EFFECT OF SLEEP AND WAKEFULNESS ON CIRCADIAN RHYTHMS IN SELECTED BIOLOGICAL PHENOMENA

BY

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DECLARATION

This thesis was composed by the author, and is a record of work carried out by her on an original line of research. All sources of information have been specifically acknowledged by means of references. All help given by others is indicated in the acknowledgements.

None of this work has been presented in any previous application for a degree.

21/08/85 Elizabeth C. Jazwinska

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Abstract

The concepts of circadian rhythmicity and the mechanisms of sleep are introduced and the possible functions of sleep are discussed. The investigations undertaken to examine the effect of sleep and wakefulness are described in two main sections: Part One (Animal Studies) and Part Two (Human Study).

In Part One, an investigation into the effect of sleep and wakefulness on the 24 h variation in cell population kinetics in the jejunum of the male Syrian hamster (Mesocricetus auratus) is described. The proliferative and functional units in the jejunal epithelium were studied every 3 h during 24 h in groups of control and sleep deprived hamsters. In control hamsters (in which the normal rhythm of sleep and wakefulness was undisturbed) values in proliferative indices (labelling index and mitotic index), the crypt cell production rate, the crypt cell population and the size of the villus compartment were maximal during the light period when hamsters were sleeping. In sleep deprived hamsters peak values in parameters were reduced during the light period in comparison with control. The most striking effect of sleep deprivation was a reduction of the expected increase in the flux of cells from G_2 to M phases of the cell cycle during the light period, in the absence of any difference in food intake between control and sleep deprived hamsters at this time. The results are considered to be consistent with two resting phases in the cell cycle at G_1 and G_2 and the increase in G_2 to M flux is considered to be associated with sleep onset.

Also in Part One, an investigation into the 24 h variation in intraepithelial lymphocyte count in the jejunum of the male Syrian hamster and the effect of sleep and wakefulness on this rhythm is described. Maximum intraepithelial lympocyte count was evident when hamsters were active during the dark period and remained high when wakefulness was continued through the light period. Counts dropped during the light period only when sleep intervened. This variation was noted to be the reverse of the reported T lymphocyte population in peripheral blood of the mouse and human, and the implications of the results are discussed in view of the hamsters' chronic infection

with Giardia muris.

The circadian variation of human stature is well established. In Part Two, an investigation into the effect of sleep deprivation on body length (stature, cervical, thoracic and lumbar lengths) in young and middle-aged men is described. Sleep deprivation was associated with decreased stature and it blunted the normal 24 h rhythm in young and middle-aged men. The pattern of change in vertebral lengths was more difficult to interpret and the lack of conclusive results may have been due to decreased accuracy in the measurement of these shorter bodily dimensions. It is concluded that the 24 h rhythm in height is not an endogenous rhythm but depends upon periods of recumbency over the sleep/wake cycle.

The material of the study in Part Two is in press having been accepted for publication in Experientia.

CONTENTS

CHAPTER 1

I will begin by reviewing some of the phenomena of biological rhythms and sleep, before describing my own research in this area. My principal research concerns the rhythm in cell population kinetics in the jejunum of the male Syrian hamster (Mesocricetus auratus), under normal 24 h conditions and under conditions where there was sleep deprivation. I also investigated the rhythm in intraepithelial lymphocyte count in the tissue obtained for this study. I will subsequently also be describing research into the 24 h variation in human statue which follows from earlier research I undertook as an Honours student of Anatomy at the University of Aberdeen. I believe that these researches will enable evaluation of the contribution of sleep to the maintenance of circadian rhythmicity in these phenomena. In addition, by means of my principal research topic I hope to test my supervisors belief that sleep is a time of bodily restoration.

Circadian Rhythms

The rhythmic nature of biological phenomena has interested scientists and provoked speculation for over two centuries. Of particular fascination is the ubiquity of rhythmic oscillations in that they are to be found throughout the plant and animal kingdom, from simple unicellular to complex multicellular organisms.

The fact that life on Earth exhibits rhythmicity, correlating closely with environmental conditions, can be understood in that life evolved on a rotating planet with a day-night cycle periodicity close to 24 h. Rhythms may well have originated through evolution as an aid to survival of species on Earth.

The evolutionary aspect of rhythms is amply illustrated in species of animals having evolved to be either nocturnally or diurnally active - in some cases their wakefulness evolving to coincide with the availability of food and of lessened danger from predators.

The term "circadian" (circa = about, dies = day) is used to describe biological rhythmic oscillations which show a pattern roughly coincident with the Earth's rotation, and was coined by Halberg et al. (1959a).

The study of rhythms of living organisms, which began as a biological curiosity, has now developed into a necessity with implications for the clinician and research biologist. If biomedical variables are to be studied through drug therapy, or under a variety of experimental conditions, it is of importance that every physiological measurement and laboratory value is interpreted according to the circadian phase at which it was obtained. The rate of urinary potassium excretion, for example, varies by a factor of 5 across the day, independant of day/night variations in posture, activity or food-intake (Moore-Ede et al., 1975).

As the majority of rhythms are circadian, roughly coinciding with the oscillation of the solar day conferred by the rotation of the Earth, it might be argued that all rhythms are exogenous.

A true exogenous rhythm would not continue in the absence of an external oscillation (termed a "zeitgeber" meaning time-giver; after Aschoff, 1954). Control over rhythmic oscillations by environmental conditions alone was first refuted by De Marian (1729) when he reported that the up and down-ward movement in leaves of the Heliotrope plant were coincident with light/dark phases but continued in constant darkness. Later reports showed that the rhythmic nature of this phenomenon persisted in the absence of changes in temperature (Zinn, 1759) and in constant conditions it ceased to be precisely 24 h (De Candolle, 1832).

These early findings led to the realisation that some rhythms are driven by internal timing mechanisms, they will continue in the absence of zeitgebers and are thus endogenous.

Biological rhythms in marine organisms are not timed to the solar day but to another environmental condition imposed by the Earth's rotation: tidal cycles. This tidal rhythmicity is also endogenous. Bohn (1906) reported that the sea anemone, which expands at high tide and retracts when exposed to the air by falling tide, continued to show rhythmic oscillations for 3-8 days in constant water submersion in the laboratory. Bohn and Piéron (1906) also observed that this

tidal rhythm in the sea anemone occured a little in advance of tidal changes, suggesting the interesting possibility that rhythms evolved in organisms to ensure optimal time response with minimal time lag.

The timing of rhythms, conferring on an organism a means of optimal response, can be further illustrated by the study of cortisol levels. The endogenous rhythm in cortisol levels normally shows a trough early in sleep, rising in the later hours of sleep and reaching a peak soon after waking, possibly providing animals and man (Weitzman et al., 1971) with an endogenous 'alarm clock' as a means of preparation for the activity period ahead.

Studies of circadian rhythms, investigating their relationship to time of day and to zeitgebers, have begun to unravel the complex mechanisms of circadian rhythm control.

Animal Studies

The most fundamental rhythm in animals is that of the alternation between rest-sleep and activity-wakefulness. The entrainment of the rest/activity rhythm in nocturnal animals to the zeitgeber of alternating light/dark phases of the solar day was recognised by Halberg et al. (1958). By investigating the timing of rhythms in mice under a reversed lighting schedule, it was found that the timing of rhythms shifted to the new timing of the light/dark phase, following an adaption period. The rate of mitotic division in mouse pinnal epidermis, for example, shifted after 23 days under reversed lighting.

Similarily Alov (1959) reported a reversal in the rhythm of mitotic division in the epithelium of the cornea, tongue, oesophagus and the epidermis of mice under reversed lighting. This reversal was accompanied by a reversal in the rhythm of bodily activity. Recognising that endocrine glands play an important role in cell division, Alov (1963) studied the rhythm of mitosis in mouse corneal epithelium after extraction of adrenal and thyroid glands. The 24 h variations persisted, but the range in values decreased, suggesting

that adrenal and thyroid glands were involved in the mechanism of the 24 h rhythm but did not have absolute control.

As the light/dark zeitgeber is so important in the timing of rhythms in nocturnal animals, the test for an endogenous element in rhythms requires removal of the zeitgeber by imposing constant conditions of light (LL) or dark (DD).

The activity pattern in hamsters is strictly entrained to the dark period (casual observations of Aschoff and Meyer-Lohman, 1954 - reported by Chaudhry et al., 1958; De Coursey, 1964; Aschoff et al., 1973). Bünning (1967) reported that the spontaneous running activity rhythm in hamsters was endogenous for it persisted with a period of approximately 24 h in continuous dim illumination.

The rhythm in mitotic division also persists in constant dark (DD) or light (LL). Scheving et al. (1974b) reported that in DD the mitotic rhythm in rat corneal epithelium continued to show a regular rhythm with a period of about 24 h and with a peak and trough 12 h apart. The results in LL are, however, not so easily interpreted, although a basic rhythm persisted, two peaks and one trough were noted over 24 h.

The control imposed by light/dark periods on circadian rhythms in hamsters was further elucidated by studies on the effect of light pulses in DD, and of dark pulses in LL. Ellis et al. (1982) reported that light pulses in DD had no significant effect, for they yielded advances, delays, or no change in the activity rhythm of hamsters. Dark pulses in LL, however, did significantly peturb the circadian system, and suggested the possibility that nocturnal animals rely on sensing the dark period to entrain rhythms.

The rhythm of feeding in animals is naturally closely linked to the period of motor activity, and introduces the possibility that food intake times circadian rhythms. This has, however, been refuted. Scheving, et al. (1974a) found that the lighting regimen dominated interacting meal schedules and synchronized the mitotic rhythm in corneal epithelium of mice. Pauly et al. (1975) investigated the

same rhythm in mice, but with restricted feeding where access to food was limited to a daily 4 h span, and found that the mitotic rhythm was not phase shifted or altered. Consistent with this are the findings of Philippens et al. (1977) that the mitotic index in rat corneal epithelium altered only minimally when meal timing was restricted. The authors warn against the use of meal timing to control circadian rhythms as the light/dark zeitgeber remained the main synchronizer of rhythms. Furthermore, Morimoto et al. (1979) reported that the periodicity in activity and eating behaviour plays a direct and potent role as an entrainer of circadian adrenocortical rhythms in the rat, but the solar day plays an indirect but predominant role as an overall synchronizer of all biological rhythms.

Under normal 24 h conditions, circadian rhythms in animals are thus synchronized by the light/dark zeitgeber. It is now evident that despite showing synchrony, rhythms are controlled by a complex series of interacting oscillators which show a varying degree of responsiveness to zeitgebers. An excellent example of this phenomenon is shown by desynchrony of rhythms in mice, displayed during adaptation to a reversed light/dark cycle. Halberg et al. (1958) found that after 9 days of reversed lighting there was a concomitant reversal in the rate of mitotic division in liver parenchyma cells, but not in pinnal epidermis. Thus, due to the interrelationships between rhythms and their differing degrees of responsiveness to zeitgebers, rhythms within one animal may require different adaption periods.

Desynchrony of rhythms under abnormal conditions has been informative in aiding the understanding of circadian rhythm control mechanisms and will be discussed further when dealing with the internal 'biological clock'.

Human Studies

A growth of interest in circadian rhythms came with the realization that rhythms are not confined to plants and animals but are evident in humans too.

Man has achieved a degree of mastery over environment. He has no fear of predators, and the advent of artificial light enabled him to dictate his own light phase. Despite this, man has remained rhythmic over 24 ± 4 h and is thus termed circadian (Halberg et al., 1959a).

Possible explanations for man's circadian behaviour are his strong dependence on watches and clocks and, interrelated to this, his complex social organization patterning behaviour around 24 h.

Broadly summarized, investigations into the importance of zeitgebers to man's circadian rhythmicity have involved the following approaches: a) temporal isolation units, b) shift work and c) time zone travel.

These different approaches each have inherent advantages and disadvantages (Minors and Waterhouse, 1981b). Taken as a whole, however, the findings have been informative in aiding the understanding of strict circadian periodicity in the human.

a) Temporal Isolation Units

To investigate the effect of time-pieces and environmental cues (dictating social behaviour) on the timing of circadian rhythms, many investigators have utilized temporal isolation units. This procedure involves the removal of zeitgebers, and maintenance of rhythmicity under these conditions is considered to be due to endogenous control or an internal body clock.

Certain discrepancies have arisen in studies involving temporal isolation, and some of the anomalous results are reviewed by Minors and Waterhouse (1981b), and are believed to be due to the small numbers of subjects participating in such studies.

Lewis and Lobban (1957a,b) performed some classic experiments on circadian rhythms in two isolated communities during the artic summer at Spitzbergen. Each community made use of time-pieces that indicated the passage of 24 h, when 21 or 27 real hours had elapsed, and

involved 7 and 5 subjects respectively. Excretory rhythms (the urinary flow, and potassium and chloride urinary constituents) of subjects were monitored. Results showed that initial adaption of rhythms to the new time routine was uncommon. Although there was progressive adaption during the course of the experiment, in the majority of subjects the degree of adaption was seldom complete even after 6 weeks on the abnormal routine. The authors concluded that 24 h excretory rhythms in man are under strong endogenous control. In the same subjects the rhythm of body temperature was measured (Lewis and Lobban, 1957b). Body temperature adapted almost immediately in 11 out of 12 subjects and was strongly associated with the sleep/wakefulness rhythm. The desynchrony occuring between rhythms in urinary excretion and body temperature was suggested by the authors to be due to the existence of two timing mechanisms with varying responsiveness to zeitgeber. This desynchrony may indeed be similar to that occuring in animals (Halberg et al., 1958).

The protocol used by Lewis and Lobban (1957a,b) makes their investigation particularly informative. The study of isolated communities enables comparison of data from larger subject numbers than is possible in temporal isolation units. Furthermore, the zeitgebers of time-pieces and social organization are added, motivating subjects towards full adaption to an abnormal time routine. The endogenous rhythm in urinary excretion persisting in spite of zeitgeber set at 21 or 27 h would suggest that this rhythm is under particularly strong endogenous control.

Studies in temporal isolation units have also indicated the strong exogenous control over circadian rhythms in humans, conferred by the need to make social contact. Aschoff (1969) and Wever (1975) found that when pairs of subjects were studied in isolation, their sleep/wakefulness and temperature rhythms were synchronous, even though each subject exhibited internal desynchronization between the two rhythms. Subjects synchronized to each other suggests the influence of social interaction.

b) Shift Work

Although it can be argued that only under conditions of temporal isolation will the true exogenous/endogenous components of circadian rhythms become apparent, the need for shift work enables the study of rhythms 'in the field'. Shift work demands a change in the sleep/wake cycle over 24 h either by varying degrees (i.e. early morning, late afternoon shifts) or a complete reversal for diurnal man (i.e. night work). The study of shift workers thus enables the study of flexibility of rhythms by imposing phase shifts.

Shift work is, as a rule, regarded with some dislike; night shift being the most unpopular (Wedderburn, 1978). The major problem for shift workers being that they are a nocturnal minority in a diurnal majority population. They find themselves in a paradoxical situation whereby their work demands activity, when the rest of the population is sleeping, and demands that they sleep when there are exogenous disturbances.

Continuing on the same shift (as opposed to rotating shifts) does lead to some adaption in physiological rhythms to the new phasing. Sharp (1961) studied a community of 6 men who altered their phase by 12 h. It was found to take a minimum of 2 and a maximum of 9 days before their sleeping pattern had satisfactorily adjusted to the new timing. Meers (1975) reported that physiological variables, for example oral temperature, in night workers adapted over the course of a week to the new subjective day/night time. This adaption was slow compared to the reversal back to normal timing - noted after a diurnal routine had been adopted for only one day. It is evident that this is another example of the strength of the social interaction zeitgeber. During night shift the worker is still aware of the diurnal pattern exhibited by the rest of the population and is thus strongly motivated towards a diurnal timing in his free days.

The major complaint that shift work is tiring, together with the difficulty workers experience in phase shifting their sleep to an exogenously disruptive time, is perhaps an indication of the importance of sleep to the physical and mental well-being by perhaps

conferring rhythmicity in physiological variables. Folkard et al. (1978) concluded that subjects most suited to night work were those able to take day-time naps and to sleep through noise. By an interesting series of experiments, Minors and Waterhouse (1980) proposed that an anchor sleep of 4 hours taken regularly could confer a stable period of 24 h on circadian rhythms, even though another 4 hours of sleep was taken randomly.

The final investigatory method to be reviewed is one that overcomes the problems inherent in the study of temporal isolation and shift work, and is that of time zone travel.

c) Time Zone Travel

Subjects undergoing time zone travel (now commonplace with the advent of international air travel) are forced to change their circadian rhythmicity to a new phase. Thus adaption of rhythms can be studied when all zeitgeber are acting together, aiding (rather than conflicting with) an adaption. Studies have shown that under these conditions, circadian rhythms can change phase and once again become stable, despite being greatly out of phase with previous time experience.

As previously discussed, the exogenous and endogenous components of circadian rhythms normally occur in synchrony but show desynchrony when external oscillations are removed. Travel across time zones also causes desynchronization between exogenous and endogenous components during an adaption period (Strughold, 1971). This desynchronization has become known colloquially as 'Jet-Lag'.

The strength of endogenous control over rhythms in urinary potassium excretion and 17-hydroxycorticosteroids, despite exogenous influence to change was shown by Lafontaine et al. (1967). Pre- and post-flight measurements were carried out in subjects flying across 11 time zones and urinary constituents only adapted to the new timing after 5 days.

The slow adaption of rhythms is not caused by the event of travel

itself. In a series of classic experiments Hauty and Adams (1966a,b,c) found that during the first post-flight day (following a flight South with no time zone shift) subjects showed a decrement in performance in a number of psychometric tests; changes in temperature, respiration and cardiovascular rhythms and an increase in fatigue. By the second post-flight day these rhythms had recovered. Following a flight East or West (involving a time zone shift) rhythms only changed to the new time phase 4-8 days later.

It is evident that although endogenous rhythms are able to phase shift, they require a significant adaption period and adaption is aided by the altered zeitgebers. Klein and Wegmann (1974) reported that adaption of rhythms is speeded up by strengthening the zeitgeber (i.e. by increased activity) in the new time zone.

Studies of circadian rhythms in animals and humans have elucidated some of the factors controlling synchrony of exogenous and endogenous rhythms into circadian periodicity. Furthermore, they have shown that interrelationships and control of rhythms is complex and point to the possibility of the existence of an internal 'clock' controlling rhythms and homeostasis.

CHAPTER 2

The Search for an Internal 'Biological Clock'

The first evidence for specific neuroanatomic sites acting as circadian pacemakers came from the work of Richter (1965, 1967). To remove the zeitgeber of light/dark (which could be a masking influence on circadian rhythms) Richter used blinded rats. The rats were subjected to a variety of experimental procedures: removal of gonads; pancreas, adrenal, pituitary, thyroid and pineal glands; electroshock therapy; induced convulsions; prolonged anaesthesia and alcoholic stupor. None of these procedures affected the circadian rhythms in feeding, drinking and locomotor activity. Richter then carried out a series of lesions in the rat brain and found the only location where lesions affected circadian rhythms was in the hypothalamus.

The hypothalamus in the animal and human brain has a functional importance out of proportion with its small size. It receives neuronal input of special emotional significance from the thalamus and limbic system, and information of largely visceral origin (conveyed by the ascending fiber systems of the brain stem and spinal cord). In addition, the hypothalamus is under systemic influence, for its constituent nerve cells respond to properties of the circulating blood (including its temperature, osmotic pressure and hormonal levels).

Efferent pathways from the hypothalamus are to autonomic nuclei in the brain stem and spinal cord and to the pituitary gland by means of neurosecretory cells. These neurosecretory cells elaborate the hormones of the neurohypophysis and produce releasing factors controlling hormonal output of the adenohypophysis (Barr, 1979).

By these means the hypothalamus is able to produce responses to emotional changes and to needs signalled by hunger and thirst, and is thus instrumental in homeostasis - the maintenance of a constant internal environment.

Nocturnal animals show entrainment to the light/dark cycle. The most reliable way of detecting light/dark cues is optically. An indication or visual input to an internal 'clock' came from the findings of Halberg (1969) that vision played an important role in neuroendocrine regulation.

Moore and Lenn (1972) reported that labelled amino acids, injected into the vitreous humour of the eye, were taken up by retinal ganglion cells and transported through neural projections from the retina to the suprachiasmatic nuclei (SCN) of the hypothalamus. No other pathway, to the supraoptic or any other hypothalamic nucleus, was observed. Identification of this unique monosynaptic retinohypothalamic pathway suggested the possibility of direct visual input to the SCN pacemaker.

Shortly after identifying a retinohypothalamic tract Moore and Eichler (1972) found that SCN lesions in the rat caused a loss of the adrenal corticosterone rhythm, suggesting that the SCN or closely adjacent neuronal groups participate in the production of rhythmic events controlling the hypothalamo-pituitary regulation of adrenal function.

Independent to the work of Moore and Eichler, Stephan and Zucker (1972) found that lesions interupting primary and accessory visual pathways failed to disrupt nocturnal drinking behaviour in albino rats. Bilateral electrolytic lesions in the SCN, however, permanently eliminated the nocturnal drinking and locomotor activity. Destruction of medial and preoptic areas had no effect. In conclusion, Stephan and Zucker suggested that the generation of rhythms entrained to the light/dark cycle may be co-ordinated by neurons in the suprachiasmatic region, and that normal sleep is modified by the SCN.

A retinohypothalamic tract also exists in the hamster (Moore, 1973) and, as in rats, the SCN functions as a circadian pacemaker. Stetson and Watson-Whitmyre (1976) found that destruction of the SCN abolished circadian rhythmicity in locomotor activity, oestrous cyclicity and photoperiodic photosensitivity in the hamster. Rusak

(1977) carried out an informative series of experiments elucidating hypothalamic control of the circadian rhythmicity in running activity of hamsters. Following destruction of the SCN, leaving visual connections intact, some persistent clustering of wheel-running activity was seen entrained to the light/dark cycle which may have been due to a masking effect (Aschoff, 1960) as no circadian rhythmicity occured in constant conditions. When the optic tracts were severed behind the optic chiasm leaving the pathway to the SCN intact, circadian rhythmicity entrained to the light/dark cycle was evident although the animal was behaviourally blind. From these findings Rusak suggested that the SCN may function as a pacemaker by generating a circadian rhythm of neural activity, entrained by photic input, which regulates activity of the oscillators in the circadian system.

The possible function of the SCN as circadian pacemaker by means of its neural activity is consistent with results reported by Inouye and Kawamura (1979). Measurements were made of electrophysiological activity in hypothalmic islands (including the SCN) which had been surgically isolated from neural connections with the rest of the brain. Persistence of circadian rhythmicity in electrophysiological activity was found in these isolated islands, but not in adjacent separated regions of the brain.

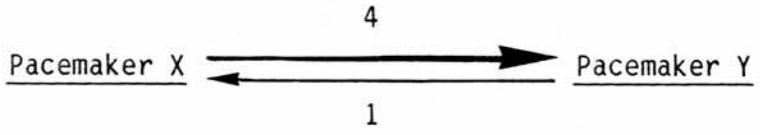
Circadian rhythms in neural activity are also evident in slices of rat hypothalamic tissue containing the SCN (Groos and Hendricks, 1982; Green and Gillette, 1982).

In addition to the retinohypothalamic tract, other neural pathways may play a part in light/dark entrainment. Direct connections exist between neurones in the SCN and axons in the optic chiasma (Groos and Mason, 1980). In addition, projections from the lateral geniculate nucleus have been found to connect with the SCN (Ribak and Peters, 1975; Swanson et al., 1974).

