NMR: 1·17 (t, J = 7·0 Hz, 3H, CH_3–CH_2–O–C =C–), 1·91 (s, 3H, CH_3– C=C–), 3·31 (q, J = 7·0 Hz, 2H, CH_3–CH_2–O–C=C–) and 7·62–8·25 (m,8H,ArH).

Hydrolysis of compound D with methanolic potash : Formation of 2-hydroxy-2′-methyl-3,3′-bi(1,4-naphthoquinone) (VI)

Compound D (150 mg) was heated with methanolic potassium hydroxide (2%, 10 ml) on a water bath. The heating was discontinued after 20 min when whole of the compound dissolved and the solution acquired a pink colour. It was cooled, diluted with water and acidified with dil HCl. The acidified solution was then extracted with chloroform, the latter washed with water and dried (anhyd. Na_2SO_4). It was concentrated and the residue crystallised from ethanol as light orange needles (50 mg), m.p. 294–98° (decomp.) (Found : C, 73·5; H, 3·9; $C_{21}H_{12}O_5$ requires: C, 73·3; H, 3·5%).

IR: 3400, 1668; 1650; UV : 253, 258, 290 and 335 (4·54, 4·61, 3·86 and 3·62).

NMR: $CDCl_3$ + $(CD_3)_2$ CO; 1·89 (s, 3H, CH_3–C= C–) and 7·63–8·28 (m,8H,ArH).

On the basis of the above data, the structure of this compound was assigned to be 2-hydroxy-2′-methyl-3,3′-bi(1,4-naphthoquinone (VI), since it could be re-acetylated to 2-acetoxy 2′-methyl-3,3′-bi(1,4-naphthoquinone) (IV) in pyridine solution.

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STUDIES ON THE *IN VITRO* EFFECT OF DIFFERENT BENZODIAZEPINES ON CHOLINESTERASE ACTIVITY OF HUMAN FETAL BRAIN

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ABSTRACT

An investigation on the *in vitro* effects of different benzodiazepines on human fetal brain cholinesterase activity at different gestational ages were studied. The enzyme activity was found to be inhibited in the cerebrum and cerebellum in a characteristic manner. Results of the kinetic pattern indicated that the catalytic function of the enzyme is mainly affected.

INTRODUCTION

CONSIDERABLE volume of evidence have accumulated regarding the pharmacological effects of different benzodiazepines[1–3]. Recently, this group of drugs has been used in early pregnancy for their minimal sedation and relief of tension. It has also been reported that diazepam can cross the placental barrier[4] in early pregnancy and thereby accumulate in fetal blood[5] and fetal tissues, particularly in brain[6–8]. Piercy *et al.*[9], observed that diazepam arrests the 'fetal respiratory like activity' by affecting fetal central nervous system (CNS), when injected intravenously during pregnancy. Different drugs[10–11] and stress conditions[12,13] which retard the normal brain development have been found to inhibit the acetylcholinesterase (AChE, EC 3·1·1·7) activity. Besides this, cholinesterase (ChE) plays important role in neurogenesis, maturation of synaptic organisations and in normal brain development[14–18]. However, no reports are available on the effects of different benzodiazepines on cholinesterase activity of developing brain. In the present communication, an attempt has been made to study the *in vitro* effects of different benzodiazepines like diazepam (DZ), desmethyldiazepam (DDZ), nitrazepam (NZ) and chlordiazepoxide (CZ) on cholinesterase activity of human fetal brain.

Materials and Methods

Collection of Samples

Brain samples from respective fetuses were obtained after therapeutic abortions upto 24 weeks from different nursing homes and MTP clinics, Calcutta. Fetuses above 24 weeks were collected as still-born. The ages were assayed from mother's menstrual cycle history. This method provides data to ± 1 week in the majority of cases[18]. According to gestational ages, 27 fetal samples were distributed as follows: 12–14 weeks (8), 16–18 weeks (8), 20–22 weeks (4), 24–26 weeks (4) and 28–30 weeks (3). The bodies of the fetuses were put into a freezer (0–4° C) immediately after death and brain tissues were removed as quick as possible. The cerebrum and cerebellum portions were separated accordingly.

