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Chemical Studies in Relation to Convulsive Conditions

EFFECT OF TELODRIN ON THE LIBERATION AND UTILIZATION OF AMMONIA IN RAT BRAIN

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Acute Telodrin (1,3,4,5,6,7,8,8-octachloro-1,3,-3*a*,4,7,7*a*-hexahydro-4,7-methanoisobenzofuran) intoxication causes an increase in alertness, blood pressure, depth and rate of respiration and in motor activity. Piloerection and decrease in heart rate precede the onset of seizures, during which ptosis and salivation occur; the rat is apparently oblivious of its surroundings. The pharmacological effects of Telodrin relate to the central nervous system and biochemical investigations have therefore been confined to the brain.

In brain, amino acids serve as units for protein synthesis and for the production of amines and enzymes which are important to the metabolism of this organ. The exceptional ability of glutamic acid to undergo oxidation, transamination and decarboxylation, the relatively high concentrations of glutamic acid, glutamine and γ-aminobutyric acid in the brain as compared with the other organs, and the possibilities of their mutual transformations, emphasize the importance of these compounds in cerebral metabolism. These substances are intimately concerned with the transfer of ammonia in brain. Work on the formation of ammonia in brain slices (Vrba, Folbergr & Kantůrek, 1957) and on the perfusion of brain (Geiger, 1958) indicates that the turnover of glutamate and related amino acids is rapid during seizures. This finding, together with the induction of seizures by administration of ammonium salts and the increased formation of cerebral ammonia in seizures, suggest a possible association between convulsions and the metabolism of the glutamate and related amino acids in brain.

Convulsants such as methionine sulphoxime, 3-methyl-3-ethylglutaramide and thiosemicarbazide (Killam, Dasgupta & Killam, 1960; Tower, 1957) reduce the concentration of γ-aminobutyric acid and glutamic acid in cortical slices and inhibit glutamine synthesis. Similarly, both human epileptogenic brain and epileptogenic lesions of animal brain produced by freezing cortex with ethyl chloride are unable to maintain the normal concentration of glutamic acid (Berl, Purpura, Gonzalez-Monteagudo & Waelsch, 1960; Tower, 1960). These observations have therefore prompted the present investigation of possible disturbances in the cerebral amino acids and related substances which may be associated with Telodrin-induced behavioural changes.

MATERIALS AND METHODS

Telodrin. 1,3,4,5,6,7,8,8-Octachloro-1,3,3*a*,4,7,7*a*-hexahydro-4,7-methanoisobenzofuran) was crystallized from hexane–methanol and hexane–acetone mixed solvents to constant m.p. 123°, when the substance ran as a single spot in two complementary solvent systems by using reverse-phase chromatography, and phenoxyethanol–$AgNO_3$ as location reagent (Mitchell, 1958).

Dimethyl sulphoxide. Laboratory-reagent grade was redistilled, and a fraction, b.p. 66–69°/10 mm., collected.

Amino acids and peptides. L(+)-Alanine, γ-aminobutyric acid, L(+)-aspartic acid, L(+)-cysteic acid, L(+)-glutamic acid, L(+)-glutamine, glycine, DL-serine, taurine, DL-threonine and glutathione (Biochemicals Roche, and Fluka A.-G., Buchs, Switzerland) were used as marker substances.

Vitamins. Nicotinamide and pyridoxine hydrochloride (Biochemicals Roche) were used.

Codecarboxylase. This preparation (Sigma Chemical Co., St Louis, Mo., U.S.A.) had $\lambda_{max.}$ 387 mμ (pH 7), $E^{1\%}_{1cm.}$ 200.

Urease. A 1% (w/v) soln. in aq. 30% (v/v) ethanol was prepared from a Sigma Chemical Co. preparation, of activity 800–1000 modified (30°) Sumner units/g.

Except where otherwise stated, all reagents were AnalaR grade (Hopkin and Williams Ltd.) or, if this grade was unavailable, laboratory-reagent grade was used.

Ninhydrin reagents. Two solutions were prepared separately by dissolving 125 mg. of ninhydrin (Koch Laboratories) in 500 ml. of acetone–acetic acid–*s*-collidine (193:5:2, by vol.) mixture (Hanes, Harris, Moscarello & Tigane, 1961) and in 1 l. of ethyl acetate–acetic acid–*s*-collidine (191:5:4, by vol.) mixture (Tigane, Wade, Wong & Hanes, 1961). A third reagent was prepared by admixture of a solution of ninhydrin (4 g.) and hydrindantin (80 mg.) in peroxide-free methylCellosolve (100 ml.) and a solution of $SnCl_2,2H_2O$ (160 mg.) in 100 ml. of 0·33M-citrate buffer, pH 5 (Fowden, 1951). After standing for a few hours, excess of precipitated hydrindantin was removed.

Cadmium acetate–ninhydrin reagent. Fresh reagent was prepared daily by mixing a solution of $Cd(CH_3CO_2)_2,2H_2O$ (50 mg.) in aq. acetic acid (5:1, v/v, mixture) (6 ml.) and a solution of ninhydrin (0·5 g.) in acetone (50 ml.).

Ehrlich reagent. A 10% (w/v) soln. (20 ml.) of *p*-dimethylaminobenzaldehyde in 12N-HCl was mixed with acetone (80 ml.) immediately before use.

Methanolic borate buffer. A soln. of H_3BO_3 (1·86 g.) with 6·5 ml. of 6N-KOH was prepared in 93·5 ml. of methanol.

