CONVERSION OF ORALLY ADMINISTERED 2-n.PENTYLAMINOACETAMIDE INTO GLYCINAMIDE
AND GLYCINE IN THE RAT BRAIN.

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Summary

Male Sprague Dawley albino rats were treated orally with 2-n.pentylaminoacetamide (10 to 100 mg/kg b.wt). This oral administration provoked a dose-related and time-dependent accumulation of glycinamide in forebrain, cerebellum, and medulla, and to increased levels of glycine in the three brain areas, and of serine in medulla. In kidney, liver and plasma, the accumulation of glycinamide was lower and there was no increase in glycine and serine levels. With a dose of 100 mg/kg b.wt, 28 % of the drug were eliminated unchanged and 16 % as glycinamide, in urines collected for 24 h. In all tissues examined, 2-n.pentylaminoacetamide and glycinamide levels peaked at 1 h and were nil again after 24 h, the ratio of 2-n.pentylaminoacetamide over glycinamide decreasing more rapidly in brain than in kidney and liver. Contrasting with the effects of 2-n.pentylaminoacetamide, the oral administration of glycinamide (66 mg/b.wt) led, 2 hours later, to similar low rises of glycinamide in plasma and brain. In another control experiment, the intraperitoneal injection of a large dose of glycine (450 mg/kg b.wt) provoked, 30 min later, modest rises of glycine levels in the central nervous system that merely reflected a contamination by plasma glycine.

Glycine is a major inhibitory neurotransmitter which binds to receptors (distinct from GABA receptors) in target neurons thereby provoking increased influx of chloride and hyperpolarization [1-5]. This role of glycine is predominant in the spinal cord [6] but is also observed in certain areas of brain such as the substantia nigra [7,8].

In general, the penetrance of orally taken polar amino acids into brain neurons depends on several factors including the gut epithelium barrier, the blood-brain barrier and amino acid transport. In particular, the transfer of glycine through the blood-brain barrier is slow [9] so that a less polar derivative of glycine might conceivably penetrate the brain more readily. 2-n.pentylaminoacetamide (=N-pentylglycinamide = NPG = milacemide CP 1552 S) is being developed as a new anticonvulsant agent by Continental Pharma Research Laboratories (Mont-Saint-Guibert, Belgium) [10,11]. The glycine residue in this compound is both N-pentylated and amidated. It was felt of interest, therefore, to compare in rat the metabolism of a single oral dose

of 2-n.pentylaminoacétamide in brain and other tissues. It is concluded that orally taken 2-n.pentylaminoacetamide provokes an efficient accumulation of glycinamide and glycine in the rat brain.

Materials and methods

Animal treatments.

Experiments were conducted on male Sprague Dawley albino (150-200 g) rats. A majority of animals, fasted overnight, received a single dose of NPG, by oral intubation, in 1 ml water. Control animals received an equivalent volume of vehicle. These rats were decapitated 2 hours later, between 10 and 11 a.m., unless stated otherwise. In one experiment, a time study was conducted after the oral administration of 100 mg/kg body wt of NPG: batches of 4 animals were sacrificed 1, 2, 4, and 24 hours after drug administration. Rats in the last group of 4 were refed ad libitum after treatment and their urines were collected. In one control experiment, 5 fasted rats received 66 mg/kg b.wt glycinamide (Gly-NH₂) in 1 ml water (i.e. 0.60 mmol/kg) at 9.00-10.00 a.m. and were sacrificed 2 hours later. In another control experiment, 4 fasted rats were injected intraperitoneally at 9.00-10.00 a.m. with glycine in 0.15 M NaCl: the concentration of glycine was 450 mg/kg b.wt (i.e. 5.9 mmol/kg), the volume injected was about 0.5 ml, and the animals were sacrificed 30 min later.

Biological specimens.

The blood was collected, immediately centrifuged in the presence of sodium heparin, and deproteinized by adding an equal volume of 10 % trichloroacetic acid. The forebrain, cerebellum, medulla spinalis (exposed by laminectomy from medulla oblongata to dorsal cord), liver, one kidney, and a sample of the gastrocnemius of the hindleg were dissected out as rapidly as possible and chilled in liquid nitrogen then stored at -90°C. After thawing, the tissues were homogenized at 2°C in enough 5 % trichloroacetic acid to make 5 to 20 % homogenates. Urines, whether collected by puncture of the bladder (after sacrifice) or for a 24 h period, were treated like plasma.

