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Studies on the interaction between cerebral 5-hydroxytryptamine and γ-aminobutyric acid in the mode of action of diazepam in the rat

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1 The effect of the benzodiazepine, diazepam, administered for 7 days in doses between 1.25 and 5 mg kg^{-1} was studied on the turnover of 5-hydroxytryptamine (5-HT) in rat cerebral cortex. 5-HT turnover was assessed by calculating the ratio of the concentration of the major metabolite 5-hydroxyindoleacetic acid (5-HIAA) to that of 5-HT (i.e., 5-HIAA:5-HT). Diazepam (2.5 and 5 mg kg^{-1} i.p. daily for 7 days) significantly reduced cerebral cortical 5-HT turnover.

2 The effect of manipulating cerebral γ-aminobutyric acid (GABA) mechanisms on this action of diazepam was studied. Treatment of animals with a subconvulsive dose of picrotoxin (3 mg kg^{-1} i.p.) reversed the fall in cortical 5-HT turnover seen following diazepam. In contrast, however, treatment with the GABA transaminase inhibitors, amino-oxyacetic acid (25 mg kg^{-1}) or ethanolamine-*O*-sulphate (250 mg kg^{-1}, 7 days) which elevated cerebral GABA concentrations, enhanced the reduction in cortical 5-HT turnover following diazepam.

3 Focal injection of picrotoxin (0.1 μg) into the region of the dorsal raphé nucleus reversed the decrease in cortical 5-HT turnover caused by diazepam.

4 The hypothesis that doses of diazepam which result in total plasma concentrations comparable to those observed in man produce a reduction in 5-HT turnover mediated via GABA neurones is discussed.

Introduction

Current thinking in the field of benzodiazepine research is now dominated by the idea of some form of interaction with the γ-aminobutyric acid (GABA) receptor complex. Benzodiazepines are believed to act at specific binding sites which are closely linked to GABA receptors. The binding of benzodiazepines increases the affinity of the GABA receptor for GABA, thereby enhancing GABAergic transmission (Costa, Guidotti & Mao, 1976). Recent reviews have thus tended to explain the pharmacological properties of the benzodiazepines in terms of this proposed modulatory effect on GABA receptors, and to dismiss the possible involvement of other neurotransmitter systems (e.g. Costa, 1980).

However, with regard to the anxiolytic action of benzodiazepines, there is also considerable evidence implicating the involvement of 5-hydroxytryptamine (5-HT) (e.g. Robichaud & Sledge, 1969; Geller & Blum, 1970; Wise, Berger & Stein, 1972; Graeff, 1974; Cook & Sepinwall, 1975; Schoenfeld, 1976; Tye, Everitt & Iversen, 1977). Early reports that benzodiazepines reduce brain 5-HT turnover (e.g. Chase, Katz & Kopin, 1970) have been largely discounted since the doses of benzodiazepines used in those animal studies were much higher than those employed clinically.

In view of the recent neurochemical evidence that GABAergic neurones of the median raphé region exert a tonic control over ascending 5-HT neurones in the same region (Forchetti & Meek, 1981), this investigation has aimed to test the hypothesis that therapeutically relevant doses of diazepam can reduce brain 5-HT turnover. We have also investigated whether or not such changes in 5-HT turnover might be secondary to an effect of the benzodiazepine via GABAergic mechanisms. Rats have been treated chronically (7 days) with diazepam and cortical 5-HT turnover monitored. The effect of subsequently manipulating cerebral GABA systems by administering either a GABA receptor antagonist (picrotoxin) or GABA-transaminase inhibitors (amino-oxyacetic acid, ethanolamine-*O*-sulphate) on turnover was investigated. Also, since it is known that cortical 5-HT terminals originate from cell bodies in midbrain

raphé nuclei (Bobillier, Seguin, Petitjean, Salvert, Touret & Jouvet, 1976), the effect of locally manipulating GABA within this site with picrotoxin was studied in order to attempt to assess a possible site of action of the benzodiazepines. The identification of locally organised GABA-accumulating neurones in the dorsal raphé (Belin, Aguera, Tappaz, McRae-Degueurce, Bobillier & Pujol, 1979) and the iontophoretic studies of Gallagher (1978) demonstrating that benzodiazepines potentiate the inhibitory response on dorsal raphé firing induced by GABA, support the hypothesis that this midbrain area may constitute an important site of action for the benzodiazepine drugs.