Boric acid reagent. To 100 ml. of aq. 0·2% H_3BO_3 soln. there was added 2 ml. of mixed indicator, prepared by admixture of 25 ml. of 0·2% methyl red and 75 ml. of 0·1% bromocresol green.

Hydrochloric acid. Stock N-HCl was prepared from AnalaR hydrochloric acid (sp.gr. 1·18) and diluted to 0·01N as required.

Experiments with animals

Animals. Except where otherwise stated, young rats (approx. 1 month old) were used (albino, Carworth Farm strain, maintained for 2–3 years as a closed colony in this Laboratory). The animals were fed with rat cubes of diet no. 86 and given unrestricted water until the time of the experiments. A random selection of animals was made for each experiment and, with few exceptions, the average weight was 120 g.

Method of dosing. Rats weighing 120 g. were given a single intraperitoneal injection of 0·90 ml. of a solution (1 mg./ml.) of Telodrin in dimethyl sulphoxide. The toxicant was administered as a 7·5 mg./kg. dose. Control rats were injected with the same volume of solvent as was used for the test animals.

Seizure pattern for acute Telodrin intoxication

The threshold of seizures, which occurred 19 min. after dosage, was followed by three or four major convulsions, coma and death; death occurred 50 min. after dosage. There was no difference in the response to intoxication due to sex or size. In some experiments Telodrin-treated animals were killed either at 15–16 min. after dosage or after the onset of the second convulsion, which usually occurred 25–30 min. after dosage. Control rats were killed at the same time, as appropriate.

Fixation of the brain in situ. Conscious animals were killed by holding them head first in liquid N_2, which froze them solid in a few seconds and effected a rapid fixation of cerebral metabolites (Gell, Cranmore & Crosbie, 1956; Richter & Dawson, 1948*b*). The frozen brains were rapidly dissected out without allowing them to thaw, care being taken to remove splinters of bone from the surface of frozen brain tissue. Experiments were made with pairs of whole brains (2·5–3·0 g. of tissue), which were crushed in a porcelain mortar under liquid N_2.

Antidotal trials. Groups of six rats (each 150–250 g.) were given an intraperitoneal administration of 50 mg. of glutamine/kg.; after 20 or 40 min. respectively they were given an intraperitoneal injection of 7·5 mg. of Telodrin/kg. The surviving animals had access to unrestricted food and water.

Similar groups of rats were given an intraperitoneal injection of 7·5 mg. of Telodrin/kg. 0, 10 or 30 min. after an intraperitoneal injection of 300 mg. of pyridoxime hydrochloride/kg.

Similar experiments were made with groups of rats that had been given an initial intraperitoneal injection of 500 mg. of nicotinamide/kg.

In other experiments the vitamin was injected intramuscularly in one thigh and the toxicant into the other thigh and, in further experiments, the vitamin was administered intramuscularly and the toxicant intraperitoneally.

Groups of six rats were given an intraperitoneal injection of 7·5 mg. of Telodrin/kg. 15 or 30 min. after an intraperitoneal injection of 400 mg. of codecarboxylase/kg.

The prescribed doses of glutamine, nicotinamide and pyridoxine hydrochloride were administered in 1 ml. of water and doses of codecarboxylase in 1 ml. of dimethyl sulphoxide.

Seizure pattern for acute picrotoxin intoxication

Rats weighing 100 g. were given a single intraperitoneal injection of 0·66 ml. of a solution containing 3 mg. of picrotoxin/ml. of 10% ethanol. The dose of the toxicant was 19 mg./kg. The threshold of convulsions occurred 5–7 min. after dosage, and the duration of convulsions was 10–15 min. This dosage was 100% lethal, all dead in 20 min., and there was no difference in the response to intoxication due to sex or size. Groups of six rats (150–250 g.) that had been given an intraperitoneal injection of

50 mg. of glutamine/kg., after 40 min. were given an intraperitoneal injection of 19 mg. of picrotoxin/kg. The surviving animals had access to unrestricted food and water.

Electrical stimulation

Electrical stimulation of the brain was carried out with stainless-steel clips as electrodes, which were applied to the scalp (0·5 cm. posterior to the eyes) of animals that had been given subcutaneous injections of Unacaine (4 mg.) soln. (Novocol Chemical Man. Co. Inc., Brooklyn 7, N.Y.). Contact was obtained by wetting the skin with electrode jelly. The current used was 50 cycles/sec. a.c. at 14 v. Stimulation for 5 sec. produced a satisfactory convulsion in a rat (150 g. wt.) after a latent period of 10–12 sec. Groups of six rats (150 g. wt.) that had been given an intraperitoneal injection of 50 mg. of glutamine/kg., after 40 min. were also subjected to electrical stimulation of the brain.

Systematic separation from rat brain of an amino acid fraction suitable for paper chromatography

Pulverized frozen brain (2·0–3·0 g.) was dropped into the weighed tube of a tissue homogenizer (Aldridge, Emery & Street, 1960), which contained 40 ml. of 12% (w/v) trichloroacetic acid at 0°. After vigorous shaking the tube was weighed and the tissue rapidly brought into a fine suspension by mechanical rotation of the fitting pestle. The suspension was centrifuged at 35000*g* at 0° in the super-speed head of a MSE Major refrigerated centrifuge for 15 min. to separate completely phospholipids from the trichloroacetic acid extract (Heald, 1956). The sediment was re-extracted with 20 ml. of 12% (w/v) trichloroacetic acid in the tissue homogenizer, and the combined supernatants were extracted with ether (4 × 60 ml.), which removed 98% of the trichloroacetic acid.

