variable effectiveness of treatments that may augment cholinergic function must be considered in the light of the multiple neuronal deficits, including GABAergic and substance P pathways [30], involved in the pathophysiology of the extrapyramidal symptons of Huntington's disease. Possibly, those individuals who prove unresponsive to cholinoceptive agents alone may respond to a combined treatment program directed at enhancing both cholinergic and GABAergic function.

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Effect of methylxanthines and elevated external potassium on high energy phosphate content in frog skeletal muscle*

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The active transport of sodium from frog skeletal muscle fibers can be stimulated by the methylxanthine, theophylline [1]. At a concentration of 2 mM the stimulation of sodium efflux appears reversible, whereas with higher doses of theophylline irreversible stimulation of sodium efflux, often associated with contractures, results. Since the irreversible stimulation of efflux was observed in sodium-free, strophanthidin-containing solutions, Hays *et al.* [1] speculated that this stimulation of sodium efflux might be a consequence of a significant decrease in the high energy phosphate content (PE) in these muscles. In order to test this idea, PE was determined in frog skeletal muscle after exposure to theophylline. Although few studies of the effects of theophylline on the functioning of frog skeletal muscle have been reported, another methylxanthine, caffeine, has been used extensively in frog skeletal muscle to study calcium movements and tension development. Therefore, for comparative purposes, PE was determined in muscles which had been exposed to levels of caffeine which in this preparation are either subthreshold or suprathreshold for contracture. In frog skeletal muscle, procaine blocks contractures elicited by suprathreshold levels of caffeine [2], blocks the increased oxygen consumption elicited by both sub- and suprathreshold levels of caffeine [3, 4] and blocks the alkalinization caused by subthreshold levels of both theophylline and caffeine [5]. Therefore, measurements were also made to determine if procaine could block the fall in PE seen in muscles exposed to subthreshold levels of theophylline and caffeine.

All measurements were made on whole sartorius muscles from the frog, *Rana pipiens*. Muscles were dissected, attached to stainless steel holders at resting length and allowed to

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Table 1. Effect of theophylline and caffeine on high energy phosphate content*

	No. of muscles	G-6-P†	CrP	ATP	PE
		(μmoles/μmole total creatine)			
Control	34	0.006 ± 0.001‡	0.678 ± 0.022	0.147 ± 0.007	0.973 ± 0.030
Theophylline (1 mM)	3	0.002 ± 0.001	0.750 ± 0.022	0.142 ± 0.009	1.034 ± 0.039
Theophylline (2 mM)	14	0.021 ± 0.005	0.333 ± 0.035	0.106 ± 0.009	0.544 ± 0.043
Theophylline (3 mM)	7	0.038 ± 0.009	0.213 ± 0.027	0.107 ± 0.013	0.433 ± 0.022
Theophylline (4 mM)	3	0.069 ± 0.016	0.143 ± 0.041	0.074 ± 0.011	0.290 ± 0.061
Caffeine (2 mM)	11	0.006 ± 0.001	0.333 ± 0.048	0.123 ± 0.010	0.579 ± 0.063
Caffeine (2 mM) + procaine (1 mM)	6	0.002 ± 0.001	0.773 ± 0.020	0.187 ± 0.015	1.146 ± 0.031
Theophylline (2 mM) + procaine (1 mM)	4	0.003 ± 0.002	0.676 ± 0.039	0.089 ± 0.004	0.854 ± 0.043

* Frog sartorius muscles were dissected and incubated for 2 hr in normal Ringer at 21°. Muscles were exposed to the theophylline- or caffeine-containing Ringer for 60 min before preparation for analysis. Control muscles remained in normal Ringer for an additional 60 min.

† Glucose 6-phosphate (G-6-P), creatine phosphate (CrP) and PE where PE = CrP + 2 ATP.

‡ All values are expressed as means ± S. E.

recover from dissection for at least 2 hr in normal frog Ringer at 21–22°. In all experiments, paired muscles were used. In general, one member of the muscle pair remained in normal Ringer while the other member was transferred to the experimental Ringer. For the procaine experiments, both members of the pair were exposed to caffeine- or theophylline-containing Ringer with or without procaine. Theophylline and caffeine were added to the Ringer as a powder and the pH was adjusted to 7.2. All solutions contained 0.9 mg/ml of curare. At the end of the incubation period the muscles were frozen, weighed and analyzed, as described in detail by Connett and Hays [6]. The contribution of ADP to the total available phosphate bond energy (PE) is very small. In the presence of methylxanthines, ADP increases are not larger than those predicted by the action of creatine kinase (EC 2.7.3.2) and adenylate kinase (EC 2.7.4.3) operating at equilibrium with the measured creatine phosphate [7, *]. Therefore, all results are expressed as high energy phosphate content (PE) where PE = creatine phosphate + 2 ATP. For the ion content measurements, the methods of Connett and Hays [6] were used.

