# EFFECT OF ANTICONVULSANT AGENTS ON PINEAL GLAND INDOLE METABOLISM

Dissertation

Submitted in Partial Fulfilment of the

Requirements for the Degree of

DOCTOR OF PHILOSOPHY

of Rhodes University

by

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November 1982

Goya: The Sleep of Reason gives birth to Monsters (c1792)

To Jenny

The evil demon disappears like the sudden ceasing of the basso parts in music, which hitherto wildly permeated the piece; what before seemed beyond control is now ordered as by magic....

L. Boltzman

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# ACKNOWLEDGEMENTS

Firstly I would like to thank my wife, Marion, for her constant support and encouragement throughout this study.

I would also like to thank my supervisor, Professor B. Potgieter, for his assistance and encouragement during this study.

Dr B.J. Wilson and Mr S. Daya deserve thanks for numerous often enlightening discussions on various aspects of pineal biochemistry and physiology. I am especially grateful to Professor J.T.-F. Wong of the University of Toronto for offering his assistance and advice on enzyme kinetics and to Dr P.A. Adams of the University of Cape Town for constructive critisism regarding kinetic behaviour and for supplying computer programs to calculate kinetic data. In this regard I would also like to thank Mr E. de Jager and Mr R.W. Gibbs for assistance in the use of computer software and with programming.

I would also like to thank the following-

Pharmaceutical industries for supplying the drugs used.

Bayer (SA) (Pty) Ltd., RSA
Ciba-Geigy (Pty) Ltd., RSA
ICI (SA) (Pharmaceuticals) (Pty) Ltd., RSA
Maybaker (SA) (Pty) Ltd., RSA
Parke-Davis (Pty) Ltd., RSA
R+C Pharmaceuticals (Pty) Ltd., RSA
Roche Products (Pty) Ltd., RSA
Rona Labs Ltd., UK
Sapos SA, Switzerland

Hoffman La Roche, Switzerland for radioactive clonazepam and diazepam which greatly facilitated pharmacokinetic determinations.

Mr G. Botha, Grahamstowm Abbatoir, for supplying bovine pineal glands, without which much of the kinetic work would have been impossible.

Mr D McGough, Syva Diagnostics (Pty) Ltd. for the loan of an Emit/Lab 5000 system.

The following firms and institutions for granting research funds which enabled this study to be completed.

| | R |
|---|---|
| Ciba-Geigy (Pty) Ltd., RSA | 750.00 |
| Council for Scientific and Industrial Research | 500.00 |
| Parke-Davis (Pty) Ltd., RSA | 250.00 |
| R+C Pharmaceuticals (Pty) Ltd., RSA | 125.00 |
| Rhodes University | 2120.00 |

# PREFACE

The general indications that the pineal gland might be involved in homeostasis, and more specifically the evidence suggesting a role in amelioration of seizure states warranted further investigation. No reports had examined a possible link between anticonvulsant drug administration and pineal gland function, and few enabled any type of presumption to be made as to possible effects. This study was an attempt to evaluate in which ways anticonvulsant drugs might alter pineal gland indole metabolism, with a view to increasing understanding of the role of the pineal in modulation of epileptic discharges.

In order to make the study as meaningful as possible extensive preliminary investigations were necessary.

Pharmacokinetic determinations gave an indication of tissue concentrations of the drugs, which could then be related to observed effects. As far as possible, where existing information was lacking, the catalytic behaviour of the various enzymes was characterised in order to explain any observed effects at a molecular level.

An attempt was also made to characterise the regulatory mechanisms controlling indole metabolism, again in order to define the pharmacological effects exerted by the drugs used. The complexity of the system made it impossible to suggest a single uniform regulatory hypothesis, although some significant observations were made.

Finally, the studies involving the anticonvulsant drugs were conducted on intact animals, isolated organs and individual enzymes in an attempt to determine whether the observed effects were occuring at a molecular, local or central level.

# CHAPTER 1

## LITERATURE REVIEW

The pineal gland is unique in that it enjoys a large measure of protection by virtue of its location and yet is directly exposed to the vascular system. This provides it with an ideal environment in which to monitor homeostasis and, considering the many physiological effects attributed to the pineal gland [1], it is indeed possible that it is involved in such a function. Another observation which underlines the possible importance of the pineal gland in the control of physiologic mechanisms is that, of all tissues except the kidney, it is the most richly perfused by the vascular system [2]. This blood supply originates from the posterior cerebral artery [3].

The pineal has been claimed to be part of the diffuse neuroendocrine system [4] and fulfils the requirements of an APUD organ [5] which suggests neuroendocrine modulatory functions. The pineal consists primarily of interstitial cells, possibly macroglia [6] and pinealocytes which are the recepto-sensory components [7]. It is the pinealocyte activity which makes the pineal part of the APUD system and enables it to function in the diffuse neuroendocrine system.

The physiologically significant pineal products are considered to be mainly the methoxyindoles and peptides, although hydroxyindoles have been thought to exert physiological effects [8,9,10].

There appear to be numerous nervous connections between the pineal and various parts of the central nervous system. The best documented pathway is that which conveys information from the retina to the pineal along the retinohypothalamic tract, through the suprachiasmatic nucleus, medial forebrain bundle and superior cervical ganglion [11,12]. Photic information also travels to the pineal via the habenular posterior commissure complex [12,13,14]. This pathway is faster than that via the suprachiasmatic nucleus [13]. The suprachiasmatic nucleus is thought to be important for the generation of normal photic rhythms in the pineal [15,16,17] and acetyl choline may be involved in the transmission of such

information [18]. Fibres containing oxytocin and vasopressin which extend from the suprachiasmatic nucleus to the pineal via the habenular commisure, also appear to exist, and may indicate a role in fluid and electrolyte balance [19,20]. It has also been suggested that a link exists between the pineal and diencephalon [21]. Evidence, however, suggests that the bulk of inputs arrive through the stalk [22].

The sympathetic nervous connections to the pineal have received the most attention and, apart from the classic transmitter noradrenaline, it has been suggested that serotonin may serve some transmitter functions [23,24] although it has been shown not to stimulate pineal activity [25]. Noradrenaline was found to act on highly specific receptors [26] which have been identified as β-adrenergic receptors [27]. These probably belong to the $\beta_1$-subgroup [28]. Information which results in stimulation of these receptors travels along sympathetic nerves from the superior cervical ganglion [29,30]. Activation of these receptors stimulates adenyl cyclase production of cyclic AMP which, in turn, is responsible for increased synthesis of the enzyme serotonin N-acetyltransferase [31,32,33]. This β-stimulated induction of enzyme levels has been suggested to occur via two routes; increase in transcription, and enhancement of post-transcriptional events. Transcription, however, is not always necessary for enzyme induction [32]. Stimulation of transcriptional events results in the synthesis of messenger RNA [34], presumably involving phosphorylation of nuclear proteins [35,36] and resulting in increased synthesis of serotonin N-acetyltransferase [32]. It is interesting to note that β-stimulation regulates activity of cyclic AMP phosphodiesterase [37,38] and appears to stimulate serotonin synthesis [39].

Although it has been shown that adenyl cyclase is linked to postsynaptic β-receptors [40] effects of β-stimulation may not be mediated exclusively by cyclic AMP. Hyperpolarization of pinealocyte membranes resulting from activation of β-receptors might be involved in such effects, possibly independently of cyclic AMP [41]. It is possible that both increase in cyclic AMP and hyperpolarization are necessary for complete expression of the β-stimulatory effects in the pineal [41] and it has been shown that calcium ions are essential for such activity [42,43].

Activation of β-receptors also leads to a release of taurine from pinealocytes; a process presumed to occur as a result of membrane hyperpolarization [44] and a cyclic AMP mediated mechanism [45]. This released taurine has been implicated as an extracellular feedback messenger [44] and has been shown to stimulate β-receptors [46].

To further complicate the matter sensitivity of the β-receptors to stimulation varies. These receptors exhibit supersensitivity at the onset of the dark phase, presumably as a result of decreased noradrenaline release during the light phase [47 48]. Conversely, increased transmitter release during the dark phase leads to the developement of β-receptor subsensitivity [47]. Receptor stimulation causes a decrease in the quantity of receptors and in the activity of hormone sensitive adenyl cyclase [49], suggesting that receptor sensitivity is dependent, at least to a certain extent, on the sensitivity of adenyl cyclase to stimulation. Lack of stimulation, on the other hand, leads to an increase in sensitivity of adenyl cyclase [49]. Apart from this link between β-receptor and adenyl cyclase sensitivity it appears that receptor sensitivity might be affected by changes in phosphodiesterase activity [50]. Calcium ions and cyclic GMP have also been implicated as mediators in the process of developement of β-receptor subsensitivity [51]. The number of β-receptors has been found to be highest at the start and lowest at the end of the dark phase [52] which parallels changes in sensitivity and may contribute to the developement of sub- or super-sensitivity. Under certain circumstances sex hormones can increase sensitivity of β-receptors to stimulation [53].

Presynaptic calcium dependent α-adrenergic receptors appear to exist in the pineal gland, stimulation leading to production of cyclic GMP [54,55]. This effect appears to be inhibitory leading to decreased noradrenaline release [55]. Generation of cyclic GMP has also been shown to occur as a result of some postsynaptic mechanism and might act as a physiological antagonist of cyclic AMP [56]. Stimulation of these postsynaptic α-receptors enhances phosphorylation of phosphatidylinositol [57,58]. Both pre- and post-synaptic α-receptors present in the pineal fulfil the requirements for $\alpha_1$-adrenergic receptors [59]. There has been a suggestion that noradrenaline may act on both receptors, with the

effect on α-receptors leading to a decrease in β-receptor sensitivity [60]. Such dual receptor activation provides a means whereby the pineal may control a number of responses concurrently [60]. Apart from the sympathetic connections it appears that there are parasympathetic fibres which cause depletion of pinealocyte serotonin stores [61], possibly by action on muscarinic receptors [62]. These muscarinic cholinergic receptors seem to be located mainly on sympathetic terminals [61,62], although some may occur on pinealocytes [62]. Parasympathetic fibres apparently pass through the superior cervical ganglion [61], cause release of arginine vasotocin [63] and seem to be involved to some extent in the mediation of the effects of light on the pineal gland [64]. Muscarinic receptors are regulated by guanyl nucleotides [65,66] which suggests that α-receptor activation might modify cholinergic transmission in the pineal gland. Carbachol has, however, been shown to have no stimulatory action on the pineal [25] which tends to favour a modulatory role for the para-sympathetic nervous system in pineal function.

The enzymes involved in synthesis and degradation of gamma-amino butyric acid (GABA) are present in the pineal gland [67] which is suggestive of a transmitter role. Although such a role is not favoured by some reports [25,67] GABA has been shown to inhibit noradrenergic stimulation of pineal β-receptors [68]. GABA itself, although not affected by catecholamines or photic inputs [69], may in some way modulate pineal function.

Noradrenaline in pineal sympathetic nerves appears to be regulated by dopamine levels [70] and dopamine has been shown to affect phospholipid metabolism [71]. This latter effect is apparently mediated via α-receptors and not true dopaminergic receptors [58] which suggests that, although it may exert some modulatory actions, it is unlikely that a dopaminergic innervation per se has any influence on pineal activity.

The presence of a circadian rhythm in pineal histamine [71] may indicate that this biogenic amine has some transmitter function in the pineal although evidence in favour of such a proposal is lacking and it has been shown to have no effect on pineal activity [25].

Apart from the nervous stimuli the pineal is affected by adrenal hormones with stress leading to an increase in adrenaline and dopamine content of the pineal [72].

From the foregoing discussion it is apparent that β-adrenergic receptors exert the most stiking effect on pineal indole metabolism. Modulation of such activity, however, may occur as a result of α-adrenergic, muscarinic cholinergic or GABA receptors. Although evidence for the participation of other transmitters is lacking it cannot be completely rejected until disproved.

Metabolically the pineal gland is very active with large amounts of oxidative enzymes [73]. Of these enzymes glucose-6-phosphate dehydrogenase and lactate dehydrogenase appear to be present in the highest concentrations [73]. The former may be important for synthesis of pentose nucleotides or for production of NADPH while the presence of relatively large amounts of cytochrome oxidase [73] implies that the pineal gland normally requires a large amount of oxygen.

Although a highly specific allosteric serotonin carrier has been characterised in pinealocyte membranes [74], pineal indole metabolism probably begins with uptake of dietary tryptophan. The uptake of tryptophan and its conversion to 5-hydroxytryptophan in the pineal appears to be unaffected by serotonin [75] unlike the carrier system which is modulated by serotonin concentrations [74]. Certain aspects of indole metabolism are contentious but by and large can be summarized (Figure 1)[from 76-82].

Although it has not been documented 6-hydroxymelatonin may be formed in the pineal gland (unpublished results).

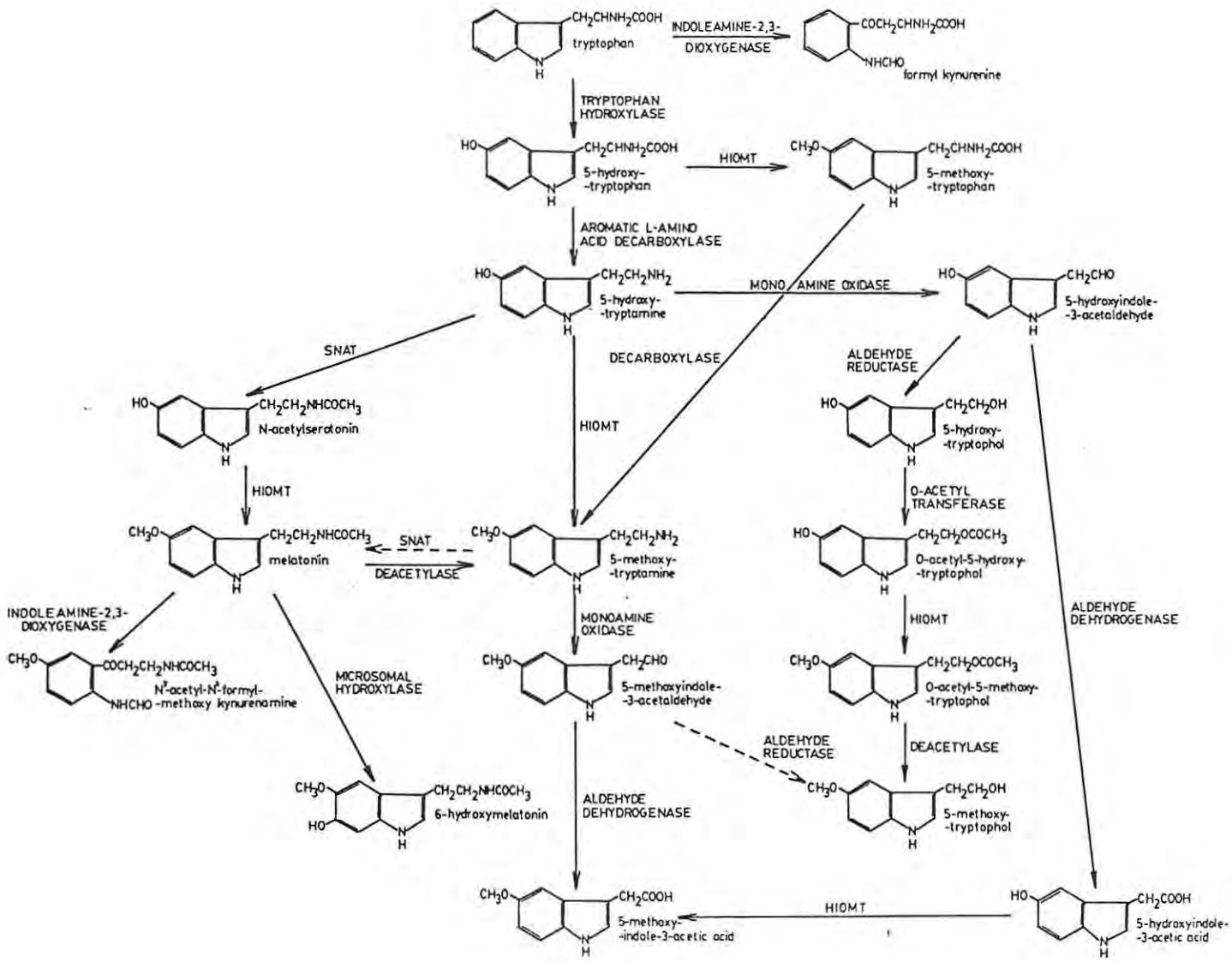


Figure 1: Indole metabolism in the pineal gland [from 76-82]. HIOMT = hydroxyindole-O-methyltransferase and SNAT = (serotonin) N-acetyltransferase

Since the discovery of melatonin [83] considerable work has suggested physiological roles for pineal indolic products, especially the methoxyindoles. Originally melatonin was thought to be a unique product of the pineal gland. This view has, however, been disputed and melatonin has been found in the retina [84] and enterochromaffin cells [85]. The presence of melatonin in the plasma of pinealectomized animals [86,87,88] supports an extra-pineal source, although complete abolition of melatonin following pinealectomy has also been reported [89].

Pineal tryptophan hydroxylase appears to be different from the cerebral enzyme [90] and may be involved in regulation of indole synthesis although contradictory evidence exists. Tryptophan hydroxylase appears to be localized in pinealocytes and may not undergo a circadian rhythm [91] although other reports suggest the presence of a rhythm with peak activity during the dark phase [92,93]. This nocturnal increase in enzyme activity may be under β-adrenergic control [93] although, again, there is evidence to the contrary [94]. The activity of hydroxytryptophan decarboxylase also appears to be under sympathetic nervous control with peak activity during the light phase [95].

Monoamine oxidase is found in the outer mitochondrial membrane [96,97] in association with lipid [97] and activity has been shown to increase with oxygen concentration [98]. Two types of monoamine oxidase have been isolated [99] and only one of these (type A) can utilize serotonin and 5-methoxytryptamine as substrates [100]. This implies that it is the type A monoamine oxidase which is involved in pineal indole metabolism. Monoamine oxidase activity may be regulated to a certain extent by phospholipids [101] which could indicate an involvement of the sympathetic nervous system. It is of interest that administration of monoamine oxidase inhibitors leads to increased production of N-acetylserotonin in the pineal, an effect probably resulting from increased serotonin levels [102,103]. The presence of endogenous monoamine oxidase inhibitors [104,105] may indicate a function in control of pineal indole metabolism.

Aldehyde dehydrogenase occurs as several isozymes [106] located in mitochondria [107]. The catalytic mechanism is ordered with NAD being the obligatory first substrate to bind; such binding

allowing aldehyde entry into the catalytic site, and acid formation preceeding NADH removal from the enzyme [108,109]. Hormonal modification of aldehyde dehydrogenase activity [110,111] may participate in regulation of indole output from the pineal.

The catalytic mechanism for alcohol dehydrogenase (aldehyde reductase) may be ordered Bi-Bi [112,113] or ordered Theorell-Chance [114] but in both cases NAD is the first substrate to bind to the enzyme followed by the alcohol. In the pineal gland this reaction proceeds in the opposite direction to produce 5-hydroxytryptophol from 5-hydroxyindole-3-acetaldehyde and by inference catalysis can be assumed to begin with binding of NADH to the enzyme. Different forms of this enzyme exist, some of which are dependent on NAD/NADH [112], the others on NADP/NADPH [115]. The NADPH dependent aldehyde reductase can be inhibited by both 5-hydroxyindole-3-acetic acid and 5-hydroxytryptophol [115] and may be the type present in the pineal. If this is indeed the case then aldehyde reductase activity could be modulated by glucose-6-phosphate dehydrogenase activity and by hydroxyindole concentration. Activity of aldehyde reductase may also be modulated by pyridoxal compounds [116,117] and hormones [118].

Serotonin N-acetyltransferase (SNAT), the enzyme responsible for formation of N-acetylserotonin in the melatonin biosynthetic pathway, undergoes a circadian rhythm in the pineal with peak activity during the dark phase [26]. The rhythm in SNAT activity is apparently under the control of a complex two-oscillator pacemaking system [119]. A similar rhythm has been found for the enzyme present in the eyes [120], suggesting that the plasma melatonin rythm may not be due solely to pineal gland activity. N-acetyltransferase activity has been found in fifteen brain areas [121], raising the possibility that melatonin may be synthesized in the brain.

The nocturnal induction of SNAT in the pineal is controlled by a β-adrenergic cyclic AMP dependent mechanism causing increased synthesis of enzyme molecules [33,68,122]. This process may be under dual regulatory mechanisms - firstly induction is dependent on increased cyclic AMP levels and secondly intracellular mechanisms must be capable of responding to this increase [123].

The effects of light on SNAT activity [124,125] are probably mediated by this mechanism. Cyclic AMP may be necessary for maintaining SNAT in a stable form and any decrease in cyclic AMP levels would result in reduction of SNAT activity [126,127]. Likewise both acetyl coenzyme A and sulphhydryl protecting compounds have been shown to exert a stabilizing effect on the thermolabile enzyme [128,129,130]. This has led to the suggestion that a disulphide containing peptide might be involved in regulation of SNAT activity [130] through a process of protein thiol:disulphide exchange [130,131]. The participation of acetyl coenzyme A in stabilizing SNAT may also represent an important regulatory mechanism [132]. It may be significant that cystamine, a compound capable of inactivating SNAT by thiol-disulphide exchange [130], can also activate acetyl coenzyme A hydrolase by a similar mechanism [133]. The inactivation exerted by cystamine on SNAT activity directly would thus be enhanced by reduced acetyl coenzyme A concentration resulting from increased acetyl coenzyme A hydrolase activity. Prostaglandin stimulation of SNAT activity [134,135] may represent yet another mechanism for regulating melatonin production by the pineal gland.

Centrally mediated mechanisms are also capable of modifying SNAT activity. The suprachiasmatic nuclei have been implicated as the control centre for maintenance of normal SNAT rhythm [136] and there is evidence to suggest involvement of centrally mediated $\alpha$-adrenergic mechanisms in control of the enzyme [137].

While melatonin is capable of inhibiting both rat liver and bovine pineal SNAT, no effect has been observed on the rat pineal enzyme [138]. SNAT has been thought to control melatonin production [139,140] and this may represent a negative feedback mechanism whereby increased melatonin levels would reduce output through inhibition of the enzyme.

An O-acetyltransferase enzyme has been found in the pineal gland which catalyses formation of O-acetyl-5-methoxytryptophol [125].

The final enzyme involved in synthesis of melatonin, hydroxyindole-O-methyltransferase (HIOMT), was originally thought to be unique to the pineal [141] although it has now been found in the retina and harderian gland [142] and may be present in

enterochromaffin cells [85]. The enzymes from several mammals, including man, are immunochemically similar [143]. It has been localized in mitochondria [144] and is capable of methylating a variety of hydroxyindoles [76,141,145,146]. HIOMT activity is highest during the dark phase [147,148,149], a phenomenon presumed to be controlled by noradrenaline [149], although sympathomimetic action has also been shown to decrease HIOMT levels in the pineal [150]. No nocturnal increase in HIOMT levels were found in this study (unpublished data) which suggests that increased melatonin production during the dark phase results from increased substrate availability.

HIOMT may not be involved solely in production of methoxyindoles as it has been shown to methylate oestradiol [151] although no functional significance has been attached to this activity. Several reports have suggested that more than one HIOMT might exist [152,153,154] and differences in activity might result from association-dissociation of the various forms [152]. In this regard it is interesting to note that at night HIOMT appears to produce more melatonin than 5-methoxytryptophol, the reverse occurring during the day [155,156]. Such a difference might result from changes in association. Several enzymes with different isoelectric points have been found in the pineal [154] and this might also play a role in differential methylation. Several citrate metabolites and some anions have been shown to activate HIOMT, presumably by an allosteric mechanism, while several compounds including cysteic and homocysteic acids, glutathione and S-adenosylhomocysteine inhibit the enzyme [153,157,158]. This raises the possibility that changes in metabolic status of the pineal might serve to regulate methylation of hydroxyindoles. Inhibition of HIOMT by S-adenosylhomocysteine is competitive with respect to the substrate S-adenosylmethionine, implying that the catalytic mechanism is ordered Bi-Bi with S-adenosylmethionine the first substrate to bind to the enzyme [159].

S-adenosylmethionine is capable of protecting HIOMT from proteolysis [160] and the enzyme responsible for its formation, methionine S-adenosyltransferase, undergoes a circadian rhythm in the pineal with peak activity during the dark phase [161]. Changes in HIOMT activity may therefore result from alteration in

synthesis of S-adenosylmethionine, a process which may be controlled by glucocorticoids [161].

Biogenic amines such as histamine and dopamine also inhibit HIOMT [162,163], as can pyridoxal 5'-phosphate [164,165] making the potential regulatory mechanisms extremely complex.

A variety of drugs affect HIOMT, tranquilizers inhibiting [166] and psychotomimetics stimulating activity [163], which might affect normal levels of methoxyindoles.

Moreover, the fact that seasonal variations exist in HIOMT activity [167,168] illustrates the complexity of indole metabolism in the pineal.

It is apparent that melatonin synthesis can be controlled by hormones [169,170,171] presumably by an effect on HIOMT or possibly cyclic AMP phosphodiesterase [172]. Melatonin may well regulate its own production [173], and another pineal product, arginine vasotocin, has been shown to inhibit indole metabolism [174]. The rhythm in methylation of hydroxyindoles by HIOMT is also regulated by pteridines and pterins [175-178]. It may be significant that pineal tyrosine hydroxylase uses tetrahydrobiopterin as a cofactor [179] and synthesis of biopterin in the pineal is mediated by a noradrenergic cyclic AMP mechanism [180]. Vasoactive intestinal peptide can stimulate cyclic AMP production in the pineal [181] and may be involved in some control mechanisms. De-Tyr- -endorphin can cause elevated pineal melatonin [182,183] and may represent an endogenous control mechanism assisting in integration of information.

Methoxyindoles might be involved with integration of various information thus enabling maintenance of a normal environment [5,184]. The manner in which the methoxyindoles mediate their action is not fully understood. Melatonin appears to stimulate cyclic GMP [185] and may affect secretory processes by impairing certain microtubule-dependent mechanisms [186].

The importance of the pineal gland as a neuroendocrine modulator becomes apparent when one considers the extent of its physiological effects.

Isolated reports have indicated many potential functions for the pineal gland. Among these are the findings that melatonin enhances wound healing [187], is possibly involved in intestinal physiology [188], affects melanin synthesis [189] and may serve a neuromodulatory role in the salivary glands [190]. Pineal hypertrophy results from isolation [191], activity decreases with age [192], some pineal product has the ability to inhibit placental chorionic gonadotrophin [193] and affects ornithine decarboxylase activity in a number of tissues [194]. The pineal gland is involved in tumour growth [195] and abnormal melatonin rhythms have been found in 47 disease states [196].

Possibly the most extensively documented effects of the pineal are those on the reproductive system. Melatonin is capable of suppressing reproduction [197,198,199] an effect that apparently requires an intact pineal [199]. It has been postulated that changing photoperiod is translated into melatonin production by the pineal and that this conveys seasonal information to the gonads [200,201]. Apart from melatonin, 5-methoxytryptophol [202] and 5-methoxytryptamine [203] also exert antigonadotrophic effects. The effect of 5-methoxytrytpamine, unlike that of melatonin, does not require the presence of an intact pineal [203]. Moreover, the discovery that levels vary with the menstrual cycle [204] also implies that 5-methoxytryptophol has an effect upon reproduction.

Conflicting evidence exists as to the mode of action of these methoxyindoles on reproduction. It has been suggested that while melatonin inhibits luteinising hormone release, 5-methoxy-tryptophol inhibits follicle stimulating hormone and so suppresses reproductive axis by alteration of gonadotrophin release [205]. Contradictory findings indicate that melatonin inhibits follicle stimulating hormone release [206]. The pineal, through melatonin action, participates in linking luteinising hormone surge to photoperiod [207]. Despite the inconsistencies, it appears that the antigonadotrophic effects are mediated at a central level [206,208]. An antigonadotrophic effect has been shown with a melatonin free pineal extract [209], which raises the possibility that products other than the methoxyindoles might control reproduction. For instance both pteridines and pterins have been found to be antigonadotrophic [210].

It is interesting to note that the pineal has been implicated as a target organ for androgens [211,212] and pinealectomy causes increased levels of testosterone and 17-ketosteroids [213], which tends to suggest that a pineal-gonadal feedback mechanism exists.

The hypothalamo-hypophyseal system may serve as a target area for pineal melatonin [214,215,216] and a feedback mechanism between the pineal and the hypothalamo-hypophyseal system has been suggested [217]. This control by the pineal may be mediated via the third ventricle [218], although melatonin affecting the hypothalamus may also originate from the retina [219]. Some effects exerted by the pineal on the hypothalamus are mediated by methoxyindoles [10,220,221], whilst other pineal products such as arginine vasotocin may also play a role [222].

The fact that the pineal has the capacity to activate the paraventricular nuclei [223] and that a link between melatonin rhythm and drinking behaviour has been suggested [224] may indicate yet another feedback mechanism.

Although it has been reported that there is no significant interaction between the pineal and hypothalamo-hypophyseal-thyroid axis [225], it appears that melatonin is capable of controlling thyrotropin releasing hormone release [226] and is able to potentiate thyroid stimulating hormone action [227].

Apart from inhibition of thyrotropin releasing hormone production, a pineal compound has been found to inhibit prolactin releasing hormone [228]. This compound may be melatonin, which causes increased dopamine levels in the medial basal hypothalamus resulting in decreased prolactin secretion [229], or it may be arginine vasotocin, which can modulate prolactin secretion [230]. This implicates a number of pineal secretions in the control of pituitary hormone production.

Melatonin does not appear to affect growth hormone in man [231], but with serotonin may modulate hypothalamic release of somatostatin [232]. Whereas pinealectomy causes a reduction in growth hormone secretion [233], so melatonin increases it [234]. This may be due to melatonin inhibition of dopamine release from the hypothalamus [235], an effect which would reduce somatostatin

release [236] and hence increase growth hormone secretion. Pinealectomy also leads to increased follicle stimulating hormone and luteinising hormone levels [213,237], seeming to desynchronise their release [237], while both melatonin and 5-methoxytryptamine cause a decrease in luteinising hormone release [221]. The effect of melatonin on luteinising hormone may result from inhibition of prostaglandin E synthesis [238]. The presence of luteinising hormone releasing hormone in the pineal [239] may suggest the presence of a feedback mechanism controlling gonadotrophin release.

Melatonin can also act on the pars intermedia to decrease melanocyte stimulating hormone release [240] and may indirectly affect EEG patterns by an effect on the hypothalamus [241].

Although reports have disputed any link between the pineal and pituitary-thyroid axis [242] there is considerable evidence suggesting a relationship [226,243-245].

Pinealectomy increases iodine accumulation by the thyroid, an effect reversed by melatonin [243,244], while thyroid hormones increase pineal melatonin [244,245,246]. It has been suggested that a receptor exists in the thyroid gland which is responsible for the effects elicited by melatonin [244]. The fact that melatonin can alter calcium and phosphate levels suggests an effect on the parathyroid gland [247] and it has been postulated that there is a feedback between the pineal and both the thyroid and parathyroid glands [248]. In this regard it is interesting to note that pineal cells show a rhythm in sensitivity to thyroxine, being more responsive to excitation during the day [212].

Thyroid hormones may also alter pineal activity by influencing monoamine oxidase activity, either by affecting synthesis [249] or by suppression of an endogenous inhibitor and/or dissociation of less active aggregates [250].

There is evidence that favours the presence of an adrenal-pineal axis. Pinealectomy increases corticosterone levels [213,251] while melatonin administration reverses this effect [251,252]. The pineal induced decrease in corticosterone production appears to be an effect on protein synthesis rather than competition for

ACTH receptor sites [253].

Apart from melatonin, N-acetylserotonin [254], 5-methoxytryptophol [255] and in certain instances serotonin, 5-hydroxytryptophol and 5-hydroxyindole-3-acetic acid [256] have been shown to regulate adrenocortical function. 5-methoxytryptophol enhanced release of corticosterone [255], an effect opposite to that of melatonin, may indicate that changes in pineal output can alter adrenal hormonal status.

Adrenolectomy activates the pineal, an effect which may be due to increased concentration of hypothalamic or pituitary hormones, decrease in adrenal hormones or β-adrenergic receptor supersensitivity resulting from decreased adrenal catecholamines [257].

The adrenal-pineal axis may be involved primarily in modulation of stress. The adrenal gland has been reported to mediate stress by increasing pineal melatonin [258] while stress has been shown to increase uptake of tryptophan by the pineal [259] and to increase pineal adrenaline [72]. The adrenaline is presumed to originate from the adrenal gland [72].