To concentrate the amines, amino acids and inorganic cations in the tissue extract, the resulting solution (60 ml.) was adjusted to pH 2·0–2·5 by addition of a small volume of 8N-acetic acid and percolated (0·5 ml./min.) through a column (bed-volume, 8 ml.; height:diameter >9:1) of analytical grade Dowex 50W (H^+ form; X8; 100–200 mesh) (Bio-Rad Laboratories, Richmond, Calif., U.S.A.) cation-exchange resin (1·7 m-equiv./ml.) (Hathway, 1956). The column was treated with 2 bed-volumes of water and 75 ml. of 2N-triethylamine soln. in acetone–water (1:4, v/v; Harris, Tigane & Hanes, 1961) successively. The aqueous eluate, which did not react with sensitive ninhydrin reagent (Fowden, 1951), was discarded and the triethylamine eluate was evaporated (<38°/10 mm. Hg) to dryness in a Rotating High Vacuum Type Evaporator (Rinco Instrument Co. Inc., Greenville, Illinois). This residue, which was stored over P_2O_5 at 0°/0·01 mm. Hg, was quantitatively transferred with water to a 1 ml. standard flask, and the resulting solution was used for estimations of amino acids.

Harris, Tigane & Hanes's (1961) triethylamine solvent system was found advantageous for the displacement of cerebral amines and amino acids from a resin column, since only traces of the accompanying inorganic cations were released, and the residue from the eluate could be used direct for paper-chromatographic estimation of amino acids; whereas when aq. 2N-NH_3 was used as eluent an aqueous solution of the residue had to be electrolytically desalted before chromatography. In such desalting the test solution (1 ml.) was kept cold, and, when the apparatus (Consden, Martin & Gordon, 1947; Hathway, 1956) was supplied with d.c. at 230 v, the current decreased from 500 mA to a steady minimal value of 130 mA in 5 min. The use of aq. NH_3 soln. as eluent was also undesirable in a room where amino acid chromatograms are quantitatively analysed, since unlike triethylamine ammonia interferes with the reaction of amino acids with ninhydrin. The triethylamine method was accordingly used in the present work.

In some of the work that has been reported (Porcellati & Thompson, 1957; Roberts & Frankel, 1950; Soep & Janssen, 1961) on the estimation of amino acids by paper chromatography, homogenates were prepared in aq. 75% ethanol, the supernatants were successively evaporated, dissolved in water and centrifuged, and the second supernatants evaporated. A major disadvantage incurred by using organic solvents for the extraction of brain is the extraction of a proportion of the high lipid content, and our early experiments established that the deposition of lipid caused considerable difficulty at all stages in this procedure. The attendant difficulties of precipitation could possibly be overcome by acidification and repeated extraction with a fat solvent, followed by high-speed centrifuging, but we have preferred to work throughout with a clean extract prepared in trichloroacetic acid.

Paper chromatography

Solvents. Two-dimensional chromatograms were run on Whatman no. 1 filter papers, 25·5 cm. square, by the ascending method in a constant-temperature enclosure at 29°. Butan-1-ol–acetic acid–water (4:1:5, by vol.) was used as first-way solvent, and some of the aqueous phase was introduced in a separate vessel into the tank. Phenol saturated with water was used as second-way solvent, and some of the upper phase and 3 ml. of 17N-NH_3 were introduced in a separate vessel into the tank. After irrigation with the first-way solvent, traces of acetic acid were removed from air-dried papers at 40° for 2 hr., and, after the second-way solvent, phenol was removed by three successive washes with anhydrous diethyl ether.

These solvents gave good chromatographic solution of cerebral amino acids, but improved separation of taurine from glycine and serine was obtained by using butan-1-ol–pyridine–water (1:1:1, by vol.) (Morrison, 1953) as first-way solvent. Hanes and co-workers (Hanes *et al.* 1961; Wade, Matheson & Hanes, 1961) have developed new chromatographic systems for the separation of amino acids, and, although we have successfully applied them to the separation of cerebral amino acids, the advantage was slight, and at the cost of a relatively complex technique. Wolfe's (1957) butan-1-ol–butan-2-one–17N-NH_3–water (5:3:1:1, by vol.) solvent did not give good separations of brain amino acids.

Detection of amino acids and removal of ammonia. The amino acids were located by weak ninhydrin solutions in either acetone–acetic acid–*s*-collidine (Hanes *et al.* 1961) or ethyl acetate–acetic acid–*s*-collidine (Tigane *et al.* 1961), which were used as dip reagents. Coloured spots developed within 16 hr. Ammonia was removed from excised pieces of paper by treatment with 0·1 ml. of methanolic borate buffer, followed by rapid drying at 40°. Urea was located by means of Ehrlich reagent.

Development of colour. Into separate tubes containing the excised pieces of paper, 1·5 ml. of sensitive ninhydrin reagent (Fowden, 1951) was added, and the tubes were shaken for 10 min. to extract the amino acids. The production of colour was completed by placing the tubes in boiling water for 25 min. After cooling, the coloured solutions were quantitatively transferred with acetone–water (1:1, v/v) to 10 ml. standard flasks; 25 ml. flasks were used where intense colours were encountered. The cadmium–ninhydrin reagent (Atfield & Morris, 1961; Heilmann, Barollier & Watzke, 1957) gave very weak reactions with γ-aminobutyric acid and taurine, and was accordingly less useful in this context. The extinction of the bluish-purple solutions was measured at 570 mμ in 10 mm. cells with a Unicam spectrophotometer SP. 500. No correction was made for the negligible extinction of the reagent but a correction was required for the colours produced from the paper alone.