Amino acid analysis.

The amino acid supernatant analysis was conducted according to Moore et al. [12] on two ion exchange columns in a Multichrom B automatic amino acid analyzer (Beckman, Munich, F.R.G.) coupled to a data processor (Chromatopac C-RIA from Shimadzu Co., Kyoto, Japan). The elution of acidic and neutral amino acids was carried out following the manual instructions for biological fluids, using a 55 x 0.9-cm ion exchange column filled with M-72 cationic exchange resin (Beckman) and lithium citrate buffers at a flow rate of 70 ml/hour. For the separation of basic amino acids, glycinamide, and NPG, a 22 x 0.9-cm column filled with M-71 cation-exchange resin (Beckman) was used. This column was operated at 50 ml/hour with 0.38 N sodium citrate (pH 4.15) buffer until after histidine was eluted; the temperature being 30°C for 150 min then rising progressively to 60°C during the next 55 min. After 205 min, a 1.2 N sodium citrate (pH 6.28) buffer was used for 155 min at 60°C and at a flow rate of 50 ml/hour, that allowed the elution of NPG following arginine. The column was washed at 60°C with 0.2 N NaOH (pH 13.3) for 10 min between each analysis. Under these conditions, a glycinamide standard (color factor of 6.64 as compared to 4.95 for lysine at 570 nm) elutes at 143 min i.e. clearly separated from NH₃ (133 min) and well before lysine (165 min), while a NPG standard (color factor of 19.33 at 570 nm) elutes at 360 min and well behind arginine (255 min).

Protein determination.

Tissue proteins were purified according to Schneider [13], then determined according to Lowry et al. [14] using bovine serum albumin as a standard.

Chemicals.

2-n.pentylaminoacetamide.HCl (milacemide, mol.wt of the chlorhydrate : 180.7 g) was synthesized by Continental Pharma (Mont-Saint-Guibert, Belgium). glycynamide was obtained from Bachem Feinchemikalien AG (Bubendorf, Switzerland), GABA, A grade was obtained from Calbiochem (Los Angeles, CA, U.S.A.), and glycine pro anal. was purchased from Merck (Darmstadt, F.R.G.).

Results

A ninhydrine-positive peak, not normally detected during the basic amino acid analysis of various rat tissue extracts, was present in the forebrain, two hours after a single oral administration of 100 mg NPG/kg b.wt (i.e. 552 $\mu\text{mol/kg}$). This unknown compound eluted after ammonia and before lysine from the 22-cm column.

FIG. 1

Automatically recorded chromatographic analysis of basic amino acids, glycynamide, and 2-n.pentylaminoacetamide on a 22 x 0.9-cm column of M-71 cation-exchange resin. The sample was a synthetic calibration mixture from Beckman Instruments (Palo Alto, CA, U.S.A.) containing 17 amino acids (50 nanomoles of each) enriched with GABA (25 nanomoles), glycynamide (33 nanomoles), and 2-n.pentylaminoacetamide (300 nanomoles). The elution was carried out with two sodium citrate buffers at successively higher pH and temperature, as detailed under Materials and Methods.

Its identity was established by collecting enough material directly from the column (i.e. preventing the ninhydrine reaction) during an appropriate 10 min period. This material was hydrolyzed for 20 h at 110°C with 6 N HCl under vacuum : the hydrolyzate yielded only glycine by rechromatography. The identification of the unknown peak as glycineamide was confirmed by showing that it coeluted exactly with a glycineamide standard (Fig. 1). Having also observed that NPG reacts feebly with ninhydrine, we took advantage of the same 22-cm column for detecting NPG in biological fluids. Chromatographic conditions were adapted, as indicated under Materials and Methods, in order to achieve the elution of this secondary amine : at time 205 min, and with the temperature kept at 60°C, the pH of the sodium citrate buffer was raised to 6.28 and the ionic strength to 1.2 N (instead of employing the usual 0.35 N sodium (pH 5.28) citrate buffer for basic amino acids (Fig. 1)).

FIG. 2

Effects of increasing doses of 2-n.pentylaminoacetamide (NPG) given orally to fasted rats on glycineamide levels in forebrain (▲), cerebellum (■), medulla (●), kidney (△), liver (▽), and plasma (□), 2 hours after drug administration. The results, expressed as nanomoles/g wet wt. are the means $\pm$ SEM from 4-12 rats.

TABLE 1.

