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Neurotoxicity of a non-metabolizable amino acid, 1-aminocyclopentane-1-carboxylic acid (ACPC): ACPC transport mechanisms in tissues

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1-Aminocyclopentane-1-carboxylic acid is a non-metabolizable amino acid [1], sharing the same mechanism for intracellular transport as naturally occurring amino acids of the 'large neutral' class [2] which includes phenylalanine, leucine, isoleucine, valine, tryptophan, tyrosine and methionine [2–4]. Similar to these endogenous amino acids, ACPC, in high doses, markedly alters tissue amino acid pools *in vivo* [5, 6] and disrupts protein synthesis [7–9] by competitively inhibiting intracellular transport and possibly activation of amino acids [9]. Recently it was found that ACPC induces degeneration of axons in adult mice [10] and reduces the protein and sulfatide content of histologically affected areas.* Because of these properties, ACPC is a particularly valuable tool for studying *in vivo* responses to an excess of an amino acid independently of its metabolites. Such studies may clarify the neurotoxic role of the parent amino acid(s) in amino acid-dependent brain dysfunctions as well as further define regulatory mechanisms of monoamine and protein metabolism which are mediated by the ratio of particular amino acids in plasma [11–16]. The present study provides pharmacological background for further use of the ACPC model by characterizing changes in the *in vivo* distribution and excretion of ACPC over time at neurotoxic and subneurotoxic doses.

Male mice of the C57BL/6J inbred line (Jackson Laboratories, Bar Harbor) weighing 19–20 g were used exclusively. Their age at the time of analysis was 11–13 weeks. The mice were fed Formulab chow (protein 23, fat 6.5, fiber 4 per cent) (Ralston Purina) and, during treatment, food and water were supplied *ad lib.* close to the floor of the cage. Animals were housed in rooms maintained at 23° on a 12 hr light–dark schedule.

ACPC (Calbiochem) was dissolved in phosphate-buffered (pH 7.3) isotonic saline (Difco) at 20 mg/0.3 ml. Carboxy-[^{14}C] ACPC (New England Nuclear Corp. sp. act. 9.29 mCi/m-mole; greater than 99 per cent purity) (0.5 μCi) was added to appropriate aliquots of this solution. These solutions, 0.075–0.90 ml depending on dose, were prewarmed to 37° and injected intraperitoneally (i.p.). Blood (0.05–0.1 ml) from the capillary plexus behind the eye was drawn from mice lightly anesthetized with ether into heparinized blood collecting tubes. Radioactivity was measured on 10-μl aliquots of plasma obtained by centrifugation. Urination was induced by rapidly picking the mouse up by the tail and the scruff of the neck. Radioactivity of 10-μl urine samples was measured. Mice were voided 2 hr prior to the first urine collection and voided as completely as possible during each subsequent collection to ensure the optimal reflection of urinary excretion during each interval. Mice were killed by cervical dislocation followed by decapitation. The water content of each tissue analyzed was measured by drying a representative weighed portion to constant weight under reduced pressure over potassium hydroxide. The remaining portion of tissue was weighed, homogenized in 10 vol of 10% ice-cold trichloroacetic acid (TCA) and centrifuged at 7000 *g*. The supernatants were aspirated and saved and the pellets were washed with 5 vol of 10% TCA. Following centrifugation, each supernatant of the TCA wash was pooled with its respective original supernatant and radioactivity in 1-ml aliquots was determined. Radioactivity was measured in a Beckman LS 233 liquid scintillation counter following the addition of 12 ml of tT21 scintillation cocktail [17] to each sample. Counting efficiencies were determined using [^{14}C]toluene (New England Nuclear Corp.) as an internal standard and the weight of ACPC (mg) was calculated from the dis/min and appropriate specific activity.

