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Metabolic Disposition of β-Aminopropionitrile in the Rat[1, 2] (39420)

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Controlled osteolathyrism, for many years a laboratory curiosity, is now emerging as a clinical tool of potential importance. The successful use of beta-aminopropionitrile (BAPN) in prevention of esophageal stricture and in overcoming the restrictive effects of peritendinous adhesions in animal experiments (1-3) had led to human clinical trials. Results with BAPN in treatment of patients with urethral stricture[3] and tendon adhesions indicate that the physical properties of scar tissue may be amenable to biochemical control (4, 5).

In these preliminary trials, discrepancies have been noted between the clinical data and the predicted biochemical reactions from the dose of BAPN administered. These observations have raised questions concerning the uptake, metabolism, and excretion of BAPN. Information in such areas would contribute to safer and more effective dosage of BAPN as well as to the interpretation of future clinical studies.

This report describes a method based upon thin-layer chromatography (tlc) for measurement of BAPN directly in urine and body tissues of rats. Because the major metabolite of BAPN in the rat is cyanoacetic acid (CAA) (6), which is nonlathyrogenic (7), CAA was measured concurrently by a modification of the method of Sievert *et al.* (8). Utilization of [^{14}C]nitrile-labeled BAPN made it possible to measure the rate of conversion of BAPN to CAA by tissue homogenates.

Materials and methods. Beta-aminopropionitrile fumarate and CAA were purchased from commercial sources. Plastic precoated silica gel sheets (250 μM) without $CaSO_4$ binder (Macherey Nagel) and ninhydrin aerosol spray[4] were purchased from Brinkmann Instruments, Westbury, New York. [^{14}C]Nitrile-labeled BAPN (2.03 mCi/mmole) and [2-^{14}C]CAA (55.5 mCi/mmole) were obtained from Hoffman-LaRoche, Inc., Nutley, N.J.,[5] and ICN Isotope and Nuclear Division, Irvine, Calif., respectively. Both gave single radioactive peaks by tlc.

Female Sprague-Dawley rats weighing 200-250 g were used in groups of three for the *in vivo* studies. BAPN was diluted in sterile saline and injected ip at 400 mg/kg into one group. Diuresis was produced in a second group by administering 15 ml of saline sc prior to ip injection of BAPN. A third group was injected ip with 456 mg/kg of CAA (equivalent to BAPN dosage). Urine was collected under toluene at 2, 3, 4, 6, 12, and 48 hr, the volume was recorded, and the sample frozen at −20° until analyzed for BAPN and CAA.

Tlc of BAPN in urine. Urine samples were centrifuged and an aliquot of the supernatant from samples collected at 2, 3, 4, and 6 hr was diluted 1:10 with 80% ethanol. The 12-hr sample was diluted 1:5 with 100% ethanol. The 24- and 48-hr samples were not diluted. Two-microliter aliquots of all of the samples except the 48-hr sample were spotted on a tlc plate. The 48-hr sample required a 10-μl aliquot for assay. A BAPN standard in 80% ethanol (100 μg/ml) and control urine taken prior to administration of BAPN were cochromatographed. The tlc plate was developed in acetone, 1 M NH_4OH (9:1) for 10 cm, removed, dried in

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[3] E. E. Peacock, Jr., unpublished data.

[4] Ninhydrin aerosol spray (0.1% in isopropanol) from E. Merck (Catalog no. 6758).

[5] We are grateful to Hoffmann-LaRoche, Inc., of Nutley, N.J., for a supply of [^{14}C]nitrile-labeled BAPN.

air, then heated at 90–100° for 30 min. The plate was sprayed with ninhydrin aerosol and reheated for 8–10 min at 90–100°. The violet spots corresponding to an R_f of 0.6 were scanned with a Beckman R-110 Microzone Densitometer after cutting the tlc plate into suitable strips. The tracings were cut out, weighed on an analytical balance, and the BAPN in the urine sample calculated utilizing the formula:

$$\frac{\text{experimental trace (mg)}}{\text{standard trace (mg)}} \times \text{BAPN standard spotted } (\mu g) \times \text{dilution factor} \times \frac{\text{vol of urine (ml)}}{\text{vol spotted (ml} \times 10^{-3})}.$$