Elimination of the contribution of paper to the colour developed. Quantities (10, 20, 30, 40 and 50 μg.) of each cerebral amino acid were chromatographed separately and the largest spots detected were outlined in pencil. Identical areas were traced round the spots due to particular amino acids on the remaining chromatograms. These areas were then excised and maximum colour was developed. For each amino acid linear plots were drawn of extinction versus amount of amino acid used. Each line plot was extrapolated, and the intercept on the ordinate represented the contribution of each piece of paper to the measured values of extinction (Fig. 1). A parallel line was drawn through the origin and was used as the standard linear relationship between quantity of amino acid and the extinction of colour developed. Similar chromatography with volumes (5, 10, 20, 30 and 40 μl.) of the prepared brain extract, followed by the tracing of equal areas round spots due to particular amino acids and the subsequent development of maximum colour with ninhydrin, afforded a set of linear plots of extinction versus volume of extract used. These plots were extrapolated and parallel lines drawn through the origin. In this way, the extinction produced by a particular amino acid could be related to the corresponding volume of extract and, from Fig. 1, to the quantity of amino acid implicated.

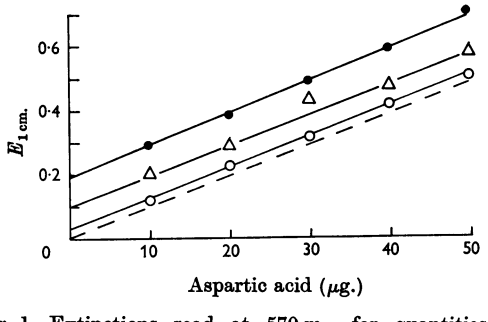

Fig. 1. Extinctions read at 570 mμ for quantities of chromatographed aspartic acid. ●, △, ○, Linear plots with the same slope and different blank values for paper were obtained on separate occasions. The broken line represents the standard linear relationship (for details see the Materials and Methods section).

Microdiffusion analysis

Whole brain (2·5–3·0 g.) from two young rats, killed in liquid N_2, was homogenized in 10 ml. of 12% (w/v) trichloroacetic acid and centrifuged at 0° as previously described.

Ammonia estimation. Portions (1 ml.) of the supernatant were transferred to no. 1 Conway units (Conway, 1962), which contained 2 ml. of boric acid reagent in the central chamber. After diffusion of the NH_3 (2 hr.) the contents of the central chamber were titrated to the original colour of the indicator with 0·01 N-HCl. An allowance was made in the calculation for the water content of brain, taken as 80% of the weight of tissue, and for the rate of hydrolysis of cerebral glutamine. Preliminary tests showed that the rate of liberation of NH_3 by glutamine hydrolysis was 0·001 mg. of NH_3/mg. of glutamine/hr. in contact with aq. 50% saturated K_2CO_3 at 25° under the experimental conditions of the Conway method; this value is in agreement with that of Richter & Dawson (1948*a*).

Glutamine estimation (*Harris*, 1943). For this estimation the trichloroacetic acid supernatant was heated at 70° for 75 min. to complete hydrolysis of the glutamine and 1 ml. portions were transferred to Conway units, as described for the estimation of NH_3. An allowance was made in the calculation for cerebral ammonia.

Urea estimation. A known volume of supernatant was neutralized with 5 N-NaOH and diluted to twice the original volume with 0·1 M-phosphate buffer, pH 7·0. Portions (1 ml.) were transferred to Conway units and incubated with 0·2 ml. of urease soln. for 30 min., before microdiffusion of NH_3. An allowance was made in the calculation for free ammonia and the rate of hydrolysis of glutamine.

Results were compared with estimations on standard solutions of $(NH_4)_2SO_4$, glutamine or urea as appropriate.

Determination of α-oxo acids

α-Oxoglutaric acid and pyruvic acid were determined spectrophotometrically after separation as their 2,4-dinitrophenylhydrazones (Friedemann, 1957). For each determination, 3·0 ml. of the aforementioned 12% trichloroacetic acid extract (representing approx. 600 mg. of original fresh brain tissue) was used.

RESULTS

The present work represents an exploratory investigation of the action of Telodrin on cerebral metabolism, and most of the observations have been limited to experiments on immature rats, since the method of killing restricts the size of animals that can be used.

Although it has now been found that pretreatment with glutamine did not affect electroshock or picrotoxin convulsions, when a convulsive dose of Telodrin was administered to rats that had been treated 40 min. previously with 50 mg. of glutamine/kg., one-third of the animals resisted convulsions and recovered. This observation suggested that cerebral amino acids are important in Telodrin intoxication, because of the significance of

glutamine as the main nitrogenous nutrient passing the blood–brain barrier (Schwerin, Bessman & Waelsch, 1950). It has, however, been suggested that the favourable effect of glutamate in certain disorders of brain is indirect (Weil-Malherbe, 1952).