Content in 3 amino acids and of glycinamide in forebrain, cerebellum, medulla, liver, kidney, and plasma 2 h after the oral administration of 100 mg/kg b.wt of 2-n.pentylaminoacetamide. Results (mean $\pm$ SEM, n=12) are expressed as nmol/g fresh weight for tissues and as nmol/ml for plasma. * $p < 0.05$ using Student's t test on control unpaired values.

TREATMENT	FOREBRAIN		CEREBELLUM		MEDULLA		LIVER		KIDNEY		PLASMA	
	0	+	0	+	0	+	0	+	0	+	0	+
SER	1109 $\pm$ 50	1204 $\pm$ 8	1013 $\pm$ 30	1096 $\pm$ 65	743 $\pm$ 24	980 $\pm$ 104*	1174 $\pm$ 83	1061 $\pm$ 57	1288 $\pm$ 61	1187 $\pm$ 83	378 $\pm$ 13	346 $\pm$ 9
GLY	962 $\pm$ 48	1250 $\pm$ 64*	915 $\pm$ 56	1159 $\pm$ 78*	4589 $\pm$ 146	5131 $\pm$ 120*	3658 $\pm$ 175	3464 $\pm$ 164	6308 $\pm$ 255	5339 $\pm$ 293*	693 $\pm$ 34	671 $\pm$ 20
GABA	2197 $\pm$ 39	2260 $\pm$ 71	1266 $\pm$ 45	1400 $\pm$ 132	749 $\pm$ 21	792 $\pm$ 58	0	0	36 $\pm$ 3	34 $\pm$ 4	0	0
GLY-NH ₂	0	261 $\pm$ 19*	0	218 $\pm$ 17*	0	113 $\pm$ 10*	0	52 $\pm$ 3*	0	76 $\pm$ 6*	0	27 $\pm$ 3*

The Table is restricted to those amino acids that were metabolically related to 2-n.pentylaminoacetamide.

The glycinamide concentration in the forebrain was 261 nmol/g wet wt, on an average, 2 hours after NPG administration at a dose of 100 mg/kg b.wt (Table I). In addition, glycine concentration increased by 30 % over control value. There were no significant changes in the levels of other amino acids (Table I and data not shown). In the cerebellum, glycinamide concentration was 218 nmol/g wet wt and glycine levels increased by 27 %. In the medulla, glycinamide concentration was somewhat less (113 nmol/g wet wt) and glycine and serine levels increased by 12 % and 32 %, respectively (Table I). In liver, kidney, and plasma, glycinamide levels were lower than in the central nervous system, and the levels of amino acids were not significantly altered except for a 15 % decrease in glycine levels in the kidney (Table I and data not shown). In urines directly collected from the bladder after sacrifice, 2 hours after NPG administration, the concentration of glycinamide (5.00 ± 0.81 $\mu\text{mol/ml}$, mean $\pm$ SEM, $n=6$) was 7-fold higher than that of glycine (727 ± 189 nmol/ml).

In the next experiment, NPG was administered orally at increasing doses (10, 33, 66, and 100 mg/kg b.wt). Two hours later, the elevation of glycinamide in all tissues examined and in the plasma was roughly proportional to the dose of NPG received (Fig. 2). The prominent capacity of the forebrain and cerebellum to accumulate glycinamide was evident again, as demonstrated by the presence of significant amounts of this compound after a low 10 mg/kg b.wt dose of NPG. By contrast, a dose of 33 mg NPG/kg b.wt was required for observing significant levels of glycinamide in medulla, liver and plasma, and an even higher dose of NPG (66 mg/kg b.wt) for noting the same phenomenon in kidney.

FIG. 3

Time course of 2-n.pentylaminoacetamide (NPG : ○) and glycinamide (●) levels in forebrain, cerebellum, medulla, plasma, liver, muscle (gastrocnemius), and kidney, following the oral administration of 100 mg/kg body weight *n*-pentylglycinamide. The results, expressed in nanomoles/g wet wt, are the means $\pm$ SEM from 4 rats.

