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DISTINCTION BETWEEN THE EFFECTS OF BARBITURATES, BENZODIAZEPINES AND PHENYTOIN ON RESPONSES TO γ-AMINOBUTYRIC ACID RECEPTOR ACTIVATION AND ANTAGONISM BY BICUCULLINE AND PICROTOXIN

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1 Interactions of depressant and anticonvulsant drugs with the neuronal γ-aminobutyric acid (GABA) receptor + effector system have been examined on afferent fibres to the rat cuneate nucleus *in vitro*. Three types of interaction have been measured: (a) potentiation of depolarizing responses to the GABA analogue, muscimol: (b) reduction in the potency of bicuculline as an antagonist of muscimol at the GABA receptor: (c) reduction in the potency of picrotoxin as an antagonist of muscimol acting on the effector mechanism.

2 Phenobarbitone reduced the potency of picrotoxin in doses which did not affect the potency of bicuculline and which caused only a small potentiation of muscimol. Pentobarbitone did not show such selectivity, a reduction in potency of picrotoxin always being accompanied by a reduction in potency of bicuculline and a substantial potentiation of muscimol.

3 Flurazepam and lorazepam both reduced the potency of picrotoxin without affecting that of bicuculline and with very little potentiation of muscimol. Phenytoin had no effect on the potency of picrotoxin whilst potentiating muscimol to the same extent as phenobarbitone.

4 The spectrum of drug activity in reducing the potency of picrotoxin correlates well with the reported anticonvulsant effects of these drugs against kindled amygdaloid seizures. Potentiation of muscimol and reduction of bicuculline potency appear more closely related to hypnotic properties.

Introduction

Evidence from several laboratories indicates that the responses of neurones to γ-aminobutyric acid (GABA) can be potentiated by a variety of depressant and anticonvulsant drugs, including barbiturates (Nicoll, 1975; Ransom & Barker, 1976; Barker & Ransom, 1978; Brown & Constanti, 1978; Schlosser & Franco, 1979), benzodiazepines (Choi, Farb & Fischbach, 1977; Macdonald & Barker, 1978a, 1979; Okamoto & Sakai, 1979) and sometimes phenytoin (Ayala, Johnston, Lin & Dichter, 1977; Adams & Banks, 1980; Nicoll & Wojtowicz, 1980). The basis of this effect for the barbiturates and phenytoin seems likely to be a prolongation of the open time of the chloride channels operated by GABA (Barker & McBurney, 1979; Mathers & Barker, 1980; Adams & Banks, 1980). Blockade of GABA uptake mechanisms appears to be a less likely explanation (Jessel & Richards, 1977; Brown & Constanti, 1978).

When these drugs are combined with antagonists of GABA such as bicuculline and picrotoxin, the potentiation of responses to GABA tends to obscure the antagonism (Brown & Constanti, 1978; Evans, 1979; Nicoll & Wojtowicz, 1980). This may be due largely to simple addition of effects. There could, however, be a more specific reduction of antagonism (Bowery & Dray, 1978; Nicoll & Wojtowicz, 1980). In that case, the interaction with bicuculline might differ from the interaction with picrotoxin since these antagonists act at separate sites in the neuronal GABA receptor + effector system (Simmonds, 1980a). Indeed, it would appear from binding studies that the picrotoxin site ought to be a more likely target for depressant and anticonvulsant drugs than the GABA receptor itself (Ticku & Olsen, 1978; Olsen, Ticku, Greenlee & Van Ness, 1979).

The present experiments were carried out to determine whether there is any distinction between the abilities of pentobarbitone, phenobarbitone, flurazepam, lorazepam and phenytoin to potentiate responses to the GABA analogue muscimol and their ability to attenuate the actions of bicuculline and picrotoxin as antagonists of muscimol. Some of the results have been reported in preliminary form (Simmonds, 1980b; 1981).

