Biochemical Pharmacology, Vol. 52, pp. 1613–1620, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00567-9

Longitudinal Distribution of Arylamine *N*-Acetyltransferases in the Intestine of the Hamster, Mouse, and Rat

EVIDENCE FOR MULTIPLICITY OF *N*-ACETYLTRANSFERASES IN THE INTESTINE*

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ABSTRACT. Experimental and clinical evidence indicates that AcCoA:arylamine *N*-acetyltransferases (NATs; EC 2.3.1.5) are involved in the bioactivation and inactivation of a wide variety of arylamine, hydrazine, and carcinogenic arylamine xenobiotics. Longitudinal distribution of NATs in the intestine of the hamster, mouse, and two strains of rat was examined utilizing the model arylamine substrates procainamide (PA) and *p*-aminobenzoic acid (PABA) for the monomorphic (NAT1) and polymorphic (NAT2) enzymes in the rodent. NAT1 and NAT2 were distributed quite differently in each species examined. In particular, rat intestinal NATs were distributed equally throughout the intestinal tract. In contrast, hamster intestinal NATs decreased in activity from the proximal small intestine to the distal large intestine. Mouse NAT2 activity was highest in the cecum, whereas NAT1 was highest in the proximal small intestine. Although these model substrates have been shown to be selective for NATs, they are not specific. Therefore, a series of biochemical studies were undertaken to evaluate NAT multiplicity in the intestine of the F-344 rat. To assess multiplicity of NAT expression, selective inhibition, differential sensitivity to heat inactivation, and kinetic analysis were performed on intestinal cytosol. Eadie–Hofstee transformation of PA N-acetylation yielded a curvilinear plot indicative that a low affinity–high capacity enzyme aside from NAT1 (presumably NAT2) was contributing to PA N-acetylation activity. PA activity was found to exhibit approximately 4- to 5-fold greater thermostability than PABA activity. Furthermore, PA acetylation could be inhibited selectively with vinyl fluorenyl ketone (2.5 to 5 μM) but not with methotrexate (up to 2 mM). Taken together, these studies suggest the expression of both NAT1 and NAT2 in the intestine of the F-344 rat. BIOCHEM PHARMACOL 52;10:1613–1620, 1996. *Copyright © 1996 Elsevier Science Inc.*

KEY WORDS. *N*-acetyltransferase; procainamide, *p*-aminobenzoic acid; intestinal metabolism; rats, mice; hamsters

Epidemiological and experimental evidence suggests an association between HA‡ ingestion and colorectal cancers [1–3]. These HAs are believed to undergo bioactivation prior to initiating the cascade of events that leads to tumor development [4]. Numerous enzymes have been implicated in this bioactivation scheme, including NAT [5–7]. NATs are known to metabolize numerous arylamine, hydrazine, and heterocyclic amine xenobiotics. Importantly, metabolism via these enzymes may serve to protect or predispose individuals to xenobiotic-induced toxicity [8]. Several epidemiological studies indicate that fast acetylators may be at higher risk for the development of colorectal cancer than slow acetylators [9–11].

While colorectal cancer is the third most common form of cancer in the United States, cancer of the upper intestinal region is rare [12]. It has been shown that administration of HAs such as PhIP to male F-344 rats results in colorectal tumor formation at a 55% incidence [13], as well as PhIP–DNA adducts that are preferentially distributed in the colon [14]. Most recently, it was shown that hamsters congenic in the NAT2 locus have a higher propensity to form aberrant crypt foci in the cecum and colon than hamsters that are homozygous for the slow acetylator genotype after administration of the model colon carcinogen 3,2′-dimethyl-4-aminobiphenyl [15]. The reason for the propensity of tumor development in the colorectal region, both

* Portions of this work were presented at the Sixth North American Meeting of the International Society for the Study of Xenobiotics in Raleigh, North Carolina, October 23–27, 1994.

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‡ *Abbreviations:* HAs, heterocyclic amines; NATs, AcCoA:arylamine *N*-acetyltransferases; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; F-344, Fischer-344; AcCoA, acetyl coenzyme A; L-CYS, L-cysteine; DTT, dithiothreitol; MTX, methotrexate; PA, procainamide; NAPA, *N*-acetylprocainamide; PABA, *p*-aminobenzoic acid; NAPABA, *N*-acetyl-*p*-aminobenzoic acid; NaPPi, sodium pyrophosphate; WKY, Wistar Kyoto; SED, Sorensen's phosphate buffer containing 1 mM DTT and EDTA; PMSF, phenylmethylsulfonyl fluoride; RMANOVA, repeated measures analysis of variance; SNK, Student–Newman–Keuls; and VFK, vinyl fluorenyl ketone.

Received 27 February 1996; accepted 10 June 1996.

clinically and experimentally, is unknown. Recognizing that bioactivation is an important determinant of tumor formation [16], the regional distribution of adducts and neoplasia may be a reflection of heterogeneity in the longitudinal distribution of NATs, which are known to bioactivate these compounds to mutagenic agents [17–19].

