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Effect of anticonvulsant drugs *in vitro* on pineal gland indole metabolism in organ culture

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The pineal gland may play a role in seizure states [1, 2] and has been suggested to exert an anticonvulsant effect through the actions of melatonin [2–5]. Few reports have investigated the effect of anticonvulsant drugs on the pineal gland. These reports indicate that diazepam but not phenobarbitone causes a significant reduction in nocturnal Acetyl Co A: Arylamine *N*-Acetyltransferase (EC 2.3.1.5) (SNAT) activity [6] and that sulthiame is a mixed non-competitive inhibitor of SNAT *in vitro* [7]. As an extension to these studies the effect of 12 anticonvulsants *in vitro* on pineal gland indole metabolism was investigated.

Materials and Methods

Treatment of rats. Female rats (180–220 g) kept under constant light conditions (04.00–16.00 light) were used. The rats were allowed at least 2 weeks in which to become acclimatized to the light cycle before being used. Before use it was ascertained that the rats were not pregnant and oestrous smears were taken to determine the phase of the sexual cycle. The osestrous smears were taken at the same time every day (11.30) and only rats showing positive cornification were used that afternoon. Rats were sacrificed at 15.00 and pineal glands removed with minimum delay using sterile forceps and placed into culture media.

Organ culture. Pineal glands were incubated individually in 50 μl culture medium (B G.J.b. medium, Fitton–Jackson modification, Gibco Europe, U.K.) containing 5-hydroxy [G-^{3}H] tryptamine creatinine sulphate (1.6×10^{-5} M, 370 GBq/mmole, Amersham, U.K.) and the drugs in varying concentration (1×10^{-6}–$\times 10^{-3}$ M). Pineal glands were placed into culture shortly after sacrifice, tubes were gassed with 95% O_2/5% CO_2, sealed and incubated at 37° for 24 hr. After incubation pineal glands were removed and 5 μl aliquots of the culture medium spotted onto pretreated thin layer chromatography (TLC) plates (Silica gel 60, Merck, Darmstadt, F.R.G). The pretreating involved applying 5 μl of 95% ethanol containing 2.5 μg of each of the serotonin metabolites. These spots were dried prior to application of the culture media and served to localise the various metabolites after two dimensional development.

Control tubes containing medium and 5-hydroxy[G-^{3}H] tryptamine were treated in the same way except pineal glands were omitted and served to calculate background values for each metabolite.

TLC separation and quantitation. The spotted plates were dried and first developed in chloroform:methanol (9:1) followed by development in ethyl acetate at right angles to the first run. Before development and after each run the plates were dried by brief exposure to heat (65°). After development in ethyl acetate the plates were dried and sprayed with Van Urk's reagent (1 g 4-dimethylaminobenzaldehyde in 50 ml 25% hydrochloric acid and 50 ml ethanol) followed by heating at 95° for a short while. The coloured spots which resulted were scraped into scintillation cocktail and radioactivity present quantitated. Counting efficiency was determined using the external standards channel ratio and exceeded 20% in all cases.

Control incubations were treated in the same way, radioactivity resulting being substracted from pineal values to obtain a true quantity for the metabolies produced.

Analysis of data. Counts per minute (cpm) were converted into nanograms (ng) produced/pineal/24 hr with standard error of the mean (SEM) using computer assisted analysis. The specific activity of the metabolites varied due to removal of some of the original tritiated hydrogen atoms on serotonin. As a result a correction factor was built into the programs to enable accurate calculation of the quantities of metabolites produced. All determinations were repeated four times on separate occasions. The Student-*t* distribution was used to calculate probabilities.

Results and discussion

Most of the drugs used caused a significant increase in both hydroxyindole acetic acid and hydroxytryptophol production (Table 1) which possibly resulted from increased substrate availability due to either enhanced serotonin uptake and/or reduced efflux. An increase in the concentration of diazepam and diphenylhydantoin caused a reduction in both hydroxyindole acetic acid and hydroxytryptophol production which may result from decreased MAO activity as both these drugs have been shown to exert MAO inhibitory activity [8, 9].