Amino acids. The amino acid contents of the whole brain of the adult rats are well established. Average values (μmoles/100 g. of fresh tissue) were: glutamic acid 900; γ-aminobutyric acid 400; glutamine 380; aspartic acid 248. With the exception of glutamine (492; range 459–526) the corresponding concentrations in the brain of the young rats were considerably lower: glutamic acid 486 (range 483–502), γ-aminobutyric acid 199 (range 176–216) and aspartic acid 114 (range 102–127). These values for the cerebral amino acids of immature rats are comparable with the corresponding concentrations found by Häkkinen & Kulonen (1961) for young Wistar rats.

In Table 1 are listed the changes observed in γ-aminobutyric acid, glutamic acid, aspartic acid and glutamine in the brain of young rats, killed at the time of the second convulsion after administration of a dimethyl sulphoxide solution of Telodrin, and the corresponding changes produced at the same interval of time (25 min.) after administration of the solvent. The administration of dimethyl sulphoxide produced no significant changes in the concentrations of the four cerebral amino acids, but the changes in glutamic acid and glutamine due to Telodrin are significant, whereas the changes in γ-aminobutyric acid and aspartic acid are not significant. A significant elevation in the concentration of cerebral glutamine and a fall in that of glutamic acid have therefore been demonstrated at this time in the pattern of Telodrin-induced seizures. No change occurred in the concentration of taurine, which was measured in some of the experiments.

The short interval of time between dosage and onset of convulsions caused some experimental difficulty in the measurement of cerebral glutamine in animals that were killed 15–16 min. after administration of Telodrin, with the result that some of the animals may have been killed before the toxicant had had time to be effective and others may have just begun a first convulsion during the second or so required for transfer from the cage to liquid nitrogen. The concentration (μmoles/100 g. of fresh tissue) of cerebral glutamine at that time

Table 1. *Effect of Telodrin on the free amino acids and related substances of brain of young rats*

Rats were killed 25 min. after the administration of a dimethyl sulphoxide solution of Telodrin or the solvent. Figures give concentrations in μmoles/100 g. of fresh whole brain. The first row of probabilities (*P*) refers to results for the dimethyl sulphoxide-treated animals versus the untreated animals, and the second row of *P* values to results for Telodrin-treated animals versus the other animals (dimethyl sulphoxide-treated plus untreated).

Substance administered	γ-Amino-butyric acid	Glutamic acid	Aspartic acid	Glutamine	Ammonia	Urea
None	216	502	127	326*	57	443
	205	473	102	459	51	550
	176	483	113	523	95	468
				477	61	—
				476	67	—
				526	—	—
Mean	199	486	114	492	66	487
Dimethyl sulphoxide	175	462	138	521	38	523
	141	430	105	476	51	425
	158	446	120	580	56	432
					48	—
Mean	158	446	122	525	48	493
Change (%)	−20·6	−8·2	+7·0	+6·5	−27·2	+1·2
P of chance occurrence of the differences	>0·1	>0·1	>0·5	>0·25	>0·05	>0·8
Telodrin in dimethyl sulphoxide	230	411	113	580	44	526
	176	375	83	782	40	541
	211	448	105	562	51	448
	148	422	109	685	72	—
				577	63	—
Mean	191	414	102	637	54	505
Change (%)	+20·9	−7·3	−16·4	+21·3	+12·5	+2·4
P of chance occurrence of the differences	>0·5	<0·025	>0·10	<0·005	>0·10	>0·25

* This value was thought to be in error and has been omitted from the calculations, since it falls outside the 1% confidence band (389–581) for the mean of these values.

was 508, 612, 635, 658, 536 and 568. These experiments showed that a rise in cerebral glutamine (% change +11·6; $P < 0·025$) occurred in the preconvulsive state or at the beginning of the first convulsion, and this suggested that the increased concentration of cerebral glutamine might be associated with increased cerebral irritability due to Telodrin rather than with the convulsions themselves.

Since this evidence was somewhat inconclusive, the time-course for the change in glutamine content of rat brain after administration of Telodrin was determined (Fig. 2). This shows unequivocally that cerebral glutamine rose to the maximum concentration during the preconvulsive period, and the shape of this curve leads us to believe that the changes in glutamine are significantly related to the action of Telodrin. After the first convulsion, the concentration remained approximately constant, which suggested that a limiting value had been attained for the synthesis and retention of glutamine.

Evidence of preconvulsive changes in the electrical activity of rat brain is afforded by the electroencephalographic recordings taken 15 min. after administration of Telodrin. These show periodic slow waves and some sharp elements. The conclusion is drawn that the changes in glutamine are significantly related to the action of Telodrin on the brain.

Ammonia. The mean value (μmoles/100 g. of fresh brain) for free ammonia in the whole brain of young rats was found to be 66, and similar values were obtained for hooded Lister and Carworth Farm strain E animals, belonging to the same weight group (Table 2). The concentration of free ammonia in the brain of 2-week-old weanlings belonging to the hooded Lister and Carworth Farm strain E animals was strictly comparable with Richter & Dawson's (1948*a*) value of 17 for Wistar rats of this weight group (Table 2). The conclusion is drawn that a higher concentration of cerebral ammonia is associated with the larger animals, whereas the fact that the concentration of cerebral ammonia in young rats and 2-week-old Carworth Farm weanlings is approximately the same may be due to a species variation.

A decrease in the concentration of cerebral ammonia (% change 27·2) was registered 25 min. after administration of dimethyl sulphoxide, but this was barely significant ($P > 0·05$) because of the variability in experimental results. Telodrin produced no significant change in cerebral ammonia at the time of the second convulsion (Table 1).