Internal desynchronization of rhythms under abnormal conditions reported in man (Jouvet et al., 1975; Aschoff, 1965; Wever, 1979) and animals (Halberg et al., 1958), suggested that circadian rhythms are

controlled not by one, but two internal clocks.

Czeisler et al., (1980) analysed circadian rhythmic variables and found that variables fell into two main groups which they termed 'X' and 'Y', suggesting the possibility that each group had its own pacemaker. Circadian variables falling into these two main groups are reproduced below, after Moore-Ede (1983).



REM Sleep	Rest/Activity Cycle and Slow Wave Sleep
Core Body Temperature	Skin Temperature
Plasma Cortisol	Plasma Growth Hormone
Urine Potassium Excretion	Urine Calcium Excretion

The coupling force exerted by 'X' on 'Y' is approximately four times greater than that of 'Y' on 'X' (Czeisler et al., 1980). The authors arrived at these figures following investigation of the 'X' driven body temperature rhythm and the 'Y' driven sleep/wake cycle in a human subject under temporal isolation. Rhythms, when 'free-running' and synchronous, ran at 25.3 h. Following desynchronization the sleep/wake cycle ran at 29.3 h and the rhythm in body temperature at 24.5 h. It was the fact that the rhythm in body temperature remained so much closer to the 'free-running' conditions that led the authors to suggest the strength of the coupling force of 'X' on 'Y'.

Measurements of variables following desynchronization obtained by Wever (1979) suggest the coupling force between 'X' and 'Y' to be 12:1. Kronauer et al. (1982), however, support the coupling force between 'X' and 'Y' of 4:1, pointing out that even following internal desynchronization there will be continued interaction between 'X' and 'Y'.

The role of the SCN in the theory of 'X' and 'Y' pacemakers is that they are most probably the 'Y' pacemaker. Indeed the control of the SCN has been shown to be confined to those variables falling into the 'Y' group. Following destruction of the SCN in squirrel monkeys, the behavioural rhythms of activity, feeding, and drinking were disrupted but the rhythm in body temperature persisted (Fuller et al., 1981). In addition the circadian rhythm in cortisol persists following total ablation of the SCN in rhesus monkeys (Reppert et al., 1981).

As the SCN are coupled to the retinas by means of the retinohypothalamic tract, Moore-Ede (1983) suggests that the light/dark cycle has a predominant effect on the 'Y' variables.

Although circadian rhythms have been studied so extensively it is interesting to note that the siting of the 'X' pacemaker(s) remains unknown, and with this comes the realisation that there is still an enormous amount of work to be carried out on the control of circadian oscillating systems.

The study of mammalian tissues in vitro has shown that various tissues can independently maintain circadian rhythmicity, for example, hamster adrenal glands (Andrews and Folk, 1964) and rat liver cells (Hardeland, 1973). This self regulation has been termed secondary-oscillation (Moore-Ede et al., 1982).

The work of Rusak (1977) showed that following SCN destruction, hamsters' wheel-running activity in constant conditions did not exhibit circadian periodicity (as previously discussed) but tended to be clustered into 2 or 3 peaks. This suggests that wheel running activity is driven by secondary oscillators, and without the SCN no internal coupling could be maintained to produce a coherent circadian rhythm.

Albers et al. (1981) found that drinking behaviour in squirrel monkeys (normally occurring in the light period) 'free-runs' in constant conditions with a period up to 25 h. Following SCN lesions there was a persistence in the circadian rhythm for several months, with gradually decreasing amplitude, until it occured randomly across

the day and night. These results suggested that the SCN may be a pacemaker, or co-ordinator, of a group of weakly coupled secondary oscillators that drive drinking behaviour.

Until recently there was some doubt as to whether humans possessed SCN. Lydic et al., (1980) reported that a pair of neuronal clusters, apparently homologous to the SCN in animals, can be found in the human brain, but they appear to be smaller and more diffuse than those in other mammalian species. There are obvious ethical problems in the study of human SCN, but tumours in this region of the brain have been found to disrupt the sleep-wake cycle (Fulton and Bailey, 1929).

Drawing parallels between the circadian rhythm control in animals and humans is dependant upon comparison of variables showing circadian rhythmicity. There do, in fact, seem to be significant similarities between humans and infrahuman mammals. Moore-Ede (1983) reviewed the similarity between circadian rhythms in squirrel monkeys and humans. In both, internal desynchronization can occur between rest/activity and temperature rhythms, but only after several months. In addition, phase shifts in light/dark periods causes internal desynchronization in the rest/activity and body temperature rhythm in squirrel monkeys and humans. Furthermore, the rhythm in body temperature has a more limited range of entrainment than the rest/activity rhythm in both human (Minors and Waterhouse, 1981a; Wever, 1979) and the squirrel monkey (Gander and Moore-Ede, 1982).

Moore-Ede (1983) proposed that the organization of the circadian system in humans and animals is similar, but that 'X' and 'Y' pacemakers are stronger in animals so that internal desynchronization in the absence of time cues is not so readily observed. Consistent with this is the relatively larger sized and more closely organized SCN in animals, and man's strong dependance on time pieces and social organization in the control of circadian rhythms.

CHAPTER 3

Sleep

Sleep in animals and man under natural day/night conditions is a cyclic 24 h phenomenon. The main sleep periods coincide with one particular stretch of the 24 h and alternate with periods containing little sleep in the remainder.

The rhythm of sleep/wakefulness is a fundamental rhythm in living organisms. It differs from the circadian rhythm pattern noted in other biological variables, in that is it more an alternation between two states, and can thus be described mathematically by a rectangular wave as opposed to the cosinor curve of circadian rhythmicity (Minors and Waterhouse, 1981b).

Despite these differences, strong coupling forces exist between sleep/wakefulness and circadian variables as shown by the interaction of 'X' and 'Y' circadian oscillating systems (Czeisler et al., 1980), and the experiments of Minors and Waterhouse (1980) which suggested the importance of anchor sleep for stability in the circadian system.

In this chapter the organization and regulation of sleep will be described, concentrating on sleep in man owing to the greater extent of the literature. The role of sleep in patterning hormone secretion will be discussed and the proposed functions of sleep reviewed.

In a review of the organization and regulation of sleep, Koella (1984) stated:

" ..... sleep cannot be explained by a mere passive dropping away from the waking state; it must be viewed as an actively induced and homeostatically controlled, organized function."

Behavioural observations, psychophysiological test procedures and the measurement of brain activity by electroencephalographic (EEG) recordings has led to the definition of two distinct phases of sleep in man and infrahuman mammals: non-rapid-eye-movement (NREM) and rapid-eye-movement (REM).

In man there are usually taken to be 4 different stages within the NREM phase (stages 1-4) based on EEG appearances; stages 3 and 4, deep sleep, are usually referred to as slow-wave sleep (SWS). In infrahuman mammals the whole NREM phase is usually called SWS; some investigators referring to low amplitude and high amplitude SWS, depending on whether less or more then 50% of slow-wave proportion is noted.

Much of the work on sleep has been carried out within the environs of sleep laboratories. It could be argued that only investigations 'in the field' will give a true indication of normal sleep patterns. This is, however, not true as investigations involving home studies have produced the same results as those in sleep laboratories.

Koella (1984) gave a particularly detailed exposition of the appearance and organization of sleep stages in man, the outlines of his review are presented below.

In man the onset of sleep is characterized by drowsiness and a gradual drift into stage 1 sleep. Vigilance in the sensory and motor systems drops as is indicated by the decreases in arousability, heart rate, blood pressure, respiratory activity, muscular tone and reflex excitability.

As sleep progresses into stages 2,3 and 4; vigilance in higher functions decreases. Consciousness is virtually lost and it becomes increasingly difficult to waken an individual. During the deep stages of sleep, man and animals are still capable of differentiating between familiar and unfamiliar sensory stimuli, and continue to sleep or wake accordingly.

Upon entering NREM sleep, the patterns in cortical EEG show an initial desynchronization, the patterns becoming increasingly synchronized with the shift to deep sleep. the synchronized EEG pattern reflects a drop in reactivity of cortical networks.

During REM sleep, responsiveness in the majority of motor functions

drops to very low levels and muscle tone and reflex excitability are virtually absent. By contrast, rapid-eye movement is evident (Aserinsky and Kleitman, 1955), blood flow to the brain is increased far above waking levels (Townsend et al., 1973) and cortical EEG shows an arousal pattern indicating enhancement of cortical network reactivity. Subjects awakened from REM sleep usually report they have been dreaming (Dement, 1960).

In man and infrahuman mammals, the phases of SWS and REM sleep are cyclic. In man the first REM episode is normally recorded 60-70 minutes after sleep onset. The alternation between stage 4 (of SWS) and REM during the sleep period then assumes cyclic periods of approximately 90-100 minutes duration (Webb, 1974). The REM period constitutes approximately 20 minutes, as such there may be 4 or 5 REM periods during a normal 8 h sleep in man.

There is, however, a large scatter about the average phase durations and alternations within a sleep episode. Under normal 24 h conditions, NREM is more pronounced at the beginning, with a preponderance of REM in the latter part of the sleep period (Feinberg and Floyd, 1979).

Experimental evidence supporting the proposal that SWS and REM are controlled by separate pacemakers (Czeisler et al., 1980) has come from studies of subjects maintained on non-24 h schedules of sleep and wakefulness. Webb and Agnew (1977) measured sleep stages in groups of male subjects on a variety of sleep/wakefulness regimes (hours asleep: hours awake - 3:6, 4:8, 6:12, 10:20, 12:24). Despite such widely ranging regimes, SWS still occured before REM in each sleep episode and is evidence of the sleep infrastructure stability. In addition, the amount of SWS correlated with the amount of prior wakefulness rather than time of day, whereas REM depended on time of day rather than amount or prior wakefulness. Similar findings were reported by Hume and Mills (1977) and suggest separate control of SWS and REM propensity (i.e. 'γ' and 'χ' pacemaker control).

Minors and Waterhouse (1981a) instructed a subject, kept in isolation, to live a regular regimen of sleep/wakefulness and meal time in accord

with a clock. For a control period this clock ran at 24 h before being changed, without the subject's knowledge, to 21 h for 4 days. The time piece was then removed and the subject instructed to continue estimating time. The rhythm of sleep/wakefulness continued at 21 h for several days, whilst the rhythm of deep body temperature ran at 25 h. Although illustrating 'X' and 'Y' control very neatly, these results must be interpreted with some trepidation owing to the fact that only one subject was investigated.

The strong coupling force between oscillating systems is evident from studies of man under natural day/night conditions. Adult man starts sleeping when his deep body temperature is on its descending limb and wakes when it begins to rise (Zulley et al., 1981). Even under conditions of temporal isolation, when desynchrony of the circadian system becomes apparent, a coupling force between sleep/wakefulness and deep body temperature is still evident. In 10 'desynchronized' subjects, Zulley et al. (1981) noted that 8 were still sleeping at the time of body temperature minima, and only one slept at the time of body temperature maxima. Mills (1968) reported that in a subject living a 12 h day, with a 4 h sleep period, each sleep period was associated with a fall in deep body temperature. In a later study Mills et al. (1978) demonstrated that the effect of sleep in lowering body temperature, was in itself subject to circadian variation, being zero in the morning at, or shortly before, 12.00 h and maximal at night. In addition Mills, et al. (1974) suggested that the body temperature rhythm in subjects kept in isolation (under conditions of desynchronization) showed two beats; one related to sleep/wakefulness and the other endogenous.

During sleep, energy requirements are reduced and thus body temperature decreases; the rise in body temperature at the time of waking indicates preparation for the rigours of activity that lie ahead. This is possibily another example of the synchronous circadian system conferring a means of optimal response with minimal time lag.

Sleep and Hormone Secretion

Hormone secretion in man and animals shows rhythmic oscillations. In

the specific cases of growth hormone (GH) and prolactin, rhythmicity is conferred by the event of sleep. This suggests that sleep/wakefulness, rather than merely being a 'Y' oscillator variable, may actually participate in 'Y' oscillator control.

Evidence for the sleep (in particular SWS) dependance of GH secretion in man has come from studies in which the sleep/wake cycle is altered. When sleep is delayed or advanced (Takahashi et al., 1968; Honda et al., 1969), the sleep/wake cycle reversed (Sassin et al., 1969a) or naps taken during the day-time (Weitzman, 1975), the changed time of sleep is associated with an immediate change in GH release. Even following the internal desynchronization of sleep/wakefulness and deep body temperature rhythms, there is no dissociation between sleep and GH secretion (Czeisler, 1978).

Prolactin secretion in man is at a maximum during the night, showing increasing peaks as sleep progresses to reach highest levels at, or shortly after, waking (Sassin et al., 1972). When sleep is delayed by 3 or 6h, or the sleep/wakefulness cycle reversed, there is an immediate change in the timing of prolactin secretion so that it retains correlation with new sleep timing (Sassin et al., 1973). Furthermore, day-time naps cause a rise in prolactin secretion (Parker et al., 1973).

Thyrotrophic hormone (TSH) and cortisol hormone secretion are strongly endogenous. Under normal 24 h conditions, these hormones show minimum values at the time of sleep and are maximum during waking hours. Although endogenous control is strong, the inverse correlation between sleep episodes and secretion suggests sleep may play a role in conferring secretory rhythmicity.

In man, if the time of sleep is advanced, the concomitant fall in TSH starts earlier, and if sleep is delayed this fall is later (Parker et al., 1976). The initiated fall in TSH is not conferred purely by absence of activity for a persistence of the 24 h rhythm is found in subjects recumbent during 24 h (Vanhaelst et al., 1972).

Under normal 24 h conditions, minimal levels in cortisol secretion are

noted at the time of sleep, there is a rise towards the end of the sleep episode and a peak during first waking hours (Ritchie et al., 1983). The strong endogenous control of this rhythm is evident; it is not affected by food intake or activity (Reinberg et al., 1970), it is desynchronous with the sleep/wake cycle under conditions of temporal isolation (Weitzman et al., 1979), the rate of rhythm adaption following time shifts is slow (Aschoff et al., 1975) and it does not adapt immediately or fully to any abnormal schedules of sleep/wakefulness (Orth, et al.,1967). The rhythm of sleep/wakefulness does, however, have a degree of influence on cortisol secretion for it can confer an ultradian rhythm on the circadian rhythm (Weitzman et al., 1974), and although low at the expected time of sleep (even if wakefulness continues) it is dependant on sleep to reach its lowest levels (Weitzman et al., 1983).

Adrenalin and testosterone secretion in man show a temporal relation to the normal sleep/wake cycle but are both under strong endogenous control. Adrenalin secretion is characterised by peak values during the day-time and minimum values at night time. Its strong endogenous control is evident from the finding that even after the sleep/wake cycle has been reversed for 7 days, no concomitant reversal in the timing of the rhythm is noted (Akerstedt, 1977) and that the rhythm is not altered by prolonged sleep deprivation(Fröberg et al ., 1975; Akerstedt and Fröberg, 1979).

Testosterone secretion is maximal at night in the latter half of the sleep period (Parker et al., 1980) Miyatake et al., (1980) however, have shown that the peak timing of testosterone secretion is complementary with sleep rather than dependent on sleep as the rhythm is not altered during sleep deprivation, or by reversal of the sleep/wake cycle.

Proposed Functions of Sleep

Sleep constitutes a significant proportion of life in animals and man, as such the proposed functions of sleep are particularly interesting to review.

In a review of the functions of mammalian sleep, Horne (1982) stated:

"The putative functions of mammalian sleep range from the non-restorative 'non-behaviour' occupying unproductive hours, providing safety and conserving energy, to an essential and special restitutive process for the body and brain."

Controversy surrounds sleep function, as the two main schools of thought are that sleep is 1) restorative or 2) adaptive 'non-behaviour'.

In the first instance, the view that sleep plays an important physiological role by promoting restoration of body and brain will be reviewed.

Elucidation of sleep function was impeded by the tendency for early investigators to regard sleep as a single event with a function. For example, Hebb (1949) suggested that sleep might involve a reorganization, rather than an absence, of neuronal activity. It was Roffwarg et al.(1966) who proposed that the function of sleep should no longer be discussed as one concept. With increasingly elegant methodology in the measurement of sleep it had become evident that the physiological characteristics of the alternating phases of NREM and REM were so different that a 'dualistic' approach to sleep function should be adopted.

The need for REM sleep was first indicated by the classic experiments of Dement (1960) on the effects of specific REM deprivation (which Dement termed 'dream deprivation'). The effect of REM deprivation was compared to a control situation, whereby the same subjects were given control awakenings not associated with REM sleep. Over the course of the REM deprivation period (on average 5 nights) subjects showed an increasing frequency in "attempts" to enter REM. During subsequent 'recovery' nights, total REM time and percentage of REM sleep increased. Dement suggested that a 'rebound' effect such as this may last as long as 5 nights, following 5 deprived nights. When the same subjects were given 'control awakenings', no increase in REM sleep during the distrubed period, nor during subsequent 'recovery' nights, was noted. Dement suggested that the results were indicative of the human need for a certain amount of REM sleep each night.

The proposed physiological need for REM sleep was developed further by Roffwarg et al. (1964). In a study of sleep phases in children, a large amount of REM sleep was noted, this amount decreasing with maturation. In a study of premature infants, Parmelee et al., (1964) found that twice as much REM sleep occured in the month or two prior to the expected time of birth. This is a time when the brain is growing rapidly, and as REM sleep is characterized by considerable excitation of neurons in the brain, suggested that the REM phase of sleep may play a role in stimulating structural maturation and maintenance within the central nervous system (CNS) (Roffwarg et al. 1966).

In support of the brain's need for REM sleep, are the findings that below average amounts of REM sleep are noted in senile subjects (Feinberg, 1968a) and mental defectives (Feinberg, 1968b).

Oswald (1970, 1976) proposed the 'dualistic' function of sleep to be restoration and repair of body and brain; specifically citing REM sleep as the time for brain growth and renewal and SWS for growth and renewal of general bodily tissues.

The need for NREM sleep, or more particularly SWS, for body restoration was first indicated by the finding that the amount of SWS was associated with the amount and quality of prior wakefulness, later a relation with exercise was claimed when it was found that college atheletes who had exercised in the afternoon, showed an increased amount of SWS in the subsequent sleep period (Baekeland and Lasky, 1966). Similar results have been reported in cats (Hobson, 1968). This taken in conjunction with the sleep dependance of GH secretion (an anabolic hormone) and the fact that SWS and GH secretion are assigned to 'Y' oscillator control (Czeisler et al., 1980) suggests that the SWS phase is intimately involved in body growth, maintenance and repair. Accordingly, Adamson et al., (1974) reported increased GH secretion after a period of exercise in adults.

To explain the association of sleep with body restoration, Adam (1980) proposed that it is the differing energy demands of the

activity/inactivity rhythm that chiefly determine the degradative/synthetic rhythm. Closely related to these rhythms is cellular energy charge (EC), a measure of the available free energy in the form of Adenosine Triphosphate (ATP), a fundamental metabolic co-ordinator.

During activity under normal conditions, cellular work depletes EC and degradative processes are enhanced by complementary timing of the rhythm of the catabolic hormones cortisol and adrenalin, high levels being noted during waking hours (Ritchie et al., 1983; Prinz et al., 1979). In addition, brief exercise has been found to reduce the EC in rat skeletal muscle (Wojciechowska et al., 1975) and ATP in human muscle (Karlson and Saltin, 1970).

During sleep, the rate of muscle activity and body heat production is reduced, and the activity of the Na+/K+ ion pump is also reduced. As a result of these lessened catabolic demands, EC reaches higher levels and, augmented by the complementary timing of anabolic hormones: testosterone, GH, and prolactin, synthesis is favoured and degradative processes diminish.

Several investigations support Adam's hypothesis. Atkinson (1969) found that low values of EC in tissues favours ATP producing pathways, whilst high values of EC promote ATP utilizing sequences (tissue restoration). Durie et al., (1978) found that the rise in EC was sleep dependant, and enhanced mitotic activity has been found to be dependant on higher concentrations of ATP (Guttes and Guttes, 1959; Epel, 1963).

In an extensive review of the literature documenting mitotic activity in nocturnal animals, Adam and Oswald (1983) suggest that although mitotic activity is noted throughout 24 h a great proportion of cells remain primed for mitosis until the event of sleep when mitosis in these primed cells is initiated, and the authors used this as an indication of the importance of sleep in tissue restoration.

If, as suggested, sleep is a time of restoration, the event of sleep could be considered as a highly evolved form of rest ensuring that

body and brain have a chance to recuperate after activity (Adam, 1980).

The second view of sleep, that it is adaptive non-behaviour, is not incompatible with the first view.

Horne (1983) challenged the view that sleep fulfils a restorative role, augmented by hormonal levels, by drawing attention to the control of protein synthesis. For example, Waterlow et al., (1978) claimed that the main stimulus to protein synthesis, and to an increase in the protein content of cells, is food absorption. Horne (1983) thus suggested that sleep may be characterized by a state of tissue degradation during the night-time fast. Garlick et al., (1980) measured plasma free amino acids in man and reported that high rates of protein synthesis were evident in the day, falling at night. These authors were criticized by Adam and Oswald (1981) for using an indirect method of protein synthesis measurement (by the findings of Fern and Garlick, 1974), and for defining night-time as 20.00 h-08.00 h - for this is a time when anabolic and catabolic hormone secretion (for example GH and cortisol) change considerably.

The work of Quabbe (1978) and Quabbe et al. (1981) supported the view that sleep is not related to body restoration, by showing no sleep-linked release of GH in the normal sleep of the rat, cat, dog or rhesus monkey. These findings are contrary to the results in man (Sassin et al., 1969a,b; Schnure et al., 1971). More animal investigations are obviously needed to make the evidence more convincing.

Adam (1980) proposed that oscillations in EC determine the degradative/synthetic rhythm. Horne (1983), however, proposed that high EC does not stimulate anabolism, it only promotes it given adequate amino acid substrate - not found in human sleep and cited the reports by Garlick et al. (1981) and Clugston and Garlick (1982). These authors, however, did not report on the level of intracellular amino acids which have 24 h variation, peaking during rest or sleep, independent of food intake (Adam and Oswald, 1981).

As previously discussed, a period of exercise has been associated with a subsequent increase in SWS (Baekeland and Lasky 1966; Hobson 1968) Horne et al.(1983), however, reported that an increase in SWS following exercise was only apparent in fit trained subjects. Horne et al. suggested that this was a measure of their endurance of high rates of exercise for much longer periods than untrained subjects, and indicated an ability to sustain more prolonged thermal loads. This, the authors proposed may have had a significant effect on cerebral metabolism and be a key to the subsequent SWS increase.

It is difficult to reconcile the proposal that sleep does not play a part in stimulating bodily restoration in man, with the extensive review by Adam and Oswald (1983) that tissue restoration in nocturnal animals is favoured during the light period when sleep predominates. It is important to realise, however, that these authors were not able to conclude unequivocably that it was sleep, rather than time of day, that was the more important factor - owing to the lack of information on the sleep pattern of experimental animals. Hence this Thesis had its starting point and gives an answer.

Studies of sleep deprivation can perhaps solve the controversies surrounding sleep function. Rechtschaffen et al. (1983) reported the deleterious effect of prolonged sleep deprivation in rats. This was an elegantly controlled study in which control and experimental (sleep deprived) rats received the same physical stimuli, but stimulus presentations were linked to sleep onset in only the experimental rats. Prolonged sleep deprivation caused weight loss (despite increased food-intake), severe pathological changes in tissues (for example skin lesions, fluid in lungs and trachea, stomach ulcers and severe oedema in limbs) and death.

