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The 'Neurotoxicity' of L-2,4-Diaminobutyric Acid

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The neurolathyrogen L-2,4-diaminobutyric acid is concentrated by liver, and liver damage can yield neurotoxicity; thus the neurotoxicity caused by this compound may be due to liver damage followed by secondary brain damage. 1. The intraperitoneal administration of toxic doses of L-2,4-diaminobutyric acid to rats resulted in hyperirritability, tremors and convulsions in 12–20hr. and increased the concentration of ammonia of blood and brain slightly and the concentration of glutamine of brain two- to three-fold. By contrast, toxic doses of L-homoarginine, L-lysine, L-leucine and ammonium acetate caused dyspnoea, extreme prostration, and in some cases coma in 15–30min., and increased the concentration of ammonia of blood significantly and the concentration of glutamine of brain slightly. These results indicate that L-2,4-diaminobutyric acid caused a chronic ammonia toxicity, whereas the other amino acids and ammonium acetate resulted in an acute ammonia toxicity. 2. Liver slices from L-2,4-diaminobutyric acid-treated animals and normal liver slices preincubated with L-2,4-diaminobutyric acid utilized ammonia and formed urea at a lower rate than control slices from normal rats. 3. L-2,4-Diaminobutyric acid inhibited competitively ornithine carbamoyltransferase of rat liver homogenates, thus demonstrating that this reaction is a primary site of toxicity for this neurolathyrogen. Although we were unable to show marked elevations of blood ammonia concentration after treatment with L-2,4-diaminobutyric acid, these results are interpreted to mean that ammonia utilization (urea synthesis) in liver is inhibited by L-2,4-diaminobutyric acid and that at least part of the neurotoxicity is due to a prolonged slight increase in body ammonia concentration.

The L-isomer of DBA* (L-DBA), a major component of the polymixin group of antibiotics (Rebstock, 1960), a component of bacterial cell walls (Perkins & Cummins, 1964) and of certain *Lathyrus* and related seeds (Ressler, Redstone & Erenberg, 1961; Bell, 1962*a*; Van Etten & Miller, 1963; *Nutrition Reviews*, 1963; Bell & Tirimanna, 1965), appears to be neurotoxic in rats and mice, although it may not be the neurotoxic agent of human lathyrism (*Nutrition Reviews*, 1963; Riggs, Coyne & Christensen, 1954; Kessel, 1959). Mouse and rat liver (Kessel, 1959; Christensen, Riggs, Fischer & Palatine, 1952; Mushahwar & Koeppe, 1963) and ascites-tumour cells (Christensen *et al.* 1952; Christensen & Riggs, 1956; Pal & Christensen, 1959) concentrate DBA strongly. This ability to concentrate DBA results in a severe loss of K^+ ions by both liver and ascites-tumour cells. Studies with labelled DBA (Mushahwar & Koeppe, 1963) have shown that rats can oxidize both isomers to carbon dioxide and that β-alanine is a major catabolite.

* Abbreviation: DBA, 2,4-diaminobutyric acid.

The increasing evidence that suggests a wide distribution of DBA in Nature (Rebstock, 1960; Perkins & Cummins, 1964; Ressler *et al.* 1961; Bell, 1962*a*; Van Etten & Miller, 1963; *Nutrition Reviews*, 1963; Bell & Tirimanna, 1965) has encouraged us to study the mechanism of its toxicity. Because the toxic symptoms after administration of lethal doses of L-DBA appear only after 12–20hr. and because liver is known to concentrate DBA with a consequent loss of K^+ (Christensen *et al.* 1952), the neurotoxicity might have been due to liver damage followed by secondary brain damage. Severe liver damage could result in hypoglycaemia or in increased blood and brain ammonia concentration; either of these conditions would be neurotoxic. An elevated blood and brain ammonia concentration would be expected to result in an increase in brain glutamine concentration (Flock, Block, Grindlay, Mann & Bollman, 1953; DuRuisseau, Greenstein, Winitz & Birnbaum, 1957; Clark & Eiseman, 1958; Berl, Takagaki, Clarke & Waelsch, 1962). Because the administration of toxic doses of L-DBA did not

markedly lower blood sugar concentration, hepatic formation of urea might have been inhibited, causing an elevation in blood and brain ammonia concentration and hence neurotoxicity. To test this hypothesis the syndrome of L-DBA toxicity and the effect of L-DBA on concentrations of glutamate, aspartate, glutamine and ammonia in brain and of ammonia in blood were compared with the effects of other amino acids and ammonia. The effects of L-DBA on the utilization of ammonia and synthesis of urea by liver slices and the activity of liver ornithine carbamoyltransferase are also reported.

A preliminary communication has been published (Koeppe, O'Neal, Chen & Meghal, 1966).