In the case of clonazepam, diazepam, diphenylhydantoin, ethosuximide, phenobarbitone and valproate an increase in drug concentration led to a decrease in hydroxytryptophol production (Table 1) which may also be attributed to inhibition of synthesis as all these drugs have been shown to be aldehyde reductase inhibitors [10–15].

That acetazolamide, clonazepam, diazepam, diphenylhydantoin and phenobarbitone all increase brain serotonin [16–20] might indicate that serotonin uptake into the pineal gland was likewise enhanced by treatment with the anticonvulsant drugs, thus increasing substrate availability and production of hydroxyindole acetic acid and hydroxytryptophol above control levels despite the existence of MAO and aldehyde reductase inhibitory activity.

Acetazolamide, carbamazepine, clonazepam, diazepam, diphenylhydantoin, sulthiame and valproate all caused a decrease in *N*-acetylserotonin production and as only sulthiame has been found to affect SNAT *in vitro* [7] some effect/s other than enzyme inhibition must be responsible for the reduced production of *N*-acetylserotonin observed with the other drugs. These effect/s possibly result in decreased SNAT levels supported by the observation that administration of the anticonvulsant drugs *in vivo* led to decreased SNAT activity (unpublished data). The mechanism/s responsible for the reduced levels are unclear but may involve interference with calcium influx or changes in membrane polarization, both of which would interfere with SNAT induction [17, 18, 21].

Melatonin synthesis was reduced by acetozalamide, carbamazepine, clonazepam, diazepam, diphenylhydantoin, primidone, sulthiame and valproate (Table 2) and tended to parallel decrease in *N*-acetylserotonin levels. The reduction in substrate available for methylation was therefore probably responsible for reduction in melatonin production, confirming earlier reports [22–26].

Half the drugs examined caused a significant increase in methoxytrytophol production (Table 2). This may be partially explained by increased substrate availability and

Table 1. Effect of various anticonvulsant drugs *in vitro* on amounts of hydroxyindoles formed

Drug		Hydroxyindole formed (ng/pineal/24 hr) NAS	HIAA	HTOL
No drug		4.9 ± 0.4	71 ± 05	13 ± 2
Acetazolamide	1	3.3 ± 0.9	97 ± 08**	20 ± 4
	2	2.3 ± 0.3****	98 ± 07**	21 ± 3*
	3	3.1 ± 0.7*	102 ± 11**	22 ± 4*
	4	3.7 ± 0.6*	108 ± 23	20 ± 2**
Beclamide	1	3.7 ± 1.0	97 ± 15	20 ± 4
	2	4.2 ± 1.3	86 ± 13	17 ± 4
	3	4.8 ± 0.9	91 ± 12	18 ± 3
	4	4.3 ± 1.1	105 ± 22	21 ± 4
Carbamazepine	1	2.8 ± 0.4***	72 ± 07	28 ± 2****
	2	2.1 ± 0.5***	87 ± 11	24 ± 2***
	3	1.6 ± 0.3*****	101 ± 12*	22 ± 1***
	4	1.8 ± 0.3*****	114 ± 23*	21 ± 3*
Clonazepam	1	1.7 ± 0.2*****	101 ± 12*	25 ± 4**
	2	3.2 ± 0.7*	106 ± 11**	24 ± 3**
	3	3.0 ± 0.9*	140 ± 16***	28 ± 5**
	4	3.1 ± 0.8*	149 ± 23***	29 ± 4***
Diazepam	1	1.5 ± 0.5****	58 ± 13	12 ± 4
	2	3.8 ± 0.8	74 ± 14	13 ± 3
	3	4.1 ± 0.7	98 ± 21	14 ± 3
	4	4.8 ± 0.5	101 ± 18	14 ± 3
Diphenylhydantoin	1	3.4 ± 0.9	56 ± 18	11 ± 4
	2	3.5 ± 0.8	89 ± 15	13 ± 3
	3	3.3 ± 1.0	108 ± 16*	19 ± 5
	4	2.9 ± 0.7**	138 ± 22**	21 ± 7
Ethosuximide	1	6.4 ± 1.2	114 ± 11***	16 ± 2
	2	5.2 ± 1.3	122 ± 13***	19 ± 3*
	3	4.9 ± 1.2	144 ± 20***	21 ± 4*
	4	3.4 ± 0.9	146 ± 15****	20 ± 5
Pheneturide	1	5.0 ± 1.8	130 ± 18***	22 ± 3**
	2	3.6 ± 1.5	132 ± 19**	24 ± 3**
	3	3.2 ± 1.1	141 ± 15***	25 ± 2***
	4	4.0 ± 1.0	128 ± 23**	24 ± 2***
Phenobarbitone	1	6.2 ± 1.2	146 ± 22***	24 ± 2***
	2	4.9 ± 0.3	168 ± 28***	31 ± 4***
	3	6.8 ± 2.1	170 ± 25***	32 ± 4***
	4	5.5 ± 1.2	169 ± 19****	24 ± 3**
Primidone	1	3.6 ± 0.9	186 ± 18*****	33 ± 3****
	2	3.5 ± 0.5	209 ± 22***	32 ± 3****
	3	3.4 ± 0.6	195 ± 24****	28 ± 3***
	4	3.7 ± 1.0	217 ± 26****	22 ± 4*
Sulthiame	1	1.6 ± 0.3*****	112 ± 16**	34 ± 3****
	2	2.8 ± 0.6**	148 ± 26**	30 ± 2*****
	3	3.9 ± 0.9	148 ± 23***	24 ± 3**
	4	5.8 ± 1.2	125 ± 22**	21 ± 2**
Valproate	1	2.3 ± 0.3****	134 ± 28*	21 ± 2**
	2	2.2 ± 0.4****	127 ± 29*	25 ± 3***
	3	2.2 ± 0.3****	142 ± 15***	31 ± 4***
	4	2.4 ± 0.3****	140 ± 21***	32 ± 5***