In Fig. 3, the time-course for the change in ammonia content of rat brain after the administration of a dimethyl sulphoxide solution of Telodrin is recorded. The low concentrations of ammonia just before the first convulsions are probably due to the influence of dimethyl sulphoxide. Later in the seizure pattern and before death, the high concentrations of free ammonia observed in brain are consistent with ammonia poisoning, and with an overwhelming of glutamic acid–glutamine synthesis.

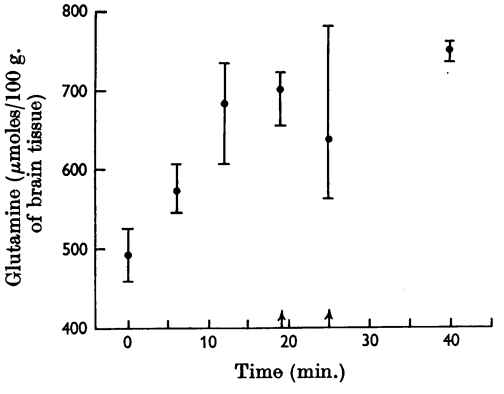

Fig. 2. Change in the glutamine content of rat brain after administration of Telodrin. Each range of values is derived from at least three pairs of animals. Arrows on the abscissa mark the times of the first and second convulsions; death occurred 50 min. after dosage.

Table 2. *Effect of age on the free ammonia of brain of young rats*

Figures give concentrations in μmoles/100 g. of fresh whole brain.

Strain of rat	Young rats (100–150 g.)	Weanling rats (25–40 g.)
Albino Carworth Farm strain E	67	24
Hooded Lister	61	22
Albino Carworth Farm	66	57
Albino Wistar	—	17*

* Value from Richter & Dawson (1948*a*).

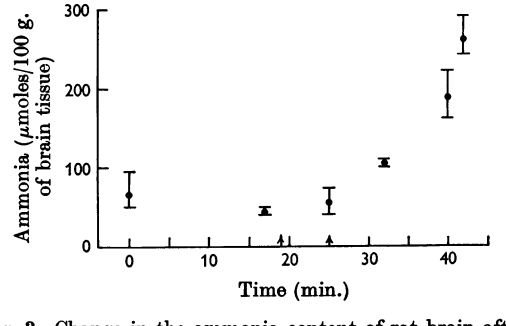

Fig. 3. Change in the ammonia content of rat brain after administration of Telodrin. Each range of values is derived from at least three pairs of animals. Arrows on the abscissa have the same significance as in Fig. 2; death occurred 50 min. after dosage.

Table 3. *Effect of Telodrin on the α-oxo acids of brain of young rats*

Rats were killed 25 min. after administration of a dimethyl sulphoxide solution of Telodrin or the solvent. Figures give the concentration in μmoles/100 g. of fresh whole brain. The first row of probabilities (*P*) refers to results for dimethyl sulphoxide-treated animals versus the untreated animals, and the second row of *P* values to results for Telodrin-treated animals versus the other animals (dimethyl sulphoxide-treated and untreated).

Substance administered	Pyruvic acid	α-Oxo-glutaric acid
None	14·1	4·7
	12·8	5·7
	10·7	4·9
	12·8	4·5
Mean	12·6	4·9
Dimethyl sulphoxide	13·7	7·8
	12·6	8·6
	13·6	14·8
Mean	13·3	10·4
Change (%)	+5·6	+112·3
P of chance occurrence of the differences	>0·5	<0·05
Telodrin in dimethyl sulphoxide	24·3	12·7
	23·8	15·5
	20·0	12·8
	21·9	22·0
Mean	22·5	15·7
Change (%)	+78·6	—
P of chance occurrence of the differences	<0·001	—

Urea. It is of interest that the concentration (487 μmoles/100 g. of fresh brain) of cerebral urea for young rats is in the same range as the value 476, reported by Sporn, Dingman, Defalco & Davies (1959), for more mature, 3-month-old Sprague–Dawley animals. The separate administration of dimethyl sulphoxide and of a dimethyl sulphoxide solution of Telodrin produced no significant change in the concentration of cerebral urea in young rats at the time of the second convulsion (Table 1).

Pyruvic acid and α-oxoglutaric acid. In Table 3 are listed the changes observed in pyruvic acid and α-oxoglutaric acid in the brain of young rats, killed at the time of the second convulsion after administration of a dimethyl sulphoxide solution of Telodrin, and the corresponding changes produced at the same interval of time (25 min.) after administration of solvent. The average value (μmoles/100 g. of fresh tissue) for pyruvic acid (12·6) in the brain of the young rats was lower than the corresponding concentration (19·5) in the brain of adult rats (Frohman, Orten & Smith, 1951), but this value was not obtained under comparable conditions. The administration of dimethyl sulphoxide produced no significant change in the concentration of cerebral pyruvic acid, but after Telodrin the change in pyruvic acid is highly significant. For cerebral α-oxoglutaric acid, the concentration in the group of young rats treated with dimethyl sulphoxide is probably different ($P < 0·05$) from that in normal animals, whereas the Telodrin-treated animals are not significantly different ($P > 0·05$) from the dimethyl sulphoxide-treated rats. Because of the interference of the solvent in this way, no acceptable conclusion can be reached about the effect of Telodrin on cerebral α-oxoglutaric acid.