To date, little is known about the intestinal distribution of NATs. The presence of NATs in humans has been reported in isolated segments of the small and large intestine [20–22]. Reports of significant NAT activity in the gut-wall of the rabbit were described by Hearse and Weber [23]. The longitudinal distribution of NATs within the rabbit has been reported to decrease approximately 10-fold for NAT2 expression along the duodenal-colonic axis, with the highest activity residing proximally. NAT1 expression was found to be relatively constant [24]. Chung *et al.* [25] have reported a homogenous distribution of NAT2 in the gastrointestinal tract of the inbred rapid acetylator mouse strain C57BL/6J (B6). On the other hand, reduced NAT activity has been demonstrated in the colon versus the small intestine of the hamster [26, 27]. Moreover, numerous NAT enzymes are known to bioactivate arylhydroxamic derivatives in varied capacities in this species [28].

The objective of the present study was to determine the longitudinal distribution of NAT1 and NAT2 in the intestine of the mouse, hamster, and rat. The F-344 and WKY strains of rat were used as these have been demonstrated to exhibit the fast and slow acetylator phenotype, respectively [29]. Common strains of mouse (Swiss albino) and of hamster (Syrian Golden) were examined to determine if species differences in distribution of NAT exist. Since the F-344 rat has been suggested as a model strain for HA-induced colorectal cancer [30], further studies were undertaken in this rat strain to confirm that a multiplicity of NAT expression exists in the intestine.

MATERIALS AND METHODS

Materials

AcCoA, L-CYS, DTT, MTX, PA, PABA, NAPABA, and NaPPi were obtained from commercial suppliers and used as obtained. NAPA was a gift of E.R. Squibb & Sons, Inc. (Princeton, NJ). VFK was donated by Dr. Patrick Hanna, University of Minnesota, and was synthesized by his laboratory as previously described [31]. Date of birth-matched male F-344 (130 g), WKY (250 g), Syrian Golden hamsters (100 g) and Swiss albino mice (25 g) were obtained from Charles River (Wilmington, MA) and acclimated in a 12-hr light/dark cycle, humidity- and temperature-controlled environment for at least 72 hr before experimentation. Animals had access to standard rodent chow and water *ad lib.* until 12 hr prior to organ harvest when food was removed to facilitate intestinal removal. All animals were killed between 7:30 and 9:30 a.m. on study days.

Longitudinal Distribution of NAT

Four F-344 (130 g) and WKY (250 g) rats, Syrian Golden hamsters (100 g), and Swiss albino mice (25 g) were killed via CO_2 asphyxiation. The intestinal tract was removed from the pyloric sphincter to the anus, with care taken to remove the pancreas and external Peyer's patches from the intestinal lumen. Ingesta was gently flushed out of each lumen with 12–15 mL of Sorensen's phosphate buffer (0.067 M, pH 7.4). For the rat studies, the small intestine was divided into equal segments (each 20 cm) that were designated as the duodenum, jejunum, and ileum, though these are approximations. The large intestine was divided into the cecum and colorectum (segments 4 and 5, respectively). The small and large intestines of the hamsters were divided into equal lengths (20 cm) excluding the cecum (it was found that this region, despite overnight fasting, was packed full of ingesta and could not be successfully cleansed without significant damage to the tissue), while the small intestines of mice were divided into equal-length segments (20 cm) with the large intestine separated into the cecum and colorectum. All tissues were frozen in liquid nitrogen and stored at −70° until used for enzyme assays. On the day of NAT activity determination, organs were thawed on ice, minced, and homogenized with SED buffer containing 50 μM PMSF and 10 μM leupeptin (which were added immediately prior to use). The 100,000 *g* supernatant served as the cytosolic source of NAT. NAT1 and NAT2 activities were determined using the probe substrates PA and PABA, respectively, following methods previously described in this laboratory which quantify acetylated metabolites of each substrate via HPLC [32]. All rates of metabolite formation were normalized per milligram of protein as determined by the method of Bradford [33], using BSA to prepare a standard curve. Unless otherwise stated, all incubations were conducted in triplicate.

Studies to Determine NAT Multiplicity in the Intestine

Recent studies would indicate that NAT1 and NAT2 contribute to the N-acetylation of model carcinogens with preference given to NAT2 [29, 34]. In the rat and other rodent species, PABA is selective for NAT2 and PA is selective for NAT1 [35, 36]. It is important to recognize that substrate selectivity does not imply specificity of catalysis. Therefore, activity towards either of these substrates is not definitive proof of the presence of the enzyme for which they are selective substrates. Hence, several biochemical tests were conducted to confirm the presence of both NATs in the intestine.

