

**THE EFFECTS OF
PULSED STATIC AND OSCILLATING
MAGNETIC FIELDS ON RAT PINEAL
SEROTONIN N - ACETYLTRANSFERASE ACTIVITY**

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ABSTRACT

Concerns about the possible hazardous effects of electromagnetic fields (EMF's) has resulted in attempts to address this issue. Several authors have shown that EMF exposure affects biological systems and more specifically the pineal enzyme, serotonin N-acetyltransferase (SNAT), inhibiting the enzyme and thus melatonin production. The enzyme assay required, for performing this type of work is crucial, as the natural decay of the enzyme could bias experimental results.

An assay for measuring SNAT activity was assessed and then improved. The Ca^{2+} ion chelator, EGTA (2mM), preserved enzyme activity during pineal homogenisation. The enzyme is heat sensitive and thus keeping the homogenates on ice facilitated enzyme preservation. Not only was preservation of the enzyme crucial, but optimum substrate concentrations were required to measure maximum levels of SNAT activity. Maximum levels of activity were measured when 1/10th of a pineal gland was incubated with 12mM tryptamine. At the specified tryptamine concentration, SNAT activity increased as the concentration of Acetyl CoA increased. The enzyme efficiency, as determined from its K_m (8.19×10^{-4} M), and the level of activity exceeded those measured in other laboratories. This assay was used to determine SNAT activity following magnetic field exposure.

The magnetic field studies investigated the effects of pulsed static and oscillating magnetic fields on SNAT activity. Male Long Evans rats were housed in short and long photoperiods and were exposed for either 60mins., 30mins. or 15mins. to magnetic fields at different times during the dark phase. Rats were exposed to regularly repeated inversion of either the vertical or horizontal component of the earth's magnetic field or to regularly repeated horizontal or vertical $100\mu\text{T}$ DC field inversions. Pulsed static magnetic field exposure in all conditions had no significant inhibitory effect on SNAT activity.

Oscillating magnetic field studies investigated the effect of exposure of rats to magnetic fields tuned to ion parametric resonance conditions for Ca^{2+} . The ion parametric resonance model of Blanchard and Blackman (14) was used to determine the exposure parameters. Rats were thus exposed for an hour to a vertical AC field, of strength $14.3\mu\text{T}$ (rms), frequency was 17.2Hz while the strength of the vertical DC field was $22.55\mu\text{T}$, the existing vertical geomagnetic field strength. Under these conditions oscillating magnetic field exposure also had no significant inhibitory effect on SNAT activity.

ABBREVIATIONS

α	Alpha
AC	Alternating current
amps	Amperes
APUD	Amine Precursor Uptake and Decarboxylation
ATP	Adenosine triphosphate
AVT	Arginine vasotocin
β	Beta
Ca^{2+}	Calcium ion
CoA	coenzyme A
dB / dt	Rate of change in magnetic field per change in time
DC	Direct current
dpm	Decays per minute
DSIP	Delta sleep inducing peptide
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
ECF	Extracellular fluid
EGTA	Ethyleneglycolbis (aminoethyl ether) - tetra - acetic acid
EMF's	Electromagnetic fields
HIOMT	Hydroxy indole - O - methyltransferase
H.C.	Horizontal component
Hz	Hertz
hr	Hour
ICR	Ion cyclotron resonance
i.p.	Intra - peritoneal
IPR	Ion parametric resonance
kg / bw	Kilogram per bodyweight
L/D	Light / dark cycle
MF	Magnetic field
min	Minute
NE	Norepinephrine
nmol / p / hr	nmol of N - acetylatedtryptamine formed per hour per pineal gland
nT	nanoTesla
PKC	Protein kinase C
RHT	Retino - hypothalamic tract
rms	Root mean square
rpm	Revolutions per minute
secs	Seconds
SCG	Superior cervical ganglion
SCN	Suprachiasmatic nucleus
SNAT	Serotonin N-acetyltransferase
μT	micro Tesla
VC	Vertical component

CHAPTER 1

SEROTONIN N-ACETYLTRANSFERASE AND ELECTROMAGNETIC FIELDS

(REVIEW)

Modern technology and industrialisation has made life much easier and more entertaining with the advent of computers, satellites, high powered tools, television sets to name but a few. Most if not all of these advantages are made possible with the use of electricity. This power source could have deleterious effects on human health as a result of the development of an unnatural environment with bright lights at night and the generation of electromagnetic fields ("EMF's"). While concern by the public for safe electricity is the main driving force behind much of the research there are many other reasons for investigating EMF effects which are far more fundamental. Research has focused on the pineal gland responses to electromagnetic fields as it is believed to be responsible for "magnetosensitivity" (as initially illustrated in birds). Apart from the pineal gland other research is centred on Ca^{2+} metabolism, RNA and protein synthesis and tumourogenesis to name but a few.

The major hormone synthesised in the pineal gland is melatonin. Its biochemistry has become the centre of intense studies as it has been implicated in the inhibition of certain forms of cancer such as breast cancer (155) but also depression (176). Melatonin is produced in a circadian fashion with high levels being produced at night and low steady state levels during the day. The rate limiting enzyme involved in the synthesis of melatonin is serotonin N-acetyltransferase (SNAT) (8,57). Any perturbation of SNAT activity would thus alter the production of melatonin. There are many factors (discussed later) which affect the activity of SNAT but a great deal of emphasis has been placed on the effect of electromagnetic fields. Advances in scientific techniques and instrumentation have resulted in the rapid accumulation of data and information pertaining to the regulation of SNAT activity and thus melatonin synthesis.

This review will discuss the anatomy and physiology of the pineal gland and address the influence of various forms of EMF's on pineal biochemistry. The major emphasis, however, is centred on the effect of EMF's on SNAT activity.

1.1. INTRODUCTION

Pineal research can be divided into three periods of interest (154); 1): about 300BC the human pineal was discovered by Herophilus, but no concrete information and knowledge was gained during this period 2): from the middle of the 19th century to the present period which is characterised by interest in the study of comparative histology, anatomy and embryology 3): the past 40-50 years which has seen the rapid development in pineal endocrinology, pharmacology and biochemistry. During this period Lerner et al. (65) identified the major pineal hormone, melatonin, which earlier investigators thought was responsible for lightening of the skin of amphibians.

1.2. THE PINEAL GLAND

1.2.1. Structure

1.2.1.1. Submammalian anatomy

Very little is known about the physiology and biochemistry of the pineal gland of submammalian species as most of the work has focused on that of mammals. This subject was addressed in an earlier review (110) and this section only attempts to outline and summarize some of the points made. The pineal is unique in both morphology and phylogeny which makes the study of this gland and its functions complicated. These variations are seen not only between species but also within species. The pineal gland of fish, amphibians and lacertilian reptiles are mainly photoreceptive structures with epithalamic outgrowths to assist in its function (110). The pineals of fish are also capable of producing indoleamines and in some cases show an affiliation with reproduction (110). Birds, turtles and snakes, on the other hand, have lost this photoreceptive capability (110). The pineal gland of birds has been shown to have secretory and photoreceptive elements and thus appears to be an intermediate between the pineal of submammalian animals and mammals (110).

It is at this point that we observe a digression from submammalians as the pineal gland of birds is innervated and shows similar biochemistry to that of mammals.

1.2.1.2. Mammalian anatomy

Location and structure :

The pineal gland has been referred to by several descriptive names but the most recent and probably most appropriate is that of a neuroendocrine transducer which is synchronised to the light-dark cycle (8, 178, 179). The pineal has several unique features. These include being part of the central nervous system yet lying outside the blood brain barrier and receiving sympathetic and central innervation (110). The pineal gland is highly vascularized; its arterial blood supply is via the posterior choroid arteries and the blood is drained by the internal cerebral veins which run dorsally from the pineal (110). The gland is in contact with two recesses of the third ventricle and thus consists of the pineal recess and the dorsal suprapineal recess (110). It is located between the posterior commissure and the habenular and is attached to the posterior roof of the third ventricle (110).

Histology :

There are two cell types in the mammalian pineal gland: parenchymal (pinealocytes) and interstitial cells. The pinealocyte is the major cell type as it is the factory for melatonin production. Pinealocytes show the characteristics of a paraneuron (as noted in 150) and a cell belonging to the amine precursor uptake and decarboxylation series (APUD) (154) which would be expected if the cell is involved in the synthesis of melatonin from tryptophan. The main portion of the pinealocyte is the perikaryon with one or two processes which end close to the precapillary space or interstitial lacunae. Serotonin (5 - Hydroxytryptamine) has been identified in pinealocytes, interstitial cells and sympathetic nerve endings of the pineal further substantiating the synthetic role of the pineal (89). Synaptic ribbons and dense cored vesicles have also been identified in the pinealocyte which are associated with a secretory function (110).

The pinealocytes have been divided into two groups; those predominantly under the influence of central commissural fibres and responding to olfactory, acoustic and short term optic stimuli while the second group is exclusively under the influence of sympathetic fibres from the superior cervical ganglion (SCG) (154).

The peripheral fibres are postganglionic and release norepinephrine (NE) but its activity increases after dark onset in response to acetylcholine (110). The interstitial cells are not involved in the production of melatonin and will not be dealt with in this review.

The human pineal gland:

The human pineal has a similar morphology and innervation to that of lower mammals (154). The synaptic terminal of the sympathetic fibres from the SCG form synapses in the perivascular region and so innervate the pineal (154). These junctions vary from one species to another and it is possible that the type of synaptic junction determines the accessibility of the pinealocytes to the neurotransmitters released from the SCG (110). The human pineal undergoes significant morphological changes during early childhood. There are two groups of cell types distinguishable at birth (82), type I and type II. At birth the type I cells (glial cells) are dominant. However after 1 year the type II cells (pinealocytes) are dominant (82). The glial cells, which are mainly fibrous astrocytes, are fewer in number. The two cell types are easily distinguishable as the pinealocytes have a large nucleus and nucleolus with several cytoplasmic processes. The glial cells on the other hand have a smaller nucleus.

1.2.2. Pineal and environmental light

It is well established that photosensory information at the retina is transmitted to the pineal via a complex neural path (154). Sensory information from the retina is conveyed via the retinohypothalamic tract to the suprachiasmatic nucleus (SCN) (154) and some information from the primary optic tract also reaches the SCN via the lateral geniculate nucleus (154).

Since the retina responds to light / dark it is not hard to understand that the retinohypothalamic tract is responsible for mediating the L/D entrainment of the SCN (89). From the SCN the signals are conveyed to the lateral hypothalamus via the medial forebrain bundle (87). The path continues over the reticular formation and upper thorax and passes through the intermediolateral column to synapse on the superior cervical ganglion (SCG) (18).

Postganglionic sympathetic fibres then travel along the tentorium cerebelli and enter the pineal via the nervi conarii (25). Recent studies however suggest that there may be direct synaptic communication between the brain or habenular nucleus and the pineal gland (83), but more studies need to be done in this area.

It follows from this that light and the duration thereof might be the main initiator of seasonal behavioural activities which are influenced by the circulating levels of melatonin. There are some flaws, however in this trend of thought, which will be discussed. No known photoreceptors, exist in adult mammals, other than the retina but this is apparently not the case in young rats and maybe other young mammals. Light influences the rhythms of pineal melatonin production for up to one month after birth in blinded new-born rats (89). The path of information transfer in humans is less well defined, but a study of the melatonin rhythms in quadriplegics (53) suggests that the pineal could be regulated by sympathetic nervous connections.

1.2.3. Functions

The pineal gland, or more specifically melatonin, has been implicated in many physiological functions as reviewed by Ebadi (25). However its functioning is sensitive to external influences such as day-night cycles, season-to-season variation, physiological state (pregnancy, development, maturation) and stress conditions which affect its activity (154).

The pineal gland is responsible for the synthesis of melatonin and several other indoleamines which are released via diffusion. Melatonin is a lipid soluble circulating indoleamine and is taken up by all organs, including the brain, as there is no effective blood brain barrier (178, 179). Melatonin is considered to be responsible for mediating most of the functions attributed to the pineal gland (less is known about the functions of other indoleamines). It has direct cellular effects on the hypothalamus (87), gonads (87), pituitary gland (111) by affecting the production of the releasing factors for anterior pituitary prolactin, luteinising hormone and follicle stimulating hormone.

The pineal gland and melatonin have been implicated in immune function (75, 76) since ablation of the pineal gland results in immunosuppression (4). *In vitro* studies have shown that melatonin decreases the proliferation of oestrogen - sensitive breast cancer cells (70). These results corroborate claims linking different forms of cancer and childhood leukaemia to EMF exposure, which has in turn been shown to inhibit melatonin production (50, 51). Melatonin is also thought to affect aging. This theory stems from the fact that nighttime administration of melatonin to rats resulted in a prolongation of their life-span (76). The pineal is also involved in hibernation and thermoregulation (53). Changes in external temperatures can influence pineal activity resulting in changes which would in turn influence the core body temperatures. Glucose and energy metabolism, food intake, porphyrin content of the Harderian gland (41), G.I.T, kidney, liver function, hair colour and nerve growth could also be influenced (154). Several other functions have been allocated to the pineal. Most of the functions are effected by melatonin. Some of these effects are listed in the table provided by Ebadi (25). The pineal gland is thus of considerable physiological importance. The possibility that its function is sensitive to EMF's is important as suppression of melatonin production could have deleterious effects on the health of the individual.

1.2.4. Pineal biochemistry

1.2.4.1. Melatonin biosynthesis

Melatonin is the major known hormone synthesised in the pineal and its synthesis, regulation and effects have been studied in great detail. Fig. 1.2.4.1. illustrates the biochemical path by which melatonin is synthesised in the pineal gland.

The melatonin precursor, tryptophan, is actively transported across the pinealocyte membrane where it is hydroxylated at position 5, by tryptophan hydroxylase, to form 5-hydroxytryptamine. The latter is decarboxylated to form 5-hydroxytryptamine (serotonin) using pyridoxyl phosphate as a co-factor (25). The concentration of serotonin during the light period is higher than that found in any other organ (or brain region) analysed (25), with a concentration of 0.5mM but it drops drastically during the dark period when it is converted to N- acetylserotonin by serotonin

N-acetyltransferase (SNAT).

N-acetylserotonin is then methylated via hydroxyindole-O-methyltransferase (HIOMT), an S-adenosylmethionine requiring enzyme, to form melatonin.

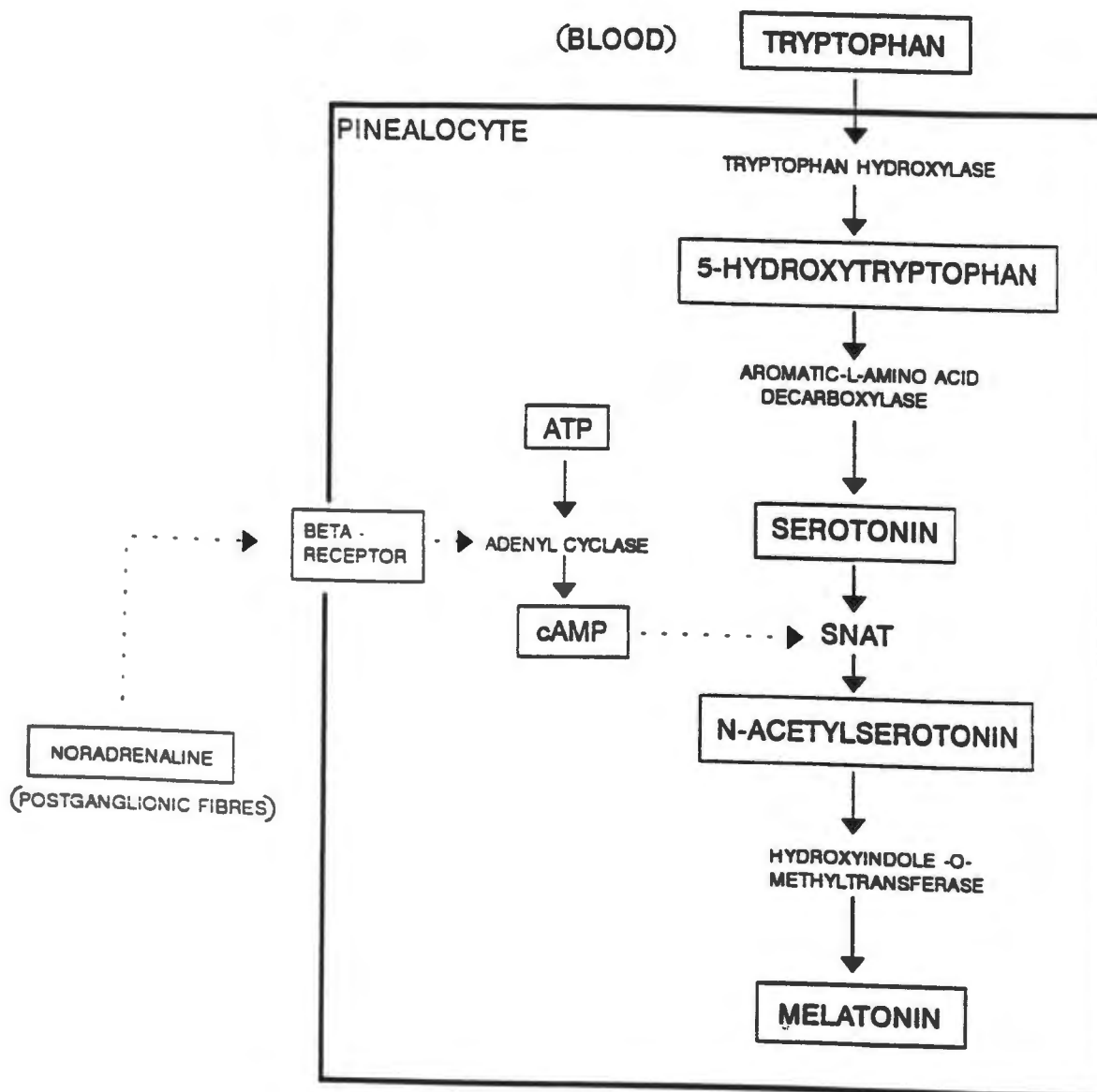


Figure 1.2.4.1. : Biochemical path for melatonin synthesis (25).

1.2.5. Pineal secretions

1.2.5.1. Melatonin

The purpose of this section is to provide an overview of melatonin functions and to comment on other active agents released by the pineal gland.

Melatonin (N-acetyl-5-methoxytryptamine; mp: 116-118°C) is the major indoleamine. It is of great physiological significance as it mediates most of the biological functions attributed to the pineal gland (182).

The melatonin generating system could be established before birth (56), however melatonin formation does not occur in neonatal rat pineals (17) as the enzyme HIOMT appears only in the second week of development. Pineal and serum melatonin levels exhibit a distinctive peak during the scotophase of the diurnal cycle in all vertebrates (42). Melatonin levels are low during the day. The synthesis of melatonin is regulated by the release of norepinephrine (NE) (at night) which interacts with β - (and in some cases α -) receptors resulting in a cascade effect. This results in increased SNAT activity which is the rate limiting step involved in the production of melatonin. Melatonin is not stored but is secreted into the bloodstream and this is seen in the serum levels that are higher than those in the lamellar or ventricular cerebrospinal fluid (150). There is no effective blood brain barrier for melatonin which is taken up by all organs. Seventy percent of the circulating melatonin is weakly bound to albumin (102). Even though membrane bound melatonin receptors have been identified, melatonin is still able to diffuse through the membrane and could affect biochemical reactions directly such as modifying cell calmodulin levels. Melatonin receptor numbers also vary in different tissues and at different times during the light / dark (L/D) period. Peak numbers occur during the early part of the dark phase. The levels then decrease and reach a steady state during the light phase. This variation in melatonin receptor numbers has given rise to the idea of receptor down- / upregulation (111), with the result that as the circulating levels of melatonin increase so the number of receptors decrease with a resultant attenuation of response to the hormone and visa versa. The pineal is not the only source of melatonin since its ablation fails to eliminate melatonin in the circulation (66). Furthermore, melatonin has been identified in alligators and armadillos (130) which do not possess a pineal gland. Melatonin has been demonstrated in the hypothalamus, brainstem, SCN, Harderian gland, retina and intestine (133) in some mammals the retina may be the main source of melatonin (108).

The hormone generally has suppressive effects on other endocrine glands, is believed to decrease tumourogenesis in animals, has antigonadotrophic effects (not consistently) (111) and its reproductive effects have been extensively studied and summarised (65, 112). The adrenal and thyroid glands are also targets of melatonin (145), it affects locomotor activity (109), acts on neurones which utilise serotonin as a neurotransmitter (6), and is implicated in immune responses (75, 76).

1.2.5.2. Arginine vasotocin

Arginine vasotocin (AVT) is a peptide hormone which was first isolated from bovine pineal extracts in 1963 (81). While present in some mammalian species, the extent of its distribution remains to be determined. It is normally synthesised in the pituitary gland of lower animals. Bovine AVT is synthesised in the pineal recess and subcommissural organ and is stored in undefined cells in the pineal gland (105).

Its amino acid sequence shares a high degree of identity with arginine vasopressin as it differs only by one amino acid residue with an isoleucine instead of 3-phenylalanine. It also differs from oxytocin in that it has an arginine residue instead of 8-leucine. As far as its functions are concerned, AVT (synthetic) is similar to melatonin in terms of its effector targets but is more potent than melatonin in several functions such as sleep induction and anticonvulsant effects. AVT increases rapid eye movements during sleep episodes (16). The peptide is also believed to act via serotonergic pathways (106) and could possibly interfere with the release of serotonin at postsynaptic sites and in addition it has been implicated in delaying brain maturation (26). Another crucial function (possibly) is its link with the inactivation of SNAT activity as discussed in section 1.3.4.6.

1.2.5.3. Other hormones and chemical factors

Several tripeptides have been isolated from the pineal gland and characterised by Orts and co-workers (100), as having antigonadotrophic activity. A nonapeptide, delta-sleep-inducing peptide (DSIP) which like melatonin, also exhibits a circadian rhythm (29) which parallels that of corticosterone.

