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SHORT COMMUNICATIONS

An intestinal arylamidase that selectively hydrolyzes certain aromatic amides

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Although a recent comprehensive review article discusses the participation of gastrointestinal micro-organisms in the metabolism of xenobiotic substances [1], the role played by the enzymes of the gastrointestinal tract itself in the metabolism of ingested drugs or toxic agents has not been fully appreciated or adequately explored.

The presence of an arylamidase in the intestine that could hydrolyze drugs that contain an amide functional group was suggested by the work of Michel *et al.* [2], who first reported that acetanilide was hydrolyzed by rat intestinal homogenates, albeit at only a slow rate compared to liver. Krebs *et al.* [3] observed that mucosa from sheep jejunum were capable of hydrolyzing the acetyl group from acetylsulfomezathine, while Gomori [4] presented histochemical evidence that rat and guinea pig intestinal mucosa contain an amidase that hydrolyzes the amide group of *N*-chloroacetyl-α (or β)-naphthylamines. Patki and Shirsat [5] concluded from indirect evidence after oral feeding of a number of homologous N^4-aliphatic acyl derivatives of sulfadiazine to mice that deacylation occurred in the gastrointestinal tract, the propionyl and butyrl group being the most rapidly deacylated. The presence of an enzyme in rat jejunal strips that readily hydrolyzed the butyryl group from the carcinogenic *N*-2-fluorenylbutyramide (FBA) was demonstrated by Nagasawa and Gutmann [6]. Recently, Wang and Bryan [7] noted that homogenates prepared from the small intestine of rats, mice, hamsters, and guinea pigs hydrolyzed the formyl group from *N*-formylated nitrofurylthiazolamines.

We wish to report that intestinal mucosa from a number of different species contain an arylamidase– which can be isolated from whole intestinal homogenates conveniently in the form of acetone powders of the soluble fraction – that is especially active in deacylating the butyryl group from FBA. Some of the properties and specificities of this arylamidase have also been studied.

Materials and methods. *N*-2-Fluoroenylbutyramide, *N*-2-fluorenylacetamide, *N*-2-fluorenylsuccinamic acid, *N*-2-fluorenylbenzamide, and *N*-2-fluorenyl-*p*-toluenesulfonamide, all prepared in this laboratory, were available from an earlier study [6]. 4-Acetamidobiphenyl, *p*-nitroacetanilide, 4-acetamidobenzoic acid, and N^4-acetylsulfanilamide were prepared by acetylation of 4-aminobiphenyl, *p*-nitroaniline, *p*-aminobenzoic acid and sulfanilamide, respectively, by standard procedures followed by at least two crystallizations from appropriate solvents to give products with melting points corresponding to the literature values.

When possible, the animals were fasted 24 hr before sacrifice and subsequent excision of the small intestine. The longitudinally-slit intestine (jejunum and ileum, pooled from 2–4 animals when necessary) was washed gently with cold isotonic saline, cut into small squares, blotted on filter paper, and weighed. A 10% (w/v) homogenate was prepared in ice-cold demineralized water by blending in a Waring blender for 5–6 min. The mince was further homogenized in a perfluorocarbon-on-glass homogenizer until all of the large pieces of tissue were disintegrated. The heavier particulate fraction was first sedimented by centrifugation at 9000 *g* (R_{max}) for 20 min at +5°, the sediment was washed with 20 ml of fresh demineralized water by homogenization, and the centrifugation repeated. The combined supernatant fractions were then centrifuged at 110,000 *g* (R_{max}) for at least 60 min to remove the microsomes. To precipitate the enzyme, the 110,000 *g* supernatant was diluted with 10 vol of ice-cold acetone. After standing several hours or overnight, the protein precipitate was sedimented by centrifugation at 700 *g* for 5 min, washed once with 1 vol of cold acetone, resedimented, and collected by filtration (suction) on a sintered glass filter. No further attempts were made to purify the enzyme beyond this stage. The acetone powders can be kept at −5° in tightly capped vials for several months without detectable loss of enzymic activity. The nitrogen content of these acetone powders as determined by a semi-micro Kjehldahl procedure was 0.12 ± 0.01 mg nitrogen/mg of preparation for all of the species studied, *viz.*, rat (Holtzman, male, female), mice (CF-1, m), guinea pig (Mayo Clinic strain, m, f), rabbit (albino laboratory stock, m, f), dog (mongrel, m). The smaller animals were sacrificed by cervical dislocation, the rabbits were killed by exsanguination from the heart, and the dog intestines were obtained during experimental surgery. The human specimen was a male adult patient who had died from Hodgkins disease; the intestine was removed 3 hr after death.

