	Inclination (% grade)	Duration at Each Speed (min)
1	8.2, 15.2, 19.3	6	10
2	8.2, 15.2, 19.3	6	10

Rats from Gr III and IV were not trained but were maintained under similar conditions to those of the trained rats. Each rat's weight was recorded daily before exercising the rats on the treadmill in order to determine the body weight changes during the entire period of training.

After completing the preceding protocols, rats were decapitated and different regions of brain (corpus striatum, cerebral cortex, brain stem, and hippocampus) were removed and frozen in liquid nitrogen. Tissues were stored at -70°C until analysis.

Enzyme Preparation: Frozen tissues were thawed and 10% homogenates were prepared in 10 mM EDTA phosphate buffer (pH 7) using an ultrasonic processor. Fifty µl of this aliquot was transferred to Eppendorf tubes for protein assay. To the remaining homogenate, equal volume of Triton X-100 (0.4%) and bovine serum albumin (0.2%) was added to release full enzyme activity. The concentration of the homogenate was diluted for each brain region until activities obtained were linear with tissue concentration.

Choline Acetyltransferase (ChAT) Assay: ChAT activity was determined using the method of Fonnum (113), recently modified by Arneric and Reis (114). The incubation mixture contained 0.1 µCi [^{3}H]acetyl CoA, 300 mM NaCl, 50 mM Na phosphate buffer, (pH 7.4), 8 mM choline chloride, 5mM EDTA, and 0.1 mM Phy sulfate. The mixture was incubated for 40 min at 37°C, and the reaction was stopped with 0.5 ml of 2-heptanone containing sodium tetraphenylboron (10 mg/ml). The

contents were vortexed, and centrifuged, and the organic phase was removed into scintillation vials. This step was repeated, and to 1 ml of organic phase 15 ml of Ready-Solve (Beckman) was added. The contents were vortexed and counted in a Beckman liquid scintillation counter (LS 5800). ChAT activity was calculated as nmoles ACh synthesized per hour per mg protein.

Acetylcholinesterase (AChE) Assay: The AChE assay was performed using the method of Fonnum (115), with slight modifications. The homogenate was pre-incubated for 30 min at 37°C with iso-OMPA Cl x 10^{-5}M, a selective inhibitor of BuChE activity. The incubation mixture contained 0.1 µCi [^{3}H] acetylcholine (0.5 mM), 20 mM sodium phosphate buffer, (pH 7.2), and bovine serum albumin (0.8 mg/ml). The final incubation volume was 100 µl, and incubation was carried out at 30°C for 30 min. After incubation, 0.4 ml of ice-cold 10 mM sodium phosphate buffer, pH 7.4, was added, and tubes were placed on ice. Acetylcholine was removed by shaking with 0.5 ml of 2-heptanone containing of sodium tetraphenylboron (10 mg/ml). After centrifugation, the ketone layer was aspirated, and the aqueous phase was washed once more with ketonic sodium tetraphenylboron. The final aqueous layer was transferred into scintillation vials and 16 ml of scintillation cocktail was added. The vials were vortexed, and counted, and AChE activity was calculated as µmole per hour per mg protein.

Protein concentrations were determined with the Coomassie blue protein-binding method (116), using bovine serum albumin as standard.

Calculations and Statistics: The data were expressed as the mean ± S.E.M. of four rats. The statistical analysis was performed on the absolute values of the data obtained from each experimental group. Data was analyzed, and the differences detected by Student's two-tailed t-test. The criterion of statistical significance was $p < 0.05$.

Results

The results of ChAT and AChE activities are expressed as nmol/mg of protein/hr and µmol/mg of protein/hr, respectively, for the different brain regions studied and are shown in Figs. 21 to 24 and Tables 12 to 15.

Corpus Striatum: ChAT activity did not change in corpus striatum (98% of control) due to training (Gr II). ChAT activity significantly decreased to 76% 95%, and 91% of control in Gr IIIa, IVa, and Va rats, respectively, which were sacrificed after 20 min of Phy administration (Fig. 21; Tables 12 and 13). ChAT activity also decreased to 88%, 68%, and 88% of control in Gr IIIb, IVb, and Vb rats, respectively, which were sacrificed after 24 hr of Phy administration (Fig. 22 and Tables 12 and 13).

AChE activity significantly decreased in subacute Phy-administered (Gr IIIa) and subacute Phy + acute exercise or trained exercise (Gr IVa and Gr Va) rats sacrificed 20 min after Phy administration (Fig. 23; Tables 14 and 15). AChE activity remained at 89%, 87%, and 90% of control in Gr IIIb, IVb, and Vb, respectively, even after 24 hr (Fig. 24; Tables 14 and 15).

Cerebral Cortex: ChAT activity in cerebral cortex of trained rat (Gr II) was low (84% of control). There was no significant change in ChAT activity after

subacute Phy + acute exercise (Gr IVa and b) in cerebral cortex of rats sacrificed at 20 min or 24 hr after Phy administration (Figs. 21 and 22; Tables 12 and 13). However, ChAT activity decreased to 89% of control in cerebral cortex of rats sacrificed after 24 hr of Phy administration (Gr IIIb). This enzyme activity decreased significantly ($p < 0.05$) in rats sacrificed 24 hr after subacute Phy + training (Gr Vb).

AChE activity was low in trained rats (Gr II) and in subacute Phy (Gr IIIa), as shown in Fig. 23 and Tables 14 and 15. AChE activity remained at 104, 87, and 94% of control in Gr IIIb, IVb, and Vb, respectively, even after 24 hr (Fig. 24; Tables 14 and 15). However, AChE activity significantly decreased to 72% and 75% of control in subacute Phy + acute exercise (Gr IVa) and in subacute Phy + trained groups (Gr Va) (Fig. 24). AChE activity recovered in all groups by 24 hr after Phy administration (Fig. 23). These results indicate that AChE activity in cerebral cortex was inhibited by Phy + exercise (acute or trained) at 20 min of Phy administration and recovered to control level by 24 hr.

Table 12: Effect of repeated dose of physostigmine (70 µg/kg,i.m.) daily for 2 wk and endurance training for 2 wk on choline acetyl transferase (ChAT) activity (in nmoles/mg of protein/hr) in different brain regions of rat. Values are mean+S.E.M. (n=4).

Groups	Treatment	Corpus Striatum	Cerebral Cortex	Brainstem	Hippocampus
I	Sedentary Control	484.7 ± 7.8	128.1 ± 9.7	266.5 ± 12.4	146.1 ± 19.0
II	Trained Exercise	475.4 ± 19.4	107.6 ± 2.8	194.0* ± 5.8	113.7 ± 3.2
III	**Subacute Phy**				
IIIa	Sacrificed 20 min after Phy	370.1* ± 3.4	122.6 ± 11.0	215.9* ± 18.8	124.9 ± 9.1
IIIb	Sacrificed 24 hr after Phy	430.1* ± 14.3	114.0 ± 4.4	207.9* ± 8.7	110.9 ± 6.1
IV	**Subacute Phy + Acute Exercise**				
IVa	Sacrificed 20 min after Phy	461.1* ± 5.9	122.0 ± 5.9	224.7 ± 22.0	109.5 ± 2.0
IVb	Sacrificed 24 hr after Phy	332.2 ± 1.3	118.4 ± 3.1	218.3* ± 9.4	105.3* ± 4.6
V	**Subacute Phy + trained exercise**				
Va	Sacrificed 20 min after Phy	445.6* ± 15.9	105.5 ± 13.7	213.4* ± 17.2	110.1 ± 5.2
Vb	Sacrificed 24 hr after Phy	428.8 ± 1.0	101.7* ± 3.0	216.0* ± 3.3	107.2* ± 3.1

* = Significant at $p < 0.05$

Table 13: Effect of repeated dose of physostigmine (70 µg/kg, i.m.) daily for 2 weeks and trained exercise for 2 weeks on choline acetyltransferase (ChAT) activity (% of control) in different brain regions of rats. Values are mean ± S.E.M. (n = 4).

Group	Treatment	Corpus Striatum	Cerebral Cortex	Brainstem	Hippocampus
II	Trained Exercise	98.1 ± 4.0	84.0 ± 2.2	72.8* ± 2.2	77.8 ± 2.2
III	**Subacute Phy**				
IIIa	Sacrificed 20 min after Phy	76.3* ± 0.7	95.6 ± 8.6	81.0* ± 7.0	85.5 ± 6.2
IIIb	Sacrificed 24 hr after Phy	88.7* ± 2.9	89.0 ± 3.4	78.0* ± 3.2	75.9 ± 4.2
IV	**Subacute Phy + Acute Exercise**				
IVa	Sacrificed 20 min after Phy	95.1* ± 1.2	95.3 ± 4.6	84.3 ± 8.2	74.9 ± 1.3
IVb	Sacrificed 24 hr after Phy	68.5* ± 0.3	92.5 ± 4.6	81.9* ± 3.5	72.1* ± 3.2
V	**Subacute Phy + Trained Exercise**				
Va	Sacrificed 20 min after Phy	91.9* ± 3.3	82.4 ± 10.7	80.1* ± 6.4	75.3 ± 3.5
Vb	Sacrificed 24 hr after Phy	88.5* ± 0.2	79.4* ± 2.3	81.1* ± 1.2	73.3* ± 2.1

*Significant at $p < 0.05$

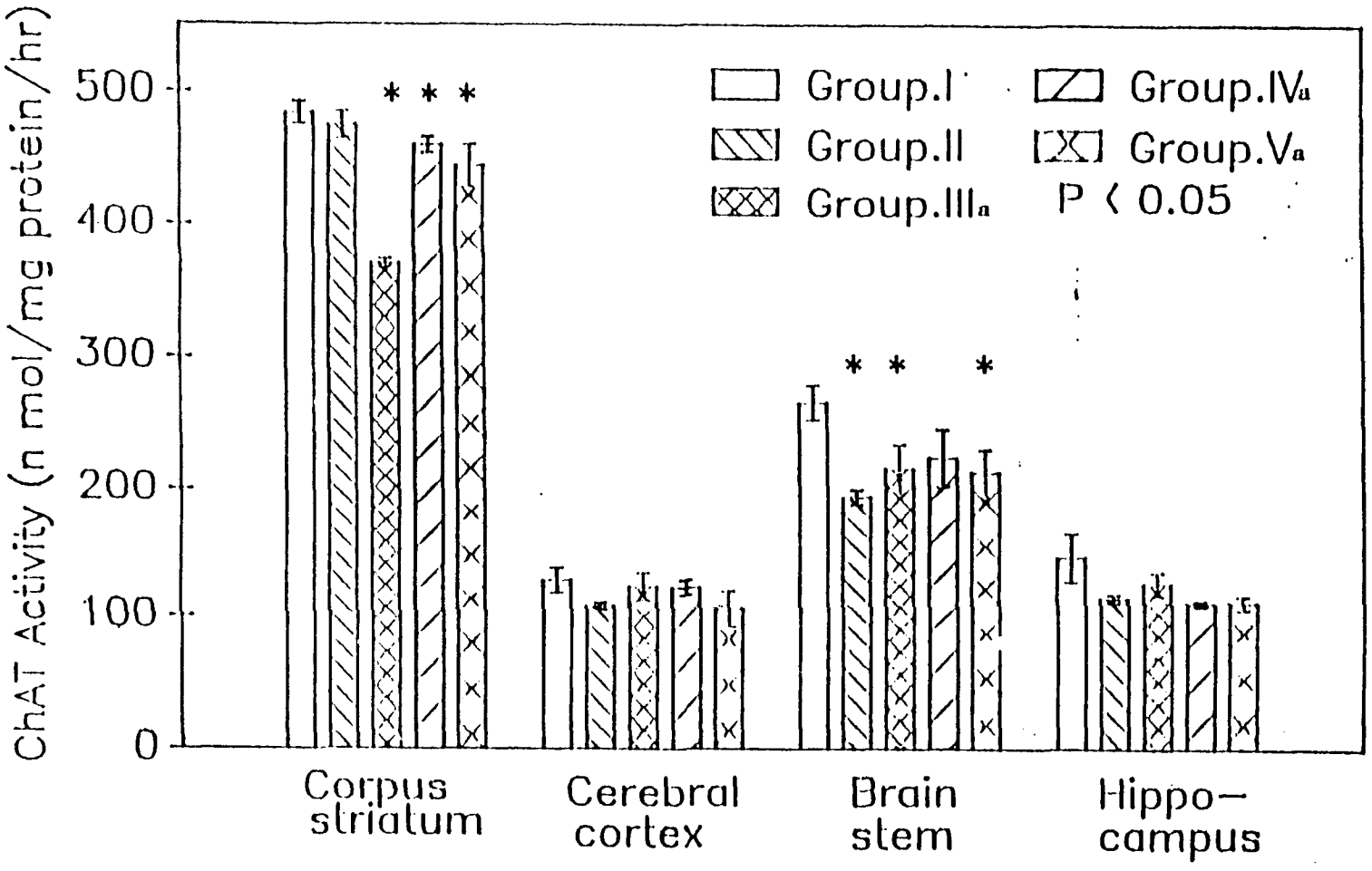


Fig. 21. Effect of trained exercise for two weeks (Gr II), subacute physostigmine administration (70 µg/kg, i.m.) for two weeks (Gr IIIa), subacute Phy administration (70 µg/kg, i.m.) for two weeks + single acute bout of exercise (Gr IV) and subacute Phy administration (70 µg/kg, i.m.) for two weeks + trained exercise for two weeks (Gr Va) on ChAT activity in different regions of brain in rat. Rats were sacrificed 20 min after the last dose of Phy administration.

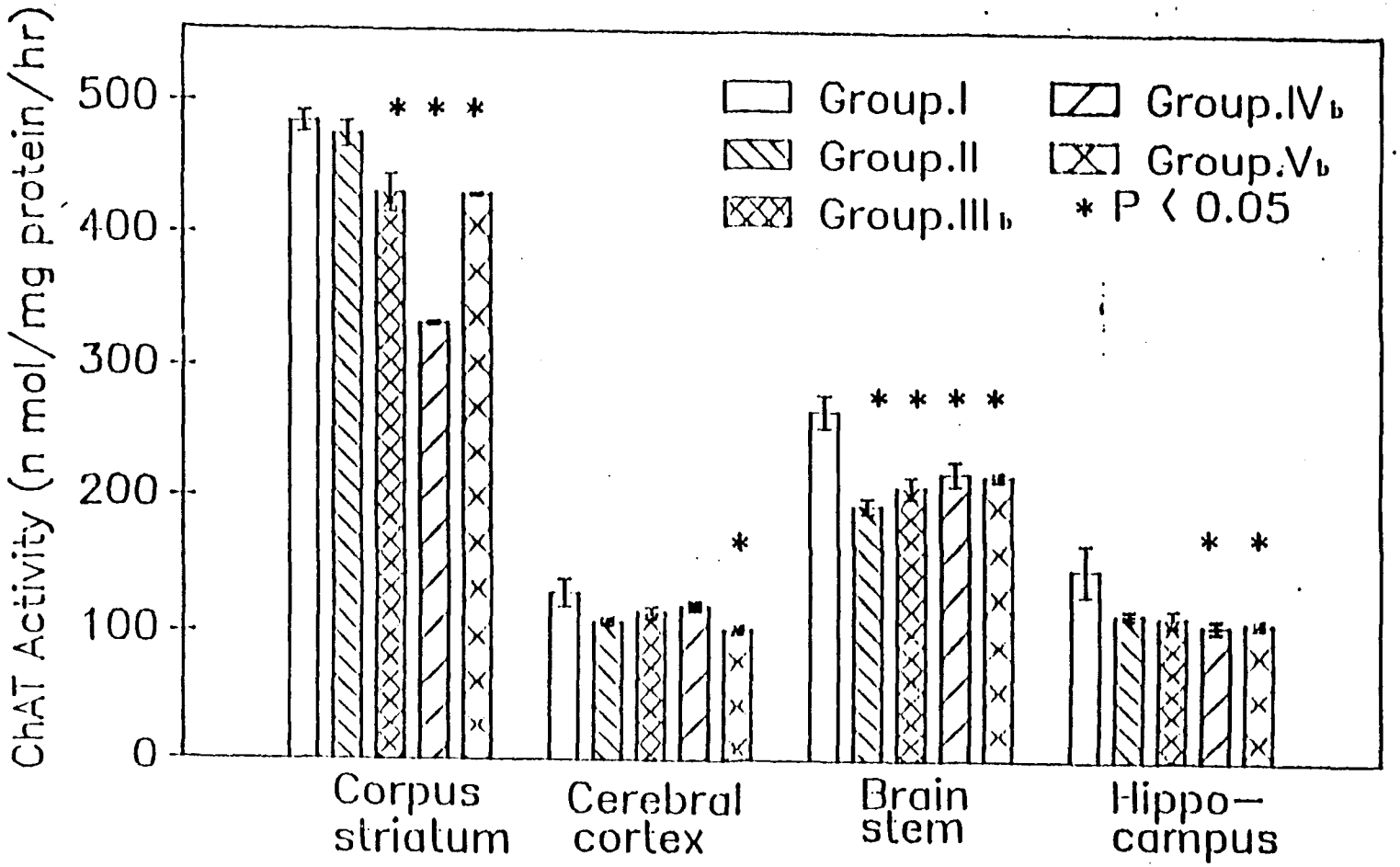


Fig. 22. Effect of trained exercise for two weeks (Gr II), subacute Phy administration (70 µg/kg, i.m.) for two weeks (Gr IIIb), subacute Phy administration (70 µg/kg, i.m.) for two weeks + single acute bout of exercise (Gr IVb) and subacute Phy administration (70 µg/kg, i.m.) for two weeks + trained exercise for two weeks (Gr Vb) on ChAT activity in different regions of brain in rat. Rats were sacrificed 24 hr after the last dose of Phy administration.

Table 14: Effect of repeated dose of physostigmine (70 µg/kg, i.m.) daily for 2 weeks and trained exercise for 2 weeks on AChE activity (µmole/hr/mg of protein) in different brain regions of rats. Values are mean ± S.E.M. (n = 4).

Group	Treatment	Corpus Striatum	Cerebral Cortex	Brainstem	Hippocampus
I	Sedentary Control	54.3 ± 1.3	5.9 ± 0.3	7.7 ± 0.3	7.9 ± 0.3
II	Trained Exercise	51.1 ± 2.2	5.0 ± 0.1	6.2 ± 0.2*	7.5 ± 0.3
III	**Subacute Phy**				
IIIa	Sacrificed 20 min after Phy	44.5 ± 1.3	5.4 ± 0.2	4.7* ± 0.2	6.0* ± 0.4
IIIb	Sacrificed 24 hr after Phy	48.5 ± 2.9	6.2 ± 0.2	6.4* ± 0.4	7.6 ± 0.2
IV	**Subacute Phy + Acute Exercise**				
IVa	Sacrificed 20 min after Phy	44.2* ± 1.3	4.2* ± 0.1	6.1* ± 0.1	5.5* ± 0.2
IVb	Sacrificed 24 hr after Phy	47.5 ± 0.6	5.2 ± 0.3	7.2 ± 0.4	7.2* ± 0.4
V	**Subacute Phy + Trained Exercise**				
Va	Sacrificed 20 min after Phy	42.4* ± 2.1	4.4 ± 0.3	5.5* ± 0.2	5.8* ± 0.2
Vb	Sacrificed 24 hr after Phy	48.8 ± 1.7	5.6 ± 0.3	7.0 ± 0.4	7.2* ± 0.1

*Significant at $p < 0.05$

Table 15: Effect of repeated dose of physostigmine (70 µg/kg, i.m.) daily for 2 weeks and trained exercise for 2 weeks on AChE activity (% of control) in different brain regions of rats. Values are mean ± S.E.M. (n = 4).