* R. J. Connett, E. T. Hays and F. J. Pearce, unpublished observations.

Effect of methylxanthines on high energy phosphate contents. Resting frog sartorius muscle PE averages about 1 μmole/μmole of total creatine [6, 8, 9]. In this series of experiments, resting PE values are not significantly different from previously reported values (Table 1). After 1 hr of exposure, significant reductions in PE were seen with theophylline concentrations of 2 mM or greater. One mM theophylline, which is below threshold for the stimulation of sodium transport in these muscles [1], also has no significant effect on energy content. In addition, 1 hr of exposure to 2 mM caffeine results in a PE content no different from that produced by 2 mM theophylline. As part of the assay procedure the glucose 6-phosphate content was also obtained. The results in Table 1 indicate that, while theophylline treatment results in a significant increase in the glucose 6-phosphate level, the increase is not seen in muscles exposed to caffeine.

Time course of theophylline effect. In paired muscles PE was measured after the muscles had been exposed to theophylline for 20, 40, 60 or 80 min. Figure 1 indicates that, during the 80-min exposure to theophylline, the fall in PE appears to be continuous, with a significant fall occurring in the first 20 min of exposure.

Correlation of methylxanthine effects with internal calcium levels. Caffeine has been used extensively to study calcium fluxes in frog skeletal muscle [2, 10]. Although 2 mM

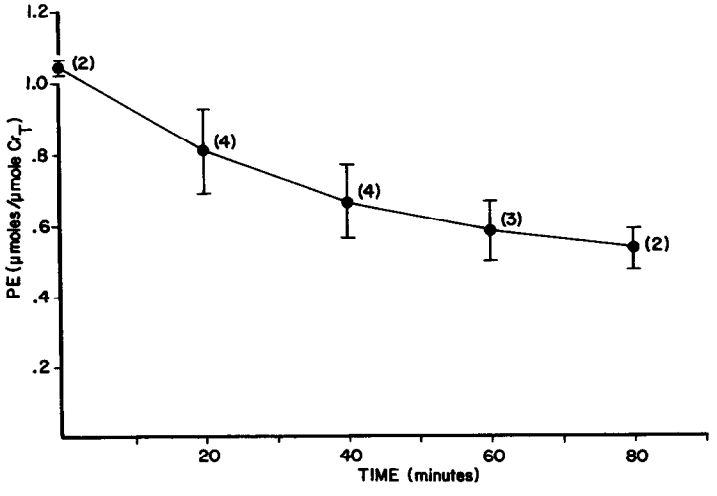

Fig. 1. Time dependency of the theophylline-induced fall in energy stores. High energy phosphate (PE) content was measured in paired muscles in normal Ringer. One member of the pair was analyzed after 0, 20, 40 or 60 min of exposure to 2 mM theophylline while the other member of the pair was analyzed after an additional 20 min of exposure to theophylline. All values are means ± S. E. expressed as a fraction of the total creatine (Cr_T), with the number of muscles indicated in parentheses.

caffeine potentiates twitch tension in skeletal muscle [11], this level is generally considered to be subthreshold for tension development [2, 10, 12]. Subthreshold levels of caffeine have been shown to release calcium from the internal calcium stores and also to increase sarcolemmal calcium movements [13]. Local anesthetics, such as procaine, have been shown to suppress these effects of caffeine, as well as to block the internal alkalinization produced in frog muscle by 2 mM caffeine or theophylline [5]. PE was measured in muscles which had been exposed to 2 mM theophylline or caffeine, with and without the addition of 1 mM procaine. Table 1 shows that procaine prevents the fall in PE produced by these levels of theophylline and caffeine. These results suggest that the effects of theophylline and caffeine on PE may be correlated with the altered calcium levels or increased internal pH in these muscles.

