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DIAZEPAM METABOLISM AND ANTICONVULSANT ACTIVITY IN NEWBORN ANIMALS

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Abstract—Diazepam metabolism was studied *in vivo* and *in vitro* in newborn and adult rats and guinea-pigs. Both newborn rats and guinea-pigs are more sensitive to the antimetrazol action of diazepam than the adult animals. The difference in activity was about fifteen times for rats but only twice for guinea-pigs. This difference may be explained partly by the different levels of diazepam and its metabolites in the brain of newborn and adult animals. The total benzodiazepines present in the brain of newborn rats and guinea-pigs were always higher than in adult animals. Studies carried out with liver microsomal enzymes indicate that the metabolism in newborn animals is considerably reduced in respect to adult rats and guinea-pigs.

IT HAS been reported previously that age may be an important factor in determining the effect of a drug. This has been ascribed partly to the fact that age influences the liver microsomal enzymes which are considered important for drug metabolism.[1] In particular it is known that liver microsomal enzyme activity in newborn animals, including rats and guinea-pigs, is deficient.[2,3] As a part of a systemic study on diazepam metabolism in several animal species and under different experimental conditions[4,5] we have investigated the metabolism *in vitro* and *in vivo* as well as the anticonvulsant activity of diazepam in newborn and adult rats and guinea-pigs. Previous studies have established that in adult rats diazepam is metabolized *in vitro* by a process of N_1-demethylation and C_3-hydroxylation[6] while adult guinea-pigs show only the N_1-demethylation.[7] *In vivo* diazepam administration results in the accumulation of *N*-demethyldiazepam in the brain of guinea-pigs[8] but not of rats.[9] Consequently, due to the pharmacological activity of *N*-demethyldiazepam[4] the duration of the antimetrazol effect after diazepam administration is longer lasting in guinea-pigs than in rats.[8]

MATERIALS AND METHODS

Animals

Newborn (body wt 7 g) and adult (body wt 150 g) male Sprague–Dawley rats and newborn (body wt 90 g) and adult (body wt 250 g) male Albino guinea-pigs were used in all experiments.

Preparation of liver microsomes

Animals were killed and the livers were immediately removed and homogenized in ice-cold 1·15 per cent KCl solution (1:4 w/v) with a Teflon-glass homogenizer. The homogenate was centrifuged at 9000 *g* for 20 min and the supernatant fraction

was centrifuged again at 105,000 *g* for 1 hr (rotor 40'-Beckman Model L ultracentrifuge).

Incubation in vitro

The method used was similar to that described by Kato *et al.*[10] The incubation mixture (5 ml) consisted of NADP (1·5 μmoles), glucose-6-phosphate (50 μmoles), glucose-6-phosphate dehydrogenase (0·5 units), magnesium chloride (25 μmoles), nicotinamide (50 μmoles), 1·4 ml of 0·2 M phosphate buffer, the substrate (diazepam; *N*-demethyldiazepam; *N*-methyloxazepam or oxazepam) dissolved in ethanol and added in amounts ranging from 10 to 500 μg and 0·45 ml of 1·15 per cent KCl. The microsomal suspension added to the incubation mixture was 2·5 ml, equivalent to 1 g of liver, for adult animals and 4 ml, equivalent to 1·6 g of liver, for newborn animals. The amount of microsomal proteins incubated was the same in both cases (24 mg). The incubation was carried out at 37° for 10 min under air in a Dubnoff metabolic shaker.

Drug administration

In the experiments performed *in vivo* newborn and adult rats or guinea-pigs were injected subcutaneously with diazepam at a dose of 5 mg/kg. In the experiments performed in order to establish a correlation between antimetrazol activity and brain levels of diazepam and its metabolites, diazepam was dissolved in a solvent containing propyl glycol, glycofurol, benzyl alcohol and water (30:30:2:48) and administered by subcutaneous injection at doses corresponding to the ED_{50} of metrazol. The ED_{50} is the dose in mg/kg s.c. protecting 50 per cent of the animals from the convulsions induced by metrazol at a dose corresponding to ED_{100} i.p., the miminal dose able to induce convulsions in 100 per cent of the treated animals.

The method of Litchfield *et al.*[11] was used for the calculation of the fiducial limits. The determinations and the treatments of the animals were performed always at 2 p.m. to avoid the effect of diurnal rhythm.