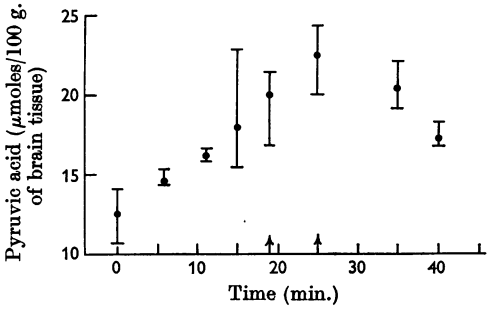

Fig. 4. Change in the pyruvic acid content of rat brain after administration of Telodrin. Each range of values is derived from at least three pairs of animals. Arrows on the abscissa have the same significance as in Fig. 2; death occurred 50 min. after dosage.

The time-course for the change in pyruvic acid content of rat brain after administration of Telodrin (Fig. 4) shows that pyruvic acid rose to near the maximum concentration during the pre-convulsive period. The shape of this curve, in conjunction with the statistical values, suggests that the changes in pyruvic acid are significantly related to the action of Telodrin. An enhanced

concentration of pyruvic acid accompanied the initial increased cerebral irritability due to Telodrin, but in the period of coma and less violent seizures that follow the tonic extensor seizure the concentration fell. The rapid increase in pyruvic acid concentration (Fig. 4) follows and parallels the similar change in concentration of glutamine (Fig. 2).

Antidotal experiments. The usual pattern of seizures occurred when a convulsive dose of Telodrin was administered to rats previously treated by various parenteral routes with massive doses of nicotinamide and pyridoxine hydrochloride respectively, and one-third of the animals resisted convulsions and appeared to recover when Telodrin was administered 15 min. after administration of 400 mg. of codecarboxylase/kg. Nicotinamide and pyridoxine do not therefore behave as biochemical antagonists for Telodrin, whereas these substances are effective antidotes for convulsions induced by the carbonyl-trapping hydrazines and thiosemicarbazides (Jenney & Pfeiffer, 1958; Jenney, Smith & Pfeiffer, 1953), which also reduce the concentrations of cerebral γ-aminobutyric acid and glutamic acid (Balzer, Holtz & Palm, 1960). In contrast with the observation that codecarboxylase accelerates the onset of isonicotinic acid hydrazide-induced convulsions (Balzer *et al.* 1960), codecarboxylase has now been shown to possess antidotal properties in acute Telodrin intoxication, and this behaviour may be related to the requirement of glutamic decarboxylase and γ-aminobutyric acid transaminase for the coenzyme.

DISCUSSION

There is strong evidence that the action of Telodrin leads to liberation of ammonia in the brain, and that this occurs before the onset of and throughout convulsions. Glutamic acid, glutamine and α-oxoglutaric acid are utilized in the ammonia-binding mechanism, which is overwhelmed later in the seizure pattern when free ammonia accumulates in cerebral tissues. The origin of the liberation of ammonia and its significance for nervous function are outside the scope of the present investigation; present findings are concerned with the utilization of cerebral ammonia in acute Telodrin intoxication, and they conform with the background of knowledge on disposal of ammonia in the brain.

One molecule of ammonia combines with α-oxoglutaric acid to form glutamic acid, and a second molecule of ammonia combines with glutamic acid to form glutamine. This electrically neutral compound behaves as an acceptable form of transport for ammonia. Whereas reductive amination of α-oxoglutaric acid occurs spontaneously in the presence of glutamic dehydrogenase, glutamine synthesis requires ATP and is inhibited by dinitrophenol (Elliott, 1948). The concentration of high-energy phosphates is lowered by the production of ammonia (Muntz, 1953), and glycolysis and respiration are accordingly accelerated. Continued withdrawal of α-oxoglutaric acid towards glutamic acid reduces the supply of dicarboxylic acids necessary for the functioning of the tricarboxylic acid cycle (Recknagel & Potter, 1951), and causes an accumulation of pyruvic acid. Pyruvic acid accumulated in the brains of dogs that had been treated with ammonia (Clark & Eiseman, 1958), and in the brains of rats during intoxication with Telodrin.

Other methods for the utilization of ammonia in the brain should also be considered. Transaminations involving oxaloacetic acid and pyruvic acid may contribute to the main method for ammonia disposal, since in cerebral tissues there is a high activity of the relevant transaminases (Awapara & Seale, 1952). The identification of urea in our cerebral extracts, together with recent evidence that cerebral urea concentrations are independent of blood urea and that the synthesis of urea from L-arginine occurs endogenously in the brain (Davies, Defalco, Shander, Kopelman & Kujasu, 1961; Sporn *et al.* 1959), led us to consider whether a close integration of the enzymes of the cerebral glutamic acid–glutamine system and of those that participate in urea synthesis might contribute to ammonia disposal in the brain, but present work showed that urea synthesis was not utilized by brain as a method for ammonia disposal in acute Telodrin intoxication. This observation is consistent with the higher turnover of glutamic acid substances compared with urea intermediates. Brain synthesizes urea at a rate of 1 μmole/g. of tissue/hr. (Blass, 1961), whereas the maximum rate of glutamine synthesis is 10–20 μmoles/g. of tissue/hr. (McIlwain, 1959). Hence, more of the ammonia of the brain would be expected to enter glutamine than urea.