The ACPC distribution ratio was defined as the weight of ACPC (mg) in tissue water/weight of ACPC (mg) in plasma water. Plasma water content was determined to be 95 per cent of the total plasma volume on the basis of five individual measurements. For CNS tissue, kidney, spleen and pancreas, where data on ACPC accumulation approximated a straight line, the distribution ratio was represented statistically by the slope of the linear regression through the origin calculated by the method of least squares [18] for the dependent variable y (ACPC in tissue water) on the values of the independent variable x (ACPC in plasma water). A 95 per cent confidence interval for the slope of each line was computed. Accumulation of ACPC against a concentration gradient, (tissue-water ACPC)/(plasma-water ACPC) > 1, was considered statistically significant if the regression line slope minus the lower 95 per cent confidence interval limit exceeded unity. Curved lines were fitted by eye to the data of Figs 3 and 4. Nonparametric correlations between behavioral states and liver or plasma levels of ACPC were performed on the data in Fig. 3. Behavioral state was evaluated on the basis of two nonspecific signs of ACPC toxicity, decreased spontaneous movement (expressed as per cent of normal activity) and generalized weakness (estimated from the ability to maintain a certain position on the rungs of a cage top). The behavioral classifications of 'no symptoms', 'mildly affected' (activity reduced 25–50 per cent; difficulty maintaining an upside down position), 'moderately affected' (activity reduced 50–90 per cent; unable to main-

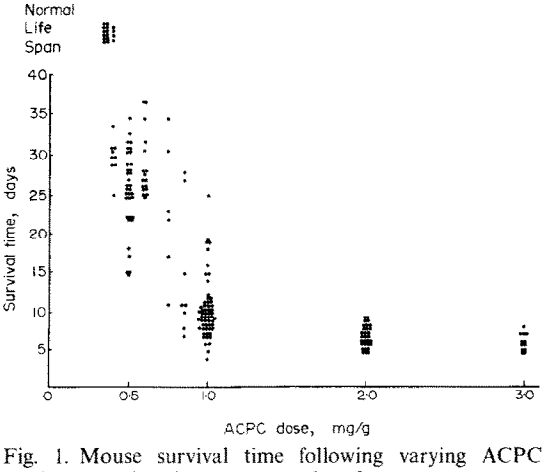

Fig. 1. Mouse survival time following varying ACPC doses. Each point represents data from one mouse.

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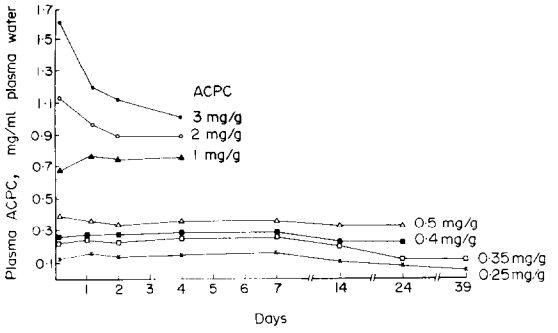

Fig. 2. Plasma levels of ACPC in mice at different times following varying ACPC doses. Each point represents the mean from 4–5 mice. The standard deviations are less than 15 per cent.

tain an upside down position) and 'severely affected' (activity reduced 90–100 per cent; unable to maintain a vertical position) were assigned the numbers 1, 2, 3, and 4 respectively. Kendall's rank correlation coefficients (τ) and their probability (P-value) of occurrence under the null hypothesis (i.e. no correlation) were then computed [19].

The LD_{50} range for ACPC lies between 0.35 and 0.40 mg/g (Fig. 1). The latter was also the concentration required for behavioral signs of neurotoxicity [10]. Most mice died in the fourth and fifth weeks following 0.4–0.6 mg/g of ACPC or in the second week following 1 mg/g or more of ACPC. The half-life of ACPC in the plasma was extremely long (Fig. 2). At the threshold lethal concentration (0.4–0.5 mg/g) the plasma ACPC level at 24 days was not significantly different from that at 1 day. The highest ACPC doses of (1–3 mg/g) resulted in nearly similar ACPC plasma levels by the fourth day.

The accumulation of ACPC in tissues was measured at plasma ACPC levels between 0.02 and 1.29 mg/ml (Table 1). More than 98 per cent of the tissue ACPC was TCA-soluble. Most of the remaining 2 per cent was associated with protein and was resistant to solubilization by organic solvent extraction or by heating briefly at acid or alkaline pH. The ACPC distribution ratios in brain and spinal cord were not significantly different from unity. Accumulation against a concentration gradient occurred in kidney, in spleen to a greater degree, and to a much greater degree in pancreas. The distribution ratios for CNS tissue, kidney and spleen did not change as a function of plasma ACPC concentration or of time between 4 and 40 days after ACPC administration.