Chemical determinations

Assay of benzodiazepines. The samples to be analyzed (microsomal preparations or brain extracts) were prepared according to the method previously described for the analysis of several benzodiazepines.[12,13] The analyses were carried out by using a gas chromatograph (Model GI Carlo Erba, Milan) equipped with a Ni 63 electron capture detector (Voltage 42 V). The stationary phase was OV_{17} 3 per cent on Gas Chrom Q (100–120 mesh) packed into a 1 m glass column (2 mm i.d., 4 mm o.d.). The flow rate of the carrier gas (nitrogen) was 42 ml/min and the column temperature was 240°.

Assay of metrazol (pentylenetetrazol). The animals were sacrificed by decapitation 10 min after i.p. injection of metrazol; one group of animals was pretreated with diazepam at doses corresponding to the ED_{50} 60 min before sacrifice. The brain was rapidly removed and homogenized in ice cold absolute ethanol 1:10 (w/v) as previously described.[14]

The gas chromatographic equipment was a Model GI (Carlo Erba, Milan) equipped with a hydrogen flame ionization detector.

The stationary phase was represented by OV_1 3 per cent on Gas Chrom Q (60–80 mesh) packed into a 2 m glass column (2 mm i.d., 4 mm o.d.). The flow rate of the carrier gas (nitrogen) was 41 ml/min, the column and the injector temperature were respectively 160 and 260°.

RESULTS

Comparison of the activity of the liver microsomal enzymes in newborn and in adult animals

In newborn rats there is relatively more C_3-hydroxylation than N_1-demethylation in respect to adult rats (Table 1), while in newborn guinea-pigs the N_1-demethylation is reduced in respect to adult guinea-pigs (Table 2). It is remarkable that newborn guinea-pigs have the capacity to hydroxylate diazepam while in adult guinea-pigs *N*-methyloxazepam was never found. Over 90 per cent of the diazepam incubated with liver microsomes from adult animals was recovered, partly unmetabolized and partly as metabolized products. In contrast to these results only 60 per cent of the diazepam was recovered after incubation of small amounts of diazepam (20–30 μg) with liver microsomes from newborn liver of rats or guinea-pigs. The incubation of amounts of diazepam greater than 100 μg, when the metabolic pathways of both *N*-demethylation and C_3-hydroxylation were operational, led to an increased recovery of the total benzodiazepines (about 80 per cent) for the liver of newborn animals.

TABLE 1. METABOLISM *in vitro* OF DIAZEPAM BY LIVER MICROSOMES OF NEWBORN AND ADULT RATS

Rat	Diazepam added (μg)	Substrate and metabolites recovered after 10 min incubation at 37°		
		Diazepam* (μg)	*N*-Demethyl diazepam† (μg)	*N*-Methyl oxazepam‡ (μg)
Newborn	20	9·65 ± 0·01	—	< 0·05
	30	14·74 ± 0·03	—	1·94 ± 0·02
	40	20·66 ± 0·03	—	3·73 ± 0·01
	60	32·99 ± 0·05	< 0·05	7·16 ± 0·01
	100	63·94 ± 0·01	0·39 ± 0·01	15·57 ± 0·03
	200	125·50 ± 0·03	2·03 ± 0·01	42·15 ± 0·01
	300	213·24 ± 0·03	3·87 ± 0·02	48·14 ± 0·02
	500	400·11 ± 0·05	1·23 ± 0·01	40·00 ± 0·02
Adult	20	14·50 ± 0·05	0·52 ± 0·01	4·10 ± 0·02
	30	21·85 ± 0·03	0·79 ± 0·01	5·60 ± 0·02
	40	30·40 ± 0·03	1·05 ± 0·02	6·80 ± 0·01
	60	47·32 ± 0·01	1·35 ± 0·02	9·31 ± 0·10
	100	82·50 ± 0·01	1·97 ± 0·01	14·00 ± 0·08
	200	166·60 ± 0·02	3·92 ± 0·03	25·62 ± 0·05
	300	253·12 ± 0·10	5·57 ± 0·02	37·12 ± 0·10
	500	461·12 ± 0·08	5·00 ± 0·02	35·00 ± 0·12

* Substrate unmetabolized.
† Substrate metabolized by *N*-demethylation.
‡ Substrate metabolized by C_3-hydroxylation.

Tables 3 and 4 show the *in vitro* metabolism of *N*-methyloxazepam by liver microsomes of newborn and adult rats and guinea-pigs. The liver from both newborn, rats

and guinea-pigs showed a *N*-demethylation activity which was lower than the liver from adult animals. *N*-Demethyldiazepam and oxazepam tested at 10–500 μg are not metabolized by the liver of newborn rats or guinea-pigs.