Exposure to constant illumination inhibits its activity or reduces its level. The link between DSIP and corticosterone is as yet of unknown significance. Intravenous injection of DSIP results in a decrease in the nocturnal peak in SNAT activity (35) and thus it may play a role in the regulation of SNAT activity. Luteinizing hormone releasing hormone peptides have also been isolated from ovine pineal extracts. Several methoxyindoleamines have also been found in the pineal gland, some of which are: 5-methoxytryptophan, 5-methoxytryptamine, 5-methoxyindoleacetaldehyde, 5-methoxyindoleacetic acid, 5-methoxytryptophol, O-acetyl-5-methoxytryptophol. Other indoleamines synthesised in the pineal gland include: 5-hydroxyindoleacetaldehyde, 5-hydroxyindolacetic acid, 5-hydroxytryptophol and O-acetyl-5-hydroxytryptophol. The functions of most of these indoleamines are unknown. Other chemical compounds in the pineal gland such as taurine, S-adenosyl-L-methionine and S-adenosylhomocystein may also be involved in the regulation of melatonin synthesis. Taurine levels rise and reach a maximum level in the late dark period (36) and has a stimulatory effect on SNAT activity (173), but since it is in competition with NE. S-adenosyl-L-methionine stimulates melatonin synthesis but when the amino group interacts with pyrodoxyl phosphate (183), then it loses this ability. S-adenosylhomocystein also has a stimulatory effect on indole metabolism but inhibits HIOMT activity (60). The neurotransmitter gamma amino butyric acid (GABA) inhibits NE induced stimulation of SNAT activity (24). Pteridines, are light sensitive chemicals and derivatives thereof have been isolated in ovine pineal extracts (161). Their production may be regulated by an adrenergic cAMP-dependent mechanism. These pteridine derivatives have an inhibitory effect on HIOMT activity, but the effects depend on the time of year of administration (26).

1.3. SEROTONIN N-ACETYLTRANSFERASE

1.3.1. General characteristics

Two different forms of acetyltransferases can occur in the same animal.

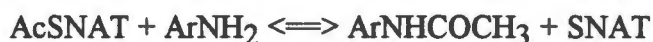
The enzyme of relevance to this review serotonin (arylalkylamine) N-acetyltransferase (SNAT) is located in the pineal gland, retina and liver. SNAT is a cytosolic protein with a molecular weight of about 10 kDa (as in 182). It is unstable in its pure form and has not yet been crystallised and thus cDNA clones cannot be made. Hence the exact structure of this enzyme is unknown. It is for these reasons that molecular biological studies of the enzyme are incomplete and little is known about its regulation, expression and activity. However SNAT activity undergoes a circadian rhythm with dark period levels increasing up to 100 times that of daylight levels (42). It is known that this increased activity requires new mRNA synthesis which occurs within about 90 minutes after the start of dark stimulated noradrenergic (57) activation of pinealocytes.

1.3.2. Function

SNAT is responsible for the acetylation of serotonin to form N-acetylserotonin. The mechanism involved in the acetylation of serotonin is catalysed by a covalent acetyl coenzyme A (74) as shown earlier.

1.3.2.1. SNAT as the rate limiting enzyme

SNAT is the rate limiting enzyme involved in the synthesis of melatonin (8). It is responsible for the N-acetylation of serotonin to form N-acetylserotonin. This path is the major synthetic route for the synthesis of melatonin as SNAT and HIOMT activity have been shown to increase at night. This was confirmed when Reiter et. al. (114, 120) who showed that a 1min. light pulse inhibited SNAT activity and subsequently melatonin production irrespective of the levels of serotonin. SNAT transfers an acetyl group from Acetyl CoA to the amino group of serotonin probably utilising the "ping-pong Bi-Bi" mechanism as described by Hanna (40) (for most N-acetyltransferases) as illustrated in the reaction equation below. This means that the reaction requires 2 substrates, yields 2 products and one or more products are released before all substrates have been added.



Tryptophan and serotonin levels increase during the light phase as a result of the low SNAT activity (57) but serotonin levels decrease during the dark phase (178, 179) when SNAT activity is at its peak and when NE stimulation is maximal.

1.3.3. Rhythmic activity

SNAT activity exhibits a circadian rhythm with low levels occurring during the light period and up to a 100 fold increase during the dark period. This rhythm is well established in all animals studied whether active at night or during daylight. SNAT levels reach their peak values at night about 4-5 hours after lights off yet a single 1min. light pulse (114) can decrease SNAT activity to daytime levels and thus can offset the circadian signals. Towards the end of the dark phase, SNAT levels decrease prior to the onset of light. This could possibly be due to the effect of some chemical agent, synthesised (21) in the pineal, which acts as an enzyme deactivator. Light also has a phase delaying effect while melatonin administration at night has a phase advancing effect (reviewed in 182) on peak SNAT activity at night, but light is an overriding factor compared to melatonin.

Light is not, however, the sole regulator of the pineal circadian activity, since blinded rats are initially synchronised to the L/D cycle with respect to SNAT activity. This would indicate that there might be some inherent biological clock regulating its activity. The pineal and its rhythmic activity is not rigidly controlled by any one variable as it has been shown to exhibit: ultradian (<24hrs.) (125), infradian (>24hrs.), circannual (seasonal) (referred to in 183) and oestrus cycle rhythms (111) which confirms that there may be many innate synchronisation mechanisms. Temperature is another factor involved in pineal activity as cold ambient temperatures have been shown to decrease the sensitivity of the pineal to melatonin (183). This would result in higher levels of melatonin being produced. Seasons of the year also play a role in regulation of SNAT activity. Rats examined during winter have greater nocturnal melatonin production as opposed to those examined during spring (see reference 26).

1.3.4. Regulation

1.3.4.1. Light

Light is perceived by the retina and acts on the SCN via the retino-hypothalamic tract (RHT) leading to modifications of the "clock" function of the SCN. Action potentials are then transmitted via the medial forebrain bundle and upper thoracic spinal cord where preganglionic fibres innervate the SCG. The postganglionic sympathetic nerve fibres terminate on the pinealocytes and release NE which stimulates the α - but more specifically β -receptors on cell membrane (88, 104). This activates adenylate cyclase, resulting in increased cAMP production and increased SNAT activity. NE is the major neurotransmitter but it is possible that other neuropeptides may be involved in the regulation of melatonin production (see below). The message for increased melatonin production thus originates in the SCN when the neurones are relieved of the inhibitory effects of retinal activity as light stimulation decreases. In these lower animals (fish, amphibians and birds) the pineal is photosensitive *in vitro* (12) as reflected in the variation of the levels of SNAT activity in light and dark environments. In higher animals such as rats photosensitivity does not reside in the pineal gland (8). Rhodopsin could mediate susceptibility of the pineal to light as photosensitivity has been reported in chick pineal glands. It has been shown that 11-cis retinal undergoes a conversion to all-trans retinal in chick pineals which could explain how pineal glands could be directly sensitive to light. This could also explain the persistence of a circadian rhythm in cultured chick pineal glands (as reviewed in chapter 1 of 182). The eyes are thus not crucial in all animals for the control of SNAT activity. This may be the case where the pineal is located near the surface of the body, as in the case of amphibians. The sensitivity of the pineal gland to light varies among species and is highly species specific. Syrian hamsters have been shown to respond to light intensities of 0.1mW/cm^2 and albino rats respond to a lower light intensity (74, 166). The cotton rat has greater sensitivity compared to the Syrian hamster (mentioned in 182). Since SNAT is the rate limiting enzyme involved in the production of melatonin it is quite probable that alterations in melatonin is a result of alterations in SNAT activity.

The duration of the dark period to which rats are acclimatised also affects pineal melatonin production. The duration of the melatonin peak is longer during long dark periods and shorter for short scotophase. Extension of light into the dark period suppresses SNAT activity and therefore the rise in nighttime melatonin levels (101). However not all wavelengths of light affect pineal SNAT activity as neither red nor yellow light has been shown to decrease pineal SNAT activity (17). For example blue light ($\lambda = 500\text{nm}$) has a greater inhibitory effect on pineal melatonin production in the Syrian hamster. The order of effectiveness of light wavelengths which inhibit pineal melatonin production has been demonstrated to be blue > green > yellow > ultraviolet > red (17) for a given period of time. Since light affects pineal melatonin and SNAT it is inevitable that L/D cycles or photoperiodicity also affects the regulation of melatonin production. Constant darkness results in a free run and persistence of a circadian rhythm in serum melatonin levels in sheep and horses (129). Constant light has been shown to result in arrhythmia in chickens (10). Reversal of the normal photoperiods also affect melatonin production as has been demonstrated in rats. Reversal causes a depression of middark melatonin synthesis and secretion to that of midlight levels for 2 days after the reversal. Adaptation to a reversed L/D cycle occurs within 4 days (5). Light is thus a crucial factor in the regulation of pineal melatonin production. Specific characteristics of light however dictate to a large extent the response of the pineal. This should also be kept in mind when considering EMF effects on pineal activity as specific EMF characteristics might affect pineal function while others may not.

1.3.4.2. Receptors

Two types of receptors are involved in the regulation of SNAT activity in the pinealocyte. The most important is the β -adrenergic and to a lesser extent the α -adrenergic receptors. These respond to NE released from postganglionic sympathetic nerve endings which arise in the SCG. Both α - and β -receptor densities but not their affinity exhibit circadian rhythms similar to that of SNAT activity (154, 182).

Continual stimulation of the receptors results in receptor downregulation and a decrease in SNAT activity (111). It has been demonstrated in humans that β -receptors respond to NE, since propranolol, a β -receptor antagonist, inhibits the nocturnal increase in circulating melatonin (162). Stimulation of α -2 adrenergic receptors in certain species also gives rise to an increase in cAMP and hence SNAT (183). However in humans α -2 receptors seem to mediate an inhibitory effect as clonidine, an α -2 adrenoreceptor agonist, reduces nocturnal plasma melatonin (see 182). Receptors and their functions vary with age in that α -1 receptors are more sensitive to agonists in young rats as opposed to that of adults. Thus it may be more important in regulating SNAT activity during development compared to β -receptors which are less sensitive to agonists during early development (17). The reverse is true once the animal reaches maturity. However a recent study in which the authors measured K^+ efflux from the pineal cell (21), both the α - and β -receptors were found to be involved in this process which resulted in hyperpolarization of the cells. This hyperpolarization is thought to be involved in the stimulation of SNAT activity (57). Other receptors located on pinealocyte membranes are GABAergic, glutamate, D1 & D2 Dopaminergic, muscarinic cholinergic and Serotonergic (25). The functions of these receptors are as yet not fully understood. Vasoactive intestinal peptide (VIP) receptors have also been identified on the membrane of pinealocytes and their activation results in the stimulation of cAMP and cGMP production (184).

1.3.4.3. cAMP

Stimulation of SNAT activity is mediated by the second messenger cAMP. Ca^{2+} potentiates an increase in cAMP by stimulating adenylate cyclase. The increase in cAMP levels in the pinealocyte results in a 30-100 fold increase in SNAT activity but this activation requires protein synthesis (directly or indirectly), since D-actinomycin blocks adrenergic stimulation of SNAT activity (17). This indicates that cAMP affects not only the level of translation but maybe also transcription resulting ultimately in increased SNAT activity. This process is believed to take about 90min. cAMP derivatives are evidently also involved in the stimulation of SNAT activity. A derivative of cAMP is 5-AMP and inhibition of its synthesis from cAMP results in

decreased SNAT activity (99). cAMP also activates protein kinases in the cell resulting in stimulation of SNAT activity; further details of which are not clear (57). Low concentrations of Lithium have been shown to inhibit SNAT activity by inhibiting cAMP production (185). cAMP also acts in collaboration with Ca^{2+} (see section 1.3.4.4) to ensure the hyperpolarization of the pinealocyte keeping SNAT in its reduced and therefore active form (57). Thus cAMP plays a major role in the activation of SNAT activity. However not much is known about the mechanisms of action.

1.3.4.4. Cations

Several ions are involved in the regulation of SNAT activity, but Ca^{2+} ions are dominant in this regard. The intracellular Ca^{2+} concentration is increased by α -1 receptor stimulation. This in turn causes the activation of protein kinase C (PKC) which enhances the β -adrenergic stimulation of cAMP. Thus the effect of stimulation of the β -receptors by NE is potentiated. This system of activation is the basis for the PKC/G-protein "AND" gate mechanism i.e. requiring concurrent activation by two inputs to ensure maximum stimulation (21) of SNAT activity. In keeping with the "AND" gate mechanism electrophysiological studies of Cena et. al. showed that the increase in Ca^{2+} concentration stimulated K^+ efflux from the pinealocyte (referred to in 21). The resulting hyperpolarization is believed to keep SNAT in a reduced and therefore active form. Tests using K^+ channel blockers (tetraethylamine - 10mM & scorpion venom - 10ng/ml) confirmed that K^+ channels specifically were activated by Ca^{2+} . Cell attachment studies were used to show that NE acting via intracellular mechanisms mediated this effect. The study also revealed that an increase in Ca^{2+} from 10nM to 1mM at the inner face of the membrane also increased the frequency of opening of the K^+ channels. A rise in intracellular Ca^{2+} is responsible for the conversion of protein kinase C to an activated form, which in turn activates adenylate cyclase. This could compliment the other actions of Ca^{2+} as it has already been shown that cAMP or bits of its catalytic subunit increases the activity of $\text{K}(\text{Ca})$ channels in other systems (28). Ca^{2+} and Mg^{2+} together have been shown to stimulate SNAT activity (at a molecular level) (85) by binding to the enzyme, so increasing its

affinity for tryptamine which binds to the enzyme prior to acetyl-Co A (in rats). Thus Ca^{2+} can activate SNAT directly and is clearly a crucial factor in the regulation of SNAT activity. Ca^{2+} can also have inhibitory effects on SNAT. The pineal glands of cardiomyopathic hamsters have deficient Ca^{2+} pumps which result in accumulation of intracellular Ca^{2+} but a decrease in SNAT activity (122). This is possibly caused by the induction of an inhibitory molecule at relatively high Ca^{2+} levels (21). Intracellular Ca^{2+} levels are usually 10000 times lower than in the extracellular fluid (ECF) which is achieved via intracellular storage mechanisms and efflux pumps (122). A slight influx of Ca^{2+} will affect the cells biochemistry. Rodriguez-Cabello et al. (128) have shown, however, that ethyleneglycolbis (aminoethyl ether) - tetra - acetic acid (EGTA), a Ca^{2+} specific ion chelator, prevents the loss of SNAT activity during tissue homogenisation and incubation. This would seem contradictory to the point made above that Ca^{2+} stimulates SNAT activity. It is probable, however, that homogenisation exposes the enzyme to exceedingly high concentrations of Ca^{2+} which would be toxic to the enzyme. Thus it is apparent that Ca^{2+} is essential to the regulation of SNAT activity and other di- and monovalent ions potentiate its effects.

1.3.4.5. Inactivation

How SNAT activity is suppressed or inhibited towards the end of the dark period is unknown. It is clear, however, from many reports that its high nocturnal level of activity declines sharply, well before the onset of the light period. It has already been speculated that some compound is formed in the pineal at the end of the dark phase which is responsible for the inactivation (21) of SNAT. However the details of this process are not known.

Another possible deactivation mechanism for SNAT is protein thiol : disulphide exchange (91, 92) which causes a rapid decrease in enzyme activity in intact cells. It has been speculated (57) that cAMP could control another enzyme within the pinealocyte which is responsible for the conversion of a pool of free thiols into disulphides which then cause SNAT inactivation. This mechanism is supported

by the fact that rabbit liver SNAT showed the loss of a single cysteine residue after being treated with an active site directed irreversible inhibitor (4). Further evidence to support this theory of thiol : disulphide exchange is the discovery that pineal SNAT is inactivated by disulphide containing peptides such as AVT and cystamine and that this effect is reversed when treated with dithiothreitol (93). Not all disulphide containing peptides have this efficient inactivating ability. Penicillin disulphide and diacetylcystamine are less effective, the reason for which is not clear.

Membrane potential changes (104) could also be involved in the activation / inactivation of SNAT activity, since activation of SNAT activity requires hyperpolarization of the cell membrane. The significance of the hyperpolarization is not yet clear. The mechanism is mediated by the cAMP / Ca^{2+} activation of K^+ channels which would result in a change in some transmembrane kinase or reductase to keep SNAT in an active state (57). Furthermore Morton (85) showed that, in addition to Ca^{2+} , K^+ and Na^+ also increase SNAT activity. Morton however goes on to suggest that an excess of these cations would inhibit SNAT activity which like Ca^{2+} could be activating a SNAT inactivation molecule. This theory conflicts with the proposal that hyperpolarization maintains SNAT activity and more work needs to be done in order to clarify this matter.

Contrary to most studies it has been speculated that cAMP and NE are capable of inhibiting SNAT activity depending on the time of year (13) and substrate concentration, but the mechanisms are not clear (152, 153). It is thus evident that regulation of SNAT activity is complex and requires more research. The complexity is aggravated by the fact that variation in results with respect to different species is significant. Apart from this variations (96), time of day, seasons, gender, age and physiological status affects adaptive mechanisms of the pineal and so too SNAT activity (26, 154).

1.4. ELECTRIC AND MAGNETIC FIELDS

1.4.1. Electromagnetic fields (EMF's)

Electromagnetic fields consist of two components namely electric fields (EF's) and magnetic fields (MF's). Visible light forms part of the electromagnetic spectrum which consists of electromagnetic waves each with its characteristic wavelength. The spectrum of electromagnetic radiation ranges from 10nm to 10^5 km. The spectrum can be divided into an ionisation and non-ionisation region. EMF's of wavelengths less than 380nm can cause molecular ionisation (182). Molecular vibration, induced charge flow and molecular rotation occurs with exposure to longer wavelengths (see 182). Wavelength can also be expressed in terms of frequency which is the number of waveforms which pass a given point in 1sec. Electric fields (symbol E; SI unit V/m) are present whenever differentially charged particles (ions) which are usually interdependent or charged masses are located in different places. From this it follows that between a power line and the earth's surface an electric field exists. Magnetic fields (symbol B; SI unit T (Tesla)) originate from permanent magnets or as a result of charged particles that have been moved. Every electric field generates a magnetic field (with a proportional flux density). Changes in magnetic field density per unit time (dB/dt) generates an electric field which can induce electric currents (182). This results in the generation of eddy currents in conducting matter, hence electromagnetic fields.

During the project animals would be exposed to static MF's and 50 / 60Hz oscillating fields as those are the prevailing frequencies in the environment. Artificial MF's can be generated by using a pair of Helmholtz and Merrit coils which is connected to a power supply. This provides a means of generating homogenous MF's with a desired field strength.

1.5. EMF's AND THE PINEAL GLAND

1.5.1. Introduction

Naturally occurring electric field potentials seldom exceed 100mV while the MF strengths are in the femtotesla range.

Whether artificial electric and MF's could affect biological systems is of interest as it could pose a health risk. Several studies have been undertaken to attempt to address this issue. The results obtained from these studies demonstrated that EMF's affect pineal physiology and biochemistry and more specifically the production of melatonin. Several studies have however failed to demonstrate an EMF effect on melatonin production (33,47). The reasons for the contradictory evidence could be due to the variations in animal species, age of the animals, seasonal changes and experimental exposure conditions. A consequence of the EMF effect on melatonin production is the increased risk of breast cancer (154, 154a, 155, 162). Stevens proposed the melatonin hypothesis to explain the connection between EMF exposure and breast cancer. It has been hypothesized that low frequency EMF exposure reduces nocturnal pineal melatonin production (141). This results in increased oestrogen and prolactin production and causes an increase in the proliferation of breast epithelial stem cells (154a). This is confirmed by the fact that 60Hz EMF's block melatonin's oncostatic effect on oestrogen positive breast cancer cells (70). The suppressed immune response as a result of EMF exposure (75, 76) to tumor formation leads to the increased risk in breast cancer.

1.5.2. The characteristics of EMF's that affect pineal activity

1.5.2.1. The magnetic field

The pineal is believed to be involved in the detection of MF's for several reasons (as proposed in reference 144) : 1) The gland is involved in the regulation of circadian rhythms which can be prevented from phase shifts and can be influenced by artificial MF's. 2) The pineal is under the influence of sympathetic innervation. The sympatho-adrenergic system is sensitive to MF stimuli. This means that MF's could affect pineal activity via its neural connections. 3) The pineal is a time keeping organ as it is sensitive to L/D rhythms which makes it suitable for being part of a compass - solar - clock system for maintaining orientation in birds. These observations were strengthened by the fact that the electrical activity of pinealocytes was depressed by

exposure to a DC MF but was restored on removal of the stimulus (144). Another study which laid the foundation for the present investigations showed a decrease in electrical activity in the pineal of both the guinea pig and pigeon in response to a change in direction and intensity of the natural MF. More specifically the authors showed that inversion (using Helmholtz coils) of the vertical component of the natural magnetic field resulted in a decrease in SNAT activity (143). There are two types of MF's to consider namely static and oscillating MF's.

1.5.2.1.1. Static Magnetic fields

Static MF's are time invariant and influences biological systems only at field intensities of 1T or greater. However effects have been shown to occur at lower field strengths when the direction of the field is changed suddenly. Thus inverting the horizontal component of the earth's natural MF caused a decrease in pineal cAMP (132). A decreased cAMP could be translated to a decrease in SNAT activity as discussed above (1.3.4.3.). The study by Welker et. al. (169) showed that alterations of the local geomagnetic field decreased SNAT activity. Helmholtz coils were used to invert the horizontal component of the earth's MF at night. A decrease in SNAT activity and melatonin production was measured at 15min. and up to 2hrs after a single field inversion. However exposure of the animals to the inverted MF for 24hrs had no lasting effect on SNAT activity. When the field was switched off there was a renewed depression of SNAT activity suggesting that the inversion *per se* is not responsible for the observed effect but rather its change in direction. This effect was observed at inclinations varying from 58-78° for a 30min. exposure period (97). Olcese et. al. (96) have shown that 50° rotation of the horizontal component of the earth's MF significantly decreased SNAT activity and melatonin production in Long Evans (hooded) and Sprague-Dawley rats. These rats were exposed for 30min. at night to the altered MF. However no effects were observed in blinded rats. From this they concluded that the eyes (retina) mediated the MF effects on SNAT activity. Richardson et. al. (127) also showed that pulsed static MF's resulted in inhibition of SNAT activity *in vitro* after pineal glands were exposed to isoproterenol (which stimulates SNAT activity). The MF's (40 μ T) were inverted at 1min. intervals for 1

and 2hrs respectively. Decreased SNAT activity was observed after 1hr. Yaga et. al. (180) also showed that exposure of rats to pulsed static MF at 1 min. intervals for 45min. during the dark phase decreased SNAT activity and melatonin production *in vivo*. Lerchl et. al. (62) exposed rats to pulsed fields by repeatedly inverting the horizontal component of the earth's MF ($40\mu\text{T}$) at 1min. intervals. This was achieved with a pair of Helmholtz coils which were connected to a power supply and the flow of current in the coils were inverted either automatically or manually. Automatic inversions of the geomagnetic field occurred rapidly (within 25msec.) while manual inversion of the geomagnetic field occurred over a 1sec. period. A reduction in SNAT activity occurred only in pineals of rats exposed to automatically inverted fields. These findings suggested that induced eddy currents generated during the rapid switching process caused the effects. These results were confirmed in studies conducted by Richardson et. al. (126) which demonstrated that only in animals exposed to both inverted MF's and induced eddy currents was there a decrease in SNAT activity and melatonin production.