MATERIALS AND METHODS

Animals. Male albino rats, obtained from the Holtzman Rat Co., Madison, Wis., U.S.A., were given a commercial 25% protein rat diet and water *ad libitum* before the experiments. Leghorn cockerels, obtained from the Stillwater Hatchery, Stillwater, Okla., U.S.A., were raised on a commercial chick starter ration. Rats weighing 165–315g. and chicks weighing 190–250g. after a 24hr. period without food were utilized for all experiments. After the intraperitoneal administration of the test compounds dissolved in 5ml. of water, or an equal volume of water (controls), the animals were allowed to drink water *ad libitum* for the duration of the experiment; however, in three experiments urine was collected over experimental periods during which no water was provided for the rats. At the termination of experiments, the animals were decapitated and the desired tissues were collected.

Isolation and assay of amino acids. Tissues to be analysed for amino acids were blotted with filter paper and frozen in liquid N_2 immediately after dissection. Glutamate, aspartate and glutamate from glutamine were isolated from the frozen tissue by the method described by O'Neal & Koeppe (1966). Dowex 1 (X8; acetate form; 100–200 mesh) columns (1·0cm. × 12cm.) were used. Aspartate and glutamate were quantitatively determined by the ninhydrin method of Rosen (1957) as modified by Grant (1963).

The basic free amino acids of brain, liver and kidney were isolated from perchlorate filtrates (O'Neal & Koeppe, 1966) of these tissues on columns (1·0cm. × 12cm.) of Amberlite CG-50 (Na^+ form; 100–200 mesh). Five volumes of 1·0M-acetate buffer, pH4·7, per volume of resin were allowed to flow through the column at 0·5ml./min. When the pH of the effluent equalled that of the influent buffer, the column was rinsed free of excess of buffer with water. A portion of the neutral perchlorate filtrate was diluted with water to 25ml. and passed over the buffered Amberlite CG-50 column at approx. 0·5ml./min. The column was washed with water until the acidic and neutral amino acids had passed through. The basic amino acids were then eluted with 1·0N-HCl. To remove ammonia from the eluates, the pH was adjusted to 11·5 with 2·0N-KOH and the solution was placed overnight in an evacuated desiccator containing 200ml. of conc. H_2SO_4 (Moore & Stein, 1954). The solution was then neutralized with 1·0N-HCl and the basic amino acids were quantitatively determined by the method (Rosen, 1957; Grant, 1963) used for the acidic amino acids, with L-DBA hydrochloride as the standard. A portion of the isolated basic amino acid solution was desalted by passing it over a Dowex 1 (X8; OH^- form; 100–200 mesh) column (1·0cm. × 12cm.) at a rate of about 20ml./hr. The column was washed slowly with 100ml. of water and eluted with 0·5N-acetic acid, and the acetic acid was removed by evaporation to dryness. The free basic amino acids were dissolved in water and assayed by ascending chromatography on Whatman no. 1 filter paper with 2-methylpropan-2-ol–butan-2-one–water–aq. NH_3 (sp.gr. 0·88) (4:3:2:1, by vol.) solvent (Fink, Cline & Fink, 1963).

Tissue K^+ *and* Na^+ *analysis.* Liver, brain and kidney tissues obtained from rats given L-DBA or controls were blotted, weighed and ashed at 550° in a muffle furnace. The residue was dissolved in 3·6N-HCl and analysed for K^+ and Na^+ with an atomic absorption spectrophotometer (Perkin–Elmer model 303) (*Analytical Methods for Atomic Absorption Spectrophotometry*, 1964).

Determination of tissue ammonia and urea. Protein-free filtrates for ammonia analysis were prepared by precipitation with 20% (blood) or 10% (brain) (w/v) trichloroacetic acid (Nathan & Rodkey, 1957; Tews, Carter, Roa & Stone, 1963). For determination of ammonia the microdiffusion method described by Seligson & Seligson (1951) was combined with the ninhydrin colorimetry of Rosen (1957) and Grant (1963). It was necessary to diffuse the samples for at least 3hr. to get quantitative recovery from prepared ammonia standards. Protein-free filtrates of blood, brain and liver were prepared as described by Somogyi (1945). Portions of these filtrates and samples of urine preserved with toluene were analysed for urea by the method of Archibald (1945).

Histopathological examination of tissue. Rats were given 4·4m-moles of L-DBA/kg. body wt., or 5ml. of water, intraperitoneally. Then 30hr. later they were decapitated and the brain, liver and kidney were dissected, blotted, cut into 5–10mm. pieces and fixed in 10% (w/v) formalin. Sections 4–6μ thick were prepared and stained with haematoxylin–eosin for examination.

