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The Neurotoxicity of β-*N*-Oxalyl-L-αβ-diaminopropionic Acid, the Neurotoxin from the Pulse *Lathyrus sativus*

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Intraperitoneal administration of β-*N*-oxalyl-L-αβ-diaminopropionic acid, the neurotoxin from *Lathyrus sativus*, to 12-day-old rats causes typical convulsions within 10min. There is a striking accumulation of glutamine in the brain, and chronic ammonia toxicity is indicated. There are no changes in the amounts of urea, aspartic acid and glutamic acid in the brain. Adult rats, even when injected with a dose of excess of β-*N*-oxalyl-L-αβ-diaminopropionic acid, do not develop symptoms, and there are no changes in the amounts of glutamine or ammonia in the brain. A significant concentration of β-*N*-oxalyl-L-αβ-diaminopropionic acid can be detected in the brain of the young rat but not in that of the adult animal. It is concluded that β-*N*-oxalyl-L-αβ-diaminopropionic acid interferes with the ammonia-generating or -fixing mechanisms in the brain and leads to chronic ammonia toxicity.

The consumption of the seeds of *Lathyrus sativus* has been known to cause the disease neurolathyrism in man (Selye, 1957). A neurotoxin, ODAP,* has been isolated from the seeds of *L. sativus* (Rao, Adiga & Sarma, 1964; Murti, Seshadri & Venkitasubramanian, 1964). ODAP induces typical neurological symptoms in young chicks and rats when administered intraperitoneally (Adiga, Rao & Sarma, 1963; Rao & Sarma, 1967). Permanent paralysis of the hind legs is produced in the adult monkey by the administration of this compound intrathecally through the lumbar route (Rao, Sarma, Mani, Raghunatha Rao & Sriramachari, 1967). However, no information is as yet available on the biochemical mechanism of the action of ODAP.

O'Neal, Chen, Reynolds, Meghal & Koeppe (1968) reported that the neurolathyrogen L-αγ-diaminobutyric acid, a higher homologue of the diamino acid moiety of ODAP and a constituent of certain *Lathyrus* and related seeds, causes chronic ammonia toxicity in rats. However, L-αγ-diaminobutyric acid is not present in *L. sativus* seeds and is probably not the neurotoxic agent of human lathyrism (*Nutrition Reviews*, 1963). In the present investigation, the possibility of ammonia toxicity in young rats given ODAP was studied. The mechanism of action of ODAP is discussed in relation to that reported for L-αγ-diaminobutyric acid (O'Neal *et al.* 1968).

* Abbreviation: ODAP, β-*N*-oxalyl-L-αβ-diaminopropionic acid.

MATERIALS AND METHODS

Animals. Young (12-day-old) albino rats weighing 15–20g. were used for the experiments. Adult rats used in some experiments weighed 150–200g. In the young rats ODAP was administered intraperitoneally in 0·2ml. of water. In the adult rats ODAP was given intraperitoneally in 1·0ml. of water. Control rats were given 0·9% NaCl in the same manner. At the termination of experiments, the animals were decapitated and the desired tissues were collected.

Processing of tissues. The brain and liver tissues were quickly washed in cold 0·9% NaCl and blotted with a filter paper, and the fresh weights were recorded. Normally samples from three young rats were pooled and homogenized with 12% (w/v) trichloroacetic acid (5ml./g. of tissue). All operations were carried out at 0°. The trichloroacetic acid homogenates were centrifuged at 15000*g* for 20min. and the clear supernatants were used for the determination of ammonia, glutamine and urea, essentially by the procedures described by Hathway & Mallinson (1964).

Microdiffusion of ammonia. Ammonia was measured by the Conway technique. Portions (1ml.) of the supernatant were transferred to Conway units containing 1ml. of 0·02N-HCl in the central chamber. The ammonia was liberated by the addition of 1ml. of satd. K_2CO_3 solution. Diffusion was allowed to proceed for 2hr. Allowance was made for the hydrolysis of glutamine under these conditions.