From the work done thus far it is apparent that MF effects are a consequence of at least two interacting factors. The change in the static MF and the induced eddy currents are crucial in mediating MF effects. The switching mechanism used when investigating the effects of static MF's thus needs to be specified. This was not the case in earlier studies performed and thus the effect of the rate of current flow inversion cannot be assessed. The time of day / night of exposure could also be relevant as the MF effect depends on photoperiod. The pineal was shown to be more sensitive to MF effects later into the dark period as opposed to the early dark period (180). Cutaneous pigmentation could prove to be another factor affecting pineal sensitivity. Albino gerbils responded and were sensitive to MF's while pigmented members showed no response to a 60° rotation of the horizontal component of the earth's MF (151). This would suggest that loss of pigmentation increases sensitivity to light and MF's. Apparently static MF effects are not confined to a specific strain of rat. However more evidence is needed about species specificity and further studies are required to elucidate the precise mechanism by which static MF's affect SNAT activity.

It will also become clear as more work is done concerning the static MF's that the precise means by which MF's affect SNAT activity will be elucidated. Static MF's have been shown to decrease SNAT activity by 20-50% but the description of the exposure conditions in each experiment was not always clear making it very difficult to repeat the experiment exactly. The problems encountered are discussed in detail in the discussions in chapters 4 and 5.

1.5.2.1.2. Linearly polarised oscillating MF's

Oscillating MF's can cause biological effects at flux densities in the range 20-70 μ T (reviewed in 9). Recently attention has focused on the effects of this type of MF on pineal activity as it is relevant to everyday exposure conditions. Oscillating MF's have been shown to affect pineal biochemistry but under specific conditions. Effects occur at different field intensities and exposure duration. This was demonstrated when a significant decrease in the number of pineal synaptic ribbons was observed after exposure to a 5.2 μ T (50Hz) MF. In this study male Wistar rats were exposed for 30mins. per day for 7, 15 and 21 days after which time an effect was observed. A significant drop in serum melatonin was also measured after 15 days. Kato (52) exposed Wistar rats for 6 weeks to horizontally or vertically orientated 1 μ T, 50Hz MF's. The results of this experiment showed that chronic exposure of rats to the oscillating MF's had no effect on pineal gland or plasma melatonin levels. Selmaoui (142) investigated the effects of both the intensity and the duration of exposure. Two groups of Wistar rats were exposed to 50Hz MF's of either 1, 10 or 100 μ T. The first group of rats were exposed for 12hrs and the second group for 18hrs per day for 30 days. The shorter-term exposure reduced pineal SNAT activity and serum melatonin levels at a field intensity of 100 μ T only. Longer-term exposure had the same effect but at field strengths of both 10 and 100 μ T. From these results it is apparent that the MF effects are related to both duration of exposure and field intensity. The duration of the photoperiod could also alter the sensitivity of the pineal to oscillating MF's. Matt (78) exposed Siberian dwarf hamsters to a 100 μ T 60Hz MF for 15min., 2hours before dark onset and animals were killed 3hrs and 5hrs after lights off and pineal melatonin content assayed. From the results obtained it appeared that EMF effects

were most dramatically demonstrated in animals in short daylengths and long night phases in which a delay in the onset of the melatonin peak occurs. The amplitude of the peak was also lower. The exact mechanism as to how oscillating fields interact with the pineal gland *in vivo* is not clear. However oscillating MF's have been shown to decrease SNAT activity in organ culture as well (64). Pineal glands were exposed for one hour to 33Hz oscillating MF's at field strengths tuned to resonance conditions for calcium ions. The fields significantly decreased SNAT activity. The conclusion from this study was that MF's specifically affected pineal biochemistry thus bypassing mechanisms requiring retinal stimulation. Yellon (181) showed that when male and female Djungarian hamsters were exposed to a 100 μ T, 60Hz horizontal MF for 15min. 2hrs before dark onset (dim red light less than 3 lux was present) the rise in the melatonin nighttime melatonin peak was delayed by 4hrs. The results of the study indicates that daytime exposure to MF's also affects melatonin production. A second study failed to reproduce the results of the first but they have apparently been confirmed in an independent laboratory (reviewed in 116). Preliminary studies claimed (116) that time varying MF's lower blood melatonin levels as well. Humans were exposed to a 60Hz, 20 μ T MF that was intermittently turned on and off during the night. The suppression of melatonin in the blood circulation was noted in individuals who had an inherently dampened nighttime melatonin level. Maximum suppression also occurred late in the dark phase. This has not been confirmed.

1.5.2.1.3. Circularly polarised oscillating Magnetic fields

MF's can be envisioned to be a vector quantity with both magnitude and direction. The vector can change direction or magnitude. Hence a vector of fixed magnitude but a change in direction only (a rotating vector) is called circular polarisation. In order to generate a circularly polarised MF two sets of coils with axes at right angles are required. The current in the two coils should also be 90° out of phase. The effects of this type of MF exposure on pineal function has recently been investigated. Kato et. al. (50) exposed rats for 6 weeks to a 50Hz circularly polarised MF (1-250 μ T) and observed a significant decrease in melatonin production at flux densities in excess of 1 μ T. These results were reproduced in both Wistar-King and Long-Evans (51) rats.

Since Long Evans rats are pigmented and Wistar King rats are not, the results of this study would suggest that pigmentation had no effect on pineal sensitivity to EMF's. This is in contrast to findings of Olcese et. al. (96) showed that MF stimulation did not depress melatonin production in ACI (fully pigmented) rats. These results show that melatonin production (and probably SNAT activity) is sensitive to time-varying MF's. Although circularly and linearly polarised MF's are both oscillating MF's the type of polarisation could be an additional variable affecting pineal biochemistry. This is quite clear when comparing the results obtained from exposure to circularly polarised fields to vertical or horizontal 50Hz 1 μ T MF's (52).

1.5.2.2. Electric fields

Very few studies have been reported on the effects of electric fields on pineal gland function. An early study showed apparently dramatic effects on pineal activity due to sinusoidal EF exposure (175). In this study rats were exposed for 3 weeks to a 60Hz electric field (39kV/m). One and 2 weeks of exposure had no effect on pineal SNAT activity or melatonin production. There was a significant decrease in pineal melatonin production and SNAT activity as measured 4hrs after lights off after 3 and 4 weeks exposure. In a more recent study (37), which closely replicates the above mentioned, no effect on pineal melatonin content was found although the serum levels of the hormone were reduced in exposed animals. Rats were exposed for 20hr/ day for 30 days to a 65kV/m, 60Hz and the levels of pineal melatonin were measured 4 hours after lights off. The experiment does not simulate the first exactly with regard to the strength of the electric field and the time of lights on and off. In this experiment the time of lights off was 12:00 as opposed to 22:00. It is not known whether the duration of light and darkness could affect pineal sensitivity but the possibility cannot be ruled out. The electric field intensity could be a crucial factor as a higher field strength was used in the latter study. Inevitably the effect of field intensity and pineal sensitivity would need to be investigated before conclusive statements can be made.

1.6. MECHANISMS BY WHICH EMF's AFFECT PINEAL BIOCHEMISTRY

1.6.1. Induced Eddy Currents

From experimental observations made thus far it is evident that MF's could interact "directly" and / or "indirectly" with the pineal gland (51, 52). Direct effects on SNAT activity are mediated by the retina. Indirect effects may be the result of electrical (eddy) currents induced within the pineal gland itself by the rapidly changing MF flux density. Recent evidence shows that eddy currents which arise during MF inversion exposure could in fact be responsible for the observed inhibitory effects on SNAT activity.

Every electric field generates a MF and every MF generates an electric field and thus the electric field cannot be discarded as not being able to affect pineal activity. Early studies by Liboff et. al. (68) illustrated that weak induced currents produced essentially the same effects as strong induced currents. These authors concluded that it was unlikely that the MF effects were caused by circulating or eddy currents which arise, due to magnetic induction, when extremely low frequency (<500Hz) MF's interact with living tissue. To the contrary Lerchl et. al. (62) showed that when rats were exposed to repeatedly inverted MF's (40 μ T) in the dark (no red light at night), only animals exposed to automatically activated fields (instantaneous as opposed to the manual switch which took 1sec. for activating and deactivating the MF) responded with reduced SNAT activity. From this it was concluded that exposure to MF's *per se* does not affect pineal SNAT activity, leaving induced eddy currents arising from the rapid on/off switching of the MF's as the cause of the observed effects (62). As for oscillating MF's at 50 or 60Hz it could be that the "sweeping" or "rotating" MF with its constantly changing vector interacts with the pineal tissue.

Recent studies however showed that the eddy currents are not solely responsible for the apparent MF effects since animals exposed to eddy currents alone did not show a decrease in SNAT activity (127). These animals were exposed at night to an pulsed

MF (automatic inversion of the horizontal component of the earth's MF $-40\mu\text{T}$) by turning the Helmholtz coils on and off at 1min. intervals for 30-60mins. The induced eddy currents together with the changing MF caused a decrease in SNAT activity indicating that there is some interactive mechanism, between these two components, which is responsible for mediating effects on SNAT activity.

Many studies in which investigators have demonstrated that DC MF inversion decreases SNAT activity have failed to document the switching method. It is thus not clear in those studies whether eddy currents could be involved in mediating the MF effects. The induced currents would affect the pineal directly but if induced currents were not a factor in those studies then an alternative mechanism should exist. How exactly the eddy currents produce their effects is not known. It is possible that eddy currents are produced in the eyes stimulating photoreceptors. This in turn is interpreted as light and is relayed to the pineal via the neural connections between the retina and the pineal.

1.6.2. The role of the eyes

The connection between the visual system and the pineal gland is well established and retinal stimulation could be involved in mediating EMF effects on the pineal. In one study, blinded rats did not respond to MF's (97). Rats were exposed for 30min. at night to a rotation by 50° of the horizontal component of the earth's MF ($50-100\mu\text{T}$). No significant decrease in SNAT activity and melatonin production was observed in blinded rats. Olcese et. al. (95) again showed in 1990 that surgical removal of the eyes prevented the inhibitory effect of MF's on SNAT activity. For this reason it has been concluded by some authors (123) that the eyes mediated the MF effect. Reiter and Yaga (1993) proposed that the isomerisation of 11-cis-retinal to an all-trans configuration mediates the inhibitory effect on SNAT activity but the exact mechanism is not clear. The authors proposed that the eddy currents produced by MF's induces the isomerisation of the photopigment leading to photoactivation of rhodopsin which is perceived as "light". MF's could activate magnetophosphenes

(visual sensations of light) which in turn could inhibit melatonin production via the retinal path. The field strengths are, however, too low since magnetophosphenes are generated in humans at field strengths greater than 10mT and at frequencies above 20Hz. The role of the eyes, thus, remains ambiguous in the mediation of MF effects since pineal organ culture studies (50) demonstrated an attenuated melatonin response to isoproterenol stimulation in the presence of a pulsed magnetic field. Thus the eyes could be involved in the mediation of EMF effects but other mechanisms may be involved too.

1.6.3. Role of sympathetic innervation

It has been known that MF's affect the sympatho-adrenergic system (143). MF's could in fact affect the release of NE as EMF exposure has been shown to change the concentration of neurotransmitters in humans and animals (176). The role of the sympathetic innervation of the pineal gland is to transfer visual stimulation to the pineal in response to dark exposure. This results in an increase in NE release with a concomitant increase in SNAT activity and melatonin secretion. During the dark the increased NE release produces an increase in cAMP which stimulates SNAT activity. Studies have shown that cAMP levels in the pineal decrease when rats are exposed to MF's (132). Rats were exposed for 1 hour to repeated inversion of the horizontal component of the earth's MF. Exposed animals showed a 38% decrease in pineal cAMP levels which suggests that MF's act upstream from adenylyl cyclase. Synaptic transmission at the β -receptor is thought to be impaired by MF (44) or more specifically the eddy currents generated from rapidly inverted MF's.

1.6.4. Ca²⁺ as a mediator

Ca²⁺ has been implicated in the regulation of SNAT activity as it has been shown to stimulate its activity at certain concentrations (86). High levels of calcium could however have an inhibitory effect. Extracellular Ca²⁺ is needed for the full induction of SNAT activity and the stimulation of β -adrenergic receptors (85). A circadian rhythm in the calcium content of the pineal gland has been reported

(referred to in 86) with peak levels occurring at the onset of the dark period. The increase in Ca^{2+} in the cell enhances melatonin production while an increase in plasma melatonin is associated with a reduction in circulating plasma Ca^{2+} (86). This could be the basis of a negative feedback mechanism. Long term exposure to elevated levels of Ca^{2+} however has an inhibitory effect providing evidence for both a stimulatory and inhibitory role for Ca^{2+} . This inhibitory effect could be a consequence of the activation of a SNAT inactivating compound (21). Alpha-1 adrenergic receptors are involved in the influx of extracellular Ca^{2+} (86) which is essential for α -1 adrenergic potentiation of β - adrenergic stimulation of cAMP (19). At a molecular level Morton has shown that Ca^{2+} ions activates SNAT activity (85) by binding directly to the enzyme. This increases the binding affinity of the enzyme for tryptamine. Calcium ions are also responsible for the opening of K^+ efflux channels (19) resulting in membrane hyperpolarization which might be needed to keep the enzyme in an activated state. Calcium ions are thus crucial in the regulation of SNAT activity and variation in their level would most likely modify the activity of the enzyme. Since several studies have shown that exposure of biological systems to MF's tuned to Ca^{2+} cyclotron resonance conditions resulted in a variety of effects (48), it is possible that SNAT activity could also be influenced by similar exposures. Lerchl et. al. (64) showed that low frequency MF's tuned to the resonance conditions for Ca^{2+} resulted in a decrease in melatonin production when pineals were exposed for 2.5 hrs *in vitro*. These authors proposed, however, that the mechanism by which MF's induced an effect was via receptor internalisation. A group of investigators (72) have shown that β -receptors could be internalised when cells in culture were exposed to EMF's. This internalisation of the β - receptors would also explain the decrease in cAMP levels (132) following exposure to a pulsed MF. More extensive studies are required to confirm the results obtained by Lerchl et. al. and to extend investigations to confirm whether EMF's affect calcium levels and so too SNAT activity.

1.7. THE ION CYCLOTRON & ION PARAMETRIC RESONANCE MODELS

1.7.1. The need for a theoretical model

Laboratories in many countries have investigated EMF effects on biological systems. Some have shown EMF effects while others have failed to do so. Most of the effects are due to exposure to particular magnetic field characteristics, unique to that group of investigators. Replication of many of the studies is difficult if not impossible. The lack of robustness and thus transferability of experiments has been a major stumbling block towards further progress. A need thus arose to develop a mechanism by which EMF's affect biological systems. Investigators have claimed that resonance mechanisms may account for some of the observed effects. Liboff (69) formulated the ion cyclotron resonance model (ICR) to predict the response of a biologically active ion to EMF's. This model describes how unhydrated ions may exhibit a distinctive resonance response established by combined AC and DC MF's. However the limitations of the ICR model (14) lead Blanchard et al (14) to developed the ion parametric resonance (IPR) model to predict biological responses to EMF's.

1.7.2. The ion cyclotron resonance model

According to this model an oscillating magnetic field at a specific frequency, together with a specific static field (DC magnetic field) of predetermined flux density culminates in an excitatory frequency, specific to each ion. The specific frequency of an ion is given by the equation:

$$f_c = (1/2 \pi) \times (q/m) \times B_0$$

where f_c is the specific resonance frequency of a particular ion, q (in coulombs) is the charge of the ion, m (in Kg) is the ion mass and B_0 (in mG) is the magnetic field flux density. This model applies to a variety of biologically active ions when they approach the anhydrous environment of their binding sites. According to the ICR model, the specific MF conditions for a given ion results in weak oscillations which impede

the action of the ion and produce the biological effects. To test the model Liboff exposed human lymphocytes to Ca^{2+} cyclotron resonance conditions for 60min. and demonstrated increased $^{45}\text{Ca}^{2+}$ uptake. Lerchl et. al. (64) exposed whole pineal glands to cyclotron resonance conditions for Ca^{2+} . It was demonstrated that under these conditions MF's caused a reduction in SNAT activity and melatonin production.

1.7.3. The ion parametric resonance model

Biologically active ions are susceptible to different AC flux densities, which was not accounted for by the ICR model as opposed to the ion parametric resonance (IPR) model (14). The IPR model is the most recent predictive theory and is based on an ion resonance model described by Lednev (61). The model examines the MF effects on any unhydrated ions within a molecular structure (protein, nucleic acids or lipids) which requires an ionic cofactor to function i.e. to drive the chemical reaction. The IPR model takes into account most (if not all) experimental variables and their combined effect on the biological system, including the parallel components of the exogenous AC and DC MF's. The interaction of an ion with its ligand can be viewed as an oscillator with a characteristic vibrational frequency (same as f_c in ICR) and thermal energy may play a role in the transition between various energy states. It is speculated (13) that the natural vibrational modes of the molecular structure particularly the ion cavity and active site have evolved in tune with the vibrational modes of the specific ion cofactor. Hence during exposure at a specific ion resonance, interactions of the magnetic field with a critically bound ion could conceivably alter the vibrational dynamics between the ion and its molecular ligand structure by splitting and modulating the energy states of the complex. This results in spatial and temporal changes leading to a change in the dynamics of the active state and inevitably in altered biochemical activity. It is the alteration in dynamics which is crucial in explaining magnetic field effects on biological systems.

The IPR model has its basis as far back as 1960 when Podgoretskii (see 14) showed that parallel exogenous AC and DC MF's have effects at the atomic level and then postulated that an external DC magnetic field creates a Zeeman splitting of the quantum energy level of each ion. The split energy levels are subsequently frequency modulated by an external AC magnetic field. The fundamental parameter of the IPR model is the frequency index:

$$n = f_c / f_{ac}$$

where n is the frequency index, f_c is essentially the same as that of the ICR, f_{ac} is the frequency of the AC MF to which the system is exposed. The IPR model predicts that when an ion is exposed to its critical resonant frequency as a result of the applied magnetic DC field and AC field frequency then the probability, p , of a transition between energy states will be modified in a predetermined manner which is reflected in the biological endpoint measured.

$$p = K_1 + K_2 \cdot (-1)^n \cdot J_n(n \cdot 2B_{ac}/B_{dc})$$

where J_n is the Bessel function, K_1 is the system's response when $B_{ac} = 0$, K_2 is a real constant whose value depends on the particular biological endpoint measured and n is the frequency index. The IPR model has been tested empirically for the effect of MF's on PC-12 cells and the system was found to respond in a deterministic way (13). In the study PC-12 cells were exposed to different AC and DC MF frequencies and intensities to determine whether the IPR model is applicable (13). Cells were exposed to on and off resonance conditions and in all cases responses were consistent with the predictions of the IPR model. As Ca^{2+} is crucial in the regulation of SNAT activity it would be appropriate to expose pineals to resonance conditions for Ca^{2+} to ascertain whether the IPR model is applicable. Changes in the AC field frequency and DC field intensity would then need to be investigated to determine the biological response under the various conditions.

1.8. CONCLUDING REMARKS

The results of investigations which test the effects of EMF's on SNAT activity and melatonin are not completely convincing. The variation in the levels of activity and experimental conditions extends over both extremes namely those studies which shows that EMF's affect SNAT activity and melatonin production to studies where no effects are detected. A short-fall of many of the earlier studies was the poor documentation of EMF variables. This makes it difficult for other investigators to test the conclusions reached. Apart from these technical problems there are other factors which need to be considered when embarking on MF studies. Some of the factors which need to be considered include climate, onset of dark / light, physiological status (eg. stress) and age of the animals. No proven mathematical model has been accepted, as yet, to predict the response of biological systems to EMF stimuli. The IPR model is however the most recently formulated theoretical model to predict a biological response when animals are exposed to specific EMF conditions.

Another important question which needs clarification is the mechanism by which EMF effects are mediated and detected within the pineal cells. This is proving to be quite controversial with EMF effects being demonstrated both *in vivo* and *in vitro*. These are but a few of the many questions surrounding the MF effect on biological systems and SNAT activity specifically. It is against this background that studies were undertaken to address some aspects of this topic in an attempt to contribute further to other studies. Some of the questions addressed during the project was the effect of pulsed static MF's on SNAT activity. The effect of field intensity, orientation, exposure time and duration was also investigated. The effect of oscillating MF's was investigated. Firstly the effect of EMF's on SNAT activity *in vivo* needed to be investigated. It would however be impossible to reproduce other studies exactly. These questions were addressed by placing rats in a pair of Merrit coils and adjusting the current strength and flow direction and then measure SNAT activity. An extension of the project was to investigate EMF effects *in vitro* by exposing whole pineal glands to ICR and IPR conditions.

Lerchl's work which showed a decrease in SNAT activity and melatonin production when pineal glands were exposed to ICR conditions tuned to Ca^{2+} resonance would need to be reproduced.

CHAPTER 2

MATERIALS AND METHODS

2.1. ANIMALS

2.1.1. Enzyme assay optimisation

Male Long Evans inbred rats, obtained from the University breeding colony, were housed in air conditioned quarters in a 12 : 12hr L/D period (lights on at 06:00h). Food and water were available *ad libitum*. Temperature was regulated at ~22°C. Animals were housed 10 per cage and the cages were cleaned three times a week. Rats (200-300g) were injected with isoproterenol (i.p. 15mg of isoproterenol / kg bw) between 09:30 and 10:30. Each rat was anaesthetised by placing it for 30secs. in a sealed perspex cage which contained a swab of cottonwool saturated with halothane and decapitated 3hrs later. Pineal SNAT activity was assayed immediately thereafter.