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Methods

Experiments were done on slices of rat cuneate nucleus prepared by sectioning the excised medulla oblongata with a razor blade both sides of each dorsal funiculus. This yielded slices 0.5–0.6 mm thick (Simmonds, 1978). The caudal end of the dorsal funiculus was trimmed free of underlying tissue so that the slice could be placed in a two-compartment bath with the bulk of the slice in one compartment and the caudal end of the dorsal funiculus projecting through a greased slot from the second compartment (Hayes & Simmonds, 1978). Both compartments were continuously perfused with a modified Krebs medium but drugs were perfused through the first compartment only. The d.c. potential between the two compartments was recorded via Ag/AgCl electrodes embedded in 3% agar in saline, a high impedance amplifier and chart recorder. Drug-induced negativity in the first compartment was interpreted as a depolarization of the afferent nerve fibres of the dorsal funiculus. Responses were measured at peak amplitude.

Muscimol (Fluka) was used routinely as the agonist for GABA receptors since its much slower removal by uptake, compared with GABA, makes it more suitable for quantitative studies (Simmonds, 1980a). Muscimol dissolved in the Krebs medium was perfused for periods of 2 min. Pentobarbitone sodium (Sigma), phenobarbitone sodium (BDH) and flurazepam (Roche) were dissolved directly in the Krebs medium. Lorazepam was obtained as Ativan injection (Wyeth) and was diluted in Krebs medium. In control experiments for lorazepam, the vehicle used in Ativan injection was similarly diluted in Krebs medium. Phenytoin sodium (5,5-diphenylhydantoin sodium; Sigma) was dissolved in distilled water and added to an equal volume of double strength Krebs medium. Picrotoxin (Sigma) was dissolved directly in the Krebs medium and (+)-bicuculline (Sigma) was prepared as a 10^{-2}M solution in 0.02 M HCl and added to the Krebs medium just before use. When these drugs were tested, either singly or in combination, against responses to muscimol, they were perfused for 30 min before and during the applications of muscimol. The modified Krebs medium contained (mM): NaCl 118, KCl 2.1, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 2.2, $NaHCO_3$ 25 and glucose 11 and was continuously bubbled with 95% O_2/5% CO_2.

The Schild plots were calculated by least squares regression analysis and statistical comparisons were made by Student's *t* test.

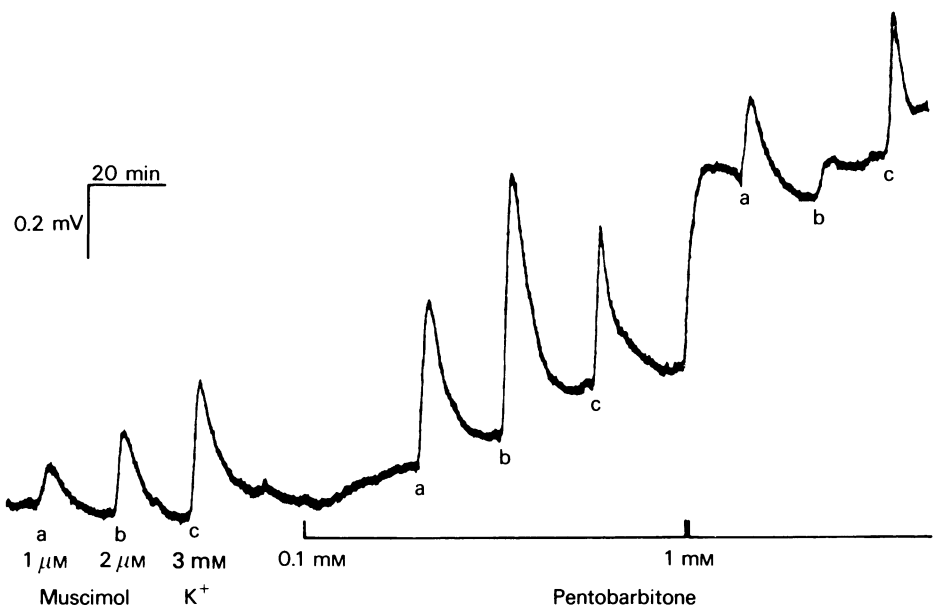

Figure 1 Potential changes in fibres of the dorsal funiculus induced by muscimol and pentobarbitone. Responses were obtained to 2 min superfusions with 1(a) and 2 μM (b) muscimol and an increment of 3 mM K^+ (c). Pentobarbitone 10^{-4}M elicited a slow depolarization and markedly enhanced responses to muscimol with little effect on the response to K^+. Pentobarbitone 10^{-3}M elicited a substantial depolarization in the presence of which the responses to muscimol were attenuated while that to K^+ was unchanged.