Determination of glutamine. The trichloroacetic acid supernatant was heated at 70° for 75min. and portions (1ml.) were transferred to Conway units for ammonia measurement. The free ammonia values were subtracted from these values, which were then taken to represent the ammonia liberated from glutamine. In some experiments the glutamic acid obtained from glutamine under these

conditions was also determined along with the liberated ammonia. Measurements of glutamine concentration based on the determination of glutamic acid and ammonia showed a good agreement.

Determination of urea. A known volume of the trichloroacetic acid supernatant was neutralized with 5N-NaOH and then diluted to twice its volume with 0·1M-sodium phosphate buffer, pH 7·0. Portions (1ml.) were transferred to Conway units and incubated with 0·2ml. of a 1% solution of urease in aq. 30% (v/v) ethanol for 45min. before microdiffusion of ammonia. An allowance was made for free ammonia and the rate of hydrolysis of glutamine.

Determination of ammonia. A portion (0·5ml.) of 0·02N-HCl from the central chamber of the Conway unit was taken and ammonia was measured by the phenol–hypochlorite method (Russel, 1944).

Amino acid analysis. A suitable volume of the trichloroacetic acid supernatant was extracted with ether repeatedly and a known volume of the aqueous layer was concentrated to dryness in vacuum. The residue was dissolved in a known volume of water and portions were spotted on Whatman no. 1 paper for two-dimensional chromatography with butan-1-ol–acetic acid–water (15:3:7, by vol.) as solvent for the first run and phenol–ethanol–water (3:1:1, by vol.) containing 8-hydroxyquinoline for the second run in an ammonia atmosphere. The papers were sprayed with 0·5% (w/v) ninhydrin in acetone. The pink spots corresponding to aspartic acid and glutamic acid were eluted in 4ml. of aq. 75% (v/v) ethanol containing 0·2mg. of $CuSO_4$ and the colour was measured in a Klett photoelectric colorimeter with no. 54 filter (Giri, Radhakrishnan & Vaidyanathan, 1952).

Determination of ODAP in tissues. A portion of the sample prepared for amino acid analysis was passed through a column (1·0cm. × 10cm.) of Dowex 50 (H^+ form; 100–200 mesh). The column was eluted with water and, after rejection of the first 20ml. of the effluent, another 100ml. of the water effluent was collected and evaporated to dryness in a flash evaporator at 40°. The residue was dissolved in a known volume of water, and portions were spotted on strips of Whatman no. 1 paper and subjected to electrophoresis at pH 3·6 with pyridine–acetic acid–water (1:10:190, by vol.) for 2hr. at 800v. ODAP was the fastest-moving acidic amino acid in these strips and was determined quantitatively by using ninhydrin spray as described above for amino acid measurements.

Materials. ODAP was isolated from *L. sativus* seed meal by extraction with aq. 75% (v/v) ethanol followed by the column-chromatographic procedure described by Rao *et al.* (1964). The final preparation was subjected to column treatment once again and recrystallized repeatedly from water–acetone. The final preparation did not contain more than 0·5% of the α-isomer.

Urease type IV (2090units/g.) was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.

RESULTS

Administration of ODAP (1·4m-moles/kg. body wt.) to 12-day-old young rats by the intraperitoneal route caused typical convulsions and tremors within 10min. The animals died between 3 and 5hr. after the administration of the neurotoxin,