2.1.2. Electromagnetic field studies (in vivo)

Thirty male Long Evans rats (per study) were acclimatised to a specific L/D cycle, in a light-tight room for 10-14 days, by which time they weighed 200-300g. Food and water were available *ad libitum*. Temperature in the room was maintained at ~22°C. Animals were housed 10 per cage and the cages were cleaned three times a week. Experiments and the decapitation of the rats were carried out during the dark phase in the presence of a dim red light which was switched on 30mins. prior to the onset of MF exposure. Ten rats were employed for each separate experiment and 2-3 experiments comprised a single study. The rats were randomly assigned to 4 plastic cages (with wooden lids) 15mins. prior to the onset of the exposure. Five rats were used for the experimental and control groups respectively. Only experimental rats were exposed to either pulsed static or oscillating MF's. The rats were calm during the exposure period. Rats were lightly anaesthetised following exposure and were decapitated immediately thereafter. The pineal glands were removed in an adjacent room under fluorescent light. Euthanasia and removal of 10 pineal glands lasted 15-

20mins. Thereafter pineal SNAT activity was measured.

2.2. CHEMICALS

Rats were injected with the β -adrenergic agonist, d,l-isoproterenol HCL (Sigma Chemical Co (St Louis, Mo)), to stimulate SNAT activity during the day.

Enzyme assay reagents / buffers :

The enzyme substrates, Acetyl CoA and tryptamine HCL, were supplied by Sigma Chemical Co (St Louis, Mo) while (^{14}C) labelled Acetyl CoA (55mCi/mmol) was obtained from Amersham International (UK). Pineal glands were homogenised in 0.1M Sodium Phosphate buffer (pH 6.5) which contained EGTA (BDH Laboratories (UK)). Clarke's borate buffer (0.2M)(pH 10) was used to stop the chemical reaction following the incubation period. Aquasafe 500 Plus scintillation fluid (Zinsser Analytic (Frankfurt)) was used for counting radioactivity of the samples.

2.3. SNAT ASSAY

This section describes the final developed assay which was used to measure SNAT activity during the studies which investigated the effects of MF exposure on the enzyme's activity.

Individual pineals were homogenised in 150 μl of ice cold 0.1M sodium phosphate buffer (pH 6.5) containing 2mM EGTA. Each pineal gland was homogenised for 30secs. The homogenates were kept in a micro glass homogeniser on ice at all times. Fifteen microlitres of the homogenate (which contained 1/10th of a pineal gland) was added to a 10 μl mixture of Acetyl CoA / tryptamine. Each pineal homogenate was assayed in triplicate. The final concentration of Acetyl CoA was 180 μM of which 24 μM was ^{14}C labelled. The concentration of tryptamine was 12mM. Samples were incubated for 30mins. at 37 $^{\circ}\text{C}$ and the chemical reaction was stopped by adding 100 μl of 0.2M borate buffer (pH 10) to the incubate. The product formed, N-acetyl tryptamine, was extracted by adding 1ml of water saturated chloroform to the samples followed by vortexing for 15secs. Immediately thereafter the samples were

centrifuged at 2500 rpm for 10mins. and the top aqueous phase was aspirated off. A second extraction procedure was carried out which again required the addition of 100µl of borate to the samples, vortexing and centrifugation. The aqueous phase was again removed. The amount of radioactive Acetyl CoA incorporated into the N-acetylated tryptamine was measured by counting (15mins.) 500µl of the chloroform phase using standard radioactive counting techniques.

2.3.1. Calculation of SNAT activity

$$\frac{(\text{Sample count (dpm)} - \text{blank count(dpm)}) \times a \times 2 \times 2 \times b}{\text{total count (dpm)}} = (\text{nmol/p/hr})^*$$

a = pineal fraction used (e.g. 1 for whole, 10 for 1/10th of a pineal)

b = total nmols of Acetyl CoA per sample

2 = conversion of 30mins. incubation to 60mins.

2 = correction factor as half chloroform extract counted

total dpm = total radioactivity per sample expressed as disintegrations per minute

* nmol of N-acetylated tryptamine formed per pineal gland per hour.

The small size of the pineal glands and the adherent fluid prevents the accurate measurement of its mass. Protein determination would also be complicated by the different proportions of adhering tissue. The size of the pineal glands of rats in a specific weight range was fairly uniform (~1mg each), hence SNAT activity was expressed per pineal gland. This is the standard notation for the expression of SNAT activity in most publications.

2.4. EXPOSURE SYSTEMS

2.4.1. Coil system

Two identical sets of 4 square Merrit coils were used in the experiments, one for exposed and the other for sham exposed rats. Each set of coils could house 2 plastic cages containing 2 to 3 rats each.

The strengths of the various currents used are shown in section 2.4.2.

Location and orientation of coils :

The two sets of coils were located approximately 2m apart and at a distance of 1.5m to 2.0m from the power supplies. Both sets of coils were orientated such that the sides of the coils were parallel to the geomagnetic north - south axis. The intensities of the components of the earth's magnetic field in the experimental and control coils were measured using a gaussmeter (F.W. Bell - model 9500). The respective strengths of the horizontal and vertical components of the earth's MF in the experimental coils were $10.60\mu\text{T}$ and $22.80\mu\text{T}$ while those in the control coils were $10.24\mu\text{T}$ and $25.26\mu\text{T}$.

Additional equipment used for the oscillating MF studies included a function generator (Philips - PM5135) coupled to a stabilized power amplifier (Rotel - RB-970BX) which had been modified to a current amplifier.

Assessment of the exposure system :

The coils were designed according to the Merrit - 4 coil design and therefore the homogeneity of the fields within the coils was assumed to be the same as that described by Kirschvink (54). The uniformity within the coils ranged from less than 0.02% variation at the centre of the coils to 200% at the periphery. The cages were located at the centre of the coils such that the perimeter of the cages corresponded to a maximum of 20% variation in field uniformity. A downward drift in the strength of the DC field was observed during the 60min. exposure period, in the static field studies. The field strength was set at $100\mu\text{T}$ before the exposure period but had dropped to $95.8\mu\text{T}$ (S.D. $6.7\mu\text{T}$) at the end. When the vertical DC field was set at $100\mu\text{T}$ the horizontal field was recorded as $6.44\mu\text{T}$ while inversion of the vertical field resulted in a horizontal field of $16.62\mu\text{T}$. On the other hand, when the horizontal DC field was set at $100\mu\text{T}$ the vertical field was $26.07\mu\text{T}$ while inversion of the horizontal DC field resulted in a vertical field of $19.48\mu\text{T}$. The magnetic field intensities and frequencies used in the experiments were weak and thus audible hum and detectable vibration was not likely to be significant.

Background alternating magnetic fields :

AC MF's in the room ranged from 2nT (rms) at sites furthest from the power supplies and other electrical equipment to 50nT (rms) at a point 1.5m from the power supply. The AC fields in the coils ranged from 50 to 80nT depending on the orientation of the probe.

2.4.2. Static Magnetic fields*Vertical fields :*

Rats were exposed to linearly polarised static MF's, the vertical component of which was inverted 180° at regular intervals. Most studies investigated the effects of either 1min. or 5min. interval repeat inversions. The strength of these fields ranged from 10µT to 100µT. Both Merrit coils were energized to generate a vertical field of 100µT (V = 9.4volts ; A = 0.22amps.). The vertical field in the experimental coil was inverted and the current strength required to generate a field of equal intensity but of opposite polarity was 0.34amps. and the voltage was 14.1volts.

Horizontal fields :

In order to investigate the effects of a horizontal DC MF the above coils were rotated 90° and orientated to produce a static DC field aligned to the geomagnetic north - south axis. The current (V = 3.3volts; A= 0.05amps.) in the coil was set to generate a field equal to that of the earth's H.C. (10.60µT) but of opposite polarity. Some experiments also investigated the effects of 100µT DC field inversions when the current used was V = 10.7volts; A = 0.25amps. and V = 13volts; A = 0.31amps for respective fields of opposite horizontal polarity.

2.4.3. Oscillating Magnetic fields*Vertical fields :*

A single *in vivo* experiment was performed to test the ion parametric resonance model. The exposure system was tuned to Ca²⁺ resonance conditions. Thus the vertical AC field strength was 14.3µT(rms) and frequency was 17.2Hz while the strength of the vertical DC MF was 22.55µT, the existing vertical geomagnetic field strength.

2.5. CHARACTERISTICS OF THE MF AND TRANSIENT INDUCED CURRENTS

2.5.1. Magnetic field changes

The Merrit coils were energised to produce a DC field of $100\mu\text{T}$ which was inverted using the solid state relay switch. The gaussmeter connected to a digital storage oscilloscope (Kikusui - DSS 6522) measured the temporal change of the static field during the inversion (Fig. 2.5.1.).

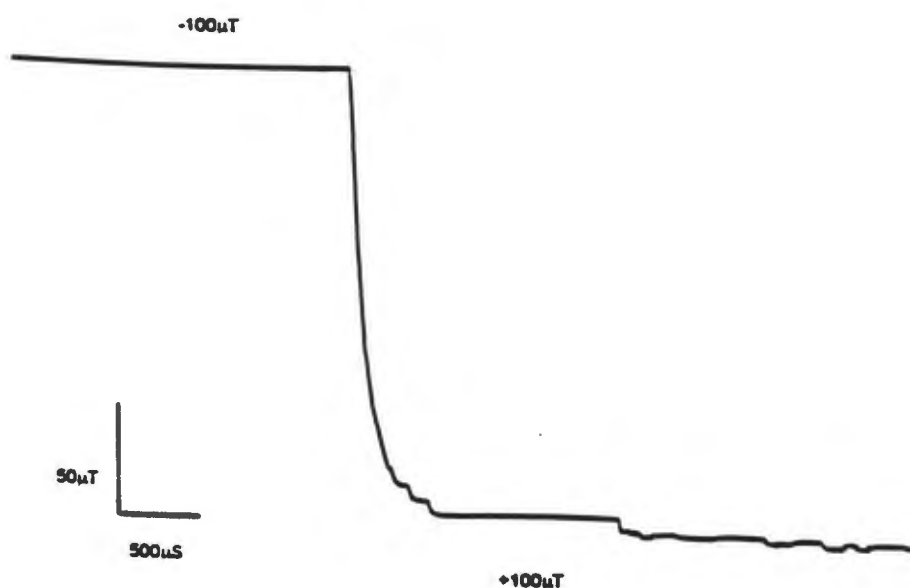


Figure 2.5.1. : Temporal characteristics of a $100\mu\text{T}$ DC magnetic field inversion. The time taken to reach the new steady state was approximately $800\mu\text{s}$.

2.5.2. Transient induced currents

The inductive effect of DC field inversion by 180° was measured by placing a search coil, consisting of 4000 turns (diameter - 7cm; type of wire - copper) within the Merrit coils.

The search coil was connected to the storage oscilloscope and its signal was recorded on an Omnigraphic 2000 X - Y Recorder.

To ascertain whether the transient induced currents generated in the experimental coils would influence the EMF environment in the control exposure system, the transients were measured at 300mm and 2000mm from the experimental coils. The characteristics of the transients generated during DC field inversions are shown in tables 2.5.2.(i) and (ii).

Table 2.5.2.(i). : Inductive effect of DC field inversion by 180°

(Search coil placed in the centre of the Merrit coil)

Field switching direction	Field strength (μT)	Duration of field transient (mS)	Amplitude of induced field (mV)
plus to minus	100	1.6 to 2.0	440
plus to minus	25	1.6	440
minus to plus	100	1.6	440
minus to plus	25	1.6	440

The data show that the switching process was very fast and would therefore cause a large value of dB/dt. The results also showed that the induced field strength was independent of the static MF strength.

Table 2.5.2.(ii). : Inductive effect of DC field inversion by 180°

(Search coil placed outside Merrit coil)

Field switching direction	Field strength (μT)	Duration of induced field (μS)	Amplitude of induced field (mV)	Distance from Merrit coils (mm)
plus to minus	100	800	450	300
plus to minus	100	600	5	2000
minus to plus	100	1200	450	300
minus to plus	100	800	5	2000

The induced field strength as measured in mV displayed a rapid decay with distance from the source. The amplitude of the induced field, however, remained significant at a distance of 2m away from the source. The durations of the induced fields were much shorter than those reported from other laboratories (25msec.) (124). A graphical representation of the transient is shown in figure 2.5.2.

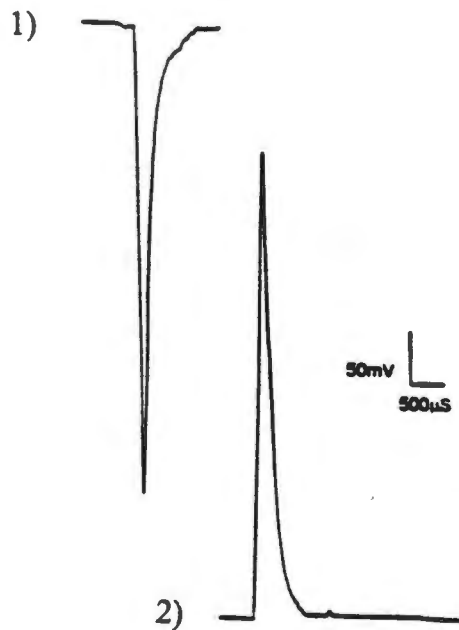


Figure 2.5.2. : Induced transients measured at 300mm from the Merrit coils. The Merrit coils produced a static magnetic field of $100\mu\text{T}$ which could be inverted rapidly with a solid state relay switch. The order of switching was minus to plus (1) then plus to minus (2).

2.6. STATISTICAL ANALYSIS

Data were expressed as means together with their standard errors. Results were analysed using one way analysis of variance followed by the Bonferroni multiple comparison test or paired or unpaired Student t-test ($P < 0.05$ was considered significant). The Instat (v 2.0) computer program was used for the statistical analysis.

CHAPTER 3

OPTIMISATION OF THE SNAT ASSAY

3.1. INTRODUCTION

SNAT is the rate limiting enzyme involved in the production of melatonin. Any alteration in the enzyme's activity would thus affect melatonin production, the significance of which has already been discussed. SNAT is however an unstable enzyme and rapidly loses its activity during and after the homogenisation of the pineal gland. Preliminary experiments, measuring SNAT activity, confirmed this result and thus low levels of activity were measured initially. The need for the optimisation of the assay for SNAT activity should be viewed in the context of the prospective MF studies to be undertaken. Many experiments carried out thus far have shown that MF's inhibited SNAT activity (50, 51, 62, 63 121, 126, 144). Widely different levels of enzyme activity have been measured in various laboratories for reasons that are not clear. Reported levels ranged from 0.2nmol/p/hr to 14.7nmol/p/hr (23, 128). Instability of the SNAT assay is associated with enzyme deactivation. This could bias the experimental results in the same direction as that expected by the actions of MF exposure. It was thus imperative to establish a stable assay for the measurement of SNAT activity. This would ensure that any inhibitory effect on SNAT activity observed could be interpreted with confidence.

Most investigators have employed techniques based on the assay developed by Deguchi and Axelrod (23) which involves the N-acetylation of tryptamine in a mixture containing the enzyme, present in the homogenate of the pineal gland, ^{14}C or ^3H labelled Acetyl CoA and tryptamine. The tryptamine was N-acetylated with the acetyl group from Acetyl CoA during the incubation period. The rate of product formation was used as an index of the enzyme's activity. Recently it was shown that the Ca^{2+} ion chelator, EGTA, preserved SNAT activity during the homogenisation of the pineal gland (128).

This part of the project thus investigated the ideal conditions for measuring SNAT activity, using EGTA and Acetyl CoA to preserve and stabilise the enzyme.

3.2. METHODS

Basic assay technique

The assay described here is the method used to measure SNAT activity at the onset of the project. It thus served as the basis on which to develop the final assay used in the EMF studies. The assay was developed by investigating separate aspects of the assay while still maintaining the basic method. Pineal SNAT activity was stimulated during the day by injecting rats with isoproterenol (15mg/kg bw). SNAT activity was then measured 3hrs later. Individual pineal glands were homogenised in 150 μ l of ice cold 0.1M sodium phosphate buffer (pH 6.5) containing 2mM EGTA. Triplicate, 15 μ l aliquots, of the homogenate (1/10th of a pineal gland) were incubated with 10 μ l of a tryptamine / Acetyl CoA mixture such that their concentrations in the final volume of 25 μ l were 10mM and 50 μ M respectively. Samples were incubated for 30mins. at 37°C after which the reaction was stopped by adding 100 μ l of 0.2M borate (pH 10). The N-acetylated tryptamine was extracted using water-saturated chloroform. The upper, aqueous, phase was removed and the wash step, using borate, was repeated. The amount of N-acetylated tryptamine formed was measured by counting 500 μ l of the organic phase and enzyme activity was calculated as shown in section 2.3.1. Each set of experiments measured SNAT activity in 4 pineal glands and the results were expressed as the mean together with its standard error.

The subsequent sections describe the improvements made to the assay which resulted in the final protocol employed in the MF studies (section 2.3.). The final assay thus resulted from repeated investigations of effects of variation in the concentration of EGTA, the various substrates and the enzyme itself. In addition, enzyme stability was assessed with respect to the preincubation time and temperature and the final assay was then used to confirm the circadian variation in SNAT activity.

3.2.1. Effect of EGTA concentration, in the homogenisation buffer, on SNAT activity measurement

Individual pineals were homogenised for 30secs. in 0.1M sodium phosphate buffer (pH 6.5) containing either 0, 1, 2 or 4mM EGTA. The basic protocol was then followed.

3.2.2. The effect of tryptamine concentration on the measurement of SNAT activity

Five pineal glands were pooled and homogenised for 30secs. in 750 μ l of ice cold buffer containing 2mM EGTA. Fifteen microlitre aliquots were incubated with 10 μ l of a tryptamine / Acetyl CoA mixture. The final concentration of Acetyl CoA, in the mixture, was kept constant (50 μ M) but the concentration of tryptamine was varied from 6mM to 48mM.

3.2.3. The effect of Acetyl CoA concentration on the measurement of SNAT activity

Five pineal glands were pooled and homogenised for 30secs. in 750 μ l of ice cold buffer containing 2mM EGTA. Separate experiments were carried out in which 15 μ l aliquots were incubated with a 10 μ l mixture of tryptamine / Acetyl CoA. The final optimal concentration of tryptamine, as determined in the previous section, was kept constant (12mM) but the Acetyl CoA concentration ranged from 32 μ M to 1.18mM. The concentration of 14 C labelled Acetyl CoA was constant for each experiment.

3.2.4. Effect of varying the ratio of pineal tissue to substrate concentration on measured SNAT activity

No native enzyme was available to investigate the effects of varying enzyme concentration on the measurement of SNAT activity. Therefore serial dilutions of pineal gland homogenates were made and assayed. Four pineal glands were pooled and homogenised in 120 μ l of buffer containing 2mM EGTA. Serial 1 in 1 dilutions of the homogenate were made using homogenisation buffer / EGTA resulting in 7 samples ranging from 0.0078 to 0.5 pineal per 15 μ l homogenate. The experiment was

repeated once. The optimised concentrations of EGTA, tryptamine and the selected concentration of Acetyl CoA were used in these experiments.

3.2.5. Stability of the enzyme under different assay conditions

Initial experience with the assay showed that SNAT was very unstable once pineal glands (and therefore pinealocytes) had been disrupted. This instability was thought to be linked to an active Ca^{2+} mediated deactivation of the enzyme which could be reduced in the presence of EGTA and by reducing the temperature of the homogenates.

Preincubation period

The stability of the enzyme was assessed, under final assay conditions, with respect to temperature of the homogenate, EGTA concentration and preincubation time i.e. the time between homogenisation of the pineal glands and addition of the substrates. Three sets of pineal glands (2 per set) were homogenised in 300 μl of 0.1M buffer containing either 0, 2 or 4mM EGTA. Aliquots (50 μl) of each of the three homogenates were distributed to 3 sets of tubes, the first of which was assayed immediately and represented the zero preincubation time control. The other 2 sets were preincubated on ice (4 $^{\circ}\text{C}$) and at 37 $^{\circ}\text{C}$ respectively for either 10 or 20mins. Enzyme activity was assayed immediately. Thereafter each aliquot was assayed for SNAT activity using the basic protocol but using 12mM tryptamine and 180 μM Acetyl CoA as derived from the above experiments. Enzyme activity was expressed as a percentage of that measured in the zero preincubation time control tubes. The raw data are shown in table A in the appendix.

Incubation period

Individual pineal glands were homogenised in ice cold buffer containing 2mM EGTA. Triplicate 15 μl aliquots of the homogenate were incubated with 12mM tryptamine and 180 μM Acetyl CoA for either 10, 20 or 30mins. Thereafter the basic protocol was followed.

3.2.6. Circadian variation in SNAT activity

Rats (150 - 200g) were acclimatized to either a normal or inverted 10 : 14 hr L/D cycle for 10 to 14 days by which time the rats weighed 200g to 300g. The time of lights on for the normal and inverted L/D cycles was 08:00 and 18:30 respectively. The circadian rhythm in SNAT activity was also measured in rats housed for 10 to 14 days in an inverted 14 : 10 hr L/D cycle (lights on at 14:30). Groups of 6 rats were decapitated at specific times during the 24hr cycle. Rats were lightly anaesthetised, using halothane anaesthetic, prior to decapitation. Decapitation of rats during the dark period was carried out under a dim red light but removal of the pineal gland occurred in an adjacent laboratory, under normal fluorescent light. The pineal glands were immediately placed in ice cold EGTA / buffer and SNAT activity was measured.

3.3. RESULTS

The results presented in figure 3.3.1. show that relatively high levels of enzyme activity were measured in pineal glands homogenized in 1, 2 and 4mM EGTA. The activity in the presence of EGTA increased significantly ($P < 0.001$) between 4 and 5 fold compared with that in the absence of EGTA. There were no significant differences between the levels of activity measured in the presence of EGTA. However the 2mM EGTA concentration was adopted for subsequent assays.