It is evident that much further work is needed to clarify the role of sleep and body restoration. The work in this thesis involves the experimental procedure of sleep deprivation. As such it is relevant to discuss this manoeuvere.

Sleep deprivation as an experimental procedure has been criticized for producing stress (i.e. emergency reactions) which could invalidate results. High levels of glucocorticoids are commonly associated with stress. Akerstedt et al., (1980) measured plasma samples of cortisol hormone in 12 men over the course of 48 h of sleep deprivation. Resultant stress would thus be indicated by elevated levels of this steroid hormone during sleep deprivation. Over the course of sleep deprivation, however, this hormone level was reduced. In addition, lower levels of both psychological and physiological activation and an increase in self rated fatigue was noted. These findings are thus inconsistent with the proposed stress (emergency reaction) consequent upon sleep deprivation.

One final interesting point is the effect of sleep deprivation in man on subsequent sleep during 'recovery nights'. A 'rebound' effect is noted following selective phase deprivation ie. REM sleep (Dement, 1960) and stage 4 sleep (Agnew et al., 1964,1967). Following total sleep deprivation, subsequent 'recovery' nights are characterized by increased SWS and reduced REM sleep in the first instance (Berger and Oswald, 1962) followed by increased REM in later recovery nights (Williams et al., 1964). Sleep deprivation may thus have a more immediate effect on general bodily tissues than the brain.

It is interesting to note the similarities between the effect of sleep deprivation in man and rats. Friedman et al., (1979) reported that rats, sleep deprived for 24 h, showed increased amounts of high amplitude SWS on the first 'recovery' night, and this was followed in later 'recovery' nights by increased REM and low amplitude SWS. The authors also noted that although one group of rats were three times more active during the sleep deprivation period, sleep deprivation had the same effect on their subsequent sleep, suggesting that changes in exercise and energy depletion may have little effect on 'rebound' sleep measure.

CHAPTER 4

Main Aims of the Work in this Thesis

By investigating the importance of sleep for the maintenance of circadian periodicity in biological phenomena, in particular cell proliferation, I hope to begin to unravel the functions of sleep. In addition I hope to answer the question: is sleep merely a phase in the alternating rhythm of activity/inactivity, or does it confer rhythmicity in biological variables.

Structure of the Work Described

The work in this thesis is divided into two sections; Part one: Animal Studies, and Part two: Human Study, the former being the more substantial.

Part One

The ethical considerations demanded in human studies, and the need for repeated measurements to overcome differences between human subjects forced constraints on the study of tissue restoration in man. To overcome these problems, tissue restoration was studied in laboratory animals.

The major investigation in this section was into the effect of time of day and the sleep/wake cycle on the cell population kinetics in the jejunum of the male Syrian hamster (Mesocricetus auratus). This investigation was carried out with the invaluable help of Dr Anne Ferguson in the Western General Hospital, Edinburgh.

Intraepithelial lymphocytes are of particular interest to Dr Ferguson and her team, and this interest motivated my investigation into the effect of time of day and the sleep/wake cycle on intraepithelial lymphocyte numbers, as this count could be performed in tissues prepared for cell population kinetic analysis.

Part Two

This section describes an investigation into the effect of sleep deprivation on the rhythm in body lengths of young and middle-aged men. This study was selected for two reasons. Firstly, the circadian rhythm in stature was of particular interest to me owing to my own preliminary investigation on this phenomenon. Secondly, controlled sleep deprivation studies were being carried out under the supervision of Professor I Oswald , Dr K Adam and Dr C Shapiro, in the Sleep Laboratory of the Royal Edinburgh Hospital and as such a ready supply of subjects was available.

PART ONE

Animal Studies

CHAPTER 5

As the principal research described in Part One concerns the circadian rhythm in cell population kinetics I will begin by outlining some of the basic concepts of cell proliferation and critically review some of the work in this area.

The Cell Cycle

In a cell population undergoing constant renewal, individual cells divide periodically. The process of cell division has been divided into four distinct phases (G_1, S, G_2, M) which together constitute the cell cycle (Howard and Pelc, 1953). A renewing tissue can be divided into two compartments, as described by Cairnie et al. (1965b):

(1) The P compartment containing proliferating (P) cells actively engaged in the cell cycle.
(2) The Q compartment containing non-proliferating (Q) cells which are either in prolonged G_1 (termed the G_0, resting phase) during which entry into the cell cycle is suspended, but the potential for cell cycle proliferation remains, or there is migration out of the proliferative zone and differentiation into functional mature cells (Aherne et al., 1977; Eastwood, 1977).

A diagrammatic representation of the cell cycle and the relationship between P and Q compartments is shown in FIG 5.1.

The G_1 Phase

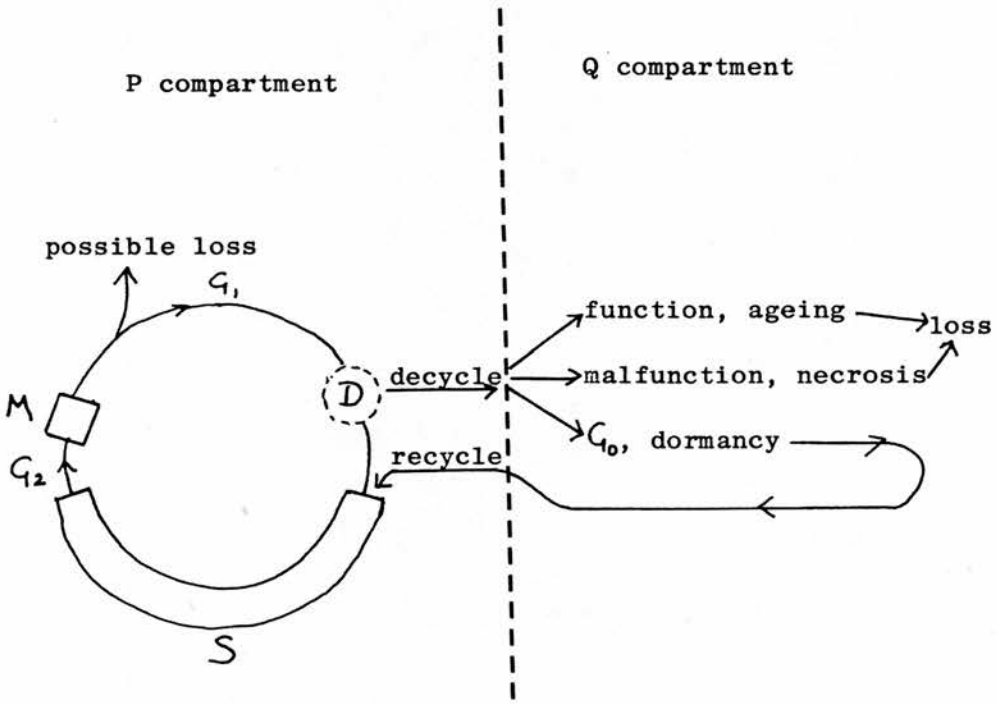
This phase is the most variable in duration (Meuller, 1971; Aherne et al., 1977). It is the 'decision point' of the cell cycle at which cells may irreversibly enter the cell cycle or exit (to either enter a resting phase or to become fully functional, Aherne et al., 1977).

Multiple control processes operate to regulate the progression of actively proliferating cells through G_1. Protein and RNA synthesis is carried out and the cell prepares to initiate the replication of its nuclear DNA and express the genes for histone systhesis (Meuller,

FIG 5.1

The Cell Division Cycle, Showing Partition of the Population into Proliferating (P Compartment) and Non-Proliferating (Q Compartment) Cells and How Cells in G_0 May Return to the Cell Cycle

Reproduced after Aherne et al. (1977)



1971).

The S Phase

DNA, histones, and non-basic proteins are replicated thus assuring there is phenotypic and genetic continuity. Synthetic processes necessary for replication proceed in a highly ordered and closely coupled manner (Prescott, 1966; Meuller, 1971). Once DNA replication has started, cells pass directly through S phase, the phase end is marked by the completion of replication processes (Meuller, 1971).

G_2 Phase

The cell prepares for initiation of mitosis and completion of division. The precursor subunits of the mitotic apparatus are assembled and division-related protein is synthesized (Tobey et al., 1971).

Cells may be arrested in G_2 and held for at least 5 days ensuring a ready availability of cells for entry into mitosis (Gelfant, 1963; Tobey et al., 1971).

The M Phase (Mitosis)

Chromosome condensation, nucleolar disaggregation and nuclear envelope fragmentation is followed by the apportioning of identical genetic material to daughter cells (Tobey et al., 1971). Each chromosome undergoes precisely orchestrated movements that result in separation of sister chromatids as the nuclear contents are divided (Alberts et al., 1983). These movements are recognised optically by the chromosomal configurations comprising the mitotic stages of prophase, metaphase, anaphase, and telophase. The end of the M phase is marked by the completion of cytoplasmic division (cytokinesis).

The Mitotic Index (I_m)

Cells in mitosis can be recognised optically so the mitotic index (I_m) can be defined simply by relating the number of cells in mitosis to the total number of cells in the population scanned.

A simple mitotic count is not a true indication of the proliferative status of a tissue for the number of cells in mitosis depends on (a) the duration of M (t_m) and (b) the rate of entry into M (Aherne et al., 1977).

The development of stathmokinetic techniques has greatly enhanced the determination of mitotic rate. Stathmokinetic agents (the drugs colchicine, Colcemid, vinblastine and vincristine) interfere with the formation of the mitotic spindle apparatus so that the process of mitosis is blocked at metaphase. Recording the number of blocked metaphases accumulating with time (after stathmokinetic agent administration) enables calculation of the rate of entry of cells into M (Lipkin, 1971; Aherne et al., 1977), the average duration of M (t_m) and the apparent cell cycle time ($\dot{t}_c$) (Aherne et al., 1977). The relative efficiency of stathmokinetic agents is reviewed by Tannock (1967).

Autoradiographic Techniques

The nucleic acids DNA and RNA have only 3 out of their 4 component bases in common. Each nucleic acid contains adenine, guanine and cytosine, but only DNA contains thymine and RNA uracil (Cleaver, 1967). A label introduced into the nucleoside thymidine thus acts as a specific tracer of DNA metabolism and consequently the S phase of the cell cycle. ^{14}C labelled thymidine became available in 1954 (Cleaver, 1967) but it was the successful synthesis of tritium (^3H) labelled thymidine in 1957 (Taylor et al., 1957; Verley and Hundbelle, 1957) and the development of high resolution autoradiography (Caro and van Tubergen, 1962) to trace its incorporation, that gave great impetus to the study of cell proliferation.

Although thymidine does not occur naturally on the main intracellular pathways of DNA synthesis, it is rapidly introduced to these pathways by the action of the enzyme thymidine kinase (Cleaver, 1967). Thymidine excess to requirements is rapidly degraded and excreted.

The isotopes ^{14}C and ^{3}H-thymidine emit β particles of radiation, and this radiation can be identified by autoradiography (ARG) (the basic principles of ARG are reported by Doniach and Pelc, 1950). ARG essentially involves placing photographic emulsion over a section of tissue which has incorporated labelled thymidine (^{14}C and/or ^{3}H) into DNA. The radiation emitted from these isotopes appears as silver grains deposited in the overlying emulsion. Identification of the morphological structures emitting radiation is dependant upon intimate contact between the emulsion and the tissue section.

The β particles emitted from ^{3}H have lower energy and shorter range than those emitted by ^{14}C (Cleaver, 1967), there is thus less scatter of electrons from ^{3}H and it is used in preference to ^{14}C for high resolution ARG.

In a review, Maurer (1981) discussed the pitfalls of labelled thymidine usage in the study of cell proliferation. ^{3}H-TdR incorporation is used as a measure of the proportion of cells engaged in the cell cycle, but DNA synthesis without cell division can occur in repair processes, for example following damage by cold (Pelc and Viola-Magni, 1969) and radiation (Rasmussen and Painter, 1966).

The successful interpretation of ^{3}H-TdR label in cells demands assumptions as to the specificity and stability of ^{3}H-TdR, Cleaver (1967) concluded there was sufficient evidence to accept the use of ^{3}H-TdR as a reliable label for the study of cell proliferation. The greatest problems arise when ^{3}H-TdR is used in high doses and/or at high specific activity. For example, cells in S phase are killed by ^{3}H-TdR given in high doses: 32-42 μ Ci/g body weight (Schreml et al., 1974), or at high specific activity; >24 Ci/mM (Gibbs and Casarett, 1969). In addition, Greulich et al. (1961) reported that ^{3}H-TdR stimulated cell proliferation and Møller et al. (1974) found that a

dose of 1 μ Ci/g body weight, stimulated G_2 cells to enter mitosis.

Most investigators use ^{3}H-TdR in low doses (< 1 μ Ci/g body weight) and at low specific activity (5 Ci/mM) for small animal work (i.e. rodents), together with histological sectioning procedures which free the metabolic products, impurities and excess amounts of thymidine (Diab and Roth, 1970) thus minimizing the pitfalls.

It is evident, however, that successful study of cell proliferation involves the use of several different assays, rather than a total reliance on ^{3}H-TdR techniques.

Cell Cycle Analysis Using ARG

An excellent review of the techniques is given by Aherne et al. (1977). The most commonly used technique (labelling index determination) is summarized below and further techniques (allowing more elaborate cell cycle analysis i.e. calculation of phase durations) are listed.

Labelling Index (I_s)

The physical half life of ^{3}H is 12.26 years (Cleaver, 1967) but its biological availability is limited to just a few minutes as it is rapidly metabolised in vivo (Rubini et al., 1960). It is thus an ideal pulse label. Exposure of a cell population to ^{3}H-TdR (given as a single pulse injection) followed by ARG, enables the fraction of cells synthesizing DNA to be determined and is termed the pulse labelling index (I_s) (Aherne et al., 1977). Its use as a proliferative index is beset by the same problems as in the simple I_m determination; the number of cells in the S phase being dependant on (a) the duration: t_s and (b) the rate of entry to S.

Additional Techniques of Cell Cycle Analysis

A number of other techniques have been developed for the study of the cell cycle and its constituent phases. Briefly these are as follows:

(1) Fraction of labelled mitoses (Quastler and Sherman, 1959; Wimber, 1963; Aherne et al., 1977).

(2) Combined metaphase blocking and continuous labelling (Puck and Steffan, 1963).

(3) Grain counting (Aherne et al., 1977).

(4) Scintillation counting (Aherne et al., 1977).

(5) Flow cytometry (Van Dilla et al., 1969; Guseman, 1978).

Circadian Rhythm in Cell Proliferation

Evidence of a circadian rhythm in mitotic activity in tissues of the cat and mouse was reported by Mrs Droogleever Fortuyn-van Leyden (1916, 1926) and later confirmed in the mouse by Carleton (1934). The circadian rhythm in cell proliferation is now well established and excellent reviews of this phenomenon in humans and animals have been carried out by Bullough (1965), Moore-Ede et al. (1976) and Moore-Ede and Sulzman (1977). Findings in humans and rodents (rat, mouse and hamster) are summarized below.

Human Studies

Owing to the relative ease of the sampling procedure the epidermis has been the subject of most investigations. The peak in mitotic index (I_m) has been reported to occur at night: between 24.00-04.00 h by Scheving (1959) and at 01.00 h by Fisher (1968). A small but significant peak in labelling index (I_s) has also been found to occur at night (24.00 h) by Kahn et al. (1968).

Schell et al (1980) and Gelfant et al (1982) failed to confirm these earlier reports, noting instead no clear circadian rhythm in epidermal proliferation, and Camplejohn et al., (1984) reported a significantly higher I_m at 21.00 h compared to 09.00 h and 15.00 h, but no significant variation in I_s with time.

The disparity in reports could be due to the different protocols governing the investigations. The main contributory factors being as follows:

(1) Sample times: Scheving (1959) and Fisher (1968) obtained skin biopsies every hour and every 2 hours respectively. These timings allow for a more accurate assessment of the change in proliferative rates with time of day than do the 6 hourly sample times utilized by Kahn et al. (1968), Schell et al. (1980), Gelfant et al. (1982) and Camplejohn et al. (1984) where a 24 h rhythm may be obscured, the peak and trough occuring outwith the sample times.

(2) Statistical methods used for data analysis: Kahn et al (1968), Schell et al. (1980), Gelfant et al, (1982) and Camplejohn et al, (1984) document their statistical procedures well. On the other hand Scheving (1959) does not mention the statistical tests used and Fisher (1968) makes no mention of statistical analysis and furthermore samples only 3 subjects at some points. Clearly larger subject numbers are required for statistical significance.

(3) Control of sleep and activity patterns of subjects: Fisher (1968) reported an inverse relationship between bodily activity and epidermal mitoses. Other authors however, do not clarify the rest/activity pattern and as such it is uncertain whether lack of control at this level may have contributed to the anomalous results.

Animal Studies

Investigators using laboratory animals are able to sample a wide range of tissues from strictly controlled populations. Consequently the literature on animal tissues is far more extensive than that in humans.

Some of the reports in rodents are listed briefly here, to give an idea of the range of tissues that have been investigated, before I go

on to discuss the timing and control of rhythms in cell proliferation.

In the rat, a circadian rhythm in cell proliferation is evident in the epidermis (Blumenfield, 1939; Scheving and Pauly, 1960) and the epithelium of small intestine (Al-Dewachi et al., 1976)

In the mouse, cell proliferation has been found to be circadian in the epidermis (Bullough, 1948a) the epithelium of the cornea (Alov, 1963; Burns and Scheving, 1975; Scheving et al., 1978), the epithelium of the tongue (Alov, 1963) the epithelium of the gut (Bullough, 1948a; Alov, 1963; Scheving et al., 1978; Sigdestad et al., 1969; Sigdestad and Lesher, 1972; Al-Nafussi: and Wright, 1982b) and the bone marrow (Scheving et al., 1978).

In the hamster, a circadian rhythm of cellular proliferation in the epithelium of the cheek pouch has been reported (Møller et al., 1974; Møller and Keiding, 1982; Rubin et al., 1983).

Cell Proliferation Control

Hormonal Influence

The effect of hormone secretion of cell proliferation has been reviewed by Bullough (1965) and Epifanova (1971).

Stress hormones (adrenalin, ACTH and the glucocorticoids) have all been shown to act as mitotic inhibitors (Ghadially and Green, 1957; Halberg et al., 1959b, Bullough and Laurence, 1961,1964a,b, 1968).

Tissue specific substances inhibiting cell proliferation are known as chalones. Bullough (1965) proposed that adrenalin acted to inhibit mitoses by augmenting chalone action. Taking this a step further, Bullough and Laurence (1968) suggested that cortisol was instrumental in reducing the rate of adrenalin loss and thus prolonged chalone-adrenalin activity. The chalone-adrenalin complex was claimed to be specific in its action on the M phase of the cell

cycle by Baden and Sviokla (1968). Bullough and Laurence (1967) further proposed that chalone was instrumental in determining the de cision phase of a cell: in the presence of sufficient chalone a cell would decycle and progress towards tissue function, and that if the chalone concentration fell, resting cells (in which the necessary genes are still potentially active) would re-enter the cell cycle.

Consistent with the hypothesis that adrenalin supplements mitotic inhibitors, were the findings of Alov (1963) that ablation of the adrenal glands did not abolish 24 h rhythmicity in cell division, which suggested that their involvement in cell proliferation control was secondary rather than decisive. Furthermore, Fisher (1968) suggested that the rhythmicity in cellular proliferation (of human epidermis) was due to corticosteroid hormones and that adrenalin produced an additional anti-mitotic effect during exercise. The inverse relationship between endogenous cortisol secretion and cellular proliferation was confirmed in a further study by Fisher (1971). More recently Schell et al. (1981) claimed a time shifted inverse correlation between endogenous cortisol secretion and I_s in human epidermis, however these results should be interpreted in light of the limited time samples (biopsies were only taken every 6 h).

Oestrogens stimulate DNA synthesis in target tissues (Perrotta et al., 1961; Perrotta, 1962) and accelerate the passage of cells through the pre-synthetic period in target and non-target tissues (Epifanova, 1971).

GH stimulates mitosis (Leblond and Carriere, 1955) and both insulin and GH initiate DNA synthesis, but do not alter its rate (Lockwood et al., 1967; Turkington, 1968).

The influcence of hormones on cell proliferation may be (1) a manifestation of 'complementary timing', cell proliferation increasing at a time when anabolic hormones are high (i.e. GH, prolactin and testosterone) and catabolic hormones are low (i.e. cortisol), and/or (2) just one of the mechanisms operating in a multifactorial system of cell proliferation control.

The Light/Dark Cycle (Nocturnal Animals)

Entrainment of circadian rhythms to the light/dark cycle is well established (Chapter 1) and reversal of the light/dark cycle causes a concomitant reversal of circadian rhythms in cell proliferation once animals are standardized to the new lighting regime (Halberg et al., 1958; Alov, 1959). This effect is now so well accepted that a group of animals housed under normal lighting have been used together with a group of animals housed under reversed lighting (each group standardized to their respective lighting schedules for 4 weeks) to obtain one 24 h set of data, effectively freeing investigators from the need for night-time vigils (Potten et al., 1977).

There is less agreement on the timing (i.e. during light or dark) of the peak rates in I_s and I_m. Some of the anomalous findings are discussed below.

(1) Peak in I_s and S phase influx at the beginning of the dark period: in the epithelium of the hamster cheek pouch (Møller and Keiding, 1982). This was a particularly well designed investigation in which 7 hamsters were sampled every 1 1/2 hours for 48 h; The timing of samples and the reputable statistical procedures, which are well documented, gives particularly strong evidence for the existence of a circadian rhythm in cell proliferation in this tissue. Although the authors took great care to control the light/dark cycle under which hamsters were housed, standardization was carried out for only 14 days prior to the start of the experiment and thus the adaption to the light/dark cycle may not have been complete (Halberg et al., 1958).

(2) Peak in I_s at the beginning of the light period: in a variety of tissues in the mouse, and a peak in I_m at the beginning of the light period: in the corneal epithelium of the mouse (Scheving et al., 1978). In this investigation mice were standardized to the light/dark cycle for 6 weeks prior to the start of the experiment and it can thus be assumed that any necessary adaption to this cycle was complete.

(3) Peaks in I_s and I_m in the latter half of the light period: in the jejunum of the rat (Al-Dewachi et al., 1976). These authors showed by clear statistical procedures that these peaks were significantly in synchrony. Rats, however, were only standardized to the light dark cycle for 14 days.

(4) Peaks in I_s and I_m in the latter half of the dark period: in the jejunum of mice (Sigdestad et al., 1969). In a second study (Sigdestad and Lesher, 1970) the I_s and I_m peaks were found to be in synchrony. These two studies, however, are open to criticism as the authors make no mention of (a) a standardization period allowing mice to adapt to the light/dark cycle and (b) whether the rhythm was statistically significant.

(5) Peak in I_s in the latter half of the dark period followed 3-6 h later by a peak in I_m: in the small intestine of the mouse, (Potten et al., 1977). These authors were particularly careful to control the light/dark cycle and mice were standardized 4 weeks prior to the start of the experiment. The results are well described but there is no mention of the statistical significance of the rhythms.

(6) Peak in I_s at the end of the light period, followed 6-8 h later by a peak in I_m: in the jejunum of the mouse, (Al-Naffussi and Wright, 1982b). These authors allowed 4 weeks for the standardization of mice to the light/dark cycle and gave a good clear account of the statistical significance of the rhythms.

(7) Peak in I_s at the beginning of the light period followed 6 h later by a peak in I_m: in the epithelium of the hamster cheek pouch, (Izquierdo and Gibbs, 1972,1974). Hamsters were standardized to the light/dark cycle for at least 4 weeks before the start of the experiment, but there is no mention of the statistical significance of the rhythms.