CAA in Urine. Aliquots (0.1 ml) of centrifuged urine corresponding to the samples used in the BAPN study were diluted to 1.0 ml with H_2O. Sixty percent H_2SO_4 (0.2 ml) was added, followed by 10 ml of ethyl acetate (EA) saturated with H_2O. Samples were extracted for 10 min on a mechanical shaker, centrifuged at 2000 rpm for 5 min, and 5 ml of supernatant was transferred to graduated tubes containing 0.5 ml of 0.1 *M* acetate buffer at pH 5.0. The contents were shaken and the ethyl acetate evaporated under N_2 at 55–65°. All samples were brought to 10 ml with H_2O. Control specimens of urine with and without 100 μg of CAA were run concurrently as a standard and specimen blank, respectively. Aliquots of the final 10-ml dilution ranging from 0.1 to 1.0 ml were analyzed colorimetrically as described by Sievert *et al.* (7). The maximum optical density obtained against a water reference at 490 nm and corrected for the specimen blank was used to calculate the CAA content in the experimental urines according to the following formula:

$$\frac{\text{OD of experimental}}{\text{OD of standard}} \times \text{standard } (\mu g) \times \frac{10 \text{ ml}}{\text{aliquot for colorimetry (ml)}} \times \frac{10 \text{ ml}}{\text{EA aliquoted (ml)}} \times \frac{\text{urine vol (ml)}}{0.1 \text{ ml}}$$

Characterization of the radioactivity in rat liver and brain after injection of [^{14}C]BAPN. Determination of CAA. In a preliminary experiment, the partition coefficient of [^{14}C]BAPN and [^{14}C]CAA was separately determined in 0.01 *M* unlabeled BAPN and CAA, respectively. The solutions were acidified with H_2SO_4 as in the previous determination. Ten volumes of diethyl ether previously saturated with H_2O were added and the mixture agitated for 10 min. After centrifugation, aliquots of the ether layer were added to scintillation vials containing 1 ml of 0.01 *M* BAPN and CAA in H_2O, and the ether was evaporated by an air current. Only 1 ± 0.2% (SD) of the radioactivity from [^{14}C]BAPN was found in the ether layer, while 80.0 ± 0.6% (SD) of the [^{14}C]CAA (six trials) was extracted by ether. Similar recoveries were obtained in the presence of liver and brain tissue as described below.

BAPN (8.3 mg) containing 4.95 μCi of [^{14}C]BAPN was injected iv into each of three rats. After 24 hr, the animals were sacrificed and the brain and liver were removed, blotted, and weighed. Tissue was stored at −20°. Thirty percent homogenates were prepared in 0.01 *M* $CaCl_2$. Two-tenths milliliter of liver and 0.5 ml of brain homogenate received 0.1 ml of 1% Triton X-100 in 0.01 *M* $CaCl_2$ and were brought to 1.0 ml with the $CaCl_2$ solution. After incubating for 1 hr at 37° to allow the Triton X-100 to further solubilize the tissue, the CAA present in the tissue was estimated by ether extraction. The radioactivity ascribable to ^{14}CAA in disintegrations per minute per gram of tissue was calculated by the following formula:

$$\frac{\text{net cpm}}{\text{counting efficiency}} \times \frac{10 \text{ ml}}{\text{vol of ether aliquoted}} \times \text{partition ratio} \times \frac{1000 \text{ mg}}{\text{wt of tissue (mg)}}.$$

The nanomoles of CAA/g equivalent to the dpm/g were calculated from the specific activity of the [^{14}C]BAPN administered.

Test for BAPN in brain tissue. One-milliliter aliquots of the 30% homogenates of

brain tissue were added to 4 ml of ice-cold ethanol. After 30 min in ice, samples were centrifuged at 2000 rpm for 10 min. Four-and-five-tenths milliliters of supernatant was reduced to less than 1.0 ml by drying under N_2 at 60–70°. All samples were adjusted to 1.0 ml with 80% ethanol. Aliquots (0.9 ml) were transferred to microvials and again evaporated to dryness under N_2 at 60–70°. The residues were suspended in 50 μl of 80% ethanol on a Vortex mixer. Radioactivity was measured by counting a 5-μl aliquot in 1 ml of H_2O containing 0.01 *M* unlabeled BAPN and CAA as carrier. Unlabeled BAPN was added to the remaining 45 μl of the experimental samples to provide 0.5 μg of BAPN per 10-μl aliquot of sample spotted on the tlc plate. A BAPN standard (0.5 μg) in 80% ethanol was spotted also. A tissue control from a rat not injected with [^{14}C]BAPN but receiving a radioactive spike *in vitro* was cochromatographed. All spots corresponding to an R_f of 0.6 were scraped into scintillation vials; 1 ml of carrier BAPN was added and the samples counted.