KINETICS OF PA. An Eadie–Hofstee plot was generated for NAPA formation to evaluate multiplicity [37]. Incubations were performed at eight different substrate (PA) concentrations (0.15 to 6 mM) with and without 100 μM PABA. To maintain constant AcCoA concentrations throughout the reaction, an AcCoA-regenerating system was used as previously described [38, 39]. Initial formation velocity of the N-acetylated metabolite was determined as previously described.

THERMAL STABILITY OF NAT. Cytosol was prepared from

intestinal segments 1–5 as previously described from a F-344 rat for each temperature studied. Cytosol was preincubated at 27°, 37°, and 45° for a period of 0, 15, 30, 45, or 60 min prior to standard incubation conditions. PA and PABA activities were then determined as described.

ISOZYME-SELECTIVE INHIBITION OF NAT1. Intestinal cytosol was prepared from segments 1–5 using a 50 mM NaPPi buffer (pH 7.0) with DTT (0.16 mM). DMSO was included in both control and treatment groups at a final concentration of 1%. Briefly, intestinal cytosol (final protein concentration 1 mg/ml) was preincubated with VFK (0.312, 0.625, 1.25, 2.5, 5, 10, and 25 μM) for 1 min at 37°. The action of VFK was terminated by the addition of L-CYS (final concentration 10 mM), which contained sufficient DTT to bring the final incubation concentration to 1 mM. Preliminary experiments confirmed the ability of 10 mM L-CYS to quench the action of VFK after a 1-min preincubation [31, 35]. Moreover, changing the order in which L-CYS was added greatly reduced the ability of VFK to inactivate NAT1 activity. The preincubated samples were placed on ice and assayed for NAT1 or NAT2 activity as previously described. Preliminary studies indicated that VFK did not interfere with the HPLC analysis of either metabolite.

INHIBITION OF NAT2 WITH MTX. MTX was reconstituted in SED buffer immediately prior to use. Intestinal cytosol was preincubated with MTX (concentration range 10–2000 μM) for 10 min at 37° prior to the addition of substrate probe. Preliminary studies indicated that MTX did not interfere with the HPLC analysis of either metabolite.

Data Analysis

The hypothesis of heterogeneous distribution of NAT1 and NAT2 among intestinal segments of the F-344 rat was tested by using RMANOVA. To isolate differences in RMANOVA, the SNK post-hoc test was used to evaluate pairwise comparisons. A value of $P < 0.05$ was considered to be significant. To compare differences in thermal stability, the paired Student's *t*-test was used to evaluate thermal inactivation constant (k_{ti}) for NAT1 and NAT2.

RESULTS

Longitudinal Distribution

Figure 1 shows the distribution of NAT1 and NAT2 activity along the duodenal-colonic axis in the F-344 and WKY intestinal tract. NAT1 activity in the F-344 did not differ between segments 1–5. RMANOVA revealed significant differences among segments for NAT2 activity in the F-344 intestinal tract ($P < 0.05$). However, when the SNK post-hoc analysis was performed to isolate which segment was different, all pairwise comparisons failed to reach the predetermined level of significance. This can be explained by the rigidity of the SNK test criteria, which is higher than RMANOVA. WKY animals were also examined since this strain of rat has been suggested to express the slow acetylation trait [29, 30, 40, 41]. For this strain, segment 5 had significantly greater NAT1 activity than segments 2, 3, or 4. Moreover, NAT2 activity was significantly greater in segments 4 and 5 versus segments 1, 2, or 3.