These anomalous reports may be due to a number of factors, for example differences in analysis of cell proliferation, the tissue

under investigation or the species, strain or sex of animals used. Potten et al (1977) used female animals and as the stage of the oestrous cycle was not controlled the well established fluctuating hormonal levels during the oestrous cycle may have invalidated comparison between groups of animals. Scheving et al, (1978) did not mention the sex of animals used and all other investigations used male animals. In addition, as standardization to the light/dark cycle varied amongst investigations from 14 days to 6 weeks, it is difficult to draw any conclusions about the effect of light and dark periods on cell proliferation.

There are, unfortunately, few definitive studies of the effects of light and dark periods on specific animals. Elliot (1981) reported that the hamsters photoperiodic clock is extremely responsive to light, but Ellis et al. (1982) reported that hamsters rely on sensing the dark period to initiate rhythms. Carleton (1934) reported that the 24 h rhythm of I_m in the epidermis of mice was lost when mice were kept in continuous light but was retained in continuous dark. Carleton, however, sampled only one mouse mice every 4 h for 24 h in continuous light, and only two mice every 4 h for 24 h in continuous dark. In addition standardization was only carried out for 10-14 days prior to the start of the experiment. Clearly sample numbers and standardization time should be increased in this type of investigation to reach conclusive results. Scheving et al., (1974b) reported that continuous light reduced the amplitude of the circadian rhythm in I_m in the corneal epithelium of the rat. One group of rats sampled in continuous light, however, had previously been standardized to continuous darkness and were unilaterally enucleated before being standardized to continuous light. It is not certain what effect this 'double sampling' could have had on mice or indeed how well they settled down in continuous light after having each lost one eye.

Rest/Activity

An inverse relationship between wakefulness (activity) and I_m in the pinnal epidermis of the mouse was reported by Bullough (1948a). In this investigation, Bullough measured feeding and drinking activity

in mice which was evidently not a true analysis of their activity level. The author claimed to confirm these preliminary results in a second study (Bullough, 1948b) which showed that inducing mice to sleep caused a rise in I_m at the expected time of low, and conversely, inducing wakefulness at the normal time of sleep caused a decrease in I_m at the expected time of its high. On the basis of these findings Bullough (1948b) claimed:

"The conclusion is now justified that the rate of mitosis normally increases during sleep, and decreases during hours of wakefulness and exercise."

Bullough's methods in these investigations are, however, open to criticism. The pinnal epidermis of 5 mice was sampled repeatedly every 2 h for 24 h, each sample being taken immediately adjacent to the preceeding one. Bullough claimed that there would be no wounding effect in the epidermis after just 2 h (this being the time between each sample). It is now evident, however, that if, as suggested by Gelfant (1971), there is a resting population of cells in G_2 which are prepared for division then these cells could well have been stimulated by tissue wounding to enter mitosis within 2 h. If so, the increased number of mitoses may well have invalidated the results. As Bullough does not mention the time of day at which sampling began, the possible wounding effect cannot be evaluated. Another important point is that I_m was determined by relating the number of mitoses per unit area of tissue. This type of count could well be affected by the fixation and sectioning procedures used, leading to incomparable areas of tissue being examined for the counts. Furthermore, to induce mice to sleep, Bullough used a dose of barbiturates without knowing what the effect of this dosage alone would have on I_m.

It is interesting that Alov (1963) was able to support Bullough's work by showing a striking inverse relationship between bodily activity and I_m in the corneal epithelium of mice.

During sleep body temperature falls (as noted in Chapter 3), but there is as yet no confirmation of a relationship between body temperature and cellular proliferation. For example, peaks in cell

proliferation have been reported to occur at the time of body temperature low in rats (Halberg et al., 1954) and hamsters (Chaudhry et al., 1958). Both groups of authors, however, used only two sample points in 24 h and clearly more frequent samples would have to be obtained before their conclusions can be justified. On the other hand Scheving and Pauly (1960) sampled rats every hour for 24 h, and found that peaks in cellular proliferation occured at the time of peaks in spontaneous activity and rectal temperature. In this investigation, however, cellular proliferation was measured on one day, and rectal temperature 5 days later (in the same rats) thus these authors conclusions too may not be fully justified.

Recently, investigations into the circadian rhythm in cellular proliferation have detailed the strict control of light/dark periods but have not included observations of sleep/wakefulness patterns in experimental animals. Thus Adam (1980), and Adam and Oswald (1983), could only propose that sleep was the time of peak rates in cellular proliferation, for a review of the literature does not allow separation between the time of day and sleep/wakefulness variables. So, I hope to investigate this point in my research.

The effects of hormones, the light/dark cycle and rest/activity on cellular proliferation may all be constituents of a multifactorial system of cell proliferation control, in turn all being part of the synchronized circadian system in animals and man (Czeisler et al., 1980; see Chapter 2).

The attribution of cell proliferation control to one circadian pacemaker is only just beginning. The strong association between the rhythm in cellular proliferation and sleep dependant GH secretion, together with its inverse relationship to cortisol secretion would seem to suggest that cell proliferation is under 'Y' pacemaker control (the SCN) according to the model of Czeisler et al. (1980). Experimental evidence on the effect of 'Y' pacemaker removal, however, does not support this proposal. Powell et al. (1980) reported that SCN ablation in the mouse did not abolish the circadian rhythm in I_m in the epithelium of the cornea, but instead caused a phase advance and amplitude reduction of this rhythm together with

that of core body temperature. Scheving et al. (1983a) reported that SCN ablation in the mouse caused a phase advance of I_s in the epithelia of the tongue and gut and of I_m in the epithelium of the cornea.

The phase advance of the cell proliferation rhythm in association with the 'X' driven core body temperature variable, rather than an elimination of this rhythm following SCN ablation, suggests the possibility that cell proliferation is an 'X' driven variable. Further investigations in this area may be particularly informative in the clarification of cell proliferation control.

Glossary of terms used to describe the parameters of cell renewal systems

(After Quastler (1959), Aherne et al. (1977) and Wright (1980)).

CELL CYCLE PHASES:

G_1 = post-mitotic pre-synthetic period.
G_o = cells arrested in G_1
S = cells synthesizing DNA.
G_2 = post-synthetic pre-mitotic period.
M = cells in mitotis.
C = cell cycle = $G_1 + S + G_2 + M$.

DURATION OF CELL CYCLE PHASES

t_{G_1} = duration of G_1 phase.
t_s = duration of S phase.
t_{G_2} = duration of G_2 phase.
t_m = duration of mitosis.
t_c = cell cycle time (sum of phase durations).

INDICES

I_m = mitotic index.
I_s = labelling index.
I_p = growth fraction.

RATE PARAMETERS

K_b = birth rate
CCPR = crypt cell production rate/hour

Chapter 6

Aims of The Investigation

To test the following hypotheses:

1 That peaks in cell proliferation are noted during sleep as claimed by Bullough (1948b) and Adam and Oswald (1983).

2 That sleep deprivation reduces the peak in cellular proliferation. Bullough and Laurence (1961) tried out the effects of sleep deprivation on mice and found that when wakefulness was induced, at a time when mice normally sleep, mitotic index remained at a low level typical of a normal mouse when awake.

Procedure to Test the Above Hypotheses

1 The study of cell population kinetics in the jejunum of the male Syrian hamster (Mesocricetus auratus) during 24 h under normal conditions (control animals).

2 Investigation of the relationship between sleep and wakefulness and cell proliferation (by imposing sleep deprivation in experimental animals).

Taking great care to (a) ensure complete standardization of animals to the light/dark cycle, (b) minimize disturbance to control animals during their sleep period, (c) impose complete sleep deprivation in all experimental animals (which Bullough and Laurence were not able to do), and (d) to use critically proven methods of studying cell proliferation rather than relying on the rather isolated measure of mitotic index as used by Bullough (1948b) and Bullough and Laurence (1961).

Reasons for the Choice of this Tissue

1 The tissue architecture of the small intestine allows detailed analysis of its proliferative and functional units. There is comprehensive literature on its kinetic organization (Quastler

and Sherman, 1959; Cairnie et al., 1965a,b; Aherne et al., 1977; Al-Mukhtar et al., 1982), and the rhythmic nature of cell proliferation in the small intestine is well established (Scheving et al., 1983b). There is, however, no definitive work on the cell population kinetics in the small intestine of the hamster.

2 The hamster is particularly useful in studies relating to the effect of sleep/wakefulness, for its strict nocturnal activity enable definition of waking and sleeping periods (casual observations of Aschoff and Meyer-Lohmann, 1954 - reported by Chaudhry et al., 1958; Pittendrigh, 1960; De Coursey, 1964; Aschoff et al., 1973).

3 High proliferative rates evident in the small intestine (Eastwood, 1977) make discrimination of any experimental effect (in this case that of sleep deprivation) more likely during a short period of time.

Chapter 7

THE EFFECT OF SLEEP AND WAKEFULNESS ON THE 24 H VARIATION IN CELL POPULATION KINETICS IN THE JEJUNUM OF THE MALE SYRIAN HAMSTER (Mesocricetus auratus)

Introduction to the Epithelium of the Small Intestine

The small intestine extends from the pyloric orifice, where it is continuous with the stomach, to the ileocaecal junction, where it continues into the large intestine.

It is customarily divided into three sections: duodenum, jejunum and ileum; but the divisions between regions are not sharply defined.

The functions of the small intestine are the transport of food material from stomach to large intestine, digestion and absorption of nutrients and the transport and diffusion of ions and water.

The epithelium of the small intestine projects into the lumen as numerous villi which greatly increase the functional surface area, so enhancing its secretory and absorptive efficiency.

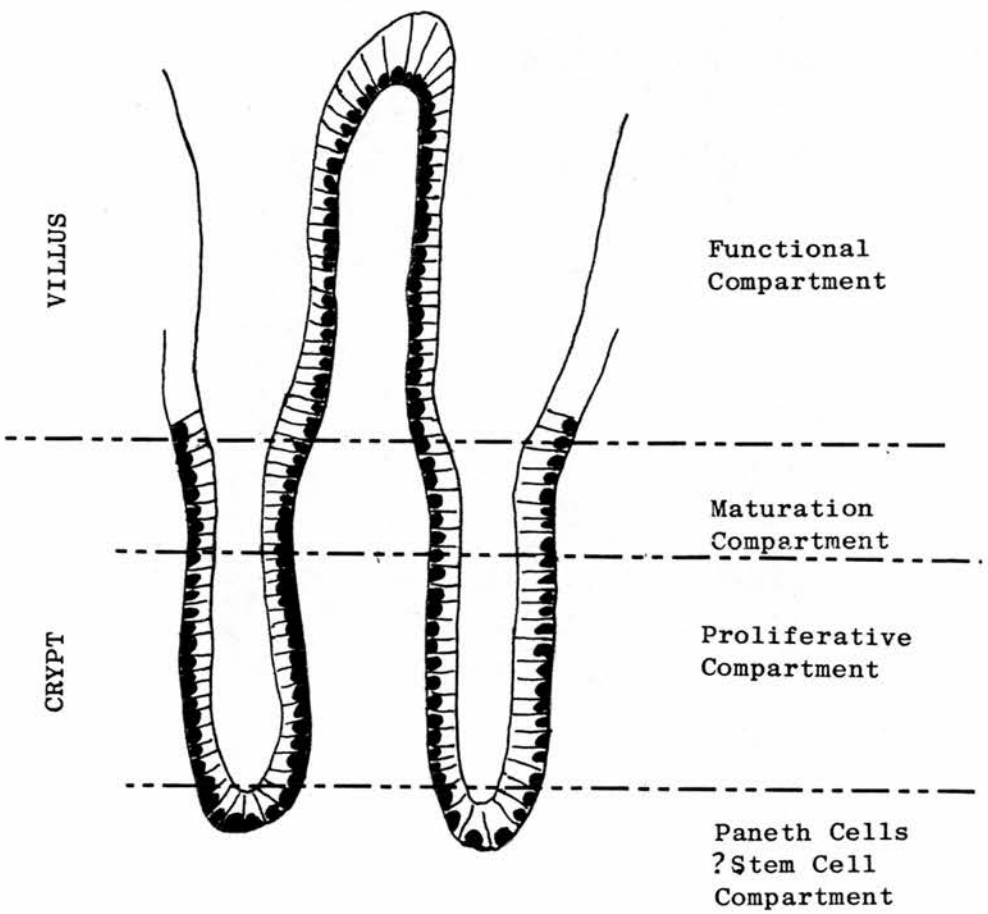
At the base of the villi are numerous crypts of Lieberkühn. Bizzozero (1892) noted a profusion of mitoses in crypts, and suggested that crypts formed the proliferative compartments and that cells migrated out to form sheets of functional villus cells. This hypothesis was confirmed by tracing the movement of goblet cells (Friedman, 1945) and labelled crypt cells (Leblond et al., 1948) from crypt to villus.

The epithelium of the small intestine is in a state of constant rapid renewal, the cell cycle time being in the range 10-14 h in most mammals (Cleaver, 1967). In simple terms, it is defined as a two-compartment system: proliferative compartment and functional compartment (crypt and villus respectively). Cell loss from the functional compartment is compensated for by cell production in the proliferative compartment (see Fig 7.1).

FIG 7.1

Diagrammatic Representaion of the Kinetic Compartments of the Small Intestinal Epithelium

Reproduced after Aherne et al. (1977)



In a series of definitive studies, Cheng and Leblond (Cheng, 1974a,b; Cheng and Leblond, 1974,a,b) described 4 main epithelial cell types in the small intestine of the mouse:

1 Columnar (absorptive)
2 Goblet
3 Paneth
4 Entero-endocrine (secretory)

Among these categories columnar cells are the most numerous.

Cairnie (1970) proposed that precursors of goblet cells lay within crypts, whereas precursors of Paneth cells lay outside crypts. It is, however, generally accepted that all 4 cell types arise from precursor cells located in the crypt basal one-third: the stem cell compartment (Potten and Hendry, 1983). Whether precursor cells are multipotential, as proposed by Cheng and Leblond (1974c) is not proven (Potten and Hendry , 1983). Differentiated columnar, goblet and entero-endocrine cells migrate onto the villus, but Paneth cells are found exclusively in the crypt basal region (Cheng, 1974b).

The fact that the function of crypt cells is proliferation and that of villus cells is absorption and elaboration of enzymes, indicates that during the movement from crypt to villus cells undergo profound changes. Quastler and Sherman (1959) proposed that these changes occured when proliferative cells passed a 'critical stage' and differentiated. Cairnie et al., (1965b) refined this hypothesis in their proposal of the gradual transition of cells during movement from crypt base to crypt mouth. At the crypt base, cells are purely proliferative and each division results in two daughter cells also destined to divide; during movement upwards, cells pass through an intermediate stage of asynchronous division, where each division results in one dividing and one non-dividing daughter cell, until cells reach a region where there is an increasing probability of a final division resulting in two non-dividing daughter cells. Crypt cells can thus be defined as having a declining self-renewal probability with advancing cell position (Potten and Hendry, 1983).

Situated within the laminia propria are subepithelial sheaths of fibroblasts which surround crypts (Pascal et al., 1968; Marsh and Trier 1974 a,b) and undergo division and synchronous migration with adjacent epithelial cells. This relationship suggests pericryptal fibroblasts serve to enhance structural integrity and functional efficiency (perhaps facilitating the exchange of materials) in the intestinal mucosa (Parker et al., 1974; Marsh and Trier, 1974a), and possibly regulate epithelial and/or mesenchymal cell proliferation, migration and differentiation (Marsh and Trier, 1974b).

Zajicek (1977) proposed the concept of an 'intestinal proliferon' composed of epithelial and connective tissue, and neural and vascular elements, which enables the displacement of the different cell populations to be closely co-ordinated. As each villus is surrounded by several attendant crypts supplying it with cells, Wright (1980) suggested that the 'intestinal proliferon' concept should be extended to cover the villus and the whole of its attendant crypt population.

The importance of the intestinal epithelium as a secretory and absorptive area has generated much interest in its adaption, for example during disease (Betts et al., 1966; Wright et al., 1973a,b; MacDonald and Ferguson, 1977; Hasan et al., 1981), drug administration (Scott and Peters, 1982) and radiation (Lesher and Bauman, 1969; Wimber and Lamerton, 1963). A stimulus to such investigations has come from the clarification of the cell population kinetics in the small intestine (Quastler and Sherman, 1959; Fry et al., 1963; Cairnie et al., 1965a,b; Aherne et al., 1977; Al-Mukhtar et al., 1982).

Control of Cell Proliferation in the Epithelium of the Small Intestine

Control of cell proliferation has previously been discussed in Chapter 5, but further mention should be made here of the control processes specifically in intestinal epithelium. Excellent reviews of the subject are given by Williamson (1978) and Scheving et al. (1983b). The constant turnover of cells is maintained by the close association between functional villus compartments and their

attendant crypts achieving a 'steady state' (Williamson, 1978; Wright and Irwin, 1982a). It is commonly accepted that cell loss from the villus is compensated for by cell production in the crypt, and that a negative feedback mechanism is in operation (with critical cell loss being the stimulator, see FIG 7.2). Increased cell proliferation in the crypt following crypt cell damage (Wright and Irwin, 1982b) and starvation (Al-Dewachi et al., 1975) are consistent with this hypothesis.

The negative feedback mechanism may operate at two levels:

(1) From villus to crypt.
(2) From differentiating crypt cells to stem cells (Wright and Irwin, 1982b) or from stem cells themselves (Britton et al., 1982).

The Effect of Hormones and Polypeptides

Clarke (1974) proposed that there was local control of cell proliferation in the small intestine (i.e. the stimulus was contact with luminal contents: ingesta). There is, however, more support for the systemic control of cell proliferation (Scheving et al., 1983b) and consistent with this are the reports of synchronous proliferation in a variety of tissues in one animal (Potten et al., 1977; Scheving et al., 1978).

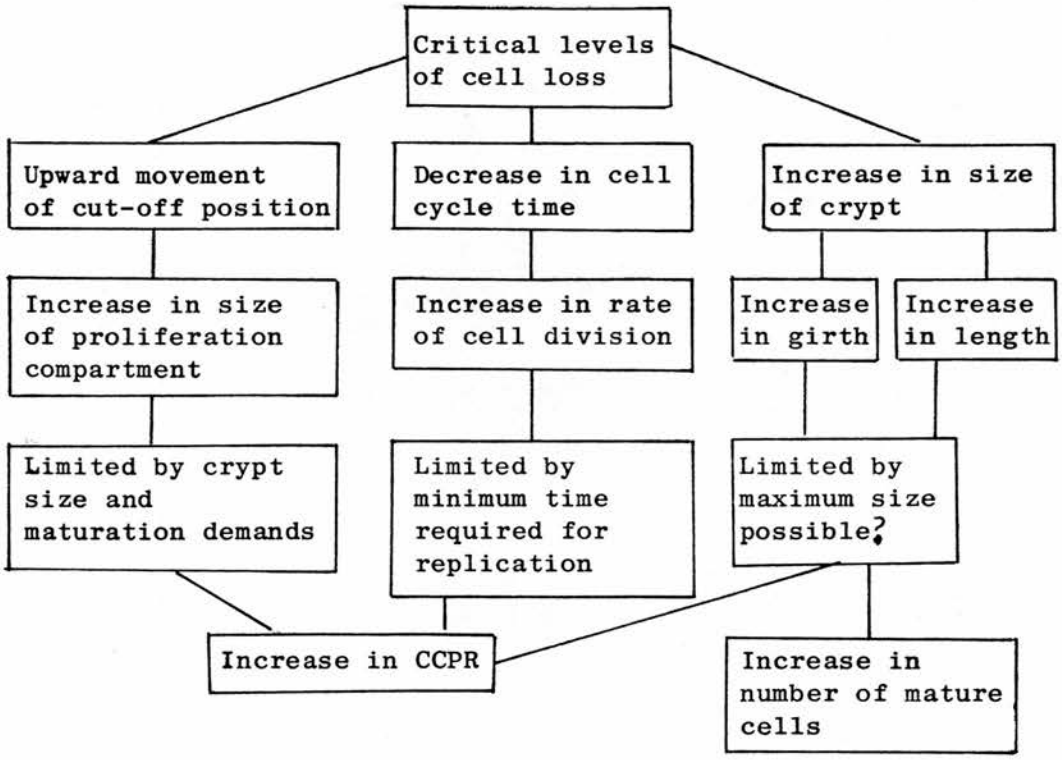
The effects of hormones and polypeptides (i.e. epidermal growth factor-EGF, insulin, glucagon and ACTH-1-17) on cell proliferation in the small intestine have been reviewed by Scheving et al. (1983b). EGF in particular has been the subject of a great deal of interest. It is a polypeptide which can be isolated from sub-mandibular salivary glands, and its trophic effects were first noted in epidermal tissue (Cohen and Elliot, 1963). A specific EGF receptor was later demonstrated in isolated intestinal epithelial cells (Forgue-Lafitt et al., 1980) and EGF was found to stimulate cell proliferation in the gastric mucosa of rats (Johnston and Guthrie, 1980), the jejunal and ileal crypts also of rats, and the duodenal, ileal and colonic crypts of mice (Al-Naffussi and Wright, 1982a).

FIG 7.2

Negative Feedback Mechanism in the Small Intestinal Crypt:

Compensatory Crypt Reactions to Increased Cell Loss

Reproduced after Wright (1980)



In addition tissue specific inhibitors of the cell cycle known as chalones may be implicated in small intestine cell proliferation control. Hagemann and Lesher (1973) proposed that G_1 was the principal modulator of cell cycle time (and thus of cell proliferation see FIG 7.2) and G_1 was, therefore, the logical site for a control signal. Consistent with this proposal was the report of an inhibitory substance present in intestinal extracts which delayed the G_1 to S transition in mouse crypt cells (Sassier and Bergeron, 1980). Further support comes from the investigation by Al-Naffussi and Wright (1982b) of the circadian rhythm in the mouse jejunal epithelium which reported partially synchronized waves of cells in S and M phases, a peak in S being followed some 6-8 h later by a peak in M.

Brugal and Pelmont (1975), however, isolated two chalone-like substances from the intestinal extract of the adult newt, one inhibiting cells in G_1 and the other inhibiting cells in G_2. Consistent with chalone effects at both G_1 and G_2 is the initial report of two distinct cell populations in the mouse epidermis that are blocked or delayed in G_1 and G_2 (Gelfant, 1963), together with the more recent review of this phenomenon in a variety of animal tissues (Gelfant, 1977) and more specifically the findings of synchronous waves of cells in S and M (peak values occuring at the same time) in the jejunal epithelium of the rat (Al-Dewachi et al., 1976) and mouse (Sigdestad and Lesher, 1970).

Chapter 8

MATERIALS AND METHODS

Hamsters

Male Syrian hamsters* (Mesocricetus auratus) aged 14 weeks, were brought into the Animal House at the Western General Hospital, Edinburgh. All were housed in one quarantine room (maintained at 20°C) and kept under a reversed light/dark cycle (light 18.00 h-06.00 h, dark 06.00 h-18.00 h) for 5 weeks before the start of the experiment (to allow for their complete adaption to the new environment and timing of light/dark periods). The daily routine working of the Animal House Technicians took place during the period 09.00 h-17.00 h, the reversed light/dark cycle thus ensured that there was minimal external disturbance to hamsters during their rest-sleep time in the light period 18.00-06.00 h).

Hamsters were caged in groups of 6 and received food (standard laboratory chow) and water ad libitum. The routine checking and cleaning of cages, and food and water replenishment, was carried out during the dark period (hamsters activity period) under the illumination of a red safety light.