Although melatonin receptors have been found in numerous peripheral tissues [260] and melatonin has been postulated to act intracellularly [260,261], the brain has been suggested as the primary target for pineal hormones [262]. It is of interest that melatonin may be able to regulate its own receptors [263].

The presence of a habenulo-pineal axis has also been suggested [264]. Melatonin affects serotonin synthesis in raphe neurones, [257,265] but the highest concentration of melatonin receptors are in the medial basal hypothalamus [266]. Receptor agonist activity is abolished by 6-hydroxylation while methoxyindoles have a greater activity than their hydroxy counterparts [266].

Melatonin enhances sleep, an effect apparently independent of changes in brain indoles, catecholamines or electrical activity [267]. Melatonin secretion associated with nocturnal arousals has been suggested to restore sleep [268] while other pineal products such as 5-hydroxytryptophol [9] and arginine vasotocin [269] may

also be involved in the sleep mechanism.

The pineal can regulate pain through a central effect [270] which may involve mediation by methionine-enkephalin [271]. Various indoles have been shown to inhibit benzodiazepine receptor binding [272,273], with the melatonin metabolite N-acetyl-5-methoxy kynurenamine being the most potent, followed by melatonin [273]. Both these indoles are more potent than the putative endogenous benzodiazepine receptor antagonists inosine and hypoxanthine [273].

Although the choroid plexus cannot transport melatonin into the cerebro-spinal fluid it may possibly regulate central melatonin levels by affecting movement into the blood [274].

There are numerous reports which indicate that the pineal gland is involved in modulation of epileptic seizures. That audiogenic seizure suseptibility is regulated by light [275] might implicate pineal involvement. Some indoles have suggested anticonvulsant activity [276] as has tryptophol [277], serotonin and 5-methoxytryptamine [278], while L-tryptophan [279] and indole aldehydes and alcohols may modulate seizure activity [280]. Most reports, however, implicate melatonin as the pineal anti-convulsant principle [281-284].

Pinealectomy causes seizures which are not prevented by administration of melatonin or pineal extract [285,286], thus implying that an intact pineal is essential for anticonvulsant activity. With an intact pineal, the removal of central melatonin causes transient seizure activity [287], hence further supporting a role for melatonin in seizure modulation. Some reports, however, find melatonin to be ameliorative in pinealectomy induced seizures [283]. These seizures may be facilitated by a decrease in telencephalic catecholamines [288], an effect which might be reversed by melatonin [289]. Melatonin has also been shown to reduce the ability of epileptic neurones to sustain activity [284,290] and may also alter EEG indirectly by an action on the hypothalamus [241]. These findings are consistent with the proposal that pinealectomy induced seizures may result from the increased gonadotrophin and oestrogen levels [291]. A report implicates decreased potassium levels in seizure generation [292]

whilst another suggests that the pineal suppresses seizures by affecting activation or by modifying the balance between inhibitory and excitatory post-synaptic potentials or, alternatively, that the effect is due to metabolic and hormonal changes [293]. The overall effect of the pineal in modifying seizure behaviour is probably a mixture of all these various effects, complicated by the observation that there is a diurnal rhythm in sensitivity to melatonin [294].

The pineal gland has been implicated as an endogenous time-keeping device [295-298] but its role appears to be that of a 'coupling device' rather than a controlling device [299]. The suprachiasmatic nuclei participate with the pineal in generation of rhythms and form an important part of the mechanism [300]. Three types of electrically distinct cells have been isolated in the pineal [301]. One type, with a constant firing rate, is presumed to be under commisural fibre control, whilst the other two types exhibit increased firing, either during the day or during the night [301]. These two cell types might be responsible for time-keeping [301] and it has been suggested that melatonin may serve to indicate the middle of the dark phase [302]. While hydroxyindoles were predominantly depressant the methoxyindoles were mostly excitatory in their effect on pineal cells [303]. The effects of melatonin and 5-methoxytryptophol, although similar, appear to be mediated on different cells [303].

Several reports indicate a role for the pineal gland in thermoregulation [304-307], and temperature has been found to be important for regulation of melatonin levels [308] thus favouring the presence of a temperature-pineal feedback mechanism.

That hypoglycaemia decreases 5-methoxytryptophol levels [204], insulin inactivates SNAT [130] and pinealectomy causes an increased insulin secretion, apparently resulting from lack of pineal secretions [309], suggests feedback between these two systems.

Reports on the effect of pineal products in self-regulation are contradictory. While some reports indicate that melatonin may influence its own synthesis [212] and affect serotonin rhythm [310], other findings fail to observe any effect [311]. There is

also evidence which indicates that 5-methoxytryptamine has no effect on pineal activity [312]. The fact that there may be melatonin receptors in the pineal [313] and that melatonin can change firing rate of pineal cells [314] suggests some role in regulating pineal secretion, possibly of arginine vasotocin [315]. Pineal influence on physiological processes and influences exerted on the pineal can be summarized as follows (Figure 2).

Although there appears to be only one report dealing with the effect of anticonvulsants on the pineal gland, many of the effects observed suggest that these drugs may exert an effect on the pineal.

Diazepam (DZP) is able to inhibit nocturnal increase in SNAT activity, possibly by an effect on the hypothalamus to decrease adrenergic stimulation of the pineal [316]. Phenobarbitone (PBT), however, has been found to have no effect [316].

Several reports show that various anticonvulsants have the ability to alter central cyclic nucleotide levels. Acetazolamide (ATZ), DZP, diphenylhydantoin (DPH) and PBT are all able to inhibit brain cyclic AMP phosphodiesterase [317,318] while DPH, PBT and carbamazepine (CBZ) decrease cerebrospinal fluid cyclic AMP levels [319] and DZP causes a decrease in cerebellar cyclic GMP [320]. Most anticonvulsant drugs appear to inhibit brain aldehyde reductase (EC 1.1.1.2). Inhibition by CBZ, DPH, ethosuximide (ETH) and PBT appears to be noncompetitive [321,322] although inhibition by DPH may be mixed [322]. Clonazepam (CLZ) and DZP [323], primidone (PRD) [324] and valproate (VPA) [323,324,326] also inhibit aldehyde reductase, VPA in an uncompetitive fashion [326].

Both DZP and DPH inhibit monoamine oxidase [327,328] and DPH inhibits noradrenaline uptake [327,329]; effects which would probably facilitate noradrenergic transmission. PBT may also exert an effect by altering noradrenaline rhythm [330].

Numerous effects on the serotoninergic system are mediated by the administration of anticonvulsants. An increase in brain serotonin levels has been shown to result from ATZ [331], CLZ [332], DZP and DPH [333,334] and PBT [328] administration, an effect which

may be due either to decreased efflux or to metabolism [328]. The findings that CLZ reduces serotoninergic activity [335] and that DZP decreases serotonin synthesis [336] add to the complexity of the issue making positive predictions difficult. PBT is capable of altering serotonin rhythm [330] while VPA has no effect on synthesis but increases 5-hydroxyindole-3-acetic acid levels, presumably resulting from inhibition of transport [337,338]. VPA may displace protein bound serum tryptophan, thus increasing brain levels [339], which may account for the increased 5-hydroxyindole-3-acetic acid levels. Increased serotonin levels resulting from CLZ or DZP administration may be due to increased tryptophan uptake [340,341].

Inhibition of calcium uptake results from administration of CBZ, DZP, DPH and PBT while ETH and VPA are ineffective [342]. Another report, however, finds high ETH concentration to increase calcium influx [343]. DPH has also been shown to inhibit calcium stimulated protein phosphorylation [344], to enhance potassium uptake [345] and to block potassium release [346]. CBZ can also decrease depolarization [347]. Although it is difficult to gauge what effect these changes might have, any change in membrane function has the potential for some response.

While PBT can decrease excitatory transmission [348], both DZP [349] and CBZ [350] have been shown to decrease neurotransmitter release, which may influence pineal function. Changes in endocrine function occur with use of several anticonvulsant drugs. DZP increases release of growth hormone from the pituitary [351] and inhibits luteinising hormone release, possibly by an action on the hypothalamus [352]. Both luteinising and follicle stimulating hormone surges are blocked by PBT [353] whilst CBZ, DPH, PBT and VPA increase plasma testosterone and sex hormone binding globulin levels [354].

Both DPH and VPA inhibit release of thyroid stimulating hormone from the pituitary [355,356] and CBZ increases extra-thyroid metabolism of thyroid hormones [357].

DPH but not PBT disrupts corticosterone output and inhibits pituitary-adrenal stress response [358].

ATZ antagonizes dopaminergic pancreatic secretion [359] and alteres insulin secretion, the effect being dependent on glucose concentration [360]. Inhibition of insulin secretion by DPH appears to result from blockade of calcium and/or sodium transport [361,362].

Inhibition of leucine enkephalin activity results from ETH and VPA but not from DPH and PBT administration [363] whilst DZP has been found to increase hypothalamic enkephalin levels [364]. VPA also inhibites oxidative phosphorylation [365], resulting in a low energy state with great potential for affecting overall metabolism.

Central taurine levels are altered by DPH, ETH, PBT, sulthiame (STH) and VPA [366]. DPH interfered with folate metabolism [367], and both DZP and PBT cause hypothermia [368,369]; all effects which might involve the pineal gland.

DZP can increase activity of prostaglandin synthetase [370] and it has been suggested to act through a non-serotoninergic indoleaminergic system [371]. This latter finding is interesting in that DZP action is presumed to be mediated through specific benzodiazepine receptors which may be regulated by endogenous ligands [372], and in that various endogenous indoles are capable of fulfilling such a role [272,273].

However attractive this evidence might appear in linking anticonvulsant drug effects with the pineal gland, it must be remembered that such a relationship may not in fact exist. The evidence does, however, give an indication of what effects might be expected, and would certainly help to explain any observed effects.

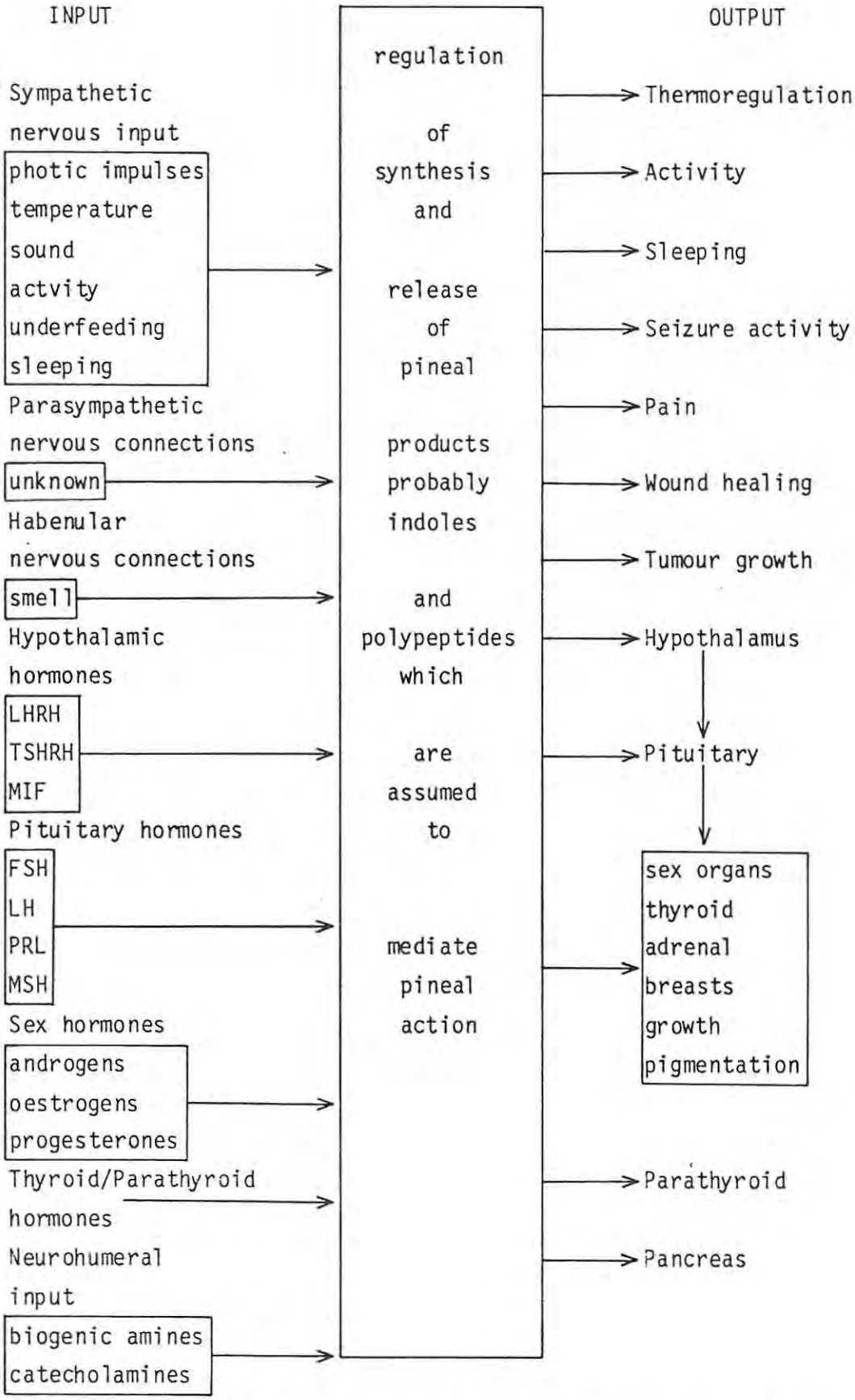


Figure 2: Schematic representation of the various inputs presumed to influence pineal activity and the numerous physiological processes upon which the pineal is thought to exert an effect (modified from [1]).

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CHAPTER 2

# PHARMACOKINETICS OF ANTICONVULSANT DRUGS

INTRODUCTION

MATERIALS

METHODS
Dosing and Sampling
Carbonic Anhydrase Inhibition
Densitometry of Thin Layers
Ultra-Violet Spectrophotometry
Radioactive Drugs
Emzyme Multiplied Immunoassay Technique
Analysis of Data

RESULTS AND DISCUSSION

REFERENCES

## INTRODUCTION

With most pharmacological studies using laboratory animals there is often the problem of deciding what quantity of a drug to administer.

The techniques frequently adopted rely on observation of gross changes in the behaviour of the animal after drug administration. In the case of anticonvulsants these methods include prevention of chemically induced convulsions [1], suppression of cobalt induced seizures [2] and prevention of components of amygdaloid kindled seizures [3].

All these methods have the same serious disadvantage in that they give an indication of the ability of the drug OR METABOLITES to prevent occurence of an event without giving an indication of the tissue concentration of drug. It is the tissue concentration that is important in order to give an indication of the amount of drug which is capable of eliciting a pharmacological response.

In view of this it was decided to determine the plasma concentrations of the various anticonvulsants used in this study. Several reports have indicated the normal therapeutic plasma concentrations of anticonvulsants [4-7] and the dosages necessary to achieve these levels were sought.

## MATERIALS

See Appendix A.

## METHODS

Dosing and Sampling. Rats of both sexes (250-300g) were dosed on a mg/kg basis by intraperitoneal injection. Drugs insoluble in water were either dissolved or suspended in propylene glycol. Beclamide and pheneturide resisted both solution and suspension and were consequently given orally as powder in a syrup simplex matrix. At set intervals after dosing rats were anaesthetised using ether and blood samples were collected by cardiac puncture. After collection the samples were centrifuged and the resultant plasma was stored at -20°C for no longer than a week prior to analysis of plasma levels.

Carbonic Anhydrase Inhibition. Acetazolamide (ATZ) and sulthiame (STH) concentrations were determined using a carbonic anhydrase assay. Phenol red (12.5mg/l) was dissolved in 0.01M carbonate-bicarbonate buffer pH10 and carbonic anhydrase (10mg/l) in distilled water, both solutions being kept on ice before use. The reaction vessel was similar to that used previously [8] except that the $CO_2$ inlet was at the bottom of the vessel. It was immersed in an ice bath and was attached to a $CO_2$ supply which purged the vessel continuously at a constant rate. Prior to assay 700ul buffer/indicator solution was mixed with 100ul sample (standard or diluted plasma sample) and 100ul carbonic anhydrase. A 700ul aliquot was pipetted into the reaction vessel and time for indicator to change from red to yellow was noted. Plasma samples were diluted (15ul/ml) with distilled water immediately prior to assay, heated at 100°C for 5 minutes to denature native carbonic anhydrase and cooled before assay. A calibration curve was obtained as above using spiked serum samples. Reaction mixtures were agitated to prevent frothing and after reaction the

vessel was emptied by applying a suction to the second inlet. After removing the reaction mixture the vessel was rinsed twice with distilled water before reuse.

Densitometry of Thin Layers. Beclamide (BCL) and pheneturide (PTR) levels were evaluated using thin layer chromatography (TLC).

For determination of BCL 1ml of plasma containing 200ug/ml ethosuximide as internal standard was mixed with 50ul saturated sodium carbonate and 2ml chloroform, vortexed for a minute and centrifuged for 10 minutes to separate the two layers. The upper plasma layer was then aspirated off and a further 3ml chloroform and sodium sulphate were added, tubes being vortexed again for a minute. The sodium sulphate was quickly filtered off and the chloroform evaporated to dryness at 40°C under nitrogen. The sides of the tubes were washed down using chloroform, the final residue being taken up in 50ul chloroform. A 10ul quantity was spotted onto TLC plates, developed in chloroform:acetone (9:1) and examined at 217nm on a densitometer after drying. The calibration curve was constructed by spiking plasma with BCL and treating as above.

Evaluation of PTR used the same procedure except diazepam (10ug/ml) was the internal standard and the scanning wavelength on the densitometer was 220nm.

Ultra-Violet Spectrophotometry. A UV assay was employed to analyse carbamazepine (CBZ) plasma levels. Plasma samples (300ul) were diluted with 500ul of 0.05M phosphate buffer pH6.8, 3ml dichloromethane was added and after vortexing for a minute samples were centrifuged for 5 minutes. A 2ml aliquot of the organic phase was then evaporated to dryness at 40°C under nitrogen, 2ml of 4M hydrochloric acid was added and the tubes heated at 150°C for 30 minutes. A further 2ml of 4M hydrochloric acid was added and the absorbance of this solution was read at 258nm on a Beckman model 25 spectrophotometer. A calibration curve was constructed by spiking serum with CBZ and treating these samples as above.

Radioactive Drugs. Clonazepam-5-$^{14}$C (1.15MBq/mg)(CLZ) and

diazepam-5-$^{14}$C (6.88GBq/mg)(DZP) were diluted using non-labelled drug and administered as outlined above. Radioactivity of plasma and, where possible, pineal glands were determined. Soluene 350 was used to solubilize pineal glands prior to introduction of scintillation cocktail.

Both CLZ and DZP were administered for three days and blood samples were collected as previously described. Samples (1ml) of plasma were mixed with 1ml 2M borate buffer pH10 and 5ml toluene, vortexed for 2 minutes and centrifuged for 10 minutes. The organic phase was aspirated off and evaporated at 40°C under nitrogen. After washing down the sides with toluene the residue was dissolved in 50ul chloroform and this entire amount spotted onto thin-layer plates. These plates were developed in chloroform:n-heptane:ethanol (50:50:5) and autoradiographs taken using Kodak Plus-X pan film negative (125 ASA). After development of the autoradiographs the spots were scraped off the thin-layer plates and quantitated.

Enzyme Multiplied Immunoassay Technique (Emit). Diphenylhydantoin (DPH), ethosuximide (ETH), phenobarbitone (PBT), primidone (PRD) and valproate (VPA) levels were determined by Emit using the Emit/Lab 5000 system and standard reagent kits according to manufacturers specifications.

Analysis of Data. For construction of calibration curves (ATZ, BCL, CBZ, PTR and STH) five concentrations of the drugs were assayed in triplicate on separate occasions and the means of these determinations were used. The best fit was obtained using linear regression analysis using the method of least squares and the coefficient of determination ($r^2$) was used as a measure of correlation. Standard error of the mean (SEM) was calculated for each value and was omitted from graphs where too small.

## RESULTS AND DISCUSSION

The only problem encountered with the carbonic anhydrase inhibition was determination of the end-point of the reaction as colour change was not abrupt. This problem was largely overcome with experience. As the reaction rate varied slighlty with each set of determinations, the calibration curves were plotted as

drug concentration versus percentage inhibition in order to standardise the procedure (Figure 1a and 1b). To ensure the accuracy of the technique standard concentrations of either ATZ or STH were included with each batch of samples. These were all treated in the same way, the standard samples serving as controls to ensure that the technique was functioning in a satisfactory fashion.

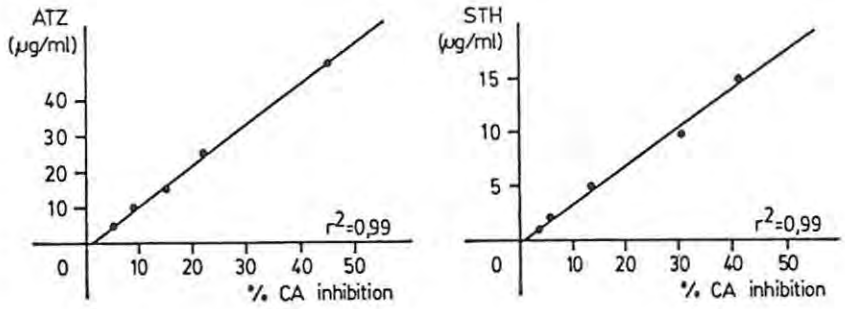


Figure 1: Calibration curves used to determine plasma concentration of a) acetazolamide (ATZ) and b) sulthiame (STH). Each point represents the mean of three determinations performed as outlined in the text. Coefficient of determination ($r^2$) given as a measure of linearity. (CA = carbonic anhydrase).

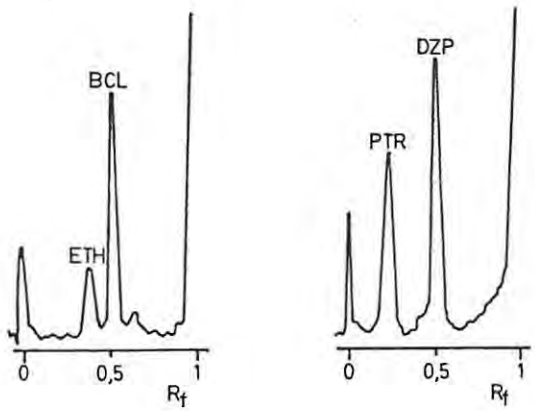


Figure 2: TLC scans from plasma samples for a) beclamide (BCL) using ethosuximide (ETH) as internal standard and b) pheneturide (PTR) with diazepam (DZP) as internal standard.

The extraction and separation technique adopted for BCL and PTR gave good resolution with little background interference (Figure 2a and 2b). Calibration curves were constructed as peak height ratios versus plasma concentration (Figure 3a and 3b).

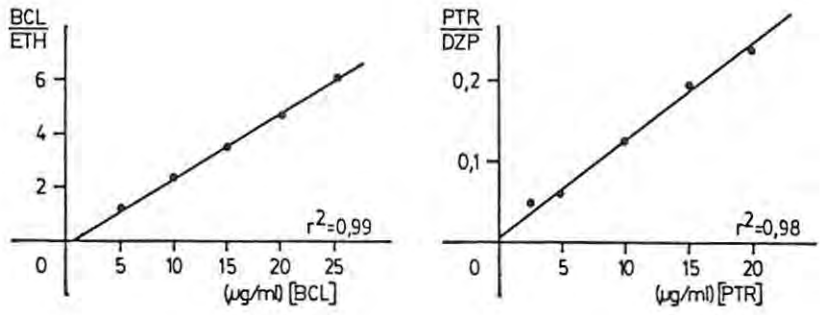


Figure 3: Calibration curves used to determine plasma concentration of a) beclamide (BCL) and b) pheneturide (PTR) using peak height ratio versus drug concentration. Each point represents the mean of three determinations performed as outlined in the text. Coefficient of determination ($r^2$) given as a measure of linearity.

Heating CBZ with hydrochloric acid resulted in the formation of 9-methylacridine which gave the characteristic UV absorption at 258nm. CBZ plasma levels were obtained using the calibration curve of absorption versus CBZ concentration (Figure 4).

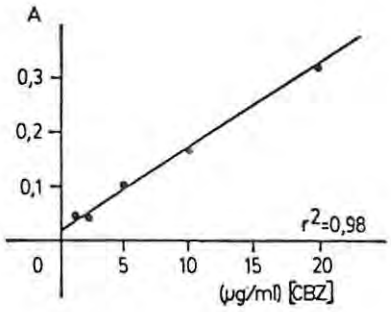


Figure 4: Calibration curve of absorption (A) versus carbamazepine (CBZ) plasma concentration obtained as outlined in the text. Each point represents the mean of three determinations. Coefficient of determination ($r^2$) given as a measure of linearity.

Using radioactive CLZ and DZP simplified evaluation and allowed determination of the metabolites with relative ease after thin layer separation using autoradiographic localisation (Figure 5a and 5b). The pineal DZP profile (Figure 6) closely parallels that of plasma DZP (Figure 11) suggesting that plasma levels give a good indication of the tissue concentrations at any time.

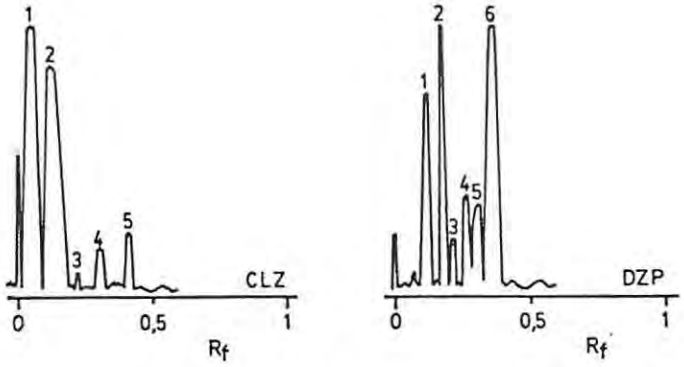


Figure 5: TLC scans constructed from autoradiographic determination of a) clonazepam (CLZ) and b) diazepam (DZP) metabolites after administration, extraction and separation of radioactive drugs as outlined in the text.
a) CLZ metabolites as follows- 1 = 7-acetamidoclonazepam, 2 = 7-aminoclonazepam, 3 and 4 = hydroxy metabolites and 5 = clonazepam
b) DZP metabolites as follows- 1 = oxazepam, 2 = N-desmethyl-diazepam, 3 = 5(p-hydroxyphenyl)oxazepam, 4 = 3-hydroxydiazepam, 5 = 5(p-hydroxyphenyl)desmethyldiazepam and 6 = diazepam.

The pharmacokinetic profiles for the various drugs are shown in Figures 7-18. Each point represents the mean of three values with the standard error of the mean (omitted where too small to display).

In order to remain effective the plasma levels of a drug must be maintained above the minimum therapeutic level at all times. These concentrations were evaluated after twice daily administration of drugs for three days, samples being taken shortly before the second dose on the third day. These trough levels (T) gave an indication of the amount of drug necessary to maintain a constantly effective plasma concentration and it was these dosages that were used in subsequent studies. These values were successfully determined for all drugs except VPA (Table 1,

Figures 7-18) in which twice daily administration was inadequate to maintain therapeutic levels due to rapid elimination. In order to maintain constant therapeutic VPA levels would require the use of a sustained release preparation or frequent dosing which is undesirable.

The half-life ($t_{1/2}$) and elimination rate constant ($K_e$) were also determined (Table 2) and it is apparent that drugs are removed from rats much faster than from human subjects [9,10,11]. This information allows accurate predictions to be made about plasma levels in rats whereas existing clinically evaluated data would give a false indication. In this regard it is interesting that the pharmacokinetic data evaluated here is similar to that obtained for the Mongolian Gerbil [12] although significant differences do occur. This serves to emphasise the observation that pharmacokinetic behaviour of drugs varies markedly with species and in order to make accurate predictions about plasma levels/elimination rate the data must be treated as species-specific.

Table 1: Plasma levels of anticonvulsant drugs after repeated administration compared to normal therapeutic levels.

| DRUG | DOSE (mg/kg)* | PLASMA TROUGH (ug/ml) | THERAPEUTIC$ LEVELS(ug/ml) |
|---|---|---|---|
| Acetazolamide | 100 | 13±1 | 10-14 |
| Beclamide | 250+ | 15±3 | 9-19 |
| Carbamazepine | 25 | 7±1 | 6-12 |
| Clonazepam | 0.1 | 0.005±0.002 | 0.005-0.05 |
| Diazepam | 10 | 1±0.2 | >0.6 |
| Diphenylhydantoin | 100 | 11±2 | 10-20 |
| Ethosuximide | 25 | 42±3 | 40-100 |
| Pheneturide | 150+ | 11±7 | ±10 |
| Phenobarbitone | 12.5 | 19±1 | 10-30 |
| Primidone | 142 | 9±1 | 5-15 |
| phenobarbitone-primidone metabolite | | 10±3 | 10-30 |
| Sulthiame | 50 | 10±1 | 3-11 |
| Valproate | 64 | 2±1 | 50-100 |

* administered twice daily intraperitoneally except + given orally. $ from [4-7].

Table 2: Half-life ($t_{1/2}$) and elimination rate constant ($K_e$) determined for the various anticonvulsant drugs.

| DRUG | $t_{1/2}$ (hr) | $K_e$ ($hr^{-1}$) |
|---|---|---|
| Acetazolamide | 1.5 | 0.46 |
| Beclamide | - | - |
| Carbamazepine | 1.8 | 0.40 |
| Clonazepam | 1.0 | 0.69 |
| Diazepam | 0.8 | 0.90 |
| Diphenylhydantoin | 1.4 | 0.50 |
| Ethosuximide | 7.5 | 0.09 |
| Pheneturide | - | - |
| Phenobarbitone | 3.6 | 0.19 |
| Primidone | 3.3 | 0.21 |
| Sulthiame | 2.5 | 0.28 |
| Valproate | 0.3 | 2.31 |

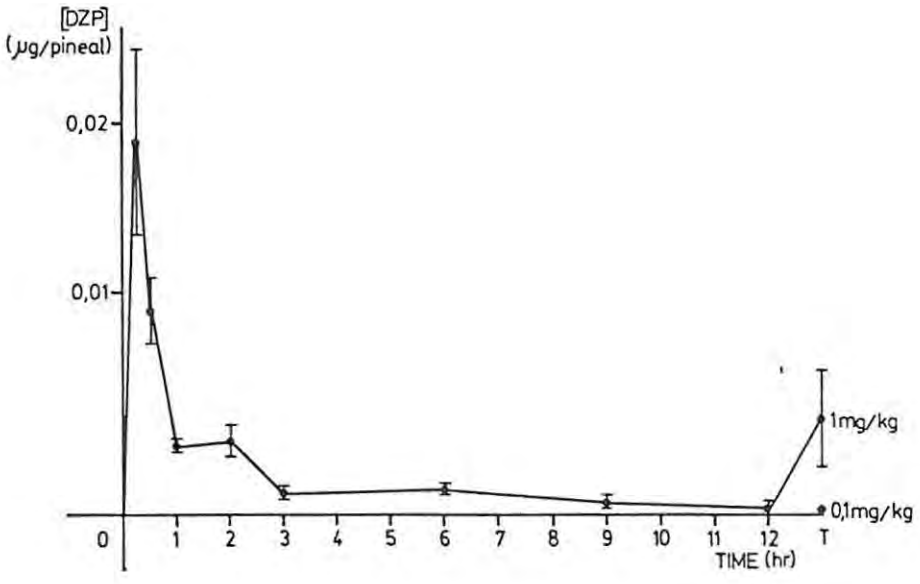


Figure 6: Pineal diazepam (DZP) concentration after administration of radioactive drug at the dose indicated at various time intervals. (T = plasma trough after administration of drug for three days).

Figures 7-18: Plasma profiles determined for the various drugs as outlined in the text. (T = plasma trough after administration of drug for three days).

