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EFFECT OF CYCLOPHOSPHAMIDE ON THE ACTIVITY AND DISTRIBUTION OF PENTOBARBITAL IN RATS

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Abstract—Cyclophosphamide (CP) injected at different treatment schedules into CD male rats, prolongs narcosis after either parenteral or oral administration of pentobarbital. However, it takes 7 days before any change appears after CP pretreatment. The level of pentobarbital in blood and brain at different times after injection was higher in CP pretreated than in untreated animals. Since previous studies reported a decreased rate of inactivation of drugs after treatment with cytotoxic agents, experiments were performed to ascertain whether, beside the impairment of the metabolism, the animals respond differently to the same brain concentration of pentobarbital.

PREVIOUS reports from these laboratories have demonstrated that cyclophosphamide (CP) and other cytotoxic drugs markedly inhibit in rats the activity of the liver microsomal enzymes *in vitro* 7 days after their administration in single or repeated doses.[1,2] Since these enzymes are important in determining the rate of metabolism and thus the duration of activity of many drugs, it was decided to carry out an investigation *in vivo*. Cyclophosphamide has been selected as a representative cytotoxic agent because of its extensive utilization in clinical practice for the chemotherapy of cancer. Pentobarbital has been chosen as a prototype of drugs that are metabolized and inactivated by liver microsomal enzymes.[3] The interaction between cyclophosphamide and pentobarbital has been studied at various schedules of treatment and under different experimental conditions, keeping in mind that these drugs may be combined also in the clinical situation.

The results obtained indicate that cyclophosphamide can markedly inhibit the disposition of pentobarbital and considerably increase the efficacy of this barbiturate.

EXPERIMENTAL

CD male rats (150 ± 20 g body wt), obtained from Charles River Italia, were used.

All animals were kept at a room temperature of 22°, and at a relative humidity of 60 per cent in cages of Makrolon (47 × 25 × 15 cm for six rats) and with free access to food (Diet Charles River 4RF21) and water during the experiments.

Cyclophosphamide was kindly supplied by the Drug Research and Development Chemotherapy, N.C.I., Bethesda, Md. U.S.A. and Zilliken Co. (Genova, Italy). Pentobarbital was a gift from Abbott, Chicago, Ill. (U.S.A.).

For intraperitoneal, intravenous and oral administration both cyclophosphamide and pentobarbital were dissolved in water (5 ml/kg for intravenous injection, and 10 ml/kg for intraperitoneal and oral administration).

The hypnotic effect of pentobarbital was determined as the time of loss of the righting reflex. The *in vivo* metabolism was determined as the rate of disappearance of pentobarbital from blood and brain. Blood and brain concentrations of pentobarbital were measured according to the spectrophotometric method described by Brodie *et al.*[4] with minor modifications. The statistical evaluation of the results was performed by an analysis of variance.

The regression line of the logarithms of the pentobarbital concentration on time was calculated using the method of least squares starting 20 min after injection. The significance of the regression, non linearity and nonparallelism was assessed using Fisher's F ratio. The half-life ($T_{\frac{1}{2}}$) and the volume of distribution (Vd) were calculated from the estimated slope of the regression line and the extrapolated intercept.

RESULTS

The results reported in Table 1 indicate that cyclophosphamide (CP), administered at the dose of 120 mg/kg i.p., markedly increases the sleeping time induced by pentobarbital, when injected at a time interval of 7 or 14 days, but not at shorter times, before pentobarbital. This increase in the duration of narcosis beginning on day 7 after CP administration is in good agreement with the impairment of the liver microsomal enzyme activity observed in previous studies[1] under the same experimental conditions.

TABLE 1. SLEEPING TIME INDUCED BY PARENTERAL ADMINISTRATION OF PENTOBARBITAL (35 mg/kg i.p.) IN RATS PRETREATED WITH CYCLOPHOSPHAMIDE (CP) (120 mg/kg i.p.)

Time between CP and pentobarbital	Sleeping rats* (%)	Sleeping time (min ± S.E.)
—	70	46 ± 3
1 hr	70	40 ± 3
4 hr	70	35 ± 3
14 hr	50	43 ± 2
1 day	50	41 ± 2
3 days	80	53 ± 3
7 days	100	122 ± 11†
14 days	80	110 ± 9†

* Ten animals per group.
† $P < 0{\cdot}01$ (analysis of variance).