NAS, *N*-acetylserotonin; HIAA, 5-hydroxyindole-3-acetic acid; HTOL, 5-hydroxytryptophol; 1 = 10^{-3} M, 2 = 10^{-4} M, 3 = 10^{-5} M and 4 = 10^{-6} M. Results represent mean ± S.E.M.

*P = 0.05, **P = 0.025, ***P = 0.01, ****P = 0.005, *****P = 0.001.

Table 2. Effect of various anticonvulsant drugs *in vitro* on indole methylation in organ culture

Drug		Methoxyindole formed (ng/pineal/24 hr) MTN	MIAA	MTOL
No drug		4.5 ± 0.4	5.8 ± 0.7	1.9 ± 0.9
Acetazolamide	1	20.0 ± 0.3****	4.8 ± 0.7	3.3 ± 0.7
	2	1.8 ± 0.3****	5.5 ± 1.2	3.5 ± 0.8
	3	2.2 ± 0.3****	5.2 ± 1.0	3.3 ± 0.7
	4	3.0 ± 0.4**	6.8 ± 0.9	3.6 ± 0.8
Beclamide	1	3.8 ± 0.3	4.6 ± 0.8	3.2 ± 0.9
	2	4.6 ± 0.2	4.8 ± 0.6	3.4 ± 0.5
	3	4.9 ± 0.3	4.9 ± 0.6	3.3 ± 0.7
	4	4.5 ± 0.3	4.9 ± 0.7	3.2 ± 0.8
Carbamazepine	1	2.1 ± 0.4***	4.8 ± 0.8	4.2 ± 1.0*
	2	1.7 ± 0.5***	4.5 ± 0.7	4.3 ± 1.1*
	3	1.2 ± 0.4****	4.8 ± 0.5	6.0 ± 1.1**
	4	1.3 ± 0.4****	5.3 ± 0.7	6.2 ± 1.2**
Clonazepam	1	1.1 ± 0.4*****	6.3 ± 0.9	2.8 ± 0.9
	2	2.5 ± 0.5**	6.2 ± 1.1	3.2 ± 0.6
	3	2.5 ± 0.4***	7.0 ± 0.8	4.7 ± 1.3**
	4	2.4 ± 0.4***	7.2 ± 1.1	5.5 ± 1.2**
Diazepam	1	1.0 ± 0.4*****	2.9 ± 0.8**	0.8 ± 0.3
	2	2.9 ± 0.4**	3.1 ± 0.8**	1.3 ± 0.9
	3	3.4 ± 0.5*	3.2 ± 0.7**	1.4 ± 0.6
	4	3.8 ± 0.8	4.2 ± 0.6	2.6 ± 0.8
Diphenylhydantoin	1	3.0 ± 0.9	3.5 ± 0.5**	1.5 ± 0.4
	2	2.9 ± 0.5**	4.9 ± 0.3	3.2 ± 0.9
	3	2.7 ± 0.4**	5.2 ± 0.6	3.3 ± 0.8
	4	2.6 ± 0.5**	6.9 ± 1.2	4.1 ± 1.1
Ethosuximide	1	5.7 ± 0.9	4.3 ± 0.7	2.2 ± 0.9
	2	4.7 ± 0.5	5.1 ± 0.8	2.3 ± 0.7
	3	4.6 ± 0.4	5.2 ± 1.0	3.8 ± 0.8
	4	3.5 ± 0.5	6.3 ± 1.1	3.9 ± 1.0
Pheneturide	1	4.5 ± 0.7	6.0 ± 0.9	2.7 ± 0.7
	2	3.9 ± 0.4	6.4 ± 1.0	3.6 ± 0.8
	3	3.4 ± 0.6	6.1 ± 1.1	3.8 ± 0.9
	4	4.1 ± 0.3	6.2 ± 1.3	4.0 ± 1.0
Phenobarbitone	1	4.8 ± 0.9	6.1 ± 0.9	3.1 ± 0.7
	2	3.9 ± 0.4	6.2 ± 0.8	4.4 ± 0.8*
	3	5.3 ± 0.6	6.4 ± 1.0	4.3 ± 1.0*