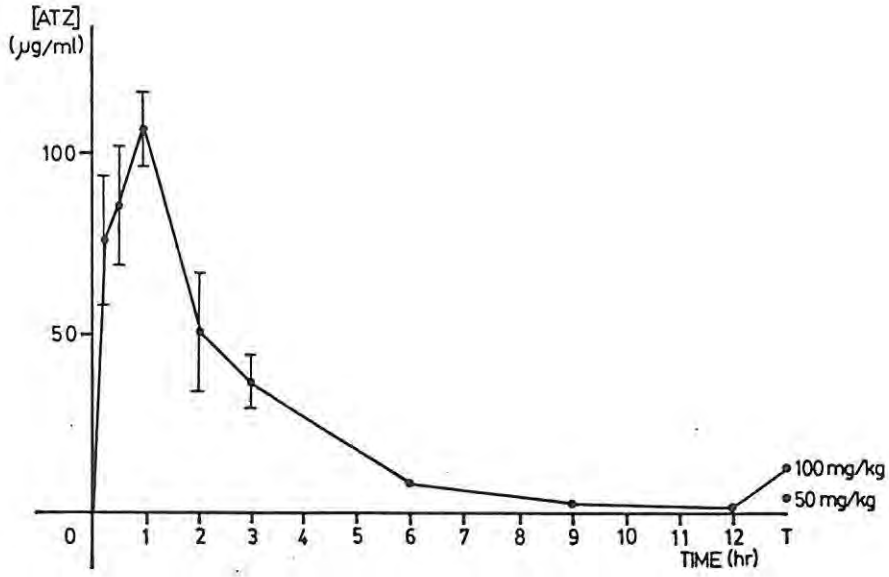


Figure 7: Plasma acetazolamide (ATZ).

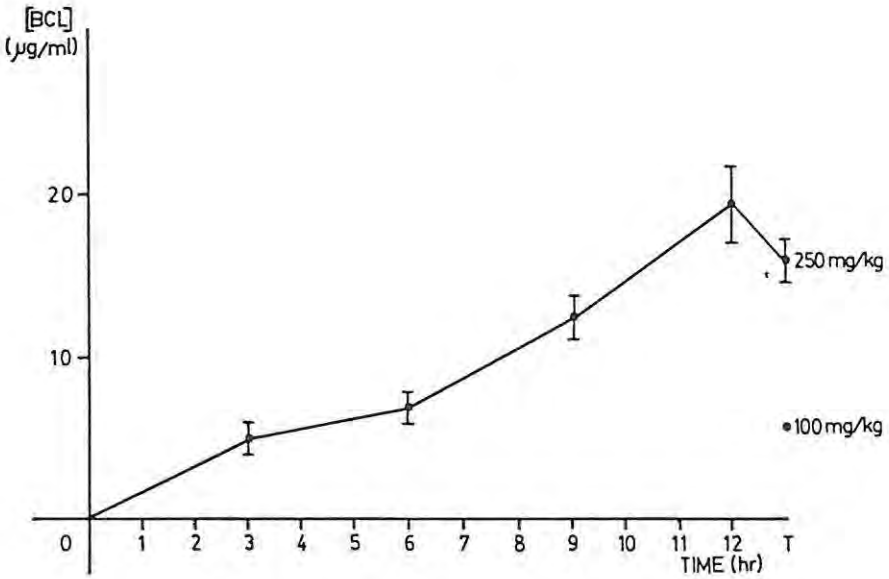


Figure 8: Plasma beclamide (BCL).

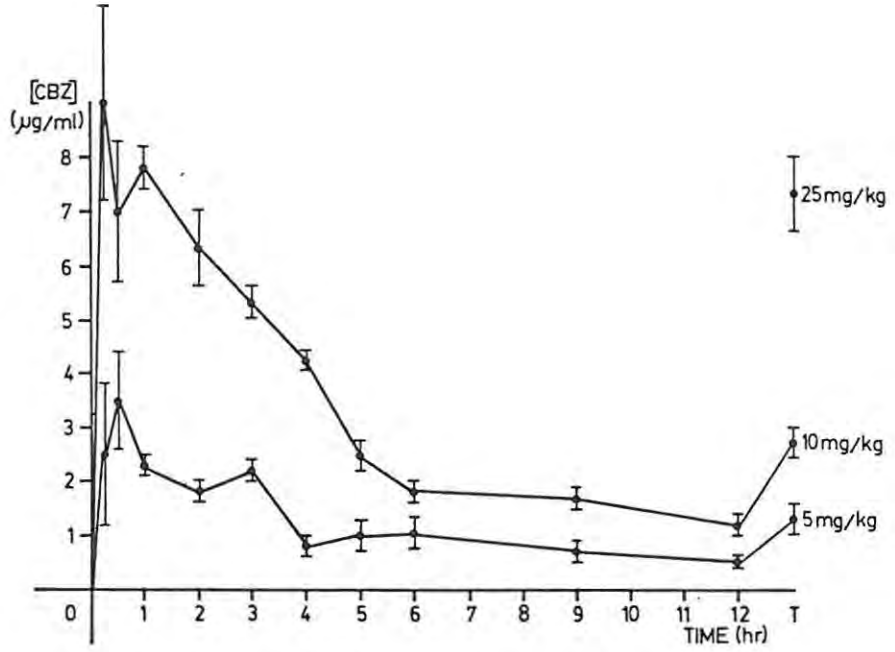


Figure 9: Plasma carbamazepine (CBZ).

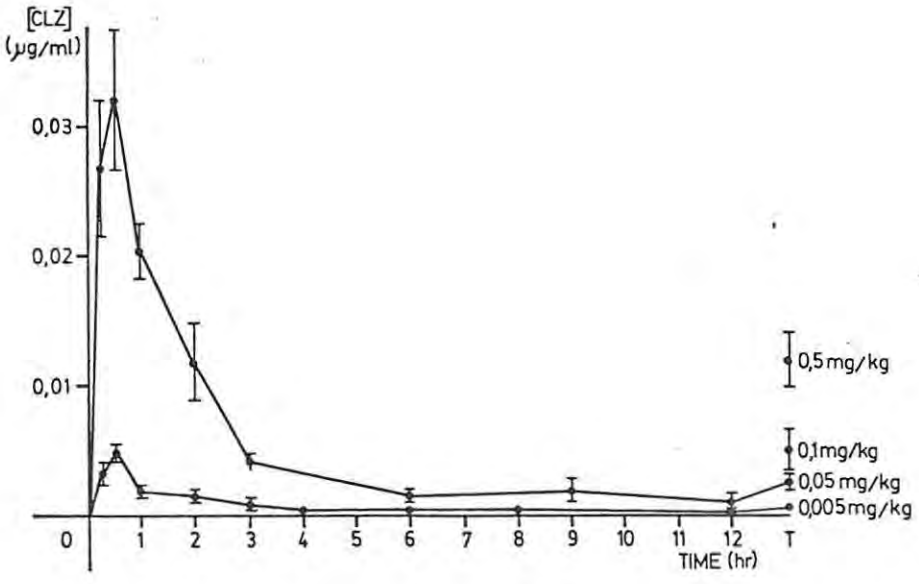


Figure 10: Plasma clonazepam (CLZ).

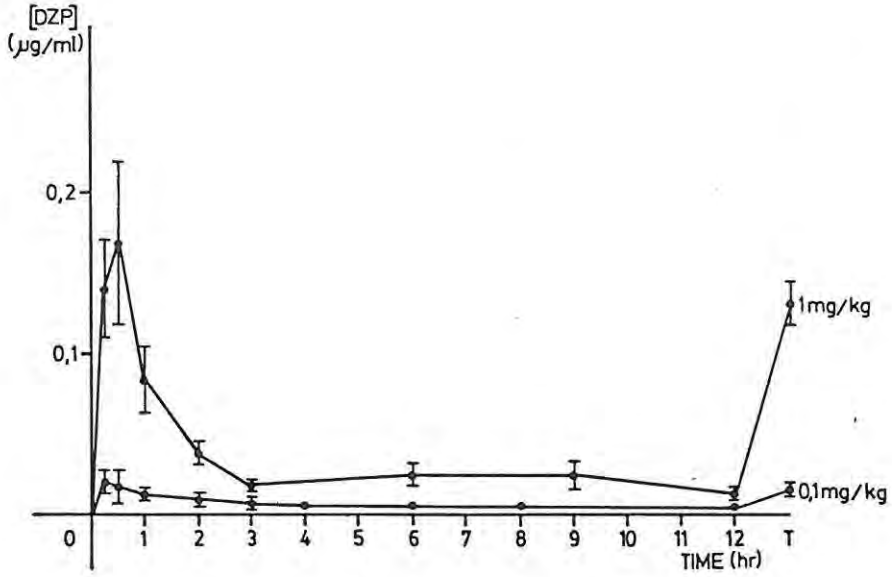


Figure 11: Plasma diazepam (DZP).

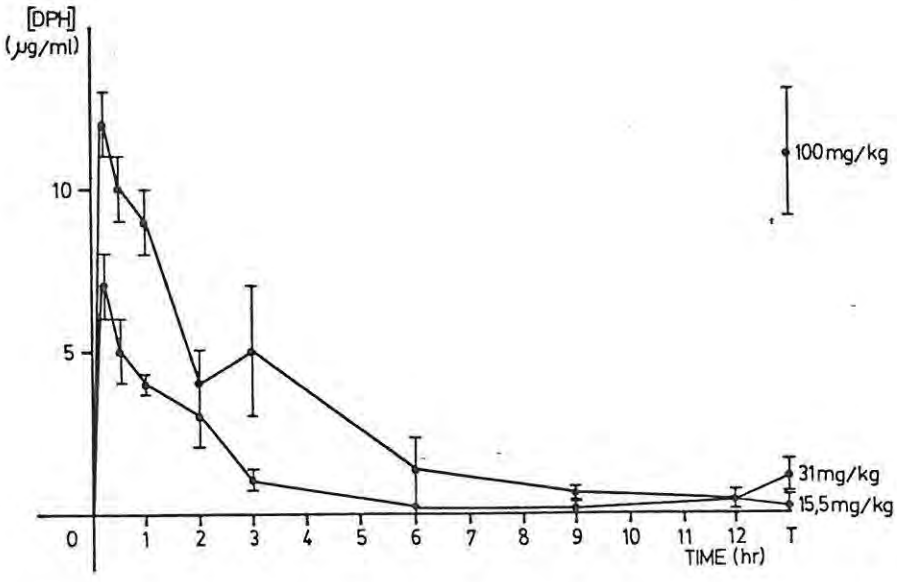


Figure 12: Plasma diphenylhydantoin (DPH).

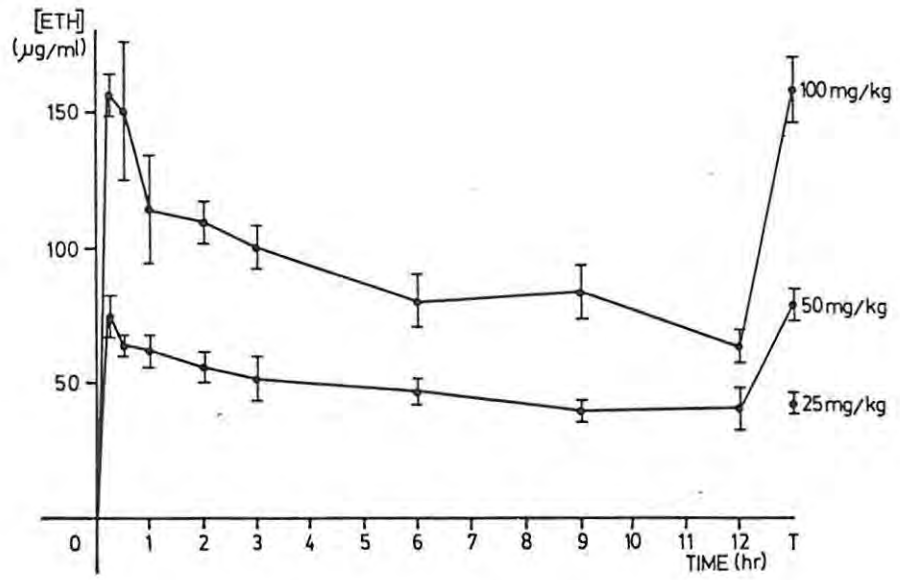


Figure 13: Plasma ethosuximide (ETH).

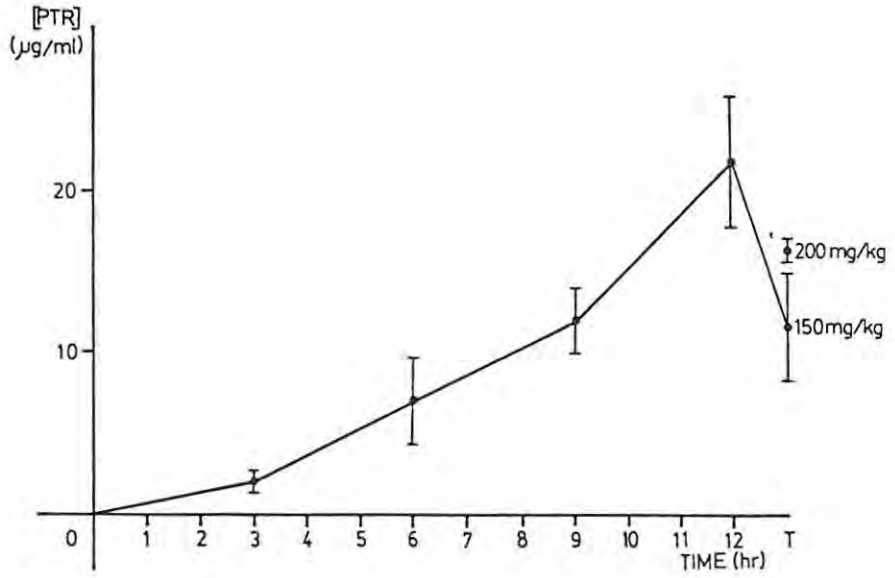


Figure 14: Plasma pheneturide (PTR).

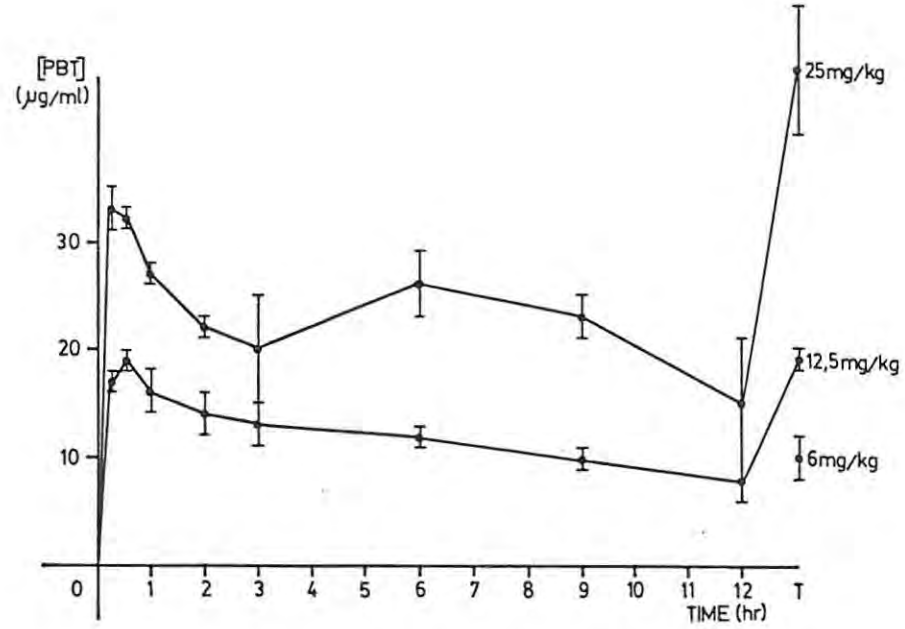


Figure 15: Plasma phenobarbitone (PBT).

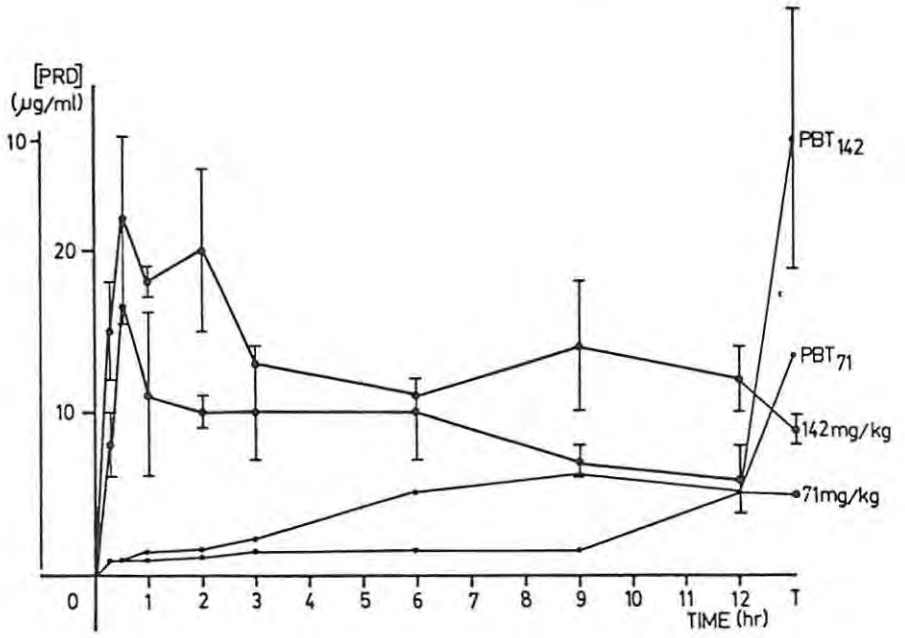


Figure 16: Plasma primidone (PRD).

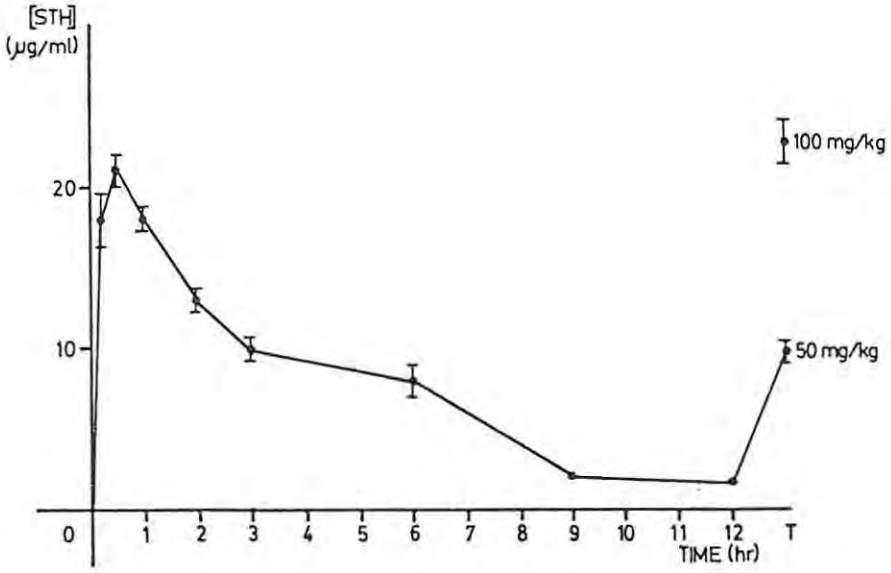


Figure 17: Plasma sulthiame (STH).

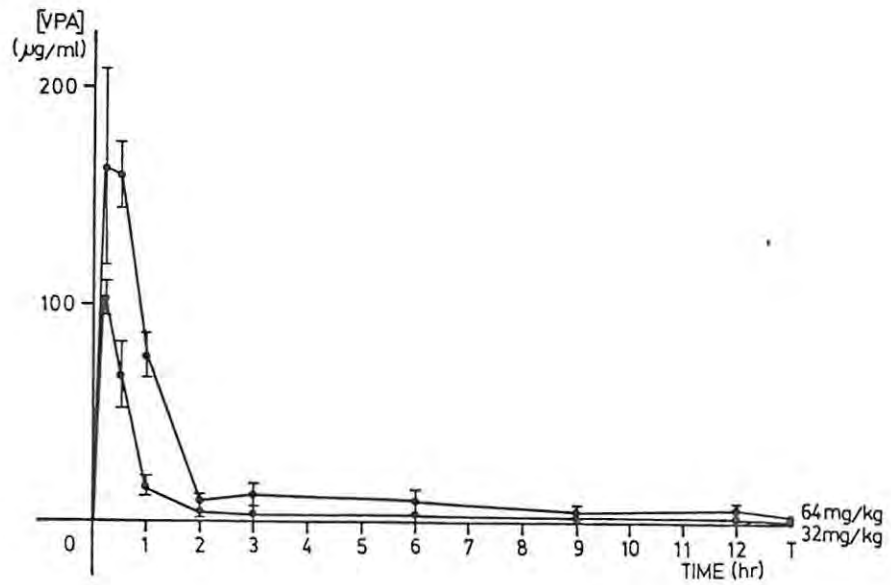


Figure 18: Plasma valproate (VPA).

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CHAPTER 3

# KINETIC PROPERTIES AND PROBABLE MECHANISM OF CATALYSIS OF ACETYL COENZYME A:ARYLAMINE N-ACEYTLTRANSFERASE (EC 2.3.1.5)

INTRODUCTION

MATERIALS

METHODS
Preparation of N-acetyltransferase (SNAT)
Assay for SNAT
Product Purity
Effect of pH
Effect of Temperature
Evaluation of Kinetic Constants
Cosubstrate Effects
Alternate Substrate Effects
Product Effects
Effect of pH on Kinetic Behaviour
Effect of Temperature on Kinetic Behaviour
Evaluation of Data

RESULTS AND DISCUSSION

REFERENCES

## INTRODUCTION

The hormone melatonin, isolated by Lerner *et al* [1] is produced in the pineal gland by O-methylation of N-acetylserotonin [2]. The enzyme responsible for O-methylation in the pineal, hydroxyindole O-methyltransferase, can only utilise serotonin one tenth as efficiently as it can use N-acetylserotonin implying that N-acetylation preceeds O-methylation [2]. Serotonin has been shown to be N-acetylated to N-acetylserotonin *in vivo* [3] and this reaction is catalysed by an N-acetyltransferase enzyme [4]. This enzyme would be more accurately termed acetyl coenzyme A: arylamine N-acetyltransferase (EC 2.3.1.5) and is not unique to the pineal gland being found in other tissues such as the liver

[5]. The pineal enzyme, however, is unique in that it is under β-adrenergic cyclic AMP control [6], levels rising during the dark phase [7]. It is the formation of N-acetylserotonin that is rate-limiting in the formation of melatonin [8].

The lability of pineal N-acetyltransferase has precluded any in-depth study and few kinetic determinations have been attempted [5]. The present study attempted to design an assay technique to permit sensitive evaluation of N-acetyltransferase activity in order to elucidate the catalytic behaviour of the enzyme. This information would allow a better understanding of pineal biochemistry and would enable more accurate interpretation of any pharmacological effects on the enzyme.

## MATERIALS

See Appendix A.

## METHODS

Preparation of N-acetyltransferase. Prior to use groups of rats of both sexes (250-300g) were pretreated with isoprenaline HCl (25mg/kg ip) in order to induce N-acetyltransferase (SNAT) levels in the pineal. Maximum induction occured after three hours when the rats were sacrificed, pineal glands removed with minimum delay and homogenised on ice in 0.05M phosphate buffer pH6.5 (unless otherwise stated). Equal aliquots of this mixture were used for the determinations. Time between sacrifice and addition of enzyme homogenates to reaction vessels was kept to the absolute minimum to prevent excessive degradation of the enzyme.

Assay for SNAT. A modification of the method of Deguchi and Axelrod [9] was used. This method relied on transfer of a $^{14}C$-acetyl group from [1-$^{14}C$]acetyl coenzyme A (AcCoA) to tryptamine HCl (Tryp) to form N-acetyltryptamine (NAT). Tryp was used in place of the natural substrate serotonin (5-HT) as SNAT could utilize it far more readily than 5-HT. Incubation mixtures contained AcCoA and either Tryp or 5-HT, concentration depending on the parameter being investigated. Variation in the incubate volume was found to affect enzyme activity and a volume of 300ul per pineal gland was chosen for all determinations. Incubation

was performed at 20°C for an hour (unless otherwise stated), reaction being terminated by the addition of an equal volume of 0.2M borate buffer pH10. The radioactive product of the reaction was extracted into 3ml toluene:isoamyl alcohol (97:3) by vortexing for a minute. Recovery using this extraction procedure was about 97%. After centrifuging for 10 minutes, 2ml of the organic phase was aspirated off into 6ml scintillation cocktail and radioactivity quantitated by scintillation spectroscopy. Counting efficiency was determined using the external standards channel ratio and exceeded 90% in all cases.

Blank incubations were performed in which only enzyme was omitted from the procedure, these values being subtracted from those of enzyme incubates to arrive at a true value for enzyme activity. That an enzyme was responsible for formation of extractable radioactivity was verified by the addition of 0.1% w/v trypsin to some reaction mixtures. Activity was abolished shortly after trypsin addition verifying protein involvement.

Product Purity. Thin-layer chromatographic (TLC) separation was used to evaluate purity of the radioactive product. After incubation toluene:isoamyl alcohol extracts were evaporated to dryness at 40°C under nitrogen, the residue being dissolved in chloroform and spotted onto TLC plates. When 5-HT was used as a substrate plates were developed in chloroform:methanol:glacial acetic acid (97:3:1) and when Tryp was the indolic substrate the solvent system used was toluene:glacial acetic acid:ethyl acetate:water (16:8:4:1).

After development these plates were dried and strips of photographic film negative (Kodak Plus-X pan, 125 ASA) were attached to the plates which were allowed to expose in light proof containers at 0°C for several weeks prior to development.

The NAT standard used to check purity of the radioactive product was synthesised by reacting equimolar quantities of Tryp and acetic anhydride in water at 50°C for an hour. The reaction mixture was acidified using hydrochloric acid (4M) and poured over ice before extraction into several aliquots of ethyl acetate which was evaporated to dryness under nitrogen at 40°C and the product purified by TLC using the solvent system above.

Effect of pH. Groups of pineal glands from isoprenaline-stimulated rats were pooled and homogenised in distilled water containing Tryp ($6x10^{-3}M$) and AcCoA ($1.2x10^{-4}M$) using 150ul per pineal gland. Equal aliquots of this mixture (150ul) were added to 150ul aliquots of 0.1M phosphate buffer of varying pH. Reaction was monitored after incubation at 20°C for three hours.

Effect of Temperature. Groups of pineal glands from isoprenaline-stimulated rats were pooled and homogenised in 0.05M phosphate buffer pH6.5 (300ul per pineal gland) containing both Tryp ($1.5x10^{-3}M$) and AcCoA ($6x10^{-5}M$). Equal aliquots (150ul) were incubated at various temperatures for varying lengths of time, activity being monitored as described above.

Evaluation of Kinetic Constants. Equal aliquots (150ul) obtained from homogenisation of pooled stimulated pineal glands were incubated at 20°C for an hour. Reaction remained linear under these conditions. Homogenates contained one substrate in constant concentration ($4.5x10^{-3}M$ for Tryp or $1.8x10^{-4}M$ for AcCoA) while the concentration of the second substrate was varied ($1.5x10^{-4}$-$3x10^{-3}M$ in the case of Tryp and 5-HT and $1.5x10^{-6}$ -$3x10^{-5}M$ in the case of AcCoA).

Cosubstrate Effects. Both Tryp and AcCoA were mixed in a ratio of their $K^s_m$ values and concentration of this mixture was varied ($5x10^{-5}$-$1x10^{-3}M$) reaction being monitored as above.

Alternate Substrate Effects. Equal aliquots of enzyme homogenates were incubated in which concentration of either 5-HT or Tryp were held constant and sub-saturating ($5x10^{-4}M$) while concentration of AcCoA was varied ($2x10^{-6}M$-$3x10^{-5}M$). Velocity was monitored at each concentration.

Product Effects. In all cases concentration of one substrate was kept constant ($3x10^{-3}M$ for Tryp and $1.8x10^{-4}M$ for AcCoA) while concentration of the second substrate was varied ($2x10^{-6}$-$3x10^{-5}M$ for AcCoA and $2x10^{-4}$-$3x10^{-3}M$ for Tryp and 5-HT). These incubations were performed in the presence and absence of the various products at several concentrations ($1x10^{-6}$ and $1x10^{-5}M$ for coenzyme A, $1x10^{-4}$ and $1x10^{-3}M$ for NAT and $1x10^{-5}$, $1x10^{-4}$ and $1x10^{-3}M$ for N-acetylserotonin (NAS)).

Effect of pH on Kinetic Behaviour. Buffer solutions of various pH were added to reaction tubes and evaporated to dryness prior to incubation. Enzyme and substrate solutions were made up in distilled water and added to these reaction vessels thus reconstituting the 0.05M phosphate buffer environment at various pH values. The tubes contained one substrate in constant concentration ($3x10^{-3}$M for Tryp and $1.8x10^{-4}$M for AcCoA) while concentration of the second substrate varied ($2x10^{-6}$-$3x10^{-5}$M for AcCoA and $2x10^{-4}$-$3x10^{-3}$M for Tryp).

Effect of temperature on Kinetic Behaviour. Incubates containing enzyme and substrates (concentrations as for effect of pH on kinetic behaviour) in 0.05M phosphate buffer pH6.5 were subjected to various incubation temperatures to monitor changes in kinetic behaviour.

Evaluation of Data. Reactions were carried out in duplicate and the means were used for analysis. The effects of temperature on reaction rate with time were analysed using linear regression, the coefficient of determination ($r^2$) being used as a measure of linearity. All kinetic determinations were evaluated using computer assisted non-linear iterative regression analysis.

## RESULTS AND DISCUSSION

The exposed areas on the autoradiographs corresponded to pure NAT or NAS respectively verifying purity of the product.

Activity of SNAT peaked at a pH of 6.4 (Figure 1). A tri-linear function was created by plotting log velocity versus pH (Figure 2), the points of inflection giving the pK values for ionizing groups which influence catalytic activity [10]. The points of inflection (6.4 and 6.8) imply participation of imidazolium groups and possibly α-ammonium groups of cystine in maintaining the catalytic environment of the active sites. It is not known in what way these sites might affect activity but may influence active sites directly, alter substrate binding, enzyme conformation or some other mechanism.

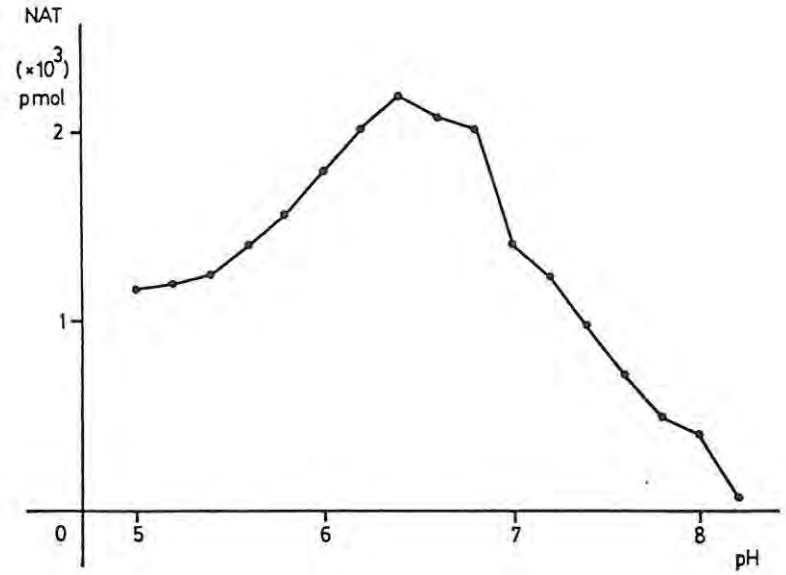



Figure 1: Effect of change in pH on SNAT activity. Points are the means of duplicates. Incubation at 20°C for three hours.

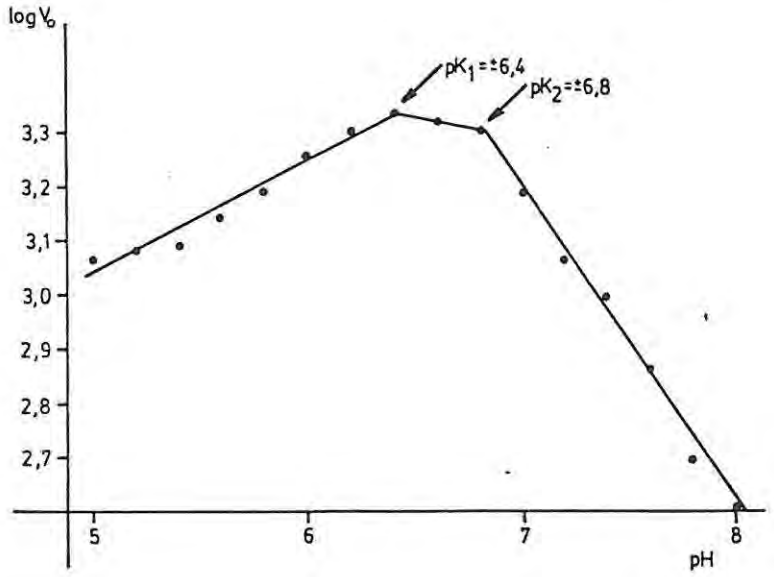



Figure 2: Plot of log V versus pH to determine pK values of groups important to SNAT activity. (pK = points of inflection).

