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BRIEF COMMUNICATION

D-Proline: Stereospecificity and Sodium Chloride Dependence of Lethal Convulsant Activity in the Chick

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CHERKIN, A., J. L. DAVIS AND M. W. GARMAN. *D-proline: stereospecificity and sodium chloride dependence of lethal convulsant activity in the chick.* PHARMAC. BIOCHEM. BEHAV. 8(5) 623–625, 1978. – Two- and five-day old chicks were injected intraventricularly with D-proline and structurally related compounds. D-proline produced convulsions and lethality, but was non-amnestic, whereas the naturally-occuring isomer, L-proline, was non-convulsant and non-toxic but amnestic. D-proline convulsions were accompanied by decreased high frequency in the EEG and increased slow wave activity. High amplitude spiking was not observed. The lethality of D-proline was saline-dependent. Control experiments ruled out possible toxic factors such as hypertonicity, pH, pyrogens, injection volume, or needle misplacement. The results demonstrate that saline and distilled water are not equivalent injection vehicles. A sodium-free vehicle may lead to artifacts but is advantageous in experiments in which amino acid transport must be minimized.

D-proline Chicks Convulsions Amino acid transport Amino acid toxicity

NEUROTOXIC effects of amino acids and structurally related compounds have been demonstrated with glutamic acid and its analogs [7] and seizure activity has been observed with several amino acid analogs [1]. The usefulness of determining the structure-activity relationships of convulsant compounds has been emphasized [8]. We now report the lethal convulsant activity of D-proline injected intraventricularly, because of its stereospecificity and its apparent dependence upon sodium chloride. In addition, we report mortality data for other compounds structurally related to proline. These observations arose during our study of the structure-activity relationships of the amnesic effect of L-proline [2,3].

The animals were male White Leghorn chicks of Strains K-137, K-163, and DeKalb XL (Pace/Setter Products, Alta Loma, CA). They were received on Day 2 post-hatch and injected either on the same day or on Day 5. In the latter case, they received starter mash and water ad lib during Day 2 to Day 5.

All compounds were used as received from the supplier: D-proline, L-proline, 3,4-dehydro-DL-proline, L-baikiain, and L-isoleucine were Calbiochem A Grade and all other compounds (Table 1) were Sigma Chemical Products. Potassium chloride, sodium chloride, and sodium bicarbonate were analytical reagents (Mallinckrodt) and the distilled water was Sterile Water for injection, USP (Abbott). Solutions were freshly prepared within 1 hr of starting each experiment and the pH was brought to 7.2 ± 0.5 with $NaHCO_3$ crystals. Solutions were injected using a 500 μl syringe fitted with a repeating dispenser and a 27 ga needle fitted with a plastic sleeve which left 4.0 mm of the needle exposed. An injection of 10 μl was made into each cerebral hemisphere with placement 4 mm posterior to the orbital suture and 1.5 mm lateral to the midline. Trials with India ink injections showed distribution in both lateral ventricles in 70% of chicks (N = 20), in one ventricle in 20%, and in neither ventricle in 10%. The mortality rate 24 hr after injection was recorded. Statistical comparisons are based on the χ^2 test with the Yates correction, with a significance level of $p<0.05$. In experiments using 300 mM D-proline in 75 and 150 mM NaCl, and 600 mM D-proline in 150 mM NaCl, the behavior of chicks was observed at intervals after injection, with recording of: convulsions, body posture, head posture, head position, eye closure, and cessation of breathing.

The mean mortality rate (Table 1) of 300 mM D-proline in the absence of NaCl was low (4.2%) and not significantly different from that of L-proline (0.4%). In 75 mM NaCl, 300 mM D-proline produced no immediate behavioral reaction but within 2 min astasia was observed and the chicks assumed a squatting posture with eyes closed and head drooping; this posture lasted up to 1 hr. In a few cases, the chicks spent the first few post-injection minutes on their backs, paddling their feet. Half the chicks injected with 300 mM D-proline in 150 mM NaCl showed convulsions, primarily opisthotonic, within 2 min; the others

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TABLE 1

MORTALITY RATES IN CHICKS INJECTED WITH SOLUTIONS OF PROLINE AND OTHER COMPOUNDS (10 μl PER BRAIN HEMISPHERE)