Preparation of Enzymes

Cerebrum and cerebellum portions of the brain tissue were homogenised in a glass homogenizer (0–4° C) in a 9 volume of 0·32 (M) sucrose. The cell debris and nuclear residue were removed by centrifugation for 10 min in a cold centrifuge (Model, K–24) at 1,500 g. The supernatant fraction was used as the enzyme source.

TABLE I

Percentage of inhibition of cholinesterases (ChE) activity of Human Fetal Brain (Cerebrum)

(Enzyme system was first pre-incubated with vehicle or drugs in vehicle for 30 minutes at 37° C. Figures in the parenthesis indicate the number of cases studied. Results have been expressed in percentage of inhibition of the enzyme activity, considering the activity of control as 100%).

Gestational ages in weeks	Doses in μg/mg protein	Drugs			
		Diazepam	Desmethyl-diazepam	Nitrazepam	Chlordia-zepoxide
12–14 (8)	10	10·0	15·5	16·1	13·0
	20	30·2	32·5	26·8	22·8
	50	40·6	45·2	35·4	26·5
	100	50·8	55·2	45·0	37·2
	200	70·0	66·6	65·0	55·1
16–18 (8)	10	15·5	12·1	14·8	10·0
	20	32·5	25·6	22·9	20·0
	50	45·5	40·2	38·7	28·5
	100	52·0	50·2	48·1	35·1
	200	61·1	63·3	60·0	50·0
20–22 (4)	10	19·3	17·6	22·5	8·7
	20	31·9	30·5	32·5	17·6
	50	38·7	35·2	44·6	25·0
	100	54·4	58·8	58·8	40·8
	200	62·2	60·0	66·6	49·4
24–26 (4)	10	18·4	20·0	13·0	9·6
	20	33·3	33·8	33·5	19·3
	50	43·4	40·7	46·6	24·2
	100	53·3	50·0	52·4	42·0
	200	66·7	60·0	66·6	57·8
28–30 (3)	10	12·5	14·5	15·0	12·5
	20	32·5	37·5	34·5	25·4
	50	40·2	45·0	45·8	27·8
	100	50·0	55·0	60·0	40·0
	200	65·6	60·0	70·2	52·1

TABLE II

Percentage of inhibition of cholinesterases (ChE) activity of Human Fetal Brain (Cerebellum)

(Enzyme system was first pre-incubated with vehicle or drugs in vehicle for 30 min at 37° C. Figures in the parenthesis indicate the number of cases studied. Results have been expressed in percentage of inhibition of the enzyme activity, considering the activity of control system as 100%).

Gestational ages in weeks	Doses in μg/mg protein	Drugs			
		Diazepam	Desmethyl-diazepam	Nitrazepam	Chlordiaze-poxide
12–14 (8)	10	15·0	10·0	12·0	11·5
	20	22·0	20·2	22·0	26·6
	50	30·0	30·8	38·0	32·8
	100	40·5	50·4	48·0	47·2
	200	80·0	77·5	72·0	74·0
16–18 (8)	10	10·2	16·0	16·2	15·0
	20	25·6	28·2	22·5	25·0
	50	34·2	35·0	38·4	35·5
	100	45·0	55·4	50·5	50·1
	200	75·5	82·8	75·0	75·8
20–22 (4)	10	12·2	16·4	14·0	18·4
	20	20·6	25·5	21·5	26·2
	50	30·8	40·0	48·0	40·4
	100	50·0	50·0	55·6	60·1
	200	70·0	81·2	75·2	76·4
24–26 (4)	10	13·2	19·1	14·2	16·8
	20	24·3	28·7	24·8	25·0
	50	35·1	39·4	32·9	35·2
	100	47·2	50·6	54·2	55·1
	200	75·1	72·5	70·7	73·1
28–30 (3)	10	10·8	18·6	19·2	15·6
	20	22·1	26·7	29·9	26·2
	50	38·2	40·2	35·7	43·6
	100	52·4	58·8	54·4	59·1
	200	70·2	72·2	73·0	70·2

