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.

KH_2PO_4 buffer (pH 7.25). All animals were killed at approximately the same time of day. Tissue was homogenized in 3 vol. of KH_2PO_4 buffer with a Polytron (Brinkmann Instr., Westbury, NY) and the homogenate was centrifuged at 15,000 *g* for 15 min. The resulting supernatant fluid was collected and centrifuged at 105,000 *g* for 60 min to obtain a microsomal pellet which then was resuspended in 0.25 M glycerol-phosphate buffer, pH 7.25. All procedures were performed at 0–4°. Protein in microsomal preparations was determined by the method of Lowry *et al.* [9] with BSA as the reference standard. To obtain sufficient microsomal protein, mammary tissue from two rats was pooled until age 62 days. Sufficient tissue was obtained to make determinations on individual rats at all later ages.

AHH activity in mammary microsomes was measured essentially by the method of Nebert and Gelboin [10]. The reaction was carried out using 100 μl of mammary microsomal suspension (3–8 mg protein/ml) in a final incubation volume of 1.05 ml. The incubation mixture contained: 0.25 ml of 0.2 M Tris (pH 7.25), 0.03 ml of 0.1 M $MgCl_2$, 0.62 ml H_2O, 0.3 mg NADH, 0.3 mg NADPH and 0.7 mg BSA. In experiments testing the effect of ANF on AHH, ANF was added in methanol to give a final concentration of 10 μM in the reaction vessel. The reaction was initiated by adding 0.1 μmole BP in 50 μl methanol to each vessel. Mammary samples were incubated for 60 min at 37°. The assay was performed under conditions of linearity with respect to time and protein concentration.

AHH activity is expressed as pmoles product formed/mg of microsomal protein/min. "Product" refers to the alkali-extractable metabolites of BP that are measured spectrophotofluorometrically; 3-hydroxybenzo(a) pyrene, extracted under conditions identical to those of samples, was used as a standard and was a generous gift from Dr. H. V. Gelboin, National Cancer Institute, to Dr. W. T. Allaben.

Because the proportion of epithelial cells to fat changes with development in mammary tissue, AHH activity also is expressed as pmoles product formed/mg of DNA/min. DNA