Results

Pentobarbitone and phenobarbitone

The initial effect of adding barbiturate to the tissue was a dose-dependent depolarization. A small effect was nearly always seen with 10^{-4}M pentobarbitone and a substantial depolarization with 10^{-3}M (Figure 1). Phenobarbitone was less potent in this respect; 10^{-4}M had no consistent effect and 10^{-3}M was comparable with pentobarbitone 10^{-4}M. These depolarizations could be antagonized by bicuculline or picrotoxin.

The most marked effect of pentobarbitone was the potentiation of depolarizing responses to muscimol (Figure 1). This was dose-related up to 10^{-4}M pentobarbitone but at 10^{-3}M the responses to muscimol were substantially reduced by occlusion or desensitization. The same phenomenon could have occurred to a lesser degree at the lower concentrations of pentobarbitone, even though the net effect was a marked potentiation of muscimol. To test this idea, an attempt was made to simulate the depolarization induced by pentobarbitone 10^{-4}M with a continuous perfusion of 0.25 μM muscimol. When the usual 2 min acute applications of muscimol were superimposed, the responses were smaller than under control conditions, to the extent that the potency of the acute muscimol was reduced by a factor of 2. Thus, it is possible that the full potentiating activity of pentobarbitone may have been underestimated.

To measure the degree of potentiation of muscimol, two-point muscimol dose-response lines were constructed with the higher dose being twice the lower. The doses were selected to be in the low to middle range of the full dose-response curve (Simmonds, 1980a). The displacement of the dose-response line to the left by pentobarbitone and phenobarbitone was always approximately parallel so that potentiation was expressed as the equipotent muscimol dose-ratio (Figure 2). These dose-ratios are plotted as logarithms in Figure 4(a) and show that pentobarbitone was about 13 times more potent than phenobarbitone in potentiating muscimol.

With the barbiturate still present, either bicuculline or picrotoxin was added to the superfusion medium and the muscimol dose-response line redetermined (Figure 2). The effect of the antagonist was expressed as the equipotent muscimol dose-ratio with reference to the line obtained in the presence of barbiturate alone. Values of log (muscimol dose-ratio − 1) were plotted against log concentration of antagonist (Schild plot, Figure 3). These Schild plots were regarded as log dose-effect curves for the antagonists and the effect of barbiturates upon them was determined by comparison with data previously obtained in the absence of barbiturates (Simmonds,

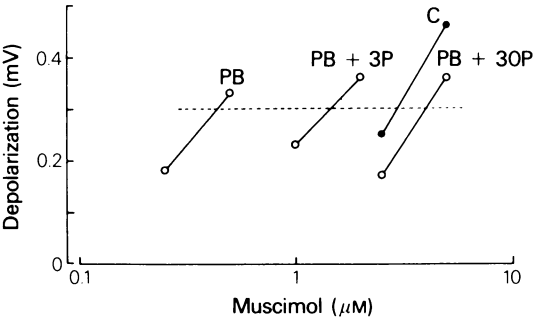

Figure 2 Effects of pentobarbitone and picrotoxin on log dose-response lines for muscimol. The results represent a single experiment and each point is the amplitude of a single response. Pentobarbitone 10^{-4}M (PB) shifted the control (C) muscimol dose-response line to the left by a factor of 6.8 in an approximately parallel fashion. Addition of picrotoxin 3×10^{-6}M (PB + 3P) shifted the line back to the right by a factor of 3.3 and an increase in picrotoxin concentration to 3×10^{-5}M (PB + 30P) increased this shift to 9.1. The muscimol dose-ratios were measured at an arbitrarily selected response level (0.3 mV in this experiment).