	4	4.5 ± 0.4	6.3 ± 1.1	4.2 ± 1.1
Primidone	1	2.7 ± 0.4**	7.6 ± 1.1	6.1 ± 1.4**
	2	2.4 ± 0.5***	7.5 ± 1.2	6.4 ± 1.3**
	3	2.3 ± 0.4***	7.1 ± 1.0	5.9 ± 1.1**
	4	3.6 ± 0.6	7.0 ± 0.9	4.6 ± 1.2*
Sulthiame	1	0.9 ± 0.3*****	6.1 ± 1.1	6.2 ± 0.7*****
	2	2.1 ± 0.6***	5.6 ± 0.8	4.8 ± 0.6***
	3	3.4 ± 0.4*	5.2 ± 1.2	3.0 ± 0.9
	4	4.3 ± 0.7	4.5 ± 1.1	2.4 ± 0.9
Valproate	1	1.6 ± 0.3****	5.8 ± 1.1	6.3 ± 1.0***
	2	1.7 ± 0.3****	4.7 ± 0.7	6.4 ± 1.1**
	3	1.8 ± 0.3****	4.6 ± 0.8	6.7 ± 1.2**
	4	1.7 ± 0.3****	4.4 ± 0.5	7.0 ± 1.4**

MTN, melatonin; MIAA, 5-methoxyindole-3-acetic acid; MTOL, 5-methoxytryptophol; 1 = 10^{-3} M, 2 = 10^{-4} M, 3 = 10^{-5} M and 4 = 10^{-6} M. Results represent mean ± S.E.M.
* P = 0.05, ** P = 0.025, *** P = 0.01, *** P = 0.005, ***** P = 0.001.

probably also results from an increase in the number of catalytic sites on HIOMT available for methylation. *N*-acetylserotonin appears to have the greatest affinity for HIOMT [26] and any decrease in *N*-acetylserotonin availability would increase the amount of other hydroxyindoles that could be processed by HIOMT.

The reduction in methoxyindole acetic acid caused by diazepam and diphenylhydantoin at 10^{-3} M may be due to reduced substrate availability, while the lower methoxyindole acetic acid production at diazepam concentrations below 10^{-3} M cannot be explained.

It is evident from the foregoing discussion that anticonvulsant drugs are capable of causing significant changes in pineal gland indole metabolism. What effect this would have on a seizure states is unclear but considering the many functions attributed to the pineal gland [27] some changes are likely to occur. Further studies would be required to clarify the relationship between anticonvulsant drugs and the pineal gland.

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