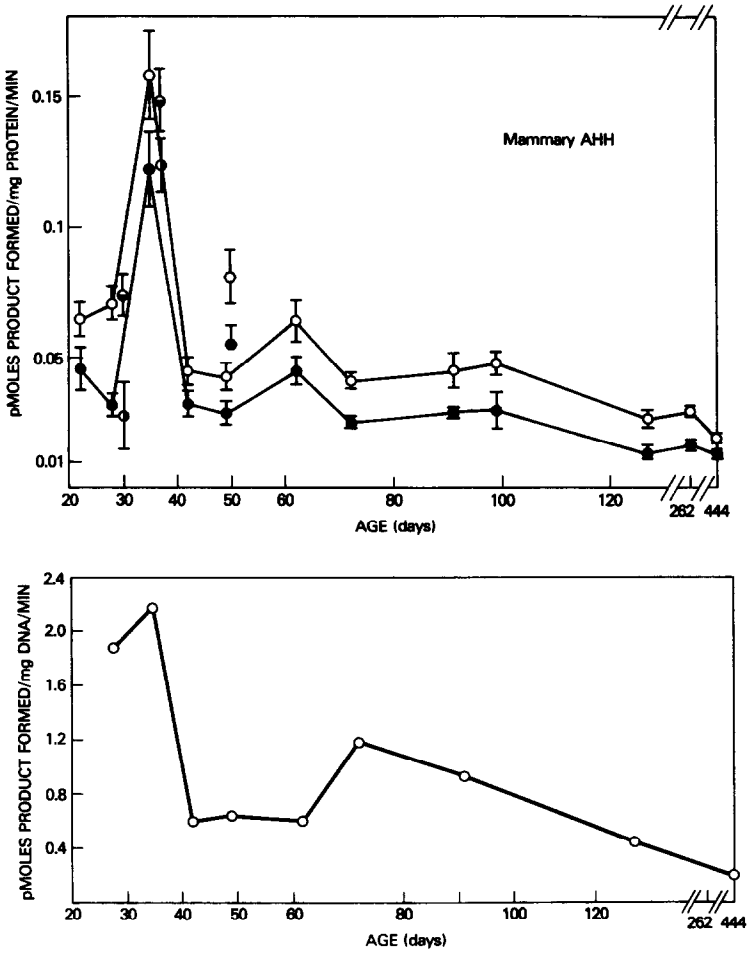

Fig. 1. Mammary aryl hydrocarbon hydrolyase—development in control (corn oil-treated) female Sprague–Dawley rats from age 22 days to age 444 days. Top panel: AHH activity is expressed as pmoles product formed/mg of microsomal protein/min. Key: (○—○) mammary microsomes from control rats; (●—●) microsomes from the same rats incubated in the presence of 10 μM ANF; (◇) microsomes prepared from rats ovariectomized 2 weeks before AHH assay at age 50 days; (◆) microsomes from the same ovariectomized rats incubated in the presence of 10 μM ANF; (⊖) repeated assays on a new group of control rats at 28 and 35 days of age; and (◑) effect of 10 μM ANF on repeated assay points. Each point represents the mean activity from eight individual determinations; vertical bars indicate the standard error of the mean. Bottom panel: AHH activity in control microsomes expressed as pmoles product formed/mg of DNA/min. Activity per unit of DNA was calculated by dividing the total activity in all microsomal protein from a given sample by the total DNA obtained from the same tissue. The standard error of the mean was less than 0.25 at each age.

concentration in mammary tissue was determined with ethidium bromide, as described by Beers and Wittliff [11].

The development of constitutive mammary AHH in control (corn oil-treated) female Sprague–Dawley rats shows a significant ($P < 0.05$) 2-fold increase in specific activity expressed as product formed/unit of protein) at age 35 days, then returns to pre-35-day values by age 42 days (Fig. 1, top). When expressed as product formed/unit of DNA in the tissue, activity remains high at ages 28 and 35 days but also shows a decrease to low levels by age 42 days. When expressed per unit of DNA, activity remains low through age 62 days (Fig. 1, bottom). Time points of 28 and 35 days of age were reassayed with a new batch of rats obtained approximately 3 months after the original group; the same pattern of AHH activity was shown again (Fig. 1, top).

Addition of 10 μM ANF to the microsomal incubation mixture produced a consistent ~35 per cent inhibition of basal AHH activity in mammary microsomes throughout development (Fig. 1, top). Ovariectomy of control rats 2 weeks prior to assay at age 50 days produced a significant ($P < 0.05$) 2-fold increase in basal AHH activity compared to intact rats when assayed at age 49 days (Fig. 1, top), whereas ovariectomy had no effect on inducibility of AHH by BNF (Fig. 2, top).

Pretreatment with phenobarbital (80 mg/kg body weight, injected i.p. in 0.9% saline for 3 consecutive days prior to assay) only slightly increased mammary AHH activity; at 51 days of age AHH activity in PB-treated rats was 1.35 times the control (corn oil) value and at 99 days of age PB treatment increased mammary AHH only to 1.05 times the control (data not shown).

The ratio of BNF-induced activity:control activity ("fold inducibility") varied with the age of the rats, ranging from 6- to 70-fold for mammary tissue compared with 11- to 36-fold for livers assayed from the same animals. When expressed as activity/unit of protein, inducibility appears high around 50 days of age and from 100 to 262 days of age (Fig. 2, top). However, when expressed as activity/unit of DNA, inducibility at age 50 days is no higher than at any earlier ages (cf. Fig. 1, bottom and Fig. 2, bottom). Inducibility in mammary