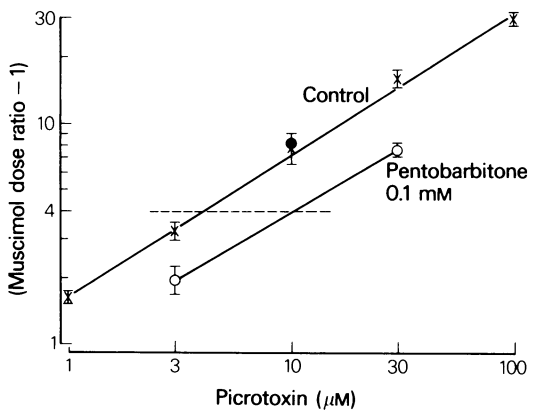

Figure 3 Schild plots of log (muscimol dose-ratio − 1) versus log concentration of picrotoxin. The control line (×) was taken from experiments previously reported (Simmonds, 1980a); 12 further control experiments were done at intervals during the present series (●) and the mean results are shown. Results obtained in the presence of pentobarbitone 10^{-4}M (○) are the mean of 5 values at each point obtained from experiments similar to that depicted in Figure 2. The two lines are approximately parallel having slopes of 0.635 ± 0.021 (control) and 0.590 ± 0.068 (pentobarbitone). Pentobarbitone 10^{-4}M significantly reduced the potency of picrotoxin as an antagonist of muscimol, shown by the shift to the right by 0.404 ± 0.053 log unit (factor of 2.5) at the level (muscimol dose-ratio − 1) = 4. The Schild plot for picrotoxin in the presence of pentobarbitone 10^{-5}M was visually indistinguishable from the control line. Vertical lines indicate s.e.mean for all points.

1980a). In each comparison, the slopes of the Schild plots were not significantly different ($P > 0.05$). The lateral displacement of the Schild plot by barbiturate was measured at the level (muscimol dose-ratio -1) $= 4$ and expressed in terms of log units of antagonist concentration, i.e. the difference in pA_5 values. These results are shown in Figure 4(b) for bicuculline and Figure 4(c) for picrotoxin.

The concentration of phenobarbitone required to reduce the potency of bicuculline was about 10 times the concentration of pentobarbitone for the same effect. This difference corresponded closely with the relative potencies of these two barbiturates in potentiating muscimol (Figure 4a). As regards the reduction in potency of picrotoxin, phenobarbitone and pentobarbitone were approximately equipotent.