Enzymes exist in different protonation states in apparent equilibrium [11] which can be represented as follows.

$$H_2{}^+E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} H_1{}^+E \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} H_0{}^+E$$

The pH profile for SNAT (Figure 1) suggests that only the $H_1{}^+E$ form of the enzyme is active and either increase or decrease in protonation leads to loss of catalytic activity. If it is accepted that at optimal pH all the enzyme exists in the active form $H_1{}^+E$ and as pH increases or decreases so $H_1{}^+E$ levels decrease in direct proportion to decrease in activity, then the proportions of the three protonation states of the enzyme can be depicted as a function of pH (Figure 3).

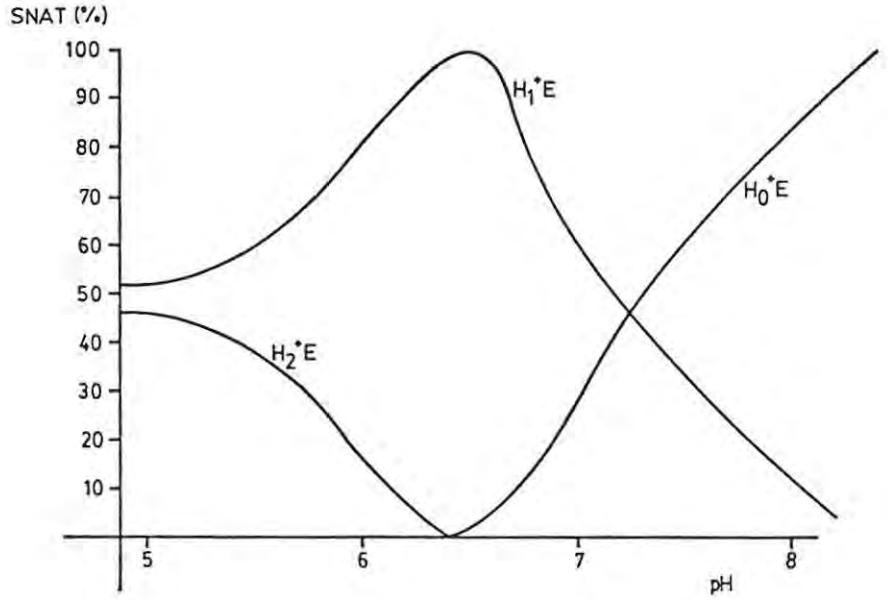



Figure 3: Hypothetical plot of change in proportion of the various protonation forms of SNAT with variation in pH. Determination as outlined in the text.

Several inferences can be drawn from this information. Firstly, the rate constant $k_2$ has a greater magnitude than $k_{-1}$ as $H_1{}^+E$ changes to $H_0{}^+E$ with greater rapidity as pH decreases. Secondly, as rate of change of $H_1{}^+E$ to $H_0{}^+E$ is relatively uniform and goes essentially to completion with increase in pH implies that there are no ionizing groups with pK values in the

range 6.8-8.3 that directly affect the active site. On the other hand, as $H_1{}^+E$ changes to $H_2{}^+E$ with decrease in pH, the profile of the rate of change varies. Rate of change remains constant as pH falls to about 5.6 then decreases with further reduction in pH implying that below pH5.6 conversion to inactive form is being resisted, indicative of an involvement of ionizable groups with pK values below 5.6 in maintaining a measure of catalytic activity. The limitations of the phosphate buffer did not allow determinations below pH5.0.

Rearrangement of the Arrhenius equation allows a plot of log initial velocity versus reciprocal of absolute temperature to yield activation energies for the reaction (Figure 4).

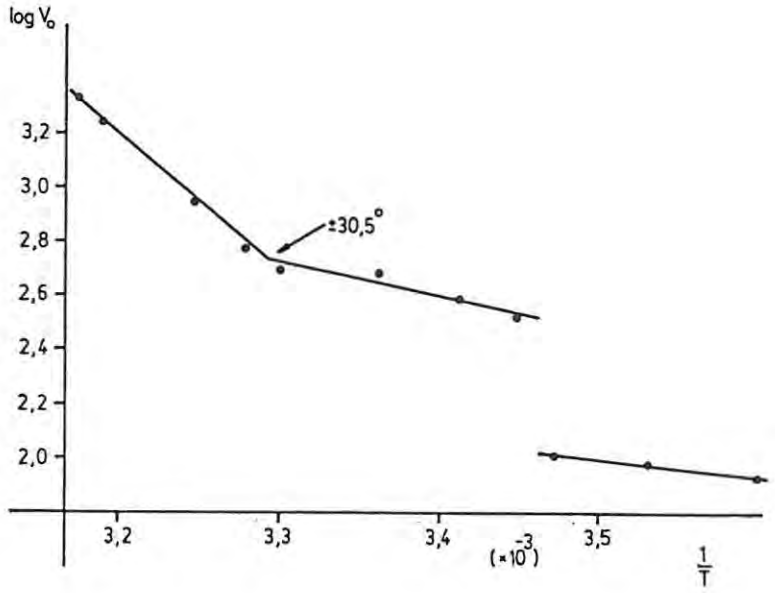



Figure 4: Arrhenius plot of log initial velocity ($V_0$) versus reciprocal of absolute temperature illustrating change in activation energy with temperature. POINTS ARE DUPLICATES.

Between 17 and 42°C the function was bi-linear with a discontinuity at about 30.5°C. Below this temperature activation energy was low (about 2.1Kj $mol^{-1}$) while above 30.5°C activation energy rose to about 10.5Kj $mol^{-1}$. This change probably results from denaturation of the enzyme which becomes more pronounced above 30.5°C.

Between 5 and 15°C, although the plot was linear, it was independent of the other linear sections of the graph with an activation energy of about 1.7Kj $mol^{-1}$. The fact that this section of the plot is almost parallel to that between 17 and 30.5°C suggests that, although there is a break in continuity of the plot, this is not due to a change in the catalytic process as such. This difference could possibly be explained by an effect of a decrease in temperature on the primary hydration shell surrounding the enzyme. As incubation temperature falls from 17 to 15°C the increase in density and decrease in degree of autoprotolysis causes the primary hydration shell to become more tightly bound thus impeding substrate entry into the active site. Enzyme, although still present in the active form, has a reduced turnover number hence the velocity versus time profile would be expected to differ from those at higher temperature which is indeed the case (Figure 5).

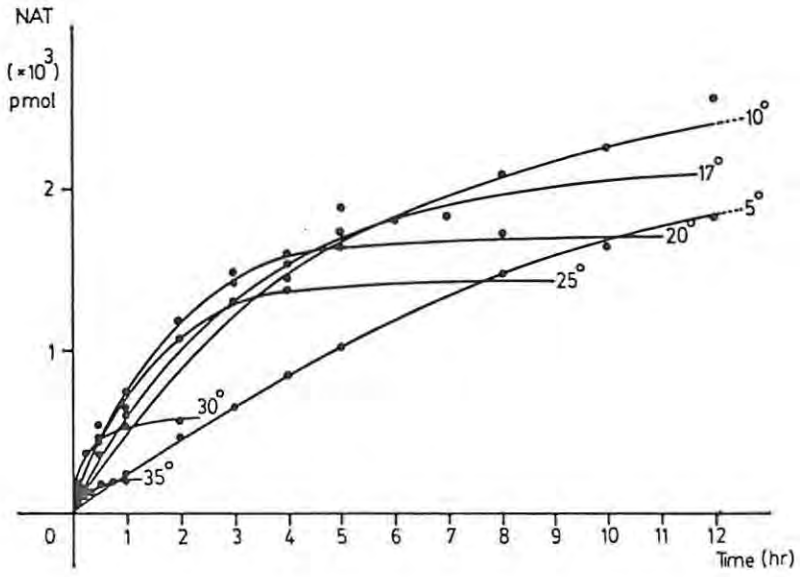



Figure 5: Product formation (NAT) with time at various incubation temperatures shown on the graph. POINTS ARE DUPLICATES.

The anomalies observed in the Arrhenius plot could, however, be due to a phase transition in the enzyme [12,13]. The transition in this case would take place between 15 and 17°C, the result being a more accessible catalytic site, hence an increased turnover number.

SNAT activity was found to obey the following first order function closely.

$$\log([P]^{\infty}-[P]) = -kt + \log[P]$$

where k is a constant, $[P]^{\infty}$ is the maximum product concentration and [P] is product concentration at time t, with temperature remaining constant.

The estimated regression coefficients k and $\log[P]^{\infty}$ were obtained for each incubation temperature employed in order to obtain the half-life ($t_{1/2}$) of the enzyme (Table 1, Figure 5). From these results it can be seen that decrease in incubation temperature led to a significant increase in enzyme activity allowing more sensitive determinations. This increase in activity can be presumed to be the result of decreased thermal degradation of SNAT encountered at higher temperatures.

Table 1: Values derived for the function $\log([P]^{\infty}-[P]) = -kt + \log[P]$ where k is a constant, $[P]^{\infty}$ is maximum product concentration and [P] is product concentration at time t, with temperature remaining constant. The coefficient of determination ($r^2$) illustrates the linearity of the plots.

| INCUBATION TEMP (°C) | K ($sec^{-1}$) | LOG $[P]^{\infty}$ | HALF-LIFE (t1/2)(sec) | $r^2$ |
|---|---|---|---|---|
| 5 | $1.3\times10^{-5}$ | 3.401 | $2.3\times10^{4}$ | 0.99 |
| 10 | $2.5\times10^{-5}$ | 3.413 | $1.2\times10^{4}$ | 0.98 |
| 17 | $8.0\times10^{-5}$ | 3.350 | $3.8\times10^{3}$ | 0.92 |
| 20 | $8.1\times10^{-5}$ | 3.242 | $3.7\times10^{3}$ | 0.99 |
| 25 | $9.6\times10^{-5}$ | 3.136 | $3.1\times10^{3}$ | 0.99 |
| 30 | $3.2\times10^{-4}$ | 2.676 | $9.5\times10^{2}$ | 0.98 |
| 35 | $4.6\times10^{-4}$ | 2.088 | $6.5\times10^{2}$ | 0.93 |

Kinetic evaluations were based on the theory of Michaelis and Menten [14] resulting in determination of the Michaelis-Menten constant ($K^S_m$) and saturation velocity ($V_S$) for the three substrates used (Table 2, Figure 6a, 6b and 6c). The $K^S_m$ values were similar to those previously reported while the $V_S$ values differed [5].

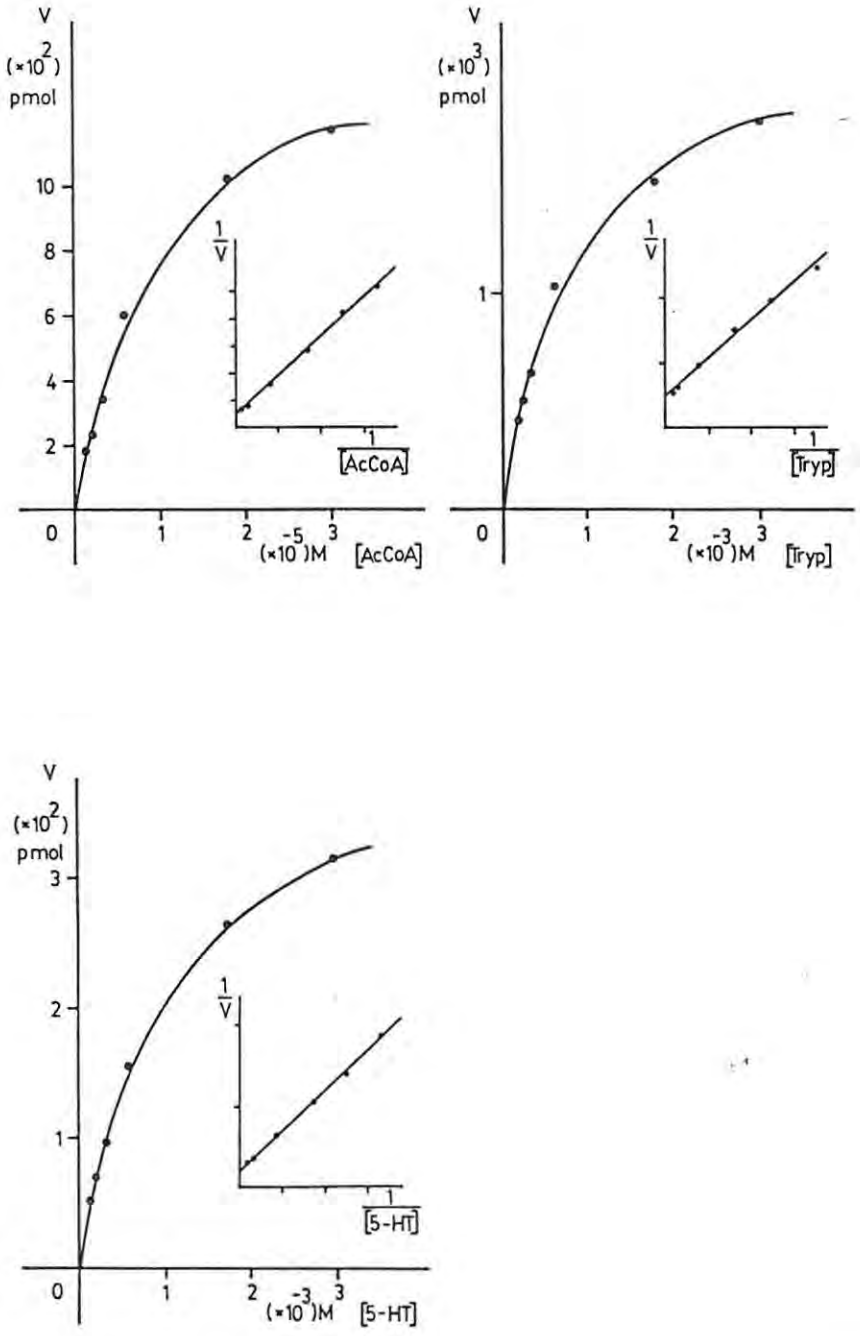



Figure 6: Effect of substrate concentration on velocity of reaction (V) with a) acetyl coenzyme A (AcCoA), b) tryptamine (Tryp) and c) serotonin (5-HT) as the varied substrate. Double reciprocal plots inset to illustrate linearity.

Table 2: Michaelis-Menten kinetic constants ($K^S_m$) and saturation velocities ($V_S$) determined for SNAT substrates using non-linear regression analysis.

| VARIED SUBSTRATE | $K^S_m$(uM) | $V_S$(pmol/pineal/hr) |
|---|---|---|
| Acetyl Co A | 10.6±0.9 | 1607±55 |
| Tryptamine | 672.6±59.4 | 2157±71 |
| Serotonin | 973.0±44.4 | 415±8 |

When velocity was monitored as the concentration of both substrates varied in a constant ratio, the resulting double reciprocal plot was found to be parabolic-up, indicative of a sequential rather than ping-pong catalytic mechanism [15]. Using alternate substrates (Tryp and 5-HT) produced double reciprocal plots with dissimilar gradients also indicative of a sequential mechanism [16].

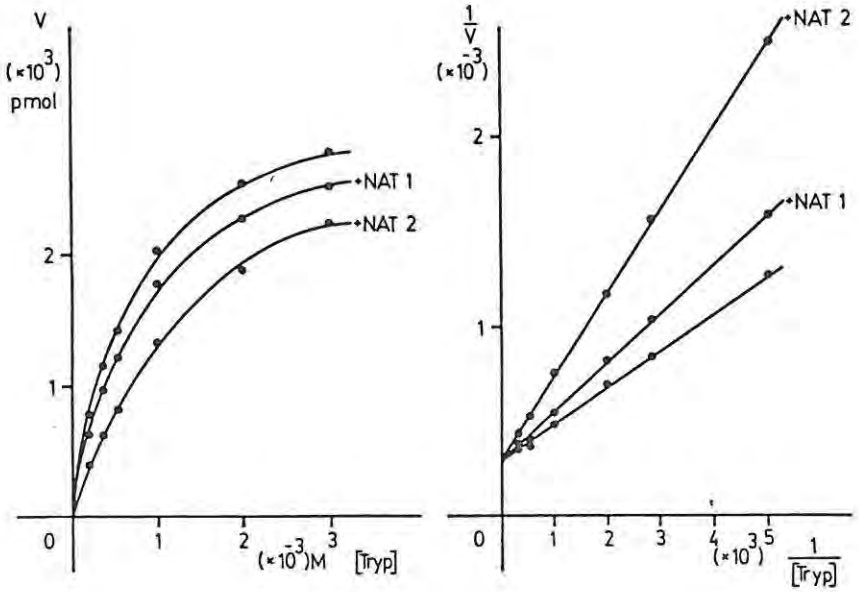



Figure 7: Effect of N-acetyltryptamine (NAT) on catalytic behaviour of SNAT with tryptamine (Tryp) as the varied substrate (NAT 1 = 0.1mM, NAT 2 = 1mM). Double reciprocal plot illustrates the competitive nature of the interaction.

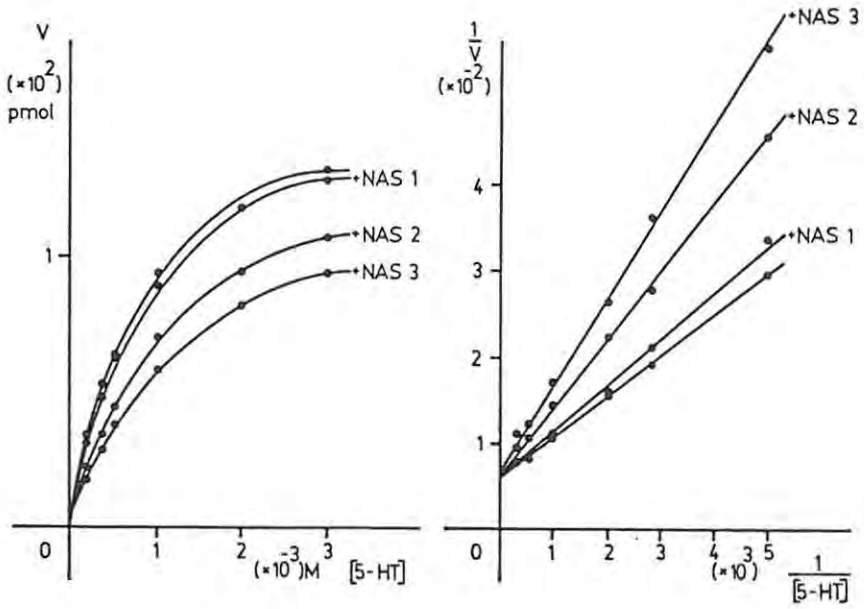
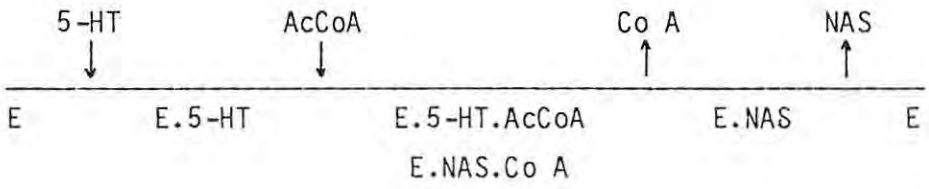



Figure 8: Effect of N-acetylserotonin (NAS) on catalytic behaviour of SNAT with serotonin (5-HT) as the varied substrate (NAS 1 = 0.01mM, NAS 2 = 0.1mM, NAS 3 = 1mM). Double reciprocal plot illustrates the competitive nature of the interaction.

All the product inhibition patterns were mixed non-competetive except the effect of NAT and NAS on the enzyme in which case competitive inhibition was observed (Figure 7 and 8). This is indicative of an ordered Bi-Bi catalytic mechanism [17] which can be represented as follows.




The binding of 5-HT to the enzyme causes some conformational change which allows binding of AcCoA, acetyl transfer then occurs and products leave, also in a sequential fashion.

It is interesting that this catalytic mechanism appears to be similar to that of liver N-acetyltransferase [18] and yet the liver enzyme is unaffected by sympathetic stimulation [5]. This

suggests that while β-adrenergic stimulation is capable of increasing pineal SNAT levels it does not influence catalysis per se.

The effects of changing pH on kinetic behaviour of SNAT were varied (Table 3). An increase in pH led to increased affinity of the enzyme for Tryp while the saturation velocity rose to a maximum at pH6.5 before decreasing with further increase in pH. Affinity for AcCoA was greatest at pH6.5, saturation velocity decreasing with increase in pH. This suggests that change in activity does not result from change in affinity but from an effect on acetyl transfer thus hindering product formation.

Table 3: Effect of varying pH on kinetic constants for SNAT substrates.

| VARIED SUBSTRATE | pH | $K^S_m$(uM) | $V_S$(pmol/incubate/hr) |
|---|---|---|---|
| Acetyl Co A | 6.0 | 29.7±0.9 | 525±9 |
| " | 6.5 | 13.1±0.5 | 226±4 |
| " | 6.8 | 32.4±2.2 | 250±10 |
| Tryptamine | 6.0 | 1510.4±31.5 | 705±7 |
| " | 6.5 | 987.3±15.1 | 967±6 |
| " | 6.8 | 444.0±121.3 | 682±6 |
| " | 7.0 | 43.8±6.0 | 201±2 |

Increase in incubation temperature tended to increase affinity of enzyme for Tryp while the saturation velocities for both Tryp and AcCoA decreased (Table 4). The apparent increase in affinity may be the result of increased thermal agitation as incubation temperature rises resulting in increased particle collisions. The decrease in velocity probably results from denaturation with increased temperature.

Table 4: Effect of varying incubation temperature on kinetic constants for SNAT substrates.

| VARIED SUBSTRATE | TEMP (°C) | $K^S_m$(uM) | $V_s$(pmol/incubate/hr) |
|---|---|---|---|
| Acetyl Co A | 35 | 21.5±1.2 | 43±1 |
| " | 30 | 45.6±3.8 | 234±13 |
| " | 25 | 39.7±1.3 | 557±11 |
| " | 20 | 11.9±0.4 | 798±11 |
| " | 15 | 70.1±2.2 | 1311±31 |
| " | 5 | 12.1±0.6 | 249±5 |
| Tryptamine | 35 | 828.2±52.2 | 269±7 |
| " | 30 | 1550.0±36.4 | 891±10 |
| " | 25 | 937.1±18.0 | 791±6 |
| " | 20 | 1100.3±14.0 | 1285±7 |
| " | 15 | 2680.5±32.7 | 2234±16 |
| " | 5 | 10108.3±235.1 | 2115±40 |

Although it was not possible to purify SNAT due to its thermolability, the purity of the product indicates that the observed effects were the exclusive result of SNAT activity and and hence gave a reasonable indication of the catalytic behaviour.

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CHAPTER 4

# KINETIC PROPERTIES AND PROBABLE MECHANISM OF CATALYSIS OF HYDROXYINDOLE-O-METHYLTRANSFERASE (EC 2.1.1.4)

INTRODUCTION

MATERIALS

METHODS
Preparation of Hydroxyindole-O-methyltransferase (HIOMT)
Assay for HIOMT
Product Purity
Effect of pH
Effect of Temperature
Evaluation of Kinetic Constants
Cosubstrate Effects
Product Effects
Effect of pH on Kinetic Behaviour
Effect of Temperature on Kinetic Behaviour
Evaluation of Data

RESULTS AND DISCUSSION

REFERENCES

## INTRODUCTION

Hydroxyindole-O-methyltransferase was first detected in bovine pineal gland [1] and has since been found in the pineal glands of other mammals [2], birds, reptiles and amphibians [3]. Until recently it was thought to be unique to the pineal gland but has since been found in the retina and harderian gland [4] and may occur in enterochromaffin cells [5]. Retinal hydroxyindole-O-methyltransferase exhibits similar properties to the pineal enzyme while that from the harderian gland is notably different, being stimulated by magnesium ions and showing a different spectrum of substrate specificities [4].

Hydroxyindole-O-methyltransferase catalyses the formation of a

variety of methoxyindoles including 5-methoxytryptophan and 5-methoxytryptamine [6], melatonin [1], 5-methoxytryptophol [7] and 5-methoxyindole-3-acetic acid [8]. It has been suggested that several such enzymes exist in the pineal [9], activity being regulated by pterins and pteridines [9,10]

Although studies have evaluated some kinetic parameters [4] and several groups have obtained pure enzyme [11,12] only one report has suggested a catalytic mechanism [13]. This report proposed an ordered Bi-Bi mechanism for the enzyme [13].

The present study attempted to increase sensitivity of determination of the enzyme and to further characterise the catalytic mechanism.

## MATERIALS

See Appendix A.

## METHODS

Preparation of Hydroxyindole-O-methyltransferase (HIOMT). Both rat and bovine enzymes were used.

Rats of both sexes (250-300g) were sacrificed, pineal glands were removed and homogenised on ice in 0.05M phosphate buffer pH7.9 (unless otherwise stated) using 160ul per pineal gland. These homogenates contained substrates in varying concentration depending on the parameter being investigated.

Bovine pineal glands were collected within an hour of death and homogenised in 0.15% w/v KCl (1ml per 100mg tissue), centrifuged for 30 minutes at 4°C to remove cell debris and the resultant supernatant was lyophilised. The lyophilisate (1mg per 12.37 1.34 mg wet tissue) was stored at -20°C and was used as a source of HIOMT. Prior to use this lyophilisate was dissolved in 0.05M phosphate buffer pH7.9 (unless otherwise stated) using 1mg per 100ul final incubate volume.

Assay for HIOMT. The assay technique adopted was modified from that of Axelrod et al [14] which relied on transfer of a 14C-

methyl group from S-adenosylmethionine (AMe) to N-acetylserotonin (NAS) forming melatonin (MTN). Incubation was carried out at 42°C for an hour (unless otherwise stated) and reaction was halted by the addition of an equal volume of 0.2M borate buffer pH10. Reaction remained linear under these conditions. Radioactive MTN was extracted into 3ml toluene:isoamyl alcohol (97:3) mixture by vortexing for a minute. After centrifugation, 2ml of the organic phase was aspirated into 6ml scintillation cocktail and quantitated. Extraction led to about 98% recovery. Counting efficiency was determined using the external standards channel ratio and exceeded 90% in all cases.

Blank incubations were performed in which only enzyme was omitted from the procedure, these values being subtracted from those of enzyme incubates to arrive at a true value for enzyme activity.

Trypsin addition (0.1% w/v) to some incubates led to a rapid decrease in activity, verifying protein involvement in the formation of $^{14}$C-MTN.

Product Purity. Toluene:isoamyl alcohol extracts were evaporated to dryness under nitrogen at 40°C, the residue dissolved in 20ul chloroform and spotted onto thin-layer (TLC) plates. These TLC plates were developed in chloroform:methanol:glacial acetic acid (97:3:1).

After development the plates were dried and strips of photographic film negative (Kodak Plus-X pan, 125 ASA) were attached and left to expose in light proof containers at 0°C for several weeks prior to development.

Effect of pH. Groups of rat pineal glands were pooled and homogenised in distilled water (80ul per pineal) and 80ul aliquots were added to an equal volume of 0.1M buffer solutions. These mixtures contained AMe ($4\times10^{-4}$M) and NAS ($7.6\times10^{-4}$M) and velocity was monitored for an hour at 38°C.

Effect of Temperature. Pooled rat pineal gland homogenates containing AMe ($4\times10^{-4}$M) and NAS ($7.6\times10^{-4}$M) in 0.05M phosphate buffer pH7.9 were divided into equal aliquots (160ul) and incubated at various temperatures for different times.

Evaluation of Kinetic Constants. The Michaelis-Menten kinetic constants [15] were determined for both rat and bovine pineal HIOMT. While concentration of one substrate was kept constant ($8x10^{-4}M$) the concentration of the second substrate varied ($1.5x10^{-5}$-$3.2x10^{-4}M$) velocity being monitored at each concentration.

Cosubstrate Effects. Both AMe and NAS were mixed in a ratio of their $K^s_m$ values and velocity was monitored as concentration of this mixture varied ($5x10^{-5}$-$1x10^{-3}M$).

Product Effects. The effects of MTN and S-adenosylhomocysteine (SAH) on the kinetic behaviour of bovine pineal HIOMT were evaluated. In all cases concentration of one substrate was kept constant ($8x10^{-4}M$) while the concentration of the second substrate was varied ($2x10^{-5}$-$3x10^{-4}M$). Such determinations were carried out in the presence and absence of both products (saturating MTN and $1x10^{-5}$, $5x10^{-5}$ and $1x10^{-4}M$ SAH).

Effect of pH on Kinetic Behaviour. Buffer solutions of various pH were added to reaction tubes and evporated to dryness prior to incubation. Enzyme and substrate solutions were made up in distilled water and added to these reaction vessels thus reconstituting the 0.05M phosphate buffer environment at various pH values. The tubes contained one substrate in constant concentration ($8x10^{-4}M$) while the concentration of the second substrate varied ($2x10^{-5}$-$3x10^{-4}M$).

Effect of Temperature on Kinetic Behaviour. Incubates in 0.05M phosphate buffer pH7.9 containing one substrate in constant concentration ($8x10^{-4}M$) and the second substrate at various concentrations ($2x10^{-5}$-$3x10^{-4}M$) were incubated at different temperatures.

Evaluation of Data. Reactions were carried out in duplicate, the means being used for analysis. The effects of temperature on reaction rate with time were analysed using linear regression, the coefficient of determination ($r^2$) being used as a measure of linearity. All kinetic determinations were evaluated using computer assisted non-linear iterative regression analysis.

On development of the autoradiographs only one exposed area was detected which corresponded to pure MTN verifying product purity.

Although HIOMT activity increased by about 25% when determined in glycine buffer pH9 instead of phosphate buffer pH7.9 at 38°C (Figure 1) this increase was not maintained at higher temperatures. Consequently, it was concluded that the best buffer system was 0.05m phosphate at pH7.9 and the optimum conditions for determination of HIOMT activity in vitro are incubation at 42°C using a final incubate volume of 160ul/rat pineal gland or 100ul/mg of bovine pineal lyophilisate.

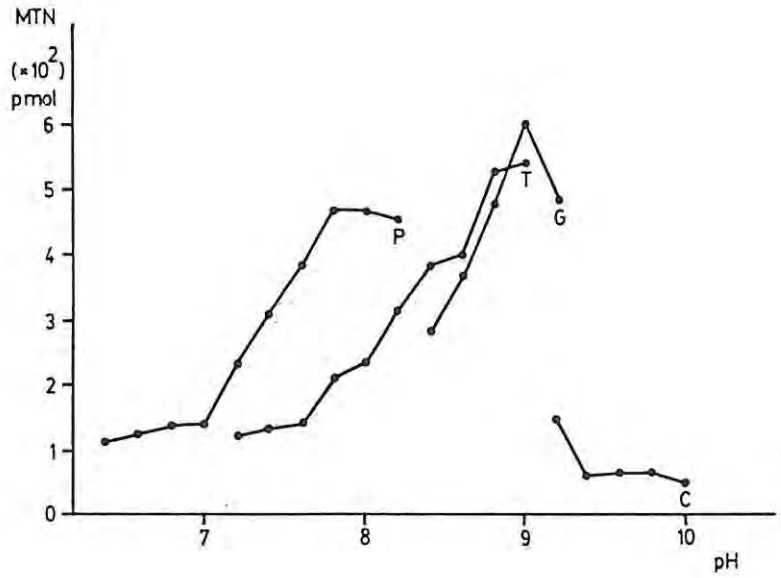



Figure 1: Effect of different buffer systems on rat HIOMT activity. Incubation at 38°C for one hour in 0.05M buffer. C = carbonate, G = glycine, P = phosphate and T = tris buffer.

A plot of log velocity versus pH gave a bi-linear function with the point of inflection at 7.8 (Figure 2). This value represents the pK value of ionizing groups which influence activity [16] and these groups may be α-ammonium groups [17]. It is not known in what way these groups influence activity but may affect substrate binding, enzyme conformation or exert a direct effect on the

active site. The pH profile suggests that the higher protonation states of HIOMT are inactive and decrease in protonation leads to an increase in activity.