Hamsters were killed, in groups of 6, by cervical dislocation in an adjacent post-mortem room. The timing of death is best seen in the time table: FIG 8.1. In all, 12 hamsters were killed every 3 h over 24 h under control conditions; and 12 hamsters every 3 h over the light period under sleep deprivation conditions.

* obtained from Wrights of Essex
Latchingdon
Chelmsford
CM3 6HD
England

FIG 8.1

Time Table: Number of Male Syrian Hamsters Killed at Each Time Point

During the Experimental Period in December 1982

DATE	TIME (h)								
	06.00	09.00	12.00	15.00	18.00	21.00	24.00	03.00	06.00
13th	6 *	6 **	6 *	6 **					
14th					6 * 6sd *	6 ** 6sd **	6 * 6sd *	6 ** 6sd **	6 * 6sd *
15th									
16th					6 ** 6sd **	6 * 6sd *	6 ** 6sd **	6 * 6sd *	6** 6sd **
17th									
18th	6 **	6 *	6 **	6 *					

sd: sleep deprived hamsters * : intraperitoneal injection of ^{3}H-TdR (*one hamster, ** two hamsters)

Sleep Deprivation

Cages of hamsters selected (at random) for this section were moved into an adjacent quarantine room at 16.30 h (90 mins before the first killing) and were kept awake by gentle handling procedures carried out by experienced Animal House Technicians. This room was also under reversed lighting, the light and dark periods occuring at the same time as in the quarantine room in which hamsters had been standardized for 5 weeks. The removal of sleep deprived hamsters from their original quarantine room was necessary to ensure that sleep deprivation was complete (during the standardization period I had noted that hamsters began to rest and sleep a little in advance of the start of the light period) and in addition to minimize disturbance to the control hamsters permitted to sleep.

Injection of Tritiated Thymidine

3 hamsters (selected at random) in each group of 12 received [6-^{3}H] Thymidine* by intraperitoneal injection one hour before killing, at a dosage of 0.5 μ Ci** /g body weight***.

*Obtained from Amersham International
Radiochemical Centre
PO Box 16
Amersham
Buckinghamshire
England

**At specific activity 5.0 Ci/mM, radioactive concentration 100 μ Ci/ml (10% of the original concentration in isotonic saline).

***Mean body weight 120 g (range 112-125 g). Each hamster thus received 60 μ Ci [^{3}H]-TdR (0.6 ml of solution prepared as above**).

Collection of Tissue Samples

1 Immediately following cervical dislocation, the hamster was pinned dorsal side down on a cork dissecting board.

2 Incision* was made through the abdominal wall and the exposed gut severed immediately below the ligament of Trietz.

3 A second cut** was made approximately 4 cm distal to the first, the section of jejunum removed and flushed with isotonic saline using a 1 ml syringe.

4 The proximal 1 cm, and the distal 1 cm, of the flushed section were removed and discarded. The remaining 2 cm was placed on filter paper, opened* and gently spread, lumen side up.

5 This 2 cm section was cut* into 2 sections:
1 cm :- placed in Carnoy's fixative for 24-36 h to await processing.
1 cm :- placed in Clarke's fixative for 24-36 h, stored in 75% ethyl alcohol to await microdissection.

Points 3-5 shown in FIG 8.2

* Using fine dissecting scissors

** Carnoy's fixative:

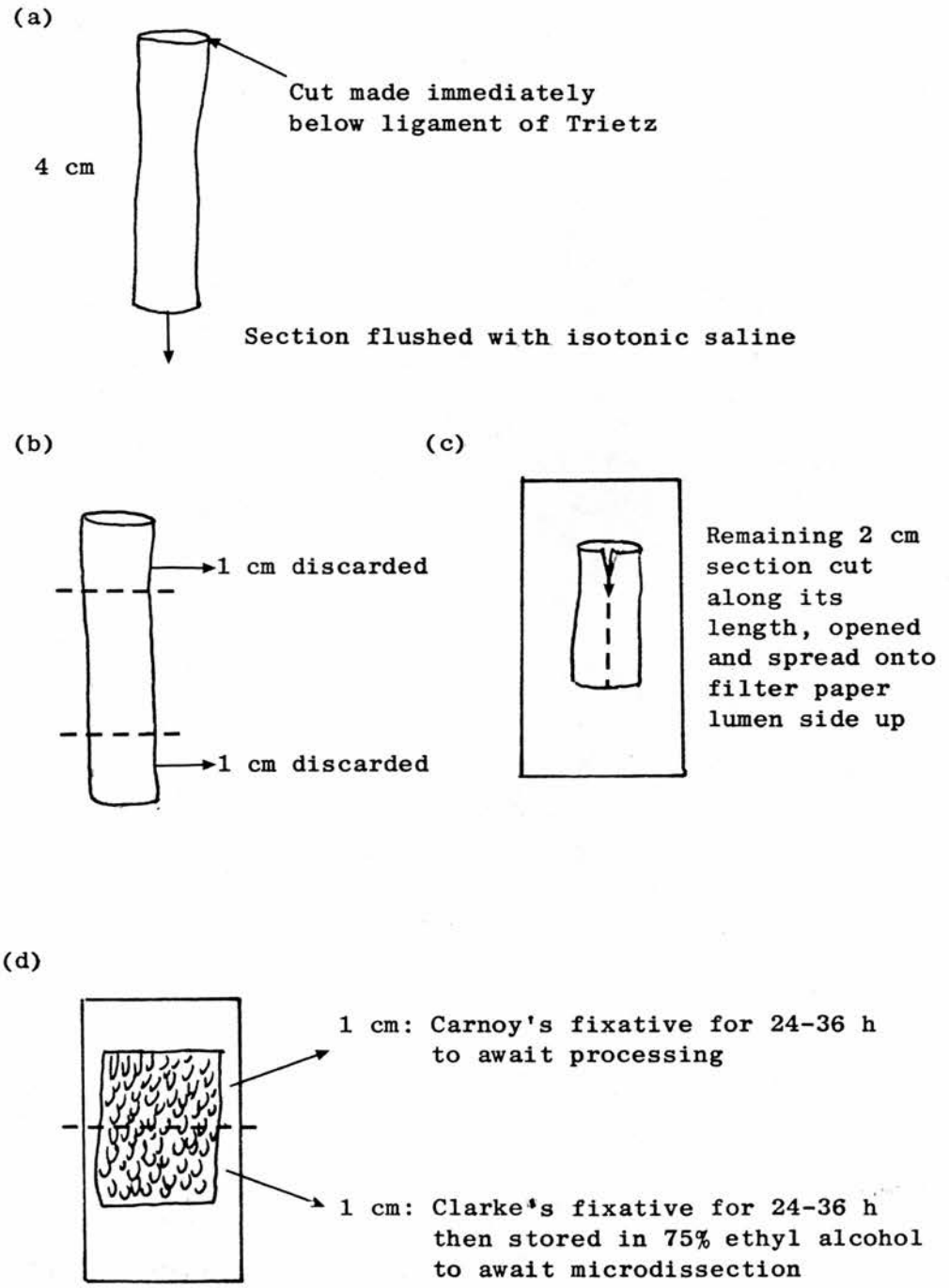
Absolute ehtyl alcohol	(600 mls)
Chloroform	(300 mls)
Glacial acetic acid	(100 mls)

*** Clarke's fixative

Absolute ethyl alcohol	(750 mls)
Glacial acetic acid	(250 mls)

FIG 8.2

Diagrammatic Representation of the Series of Cuts Made in the Section of Hamster Jejunum Removed For Collection of Samples



Processing of Samples

I Basic Histology - Carnoy's Fixed Samples

Note: This basic histology procedure was for samples obtained from hamsters not receiving [^{3}H]-TdR before death. Treatment of samples from hamsters which did receive [^{3}H]-TdR before death is described later in the section on autoradiography.

The following embedding, sectioning and staining procedures were carried out by an experienced Laboratory Technician in the Gastro-Intestinal laboratory at the Western General Hospital who used the following methods.

Embedding and Sectioning

1 Samples, wrapped in gauze and put inside metal capsules, were placed in a histokinette set for a 48 h cycle (4 changes absolute alcohol, 4 changes chloroform, 2 changes paraffin wax - at 52^{o}C).
2 Samples impregnated with pure paraplast inside a vacuum embedding flask.
3 Embedded (villi facing up) in pure paraplast.
4 Fully set tissue blocks trimmed, placed on wooden chocks and frozen.
5 Sections were cut in which the long axis of the villi and crypts were all in the plane of the section, at a thickness of 3 μm using a Jung Rotary Microtome, (sections were cut 100 μm apart.)
6 Tissue blocks realigned and serial sections were cut tangentially to the bowel lumen at a thickness of 3 μm.
7 Cut sections floated in a waterbath (45^{o}C) and lifted onto pre-cleaned glass slides.
8 Air dried for 10 mins.
9 Placed in incubator (37^{o}C) overnight to ensure thorough drying and adhesion of tissue section to slide.

Staining with Harris's Haematoxylin

1 Deparaffinize in xylene (10 mins).
2 Hydrate through descending grades of alcohol.
3 Wash in running tap water (10 mins).
4 Stain with Harris's Haematoxylin* (5 minutes).
5 Wash in running tap water and "blue"** in saturated lithium carbonate solution.
6 Wash in running tap water.
7 Examine degree of staining using a light microscope. If overstained, differentiate in acid alcohol*** (4-5 seconds), and repeat stages 5 and 6.
8 Dehydrate through ascending grades of alcohol, 2 changes absolute alcohol and finally 2 changes of xylene.
10 Mount coverslip over section with DPX mounting medium.
11 Leave slide flat to allow DPX to set for at least 24 hours.

* Harris's Haematoxylin

Haematoxylin	(12.5 g)
Absolute ethyl alcohol	(125 ml)
Potassium alum	(250 g)
Distilled water	(2500 ml)
Mecuric oxide	(6.25 g)
Glacial acetic acid	(100 ml)

** Haematoxylins stain nuclei a red colour which is converted to blue-black when the section is immersed in saturated lithium carbonate solution.

*** Acid alcohol

70% ethyl alcohol	(99 ml)
Concentrated hydrochloric acid	(1 ml)

II Carnoy's Fixed Samples - Autoradiography

The following procedures were carried out on tissue samples from hamsters which received [^{3}H]-TdR before death.

Preparation for Autoradiography.

1 Embedding and sectioning as described for basic histology of unlabelled tissue, points 1 to 5 inclusive*.

2 Cut sections floated in a waterbath (45^{o}C) and lifted onto slides which had been subbed (pre-coated with a thin film of gelatin and chrome alum)**. "Subbing" ensures a high degree of adhesion between the slide and photographic emulsion.

3 Air dry slides (10 mins).

4 Place in incubator (37^{o}C) overnight to ensure thorough drying and section adhesion.

5 Deparaffinize sections in xylene (10 minutes) and hydrate through descending grades of alcohol. Wash in running tap water (10 minutes). Air dry.

* Serial sections were also cut tangentially to the bowel lumen and stained with Harris's Haematoxylin as described in the section on basic histology.

** "Subbing" procedure

1 Pre-cleaned slides immersed (3 times) in hot (but not boiling) potassium dichromate solution (100 g potassium dichromate, 850 mls distilled water, 100 mls concentrated sulphuric acid).

2 Rinse slides in distilled water and immerse in a solution of gelatin and chrome alum (1 g gelatin, 200 mls distilled water, 0.5 g chrome alum).

3 Dry in warm air.

Dipping Procedures

Procedures were carried out by myself with the invaluable assistance of Yvonne Barlow, using the facilities in the Department of Pathology, Medical School, Teviot Place, Edinburgh.

Note: Points 1-7 inclusive were carried out in the dark room under the illumination of a red safety light.

1 K2 ARG emulsion*, obtained in gel form, was melted in a beaker in a waterbath (47°C) without stirring (to prevent bubbles forming).

2 The melted emulsion was poured into a measuring cylinder and 2% glycerol (pre-warmed to 47°C) added to give a final concentration of 2 parts emulsion: 1 part glycerol.

3 A dipping vial was filled with the emulsion + glycerol, and this dipping vial together with remaining emulsion + glycerol was kept in the waterbath (47°C).

4 Each slide was dipped smoothly into the dipping vial and removed slowly and evenly, the back of the slide was wiped on a clean tissue, and slides stacked in a rack.

5 Slides were placed in a light proof box containing dessicant (Silca gel) and left to air dry for at least 7 hours.

6 When dry, slides were packed into dry plastic containers with dessicant added (Silica gel).

7 The containers were light proofed by wrapping in silver foil, and

8 Stored at 4°C for a set exposure period.

Practice slides were used to perfect the dipping technique and to determine optimum exposure time. Best results were found after an exposure time of 28 days.

* Obtained from: Hamilton Tait
Eastfield Drive
Penicuik
Midlothian
Scotland

The temperature of the emulsion, relative humidity of the dark room and the rate at which slides are withdrawn from the emulsion can all affect the thickness of the emulsion to some degree (Flitney, 1977). For this reason all slides to be used for analysis were all taken through the procedure at the same time (together with some control slides bearing unlabelled tissue sections to check for excessive background grain count and light leakage).

Developing and Fixing of Autoradiographs

Note: Points 1-4 all carried out in the dark room at 20°C under illumination from a red safety light.

Practice runs were developed and fixed in coplin jars, for the main run, plastic trays were used.

1 Placed in Kodak D19 developer for 3 1/2 mins.
2 Washed well in running water (2 minutes).
3 Placed in Kodak Unifix fixer for 9 mins.
4 Washed well in running water (10 minutes).
5 Air dried for 24 hours.

Staining of ARGS

Sections were stained through the emulsion with Harris's Haematoxylin according to the methods detailed previously in the section on basic histology with the following amendments:

Deparaffinize and hydrate (stages 1-2) omitted.
Staining time in Harris's Haematoxylin (stage 4) - 30 minutes.

III Clarke's Fixed Samples - Preparation for Microdissection

Staining by Feulgen Reaction

Procedure was carried out on tissue samples by myself, in the Gastro-Intestinal Laboratory at the Western General Hospital, according to the methods of Ferguson et al. (1977).

1 50% ethyl alcohol for 10 minutes.
2 Tap water for 10 minutes with 3 changes.
3 1 M HCL at 60°C (waterbath) for 6 minutes.
4 Tap water for 10 minutes (3 changes).
5 Schiff reagent at room temperature (1 hour).
6 Several rinses in tap water to remove excess Schiff.
7 Specimen stored in 75% ethyl alcohol to await microdissection (carried out within 24 h).

Microscopes Used for Examination of Histological Sections and Microdissected Samples

1 Light microscope: Leitz Dialux (20 EB); lenses x 25, x 40, x 100, eyepieces x 10.
2 Dissecting microscope: Zeiss binocular microscope; x 1.6 magnification.
3 Projecting microscope: Gillette and Sibert; lenses x 10, x 40.

Determination of Cell Population Kinetics: Blind Measurements

A Technician gave all the samples to be examined (labelled and unlabelled sections, and Feulgen stained tissue) a random code, in place of the time, to ensure that I made all counts and measurements 'blind'. Other authors reporting their investigations on cell population kinetics (for example, Bullough 1948a,b; Scheving and Pauly, 1960; Sigdestad and Lesher, 1970; Al-Dewachi; et al., 1976; Al-Naffussi and Wright, 1982b) have failed to mention whether measurements were made 'blind' and may have subconsciously allowed their expectations to bias their choices of tissue areas for analysis.

Labelling Index (I_S) and Distribution of Labelled Cells

This was determined in autoradiographs.

Counts

Using a light microscope (x 250 magnification) 30 axially sectioned crypts were selected in each sample according to the criteria of Al-Dewachi et al. (1974). (Crypts in which the base, middle and top were all in the plane of section).

Starting at the mid-base of the crypt, the total number of cells lining the left-hand side of the crypt known as the left-hand crypt column, and the cell positions of labelled nuclei in this column were recorded (x 1000 magnification) according to the methods of Cairnie et al. (1965a). See FIG 8.3. The spacing of sections (100 μm apart) was such that no crypt was counted twice.

The criterion used to establish whether a cell was labelled, was more than 5 grains over the nucleus, each grain being within one grain's diameter of the nucleus (Cleaver, 1967; Wright, 1971). Background noted in control slides was minimal, contributing less than one grain per nucleus.

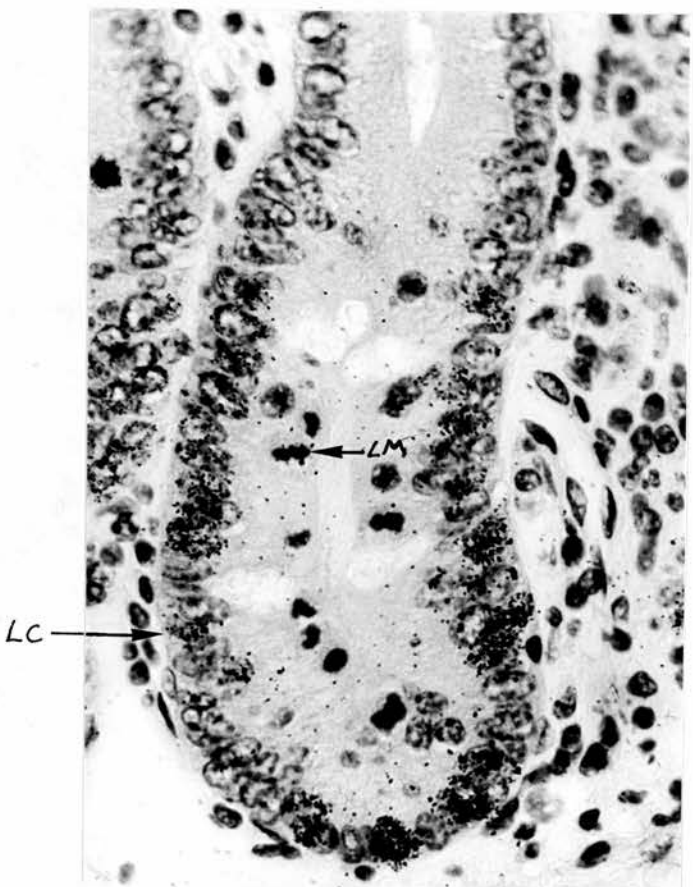
Distribution of Labelled Cells

The data from 3 hamsters per group was combined and analysed using the standardization program of Wright et al. (1972). This program, a modified version of the one designed by Cairnie and Bentley (1967) allows direct comparison of the labelled cell distribution in long and short crypts. Labelled cells in a crypt are computed to correspond to a number of units in a standardized crypt of 1000 units. The standardized results of 90 crypts, counted in each group of hamsters, are then combined and projected onto a crypt of mean length (mean left-hand crypt column length of all crypts recorded). This (ALGOL) program was run by Dr D.R. Appleton on an IBM 360/67 computer (in the Department of Medical Statistics, Newcastle University, Newcastle-upon-Tyne). The results were obtained as graphical output showing the distribution of labelled cells in a crypt of mean size (found to be 40 cells) in each group of hamsters see (FIG 8.4).

FIG 8.3

Appearance of Labelled Crypt Cells and Mitoses in the Hamster Jejunal Crypt

(ARG stained through emulsion with Harris's Haematoxylin x500)

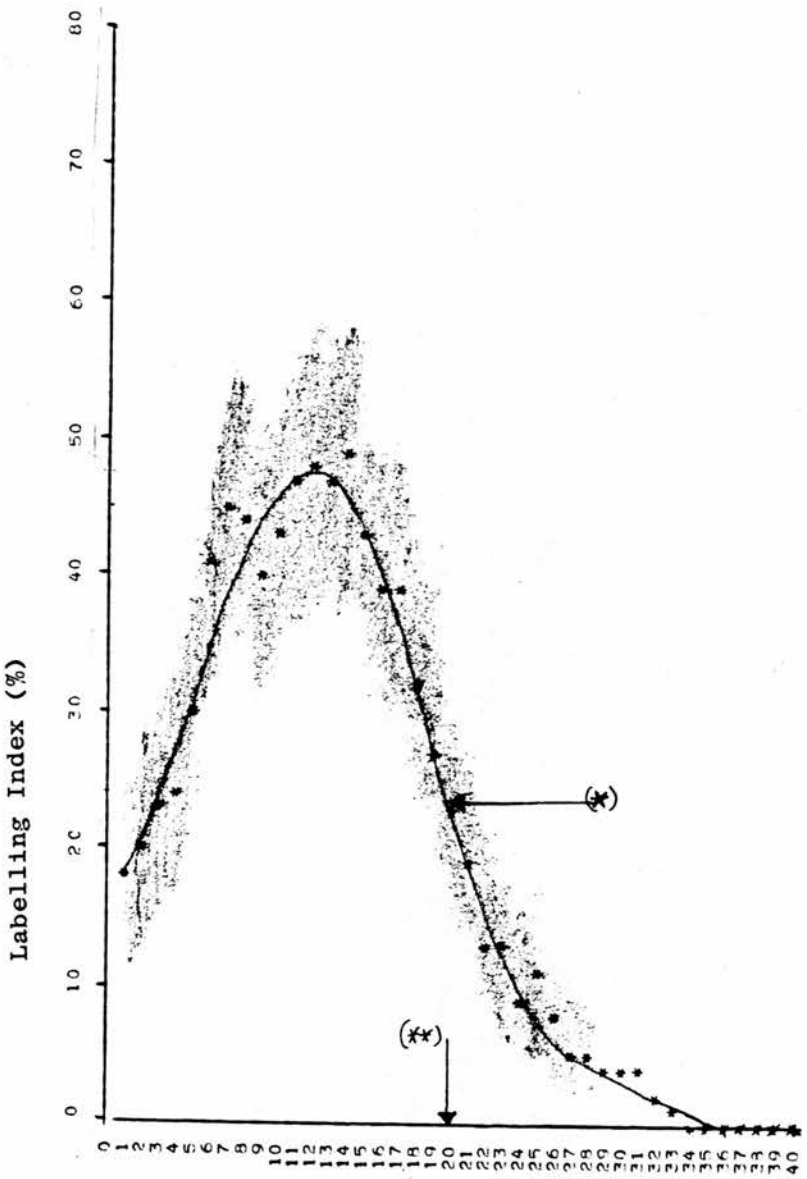


Lc : Labelled Crypt Cell

LM : Labelled Mitotic Figure

FIG 8.4

The Labelling Index Distribution Curve for the Hamster Jejunal Crypt



Shaded area indicates 95% confidence limits

Points used for calculation of crypt growth fraction (I_p):

(*) → Half maximum value of labelling index

(**) → Corresponding cell position

Labelling Index

In addition to standardization computation, the program of Wright et al. (1972) computes the number of labelled cells and the total number of cells counted in each hamster. The number of labelled cells divided by the total cells counted gave the pulse labelling index (I_s) in each hamster.

The Mitotic Index (I_m) and Distribution of Mitotic Cells

Counts

In the 3 hamsters per group which received [^{3}H]-TdR before death, mitotic cells were counted in 30 axially sectioned crypts in autoradiographs. In the remaining 9 hamsters per group, a larger number of tissue sections were prepared so 60 axially sectioned crypts were selected for counts. All counts were made using a light microscope (x 1000 magnification).

Cells were identified as mitotic when in stages from late prophase to early telophase, according to the criteria of Fisker et al., (1982). Late prophase: appearance of chromatin as threads contrasting with the clear nucleoplasm. Metaphase: disappearance of nuclear membrane, chromatin material formed into intensely stained clumped masses. Anaphase/early telophase: from the first distinct signs of separation of the chromatids until the chromosomes of daughter nuclei appear as darkly stained masses.