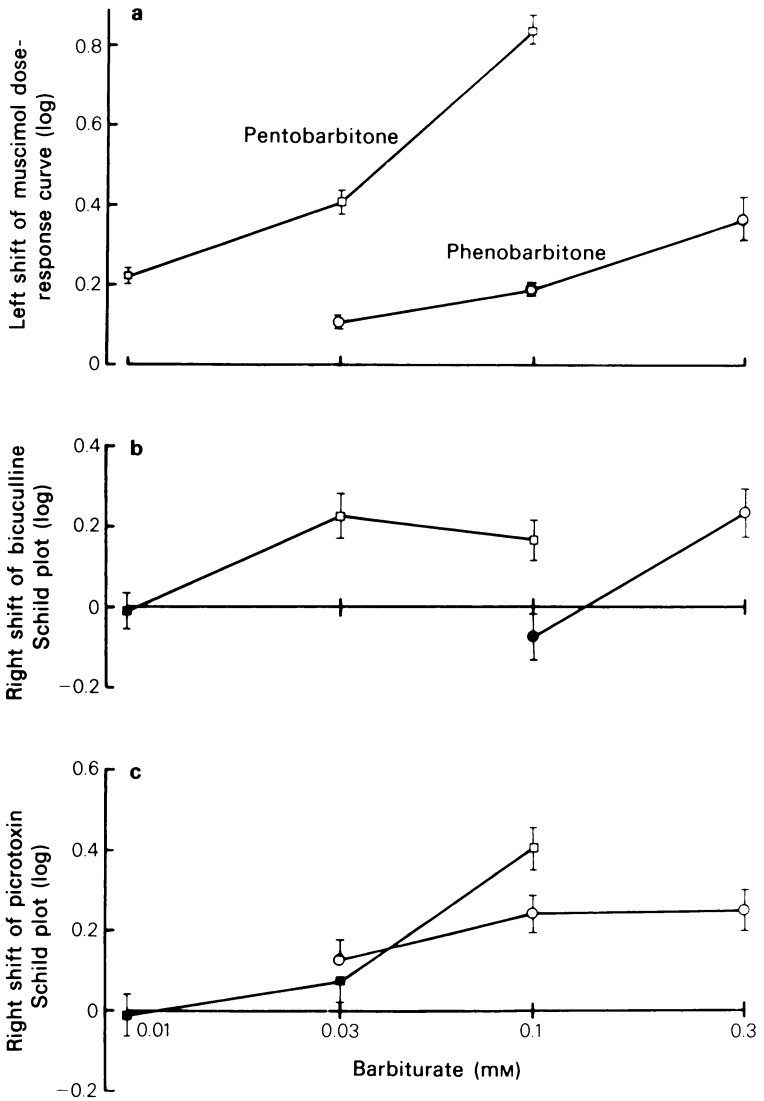

Figure 4 (a) Potentiation of muscimol by pentobarbitone and phenobarbitone. The shift to the left of the muscimol dose-response line is plotted in log units (mean ± s.e.mean of 6–16 values) versus log concentration of barbiturate. (b) Reduction in potency of bicuculline by pentobarbitone and phenobarbitone. The shift to the right of the Schild plot for bicuculline, measured at the level of (muscimol dose-ratio -1) $= 4$, is plotted in log units (mean ± s.e.mean) versus log concentration of barbiturate. (c) Reduction in potency of picrotoxin by pentobarbitone and phenobarbitone. The plot is constructed in a similar way to that for bicuculline in (b). Open symbols indicate significant difference from zero ($P < 0.05$).

Thus, a concentration of phenobarbitone can be selected (10^{-4}M) which reduces the potency of picrotoxin while causing only a small potentiation of muscimol. With pentobarbitone, no such selective effect can be obtained, the concentration required to reduce the potency of picrotoxin also causing a substantial potentiation of muscimol.

In these analyses it has been assumed that bicuculline and picrotoxin did not antagonize the potentiating action of the barbiturates. Such an interaction would, on its own, tend to *increase* the apparent potencies of the antagonists. Since this was never observed, it seems either that the original assumption was correct or that the interactions tending to decrease antagonist potency always dominated any increase in antagonist potency. If the latter situation did obtain, the reported reduction in antagonist potency (Figure 4b, c) would be underestimates. On the other hand, there might be a tendency to overestimate the reduction in antagonist potency, particularly with 10^{-4}M pentobarbitone, if the full potentiating activity of the barbiturates was obscured by partial occlusion or desensitization of responses to muscimol.

Flurazepam and lorazepam

Similar experiments to those with the barbiturates were carried out with these benzodiazepines. Neither flurazepam nor lorazepam evoked a direct response but they both caused parallel shifts of the muscimol dose-response line to the left (see Simmonds, 1980b). The potentiation by flurazepam 10^{-6}M was quite small (Figure 5a) and there was no further increase at 10^{-5}M. With lorazepam 10^{-6}M, the potentiation of muscimol was a little bigger although part of this was due to a significant effect of the vehicle for lorazepam (Figure 5a). An increase in lorazepam concentration to 10^{-5}M caused no further increase in the potentiation of muscimol. Thus, neither of the benzodiazepines was able to potentiate muscimol to the same extent as the barbiturates.