Table 1. The distribution ratio of ACPC in various tissues

Tissue (N)*	Time after ACPC administration	Distribution ratio
whole brain (19)	4–40 days	1.13 ± 0.16‡
spinal cord (8)	4–40	1.17 ± 0.24
kidney (19)	4–40	1.22 ± 0.12
spleen (18)	4–40	1.58 ± 0.10
pancreas (35)	5	5.28 ± 1.78
liver (10)	5†	2.14 ± 0.46
liver (9)	24–40†	1.11 ± 0.16

* Number of animals.

† For plasma ACPC concentrations less than 0.5 mg/ml of plasma water. Above this concentration, the distribution ratio changes (see Fig. 3 and text) as a function of ACPC concentration.

‡ 95 Per cent confidence interval of the distribution ratio.

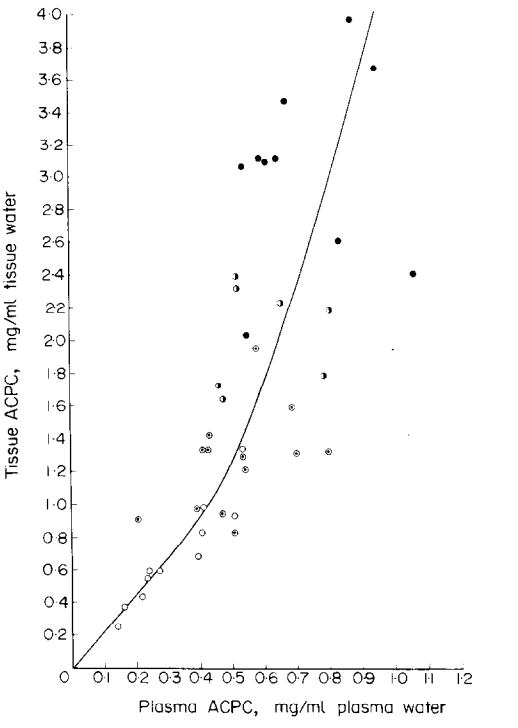

Fig. 3. ACPC accumulation in mouse liver and behavioral state of mice at varying plasma ACPC levels. Each point represents data from one mouse analyzed 5 days after ACPC administration. The clinical state is characterized as not affected ○, mildly ⊙, moderately ◑ or severely ● affected.

In liver, by contrast, the ACPC distribution ratio decreased significantly over time after ACPC injection from 2.14 ± 0.46 at 5 days after injection to an apparently stable ratio of 1.11 ± 0.16 beyond 24 days after injection (Table 1). Because of this variability in ACPC accumulation with time, a fixed interval of 5 days after ACPC administration, was used in further studies of liver (Fig. 3). The hepatic ACPC distribution ratio was considerably greater than unity at the lowest doses and increased, though quite variably, with increasing plasma ACPC levels. Since liver ACPC and mouse survival time (Fig. 1) were both highly variable at certain ACPC doses, their relationship was investigated using the observation that the severity of two non-specific behavioral signs of toxicity, weakness and diminished spontaneous activity, reliably forecasts the survival time of ACPC-treated mice. Categorized as severe, moderate, mild or absent (see above), these behavioral signs were highly correlated with liver ACPC concentration ($\tau = 0.88$, $P < 10^{-6}$), considerably higher than with plasma ACPC concentration ($\tau = 0.61$, $P < 10^{-5}$).

The elimination of ACPC in the urine was measured at intervals during the first 2 days following its administration in doses of 0.25–3 mg/g (Fig. 4). Inducing urination after appropriately short time intervals permitted transient changes in ACPC excretion to be detected and correlated with fluctuations in plasma ACPC levels. Below 0.5 mg/g, ACPC appeared in exceedingly low concentrations in the urine. The urinary ACPC level was considerably higher at 1 mg/g but still represented a relatively small fraction of the injected dose. The 2–3 mg/g doses of ACPC marked a turning point in the renal handling of ACPC in that large quantities appeared in the urine following these doses. Peaks of urinary ACPC concentration were observed 0–6 hr and 9–20 hr after ACPC injections, occurring concomitantly with elevations of plasma ACPC. When