Hamster NAT1 and NAT2 activities both decreased ap-

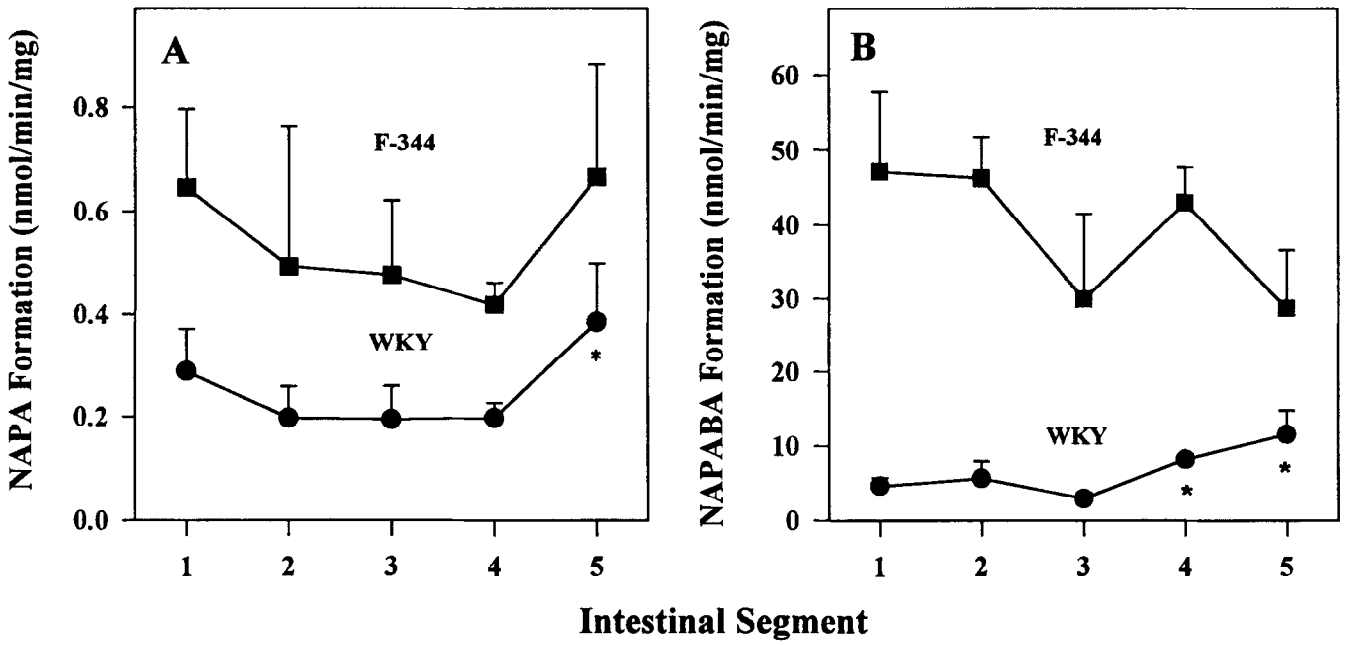

FIG. 1. Intestinal longitudinal distribution of (A) NAT1 and (B) NAT2 in the F-344 and WKY rat. Each point represents the mean ± SD of 4 animals. Key: (*)$P < 0.05$ for (A) segment 5 vs segment 2, 3, or 4 and (B) segment 4 or 5 vs segment 1, 2, or 3.

proximately 3- to 4-fold from proximal to distal along the duodenal-colonic axis (Fig. 2). NAT1 activity in segments 1 or 2 was found to be significantly greater than that in segments 3 or 4 ($P < 0.05$), while NAT2 activity was found to be significantly greater in segment 1 versus all other segments.

In contrast to the rat and hamster distribution patterns of NAT, the mouse had measurable NAT1 activity only in the small intestine (Fig. 2A). The highest NAT2 activity (Fig. 2B) was found to be in the cecum ($P < 0.05$ vs all other segments) with a decrease noted from proximal to distal segments of the small intestine ($P < 0.05$ for segment 1 vs segment 2).

Evaluation of NAT Multiplicity

Kinetics of selective substrates. As shown in Fig. 3, the curvilinear shape of the Eadie–Hofstee plot of NAPA formation was consistent with two enzymes that catalyze a single substrate. Moreover, in the presence of 100 μM PABA, a linear transformation was observed, which suggests that NAT2 contributes to the overall catalysis of PA to NAPA, as shown by the net velocity difference (low affinity–high capacity enzyme).

THERMAL STABILITY OF NAT. Figure 4 depicts the thermal stability of segments 1–5, which were preincubated at 45° for the indicated times. Because the rates of thermal inactivation appeared to follow an apparent first order rate of decay, the k_{ti} for NAPA and NAPABA formation was determined via regression analysis of the ln% activity versus time curve and found to be 0.015 and 0.068 min^{-1}, respec-

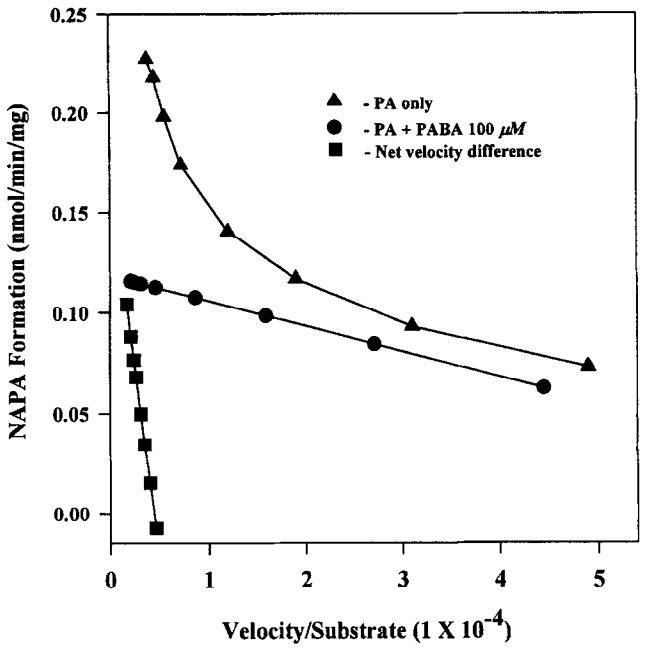

FIG. 3. Eadie–Hofstee transformation of NAPA formation in F-344 segment 2.