Group	Treatment	Corpus Striatum	Cerebral Cortex	Brainstem	Hippocampus
II	Trained Exercise	94.0 ± 4.0	84.3 ± 2.51	80.5* ± 2.6	94.1 ± 3.6
III	**Subacute Phy**				
IIIa	Sacrificed 20 min after Phy	81.8 ± 2.4	90.8 ± 4.1	61.2* ± 3.1	75.6 ± 4.9
IIIb	Sacrificed 24 hr after Phy	89.3 ± 6.0	104.1 ± 3.2	82.1* ± 4.7	94.7 ± 2.2
IV	**Subacute Phy + Acute Exercise**				
IVa	Sacrificed 20 min after Phy	81.4* ± 2.4	72.6* ± 2.0	79.8* ± 1.8	68.8* ± 2.7
IVb	Sacrificed 24 hr after Phy	103.8 ± 1.0	87.0 ± 4.5	93.2 ± 4.9	90.4* ± 5.5
V	**Subacute Phy + Trained Exercise**				
Va	Sacrificed 20 min after Phy	77.9* ± 3.8	74.8* ± 4.9	71.6* ± 2.1	72.2* ± 2.7
Vb	Sacrificed 24 hr after Phy	104.5 ± 3.0	93.6 ± 5.7	90.5 ± 5.9	90.9* ± 1.5

*Significant at $p < 0.05$

Brainstem: ChAT activity decreased significantly in brainstem in all groups indicating that this region is more sensitive to ChAT activity to both Phy as well as exercise. ChAT activity decreased to 73% of control ($p < 0.05$) in trained exercise rats (Gr II). ChAT activity also decreased significantly in all groups of rats sacrificed after 20 min or 24 hr of Phy administration with or without exercise (Fig. 22; Tables 12 and 13).

AChE activity significantly decreased ($p < 0.05$) in brainstem in all groups except IVb and Vb (Fig. 24; Tables 14 and 15). AChE activity decreased to 81, 61, and 82% of control in trained exercise Gr II subacute Phy rats sacrificed after 20 min (Gr IIIa) and subacute Phy rats sacrificed after 24 hr (Gr IIIb), respectively (Figs. 23 and 24). AChE activity also decreased significantly to 79% of control in subacute Phy + acute exercise (Gr IVa) and 72% of control in

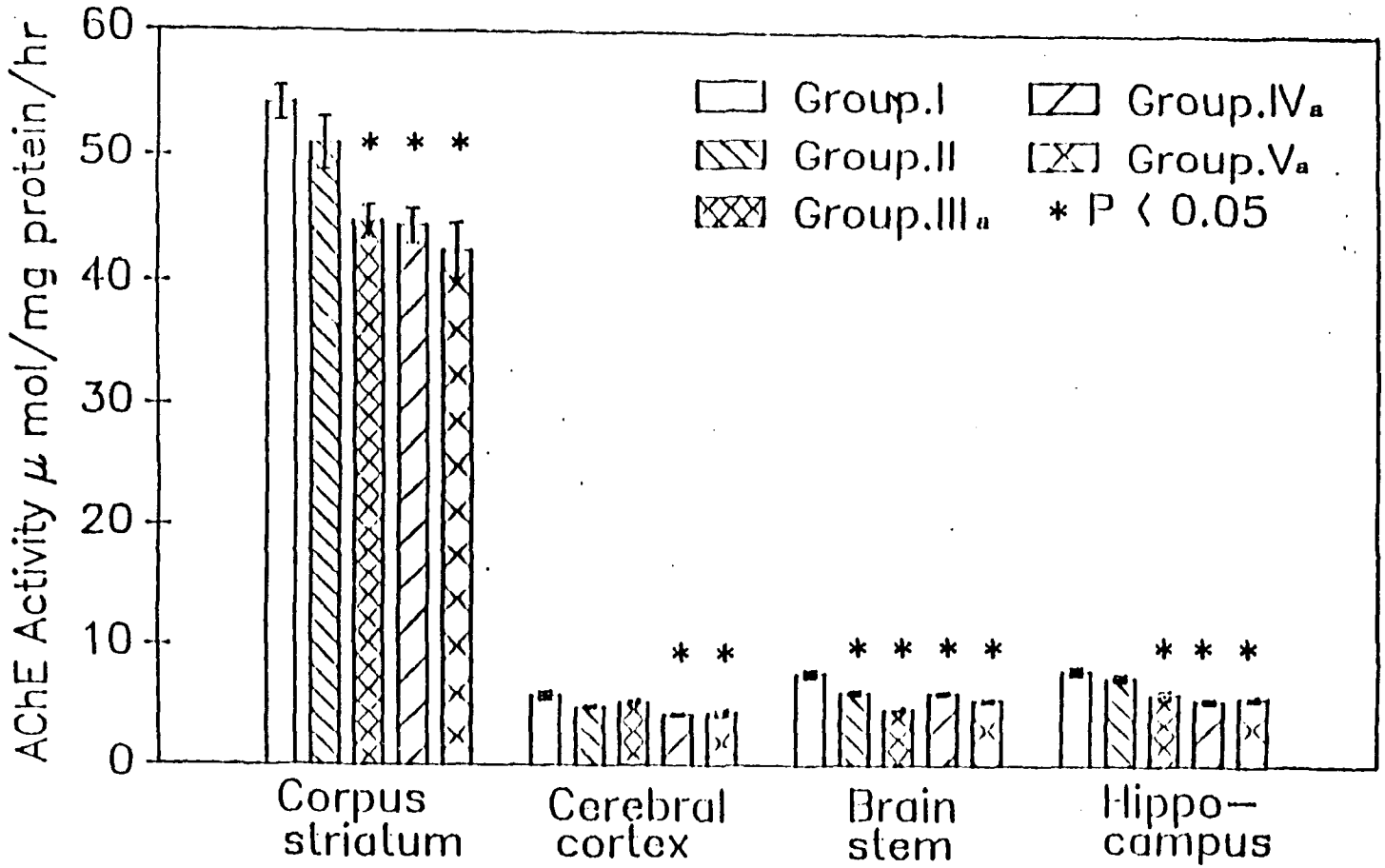


Fig. 23. Effect of trained exercise for two weeks (Gr II), subacute Phy administration 70 µg/kg, i.m.) for two weeks (Gr IIIa), subacute Phy administration (70 µg/kg, i.m.) for two weeks + single acute bout of exercise (Gr IVa) and subacute Phy administration (70 µg/kg, i.m.) for two weeks + trained exercise for two weeks (Gr Va) on AChE activity in different regions of brain in rat. Rats were sacrificed 20 min after the last dose of Phy administration.

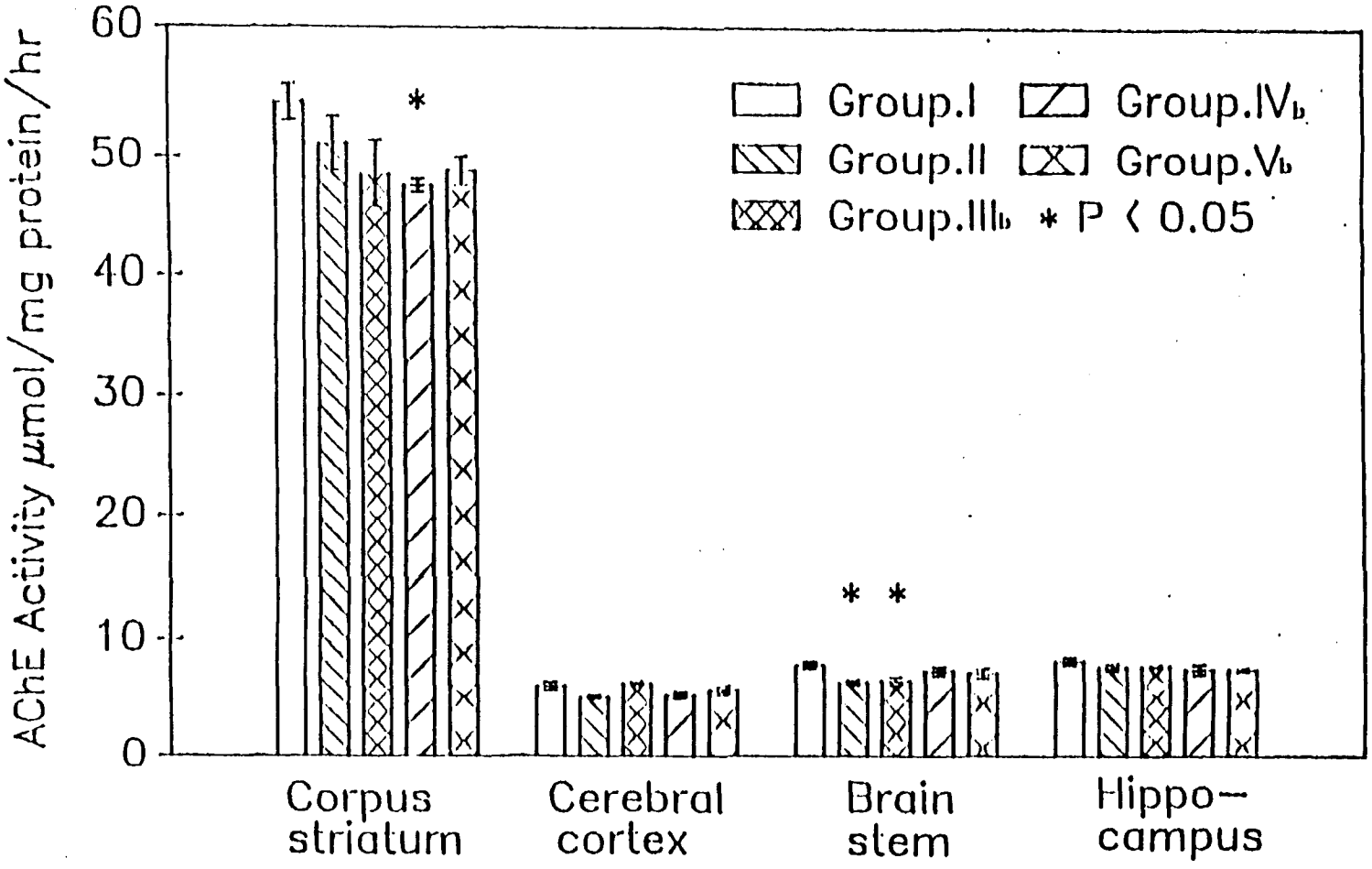


Fig. 24. Effect of trained exercise for two weeks (Gr II), subacute Phy administration (70 µg/kg, i.m.) for two weeks (Gr IIIb), subacute Phy administration (70 µg/kg, i.m.) for two weeks + single acute bout of exercise (Gr IVb) and subacute Phy administration (70 µg/kg, i.m.) for two weeks + trained exercise for two weeks (Gr Vb) on AChE activity in different regions of brain in rat. Rats were sacrificed 24 hr after the last dose of Phy administration.

subacute Phy + trained exercise (Gr Va) rats sacrificed after 20 min of Phy administration (Fig. 23; Tables 14 and 15). AChE activity was significantly low in subacute Phy (Gr IIIb) rats sacrificed after 24 hr of Phy administration (Fig. 24). However, AChE activity recovered to 93% and 90% of control in Gr IVb and Gr Vb, respectively (Fig. 24; Tables 14 and 15).

Hippocampus: ChAT activity decreased to 77% of control in hippocampus in trained rats (Gr II). ChAT activity significantly decreased ($p < 0.05$) to 72% of control in subacute Phy + acute exercise (Gr IVb) and 73% in subacute Phy + trained exercise (Gr Vb) rats sacrificed after 24 hr of Phy administration (Fig. 22; Table 13). ChAT activity was low in all other groups (Figs. 21 and 22; Tables 12 and 13).

AChE activity decreased in all groups but significantly decreased to 76%, 69%, and 72% of control ($p < 0.05$) in Gr IIIa, IVa, and Va, respectively (Fig. 23; Tables 14 and 15).

Discussion

This study suggests that the biosynthetic and degradative enzymes for ACh in brain regions involved with control of motor, autonomic, and cognitive functions are affected by trained exercise and subacute Phy in a regionally selective pattern that appears to depend on the type and interaction of these two stressors. Rats were sacrificed at 20 min and 24 hr after subacute administration of Phy in order to observe short- and long-term effects of the drug on ChAT and AChE activities, as well as the effects of single acute exercise or trained exercise. The data are consistent with the hypothesis that the responsiveness of these brain regions to these different stressors is a function of the level of ongoing cholinergic transmission and that elevations in ACh levels due to AChE inhibition may have long-term effects on ChAT and AChE activities through a negative feedback mechanism.

Previous work from this laboratory (57) suggested that inhibition of whole brain ChE activity by Phy was enhanced with exercise. However, it was unclear whether all brain regions responded similarly, since both the spontaneous activity and the regulation of that activity is differentially controlled in brain (117,118). Moreover, it was uncertain whether other enzymes involved in cholinergic transmission may also be affected.

This study observed marked (up to 5 to 20-fold) differences in the regional distribution of ChAT and AChE consistent with the known cholinergic innervation to the areas examined (119). Remarkably, the short- and long-term changes in ChAT and AChE activity elicited by the different stressors also showed regional selectivity. For example, the only brain region where ChAT activity responded to trained exercise was the brainstem, a region which is involved in maintaining critical autonomic functions related to the cardiopulmonary system and where ACh has potent actions (120). ChAT activity in cerebral cortex is not affected by any individual stressor, which may suggest the relative sparing of cholinergic systems in higher association centers involved with cognitive function. However, combination of these two stressors (Phy + trained exercise) did show an interaction to reduce ChAT activity in cortex and in hippocampus, an area of brain involved in learning and memory processes. In contrast, ChAT activity in corpus striatum was depressed significantly in all groups receiving Phy, and remained depressed even 24 hr following withdrawal of Phy. Thus, the cholinergic system in corpus striatum, which is normally involved in motor control, is

essentially unaffected by exercise, but susceptible to a chemical stressor such as Phy.

Results by others examining the effects of Phy have been mixed. In contrast to this study, Miyamoto et al. (121) showed decreased ChAT in cortical regions, but no significant changes were shown in hippocampus and striatum after continuous minipump infusion of Phy for 2 wk. Mandel and Thal (122) similarly reported a decrease in ChAT and AChE in cerebral cortex due to Phy administration. It is unclear whether the differences in these studies may be explained by the doses used or the continuous nature of the Phy administered.

However, Hata et al. (123) reported that cholinergic parameters in various regions of brain react differently to altered stress conditions, such as cold and swimming. This study supports this concept since regional differences in cholinergic activities were also observed for Phy and exercise.

The molecular/physiological mechanisms that govern the lasting changes in ChAT and AChE activity remain equivocal at this time. However, three comments can be made. First, it is unlikely that markedly decreased clearance of Phy following trained exercise then single dose of [^{3}H]-Phy (unpublished data from our lab) can fully account for the inhibition of AChE activity seen at the 24 hr period since trained exercise alone reduces AChE. Second, in brainstem the mechanism to decrease ChAT may be the same for exercise and Phy, since the combination of the two stressors did not show additive effects. Third, elevating tissue concentrations of ACh may ultimately govern these long-term effects independent of the mechanisms that initiate these effects. This premise is best illustrated in the brainstem where respiratory and cardiovascular functions are regulated. Trained exercise alone increases blood pressure and heart rate (124) and down regulates ChAT activity. Increases in medullary tissue concentrations of ACh by ChE inhibitors also elevates blood pressure and respiration (125). It is plausible that when tissue levels of ACh rise ChAT activity is down regulated as a compensatory mechanism to normalize cholinergic transmission and, hence, blood pressure. In order to verify this hypothesis, both tissue concentrations and the turnover of ACh need to be measured. Experiments are in progress to further support this hypothesis.

To summarize, Phy, exercise or the combination of each decreases brain ChAT and/or AChE activities in a regionally selective pattern. These data are consistent with the hypothesis that elevations in ACh concentrations down-regulate the level of ongoing cholinergic neurotransmission via a negative feedback mechanism. A schematic of this concept is depicted in Fig. 25.

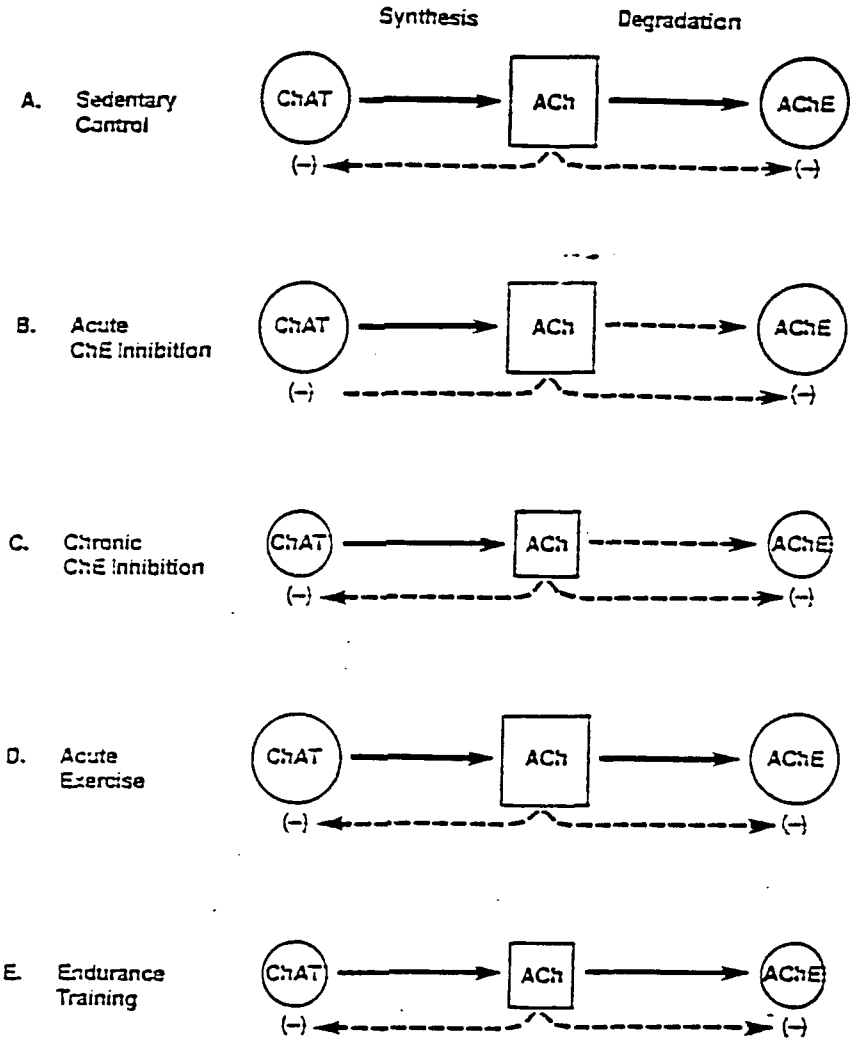


Fig. 25: A schematic depicting the responsiveness of the brainstem cholinergic system to Phy, a ChE inhibitor, and excercise. **PANEL A** - Under control conditions tissue levels of ACh are regulated by the net systhesis and degradation of ACh by ChAT and AChE, respectively. **PANEL B** - Immediately following ChE inhibition, tissue levels of ACh rise and initiate mechanisms that decrease both ChAT and AChE activity. **PANEL C** - Chronically elevated ACh concentrations eventually down-regulate ChAT and AChE activities to normalize cholinergic transmission. **PANEL D** - Acute exercise initially increases tissue ACh levels by enhancing biosynthesis without affecting degradation. This phenomenon does not appear to be expressed in corpus striatum, hippocampus or cerebral cortex. **PANEL E** - However, chronically elevated levels of ACh still down-regulate ChAT and AChE activities, although initaited via different mechanisms.