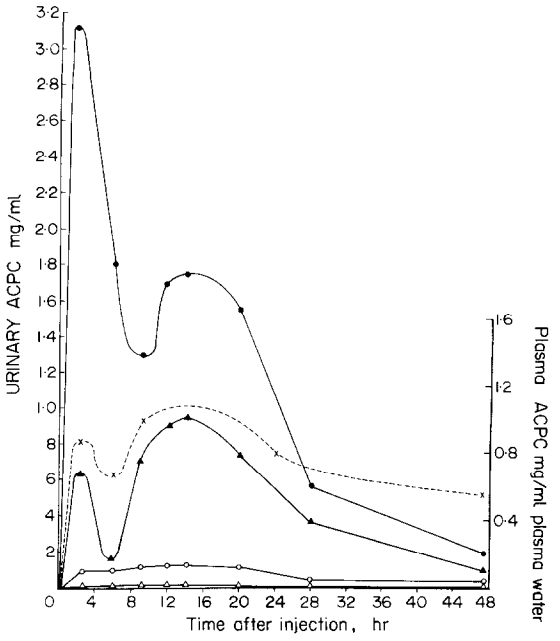

Fig. 4. Urinary ACPC at different times following 0.5 △, 1.0 ○, 2.0 ▲ or 3.0 ● mg/g of ACPC. Each point is the mean from 3–5 mice. The dotted line ×----------× represents plasma ACPC following 2 mg/g of ACPC and is the mean from 4 mice. Standard deviations are less than 25 per cent

individual values of urinary ACPC concentration at 3 hr were plotted against the corresponding plasma values (not shown), an ACPC concentration of 0.5 mg/ml (4.1 mM) was found to be the threshold above which significant fractions of the injected ACPC dose appeared in the urine.

These findings represent the first detailed information on the *in vivo* behavior of ACPC at concentrations which are comparable to or higher than physiological levels of endogenous amino acids. Earlier *in vivo* studies of ACPC have been limited to the use of trace quantities of radiolabeled ACPC and short intervals after administration [20–24] at which time the ACPC levels in certain organs are labile [23]. The neurotoxic actions of ACPC, by contrast, require high doses, are manifested behaviorally only after days or weeks [10] and are best understood, therefore, when studied under such conditions.

The accumulation of ACPC in whole brain was found to be comparable to that in spinal cord where histological [10] and biochemical* lesions are prominent. The cerebellum, another CNS region histologically affected by ACPC, also does not preferentially accumulate ACPC [21]. These tissues did not accumulate ACPC against a concentration gradient but did achieve levels equivalent to the plasma ACPC concentration, consistent with recent evidence that 'large neutral' amino acids are transported in neural tissue by facilitated diffusion [4].

The pancreas was much more active in accumulating ACPC than other tissues [24, 26]. It is unlikely, however, that the high pancreatic accumulation of ACPC is critical for the development of toxicity since ACPC toxicity in dogs is comparable to that in mice, even though ACPC is not selectively concentrated in dog pancreas [26]. The liver initially accumulated ACPC to concentrations much higher than those in plasma, but over the next 3 weeks, tissue levels of ACPC fell relative to plasma ACPC levels and a stable distribution ratio of 1 became established. This decrease in the ACPC distribution ratio over time can be likened to the changes in hepatic levels of natural amino acids which occur as a result of postprandial hepatic regulation [27]. The liver, in this context, acts as a buffer between the gut and arterial circulation by accumulating the suddenly increased concentration of amino acids in portal blood during feeding and then gradually replenishing the systemic circulation as amino acids are utilized in the tissues [27]. If this process were applicable for ACPC, it would be considerably prolonged since ACPC is not metabolized [1] and its elimination from the systemic circulation is exceedingly slow. The marked variability of individual hepatic ACPC levels is comparable to that observed in studies with natural amino acids. The rise in the ACPC distribution ratio with higher plasma concentrations is likely related to the dose-dependent reduction in food intake by mice following ACPC administration. Starvation has been shown to considerably increase the hepatic ACPC distribution ratio [7, 22].