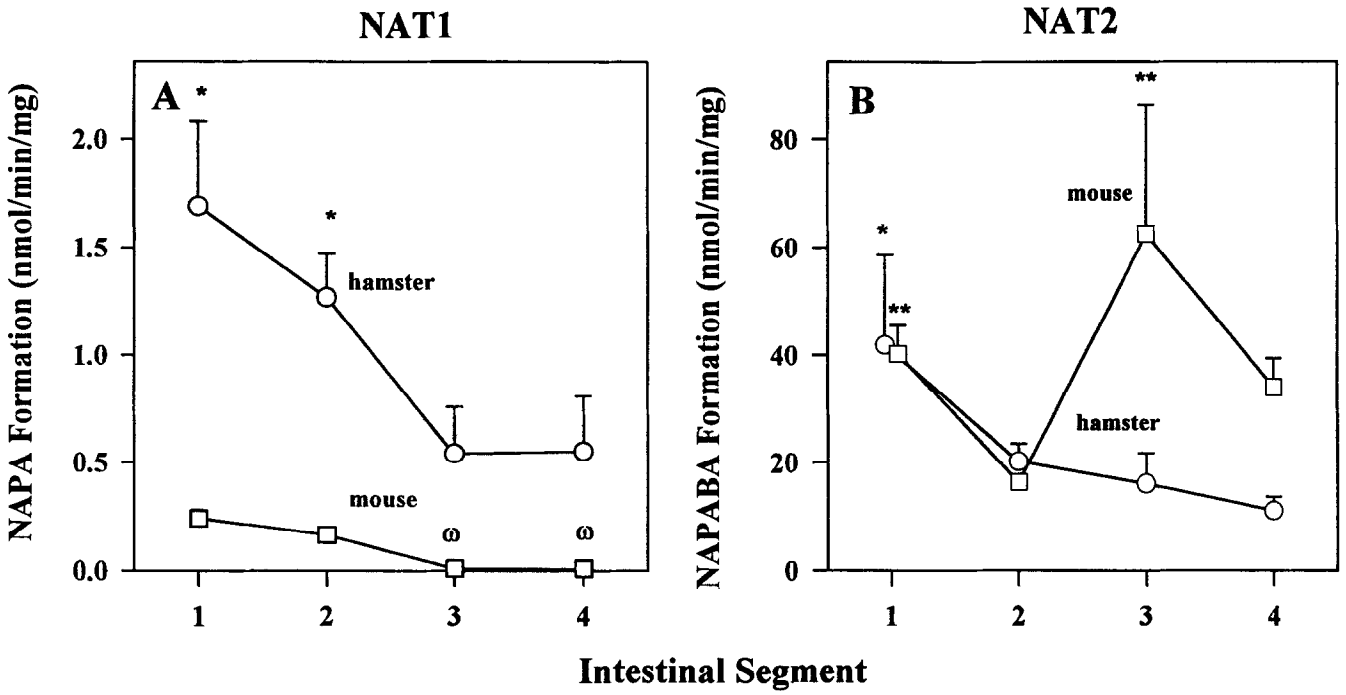

FIG. 2. Intestinal longitudinal distribution of (A) NAT1 and (B) NAT2 in the hamster and mouse. For the hamster, segments 1 and 2 represent proximal and distal small intestine, while 3 and 4 represent proximal and distal colon, excluding the cecum. Key: (*)$P < 0.05$ for (A) segment 1 or 2 vs segment 3 or 4 and (B) for segment 1 vs all other segments. For the mouse, segments 1 and 2 represent the proximal and distal segments of the small intestine, while segments 3 and 4 represent the cecum and colon, respectively. The symbol ω below indicates below detectable limits (0.04 nmol on column). Key: (**)$P < 0.05$ for (B) segment 3 vs all other segments and for segment 1 vs segment 2. Each point represents the mean ± SD of 4 animals.