Starting at the mid-base of the crypt, the number of cells in the left hand crypt column were recorded. Mitotic figures are found out of line with the interphase nuclei (Tannock, 1967) so each cell position in the crypt column was assigned a score (0, 1/2, 1, 1 1/2 or 2) according to the presence of mitotic figures (0 = no mitotic figure, 1/2 = mitotic figure situated mid-way between left and right-hand crypt columns, 1 = one mitotic figure directly opposite crypt column cell, etc.).

Distribution of Mitotic Cells

Mitotic index distribution diagrams were prepared using data from 9 (unlabelled) hamsters per group according to the methods detailed in distribution of labelled cells. Mitotic distribution diagrams were also prepared using the data from 3 ^{3}HTdR-labelled hamsters per group in order that mitotic index could be evaluated in a total of 12 hamsters per group.

Mitotic Index

Using the computer output of the standardization program the number of mitotic figures was divided by the total number of cells counted to give the mitotic index (I_m) in each hamster (in the same way as I_s was calculated). In addition the numbers of labelled mitoses were recorded in autoradiographs of the 3 hamsters per group, in order to calculate the percentage of labelled mitoses.

Growth Fraction (I_p)

A growth fraction calculation for the whole crypt represents the fraction of the crypt that is in the proliferative compartment. It is the region over which labelled and mitotic cells are observed and may be considered to end approximately at the position where I_s falls to half its maximum or peak value (Cleaver, 1967).

The growth fractions of crypts was approximated from the labelling index distribution diagrams for each group of hamsters. By assuming

equal numbers of cells at each position the growth fraction was taken to be equal to the ratio of the number of cell positions in the proliferative compartment to the total number of cells in the crypt column. (For example: cell position at which I_S falls to half its peak value = 20, total number of cells in crypt column = 40, growth fraction = 20/40 = 0.50 (or 50%), see FIG 8.4).

This calculation assumes that all cells in the proliferative compartment are cycling and thus that the growth fraction in this region equals unity. This is likely to be the case for the mid-crypt, but not the crypt base where there are differentiated Paneth cells and a stem cell compartment characterised by low proliferative indicies (Cleaver, 1967). Given these limitations, Wright et al. (1973b) claimed that growth fraction calculation by this method provides a simple means of estimating the size of the proliferative compartment in intestinal crypts.

Crypt Morphology

The most accurate method of measuring crypt morphology is to perform counts of cells in crypt squash preparations. This is, however, an extremely tedious method when the sample number is large, as it is here (with 12 hamsters per group). For this reason, the crypt column length and the crypt column count were used to approximate the crypt population.

Crypt column length

This refers to the number of cells along the length of the crypt and was the number of cells in the left hand columns of crypts as selected for, and recorded in, determination of mitotic index in each hamster.

Crypt column count

This is the number of cells in the crypt circumference and is thus a measure of the number of crypt columns in the crypt (Wright, 1980). In tangentially sectioned tissue, approximately circular crypt

cross-sections were selected in which the peripheral band of crypt cells and the crypt lumen were visible (see FIG 8.5), the number of cells in the peripheral band was counted. Counts were made in 30 crypt cross sections in each hamster, using a light microscope (x 250 magnfication).

Tannock (1967) warned that the spatial arrangement of mitotic cells in the crypt lumen would lead to an over-estimation of I_m. The examination of crypt cross-sections at this stage in the proceedings allowed estimation of Tannock's constant in control and sleep deprived hamsters. 10 crypt cross-sections containing metaphases were selected in each hamster, and the ratio a/b,termed Tannocks constant,calculated (a= distance from crypt axis of mitotic figure in metaphase, b= distance from crypt axis of interphase nucleus) under x 400 magnification.

Crypt population

An estimate of the crypt population was obtained from the product of the mean crypt column length and the mean crypt column count in each hamster (Wimber and Lamerton, 1963).

Number of Proliferating Cells (N_p)

The mean proportion of the crypt occupied by the proliferative compartment (the estimated crypt growth fraction) multiplied by the mean crypt population gave the approximate number of proliferating cells in each group of hamsters.

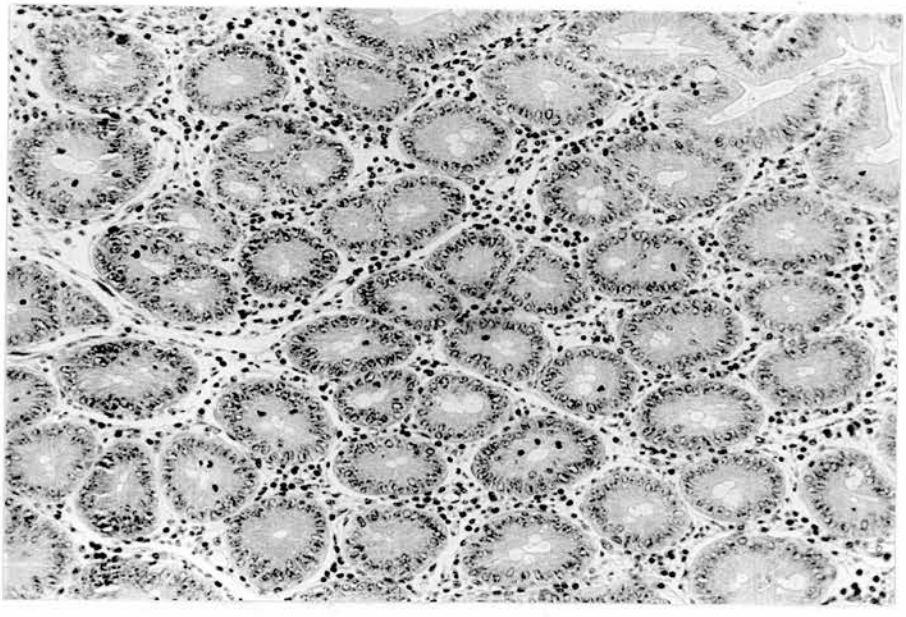
Crypt Cell Production Rate (CCPR)

The crypt cell production rate is sensitive to N_p and I_m (see FIG 7.2), and is thus a very useful parameter in the determination of the kinetic state of the intestinal epithelium (Al-Mukhtar et al., 1982). CCPR is commonly calculated from a combination of metaphase arrest and crypt microdissection. In the present investigation, metaphase arrest at each 3 h point was not practicable so the birth rate of

FIG 8.5

Hamster Jejunal Crypts Sectioned Tangentially to the Bowel Lumen

Used for crypt column count (Harris's Haematoxylin x 160)



cells (K_b) was used as a measure of the CCPR (Al-Mukhtar et al. 1982) where:

$$K_b = \frac{I_m}{t_m} \times N_p$$

I_m = mitotic index

t_m = duration of mitosis, normally calculated by metaphase arrest but as this technique was not used in the present investigation the duration of mitosis was taken to be 1 h according to results in the hamster jejunum reported by Betts et al. (1966).

N_p = number of proliferating cells in the crypt.

CCPR was calculated in each hamster according to this methodology.

Villus Area

Counts of functional villus cells give an accurate assessment of the size of the villus compartment. This technique is, however, extremely tedious, so an estimate of villus compartment size was obtained by measurement of projected villus area. Al-Mukhtar et al. (1982) warned that use of an indirect measurement of the villus compartment size is only meaningful if the parameter is compared to the villus cell population, and thus its accuracy evaluated. Measurement of projected villus area shows high correlation ($r = 0.96$, $p<0.001$) with counts of villus cells in squash preparations (Hasan and Ferguson, 1981) so it was considered to be an excellent method for studying the large sample numbers. The procedure of microdissection, according to Hasan and Ferguson (1981), was followed:

Under a binocular microscope (x 1.6 magnification) and with the use of an orange stick and fine forceps, the muscularis propria was removed from tissue samples stained by the Feulgen reaction. Using a fine needle, individual villi and their associated crypts were dissected out. Villi were cut free at their base (see FIG 8.6), placed on a glass slide with 75% ethyl alcohol, covered gently with a coverslip and their image projected onto a screen at a fixed distance (using a projecting microscope which was composed of a light microscope, at x 100 magnification, with a camera lucida attachment).

FIG 8.6

Microdissected Single Villus From Hamster Jejunum

(Schiff x 160)

NOTE: I made several unsuccessful attempts to transport microdissected villi to the Medical Photography Premises without damaging the villi. The villus shown above has part of its base missing and is not representative of villi used for area counts, it is included here to indicate the characteristic finger shaped villi in the hamster jejunum.

The outlines of villi were mapped onto paper and their areas measured (cm^2) using a planimeter. Measurements were made of 10 villi in each hamster.

Crypt: Villus Ratio

To obtain a clear picture of the cell population kinetics in the small intestine it is important to evaluate the number of crypts producing cells for the villus compartment. This was carried out by calculating the crypt: villus ratio in each hamster. The Feulgen stained tissue was viewed from the serosal aspect with strong transmitted light (light microscope x 250 magnification; counting grid fitted into eyepiece). Focusing on the crypt fundi, the number of crypts in the area of the grid were counted (using standard stereometry methods). Focusing through the tissue to reach the crypt-villus junction, the number of villi in the same area was counted. Three such counts were carried out in each hamster and their mean crypt:villus ratio calculated.

Crypt Cell Mass per Villus

The total crypt cell mass supplying each villus was calculated from the crypt cell population multiplied by the crypt:villus ratio in each hamster (Wright and Irwin, 1982a).

Net Villus Influx

The rate at which cells enter the villus from the crypts can be evaluated as the product of CCPR and the crypt: villus ratio. The method of Wright and Irwin (1982a) was modified to take into account the number of cells produced in crypts (CCPR) taking part in any repopulation of crypts over the time periods.

For example net villus influx between 06.00 h and 09.00 h =

$$\left(A\ \frac{06.00\ h + A\ 09.00\ h}{2}\right) - \left(B\ \frac{09.00\ h - B\ 06.00\ h}{3}\right) \times \left(C\ \frac{06.00\ h + C\ 09.00 h}{2}\right)$$

where A = mean CCPR

B = mean crypt population

C = mean crypt:villus ratio

(mean values in 12 hamsters per group).

Food Intake

The amount of food eaten by groups of 6 hamsters (control - every 3 h over 24 h, sleep deprived - every 3 h over the light period) was measured by simple subtraction of the weight* of food in cages at the time when animals were killed from the weight* of food at the start of each experimental occasion. From these weights the approximate amount of food eaten every 3 h by the groups of hamsters was calculated.

* Food weighed on a top pan electronic balance, accurate to the nearest 100 mg

Note:

Schiff reagent (Feulgen stain) was obtained from
Koch-Light Laboratories Ltd
Poyle Estate
Willow Road
Colnbrook
Slough
Berkshire

All other reagents (unless otherwise stated) were obtained from
BDH Chemicals Ltd
Macfarlane Robson Ltd
Burnfield Avenue
Thornliebank
Glasgow

Data Analysis

Both the labelled and mitotic cell counts were obtained from differently sized cell samples owing to the variation in crypt column lengths (indicative of the variation between short and long crypts). The variation in total cell sample for each group of animals was more pronounced in mitotic counts as 30 crypts were examined in 3 hamsters, and 60 crypts in the remaining 9 hamsters per group. For this reason weighted means of I_s and I_m were calculated, weighting by the total number of cells in each sample (Appleton, 1984). Standard errors of I_m means were calculated (Snedcor and Cochran, 1967), standard errors of I_s means were not calculated owing to the smaller n (3 hamsters per group).

The means ($\pm$ SEM) in all other parameters were calculated using the BMDP 1982 statistical package developed at the Health Sciences Computing Facility, University of California, Los Angeles.

The pattern of some circadian rhythms can be approximated by a sine wave of period 24 h. To see whether variables followed this pattern, sine waves were fitted to group means in each variable using the methods of Nelson et al. (1979) and the fit analysed by multiple regression using GLIM: GLIM is a package for the interactive analysis of 'generalized linear models' written by Baker and Nelder (1978) and distributed by the Numerical Algorithms group for the Royal Statistical Society. This analysis is particularly useful as it enables calculation of the percentage change in variables, thus indicating the degree of the variation over 24 h. In the absence of fit to sine waves, the effect of time of day was determined by analysis of variance, using GLIM for I_s and I_m as this allows for weighting of the values - see above. ANOVA was carried out using BMDP in all other parameters).

Circadian rhythms may follow a variety of wave forms and another wave which can be tested for is a rectangular wave. In addition this wave form is particularly useful in evaluating the relationship between sleep and wakefulness and variables investigated (Minors and Waterhouse, 1981b). This method involves examining the 24 h time

span as 3 sampling periods:

Period 1 = overall 24 h time span (06.00 h - 06.00 h)
Period 2 = dark period (09.00 h - 18.00 h)
Period 3 = light period (21.00 h - 06.00 h)

Calculation of a rectangular wave fit (F value) was as follows.

$$F = n\text{-}2x \left[\frac{SS_1 - (SS_2 + SS_3)}{(SS_2 + SS_3)}\right]$$

where n = number of time points in Period 1, SS = sum of squares calculated for each variable according to the specified periods (1,2 and 3). As rectangular wave fit does not allow the same quantitative measurements as in sine wave fit, ANOVA was carried out even when a significant fit was evident.

The effect of sleep deprivation was assessed by use of analysis of variance to determine (i) whether there were significant time effects within the 24 h control group and (ii) whether over the light period the sleep deprived groups exhibited a significantly different pattern in comparison to the time-matched control (sleeping) groups. (ANOVA was carried out using GLIM for I_s and I_m and BMDP for remaining parameters).

Differences between means in variables (maximum/minimum in control, and control/sleep deprived hamsters) were analysed using the two sample t-test. This calculation was not used when variables were determined in 3 hamsters per group (for example I_s and percentage of labelled mitoses) as an n of 3 is too small for this test to be acceptable.

CHAPTER 9

RESULTS

As soon as the first histological sections were examined it became evident that the hamsters were chronically infected with the protozoan Giardia muris. This lumen-dwelling flagellate is found in the small intestine of man and animals. In the human, mild infection with Giardia muris is often asymptomatic but heavy infection can cause shortening of villi and associated malabsorption (Hoskins et al., 1967). In mice, Giardia muris infection has been found to produce a doubling of the CCPR and an increase of approximately 60% in net villus influx, but no significant villus atrophy (MacDonald and Ferguson, 1978). Following the disconcerting discovery that Giardia muris was present in the jejunum of hamsters to be used for cell population kinetic analysis, tissue sections from all hamsters were immediately examined under a light microscope x 1000 magnification. All hamsters were found to be infected, (identified as so by the presence of trophozoites - see FIG 9.1) so although cell population kinetic values would be expected to differ from those in an uninfected hamster population in the ways described above, the universal nature of the infection suggested that effects would be consistent in the population under study. It was thus thought reasonable that the cell population kinetic analysis of these Giardia muris infected hamsters should proceed.

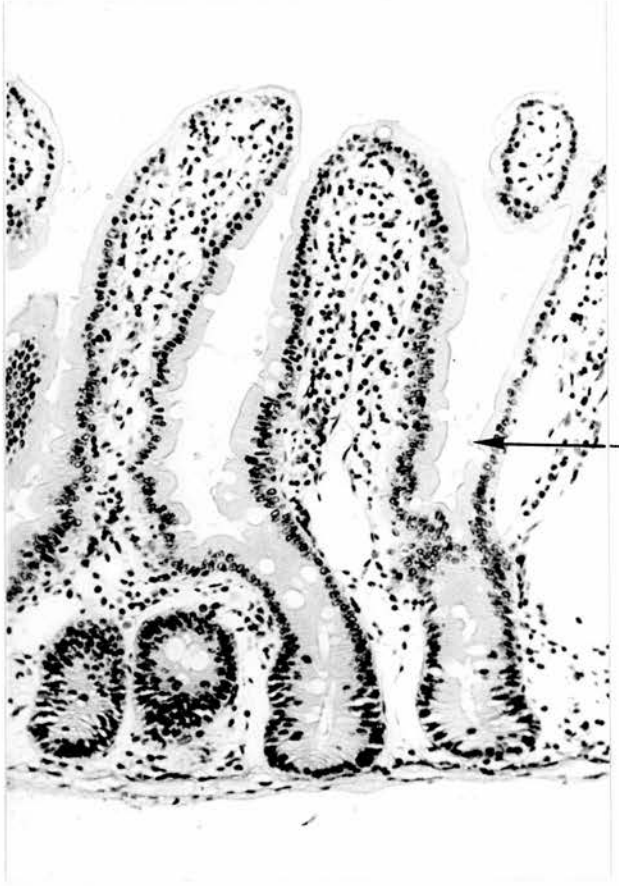
Food Intake

Food intake during the dark period (06.00 h - 18.00 h) was on average 8.0 g/h/cage of 6 hamsters. During the light period (18.00 h - 06.00 h) food intake in control hamsters was on average 2.5 g/h/cage, and a similar intake of 2.8 g/h/cage was noted in sleep deprived hamsters. (Food intake is shown in Appendix 1.1).

FIG 9.1

Presence of *Giardia muris* Trophozoites in Histological Sections of the Hamster Jejunum

(Harris's Haematoxylin x 160)



The Proliferative Indices

a) Labelling Index (I_s)

Counts of labelled cells are presented in Appendix 1.2, weighted means in Table 9.1.

Control

I_s values showed a significant fit to a rectangular wave ($F=7.48$, $df=1,7$, $p<0.05$).

A significant variation in I_s with time of day during 24 h was evident ($F=3.09$, $df=8,18$ $p<0.025$ with ANOVA) a broad peak occurring during the light period (see FIG 9.2).

Effect of Sleep Deprivation

Sleep deprivation significantly altered I_s values in relation to time over the light period ($F=40.86$, $df=1,20$, $p<0.001$ with ANOVA) and destroyed the rectangular wave fit. I_s in sleep deprived hamsters fell to half the control value during the latter part of the light period, (see FIG 9.2)

b) Mitotic Index

Counts of mitotic cells are presented in Appendix 1.3, and weighted means ($\pm$ SEM) in Table 9.1.

The ratio desribing the spatial arrangement of mitotic cells in crypt sections (called Tannock's constant; after Tannock, 1967) multiplied by I_m enables an accurate measure of the mitotic index in crypts. Tannock's constant was calculated as 0.57 and showed remarkable consistency with time of day in control and sleep deprived hamsters (see Table 9.1). Consequently, it was decided that its exclusion in the calculations of I_m should not affect my analysis.

Table 9.1

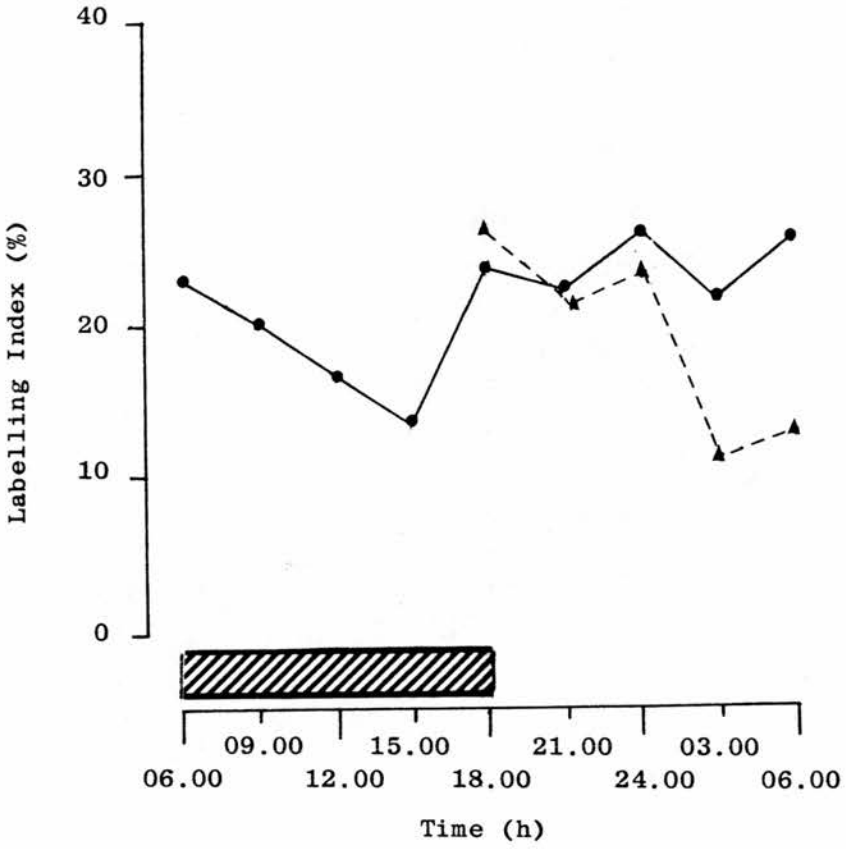
Crypt Cell Population Kinetics in the Jejunum of the Male Syrian Hamster During 24 h With Sleep or Sleep Deprivation

	Time (h)	Labelling Index (I_s) % $\overline{X}$	Mitotic Index (I_m) % $\overline{X}$ (S.E.M)	Percentage Labelled Mitoses $\overline{X}$	Tannocks Constant $\overline{X}$ (S.E.M)
Control Hamsters	06.00	23.0	4.7 (0.2)	31.4	0.57 (0.01)
	09.00	20.0	4.2 (0.3)	22.5	0.57 (0.01)
	12.00	16.9	4.9 (0.4)	16.7	0.57 (0.02)
	15.00	13.5	4.8 (0.5)	28.4	0.57 (0.02)
	18.00	23.9	8.5 (0.5)	41.0	0.57 (0.01)
	21.00	22.3	9.1 (0.3)	25.7	0.57 (0.03)
	24.00	26.0	6.9 (0.6)	39.5	0.57 (0.01)
	03.00	21.9	6.8 (0.6)	35.5	0.57 (0.01)
	06.00	25.8	4.5 (0.4)	33.3	0.57 (0.00)
Sleep Deprived Hamsters	18.00	26.4	5.3 (0.5)	36.3	0.57 (0.01)
	21.00	21.6	6.1 (0.5)	38.4	0.57 (0.03)
	24.00	23.3	5.6 (0.3)	19.6	0.57 (0.02)
	03.00	11.1	5.2 (0.3)	18.2	0.57 (0.01)
	06.00	13.1	5.4 (0.4)	20.3	0.57 (0.01)

FIG 9.2

Changes in Labelling Index With Time of Day in Control (●——●) and Sleep Deprived (▲- - -▲) Hamsters.

Weighted means of 3 hamsters are shown at each point



= Dark period

Control

I_m showed a significant fit to a sine wave describing a circadian rhythm ($F=16.80$, $df=2,6$, $p<0.01$ with GLIM) and the circadian variation was 74% (see FIG 9.3). The maximum value (9.1% $\pm$ 0.3 at 21.00 h) was significantly different from the minimum value (4.2 % $\pm$ 0.3 at 09.00 h), ($t = 12.06$, $df=22$, $p<0.001$).

Effect of Sleep Deprivation

Values maintained a significant fit to a sine wave ($F=7.22$, $df=2,6$, $p<0.05$ with GLIM) but the circadian variation was reduced to 25% (FIG 9.3). ANOVA revealed that sleep deprivation significantly altered values in relationship to time over the light period ($F=39.38$, $df=1,110$, $p<0.001$). Using t-tests, sleep deprivation values were found to be significantly below control at 18.00 h ($t = 4.47$, $df = 22$, $p<0.001$), 21.00 h ($t = 5.21$ $df=22$. $p<0.001$) and 03.00 h ($t = 2.29$, $df=22$, $p<0.05$).

Percentage of Labelled Mitotes

Means are presented in Table 9.1.

Control

ANOVA revealed that values did not vary significantly with time of day.

Effect of Sleep Deprivation

In sleep deprived hamsters the variation with time over the light period was not significantly different from that in control hamsters (as tested by ANOVA).