3.3.1. Effect of EGTA concentration, in the homogenisation buffer, on SNAT activity measurement

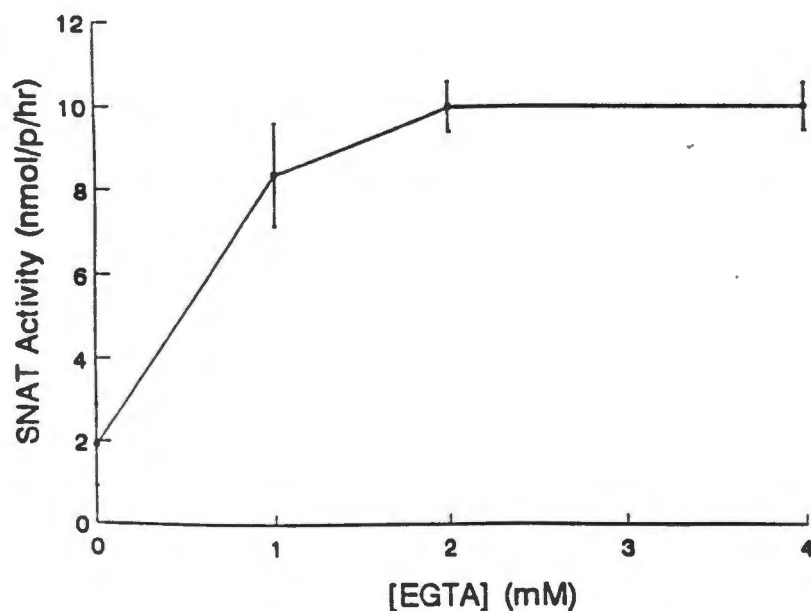


Figure 3.3.1. : The effect of EGTA on the measurement of SNAT activity.
(n = 4 in each case)

3.3.2. The effect of tryptamine concentration on the measurement of SNAT activity

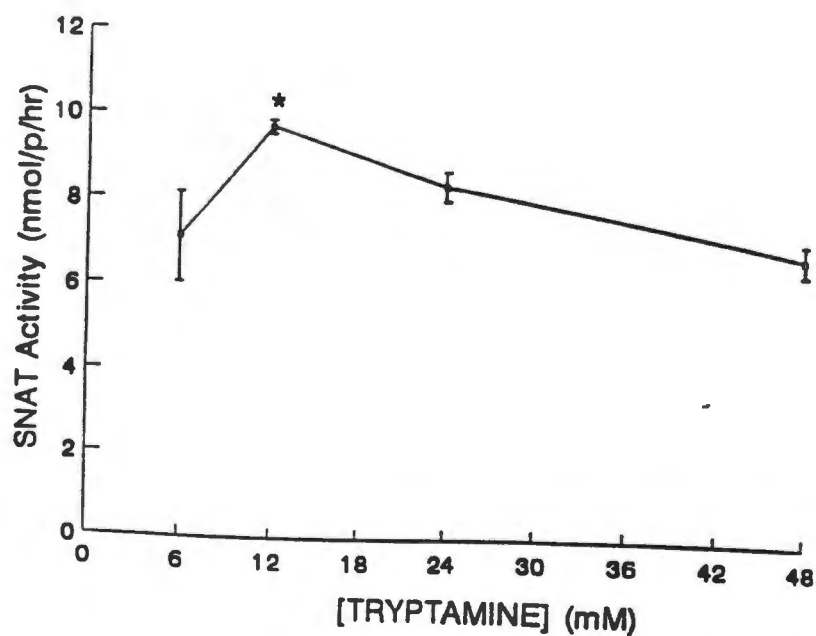


Figure 3.3.2. : The effect of tryptamine concentration on the measurement of SNAT activity. * P < 0.05 compared with the other three points.
(n = 4 in each case)

The result in figure 3.3.2. shows significant peak SNAT activity at 12mM tryptamine. Concentrations of tryptamine higher than 12mM appeared to have an inhibitory effect on SNAT activity while those lower than 12mM appeared to be rate limiting. SNAT activity in the presence of 12mM tryptamine was significantly ($P < 0.05$) higher than the others.

3.3.3. The effect of Acetyl CoA concentration on the measurement of SNAT activity

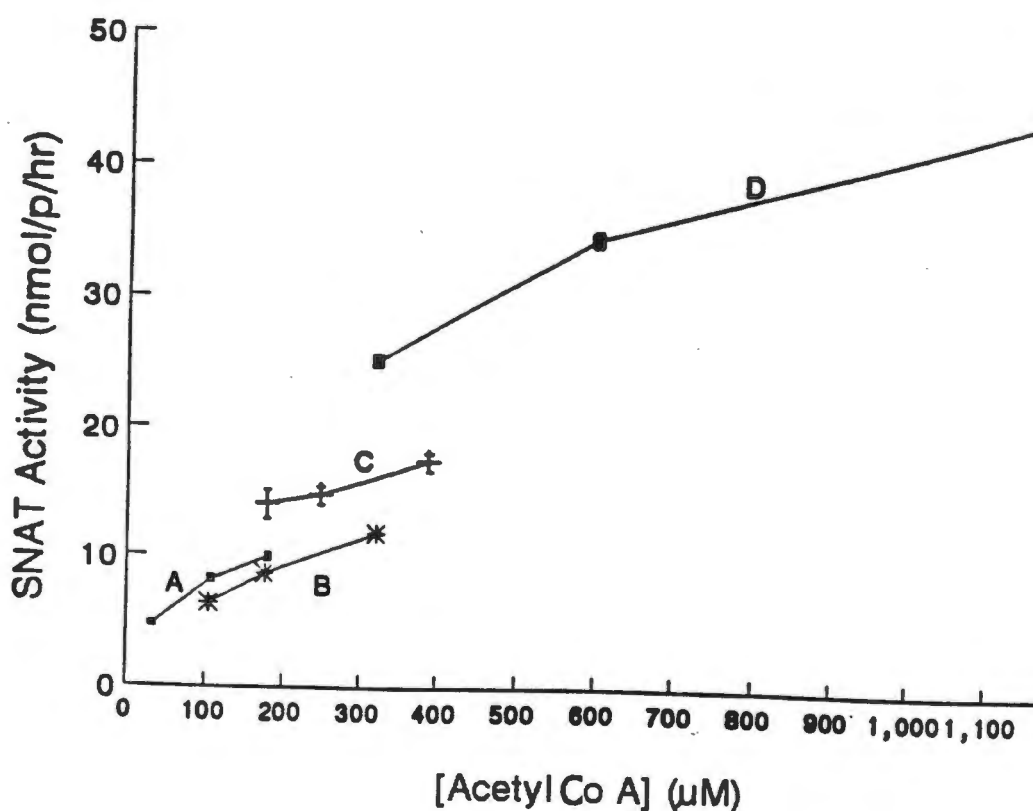


Figure 3.3.3.(i) : The effect of Acetyl CoA concentration on the measurement of SNAT activity as assessed in 4 separate experiments (A, B, C, D). ($n = 4$ in each case)

The results in figure 3.3.3.(i). show that SNAT activity increases with an increase in the concentration of Acetyl CoA. Under the prevailing conditions of these experiments the enzyme did not appear to be saturated even at the high concentration of Acetyl CoA of 1.18mM.

These data were used to derive the K_m of the enzyme for Acetyl CoA using the Lineweaver-Burk plot. The data points were pooled and a best fit line was generated using simple linear regression. The results are shown in figure 3.3.3.(ii). and indicated a K_m of 8.19×10^{-4} M and a V_{max} 58.8nmol/p/hr.

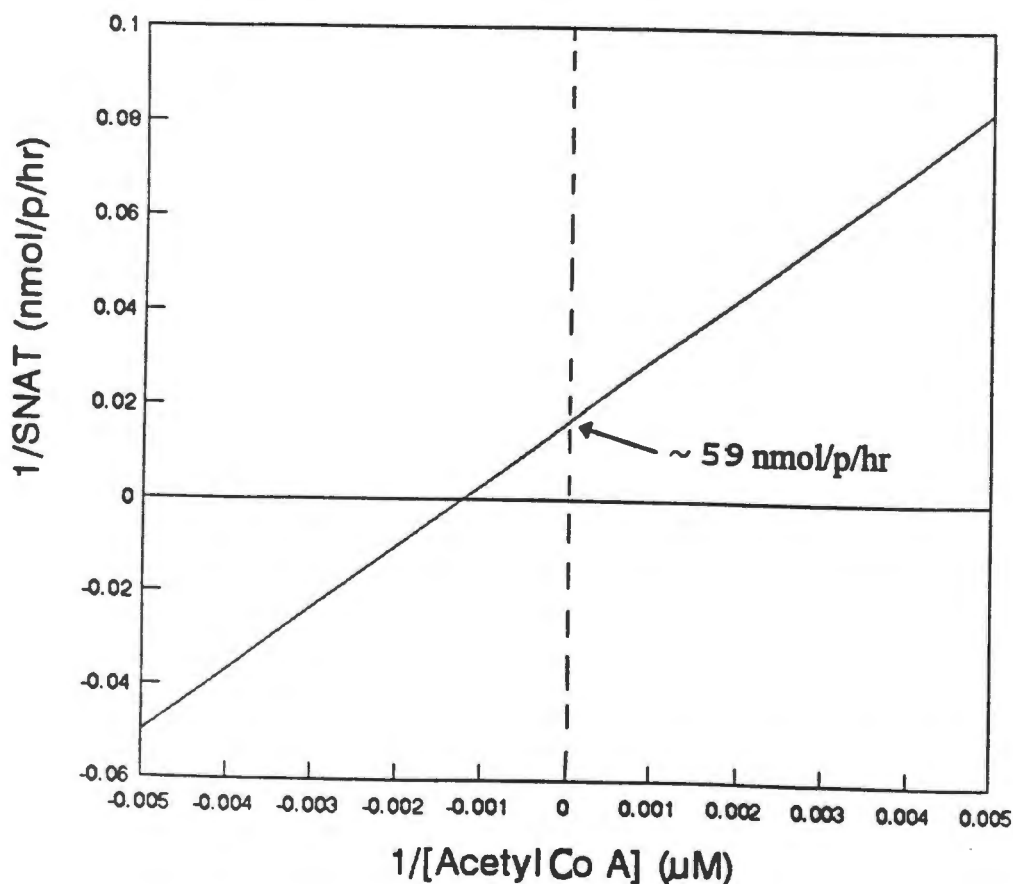


Figure 3.3.3.(ii) : Plot of $1/\text{Acetyl CoA}$ versus $1/\text{SNAT activity}$. The regression equation was $y = 13.39x + 0.017$ and the correlation coefficient was 0.912.

3.3.4. Effect of varying the ratio of pineal tissue to substrate concentration on measured SNAT activity

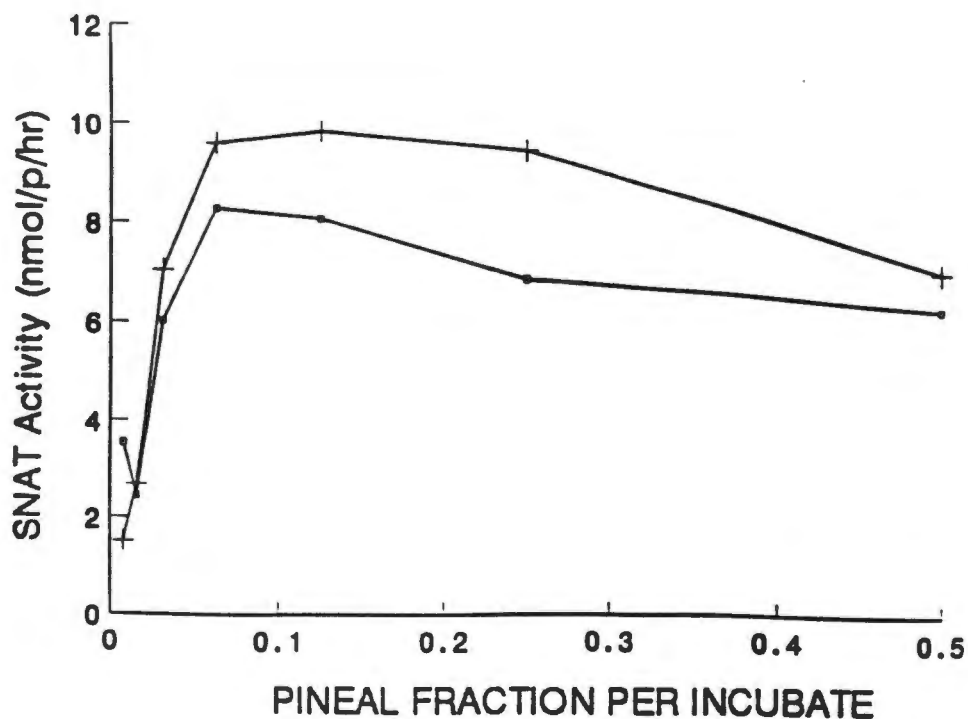


Figure 3.3.4. : The relationship between pineal tissue fraction per incubate and SNAT activity. The two graphs represents the results obtained from two separate experiments.

Figure 3.3.4.: Shows the influence of varying the quantity of pineal tissue and thus enzyme concentration on the measured SNAT activity. The concentration of the enzyme was varied over nearly 2 orders of magnitude while the effective substrate concentrations remained constant. The graph shows a dramatic decrease in enzyme activity at low pineal fractions. Although the data could not be analysed statistically, the sharp decrease in SNAT activity observed when very small fractions of pineal gland was analysed, suggested that enzyme inhibition had occurred to an increasing extent as the ratio of moles of substrate or of EGTA to enzyme quantity increased.

Peak SNAT activity was measured within the range 0.06 to 0.125 of a pineal gland per incubate.

The concentrations of substrates and EGTA relative to whole pineal in this study were calculated. The aforementioned relationships to the pineal fraction assayed and the resultant SNAT activity measured are shown in Table 3.3.4. This shows the sharp decline in SNAT activity as the concentration of substrates and of EGTA per pineal gland increases exponentially with each dilution of homogenate. The substrate and EGTA concentrations relative to pineal tissue increased more than 60 fold.

Table 3.3.4. The relationship between the concentration of EGTA , tryptamine, Acetyl CoA and SNAT activity in serially diluted pineal homogenates

Pineal Fraction	[Trypt]/Pineal ($\mu\text{mol/p}$)	[Acetyl co A]/Pineal (nmol/p)	[EGTA]/Pineal ($\mu\text{mol/p}$)	SNAT Activity nmol/p/hr *
0.5	0.6	8.4	0.06	6.61
0.25	1.2	16.8	0.12	8.13
0.125	2.4	33.6	0.24	8.96
0.0625	4.8	67.2	0.48	8.94
0.03125	9.6	134.4	0.96	6.54
0.0156	19.2	268.8	1.92	2.56
0.0078	38.4	537.6	3.84	2.53

* Means of the 2 sets of data

3.3.5. Stability of the enzyme

Preincubation period

This experiment was designed to assess the stability of the enzyme prior to the addition of substrates (the preincubation period). Three variables were introduced, namely concentration of EGTA in the homogenate, temperature of the homogenate and duration of the preincubation period.

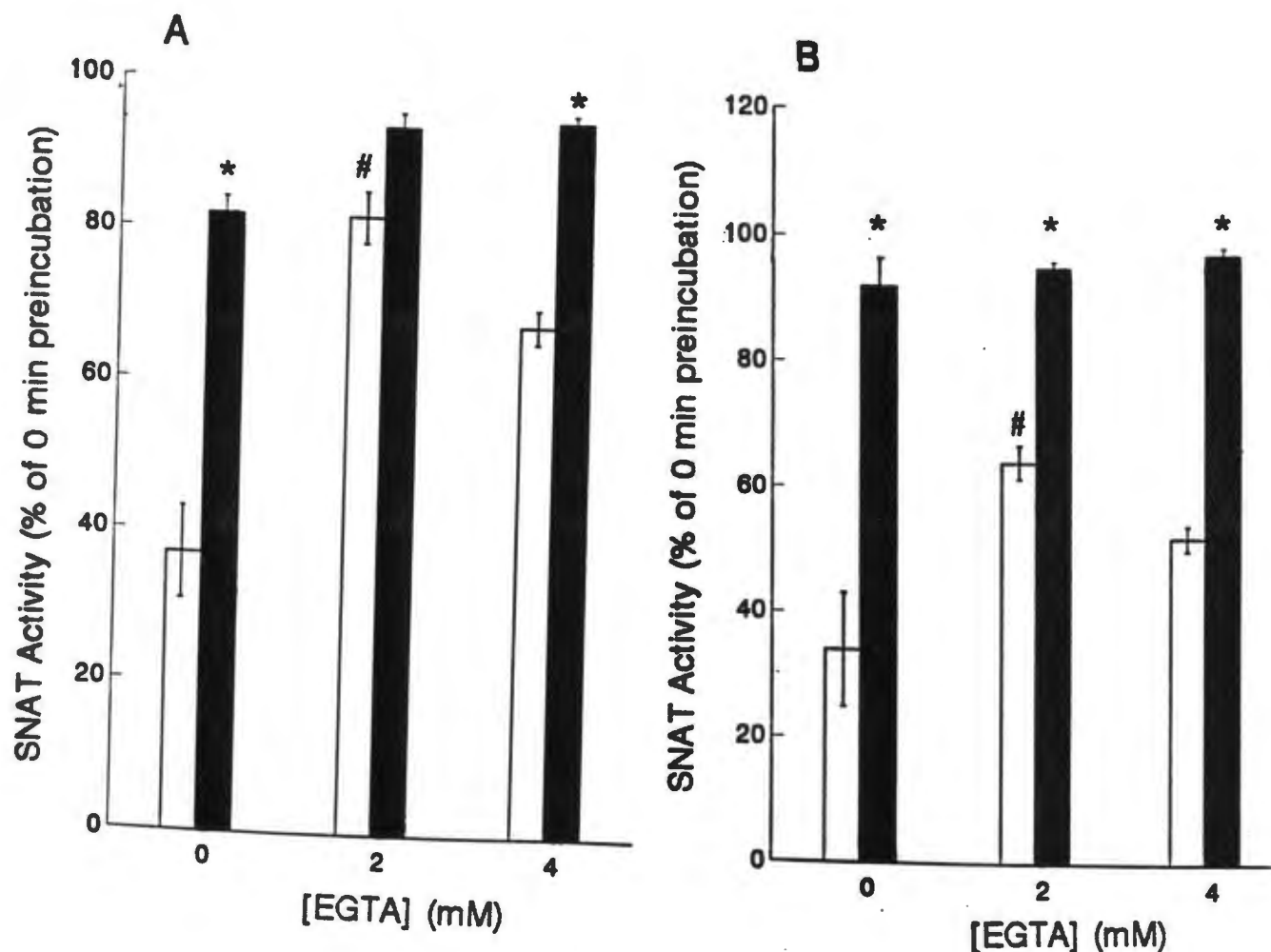


Figure 3.3.5. : The effect of preincubation period, temperature and EGTA concentration on SNAT activity. A = 10min.; B = 20min. preincubation period. Open bars and black bars represent 37°C and 4°C preincubation temperatures respectively. * P < 0.01 vs 37°C. # P < 0.01 vs 0mM EGTA.

It is clear from figure 3.3.5. (A) and (B) that EGTA had a protective effect on SNAT activity which was evident at a concentration of 2mM even when samples were preincubated at 37°C. Low preincubation temperatures caused a statistically significant elevation of the levels of SNAT activity irrespective of the duration of preincubation. The temperature effect was most evident when EGTA was absent from the homogenisation buffer as more than 60% of enzyme activity was lost at 37°C irrespective of preincubation time. The data suggested, therefore, that all tubes containing the homogenate were to be stored on ice, that the homogenates should contain 2-4mM EGTA and that under these conditions, less than 5% of SNAT activity would be lost during a 20min. preincubation period. This provided ample time for the processing of 10-12 pineal glands before initiation of the assay by addition of substrates.

Incubation period

Pineal glands were homogenised under optimum preincubation conditions as derived at above. Pineal homogenates were then incubated for either 10, 20 or 30 mins. and SNAT activity was measured. The results are presented in Table 3.3.5.

Table 3.3.5.: The effect of incubation period on SNAT activity

Incubation Time (mins)	Mean SNAT Activity (nmol/p/hr)	S.E.M.
10	18	1.2
20	17.3	1.3
30	16.2	1.6

From the results presented in Table 3.3.5., no significant differences between the levels of SNAT activity measured in pineal homogenates incubated for either 10, 20 or 30mins. There does however, appear to be a gradual downward trend in the level of activity measured as the incubation period increased.

3.3.6. Circadian variation in SNAT activity

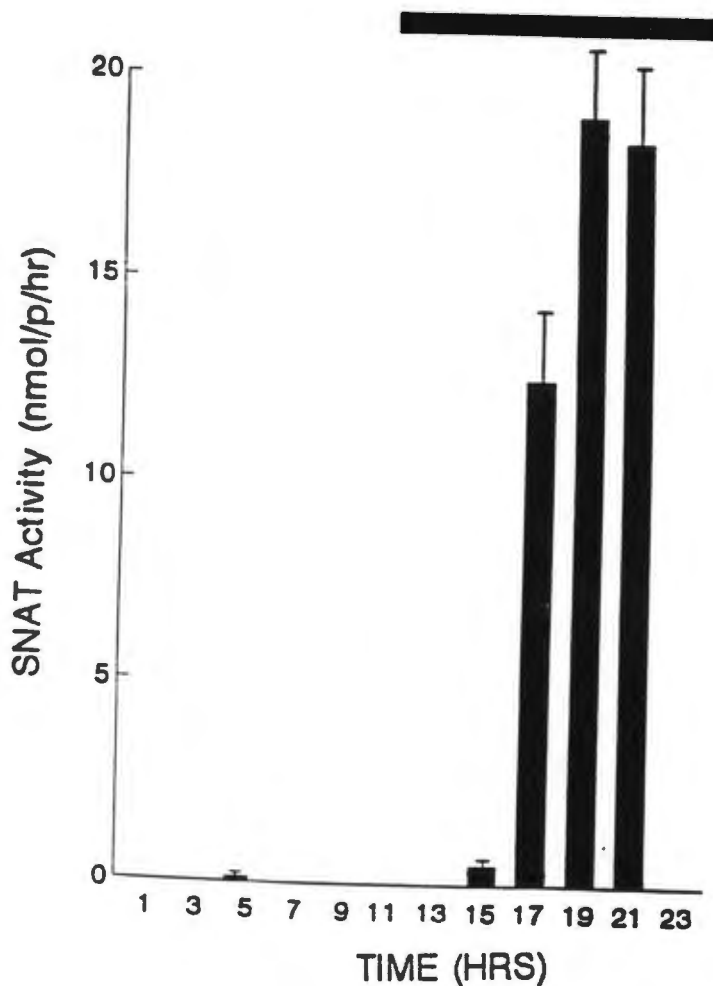


Figure 3.3.6.(i) : SNAT activity measured in rats housed in a normal 10 : 14 hr L/D cycle at the times shown during a 24hr period. The shaded bar at the top of the graph indicates the dark phase of the 24hr period beginning 10hrs after lights on. Time of lights on corresponds to time zero. (n = 6 in each case).