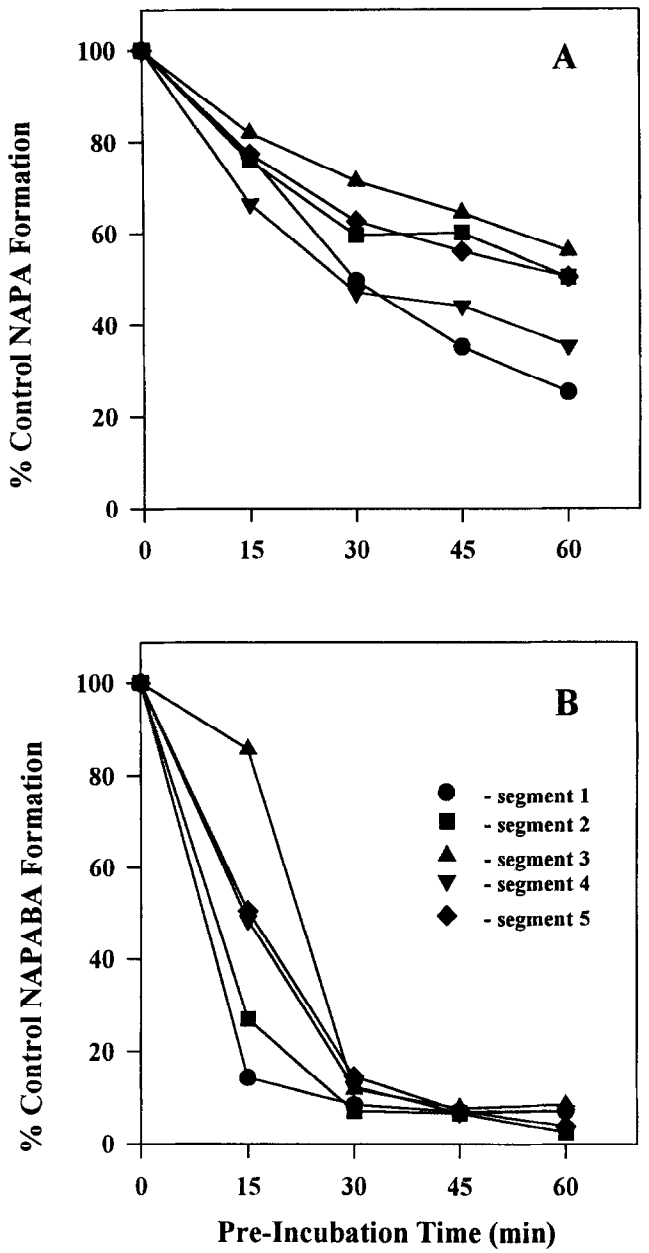

FIG. 4. Thermal stability of (A) NAPA formation or (B) NAPABA formation following preincubation of intestinal F-344 cytosol at 45°. Data shown represent the mean data of triplicate determinations from cytosol obtained from the same rat intestine. Control levels for NAPA formation for segments 1–5 were: 1.09, 0.79, 0.82, 0.96, and 0.76 nmol/min/mg, respectively. Control NAPABA formation for segments 1–5 were: 50.4, 31.4, 25.9, 46.83, and 32.75 nmol/min/mg, respectively.

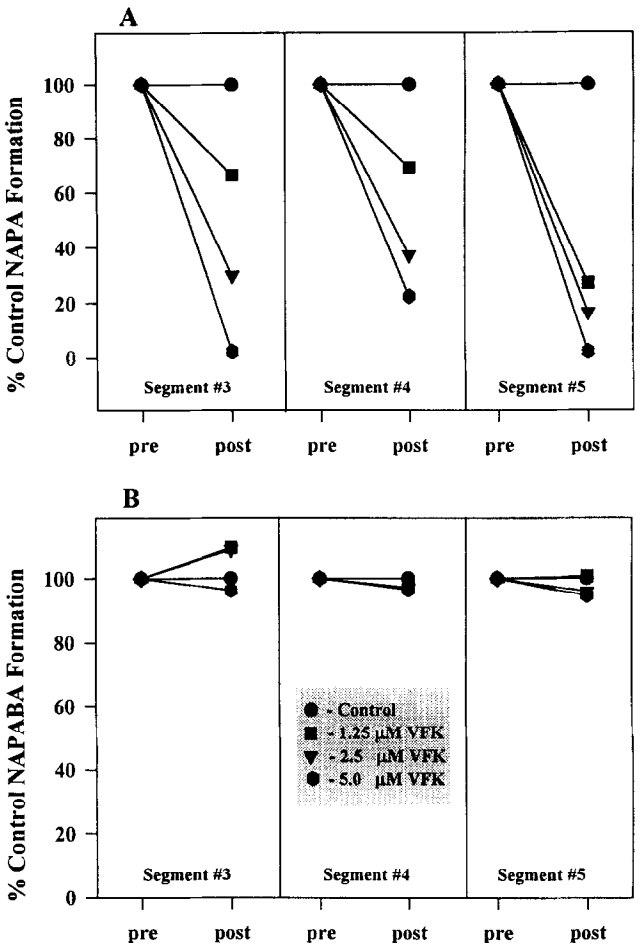

FIG. 5. Effect of VFK (concentration 1.25, 2.5, or 5 μM) preincubation for 1 min on (A) NAPA formation or (B) NAPABA formation in segments 3, 4, and 5 from intestinal F-344 cytosol. Data shown represent the average of duplicate determinations in cytosol from various segments of the same rat intestine. NAPA control activities for segments 3–5 were 0.13, 0.24, and 0.15 nmol/min/mg, respectively. NAPABA control activities for the same segments were 4.28, 15.05, and 4.32 nmol/min/mg, respectively.

tively ($P < 0.05$). This 4- to 5-fold difference between NAPABA and NAPA thermal inactivation constants was also seen at 37° and 27° for both small and large intestinal segments (data not shown).