Doses of CP as low as 40 mg/kg i.p. are still effective in prolonging the activity of pentobarbital (see Table 2). Also repeated treatment with CP (20 mg/kg i.p. daily for 6 days) increases the duration of narcosis induced by pentobarbital with a peak of effect occurring 9 days after the last CP treatment (see Table 3).

Since barbiturates are often administered by oral route, the influence of CP on pentobarbital administered orally has been investigated. Again, 7 days after a single treatment with CP, rats slept significantly longer than controls (see Table 4).

It is evident from Tables 5 and 6 that pentobarbital levels are higher in blood and brain of animals pretreated 7 days before with CP, both intraperitoneally (Table 5) and orally (Table 6). The half-life of pentobarbital in blood has been increased by the i.p. treatment with CP, while the volume of distribution was not changed.

TABLE 2. SLEEPING TIME INDUCED BY PENTOBARBITAL 7 DAYS AFTER A SINGLE TREATMENT OF CYCLOPHOSPHAMIDE (CP) INJECTED AT DIFFERENT DOSES

CP (mg/kg i.p.)	Sleeping rats* (%)	Sleeping time (min ± S.E.)
—	70	46 ± 3
40	60	81 ± 5†
80	90	78 ± 8†
120	100	122 ± 11†

* Ten animals per group.
† $P < 0.01$ (analysis of variance).

TABLE 3. SLEEPING TIME INDUCED BY PENTOBARBITAL (35 mg/kg i.p.) AT DIFFERENT TIME INTERVALS AFTER REPEATED TREATMENTS WITH CYCLOPHOSPHAMIDE (CP) (20 mg/kg i.p. × 6 days)

Time between last CP treatment and pentobarbital (days)	Sleeping rats* (%)	Sleeping time (min ± S.E.)
—	80	44 ± 3
1	90	69 ± 2†
6	80	98 ± 8†
9	100	142 ± 10†
12	60	74 ± 8†

* Ten animals per group.
† $P < 0.01$ (analysis of variance).

TABLE 4. SLEEPING TIME INDUCED BY ORAL ADMINISTRATION OF PENTOBARBITAL (80 mg/kg) IN RATS PRETREATED WITH CYCLOPHOSPHAMIDE (CP) (120 mg/kg i.p.)

Time between CP and pentobarbital	Sleeping rats* (%)	Sleeping time (min ± S.E.)
—	70	103 ± 9
1 hr	100	94 ± 10
3 days	100	140 ± 15
7 days	100	175 ± 16†

* Ten animals per group.
† $P < 0.01$ (analysis of variance).

TABLE 5. BLOOD AND BRAIN LEVELS OF PENTOBARBITAL AFTER PARENTERAL ADMINISTRATION (35 mg/kg i.v.) IN RATS 7 DAYS AFTER CYCLOPHOSPHAMIDE (CP) (120 mg/kg i.p.)

Time after pentobarbital (min)	Blood pentobarbital (mean ± S.E.)		Brain pentobarbital (mean ± S.E.)	
	Controls (μg/ml)	CP (μg/ml)	Controls (μg/ml)	CP (μg/ml)
1	26·6 ± 0·4	26·0 ± 0·3	24·4 ± 1·8	28·1 ± 1·9*
20	14·4 ± 0·3	19·3 ± 0·3†	19·7 ± 0·9	22·7 ± 0·7*
40	11·2 ± 0·4	14·3 ± 0·3†	14·6 ± 0·6	18·4 ± 0·7†
60	8·4 ± 0·5	11·3 ± 0·2†	10·4 ± 0·6	14·6 ± 0·3†
90	3·7 ± 0·3	7·4 ± 0·5†	4·5 ± 1	7·4 ± 0·7*
$T_{\frac{1}{2}}$ (min)	35	50‡	31	42
Vd (l/kg)	1·07	0·99	—	—

Each figure is the mean of five determinations.
* $P < 0.05$; † $P < 0.01$; relative to controls after analysis of variance.
‡ Fisher's *F* ratio for non parallelism is significant ($P < 0.01$).