The efficacy of the final assay was assessed by measuring the levels of SNAT activity at specific times during a 24hr period. Groups of 6 rats were decapitated 5hrs after lights on and at 4 or 5 time points at 2hr intervals during the dark period. Experiments were performed on rats housed in either normal or inverted 10 : 14 hr L/D cycles and inverted 14 : 10 hr L/D cycles.

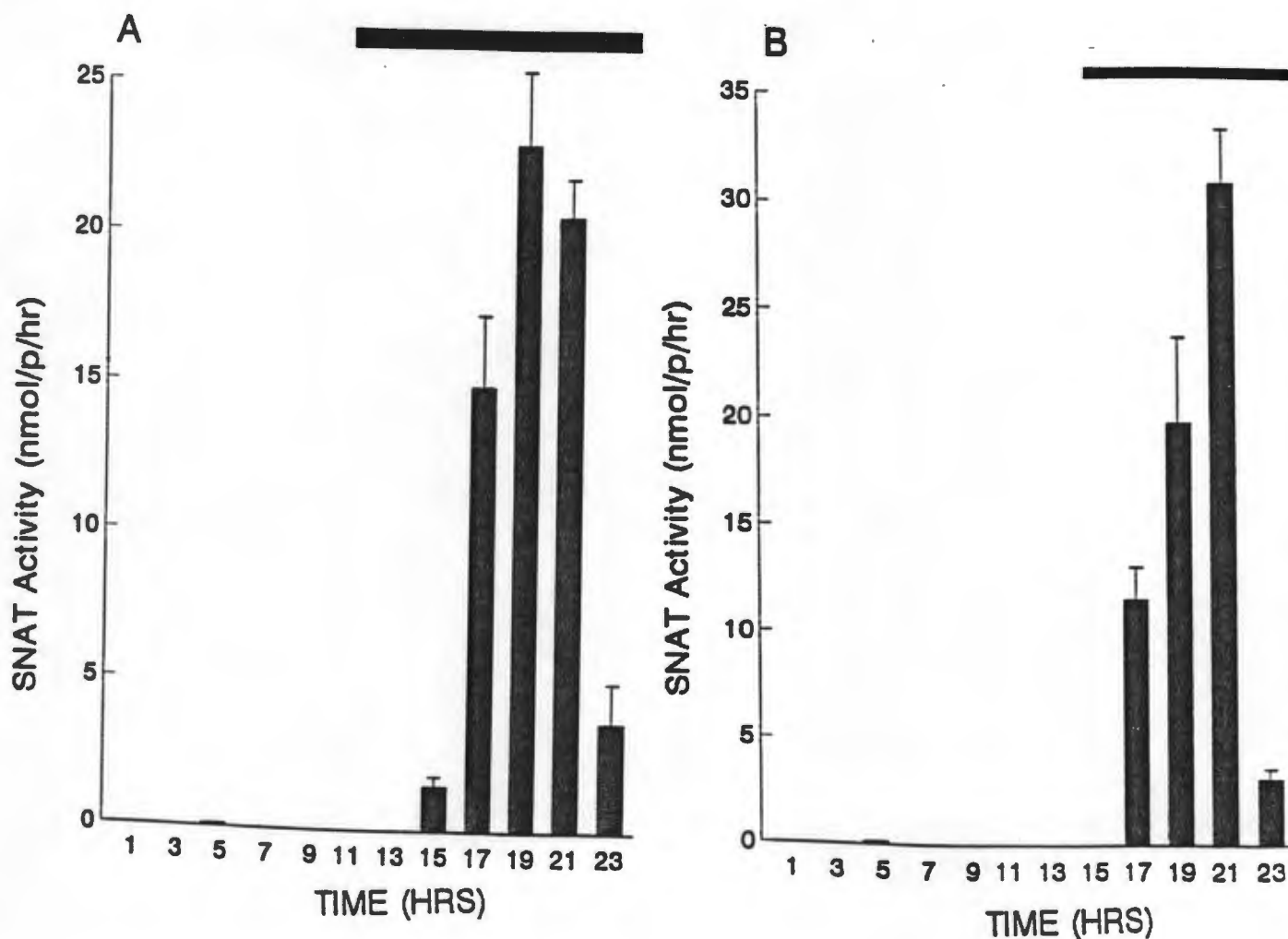


Figure 3.3.6(ii). : SNAT activity, in rats housed in an inverted 10 : 14hr L/D cycle (A) and 14 : 10hr L/D (B) cycle at the times shown during a 24hr period. The shaded bar above the graph indicates the dark phase of the 24hr period which began 10 and 14hrs after lights off respectively. Time of lights on corresponds to time zero. (n = 6 in each case).

3.4. DISCUSSION

The experiments performed in this part of the project resulted in the optimisation of a stable enzyme assay. The subsequent levels of activity measured exceeded those measured in other laboratories by at least 2 fold. The enzyme's activity was preserved by homogenising the pineal glands in 2mM EGTA and keeping the homogenates on ice. The substrate concentrations were varied for optimum levels of activity.

SNAT is an unstable enzyme once released from the interior of the cell. Rodriguez et. al. (128) claimed that the Ca^{2+} ion chelator, EGTA, preserved SNAT activity during homogenisation of the pineal gland, which is predominantly the stage during which the enzyme loses its activity. This finding was confirmed as 1, 2 and 4mM EGTA preserved SNAT activity (fig. 3.3.1.). The levels of activity measured in the absence of EGTA was significantly lower ($P < 0.001$) than when pineals were homogenised in 1mM EGTA. A 4 to 5 fold increase in the levels of SNAT activity was measured when pineal glands were homogenised in buffer containing EGTA. These results could however appear to be contradictory to those found by Morton (85) who showed that at the molecular level Ca^{2+} ions stimulated SNAT activity. The explanation for this apparent paradox could be that release of free Ca^{2+} , within the pinealocyte, had a stimulatory effect. Homogenisation of the pineals however exposed the enzyme to excessively high levels of extracellular Ca^{2+} with the opposite effect. The greatly increased Ca^{2+} could actively stimulate a SNAT inactivating substance (21). Rodriguez et. al. (128) showed that EGTA, which chelates Ca^{2+} , prevented this reaction. On the other hand, high levels (10mM) of EGTA was also shown to be toxic. Accurate recovery and measurement of SNAT activity relied not only on its preservation but also on optimal substrate concentrations. Thus maximal levels of SNAT activity were measured when the pineal homogenate was incubated with 12mM tryptamine (fig. 3.3.2.). The level of activity at this concentration was significantly higher ($P < 0.05$) than that measured when the enzyme was incubated with lower or higher tryptamine concentrations. It was also observed that low

concentrations of tryptamine were rate limiting over the 30min. incubation period but concentrations exceeding 12mM appeared to exert an inhibitory effect on the enzyme.

For reasons which will be discussed later it is important to note that this effect was demonstrated using a fixed concentration (50 μ M) of Acetyl CoA. The effect of Acetyl CoA, on SNAT activity measurement, was investigated by incubating 1/10th of a pineal gland with 12mM tryptamine at various concentrations of Acetyl CoA (fig. 3.3.3.(i)). In all, but one experiment, the level of activity increased significantly as the concentration of Acetyl CoA increased. Levels of enzyme activity, not yet reported in the literature, in the range of 30nmol/p/hr to 40nmol/p/hr at Acetyl CoA concentrations exceeding 500 μ M were measured. From the graph (fig. 3.3.3.(ii)) it is apparent that Acetyl CoA had a significant stimulatory effect which had not been the case for tryptamine. The preservation and viability of the enzyme was confirmed by measuring its K_m , using the Lineweaver-Burk plot. K_m is the substrate concentration at which the reaction velocity is half maximal. Thus, an enzyme with a low K_m , achieves maximal catalytic efficiency at low substrate concentrations. The K_m was measured and found to be 8.19×10^{-4} M. This meant that the chemical reaction which was the basis of the assay proceeded to completion and the levels of activity measured were a true reflection of the enzyme's activity. The graph presented in figure 3.3.3.(i) suggested that no maximal rate of the reaction had been reached as the rate of the reaction continually increased as the Acetyl CoA concentration increased. The maximum level of activity measured then was 40nmol/p/hr at a concentration of 1.18mM Acetyl CoA. On the other hand, the V_{max} for the enzyme was 58.8nmol/p/hr. Acetyl CoA concentration was thus not increased to saturation. However the V_{max} , as measured, would suggest that the optimal Acetyl CoA concentration would be well in excess of 1.18mM. Budgeting constraints restricted the use of this substrate at 180 μ M. A constant pineal fraction (1/10th of a pineal gland) was used for the above experiments. Variation of pineal fraction and keeping the substrate concentration fixed (section 3.3.4.) would permit assessment of the

limits of the assay to a certain extent since the effective concentration of the substrate would increase, relatively, as the pineal homogenate was diluted.

From table 3.3.4. it was clear that maximum levels of SNAT activity were measured under specific conditions of EGTA and substrate concentrations (2mM EGTA, 12mM tryptamine, 180 μ M Acetyl CoA). Outside of these parameters low levels of SNAT activity were measured. The results from these experiments (fig. 3.3.4.) suggested that maximum levels of activity were measured when fractions of a pineal gland in a range of 0.06 to 0.125 was used in the assay. Low levels of activity were measured when the ratio of substrate to pineal gland was high. It thus appeared that high substrate concentrations were inhibitory during the 30min. incubation period. On the other hand, as the ratio of substrate concentration to pineal gland decreased below 33.6nmol/p and 2.4mmol/p for Acetyl CoA and tryptamine respectively the levels of enzyme activity also decreased suggesting that the levels of one or more substrates became limiting. This confirmed the results found earlier (fig. 3.3.2.) which illustrated the inhibitory effect of tryptamine at high concentrations. The increased relative concentration of Acetyl CoA should have had a stimulatory effect on enzyme activity as shown in (fig. 3.3.3.(i)), however the increased tryptamine concentration probably overrode this effect. The relative EGTA concentration also increased with pineal homogenate dilution. High concentrations of EGTA would also be expected to have inhibited SNAT activity as demonstrated by Rodriguez et. al. (128). The authors showed that SNAT activity was inhibited when pineal glands were homogenised in 10mM EGTA which corresponded to 3.75 μ moles of EGTA per pineal which in turn was comparable to the incubation of 0.0078 of a pineal gland in the present assay.

Having developed the assay it was imperative that the stability of the enzyme was assessed as time delays resulted when a series of pineals were processed in a single experiment. The stability of the enzyme was assessed with respect to temperature of the homogenate, EGTA concentration and preincubation time. The results of these studies (fig. 3.3.5.(i)) demonstrated the preservative effect of both EGTA and low temperatures during the preincubation period.

The effect of EGTA alone was clearly shown when samples were preincubated in 2mM EGTA at 37°C. Thus removal of Ca²⁺ ions from the homogenate appeared to be more important in preserving enzyme activity than a reduction in temperature. The enzyme was also temperature sensitive as low preincubation temperatures exerted a statistically significant preservative effect on the measured levels of activity irrespective of the preincubation time. The temperature effect was most evident when EGTA was absent from the homogenate as more than 80% of enzyme activity was retained at 4°C while less than 40% was retained at 37°C irrespective of the preincubation time. It should be remembered, however that the levels of activity were expressed as a percentage of those measured, at the specific EGTA concentration, for zero preincubation time. Thus even though the percentage preservation for 0, 2 and 4mM EGTA was comparable for 10min. and 20min. preincubation, the absolute levels of SNAT activity with and without EGTA were significantly different. See table (appendix : table A) for absolute values. Thus the enzyme's activity is well preserved when pineal glands were homogenised in buffer containing 2mM EGTA and then keeping the homogenates on ice prior to incubation. The stability of the enzyme during the incubation period was also assessed (Table 3.3.5.). Under the optimised preincubation conditions and substrate concentrations the enzyme's stability during the incubation period did not change significantly as the incubation period increased from 10 to 30mins. There appeared, however, to be a gradual downward trend in the levels of activity measured as the incubation period increased. These experiments thus resulted in the development of an optimised assay which measured levels of activity which exceeded those reported in the scientific literature by at least 2 fold. The enzyme was shown to be stable for 20mins. provided the pineal homogenate was stored on ice and contained 2mM EGTA in the homogenisation buffer. It appeared not to deteriorate significantly during a 30min. incubation with substrate at 37°C.

The efficacy of the assay however needed to be tested under physiological conditions and thus the circadian variation in SNAT activity was measured in rats housed in

different photoperiods.

A consistent feature of all the measurements was the low levels of activity recorded during the daylight and the high levels of activity measured during the dark phase. Peak levels of activity at night were in the range of 17.9 to 33.0 nmol/p/hr which exceeded that measured 5hrs after lights on by 100 to 200 fold compared to the 60 to 100 fold described in the literature (57). There were however slight variations between the patterns of activity observed for different photoperiods. Rats housed in either normal or inverted 10 : 14hr L/D cycles showed peak levels of activity at 9hrs and 11hrs after lights off respectively. The results for these time points were not significantly different. This was then considered to be the duration of the elevated levels of activity for the normal and inverted 10 : 14hr L/D cycles. Phrased differently, the elevated levels of activity persisted for at least 2hrs. in animals housed in long dark periods. This was compared to rats housed in short dark periods (fig. 3.3.6.(ii)). Peak levels of SNAT activity occurred 7hrs after light off in rats housed in short dark periods (14 : 10hr L/D cycle). This peak was only measured at the single time point, compared to rats housed in longer dark periods. It was thus concluded that the duration of the elevated levels of activity was shorter in rats housed in short dark periods. The peak level of activity in rats housed in short dark periods was, however, significantly higher than those measured in rats housed in long normal or inverted L/D cycles. These results thus confirm the general pattern of variation observed by other workers (42) between short and long dark periods. Illnerova et. al. (42) demonstrated that rats housed in short dark periods displayed high levels of SNAT activity but of shorter duration. The peak levels of enzyme activity of rats housed in long dark periods were lower but of longer duration than for those housed in short dark periods.

The efficacy of the assay was thus established under physiological conditions. The assay was sensitive enough to detect low and high levels of activity which

differed by 100 to 200 fold. The assay could thus be employed to investigate the inhibitory effects of MF's on SNAT activity as any decrease in SNAT activity would be readily detectable. The documented decrease in SNAT activity is only 25% to 50% in the majority of studies. The stability of enzyme during the assay would thus reduce the possibility of the introduction of an artifact should an inhibitory effect occur.

CHAPTER 4

EFFECTS OF PULSED STATIC MAGNETIC FIELDS ON SNAT ACTIVITY

A) THE EFFECTS OF EARTH STRENGTH MF's ON SNAT ACTIVITY

4.1. INTRODUCTION

Most EMF studies relating to effects on pineal SNAT activity have investigated the effects of variation of one of the components of the earth's MF. The investigations have focused predominantly on the effects of inversion of the horizontal component of the earth's MF on the enzyme's activity (63, 126, 127, 152). Results from such studies indicated that exposure of rats, at night, to repeated inversions of the horizontal component inhibited SNAT activity, pineal melatonin production and in some studies depressed serum melatonin concentration. Very few studies have investigated effects of inversion of the vertical component of the earth's field on SNAT activity. Semm et. al. (144) however showed that inversion of the vertical component of the earth's MF (50 μ T) resulted in decreased electrical activity in guinea pig pineal cells. The reduced electrical activity was however demonstrated in only 21% of the cells exposed.

Several aspects relating to the MF characteristics and the environmental conditions need to be considered when performing MF's studies. Recent reports suggested that the MF was not necessarily the cause of the inhibitory effect on SNAT activity. The rapid switching which occurred when the MF was inverted resulted in the flow of transient currents (eddy currents) which were thought to have caused the biological responses (62). Richardson et. al. (126) confirmed this proposal by showing that SNAT activity was inhibited only when rats were exposed to MF inversions that caused such transients.

In European and North American laboratories, where most of these studies were undertaken, the horizontal component of the geomagnetic field lies between $40\mu\text{T}$ and $50\mu\text{T}$. This is in contrast to the Southern African regions, where the horizontal MF is about $11\mu\text{T}$.

The time of day at which animals are exposed is another aspect which has become relevant to MF studies. Yaga et. al. (180) showed that enzyme sensitivity to repeatedly inverted MF exposure changed during the photoperiod; increasing towards the end of the dark period. However studies performed prior to the postulation of this hypothesis demonstrated inhibitory effects 3-6hrs after lights off which corresponded to the period before peak SNAT activity. Not only was it thought that the time after lights off affected pineal sensitivity to MF's but also the duration of the dark period to which the rats were adapted. Matt et. al. (78) showed that 60 Hz MF's had a greater effect on SNAT activity, in hamsters, when the animals were housed in long dark periods compared to short dark periods. Furthermore pineal sensitivity to MF exposure is possibly influenced by the duration of the exposure period (126). Richardson et. al. (128) demonstrated that rats became adapted to the stimulus after 60min. of exposure whereas exposure for 30mins. caused significant inhibition of SNAT activity. Such findings are in contrast to most of the earlier studies, by the same authors, which showed significant effects after a 60min. exposure period.

The present study was divided into two sections (A & B). The first investigated the effects of inversions of the horizontal and vertical components of the earth's MF. The second section investigated the effects of inversions of $100\mu\text{T}$ DC fields on SNAT activity. Experiments were carried out on rats housed in either normal or inverted L/D cycles. The effects of time of exposure and duration of the dark phase were incorporated into the experimental protocols. From the outset, however, it was impossible to exactly reproduce any study as there were inherent differences such as the variation in the intensities of the vertical and horizontal components of the earth's MF. These differences are discussed in more detail in the discussion.

4.2. EXPERIMENTAL METHODS

4.2.1. Inversion of the horizontal component of the earth's MF

Rats were acclimatised to a normal 10 : 14hr L/D cycle. The time of lights on was 08:00 and the time of lights off was 18:00. Rats (exposed and sham exposed) were placed in their respective sets of coils at 05:30 i.e. 11.5hrs after lights off. The horizontal component of the earth's MF was inverted at 1min. intervals for 30min.

4.2.2. Inversion of the vertical component of the earth's MF

Rats were acclimatised to an inverted 14 :10hr L/D cycle. The time of lights on was 14:30 and lights off was 04:30. Rats were either exposed to a single or to repeated inversions of the vertical component of the earth's MF.

Effect of a single inversion :

Rats (experimental and control) were placed in their respective coils at 10:30. The vertical component of the earth's MF was inverted at 11:15 and the rats were exposed to the inverted field for 15mins.

Effect of repeated inversions :

Rats (experimental and control) were placed in the coils at 10:30. The vertical component of the earth's MF was inverted at 1min. intervals for 60mins.

4.3. RESULTS

4.3.1. Effect of the inversion of the horizontal component of the earth's MF on SNAT activity

Figure 4.3.1. Shows that control and exposed rats had SNAT activities of (16.2 +- 3.2nmol/p/hr) and (15.5 +- 5.7nmol/p/hr) respectively. The effect of a 30min. exposure to repeat 1min. inversions was not statistically significant.

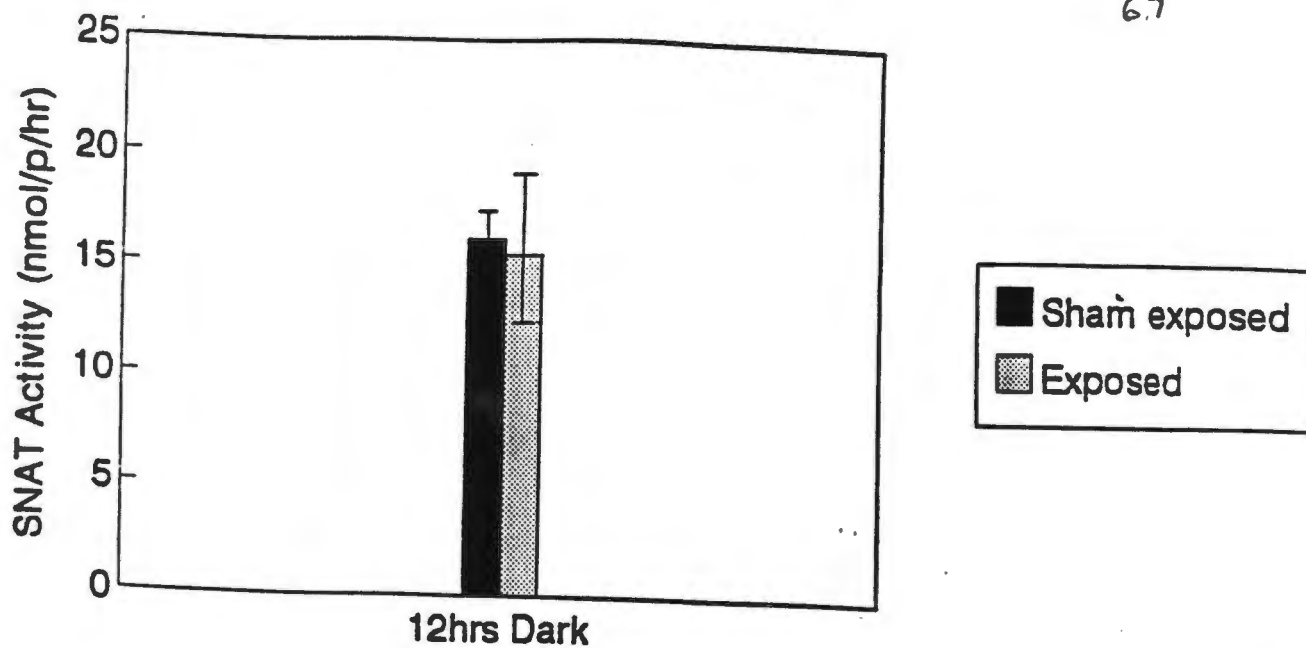


Figure 4.3.1.: The effect of repeated inversions, at 1min. intervals for 30mins., of the horizontal component of the earth's MF on SNAT activity. Rats were decapitated and SNAT activity was assayed 12hrs after lights off. (n = 5 in each case)

4.3.2.