The antagonism of muscimol by bicuculline was not significantly affected by either flurazepam or lorazepam at 10^{-6}M (Figure 5b) but the Schild plots for picrotoxin were displaced to the right in a parallel fashion. Thus the potency of picrotoxin as a muscimol antagonist was significantly reduced by both benzodiazepines to similar extents (Figure 5c). The vehicle for lorazepam also caused a small but significant reduction in the potency of picrotoxin. An increase in the concentration of flurazepam and lorazepam to 10^{-5}M did not further reduce the potency of picrotoxin.

Further experiments were done to determine whether the maximum reduction in picrotoxin potency with a benzodiazepine alone or phenobarbitone alone could be exceeded with a combination of flurazepam 10^{-6}M and phenobarbitone 10^{-4}M. As shown in Figure 5, this combination reduced the potency of picrotoxin to about the average of the effects of flurazepam alone and phenobarbitone alone. In contrast, the potentiation of muscimol by the combination approximated the sum of the effects of flurazepam alone and phenobarbitone alone.

Phenytoin

Superfusion of the tissue with phenytoin 10^{-4}M always caused a clear hyperpolarization which was sustained. Muscimol was potentiated to the same extent as it was by phenobarbitone (Figure 5a) but in contrast to phenobarbitone, phenytoin did not significantly affect the potency of picrotoxin. Phenytoin 10^{-4}M was close to the limit of solubility in the Krebs medium.

Discussion

Drugs with depressant and anticonvulsant properties interacted with the GABA receptor + effector system in rat cuneate afferents in two separate ways.

Firstly, there was an interaction reflected in the potentiation of responses to muscimol. This was most marked with pentobarbitone while phenobarbitone was about 13 times less potent. Phenytoin was equipotent with phenobarbitone and the benzodiazepines caused only a weak potentiation. A similar order of activity was seen with respect to the reduction in potency of bicuculline as a GABA receptor antagonist, significant effects being obtained only with barbiturates at concentrations that caused a substantial potentiation of muscimol. This might suggest that the potentiating action was due to an effect, either direct or indirect, on the GABA receptor.

Secondly, there was a different spectrum of activity seen with regard to the reduction in potency of picrotoxin. Flurazepam and lorazepam were particularly effective, followed by phenobarbitone and pentobarbitone which were approximately equipotent. Phenytoin was inactive in this respect. Since it appears that the site of action of picrotoxin as a GABA antagonist is distinct from the GABA receptor (Simmonds, 1980a), it is perhaps not surprizing that the order of drug activity at the picrotoxin site was different from that for an action thought to be at the GABA receptor.

Certain features of this pattern of drug interaction with the GABA system have been described before in other preparations but there are also some contradictory results. In the frog spinal cord, for example, both phenobarbitone and pentobarbitone reduced the potency of picrotoxin as a GABA antagonist on motoneurones without affecting that of