TABLE 6. BLOOD AND BRAIN LEVELS OF PENTOBARBITAL AFTER ORAL ADMINISTRATION (80 mg/kg) IN RATS PRETREATED WITH CYCLOPHOSPHAMIDE (CP) (120 mg/kg i.p.)

Time after pentobarbital (min)		Pentobarbital (μg/ml or g; mean ± S.E.)			
		—	1 day‡	3 days‡	7 days‡
Blood	15	16·1 ± 0·6	16·6 ± 0·4	16·4 ± 0·7	15·4 ± 0·4
	45	18·0 ± 0·8	17·6 ± 0·4	18·0 ± 0·7	15·1 ± 0·6*
	90	14·1 ± 1·0	13·9 ± 0·5	17·7 ± 0·6*	14·0 ± 0·2
	180	13·2 ± 0·9	12·5 ± 0·7	15·2 ± 0·2	16·2 ± 0·2*
	240	7·0 ± 0·5	ND	5·9 ± 0·6	14·4 ± 0·4†
Brain	15	22·5 ± 1·5	ND	24·0 ± 1·0	28·4 ± 1·2*
	45	26·5 ± 0·6	ND	26·2 ± 0·7	32·6 ± 1·5†
	240	18·8 ± 1·0	ND	20·3 ± 0·9	25·3 ± 1·2†

ND—not determined.
Each figure is the mean of five determinations.
* $P < 0.05$; † $P < 0.01$; relative to controls after analysis of variance.
‡ Pentobarbital was given at the dose of 80 mg/kg per os on day 1, 3 or 7 after CP treatment.

In order to establish if the decrease of food intake and the loss of body weight induced by CP could be responsible for the increased efficacy of pentobarbital, the sleeping time of animals pair-fed with the group treated with CP (120 mg/kg i.p.) was compared with controls. Table 7 shows that, although an increase in sleeping time occurs in starved rats, the difference in sleeping time between pair-fed and CP treated rats is significant after analysis of variance.

Since it may be possible that, besides a reduced metabolism, also a change in sensitivity at the receptor sites could be involved in this prolonged effect of pentobarbital induced by CP, two groups of animals were treated with equiactive doses of pentobarbital (25 and 35 mg/kg for CP treated and control rats, respectively). Brain levels

TABLE 7. INFLUENCE OF BODY WEIGHT AND FOOD INTAKE ON THE SLEEPING TIME INDUCED BY PENTOBARBITAL (35 mg/kg i.p.)

Group	Treatment (mg/kg i.p.)	Body wt % increase after 7 days	Av. daily food intake (g/rat ± S.E.)	Sleeping rats* (%)	Sleeping time (min ± S.E.)
Controls	—	100	20 ± 2	60	43 ± 4
(diet *ad lib.*)	40	42	ND	60	81 ± 5†
	80	42	ND	90	78 ± 8†
	120	15	13 ± 4	90	105 ± 9§
Pair-fed‡	—	53	—	80	68 ± 11

ND—not determined.
* Ten animals per group.
† $P < 0.01$ (analysis of variance) in relation to controls.
‡ Pair-fed with the group receiving CP 120 mg/kg i.p.
§ $P < 0.01$ (analysis of variance) in relation to pair-fed rats.

At the beginning of the experiment, i.e. on the day of CP treatment, the body wt of the rats was 150 ± 5 g and after 7 days, on the day of pentobarbital treatment, the body wt of controls was 212 ± 3 g.

of pentobarbital were measured at the beginning of narcosis, at the end of narcosis, and 90 min after the injection of pentobarbital (Table 8).

CP pretreated animals showed no significant differences in brain drug levels compared with controls in any of the three experimental conditions.