In vitro *Drug Treatment*

Enzymes were pre-incubated with 0·01 ml of the vehicle containing diazepam (DZ), desmethyldiazepam (DDZ), nitrazepam (NZ) and chlordiazepoxide (CZ) at the doses of 10 μg, 20 μg, 50 μg, 100 μg and 200μg per mg protein in a volume of 1 ml of the incubation system at 37° C for 30 minutes. The control system contained 0·01 ml of the vehicle instead of drugs.

Estimation of Cholinesterase Activity

Cholinesterase activity was estimated according to the method of Hestrin *et al.*,[19] using acetylcholine-chloride (Sigma Chemical Co. U.S.A.) as a substrate The assay medium contained Na_2HPO_4/KH_2PO_4 (M/15), pH 7·8, 0·003 (M) $MgCl_2$ and 0·001 (M) substrate in a final volume of 1 ml. Protein was estimated according to the method of Lowry *et al.*[20].

Results and Discussion

From the present investigation it is clear that the benzodiazepine derivatives inhibit the ChE activity of human fetal brain in a dose dependent manner (Tables I and II). However, the rate of inhibition showed no variation with respect to prenatal ages. But the effect is more prominent in cerebellum than cerebrum at higher doses. This may be due to the high lipophilicity of the drugs in more lipid rich region of the brain, *i.e.*, cerebellum. Several workers[21–23] have reported that DZ and other benzodiazepines did not alter the AChI activity of rat brain at very low

TABLE III

Kinetic Studies on the in vitro effect of different benzodiazepines on cholinesterase activity of Human Fetal Brain

Drugs (200 μg per mg. protein)	Cerebrum		Cerebellum	
	K_m in 10^{-4}(M)	V_{max} ΔOD_{540nm}/mg Pr/hr	K_m in 10^{-4}(M)	V_{max} ΔOD_{540nm}/mg Pr/hr
Control	4·0	0·22	5·0	0·4
Diazepam	8·7	0·22	10·0	0·4
Desmethyldiazepam	13·0	0·22	6·0	0·4
Nitrazepam	11·0	0·16	6·0	0·4
Chlordiazepoxide	6·0	0·16	15·0	0·4

doses (1–10 μg/100 mg tissue) under *in vitro* conditions. However, the inhibitory effects of DZ and other benzodiazepines observed in our investigation may be responsible for the higher doses of drugs taken for the *in vitro* investigation.

Further, Lineweaver and Burk plot of kinetic study on ChE revealed that DZ and DDZ altered the K_m in both cerebrum and cerebellum without affecting V_{max} indicating the disturbed catalytic function of the enzyme. But the effects of NZ and CZ are found to be somewhat peculiar. In cerebellum NZ and CZ like DZ and DDZ altered the kinetic pattern. But these drugs (NZ and CZ) in cerebrum affect both K_m and V_{max} and thereby alter both the catalytic function and the substrate binding capacity. Although the present work is insufficient to explain the differential inhibitory effects of NZ and CZ in cerebrum and cerebellum, it is possibly due to the differences in the biochemical environments such as lipid make up and other factors in the two region of the brain which controls the neuropharmacological effects of this group of depressive drugs.

In the light of the above results it may be concluded that the inhibitions of ChE, the membrane-bound enzyme, by the benzodiazepine group of transquilisers alter the membrane-enzyme relationship[24]. Thus the drug-membrane interactions as evidenced by the inhibition of ChE in the fetal and developing brain may produce the alterations, in structural and functional maturation of CNS and, as a result, the normal brain development is disturbed[25].

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