The recent observation in this Laboratory that Telodrin stimulates the medulla oblongata (D. E. Stevenson, P. L. Chambers & B. Reiff, personal communication) is of interest, since ammonium ions are also a medullary stimulant (Goodman & Gilman, 1955) and since the present work suggests that liberation of ammonia in the brain occurred before the onset of and throughout Telodrin-induced convulsions. Medullary stimulation by Telodrin might accordingly be associated with liberation of cerebral ammonia, and in this connexion a similar liberation of cerebral ammonia occurs in epileptic convusions. Electrical stimulation of the brain (Richter & Dawson, 1948*a*) caused a significant rise in the concentration of ammonia in the brain in the preconvulsive period.

A significant rise in the concentration of free ammonia in rat brain has also been found after convulsions produced by administration of camphor (Vladimirova, 1938), pentamethylenetetrazole (Torda, 1953) and picrotoxin (Richter & Dawson, 1948*a*), and these compounds also stimulate the medulla oblongata (Gaddum, 1959). Atropine should not be administered during acute Telodrin intoxication, since it stimulates the medulla and higher centres.

Richter & Crossland (1949) have found that the total acetylcholine content of the rat brain varies inversely with the activity of the animal. Thus the concentration of acetylcholine is reduced below the normal during seizures induced by pentamethylenetetrazole or picrotoxin. It is also known that sodium pyruvate brings about a definite inhibition of acetylcholine synthesis, and acetylcholine should therefore be affected in acute intoxication with Telodrin.

SUMMARY

1. During acute Telodrin intoxication an increase is observed in the content of glutamine in the brain and a decrease in glutamic acid. Aspartic acid, γ-aminobutyric acid and urea remain unchanged. Some animals previously treated with glutamine resisted Telodrin convulsions.

2. During acute Telodrin intoxication, cerebral pyruvic acid accumulates until the tonic extensor seizure, after which the concentration diminishes.

3. In the early stages of acute Telodrin intoxication the content of free ammonia in the brain remains unchanged, but an increase is observed later in the seizure pattern.

4. The conclusion is drawn that the action of Telodrin causes liberation of ammonia in the brain and that this occurs before the onset of convulsions and throughout their course. Glutamic acid, glutamine and α-oxoglutaric acid are utilized in an ammonia-binding mechanism, which later becomes overwhelmed and free ammonia accumulates in the cerebral tissues.

5. With the exception of glutamine, the content of the free amino acids in brain was significantly lower in the young rats than in adult rats. The concentration of cerebral ammonia was significantly higher in young rats than in 2-week-old weanlings.

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Role of Pyridoxine in Glutathione Metabolism

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Binkley, Christensen & Jensen (1952) demonstrated that pyridoxal phosphate is a cofactor in the biosynthesis of cysteine from methionine. Blaschko, Datta & Harris (1953) reported that the decrease in urinary excretion of taurine in vitamin B_6 deficiency is in part due to the decrease in the concentration of the pyridoxal phosphate-containing enzyme, cysteinesulphinate decarboxylase, which catalyses the formation of hypotaurine. On the basis of the decrease of pantothenic acid content in the liver of pyridoxine-deficient rats, Williams & Hata (1959) observed that its CoA activity is also decreased. As a result of these findings which point to the existence of metabolic interrelationships between vitamin B_6 and sulphur-containing amino acids, we studied the role of pyridoxine on the metabolism of another physiologically important thiol compound, namely reduced glutathione (GSH). The present paper gives results to show that pyridoxine deficiency induced under various dietary conditions brings about a consistent increase in the concentration of GSH in liver and, to a less extent, in erythrocytes, and accelerates the incorporation of [2-^{14}C]glycine into liver GSH.

EXPERIMENTAL

Preparation of experimental animals and diet. Weanling or 6–8 week-old rats of both sexes of the McCollum strain were used. They were placed in an air-conditioned room maintained at 22–24° and housed in individual screen-bottom cages for 5–6 weeks. A high-fat (Hsu & Chow, 1957) or a high-protein (Hsu & Kawin, 1962) basal diet deficient in pyridoxine was used to induce vitamin B_6 deficiencies. For control, another group of animals was given the same pyridoxine-deficient diet supplemented with pyridoxine hydrochloride (20 mg./kg.). In some studies deoxypyridoxine, an antagonist of pyridoxine, was also administered to groups of rats on the deficient diet to accelerate pyridoxine deficiency.

A complete synthetic diet was also used for certain experiments, and its percentage composition was as follows: essential amino acids, 10·9; non-essential amino acids, 8·0; corn oil, 5·0; sucrose, 72·0; salt mixture, 4·0. All known vitamins except pyridoxine were added to the synthetic diet in a manner similar to the high-fat diet described above. The amino acid composition of the diet, expressed as g./kg., is shown in Table 1. In the preparation of cysteine-free or

Table 1. *Amino acid composition of the diet*

The amino acid contents are expressed as g./kg. of diet.

L-Arginine hydrochloride	8·55
L-Histidine hydrochloride	6·27
DL-Isoleucine	15·39
DL-Leucine	18·24
DL-Methionine	6·84
Cysteine*	3·42
DL-Phenylalanine	10·26
DL-Tryptophan	2·28
DL-Threonine	11·40
DL-Valine	15·96
DL-Alanine	13·68
DL-Aspartic acid	13·68
DL-Glutamic acid†	20·00
DL-Tyrosine	15·96
Glycine	7·98
DL-Lysine hydrochloride	14·25
DL-Serine	5·70
Total	189·86

* Cysteine was omitted in the preparation of the cysteine-free diet, and the DL-methionine content was increased to 9·0 g./kg.

† Glutamic acid was omitted in the preparation of the glutamic acid-free diet.