VIII. EFFECT OF CHOLINESTERASE INHIBITOR AND EXERCISE ON CHOLINE ACETYLTRANSFERASE AND ACETYLCHOLINESTERASE ACTIVITIES IN RAT EDL AND SOLEUS MUSCLE

Introduction

This section describes the modulatory effects of subacute Phy on choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) activities in fast and slow muscles of rat after training and acute exercise. Recently, we have reported the effect of subacute Phy and exercise on ChAT and AChE activities in different brain regions of rat (126). In continuation of this work, we have studied ChAT–a specific marker of the cholinergic system, and AChE activities in fast muscle (Extensor digitorium longus; EDL) and slow muscle (Soleus) of rat.

Some of the muscle characteristics are influenced by the neurotransmitter acetylcholine (ACh), neurogenic substances conveyed by axoplasmic transport, and muscle electromechanical activity (127). These regulatory factors are affected by changes in motor activity. The experimentally induced enhancement of neuromuscular use or disuse invariably leads to dramatic modifications in the muscle general metabolism, contractile properties, and neuromuscular transmission-related molecules (128,129). ACh, ACh receptors, ChAT, and AChE have received much attention because of their usefulness as a sensitive indicator of normal nerve-muscle interactions (130). AChE at neuromuscular junction has an essential role of ending synaptic transmission (131), its regulation in skeletal muscles partly depends on nerve evoked muscle activity (132). Recently, Fernandez and Donoso (65) showed that treadmill exercise significantly increases G_4 form of AChE but not other AChE forms and predominantly in fast-twitch but not in slow-twitch muscle of rat. Contrary to this, we have observed a decrease in total AChE in thigh muscle (57). Endurance training also induces several ultrastructural and protein metabolic changes in exercised skeletal muscle (133,134). However, to date, no information is available on simultaneous changes in synthetic ChAT and degradative AChE enzyme activities in fast twitch EDL and slow twitch soleus muscles due to treadmill exercise and/or ChE inhibitor. We have chosen to study AChE and ChAT in order to clearly delineate the effects of Phy and treadmill exercise on EDL and soleus cholinergic system. This study sought to determine whether subacute Phy and trained treadmill exercise or the combination of these two regulate the biosynthetic and degradative enzymes for ACh.

Materials and Methods

Phy free base, acetyl-Co A, and acetylcholine chloride were obtained from Sigma Chemical Co. (St. Louis, MO). [^{3}H]-Acetyl-Co A and [^{3}H]acetyl choline iodide were obtained from Amersham Corp. (Chicago, IL). Ready-solv was procured from Beckman Instruments, Inc. (Fullerton, CA). All other chemicals were analytical grade and were obtained from the usual commercial sources.

Animals: Male Sprague-Dawley rats (weight 150-175 g) were obtained from Harlan Industries, Indianapolis, IN. These rats weighed 250-300 g at the time of sacrifice. The rats 14-15 wk old, and they are young adults. The rats were divided into five (5) groups (Gr).

Gr I: received saline and served as sedentary controls. These rats were sacrificed on the day of the experiment.

Gr II: was trained for 2 wk as per the protocol (Table 11) and exercise was stopped 24 hr prior to sacrifice.

Gr III: received Phy (70µg/kg, i.m.) twice daily for 2 wk. This Gr was divided into two subgroups. Gr IIIa was given Phy on the day of the experiment and sacrificed after 20 min; Gr IIIb was given Phy 24 hr prior to sacrifice.

Gr IV: Phy was administered (70 µg/kg, i.m.) twice daily for 2 wk. Acute exercise (100% $VO_{2\ max}$) was given 24 hr before sacrifice. These rats were divided into two subgroups. Gr IVa was given Phy on the day of the experiment and sacrificed after 20 min; Gr IVb was given Phy 24 hr prior to sacrifice.

Gr V: Phy was administered (70 µg/kg, i.m.) twice daily for 2 wk and trained every morning for 30 min as per protocol (Table 1). This group was divided into two subgroups. Gr Va received Phy on the day of experiment and sacrificed after 20 min, Gr Vb received Phy 24 hr prior to sacrifice.

Training of Rats: Rats from Gr II and Gr V were acclimatized to treadmill in the beginning and were trained on a 9-channel motor driven treadmill (custom built at SIU), using an incremental exercise program. During this program of exercising, the speed (meters/min), angle of inclination (% grade) and the duration (min) of exercise were varied to obtain different levels of exercise intensity as shown in Table 11.

Rats from Gr III and IV were not trained but were maintained under similar conditions to those of the endurance-trained rats. Each rat's weight was recorded daily before exercising the rats on the treadmill in order to determine the body weight changes during the entire period of training.

After completing the preceding protocols, rats were decapitated, and soleus and EDL muscles were isolated from 4 animals in each group. Tissues were frozen in liquid nitrogen and were stored at -70°C until analysis.

Enzyme Preparation: The soleus and EDL were freed from tendon and were powdered under liquid nitrogen. The powdered muscle was weighed and 5% homogenate was prepared in 10 mM EDTA phosphate buffer (pH 7) using an ultrasonic processor. Fifty µl of this aliquot was transferred to Eppendorf tubes for protein assay. To the remaining homogenate equal volume of Triton x-100 (0.4%) and bovine serum albumin (0.2%) was added to release full enzyme activity. The concentration of the homogenate was diluted until activities obtained were linear with tissue concentration.

Choline Acetyltransferase (ChAT) Assay: ChAT activity was determined using the method of Fonnum (113), recently modified by Arneric et al. (120). The incubation mixture contained 0.1 µCi [^{3}H] acetyl CoA, 300 mM NaCl, 50 mM Na phosphate buffer, (pH 7.4), 8 mM choline chloride, 5mM EDTA, and 0.1 mM Phy sulfate. The mixture was incubated for 40 min at 37°C. After incubation, the reaction was stopped with 0.5 ml of 2-heptanone containing 5 mg sodium tetraphenylboron. The contents were vortexed and organic phase was removed into scintillation vials after centrifugation. This step was repeated again and to 1 ml of organic phase 15 ml of Ready-Solve (Beckman) was added. The contents were vortexed and counted in a Beckman liquid scintillation counter (LS 5800). ChAT activity was calculated as nmoles ACh synthesized per hour per mg protein.

Acetylcholinesterase (AChE) Assay: The AChE assay was carried out as per the method of Fonnum (115), with slight modifications. The homogenate was pre-incubated for 30 min at 37°C with iso-OMPA Cl x 10^{-5} M, a selective inhibitor of BuChE activity. The incubation mixture contained 0.1 µCi [^{3}H]acetylcholine (0.5 mM), 20 mM sodium phosphate buffer, (pH 7.2), and bovine serum albumin (0.8 mg/ml). The final incubation volume was 100 µl and incubation was carried out

at 30°C for 30 min. After incubation, 0.4 ml of ice-cold 10 mM sodium phosphate buffer, pH 7.4 was added and tubes were placed on ice. The acetylcholine was removed by shaking with 0.5 ml of 2-heptanone containing 5 mg of sodium tetraphenylboron. After centrifugation, the ketone layer was removed and the aqueous phase was washed once more with ketonic sodium tetraphenylboron. The final aqueous layer was transferred into scintillation vials and 16 ml of scintillation cocktail was added. The vials were vortexed and counted. The AChE was calculated and expressed a μmole per hour per mg protein.

Protein concentrations were determined with the Coomassie blue protein-binding method (116) using bovine serum albumin as standard.

Calculations and Statistics: The data were expressed as the mean ± S.E.M. of four rats. The statistical analysis was performed on the absolute values of the data obtained from each experiment group. Data were analyzed and the differences detected by Student's paired t-test. The criterion of statistical significance was determined at 10, 5, 1, and 0.1% level.

Results

The results of ChAT and AChE activities are expressed as nmol of ACh synthesized/mg protein/or and μmol of ACh hydrolyzed/mg protein/hr and are presented in Figs. 26-29 (Tables 16 and 17). The percent of control was calculated for ChAT and AChE and are presented in Table 18. Data presented in the tables show the activities of these 2 enzymes in both muscles at 20 min and 24 hr after treatment. Trained exercise (Gr II) decreased ChAT activity significantly ($p < 0.05$) to 68% of control in EDL, whereas in soleus ChAT activity increased to 124% of control (Figs. 26-27; Table 18). AChE activity was decreased significantly to 57% and 55% of control ($p < 0.05$) in both muscles due to trained exercise (Figs. 28-29). Subacute Phy (Gr III) decreased ChAT to 89% and 94% of control in EDL and soleus at 20 min but increased to 110% and 116% of control by 24 hr. Subacute administration of Phy decreased AChE activity significantly to 56% and 57% of control in EDL ($p < 0.05$) and soleus ($p < 0.01$) at 20 min and remained depressed at 68% and 67% of control even after 24 hr (Table 18). Subacute Phy + single acute exercise (Gr IV) decreased ChAT activity in EDL and soleus to 96% and 90% of control, which increased to 105% and 111% of control by 24 hr, respectively. In this group, AChE activity in EDL and soleus decreased significantly to 63% ($p < 0.1$) and 32% ($p < 0.001$) of control at 20 min, respectively. However, AChE values recovered to 88% of control in EDL and 50% of control in soleus at 24 hr. Subacute Phy + trained exercise (Gr V) decreased ChAT activity significantly ($p < 0.05$) to 65% of control in EDL and remained depressed up to 24 hr. ChAT activity in soleus has not shown any significant change in this group. AChE activity decreased significantly ($p < 0.001$) to 42% ($p < 0.001$) and 29% ($p < 0.01$) of control at 20 min in EDL and soleus, respectively. AChE activity remained depressed at 62% and 65% of control, even after 24 hr in EDL and soleus muscle, respectively. These results showed a constant decrease in AChE activity in both muscle in all groups at 20 min, which did not recover even after 24 hr. On the other hand, ChAT showed a transient decrease at 20 min in both muscles and recovered to control level by 24 hr. Only in subacute Phy + trained exercise group ChAT remained depressed even after 24 hr, in EDL muscle.

Our ChAT and AChE activities in sedentary control rat compare well with the literature values (135,136,128).

Discussion

ChAT activity was predominantly found in cholinergic neurons (118) and specifically tends to be high at cholinergic synapses and cholinergic neurons (137). A significant decrease was observed in ChAT in fast-twitch EDL muscle, but not in soleus muscle. This finding may be explained by considering the animal locomotion and the muscles involved in locomotion. Fast-twitch muscles are primarily active during locomotion, whereas slow-twitch muscles are active while the animals are standing, regardless of their locomotive state (138,139). This active involvement of EDL during exercise may lead to a decrease in ChAT. But AChE was inhibited in both fast and slow muscles significantly due to trained exercise. This is contrary to Fernandez and Donoso (65), who reported the increase in G_4 form AChE in fast-twitch muscle due to exercise. We studied the total AChE instead of molecular forms and, moreover, the treadmill exercise was different from the protocol followed by the above authors. Their exercise protocols consisted of 1-2 hr with a speed of 30-35 m/min once or twice a day, whereas our protocol is 20 min at progressive speeds only once a day. The difference in exercise protocols may alter the enzyme activity. The total AChE and not the molecular forms decreased even if G_4 form showed increase. Subacute Phy decreased ChAT activity in both muscle, but increased to above control level by 24 hr. The initial decrease in ChAT indicates that Phy decreases the synthesis of ACh also. AChE was significantly decreased in both muscles and remained depressed even after 24 hr possibly due to accumulation of Phy after subacute Phy administration. Phy is a tertiary amine compound and is metabolized in peripheral tissue (140). Since Phy is a reversible ChE inhibitor, the AChE activity has to return to control levels after a short time. But a sustained inhibition of AChE was observed even after 24 hr. The subacute administration of Phy seems to alter the regulation of AChE. In subacute Phy + single acute exercise group only a transient decrease in ChAT was observed, whereas significant decrease was found in AChE in both muscles. In subacute Phy + trained exercise group a significant decrease in ChAT was observed in EDL but not in soleus, and remained depressed even after 24 hr. Phy decreased ChAT activity significantly in EDL, but not in soleus, and the exercise prolonged this effect up to 24 hr. Soleus is not actively involved during exercise; hence, a transient decrease in ChAT was observed in this muscle. AChE was significantly decreased in both muscle in subacute Phy + trained exercise and remained depressed even after 24 hr. Several authors reported the altered ChAT and AChE activity due to Phy administration (122,123,121,126) in different regions of brain and tissues. However, this is the first report to show the changes in ChAT and AChE activities in fast and slow muscle due to combination of Phy and exercise. Phy and exercise seem to have a transient effect on ChAT but has a profound influence on AChE activity. Subacute Phy and trained exercise seems to regulate the AChE but not the ChAT

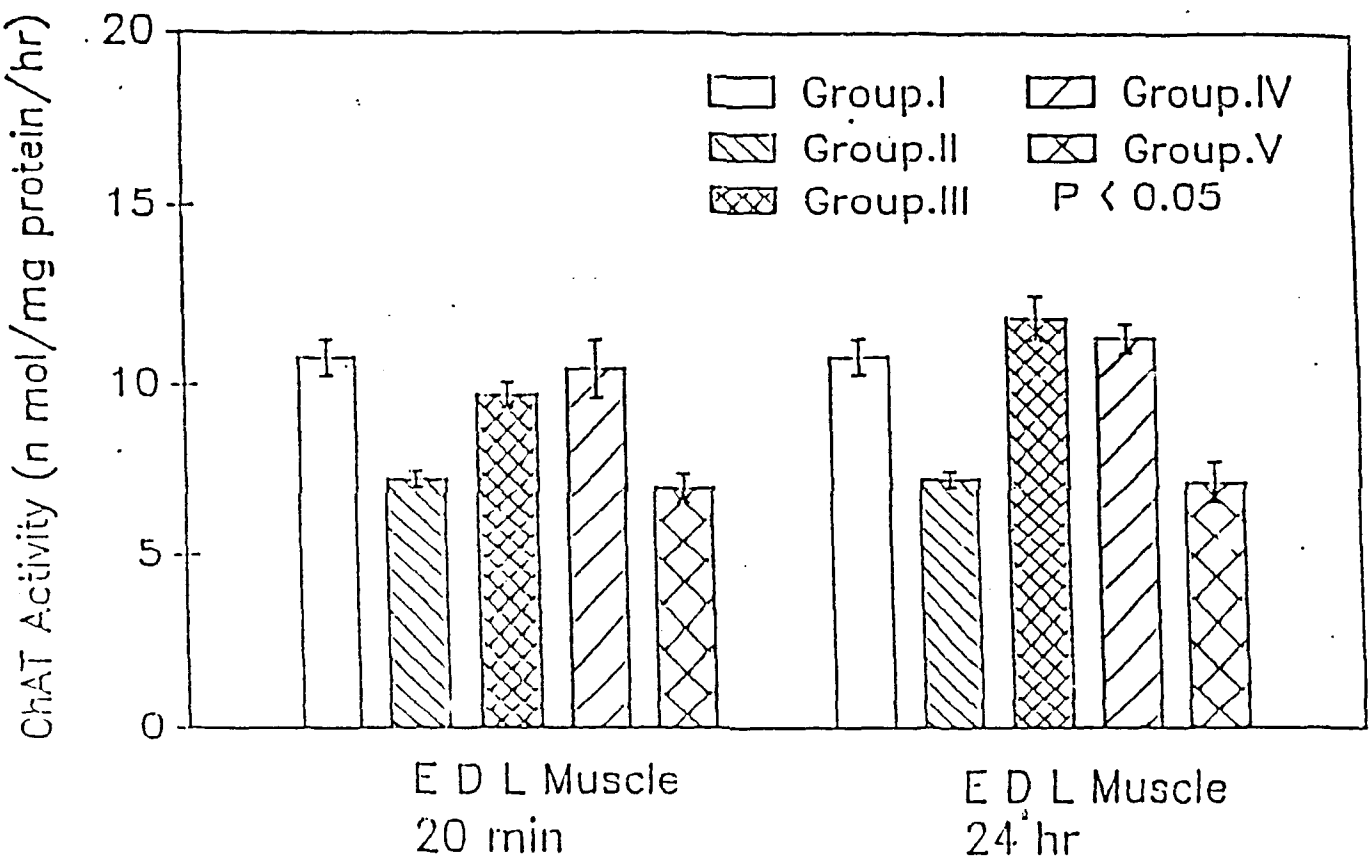


Fig. 26: Effect of endurance training for 2 wk (GR II), subacute Phy administration (70 µg/kg, i.m.) for 2 wk (Gr III), subacute Phy administration (70 µg/kg, i.m.) for 2 wk + single acute bout of exercise (Gr IV) and subacute Phy administration (70 µg/kg, i.m.) for 2 wk + endurance training for 2 wk (Gr V) on ChAT activity in EDL muscle of rat. Rats were sacrificed 20 min or 24 hr after the last dose of Phy administration.