ISOZYME-SELECTIVE INHIBITION OF NAT1. As shown in Fig. 5, preincubation of VFK for 1 min at a concentration of 1.25, 2.5 and 5 μM was found to selectively inhibit NAPA but not NAPABA formation. Moreover, concentrations of 10 and 25 μM VFK did not influence NAPABA formation activity, while NAPA formation was below limits of detection at these concentrations of VFK (data not shown).

INHIBITION OF NAT2 ACTIVITY. Preincubation of MTX with intestinal cytosol inhibited NAPABA formation with an approximate IC_{50} of 25 μM (Fig. 6). While an IC_{50} was not attainable for inhibition of NAPA formation, up to 40% inhibition was noted.

DISCUSSION

Several lines of evidence suggest that heterogeneity in the longitudinal expression of NATs in the intestine may be an important factor in the development of environmentally induced colorectal cancer [11, 13, 19]. Moreover, the reactivity of the proximate carcinogen/mutagen from HAs sug-

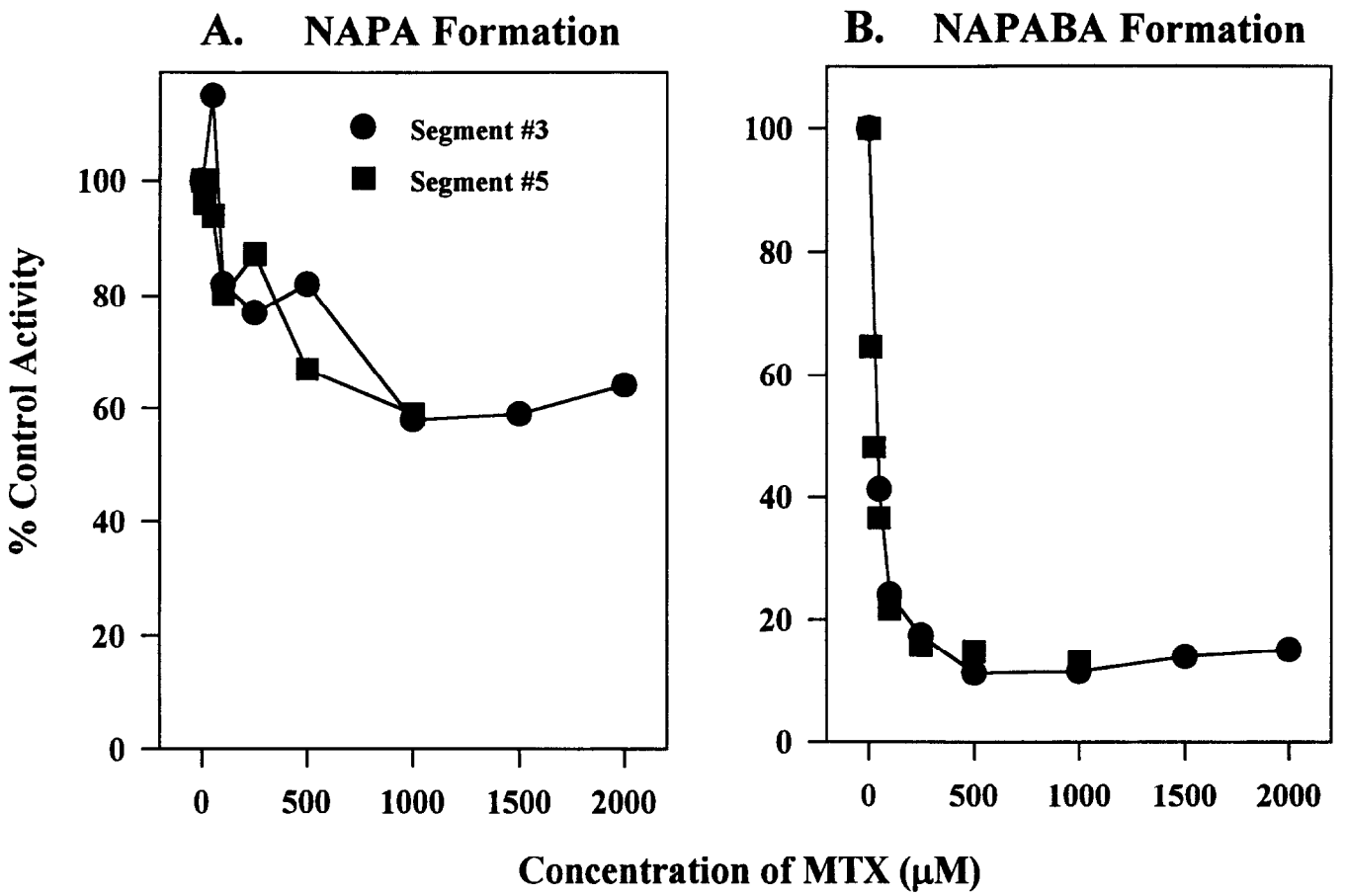

FIG. 6. Effect of MTX on (A) NAPA formation or (B) NAPABA formation on segments 3 and 5 from intestinal F-344 cytosol (control NAPA formation values for segments 3 and 5 were 0.84 and 1.5 nmol/min/mg; control NAPABA formation values for the same segments were 4.97 and 21.61 nmol/min/mg). Data represent the mean value of triplicate determinations in cytosol obtained from segments of the same rat intestine.