Methods

Drugs and injection regime

For intraperitoneal injection, diazepam (Valium, Roche) was suspended in 1% v/v Tween 80 (Sigma) in distilled water. All other drugs, picrotoxin (Sigma), amino-oxyacetic acid (AOAA, Sigma) and ethanolamine-*O*-sulphate (EOS) were dissolved in 0.9% w/v NaCl solution (saline) and administered intraperitoneally.

Male Porton rats (400–450 g) were used in this study. Each animal was housed individually. Animals received daily injections of diazepam (5, or 2.5 mg kg^{-1}) or vehicle for 7 days. Rats were killed 1 h after final injection.

In other studies picrotoxin (3 mg kg^{-1}) was administered acutely immediately after the final diazepam (or vehicle) injection and animals killed 1 h later. In the case of AOAA (25 mg kg^{-1}; Collins, 1973), this drug was given 4 h before killing (i.e. 3 h before the final dose of diazepam). For EOS (250 mg kg^{-1}) this agent was also administered chronically being given daily for 7 days into diazepam or vehicle-injected rats (Fletcher & Fowler, 1980). EOS was synthesized by the procedure of Lloyd, Tudball & Dodgson (1961).

Estimation of 5-hydroxytryptamine turnover

The ratio of the concentration of the major metabolite of 5-HT, 5-hydroxyindoleacetic acid (5-HIAA) and of 5-HT itself (i.e. 5-HIAA: 5-HT) was determined for each animal and taken as an index of 5-HT turnover (Curzon, 1981). Rats were killed by cervical dislocation and approximately 200 mg of frontoparietal cortex removed. Tissue was weighed and indoleamines extracted into acidified butanol as described by Maickel, Cox, Saillant & Miller (1968). Fluorimetric analysis of 5-HT and 5-HIAA followed the method of Curzon & Green (1970).

Estimation of γ-aminobutyric acid concentrations

The biochemical effect of treating animals with the GABA-transaminase inhibitors AOAA and EOS was monitored by recording cortical and raphé GABA concentrations 4 h after a single AOAA injection and 1 h after the final EOS dosage. Animals were killed by cervical dislocation and both cerebral cortex and raphé regions were rapidly dissected out and immediately frozen on dry ice. The raphé nucleus was dissected by the procedure previously described (Kerwin & Pycock, 1979). All groups of animals (vehicle-injected controls, AOAA-injected and EOS-injected) were killed and brain regions dissected in an identical manner so that the time elapsed between killing the animals and freezing the tissue for each group (30 s) was the same. Tissue samples were weighed, homogenized in 95% ethanol, and the GABA content estimated fluorimetrically by coupled assay with GABA aminotransferase and succinic semialdehyde dehydrogenase (GABASE, Sigma Ltd) using the method of Scott & Jakoby (1959).

Raphé injection studies

In a final series of experiments, picrotoxin or saline was stereotaxically injected into the region of the dorsal raphé in chronic diazepam (5 mg kg^{-1}, 7 days) or vehicle-injected rats. Animals were anaesthetized with halothane/air mixture 30 min after the final injection of diazepam and immobilized in a Kopf stereotaxic frame. Picrotoxin (0.1 μg in 0.5 μl saline) or saline was injected over a 1-min interval at the stereotaxic co-ordinates (A+0.35, LO, V-0.8, König & Klippel, 1963). The skin was clipped back in position over the skull and the anaesthesia was discontinued. Animals recovered from the effects of the anaesthesia in about 2 min and were subsequently killed 30 min later for the estimation of corticol 5-HT turnover. Verification of the site of injection was made by both macroscopic and microscopic examination of the midbrain raphé nuclei.