Effect of the inversion of the vertical component of the earth's MF

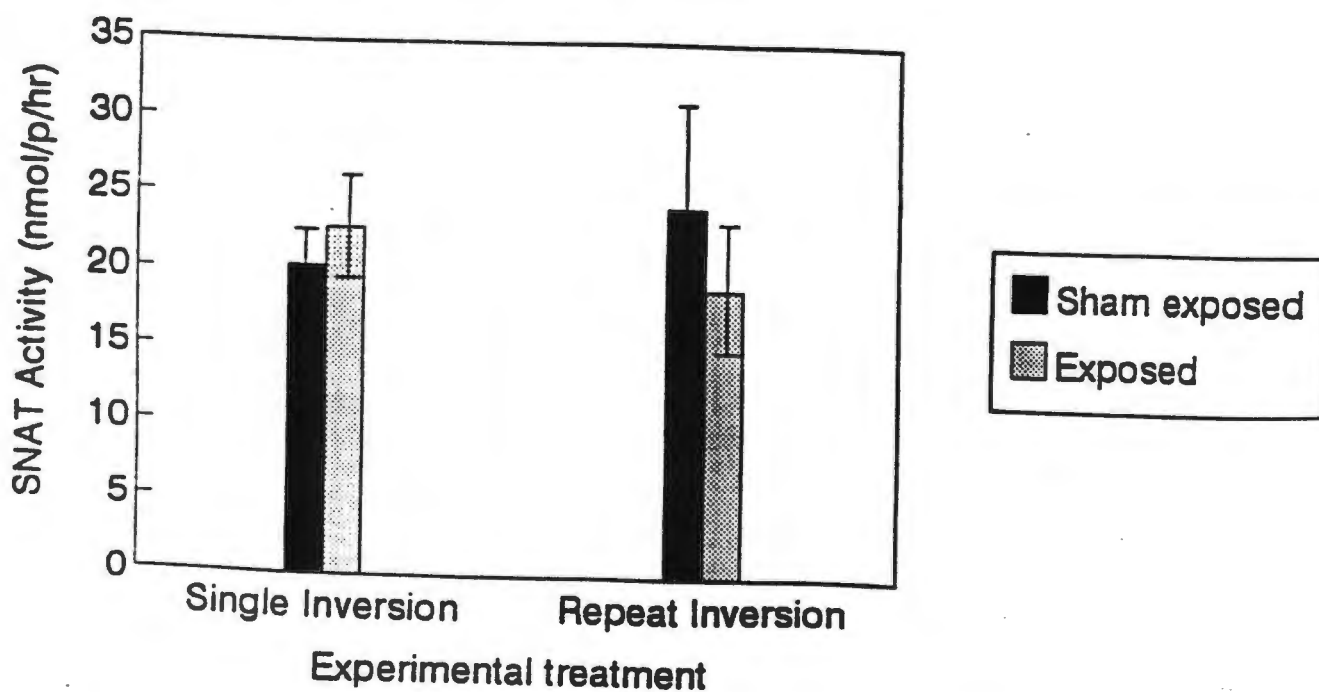


Figure 4.3.2. : The effect of single and repeated (1min. intervals) inversions of the vertical component of the earth's MF on SNAT activity. Rats were decapitated and SNAT activity was assayed 7hrs after lights off following a 60mins. exposure period. (n = 5 in each case)

Figure 4.3.2.: Shows no significant effect of either single or repeated inversions of the vertical component of the earth's MF on SNAT activity. In this experiment measurement of SNAT activity was made at 7hrs after the onset of darkness.

B) THE EFFECT OF 100 μ T STATIC FIELD INVERSIONS ON SNAT ACTIVITY

4.4. INTRODUCTION

Since the inversion of earth strength MF's had no significant inhibitory effect on SNAT activity, the experiments were repeated using 100 μ T DC MF's. The selected DC field strength was twice that of the vertical component of the earth's MF as employed by Semm et. al. (144).

A preliminary study was performed to determine the possible effect of 100 μ T horizontal DC MF inversions on SNAT activity. Subsequent experiments concentrated on the effects of inversion of the vertical DC field. This section describes, in addition, the effects of exposure duration, number of field inversions and the time of exposure during the dark phase on SNAT activity.

4.5. EXPERIMENTAL METHODS

4.5.1. Effect of inversion of a 100 μ T horizontal DC field on SNAT activity

Animals were acclimatised to an inverted 14 : 10hr L/D cycle (lights on at 14:30). Rats (experimental and control) were placed in their respective coils 3 or 6hrs after lights off and were exposed for 60mins., at 1min. intervals, to inversions of the horizontal DC field.

4.5.2. Effect of the frequency of inversion of a 100 μ T vertical DC field on SNAT activity

Animals were acclimatised to an inverted 14 : 10hr L/D cycle (lights on at 14:30). Rats (experimental and control) were placed in their respective coils 3 or 6hrs after lights off. Experimental animals were exposed to either a single inversion, 5min. repeat inversions or 1min. repeat inversions of the vertical field.

Effect of a Single inversion :

Control and Experimental rats were exposed to a 100 μ T vertical DC field, 6hrs after lights off. The field in the experimental coils was inverted 45mins. after the onset of exposure period and the rats were kept to the inverted field for the next 15mins. and then decapitated.

Effect of 5min. and 1min. repeat inversions :

Rats (experimental and control) were placed in their respective coils and exposed to a 100 μ T vertical DC field, either 3 or 6 hrs after lights off. The field in the experimental coils was inverted at 5min. or 1min. intervals so that the rats were exposed to either 12 or 60 field inversions respectively.

4.5.3. Effect of the duration of the exposure period on SNAT activity

Rats were acclimatised to a normal 10 : 14hr L/D cycle (lights on at 08:00). In all these experiments rats were decapitated 12hrs after light off. The effect of three different exposure durations, 60mins., 30mins. and 15mins. were investigated. Since the MF was inverted at 1min. intervals for each time period the numbers of inversions were 60, 30 and 15 respectively. Each experiment was repeated at least once.

Effect of 60mins. and 30mins. exposure periods :

Rats (experimental and control) were exposed to a 100 μ T vertical DC field starting at either 05:00 or 05:30. The vertical field in the experimental coils was inverted at 1min. intervals for either 60mins. or 30 mins.

Effect of 15min. exposure period :

Rats (experimental and control) were placed in their respective coils at 05:30 and exposed to a vertical DC field of $100\mu\text{T}$. The field in the experimental coils was inverted at 1min. intervals for 15mins. after which the experimental and control rats were removed (05:45) from the coils. The decapitation procedure was started 15mins. after the removal from the coils.

4.5.4. Pineal SNAT sensitivity to pulsed MF's during the dark period

Rats were acclimatised to an inverted 10 : 14 L/D cycle (lights on at 16:30). Groups of rats (experimental and control) were placed in their respective coils 6, 10, 11, 11.5 or 12 hrs after lights off and exposed to a $100\mu\text{T}$ vertical DC field. The field in the experimental coils was inverted at 1min. intervals for 60mins. Each study was repeated at least once.

4.6. RESULTS

The results presented in figure 4.6.1., show that 1min. repeat inversions of a $100\mu\text{T}$ horizontal DC field had no significant effect on SNAT activity as measured at 4 and 7hrs after lights off.

4.6.1. Effect of inversion of a 100 μ T horizontal DC field on SNAT activity

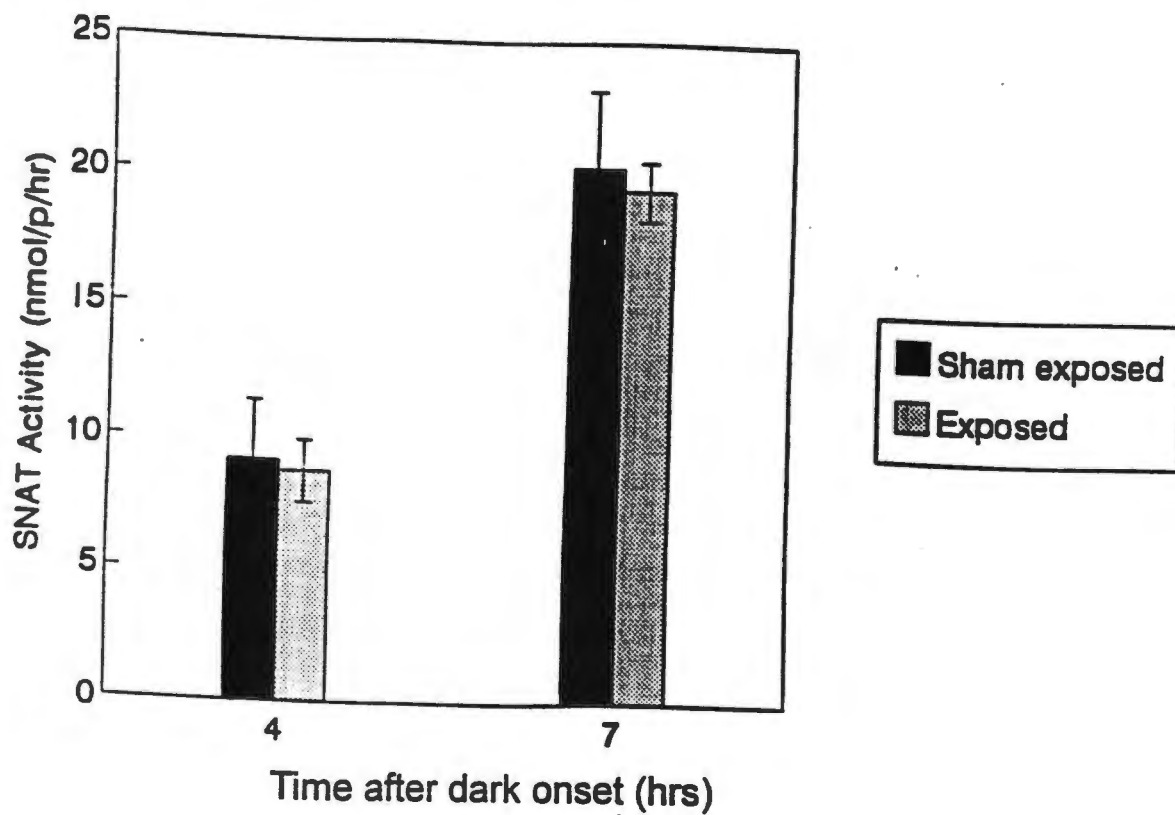


Figure 4.6.1. : The effect of repeated inversions, at 1min. intervals, of a 100 μ T horizontal DC field on SNAT activity. The exposure period was 60mins. starting at 3 or 6hrs after lights off. Rats were decapitated and SNAT activity assayed immediately after the exposure period (i.e. 4 and 7hrs respectively after lights off). (n = 10 in each case)

The results presented in figure 4.6.2. show that the patterns of inversion of the vertical DC field had no significant effect on SNAT activity as measured 4 and 7hrs after lights off. It was evident that neither single nor repeated inversions of the 100 μ T vertical DC field inhibited SNAT activity.

4.6.2. Effect of the frequency of field inversions of a 100 μ T vertical DC field on SNAT activity

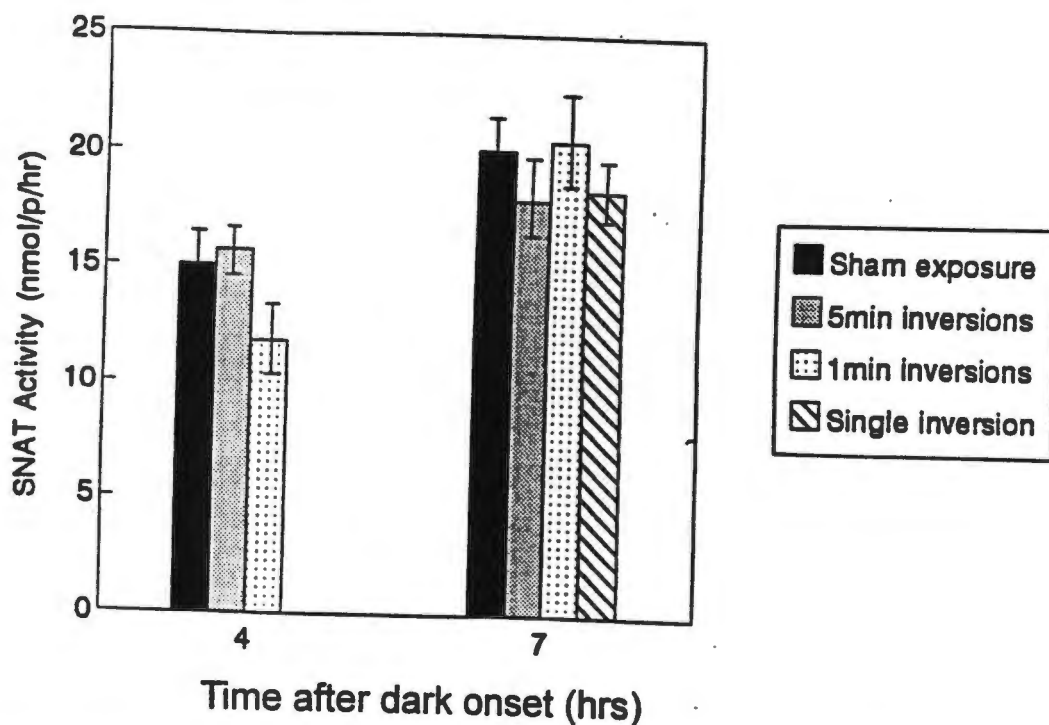


Figure 4.6.2. : The effect of single or repeated inversions of a 100 μ T vertical DC field on SNAT activity. Rats were exposed to the fields at either 3 or 6hrs after lights off and the vertical field in the experimental coils was inverted once or at either 1min. or 5min. intervals during the 60min. exposure period. Rats were decapitated and SNAT activity was assayed immediately after the exposure period (i.e. 4 and 7hrs respectively after lights off). (n = 5 for single inversion; n = 10 for 5min. and 1min. inversions)

4.6.3. Effect of the duration of the exposure period on SNAT activity

From the results presented in figure 4.6.2. there was no significant difference between different patterns of MF exposure. One minute repeat inversions of the earth's field have been used in the majority of experiments in earlier studies to illustrate that MF's inhibited SNAT activity. Subsequent experiments thus investigated MF effects by exposing rats to 1min. repeat inversions of the static vertical field.

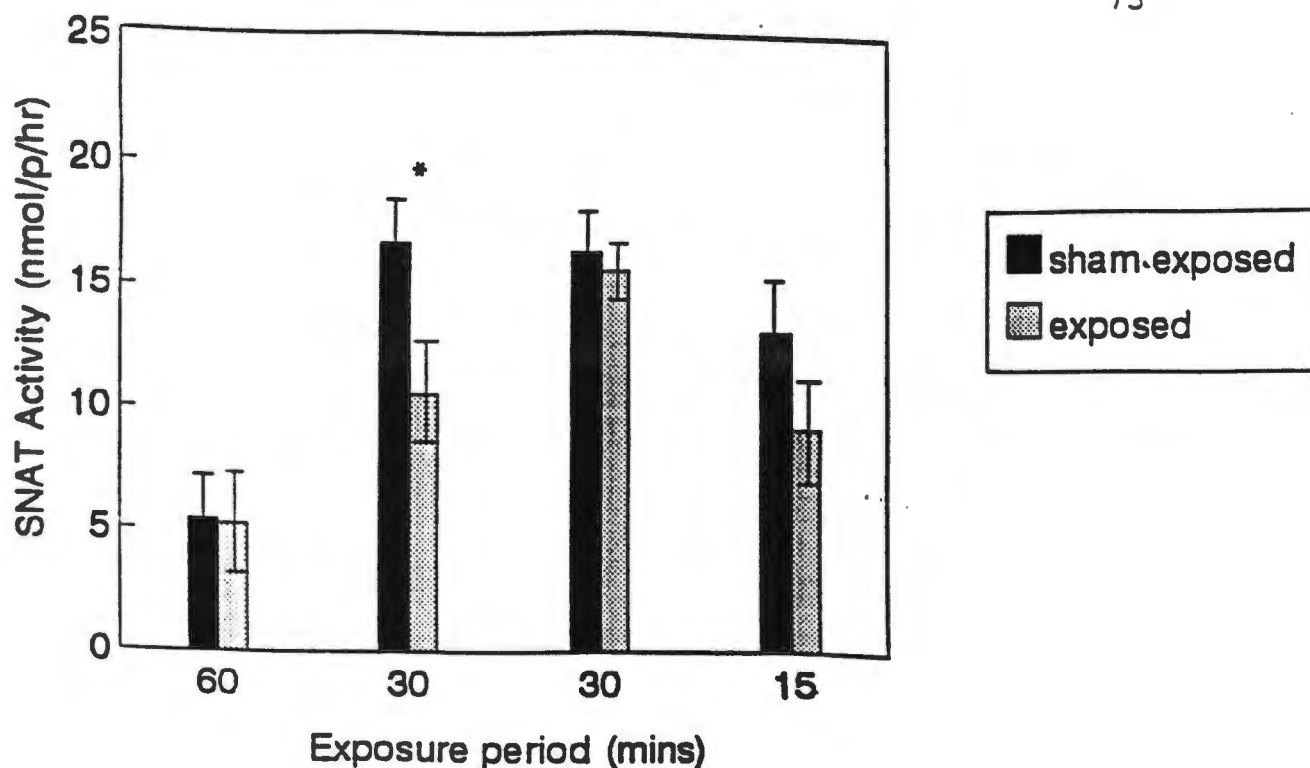


Figure 4.6.3. : The effect of the duration of exposure to a pulsed $100\mu\text{T}$ vertical DC fields on SNAT activity. Rats were exposed for 60 and 30mins. to repeated inversions, at 1min. intervals, of the vertical field. The 30min. exposure period experiment was repeated 1 month after the completion of the first study. Rats were also exposed for 15mins. to repeated inversions of the vertical DC field but the exposure period was followed by a delay of 15mins. prior to decapitation. (n = 15 in each case). * $P < 0.05$

Both the exposed and control levels of SNAT activity were significantly lower than in other experiments when rats were exposed for 60mins. as well as the other groups ($P < 0.05$). There was however no significant difference between the levels of enzyme activity in the control and exposed groups. A significant ($P < 0.05$) inhibitory effect on SNAT activity was measured in one experiment when rats were exposed to the pulsed field for 30mins. The apparent response was a 35% decrease in SNAT activity compared to the controls. This study was repeated one month later when the experimental animals failed to respond to the pulsed fields. Exposure of the rats to pulsed fields for 15mins., followed by a delay of 15mins. also had no significant inhibitory effect on SNAT activity.

4.6.4. Pineal SNAT sensitivity to pulsed MF's during the dark period

The results presented in figure 4.6.4. show no significant differences in the level of SNAT activity between exposed and sham exposed rats at various times during the dark period.

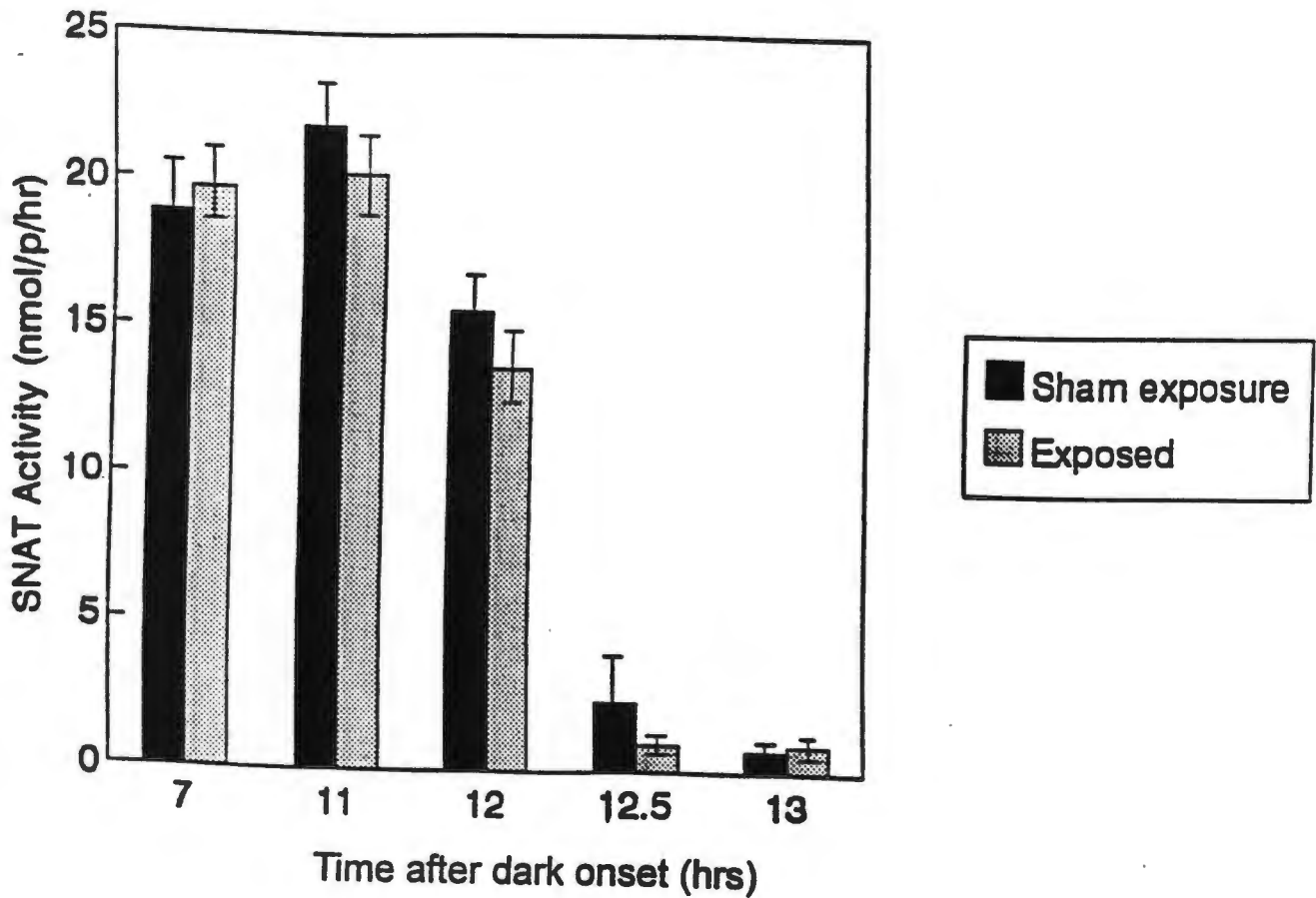


Figure 4.6.4. : The effect of exposure to pulsed static fields, at different times during the dark phase, on SNAT activity. Rats were exposed to 1min. repeated inversions of the vertical $100\mu\text{T}$ DC fields for 60mins. The times of exposure started 6, 10, 11, 11.5 and 12hrs after lights off and the rats were decapitated 1hr later immediately after the exposure period. (n = 15 in each case)

4.7. DISCUSSION

The results from the present experiments failed to demonstrate any inhibitory effects of MF exposure on rat pineal SNAT activity. This lack of a response was consistently repeated under various exposure conditions. Rats were exposed to pulsed static MF's of either earth strength or 100 μ T DC field inversions. The frequency of these field inversions was varied from a single one to 60 during a 60min. exposure period. The duration of the exposure period and the time of exposure during the dark phase also had no significant effect on pineal SNAT activity. These results thus failed to confirm the observations of many of the recent studies in which rats were exposed to pulsed static MF's.