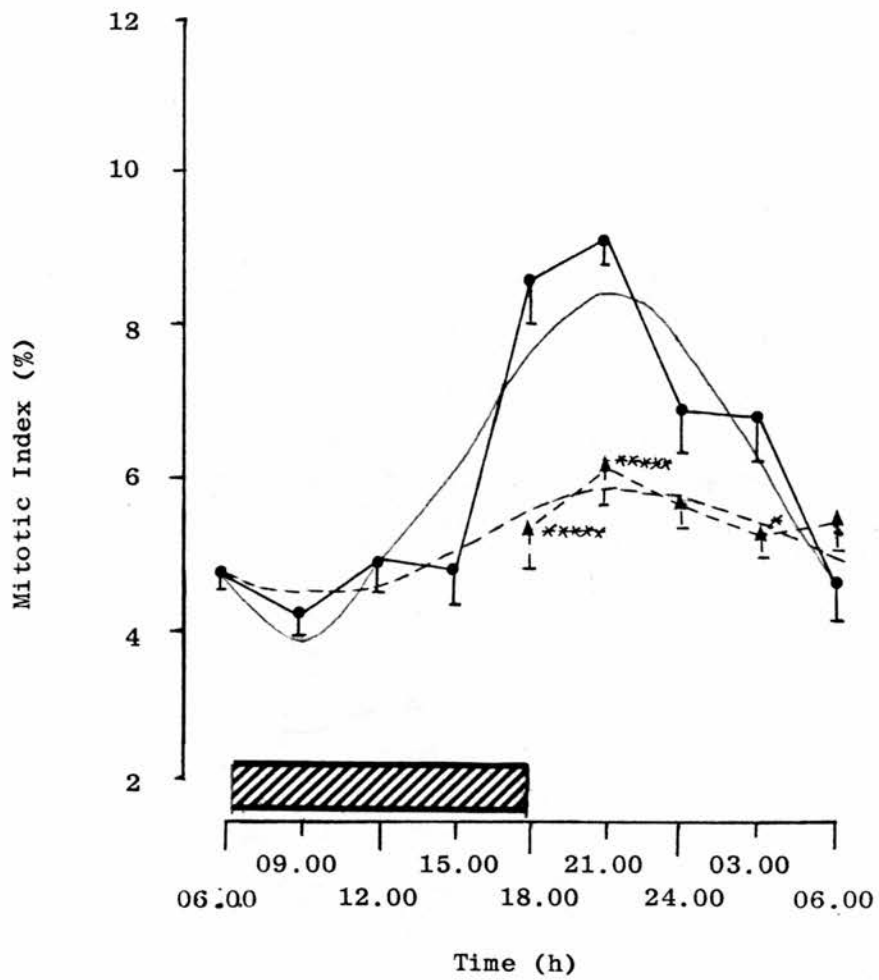
There was a significant linear correlation between percentage of labelled mitoses and I_s over 24 h in all 14 groups of hamsters ($r = 0.65$, $df = 12$, $p<0.02$).

FIG 9.3

Changes in Mitotic Index With Time of Day in Control (●——●)

and Sleep Deprived (▲ - - ▲) Hamsters

Weighted means of 12 hamsters (±S.E.M) are shown at each point



Sine wave fit: (∿) control hamsters
(∿) sleep deprived hamsters

▨ = Dark period

Values in control and sleep deprived hamsters significantly different with t-test: * $p < 0.05$, ***** $p < 0.001$

Distribution of Labelled Cells in Crypts

Labelling index distribution diagrams are presented in Appendix 1.4 and show the variation in I_s with crypt cell position. It is evident that highest values in I_s occur in the bottom two thirds of the crypt and lowest in the top one third of the crypt. The top 6 cell positions of standardized crypts did not contain labelled cells. Mean values of I_s were calculated for each one third of the crypt (bottom one third, cell positions 1-13; middle one third, cell positions 14-26; top one third cell positions 27-40). No significant fit was found, in any of these regions, in control or sleep deprived hamsters to a sine wave or rectangular wave over the 24 h period.

Distribution of Mitotic Cells in Crypts

Mitotic index distribution curves are presented in Appendix 1.5. Highest I_m values were evident in the mid region of the crypt and very low values in the top one third of the crypt. Mitotic figures were absent in the top 6 cell positions of standardized crypts.

The standardized crypts were divided into thirds according to cell position (as detailed previously) and it was evident that mean I_m in each one third of the crypts of control hamsters exhibited a significant fit to a sine wave over 24 h (analysed with GLIM): Bottom one third ($F = 26.97$, $df = 2,6$ $p<0.01$) circadian rhythm 67%; middle one third ($F = 23.80$, $df = 2,6$ $p<0.01$) circadian rhythm 74%; top 1/3 ($F = 8.20$, df 2,6, $p<0.025$) circadian rhythm 129%. In sleep deprived hamsters none of the crypt regions exhibited a significant fit to a sine wave over 24 h.

The Growth Fraction

The values of I_p (Table 9.2) as a function of time are shown in FIG 9.4.

Control

The proportion of the crypt occupied by the proliferative compartment

Table 9.2

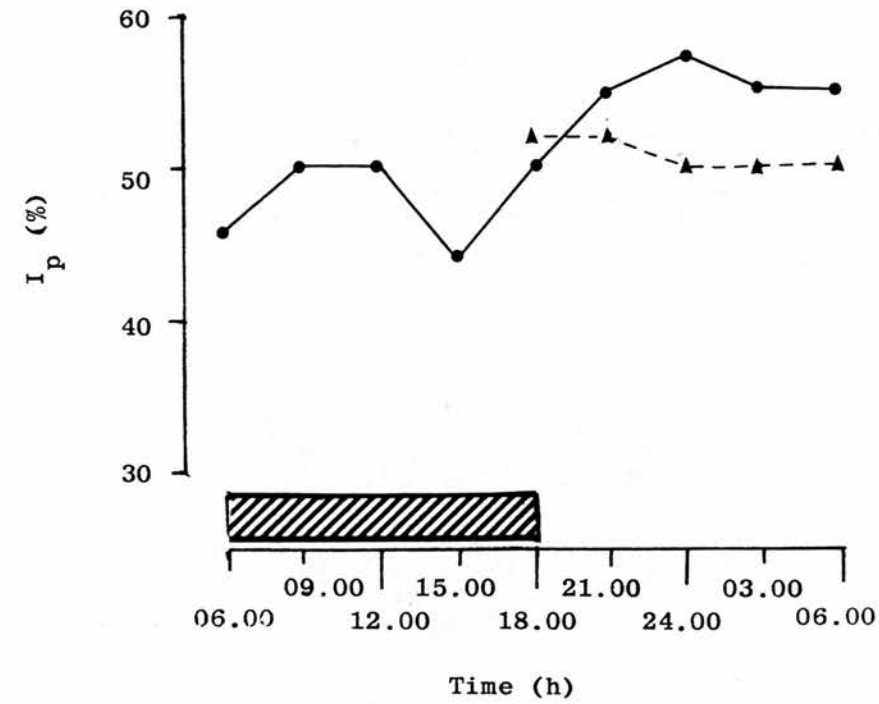
Crypt Cell Population Kinetics in the Jejunum of the Male Syrian Hamster During 24 h With Sleep or Sleep Deprivation

	Time (h)	Crypt Column Length (cells) $\bar{X}$ (S.E.M)	Crypt Column Count (cells) $\bar{X}$ (S.E.M)	Crypt Population (cells) $\bar{X}$ (S.E.M)	I_p (%)	N_p (cells)	CCPR $\bar{X}$ (S.E.M)
Control Hamsters	06.00	40.8 (0.7)	23.4 (0.2)	954.1 (17.9)	46	438.9	21.0 (1.5)
	09.00	40.4 (0.6)	22.3 (0.3)	897.5 (13.3)	50	448.8	21.2 (2.2)
	12.00	39.3 (1.3)	23.1 (0.2)	908.4 (36.1)	50	454.2	22.5 (1.4)
	15.00	36.6 (1.1)	24.1 (0.5)	868.2 (26.1)	44	382.0	19.7 (2.1)
	18.00	38.0 (1.2)	24.7 (0.3)	938.3 (32.8)	50	469.2	39.1 (2.4)
	21.00	41.1 (0.8)	25.7 (0.3)	1047.8 (42.3)	55	576.3	51.8 (1.6)
	24.00	39.8 (0.9)	23.9 (0.2)	952.3 (22.7)	57	542.8	40.9 (4.2)
	03.00	39.5 (0.5)	24.4 (0.2)	964.0 (13.8)	55	530.2	38.5 (4.0)
	06.00	41.2 (1.0)	23.3 (0.2)	960.3 (29.0)	55	528.1	24.6 (2.2)
Sleep Deprived Hamsters	18.00	41.1 (0.9)	23.2 (0.2)	954.5 (24.0)	52	496.3	26.2 (2.1)
	21.00	40.9 (1.0)	24.4 (0.3)	1007.2 (28.7)	52	523.8	33.2 (2.7)
	24.00	42.5 (0.8)	24.2 (0.2)	1043.8 (31.4)	50	521.9	26.3 (1.4)
	03.00	41.5 (1.0)	22.4 (0.2)	926.9 (24.8)	50	463.5	25.0 (1.8)
	06.00	40.7 (0.7)	21.5 (0.3)	876.4 (16.6)	50	438.2	25.6 (2.4)

FIG 9.4

Changes in Crypt Growth Fraction (I_p) With Time of Day in Control (●——●) and Sleep Deprived (▲- - -▲) Hamsters

I_p in 3 hamsters is shown at each point



= Dark period

was greatest at 24.00 h (57%) and least at 15.00 h (44%).

Effect of Sleep Deprivation

During sleep deprivation, the proliferative compartment of the crypt was reduced (in comparison with control) and remained at around 50%.

The Crypt Morphology

The crypt morphology parameters are given in Table 9.2.

a) Column Length

Control

Significant variation with time of day was evident ($F=2.65$, $df=8,99$ $p<0.025$ with ANOVA), with a broad peak 21.00 h - 09.00 h and a trough at 15.00 h). The maximum value (41.1 cells $\pm$ 0.8 at 21.00 h) was significantly different from the minimum value (36.6 cells $\pm$ 1.1 at 15.00 h), ($t = 3.31$, $df = 22$, $p<0.01$).

Effect of Sleep Deprivation

Sleep deprivation significantly altered the variation in column length during the light period ($F=6.23$, $df=1,110$ $p<0.025$ with ANOVA) and values were significantly above control at 24.00 h ($t = 2.17$, $df = 22$, $p<0.05$).

b) Column Count

Control

Values showed a significant fit to a sine wave over 24 h ($F=11.33$, $df=2,6$, $p<0.01$ with GLIM) and the circadian rhythm was 10%. The maximum value (25.7 cells $\pm$ 0.3 at 21.00 h) was significantly different from the minimum value (22.3 cells $\pm$ 0.3 at 09.00 h), ($t = 3.64$, $df = 22$, $p<0.002$).

Effect of Sleep Deprivation

With sleep deprivation there was no significant fit of values to a sine wave over 24 h and the variation in values over the light period was altered significantly in comparison with control hamsters ($F=56.86$, $df=1,110$, $P<0.001$ with ANOVA). Values in sleep deprived animals were significantly below control at 18.00 h ($t = 2.66$, $df = 22$, $p<0.02$), 03.00 h ($t = 4.22$, $df = 22$, $p<0.001$) and 06.00 h ($t = 3.02$, $df = 22$, $p<0.01$).

c) Total Crypt Population

The variation in crypt population with time of day is shown in FIG 9.5.

Control

Values showed a significant fit to a rectangular wave ($F=8.98$, $df=1,7$, $p<0.025$) and the variation with time of day was significant ($F=4.18$, $df=8,99$, $p<0.001$ with ANOVA). The maximum value (1047.8 cells $\pm$ 42.3 at 21.00 h) was significantly different from the minimum value (868.2 cells $\pm$ 26.1 at 15.00 h), ($t = 5.23$, $df = 22$, $p<0.001$).

Effect of Sleep Deprivation

The rectangular wave fit was no longer found but ANOVA revealed that the variation in values during the light period was not altered significantly by sleep deprivation.

Number of Proliferating Cells (N_p)

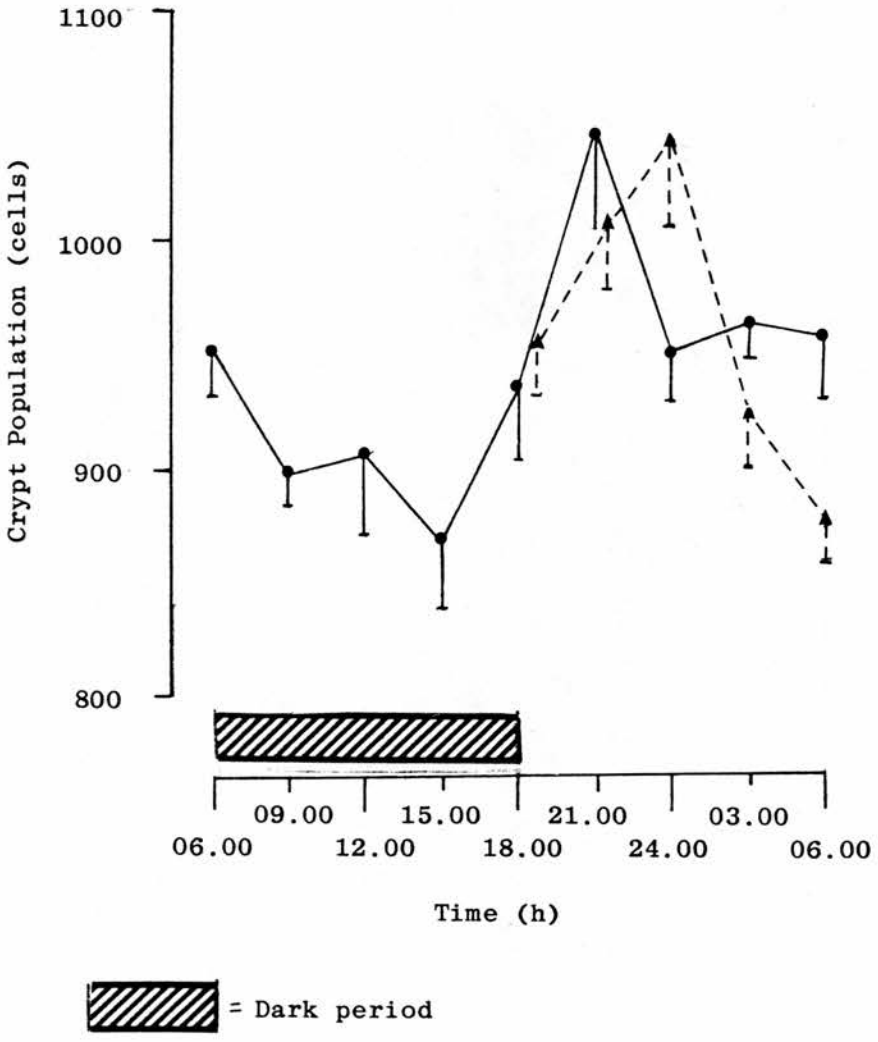
The mean number of proliferating cells per crypt is given in table 9.2.

In control animals a significant fit to a rectangular wave over 24 h was evident ($F=7.51$, $df=1,7$, $p<0.05$). The maximum value occured at 21.00 h (576.3 cells) and the minimum value (382.0 cells) at 15.00 h.

FIG 9.5

Changes in Crypt Population With Time of Day in Control (●——●) and Sleep Deprived (▲- - -▲) Hamsters

Means of 12 hamsters (±S.E.M) shown at each point



With sleep deprivation the rectangular wave fit was no longer found and the number of proliferating cells was reduced in comparison to control. N_p used in conjunction with I_m and t_m (i.e. as in the calculation of CCPR) gives an indication of the proliferative status of crypts.

The Crypt Cell Production Rate (CCPR)

Mean values (± SEM) are given in table 9.2, and are presented graphically in FIg 9.6.

Control

A significant fit to a sine wave over 24 h was evident ($F=17.70$, $df=2,6$, $p<0.01$, with GLIM) and the circadian variation was 94%. The maximum value (51.8 cells ± 1.6 at 21.00 h) was significantly different from the minimum value (19.7 cells ± 2.1 at 15.00 h), ($t = 8.37$, $df = 22$, $p<0.001$).

Effect of Sleep Deprivation

The significant fit to a sine wave was no longer found, and sleep deprivation significantly altered the variation in values during the light period ($F=49.68$, $df=1,110$ $p<0.001$ with ANOVA). Values in sleep deprived animals were significantly below control at 18.00 h ($t = 2.73$, $df = 22$, $p<0.02$), 21.00 h ($t = 4.01$, $df = 22$, $p<0.001$), 24.00 h ($t = 2.24$, $df = 22$, $p<0.05$) and 03.00 h ($t = 2.07$, $df = 22$, $p<0.05$).

Crypt: Villus Ratio

In both control and sleep deprived hamsters approximately 3 crypts supplied each villus (mean values ± SEM are given in Table 9.3).

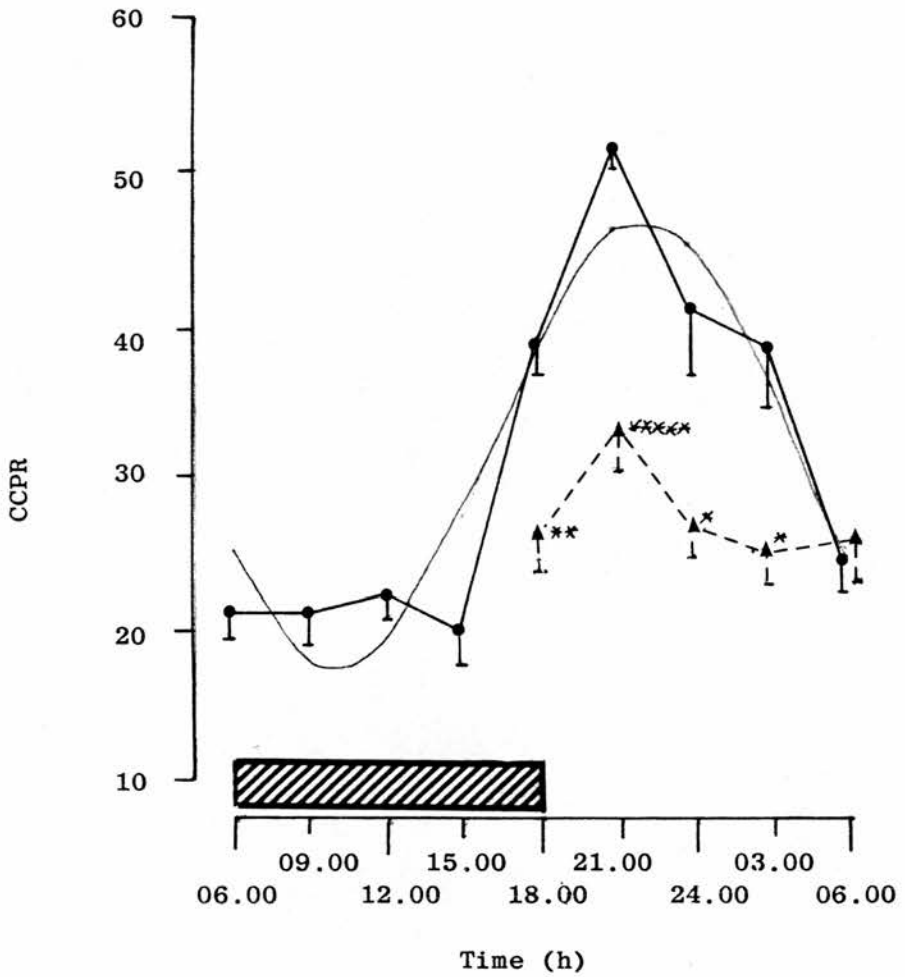
Crypt Cell Mass per Villus

Mean values (± SEM) are given in Table 9.3.

FIG 9.6

Changes in Crypt Cell Production Rate (CCPR) With Time of Day in Control (●——●) and Sleep Deprived (▲- - -▲) Hamsters

Means of 12 hamsters (±S.E.M) shown at each point



Sine wave fit: (∿) control hamsters

▨ = Dark period

Values in control and sleep deprived hamsters significantly different with t-test: * $p < 0.05$, ** $p < 0.02$, ***** $p < 0.001$

Table 9.3

Crypt Cell Population Kinetics in the Jejunum of the Male Syrian Hamster During 24 h With Sleep or Sleep Deprivation

	Time (h)	Projected Villus Area (cm^2) $\bar{X}$ (S.E.M)	Crypt:Villus Ratio $\bar{X}$ (S.E.M)	Crypt Cell Mass per Villus $\bar{X}$ (S.E.M)	Net Villus Influx (cells)
Control Hamsters	06.00	42.1 (2.4)	2.76 (0.15)	2622.5 (138.2)	
	09.00	53.5 (2.8)	3.06 (0.18)	2753.9 (182.3)	116.3
	12.00	39.9 (1.5)	2.99 (0.14)	2618.6 (95.7)	55.1
	15.00	43.9 (1.8)	2.83 (0.14)	2353.5 (63.7)	100.4
	18.00	38.3 (1.2)	2.83 (0.08)	2653.7 (111.9)	17.1
	21.00	40.9 (1.9)	2.85 (0.14)	2987.1 (161.9)	25.4
	24.00	59.0 (2.3)	2.89 (0.09)	2757.5 (128.2)	224.4
	03.00	48.5 (2.4)	2.74 (0.10)	2641.2 (96.7)	100.8
	06.00	50.1 (0.9)	2.70 (0.09)	2587.8 (104.3)	89.2
Sleep Deprived Hamsters	18.00	46.3 (1.2)	2.61 (0.12)	2488.9 (129.9)	
	21.00	40.3 (2.4)	2.86 (0.18)	2905.1 (240.8)	33.2
	24.00	48.2 (2.0)	2.50 (0.10)	2631.5 (164.7)	47.0
	03.00	50.1 (2.3)	2.58 (0.09)	2380.2 (83.6)	164.1
	06.00	39.4 (1.3)	2.58 (0.07)	2251.9 (61.6)	108.7

Control

No significant fit was found to a sine wave or rectangular wave and ANOVA showed no significant variation in values with time of day.

Effect of Sleep Deprivation

Sleep deprivation significantly altered values during the light period ($F = 5.00$, $df = 1,110$, $p<0.05$ with ANOVA) and at the end of the sleep deprivation period (at 06.00 h) the value in sleep deprivation hamsters was significantly below control ($t = 2.77$, $df = 22$, $p<0.02$).

Villus Compartment Size

Projected villus areas (mean $\pm$ SEM) are given in Table 9.3. The variation in projected villus area with time of day is shown in FIG 9.7.

Control

Significant variation with time of day was evident ($F=12.16$, $df=8,99$, $p<0.001$ with ANOVA). The maximum value (59.0 cm^2 $\pm$ 2.3 at 24.00 h) was significantly different from the minimum value (38.3 cm^2 $\pm$ 1.2 at 18.00 h), ($t = 5.40$, $df = 22$, $p<0.001$).