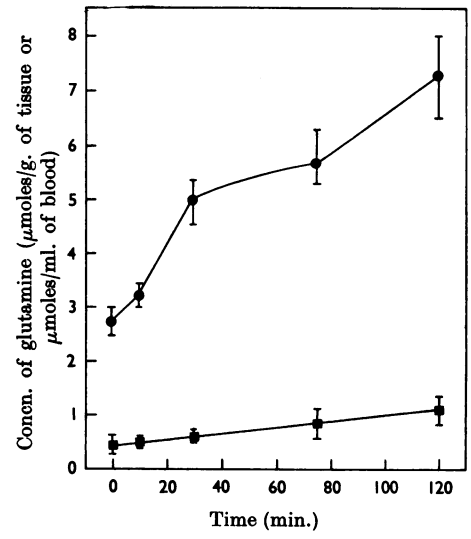

Fig. 1. Effect of ODAP on glutamine concentrations in blood and brain of young rats. Young (12-day-old) rats (15–20g.) were injected intraperitoneally with ODAP (1·4m-moles/kg. body wt.). The animals were killed at various times and the tissues were analysed for glutamine content as described in the Materials and Methods section. The range of values indicated for each point were obtained from five independent experiments. ■, Concn. of glutamine in blood (μmoles/ml.); ●, concn. in brain (μmoles/g.).

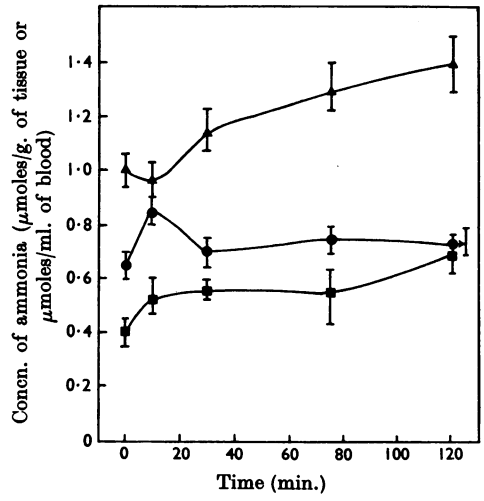

Fig. 2. Effect of ODAP on free ammonia concentrations in blood, liver and brain of young rats. The details are as described in Fig. 1. The range of values indicated for each point was obtained from five independent experiments. ■, Concn. of ammonia in blood (μmoles/ml.); ▲, concn. in liver (μmoles/g.); ●, concn. in brain (μmoles/g.).

Table 1. *Effect of ODAP on urea concentrations in blood, liver and brain of young rats*

Young (12-day-old) rats (15–20g.) were injected intraperitoneally with ODAP (1·4m-moles/kg. body wt.). The animals were killed at 30min. or 2hr. after ODAP administration and the tissues were analysed for urea content as described in the Materials and Methods section.

	Concn. of urea in blood (μmoles/ml.)		Concn. of urea in liver (μmoles/g.)		Concn. of urea in brain (μmoles/g.)	
Treatment Time	30min.	2hr.	30min.	2hr.	30min.	2hr.
0·9% NaCl (control)	3·1	4·2	5·4	5·2	2·3	2·8
	2·9	2·8	4·7	5·5	2·6	2·5
ODAP	3·5	3·0	5·5	5·2	1·9	2·9
	2·8	3·6	4·8	4·9	2·2	2·2

Table 2. *Effect of ODAP on aspartic acid and glutamic acid concentrations in liver and brain of young rats*

Young (12-day-old) rats (15–20g.) were injected intraperitoneally with ODAP (1·4m-moles/kg. body wt.). The animals were killed 2hr. after ODAP administration and the tissues were analysed for the amino acids as described in the Materials and Methods section.

	Concn. in liver (μmoles/g.)		Concn. in brain (μmoles/g.)	
Treatment	Aspartic acid	Glutamic acid	Aspartic acid	Glutamic acid
0·9% NaCl (control)	1·0	2·3	1·8	4·8
	1·1	2·7	2·5	4·2
ODAP	1·7	2·4	1·7	4·3
	1·2	2·5	2·2	3·7

depending on the severity of the convulsions produced. Brain glutamine concentration showed a striking increase under these conditions (Fig. 1). This increase was evident as early as 10min. after ODAP administration, with a progressive increase with time. The blood glutamine concentration also showed a steady but small increase.