Determination of plasma levels of diazepam

Electron-capture-gas-liquid-chromatography (EC-GLC) was used under the following conditions: The 1.5 m borosilicate glass column contained 3% OV-17 on 100–120 mesh Gas Chrom Q (Perkin-Elmer) as support phase. The column was conditioned by 4 h heating at 325°C with no gas flow, followed by 12 h at 275°C with carrier gas flow at 40 ml min^{-1}. A Pye-Unicam 104 gas chromatograph equipped with a 10 mCi ^{63}Ni electron capture detector was used. The

carrier gas was nitrogen; and the flow rate 75 ml min^{-1}; injection port: 290°C, detector: 320°C and column temperature 230°C.

Extraction of diazepam from rat plasma followed the method of de Silva, Bekersky, Puglisi, Brooks & Weinfeld (1976). The column was primed with three 5 μl aliquots of control plasma extract and temazepam was used as internal standard. Overall recovery was between 69 and 76%.

Statistical analysis of results

No objection could be seen to taking brain concentrations of 5-HT and 5-HIAA to be normally distributed, and therefore means, s.e.means and Student's *t* values have been calculated. It may not be safe to assume, however, that the ratio of these two variables, 5-HIAA: 5-HT, is normally distributed, since the variance of a ratio tends to increase as the ratio itself increases. Means and s.e.means were nevertheless chosen as a convenient method of displaying the results but Mann-Whitney U-tests as well as Student's *t* tests were performed on the data to assess significance of difference between groups. Two-factor analysis of variance has been carried out on some of the data to assess possible interaction between two different drug treatments.

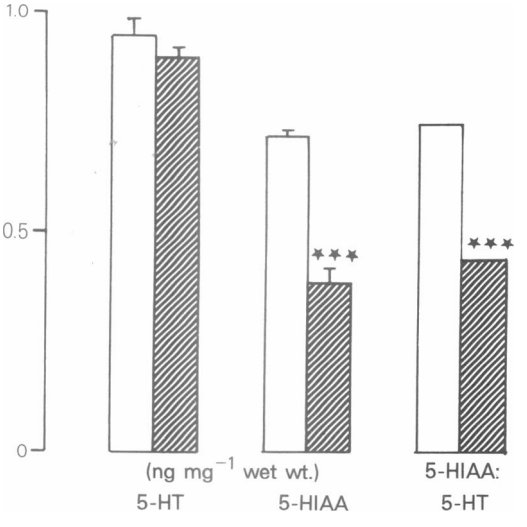

Figure 1 The effect of chronic diazepam treatment in rats on cortical 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) concentrations, and on the cortical 5-HIAA: 5-HT ratio. Rats received either 1% Tween (open columns) or diazepam (5 mg/kg) (hatched columns) daily for 7 days. Animals were killed 1 h after final intraperitoneal injection. 5-HT and 5-HIAA concentrations expressed as ng mg^{-1} wet weight tissue: vertical bars indicate s.e.mean. $n = 9$; *** denotes significant difference between vehicle and drug-treated groups at the level $P < 0.001$ (2-tailed *t* test and Mann-Whitney U test).

Results

Initially, the doses of diazepam produced a marked loss of spontaneous exploratory behaviour in the rats lasting for up to 2 h post-injection. There was, however, no loss of the righting reflex observed in any animals. By the seventh day of treatment, no sedation or reduction of spontaneous activity was apparent so that the rats had clearly become tolerant to the sedative action of the diazepam. It was at this stage, following the seventh dose of diazepam that the effects of the drug on 5-HT turnover were assessed.

Chronic diazepam on 5-hydroxytryptamine turnover

The effect of chronic treatment of diazepam (5 mg kg^{-1}) for 7 days on cortical 5-HT turnover is illustrated in Figure 1. As indicated in the Methods, 5-HT turnover was estimated by calculating the 5-HIAA: 5-HT ratio, and chronic diazepam significantly reduced this parameter ($P < 0.001$). Analysis of 5-HT and 5-HIAA levels indicates that the changes induced by diazepam are focussed on reduced 5-HIAA concentrations compared with control values (from 0.73 ± 0.02 to 0.39 ± 0.04 ng mg^{-1} wet weight of tissue, $P < 0.001$) (Figure 1). In contrast, no significant changes in cortical 5-HT concentrations were observed following drug treatment.