Fig. 3 illustrates the time course of variations of NPG and glycineamide levels in forebrain, cerebellum, medulla, plasma, liver, muscle and kidney, after the oral 100 mg/kg b.wt dose of NPG. The concentrations of both NPG and glycineamide were maximal after 1 hour in all tissues examined and in plasma while neither NPG nor glycineamide could be detected anymore after one day. Interestingly, the concentration of glycineamide was at least 2-fold higher than that of NPG, after 1 hour only, in the forebrain and cerebellum, and after 2 hours in the medulla. By contrast, the concentration of NPG remained markedly higher than that of glycineamide, for at least 4 hours, in the kidney, and somewhat higher, for 1-2 hours, in liver and muscle. When considering the relatively low plasma levels of NPG and glycineamide at all times, it is clear that the tissues investigated concentrated both compounds actively (Fig. 3). Finally, the concentrations of NPG and glycineamide in urines collected over a 24 hours period were, respectively, 11.71 ± 1.27 and 6.60 ± 0.36 $\mu\text{mol/ml}$ (mean $\pm$ SEM, $n = 4$), i.e. much higher than in all body tissues tested, including kidney.

TABLE 2

Content of forebrain, cerebellum, medulla, liver, muscle, plasma, kidney and urines in A/ glycineamide, glycine and serine 2 h after the oral administration of 66 mg/kg b.wt glycineamide, and B/ glycine and serine, 30 min after the intraperitoneal injection of 450 mg/kg b.wt glycine. Results (mean $\pm$ SEM, $n=5$ in A, and 4 in B) are expressed as nmol/g fresh weight for tissues and as nmol/ml for plasma and urines. * $p < 0.05$ using Student's t test on control unpaired values in Table 1.

TREATMENT	A: GLYCINAMIDE (orally)			B: GLYCINE (intraperitoneally)	
	GLY- NH_2	GLY	SER	GLY	SER
FOREBRAIN	18 $\pm$ 3	967 $\pm$ 27	1155 $\pm$ 40	1257 $\pm$ 100*	1392 $\pm$ 76*
CEREBELLUM	8 $\pm$ 4	884 $\pm$ 82	1000 $\pm$ 51	1426 $\pm$ 46	1210 $\pm$ 98
MEDULLA	7 $\pm$ 5	4899 $\pm$ 186	763 $\pm$ 44	4831 $\pm$ 252*	783 $\pm$ 32*
LIVER	49 $\pm$ 5	3457 $\pm$ 328	1271 $\pm$ 167	19190 $\pm$ 1342	4658 $\pm$ 662
MUSCLE	38 $\pm$ 4	3059 $\pm$ 257	714 $\pm$ 89	ND	ND
PLASMA	19 $\pm$ 2	605 $\pm$ 57	356 $\pm$ 21	3732 $\pm$ 186*	631 $\pm$ 35*
KIDNEY	60 $\pm$ 4	5568 $\pm$ 408	1247 $\pm$ 60	17193 $\pm$ 338	2570 $\pm$ 85
URINES	2896 $\pm$ 984	5090 $\pm$ 1343	106 $\pm$ 22	1675 $\pm$ 203	198 $\pm$ 21

ND : not determined

In a first control experiment, the oral administration of glycineamide at a dose of 66 mg/kg b.wt. (equimolar to a 100 mg/kg dose of NPG) led, 2 h later, to the elimination of relatively large quantities of glycineamide in urines directly collected from the bladder (2.896 $\mu\text{mol/ml}$ on an average : Table 2). At the same time, the levels of glycineamide were low in the plasma (19 nmol/ml). The ability of tissues to store glycineamide was mediocre, as indicated by the fact that glycineamide concentrations in brain, cerebellum, and medulla were similar to those in the plasma, and those in liver and voluntary muscle 2-fold higher only (Table 2). Glycineamide exerted no significant effects on the free amino acid spectrum of all biological fluids examined (Tables 1 and 2, and data not shown).

In a last control experiment, a limited attempt was made to introduce exogenous glycine directly into the central nervous system : the dose of 450 mg/kg b.wt. of glycine injected intraperitoneally was 10 times higher, on a molar basis, than the oral dose of 100 mg/kg b.wt. of NPG currently

utilized. When compared to control animals (Table 1), those sacrificed 30 min after the glycine injection showed (Table 2) 5.4-, 5.2-, and 2.7-fold rises in glycine levels and 1.7-, 4.0-, and 2.0-fold rises in serine levels in, respectively, plasma, liver, and kidney. Glycine and serine levels in forebrain and cerebellum increased by 19 to 56 % only, these modest changes being probably due largely, if not exclusively, to a contamination by plasma amino acids. There were no significant changes in the levels of other amino acids (Tables 1 and 2, and data not shown).