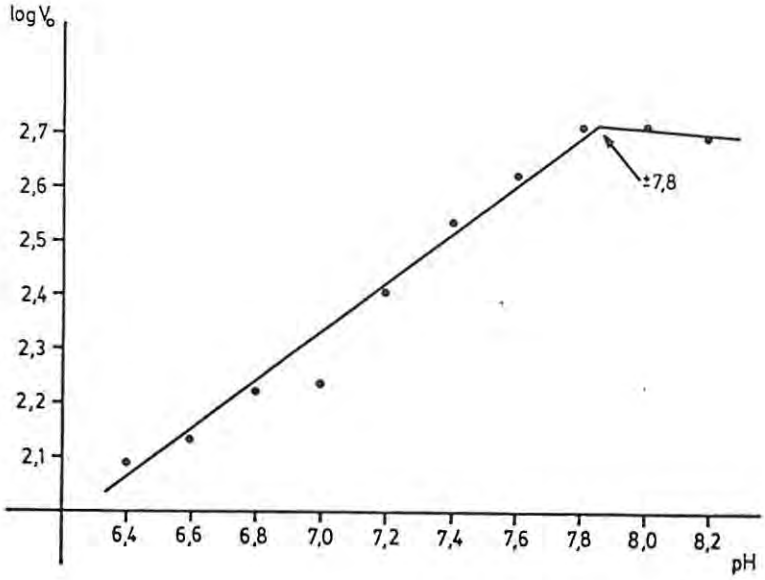



Figure 2: Plot of log V versus pH to determine pK values of groups important for HIOMT activity. The point of inflection indicates the pK value. Each point represents the mean of duplicates.

The Arrhenius plot of reaction rate versus reciprocal of absolute temperature yielded a bi-linear function with the point of inflection at 32°C (Figure 3). At lower temperatures the activation energy was about 10.5Kj $mol^{-1}$ while above 32°C the activation energy doubled. This discontituity may indicate that the enzyme is present in two active forms in equilibrium with one another, change in activity occuring with changes in incubation temperature. Evidence exists suggesting that HIOMT occurs in different molecular forms with differing activity [11] which would support this proposal. The discontinuity may also indicate a phase transition in the enzyme [18,19].

HIOMT activity with time was found to be first order for all temperatures tested and obeyed the following function closely where k is a constant, $[P]^{oo}$ is maximum product concentration

at any one temperature and [P] is product concentration at time t, temperature remaining constant.

$$\log([P]^{\infty}-[P]) = -kt + \log[P]$$

This data was evaluated in order to obtain the estimated regression coefficients k and $\log[P]^{\infty}$ which enabled calculation of half-life ($t_{1/2}$) and consequently rate constants for the reaction at all temperatures used. The coefficient of determination ($r^2$) was used to illustrate linearity (Table 1, Figure 4).

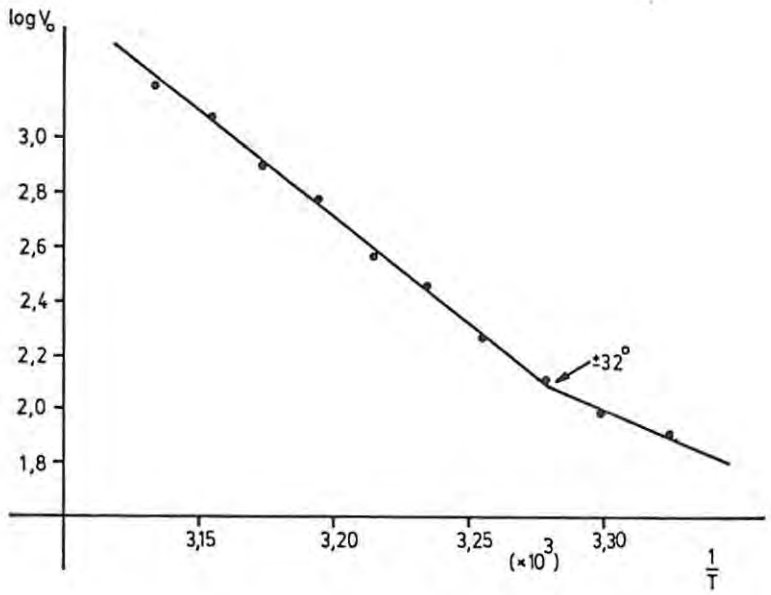



Figure 3: Arrhenius plot of log initial velocity ($V_0$) versus reciprocal of absolute temperature illustrating change in activation energy with temperature.

The Michaelis-Menten constant ($K^S_m$) and saturation velocity ($V_S$) were calculated for both substrates using rat and bovine pineal HIOMT (Table 2, Figure 5a and 5b, 6a and 6b). These values differ slightly from those previously reported [4] probably as a result of differences in assay conditions employed.

Table 1: Values derived for the function $\log([P]^{oo}-[P]) = -kt + \log[P]$ where k is a constant, $[P]^{oo}$ is maximum product concentration and [P] is product concentration at time t, temperature remaining constant. Values obtained from incubation of rat pineal incubates. The coefficient of determination ($r^2$) illustrates the linearity of the plots.

| INCUBATION TEMP (°C) | K ($sec^{-1}$) | LOG $[P]^{oo}$ | HALF-LIFE (t1/2)(sec) | $r^2$ |
|---|---|---|---|---|
| 34 | $2.7x10^{-5}$ | 2.931 | $1.5x10^4$ | 0.99 |
| 38 | $3.7x10^{-5}$ | 2.908 | $1.1x10^4$ | 0.99 |
| 42 | $6.8x10^{-5}$ | 2.858 | $6.0x10^3$ | 0.98 |
| 44 | $1.5x10^{-4}$ | 2.736 | $2.7x10^3$ | 0.94 |
| 46 | $2.3x10^{-4}$ | 2.599 | $1.7x10^3$ | 0.90 |

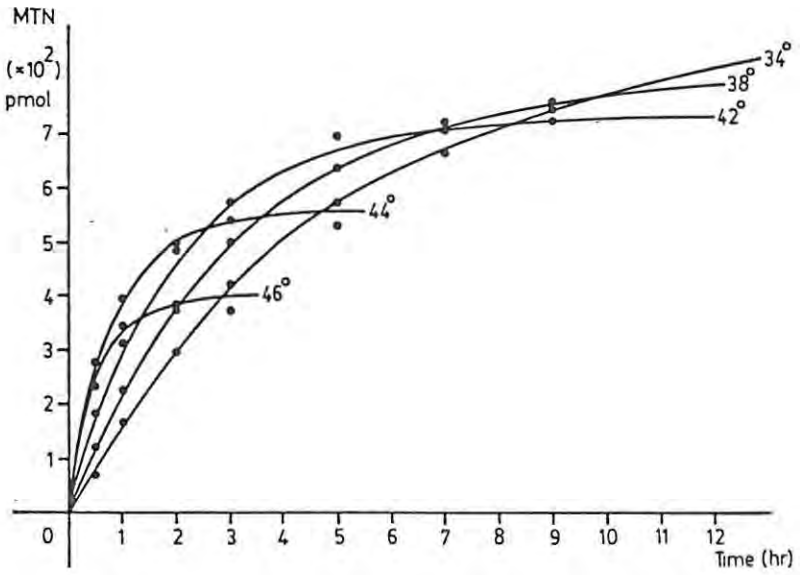



Figure 4: Effect of different incubation temperatures and times on melatonin formation. Incubation was in 0.05M phosphate buffer (pH7.9) for varying lengths of time at the temperatures shown on the graph.

Table 2: Michaelis-Menten kinetic constants ($K^S_m$) and saturation velocities ($V_S$) determined for rat and bovine pineal HIOMT using non-linear regression analysis.

| TYPE OF HIOMT | VARIED SUBSTRATE | $K^S_m$(uM) | $V_S$(pmol/incubate/hr) |
|---|---|---|---|
| Rat | AMe | 75.8±3.2 | 708±11 |
| " | NAS | 129.2±21.5 | 770±56 |
| Bovine | AMe | 55.3±2.3 | 4722±70 |
| " | NAS | 35.6±3.2 | 6138±176 |

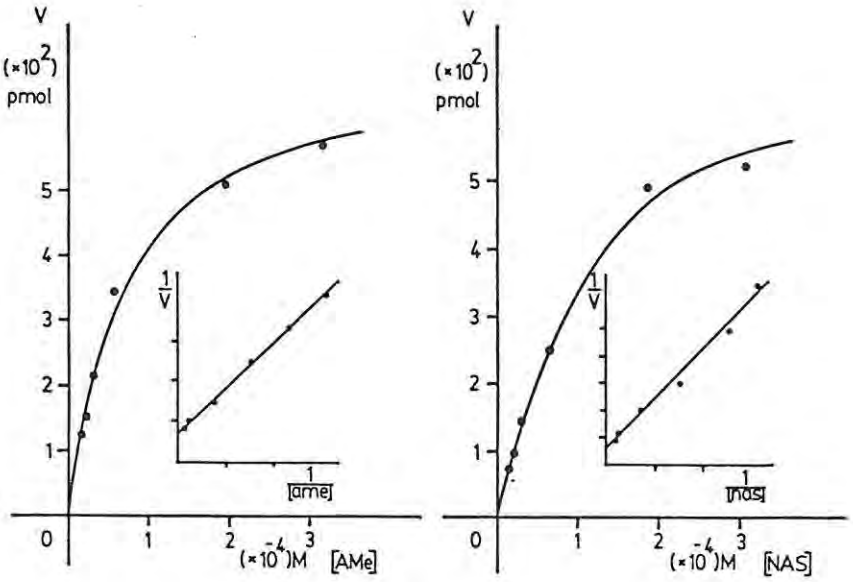



Figure 5: Effect of substrate concentration on velocity of reaction (V) for rat pineal HIOMT with a) S-adenosylmethionine (AMe) and b) N-acetylserotonin (NAS) as the varied substrate. Double reciprocal plots inset to illustrate linearity.

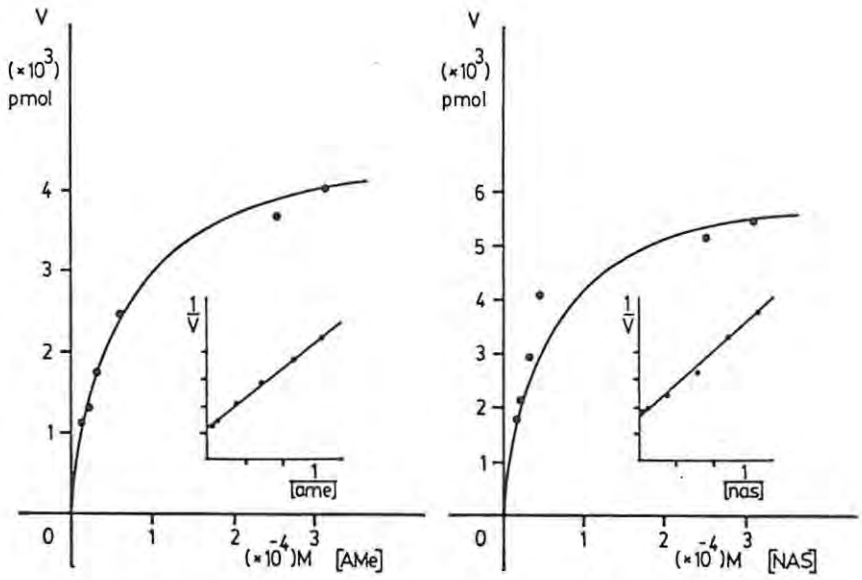
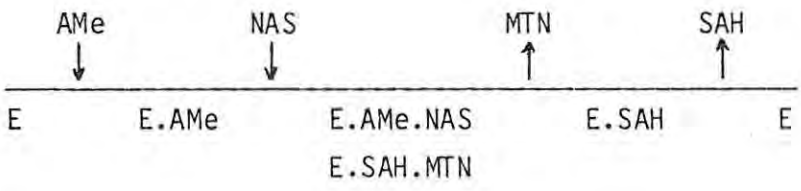



Figure 6: Effect of substrate concentration on velocity of reaction (V) for bovine HIOMT with a) S-adenosylmethionine (AMe) and b) N-acetylserotonin (NAS) as the varied substrate. Double reciprocal plots inset to illustrate linearity.

When concentration of both substrates was varied in a constant ratio the double reciprocal plot was parabolic-up, indicative of a sequential rather than ping-pong catalytic mechanism [20].

Melatonin was an uncompetitive inhibitor of HIOMT with respect to NAS and a mixed non-competitive inhibitor with respect to AMe. On the other hand, SAH was a mixed non-competitive inhibitor with NAS as the varied substrate and exerted a competitive inhibition when AMe was the varied substrate (Figure 7). This product inhibition pattern is indicative of an ordered Bi-Bi mechanism [21] which is in agreement with a previous report [13] and may be represented as follows.



Catalysis begins with binding of AMe to HIOMT, such binding causing a change in conformation which allows NAS to bind to the enzyme, methyl transfer then occurs and products leave in a sequential fashion.

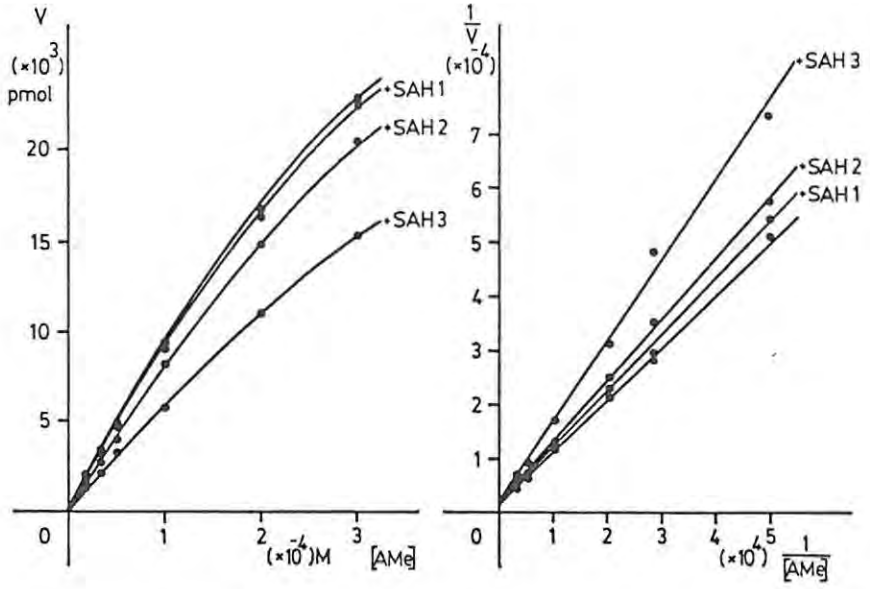



Figure 7: Effect of S-adenosylhomocysteine (SAH) on catalytic behaviour of HIOMT with S-adenosylmethionine (AMe) as the varied substrate. (SAH 1 = 10uM, SAH 2 = 50uM, SAH 3 = 100uM). Double reciprocal plot illustrates the competitive nature of the interaction.

Change in pH appeared to have little effect on NAS utilization by HIOMT while affecting both intrinsic activity and affinity for AMe (Table 3). At the lower pH affinity for AMe was about double that at higher pH while the intrinsic activity was less than half that at higher pH. The ratios between $K^{AMe}_m$ and $V_{AMe}$ varied little, suggesting an effect on the rate constant for dissociation of enzyme-substrate complex to yield products. This effect probably results from a change in protonation in the vicinity of the active site allowing product to leave more readily at a higher pH. The fact that affinity of HIOMT for AMe increased with decrease in pH implied that an electron rich area of the AMe molecule was attracted to the active site. That velocity was reduced under these conditions implied that positive

charge in the vicinity of the active site inhibited transfer of the methyl group to NAS. As enzyme activity remained constant with increase in pH once a certain level of deprotonation had been reached supports the proposal that positive charge in the vicinity of the active site enhanced binding of AMe but interfered with the catalytic process as such. Removal of positive charge from the active site allowed transfer of the methyl group.

Table 3: Effect of varying pH on kinetic constants for bovine pineal HIOMT substrates S-adenosylmethionine (AMe) and N-acetylserotonin (NAS).

| VARIED SUBSTRATE | pH | $K^S_m$(uM) | $V_S$(pmol/incubate/hr) |
|---|---|---|---|
| AMe | 7.5 | 269.6±1.0 | 16868±36 |
| " | 7.9 | 570.1±2.8 | 36078±128 |
| " | 8.8 | 517.0±1.0 | 36264±51 |
| NAS | 7.5 | 109.9±0.2 | 21503±13 |
| " | 7.9 | 109.8±0.3 | 21239±25 |
| " | 8.8 | 130.0±0.2 | 23281±13 |

It is probable, therefore, that the electron rich flat adenosyl ring structure of AMe is attracted to the active site on HIOMT and this binding then enables the enzyme to accept NAS. Methyl transfer could be a nucleophilic substitution in which case presence of a group such as the imidazole side chain of histidine would enhance the nucleophilicity of the hydroxyl group on NAS. The presence of such a group would also account for the observed pH effects. That histidine is present in HIOMT [11,12] may indicate such a role although it is largely conjectural at this stage.

The lower the incubation temperature the greater the affinity of HIOMT for its substrates while the saturation velocity decreased (Table 4) implying that activity of the enzyme was largely dependent on particle collisions. With decrease in temperature particle-particle interactions decrease and activity followed a

similar pattern despite the increased affinity for substrates.

Table 4: Effect of varying incubation temperature on kinetic constants for bovine pineal HIOMT substrates S-adenosylmethionine (AMe) and N-acetylserotonin (NAS).

| VARIED SUBSTRATE | TEMP (°C) | $K^S_m$(uM) | $V_s$(pmol/incubate/hr) |
|---|---|---|---|
| AMe | 42 | 470.6±3.0 | 29907±129 |
| " | 35 | 109.1±1.2 | 7018±32 |
| " | 30 | 40.3±6.1 | 1883±357 |
| " | 25 | 9.8±0.1 | 1341±1 |
| NAS | 42 | 381.7±1.7 | 25043±73 |
| " | 35 | 643.6±0.1 | 2559±1 |
| " | 30 | 70.1±0.8 | 905±4 |
| " | 25 | 68.9±0.9 | 509±2 |

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CHAPTER 5

# EFFECT OF ANTICONVULSANT DRUGS *in vitro* ON THE ENZYMES INVOLVED IN PINEAL GLAND INDOLE METABOLISM

INTRODUCTION

MATERIALS

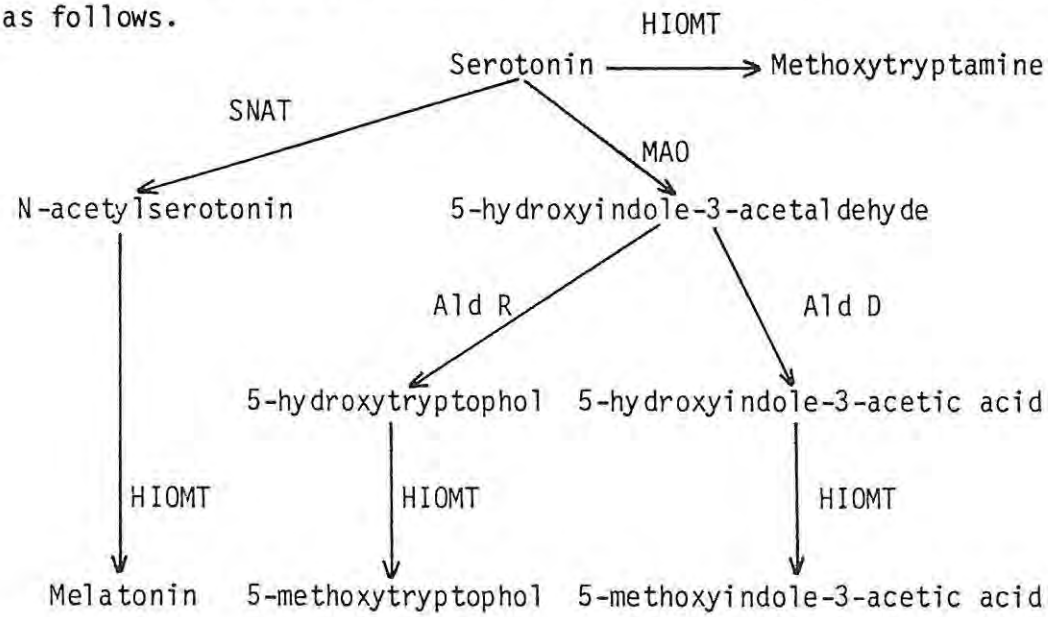
METHODS
Assay for Acetyl Co A:Arylamine N-acetyltransferase
Assay for Hydroxyindole-O-methyltransferase
Assay for Monoamine Oxidase
Assay for Aldehyde Dehydrogenase
Assay for Aldehyde Reductase
Evaluation of Data

RESULTS AND DISCUSSION

REFERENCES

## INTRODUCTION

The metabolic fate of serotonin is complex and may be summarized as follows.




SNAT = N-acetyltransferase, MAO = Monoamine oxidase, Ald D and Ald R = Aldehyde dehydrogenase and reductase respectively and HIOMT = Hydroxyindole-O-methyltransferase.

Although it has been suggested that a deacetylase [1] and O-acetyltransferase [2] also participate in indole metabolism, the vast majority of metabolised serotonin proceeds along the paths outlined above.

There is evidence implicating the involvement of the pineal gland in seizure states [3,4]. A number of reports attribute the anti-seizure activity of the pineal gland to the hormone melatonin [4-7]. In the light of these findings it is interesting that no reports have studied the effect of anticonvulsant drugs on the pineal gland and more specifically on the enzymes responsible for the synthesis of melatonin. It is not possible, however, to consider any part of this scheme in isolation as any change in one pathway is bound to affect the others to a certain extent.

An attempt was made to ascertain to what extent anticonvulsant drugs affected these enzymes in vitro. This information would give a valuable insight into what changes to expect in the in vivo situation, although not all in vitro effects would necessarily become manifest in vivo, SINCE IN VITRO DOSE MAY NOT REFLECT IN VIVO CONCENTRATIONS.

## MATERIALS

See Appendix A.

## METHODS

Assay for Acetyl Co A:Arylamine N-acetyltransferase. Rats of both sexes (200-300g) were treated with isoprenaline HCl (25mg/kg ip) three hours before sacrifice in order to induce levels of N-acetyltransferase (SNAT). Pineals were removed with minimum delay and homogenised in 0.05M phosphate buffer pH6.5 containing one substrate in constant concentration ($3x10^{-3}$M in the case of tryptamine HCl and $1x10^{-4}$M in the case of acetyl coenzyme A) while the concentration of the second substrate varied ($2x10^{-6}$-$3x10^{-5}$M in the case of acetyl coenzyme A and $2x10^{-4}$-$3x10^{-3}$M in the case of tryptamine HCl). These homogenates were incubated at 20°C for an hour in the presence and absence of the drugs used ($1x10^{-6}$-$1x10^{-3}$M). Reaction remained linear under these conditions. After incubation reaction was terminated by addition of an equal volume of 0.2M borate buffer and $^{14}$C-N-acetyl-

tryptamine was extracted into 3ml toluene:isoamyl alcohol (97:3) mixture by vortexing for a minute. After centrifugation 2ml of the organic phase was aspirated off into 6ml scintillation cocktail and radioactivity quantitated. External standards channel ratio was used to calculate counting efficiency which exceeded 90% in all cases.

Assay for Hydroxyindole-O-methyltransferase. Bovine pineal glands were collected shortly after sacrifice, homogenised in 0.15% KCl (1ml per 100mg tissue), centrifuged at 4°C for 30 minutes in order to remove cell debris and the resultant supernatant was lyophilised and stored frozen. This lyophilisate served as a source of hydroxyindole-O-methyltransferase (HIOMT) and prior to assay was dissolved in 0.05M phosphate buffer pH7.9 (1mg in 100ul). The assay relied on transfer of a $^{14}C$-methyl group from S-adenosylmethionine to N-acetylserotonin, the $^{14}C$-melatonin formed being extracted by vortexing for a minute with 3ml toluene:isoamyl alcohol (97:3) mixture. After centrifugation, 2ml of the organic phase was transfered to 6ml scintillation cocktail and quantitated. Incubation was performed at 42°C for an hour, during which time reaction remained linear. Enzyme incubates contained one substrate in constant concentration ($4x10^{-4}M$) while the concentration of the second substrate varied ($2x10^{-5}$-$3x10^{-4}M$) reaction being monitored in the presence and absence of the various drugs ($1x10^{-6}$-$1x10^{-3}M$). Counting efficiency was determined using the external standards channel ratio and exceeded 90% in all cases.

Assay for Monoamine Oxidase. Commercial monoamine oxidase (MAO) was obtained as a suspension in 2M ammonium sulphate (1unit/1.5ml). A spectrophotometric assay was employed which monitored formation of benzaldehyde from benzylamine at 250nm. Incubation was performed at 25°C for 30 minutes in 0.1M phosphate buffer pH7.4, the reaction remaining linear under these conditions. A benzylamine concentration of $5x10^{-4}M$ was used and activity monitored in the presence and absence of the anticonvulsant drugs ($1x10^{-7}$-$1x10^{-4}M$). Where the drug absorbed significantly at the wavelength used the maximum concentration tested was dictated by the interfering absorption. Reaction was initiated by addition of 40ul MAO suspension to 3ml buffer/benzylamine mixture preheated to 25°C. Absorption was

determined after 30 minutes without terminating the reaction

Assay for Aldehyde Dehydrogenase. This assay was conducted spectrophotometrically by monitoring formation of NADH at 340nm. Commercial aldehyde dehydrogenase (Ald D) lyophilisate was dissolved in distilled water (1mg/10ml) and 100ul was added to 2.8ml buffer/NAD mixture which was preincubated for 2 minutes prior to start of the assay. A 0.1M tris buffer pH9 containing 0.05M potassium dihydrogen phosphate, dithiothreitol (0.15mg/ml) and EDTA (0.4mg/ml) was employed and reaction was initiated by addition of 50ul acetaldehyde solution to give a final concentration of $2x10^{-4}$M. The final incubate concentration of NAD was $2x10^{-3}$M. Incubation was performed at 25°C for 30 minutes, reaction remaining linear and absorption was determined without terminating the reaction. Reaction was monitored in the presence and absence of the various drugs ($1x10^{-6}$-$1x10^{-4}$M).

Assay for Aldehyde Reductase. Commercial lyophilised aldehyde reductase (Ald R) was dissolved in distilled water (0.02mg/ml) while the substrates were dissolved in 0.1M phosphate buffer pH7.2. Reaction was monitored spectrophotometrically by observing decrease in absorption at 340nm as NADH was converted to NAD. Incubates contained NADH ($1x10^{-5}$M) and acetaldehyde ($5x10^{-2}$) and reaction was monitored in the presence and absence of the various drugs ($1x10^{-6}$-$1x10^{-3}$M). Reaction was initiated by the addition of 50ul Ald R solution to 2.95ml buffer/substrate mixture and was incubated at 25°C for 5 minutes, reaction remaining linear under these conditions.

Evaluation of Data. Kinetic constants were determined using computer assisted non-linear regression analysis. As the MAO, Ald D and Ald R assays were not sensitive enough and the enzymes were not of pineal origin, data was used to calculate gross changes in activity rather than kinetic effects. In all cases points represent the mean of duplicates.

## RESULTS AND DISCUSSION

None of the drugs tested affected HIOMT and only sulthiame (STH) had an effect on SNAT (figure 1 and 2).

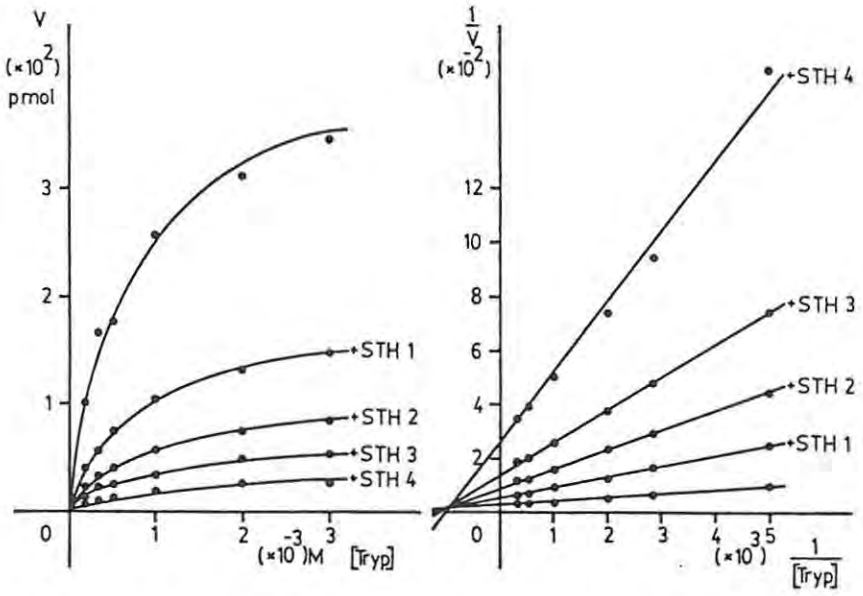



Figure 1: Effect of sulthiame (STH) on SNAT activity as concentration of tryptamine (Tryp) varied. Double reciprocal plot shown to indicate the nature of the interaction (STH 1 = 2ug/ml, STH 2 = 5ug/ml, STH 3 = 10ug/ml and STH 4 = 20ug/ml).

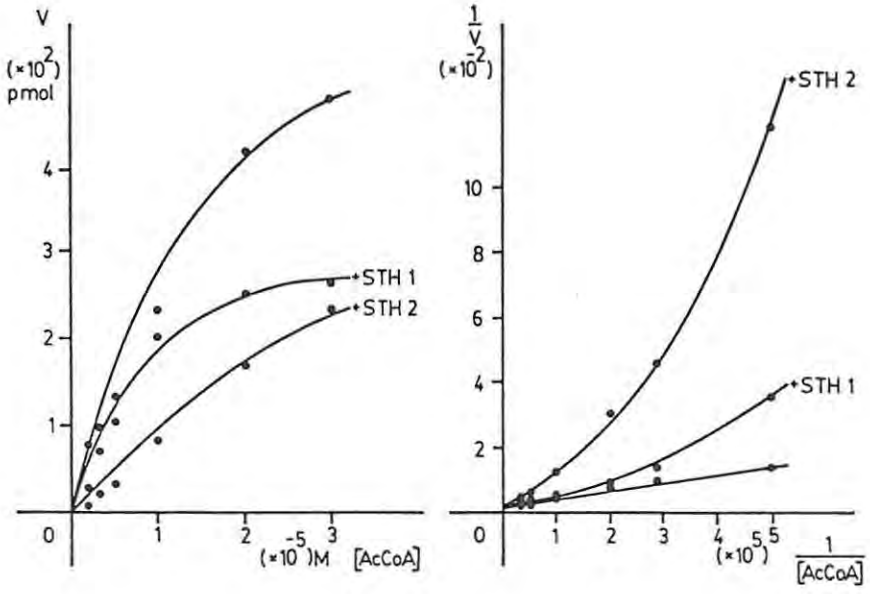



Figure 2: Effect of sulthiame (STH) on SNAT activity as acetyl coenzyme A (AcCoA) concentration varied. Double reciprocal plots shown to indicate the nature of the interaction. (STH 1 = 2ug/ml, STH 2 = 5ug/ml, STH 3 = 10ug/ml and STH 4 = 20 ug/ml).

This effect was mixed non-competitive inhibition with respect to tryptamine and the rate expression can be represented as follows [8].

$$\frac{1}{V} = \frac{1}{V_S}\left(1 + \frac{STH}{Kii}\right) + \frac{Kia}{V_S}\left(1 + \frac{STH}{Kis}\right)\frac{1}{[tryptamine]} \quad ....(1)$$

where V is velocity at a certain tryptamine concentration ([tryptamine]), $V_S$ is saturation velocity, Kii is dissociation constant for STH from E.tryptamine.STH complex, Kia is dissociation constant for E.tryptamine complex and Kis is the dissociation constant for E.STH complex.

These dissociation constants are related to Kiii (dissociation constant for tryptamine from E.tryptamine.STH complex) in the following way [8].

$$Kiii = \frac{Kia.Kii}{Kis} \quad ....(2)$$

As the double reciprocal plots are linear it may be assumed that the rapid equilibrium rather than the steady-state condition is met for the reaction.

Using the Dixon plot [9] in which reciprocal of velocity is plotted against inhibitor concentration at different fixed levels of tryptamine it is possible to obtain values for Kii and Kis in that the coordinates of the points of intersection of the lines are $(-Kis, 1/V_S[1-Kis/Kii])$. From these values it is possible to calculate Kia using equation 1 and hence Kiii using equation 2 (Table 1).