The acetone powders, although prepared from the soluble fraction of the intestines, did not completely redissolve in M/15 phosphate buffer, pH 6.8; therefore, the enzyme suspensions (0.40–4.0%, w/v) were homogenized just before use in order that representative aliquots could be taken. The incubation system contained 0.20–2.0 ml of the enzyme suspensions in buffer corresponding to protein nitrogens ranging from 0.10 to 9.6 mg per tube, 200 nmoles of the acetylated aromatic amine (added as 200 μl of a 1.00×10^{-3} M stock solution of the substrate in 95% ethanol), and M/15 phosphate buffer, pH 6.8, to a total vol of 3.0 ml. After incubation at 37° in air for 1 or 2 hr, the reaction was stopped by the addition of 4.0 ml of 5% perchloric acid, and the precipitated proteins sedimented by centrifugation at 11,000 *g* (R_{max}) in the cold for 30 min. Control tubes which were also incubated included one where enzyme was omitted, one where substrate was omitted, and one to serve as internal standard where 200 nmoles (theoretical amount liberated by complete hydrolysis) of the corresponding free amine replaced the acetylated substrate.

The aromatic amines liberated by the action of the intestinal amidase were quantitatively determined by diazotization and coupling with sodium 2-naphthol-3,6-disulfonate (R-salt) using a procedure patterned after both the Bratton and Marshall test [8] and the R-salt method of Westfall [9] as follows: to 3.0 ml of the deproteinized incubation mixtures, 1.0 ml of 2N HCl and 1.0 ml of 0.10% (w/v) aqueous $NaNO_2$ were added successively. After mixing for 3 min, 1.0 ml of a freshly-prepared 1.0% (w/v) solution of sodium 2-naphthol-3,6-disulfonate (which had been re-

Table 1. Comparison of the intestinal arylamidase from various species

Substrate	Activity: nmoles FBA hydrolyzed/2 hr/mg N*									
	100,000 *g* supernatant	Acetone powder								
	Rat	Rat		Mouse	Guinea pig		Rabbit		Man	Dog
		m	f	m	m	f	m	f	m	m
N-2-Fluorenylbutyramide (FBA)	155 (f)	≥200†	≥200†	170	83	84	70	34	63	<5
N-2-Fluorenylacetamide	44 (m)	63	77	88	42	28	18	17	<5	<5
N-2-Fluorenylsuccinamic Acid	<5	10	15	9	5.0			35	<5	<5
N-2-Fluorenylbenzamide	<5	11		<5	<5			<5	<5	<5
4-Acetamidobiphenyl	14 (m)	15		46	14		5.6	6.1	<5	<5
N-Acetylglycine			(5100)‡							

* Results of two or more separate determinations, except for mouse, female guinea pig and female rabbit. For low activities, the enzyme concentrations were raised 2 to 10-fold in order to increase the sensitivity of the assays. Acetanilide, *p*-nitroacetanilide, 4-acetamidobenzoic acid, N^4-acetylsulfanilamide or *N*-2-fluorenyl-*p*-toluenesulfonamide were not hydrolyzed under these conditions by the 10^5 *g* supernatant fraction of the rat or by the acetone powders of any of the species listed here.

† See footnote * under Materials and Methods.

‡ Amount of glycine liberated as described under Materials and Methods.

crystallized from water using charcoal for decolorization) in 10 M NH_4OH was added. The absorbance was read on a Beckman DU spectrophotometer at the following wavelengths: 520 nm for 2-fluorenamine, 515 nm for 4-aminobiphenyl, 500 nm for *p*-nitroaniline, and 490 nm for sulfanilamide and aniline. These wavelengths lie at or very close to the absorption maxima of the 1-arylazo-2-naphthol-3,6-disulfonate chromophores developed with the different amines under the above conditions. Beer's law was followed by all of the azo dyes formed within the range of concentration used, provided that the proteins in the incubation system were first sedimented by strong centrifugation. However, with increasing protein concentrations, the absolute color yield diminished due to loss of some aromatic amine by adsorption on the sedimented protein, and the use of internal standards was adopted to correct for this error. In these cases, the amount of *N*-acylaromatic amine hydrolyzed was calculated from the expression:

$$\text{nmoles hydrolyzed} = (200)\frac{A_S - A_B}{A_{IS} - A_B}$$

where A_S and A_{IS} are the absorbancies of the sample and the internal standard, respectively, and A_B, the absorbance of the blank which contained no substrate. Activities are expressed as the amount of substrate hydrolyzed/time/mg protein nitrogen. In no case was hydrolysis observed in the absence of enzyme. The amount of glycine liberated from acetylglycine was determined by the ninhydrin method of Troll and Cannan [10].