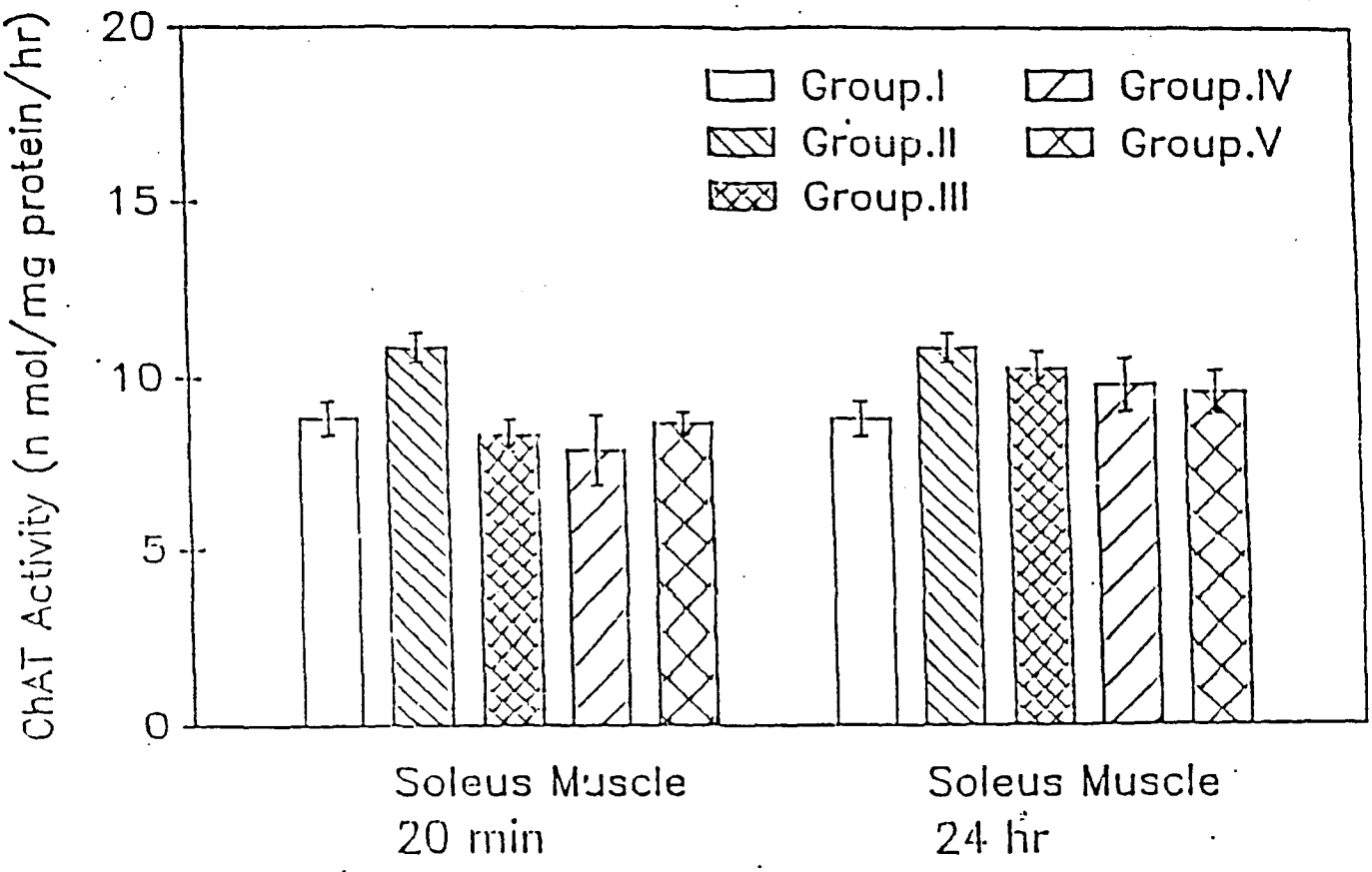


Fig. 27: Effect of endurance training for 2 wk (Gr II), subacute Phy administration 70 µg/kg, i.m.) for 2 wk (Gr III), subacute Phy administration (70 µg/kg, i.m.) for 2 wk _ single acute bout of exercise (Gr IV) and subacute Phy administration (70 µg/kg, i.m.) for 2 wk + endurance training for 2 wk (Gr V) on ChAT activity in soleus muscle of rat. Rats were sacrificed 20 min or 24 hr after the last dose of Phy administration.

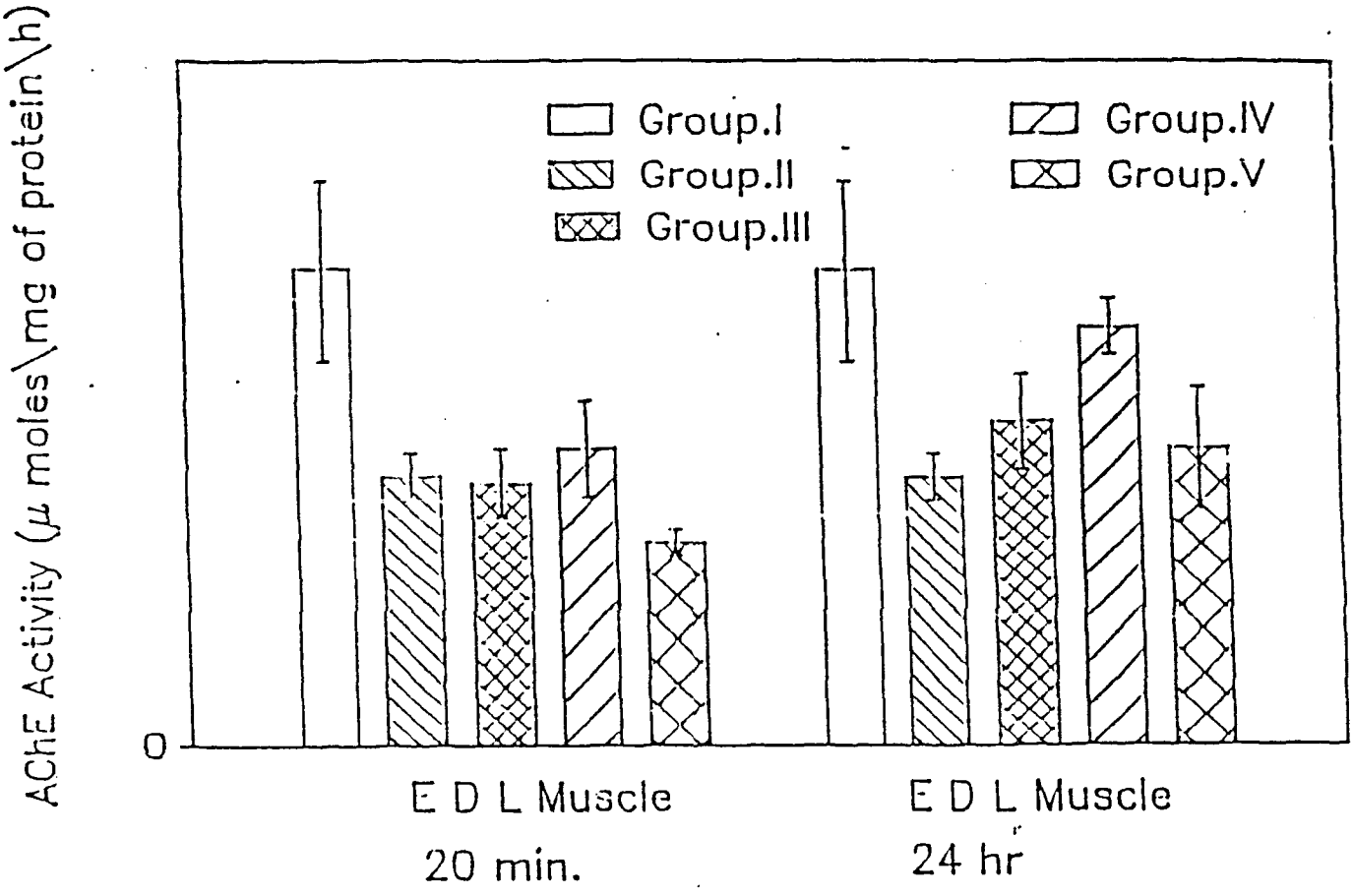


Fig. 28: Effect of endurance training for 2 wk (Gr II), subacute Phy administration 70 µg/kg, i.m.) for 2 wk (Gr III), subacute Phy administration (70 µg/kg, i.m.) for 2 wk _ single acute bout of exercise (Gr IV) and subacute Phy administration (70 µg/kg, i.m.) for 2 wk + endurance training for 2 wk (Gr V) on AChE activity in EDL muscle of rat. Rats were sacrificed 20 min or 24 hr after the last dose of Phy administration.

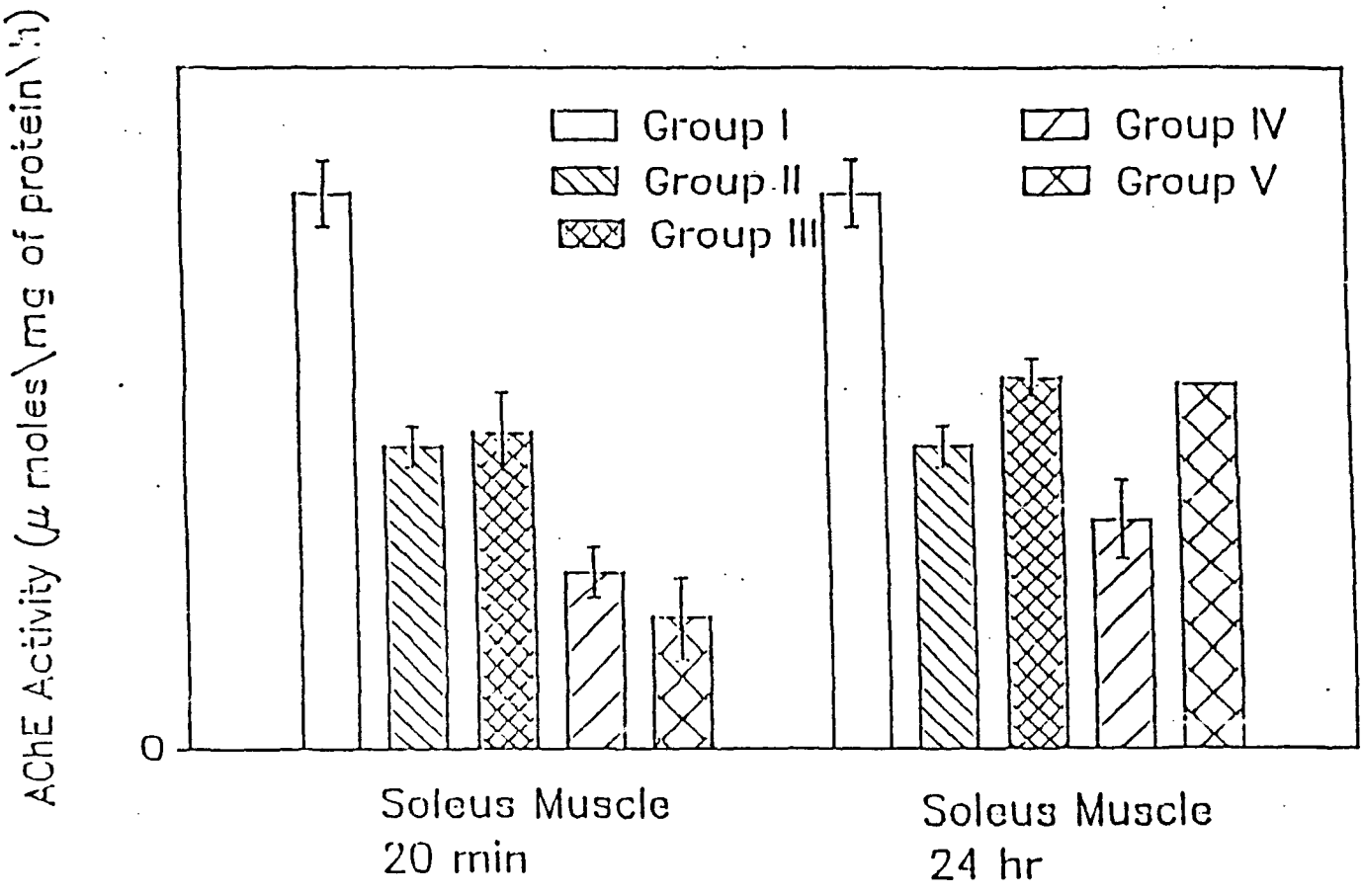


Fig. 29: Effect of endurance training for 2 wk (Gr II), subacute Phy administration 70 µg/kg, i.m.) for 2 wk (Gr III), subacute Phy administration (70 µg/kg, i.m.) for 2 wk _ single acute bout of exercise (Gr IV) and subacute Phy administration (70 µg/kg, i.m.) for 2 wk + endurance training for 2 wk (Gr V) on AChE activity in soleus muscle of rat. Rats were sacrificed 20 min or 24 hr after the last dose of Phy administration.

Table 16: Effect of subacute Phy (70 µg/kg, i.m.) and trained exercise on ChAT activities in EDL and soleus muscles of rat.

			ChAT Activity	
Group	Treatment	Time	EDL	Soleus
I	Sedentary Control		10.8 ± 0.5	8.8 ± 0.5
II	Trained Exercise		7.3 ± 0.2*	10.9 ± 0.4*
III	Subacute Phy	20 min	9.7 ± 0.4++	8.3 ± 0.4
		24 hr	11.9 ± 0.6	10.3 ± 0.5++
IV	Subacute Phy + Acute Exercise	20 min	10.4 ± 0.8	7.9 ± 1.00++
		24 hr	11.3 ± 0.4	9.8 ± 0.8
V	Subacute Phy + Trained Exercise	20 min	7.0 ± 0.4*	8.7 ± 0.3
		24 hr	7.2 ± 0.6*	9.6 ± 0.6

Values are mean of 4 observations ± S.E.M.. ChAT activity was expressed as nmoles of ACh synthesized/mg protein/hr.

++ $p < 0.1$
* $p < 0.05$

Table 17: Effect on subacute Phy (70 µg/kg, i.m.) and trained exercise on AChE activities in EDL and soleus muscles of rat.

			AChE Activity	
Group	Treatment	Time	EDL	Soleus
I	Sedentary Control		0.7 ± 0.1	0.8 ± 0.1
II	Trained Exercise		0.4 ± 0.03*	0.4 ± 0.1***
III	Subacute Phy	20 min	0.4 ± 0.1*	0.5 ± 0.1**
		24 hr	0.5 ± 0.1	0.5 ± 0.03***
IV	Subacute Phy + Acute Exercise	20 min	0.4 ± 0.1	0.3 ± 0.04***
		24 hr	0.6 ± 0.04	0.3 ± 0.1
V	Subacute Phy + Trained Exercise	20 min	0.3 ± 0.02***	0.24 ± 0.1***
		24 hr	0.4 ± 0.1	0.5 ± 0.04**

Values are mean of 4 observations ± S.E.M. AChE activity was expressed as nmoles of ACh hydrolyzed/mg protein/hr.

++ $p < 0.1$
* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

Table 18: ChAT and AChE activities (% of control) in fast (EDL) and slow (soleus) muscle in subacute Phy (70 µg/kg, i.m., twice daily 2 wk) and/or trained exercise. Values are mean ± S.E.M. (n=4).

			Choline Acetyltransferase		Acetylcholinesterase	
Group	Treatment	Time of Sacrifice After Treatment	EDL	Soleus	EDL	Soleus
II	Trained Exercise		67.8 ± 2.1*	123.9 ± 4.9*	57.1 ± 4.5*	54.9 ± 3.6***
III	Subacute Phy	20 min	89.6 ± 3.3++	94.4 ± 4.9*	55.7 ± 7.1*	57.3 ± 7.3**
		24 hr	110.5 ± 6.0	116.4 ± 4.8++	68.6 ± 10.0	67.1 ± 3.6***
IV	Subacute Phy + Acute Exercise	20 min	96.7 ± 7.8	89.8 ± 11.1++	62.9 ± 10.0++	31.7 ± 4.9***
		24 hr	105.1 ± 3.9	111.1 ± 8.9	88.6 ± 5.7	50.6 ± 7.3***
V	Subacute Phy + Trained Exercise	20 min	65.3 ± 3.8*	98.4 ± 3.5	42.9 ± 2.85***	29.2 ± 4.8**
		24 hr	66.7 ± 5.6*	108.5 ± 7.2	62.9 ± 12.8	65.8 ± 4.8**

IX. EFFECT OF PHY AND CONCURRENT ACUTE EXERCISE ON TIME COURSE OF ChE ACTIVITY IN RAT

Introduction

Phy, a centrally acting reversible anti-ChE agent, is considered to be a potential prophylactic agent against organophosphate intoxication (34).

The pharmacodynamics of Phy (inhibition of ChE activity) are likely to be altered by concurrent exercise due to altered blood flow rates to liver and pH of muscles. During exercise, cardiac output increases with the increase in intensity of workload, and concomitant changes in regional blood flow distribution occurs. Thus, the blood flow to skeletal muscles and skin is greatly increased, while the hepatic blood flow decreases during exercise (141,142).

The changes that occur in cholinergic system due to physical exercise have not received much attention. The rates of absorption, distribution, metabolism, and excretion are most important in determining the duration of action of Phy, which are likely to be altered during exercise. In turn, these processes will also affect the ChE activity due to Phy.

The combined effects of Phy and exercise on cholinergic system have not received much attention. Since intense fitness is required in the battlefield, how the physical exercise would influence the Phy-induced ChE activity needs to be considered during the development of potential pretreatment agent and therapy regimen. We have previously reported that the rate of decarbamylation decreased significantly in RBC and tissues due to postendurance training and administration of Phy compared to Phy alone. However, the postexercise has an opposite effect to that of postendurance training. The rate of decarbamylation of ChE increased in RBC, brain, and diaphragm and decreased in muscle after postacute exercise administration of Phy compared to Phy alone. The present study examined the effects of prior administration of Phy and then concurrent acute exercise on time course of ChE activity in RBC and tissues of rat.

Materials and Methods

Chemicals: Phy free base was obtained from Sigma Chemical Co. (St. Louis, MO). [^{3}H]-Phy (13 Ci/mmol) was custom-synthesized by Amersham Corp. (Chicago, IL). Ready-Solv EP was procured from Beckman Instruments Inc. (Fullerton, CA). Drierite (anhydrous $CaSO_4$), procured from W.A. Hammond Drierite Co. (Xenia, Ohio), was used. Diagnostic kit was purchased from Sigma Chemical Co. (St. Louis, MO) for the determination of blood Hb. All other chemicals were analytical grade and were obtained from the usual commercial sources.

Preparation of the [^{3}H]-Phy Solution: Phy was labeled with tritium on both ortho positions to the carbamate chain on the aromatic ring of Phy. [^{3}H]-Phy was diluted with unlabeled Phy (162.07 μCi/140μg/ml). The solution was prepared using physiological saline (0.9% w/v) in which 10 μl of hydrochloric acid was added to ensure that the solution was in an acidic pH change. The purity of Phy was assessed using high-performance liquid chromatography (HPLC) using an ultraviolet detector and by also monitoring the [^{3}H]-Phy in the eluant. The solution used in all experiments was greater than 95% pure.

Animals: Male Sprague-Dawley rats (Harlan Industries, Indianapolis, IN) weighing 175-200 g were used in this study. The rats were divided in to four

groups: sedentary control (Gr I); acute exercise (80% $VO_{2\ max}$) (Gr II); Phy administration (70 µg/kg, i.m.) (Gr III); and Phy + concurrent acute exercise (Gr IV).

Phy Administration and Exercising of Rats in Treadmill: The Oxyscan System and Omnipacer Treadmill (Omnitech. Inc., Columbus, Ohio) were used to monitor the maximal oxygen consumption ($VO_{2\ max}$). The rats from Gr II and IV were exercised on treadmill as described in Table 2 to obtain the 100% $VO_{2\ max}$ for each rat. After 3 days, the rats from Gr II were exercised at different speeds and inclinations for 20 min, corresponding to approximately 80% $VO_{2\ max}$. Rats from Gr IV were administered Phy and then were exercised for 20 min. The rats from Gr II and IV were sacrificed at 20, 22, 25, 30, 35, and 50 min after Phy administration or start of exercise. Rats from Gr III were administered Phy (70 µg/kg, i.m.) and were sacrificed at 20, 22, 25, 30, 35, and 50 min. Sedentary control rats (Gr I) were administered saline and were sacrificed after 15 min.

All animals were sacrificed between 8:00 and 11:00 AM to minimize circadian cycle effects. Each Gr consisted of 4 rats. Soon after decapitation, blood, brain, heart, diaphragm, and thigh muscle were collected. The blood was processed for determination of RBC-ChE. The tissues were stored at -70°C until analysis for ChE determination.