	Compound	Conc. (mM)	NaCl (mM)	Exps. (N)	Range Chicks (N)	Range Mortality (%)	Total Chicks (N)	Total Mortality (%)
1.	D-Proline	300	–	13	20–60	0–20.0	425	4.2
		300	75	1	–	–	30	0.0
		300	150	2	30–30	26.7–26.7	60	26.7
		300	300	2	30–90	23.3–52.5	120	45.0
		300	562–600	3	30–30	23.3–43.4	90	30.0
		600	–	2	30–80	3.8–53.3	110	17.3
		600	150	1	–	–	30	16.7
2.	L-Proline	300	–	25	20–135	0–5.0	1379	0.4
		300	562	2	–	–	60	0.0
		600	–	7	30–160	0–10.0	460	2.4
3.	L-PRO methyl ester·HCl	300	180	1	–	–	20	0.0
4.	L-PRO benzyl ester·HCl	300	180	1	–	–	20	0.0
5.	Hydroxy-L-proline	300	–	2	–	–	90	0.0
6.	Sodium L-pyroglutamate	300–600	–*	2	–	–	60	0.0
7.	3,4 Dehydro-DL-proline	100	–	1	–	–	60	8.3
		300	–	2	30–30	33.3–63.3	60	48.3
8.	DL-Pipecolic acid·HCl	600	590	3	–	–	90	0.0
9.	L-Baikiain	150	–	1	–	–	30	26.7
		300	–	1	–	–	30	73.3
10.	L-Azetidine-2-COOH	300–600	–	2	30–30	0–3.3	60	1.7
11.	Monosodium L-glutamate	75–300	–*	3	–	–	60	0.0
12.	L-GLU diethyl ester·HCl	100–300	66–238	5	–	–	140	0.0
13.	L-Isoleucine	300	–	12	20–135	0–2.5	745	0.1
14.	Glycine	300	–	1	–	–	30	0.0
15.	Dibutyryl c-GMP	0.2–14.4	154	4	–	–	80	0.0
16.	KCl	125–200	–	3	20–40	0–5.0	90	1.1
		250	–	4	20–80	0–3.3	260	1.2
		250	154	1	–	–	40	0.0
		500	–	1	–	–	30	6.7
		750	–	1	–	–	30	46.7
17.	NaCl	–	154–404	3	–	–	150	0.0

*Contains Na^+ in equimolar concentration

exhibited astasia or fell on their sides. Thirty-two min after injection, a few chicks appeared normal but most exhibited dyspnea, closed eyes, and head droop.

In the first 2 min after injection, 600 mM D-proline in 150 mM NaCl produced results similar to those with 300 mM D-proline in 150 mM NaCl. However, within 3 min all chicks had eyes closed and were either astatic with head down, or on their sides. Most exhibited intermittent leg and wing thrashing, convulsions and dyspnea. When mortality was noted, it occurred within 128 min.

The NaCl interaction effect was not observed with L-proline, DL-pipecolic acid, L-glutamic acid diethyl ester, dibutyryl c-GMP, or KCl. No mortality was observed with NaCl alone (154, 250, or 404 mM) or with 154 mM NaCl plus 250 mM KCl. KCl at 125–500 mM had low mortality rates (0.0–6.7%) but 750 mM KCl had a high rate (46.7%). These facts rule out ascribing the lethality of D-proline in saline solution to such single factors as: sodium chloride, hypertonicity, pH, or mechanical factors such as injection volume or needle misplacement. The large variability of mortality rates is indicated by the ranges observed (Table 1). This variability may account for the higher mortality

with 300 mM D-proline compared to 600 mM D-proline, each in 150 mM NaCl. The difference is however not statistically significant ($p>0.5$). The marked toxicity of the distilled water solutions of two unsaturated analogs of proline, namely 3,4-dehydro-DL-proline and L-baikian (4,5-dehydro-L-pipecolic acid), is noteworthy.