For *in vivo* administration of agents affecting protein synthesis, littermates (Holtzman rats, males) weighing approximately 100–150 g were used for each experimental group. Sets of 2 animals received a single agent and 2 animals from the same litter served as controls. Sodium phenobarbital (40 mg/kg) was administered intraperitoneally once daily for 3 days. Puromycin (7 mg/kg and 35 mg/kg) and DL-ethionine (175 mg/kg) were administered orally twice a day for 3 days, while phenothiazine in peanut oil (100 mg/kg) was administered orally once daily for 2 days. All control animals received the corresponding vehicle for equal periods of time. When not specified, the vehicle was water. The animals were sacrificed 18–24 hr after the last dose, and food was withheld during this period.

Results. The amidase, of mucosal origin [6] was isolated from the soluble 110,000 *g* supernatant fraction of the rat small intestine. The mitochondrial and microsomal fractions had essentially no activity, while the 110,000 *g* supernatant fraction hydrolyzed up to 155 nmoles of FBA in 2 hr per mg of protein nitrogen (Table 1), compared to 34 nmoles/2 hr/mg N for a 10 per cent homogenate of the whole small intestine. The acetone powders prepared from the 110,000 *g* supernatant fraction from the rat intestine always hydrolyzed FBA at a rate ≥ 200 nmoles/2 hr/mg N under the standard conditions of assay* and at optimal enzyme and substrate concentrations deacylated FBA at a calculated rate of 480 ± 80 nmoles/hr/mg N. This enzyme preparation exhibited a broad pH optimum over the range pH 6.5–8.5. Although solubility problems mitigated against precise enzyme kinetic measurements, the approximate K_m of the rat enzyme for FBA was calculated from Lineweaver–Burk plots[11] to be $1.15 \pm 0.19 \times 10^{-4}$ M.

The deacylation of variously acylated 2-fluorenamines as well as the deacetylation of the acetyl derivatives of some representative amines are compared in Table 1. The specificity of the 110,000 *g* supernatant fraction of rat intestine was not different from the acetone powder derived therefrom, indicating that the acetone treatment had no effect on this property of the enzyme. Also, there appeared to be no differences between acetone powder preparations from male and female rats. Of the *N*-acylated aromatic amines tested, FBA was hydrolyzed by far the most readily, followed by *N*-2-fluorenylacetamide (Table 1). *N*-2-Fluorenylsuccinamic acid and *N*-2-fluorenylbenzamide were only slowly hydrolyzed. The butyryl group is therefore more susceptible to hydrolysis by this rat enzyme followed by the acetyl group. However, 4-acetamidobiphenyl, which differs in structure from *N*-2-fluorenylacetamide only in the lack of a methylene bridge between the two benzene rings, was only feebly hydrolyzed; and acetanilide or other electronegatively *para* substituted acetanilides were not at all hydrolyzed by this enzyme. As expected, the intestinal preparation from the rat had considerable acetylglycine deacetylase activity [10].

The enzyme that hydrolyzes FBA was present in the intestine of all the species examined except the dog, *viz.*,

*In order to detect the hydrolysis of substances which were much less readily hydrolyzed than FBA, enough enzyme was added to the test system to hydrolyze completely the added 200 nmoles of FBA in 2 hr.

Table 2. Effect of various substances on the hydrolysis of FBA by rat intestinal arylamidase *in vitro*

Compound*	Concentration in medium	Per cent inhibition
NaF	1.5×10^{-3} M	67 ± 6 (9)‡
NaF	3.0×10^{-3} M	72 ± 2 (3)
o-Phenanthroline	1.0×10^{-2} M†	51 ± 9 (8)
KCN	5.0×10^{-3}M	12 ± 9 (6)
NaN_3	5.0×10^{-3} M	12 ± 6 (5)
Iodoacetamide	1.0×10^{-3} M	13 ± 7 (3)
p-Chloromercuri-benzoate	1.0×10^{-3} M†	6 ± 5 (6)
EDTA	1.0×10^{-2} M	9 ± 2 (3)
Ethanol	10% by vol	26 ± 4 (9)
Ethanol	13% by vol	56 ± 2 (12)
Ethanol	20% by vol	87 ± 1 (3)
EGME	13% by vol	15 ± 3 (3)
EGME	17% by vol	43 ± 2 (6)
EGME	27% by vol	89 ± 1 (3)
Acetylglycine	2.5×10^{-3} M	14 ± 3 (3)

* Except for the alcohols which were added together with the substrate, these compounds were pre-mixed with the enzyme for 30 min at room temperature before addition of the substrate and incubation at 37°. In all cases except where noted, the ethanol concentration in the incubation medium was 6.7% by vol.