Effect of suprathreshold levels of caffeine on high energy phosphate content. In single muscle fibers and small bundles, 5 mM caffeine produces maximum tension [12]. In whole muscle, 5 mM caffeine increases calcium influx and efflux two to three times [2, 10]. These increases in fluxes usually occur during the first 10 min of exposure to caffeine. High energy phosphate content was measured in paired muscles in which one member of the pair remained in normal Ringer. For the five muscles exposed to caffeine for 60 min and the three muscles exposed for 30 min, PE was essentially zero (less than 0.05 μmole/μmole of total creatine). In another experiment on eight muscle pairs, one member of the muscle pair was exposed to 5 mM caffeine for 10 min. For four of these muscle pairs, 3 mM glucose was added to both the caffeine Ringer and the normal Ringer, and the solutions were bubbled with 95% oxygen. In all of these caffeine-treated muscles, the PE content was also zero. The presence of substrate and oxygen did not prevent the fall in PE to zero in the caffeine-treated muscles. Thus, treatment with 5 mM caffeine very rapidly depletes frog sartorius muscles of their high energy phosphate compounds.

Potassium effects on high energy phosphate content. Like the methylxanthines, elevated external potassium (K_0) stimulates active sodium transport [14], oxygen consumption [3, 15] and causes an alkalinization [5] of frog sartorius muscles. While 12.5 mM K_0 increases sodium efflux and oxygen consumption about 2.5-fold and reduces internal hydrogen ion concentration by about one-third, this level of K_0 is well below the measured contracture threshold [16]. In six pairs of frog sartorius muscles, PE content was measured after 3 hr of exposure to 12.5 mM K_0 Ringer and compared to companion muscles which remained in 2.5 mM K_0 Ringer. The creatine phosphate and ATP contents were lower in the muscles exposed to elevated K_0 compared to the control muscles but the reductions were not significant. However, the PE content was significantly reduced by about 5 per cent ($P < 0.025$). Thus, although the effects of potassium and the methylxanthines on many cellular functions in frog muscle are qualitatively similar, the quantitative effects on PE content are very different.

Effect of theophylline on ion content. Feinstein [2] reported that a 2-hr exposure to 5 mM caffeine resulted in a large gain of internal sodium ions (Na_i) and a larger loss of internal potassium ions (K_i) in frog sartorius muscle. Hays *et al.* [1] found that, after a 1-hr exposure to 2 mM theophylline, sodium influx was about 90 per cent of normal and sodium efflux was increased by about 35 per cent, suggesting that Na_i should be lower than control values. The results presented in Table 2 indicate that after 1 hr in theophylline, the Na_i, K_i, and water content is not altered significantly from the value obtained for control muscles that remained in normal Ringer. On the other hand, while exposure to 3 mM theophylline for 1 hr resulted in no change in Na_i, the K_i and the per cent dry weight were both significantly reduced, indicating a tendency for higher levels of theophylline, like caffeine, to cause a significant change in the ionic distribution of sodium and potassium in these muscles.

The results presented in this report indicate that, upon exposure of frog skeletal muscle to levels of theophylline below contracture threshold, a gradual fall in high energy phosphate compounds occurs. Upon exposure to levels of theophylline which are capable of causing contractures in these muscles, further reductions of the PE content occur. Our results also indicate that the same subthreshold concentration of caffeine causes a quantitatively similar fall in PE. Although theophylline has not been used extensively in frog skeletal muscle, there are some similarities in the effects of these two methylxanthines. Both 2 mM theophylline and caffeine stimulate oxygen consumption [3, 4,*] and cause an internal alkalinization of frog muscle fibers [5]. Subthreshold levels of theophylline and caffeine also cause twitch potentiation in frog muscle [11, 17]. Thus, a similar effect of these two agents on PE content in muscle might be expected.

The irreversibility of caffeine contractures is well known [11]. In addition to the caffeine-induced disruptive changes in fine structure in muscle [18], our results indicate a rapid and complete loss of PE as well, supporting the idea that caffeine causes not contractures but rigor [11]. The fact that contracture subthreshold levels of caffeine and theophylline also significantly reduce the PE content suggests that any interpretation of the results of experiments in which the methylxanthanthines are used in muscle and possibly other tissues in concentrations of 2 mM or greater should take into consideration the available high energy phosphate stores.

Hays *et al.* [1] found that the strophanthidin-sensitive stimulation of sodium efflux by 2 mM theophylline was reversible whereas higher doses of theophylline, as well as stimulating the strophanthidin-sensitive sodium efflux, also caused an irreversible stimulation of both the strophanthidin-insensitive and residual efflux. Although exposure of frog sartorius muscle to 2 mM theophylline causes a significant decrease in creatine phosphate content, sufficient ATP remains available for maintaining the reversible stimulation of active sodium transport which occurs over the 1 to 1.5 hr of exposure times used by Hays *et al.* [1]. With the higher

* R. J. Connett, unpublished observations.