Incubation and analysis of liver slices. Liver slices (0·5mm. thick) were prepared with a microtome (Stadie & Riggs, 1944). Slices of approx. 0·5g. wet wt. were incubated for 30min. at 37° in 25ml. Erlenmeyer flasks containing 4ml. of Krebs–Ringer bicarbonate buffer, pH7·4 (DeLuca & Cohen, 1964). In some of the experiments liver slices were incubated with L-DBA, L-ornithine hydrochloride or L-homoarginine hydrochloride during the 30min. before the addition of the NH_4Cl. Then 1ml. of 25mM-NH_4Cl was added and the incubation continued for 1hr. (Greenstein, Winitz, Gullino, Birnbaum & Otey, 1956). During the entire experiment, the solutions were gassed with O_2+CO_2 (95:5). The addition of 0·3ml. of 3·0% (w/v) sodium lactate, pH7·3, to the incubation medium (Gornall & Hunter, 1943), which enhanced the utilization of ammonia and production of urea by the slices, was used in two of the later experiments. The experiments were terminated by addition of 1ml. of 0·6N-$HClO_4$. Control flasks were prepared similarly except that NH_4Cl was added after $HClO_4$. The mixtures were centrifuged at 15000*g* for 15min. and the clear supernatants were decanted and analysed for ammonia and urea. Ammonia was allowed to diffuse for at least 3hr. (Seligson & Seligson, 1951) and quantitative determination was obtained by nesslerization (Boutwell, 1961). The iodinated Nessler's solution was

prepared by dissolving 30g. of KI, 41g. of HgI_2 and 2g. of I_2 in water and diluting to 200ml. with water. Urea was determined by the method of Archibald (1945).

Ornithine carbamoyltransferase (EC 2.1.3.3) assay. Rats starved for 24hr. were killed by decapitation and the liver was rapidly removed and weighed. A 1g. sample of liver was passed through a cold Harvard tissue press and homogenized in 19ml. of ice-cold water (Schimke, 1962). The enzymic activity in the water homogenate was determined by measuring the rate of citrulline formation, by using the assay system of Brown & Cohen (1959). Clear supernatant solutions were analysed for citrulline by the method of Archibald (1944) as modified by Ratner (1955). Citrulline standards were added to the incubation media and were included with each set of assays. It was established that, with the quantities of homogenate used, the rate of the reaction studied was directly proportional to protein concentration and linear with time.

Materials. L-2,4-Diaminobutyric acid hydrochloride (A grade), L-homoarginine hydrochloride (A grade), L-ornithine hydrochloride (A grade), glycylglycine (A grade) and dilithium carbamoyl phosphate (B grade) were obtained from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.

RESULTS

Effect of L-DBA on blood sugar. At 16 or 26hr. after the intraperitoneal injection of 4·4m-moles of L-DBA/kg. body wt. (Ressler *et al.* 1961) to starved rats, the blood glucose concentrations as determined by the Nelson–Somogyi method (Hawk, Oser & Summerson, 1954) were comparable with those in control rats.

Effect of amino acids and ammonia on free amino acids of brain and ammonia of brain and blood. A short time (15–30min.) after the administration of $LD_{99.9}$ (Greenstein & Winitz, 1961) doses of L-leucine or L-lysine hydrochloride (64·0 and 36·0m-moles/kg. body wt. respectively) or 20m-moles of L-homoarginine hydrochloride/kg. body wt. to rats, or 22·5m-moles of ammonium acetate/kg. body wt. to chicks, the animals developed the typical syndrome of amino acid or ammonia toxicity described by Greenstein & Winitz (1961): dyspnoea, extreme prostration and in some cases coma. The administration of smaller doses of ammonium

Table 1. *Effect of amino acids on the concentration of free glutamate, aspartate and glutamine of brain and ammonia of blood of rats*

A solution of an amino acid or an equal volume of water was administered to each rat by intraperitoneal injection. At the end of the experiments the brain and blood were collected, and the free amino acids and ammonia were isolated and measured as described in the Materials and Methods section.