Similarly, 7 day treatment of rats with 2.5 mg kg^{-1} diazepam also reduced cortical 5-HT turnover by 35% ($P < 0.01$) as shown in Figure 3. However, a lower dose regime, 1.25 mg kg^{-1} diazepam for 7 days, failed to alter 5-HT or 5-HIAA concentrations significantly compared with control animals. The 5-HT turnover (expressed as the 5-HIAA: 5-HT ratio) ranged between 0.428 and 1.216 in control rats and between 0.271 and 0.596 in animals given diazepam at 2.5 mg kg^{-1} daily.

Effect of picrotoxin on diazepam-induced reduction of 5-hydroxytryptamine turnover

A subconvulsive dose of picrotoxin (3 mg kg^{-1}) was administered acutely to animals which had received diazepam (5 mg kg^{-1}) chronically for 7 days. Picrotoxin alone, after one hour, caused a slight, non-significant rise in cortical 5-HT turnover in Tween-pretreated rats (Figure 2). However, in diazepam-pretreated rats, picrotoxin reversed the significant reduction in 5-HT turnover seen with benzo-

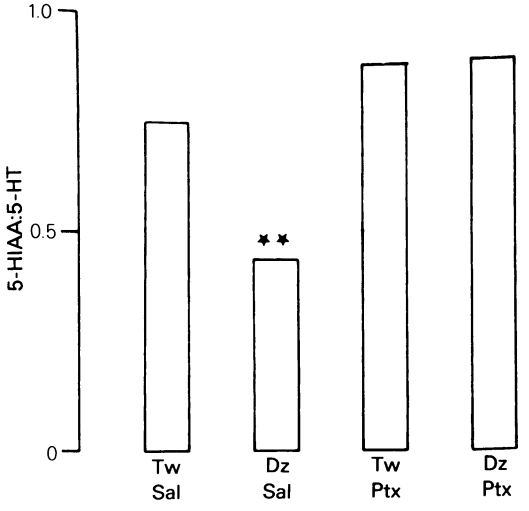

Figure 2 The effect of picrotoxin and chronic diazepam treatment in rats on cortical 5-hydroxyindoleacetic acid (5-HIAA): 5-hydroxytryptamine (5-HT) ratio. Rats received either 1% Tween (Tw) or diazepam (Dz) (5 mg kg^{-1}, i.p.) daily for 7 days. Half the animals from each group received either saline (Sal) or picrotoxin (Ptx; 3 mg kg^{-1}, i.p.) 1 h after the final dose of diazepam, and animals killed 1 h later. Results represent the mean of 9 observations; ** denotes $P<0.01$ between chronic diazepam and saline-treated groups (2-tailed *t* test and Mann-Whitney U test).

diazepine alone, and the 5-HIAA: 5-HT ratio appeared within the control range. Analysis of variance indicated a significant interaction between picrotoxin and diazepam ($F(1,32) = 7.8$, $P<0.01$).

Effect of GABA-transaminase inhibitors on diazepam-induced reduction of 5-hydroxytryptamine turnover

The effect of AOAA (25 mg kg^{-1}) on the reduction of cortical turnover of 5-HT induced by chronic diazepam treatment (2.5 mg kg^{-1}, for 7 days) is shown in Figure 3. Whereas diazepam alone induced a significantly lowered 5-HIAA: 5-HT ratio ($P<0.01$), AOAA produced only a very slight non-significant fall in this value compared with control animals. However, in combination, both treatments resulted in a highly significant decrease in cortical 5-HT turnover ($P<0.001$), the value observed with the drug combination being significantly greater than that observed with diazepam alone $P<0.05$).