† Limits of solubility; actual concentrations may have been lower as precipitates were observed. ‡ Average ± S.D.; numbers in parentheses indicate number of determinations.

rat, mouse, guinea pig, rabbit, and man, the relative activities falling in the order rat > mouse > guinea pig > man ~ rabbit > 0 (Table 1). The absence of this enzyme in the dog intestine was verified by independent observations on a second dog; no hydrolysis of FBA or any other substrate was detectable even by increasing the enzyme concentration 6.5-fold.

There appears to be some variation in the susceptibility to hydrolysis of substrates other than FBA by intestine of different species. Thus, while the rabbit enzyme deacylated FBA more slowly than the enzyme from rat or mouse, it hydrolyzed the succinyl group of *N*-2-fluorenylsuccinamic acid much more readily than the enzyme from any other species studied. The rabbit enzyme, as with the rat, mouse, and guinea pig, can also hydrolyze 4-acetamidobiphenyl, albeit at only a very slow rate. Hydrolysis of acetanilide was not detected with intestinal preparations from any of the species studied, including man.

The hydrolysis of FBA by the rat preparation was essentially uninhibited by addition of sulfhydryl blocking agents to the incubation medium (Table 2). Azide or cyanide ions likewise did not inhibit the enzyme. However, inhibition was observed with fluoride ions and by alcohols such as ethanol and ethyleneglycol monomethyl ether (EGME) in high concentrations. Whereas the metal chelating agent *o*-phenanthroline was inhibitory, EDTA was not. Acetylglycine at a concentration forty times that of FBA had only a slight effect, suggesting that different enzymes must be involved in the hydrolyses of these structurally-diverse substances.

Table 3. Effects of *in vitro* administration of agents affecting protein synthesis on FBA hydrolysis by rat intestinal arylamidase

Substance administered	Dose (mg/kg × no. days)	FBA hydrolyzed (per cent of controls)
Puromycin	14 × 3	63 ± 5*
Puromycin	70 × 3	40 ± 7
DL-Ethionine	350 × 3	33 ± 4
Phenobarbital	40 × 3	175 ± 9
Phenothiazine	100 × 2	95 ± 4

* Average ± S.D. of triplicate determinations.

Prior administration of puromycin and DL-ethionine, agents known to block protein synthesis, caused a decline in enzyme activity (Table 3), presubably by blocking the synthesis of new enzymes in the intestinal mucosa. Administration of phenothiazine, a substance which induces the synthesis of benzpyrene hydroxylase in rat intestinal mucosa [12], had no effect, while phenobarbital pretreatment had a pronounced stimulatory effect.

Discussion. Knowledge of species specificity and the organ distribution of tissue amidases that hydrolyze *N*-acylated derivatives of biologically-active amines are of intrinsic value to toxicologists and pharmacologists. Such information is also of special interest to medicinal chemists who wish to design and synthesize pharmacological agents that can be selectively activated by enzyme action [13, 14]. Despite the obvious significance of the intestine as a metabolic organ, surprisingly few studies have focused on the specific metabolism of drugs. In the case of amidases, investigators have merely included the intestine in a general screen for possible presence of isoenzymes.

The acetone powders from the small intestine of the various species described here are crude and undoubtedly contain a mixture of a number of enzymes; yet, except for FBA, *N*-acetylglycine, and, to a lesser degree, *N*-2-fluorenylacetamide, most of the acylated amines studied were only feebly or not at all hydrolyzed by these preparations. The failure of acetanilide to be hydrolyzed distinguishes this from the 'acetanilide deacetylase' described by Bray *et al.* [15]; and, since the 110,000 *g* supernatant fraction of homogenates likewise did not hydrolyze acetanilide (Table 1), this lack of activity cannot be ascribed to the known propensity of acetanilide deacetylase to inactivation by acetone treatment.

Whether the enzyme that hydrolyzes FBA is identical to the aminopeptidase found in porcine jejunum and ileum [16], to leucine aminopeptidase itself [17], or to the arylamidase found in human duodenum [18] remains to be clarified. The properties of this enzyme also suggest the possibility that it might be isozymic with the amidase isolated and purified from the soluble fraction of guinea pig livers [19], or even with the liver microsomal amidases isolated from the same species [20]. However, further purification and careful comparisons with selected additional substrates are required before such definitive relationships can be deduced.