TABLE 8. BRAIN PENTOBARBITAL LEVELS AT DIFFERENT TIMES AFTER ADMINISTRATION OF EQUIACTIVE DOSES OF PENTOBARBITAL

	Controls	CP (120 mg/kg i.p.) 7 days before
Beginning of narcosis		
Time (min ± S.E.)	7 ± 35″	8 ± 39″
Brain pentobarbital (μg/g ± S.E.)	24 ± 0·7	25 ± 0·5
End of narcosis		
Time (min ± S.E.)	48 ± 8′	56 ± 7′
Brain pentobarbital (μg/g ± S.E.)	16 ± 0·5	18 ± 0·5
90 min after pentobarbital‡		
Brain pentobarbital (μg/g ± S.E.)	12 ± 0·2	14 ± 1

Each figure is the mean of ten determinations.
‡ In this experiment controls slept 50 ± 4 min and CP pretreated rats 54 ± 6 min. Pentobarbital was given i.p. at the dose of 35 mg/kg to controls and 25 mg/kg to CP pretreated rats.

Furthermore, the time necessary to undergo sleep was the same in the two groups.

These results suggest that the increased effect of pentobarbital in CP pretreated rats is primarily the result of an impairment of the metabolic processes responsible for the disposition of pentobarbital.

DISCUSSION

Our findings show that the pharmacological effect of pentobarbital, administered either by parenteral or oral route, is markedly increased by a single or a repeated treatment with CP.

The question whether a decreased food intake and a concomitant reduction of body weight induced by CP could be relevant in explaining this effect has been only partially answered by experiments in pair-fed animals. A reduction of food intake caused by CP resulted in an increase of sleeping time. However, the effect of CP was more marked than the simple reduction of food intake. Blood and brain levels of pentobarbital after parenteral or oral administration of this drug were measured and indeed pentobarbital showed a higher plasma and brain concentration and a longer half-life in CP pretreated animals in respect to control rats.

Other data indicate that the sensitivity of CP-treated rats for pentobarbital is not changed because doses of the barbiturate equiactive in respect to controls resulted in equal brain levels of pentobarbital at the beginning and at the end of narcosis.

These observations are not in contrast with the hypothesis that a reduced activity of liver microsomal enzymes which inactivate pentobarbital may be responsible for the enhanced activity and the increased brain level of the barbiturate in CP-treated rats. Hietbrink and Du Bois[5] and Du Bois[6] showed that X-irradiation inhibits the development of the microsomal enzymes in the liver of male rats. That radiomimetic agents could behave like X-irradiation has been demonstrated by Tardiff and Du Bois[7] and by studies carried out in this Institute.[1,2] Bock *et al.*[8] report the inhibition of $\Delta^4 - 5\alpha$- and $\Delta^4 - 5\beta$-reductase activity for cortisone in the liver of rats pretreated with CP or other antitumoral agents. However, this effect on liver microsomal enzymes does not seem to be related to the level of CP itself in the liver because it takes more than 3 days before it appears. More likely the impairment of these liver microsomal enzymes is not of a competitive nature, but is related to liver toxicity due to a reduction in protein synthesis and availability of cofactors.

These studies have been carried out in normal animals but the situation may be more complex in tumor-bearing animals because it was shown that several transplantable tumors themselves inhibit the liver microsomal enzyme activity. The possible implications of these findings in the toxic and therapeutic responses to drugs in cancer patients may be of considerable significance in view of the large use of multiple drug combinations[9] in clinical oncology.

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REFERENCES

1. M. G. DONELLI, G. FRANCHI and R. ROSSO, *Eur. J. Cancer* **6**, 125 (1970).
2. M. G. DONELLI and S. GARATTINI, *Eur. J. Cancer* **7**, 361 (1971).
3. J. R. GILLETTE, *Adv. Pharmac.* **4**, 219 (1966).
4. B. B. BRODIE, J. J. BURNS, L. C. MARK, P. A. LIEF, E. BERNSTEIN and E. M. PAPPER, *J. Pharmac. exp. Ther.* **109**, 26 (1953).
5. B. E. HIETBRINK and K. P. DU BOIS, *Radiation Res.* **27**, 669 (1966).
6. K. P. DU BOIS, *Radiation Res.* **30**, 342 (1967).
7. R. G. TARDIFF and K. P. DU BOIS, *Archs int. Pharmacodyn. Ther.* **177**, 445 (1969).
8. J. BOCK, W. BRAUN, C. G. SCHMIDT and W. STAIB, *Arzneimittel-Forsch.* **21**, 2100 (1971).
9. D. P. RALL, *Cancer Res.* **27**, 2650 (1967).