Changes in free ammonia concentration with time in ODAP-treated animals are shown in Fig. 2. In the blood and liver there was a steady increase in ammonia concentration with time. In the brain, just at the time of the onset of convulsions there was a small but significant increase in free ammonia concentration; this levelled off subsequently. The urea concentration, as well as the concentrations of aspartic acid and glutamic acid, did not show any change (Tables 1 and 2).

Rao & Sarma (1967) reported that intraperitoneal administration of ODAP to adult rats, even in excess calculated on the basis of body weight, fails to cause any neurotoxic effects, and a possible blood–brain barrier to this compound in the adult animal was envisaged. In the present investigation, adult rats were injected with ODAP intraperitoneally at a concentration of 5m-moles/kg. body wt. The animals were killed at different times, and the tissues were analysed for ammonia, urea and amino acids. The results given in Table 3 reveal that the concentrations of none of the constituents changed to any appreciable extent in the tissues examined when the animals were killed 2hr. after the administration of ODAP. A similar pattern of results was obtained when the rats were killed and examined 4 and 12hr. after ODAP administration.

It appeared possible that ODAP has to enter the brain to cause neurotoxic effects, and hence an analysis was made for ODAP distribution in young and adult rats. Table 4 shows that the adult rats, even when injected with doses of excess of ODAP, contained only trace quantities of the neurotoxin in the brain. This could well be due to contamination from blood in the capillaries. However, the young rats showed a significant ODAP concentration in the brain at the time of onset of convulsions. Nearly 50% of the administered ODAP could be recovered in the urine of the adult rats within 12hr., with a substantial fall in the liver and blood concentrations.

DISCUSSION

The striking increase in the brain glutamine concentration in the ODAP-treated young animals is a clear indication of ammonia toxicity, glutamine formation being a major mechanism for the detoxification of ammonia in the brain (Berl, Takagaki, Clarke & Waelsch, 1962). At the time of onset of convulsions, a significant increase in the free ammonia concentration is evident, with a subsequent fall to normal values. This fall could be explained as being due to efficient glutamine formation. For example, Hathway & Mallinson (1964) found that in convulsions induced by Telodrin

Table 3. *Effect of ODAP on ammonia, glutamine, urea and amino acid concentrations in blood, liver and brain of adult rats*

ODAP (5·0m-moles/kg. body wt.) was injected intraperitoneally into adult rats (150–200g.). The animals were killed 2hr. after ODAP administration and the tissues were analysed for the various constituents as described in the Materials and Methods section.

		Concn. in blood (μmoles/ml.)		Concn. in liver (μmoles/g.)		Concn. in brain (μmoles/g.)	
Compound	Treatment ...	0·9% NaCl (control)	ODAP	0·9% NaCl (control)	ODAP	0·9% NaCl (control)	ODAP
Glutamine		0·53, 0·45	0·33, 0·48	—	—	3·7, 4·2	3·9, 3·4
Ammonia		0·17, 0·17	0·19, 0·23	1·6, 2·4	1·8, 2·1	1·3, 1·2	1·4, 1·5
Urea		4·2	3·8	6·8	7·2	3·2	4·0
Aspartic acid		—	—	4·0	4·4	3·9	4·2
Glutamic acid		—	—	1·5	1·4	9·3	10·0

Table 4. *Distribution of ODAP in blood, liver and brain of young and adult rats*

ODAP was injected intraperitoneally (1·4m-moles/kg. body wt.) into young rats (15–20g.) in 0·2ml. of water. Adult rats (190–200g.) received in the same manner ODAP (5m-moles/kg. body wt.) in 1·0ml. of water. The young rats were killed at 7min. and 1hr. and the adult rats at 2 and 12hr. after ODAP administration and tissues were analysed for ODAP as described in the Materials and Methods section.