TABLE 2. METABOLISM *in vitro* OF DIAZEPAM BY LIVER MICROSOMES OF NEWBORN AND ADULT GUINEA-PIGS

Guinea-pigs	Diazepam added (μg)	Substrate and metabolites recovered after 10 min incubation at 37°		
		Diazepam* (μg)	*N*-Demethyldiazepam† (μg)	*N*-Methyloxazepam‡ (μg)
Newborn	20	7·52 ± 0·02	6·00 ± 0·01	—
	30	10·86 ± 0·03	8·75 ± 0·01	—
	40	18·73 ± 0·01	12·03 ± 0·02	—
	60	30·90 ± 0·02	12·83 ± 0·01	—
	100	60·88 ± 0·03	11·50 ± 0·01	< 0·05
	200	140·74 ± 0·05	6·21 ± 0·03	3·74 ± 0·02
	300	200·40 ± 0·03	5·06 ± 0·01	5·43 ± 0·01
	500	364·41 ± 0·05	1·56 ± 0·01	6·12 ± 0·01
Adult	20	< 0·05	17·91 ± 0·08	—
	30	01·80 ± 0·03	19·00 ± 0·03	—
	40	17·90 ± 0·02	21·80 ± 0·02	—
	60	21·70 ± 0·02	35·55 ± 0·10	—
	100	38·21 ± 0·01	50·90 ± 0·12	—
	200	112·60 ± 0·01	77·40 ± 0·10	—
	300	215·10 ± 0·02	84·22 ± 0·10	—
	500	419·30 ± 0·12	80·33 ± 0·12	—

* Substrate unmetabolized.
† Substrate metabolized by *N*-demethylation.
‡ Substrate metabolized by C_3-hydroxylation.

TABLE 3. METABOLISM *in vitro* OF *N*-METHYLOXAZEPAM BY LIVER MICROSOMES OF NEWBORN AND ADULT RATS

Rat	*N*-Methyloxazepam added (μg)	Substrate and metabolites recovered after 10 min incubation at 37°	
		N-methyloxazepam* (μg)	Oxazepam† (μg)
Newborn	10	7·31 ± 0·10	< 0·01
	20	13·31 ± 0·03	< 0·01
	30	19·22 ± 0·05	< 0·01
	60	42·67 ± 0·03	< 0·01
	100	66·81 ± 0·05	1·09 ± 0·01
	200	143·12 ± 0·03	2·20 ± 0·03
	500	410·99 ± 0·10	6·97 ± 0·02
Adult	10	9·05 ± 0·01	0·80 ± 0·01
	20	18·75 ± 0·03	1·05 ± 0·02
	30	28·00 ± 0·01	1·83 ± 0·01
	60	55·33 ± 0·02	3·70 ± 0·03
	100	95·00 ± 0·01	4·12 ± 0·01
	200	193·12 ± 0·03	5·41 ± 0·06
	500	497·03 ± 0·02	2·20 ± 0·01

* Substrate unmetabolized.
† Substrate metabolized by *N*-demethylation.

TABLE 4. METABOLISM *in vitro* OF *N*-METHYLOXAZEPAM BY LIVER MICROSOMES OF NEWBORN AND ADULT GUINEA-PIGS

Guinea-pigs	*N*-Methyloxazepam added (μg)	Substrate and metabolites recovered after 10 min incubation at 37°	
		N-Methyloxazepam* (μg)	Oxazepam† (μg)
Newborn	10	—	2·05 ± 0·01
	20	—	4·62 ± 0·03
	30	< 0·01	5·75 ± 0·02
	60	38·10 ± 0·12	10·26 ± 0·11
	100	82·61 ± 0·15	7·00 ± 0·05
	200	187·70 ± 0·31	5·00 ± 0·03
	500	494·01 ± 0·50	2·78 ± 0·02
Adult	10	7·86 ± 0·01	< 0·01
	20	15·61 ± 0·02	< 0·01
	30	21·55 ± 0·03	< 0·01
	60	42·86 ± 0·01	—
	100	79·61 ± 0·02	0·56 ± 0·01
	200	165·33 ± 0·10	3·18 ± 0·02
	500	394·66 ± 0·09	5·12 ± 0·03

* Substrate unmetabolized.
† Substrate metabolized through *N*-demethylation.

In evaluating these results it is important to consider that the incubated microsomes are obtained from a wet weight of liver which is about 1·6 times greater for newborn than for adult animals. Therefore the metabolism of diazepam is considerably lower in newborn than in adult liver.

Comparison of brain levels of diazepam and its metabolites after diazepam administration to newborn and adult rats and guinea-pigs

Table 5 shows the brain levels of diazepam and its metabolites at three times after subcutaneous administration of diazepam (5 mg/kg), to newborn and adult rats.