Determination of ChE Activity: The ChE enzyme was determined by a modified radiometric method of Johnson and Russell (11). We have previously reported the details of measurements of ChE activity in RBC, brain, heart, diaphragm, and thigh muscle (36). In this procedure [^{3}H]-ACh was used as the substrate. This method measured the RA due to [^{3}H]-acetate formed by the enzymatic hydrolysis of [^{3}H]-ACh. The substrate was prepared daily by mixing 0.5 M Tris buffer (0.25 M Trizma base, 0.25 M Tris-HCl, 1.2 M NaCl, pH 7.4), AChCl (0.1 mmol for RBC, diaphragm, heart, and thigh muscle; 1 mmol for brain) and [^{3}H]-AChI (1 mCi/0.01 mmol).

The Hb content of blood was determined by Sigma Diagnostic kit, using a Beckman Spectrophotometer at 540 nm.t300R

The ChE values of RBC were expressed as µmol ACh hydrolyzed/min/g Hb content, whereas the tissue ChE values were expressed as µmol ACh hydrolyzed/min/g wet weight of tissue.

Statistical Analysis: The ChE values were subjected to Student's t-test. Significant differences were accepted at $p < 0.05$.

Results

Effect of ChE Activity: The effect of acute exercise, Phy, and Phy + acute exercise on time course of ChE activity in RBC and tissues in rats has been presented in Figs. 30-34.

RBC: Acute exercise produced ChE activity in RBC 112, 103, and 95% of control at 20, 30, and 50 min, respectively, from the start of exercise (Fig 30). Phy depressed ChE activity 74%, 79%, and 84% of control at 20, 30, and 50 min, respectively. Phy + acute exercise further depressed the ChE activity 53% and 73% of control at 20 and 30 min, respectively, and recovered to 83% of control at 50 min (Fig. 30). There was no significant change in ChE activity at 50 min between Phy alone and Phy + acute exercise in RBC (Fig. 30). Fig. 30-inset shows

the plot of % ChE inhibition vs. time in RBC on semilog graph to obtain the rate of decarbamylation (K_d) of ChE enzyme. K_d for Phy in RBC is 0 0165 min^{-1}; K_d for Phy + AE in RBC is 0.0258 min^{-1}.

Brain: Acute exercise depressed ChE activity in brain 88-97% of control from 20 to 50 min. Phy depressed ChE activity 67, 77, and 85% of control at 20,30, and 50 min, respectively. Phy + concurrent acute exercise further depressed the ChE activity 54 and 70% of control at 20 and 25 min, respectively (Fig. 31). This activity recovered to 86% of control in 50 min in Phy alone and Phy + exercise. Fig. 31-inset shows the plot of % ChE inhibition vs. time in brain on semilog graph to obtain the rate of decarbamylation (K_d) of ChE enzyme. K_d for Phy in brain is 0.0231 min^{-1}; K_d for Phy + AE in brain is 0.0385 min^{-1} min.

Heart: Acute exercise produced ChE activity in heart 87, 92, and 95% of control at 20, 30, and 50 min, respectively from the start of exercise (Fig. 32). Phy depressed ChE activity 68, 75, and 85% of control at 20, 30, and 50 min, respectively. Phy + concurrent acute exercise depressed ChE activity 78, 85, and 95% of control at 20, 30, and 50 min, respectively (Fig. 32), indicating that concurrent exercise, in fact, decreases the effect of Phy by increasing the ChE activity slightly, compared to Phy alone. Fig. 32-inset shows the plot of % ChE inhibition vs. time in heart on semilog graph to obtain the rate of decarbamylation (K_d) of ChE enzyme. K_d for Phy in heart is 0.0252 min^{-1}; K_d for Phy + AE in heart is 0.0602 min^{-1}.

Diaphragm: Acute exercise did not alter ChE activity in diaphragm (97-95% of control) from 20 to 50 min from the start of exercise (Fig. 33). Phy depressed ChE activity 67, 70, and 75% of control at 20, 30, and 50 min, respectively. Phy + acute exercise depressed ChE activity 70, 76,, and 77% of control at 20, 30, and 50 min, respectively (Fig. 33). Fig. 33-inset shows the plot of % ChE inhibition vs. time in diaphragm on semilog graph to obtain the rate of decarbamylation (K_d) of ChE enzyme. K_d for Phy in diaphragm is 0.0078 min^{-1}; K_d for Phy + AE in diaphragm is 0.0067 min^{-1}.

Thigh Muscle: Acute exercise produced ChE activity in thigh muscle, 95, 102, and 105% of control at 20, 30, and 50 min, respectively, from the start of exercise (Fig. 34). Phy depressed ChE activity 55%, 69%, and 83% of control at 20, 30, and 50 min, respectively. Phy + acute exercise produced ChE activity 58, 60, and 69% of control at 20, 30, and 50 min, respectively (Fig. 34). Concurrent exercise changes ChE activity at 30 and 50 min. Fig. 34-inset shows the plot of % ChE inhibition vs. time in muscle on semilog graph to obtain the rate of decarbamylation (K_d) of ChE enzyme. K_d for Phy in muscle is 0.0308 min^{-1}; K_d for Phy + AE in diaphragm is 0.0135 min^{-1}.

Table 19: Rate of decarbamylation (K_d) of Phy-inhibited ChE in RBC and tissues of rat. These rats were given Phy 70 µg/kg i.m., and Phy + concurrent acute exercise (80% $VO_{2\ max}$).

	Phy		Phy + Exercise	
	r	K_d (min^{-1})	r	K_d (min^{-1})
RBC	0.96	0.0165	0.91	0.0258
Brain	0.98	0.0231	0.90	0.0385
Heart	0.92	0.0252	0.98	0.0602
Diaphragm	0.85	0.0078	0.88	0.0067
Muscle	0.95	0.0308	0.94	0.0135

r = correlation coefficient of declining slope
n = 4

These results (Table 19) suggest that the rate of decarbamylation (K_d) of ChE in RBC, brain, and heart was higher and that in muscle and diaphragm the rate was lower in Phy + concurrent acute exercise than in Phy alone administered rat. These results also suggest that concurrent exercise enhances the rate of decarbamylation of Phy inhibited ChE in RBC, brain, and heart, but not in diaphragm and muscle.

Fig. 30: Effect of acute exercise (AE) (80% VO_{2max}), Phy (70 µg/kg) and Phy then concurrent AE on ChE activity in RBC as % of control after 20 min of Phy administrtion and/or exercise. Inset shows the plot of % ChE inhibition vs. time in RBC on semilog graph to obtain the rate of decarbamylation (K_d) of ChE enzyme. K_d for Phy + AE in RBC is 0.0258 min^{-1}.

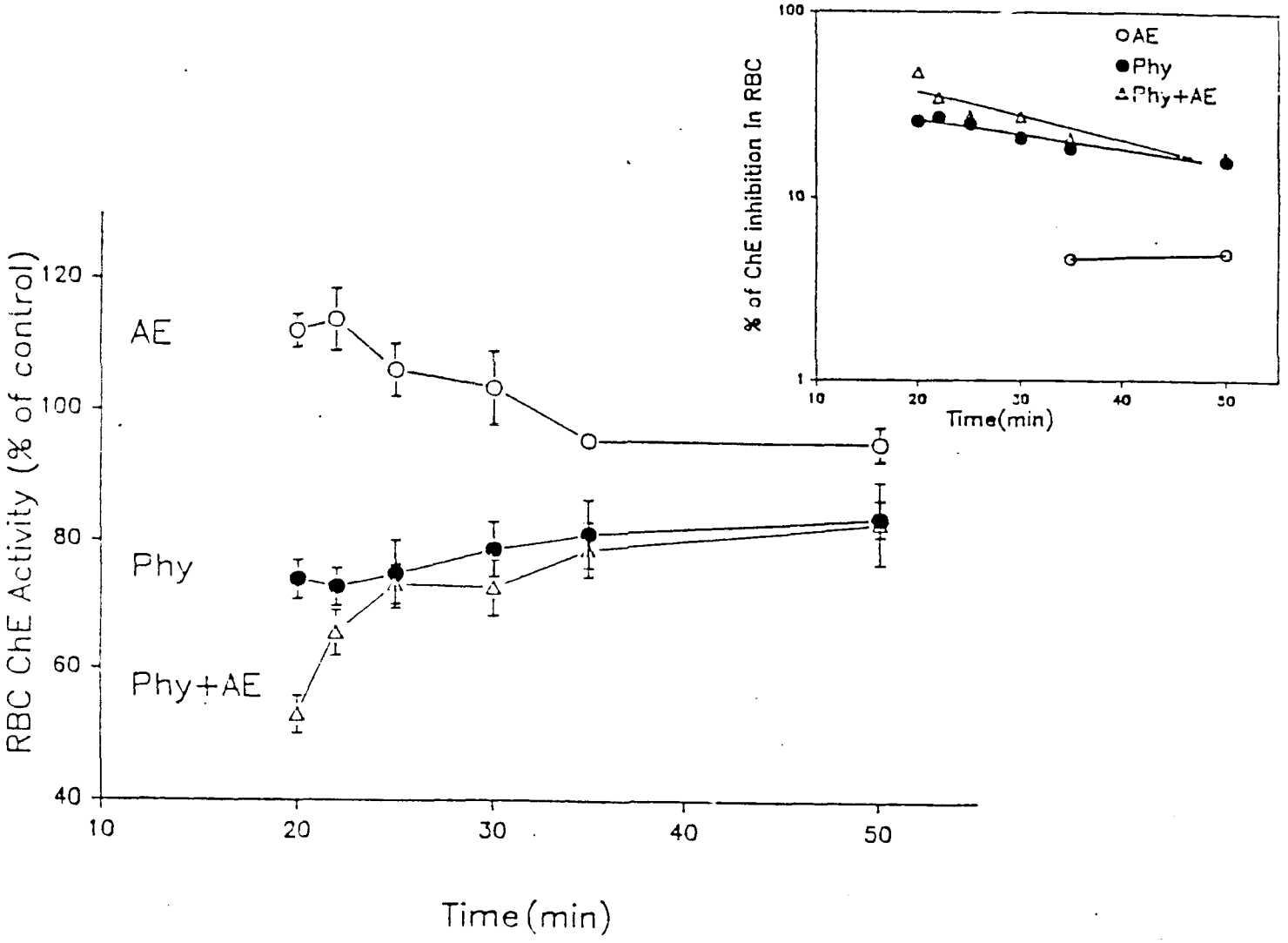
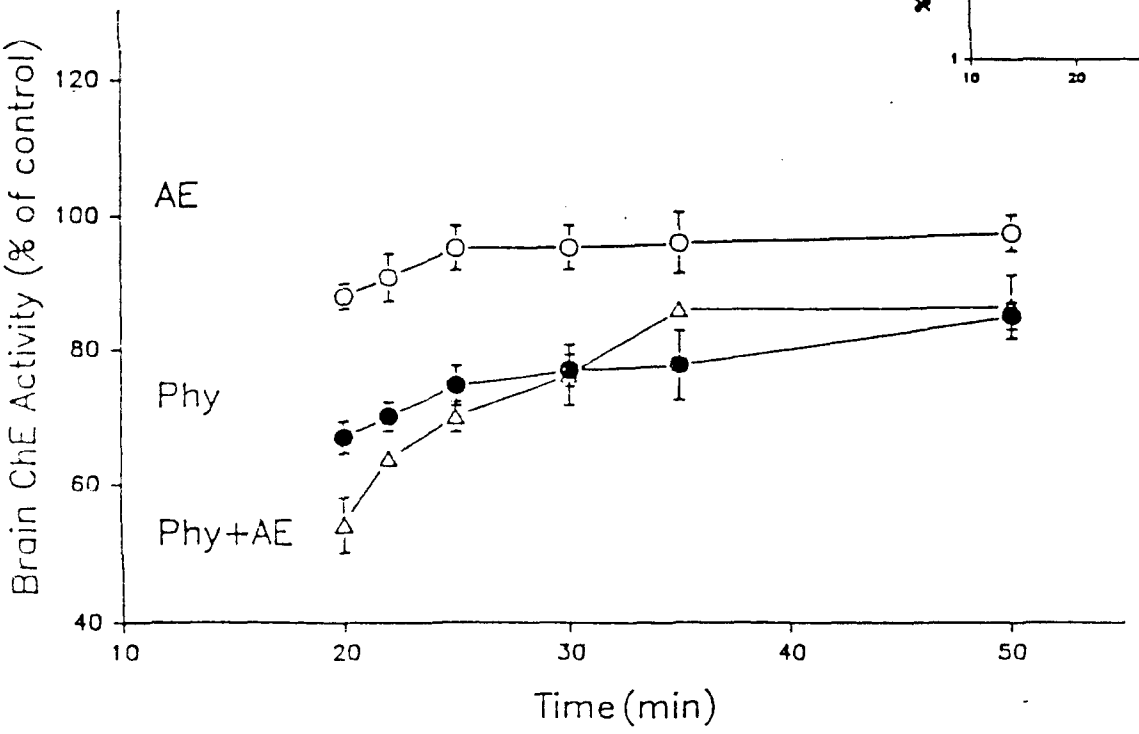


Fig. 31: Effect of acute exercise (AE) (80% VO_{2max}), Phy (70 µg/kg) and Phy then concurrent AE on ChE activity in brain as % of control after 20 min of Phy administration and/or exercise. Inset shows the plot of % ChE inhibtion vs. time in brain on semilog graph to obtain the rate of decarbamylation (K_d) of ChE enzyme. K_d for Phy in brain is 0.0231 min^{-1}; K_d for Phy + AE in brain is 0.0385 min^{-1}.



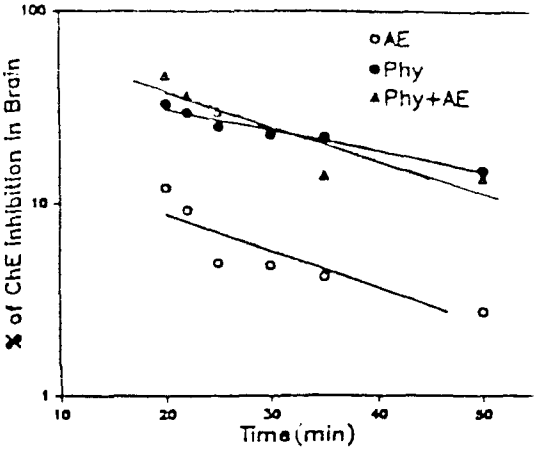


Fig. 32: Effect of acute exercise (AE) (80% $VO_{2\ max}$), Phy (70 µg,kg) and Phy then concurrent AE on ChE activity in heart as % of control after 20 min of Phy administration and/or exercise. Inset shows the plot of % ChE inhibition vs. time in heart on semilog graph to obtain the rate of decarbmylation (K_d) of ChE enzyme. K_d for Phy in heart is 0.0252 min^{-1}; K_d for Phy + AE in heart is 0.0602 min^{-1}.

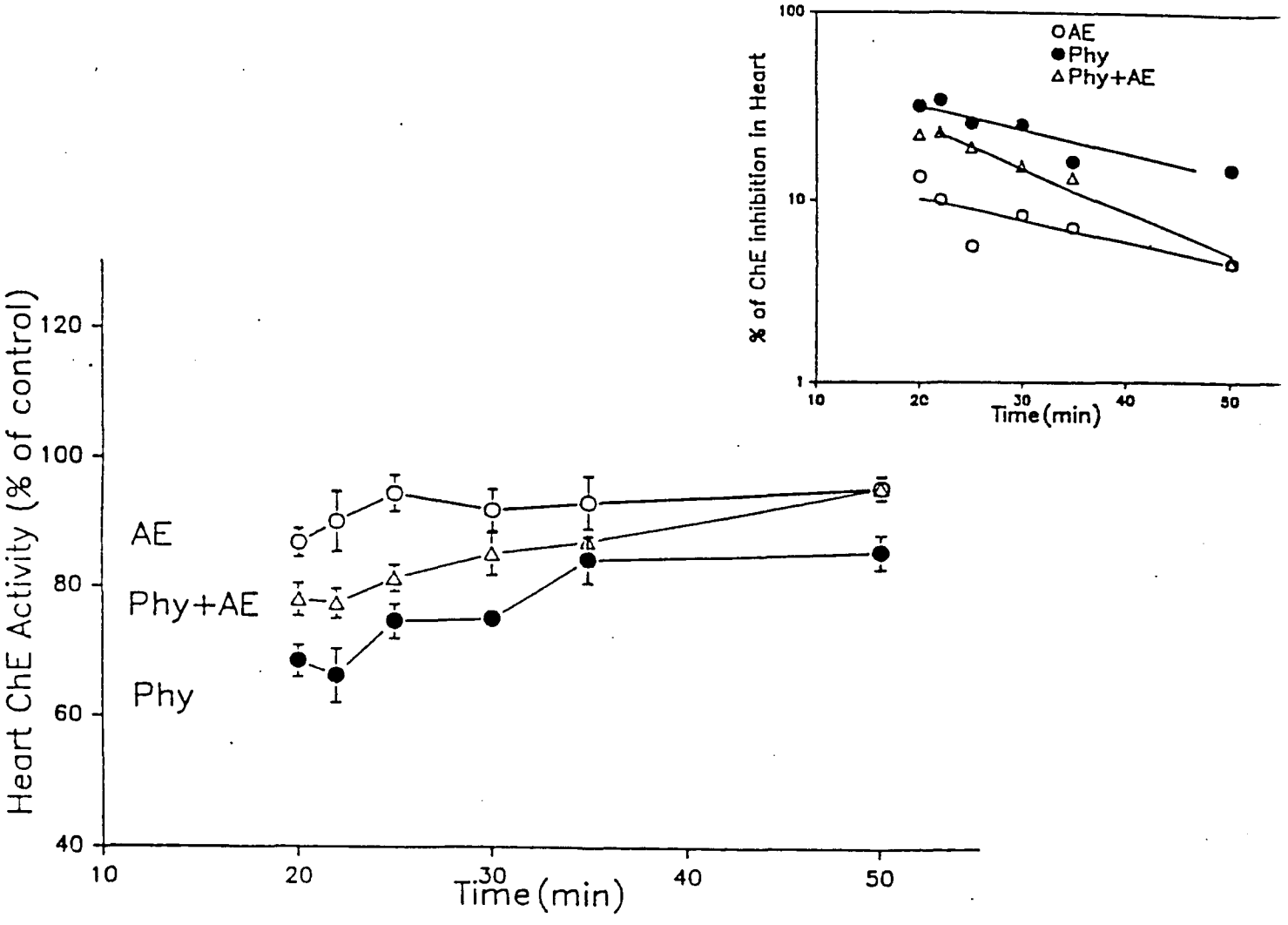


Fig. 33: Effect of acute exercise (AE) (80% $VO_{2\ max}$), Phy (70 µg/kg) and phy then concurrent AE on ChE activity in diaphragm as % of control after 20 min of Phy administration and/or exercise. Inset shows the plot of % ChE inhibition vs. time in diaphragm on semilog graph to obtain the rate of decarbamylation (K_d) of ChE enzyme. K_d for Phy in diaphragm is 0.078 min^{-1}; K_d for Phy + AE in heart is 0.0067 min^{-1}.