Compound	Dose (m-moles/kg. body wt.)	Length of amino acid treatment (hr.)	Brain			Blood ammonia (μmoles/ml.)
			Glutamate (μmoles/g.)	Aspartate (μmoles/g.)	Glutamine (μmoles/g.)	
Water		30·0	9·0	2·0	4·3	
Water		30·0	8·2	2·2	4·1	
Water		30·0	8·4	1·6	4·0	
L-DBA	4·4	30·0	8·9	2·2	10·7	
L-DBA	4·4	30·0	9·8	2·1	8·7	
L-DBA	4·4	30·0	8·8	1·9	9·0	
L-DBA	4·4	30·0	9·9	1·8	9·2	
L-DBA	4·4	25·0	10·0	1·5	7·2	
L-DBA	4·4	20·0	9·5	2·2	6·4	
L-DBA	4·4	20·0	9·7	2·4	6·7	
Water		1·0	8·1	1·5	3·0	0·12
Water		1·0	8·1	2·2	3·6	0·12
Water		1·0	9·0	1·8	4·3	0·20
L-Homoarginine hydrochloride	20·0	0·25	8·9	2·3	5·4	0·28
L-Homoarginine hydrochloride	20·0	0·25	10·2	2·0	5·5	1·72
L-Lysine hydrochloride	36·0	0·5	9·2	2·1	5·7	0·45
L-Lysine hydrochloride	36·0	0·5	8·9	2·0	3·7	0·45
L-Lysine hydrochloride	36·0	0·5	9·6	2·3	5·4	0·71
L-Leucine	64·0	0·33	8·2	2·2	10·2	0·72
L-Leucine	64·0	0·33	10·3	2·1	5·4	0·65

Table 2. *Effect of* L-*DBA and ammonia on the concentration of free glutamate, aspartate and glutamine of brain of chicks*

A solution of L-DBA or ammonium acetate or an equal volume of water was administered to each chick by intraperitoneal injection. At the end of the experiments the brain was removed, and the free amino acids were isolated and measured as described in the Materials and Methods section.

Compound	Dose (m-moles/kg. body wt.)	Length of L-DBA or ammonia treatment (hr.)	Glutamate (μmoles/g.)	Aspartate (μmoles/g.)	Glutamine (μmoles/g.)
Water		0·25	8·5	2·3	5·3
Ammonium acetate	22·5	0·25	7·6	1·5	7·2
Ammonium acetate	22·5	0·25	7·1	1·5	5·5
Water		26·0	12·7	3·0	6·2
Water		26·0	12·3	1·9	6·0
L-DBA	12·9	26·0	10·5	2·1	8·4
L-DBA	12·9	26·0	7·6	2·1	7·9

acetate (7·0 or 12·0 m-moles/kg. body wt.) to chicks resulted in no such symptoms of toxicity or inhibition of growth. Giving 5 m-moles of homoarginine/kg. body wt. caused no noticeable symptoms of toxicity in rats, but administration of 10 m-moles/kg. body wt. resulted in hypersensitivity and death after 15–20 hr.

With the exception of glutamine, the brain amino acid concentrations of the rats and chicks mentioned above were comparable with those in control animals (Tables 1 and 2). The brain glutamine concentrations of these animals were higher than those of controls, the increase being comparable with the increase in free glutamine of rat brain after treatment with a $LD_{99.9}$ dose of ammonium acetate (O'Neal & Koeppe, 1966). Blood ammonia concentration of rats increased after treatment with the above-mentioned amino acids, in agreement with the observations of Greenstein & Winitz (1961).

Intraperitoneal injection of 4·4 m-moles of L-DBA/kg. body wt. into rats resulted in hyperirritability, tremors and convulsions in 12–20 hr. followed by death in 3–8 days. At 30 hr. after treatment the glutamine concentration of brain (Table 1) was significantly higher than in control animals ($P < 0·001$) (Steel & Torrie, 1960). Shorter periods of treatment with L-DBA also resulted in increased concentrations of brain glutamine, but the increases were smaller than those of 30 hr.-treated rats. At 30 hr. after treatment with L-DBA the average ammonia concentration in brain of four treated rats was higher than in that of ten control animals, means ± S.E.M. being $1·70 \pm 0·16$ and $1·32 \pm 0·10$ μmoles/g. respectively. This same treatment resulted in a higher average ammonia concentration in blood of five treated rats than in that of 12 control rats, $0·18 \pm 0·039$ and $0·11 \pm 0·018$ μmole/ml. respectively. Blood ammonia concentrations measured 2, 6, 12 and 20 hr. after L-DBA treatment were never observed to be any higher than the average value after 30 hr. of treatment.

The administration of 3·2 or 6·5 m-moles of L-DBA/kg. body wt. to chicks resulted in no symptoms of toxicity or decrease in growth rate. Although injection of 12·9 m-moles of L-DBA/kg. body wt. to chicks did not cause the hyperirritability, tremors and convulsions observed in rats, it did appear to inhibit growth slightly and resulted in increased concentrations of brain glutamine (Table 2).