TABLE 5. BRAIN LEVELS OF DIAZEPAM AND ITS METABOLITES AFTER S.C. DIAZEPAM ADMINISTRATION (5 mg/kg) TO NEWBORN AND ADULT RATS

Time after administration (min)		Brain levels (μg/g ± S.E.)*			
		D	DD	MOX	OX
30	nb†	1·35 ± 0·03	0·83 ± 0·05	< 0·02	< 0·02
	a†	0·60 ± 0·01	0·02 ± 0·005	< 0·02	< 0·02
60	nb	2·13 ± 0·02	1·70 ± 0·01	0·09 ± 0·004	0·04 ± 0·002
	a	0·44 ± 0·01	0·28 ± 0·006	< 0·02	< 0·02
180	nb	1·06 ± 0·03	1·15 ± 0·03	0·07 ± 0·003	0·08 ± 0·001
	a	0·24 ± 0·04	0·01 ± 0·002	< 0·02	< 0·02

* D = Diazepam; DD = *N*-demethyldiazepam; MOX = *N*-methyloxazepam; OX = Oxazepam. Each point is the mean of five animals.
† nb = Newborn, a = adult.

The results obtained indicate that the brain concentration of diazepam reaches a higher value in newborn than in adult rats. Similarly *N*-demethyldiazepam accumulates more in the brain of newborn than adult rats. It is remarkable that the brain of newborn rats contains low levels of *N*-methyloxazepam and oxazepam while these metabolites were not measurable in the brain of adult rats. Table 6 shows that *N*-demethyldiazepam accumulates more in the brain of newborn than adult guinea-pigs after a subcutaneous administration of diazepam. Diazepam concentrations in brain were higher at 30 min but lower at longer times in newborn than in adult guinea-pigs. Oxazepam was present in the brain of newborn guinea-pigs but at low concentrations.

TABLE 6. BRAIN LEVELS OF DIAZEPAM AND ITS METABOLITES AFTER S.C. DIAZEPAM ADMINISTRATION (5 mg/kg) TO NEWBORN AND ADULT GUINEA-PIGS

Time after administration (min)		Brain levels (μg/g $\pm$ S.E.)*			
		D	DD	MOX	OX
30	nb†	0·43 $\pm$ 0·03	0·82 $\pm$ 0·06	< 0·02	< 0·02
	a†	0·22 $\pm$ 0·01	0·20 $\pm$ 0·02	< 0·02	< 0·02
60	nb	0·10 $\pm$ 0·01	2·69 $\pm$ 0·03	< 0·02	0·093 $\pm$ 0·02
	a	0·35 $\pm$ 0·02	0·61 $\pm$ 0·03	< 0·02	< 0·02
180	nb	0·06 $\pm$ 0·001	1·88 $\pm$ 0·09	< 0·02	0·117 $\pm$ 0·01
	a	0·11 $\pm$ 0·01	0·66 $\pm$ 0·03	< 0·02	< 0·02

* D = Diazepam, DD = *N*-demethyldiazepam, MOX = *N*-methyloxazepam, OX = Oxazepam. Each point is the mean of five animals.
† nb = Newborn, a = adult.

Antimetrazol activity of diazepam in newborn and in adult rats and guinea-pigs

Table 7 summarizes the results obtained. Newborn rats require a higher dose of metrazol than adult rats to induce convulsions. Although the administered dose of metrazol is different, the level of metrazol in the brain is similar both in newborn and in adult rats. Diazepam activity is about fifteen times higher in newborn than in adult rats but also in this case the total level of benzodiazepines in the brain is similar in the two experimental conditions.

On the contrary, in newborn and in adult guinea-pigs the convulsant dose of metrazol and its brain concentration are comparable.

Diazepam is about twice as active in newborn in respect to adult guinea-pigs. It is of interest that diazepam, *N*-demethyldiazepam and oxazepam are present in the brain of both newborn and adult guinea-pigs although the total amount of benzodiazepines necessary to exert the anticonvulsant activity is lower in the brain of newborn than adult guinea-pigs.

DISCUSSION

The results reported here confirm that newborn animals respond to drugs in a different manner than adults. Both newborn rats and guinea-pigs are more sensitive to the antimetrazol action of diazepam than the adult animal. The extent of the difference in activity was about fifteen times for rats and only twice for guinea-pigs.