gests that bioactivation may occur within the target tissue or within the liver followed by conversion to a stable intermediate that is transported to and reactivated within the target tissue [42, 43]. Inasmuch as variability in the bioactivation/inactivation pathways is a determinant of genotoxin formation, heterogeneity in the expression of xenobiotic metabolizing enzymes in a tissue-specific manner within the gut may serve to bioactivate HAs and other toxicants.

We have found that in the F-344 rat a uniform longitudinal distribution of NATs exists within the intestinal tract. Utilizing PA and PABA as probe substrates, no differences were found for the acetylation of these substrates in any intestinal region. We also examined the WKY strain, which appears to exhibit the slow acetylator phenotype [29, 30, 40, 41], to evaluate the relationship of acetylator phenotype with intestinal distribution of NATs. The WKY rat displayed greater activities in the large intestine for both NAT1 and NAT2. There was approximately a 10-fold difference in intestinal PABA acetylation between the F-344 and the WKY, while PA acetylation was roughly 2-fold greater in the F-344 rat. Hamster NAT activities decreased from proximal to distal portions of the intestinal tract, which is in agreement with other studies that have examined isolated segments of the small versus the large intestine [26, 27]. Mouse NAT activity was distributed quite differently from other rodents in that PA acetylation activity was below detectable limits in the large intestine, and PABA acetylation activity was greatest in the cecum. These results appear in contrast to those previously reported [25]; however, the apparent discrepancy may be explained, in part, by the vast strain dependency for NAT expression known to occur in this species [44], as well as differences in substrate selectivity. Clearly, these data demonstrate that NAT distribution in the intestine varies between strains (as observed in the rat) and between species (rat, hamster, and mouse). More importantly, which species most closely resembles humans remains to be investigated.

Since the substrates utilized in these studies are selective, but not specific, for NAT, catalysis of the substrate is not in itself proof of the presence of both enzymes. While western blot analysis can often be used to confirm protein presence, extensive investigation by others has found this method to produce an electrophoretically indistinguishable migration for NAT1 and NAT2 [45]. Therefore, evidence for the multiplicity of NAT expression within the intestine of the F-344 was sought, utilizing several biochemical approaches. First, a curvilinear decline in the Eadie–Hofstee transfor-

mation (Fig. 3) was observed for NAPA formation, which is consistent with significant substrate overlap of PA metabolism by NAT2 at high concentrations. Moreover, the addition of PABA (a substrate for NAT2) to the incubation led to a linearization of this curve. These data support the presence of both NATs in the intestine of the F-344 rat. Second, differences in NAT thermal stability (measured as substrate acetylation) were clearly evident. After preincubation of intestinal cytosol derived from segments 1–5, PA acetylation activity exhibited a 4- to 5-fold greater thermal stability than PABA acetylation activity. The divergent thermostability of these two substrates in identical tissue indicates a unique identity for the primary enzyme responsible for the catalysis of these two substrates [20, 47, 47]. Third, the isozyme-selective affinity label VFK was found to be a selective and potent inhibitor of intestinal cytosolic PA acetylation, in agreement with previous investigations using rat and hamster liver [31, 35, 48]. On the other hand, PABA acetylation was not inhibited to any significant degree. Moreover, MTX inhibited PABA acetylation approximately 80% at 100 μM, while PA acetylation decreased by only 20% at this same concentration. The inability of higher concentrations of MTX to further inhibit PA acetylation (Fig. 6) indicates that MTX is inhibiting the contribution of NAT2 activity (because of substrate overlap) but not the primary enzyme responsible for PA acetylation (NAT1). Taken together, these biochemical studies indicate that both NAT1 and NAT2 are expressed throughout the length of the F-344 rat intestine.

The lack of heterogeneity in the longitudinal distribution of NAT in the F-344 intestine indicates that other factors are responsible for the preferential adduct and tumor formation in the colon after exposure to HAs in this rat strain. These possibilities include, but are not limited to, differences in detoxification enzymes (such as glutathione *S*-transferase paths [49]), differences in cell turnover, and/or DNA repair [50]. Studies to elucidate these factors are currently in progress.

This work was supported, in part, by Public Health Service Grant GM45203 and by a Wayne State University Career Development Chair Award to Dr. Svensson. The authors wish to thank Dr. Patrick Hanna for his gift of VFK and helpful discussions and Dr. David K. Pitts for his advice on the statistical analysis.

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