Conversion of BAPN to CAA in vitro. [^{14}C]BAPN was added to sufficient carrier BAPN so that 2 μ*M* contained 5 × 10^5 dpm. Labeled BAPN (20 μl) was added to 0.16 ml of 0.2 *M* phosphate buffer (pH 7.4) to yield concentrations of BAPN in the range of 2.5 to 40 m*M*. Next, 20 μl of freshly prepared 5% rat liver homogenate was added to the experimental samples. The same volume of boiled homogenate was added as a control for each BAPN concentration. A blank containing tissue alone (no BAPN) and tissue containing [^{14}C]CAA to evaluate the recovery of the metabolite were also analyzed. All samples were flushed with O_2 for 15 sec, and screw caps were tightened on the centrifuge tubes containing the samples. Samples were incubated at 37° for 30 min and 0.2 ml of 2 *N* HCl added to terminate the reaction. One-tenth milliliter of carrier CAA was added to 0.01 *M* concentration, followed by 5 ml of diethyl ether equilibrated with 1 *N* HCl. Tubes were agitated mechanically for 10 min, centrifuged, and 4-ml aliquots of the ether layer transferred to scintillation vials containing 1.0 ml of 0.01 *M* CAA. Ether was evaporated with an air stream at ambient temperature, 10 ml of aquasol was added, and the samples were counted. Radioactivity was converted to μ*M* of CAA/g/hr by use of the specific activity value for the [^{14}C]BAPN added.

Results. Recovery of BAPN in rat urine. Figure 1 shows mean values for unchanged BAPN in the urine of three rats for 48 hr following an ip injection of 400 mg/kg of BAPN. Saline diuresis in the second group of rats produces earlier and more rapid excretion of urinary BAPN. The diuresed rats also excreted more BAPN in 24 hr than those not receiving diuresis; both groups showed negligible excretion of unchanged BAPN thereafter.

Recovery of CAA in the urine of rats given 400 mg/kg of BAPN or the equivalent dose of CAA directly. Mean values for CAA in urine of rats undergoing saline diuresis and treated with BAPN were compared with the urinary CAA found in similarly diuresed animals given the equivalent dose of CAA (456 mg/kg ip) (Fig. 2). Direct injection of CAA is followed by rapid and almost complete excretion of the compound within 48 hr. Almost 60% of directly injected CAA could be recovered contrasted to 2% recovery of BAPN-derived CAA in urine 6 hr following ip injection. Thereafter, urinary CAA excretion in animals given BAPN increased gradually over 48 hr to approximately 22% of the dose administered.

Recovery of CAA from rat liver and brain after injection of [^{14}C] BAPN. The partition study described in the Methods section showed 99% of [^{14}C]BAPN remained in the aqueous phase after extraction with diethyl ether; 80% of [^{14}C]CAA was extracted by ether under identical conditions. Radioactivity recovered by ether from rat brain and

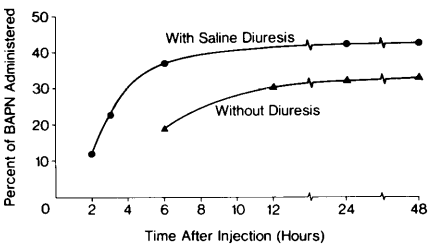

FIG. 1. Recovery of BAPN from rat urine. BAPN, 400 mg/kg, was injected into two groups of three rats each. One group received 15 ml of saline sc concurrent with BAPN dosage. The curves represent mean recoveries from each group.

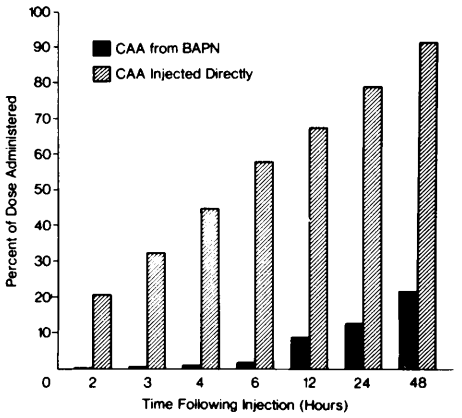

FIG. 2. Cyanoacetic acid in rat urine after injection of BAPN or cyanoacetic acid (CAA). BAPN, 400 mg/kg, or CAA, 456 mg/kg (dosage equivalent to BAPN), was injected ip into two groups of three rats given 15 ml of saline sc concurrent with drug dosage. The histograms represent mean recoveries from each group.

liver in animals injected with [^{14}C]BAPN and sacrificed 24 hr later is therefore taken as [^{14}C]CAA (Table I).

When the aqueous phase remaining from the experimental samples of rat liver was extracted with ether a second time, 80% of the radioactivity again distributed itself into the ether phase, suggesting that the BAPN-derived radioactivity remaining in the aqueous phase after the first extraction was also CAA.