TABLE 7. BRAIN LEVELS OF BENZODIAZEPINES AND METRAZOL AFTER ADMINISTRATION OF DIAZEPAM AT THE ED_{50} DOSE OF METRAZOL, TO NEWBORN AND ADULT RATS AND GUINEA-PIGS

Time* (min)		Metrazol ED_{100} i.p. (mg/kg)	Diazepam ED_{50} s.c. (mg/kg) and 95% fiducial limits	Brain levels (μg/g) ± S.E.†			
				D	DD	OX	MT
				Rats			
60	nb‡	160	0.058 (0·040–0·084)	0·207 ± 0·02	< 0·01	0·02	105·81 ± 2·3
60	a	82	0·890 (0·751–1·055)	0·082 ± 0·008	0·062 ± 0·01	0·02	196·04 ± 2·0
				Guinea-pigs			
60	nb‡	78	0·212 (0·159–0·282)	0·092 ± 0·008	0·186 ± 0·005	0·064 ± 0·005	104·16 ± 5·2
60	a	80	0·393 (0·328–0·472)	0·196 ± 0·010	0·355 ± 0·017	0·027 ± 0·002	106·08 ± 4·0

* Minutes between diazepam and metrazol administration.
† D = Diazepam, DD = *N*-demethyldiazepam, OX = Oxazepam, MT = Metrazol concentration in the brain 10 min after its administration.
‡ nb = Newborn, a = adult.

In interpreting these differences it is very important to establish to what extent the metabolism of diazepam was influenced by age as the three main metabolites of diazepam, namely *N*-demethyldiazepam, *N*-methyloxazepam and oxazepam, have anticonvulsant activity similar to the parent compound.[4] Studies with liver microsomes clearly indicate that the C_3-hydroxylation and *N*-demethylation of diazepam are considerably reduced per unit of liver weight in newborn in respect to adult rats or guinea-pigs. Similar conclusions are valid for the metabolism of *N*-methyloxazepam, while *N*-demethyldiazepam was not metabolized by the liver of newborn rats or guinea-pigs. However, the interpretation of these results is difficult because at low concentrations of diazepam or *N*-methyldiazepam the recovery in terms of known metabolites plus the unmetabolized substrate is much lower in newborn than adult animals indicating that a strong binding to proteins or alternative metabolic pathways may be present. From these results it is impossible to calculate the kinetic constant (K_m and V_{max}) for the substrates utilized. Even if the liver microsomal activity is reduced in newborn in respect to adult animals the ratio between hydroxylation and *N*-demethylation when diazepam is utilized as substrate is higher for the newborns than for the adults. The data *in vitro* cannot be easily correlated with that obtained *in vivo* when diazepam was given s.c. at the dose of 5 mg/kg. In fact *N*-demethyldiazepam accumulates in the brain of newborn rats more than in adults while *N*-methyloxazepam is present in the brain of newborns but not in adult animals. A similar situation applies also to guinea-pigs. It should be underlined that the total benzodiazepines present in the brain of newborn rats and guinea-pigs were always higher in newborns than in adult animals. This may be taken as an indication that the various enzymatic steps involved in the metabolism of diazepam are slower in newborns than in adults. The accumulation of a given diazepam metabolite in the newborn brain may be related not only to the liver metabolism but also to other factors such as the blood flow to the brain, the lipid composition, the binding to brain proteins. The importance of these factors may differ according to the administered dose. It is evident that when low doses of diazepam were injected s.c. (corresponding to the anticonvulsant ED_{50}) the level of diazepam metabolites in the brain of newborns is quite different than when high doses were given because *N*-demethyldiazepam is present in higher concentrations in the brain of adults in respect to newborn animals. It is remarkable that at equal anticonvulsant activity the brain concentrations in the various experimental groups (newborn and adult rats and guinea-pigs) are different by a factor of about 3 while the doses are different by a factor of about 15. Similarly the metrazol concentrations in the brains in the various experimental groups are identical while the dose may differ by a factor of 2 (newborn and adult rats). It may be concluded that newborn animals appear to be more sensitive to the anticonvulsant action of diazepam at an equal brain concentration of metrazol. This difference is more marked for newborn rats than for guinea-pigs. The newborn rats are, however, less sensitive, in terms of dose but not brain levels, to metrazol than adult rats or newborn or adult guinea-pigs. It should be recalled however that rats are born less mature than guinea-pigs as shown by the degree of myelinization[15] and by the brain composition of amino acids[16,17] biogenic amines[18,19] and enzymes.[20] The different sensitivity of newborn and adult rats to metrazol and diazepam may be added to the list of biochemical and functional differences found during development. Finally our findings in newborn animals support previous

clinical determinations showing that premature babies and infants metabolize diazepam to a lesser extent than adults.[21]

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