These results should be viewed in light of the negative results found in some of the experiments. There are also authors such as Adair (1) who are adamant that MF's do not affect biological systems as the field strengths are weak and probably below the detection limit of the cell. McLeod (80) also suggested that biological systems in a state of transition were likely to be affected by low frequency MF's but this would not be the case for steady state systems. In the studies performed by Semm et. al. (144) only 21% of the exposed cells responded to the inverted MF's. Richardson et. al. (126) demonstrated that 60min. exposure to pulsed static MF's had no significant inhibitory effect on SNAT activity. This is in contradiction with many experiments which demonstrated that 60min. exposures to pulsed static fields inhibited SNAT activity (62, 63, 127).

Several authors have shown that exposure of animals to inverted MF's inhibit SNAT activity. Studies have mainly focused on the effects of inversions of earth strength MF's. However the MF characteristics and parameters responsible for producing such effects remain largely unknown. Recent studies which attempted to identify these suggest that the time of exposure during the dark phase, duration of exposure periods, patterns of exposure and transient currents may all play a role in the generation of MF effects. Some authors have also questioned the significance of MF orientation while

others have investigated the effects of inversion of the horizontal component of the earth's MF. Exposure of rats to these experimental conditions resulted in significant inhibition of SNAT activity. Similar results were found in the few studies which investigated the effect of inversion of the vertical component. The experiments performed during this project thus attempted to incorporate these aspects of the exposure conditions into the experimental protocols.

The effect of orientation of earth strength MF inversions

The preliminary set of experiments investigated whether inversions of either the horizontal or vertical component of the earth's MF inhibited SNAT activity. The results from the experiments performed which investigated the effect of inversion of either the horizontal or vertical component of the earth's field (fig. 4.3.1. and fig. 4.3.2.) showed no significant inhibitory effect. Neither experimental protocol elicited an effect. These studies showed that the earth strength MF inversions together with their respective orientations had no significant effect on SNAT activity even though the field strength of the vertical component was twice that of the horizontal component of the earth's MF. The mean level of activity for rats exposed to repeated inversions were however lower than for the controls. The results from the experiment in which the vertical component of the earth's field was inverted, showed that adaptation to the MF stimulus was not crucial to short-term exposure as either single or repeated inversions of the vertical component of the earth's MF failed to produce an effect.

Several authors have shown that inversion of the horizontal component of the earth's MF inhibits SNAT activity (62, 126, 180). Fewer studies have been conducted to investigate the effect of inversion of the vertical component of the earth's field. Semm et. al. showed, however, that inversion of the vertical component inhibited electrical activity in guinea pig pinealocytes (144). The strength of the horizontal and vertical components of the earth's MF, in Europe and North America where these studies were performed, however differ significantly from those measured in South Africa.

The intensities of the horizontal and vertical components in South Africa are $10.6\mu\text{T}$ and $22.8\mu\text{T}$ respectively compared to $40\mu\text{T}$ and $50\mu\text{T}$.

There were thus inherent differences preventing the exact replication of previous studies. The obvious difference in the MF intensities, compared to those in other laboratories, to which the animals were exposed was thought to be the reason for the absence of any effect on SNAT activity. Subsequent experiments were thus performed using a higher field intensity. Animals were thus exposed to either $100\mu\text{T}$ horizontal or $100\mu\text{T}$ vertical DC fields. This was twice the strength of the vertical component of the earth's MF as measured by Semm et. al.

Effect of $100\mu\text{T}$ horizontal DC MF inversions

Yaga et. al. hypothesized that rats were more sensitive to MF's later into the dark phase (180). The aim of this study was thus two-fold. Firstly to determine whether the higher MF intensity affected SNAT activity compared to earlier results (fig. 4.3.1.) and to test Yaga's hypothesis. Initial studies investigated the effects of inversion of the $100\mu\text{T}$ horizontal DC field 3hrs and 6hrs. after lights off (fig. 4.6.1.). Rats were exposed to repeated field inversions, at 1min. intervals, for 60mins. No significant effect on SNAT activity was measured both 4 and 7hrs after lights off. The increased field intensity, compared to the earth's field (fig. 4.3.1.), thus had no significant inhibitory effect on the enzyme's activity. It was thus evident that the MF intensity was not solely responsible for eliciting MF effects. In addition the apparent increased responsiveness of the pineal cells to MF's late into the dark phase (180) could not be confirmed from these results.

Effect of the frequency of DC field inversion

Different exposure protocols, with respect to the number of MF inversions per hour, have been used in various laboratories. This section investigated the effects of the number of MF inversions per hour on pineal SNAT activity. Rudolf et. al. (132) demonstrated that a single inversion of the earth's MF inhibited SNAT activity.

Most of the more recent studies have, however, investigated effects of static field inversions at 1min. intervals (62, 126, 127, 180). Lerchl et. al. (62) exposed rats to 5min. interval repeated inversions.

In the present study exposure of rats to all of these different patterns of exposure (fig. 4.6.2.) had no significant effect on SNAT activity as measured 4 and 7hrs after lights off. There was thus no difference in the enzyme's sensitivity to the different numbers of inversions during the exposure period. The results showed that rats responded similarly to single or repeated inversions of the static field. The study provided evidence that the rats were thus not adapted to the repeated stimulus. This study also confirmed the result found earlier (fig. 4.6.1.) which showed that there was no difference in pineal sensitivity to MF exposure at different times during the dark phase. When comparing these results to those obtained in figure 4.3.2. in which rats were exposed to the vertical component of the earth's MF the increased field intensity essentially produced the same result.

Effect of exposure duration

Richardson (126) reported that animals became adapted to pulsed MF's as inhibitory effects on SNAT activity were found 30mins. after exposure but not after a 60min. exposure period. This hypothesis was tested by exposing rats to 1min. repeated inversions of a 100 μ T vertical DC field for either 60mins. or 30mins. (fig. 4.6.3.). The 60min. exposure treatment showed no significant effect on SNAT activity. The low levels of SNAT activity measured in both control and treatment groups could not be explained. The MF inversions in the experimental coils were unlikely to have affected the rats in the control coils (section 2.5.). The time of decapitation for all the experiments was 12hrs after lights off when control levels were about 15 nmol/ p/ hr. However the first study which investigated the effect of a 30min. exposure period showed a significant ($P < 0.05$) inhibitory effect on SNAT activity. This study was repeated 1 month later but the result was not reproduced. This type of variation in the results is an indication of the tentative MF effect on SNAT activity. Rats were also exposed to the repeated field inversion for 15mins.

The shorter exposure period of 15mins. followed by a 15min. delay in the decapitation failed to produce a significant inhibitory effect. The 15min. delay was incorporated into the experiment as Rudolf et. al. (132) showed that a MF effect was greatest 15mins. after the last inversion. The delay in the decapitation of the rats was thus unlikely to be significant. Semm et. al. (144) also showed that the MF inhibitory effect on SNAT activity persisted for as long as 30mins. This study failed to confirm that exposure duration (and thus number of inversions) influenced pineal SNAT sensitivity to MF inversions.

Effect of time of exposure at night

The hypothesis that rats are more sensitive to MF stimuli later during the dark phase was again investigated but using a more comprehensive study compared to the earlier studies performed in the laboratory (fig. 4.6.1. and 4.6.2.). Yaga et. al. (180) recently claimed that pineal SNAT sensitivity to MF's increased during the dark phase. This theory was tested by exposing rats to pulsed MF's at different times during the dark phase. No significant effect on SNAT activity was found at any time during the dark phase. There was however a downward trend in the means of the exposed rats as measured later into the dark phase. All the experiments showed consistently that pulsed static MF's had no significant effect on SNAT activity. Magnetic field intensities and orientations, number of inversions, time of exposure and duration of the exposure period had no significant effect on SNAT activity. Several reasons could be proposed as to why no effects were observed.

Recently it was shown that the MF *per se* was not necessarily responsible for the inhibitory effect on SNAT activity (62, 126) but rather the electrical transients (eddy currents) generated during the rapid inversion of the MF's facilitated the effect. The induced current is directly proportional to the rate of change of this MF (dB/dt). Rapid MF inversion occurred over a 25msec. period which resulted in significant dB/dt values at the time of connecting, and disconnecting, the voltage to the coils (62). The same authors showed that manual switching of the current flow in the coils resulted in a negligible inductive effect and equally negligible effect on SNAT activity.

In the present study the duration of the induced current spike generated with solid state switching was 600-800 μ s (section 2.5). This rapid rate of inversion could be of too short a duration to be detected by the biological system or to cause a significant disturbance to it. The total effective dose of induced current to which the rats were exposed was 1/30 - 1/40th of that used in other studies (62, 126). Additional studies would need to be undertaken in which the switching rates were reduced progressively to determine possible relationships not only between dB / dt and SNAT inhibition but also total current dose and SNAT inhibition.

The Long Evans strain of rats used in the present experiments differed from those used in the majority of MF studies which were Sprague Dawley and Wistar King rats. Olcese and Reuss (96) however showed that there was no difference in response to a single 30min. MF stimulus between Long Evans and Sprague Dawley rats. Both showed inhibition of pineal function. Kato et. al. (51) also demonstrated that exposure of Long Evans rats to circularly polarized MF's inhibited their pineal SNAT activity. It was thus evident that since MF effects were demonstrated in Long Evans rats in at least 2 studies, the strain of rat was unlikely to be relevant to the lack of an effect as demonstrated in this study. The failure to demonstrate inhibitory effects on SNAT activity following MF exposure was thus demonstrated consistently. This inability to reproduce results found in other laboratories could be due to the variations in the experimental parameters compared to that in other laboratories. On the other hand, unexpected as it may seem, MF exposure could have had no inhibitory effect on SNAT activity.

CHAPTER 5

EFFECTS OF OSCILLATING MAGNETIC FIELDS ON SNAT ACTIVITY

5.1. INTRODUCTION

SNAT activity is sensitive to the effects of sinusoidal MF's. Selmaoui and Touitou (142) showed that 50Hz sinusoidal MF's influenced serum melatonin concentration and pineal enzymes activities. These authors suggested that the sensitivity threshold to MF's varied with duration of exposure. Several theoretical models have attempted to describe the mechanism underlying biological responses to EMF exposure. Recently Blanchard and Blackman (14) developed the ion parametric resonance model (IPR) to predict such biological responses. The model predicted that when an ion was exposed to its critical resonant frequency, as a result of the applied DC field in the presence of an AC field of specific frequency, then the probability of a transition between energy states could be modified in a predetermined manner which would be reflected in the biological end point measured. Several experiments have been carried out to test this model and have shown positive results (see review). No attempts have been made to test this model on the pineal melatonin system. It could prove to be useful because investigators would be able to expose the pineal to specific MF conditions. Depending on whether a particular ion is involved in SNAT regulation a biological response could be predicted. The system could also be used to ascertain whether a particular ion could be involved in the regulation of SNAT if exposure to resonant conditions for that ion resulted in a biological effect.

Ca²⁺ ions are involved in the regulation of SNAT activity (21, 85, 122, 128) and alterations in its intracellular concentration would thus affect enzyme activity. The significance of Ca²⁺ ions in the regulation of SNAT activity is discussed in sections 1.3.4.4. and 1.6.4. of the review. Thus exposure of the pineal gland to parametric resonance conditions for Ca²⁺ might affect SNAT activity. The aim of this experiment was thus to determine the effect of exposure of rats to IPR conditions for Ca²⁺ on SNAT activity.

5.2. METHODS

Exposure to Ca²⁺ ion resonance conditions

Rats were acclimatised for 10 - 14 days to an inverted 14 : 10 hr L/D cycle (lights on at 14:30) in a light-tight room. Rats (5 experimental and 5 control) were placed in their respective sets of coils 3hrs after lights off (07:30) and the experimental rats were exposed for 60mins. to the specific resonant MF's. The control rats were exposed to the geomagnetic fields only. Thereafter, rats were anaesthetized, decapitated and SNAT activity was measured.

Exposure parameters

The coil system was the same as for the static MF exposure system (section 2.4.). The Merrit coils were energized and adjusted to generate a vertical AC MF of 14.3 μ T(rms) and the frequency was 17.2Hz. The strength of the vertical DC MF was 22.55 μ T. These conditions were selected as the horizontal and vertical components of the earth's MF were unaltered. The equation for the IPR model (section 1.7.3.) dictates that since the strength of the vertical DC field was 22.55 μ T for this experiment so the strength and frequency of the AC MF, for $n = 1$, needed to be 14.3 μ T and 17.2Hz respectively.

5.3. RESULTS

5.3.1. Effect of exposure, to ion parametric resonance conditions for Ca²⁺, on SNAT activity

The results presented in figure 5.3.1. show no significant difference between the levels of enzy

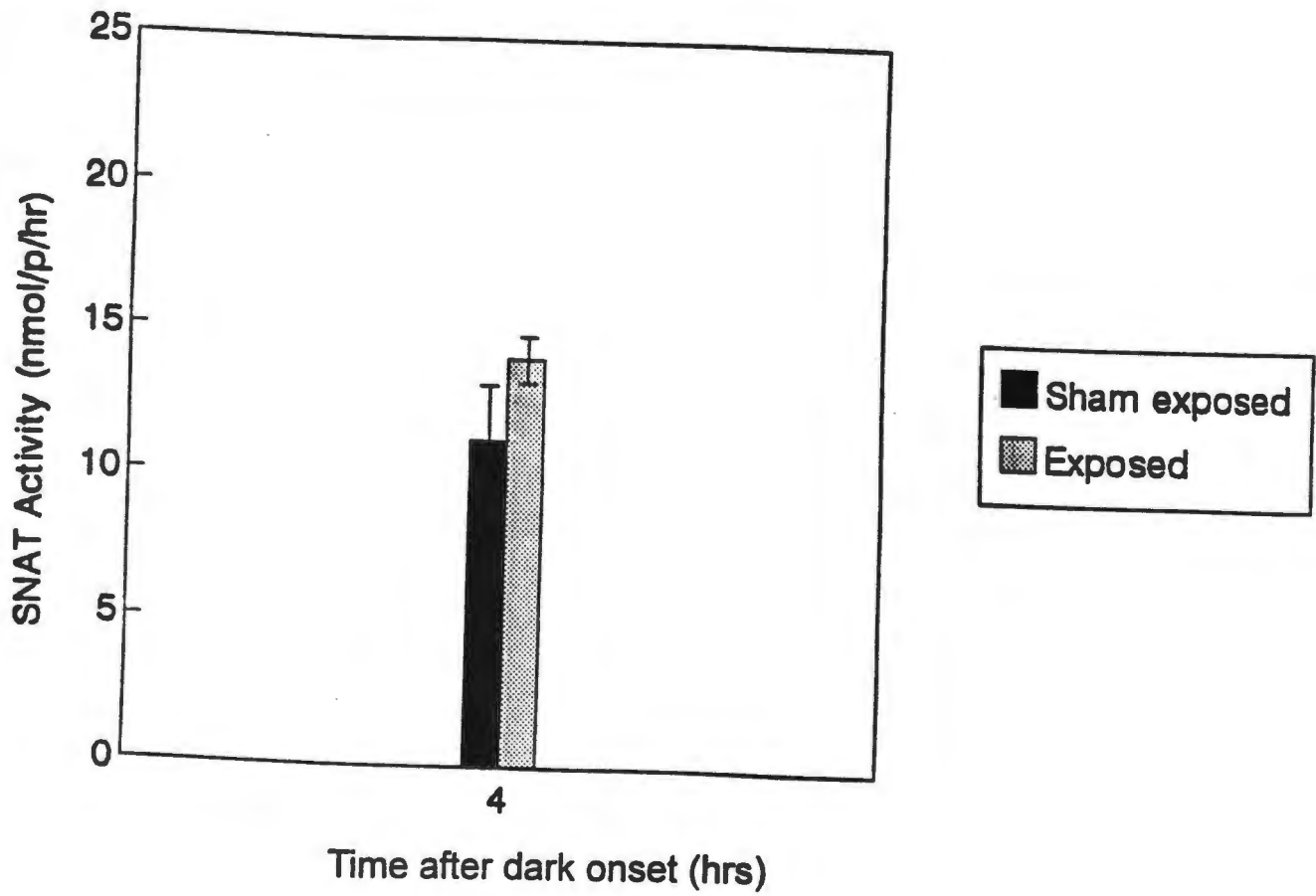


Figure 5.3.1. : Effect of exposure to ion parametric resonance conditions for Ca^{2+} ions on SNAT activity. The graph shows the levels of enzyme activity after a 1hr exposure period which started 3hrs after lights off. (n = 5 in each case)

5.4. DISCUSSION

Since Ca^{2+} ions are involved in the regulation of SNAT activity (1.3.4. and 1.6.4.) it was hypothesized that exposure of animals to resonant conditions would produce an effect on SNAT activity. The IPR model was tested in this study by exposing rats to on resonance conditions for Ca^{2+} ions. The results presented in figure 5.3.3. showed that no significant difference occurred between exposed and control rats.

The precise reason for the lack of any biological effect in the present study was not apparent. However, the complexity of the *in vivo* test system used could mask possible responses that can be demonstrated in simpler *in vitro* experiments (see below). Recently Lerchl (64) showed that exposure of rat pineal glands (in organ culture) to oscillating and static MF's tuned to cyclotron resonance conditions for the calcium ion inhibited pineal melatonin production and SNAT activity. These results have not been reproduced to date. Indeed no investigators have addressed the issue of pineal exposure to resonance conditions whether it be using the ICR or IPR models. Positive results have however been found in an experiment performed by Blackman et. al. (13) on PC 12 cells which were exposed to on and off resonance conditions for particular ions. The frequency and intensity of the AC field was also adjusted for different "n" values. In all their experiments the results were consistent with the predictions of the ICR and IPR models respectively.

The exposure parameters were however set according to the IPR model which was expected to produce an effect on SNAT activity. Not much emphasis can be placed on the results of this experiment as only a single study was performed. The generation of a MF effect was thus apparently not as obvious as one would have expected. A great deal of work needs to be performed on the significance of the IPR model as a predictor of biological responses.

CHAPTER 6

CONCLUDING REMARKS

The aims of this project were to investigate and ascertain whether pulsed static and oscillating MF's affected pineal SNAT activity in male Long Evans rats. Earlier published studies revealed that pulsed MF's inhibited SNAT activity. The effects of oscillating MF's on SNAT activity had not, however, been investigated as extensively.

SNAT is an unstable enzyme and subsequently it loses its activity during the assay. The first aim of the project was to establish an enzyme assay in which the enzyme was stabilized which would thus enable the best possible measurement of SNAT activity. A stable enzyme assay was developed which measured levels of enzyme activity which were higher than those reported in the literature. The maximum level of activity measured ranged from 40 - 50 nmol/p/hr which approached the V_{max} for the enzyme confirming the efficiency of the assay. The levels of activity measured depended on the inclusion of EGTA as a preservative, substrate concentrations and the fraction of pineal tissue assayed. Calcium ions which are released during homogenisation of the gland and which would have an inhibitory effect on SNAT activity were chelated by the EGTA. Maximum levels of activity were measured when the pineal gland fraction was incubated with 12mM tryptamine. At 12mM tryptamine the levels of activity increased further tending towards V_{max} as the concentration of Acetyl CoA increased. The low K_m value found for the enzyme was an indication of SNAT efficiency. The enzyme efficiency was probably related to its stability. The enzyme was most stable when it was homogenised in 2mM EGTA and then placed on ice for as short a preincubation time as possible. A significant decrease in enzyme activity was measured when the enzyme was preincubated for 20mins. compared to 10mins. (at 37°C). The stability of the enzyme was also demonstrated to be preserved during the actual incubation period as no significant difference occurred in the levels of activity measured when pineal homogenates were incubated for 10, 20 or 30mins.

The efficacy of the assay was established by measuring the circadian variation in SNAT activity in rats housed in normal and inverted short and long dark periods. Low levels of activity were measured during the day and high levels of activity occurred at night. These patterns of activity confirmed those found in other laboratories but the absolute levels of activity exceeded those measured in other laboratories by at least 2 fold.

The magnetic field studies entailed exposing rats to pulsed earth strength or $100\mu\text{T}$ DC MF's. A solid state relay switch was used to reverse the flow of current through the coils and thus the MF's. The transients resulting from these rapid inversions were thought to facilitate the MF effect as earlier investigators showed that the MF alone could not produce an effect on SNAT activity. No effects on SNAT activity were found in the present experiments in which rats were exposed to pulsed static MF's. The lack of response occurred in all studies including those in which rats were exposed to relatively high DC MF intensities, long dark periods or in which the times of exposure occurred towards the end of the dark phase. The lack of a response could be ascribed to the short duration of the induced current. The transient current lasted for 600 - 800ms which was much shorter than that reported in other laboratories (25msec.). It could thus be possible that the cumulative dose of induced current was too short to affect the biological system.

A single study was also conducted to investigate the effect of oscillating MF's on SNAT activity. The characteristics of the DC and AC MF's were set according to IPR conditions for Ca^{2+} ions, which are involved in the regulation of SNAT activity. A 1hr exposure of rats to these oscillating MF's, at night, had no effect on SNAT activity. It would seem that brief exposure to oscillating MF's had no effect on SNAT activity. Selmaoui and Touitou (142) showed that longer exposure durations were required to produce an effect if the MF intensity was low. The complexity of the *in vivo* biological system could also account for the lack of a response since Lerchl et. al. (58) showed that exposure of pineal glands *in vitro* to ICR conditions for Ca^{2+} inhibited SNAT activity.

APPENDIX

Table A: SNAT activity under various preincubation conditions

37°C	Preincubation time (mins)	SNAT activity (0mM EGTA)	SNAT activity (2mM EGTA)	SNAT activity (4mM EGTA)
	0	8.8	15.6	15.7
	10	3.4	12.9	10.7
	20	3.1	10.1	8.1
4°C				
	0	10.5	16.3	15.5
	10	8.6	15.2	14.7
	20	9.6	15.4	15.1

SNAT activity is expressed in nmol/p/hr and each is the mean value for three studies in which the enzyme homogenate was preincubated at 37°C or 4°C

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