Tlc for BAPN in brain tissue after iv injection of [^{14}C]BAPN into rats. An aliquot of tissue homogenate prepared from brains of rats sacrificed 24 hr after iv injection with [^{14}C]BAPN was analyzed for BAPN as described. Calculations from Table II show that 76% of the radioactivity added as [^{14}C]BAPN to the control and measured prior to chromatography could be recovered from the spot corresponding to BAPN (R_f = 0.6) after chromatography. Experimental tissues yielded no radioactivity in the corresponding spots from the unlabeled carrier BAPN added (Table II).

Conversion of [^{14}C]BAPN to CAA by rat liver homogenate in vitro. Conversion of [^{14}C]BAPN to [^{14}C]CAA appears approximately linear in the range of 2.5 to 10 m*M* inclusive of [^{14}C]BAPN added (Table III). The data indicate that a small percentage of BAPN added was changed to CAA. Table III also shows that under the conditions of this experiment addition of a starting concentration of 40 m*M* BAPN resulted in formation of a lesser amount of CAA than that obtained from a 30 m*M* BAPN concentration.

Discussion. Results of these experiments indicate that active half life of BAPN, administered to rats as a single ip dose, is short. Urinary excretion of unchanged BAPN began shortly after injection and approached maximum within 12 hr at which time approximately 33% of the administered dose was excreted (Fig. 1). This effect was exaggerated by saline diuresis, presumably by increasing renal excretion before BAPN could be deposited in tissues. Negligible BAPN was recovered in urine after 24 hr in both nondiuresed and diuresed rats (Fig. 1). The radioactivity in rat brain and

TABLE I. RECOVERY OF CAA FROM RAT LIVER AND BRAIN AFTER INTRAVENOUS INJECTION OF [^{14}C]BAPN.[a]

Tissue		[^{14}C]CAA Recovered[b]	CAA Recovered (nmoles/g)
A. Liver			
Control (no BAPN)		17	–
Experimental:	A	14,062	152
	B	8,872	96
	C	10,453	113
B. Brain			
Control (no BAPN)		15	–
Experimental:	A	7,894	85
	B	6,733	73
	C	7,198	78

[a] 8.3 mg of BAPN containing 4.95 μCi were injected. 24 × 10^3 nmole = 1 μCi (2.22 × 10^6 dpm).
[b] Corrected for 95% recovery of [^{14}C]CAA added to liver tissue from control rats (no BAPN).

TABLE II. EFFECT OF TLC ON THE RADIOACTIVITY IN BRAIN HOMOGENATES OF RATS INJECTED WITH [^{14}C]BAPN.[a]

Sample	Radioactivity (dpm/g) Before tlc	After tlc
Control + [^{14}C]BAPN[b]	1925	1472
Experimental, Rat no. 1	7105	0
Rat no. 2	4822	0
Rat no. 3	7002	0

[a] 8.3 mg of BAPN + [^{14}C]BAPN (4.95 μCi) were injected and the animal sacrificed 24 h later.
[b] Control brain homogenate received 3360 dpm of [^{14}C]BAPN and was analyzed and cochromatographed along with the experimental brain tissues.

TABLE III. CONVERSION OF [^{14}C]BAPN TO CAA BY RAT LIVER HOMOGENATE[a] *in vitro*.

Sample	[^{14}C] CAA[b] (dpm)	[^{14}C] BAPN[c] Initial concentration (m*M*)	Ether layer (A-B) (dpm)	*μM* CAA/g/hr
1	–	–	15	–
2	5875	–	4870	–
3	–	2.5	140	1.12
4	–	5.0	296	2.37
5	–	10.0	570	4.56
6	–	20.0	702	5.62
7	–	30.0	992	7.94
8	–	40.0	886	7.09

[a] Twenty microliters of fresh (A) or boiled (B) 5% rat liver homogenate equivalent to 1 mg of tissue was incubated with the concentrations of [^{14}C]BAPN listed at 37° for 30 min in 0.2 ml of 0.2 *M* phosphate buffer.

[b] The partition ratio of 4870 dpm in the ether layer from the 5875 dpm added as [^{14}C]CAA provided a correction factor for the dpm obtained with experimental samples.

[c] 5×10^5 dpm of [^{14}C]BAPN = 2 μmole of the compound.

liver in animals sacrificed 24 hr after injection of [^{14}C]BAPN appeared to be exclusively nonlathyrogenic [^{14}C]CAA (Tables I and II). Although based on a limited number of animals, the observations in the urine and tissues suggest that concentrations of BAPN could have diminished within 24 hr to levels that were not pharmacologically effective.