Effect of L-*DBA on the free basic amino acids,* Na^+ *and* K^+ *of liver and brain.* After the injection of L-DBA into rats the free basic amino acid concentration of liver was higher than that found in controls. At 1 hr. after treatment of three rats the average concentration in liver was five times that of two controls, $34·0 \pm 4·15$ and $6·6 \pm 0·35$ μmoles/g. respectively. The difference between L-DBA-treated and control rats decreased with increasing time after treatment. However, 30 hr. after L-DBA treatment, the liver still contained a small amount of DBA. Although all the difference between the concentration of free amino acids in control and L-DBA-treated rat livers has not been conclusively proved to be due to DBA, paper chromatography of the desalted basic amino acids of L-DBA-treated rat liver demonstrated the presence of significant amounts of DBA. The intensity of the colour produced by developing the paper chromatograms with ninhydrin showed that samples containing much higher concentrations of the free basic amino acids than the controls contained much more DBA than lysine and arginine. However, samples containing only slightly larger amounts of free basic amino acids than the controls contained essentially equal amounts of DBA, lysine and arginine. The R_F values of L-DBA, lysine and arginine obtained with the paper-chromatography system mentioned

Table 3. *Inhibition of ammonia utilization and urea formation by rat liver slices with* L-*DBA*

Liver slices obtained from similar-sized rats 30 hr. after the intraperitoneal administration of L-DBA hydrochloride or 5 ml. of water (controls) were used for each of the experiments. Slices obtained from a rat 30 hr. after the intraperitoneal injection of 5 ml. of water were randomly allocated to incubation flasks and incubated for 30 min. with L-DBA hydrochloride or buffer (control) before the addition of the NH_4Cl in each of the experiments *in vitro*. The incubation and analytical procedures are described in the Materials and Methods section. The results are reported as mean values with the numbers of determinations in parentheses. The *P* values were obtained by analysis of variance (Steel & Torrie, 1960).

Type of expt.	Treatment	No. of rats used	Amount of L-DBA hydrochloride (m-moles/kg. body wt.)	Ammonia used (μmoles/g. wet wt./hr.)		Urea produced (μmoles/g. wet wt./hr.)	
in vivo	Control	9	—	17·2 (20)	$P<0·025$	5·5 (6)	$P<0·10$
in vivo	L-DBA	9	4·4	11·2 (20)		0·8 (6)	
			(mM)				
in vitro	Control	4	—	19·4 (8)	$P<0·025$	6·0 (6)	$P>0·10$
in vitro	L-DBA		14·0	8·4 (8)		1·7 (6)	
in vitro	Control	2	—	24·4 (4)	$P<0·10$	5·0 (4)	$P>0·10$
in vitro	L-DBA		6·5	15·2 (4)		1·7 (4)	

in the Materials and Methods section were 0·29, 0·23 and 0·18 respectively. This accumulation of DBA by liver agrees very well with results obtained by other workers (Christensen *et al.* 1952; Mushahwar & Koeppe, 1963).

The average concentration of free basic amino acids of brain 1 hr. after L-DBA treatment of three rats was only very slightly different from that of four control rats, $4·1 \pm 1·25$ and $3·5 \pm 0·63$ μmoles/g. respectively. However, 30 hr. after L-DBA treatment of three rats the average concentration of the free basic amino acids was $5·7 \pm 0·43$ μmoles/g. and paper chromatography showed the presence of DBA. Thus it appears that a slight amount of an intraperitoneal dose of L-DBA does enter the brain and accumulation increases with time. Slight penetration of L-DBA into rodent brain has been reported previously (Kessel, 1959; Vivanco, Ramos & Jimenez-Diaz, 1966).

The concentrations of Na^+ or K^+ in brain or liver of animals 1, 15 or 30 hr. after treatment with L-DBA were not consistently different enough from those found in the controls to indicate a definite effect due to L-DBA treatment. However, 1 and 15 hr. treatment with L-DBA lowered the concentration of K^+ in liver slightly.

Effect of L-*DBA on urea synthesis of liver slices.* The injection of L-DBA into rats 30 hr. before the preparation of liver slices resulted in inhibition of ammonia utilization and urea production by these slices (Table 3). This inhibition was eliminated in one experiment by making the incubation medium 5·65 mM in L-ornithine hydrochloride. In another series of experiments the presence of either 6·5 mM- or 14·0 mM-L-DBA in the incubation medium inhibited the utilization of ammonia and the production of urea by normal liver slices (Table 3), whereas the presence of 4·2 mM-L-homoarginine hydrochloride in the incubation media had no effect.

Effect of L-*DBA on tissue urea and urine excretion.* Analysis of the urea synthesized by liver slices revealed that liver tissue of L-DBA-treated rats contained considerably more urea than that from controls. Urea determinations of tissue extracts obtained from L-DBA-treated and control rats showed that the above observation was correct and that blood and brain tissue of L-DBA-treated rats also had higher concentrations of urea than corresponding tissues of control rats (Table 4). After treatment with 4·4 m-moles of L-DBA/kg. body wt. rats excreted 5–8 ml. of urine compared with 1·0–2·0 ml. for controls over a 30 hr. period during which no water was provided for either group. However, the total urea excretion was comparable in the two groups of animals.