A similar pattern is seen with EOS, administered chronically for 7 days, as the GABA-transaminase

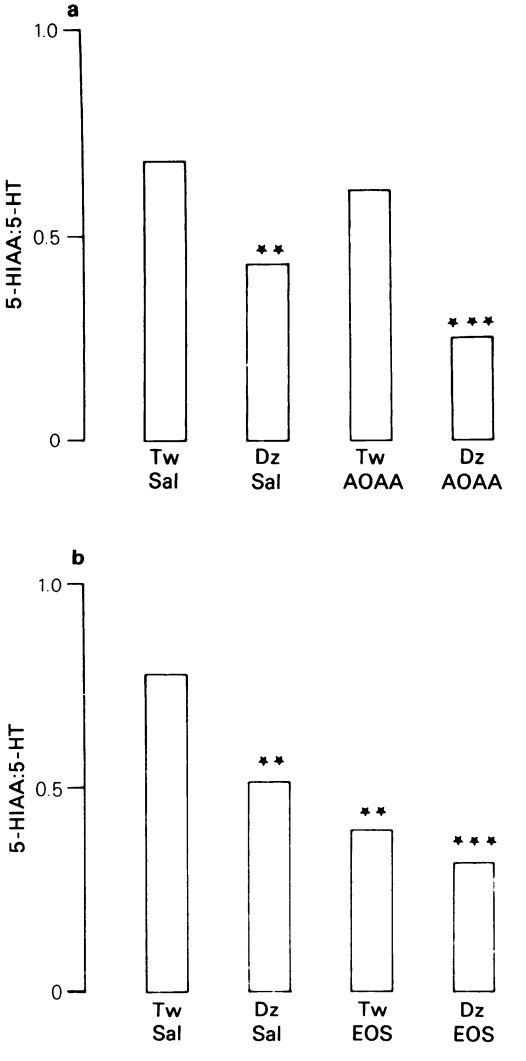

Figure 3 The effect of amino-oxyacetic acid (AOAA), ethanolamine-*O*-sulphate (EOS) and chronic diazepam treatment on cortical 5-hydroxyindoleacetic acid (5-HIAA): 5-hydroxytryptamine (5-HT) ratio. Rats received either 1% Tween (Tw) or diazepam (Dz 2.5 mg kg^{-1}, i.p.) daily for 7 days. (a) Animals from each group received either saline (Sal) or AOAA (25 mg kg^{-1}, i.p.) 3 h before the final dose of diazepam, and were killed 1 h after diazepam dose. (b) Other animals from each group received either saline (Sal) or EOS (250 mg kg^{-1}, i.p.) chronically for 7 days, and were killed 1 h after the final dose of Dz and EOS. Results represent the mean of between 4 and 9 observations. Comparison between drug-treated and control groups by 2-tailed *t* test and Mann-Whitney U test showed the following significant differences: * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

Table 1 Cerebral γ-aminobutyric acid (GABA) concentrations following treatment with GABA-transaminase inhibitors

Treatment	*Cortex*	*Raphé*
Control	1.48 ± 0.05	5.15 ± 0.50
AOAA	3.34 ± 0.20**	11.04 ± 1.82*
EOS	5.05 ± 0.25***	18.38 ± 2.38**

Control rats were treated with either amino-oxyacetic acid (AOAA; 25 mg kg^{-1}, i.p., 4 h or ethanolamine-*O*-sulphate (EOS; 250 mg kg^{-1}, i.p., 7 days, animals killed 4 h after final injection). Cortical and raphé GABA concentrations expressed as μmol μg^{-1} wet weight tissue ± s.e.mean ($n = 4$ for all groups).
*, **, ***, denotes $P < 0.05$, 0.01 and 0.001 respectively (Student's *t* test).

inhibitor. However, in the case of EOS, this drug alone significantly reduced the 5-HIAA: 5-HT ratio compared to the control situation ($P < 0.01$), although the difference between the reductions in 5-HT turnover induced by either diazepam alone or EOS alone did not prove to be statistically significant (Figure 3). In combination however, diazepam, together with EOS, reduced the 5-HT turnover ratio still further (to 41% of control values, $P < 0.001$). Analysis of variance of these data however did not provide evidence for an interaction between these two drug treatments ($F(1,12) = 0.91$) but confirmed the significance levels calculated by the *t* tests.