Table 1: Dissociation constants for the various complexes found between SNAT, tryptamine (Tryp) and sulthiame (STH) calculated as described in the text.

| DISSOCIATION CONSTANTS (uM) | |
|---|---|
| Kia | 547.4±17.6 |
| Kis | 4.4±0.1 |
| Kii | 1.6±0.4 |
| Kiii | 200.9±6.5 |

When the UV spectra of STH, tryptamine and acetyl coenzyme A were obtained in 0.05M phosphate buffer pH6.5 it was interesting to note that there appeared to be an interaction between STH and tryptamine (Figure 3).

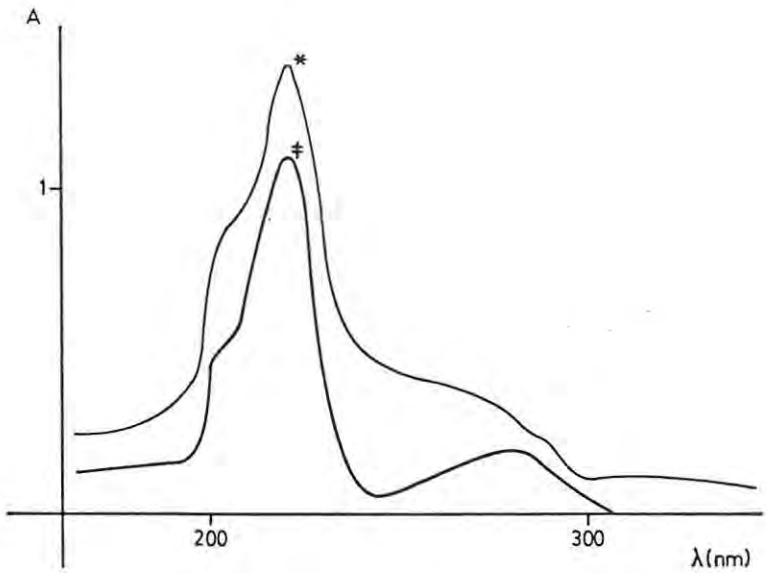



Figure 3: UV spectra obtained from sulthiame (STH) and tryptamine in 0.05M phosphate buffer pH6.5.(* = hypothetical addition of individual traces and ≠ = actual spectrum from the two together).

This interaction may also occur when tryptamine has bound to the enzyme and, although it is likely to interfere with catalysis, the major inhibitory effect of STH on SNAT can be assumed to be as a result of binding to the enzyme, such binding inhibiting the catalytic process in some way.

As the effect on catalysis is dependent on STH concentration but independent of tryptamine concentration it is likely that inhibition occurs at some stage after tryptamine has bound to the enzyme.

From the dissociation constants (Table 2) it is apparent that SNAT has a far greater affinity for STH than for tryptamine and also STH tends to bind better to the enzyme.tryptamine complex

rather than to the enzyme alone. This favours the proposal that inhibition occurs at some stage after tryptamine binding to the enzyme.

Considering that the inhibition is largely dependent on concentration of acetyl coenzyme A it is likely that STH exerts a competitive-type interaction with acetyl coenzyme A for catalytic sites. These inhibition patterns are compatible with the mechanism being random Bi-Bi although studies indicate that the catalytic mechanism is ordered Bi-Bi with tryptamine the first substrate to bind to the enzyme (see Chapter 3).

This contradiction may be resolved as follows- the increasing non-linearity of the double reciprocal plot with higher STH concentration (with acetyl coenzyme A as the varied substrate) suggests that presence of the inhibitor in the catalytic process is introducing complex second degree terms to the rate equation. As a result the plots may appear competitive whereas the interaction may not be. The interference exerted by STH probably results from the drug binding to the enzyme, such binding interfering with normal acceptance of acetyl coenzyme A at the catalytic site, affecting either acetyl transfer or product removal after catalysis.

To a certain extent the dissociation constants favour a competitive-type interaction with acetyl coenzyme A in the framework of an ordered Bi-Bi catalytic mechanism. In the envisaged ordered Bi-Bi mechanism tryptamine binding would cause a change in the catalytic site which could then accept acetyl coenzyme A, affinity of SNAT for STH follows a similar pattern in that affinity for the enzyme.tryptamine complex is greater than that for the enzyme alone. Whether this action exerted by STH occurs at the catalytic site itself or some other part of the enzyme molecule is unknown.

Inhibition of SNAT by STH occurs in the normal therapeutic range of the drug (3-11ug/ml) [10] and considering that SNAT is the rate-limiting step in production of melatonin [11-15] it might be expected that a significant reduction in melatonin production be observed in subjects taking STH. Considering the many effects postulated for melatonin (see Chapter 1) such an effect would be

bound to cause some physiological changes. Whether this occurs *in vivo* and what significance such an effect would have on anti-seizure therapy is unknown at this stage.

Of the drugs tested only clonazepam, diazepam, diphenylhydantoin, phenobarbitone and sulthiame exerted an inhibitory effect on monoamine oxidase (Table 2).

Table 2: Effect of anticonvulsant drugs on monoamine oxidase (MAO) activity. (100% activity = no inhibition).

| DRUG AND CONCENTRATION (uM) | | MAO ACTIVITY (%) |
|---|---|---|
| No drug | | 100 |
| Clonazepam | 50 | 82 |
| Diazepam | 50 | 89 |
| Diphenylhydantoin | 50 | 81 |
| Phenobarbitone | 50 | 77 |
| Sulthiame | 0.1 | 93 |

Due to interference at the wavelength employed it was not possible to get accurate results for higher STH concentrations or to determine whether the other drugs might inhibit MAO at higher concentrations. On the basis of these results, however, it appears that STH is the most powerful and diazepam the weakest inhibitor. This verifies previous reports which showed diazepam and diphenylhydantoin to be MAO inhibitors [16,17].

Ald D was weakly inhibited by diazepam, diphenylhydantoin, pheneturide and phenobarbitone while primidone, sulthiame and valproate all exerted a noticeable inhibition on the enzyme (Table 3).

Only acetazolamide exerted a powerful inhibitory effect on the Ald R used (Table 4) while carbamazepine, diazepam, diphenylhydantoin and phenobarbitone were weak inhibitors and the other drugs used showed no inhibitory action.

Table 3: Effect of anticonvulsant drugs on aldehyde dehydrogenase (Ald D) activity. (100% activity = no inhibition).

| DRUG AND CONCENTRATION (mM) | | Ald D ACTIVITY (%) |
|---|---|---|
| No drug | | 100 |
| Diazepam | 0.1 | 93 |
| Diphenylhydantoin | 0.1 | 93 |
| Pheneturide | 0.1 | 94 |
| Phenobarbitone | 0.1 | 95 |
| Primidone | 0.1 | 87 |
| Sulthiame | 0.1 | 79 |
| Valproate | 0.1 | 87 |

Table 4: Effect of anticonvulsant drugs on aldehyde reductase (Ald R) activity. (100% activity = no inhibition).

| DRUG AND CONCENTRATION (mM) | | Ald R ACTIVITY (%) |
|---|---|---|
| No drug | | 100 |
| Acetazolamide | 1.0 | 35 |
| Carbamazepine | 1.0 | 92 |
| Diazepam | 0.1 | 95 |
| Diphenylhydantoin | 0.1 | 97 |
| Phenobarbitone | 1.0 | 97 |

These results must be treated with caution, however, as the Ald R used was NADH-dependent while that in the pineal may be NADPH-dependent. Existing reports have shown carbamazepine, clonazepam, diazepam, diphenylhydantoin, ethosuximide, phenobarbitone, primidone and valproate to be inhibitors of NADPH-dependent Ald R [18-23] and this suggests that the two types of enzyme are dissimilar. The fact that different Ald R isozymes showed differences in sensitivity to valproate [23] supports this proposal.

Whether the corresponding pineal enzymes would be inhibited to the same extent or not bears further investigation and these results should be treated merely as a guide which may assist in explaination of observed effects *in vivo*.

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CHAPTER 6

# EFFECT OF ANTICONVULSANT DRUGS *in vivo* ON RAT PINEAL ACETYL CO A: ARYLAMINE N-ACETYLTRANSFERASE AND HYDROXYINDOLE-O-METHYL-TRANSFERASE LEVELS

INTRODUCTION

MATERIALS

METHODS
Dosing
Preparation of Enzyme Homogenates
Assay for SNAT
Assay for HIOMT
Extraction and Quantitation
Analysis of Data

RESULTS AND DISCUSSION

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## INTRODUCTION

Considering the evidence which implicates involvement of the pineal gland in seizure states [1,2] and especially those which attribute the anti-seizure activity to melatonin [2-5] there is little evidence of any significant investigation into the effect of anticonvulsant drugs on the pineal gland.

The present study was aimed at investigating the effect of anticonvulsant drug administration on levels of the two enyymes involved in melatonin synthesis, acetyl Co A:arylamine N-acetyl-transferase and hydroxyindole-O-methyltransferase. Theoretically any drug which could cause an increase in the activity of these enzymes, and therefore increase pineal melatonin output, would have a potentially ameliorative effect on seizures.

In the light of this hypothesis it is interesting that a report found phenobarbitone to have no effect on acetyl Co A:arylamine

N-acetyltransferase while diazepam reduced the nocturnal activity [6]. As melatonin production appears to be dependent on N-acetyltransferase activity [7-11] this diazepam mediated effect would reduce melatonin levels and presumably enhance seizure activity if melatonin is assumed to act as an endogenous anticonvulsant agent.

## MATERIALS

See Appendix A.

## METHODS

Dosing. Male rats (200-250g) were housed under constant lighting conditions (05h00-17h00 light) with access to unlimited food and water. Rats were not used until they had been exposed to the light cycle for at least two weeks. In all cases rats were dosed at 07h00 and 16h45 for three days, either with drugs or normal saline (in the case of controls) prior to sacrifice.

Drugs were injected intraperitoneally on a mg/kg basis at the doses previously determined (see Chapter 2) and where they were insoluble in water were dissolved or suspended in propylene glycol. Both beclamide and pheneturide resisted solution and suspension and were consequently administered orally as powder in a syrup simplex matrix.

Preparation of Enzyme Homogenates. Rats were sacrificed at 11h00, 22h30, 23h00 and 23h30 and pineal glands were removed with a minimum of delay and homogenised on ice in distilled water (120ul/pineal gland). When rats were sacrificed at night the entire procedure was conducted in red light (15W photographic safe light). These homogenates were divided into two equal 50ul aliquots for assay of acetyl Co A:arylamine N-acetyltransferase (SNAT) and hydroxyindole-O-methyltransferase (HIOMT) activity respectively.

Assay for SNAT. The technique adopted relied on acetylation of tryptamine HCl using $^{14}$C-acetyl coenzyme A as an acetyl donor [12]. As the quantity of enzyme present in the pineal gland increased dramatically at night different incubate volumes were

required in order to ensure optimum conditions.

With homogenates resulting from sacrifice at 1100 the final incubate volume was 75ul (50ul pineal homogenate and 25ul buffer/substrate mixture) while those from rats sacrificed during the dark phase employed a final incubate volume of 135ul (50ul pineal homogenate and 85ul buffer/substrate mixture). The final reaction mixtures were such that enzyme was dissolved in 0.05M phosphate buffer pH6.5 containing tryptamine HCl ($3x10^{-3}M$) and acetyl coenzyme A ($1.8x10^{-4}M$). These were incubated at 20°C for an hour and reaction was terminated by addition of an equal volume of 0.2M borate buffer pH10.

Assay for HIOMT. Assay relied on the formation of $^{14}C$-melatonin from N-acetylserotonin using S-adenosyl [$^{14}C$-methyl] methionine as a methyl donor [13]. In all cases the final incubate volume was 90ul (50ul pineal homogenate and 40ul buffer/substrate mixture). Reaction mixtures contained enzyme, N-acetylserotonin ($7x10^{-4}M$) and S-adenosylmethionine ($4x10^{-4}M$) in 0.05M phosphate buffer pH 7.9 and were incubated at 42°C for an hour. Reaction was terminated by the addition of an equal volume of 0.2M borate buffer pH10.

Extraction and Quantitation. The radioactive N-acetyltryptamine (resulting from SNAT activity) or melatonin (resulting from HIOMT activity) formed was extracted into 3ml toluene:isoamyl alcohol (97:3) mixture by vortexing for a minute. After centrifugation a 2ml aliquot of the organic phase was aspirated into 6ml scintillation cocktail and quantitated. Counting efficiency was determined using the external standards channel ratio and exceeded 90% in all cases.

Analysis of Data. All results were obtained in triplicate and analysed statistically in order to obtain mean values with the standard error of the mean (SEM). Probabilities were calculated using the Student-t distribution.

## RESULTS AND DISCUSSION

Although few of the results are statistically significant in almost all cases HIOMT levels were lower than controls (Table 1).

Table 1: Effect of *in vivo* administration of anticonvulsant drugs on pineal HIOMT levels at the times indicated. Results given as mean with standard error of the mean (SEM).

| DRUG AND DOSE (mg/kg) | | HIOMT ACTIVITY (pmol/pineal/hour) 11h00 | 22h30 | 23h00 | 23h30 |
|---|---|---|---|---|---|
| No drug | | 501±22 | 382±44 | 436±96 | 460±57 |
| Acetazolamide | 100 | 346±63 | 245±47 | 404±32 | 401±67 |
| Beclamide | 250 | 372±67 | 359±76 | 388±77 | 475±44 |
| Carbamazepine | 25 | 432±40 | 253±03* | 362±13 | 323±49 |
| Clonazepam | 0.1 | 334±31** | 309±26 | 270±65 | 291±77 |
| Diazepam | 5 | 313±53* | 354±31 | 222±25 | 599±168 |
| Diphenylhydantoin | 100 | 293±55* | 344±38 | 319±50 | 379±72 |
| Ethosuximide | 25 | 299±71 | 298±63 | 313±47 | 445±134 |
| Pheneturide | 150 | 94±11** | 352±51 | 327±59 | 495±23 |
| Phenobarbitone | 12.5 | 285±84 | 311±43 | 290±40 | 412±75 |
| Primidone | 142 | 327±33** | 331±38 | 373±54 | 334±53 |
| Sulthiame | 50 | 308±85 | 335±12 | 475±47 | 491±44 |
| Valproate | 64 | 345±99 | 344±33 | 303±76 | 531±40 |

* P=0.05 and ** P=0.025

Table 2: Effect of *in vivo* administration of anticonvulsant drugs on pineal SNAT levels at the times indicated.Results given as mean with standard error of the mean (SEM).

| DRUG AND DOSE (mg/kg) | | SNAT ACTIVITY (pmol/pineal/hour) 11h00 | 22h30 | 23h00 | 23h30 |
|---|---|---|---|---|---|
| No drug | | 178±08 | 1854±211 | 2795±426 | 2325±127 |
| Acetazolamide | 100 | 108±01*** | 1945±278 | 2667±237 | 2739±402 |
| Beclamide | 250 | 169±09 | 1244±054 | 1949±171 | 2195±019 |
| Carbamazepine | 25 | 36±08**** | 1640±256 | 2213±157 | 2612±023 |
| Clonazepam | 0.1 | 47±01***** | 1440±293 | 2003±701 | 2044±207 |
| Diazepam | 5 | 79±01**** | 1078±160* | 1647±482 | 2260±397 |
| Diphenylhydantoin | 100 | 89±02**** | 1737±261 | 1976±402 | 1736±559 |
| Ethosuximide | 25 | 84±07*** | 2119±333 | 2106±129 | 2709±386 |
| Pheneturide | 150 | 196±13 | 2145±470 | 2121±306 | 3354±165 |
| Phenobarbitone | 12.5 | 75±01**** | 460±117** | 671±228** | 2299±204 |
| Primidone | 142 | 129±04** | 1465±296 | 1949±228 | 2023±399 |
| Sulthiame | 50 | 213±07* | 1609±328 | 2501±372 | 2698±400 |
| Valproate | 64 | 85±09*** | 2439±223 | 1970±422 | 2488±345 |

* P=0.05. ** P=0.025, *** P=0.01, **** P=0.005 and ***** P=0.0025

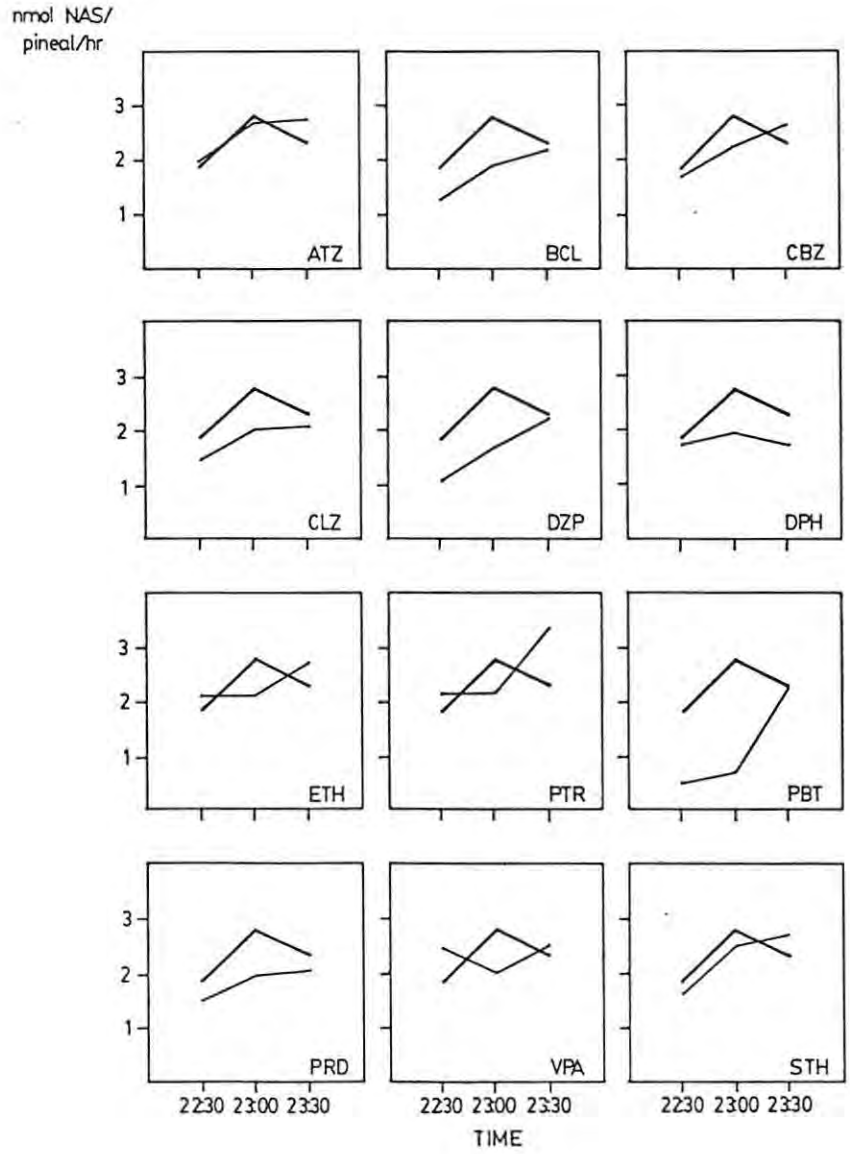


Figure 1: Graphical representation of the nocturnal increase in SNAT levels after administration of the various anticonvulsant drugs (thin lines) compared to control levels (thick lines).

The effects on SNAT were more variable than those on HIOMT and the overall trend, if a trend can be said to exist, was towards lower enzyme levels (Table 2). Both these observations imply that anticonvulsant drug treatment would lead to a reduced pineal melatonin output although this may not necessarily become manifest *in vivo*.

Regardless of the actual SNAT levels it is interesting to note that in all but two cases (diphenylhydantoin and valproate) the nocturnal peak in activity appears to be shifted towards the following light phase (Figure 1). The most significant shift was caused by phenobarbitone, an effect which is apparently abolished by high doses [6]. That increased diazepam dose caused greater reduction in SNAT activity [6] suggests that these drugs are acting, at least in part, on different mechanisms to modulate enzyme levels.

Reduction in SNAT activity and shift in rhythm caused by primidone may be the result of its metabolite phenobarbitone rather than primidone as such. That the observed effects were less pronounced than those exerted by phenobarbitone can be rationalised when one considers that the plasma concentration of phenobarbitone resulting from metabolism of primidone at the dose given would be about half that resulting from the administration of phenobarbitone at the dose used (see Chapter 2). Although primidone may be able to affect SNAT levels *per se* at least some of the effect must be considered to result from phenobarbitone action.

The shift in rhythm may be modulated to a certain extent by an effect on benzodiazepine receptors which may be present in the pineal, although they are most likely to be centrally situated. That seizures increase benzodiazepine receptor binding [14] may indicate an involvement in modulation of seizure states. It is also tempting to suggest an involvement of benzodiazepine receptors in modulation of pineal function and *vice versa* on the basis of evidence which claims that pineal indolic products can inhibit benzodiazepine receptor binding [15,16] and that pineal indoles may regulate pineal function [17,18,19]. If this were in fact the case then it could explain, to a large extent, the effects on SNAT rhythm exerted by clonazepam, diazepam,

phenobarbitone and primidone. The effect of phenobarbitone and thus of primidone may result from interaction with the barbiturate-picrotoxin component of the benzodiazepine-GABA receptor complex [20]. The effects may also involve interaction with phenobarbitone binding sites [21] and possibly with both classes of receptor.

This evidence would suggest that the pineal gland, by alteration of benzodiazepine binding, may modulate seizure states although further extensive studies would be required to verify this suggestion.

Anticonvulsant drugs may exert some of their therapeutic action by an effect on this system although a decrease in melatonin output would tend to be epileptogenic [2-5]. This is complicated by the fact that decreased melatonin levels would reduce inhibition of benzodiazepine receptor binding [15,16] which could enhance anticonvulsant effects [22].

Evidence that diphenylhydantoin may bind to benzodiazepine receptors [23] and the observation that it had no effect on SNAT rhythm while decreasing SNAT levels suggests that these receptors are not exclusively responsible for changes in SNAT rhythm and some other mechanism must be considered to exist.

Overall this evidence strongly supports some interaction between the pineal gland and benzodiazepine receptors in modulation of anticonvulsant drug effects although the entire mechanism is undoubtably multi-factorial and highly complex.

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CHAPTER 7

# DEVELOPMENT OF A SIMPLE SENSITIVE PINEAL GLAND CULTURE TECHNIQUE

INTRODUCTION

MATERIALS

METHODS
Treatment of Rats
Organ Culture using $^{14}C$-Serotonin
Organ Culture using $^{3}H$-Serotonin
TLC Separation and Quantitation
Autoradiography
Purity of Serotonin Metabolites

RESULTS AND DISCUSSION

REFERENCES

## INTRODUCTION

It is apparent that anticonvulsant drugs are capable of affecting the enzymes responsible for pineal gland indole metabolism both *in vivo* and *in vitro*. On the basis of this evidence it is not possible to state that the effects would become manifest in the intact animal although the potential for such effects exists.

Ideally a technique is required which will enable sensitive determination of normal function while allowing for drug manipulations. The fact that the rat pineal gland is small and readily accessible makes intact organ culture suitable for such purposes. Numerous organ culture techniques have been developed for the pineal gland and used with comparative success [1-5].

As none of these methods totally satisfied the requirements of this study an attempt was made to develop a new method for pineal organ culture which would allow simple sensitive determinations of all serotonin metabolites formed.

## MATERIALS

See Appendix A.

## METHODS

Treatment of Rats. Both male and female rats (150-200g) kept under constant light conditions (05h00-17h00 light) were allowed at least two weeks in which to become acclimatized to the light cycle before being used. Rats were sacrificed at 15h00 and pineal glands removed with minimum delay using sterile forceps and placed into culture medium.

Organ Culture using $^{14}$C-Serotonin. Pineal glands were incubated in pairs in 95ul culture medium (B.G.J.b. medium, Fitton-Jackson modification) containing 5-hydroxy [2-$^{14}$C] tryptamine creatinine sulphate ($1x10^{-4}$M) and in certain cases noradrenaline (NA) or gamma-aminobutyric acid (GABA) ($1x10^{-7}$-$1x10^{-4}$M). Pineal glands were placed into culture medium shortly after removal, the tubes were gassed with 95% $O_2$/5% $CO_2$ prior to sealing and were incubated at 37°C for 24 hours. After incubation pineal glands were removed and the medium evaporated to dryness under nitrogen at 40°C. Tubes were washed down with 95% ethanol and the residue taken up in 100ul 95% ethanol containing 5ug of each of the serotonin metabolites. These ethanolic solutions were then applied to thin layer chromatographic (TLC) plates, dried and metabolites separated by two dimensional development.

Control cultures were treated in the same way except pineal glands were omitted from the culture medium.

Organ Culture using $^{3}$H-Serotonin. Pineal glands were incubated individually in 50ul culture medium (B.G.J.b. medium, Fitton-Jackson modification) containing 5-hydroxy [G-$^{3}$H] tryptamine creatinine sulphate ($1.6x10^{-5}$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°C for 24 hours. After incubation pineal glands were removed and 5ul aliquots of the culture medium spotted onto pretreated TLC plates. The pretreating involved applying 5ul 95% ethanol containing 2.5ug of

each of the serotonin metabolites. These spots were dried prior to application of the 5ul culture medium and served to localise the various spots after two dimensional development.

Control tubes were treated in the same way except that no pineal glands were present and served to calculate background radioactivity for the different metabolites.

TLC Separation and Quantitation. 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°C) unless otherwise stated. After development in ethyl acetate the plates were dried and sprayed with Van Urk's reagent (1g 4-dimethylaminobenzaldehyde in 50ml of 25% hydrochloric acid and 50ml ethanol) followed by heating at 95°C for a short while. The coloured spots which resulted were scraped into scintillation cocktail and radioactivity present was quantitated. Counting efficiency was determined using the external standards channel ratio and exceeded 90% in all cases where $^{14}C$-metabolites were evaluated and 20% in all cases where $^{3}H$-metabolites were evaluated.

Plates resulting from control incubations were treated in a similar fashion, the radioactivity present at the various spots being subtracted from pineal values to obtain a true quantity for the metabolites produced.

Autoradiography. Sheets of Kodak professional film negative (25 ASA) were attached to TLC plates developed after organ culture using $^{14}C$-serotonin. After varying lengths of exposure at 0°C these negatives were developed according to the manufacturers specifications.

Purity of Serotonin Metabolites. Some 5ul aliquots of medium from cultures using $^{3}H$-serotonin were treated in the same way as other samples except that after development these plates were not sprayed with Van Urk's reagent. The areas of the plates corresponding to N-acetylserotonin, 5-hydroxyindole-3-acetic acid and 5-hydroxytryptophol were scraped off and eluted into 95% ethanol and evaporated to dryness under nitrogen at 40°C. The

sides of the tubes were washed down with successive quantities of 95% ethanol and the residue was taken up in 95% ethanol (20ul). These residues were divided in half and spotted onto TLC plates, one plate being treated according to the scheme outlined above. The other spot was scraped into scintillation cocktail and served as a control.

The various methoxyindoles were synthesised using hydroxyindole-O-methyltransferase (HIOMT). Bovine pineal lyophilisate prepared as previously outlined (see Chapter 4) was dissolved in 0.05M phosphate buffer pH7.9 (1mg lyophilisate/100ul) containing N-acetylserotonin, 5-hydroxyindole-3-acetic acid or 5-hydroxy-tryptophol ($3x10^{-4}M$) and S-adenosyl [$^{14}C$-methyl] methionine ($3x10^{-4}M$). These mixtures were incubated for an hour at 42°C and extracted into toluene:isoamyl alcohol (97:3) mixture (3ml) by vortexing for a minute. After centrifugation the organic phase was evaporated to dryness under nitrogen at 40°C, tubes were washed down with 95% ethanol and the residue taken up in 95% ethanol (20ul). This residue was divided into two equal aliquots, both being spotted onto TLC plates and treated as above.

Enzyme incubates were performed in the absence of a hydroxy substrate and were treated as above. These values were used to ascertain the level of non-indolic radioactivity extracted.

## RESULTS AND DISCUSSION

A total of 26 solvent systems were tried before choosing the systems used (see Appendix B). The resulting separation using chloroform:methanol (9:1) and ethyl acetate at right angles was excellent and reproducible (Figure 1). Changes in ambient temperature and humidity led to a change in position of the spots although separation always remained good and the position of each spot in relation to the others remained the same. The colours produced after spraying could be used for partial identification (Figure 2). Although this technique also allows separation of 6-hydroxymelatonin (Figure 1 and 2) it does not appear to be a normal pineal metabolite. Possibly the only major pineal metabolites of melatonin are 5-methoxytryptamine and possibly 6-methoxyharmalan (results not shown).

A previously reported technique used ascorbic acid to protect the indoles sensitive to oxidation [5]. The protective capacity of ascorbic acid can be disputed considering evidence which suggests that it can catalyse aromatic hydroxylation [6] and consequently was not used in this study. Solutions of the various hydroxy- and methoxy-indoles in 95% ethanol were stored at -20°C and no decomposition was detectable after 9 months indicating that inclusion of an antioxidant was unnecessary.

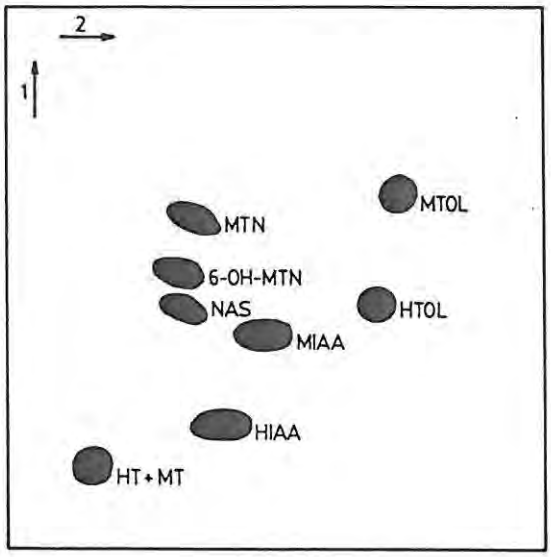


Figure 1: Typical arrangement of spots of the various serotonin metabolites after two dimensional development as outlined in the text. Development in chloroform:methanol (9:1) in direction 1 followed by development at right angles (direction 2) in ethyl acetate. (HT = serotonin, MT = 5-methoxytryptamine, NAS = N-acetylserotonin, 6-OH-MTN = 6-hydroxymelatonin, MTN = melatonin, HIAA = 5-hydroxyindole-3-acetic acid, MIAA = 5-methoxyindole-3-acetic acid, HTOL = 5-hydroxytryptophol and MTOL = 5-methoxy-tryptophol).


Figure 2: Colours produced after spraying spots of the various indoles with Van Urk's reagent, drying at 95°C and leaving for 24 hours. Colours vary with age and caution must be exercised when used for identification purposes. Spots from left to right-1 = serotonin, 2 = 5-methoxytryptamine, 3 = N-acetylserotonin, 4 = melatonin, 5 = 6-hydroxymelatonin, 6 = 5-hydroxyindole-3-acetic acid, 7 = 5-methoxyindole-3-acetic acid, 8 = 5-hydroxytryptophol and 9 = 5-methoxytryptophol.

The possibility of decomposition resulting from exposure to heat and light was also investigated. Several identical extracts from cultures were spotted onto TLC plates and developed either in the light or dark and were dried either by heating (65°C) or under a stream of nitrogen. None of these treatments had any noticeable effect on the quantities of the various metabolites extracted. As a result drying using brief heating and development in the dark were used for convenience although other methods could be used without affecting results.

Exposed areas on the developed autoradiographs corresponded exactly with the coloured spots resulting from the spray reagent, verifying that the visualised spots actually contained the radioactive metabolites produced. The absence of unaccountable exposed areas suggested that recovery was high. Using the synthesised radioactive metabolites this was verified and recovery was found to be 93±1%.

Pineal glands removed from culture media after incubation were disrupted by ultrasonication and aliquots spotted onto TLC plates, metabolites being separated and quantitated as above. The proportions of the various indoles contained by the glands were similar to those in the media (Table 1). This indicated that determination of quantities of metabolites in the medium gave an accurate indication of both pineal production and content.