Group	Time of killing	Concn. of ODAP in blood (μmoles/ml.)	Concn. of ODAP in liver (μmoles/g.)	Concn. of ODAP in brain (μmole/g.)
Young rats	7min.	0·59	0·30	0·11
	1hr.	0·60	0·28	0·08
Adult rats	2hr.	0·56	1·59	Trace
	12hr.	Trace	0·14	Nil

(1,3,4,5,6,7,8,8-octachloro-1,3,3*a*,4,7,7*a*-hexahydro-4,7-methanoisobenzofuran) in the rat there is a striking increase in brain glutamine concentration with actually a slight fall in the free ammonia concentration. However, the ammonia concentration shows an increase at later stages when the brain glutamine reaches saturation concentrations. In the present investigation, the young rats die 3–5hr. after ODAP administration and glutamine concentrations continue to increase.

O'Neal *et al.* (1968) found that the neurolathyrogen L-αγ-diaminobutyric acid at a dose of 4·4m-moles/kg. body wt. in adult rats causes a chronic ammonia toxicity. The animals show neurotoxic effects in 12–20hr. followed by death in 3–8 days. O'Neal *et al.* (1968) implicated primary liver damage followed by a secondary brain lesion. They showed that αγ-diaminobutyric acid inhibits ornithine transcarbamoylase in the liver, which explained the lowered urea synthesis in liver slices. However, animals injected with αγ-diaminobutyric acid actually show an increased tissue urea concentration, which has been explained as being due to dehydration from increased urinary output in the treated animals. The neurotoxic effects of ODAP exhibit several striking features that are quite different from those of αγ-diaminobutyric acid. ODAP at a dose of 1·4m-moles/kg. body wt. in the young rats causes convulsions within 10min. and death in 3–5hr. The urea concentrations do not show any change. The entry of ODAP into the brain appears to be essential for the neurotoxicity. The adult rat, which does not develop symptoms after intraperitoneal ODAP administration, has no detectable ODAP in the brain. These animals do not show any change in the brain glutamine or free ammonia concentrations. In support of their contention O'Neal *et al.* (1968) found that the rat is more susceptible to L-αγ-diaminobutyric acid administration than the chick. The uricotelic animal requires a higher concentration of L-αγ-diaminobutyric acid, and the symptoms observed are different. However, ODAP is equally effective in both young rats and chicks. The symptoms observed as well as the dosage requirements are similar (Adiga *et al.* 1963; Rao & Sarma, 1967). All these considerations indicate that the brain is the primary target for ODAP action.

At the same time, the picture obtained with ODAP-induced convulsions is unlike that observed with the administration of toxic doses of L-amino acids or ammonium acetate. O'Neal *et al.* (1968) observed that in these cases acute ammonia toxicity is manifested owing to a large build-up of ammonia

in blood and other tissues, ammonia being released from the injected amino acid in quantities greater than the detoxification capacity of the liver (Greenstein & Winitz, 1961). In the present investigation, the young rats showed a significant but not a high increase in free ammonia concentration in the blood and brain at the time of the onset of convulsions. Although ODAP induces convulsions and brings about changes in ammonia and glutamine concentrations in a short time, the quantitative differences are about the same as those observed for L-αγ-diaminobutyric acid. It appears that ODAP, like L-αγ-diaminobutyric acid, induces a chronic ammonia toxicity, though by a different mechanism. The delay in the onset of convulsions due to L-αγ-diaminobutyric acid administration may be due to the fact that the primary site for the build-up of ammonia is the liver. Adult rats when injected with excess of ODAP do not show any increase in tissue ammonia concentrations. These results indicate that ammonia is possibly not produced as a result of the catabolism of ODAP. The toxicity of ODAP in young rats is more likely to be due to an interference with ammonia-generating or -fixing mechanisms in the brain.

It is known that the other unusual amino acid, L-homoarginine, present in *L. sativus* does not possess neurotoxic effects (Rao, Ramachandran & Adiga, 1963; Rao *et al.* 1964). This was confirmed by O'Neal *et al.* (1968), who found that its toxicity characteristics are similar to those of other natural amino acids.

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