As previously reported by Collins (1973) and by Fletcher & Fowler (1980), biochemical analysis indicated that both AOAA and EOS significantly elevated both cortical and raphé GABA concentrations at the times studied in the above experiments (Table 1). For cortical GABA, AOAA and EOS increased concentrations by 225 and 342% respectively, while raphé GABA concentrations were raised by 215 and 357% of control values respectively.

Injection of picrotoxin into raphé nucleus on diazepam-induced reduction in 5-hydroxytryptamine turnover

A reduction in cortical 5-HIAA: 5-HT ratio was observed in rats after chronic treatment with diazepam (5 mg kg^{-1}, for 7 days) following recovery from anaesthesia and stereotaxic injection of saline into the region of the dorsal raphé nucleus ($P < 0.01$) (Figure 4). In contrast, however, injection of picrotoxin into this region resulted in a significant elevation of cortical 5-HT turnover ($P < 0.05$). Following this procedure about half the rats injected showed enhanced motor activity although none exhibited myoclonic jerks or convulsions. Focal injection of

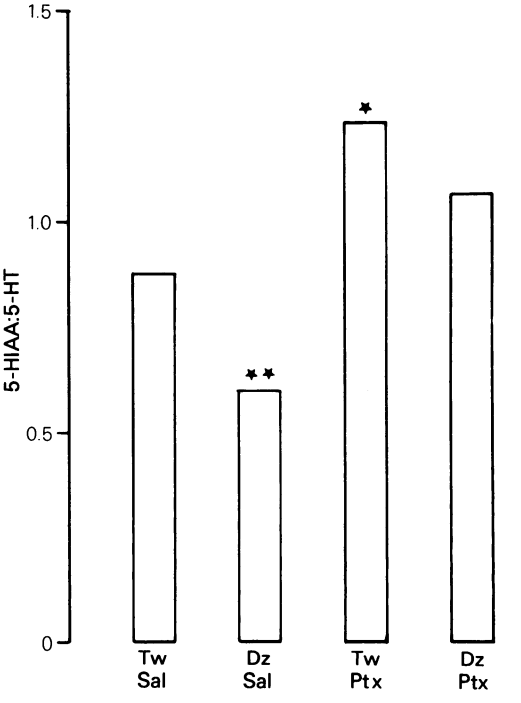

Figure 4 The effect of injections of picrotoxin into raphé and chronic diazepam treatment in rats on cortical 5-hydroxyindoleacetic acid (5-HIAA): 5-hydroxytryptamine (5-HT) ratio. Rats received either 1% Tween (Tw) or diazepam (Dz; 5 mg kg^{-1}, i.p.) daily for 7 days. Half the animals from each group received either saline (Sal; 0.5 μl) or picrotoxin (Ptx, 0.1 μg in 0.5 μl saline) stereotaxically injected into dorsal raphé nucleus. Animals were killed 30 min later. Results represent the mean of 9 observations; and * and ** denotes $P < 0.05$ and $P < 0.01$ comparison between drug-treated and control groups (2-tailed *t* test and Mann-Whitney U-test).

picrotoxin into the raphé of animals chronically treated with diazepam completely reversed the fall in 5-HT turnover seen to the benzodiazepine alone, and whereas now a slight increase in this ratio was observed it did not differ significantly from that calculated in the control animals (Figure 4). However, as Figure 4 indicates, diazepam did cause some decrease of cortical 5-HT turnover in the picrotoxin-injected rats compared to the picrotoxin-injected alone animals, although this decrease was not statistically significant.

Plasma level of diazepam

Blood samples (1 ml) were taken 60 min following the last injection of diazepam (5 mg kg^{-1}). The me-

dian plasma level was 56 ng ml^{-1} ($n = 8$) with a range between 28 and 95 ng ml^{-1}.