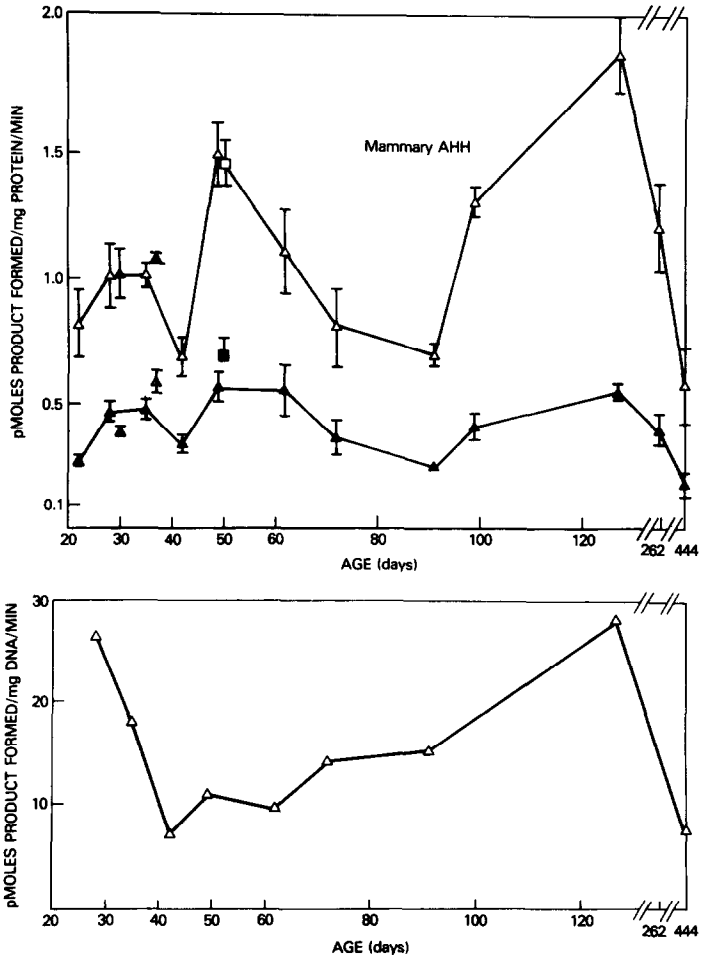

Fig. 2. Mammary aryl hydrocarbon hydroxylase—development of inducibility with BNF (80 mg/kg for 2 consecutive days before sacrifice). Top panel: AHH activity is expressed as pmoles product formed/mg of microsomal protein/min. Key: (△—△) mammary microsomes from BNF-treated rats; (▲—▲)microsomes from the same rats incubated in the presence of 10 μM ANF; (□) microsomes prepared from rats ovariectomized 2 weeks before AHH assay at age 50 days; (■) microsomes from the same ovariectomized rats incubated in the presence of 10 μM ANF; (◭) repeated assays on a new group of BNF-treated rats at 28 and 35 days of age; and (◭) effect of 10 μM ANF on repeated assay points. Each point represents the mean activity from eight individual determinations; vertical bars indicate the standard error of the mean. Bottom panel: AHH activity is expressed as pmoles product formed/mg of DNA/min. Activity per unit of DNA was calculated by dividing the total activity in all microsomal protein from a given sample by the total DNA obtained from the same tissue. The standard error of the mean was less than 3.8 at each age.

tissue is highest between ages 100 and 262 days, whether expressed as activity per unit of protein or per unit of DNA (Figs 1 and 2). Addition of 10 μM ANF to microsomal incubations from BNF-treated rats produced an inhibition of AHH activity which was about 60 per cent at most ages studied; the degree of inhibition was much greater, however, during the time when inducibility was highest (ages 91 to 262 days) (Fig. 2, top).

The concentration of DNA in mammary tissue rises markedly at puberty; in the non-pregnant rat, however, these levels return to pre-puberty values after age 62 days (Fig. 3).

The age-related development of AHH in liver microsomes has been described previously for mice [12] and rats [13, 14]. These studies indicate generally that, although some capability for hydroxylating BP exists in prenatal liver, the major increases in liver AHH activity and inducibility develop after delivery and before weaning age. Alterations in hepatic AHH activity are known to be of great significance in determining the response of an animal to the toxic or carcinogenic effects of aromatic hydrocarbons [4]. Data are more scarce on AHH development in non-hepatic tissues.

Mammary AHH potentially is of great importance in attempting to understand the exceptionally high susceptibility of mammary tissue to tumor induction by PAH. Reports have been published on AHH activity in adult mouse mammary tissue [5] and in mammary tissue of lactating rats [7, 8]. Although it is firmly established that rat mammary tissue is most susceptible to PAH carcinogenesis between 40 and 80 days of age [15], no studies have characterized mammary AHH in virgin rats during this critical period.