We have previously described chick behavioral convulsions and CNS seizures produced by amnesic doses of flurothyl [6]. Within 2 min after exposure to 1.7% v/v flurothyl, chicks underwent a period of seizure-like tonic extension, during which the frequency of neostriatal multiple-unit activity increased from two to four times the base-line activity. Opisthotonos was regularly produced, after which the EEG waned to low-amplitude slow waves, and often became nearly isoelectric before the first high-amplitude spikes occurred accompanying slow, clonic movements. Our electrophysiological records for chicks injected with 10 μl per hemisphere of 300 mM D-proline in 300 mM NaCl (N = 5) showed a postinjection decrease in neostriatal multiple-unit activity. Although slow wave activity increased and high frequency activity decreased in the EEG, slow wave amplitude typically increased. We did not observe any of the high-amplitude spikes reported for flurothy-induced convulsions [6].

It may be added that the inclusion of 600 mM NaCl in 300 mM L-proline had no effect upon its amnesic potency nor did it render 300 mM D-proline amnesic, despite the convulsive activity. These observations are consistent with the paucity of seizure spikes observed during the motor convulsions, since we have previously demonstrated the correlation between seizure spike production and amnesic potency [6].

In order to eliminate the possibility that our behavioral and EEG results were produced by pyrogenic contamination we submitted our D-proline and NaCl solutions to the limulus test, which detects gram-negative lipopolysaccharides at concentrations of 0.125 ng/ml. Our solutions were pyrogen-free.

In general, D-amino acids are less toxic than their L-enantiomers, based upon mortality following intraperitoneal injection in rats [5]. We have no explanation for the reverse relationship we have observed with intraventricular injection in chicks. In mouse brain, the distribution of D-proline was markedly different from that of L-proline, after intraperitoneal injection of the tritiated enantiomers; e.g., the D-proline labelled cerebral cortex but not cerebellum whereas the L-proline label was approximately equal in cortex and cerebellum [4]. If different distribution occurs also in the chick brain after intraventricular injection, our results might reflect differences in transport mechanisms. For example, it may be speculated that the injection of 10 μl of D-proline in distilled water into the ventricular fluid dilutes the Na^+ concentration there and in the extracellular fluid in the periventricular tissue. If D-proline is toxic, and if its transport across cell membranes is sodium-dependent, such dilution might lower the D-proline concentration at its sites of activity, with a consequent reduction in toxic effects.

In any event, our results demonstrate that normal saline and distilled water cannot always be considered as equivalent vehicles for intraventricular injections and that a vehicle low in electrolytes can lead to artifacts. We believe that chick-Ringer's solution or a mock CSF [3] would be preferable to distilled water as the injection vehicle for certain experiments in chicks, even at the expense of increased osmolarity. In other cases, such as our memory experiments [2,3], in which we believe membrane mechanisms to be most important, reduced penetrance of amino acids into cells may be desirable, in order to maintain a high extracellular concentration and to avoid possible confounding effects of elevated intracellular concentrations of the injected amino acids. For compounds with Na^+-dependent transport, a sodium-free vehicle such as distilled water provides this advantage.

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REFERENCES

1. Brady, R. O. and D. B. Tower. (Eds.) *The Neurochemistry of Nucleotides and Amino Acids.* New York: Wiley, 1969, p. 234.
2. Cherkin, A., M. J. Eckardt and L. K. Gerbrandt. Memory: proline induces retrograde amnesia in chicks. *Science* **193:** 242–244, 1976.
3. Cherkin, A. and A. Van Harreveld. L-Proline and related compounds: correlation of structure, amnesic potency and antispreading depression potency. *Brain Res.*, in press.
4. Dahnke, H. G. and G. Garweg. Das Verhalten Von D- und L-Prolin im Proteinstoffwechsel des Gross- und Kleinhirns der Maus. *Z. Physiol. Chem.* **353:** 1507, 1972.
5. Greenstein, J. P. and M. Winitz. *Chemistry of the Amino Acids,* Vol. 1. New York: Wiley, 1961, p. 370.
6. Herz, M. J., C. E. Spooner and A. Cherkin. Effects of the amnesic agent flurothyl on EEG and multiple-unit activity in the chick. *Expl Neurol.* **27:** 227–237, 1970.
7. Olney, J. W., V. Rhee and T. De Guvareff. Neurotoxic effects of glutamate on mouse area postrema. *Brain Res.* **120:** 151–157, 1977.
8. Smythies, J. R. Relationships between the chemical structure and biological activity of convulsants. *Pharmac. Rev.* **14:** 9–20, 1974.