Discussion

The analysis of urines collected for 24 hours after a single oral dose of 100 mg NPG/kg b.wt indicated that 28 ± 2 % of the dose were eliminated unchanged and 16 ± 1 % as glycinamide through the kidneys. Since no NPG and/or glycinamide could be detected in rat tissues after 24 hours (Fig. 1), it follows that a sizable proportion of the NPG administered had been converted into other derivatives. The process of NPG varied with the tissue examined. The kidney was quite active at concentrating and eliminating NPG without further metabolism (Fig. 1). By contrast, a relatively prolonged (at least 4 hours) accumulation of glycinamide was noted in the other tissues, that was most marked in the central nervous system. If we consider another index of the capacity to store glycinamide by referring the level of glycinamide to that of glycine (an obvious derivative), the ratio of glycinamide over total free glycine contents was 20.9 % in forebrain, 18.8 % in cerebellum, 2.2 % in medulla, 4.0 % in plasma, 1.5 % in liver, and 1.4 % in kidney (Table 1). The capacity of the medulla to store glycinamide may, in fact, be higher than that inferred from this ratio or from the glycinamide concentration expressed on a wet wt basis if we utilize a third index, the protein concentration. This concentration (expressed in mg/g wet wt, mean $\pm$ SEM, $n = 16$) varied largely with the tissue considered, being 82 ± 2 , 88 ± 2 , 49 ± 1 , 205 ± 7 , and 141 ± 2 in, respectively, the forebrain, cerebellum, medulla, liver, and kidney. The central nervous system appears, on this basis, to be even more efficient at storing glycinamide and, within this system, the medulla may be almost as active as the cerebellum. At any rate, the relationship between glycinamide and glycine pool(s) might obviously vary with the portion of the central nervous system investigated, when considering that glycine levels in the medulla were 4.8-fold higher than those in the forebrain (Table 1).

Fig. 2 indicates that the dose of 100 mg NPG/kg b.wt used in the preceding experiments was apparently easily metabolized *in vivo* by the tissues examined, as the conversion of NPG into glycinamide showed no sign of saturation in the 10-100 mg/kg b.wt range.

The data indicate not only that the brain was a privileged organ for concentrating glycinamide derived from NPG, they also show that glycinamide was not an end product of NPG metabolism. If we assume that the excess of glycine accumulated in the central nervous system, 2 hours after NPG administration (Table 2), was derived from locally accumulated glycinamide, it can be easily inferred that 52 %, 53 %, and as much as 83 % of this glycinamide had already been converted into glycine in, respectively, the forebrain, cerebellum, and medulla. This rough (under)estimation neglects any metabolic oxydation of the glycine produced, its conversion into serine, and/or its incorporation into proteins.

A comprehensive study of the fate of all 2-n.pentylaminoacetamide derivatives, including glycine (and serine) incorporation into proteins, will require the use of NPG radiolabelled on either the glycinamide or pentyl moieties. It is tempting, however, to outline a general scheme of NPG metabolism in rat, based on the present data. The most likely hypothesis is

that the pentyl moiety of NPG taken up orally facilitates the passive transfer of the drug through gut epithelium and the blood-brain barrier, and that its dealkylation within the nervous system then yields glycine, and serine among the major metabolites. As organs other than the central nervous system doubtless contribute to degrade NPG, glycine derived from NPG in peripheral tissues such as liver, may conceivably be also taken up by the central nervous system then converted into glycine. This mechanism is unlikely, however, considering the low capacity of tissues to concentrate plasma glycine after an oral administration of glycine (Table 2).

From the present data, it is already evident that NPG might act as a prodrug delivering glycine into the central nervous system, with glycine playing, in turn, the role of a glycine reservoir. In this respect, preliminary *in vitro* data indicate that a deprenyl-sensitive MAO-B activity present in brain mitochondria is able to liberate glycine from the secondary amine NPG and that brain microsomes slowly form glycine from glycine (not shown). The efficiency of NPG as a local glycine precursor thus contrasts with the difficulty of orally taken glycine to gain access to the brain, and with the poor brain penetration of glycine administered intraperitoneally (Table 2), the latter finding being expected as glycine is known to penetrate slowly the blood-brain barrier [9].

Further investigation will be required to explore the direct and indirect participation of orally taken NPG in the presynaptic transport, accumulation and metabolism of glycine, and in the recycling of glycine binding to postsynaptic glycine receptors.

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