From experiments reported in this paper, it is obvious that virgin rat mammary tissues possess low, but significant and reproducible levels of AHH; basal activity was 230–500 times lower than liver activity from the same animal. The type of activity resembles closely that described by Wiebel and Gelboin [13] for adult female rat liver, i.e. the activity is greatly increased by "methylcholanthrene-type" inducers (BNF) and is significantly inhibited by the presence of ANF in the reaction vessel. These general characteristics hold for the mammary enzyme at all ages studied—from 22 through 444 days. (No assays were attempted on mammary tissue of rats younger than 22 days.)

The most striking developmental change in basal, constitutive mammary AHH occurs about the time of puberty (vaginal opening between day 28 and day 35). AHH activity doubles between day 28 and day 35, then rapidly drops below pre-puberty levels by day 42 when expressed as product formed/mg of protein. However, when expressed as activity/unit of tissue DNA, activity remains high at ages 28 and 35 days, but shows a substantial decrease by age 42 days. Thus, although the mammary tissue is beginning to show increased cellularity by age 35 days (as evidenced by increased DNA concentration in the tissue, probably as a consequence of estrogen-stimulated ductile proliferation), the increase in basal AHH does not appear to be due solely to a higher concentration of epithelial cells. Although the increase in basal AHH coincides with the onset of ovarian function, the increase is not sustained by continued ovarian secretion.

Since ovariectomy produces a similar 2-fold increase in basal AHH, it appears that the increase in basal activity around puberty may be related more to hypothalamic-pituitary events than to direct stimulation by gonadal secretions.

At the ages when rats are most susceptible to PAH carcinogenesis (40–60 days) basal mammary AHH is not significantly different from that of pre-pubescent or older rats. Although basal mammary AHH is low, its presence means that mammary microsomal enzymes have the capability to locally activate (or detoxify) PAH. Dao and Sinha [16] have shown that mammary tissue, exposed in organ culture to dimethylbenz(a)anthracene, gives rise to tumors when transplanted into isologous hosts.

Inducibility of mammary AHH by BNF is highest between 91 and 262 days of age; rats in this age category have a low susceptibility to PAH-induced mammary tumors [15].

The DNA concentration in mammary tissue is highest during times of maximum susceptibility to tumor induction. No simple relationship appears to exist between mammary or liver AHH activity and age-related differential sensitivity to carcinogenesis by aromatic hydrocarbons. The susceptibility of mammary tissue to PAH carcinogenesis cannot be predicted by the fluorometric assay for AHH which detects primarily phenolic BP metabolites which generally are considered to be toxic, but not carcinogenic [4]. Whether hepatic AHH is primarily a "detoxifier" of PAH or whether significant amounts of reactive metabolites (or proximate precursors) circulate from liver to peripheral tissue such as mammary remains to be determined.

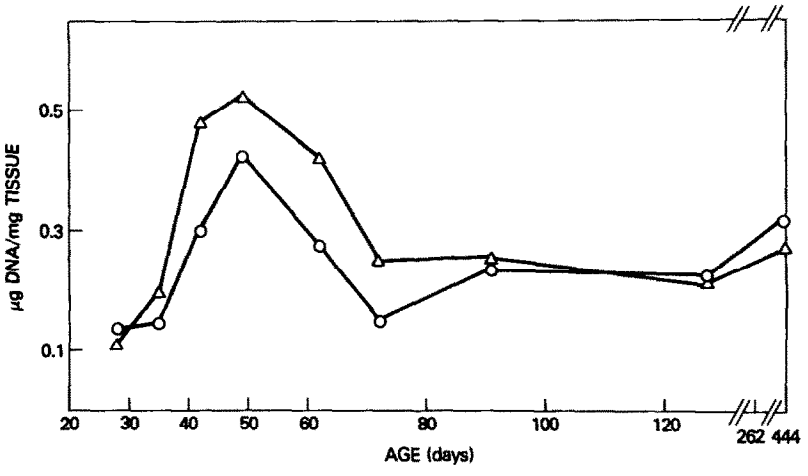

Fig. 3. Concentration of DNA in mammary tissue of rats from age 22-days through age 444 days. The ethidium bromide method of Beers and Wittliff [11] was used to measure the amount of DNA in the 15,000 *g* pellet from tissue homogenates. Key: (O—O) concentration of DNA in the mammary tissue of control (corn oil-treated) rats; and (Δ—Δ) concentration of DNA in the mammary tissue of BNF-treated rats. The standard error of the mean was less than 0.04 at each age.