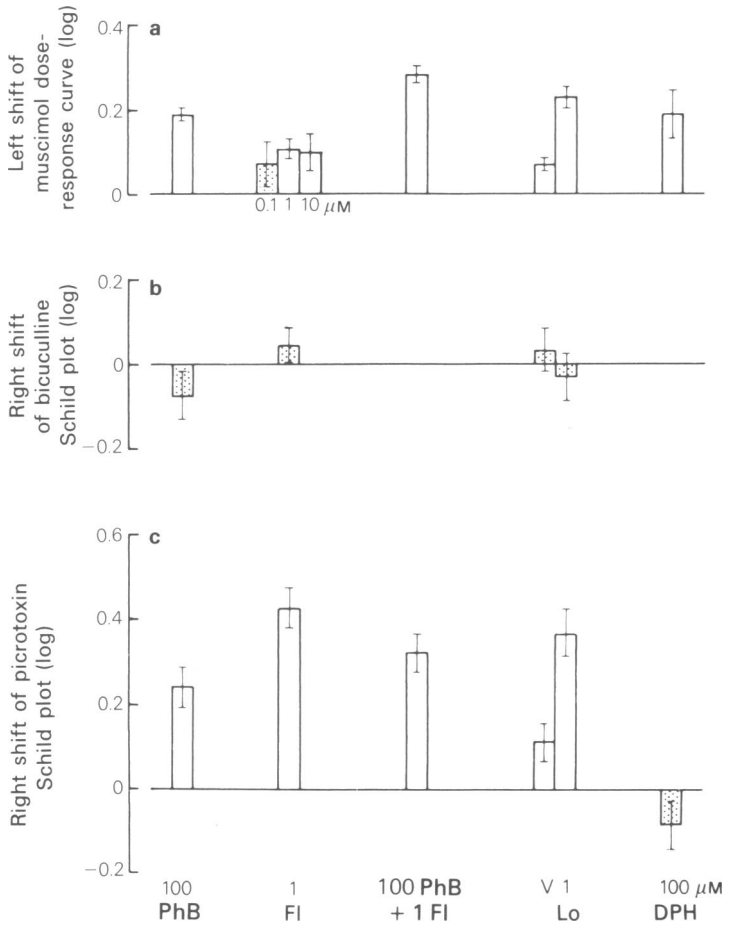

Figure 5 (a) Potentiation of muscimol, (b) reduction in potency of bicuculline and (c) reduction in potency of picrotoxin by 100 μM phenobarbitone (100 PhB), 1 μM flurazepam (1 Fl), a combination of phenobarbitone and flurazepam, 1 μM lorazepam (1 Lo), the vehicle for lorazepam (V) and 100 μM phenytoin (100 DPH). The ordinates are the same as in Figure 4. Open columns are significantly different from zero ($P<0.05$).

bicuculline (Evans, 1979; Nicoll & Wojtowicz, 1980). The only difference between these barbiturates was a five fold lower potency of phenobarbitone. Phenytoin did not affect the actions of picrotoxin in agreement with the present results but in contrast, the benzodiazepine chlordiazepoxide was also without effect (Nicoll & Wojtowicz, 1980). These discrepancies suggest some quantitative differences between frog motoneurones and rat cuneate afferents with regard to the influence of various regulatory sites on the GABA receptor + effector system. Differences of another sort are apparent in the results of Bowery & Dray (1978) on rat superior cervical ganglion, where a wide range of depressant drugs was reported to reduce the potency of bicuculline whilst causing little potentiation of GABA. However, these observations have been disputed with regard to pentobarbitone (Brown & Constanti, 1978; Evans, 1979) so it is not clear whether the properties of the GABA system in rat superior cervical ganglion really do differ from those in rat cuneate afferents. Perhaps simultaneous potentiation and occlusion or desensitization of responses to GABA may have been a factor in the experiments of Bowery & Dray (1978).

A notable feature of the present results was the modest potentiation of muscimol by the benzodiazepines compared with the much greater effect of pentobarbitone. This is in accord with the observations of Pickles & Simmonds (1978) and Riley &

Scholfield (1980) although other workers found little difference between barbiturates and benzodiazepines in potentiating GABA (Macdonald & Barker, 1979; Schlosser & Franco, 1979). However, the underlying mechanisms of potentiation may be different, since inhibitory processes thought to be mediated by GABA were enhanced by phenobarbitone *in vivo* in a distinctly different way from the enhancement by benzodiazepines (Polc & Haefely, 1976). This would be compatible with the additive effects of flurazepam and phenobarbitone in potentiating muscimol in the present experiments. A further consideration is the possibility that the limited potentiation of muscimol by the benzodiazepines might be due to a concomitant antagonism of muscimol similar to that reported to occur with GABA (Gähwiler, 1976; Macdonald & Barker, 1978a).

The quantitative detail obtained in the present study provides an opportunity to assess the predictive value of measurements of drug binding to the GABA receptor and associated sites. The enhancement of [^{3}H]-GABA binding to neuronal membranes seen with the benzodiazepines under certain conditions (Guidotti, Toffano & Costa, 1978; Lloyd & Dreksler, 1979) could provide an explanation for the small potentiation of muscimol by the benzodiazepines. However, the more marked potentiations by the barbiturates and that by phenytoin appear not to be paralleled by a change in GABA or bicuculline binding, according to most studies (Zukin, Young & Snyder, 1974; Möhler & Okada, 1977a; Lloyd & Dreksler, 1979; Olsen *et al.*, 1979). An exception is the report by Willow & Johnston (1980) that pentobarbitone can enhance GABA binding to crude synaptosomal membranes from rat brain. Since this enhancement was probably an indirect effect which could be abolished by picrotoxinin, it still does not provide a clear correlate for the potentiation of muscimol as a phenomenon not involving the picrotoxin site.