Table 2. Ion content with theophylline exposure*

Treatment	No. of muscles	Na_i (μmoles/g wet weight)	K_i (μmoles/g wet weight)	Dry weight (%)
Control	16	9.65 ± 1.02	74.23 ± 2.12	16.2 ± 0.5
Theophylline (2 mM)	8	8.70 ± 1·95	70.24 ± 1.81	16.0 ± 0.7
Theophylline (3 mM)	7	9.71 ± 2.48	64.71 ± 2.12†	14.6 ± 0.8†

* Frog sartorius muscles were dissected and incubated for 2 hr in normal Ringer at 21°. Muscles were exposed to the theophylline-containing Ringer for 60 min before preparation for analysis. Control muscles remained in normal Ringer for an additional 60 min. All values are expressed as means ± S. E.

† Significantly different from paired control muscles ($P < 0.05$).

doses of theophylline the irreversible effects on sodium efflux could be a consequence of insufficient energy stores for maintaining active transport and membrane integrity. As a consequence, an increased leakiness of the fiber membrane to ions would result, as suggested by the ion content measurements. Whereas in 1 hr 2 mM theophylline had no significant effect on sodium, potassium or water content (per cent dry weight), there was a tendency for a small decrease in internal sodium, as might be predicted from the effects of unidirectional flux measurements [1]. Also, a small but not significant loss of internal potassium ions occurred. With 3 mM theophylline, although internal sodium content was not significantly increased from the control value, a significant loss of potassium and a significant decrease in the per cent dry weight did occur. Since Hays *et al.* [1] found that the stimulation of the strophanthidin-insensitive or residual sodium efflux could not be prevented by the removal of theophylline, an increase in the passive leak of the membrane to radioactive sodium ions could explain the steadily increasing efflux with time that they observed.

In summary, whereas the methylxanthines, theophylline and caffeine, at concentrations of 2 mM or greater were shown to reduce significantly high energy phosphate content in frog sartorius muscles, elevated external potassium had little effect on high energy phosphate content. Our results suggest that both contracture subthreshold and suprathreshold levels of these two methylxanthines cause a significant imbalance between energy production and demand in muscle.

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Aryl hydrocarbon (benzo[a]pyrene) hydroxylase development in rat mammary tissue*

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It is generally accepted that polycyclic aromatic hydrocarbons (PAH) must be activated metabolically to exert their carcinogenic effects. Activation of PAH is catalyzed by the aryl hydrocarbon hydroxylase system (AHH), one of the microsomal mixed-function oxygenases [1]. The AHH system is constitutive and inducible in most tissues of many species as well as in organ and cell cultures [2–4]. Although AHH in liver is most active and has been studied extensively, it also appears that AHH in extra-hepatic tissues may be of importance in local production of reactive intermediates in tissues where tumors originate

Mammary tissue in rodents is highly susceptible to carcinogenesis by PAH, but has not been well studied for its capacity for metabolizing xenobiotic compounds. Only a few reports have appeared concerning mammary AHH: it is present and inducible with MC in several strains of mice [5], induced with MC and BNF and inhibited by ANF in mammary cell lines [6]. In lactating rats, polybrominated biphenyls induce mammary AHH and inhibit epoxide hydratase [7] and BP induces AHH [8].

In the present study, the ontogeny of AHH, its inducibility with BNF, and its inhibition by ANF were assessed, to determine the general capacity of mammary tissue to metabolize BP and to determine if AHH activity in mammary tissue shows any pronounced differences at ages when rats are most susceptible to PAH-induced carcinogenesis.

Female Sprague–Dawley rats (Holtzman Co., Madison, WI) were fed Purina Laboratory Chow and water *ad lib.* until killed; all animals were maintained under identical conditions of light and temperature. AHH was "induced" by injecting BNF intraperitoneally (80 mg/kg body weight, in corn oil) for 2 consecutive days prior to the date of death. Control animals received corn oil only. The ages at death of animals studied were: 22, 28, 35, 42, 49, 50, 62, 72, 91, 127, 262 and 444 days; repeated assays on animals received from the same supplier were performed at ages 28 and 35 days.

At selected ages animals were killed by cervical dislocation, and mammary tissue with its regional subcutaneous fat was excised, weighed, minced and placed in iced 0.25 M

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Abbreviations: AHH, aryl hydrocarbon (benzo[a]pyrene) hydroxylase; ANF, alpha-naphthoflavone (7,8-benzoflavone); BNF, beta-naphthoflavone (5,6-benzoflavone); BP, benzo[a]pyrene; BSA, bovine serum albumin; and MC, 3-methylcholanthrene.