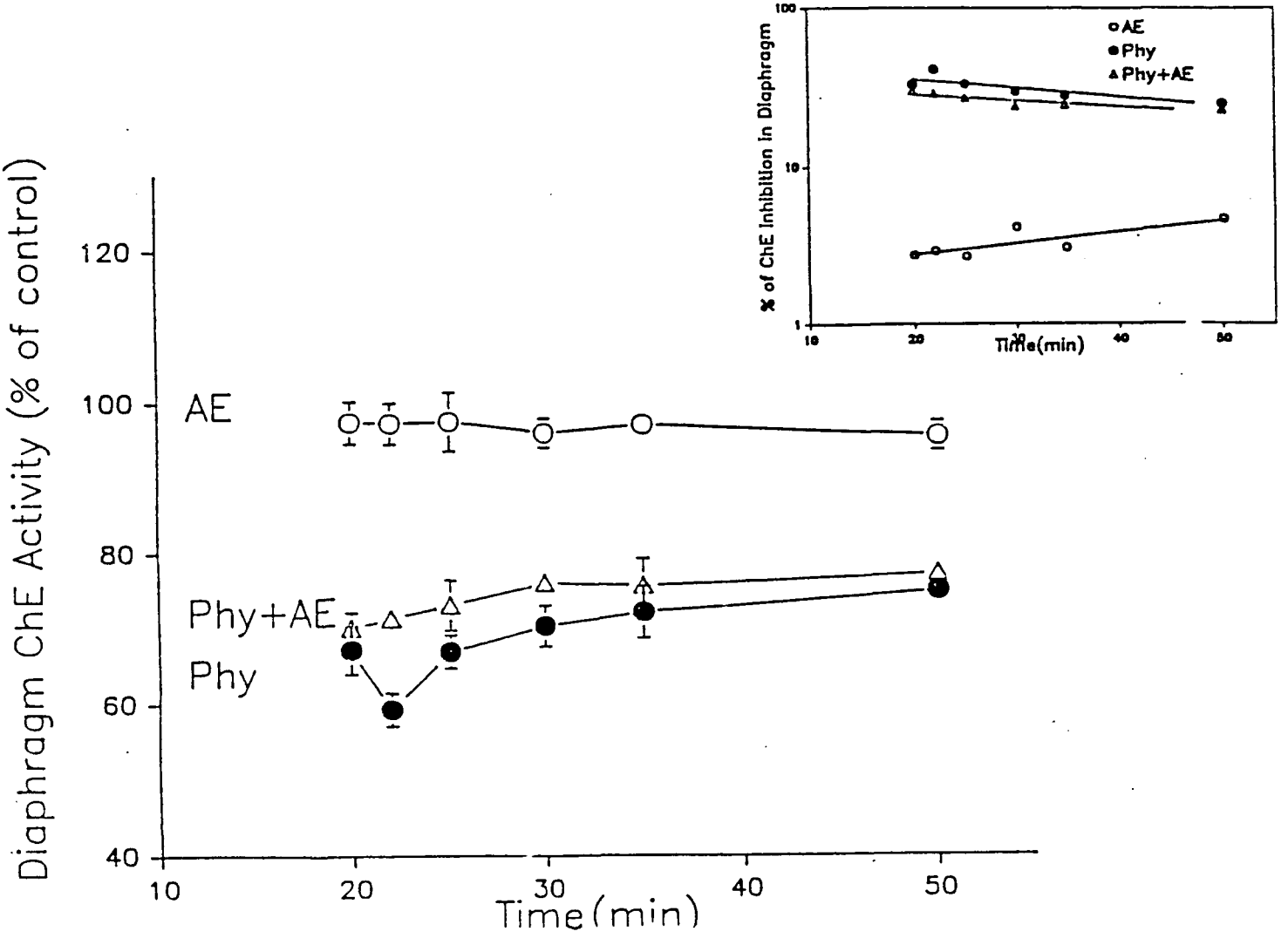
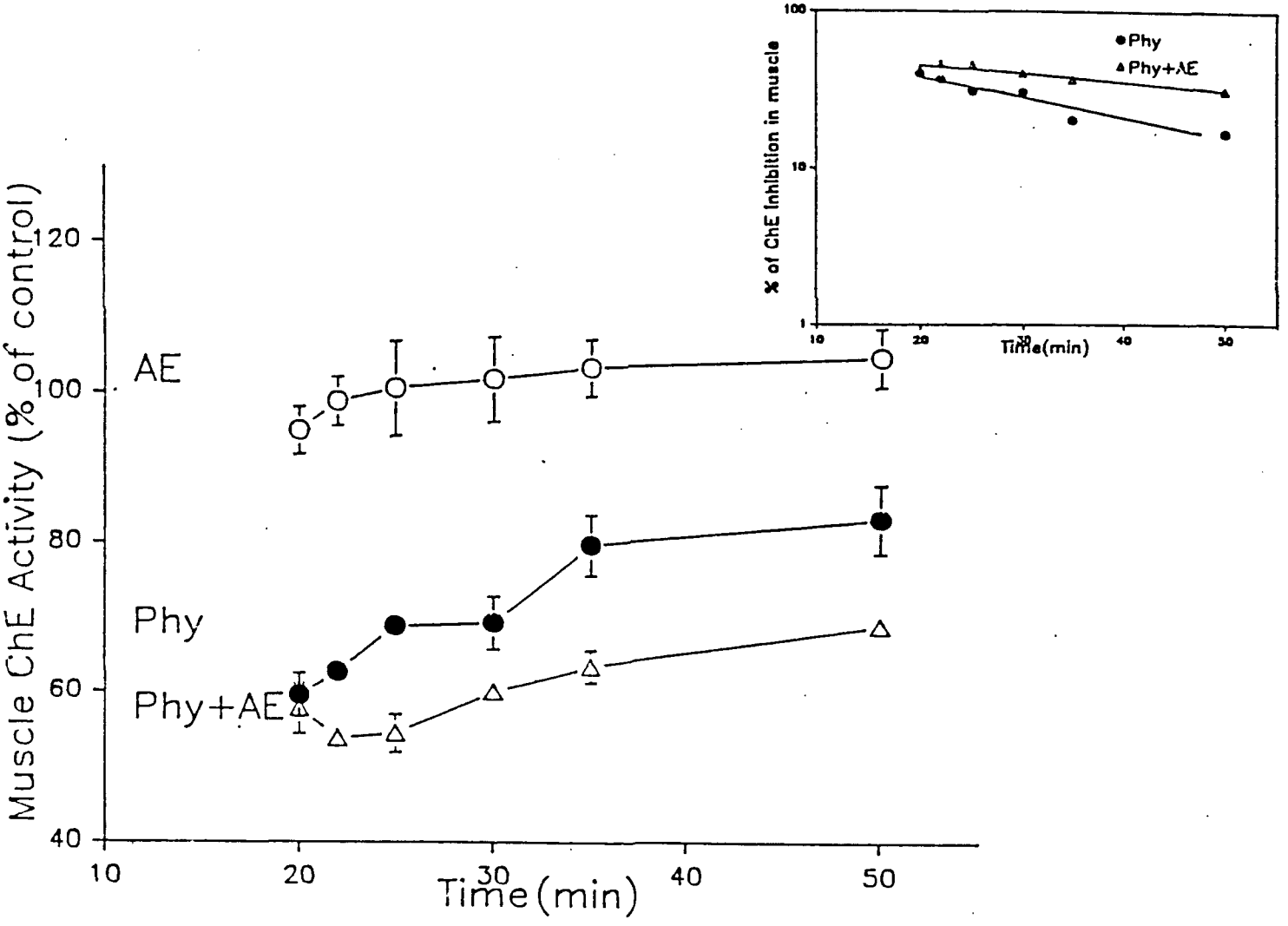


Fig. 34: Effect of acute exercise (AE) (80% $VO_{x\ max}$), Phy (70 µg,kg) and Phy then concurrent AE on ChE activity in muscle os % of control after 20 min of Phy administration and/or exercise. Inset shows the plot of % ChE inhibition vs. time in muscle on semilog graph to obtain the rate of decarbamylation (K_d) of ChE enzyme. K_d for Phy in muscle is 0.0308 min^{-1}; K_d for Phy + AE in muscle is 0.0135 min^{-1}.



X. EFFECT OF PHYSOSTIGMINE AND CONCURRENT ACUTE EXERCISE ON THE TIME COURSE OF LACTATE AND PYRUVATE IN PLASMA, AND TISSUES OF RAT

Introduction

Phy is extensively used as an antidote for tricyclic antidepressant drug overdosage. Phy, a centrally acting carbamate, and other cholinolytic compounds carry the risk of causing impairment, to some degree, of critical performances (143). Therefore, in an extensive search for a better analog of Phy for the treatment of Alzheimer patients or as an antidote for some nerve agents, a large number of Phy analogs have been synthesized (144,145). Eseroline moiety is a primary constituent of the majority of these carbamate analogs. Some of these are potent inhibitors of AChE, and eseroline may be produced by hydrolysis of the carbamate chain in these Phy analogs. Eseroline has been identified as a metabolite of Phy in plasma, brain, muscle, and liver after administration of [^{3}H]-Phy to rats (1,2,4,140). Phy is metabolized to eseroline and is converted to rubreserine (quinone-type compound). Quinones are known to disturb the mitochondrial function (69). Mitochondrial RA (Phy + metabolites) was reported to increase continuously up to 60 min in rat brain after the administration of [^{3}H]-Phy to rat (73).

Recently Somani et al. (146) have shown that eseroline induces neuronal cell death in 3 neuronal cell culture systems, mouse neuroblastoma NIE-115, rat glioma C_6 and neuroblastoma-glioma hybrid NG 108-15. It seems that eseroline causes neuronal cell death by a mechanism involving loss of cell ATP.

Phy and its metabolites may alter mitochondrial function resulting in altered redox state as reflected by L/P ratio. The Phy may also interfere with cell L/P ratio by creating an oxygen tension in the cell. The sensitivity of Phy may vary from tissue to tissue.

The role of lactate during exercise has been the subject of much interest in recent years due to its diverse metabolic role. Conflicting results were reported regarding the accumulation of lactate in blood and muscle during exercise (147). Lactate can be formed by mass conversion of pyruvate without the change in L/P ratio. The mitochondrial membrane proton shuttle may be too slow to reoxidize reduced cytosolic NAD, resulting in the conversion of pyruvate to lactate. This results in increase of L/P ratio (148). Lactate will also be formed as a net conversion when the rate of pyruvate production is higher than the citric acid cycle to metabolize it (149). The mitochondrial membrane proton shuttle oxidizes cytosolic NADH + H^+ as it transfers protons and electrons to mitochondrial O_2. This results in the conversion of pyruvate to lactate. The equilibrium of lactate dehydrogenase reaction depends on the ratio between pyruvate and lactate, the concentration of protons and the NADH concentration. An increase in the content of protons or NADH causes a shift in lactate dehydrogenase reaction towards lactate formation (150); hence, it is important to determine lactate and pyruvate concentration to evaluate the effects of drugs like Phy under different conditions.

Therefore, these studies were carried out to investigate the effect of Phy, exercise, and the combination of the two on the lactate, pyruvate, or L/P ratio in plasma, muscle, and brain.

Materials and Methods

Phy free base was obtained from Sigma Chemical Co. (St. Louis, MO). [^{3}H]-Phy (13 Ci/mmol) was custom-synthesized by Amersham Corporation (Chicago, IL). Diagnostic kits were purchased from Sigma Chemical Co. (St. Louis, MO) for the determination of lactate of pyruvate. All other chemicals were of analytical grade and were obtained from the usual commercial sources.

Preparation of the [^{3}H]-Phy solution. Phy was labeled with tritium on both ortho positions to the carbamate chain on the aromatic ring of Phy. [^{3}H]-Phy was diluted with unlabeled Phy (162.07 μCi/140 μg/ml) (1). The solution was prepared using physiological saline (0.9% w/v) in which 10 μl of hydrochloric acid was added to ensure that the solution was in an acidic pH range. The purity of Phy was assessed using high-performance liquid chromatography (HPLC) and an ultraviolet detector and also by monitoring the [^{3}H]-Phy in the eluant. The solution used in all experiments was greater than 95% pure.

Animals: Male Sprague-Dawley rats (Harlan Industries, Indianapolis, IN) weighing 175-200 g were used in this study. The rats were divided in to four groups: sedentary control (Gr I), acute exercise (80% $VO_{2\ max}$) (Gr II), Phy administration (70 μg/kg, i.m.) (Gr III), and Phy + acute exercise (Gr IV).

Phy Administration and Exercising of Rats in Treadmill: The Oxyscan System and Omnipacer Treadmill (Omnitech. Inc., Columbus, OH) were used to monitor maximal oxygen consumption ($VO_{2\ max}$). Rats from Gr II and Gr IV were exercised at different levels on treadmill to obtain 100% $VO_{2\ max}$ of each rat (126).

Determination of maximum oxygen consumption ($VO_{2\ max}$) was carried out in the beginning of the training protocol in order to determine the $VO_{2\ max}$ for each rat. Measurement of maximal oxygen consumption (100% $VO_{2\ max}$) was considered valid only if the animal ran until it could no longer maintain pace with the treadmill. During training, the fifth day of every week, $VO_{2\ max}$ was determined for each rat. After 3 days, rats from Gr II were exercised at different speeds and inclinations for 20 min, corresponding to approximately 80% $VO_{2\ max}$. Rats from Gr IV were administered Phy and then were exercised for 20 min. Rats of Gr II and Gr IV were sacrificed at 20, 22, 25, 30, 35, and 50 min after Phy administration or start of exercise. Rats from Gr III were administered Phy (70 μg/kg, i.m.) and were sacrificed at 20, 22, 25, 30, 35, and 50 min. Sedentary control rats (Gr I) were administered saline and were sacrificed after 15 min. All animals were sacrificed between 8:00 and 11:00 AM to minimize circadian cycle effects. Each group consisted of a minimum of 4 rats.

Blood was collected into precooled centrifuge tubes after decapitation. Plasma was separated from blood immediately at 4°C by centrifugation for 10 min at 5000 RPM (Jouan Inc., Winchester, Va) and deproteinized with 8% (w/v) perchloric acid immediately. The supernatant was used for the estimation of lactate and pyruvate. Lactate and pyruvate were determined by the enzymatic method of Fleischer (79) (Sigma Diagnostics, St. Louis, MO) and expressed as mmol/L.

The brain and gastrocnemius muscle of the leg were quickly dissected and plunged into liquid nitrogen. The frozen brain and muscle were wrapped in aluminum foil and stored at -70°C until analysis.

Lactate and Pyruvate Determination: Tissues were weighed in frozen condition and powdered under liquid nitrogen. To the powdered tissues, 10% (w/v) cold perchloric acid was added before thawing. Frozen tissue powder was sonicated for 30 sec with an ultrasonic processor probe in 2 intervals of 15 sec each, and 10% homogenate was prepared by adding the required amount of precooled perchloric acid. Homogenation was done below 0°C to minimize metabolic conversions in tissues. Protein precipitate was removed by centrifugation (Sorvall, Dupont) and the supernatant was neutralized with potassium carbonate. The supernatant was separated by centrifugation and used for assays. Muscle and brain lactate and pyruvate were determined by the enzymatic methods of Gutmann and Wahlefelt (151) and were expressed as μmol/g wet w of the tissue.

Statistical Analysis: The data were subjected to a parametric two-way analysis of variance for unequal n's, using a general linear model approach. This approach tested the overall effect of experimental groups with time, both as independent factors. To compare experimental groups against the control group, a one-way analysis of variance was performed at each time. In addition, each time point was compared in each group using the effect of time. Follow-up tests were performed using Duncan's multiple range test. Statistical significance was evaluated at the 5% level.

Results

The data on effect of Phy, acute exercise, and Phy + acute exercise on time course of lactate, pyruvate, and L/P ratio in plasma are given in Tables 20-22.

Phy administration initially increased lactate concentration to 123% of control, which declined up to 30 min (90% of control) (Table 21). However, the effect of exercise alone showed the opposite effect. The lactate concentration at 20 min was 119% of control which increased to 166% of control at 25 min and then declined to normal level at 35 min. The combined effect of Phy and exercise also showed an increase in lactate concentration at 22 min to 44% of control which declined steadily and returned to control level by 50 min.

Plasma pyruvate increased to 131% of control with Phy administration at 22 min and then decreased to 80% of control by 50 min (Table 22). Exercise alone showed 157% of pyruvate and decreased to 133% of control by 50 min. Phy + exercise showed 156% of control at 20 min and decreased to 87% of control by 50 min. L/P ratio, an indicator of cell redox state, will increase when the cell redox state is reduced, indicating the conversion of pyruvate to lactate. The L/P ratio showed an initial increase (34.5) with Phy at 20 min. The ratio declined to 22.4 at 30 min, then steadily increased to 41.5 at 50 min. Exercise alone did not show an increase in L/P ratio at all time points and was below control level. However, in Phy + exercise, L/P ratio was initially below control level up to 35 min, but at 50 min, it was 32.2.

Muscle: The data on effects of Phy, acute exercise, and Phy + acute exercise on the time course of muscle lactate, pyruvate, and L/P ratios are shown in Tables 23-25. Phy administration decreased lactate concentration from 104% to 71% of control from 20 to 50 min. Exercise also decreased lactate up to 74% of control. However Phy + exercise increase lactate (84-137% of control) from 20 to 50 min (Table 24).

Pyruvate concentration decreased to 71% of control at 20 min and further decreased to 33% of control by 50 min (Table 25). Though the exercised group

showed 74% of control at 20 min (which corresponds to the cessation of exercise), pyruvate increased to control level by 50 min which is an expected phenomenon. But in the Phy + exercise group, pyruvate was lowest (50% of control) compared to Phy or exercise group, and decreased further to 37% of control by 50 min. These trends were reflected in L/P ratios. Phy increased L/P ratio to 238 by 25 min but declined to 158 by 35 min. This increase in L/P ratio was due to the decrease in pyruvate at the 25-min period. The L/P ratio increased again to 216 by 50 min, indicating the conversion of pyruvate to lactate even up to 50 min. In the exercise group, a typical decreasing trend (160 to 72) in L/P ratio was observed from 20 to 50 min. The interesting observation was that Phy + exercise increased the L/P ratio to several-fold (371%) indicating the conversion of pyruvate to lactate even up to 50 min. This indicates the enhanced effect of Phy during exercise.

Brain: The data on effects of Phy, acute exercise, and Phy + acute exercise on the time course of lactate, pyruvate, and L/P ratio in brain are shown in Tables 26-28. Brain lactate has not shown much variation compared to plasma and muscle. Phy administration initially decreased lactate concentration to 84% of control up to 25 min which increased to control level by 35 min. However, exercise alone showed an increase (127% of control) at 25 min and decreased below control (76% of control) at 50 min. The combined effect of Phy + exercise showed an increase in lactate up to 30 min. (131% of control) and returned to 146% of control at 50 min. Pyruvate was found below control levels at all time points (45-57% of control) in Phy-administered rats. Exercise alone decreased pyruvate from 60 to 34% of control from 20 to 50 min. Pyruvate showed a continuous decrease (125%-50% of control) in the Phy + exercise group, from 20 to 50 min. Though lactate has not shown much variation, the decrease in pyruvate resulted in all groups. A small increase in pyruvate and a decrease in lactate decreased the L/P ratio to 4.4 at 25 min-this increased to 11.5 by 50 min. Exercise showed an opposite phenomenon in brain compared to muscle. The L/P ratio increased from 9.5 to 7.3 from 20 to 50 min. In the Phy + exercise group also the L/P ratio increased from 5.6 to 14.7 from 20 to 50 min, indicating the conversion of pyruvate to lactate continuously.