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Identification and distribution of benzylamine in tissue extracts isolated from rats pretreated with pargyline

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In the recent analyses of some biogenic amines in mammalian tissues [1–4], the monoamine oxidase inhibitor pargyline [*N*-methyl-*N*-(2-propynyl)benzylamine] has been used to increase amine levels to facilitate their identification. Chromatographic separation of the dansyl derivatives of amine extracts obtained from rats treated with pargyline revealed the presence of significant amounts of benzylamine. Since benzylamine is the shortest homologue of a series of phenylalkylamines which includes the sympathomimetic amines β-phenylethylamine and amphetamine, and may itself possess excitant properties, we have attempted to determine whether benzylamine occurs endogenously in the rat and is elevated by pargyline, or whether it originates *in vivo* as a metabolite of pargyline.

Tissues were obtained from male Wistar rats (150–200 g), either untreated, or treated with pargyline hydrochloride (i.p., 75 mg/kg body weight) or iproniazid phosphate (i.p., 100 mg/kg). Four hr after administration of the drug, the animals were stunned and decapitated, and the brain, heart, kidneys, liver, lungs and spleen were removed, weighed and homogenized in 0·4 N perchloric acid. Blood was collected in a beaker containing 0·1 ml of 1% sodium heparin solution. In those cases in which the concentration of benzylamine was found to be very low, tissues from several animals (up to eight) were pooled during subsequent experiments. Deuterated benzylamine (1,1-dideutero-1-phenylmethylamine, 25 ng of free base in the case of tissues obtained from untreated and iproniazid-treated animals, and 250–2500 ng of free base, depending on the tissue, in tissues obtained from pargyline-treated animals) was added. The suspension was mixed, and then centrifuged at 12,000 *g* for 10 min. The supernatant was decanted, an amine fraction obtained by percolating the extract through a column of Biorad AG 50W-X2[H^+], and the dansyl amines were prepared as previously reported [5]. Dansyl benzylamine was separated from the reaction mixture by successive unidimensional chromatography on 20 × 20 cm glass plates coated with Silica gel [Brinkmann Instruments (Canada) Ltd., Rexdale, Ont.] in the solvent systems chloroform–butylacetate, 4:1 (v/v); benzene–triethylamine, 8:1 (v/v); and carbon tetrachloride–triethylamine, 5:1 (v/v). The dansyl benzylamine zone was removed from the plate, and the dansyl amine eluted with 30 μl of Fisher Spectranalyzed grade ethyl acetate [2]. Dansyl benzylamine was identified by its mass spectrum and quantitated mass spectrometrically using the integrated ion current procedure [5].

To determine which, if any, tissues were metabolizing pargyline to benzylamine, several tissues were removed from untreated animals and minced by slicing into approximately 1-mm cubes. The minces were suspended in 5 ml of an isotonic solution (pH 7·2) containing NaCl (120 mM), KCl (4·8 mM), $CaCl_2$ (2·6 mM), $MgSO_4$ (1·2 mM), Na_2HPO_4 (15 mM) and glucose (10 mM), then preincubated for 10 min at 37° in a shaking water bath. Pargyline hydrochloride solution (125 μg/50 μl, final concentration $1·3 \times 10^{-4}$ M) was added, and the incubation continued for 60 min. Control samples, containing tissue minces heated for 5 min in a boiling water bath, as well as samples containing only pargyline in buffer solution, were also incubated. The incubations were terminated by adding 0·5 ml of 4 N perchloric acid to each vessel. The tissues were homogenized, deuterated benzylamine (100 ng free base) was added, and benzylamine isolated and quantitated as described.

In some cases, tissue minces were incubated with pargyline [7-^{14}C] hydrochloride (7·03 μCi/mg, Abbot Laboratories, North Chicago, Ill.). The minces were suspended in incubation medium containing unlabeled pargyline hydrochloride ($1·3 \times 10^{-4}$ M) and preincubated for 10 min at 37° in a shaking water bath. Pargyline[^{14}C] hydrochloride solution (375,000 dis./min/50 μl) was then added and the incubation continued for 90 min. Boiled tissue samples and samples containing only pargyline[^{14}C] hydrochloride were incubated in an identical manner. Incubations were ended by homogenizing the minced tissue in 0·4 N perchloric acid; unlabeled benzylamine (25 ng) was added as carrier and benzylamine isolated as above. Radioactivity was measured in a Nuclear Chicago Isocap 300 liquid scintillation counter.