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Effects of prior administration of methionine sulfoximine on the thresholds of seizures induced in mice by 3-mercaptopropionic acid or pentylenetetrazol

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Attempts to disclose the mechanism by which methionine sulfoximine (MSO) induces convulsive seizures have revealed numerous effects on neurochemical processes but have not established a relationship between such effects and the occurrence of seizures [1–3]. Among the known effects are small or moderate decreases in the activity of glutamate decarboxylase (L-glutamate 1-carboxylyase, EC 4.1.1.15, GAD) [4–6] and the γ-aminobutyrate content of the brain [5–9]. That the GABA (γ-aminobutyric acid) system plays a role in the convulsive mechanism is strongly suggested by the report of Stransky [9], who found that the period of brain GABA depletion in MSO-treated rats coincided with that of sensitivity to audiogenic seizures. DaVanzo *et al.* [10] had noted previously that amino-oxyacetic acid strongly protects cats against MSO-induced seizures, presumably by increasing the brain GABA levels. Baumel *et al.* [11] observed that hypobaric hypoxia (which also raises the GABA level) protects mice from seizures induced by MSO, as well as from those induced by the GAD inhibitor, thiosemicarbazide.

We have obtained additional evidence that the effects of MSO on the GABA system may be responsible for the excitation, DL-Methionine-DL-sulfoximine (Sigma Chemical Co., St. Louis, MO) was dissolved in 0.9% NaCl and injected i.p. into mice in doses of 300 mg/kg. The latent period preceding seizures was found to be 3.5 hr or longer. In the latter part of the latent period, a subconvulsive excitatory state exists during which the 50 per cent convulsive dose (CD_{50}) values of convulsants with short latent periods can be determined.

Pentylenetetrazol (PTZ) or 3-mercaptopropionic acid (3-MP) was injected i.p. in graded doses 165 min after the administration of MSO, and the mice were observed for at least 30 min. The criteria used to indicate a convulsive response were a generalized clonic or tonic-clonic seizure with loss of righting reflexes. Latent periods were 2 to 9.5 min for seizures induced by PTZ, and 4 to 8 min for those induced by 3-MP. Log CD_{50} values were determined from probit-log dosage regression curves. The potency of each convulsant, when given after MSO, relative to that of the convulsant alone (ratio of potencies, essentially CD_{50} of convulsant alone/CD_{50} after MSO) was calculated and the 95 per cent confidence limits of log CD_{50} values and log potency ratios were determined. Further methodological details have been described previously [12].

The convulsive action of 3-MP is attributed to its inhibitory effect on GABA synthesis [13–16], while that of PTZ does not appear to involve the GABA system directly [17, 18]. It was noted previously that each of three GAD inhibitors (allylglycine, 4-deoxypyridoxine and thiocarbohydrazide), used in such a way as to induce a subconvulsive excitatory state, had a greater potentiating effect on the convulsive action of 3-MP than on that of PTZ [12]. The data in Table 1 show that MSO behaves in the same manner. This finding strengthens the hypothesis that the excitatory effects of MSO result from an action on the GABA system.

Although the mechanism of action of PTZ remains unclarified, studies on invertebrate preparations suggest that it generates prolonged depolarization shifts by directly altering membrane properties [19]. If this is true, the threshold to PTZ presumably would be altered nonspecifically by factors that influence the level of neuronal polarization, as seems to be the case [12].

Some convulsants are known to inhibit GAD activity by interfering with the synthesis or the coenzyme function of pyridoxal phosphate [13]. Seizures induced by such a mechanism can be inhibited or delayed by systemic administration of the coenzyme [20]. Accordingly, the following experiment was done.

Pyridoxal phosphate (Sigma) was freshly dissolved in water containing NaCl and Na_2CO_3 sufficient to make the final solution isotonic at pH 6. Six mice were given MSO (300 mg/kg) and, after 2 hr, pyridoxal phosphate in doses of 60 mg/kg, i.p. All had seizures; the mean latent period ± S.D.