Discussion

In plasma all 3 groups showed an initial increase in lactate concentration which returned to control level by 35 min. Plasma lactate returned to control level by 15 min after cessation of exercise. Plasma pyruvate also increased in all 3 groups up to 15 min. In Phy and Phy + exercise groups pyruvate decreased to below control level, whereas in the exercise group it was maintained at the control level. These changes seem to be the reflection of changes taking place in muscle as reported by Brzezinska (152). It is well known that during exercise lactate formation in muscle will increase (153) and will continue to increase up to 5 min after exercise. Wasserman et al. (148) reported that increased blood lactate may be due to diffusion of lactate from muscle to blood. Lactate formed in muscle may have diffused to blood, thereby increasing blood lactate. During the early recovery period after exercise blood pyruvate was reported to rise up to 5 min (154,80), whereas blood lactate decreased after 5 min, resulting in a decrease in L/P ratio. The same trend was also observed in the exercised group. But in Phy-administered, and Phy + exercise groups the L/P ratio showed an increasing trend after 10 min of exercise, indicating that Phy may be interfering with normal functions of the cell.

After exercise, muscle lactate decreased, whereas muscle pyruvate increased resulting in linear decrease in L/P ratio up to 50 min. Phy decreased both muscle lactate and pyruvate up to 50 min, whereas Phy + exercise increased muscle lactate significantly. This resulted in the significant increase in L/P ratio in muscle. Sahlin et al. (80) reported a decrease in muscle lactate and an increase in muscle pyruvate after exercise. This results in a linear decrease in L/P ratio (148). The continuous decrease in muscle pyruvate, increase in lactate, and an increase in L/P ratio suggests that pyruvate is continuously converted into lactate, even after cessation of exercise. This is an interesting phenomenon.

Lactate can increase in cell due to mass conversion of pyruvate to lactate during increased glycolysis without change in L/P ratio. This is not the case here, since there was a steady increase in L/P ratio. Wasserman et al. (148) reported that the mitochondrial membrane proton shuttle will be slow to reoxidize reduced cytosolic NAD. This results in the conversion of pyruvate to lactate, resulting in the decrease in L/P ratio. In our experiment, it seems that Phy or its metabolite eseroline may be interfering with mitochondrial membrane proton shuttle. This may have resulted in increased formation of lactate. An accumulation of Phy and its metabolites in the mitochondria of brain was reported after Phy administration (73).

In brain pyruvate seems to be affected more than lactate. Lactate increased in brain in both exercise and Phy + exercise groups. The decrease of pyruvate in brain is significant in all groups. The L/P ratio indicates that pyruvate was converted into lactate in brain up to 30 min after cessation of exercise. In brain and muscle, Phy + exercise seem to prolong the effect of exercise on lactate and pyruvate metabolism. Since muscle is more directly involved in exercise, and blood flow to muscle will increase during exercise, Phy accumulation in muscle cells will be greater. So Phy effects are more pronounced in muscle compared to brain. The other possibility for the increase in lactate concentration in muscle and brain may be the depletion of oxygen to tissues by Phy if cellular oxygen content was reduced below the critical level necessary for mitochondrial oxidative phosphorylation. The NADH content rises leading to the reduction of pyruvate to lactate (150). Rats were exercised at 80% $VO_{2\ max}$ and the animals needed more oxygen to keep up with increased metabolic processes. Oxygen tension during exercise was created in tissues. Phy may be enhancing that tension and prolonging its effect for longer.

In *in vitro* studies with neuronal cell lines incubated with eseroline, the metabolite of Phy depleted ATP and caused leakage of lactic acid, dehydrogenase, and leakage of adenine nucleotides. These results indicated that eseroline or its oxidative product rubreserine interferes with mitochondrial function (146).

These results support that the combination of Phy administration and exercise will have an additive effect on lactate formation.

Conclusions

Based on these studies, it can be concluded that: (1) conversion of pyruvate to lactate prolonged up to 50 min (30 min after exercise) when Phy is administered before single acute exercise; (2) the effect of Phy was more pronounced on muscle than brain after exercise, which might be due to increased Phy concentration in muscle due to an increased blood flow; and (3) Phy may be creating an O_2 tension by increasing the formation of lactate after exercise.

Table 20: Effect of physostigmine (70 µg/kg, i.m.) and concurrent acute exercise (80% $VO_{2\ max}$) on the time course of lactate, pyruvate and L/P ratio in **plasma** of rat.

Time	Lactate			Pyruvate			L/P Ratio		
	Acute Exercise	Phy	Phy + Exercise	Acute Exercise	Phy	Phy + Exercise	Acute Exercise	Phy	Phy + Exercise
20	3.8 ± 0.33	3.9 ± 0.6	3.8 ± 0.05	0.2 ± 0.04	0.1 ± 0 .03	0.2 ± 0.03	23.5	34.5	19.3
22	4.2 ± 0.3	3.9 ± 0.2	4.7 ± 0.5	0.2 ± 0.01	0.12 ± 0.04	0.2 ± 0.05	21.4	23.6	23.4
25	5.4* ± 0.9	3.3 ± 0.5	4.3 ± 0.4	0.2 ± 0.4	0.1 ± 0.03	0.2 ± 0.01	22.6	23.2	22.1
30	4.5 ± 0.4	2.9 ± 0.6	4.0 ± 0.6	0.2 ± 0.03	0.1 ± 0.04	0.2 ± 0.01	23.5	22.4	20.5
35	3.4 ± 0.5	3.5 ± 0.4	3.1 ± 0.2	0.2 ± 0.03	0.1 ± 0.02	0.1 ± 0.02	18.6	32.2	24.8
50	3.3 ± 0.6	4.1 ± 0.7	3.2 ± 0.2	0.2 ± 0.03	0.1 ± 0.01	0.1 ± 0.01	19.7	41.5	32.2
Con-trol	3.2 ± 0.3	3.2 ± 0.3	3.2 ± 0.3	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.01	25.8 ±	25.8 ±	25.8 ±

Values are mean ± S.E.M. of 4 rats

Table 21: Effect of physostigmine (70 µg/kg, i.m.) and concurrent acute exercise (80% $VO_{2\ max}$) on the time course of lactate concentration (% of control) in **plasma** of rat.

Time (min)	Acute Exercise	Phy	Phy + Acute Exercise
20	119.3 ± 10.3	123.1 ± 20.1	117.0 ± 1.7
22	130.4 ± 8.8	120.6 ± 7.7	144.3 ± 15.3
25	166.5 ± 27.2	101.3 ± 15.3	134.0 ± 11.2
30	140.4 ± 11.8	90.4 ± 17.8	123.3 ± 17.8
35	104.0 ± 14.7	109.9 ± 12.8	106.1 ± 7.1
50	101.9 ± 20.0	128.0 ± 20.9	100.7 ± 7.1

Values are mean ± S.E.M. of 4 rats
Control Value: 3.2 mmol/L

Table 22: Effect of physostigmine (70 µg/kg, i.m.) and concurrent acute exercise (80% $VO_{2\ max}$) on the time course of pyruvate concentration (% of control) in **plasma** of rat.

Time (min)	Acute Exercise	Phy	Phy + Acute Exercise
20	130.9 ± 28.9	91.1 ± 31.1	156.3 ± 24.7
22	157.3 ± 9.1	131.2 ± 34.3	159.2 ± 43.2
25	189.9 ± 30.7	112.8 ± 28.6	156.2 ± 7.1
30	154.0 ± 25.6	104.1 ± 33.6	154.7 ± 10.7
35	144.6 ± 21.5	88.0 ± 13.8	97.7 ± 13.1
50	133.0 ± 22.0	79.6 ± 11.6	87.0 ± 7.2

Values are mean ± S.E.M. of 4 rats
Control Value: 0.12 ± 0.01 mmol/L

Table 23: Effect of physostigmine (70 µg/kg, i.m.) and concurrent acute exercise (80% $VO_{2\ max}$) on the time course of lactate, pyruvate and L/P ratio in **muscle** of rat

Time	Lactate			Pyruvate			L/P Ratio		
	Acute Exercise	Phy	Phy + Exercise	Acute Exercise	Phy	Phy + Exercise	Acute Exer-cise	Phy	Phy + Exer-cise
20	2.5 ± 0.5	2.2 ± 0.3	1.8 ± 0.2	0.01 ± 0.001	0.01 ± 0.003	0.01 ± 0.003	160.8	147.6	171.0
22	2.3± 0.1	2.3 ± 0.1	1.9 ± 0.2	0.1 ± 0.002	0.01 ± 0.004	0.01 ± 0.001	136.4	156.6	166.9
25	2.3 ± 0.2	2.1 ± 0.05	2.1 ± 0.3	0.1 ± 0.004	0.01 ± 0.001	0.01 ± 0.001	131.4	238.7	170.4
30	1.7 ± 0.1	2.1 ± 0.1	2.2 ± 0.3	0.02 ± 0.003	0.01 ± 0.001	0.01 ± 0.001	91.2	172.3	171.4
35	1.6± 0.1	1.6 ± 0.1	2.6 ± 0.6	0.02 ± 0.001	0.01 ± 0.001	0.01± 0.003	81.4	158.0	271.0
50	1.6± 0.1	1.5 ± 0.1	3.0 ± 0.2	0.02 ± 0.003	0.01 ± 0.000	0.01 ± 0.001	72.0	216.0	371.8
Con-trol	2.4 ± 0.4	2.4 ± 0.4	2.4 ± 0.4	0.02 ± 0.002	0.02 ± 0.002	0.12 ± 0.002	116.2	116.2	116.2

Values are mean ± S.E.M. of 4 rats

Table 24: Effect of physostigmine (70 µg/kg, i.m.) and concurrent acute exercise (80% $VO_{2\ max}$) on the time course of lactate concentration (% of control) in **muscle** of rat.

Time (min)	Acute Exercise	Phy	Phy + Acute Exercise
20	118.5 ± 22.4	104.5 ± 12.8	84.6 ± 7.9
22	106.8 ± 6.2	106.5 ± 7.3	87.4 ± 9.6
25	106.0 ± 10.1	97.9 ± 2.4	99.3 ± 15.0
30	78.9 ± 2.9	96.8 ± 3.3	104.8 ± 15.2
35	74.0 ± 3.1	76.5 ± 6.6	123.4 ± 26.2
50	74.5 ± 6.9	71.8 ± 5.0	137.9 ± 9.7

Values are mean ± S.E.M. of 4 rats
Control Value: 2.1 ± 0.4 µmol/g wet w

Table 25: Effect of physostigmine (70 µg/kg, i.m.) and concurrent acute exercise (80% $VO_{2\ max}$) on the time course of pyruvate concentration (% of control) in **muscle** of rat.

Time (min)	Acute Exercise	Phy	Phy + Acute Exercise
20	74.6 ± 8.3	71.7 ± 15.4	50.1 ± 17.6
22	79.3 ± 12.9	68.9 ± 20.3	52.9 ± 8.1
25	81.7 ± 22.4	61.4 ± 4.7	59.0 ± 2.5
30	87.7 ± 14.3	56.9 ± 4.3	62.62 ± 4.7
35	92.8 ± 6.0	49.0 ± 3.4	46.12 ± 15.1
50	104.8 ± 14.7	33.6 ± 1.1	37.6 ± 6.1

Values are mean ± S.E.M. of 4 rats
Control Value: 0.02 ± 0.003 µmol/g wet w

Table 26: Effect of physostigmine (70 µg/kg, i.m.) and concurrent acute exercise (80% $VO_{2\ max}$) on the time course of lactate, pyruvate, and L/P ratio in **brain** of rat

Time	Lactate			Pyruvate			L/P Ratio		
	Acute Exercise	Phy	Phy + Exercise	Acute Exercise	Phy	Phy + Exercise	Acute Exercise	Phy	Phy + Exercise
20	0.8 ± 0.02	0.8 ± 0.01	1.1 ± 0.03	0.1 ± 0.01	0.1 ± 0.004	0.2 ± 0.04	9.5	12.5	5.6
22	1.3 ± 0.1	0.8 ± 0.04	1.0 ± 0.1	0.1 ± 0.01	0.1 ± 0.004*	0.10 ± 0.02	8.7	11.8	9.6
25	1.2 ± 0.2	0.8 ± 0.03	1.2 ± 0.1	0.1 ± 0.02	0.1 ± 0.02	0.1 ± 0.01	9.7	8.2	12.1
30	1.0 ± 0.01	0.9 ± 0.01	1.2 ± 0.12	0.1 ± 0.005	0.1 ± 0.05	0.1 ± 0.01	9.1	8.6	13.5
35	0.7 ± 0.02	1.0 ± 0.02	1.3 ± 0.10	0.1 ± 0.002	0.1 ± 0.01	0.1 ± 0.01	7.6	12.0	16.0
50	0.7 ± 0.01	0.9 ± 0.02	1.3 ± 0.1	0.1 ± 0.004	0.1 ± 0.01	0.1 ± 0.01	7.4	18.5	14.7
Control	0.9 ± 0.03	0.9 ± 0.03	0.9 ± 0.03	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.01	6.4	6.4	6.4

Values are mean ± S.E.M. of 4 rats

Table 27: Effect of physostigmine (70 µg/kg, i.m.) and concurrent acute exercise (80% $VO_{2\ max}$) on the time course of lactate concentration (% of control) in **brain** of rat.

Time (min)	Acute Exercise	Phy	Phy + Acute Exercise
20	89.1 ± 2.7	89.1 ± 2.1	109.1 ± 3.8
22	138.4 ± 14.9	85.6 ± 4.4	111.1 ± 10.3
25	127.1 ± 21.7	84.0 ± 4.2	127.3 ± 8.3
30	108.4 ± 2.1	98.1 ± 1.5	131.5 ± 13.8
35	80.3 ± 2.7	102.7 ± 2.5	143.6 ± 11.4
50	76.9 ± 1.5	109.2 ± 2.6	146.6 ± 16.5

Values are mean ± S.E.M. of 4 rats
Control Value: 0.9 ± 0.03 µmol/g wet w

Table 28: Effect of physostigmine (70 µg/kg, i.m.) and concurrent acute exercise (80% $VO_{2\ max}$) on the time course of pyruvate concentration (% of control) in **brain** of rat.

Time (min)	Acute Exercise	Phy	Phy + Acute Exercise
20	60.4 ± 7.4	45.9 ± 3.2	125.9 ± 27.5
22	102.2 ± 5.6	46.9 ± 2.9	74.8 ± 13.6
25	85.8 ± 15.0	65.7 ± 11.2	68.0 ± 51.5
30	77.3 ± 4.1	75.1 ± 3.7	62.9 ± 10.6
35	68.5 ± 2.1**	58.9 ± 6.2	57.8 ± 7.1
50	67.2 ± 3.1	57.8 ± 11.0	50.1 ± 8.3

Values are mean ± S.E.M. of 4 rats
Control Value: 0.14 ± 0.02 µmol/g wet w

XI. EFFECT OF ENDURANCE TRAINING AND CHRONIC PHYSOSTIGMINE INFUSION ON CHOLINESTERASE ACTIVITY

Introduction

Acute or chronic administration of Phy has been effective against organophosphate intoxication (34). We have previously shown that a low dose of Phy (70 µg/kg, i.m.) and AE increase the endurance time of rat weighing 160-200 g, whereas a decrease in endurance time of rats (weighing 500 g) dosed with Phy salicylate (200 µg/kg, i.v.) has been reported (44).

Phy, or any other anti-ChE agents such as pyridostigmine, is likely to be used on a chronic basis in battlefield situations. Chronic administration of Phy leads to the development of tolerance to this drug (188). This section of the report describes the effects of continuous infusion of Phy via a chronically implanted osmotic pump (1) on a steady-state level of ChE activity in RBC in trained and untrained rat (control); and (2) on endurance time of rat.

Materials and Methods

Phy hemisulfate was obtained from Sigma Chemical Co. (St. Louis, MO). [^{3}H]-AChI was obtained from New England Nuclear Corporation. Ready-Solv was obtained from Beckman Instruments, Inc. (Fullerton, CA). Diagnostic kit for the determination of Hb was purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade and were obtained from the usual commercial sources. Alzet® osmotic pumps (Model 2002, Alza, Palo Alto, CA), which delivered 0.5 µl/hr for 14 days, were used.

Animals: Male Sprague-Dawley rats obtained from Harlan Industries, Indianapolis, IN, were kept in quarantine for 1 wk. The rats weighed 160-200 g on the first day of exercise and 300-350 g at the time of sacrifice. The rats were 15-16 wk old, and they were young adults. The rats were divided into 4 groups; Gr I served as sedentary control. Continuous infusion pumps filled with saline were implanted into the back of their necks, and they were sacrificed along with the experimental group on various days. Gr II received Phy as a base (34.5 µg/kg/hr) at a rate of 0.50 µl/hr by continuous infusion by an osmotic pump. These rats were sacrificed at the end of days 1, 2, 7, 12, and 13 of infusion. Gr III were endurance-trained for 5.5 wk. Continuous infusion osmotic pumps were implanted subcutaneously into the back of the rats' necks, and they were infused with saline until the time of sacrifice. The last exercise was given 18 hr prior to sacrifice. These rats were sacrificed at the end of days 2, 7, 12, and 13 of infusion. Gr IV were endurance-trained for 5.5 wk, then received Phy 34.5 µg/kg/hr at a rate of 0.5 µl/hr by continuous infusion, using an osmotic pump implanted subcutaneously into the back of their necks. The rats were sacrificed at the end of days 1, 2, 7, 12, and 13 of infusion.

Training of Rats: Rats from Gr III and Gr IV were acclimatized to treadmill in the beginning and were trained on a 9-channel motor-driven treadmill (custom built at SIU) using an incremental exercise program. During this program of exercising, the speed (meters/min), angle of inclination (% grade), and the duration (min) of exercise were varied to obtain different levels of exercise intensity as shown in Table 5.

The exercise protocol was selected and designed on the basis that a more demanding exercise task confronted each animal as each wk of training elapsed.

In the first 2 wk, the conveyor belt speeds were 8.3, 15.2, and 19.3 m/min, and the angle of inclination was 6°. The exercise duration for each speed was 5 min for the first wk and 10 min the second wk. In the third and fourth wk exercising speeds were increased to 19.3, 26.8, and 30.3 m/min. The duration of exercise was 10 min. The angle of inclination was 6° during the third wk and 9° in the fourth wk. The final one and one-half wk of exercising involved achieving and sustaining speeds of 35.4, 40.0 and 43.8 m/min at a 9° angle of inclination for 10 min.

The rats from Gr I and II were not trained, but were maintained under conditions similar to those of trained rats. $VO_{2\ max}$ was determined at the beginning, midpoint and at the end of the training protocol, using the Omnitech Oxyscan Analyzer.

Preparation of Phy Dose and Loading of the Osmotic Pump: Phy hemisulfate was infused subcutaneously via an osmotic pump. Due to the limited solubility and stability of Phy in saline solution, the Phy hemisulfate was dissolved in the following vehicle: 10% ethanol, 20% propylene glycol, and 70% 1:2000 glacial acetic acid, to obtain the concentration of Phy base as 34.5 µg/0.5 µl/kg/hr. The filling of the Alzet® osmotic pumps (Model 2002) was accomplished with a syringe (3 ml) and the blunt tipped, 25-gauge filling tube. The solution was drawn into the syringe and transferred carefully to the pump reservoir ensuring that the reservoir is free of air bubbles. Excess solution was wiped off, and the flow moderator was inserted until the cap was flush with the top of the pump. The excess solution displaced by the moderator was wiped off, and the loaded pumps were incubated in 0.9% saline for 4 hr at 37°C prior to insertion in the back of the neck.