Table 1: Comparison of the amounts of various serotonin metabolites present in the pineal gland and culture medium after 24 hours incubation with $^{3}H$-serotonin. Results given as the percentage of the total radioactivity present.

| METABOLITE | MEDIUM (%) | PINEAL (%) |
|---|---|---|
| Serotonin | 30 | 29 |
| N-acetylserotonin | 3 | 4 |
| Melatonin | 4 | 5 |
| 5-hydroxyindole-3-acetic acid | 49 | 45 |
| 5-methoxyindole-3-acetic acid | 2 | 3 |
| 5-hydroxytryptophol | 10 | 11 |
| 5-methoxytryptophol | 1 | 4 |

Although the technique was not used for pharmacological studies preliminary investigation with $^{14}C$-serotonin enabled autoradiographic characterisation of the procedure which was not possible using $^{3}H$-serotonin. A total of 83 cultures were performed using $^{14}C$-serotonin with pineal glands from both male and female rats. Some of the culture media contained neurotransmitters to vary quantities of metabolites. Analysis of data from these cultures revealed an interesting relationship between hydroxy- and corresponding methoxy-indoles (Figure 3). It is striking that melatonin production was found to be directly proportional to N-acetylserotonin production, while production of the other methoxyindoles was not directly related to levels of their hydroxy counterparts. This implied that melatonin production is dependent on N-acetylserotonin production and hence on N-acetyltransferase activity verifying previous reports [7-10].

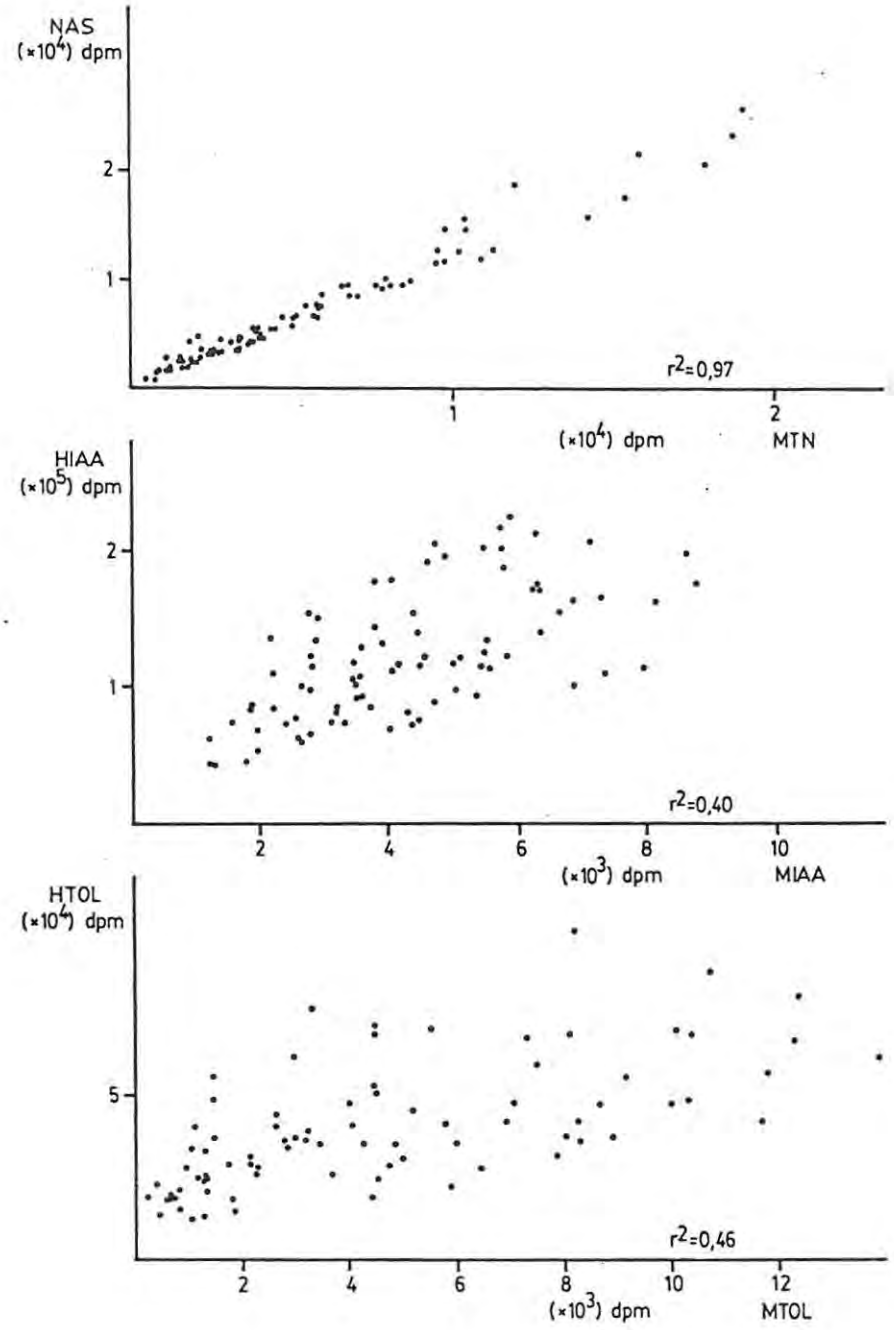


Figure 3: Relationship between hydroxy- and corresponding methoxy-indoles for rat pineal glands in organ culture. The coefficient of determination ($r^2$) is given as a measure of linearity.(See Table 2 for abbreviations).

Table 2: Amounts of methoxyindoles formed by rat pineal glands in organ culture. Values are means with standard error of the mean (SEM) for 83 cultures. (NAS = N-acetylserotonin, MTN = melatonin, HIAA = 5-hydroxyindole-3-acetic acid, MIAA = 5-methoxy-indole-3-acetic acid, HTOL = 5-hydroxytryptophol and MTOL = 5-methoxytryptophol).

| | % |
|---|---|
| [$^{14}$C]-serotonin present as methoxyindoles | 8.95±0.32 |
| NAS converted to MTN | 77.82±1.36 |
| HIAA converted to MIAA | 10.90±0.75 |
| HTOL converted to MTOL | 3.92±0.23 |

The fact that the total amount of methylation occuring in a pineal gland varied little (Table 2), despite relatively large changes in the proportions of the various methoxy products, implies that a nearly constant amount of HIOMT (or several HIOMT enzymes) is present in the pineal gland. The results may suggest the presence of one HIOMT enzyme with a greater affinity for N-acetylserotonin, methylation of the other hydroxyindoles depending on their concentration, affinity and the number of free catalytic sites on the enzyme. It is also possible that several HIOMT enzymes exist in the pineal gland although those not directly responsible for methylation of N-acetylserotonin must either be present in low concentration or have a low affinity for other hydroxyindoles since relatively small proportions are methylated when compared to N-acetylserotonin (Table 2). This is in agreement with another report which suggests the presence of multiple forms of the enzyme [11],[12].

While GABA exerted inconsistent and variable effects on pineal indole metabolism noradrenaline appeared to have a characteristic action (Figure 4). The most striking observation was the considerable increase in production of both N-acetylserotonin and melatonin by the pineal after noradrenaline treatment. The increase in melatonin closely paralleled N-acetylserotonin production which suggests that melatonin formation is dependent on N-acetyltransferase activity, again verifying previous reports [7-10].

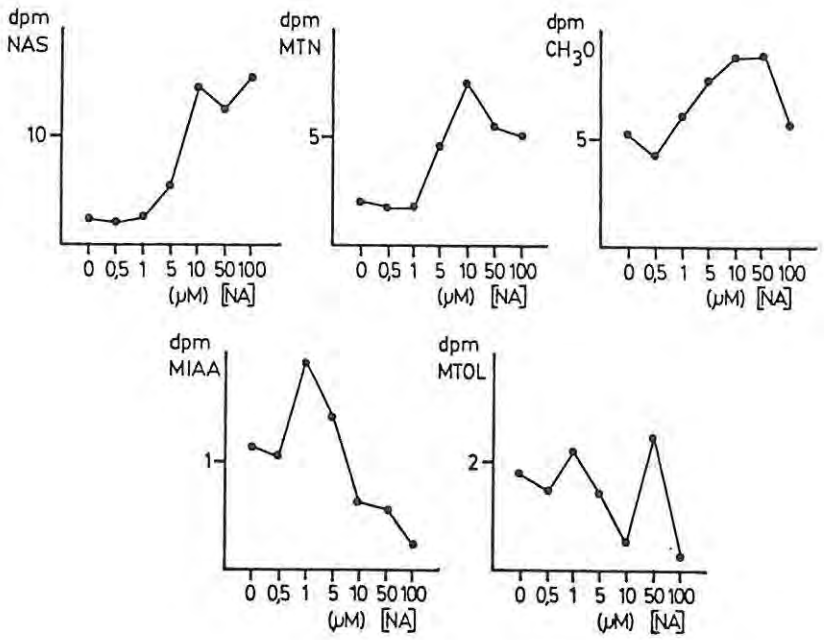


Figure 4: Effect of noradrenergic (NA) stimulation on production of pineal indoles in organ culture. (NAS = N-acetylserotonin, MTN = melatonin, $CH_3O$ = total methoxy products, MIAA = 5-methoxyindole-3-acetic acid and MTOL = 5-methoxytryptophol).

It is interesting to note that the overall production of methoxyindoles followed a very similar trend to that of N-acetylserotonin and melatonin which may suggest some degree of noradrenergic control over HIOMT levels. The increase in methoxyindole levels may, however, be due to the increased availability of substrate rather than a direct effect on HIOMT. Although 5-methoxytryptophol levels were erratic, increase in noradrenergic stimulation led, by and large, to a decrease in both 5-methoxyindole-3-acetic acid and 5-methoxytryptophol as opposed to the increase in melatonin production. This observation, coupled with the fact that HIOMT has a relatively low affinity for 5-hydroxyindole-3-acetic acid and 5-hydroxytryptophol (Table 2) supports the proposal that HIOMT is primarily involved in methylation of N-acetylserotonin, any free catalytic sites being occupied by the other hydroxyindoles dependent on their concentration and affinity.

That the overall level of methylation remains more or less constant (Table 2) and that amounts of methoxy products formed vary, to a certain extent, with variation in N-acetylserotonin levels may suggest that it is not only the absolute quantity but also the ratios between the different methoxy products that is important for mediation of pineal action.

The fact that the counts per minute (cpm) determined for the various metabolites using both isotopes of serotonin were not vastly different (Table 3) indicates that sensitivity is similar using either method. The reduction in manipulations necessary using $^{3}H$-serotonin made this method much faster and possibly more accurate and it was consequently adopted for the various pharmacological studies.

Table 3: Comparison of the values obtained using $^{14}C$- and $^{3}H$- serotonin in organ culture. (NAS = N-acetylserotonin, MTN = melatonin, HIAA = 5-hydroxyindole-3-acetic acid, MIAA = 5-methoxy-indole-3-acetic acid, HTOL = 5-hydroxytryptophol and MTOL = 5-methoxytryptophol).

| METABOLITE | CPM $^{14}C$ (total incubate) | CPM $^{3}H$ (5ul incubate) |
|---|---|---|
| NAS | 2448 | 1860 |
| MTN | 10262 | 7512 |
| HIAA | 189139 | 109985 |
| MIAA | 6415 | 3302 |
| HTOL | 44854 | 15429 |
| MTOL | 8552 | 2065 |

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CHAPTER 8

# EFFECT OF ANTICONVULSANT DRUGS ON PINEAL GLAND INDOLE METABOLISM IN ORGAN CULTURE

INTRODUCTION

MATERIALS

METHODS
Dosing and Removal of Pineal Glands
Organ Culture
TLC Separation and Quantitation
Analysis of Data

RESULTS AND DISCUSSION

REFERENCES

## INTRODUCTION

Considering the foregoing chapters it is apparent that all the anticonvulsant drugs used in this study exert effects on the pineal gland, predominantly on the melatonin synthetic pathway, although other effects also occur.

The question remained as to whether these effects were mediated locally or centrally and so the effect of the various agents *in vitro* and *in vivo* on indole metabolism was studied. This it was hoped would allow effects to be separated into local and/or central effects or indicate where interactions were possibly mediated by an action on multiple sites.

## MATERIALS

See Appendix A.

## METHODS

*Dosing and Removal of Pineal Glands*. For the *in vivo* studies male

rats (200-250g) kept under constant light conditions (04h00-16h00 light) were used. The rats were dosed with the various drugs at the dose levels previously determined (see Chapter 2) while controls were treated with normal saline. The rats were allowed at least two weeks in which to become acclimatized to the light cycle before being used. Drugs were dissolved or suspended in propylene glycol where insoluble in water and administered intraperitoneally. Both beclamide and pheneturide resisted solution and suspension and were consequently given orally as powder in a syrup simplex matrix. Rats were dosed twice daily at 06h00 and 15h00, were sacrificed at 15h00 on the third day and pineal glands removed with minimum delay using sterile forceps and placed into culture media.

Female rats (180-220g) were kept under similar conditions to male rats and were also only used after at least two weeks acclimatization . 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 oestrous smears were taken at the same time every day (11h30) and only those showing positive cornification were used that afternoon. After sacrifice at 15h00 the pineal glands were removed and treated as outlined above.

Organ Culture. Pineal glands were incubated individually in 50ul culture medium (B.G.J.b. medium, Fitton-Jackson modification) containing 5-hydroxy [G-$^{3}$H] tryptamine creatinine sulphate ($1.6 \times 10^{-5}$M). Culture media for the *in vivo* determinations contained the various drugs at a concentration in the normal therapeutic range (Table 1) while media for *in vitro* determinations contained the drugs in varying concentration ($1 \times 10^{-6}$-$1 \times 10^{-3}$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$^{o}$C for 24 hours. After incubation pineal glands were removed and 5ul aliquots of the culture medium spotted onto pretreated thin layer chromatography (TLC) plates. The pretreating involved applying 5ul of 95% ethanol containing 2.5ug 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 were treated in the same way except pineal glands were omitted and served to calculate background values.

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°C). After development in ethyl acetate the plates were dried and sprayed with Van Urk's reagent (1g 4-dimethylaminobenzaldehyde in 50ml 25% hydrochloric acid and 50ml ethanol) followed by heating at 95°C 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 subtracted from pineal values to obtain a true quantity for the metabolites produced.

Analysis of Data. Counts per minute (cpm) were converted into the required values 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. The Student-t distribution was used to calculate probabilities.

The quantities of various metabolites produced were calculated as nanograms (ng) produced/pineal gland/24 hours. In the case of monoamine oxidase (MAO) activity (5-hydroxyindole-3-acetaldehyde production), hydroxyindole-O-methyltransferase (HIOMT) activity (total methylation) and total metabolic activity, the results were calculated as the amount of serotonin oxidised, serotonin methylated and serotonin metabolised respectively. All determinations were conducted in triplicate, the three sets of results being obtained at different times.

Table 1: Concentration of anticonvulsant drugs used in culture media for *in vivo* determinations.

| DRUG | | CONCENTRATION (ug/ml) |
|---|---|---|
| Acetazolamide | (ATZ) | 10 |
| Beclamide | (BCL) | 5 |
| Carbamazepine | (CBZ) | 6 |
| Clonazepam | (CLZ) | 0.005 |
| Diazepam | (DZP) | 0.2 |
| Diphenylhydantoin | (DPH) | 10 |
| Ethosuximide | (ETH) | 40 |
| Pheneturide | (PTR) | 5 |
| Phenobarbitone | (PBT) | 10 |
| Primidone | (PRD) | 5 |
| Sulthiame | (STH) | 5 |
| Valproate | (VPA) | 50 |

## RESULTS AND DISCUSSION

The results from both *in vivo* and *in vitro* determinations are summarised in tabular form (Tables 2-5). For convenience each set of results has been divided into two sections- the first dealing with total metabolic activity and production of hydroxyindoles (Tables 2a, 2b and 4) and the second with total methylation and production of methoxyindoles (Tables 3a, 3b and 5).

In all *in vitro* determinations except high diazepam and diphenylhydantoin concentration ($10^{-3}$M) the overall indole metabolic activity of the pineal increased (Table 2a and 2b). This effect is probably due primarily to enhanced MAO activity, with increases in aldehydye dehydrogenase and aldehyde reductase output being secondary to this change as a result of increased substrate availability. Increase may be due either to increased availability of serotonin for oxidation, possibly by some effect on uptake, or may be the result of an increased quantity of MAO. In this regard it is interesting to note that acetazolamide [1], clonazepam [2], diazepam and diphenylhydantoin [3,4] and phenobarbitone [5] all increased brain serotonin levels, an effect attributable to either decreased efflux or metabolism [5]

and it is possible that in the pineal these drugs are capable of inhibiting efflux which would have the same effect as increased uptake. This statement would be correct if the drugs do not also inhibit serotonin uptake in which case an opposite effect would be expected.

The decrease in activity caused by high diazepam and diphenylhydantoin concentration is most likely due to a MAO inhibitory effect which is unable to reduce the enhanced activity below control levels at lower drug concentrations. Although both diazepam and diphenylhydantoin have been shown to inhibit MAO [5,6] the fact that diphenylhydantoin could inhibit noradrenaline uptake [6,7] may indicate an ability to block uptake systems which could contribute to the decreased activity observed. The possibility exists that both decrease in uptake and inhibition of MAO participate in the reduced activity.

Clonazepam, phenobarbitone and sulthiame are capable of inhibiting MAO in vitro (see Chapter 5) and therefore an increase in activity was most likely to be due either to increased availability of substrate for catalysis or to increased enzyme levels. Although the MAO inhibitory effect was apparent, increasing with clonazepam, phenobarbitone and sulthiame concentrations, the overall increase in metabolic activity was such that serotonin oxidation remained above control levels. Very high clonazepam levels ($10^{-3}$M) appear to have the opposite effect and MAO activity increased, possibly the result of an enhancement of oxidation such as to totally negate inhibitory effects on the enzyme itself. Such dual effects on a metabolic pathway complicate predictions regarding overall effects after drug administration especially as plasma levels are bound to vary during therapy.

Table 2a: Effect of various anticonvulsant drugs *in vitro* on total activity (as ng serotonin used/pineal/24 hours) and amounts of hydroxyindoles formed. (NAS = N-acetylserotonin, HIALD = 5-hydroxyindole-3-acetaldehyde, HIAA = 5-hydroxyindole-3-acetic acid and HTOL = 5-hydroxytryptophol).

| | TOTAL | HYDROXYINDOLE FORMED (ng/pineal/24hr) | | | |
|---|---|---|---|---|---|
| DRUG(M) | ACTIVITY | NAS | HIALD | HIAA | HTOL |
| No drug | 84±04 | 4.9±0.4 | 78±04 | 71±05 | 13±2 |
| ATZ $10^{-3}$ | 116±01*** | 3.4±1.5 | 112±03* | 97±06* | 22±4 |
| $10^{-4}$ | 111±04** | 2.3±0.2** | 108±04* | 97±05* | 19±2 |
| $10^{-5}$ | 118±21 | 3.0±0.6 | 114±22 | 100±17 | 22±7 |
| $10^{-6}$ | 125±31 | 3.6±0.7 | 121±32 | 109±32 | 20±2 |
| BCL $10^{-3}$ | 116±32 | 3.7±2.2 | 112±35 | 97±30 | 22±8 |
| $10^{-4}$ | 99±23 | 6.0±2.3 | 91±22 | 82±15 | 16±8 |
| $10^{-5}$ | 106±20 | 5.4±1.2 | 100±21 | 89±14 | 18±8 |
| $10^{-6}$ | 151±38 | 2.1±0.3** | 149±38 | 137±40 | 23±2* |
| CBZ $10^{-3}$ | 102±06 | 2.8±0.3* | 98±06 | 72±07 | 32±1**** |
| $10^{-4}$ | 105±08 | 2.1±0.6* | 102±07 | 86±10 | 23±2* |
| $10^{-5}$ | 115±12 | 1.5±0.3** | 113±12 | 100±13 | 21±1* |
| $10^{-6}$ | 125±33 | 1.8±0.3** | 122±33 | 111±33 | 21±3 |
| CLZ $10^{-3}$ | 126±23 | 1.7±0.1*** | 124±23 | 106±18 | 26±8 |
| $10^{-4}$ | 103±06 | 3.1±0.6 | 99±05 | 82±02 | 24±7 |
| $10^{-5}$ | 162±42 | 3.2±1.0 | 158±43 | 139±36 | 30±9 |
| $10^{-6}$ | 178±50 | 3.1±0.8 | 175±50 | 158±43 | 29±9 |
| DZP $10^{-3}$ | 66±23 | 1.5±0.4** | 64±22 | 56±19 | 12±5 |
| $10^{-4}$ | 86±17 | 3.8±1.1 | 81±18 | 72±13 | 14±7 |
| $10^{-5}$ | 127±28 | 4.1±0.6 | 122±28 | 117±28 | 15±3 |
| $10^{-6}$ | 106±23 | 4.9±0.2 | 100±21 | 93±21 | 14±2 |
| DPH $10^{-3}$ | 66±21 | 3.3±0.9 | 62±20 | 56±19 | 10±4 |
| $10^{-4}$ | 92±19 | 3.5±1.1 | 88±17 | 80±16 | 14±3 |
| $10^{-5}$ | 122±40 | 3.2±1.1 | 118±39 | 106±36 | 20±7 |
| $10^{-6}$ | 169±63 | 2.9±0.8 | 165±62 | 142±52 | 34±15 |

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

Table 2b: Effect of various anticonvulsant drugs *in vitro* on total activity (as ng serotonin used/pineal/24 hours) and amounts of hydroxyindoles formed. (NAS = N-acetylserotonin, HIALD = 5-hydroxyindole-3-acetaldehyde, HIAA = 5-hydroxyindole-3-acetic acid and HTOL = 5-hydroxytryptophol).

| | TOTAL | HYDROXYINDOLE FORMED (ng/pineal/24hr) | | | |
|---|---|---|---|---|---|
| DRUG(M) | ACTIVITY | NAS | HIALD | HIAA | HTOL |
| No drug | 84±04 | 4.9±0.4 | 78±04 | 71±05 | 13±2 |
| ETH $10^{-3}$ | 130±13* | 6.5±2.8 | 122±12 | 114±15 | 17±2 |
| $10^{-4}$ | 138±11** | 5.2±2.4 | 132±12* | 121±14* | 20±1* |
| $10^{-5}$ | 173±23* | 5.5±1.1 | 166±22* | 154±21* | 24±4 |
| $10^{-6}$ | 166±07*** | 2.7±1.0 | 162±06**** | 143±04**** | 31±3** |
| PTR $10^{-3}$ | 154±07*** | 5.1±1.7 | 148±07** | 135±08** | 23±3* |
| $10^{-4}$ | 153±21* | 3.5±1.4 | 148±22 | 133±21 | 26±2** |
| $10^{-5}$ | 159±17** | 3.0±0.5* | 155±17* | 142±16* | 24±2* |
| $10^{-6}$ | 145±22 | 5.7±2.2 | 138±21 | 123±23 | 24±1** |
| PBT $10^{-3}$ | 168±22* | 6.3±1.8 | 160±24 | 147±27 | 25±7 |
| $10^{-4}$ | 189±26* | 2.5±0.3** | 186±26 | 166±33 | 34±6* |
| $10^{-5}$ | 200±32* | 7.0±3.2 | 191±29* | 173±27* | 32±4* |
| $10^{-6}$ | 186±15** | 5.4±0.2 | 180±15*** | 167±09*** | 26±7 |
| PRD $10^{-3}$ | 209±17*** | 3.7±1.0 | 205±16*** | 185±15*** | 34±6* |
| $10^{-4}$ | 234±29** | 3.0±0.4* | 230±28** | 213±32** | 34±3** |
| $10^{-5}$ | 210±58 | 2.8±1.5 | 206±56 | 185±54 | 36±8 |
| $10^{-6}$ | 256±36** | 7.7±4.8 | 246±36* | 228±40* | 37±9 |
| STH $10^{-3}$ | 132±08** | 1.5±0.3** | 130±08* | 103±07* | 35±3*** |
| $10^{-4}$ | 166±33 | 2.9±0.7 | 163±32 | 145±36 | 29±2** |
| $10^{-5}$ | 174±22* | 4.0±1.1 | 169±21* | 160±23* | 22±2* |
| $10^{-6}$ | 142±19* | 7.9±1.7 | 132±21 | 121±22 | 20±1* |
| VPA $10^{-3}$ | 170±37 | 2.3±0.1** | 168±37 | 160±40 | 20±2 |
| $10^{-4}$ | 111±05** | 5.9±1.8 | 104±06 | 87±09 | 24±5 |
| $10^{-5}$ | 178±23** | 2.3±0.3** | 175±13** | 154±13** | 33±4** |
| $10^{-6}$ | 180±17** | 2.1±0.1** | 178±17* | 155±23* | 35±5** |

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

Table 3a: Effect of various anticonvulsant drugs in vitro on indole methylation in organ culture (total methylation as ng serotonin methylated/pineal/24hours). See Table 1 for drug abbreviations. (MTN = melatonin, MIAA = 5-methoxyindole-3-acetic acid and MTOL = 5-methoxytryptophol).

| | TOTAL | METHOXYINDOLE FORMED (ng/pineal/24hr) | | |
|---|---|---|---|---|
| DRUG (M) | METHYLATION | MTN | MIAA | MTOL |
| No drug | 10.1±0.3 | 4.5±0.4 | 5.8±0.7 | 1.9±0.9 |
| ATZ $10^{-3}$ | 9.3±1.8 | 3.0±1.7 | 4.7±0.3 | 3.3±0.9 |
| $10^{-4}$ | 9.2±2.1 | 1.8±0.1** | 5.5±1.4 | 3.5±1.1 |
| $10^{-5}$ | 7.9±1.5 | 2.2±0.3** | 4.8±1.1 | 2.3±0.8 |
| $10^{-6}$ | 11.5±2.3 | 3.0±0.5 | 6.8±0.9 | 3.6±1.5 |
| BCL $10^{-3}$ | 10.3±0.9 | 3.4±2.3 | 4.9±1.0 | 3.9±1.5 |
| $10^{-4}$ | 9.0±1.7 | 5.4±2.2 | 4.2±0.7 | 1.4±0.4 |
| $10^{-5}$ | 11.0±1.7 | 4.9±1.3 | 5.0±0.6 | 3.3±2.2 |
| $10^{-6}$ | 7.7±1.2 | 1.5±0.3** | 4.9±1.3 | 2.5±0.1 |
| CBZ $10^{-3}$ | 9.7±1.8 | 2.3±0.5* | 4.8±1.5 | 4.2±1.0 |
| $10^{-4}$ | 9.0±1.2 | 1.6±0.4** | 4.5±1.1 | 4.3±1.4 |
| $10^{-5}$ | 12.1±1.1 | 1.0±0.2*** | 6.8±0.4 | 6.0±0.9* |
| $10^{-6}$ | 13.3±3.5 | 1.3±0.1*** | 7.3±1.7 | 6.6±2.2 |
| CLZ $10^{-3}$ | 9.0±1.0 | 1.1±0.2*** | 6.4±0.9 | 2.9±0.9 |
| $10^{-4}$ | 10.0±0.7 | 2.5±0.7 | 6.1±1.3 | 3.1±0.5 |
| $10^{-5}$ | 12.9±1.5 | 2.5±1.0 | 7.1±0.7 | 5.4±2.3 |
| $10^{-6}$ | 14.2±3.2 | 2.4±0.7 | 7.5±1.2 | 6.4±3.2 |
| DZP $10^{-3}$ | 4.6±0.6*** | 0.9±0.3*** | 3.9±0.7 | 0.5±0.2 |
| $10^{-4}$ | 5.9±1.4* | 2.9±1.0 | 3.0±0.8 | 1.3±0.9 |
| $10^{-5}$ | 6.5±1.1* | 3.3±0.7 | 3.2±0.3 | 1.4±0.5 |
| $10^{-6}$ | 9.1±0.4 | 3.9±1.5 | 4.3±0.5 | 2.6±0.9 |
| DPH $10^{-3}$ | 6.5±1.6 | 3.0±0.9 | 3.4±0.6 | 1.4±0.4 |
| $10^{-4}$ | 9.5±1.6 | 3.0±1.2 | 5.2±0.2 | 3.0±1.2 |
| $10^{-5}$ | 9.6±1.9 | 2.8±1.0 | 5.1±0.4 | 3.4±1.4 |
| $10^{-6}$ | 13.4±2.9 | 2.4±0.9 | 7.5±1.2 | 5.5±1.6 |

* P = 0.05, ** P = 0.025, *** P = 0.01

Table 3b: Effect of various anticonvulsant drugs in vitro on indole methylation in organ culture (total methylation as ng serotonin methylated/pineal/24hours). See Table 1 for drug abbreviations. (MTN = melatonin, MIAA = 5-methoxyindole-3-acetic acid and MTOL = 5-methoxytryptophol).

| | TOTAL | METHOXYINDOLE FORMED (ng/pineal/24hr) | | |
|---|---|---|---|---|
| DRUG(M) | METHYLATION | MTN | MIAA | MTOL |
| No drug | 10.1±0.3 | 4.5±0.4 | 5.8±0.7 | 1.9±0.9 |
| ETH $10^{-3}$ | 10.6±2.0 | 5.9±3.0 | 4.3±0.9 | 2.6±0.9 |
| $10^{-4}$ | 9.7±1.0 | 4.6±2.2 | 5.2±0.7 | 2.0±0.1 |
| $10^{-5}$ | 9.7±0.6 | 4.7±0.8 | 5.3±0.8 | 1.8±0.3 |
| $10^{-6}$ | 12.4±1.2 | 2.3±0.9 | 6.7±0.4 | 5.3±0.8* |
| PTR $10^{-3}$ | 11.3±1.4 | 4.6±1.7 | 6.1±0.7 | 2.7±0.7 |
| $10^{-4}$ | 12.8±2.6 | 2.9±1.4 | 7.4±1.7 | 4.6±1.3 |
| $10^{-5}$ | 10.4±2.6 | 2.1±0.6* | 6.0±1.4 | 3.9±1.7 |
| $10^{-6}$ | 13.3±1.8 | 5.1±2.2 | 6.3±1.3 | 4.3±2.0 |
| PBT $10^{-3}$ | 12.3±0.6* | 5.4±2.2 | 6.2±0.8 | 3.0±0.9 |
| $10^{-4}$ | 14.8±0.4**** | 1.7±0.2** | 6.8±0.6 | 8.4±0.1*** |
| $10^{-5}$ | 15.8±1.6* | 6.3±3.3 | 6.8±1.2 | 5.5±1.2 |
| $10^{-6}$ | 12.7±2.7 | 4.6±0.2 | 6.4±1.2 | 4.1±1.9 |
| PRD $10^{-3}$ | 17.5±2.1* | 2.7±1.1 | 9.4±1.2 | 8.0±2.4 |
| $10^{-4}$ | 15.4±2.0 | 2.2±0.5* | 9.1±1.1 | 6.5±1.6 |
| $10^{-5}$ | 17.4±2.9 | 2.3±1.4 | 8.7±1.5 | 8.9±1.6 |
| $10^{-6}$ | 16.0±5.9 | 7.2±4.6 | 7.4±0.6 | 4.5±2.1 |
| STH $10^{-3}$ | 11.6±1.4 | 0.8±0.1*** | 6.0±1.1 | 6.4±0.6** |
| $10^{-4}$ | 11.1±1.0 | 2.2±0.6* | 5.7±0.7 | 4.9±0.2* |
| $10^{-5}$ | 10.6±0.7 | 3.5±1.3 | 6.2±1.2 | 2.9±0.9 |
| $10^{-6}$ | 11.4±0.6 | 7.3±1.7 | 4.4±1.0 | 2.3±0.9 |
| VPA $10^{-3}$ | 12.1±2.3 | 1.6±0.2** | 5.9±1.8 | 6.3±1.3 |
| $10^{-4}$ | 11.0±0.6 | 5.3±1.7 | 3.8±0.6 | 4.1±1.0 |
| $10^{-5}$ | 14.4±1.0* | 1.8±0.3** | 7.6±0.2 | 6.9±1.4* |
| $10^{-6}$ | 13.0±2.3 | 1.4±0.1*** | 6.3±0.4 | 7.0±2.4 |

* P = 0.05, ** P = 0.025, *** P = 0.01, **** P = 0.05

Table 4: Effect of *in vivo* anticonvulsant drugs on total activity (as ng serotonin used/pineal/24hours) and hydroxyindole production. See Table 1 and 2 for abbreviations.

| | TOTAL | HYDROXYINDOLE FORMED (ng/pineal/24hr) | | | |
|---|---|---|---|---|---|
| DRUG | ACTIVITY | NAS | HIALD | HIAA | HTOL |
| No drug | 88±11 | 12.3±1.8 | 73±10 | 68±09 | 10±2 |
| ATZ | 70±06 | 2.9±1.2** | 66±07 | 58±04 | 13±5 |
| BCL | 71±11 | 2.3±1.2** | 68±12 | 64±11 | 9±1 |
| CBZ | 71±06 | 3.4±1.3* | 67±07 | 54±07 | 17±1* |
| CLZ | 84±10 | 3.5±0.5** | 80±10 | 70±09 | 15±3 |
| DZP | 75±05 | 7.6±5.4 | 66±09 | 59±07 | 12±3 |
| DPH | 74±09 | 8.4±4.2 | 64±04 | 61±04 | 8±1 |
| ETH | 98±11 | 9.2±5.0 | 87±17 | 78±14 | 15±4 |
| PTR | 82±06 | 9.9±5.5 | 70±08 | 64±05 | 11±4 |
| PBT | 149±06** | 3.9±0.7** | 144±06** | 137±05** | 18±2* |
| PRD | 110±17 | 4.1±0.7** | 105±18 | 98±18 | 14±2 |
| STH | 97±01 | 4.1±0.6** | 92±01 | 82±04 | 17±4 |
| VPA | 101±04 | 8.1±3.5 | 91±05 | 84±04 | 13±1 |

* P = 0.05, ** P = 0.025

Table 5: Effect of *in vivo* anticonvulsant drugs on indole methylation (total methylation as ng serotonin methylated/pineal/24hours). See Table 1 and 3 for abbreviations.

| | TOTAL | METHOXYINDOLE FORMED (ng/pineal/24hr) | | |
|---|---|---|---|---|
| DRUG (M) | METHYLATION | MTN | MIAA | MTOL |
| No drug | 11.2±1.8 | 11.5±1.9 | 2.2±0.2 | 0.6±0.2 |
| ATZ | 6.4±1.3 | 2.1±1.1* | 3.8±1.5 | 1.6±0.7 |
| BCL | 5.6±0.7 | 1.8±1.2** | 2.5±0.9 | 2.2±0.9 |
| CBZ | 6.5±1.1 | 2.8±1.1* | 3.0±1.0 | 1.9±1.2 |
| CLZ | 6.5±0.9 | 3.1±0.6* | 3.5±1.1 | 1.2±0.3 |
| DZP | 9.7±3.7 | 7.2±5.4 | 3.1±0.4 | 1.8±0.6 |
| DPH | 9.4±2.8 | 8.0±4.0 | 2.6±0.3 | 1.2±0.3 |
| ETH | 10.3±1.8 | 6.8±0.4 | 3.6±0.7 | 2.3±1.0 |
| PTR | 7.8±3.7 | 7.8±4.9 | 1.5±0.5 | 0.7±0.3 |
| PBT | 8.3±1.3 | 3.0±0.4** | 3.5±0.7 | 3.3±1.2 |
| PRD | 7.4±1.4 | 3.3±0.5* | 3.4±0.8 | 2.1±0.9 |
| STH | 7.8±0.7 | 3.6±0.4* | 3.6±0.3* | 2.1±0.9 |
| VPA | 11.2±1.0 | 7.1±3.2 | 4.3±0.8 | 2.3±1.3 |

* P = 0.05, ** P = 0.025

In most instances, although not always significant, there was a noticeable decrease in N-acetylserotonin production from in vitro anticonvulsant drugs (Table 2a and 2b). Acetazolamide, carbamazepine, clonazepam, diazepam and diphenylhydantoin caused a reduction in N-acetylserotonin possibly due to decreased SNAT levels, decreased substrate availability resulting from increased MAO activity or a combination of these effects. The observation that carbamazepine and diphenylhydantoin could decrease cerebrospinal fluid cyclic AMP levels [8] may indicate an ability for these drugs to reduce pineal cyclic AMP and hence cause decrease in SNAT activity. Diphenylhydantoin may possibly decrease SNAT activity by an effect on protein synthesis [9]. Inhibition of calcium uptake by carbamazepine, diazepam and diphenylhydantoin [10] if it also occured in the pineal would lead to decreased cyclic AMP levels and may participate in the reduced NAS production. The fact that diphenylhydantoin increased potassium uptake [11] and decreased potassium release [12] would tend to inhibit hyperpolarization and as this has been thought to be necessary for SNAT increase [13] such an effect would account for reduced SNAT levels. Diazepam induced decrease in SNAT activity may also be the result of decreased serotonin uptake.