Histopathologic examination of liver, kidney and brain 20 hr. after L-*DBA treatment.* This examination revealed changes in the brain tissue of one of the three L-DBA-treated animals examined. This tissue showed cellular loss in all parts of the hippocampus with some demyelinization. These lesions appeared similar to those produced by some neurotoxins or by relatively acute anoxia. No lesions were observed in any of the other tissues examined. Therefore the neurotoxic effect of L-DBA is not the result of gross structural damage to any of these tissues.

Competition between L-*DBA and ornithine by ornithine carbamoyltransferase of liver.* The production of citrulline by liver ornithine carbamoyltransferase was inhibited by approx. 35% by L-DBA when the molar ratio L-DBA/ornithine was 2:1. Neither 20 nor 30 μmoles of L-homoarginine in a reaction mixture containing 15 μmoles of ornithine gave appreciable inhibition of ornithine

Table 4. *Effect of* L-*DBA on the concentration of urea of liver, brain and blood*

At 30 hr. after the intraperitoneal injection of 4·4 m-moles of L-DBA/kg. body wt. or equal volumes of water into rats, these tissues were collected and analysed for urea as described in the Materials and Methods section.

Compound	Concn. of urea: Liver (μmoles/g.)	Brain (μmoles/g.)	Blood (μmoles/ml.)
Water	7·2	3·2	4·0
		5·5	8·5
L-DBA	10·8	11·1	14·0
		10·4	11·2

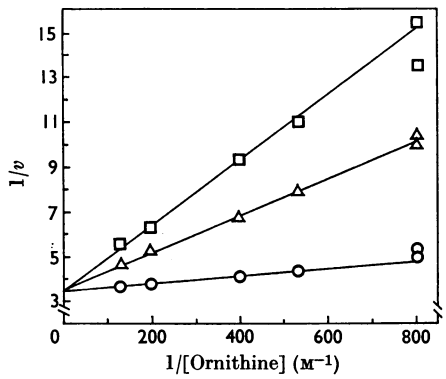

Fig. 1. Inhibition of ornithine carbamoyltransferase by L-DBA. The complete reaction mixture contained, in addition to L-DBA, pH 8·0, and L-ornithine, pH 8·0: 20 μmoles of dilithium carbamoyl phosphate, 90 μmoles of glycylglycine–NaOH buffer, pH 8·3, and 12·5 μl. of liver homogenate, in a final volume of 2·0 ml. After incubation for 15 min. at 37°, the product, citrulline, was assayed as described in the Materials and Methods section. The initial velocity, v, is expressed as μmoles of citrulline produced/min. ○, No L-DBA; △, 10 mM-L-DBA; □, 20 mM-L-DBA.

carbamoyltransferase. Fig. 1 shows Lineweaver–Burk plots of enzymic rate versus ornithine concentration in the presence of two concentrations of L-DBA. Increased L-DBA concentration resulted in an increased slope, with a common point of intersection on the ordinate. This suggests that L-DBA acts as a classical competitive inhibitor for ornithine and agrees with the studies of Herrmann, Lou & White (1966) with purified ornithine carbamoyltransferase obtained from *Neurospora crassa*.

DISCUSSION

In an effort to elucidate the mechanism of neurotoxicity of L-DBA, its physiological effects were compared with those of other amino acids and found to differ in several ways. The dose of L-DBA necessary to produce toxic effects was considerably lower than that for ammonia or for the other amino acids tested by us or by Greenstein & Winitz (1961). The symptoms of toxicity after L-DBA treatment were different and occurred more slowly than those resulting from lethal doses of other amino acids or ammonia. These observations suggest a different mechanism of toxicity for L-DBA than for the other amino acids or ammonia. The marked increase in concentration of glutamine in brain of L-DBA-treated rats indicates that the brain of these animals was detoxifying appreciable amounts of ammonia (Flock *et al.* 1953; DuRuisseau *et al.* 1957; Clark & Eiseman, 1958; Berl *et al.* 1962). The slight increase in concentration of ammonia in blood of L-DBA-treated rats relative to that of rats treated with other amino acids or ammonia shows that the L-DBA caused only a chronic ammonia toxicity whereas the other amino acids or ammonia resulted in an acute ammonia toxicity. This chronic ammonia toxicity could have been due to a low rate of release of ammonia from the relatively small dose of L-DBA combined with a rate of urea synthesis inhibited by the L-DBA in the liver. By contrast, the acute toxicity of the other amino acids is believed to result, at least in part, from such a rapid release of ammonia from the large doses of amino acid that the capacity of the liver to detoxify the ammonia by the synthesis of urea is exceeded, thus allowing the accumulation of large excesses of ammonia in the blood and other tissues (Greenstein & Winitz, 1961).