Discussion

Since the principal object of the present work was to relate the anxiolytic effects of diazepam to biochemical changes in 5-HT and GABA systems, a dose regime was chosen such that the animals became tolerant to the immediate sedative effects of the drug, and plasma levels of diazepam were comparable to those found clinically. However, it is known that benzodiazepine distribution and metabolism is different between rat and man and that the desmethylation of diazepam and chlordiazepoxide is probably less important in the rat (see, for example Koechlin, Sohwartz, Krol & Oberhausli, 1965).

After seven consecutive daily doses of diazepam the cerebral cortical level of 5-HIAA (the principal metabolite of 5-HT) was reduced whilst the level of 5-HT remained constant. This implies a reduced turnover of 5-HT neuronal systems. To obtain valid estimates of 5-HT synthesis or breakdown in absolute terms, it is necessary to employ pargyline or probenecid respectively in order to ensure that the change in 5-HIAA levels truly reflect 5-HT turnover (Curzon, 1981). However, either of these drugs could interfere with the action of diazepam and the relative values of 5-HT:5-HIAA ratios obtained in the present work are valid on the assumption that diazepam, at the dose used, has no effect on the egress of 5-HIAA from the brain.

The sub-convulsive dose of systemically-administered picrotoxin used reversed the effect of chronic diazepam, a result which supports the hypothesis that diazepam may be affecting 5-HT neurones by means of an enhancement of a GABA-mediated inhibitory input onto these systems. That picrotoxin alone was seen to increase 5-HT turnover, albeit slightly, suggests that such an inhibitory GABAergic input on to 5-HT systems may be tonically active, and indeed the model of benzodiazepine function proposed by Costa and others (e.g. Costa *et al.*, 1976) would require such a basal activity of the GABA neurones.

Conversely, increasing cerebral GABA concentrations with GABA-transaminase inhibitors might be expected to enhance the hypothesized GABA-ergic input to 5-HT systems, thereby reducing 5-HT turnover and increasing the reduction of turnover produced by diazepam. The results obtained with AOAA are consistent with this idea. Although EOS administered alone significantly reduced the turnover index of 5-HT, there was no evidence to suggest synergism with diazepam on 5-HT turnover. This could be rationalised in terms of the effect of EOS alone being maximal so that a synergistic effect with diazepam could not be shown (i.e. a 'ceiling effect'), or possibly that some adaptation to an extreme reduction in turnover in the chronic EOS plus diazepam group occurred over the 7 days of treatment, producing partial compensation and so concealing any interaction. An experiment using acute intracerebroventricular EOS or directly-acting GABA agonists rather than chronic intraperitoneal EOS treatment might be useful in resolving this point.

The plasma levels of diazepam occurring in the animals used in the present experiments are well below those which have been found to produce sedation and loss of spontaneous activity in mice (Paul & Whitehouse, 1977), and more closely correspond to the levels found in man following an oral dose of 10 mg (see de Silva *et al.*, 1976). Higher doses of diazepam administered chronically to rats (Agarwal, Lapierre, Rastogi & Singhal, 1977) produce increases in tryptophan hydroxylase activity and tryptophan levels with a rebound fall following withdrawal. This effect is associated with behavioural depression and rebound hyper-activity during withdrawal and is probably not related to the anxiolytic actions of benzodiazepines.

The experiments so far suggest that the reduction in cortical 5-HT turnover observed following chronic diazepam treatment may be exerted through a GABA mechanism, but there is no indication as to the anatomical site(s) for such an interaction. However, since the raphé cell bodies of the ascending cortical 5-HT neurones are influenced by GABA interneurones (Forchetti & Meek, 1981) this site might be particularly relevant to the results observed so far. Consequently, the GABA system in this specific area was inhibited by the focal injection of picrotoxin into the dorsal raphé nucleus. An increased cortical 5-HT turnover was observed and the focal picrotoxin treatment also reversed the effect of diazepam to reduce 5-HT turnover. These results suggest that the raphé nucleus is a likely site through which some degree of modulation of 5-HT turnover by benzodiazepines occurs. It is, in addition, an area in which benzodiazepine receptors are known to exist (Braestrup, Albrechtsen & Squires (1977).

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