At concentrations above $10^{-5}$M ethosuximide caused an increase in N-acetylserotonin production which may be due primarily to increased substrate availability resulting from increased serotonin uptake. This effect is apparently enhanced by reduction in MAO activity as ethosuximide concentration increased and may also involve increase in SNAT levels which can be rationalised when considering evidence which suggests that ethosuximide can increase calcium influx at high concentration [14].

Beclamide at low concentration ($10^{-6}$M) caused significant reduction in N-acetylserotonin production which is less marked at high concentration ($10^{-3}$M). Intermediate concentrations led to an increase in N-acetylserotonin production, these variable effects being closely related to changes in MAO output. The dose dependent variable effects of beclamide on N-acetylserotonin production can, therefore, be best explained as an effect on MAO rather than on SNAT.

Valproate caused a significant reduction in N-acetylserotonin

production at all concentrations except $10^{-4}$M, effects on MAO leading to changes in substrate availability rather than changes in SNAT activity as such.

High and low pheneturide concentrations ($10^{-3}$ and $10^{-6}$M) had little effect on SNAT while intermediate concentrations, especially $10^{-5}$M caused a significant reduction in production, an effect not entirely consistent with a primary change in MAO activity and may indicate a dose-specific alteration of SNAT levels. The variable effects may indicate effects on both membrane hyperpolarization and β-receptor stimulated adenyl cyclase activity with one effect predominating dependent on drug concentration. This, however, is merely conjecture and further investigation would be necessary to elucidate the mechanicm.

Phenobarbitone caused significant reduction in N-acetylserotonin only at concentrations of $10^{-4}$M, all higher and lower concentrations increasing activity. Changes in SNAT activity or levels are most likely responsible for these changes as differences in MAO activity do not account for the variations. Evidence that phenobarbitone could inhibit cyclic AMP phosphodiesterase [15,16] could account for increased SNAT activity by virtue of the decreased cyclic AMP degradation. A similar effect occured with primidone except low concentrations ($10^{-6}$M) enhanced while all higher concentrations decreased NAS production. The chemical similarity between the two drugs may indicate a common mode of action. The variable effects may be due to action at more than one site as discussed for beclamide. Inhibition of cyclic AMP phosphodiesterase activity is also caused by acetazolamide, diazepam and diphenylhydantoin [15,16] although an increase in SNAT levels was not observed, indicating that the actions are due to multiple effects some of which are antagonistic.

Sulthiame at low concentration ($10^{-6}$M) increased N-acetyl-serotonin production while higher concentrations caused a dose dependent decrease, apparently independent of MAO activity and serotonin uptake. This effect is most likely due to direct inhibition of SNAT (see Chapter 5) although other effects cannot be ruled out.

Increase in SNAT activity may be the result of β-receptor stimulation or membrane hyperpolarization leading to increase in cyclic AMP thus inducing SNAT levels. The observation that diphenylhydantoin, ethosuximide, phenobarbitone, sulthiame and valproate can alter brain taurine levels [17] may suggest an effect on pineal taurine which could account for effect on SNAT as taurine has been suggested to exert modulatory effects on the pineal gland [18,19].

In most cases melatonin production mirrored N-acetylserotonin production (Table 3a and 3b) and can be assumed to be due to the changes in SNAT rather than an effect on HIOMT. This would be consistent with the reports that suggest melatonin production is regulated by change in SNAT activity [20-24].

In all instances where overall methylation increased (Table 3a and 3b) the effects could be attributed to an increase in MAO activity resulting in increased substrate availability rather than an effect on HIOMT.

Increase in carbamazepine and sulthiame concentration caused an increase in 5-hydroxytryptophol production, an effect which appears to be independent of an effect on MAO and is therefore probably due to inhibition of aldehyde dehydrogenase although it may also involve increase in aldehyde reductase. This effect is interesting by virtue of the evidence that suggests carbamazepine to be a non-competitive inhibitor of brain aldehyde reductase [25]. This apparent discrepancy may be due to insensitivity of pineal aldehyde reductase to carbamazepine inhibition, or to inhibition of aldehyde dehydrogenase which may be far greater, therefore seeming to increase 5-hydroxytryptophol production merely by virtue of increased substrate available although inhibition in fact occurs.

Although clonazepam, diazepam [26], diphenylhydantoin, ethosuximide, phenobarbitone [25,27], primidone [28] and valproate [26,29,30] have all been shown to inhibit brain aldehyde reductase only diphenylhydantoin, ethosuximide and valproate exert a noticeable dose dependent inhibition on the pineal enzyme. Overall 5-hydroxytryptophol production remained above control levels (Table 2a and 2b) primarily due to increased

MAO activity. The possibility therefore exists that pineal aldehyde reductase is noticeably different from the brain enzyme although the lack of inhibitory effects may be due to other factors.

In the instances where a variable effect on N-acetylserotonin and melatonin production is exerted it is possible that the drugs are acting on at least two sites which are antagonistic, the overall effect being dependent on the drug concentration.

The foregoing discussion gives some indication of the effects exerted on indole metabolism by a direct action of the various drugs on the pineal gland. In the intact animal numerous other factors could affect activity, especially centrally mediated influences. These may enhance, abolish or otherwise modify the effects resulting from a direct local action of the drugs on the pineal. It is therefore interesting to compare these *in vitro* effects with the *in vivo* effects on indole metabolism using the same system. By and large if similar effects occur in both systems then effects can be presumed to be predominantly locally mediated while a difference would suggest central participation.

Possibly the most striking observation *in vivo* is that in all cases both N-acetylserotonin and melatonin production were decreased by anticonvulsant drug administration (Table 4 and 5). This decrease in activity was probably due primarily to lower SNAT levels although it may also have been the result of lower HIOMT levels as overall methylation was reduced (Table 5). That SNAT levels appear to be reduced by all drugs except acetazolamide and pheneturide and HIOMT levels are reduced by all drugs used (see Chapter 6) tends to favour this hypothesis.

This decrease probably results from reduced β-adrenergic cyclic AMP mediated induction of SNAT. The fact that most of the drugs could reduce N-acetylserotonin production *in vitro* indicates that this effect is probably largely mediated by local effects although central effects may also play a role. In certain instances beclamide, ethosuximide, pheneturide, phenobarbitone, primidone,sulthiame and valproate were able to increase N-acetylserotonin production *in vitro* whereas they all caused a reduction *in vivo* which suggests the involvement of central mechanisms in

causing an ultimate decrease in SNAT activity in the intact animal. The central depressant effect would be capable of overriding the locally mediated stimulant action on SNAT levels resulting in decreased N-acetylserotonin production.

In the case of sulthiame induced reduction of N-acetylserotonin and melatonin levels the best interpretation would probably be based on direct inhibition of SNAT by sulthiame (see Chapter 5), an observation borne out by the dose dependent reduction in SNAT activity observed *in vitro*. Participation of other local and even central effects, however, cannot be completely ruled out.

That methylation increased *in vitro* (Table 3a and 3b) could be explained by an effect on MAO activity leading to increased substrate availability rather than to an effect on HIOMT and suggested that these drugs did not influence HIOMT through a direct effect on the pineal. The reduction in methylation observed *in vivo* in organ culture (Table 5) and the reduction in total HIOMT levels (see Chapter 6) suggests that anticonvulsant drug-induced decrease in HIOMT levels is mediated from the central nervous system with no local component. To a certain extent decreased methylation may be the result of reduced substrate in the case where MAO activity was decreased *in vivo*. The decrease in melatonin output was accompanied by an increase in both 5-methoxyindole-3-acetic acid and 5-methoxytryptophol which suggests that the catalytic sites on HIOMT can utilise a number of substrates, probably dependent on their concentration and affinity verifying a previous report [23].

Carbamazepine administration, apart from causing a decrease in N-acetyl-serotonin and melatonin, led to a significant increase in 5-hydroxytryptophol production (Table 4) which may result from decreased activity or inhibition of aldehyde dehydrogenase or increase in aldehyde reductase levels. This effect probably originates primarily at the local level.

Ethosuximide, phenobarbitone, primidone, sulthiame and valproate caused an increase in overall metabolic activity (Table 4), an effect which probably results primarily from increased MAO activity, although levels of aldehyde dehydrogenase and aldehyde reductase may also be increased. Evidence that phenobarbitone is

capable of inhibiting MAO (see Chapter 5) suggests that either MAO levels increase to an extent that overrides inhibition or that pineal MAO is unaffected by phenobarbitone. This increased activity, although it may involve a central component, is predominantly locally mediated. The fact that phenobarbitone has been shown to increase activity of aldehyde dehydrogenase in liver [31,32] may indicate that a similar effect occurs in the pineal.

All the other drugs caused a reduction in overall activity which suggests that they exert a central effect cancelling the locally mediated increase in metabolic activity. This again indicates that dual effects may occur which are antagonistic to each other and have the potential to modulate overall activity in a complex fashion.

Sulthiame, apart from reducing melatonin production, also caused significant increase in 5-methoxyindole-3-acetic acid output, an effect probably resulting from the reduction in N-acetylserotonin output, leaving more catalytic sites available for the other hydroxyindoles.

From these results it is apparent that the most consistent effect of anticonvulsant drugs on the pineal gland is the reduction in melatonin synthesis. Whether this would influence therapeutic activity or affect normal physiological processes would require further intensive investigation. Such studies would be complicated as it would be necessary to divide observed effects into those resulting from direct action of the drug and those due to change in melatonin levels.

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# CHAPTER 9

## CONCLUSION

Of all the varied effects exerted by the anticonvulsant agents on pineal gland indole metabolism, the most striking is the reduction in melatonin production. It is significant that all the drugs used had this effect *in vivo* and all, except diphenylhydantoin and valproate, appeared to cause a shift in the nocturnal rhythm of SNAT activity. The new peak activity in the drug treated animals occured later in the dark phase. It is difficult to assign an importance to these observations and although they have potential therapeutic significance they may not contribute to the anti-seizure activity of the drugs.

The reduction in melatonin production can be largely attributed to reduction in SNAT activity which in turn appears to be mediated primarily by local effects, although in certain cases there is a definite involvement of central mechanisms. That HIOMT levels were also largely decreased *in vivo* by all the drugs might indicate that decreased melatonin could also result from decreased methylating capacity of the pineal. The reports which indicate that SNAT is rate limiting in the production of melatonin [1-5] tend to favour reduction in N-acetylserotonin production as being primarily responsible for depressed melatonin synthesis.

Any reduction in SNAT levels and hence of N-acetylserotonin production would almost certainly involve decreased cyclic AMP stimulated induction of enzyme levels. This may occur at a local level or may be initiated by depression of a central neural pathway. The ultimate effect would be reduced cyclic AMP levels which may result from decreased adenyl cyclase activity, increased cyclic AMP phosphodiesterase activity or a combination of both these effects. In this respect it is interesting to note that carbamazepine, clonazepam, diazepam, diphenylhydantoin and phenobarbitone have been shown to prevent rise in cyclic AMP [6] while acetazolamide, diazepam, diphenylhydantoin and phenobarbitone can inhibit cyclic AMP phosphodiesterase [7,8] which would tend to increase cyclic AMP levels. If these drugs

are capable of inhibiting pineal cyclic AMP phosphodiesterase then reduction in SNAT activity must result primarily from reduced adenyl cyclase activity which implies β-adrenergic receptor blockade or reduced β-stimulation.

It has been suggested that both increased cyclic AMP levels and membrane hyperpolarization are necessary for complete expression of β-adrenergic stimulation [9] and the finding that diphenylhydantoin [10,11] and carbamazepine [12] can prevent hyperpolarization may partially account for the reduced SNAT activity. Calcium ions are essential for mediation of pineal β-receptor activation and the fact that carbamazepine, diazepam, diphenylhydantoin and phenobarbitone can inhibit calcium uptake [13] may indicate that reduced β-stimulant action is involved in the decreased melatonin production resulting from anticonvulsant drug administration. SNAT induction is also dependent on phosphorylation of nuclear proteins [14,15] and diphenylhydantoin can inhibit calcium stimulated protein phosphorylation [16], again an effect which would reduce SNAT levels. Decrease in protein synthesis associated with diphenylhydantoin administration [17] would also account for the reduction in SNAT levels, again if such an effect occured in the pineal.

From this circumstantial evidence it is apparent that many of the anticonvulsant drugs have the potential for inhibiting induction of SNAT in the pineal, which would account for the reduction in melatonin production. The fact that some drugs stimulated melatonin production *in vitro* while inhibiting melatonin production *in vivo* is suggestive of a central inhibitory action on SNAT induction, presumably by decreasing pineal sympathetic stimulation, although it is conceivable that inhibitory pathways innervate the pineal directly and that these might be stimulated by the anticonvulsant drugs. ALTERNATIVELY, IN VITRO AND IN VIVO DRUG CONCENTRATIONS MAY BE DIFFERENT.

Having ascertained that the reduction in melatonin production is largely due to locally mediated effects with the participation of some central actions it is necessary to examine the possible significance of reduced melatonin levels on seizure activity. The most obvious effect would be the reduced ameliorative action on seizures exerted by melatonin directly. This, however, may not have a significant influence on seizure therapy in the short term

as the decreased effectiveness of melatonin would probably be more than compensated for by a direct anticonvulsant action of the drug concerned. With the development of tolerance to the drugs and the consequent reduction in effectiveness it is possible that the reduced ameliorative effect of melatonin may become apparent.

As melatonin levels and/or rhythm have been shown to be abnormal in 47 disease states [21] it is likely that some abnormality also exists in epileptic conditions. If this were indeed the case then the shift in melatonin rhythm caused by the various drugs might have some therapeutic importance in that the drugs could reinstate normal rhythms. This is important as circadian rhythms have been shown to have profound effects on seizure activity [22] and seizure susceptibility varies with changes in the light phase [23]. That melatonin administration can decrease sensitivity of the photosensitive baboon [24] may also suggest a link between melatonin, circadian rhythm and seizure activity.

It is possible that reduced melatonin levels can exert an indirect anticonvulsant effect which is capable of compensating for the decrease in directly ameliorative action attributable to melatonin. One of the possible indirect mechanisms involves the benzodiazepine (BZD) receptors. Both melatonin and its brain metabolite N-acetyl-5-methoxy kynurenamine have been found to be capable of inhibiting BZD receptor binding [25,26] and are more potent than the putative endogenous BZD receptors inosine and hypoxanthine [26]. A correlation has been found between BZD receptor occupancy and anticonvulsant effectiveness [27] and therefore reduced melatonin levels would increase antiseizure activity by decreasing inhibition and allowing for increased agonist binding.

If this were indeed the case then it could partially explain the effectiveness of the following four groups of drugs. Firstly the benzodiazepines clonazepam and diazepam, secondly, diphenyl-hydantoin which has been shown to compete for diazepam binding [28], thirdly, phenobarbitone which can bind to the barbiturate-picrotoxin component of the BZD-GABA receptor complex [29] and lastly primidone through the action of its metabolite phenobarbitone. Marked similarities in molecular conformation of

diazepam and diphenylhydantoin [30] also favour a similar mode of action while drugs such as sulthiame have a conformation totally different from that of other anticonvulsants [31] and would thus have a different mode of action. It is most likely that the BZD receptors involved in such a mechanism would be situated centrally, although the presence of BZD receptors in peripheral tissues [32] might suggest some effect via these areas, perhaps even the pineal although there is no evidence as such to support this proposal.

The scant evidence available supports an involvement of melatonin in modulation of BZD receptors and hence in prevention of seizure activity. Whether this is the normal role of melatonin as an endogenous modulator of seizure activity or merely a positive consequence of drug administration remains to be established. Reduction in melatonin output also has the potential for affecting a variety of endocrine and homeostatic mechanisms (see Chapter 1) which would influence seizure states to some extent. The overall effects resulting from anticonvulsant drug action are undoubtably complex and multifactorial and further extensive investigation would be necessary to fully characterise the mechanisms involved.

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# ABBREVIATIONS

A: absorption
AcCoA: acetyl coenzyme A
ACTH: adrenocorticotrophic hormone
Ald D: aldehyde dehydrogenase
Ald R: aldehyde reductase
AMe: S-adenosylmethionine
AMP: adenosine monophosphate
APUD: amine precursor uptake and decarboxylation
ATZ: acetazolamide
BCL: beclamide
BZD: benzodiazepine
CA: carbonic anhydrase
CBZ: carbamazepine
CLZ: clonazepam
CoA: coenzyme A
cpm: counts per minute
DPH: diphenylhydantoin
dpm: disintegrations per minute
DZP: diazepam
E: enzyme
EDTA: ethylenediaminetetraacetic acid
EEG: electroencephalogram
Emit: enzyme multiplied immunoassay technique
ETH: ethosuximide
FSH: follicle stimulating hormone
GABA: gamma-amino butyric acid
GBq: gigabecquerel
GMP: guanosine monophosphate
HIAA: 5-hydroxyindole-3-acetic acid
HIALD: 5-hydroxyindole-3-acetaldehyde
HIOMT: hydroxyindole-O-methyltransferase
hr: hour
5-HT: serotonin
HTOL: 5-hydroxytryptophol
ip: intraperitoneal
$K_e$: elimination rate constant
Kg: kilogram
Kia: dissociation constant for E.Tryp complex
Kii: dissociation constant for STH from E.Tryp.STH complex

Kiii: dissociation constant for Tryp from E.Tryp.STH complex

Kis: dissociation constant for E.STH complex

Kj: kilojoule

$K^s_m$: Michaelis-Menten constant

l: litre

LH: luteinising hormone

LHRH: luteinising hormone releasing hormone

M: molar

MAO: monoamine oxidase

MBq: megabecquerel

mg: milligram

MIAA: 5-methoxyindole-3-acetic acid

mol: mole

MIF: melanocyte inhibitory factor

ml: millilitre

mM: millimolar

MSH: melanocyte stimulating hormone

MT: 5-methoxytryptamine

MTN: melatonin

MTOL: 5-methoxytryptophol

NA: noradrenaline

NAD: nicotinamide adenine dinucleotide

NADH: dihydronicotinamide adenine dinucleotide

NADPH: dihydronicotinamide adenine dinucleotide phosphate

NAS: N-acetylserotonin

NAT: N-acetyltryptamine

ng: nanogram

nm: nanometre

6-OH-MTN: 6-hydroxymelatonin

PRL: prolactin

PTR: pheneturide

PBT: phenobarbitone

pmol: picomole

PRD: primidone

$r^2$: coefficient of determination

SAH: S-adenosylhomocysteine

SEM: standard error of the mean

SNAT: (serotonin) N-acetyltransferase

STH: sulthiame

$t_{1/2}$: half-life

TBq: terabecquerel

TLC: thin layer chromatography

Tryp: tryptamine

TSHRH: thyroid stimulating hormone releasing hormone

ug: microgram

ul: microlitre

uM: micromolar

UV: ultra violet

VPA: valproate

V: velocity

$V_o$: initial velocity

$V_s$: saturation velocity

# APPENDIX A

## MATERIALS

The following list summarises the various materials used in this study giving specifications where necessary and indicating supplier.

Animals. Rats of the Wistar strain kept under constant lighting conditions (12 hours light/12 hours dark) with access to unlimited supplies of food and water were used. Both male and female rats (150-300g) were used and differences are outlined in the text. As far as possible temperature of the living quarters was kept constant (15-25$^{o}$C) and an extractor fan ensured constant removal of stale air.

Anticonvulsant Drugs. These were mostly donated by the pharmaceutical manufacturers to whom I am indebted.

Acetazolamide: Sigma, USA
Beclamide: Rona Labs Ltd., UK
Carbamazepine: Ciba-Geigy (Pty) Ltd., RSA
Clonazepam: Roche Products (Pty) Ltd., RSA
Diazepam: Roche Products (Pty) Ltd., RSA
Diphenylhydantoin: Parke-Davis (Pty) Ltd., RSA
Ethosuximide: Parke-Davis (Pty) Ltd., RSA
Pheneturide: Sapos SA, Switzerland
Phenobarbitone: Maybaker (SA) (Pty) Ltd., RSA
Primidone: ICI (Pharmaceuticals) (Pty) Ltd., RSA
Sulthiame: Bayer (SA) (Pty) Ltd., RSA
Valproate: R+C Pharmaceuticals (Pty) Ltd., RSA

Biochemicals. The following were purchased from Sigma Chemical Corporation, USA.

Acetic anhydride
N-acetylserotonin
Gamma-amino butyric acid
5-hydroxyindole-3-acetic acid
6-hydroxymelatonin
5-hydroxytryptamine creatinine sulphate

5-hydroxytryptophol
Isoprenaline HCl
Melatonin
5-methoxyindole-3-acetic acid
5-methoxytryptamine
5-methoxytryptophol
β-nicotinamide adenine dinucleotide
β-nicotinamide adenine dinucleotide (reduced)
Noradrenaline HCl
Tryptamine HCl

The followimg were obtained from P-L Biochemicals, USA.

S-adenosylhomocysteine
Coenzyme A

Culture Medium. This was obtained from Gibco Europe, UK.

B.G.J.b. medium (Fitton-Jackson modification)

Enzymes. These were mostly obtained from Boehringer-Mannheim, West Germany.

Alcohol dehydrogenase
Aldehyde dehydrogenase
Carbonic anhydrase

Monoamine oxidase (Sigma, USA)

Enzyme Immunoassay Kits. These were supplied by Syva Diagnostics, USA for the following drugs.

Diphenylhydantoin
Ethosuximide
Phenobarbitone
Primidone
Valproate

Radiochemicals. The following were supplied by the Radiochemical Centre, Amersham, UK.

[1-$^{14}$C] acetyl coenzyme A (specific activity 205MBq/mmol and 2.09GBq/mmol)

S-adenosyl-L-[methyl-$^{14}$C] methionine (specific activity 18.5MBq/mmol and 2.18GBq/mmol)

S-adenosyl-L-[methyl-$^{3}$H] methionine (specific activity 2.22TBq/mmol)

5-hydroxy [side chain-2-$^{14}$C] tryptamine creatinine sulphate (specific activity 2.22GBq/mmol)

5-hydroxy [G-$^{3}$H] tryptamine creatinine sulphate (specific activity 370GBq/mmol)

Radioactive benzodiazepines were supplied by Hoffman La Roche, Switzerland as a gift for which I am grateful.

Clonazepam-5-$^{14}$C (specific activity 1.15MBq/mg)

Diazepam-5-$^{14}$C (specific activity 6.88GBq/mg)

Scintillation Cocktails. Insta-Gel, Insta-Gel II and Soluene 350 were supplied by Packard, USA.

Sundries. All solvents, chemicals and thin layer chromatographic plates (silica-gel 60) were obtained from Merck, West Germany. Photographic film used for autoradiography was Kodak Plus-X pan (125 ASA) or Kodak professional copy film (25 ASA). Double distilled deionized water was used throughout.

# APPENDIX B

## THIN LAYER CHROMATOGRAPHY SOLVENT SYSTEMS

Various solvent systems were tested for thin layer chromatographic separation of pineal serotonin metabolites and are outlined below. Rf values given for development on microscope slide sized TLC plates. It must be noted that these Rf values should be used comparatively rather than absolutely as migration distance varies with change in climatic conditions. The relative position of each spot with respect to the others, however, remains the same.(HT = serotonin, MT = 5-methoxy-tryptamine, NAS = N-acetylserotonin, MTN = melatonin, 6OHMTN = 6-hydroxymelatonin, HIAA = 5-hydroxyindole-3-acetic acid, MIAA = 5-methoxyindole-3-acetic acid, HTOL = 5-hydroxytryptophol and MTOL = 5-methoxytryptophol).

### SOLVENT SYSTEMS

1] Chloroform
2] Ethanol
3] Ethyl acetate
4] 20% KCl
5] Isoamyl alcohol
6] Chloroform : acetone (9:1)
7] Chloroform : ether (8:2)
8] Chloroform : ethanol (8:2)
9] Chloroform : methanol (9:1)
10] Dioxane : water (9:1)
11] Methanol : 25% ammonia (25:1)
12] 20% KCL : 25% ammonia
13] 20% KCl : glacial acetic acid (25:1)
14] n-Butanol : 5M acetic acid (100:35)
15] Chloroform : methanol : glacial acetic acid (92:7:1)
16] Chloroform : methanol : glacial acetic acid (75:20:5)
17] Chloroform : methanol : glacial acetic acid (80:15:5)
18] Methyl acetate : isopropanol : 25% ammonia (45:35:20)
19] Methyl acetate : isopropanol : 25% ammonia (35:45:2)
20] Butanol : ethanol : 25% ammonia (8:1:1)
21] Butanol : glacial acetic acid : water (4:1:5)-upper phase

22] Isopropanol : ethyl acetate : 25% ammonia (7:9:4)

23] Isopropanol : ethyl acetate : glacial acetic acid : water (75:25:2:3)

24] Isopropanol : water : 25% ammonia (75:20:5)

25] Methyl ethyl ketone : n-hexane (35:65)

26] Methyl ethyl ketone : acetone : 2.5M acetic acid (2:1:1)

Rf VALUES FOR SOLVENT SYSTEMS ABOVE

| | HT | MT | NAS | MTN | 6OHMTN | HIAA | MIAA | HTOL | MTOL |
|---|---|---|---|---|---|---|---|---|---|
| 1] | 0 | 0.07 | 0 | 0 | 0 | 0 | 0.03 | 0 | 0.11 |
| 2] | 0 | 0 | 0.86 | 0.86 | 0.84 | 0.77 | 0.75 | 0.88 | 0.88 |
| 3] | 0 | 0 | 0.38 | 0.41 | 0.36 | 0.81 | 0.83 | 0.76 | 0.83 |
| 4] | 0.83 | 0.70 | 0.65 | 0.38 | 0.54 | 0.78 | 0.53 | 0.83 | 0.67 |
| 5] | 0 | 0.04 | 0.47 | 0.37 | 0.27 | 0.33 | 0.20 | 0.88 | 0.81 |
| 6] | 0 | 0 | 0.05 | 0.15 | 0 | 0 | 0.08 | 0.09 | 0.35 |
| 7] | 0 | 0 | 0.05 | 0.12 | 0 | 0 | 0.08 | 0.08 | 0.30 |
| 8] | 0 | 0 | 0.67 | 0.75 | 0.73 | 0.35 | 0.67 | 0.71 | 0.91 |
| 9] | 0 | 0 | 0.30 | 0.51 | 0.38 | 0.12 | 0.40 | 0.35 | 0.57 |
| 10] | 0.98 | 0.99 | 0.98 | 0.98 | 0.70 | 0.98 | 0.99 | 0.98 | 0.99 |
| 11] | 0.35 | 0.38 | 0.89 | 0.89 | 0.87 | 0.90 | 0.91 | 0.89 | 0.89 |
| 12] | 0.92 | 0.75 | 0.83 | 0.67 | 0.74 | 0.93 | 0.80 | 0.98 | 0.82 |
| 13] | 0.92 | 0.75 | 0.79 | 0.61 | 0.67 | 0.83 | 0.74 | 0.89 | 0.74 |
| 14] | 0.42 | 0.43 | 0.82 | 0.82 | 0.78 | 0.90 | 0.90 | 0.92 | 0.92 |
| 15] | 0 | 0 | 0.34 | 0.76 | 0.38 | 0.24 | 0.76 | 0.36 | 0.80 |
| 16] | 0.13 | 0.31 | 0.81 | 0.96 | 0.86 | 0.81 | 0.96 | 0.82 | 0.97 |
| 17] | 0.13 | 0.26 | 0.72 | 0.97 | 0.80 | 0.65 | 0.96 | 0.72 | 0.97 |
| 18] | 0.63 | 0.65 | 0.91 | 0.93 | 0.55 | 0.37 | 0.56 | 0.91 | 0.93 |
| 19] | 0.13 | 0.15 | 0.90 | 0.90 | 0.85 | 0.10 | 0.11 | 0.95 | 0.95 |
| 20] | 0.40 | 0.58 | 0.82 | 0.87 | 0.69 | 0.15 | 0.23 | 0.84 | 0.89 |
| 21] | 0.67 | 0.67 | 0.80 | 0.80 | 0.75 | 0.86 | 0.86 | 0.87 | 0.87 |
| 22] | 0.48 | 0.51 | 0.85 | 0.88 | 0.78 | 0.27 | 0.35 | 0.85 | 0.88 |
| 23] | 0.78 | 0.79 | 0.95 | 0.95 | 0.94 | 0.98 | 0.98 | 0.98 | 0.98 |
| 24] | 0.47 | 0.57 | 0.89 | 0.89 | 0.85 | 0.63 | 0.67 | 0.90 | 0.90 |
| 25] | 0 | 0 | 0 | 0.08 | 0 | 0 | 0.10 | 0.12 | 0.28 |
| 26] | 0.74 | 0.75 | 0.92 | 0.92 | 0.90 | 0.96 | 0.96 | 0.96 | 0.96 |

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