Rather large doses of homoarginine were required to demonstrate the toxicity of this compound and the symptoms of toxicity and effects on concentration of blood ammonia and brain glutamine were similar to those resulting from lethal doses of other amino acids or ammonia. Therefore although significant quantities of homoarginine have been found in seeds of *Lathyrus sativus*, *L. cicera* or *L. clymenum*, the species associated with neurolathyrism in man, horses and cattle (Bell, 1962*b*), the present findings suggest that it is not a primary lathyrogenic factor in these plants, but has toxic characteristics similar to those of other amino acids. No toxic effects were observed in rats given diets containing 1·5% homoarginine hydrochloride or injected for 20 days with smaller doses than those used in the present experiment (Stevens & Bush, 1950). Whereas homoarginine caused no neurotoxic effects when given to chicks, β-*N*-oxalyl-L-2,3-diaminopropionic acid isolated from the seeds of *L. sativus* resulted in neurological symptoms when given to young chicks (Rao, Adiga & Sarma, 1964).

To get some insight into the possibility that inhibition of the rate of urea synthesis by L-DBA

is a primary mechanism of toxicity of this compound, the toxic effects of L-DBA were examined in a uricotelic animal, the chick. The increased dosage required to cause symptoms of toxicity in the chick and the differences in the symptoms observed with the chick and the rat indicate a different mechanism of toxicity for L-DBA in uricotelic and ureotelic animals. The relatively high susceptibility of the ureotelic animal to L-DBA toxicity compared with that of the uricotelic animal is consistent with the postulate that inhibition of urea synthesis is a primary site of toxicity for L-DBA in rats.

Liver, the major site of urea synthesis, known (Kessel, 1959; Christensen *et al.* 1952; Mushahwar & Koeppe, 1963) and shown here to accumulate DBA, was examined to determine what effects L-DBA had on the utilization of ammonia and the production of urea *in vitro*. The results show that L-DBA administered *in vivo* or *in vitro* inhibited ammonia utilization and urea production by rat liver slices. Addition of L-ornithine to incubation media of liver slices prepared from livers of rats treated *in vivo* with L-DBA eliminated the inhibition of ammonia utilization and urea production. As is shown in Fig. 1, L-DBA competitively inhibited ornithine carbamoyltransferase of rat liver homogenates, thereby identifying a specific site of toxicity for L-DBA. Herrmann *et al.* (1966) made similar observations with purified ornithine carbamoyltransferase obtained from *Neurospora crassa*.

One observation that tends to detract from the above hypothesis is the fact that L-DBA-treated rats had higher amounts of tissue and blood urea than did control animals. If L-DBA inhibits urea synthesis the opposite is expected. One plausible explanation for these paradoxical results is the observation that L-DBA caused a diuresis that may have dehydrated the animals enough to increase urea concentration despite a decreased rate of synthesis. The observation that L-DBA-poisoned rats excreted five times the volume of urine but an equal amount of urea as did the controls supports this possibility.

Although most of our observations are consistent with the hypothesis that the neurotoxicity of L-DBA results from ammonia toxicity caused by inhibition of urea synthesis in the liver, L-DBA may exert its neurotoxicity in other ways. Small amounts of DBA are known to penetrate rat brain. The report (Vivanco *et al.* 1966) that thyroxine treatment decreases the penetration of DBA into rat brain and protects against the neurotoxicity that it causes suggests that the entry of DBA into brain is a necessity for some of its neurotoxicity. We have confirmed the observation that treatment with L-thyroxine prevents L-DBA toxicity. Because thyroxine-protected rats had normal concentrations of brain glutamine, we believe this protective action is not due entirely to the prevention of entry of L-DBA into brain.