Excretion of BAPN-derived CAA in the urine occurred over a longer time course, appearing several hours after injection and increasing slowly and gradually throughout the 48-hr study period (Fig. 2). An equivalent dose of CAA was promptly excreted (Fig. 2). Because the pK of CAA is 2.45 (9), at physiological pH, directly injected CAA would exist as a polar anion susceptible to glomerular filtration (10). The rapid excretion of directly injected CAA (Fig. 2) is consistent with this mechanism. Slower excretion of BAPN-derived CAA supports the hypothesis that unexcreted BAPN is sequestered in tissues where it is stored and subsequently metabolised to CAA before being slowly released. This hypothesis is strengthened further by the finding that 24 hr after an injection of isotopically labeled BAPN virtually all radioactivity extracted from rat brain and liver was present as [^{14}C]CAA (Tables I and II). Similar findings were reported by Waddel *et al.* (11).

The work of Haney *et al.* (12) also points to the transient existence of the biological activity of BAPN *in vivo*. Single daily doses of BAPN (23 mg/kg) for 10 days did not inhibit totally collagen cross-linking because an additive effect was obtained by administering D-penicillamine. These data suggest that renewed lysyl oxidase activity (13) and collagen cross-linking had time to occur between BAPN doses.

The teratological properties of BAPN (14, 15) in the rat show similar pharmacokinetic limitations. Pratt and King (15) demonstrated a close correlation between cleft palate induction and inhibition of collagen cross-linking when BAPN was administered to pregnant rats on Day 15 of gestation but not on Day 14. Our data (Fig. 1; Table II) are in accord with the notion that BAPN may decrease in both maternal and fetal tissues within 24 hr following dosage on Day 14 to levels below those required to produce this abnormality in the fetus.

Although the major location, rate, and mechanism of BAPN degradation are not known, the *in situ* conversion of retained BAPN to CAA strongly suggests a metabolic system presumably including monoamine oxidase found in a number of tissues. Data reported in this paper substantiate the relatively slow kinetics of this system in the rat (Table III). Earlier findings of Sievert *et al.* (8) also show enzymatic conversion of BAPN to CAA *in vitro* by rat liver homogenate to occur more slowly than with similar preparations from seven other species. In the radiometric assay, with [^{14}C]BAPN made to contain 2.5×10^5 cpm/μm the enzyme activity of 1 mg of rat liver homogenate could be readily measured and the percentage conversion of BAPN to CAA was extremely small (Table III). Such observations are in agreement with the small conversion of BAPN to CAA noted in isolated rat livers profused with BAPN.[6] It is of interest that an initial concentration of 40 m*M* BAPN added as substrate to rat liver homogenate (Table III) showed inhibition analogous to that reported by Page and

[6] J. H. Fleisher, K. Brendel, M. Chvapil, and E. E. Peacock, Jr., submitted for publication.

Benditt (16) with pig plasma amine oxidase and kynuramine as substrate.

Because oxidative deamination appears to be the rate-limiting step in the ultimate metabolic disposition of BAPN in rats, unpredicted dose-response patterns following chronic administration of BAPN may be due to "overloading" of the degradative pathway, with storage of active BAPN and prolongation of lathyritic effect. Such data may explain prolonged elevation of collagen a:B ratios observed by Bentley *et al.* (17) after 17 days of BAPN administration. Interference with drug degradation by a specific monoamine oxidase inhibitor also might be expected to enhance osteolathyritic effect of BAPN in the rat (18).

As yet, detailed studies of BAPN metabolism in man have not been carried out. However, amine oxidase systems demonstrate enormous species variability in kinetics, substrate specificity, and cofactor requirements. In addition, the potential role of plasma amine oxidase, present in man but not in the rat, has not been explored with respect to BAPN metabolism. Further studies will be necessary to delineate the pharmacologic parameters for controlled lathyrism in humans.

Summary. Enzymatic conversion of the lathyrogen, β-aminopropionitrile (BAPN) to the deaminated, nonlathyrogenic metabolite, cyanoacetic acid (CAA) by rat liver homogenates was found to be less than 1%. Urine of rats injected with 400 mg/kg of BAPN ip contained 32% unchanged BAPN in 24 hr, with negligible recovery thereafter. BAPN-derived CAA showed 12 and 22% recovery in the urine in 24 and 48 hr, respectively. These data suggest that significant amounts of unchanged BAPN may not exist in rat tissues after 24 hr, and that BAPN-derived CAA in such tissues is gradually excreted. The observation that radioactivity in rat brain and liver 24 hr after [^{14}C]BAPN injection is present exclusively as [^{14}C]CAA supports this interpretation.

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