A better overall correlation exists between the ability of barbiturates, benzodiazepines and phenytoin to inhibit [^{3}H]-dihydropicrotoxinin binding (Ticku, Ban & Olsen, 1978; Ticku & Olsen, 1978; Olsen, Leeb-Lundberg & Napias, 1980) and their ability to reduce the potency of picrotoxin as an antagonist of muscimol. Even so, there are some quantitative discrepancies with the barbiturates and phenytoin: the order of potency in displacing [^{3}H]-dihydropicrotoxinin binding is pentobarbitone > phenytoin > phenobarbitone whereas that for reduction in picrotoxin potency was pentobarbitone = phenobarbitone > phenytoin. Further, the very high potency of (±)5-(1,3 dimethylbutyl)-5-ethylbarbituric acid (DMBB) in displacing [^{3}H]-dihydropicrotoxinin binding contrasts with its activity on the present experimental system which is little different from that of pentobarbitone (unpublished observations). The reasons for these discrepancies remain to be resolved.

With neither the benzodiazepines nor the barbiturates did the interaction with picrotoxin in the present experiments appear to be competitive since increases in the concentration of the benzodiazepines above 10^{-6}M and phenobarbitone above 10^{-4}M failed to cause any further reduction in picrotoxin potency. An indirect action of the benzodiazepines might be expected since they have their own unique binding sites (Möhler & Okada, 1977b; Squires & Braestrup, 1977; Braestrup & Nielsen, 1980). The present results suggest that the barbiturates as well may affect the picrotoxin site indirectly. There seems to be a limit to the reduction in picrotoxin potency since combination of a maximally effective dose of flurazepam with a maximally effective dose of phenobarbitone produced no greater effect than either drug alone.

The distinctly different orders of drug action in potentiating muscimol and reducing the potency of picrotoxin correspond rather well with the distinction between two behavioural properties of these drugs (Albertson, Peterson & Stark, 1980). Phenobarbitone causes a substantial reduction in both the severity and duration of kindled amygdaloid seizures in rats in doses lower than those required to cause ataxia. Pentobarbitone, on the other hand, causes a similar reduction in seizures only at doses which result consistently in ataxia. The pattern of effect of benzodiazepines is similar to that of phenobarbitone while phenytoin has very little effect on this type of seizure, even in doses which cause consistent ataxia. It is possible, therefore, that the sedative/hypnotic effects of these drugs which are described as ataxia could be due to potentiation of the action of GABA, while the anticonvulsant effect against kindled amygdaloid seizures could be due to an action at the picrotoxin site in the GABA response mechanism. A similar distinction between pentobarbitone and phenobarbitone has been described in cultured spinal cord neurones (Macdonald & Barker, 1978b) where phenobarbitone abolished picrotoxin-induced paroxysmal depolarizing events at concentrations lower than those which depressed spontaneous neuronal activity, while pentobarbitone showed no such selectivity. All these results imply that there may be an endogenous regulator of the GABA response mechanism operating at the picrotoxin site, as might also be suggested from binding studies (Olsen & Leeb-Lundberg, 1980), and that a malfunction of this regulatory action could be involved in the generation of certain types of seizure.

Overall, therefore, a pattern has emerged which suggests that a drug that potentiates GABA is likely to have hypnotic properties, while a drug that selec-

tively reduces the potency of picrotoxin as a GABA antagonist with only modest potentiation of GABA is likely to have anticonvulsant properties without too marked a sedative action. The anticonvulsant properties will be of a type shared by phenobarbitone and the benzodiazepines but not by phenytoin.

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