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REFERENCES

Analytical Methods for Atomic Absorption Spectrophotometry (1964). Norwalk, Conn.: Perkin–Elmer Corp.
Archibald, R. M. (1944). *J. biol. Chem.* **156**, 121.
Archibald, R. M. (1945). *J. biol. Chem.* **157**, 507.
Bell, E. A. (1962*a*). *Nature, Lond.*, **193**, 1078.
Bell, E. A. (1962*b*). *Biochem. J.* **83**, 225.
Bell, E. A. & Tirimanna, A. S. L. (1965). *Biochem. J.* **97**, 104.
Berl, S., Takagaki, G., Clarke, D. D. & Waelsch, H. (1962) *J. biol. Chem.* **237**, 2562.
Boutwell, J. J., jun. (1961). *Clinical Chemistry Laboratory Manual and Methods*, p. 265. Philadelphia: Lea and Febiger.
Brown, G. W., jun. & Cohen, P. P. (1959). *J. biol. Chem.* **234**, 1769.
Christensen, H. N. & Riggs, T. R. (1956). *J. biol. Chem.* **220**, 265.
Christensen, H. N., Riggs, T. R., Fischer, H. & Palatine, I. M. (1952). *J. biol. Chem.* **198**, 17.
Clark, G. M. & Eiseman, B. (1958). *New Engl. J. Med.* **259**, 178.
DeLuca, H. F. & Cohen, P. P. (1964). In *Manometric Techniques*, 4th ed., p. 132. Ed. by Umbreit, W. W., Burris, R. H. & Stauffer, J. F. Minneapolis: Burgess Publishing Co.
DuRuisseau, J. P., Greenstein, J. P., Winitz, M. & Birnbaum, S. M. (1957). *Arch. Biochem. Biophys.* **69**, 161.
Fink, K., Cline, R. E. & Fink, R. M. (1963). *Analyt. Chem.* **35**, 389.
Flock, E. V., Block, M. A., Grindlay, J. H., Mann, F. C. & Bollman, J. L. (1953). *J. biol. Chem.* **200**, 529.
Gornall, A. G. & Hunter, A. (1943). *J. biol. Chem.* **147**, 593.
Grant, D. R. (1963). *Analyt. Biochem.* **6**, 109.
Greenstein, J. P. & Winitz, M. (1961). *Chemistry of the Amino Acids*, vol. 1., p. 367. New York: John Wiley and Sons Inc.
Greenstein, J. P., Winitz, M., Gullino, P., Birnbaum, S. M. & Otey, M. C. (1956). *Arch. Biochem. Biophys.* **64**, 342.
Hawk, P. B., Oser, B. L. & Summerson, W. H. (1954). *Practical Physiological Chemistry*, 13th ed., p. 573. New York: McGraw–Hill Book Co.
Herrmann, R. L., Lou, M. F. & White, C. W. (1966), *Biochim. biophys. Acta*, **121**, 79.
Kessel, D. (1959). *Fed. Proc.* **18**, 258.
Koeppe, R. E., O'Neal, R. M., Chen, C. H. & Meghal, S. K. (1966). *Fed. Proc.* **25**, 735.
Moore, S. & Stein, W. H. (1954). *J. biol. Chem.* **211**, 893.
Mushahwar, I. K. & Koeppe, R. E. (1963). *J. biol. Chem.* **238**, 2460.

Nathan, D. G. & Rodkey, F. L. (1957). *J. Lab. clin. Med.* **49**, 779.

Nutrition Reviews (1963). **21**, 28.

O'Neal, R. M. & Koeppe, R. E. (1966). *J. Neurochem.* **13**, 835.

Pal, P. R. & Christensen, H. N. (1959). *J. biol. Chem.* **234**, 613.

Perkins, H. R. & Cummins, C. S. (1964). *Nature, Lond.*, **201**, 1105.

Rao, S. L. N., Adiga, P. R. & Sarma, P. S. (1964). *Biochemistry*, **3**, 432.

Ratner, S. (1955). In *Methods in Enzymology*, vol. 2, p. 356. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Rebstock, M. C. (1960). In *Medicinal Chemistry*, 2nd ed., p. 938. Ed. by Burger, L. New York: Interscience Publishers Inc.

Ressler, C., Redstone, P. A. & Erenberg, R. H. (1961). *Science*, **134**, 188.

Riggs, T. R., Coyne, B. A. & Christensen, H. N. (1954). *J. biol. Chem.* **209**, 395.

Rosen, H. (1957). *Arch. Biochem. Biophys.* **67**, 10.

Schimke, R. T. (1962). *J. biol. Chem.* **237**, 459.

Seligson, D. & Seligson, H. (1951). *J. Lab. clin. Med.* **38**, 324.

Somogyi, M. (1945). *J. biol. Chem.* **160**, 69.

Stadie, W. C. & Riggs, B. C. (1944). *J. biol. Chem.* **154**, 687.

Steel, R. G. D. & Torrie, J. H. (1960). *Principles and Procedures of Statistics*, p. 67. New York: McGraw–Hill Book Co.

Stevens, C. M. & Bush, J. A. (1950). *J. biol. Chem.* **183**, 139.

Tews, J. K., Carter, S. H., Roa, P. D. & Stone, W. E. (1963). *J. Neurochem.* **10**, 641.

Van Etten, C. H. & Miller, R. W. (1963). *Econ. Bot.* **17**, 107.

Vivanco, F., Ramos, F. & Jimenez-Diaz, C. (1966). *J. Neurochem.* **13**, 1461.