Implantation of Alzet® Osmotic Pumps: Alzet® osmotic pumps (Model 2002, capacity 200 µl) having a constant pumping rate of 0.5 µl/hr for 14 days were used.

The rats were anesthetized with ketamine (100 mg/kg, i.m.) and acepromazine (1 mg/kg). The back of the neck was shaved with a clipper. An incision of about 1 centimeter was made on the skin. A small pocket was formed by spreading apart the subcutaneous connective tissue. The pump was inserted into the pocket with the flow moderator pointing away from the incision. The skin incision was closed with wound clips. The rats from Gr I and Gr III were infused with 0.9% saline, while those from Group II and Gr IV were infused with Phy (34.5 µg/kg/hr) at the rate of 0.5 µl/hr.

Exercising of Rats After Implantation of Osmotic Pumps: After implantation of the osmotic pumps, the trained rats from Gr III and Gr IV were exercised daily at belt speeds of 35.4, 40.0, and 43.8 (m/min) at an inclination of 9 degrees, for a duration of 10 min at each speed. However, the rats scheduled to be sacrificed were exercised in the evening prior to their allotted day of sacrifice. Three to five rats each from Gr II, III, and IV were sacrificed by decapitation, on day 1, 2, 7, 12, and 13 after the implantation of the osmotic pump. One or two rats from Gr I were sacrificed simultaneously, along with the other rats on each day. The brain was removed; the cortex, striatum, brainstem, and hypothalamus were dissected on ice, frozen, and stored at -70°C. Heart, diaphragm, liver, kidney, and thigh muscle were removed, rinsed with saline, blotted dry, frozen in liquid nitrogen, and stored at -70°C.

Determination of ChE Activity in RBC: Blood was collected in cold heparinized tubes after decapitation. 0.5 ml of whole blood was transferred to a test tube containing 10 ml ice-cold saline. Plasma was immediately separated from whole blood by centrifugation for 10 min at 2000 rpm (Jovan Inc., Winchester, Va.) at 4°C. The plasma was then stored at -70°C.

The erythrocytes were washed in ice cold saline by centrifuging at 2000 rpm (Jovan Inc.) for 10 min at 4°C. The cells were washed three times in this manner.

The suspension was prepared by adding 2 ml of ice cold saline to the washed erythrocytes and mixing the contents on a vortex mixer for 15 sec.

The ChE enzyme estimation was carried out according to a modification of the radiometric method of Johnson and Russell (11). In this procedure [^{3}H]-ACh is used as the substrate. This method measures the RA due to [^{3}H]-acetate formed by the enzymatic hydrolysis of [^{3}H]-AChI (1 mCi/.0136 mmole).

Fifty-µl aliquots of RBC suspension and 50 µl of phosphate buffer were put in glass scintillation vials and then 50 µl of freshly prepared [^{3}H]-ACh solution (0.199 µmol/µCi) was quickly added to each vial. The total volume of reaction mixture was 150 µl. All the estimations were carried out in triplicate. Blanks representing the nonenzymatic hydrolysis of [^{3}H]-ACh were also prepared in triplicate and subtracted off as background. The reaction mixture was incubated at 37°C for 15 min in Thermolyn water bath (Syborn Corp., MI), the contents were immediately cooled on ice, and stop solution (100 µl) was added to halt the enzymatic hydrolysis. Then 4 ml of toluene scintillation cocktail (0.51% PPO, 0.03% POPOP and 10% isoamyl alcohol) was added to each sample. Ready-Solv (Beckman, Fullerton, CA) was used for measuring specific activity of the substrate. The samples were mixed on a vortex, and the RA was counted in a Beckman LS 5800 Liquid Scintillation Spectrometer.

The ChE values of RBC are expressed as µmol of ACh hydrolyzed/min/g of Hb content.

Determination of Hemoglobin: The Hb content of RBC was determined by Sigma diagnostic kit, using a Shimatzu spectrophotometer at 540 nm.

Statistical Analysis: The ChE values were subjected to one-way analysis of variance (ANOVA) followed by Duncan's multiple range follow-up tests. Significant differences were accepted at $p < 0.05$.

Results and Discussion

Effect on Endurance Time: The values for endurance time of rats after trained exercise with or without Phy administration are given in Table 29. The end point for endurance time was when the rats were completely exhausted and were not able to continue running at the given intensity of exercise. Endurance-trained rats, infused with saline (Gr III) and endurance-trained rats infused with Phy (Gr IV) ran 22.2 ± 1.4 S.E.M. (n = 9) min and 19.4 ± 0.57 S.E.M. (n = 17) min, respectively, 7 hr after insertion of pumps (Table 29). On day 2 the ET + Phy Gr IV (n = 13) ran 15.3 min compared to 24.8 min for the saline administered Gr III (n = 9). The number of rats decreased on consecutive days because of sacrifice on different days. Trained exercise and Phy infusion decreased the endurance time of rats. The first few wk of training rats mostly

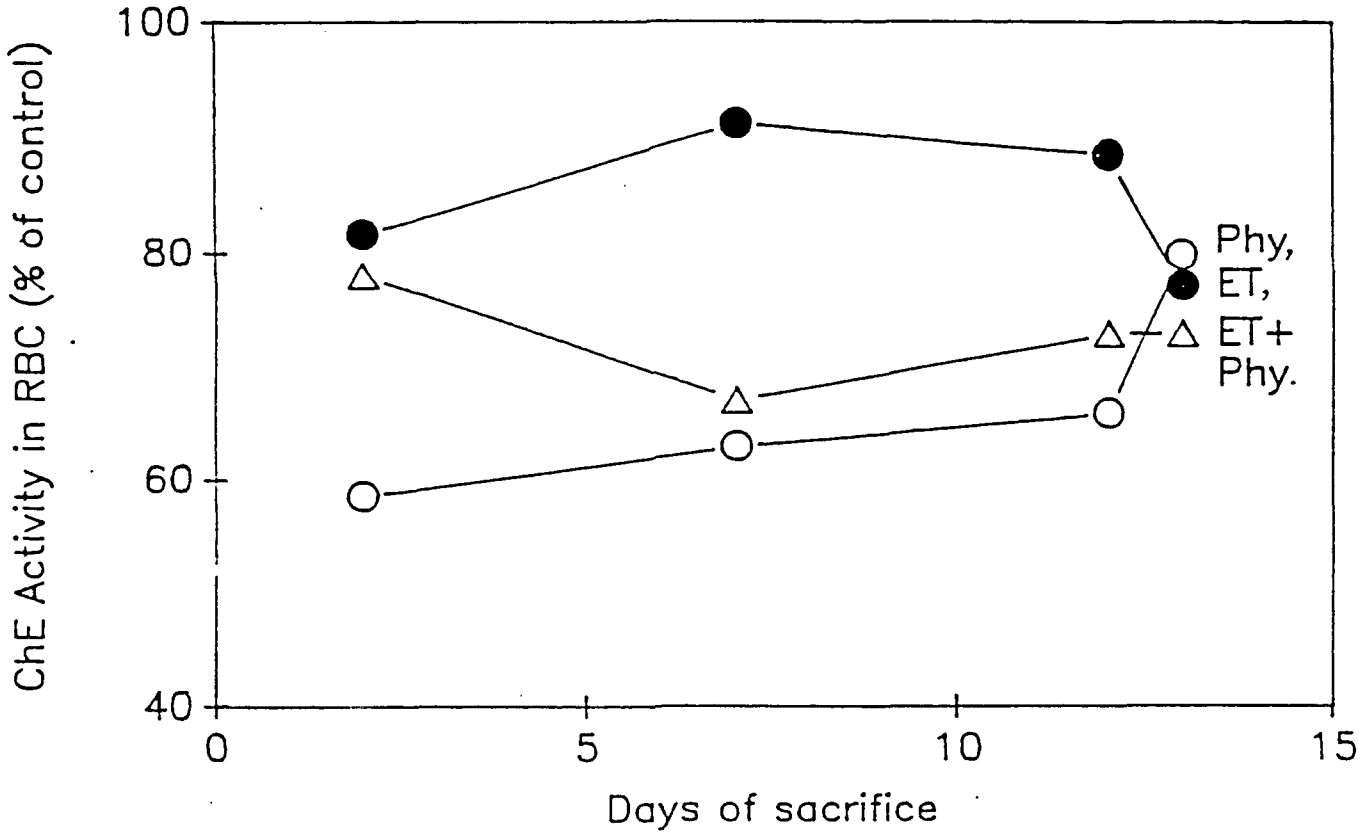
slept during the day, wrestled with their cagemates, and groomed themselves after they ran. During the last few wk of exercise, their amount of physical activity decreased and the amount of sleep increased, presumably due to stress and fatigue, particularly those which received Phy dose. They also seemed to be docile and passive for the same reasons.

The rats were endurance-trained for 5.5 wk. Then a continuous infusion osmotic pump was inserted with either saline or Phy (34.5 µg/kg/hr). Sedentary control rats, saline-administered rats or Phy-administered rats, were sacrificed on various days up to the 13th day to observe the steady-state level of ChE activity in RBC. Another Gr of rats which were infused with saline or Phy were exercised each day and were sacrificed 18 hr after the last exercise. The Phy control group showed ChE activity in RBC, 58%, 63%, 66%, and 80% of control on days 2, 7, 12, and 13, whereas endurance-trained + Phy-dosed rats showed ChE activity in RBC, 78%, 67%, 73%, and 73% of control on days 2, 7, 12, and 13 (Fig. 35). ChE activity in RBC in ET + Phy rat was 20% more on the second day, compared to Phy control rat. The ChE activity on days 7, 12, and 13 was not significantly different in ET + Phy compared to Phy control, indicating that ET decreases the Phy-induced inhibition to some extent. The number of rats sacrificed on later days was less; therefore, it becomes difficult to compare results from the second to 13th day.

Table 29: Effect of continuous infusion of physostigmine (34.5 µg/kg/hr at the rate of 0.5 µl/hr) on the endurance time of trained rats.

Days After Implantation of Osmotic Pumps	Endurance Training + Phy Endurance Time (min); mean ± S.E.M.	Endurance Training + Saline Endurance Time (min); mean S.E.M.
1	19.4 ± 0.57 (n = 17)	22.2 ± 1.4 (n = 9)
2	15.3 ± 2.69 (n = 13)	24.8 ± 2.1 (n = 9)
3	14.6 ± 2.53 (n = 6)	21.6 ± 1.5 (n = 6)
4	23.6 ± 2.4 (n = 8)	25.0 ± 1.8 (n = 8)
7	22.5 ± 4.6 (n = 2)	30.0 ± 0.0 (n = 2)
8	20.6 ± 3.5 (n = 5)	26.0 ± 2.2 (n = 5)
9	16.8 ± 3.6 (n = 5)	25.8 ± 2.3 (n = 5)
10	24.7 ± 3.5 (n = 4)	27.6 ± 1.2 (n = 3)
11	12.5 ± 5.3 (n = 2)	15.3 ± 4.2 (n = 3)
14	15.0 ± 0.0 (n = 1)	20.0 ± 3.5 (n = 2)

Fig. 35: Effect of continuous infusion of Phy by osmotic pump (Gr II), saline infusion in endurance-trained rats (Gr III) and Phy infusion in endurance-trained rats (Gr IV) on ChE activity (% of control) in RBC on different days.



CONCLUSIONS

The following conclusions are drawn from the work carried out under this contract. It is difficult to correlate some of the work, such as the relationship between biochemical parameters (lactate and pyruvate) and cholinesterase activity due to influence of exercise. We have mentioned the future scope of the work in this area. In fact, very little is known about the effect of two stressors: physical stress (exercise) and chemical stress (anticholinesterase – reversible and irreversible) (1) on the cholinergic system and (2) on the drug-metabolizing system. This area of research is quite open; some detailed thoughts are given at the end of this section. Our conclusions are as follows:

RBC, brain, and diaphragm showed dose-dependent ChE inhibition, whereas heart and muscle showed dose-related ChE inhibition at dosage studied. Phy concentration in plasma and tissues increased linearly with increase in dose.

Significant differences were found in oxygen consumption and caloric expenditure in young vs. adult rats undergoing identical acute exercise. Young rats attained a higher $VO_{2\ max}$ (81.55 ml/kg/min) compared to adult rats (68.97 ml/kg/min). The younger rats possessed a higher resting caloric expenditure (11.84 kcal/kg/hr) compared to adult rats (81.55 kcal/kg/hr). Based upon these metabolic factors, it seems inappropriate to consider 1-month and 4-month old rats as similar in their response to an incremental exercise, particularly since younger rats appear to utilize fats as a primary fuel source more readily through submaximal levels of exercise intensity compared to adult rats.

Different intensities of exercise (50%, 80%, and 100% $VO_{2\ max}$) showed a similar, but significant, inhibition of ChE activity in heart, without significantly affecting brain and thigh muscle. However, acute exercise produced a slight increase in ChE activity of RBC. Phy decreased ChE activity in RBC and tissues. The combined effect of Phy and acute exercise further decreased ChE activity in RBC and brain without significantly affecting heart, diaphragm, and thigh muscle. Exercise potentiated the effect of Phy on ChE inhibition in RBC and brain, irrespective of intensity of exercise. It seems that acute exercise affects ChE activity to a moderate degree in RBC and heart, and modifies the effect of Phy in RBC and brain.

Acute exercise transiently increased ChE activity in RBC, which returned to normal within 10-15 min. Endurance training decreased ChE activity of RBC without affecting other tissues. Acute exercise enhances the rate of decarbamylation and decreases $T_{½}$ of recovery of enzyme as compared to Phy alone. However, endurance training potentiates ChE inhibition in RBC and various tissues due to decreased metabolism of Phy, possibly due to adaptation. Endurance training for 6 wk may be beneficial to prolong and potentiate ChE inhibition by Phy. Endurance training may help in reducing the required dose of Phy and may be advantageous where Phy is used as a pretreatment drug against organophosphate intoxication.

Pharmacokinetic parameters clearly show that the absorption phase, t_{max} and C_{max} have disappeared in endurance-trained rats, indicating that increased blood flow due to exercise has caused this change compared to control rat. Area under the curve and $t_{½}$ have also increased due to endurance training. At 2 min, the amount of RA in brain, heart, lung, kidney, liver, and muscle of trained rats showed 337%, 191%, 106%, 385%, 126%, and 80% over control rat, respectively. Kidney, liver, and muscle RA remained higher up to 10 min after exercise, whereas

brain and heart RA decreased below control level within 5 min. These results indicate that training altered the distribution of [^{3}H]-Phy in tissues of rat.

Phy dosing is associated with a metabolic stress, in that elevated plasma lactate and pyruvate levels were observed in sedentary, untrained rats. Exposure to Phy, immediately after exercise in untrained animals, only resulted in a temporary metabolic stress. In the case of endurance-trained rats, the metabolic stress of both acute exercise and Phy dosing was reduced during recovery from a submaximal exercise effort.

ChAT and AChE enzyme activities in rat brain were affected in a regionally selective manner by chemical (Phy) and physical (exercise) stressors and the combinations of these 2 stressors. Corpus striatum showed significant decrease in ChAT and AChE activities due to Phy, but more so by the combination of Phy + exercise. Cerebral cortex and hippocampus were affected by the combination of the 2 stressors. Brainstem is the only region which showed inhibition of ChAT activity due to exercise as well as subacute Phy. This study suggests that the biosynthetic and degradative enzymes for ACh in brain regions involved with control of motor, autonomic, and cognitive functions area affected by trained exercise and subacute Phy in a regionally selective pattern that appears to depend on the type and interaction of these 2 stressors.

Exercise modifies ChE activity in the presence of ChE inhibitors and also alters pharmacokinetics. Therefore, these studies would be useful in the development of an appropriate therapy regimen and pretreatment agent against organophosphate intoxication.

There are primarily two thoughts for future work related to the effects of combination of physical stress (exercise) and chemical stress (anticholinesterases) on cholinergic system and drug metabolizing enzyme and pharmacokinetics.

1. Cholinergic System. Our data are consistent with the hypothesis that the responsiveness of these brain regions to different stressors is a function of the level of ongoing cholinergic activity and that elevations in ACh levels due to ACh inhibition may have long-term effects on the regulation of ChAT and AChE activities through a negative feedback mechanism.

The molecular/physiological mechanisms that govern the lasting changes in ChAT and AChE activity remain equivocal at this time. In brainstem the mechanism to decrease ChAT may be the same for exercise and Phy, since the combination of the 2 stressors did not show additive effects. Elevation of tissue concentrations of ACh may ultimately govern these long-term effects independent of the mechanisms that initiate these effects. This premise is best illustrated in the brainstem where respiratory and cardiovascular functions are regulated. Training alone increases blood pressure and heart rate and down regulates ChAT activity. Increases in medullary tissue concentrations of ACh by ChE inhibitors also elevates blood pressure and respiration. It is plausible that when tissue levels of ACh arise ChAT activity is down regulated as a compensatory mechanism to normalize cholinergic transmission and, hence, blood pressure. In order to verify this hypothesis, both brainstem concentrations and the turnover of ACh need to be measured.

2. Drug Metabolizing Enzymes and Pharmacokinetics. There is scant literature on the effect of exercise on drug disposition. From our study, we found that the pharmacokinetics of the flow-limited and poorly plasma-bound drugs

such as Phy are effected by physical exercise. However, it is not known whether trained exercise alters the metabolism of drugs, or drug metabolizing enzymes. It would be of immense help to the Army to study alterations, if any, in pharmacokinetic parameters of several drugs they use. We have recently published a review article entitled, "Effect of Exercise on Disposition and Pharmacokinetics of Drugs," (Drug Devel. and Res. 20:251-275, 1990), in which we have also discussed the future scope of work in this area.

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LIST OF ABBREVIATIONS

Absorption rate constant	Ka
Acetylcholine	ACh
Acetylcholinesterase	AChE
Acute exercise	AE
Area under the curve	AUC
Butyrylcholinesterase	BuChE
Centigrade	°C
Choline acetyltransferase	ChAT
Cholinesterase	ChE
Clearance	Cl
Concurrent acute exercise	CE
Correlation-coefficient	r
Curie	Ci
Disintegration per minute	DPM
Elimination rate constant	Ke
Endurance training	ET
Extensor digitorum longus	EDL
Gram	g
Group	Gr
Halftime	$t_{1/2}$
Hemoglobin	Hb
High-performance liquid chromatography	HPLC
Hour	hr
Intramuscular	i.m.
Kilogram	kg
Lactate/pyruvate	L/P
Meter	m
Microgram	µg
Microliter	µl
Milligram	mg
Milliliter	ml
Millimole	mmole
Minute	min
Medulla oblongata	MO
Molar	M
Nanocurie	nCi
Nanogram	ng
Organophosphate	OP
Oxygen consumption	VO_2
Percent	%
Physostigmine	Phy
Radioactivity	RA
Red blood cells	RBC
Respiratory exchange ratio	RER
Revolutions per minute	rpm
Retention time	Rt
Sedentary control	SC
Standard error of mean	S.E.M.
Trichloroacetic acid	TCA
Tritiated	[^{3}H]
Ultraviolet	u.v.
Versus	vs.
Volume of distribution	Vd
Volume	v
Week	wk
Weight	w