Systemic toxicity of ACPC was found to be highly correlated with hepatic ACPC levels even though mild to moderate fatty infiltration is the only change seen histologically in the liver (unpublished results). The mechanism of this additional toxicity is unknown at present. Since changes in amino acid pools have a marked effect on ACPC neurotoxicity*, impairment of amino acid regulation, in which the liver plays a key role, may be an important factor. In any case, it is unlikely that ACPC neurotoxicity is secondary to hepatotoxicity since ACPC is toxic to organotypic cerebellar cultures at concentrations comparable to *in vivo* brain concentrations measured here.*

Urinary excretion of ACPC at high doses (2–3 mg/g) occurred in two peaks concomitant with elevations of plasma ACPC. The inability of the tissues to accumulate ACPC at a rate comparable to that of intestinal absorption likely accounts for the initial peak of plasma ACPC. Abrupt efflux from tissues may account for the second peak since the reported distribution ratios of ACPC in the first few hours after administration in the rat are higher than those observed at later times [24].

The reabsorption system(s) in the renal proximal tubules which, in part, accounts for the long biological half-life of ACPC, is saturated by plasma ACPC concentrations above 4.1 mM. This threshold saturating concentration is lowered by the administration of other 'large neutral' amino acids which suggests that the saturating effect is not merely a reversible toxic action of ACPC on the kidney. The apparent saturating concentration of ACPC (4.1 mM) in the renal tubules is similar to the level saturating ACPC uptake systems in the choroid plexus (3 mM) [28] and in the intestine (2 mM) [29]; however, it is an underestimate of the true saturation concentration since competing amino acids were also present in the plasma perfusing the kidney.

These results show that ACPC exhibits properties similar in many respects to natural amino acids *in vivo* and offers distinct advantages as an experimental tool. A single injection of ACPC is sufficient to maintain constant reproducible plasma and tissue ACPC levels for weeks. In addition, the progression of ACPC neurotoxicity can be easily halted by inhibiting renal tubular ACPC absorption with certain neutral amino acids [30]. Finally, ACPC, a non-metabolized amino acid, is neurotoxic at concentrations comparable to those at which some natural amino acids are neurotoxic in human aminoacidurias [31]. These findings reinforce the concept [32–34] that the neurotoxicity of natural amino acids in disorders of amino acid metabolism may be partly the result of general biochemical responses to the amino acid(s) in its unmetabolized state.

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Extraneuronal uptake and metabolism of [^{3}H]L-norepinephrine by the rat duodenal mucosa*

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Recent work in our laboratory has demonstrated that rat duodenal mucosa avidly takes up circulating L-dopa and stores dopa metabolites largely in the form of glucuronide conjugates [1]. The studies reported here indicate: (i) that norepinephrine, like L-dopa, is concentrated by duodenal mucosa; (ii) that the uptake process is outside the sympathetic nerve endings; and (iii) that NE is stored in the mucosal cells as noncatechol metabolites, principally the glucuronide conjugate of NE.

The methods utilized have been previously described in detail [1]. Rats weighing 180–220 g (Charles River) were used in all experiments. L-norepinephrine-7-[^{3}H] (4–7 Ci/m-mole) and L-3,4-dihydroxyphenylalanine (6–9 Ci/m-mole) (New England Nuclear Corp.) were purified prior to use by column chromatography on alumina. They were

Table 1. NE and dopa uptake by duodenal mucosa: effect of 6-OHDA

	Total ^{3}H (nCi/g)	
	Duodenal mucosa	Heart
[^{3}H]L-NE		
Control	478.9 ± 42.8	1560.5 ± 96.4
6-OHDA	623.9* ± 46.8	307.7‡ ± 16.4
[^{3}H]-dopa		
Control	1264.7 ± 101.7	171.4 ± 6.3
6-OHDA	1675.0† ± 78.1	168.2 ± 11.4

* $P < 0.02$ † $P < 0.01$ ‡ $P < 0.001$ compared with control 5–7 animals per group; 6-OHDA administered i.v. 24 hr before (100 mg/kg). Controls received diluent. Rats were given [^{3}H]L-dopa (400 μCi (13.6 μg) per kg) or [^{3}H]L-NE (200 μCi (9 μg) per kg) i.v. and killed 10 min later. Results are means ± S.E.M. Hearts of 6-OHDA treated rats had no detectable NE by fluorescent assay.

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