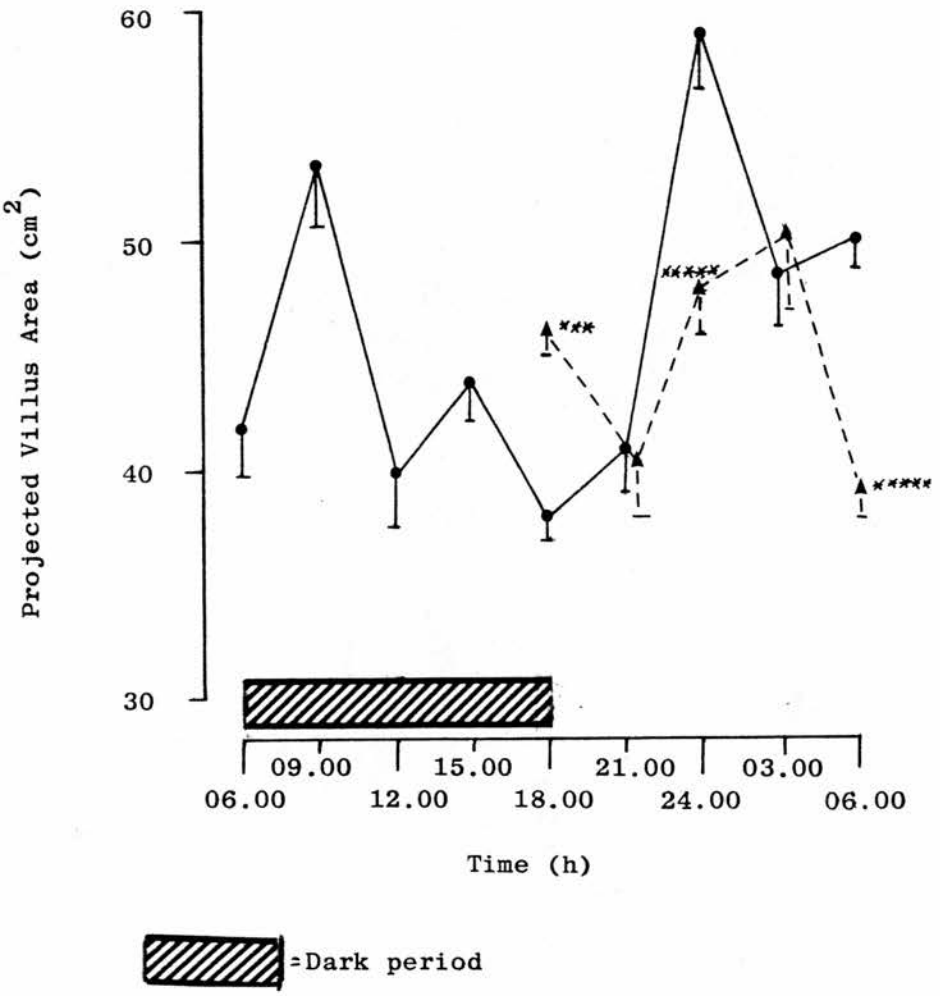
Effect of Sleep Deprivation

Sleep deprivation significantly altered the variation in values over the light period ($F=4.68$, $df=1,110$, $p<0.001$ with ANOVA). At 18.00 h villus area in sleep deprived hamsters was significantly above control ($t = 3.21$, $df = 22$, $p<0.01$) but was significantly below control at 24.00 h ($t = 6.20$, $df = 22$, $p<0.001$) and 06.00 h ($t = 4.51$, $df = 22$, $p<0.001$)

FIG 9.7

Changes in Projected Villus Area With Time of Day in Control (●——●) and Sleep Deprived (▲- - -▲) Hamsters

Means of 12 hamsters (±S.E.M) shown at each point



Values in control and sleep deprived hamsters significantly different with t-test: *** $p < 0.01$, ***** $p < 0.001$

Net Villus Influx

The influx of cells from the crypts onto the villi (Table 9.3) showed good correlation with villus area ($r=0.64$, $df=10$, $p<0.05$).

Control

The maximum flux onto the villus (224.4 cells/h) occured between 21.00 h and 24.00 h at the time when villus area was increased.

Effect of Sleep Deprivation

In sleep deprived hamsters, the flux of cells onto the villus was reduced in comparison to control, for example, between 21.00 h and 24.00 net villus influx was 47.0 cells/h in sleep deprived hamsters and 224.4 cells/h in control hamsters. This was consistent with reduced villus area evident during sleep deprivation.

The time of day at which the maximum and mimumum value in variables was noted (in control hamsters) are summarized in Table 9.4.

A summary of results indicating significant 24 h variation in variables (control hamsters) and the effect of sleep deprivation on control values over the light period is given in Table 9.5.

Table 9.4

Time of Day of Maximum and Minimum Values in Crypt Cell Population Kinetics in the Jejunum of the Male Syrian Hamster Under Control Conditions

Parameter	Time of Maximum (h)	Time of Minimum (h)	Significance *
I_s	24.00	15.00	not done
I_m	21.00	09.00	$p < 0.001$
Percentage labelled Mitoses	18.00	09.00	not done
I_p	24.00	15.00	not done
Crypt Column Length	21.00	15.00	not done
Crypt Column Count	21.00	09.00	$p < 0.002$
Crypt Population	21.00	15.00	$p < 0.001$
N_p	21.00	15.00	not done
CCPR	21.00	15.00	$p < 0.001$
Villus Area	24.00	18.00	$p < 0.001$
Crypt Cell Mass Per Villus	21.00	15.00	$p < 0.002$
Net Villus Influx	21.-24.00	15.-18.00	not done

* :Significant difference between maximum and minimum values using t-test

Table 9.5

Summary of Analysis of the Variation With Time of Day and Effect of Sleep Deprivation on Crypt Cell Population Kinetics in the Jejunum of the Male Syrian Hamster

(ANOVA: Analysis of variance. RW: Rectangular wave. SW: Sine wave.)

Parameter	Variation With Time of Day	Effect of Sleep Deprivation
Labelling Index (I_s)	ANOVA: $F=3.09$, df 8,18, $p<0.025$ RW: $F=7.48$, df 1,7, $p<0.05$	ANOVA: $F=40.86$, df 1,20, $p<0.001$ RW: destroyed
Mitotic Index (I_m)	SW: $F=16.80$, df 2,6, $p<0.01$ Circadian variation 74%	ANOVA: $F=39.38$, df 1,110, $p<0.001$ SW: Circadian variation 25%
Percentage of labelled Mitoses	Not Significant	Not Significant

Table 9.5 (Continued)

Parameter	Variation With Time of Day	Effect of Sleep Deprivation
Crypt Column Length	ANOVA: $F=2.65$, df 8,99, $p<0.025$	ANOVA: $F=6.23$, df 1,110, $p<0.025$
Crypt Column Count	SW: $F=11.33$, df 2,6, $p<0.01$	ANOVA: $F=56.86$, df 1,110, $p<0.001$ SW: destroyed
Crypt Population	ANOVA: $F=4.18$, df 8,99, $p<0.001$ RW: $F=8.98$, df 1,7, $p<0.025$	Not Significant
Number of Proliferating Cells (N_p)	RW: $F=7.51$, df 1,7, $p<0.05$	RW: destroyed
CCPR	SW: $F=17.70$, df 2,6, $p<0.01$ Circadian variation 94%	ANOVA: $F=49.68$, df 1,110, $p<0.001$ SW: destroyed
Villus Area	ANOVA: $F=12.16$, df,8,99, $p<0.001$	ANOVA: $F=4.68$, df 1,110, $p<0.001$
Crypt Cell Mass Per Villus	Not Significant	ANOVA: $F=5.00$, df 1,110, $p<0.05$

Chapter 10

DISCUSSION

Control Hamsters

This investigation has provided the first evidence of a significant variation with time of day in the proliferative indices and crypt and villus compartment size in the jejunum of the male Syrian hamster. The variation, measured over 24 h and showing significant difference between maximum and minimum values in parameters, suggests circadian rhythmicity. This assumption is supported by reports of circadian rhythmicity in the jejunum of the rat (Al-Dewachi et al., 1976) and mouse (Al-Naffussi and Wright, 1982b) and the review of circadian rhythmicity in the alimentary tract of rodents (Scheving et al., 1983b).

The maximum values in proliferative indices, and crypt and villus compartment size occurred during the light period (when hamsters were sleeping) specifically at 21.00 h and 24.00 h. The minimum values in these parameters were predominantly evident during the dark period (when hamsters were active). (See Table 9.4).

The timing of the maximum in proliferative indices during the light period and minimum during the dark period compares well with results reported for the jejunum of the rat (Al-Dewachi et al., 1976) and are consistent with the review by Adam and Oswald (1983) but are the reverse of results reported in the jejunum of the mouse (Sigdestad et al., 1969; Al-Nafussi and Wright, 1982b).

Mathematical Wave Fit to Values

There is currently a great deal of interest in the fit of values, obtained during a 24 h span, to a sine wave approximated to a period of 24 h. This wave provides a convenient calculation of the amplitude, mean value, and thus circadian variation in values (Nelson et al., 1979). Three of the parameters measured in this investigation showed significant fit to such a sine wave, and their respective percentage changes were calculated to be as follows: I_m -

74%, a sine wave also fitted significantly to each crypt third), column count - 10% and CCPR - 94%. Thus by quantifying the extent of the circadian variation it can be readily appreciated that there was a pronounced circadian rhythm in I_m and CCPR. It is interesting that a significant circadian rhythm was exhibited in column count. It is important to realise that this measure was obtained in cross-sectioned tissue giving a simplified picture of what is in effect a matrix of cells consituting the walls of intestinal crypts. Cell size increases exponentially during the first few hours after cell birth (Anderson et al., 1969). Thus the significant circadian rhythm evident in column count may be due to more cells being counted in crypt cross sections when I_m was increased. For example, Møller and Keiding (1982) noted a significant circadian variation in cells/mm in the epithelium of the hamster cheek pouch (the hamster cheek pouch is a very elastic tissue but these authors prevented any change in size of the tissue by inserting a standard plastic ring into the cheek pouch during fixation).

Al-Mukhtar et al., (1982) encouraged investigators to use the CCPR as this gives a more balanced indication of the proliferative status of crypts compared with that obtained from the estimation of the state parameters I_m and I_s alone (Aherne et al., 1977). The limitations of CCPR estimation in the present investigation are discussed later, but it should be noted here that sine wave fit to CCPR indicated an pronounced circadian variation (94%) with values peaking during the light period and as such gives further support for enhanced proliferative rates in the crypt at the time when sleep predominates.

Minors and Waterhouse (1981b) proposed that sleep and wakefulness were so sharply defined that they reflected an alternation between two states, and could thus be described by a rectangular wave form of period 24 h. To fit a rectangular wave, the 24 h must be divided into two additional periods: one where sleep predominates and the other where wakefulness predominates. Bearing in mind the strictly nocturnal activity of hamsters, the division in this investigation was made according to the light/dark cycle. Three parameters showed a significant fit to a rectangular wave: I_s, crypt population and N_p.

Although a rectangular wave does not allow for the same quantitative caclulations as in the case of the sine wave, its significant fit confirmed that the three parameters were consistently low during the dark (activity) period and consistently high during the light (sleep) period.

Possible Mechanisms Involved in Rhythmicity

The increase in the proliferative indices (I_s and I_m) and CCPR at 18.00 h coincided with the time of minimum villus compartment size. This is consistent with a negative feedback mechanism, the depleted villus area stimulating cell proliferation in the crypt. The increase in net villus influx between 21.00 h and 24.00 h and the maximum villus compartment size at 24.00 h suggests that increased proliferative rates led in turn to an increase in the migration of cells out of the crypt and onto the villus. Consistent with an increased net villus influx at the time when proliferative rates increased, is the finding that the number of crypt cells associated with each villus did not vary significantly with time of day. In addition to negative feedback from the villus compartment, it is possible that the signal for an increase in proliferation may have come from, or been maintained by, differentiating crypt cells (Wright and Irwin, 1982b) or the stem cell compartment (Britton et al., 1982).

Brown and Berry (1968) and Izquierdo and Gibbs (1972, 1974) proposed that the circadian rhythm of cell proliferation in the epithelium of the hamster cheek pouch was generated by a wave of cells passing around the cell cycle, through the S and subsequently M phase. Acceptance of this hypothesis is dependent on results which show an increase in I_s followed some hours later by an increase in I_m, for example as reported in the jejunum of the mouse by Al-Nafussi and Wright (1982b). The present investigation does not support this hypothesis for both I_s and I_m increased at 18.00 h, 77% above their respective values at 15.00 h. Similar I_s and I_m synchrony has been reported in the jejunum of the rat (Al-Dewachi et al., 1976) and mouse (Sigdestad and Lesher, 1970).

The sudden synchronous increase in the number of cells in S and M phases suggests the existence of two resting phases in the cell cycle: at G_1 and G_2, each providing a reservoir of cells which can be stimulated to re-enter the cell cycle at S and M phases respectively. This proposal is consistent with the report by Gelfant (1963) of two such populations: G_1 and G_2 resting (or blocked) cells, in the mouse epidermis, and Gelfant's review in 1977 which demonstrated that G_1 - blocked and G_2-blocked cells can be found in a variety of animal tissues.

It would appear that in the hamster jejunum both G_1 and G_2 resting cells receive their stimulus at approximately the same time: between 15.00 h and 18.00 h and thus a little in advance of the dark/light transition. From examination of the control pattern alone it is impossible to distinguish whether these G_1 and G_2 resting cells receive the same stimulus or two separate stimuli to re-enter the cell cycle. Sassier and Bergeron (1980) isolated an extract from the small intestine of the rabbit which inhibited the G_1 to S flux in the small intestine of the mouse. Its tissue specificity and inhibitory action on the cell cycle suggest its properties as a chalone. Other reports have suggested that two chalone-like substances exist which are specific in their action on the cell cycle, one inhibiting cells at G_1 and the other inhibiting cells at G_2, both in the epidermis of the mouse (Elgjo et al., 1971, 1972; Elgjo, 1974) and intestinal epithelium of the newt (Brugal and Pelmont, 1975).

At the time of the increases in I_s, I_m and CCPR (18.00 h), an increase in the growth fraction of the crypt was noted (caused by an upward movement of the "cut-off" position which effectively controls the growth fraction in this situation). Growth fraction increase alone, however, being of the order of 14% was not sufficient to account for the 77% increase in both I_s and I_m and the 32% increase in CCPR. This suggests that when I_s and I_m increased, the established proliferative compartment showed proliferative activity increased in intensity. This is further supported by the increase in CCPR (as this is sensitive to the number of proliferative cells in crypts) and the graphical results of the distribution of labelled and mitotic cells (Appendices 1.4 and 1.5).

Tutton (1973) noted that the rate of entry into mitosis and the cell cycle time in the basal portion of intestinal crypts in the rat, varied with time of day. Also working on the rat, Al-Dewachi et al., (1976), however,reported that in the whole of the jejunal crypt the rate of entry into mitosis and the cell cycle time showed only minor changes over 24 h and was therefore insufficient to account for the circadian rhythm of cell proliferation.

Measurement of cell cycle time and constituent phase durations over the 24 h period in the present investigation would have enabled evaluation of their contribution to the rhythm in cell proliferation. The best way of calculating cell cycle time and constituent phase durations is by using the FLM method (Aherne et al., 1977). Betts et al. (1966) reported that the cell cycle time in the hamster jejunum was approximately 13 h, an FLM curve would therefore have to be constructed over 13 h to obtain a single reading which is not a feasible proposition for use in studies of circadian rhythms owing to the temporal nature of the variables. The stathmokinetic method is shorter and thus more promising for the evaluation of the rate of entry into mitosis, and is one that would have been adopted in the present investigation if time and, more importantly, materials had been available.

CCPR was calculated for the enhanced information it can give on proliferative status of crypts (Al-Mukhtar et al., 1982) being sensitive to I_m, the duration of mitosis (t_m) and the number of cells involved in proliferation (N_p). It was necessary , however, to rely on an approximation of t_m (1 h) taken from the results of an investigation reported by Betts et al., (1966) of the cell cycle time in the jejunum of the hamster. The constancy of t_m over the entire 24 h period was based on an assumption, but one consistent with the reports by Izquierdo and Gibbs (1972, 1974) that in the normal cheek pouch epithelium of hamsters, the duration of S (t_s) as well as the duration of G_2 ($^tG_2$) and M(t_m) were essentially constant over 24 h despite marked circadian fluctuations in cell proliferation.

The percentage of labelled mitoses showed good correlation with I_s

over the 24 h period. It is therefore surprising that percentage of labelled mitoses did not actually vary significantly over 24 h. This inconsistency is difficult to interpret and should be taken as a warning against using only 3 animals to evaluate such a 'low incidence' parameter.

Sleep Deprived Hamsters

During sleep deprivation, I_s, I_m, CCPR and villus compartment size were reduced in comparison with control. The reduction in these parameters was noted in the absence of changes in food intake between control and sleep deprived hamsters which suggests systemic rather than local factors (e.g. food contents in the lumen) influencing proliferative indices and villus morphology. No significant alteration in total crypt cell population was evident over the whole light period, which in view of the decrease in proliferative indices, suggests that during sleep deprivation there was a reduced flux of cells out of the crypt, and this was confirmed by the reduction in net villus influx and villus compartment size. By the end of the sleep deprivation period, however, the number of crypt cells supplying each villus had decreased significantly below control and the value of net villus influx was above control. It would seem from this that there was a mechanism operating to stimulate migration of cells onto the villus (perhaps once villus compartment size had reached a critically low level) although this led to an deficit in the crypt cell mass per villus.

Effect of Sleep Deprivation on Mathematical Wave Fit

The significant sine wave fit described in column count and CCPR in control hamsters was no longer found in sleep deprived hamsters. I_m values during sleep deprivation retained a significant fit to a sine wave (indicating an increase in I_m during the light period) but circadian variation was reduced from 74% to 25%.

The significant rectangular wave fit described by I_s, crypt population and number of proliferating cells (N_p) was also lost during sleep deprivation. These parameters are therefore no longer consistently high during the light period when wakefulness is continued. The result in crypt population highlights the increased information which can be obtained from such mathematical wave fit, for although ANOVA revealed no significant alteration in values during sleep deprivation, the rectangular wave fit was sufficiently sensitive to expose the fact that during sleep deprivation, crypt

cell population did not remain as consistently high as during sleep.

Possible Mechanisms Involved in Sleep Deprivation Effects

The most striking effect of sleep deprivation was an instant reduction in I_m, but not in I_s. In fact the reduction in I_s noted later in the light period (03.00 h) suggests this was a direct consequence of the reduced population of cells passing through M 9 h earlier. This, taken together with the close phasing of I_s values in control and sleep deprived hamsters in the first half of the light period, indicated that sleep deprivation was instrumental in causing specific inhibition of G_2 to M flux alone or an increase in $^tG_2$ and is consistent with the results of Bullough (1948b) that when mice were induced to sleep mitotic rates increased at their time of expected low.

The delicately orchestrated movements of chromosomes during mitosis, whereby genetic material is separated and apportioned to daughter cells in equal amounts in a relatively short space of time (1 h compared to the 6.6 h of S phase, Betts et al., (1966)), suggests that M is a particulary sensitive phase in the cell cycle.

Fisker et al., (1982) proposed that stress induced by cage change caused a reduction in the flux of cells (in rat palatal epithelium) from G_2 to M 30 minutes later, and that this apparent blockade was lifted after a further 30 minutes (i.e. 1 h after induced stress). In the present investigation, 3 hamsters in each group of 12 were injected with [^{3}H]-TdR 1 h prior to killing. They were placed in a different cage according to stipulations of the Animal House designed to limit possible large scale contamination of cages with isotope. This procedure, whereby hamsters receiving injection of isotope were not reintroduced to their original cages, conferred the added advantage of ensuring minimal disturbance to hamsters not receiving isotope. This was particularly advantageous during the light period when control hamsters were sleeping. It is impossible to quantify the degree of stress to which isotope-labelled hamsters were subjected through receipt of intraperitoneal injection followed by cage change. The possibility that injection and cage change caused a

mitotic blockade, followed by an increased flux, is an interesting speculation, but would needed to have taken place within one hour. Measurement of mitotic stages (such as that carried out by Fisker et al., (1982)) could furnish more information on this phenomenon. It should be added that as both control and sleep deprived hamsters were subjected to the injection and cage change procedure, it is not likely to have invalidated comparison of the two groups.

One pressing question involves the stress conferred by the sleep deprivation manoeuvres. During the 5 weeks of standardization to the Animal House and the reversed light/dark cycles, hamsters were familiarized with myself and the technicians who would subsequently help me to carry out the experiment, with the intention of reducing their stress on human encounter as much as possible. Sleep deprivation of hamsters relied on their gentle frequent handling, without this type of contact wakefulness would not be ensured. Bullough and Laurence (1961) for example, found that putting mice in a rotating box did not completely prevent the mice from sleeping. Hamsters were extremely placid the during the whole of the sleep deprivation period and thus did not appear to be overtly stressed. Glucocorticoid assay can be used as one measure of stress levels; this was not carried out in the present investigation, but results in the human have suggested that stress hormones are reduced rather than elevated during sleep deprivation (Akerstedt et al., 1980).

The stress hormones all act as mitotic inhibitors (Ghadially and Green, 1957; Halberg et al., 1959b; Bullough and Laurence, 1961, 1964a,b, 1968). Although sleep deprivation appeared to exert its effect via a mitotic inhibiting mechanism, I_m was measured as higher during sleep deprivation than in the natural wakefulness predominating in the dark period. This finding is thus incompatible with elevated stress hormones during continued wakefulness and instead presents two possibilities:

(1) That stress hormones were reduced at the expected time of sleep even when wakefulness was continued. This possibility is consistent with reports of adrenalin and cortisol secretion in man. Fröberg et al. (1975) reported that the 24 h rhythm

in adrenalin secretion (maximum in the day-time and minimum at night) is maintained during 72 h of continuous sleep deprivation and Weitzman et al. (1983) noted that cortisol levels were dependent on sleep to reach their lowest levels, but were reduced even when wakefulness was continued. Furthermore, Schell et al. (1980, 1981) reported that an inverse relationship was evident between cortisol levels and I_s in human epidermis, and sleep deprivation did not confer an immediate inhibitory effect on I_s in the hamster jejunum.

(2) That stress hormones do not impose absolute control over cell proliferation. This would be consistent with the report by Alov (1963) that a circadian rhythm cell proliferation in various tissues in the mouse was still evident after adrenal gland ablation.

Does Sleep Enhance Cell Proliferation?

It is apparent that although there is an increase in I_m during the light period in both sleeping and sleep deprived hamsters, an enhanced flux of cells from G_2 to M is dependent on the intervention of sleep.

This is consistent with the hypothesis forwarded by Adam (1980) that mitotic activity is dependant on a high Energy Charge (EC), a measure of the relative concentration of ATP levels, in cells. As discussed in Chapter 3, Adam proposed that waking activity results in a low EC in cells (as reported to be the case in rat skeletal muscle by Wojciechowska et al., 1975), and that this accelerates degradative processes and decreases synthesis. During sleep, there is a reduction in muscle activity, body heat production and the activity of the Na^+/K^+ ion pump. Concomitant with these lessened catabolic demands the is a rise in EC which, augmented by sleep dependent hormone secretion (GH and prolactin) and the complementary timing of testosterone secretion, enhances anabolic processes such as cell proliferation. Durie et al., (1978) reported that EC in many tissues rose with sleep and it has been reported that I_m is dependant on high concentrations of ATP (Guttes and Guttes, 1959; Epel, 1963).

Furthermore, Leblond and Carriere (1955) reported that GH increased the mitotic rates in the rat intestine, and Chaudhry et al., (1958) reported an inverse relationship between mitotic count and deep body temperature (high during activity) in the hamster. It is evident that if there is 'whole body' control over cell proliferation, conferred by the differing energy charge of cells during sleep and wakefulness as suggested by Adam (1980) then synchrony of cell proliferation rhythms would be noted in different tissues in one animal. Scheving et al. (1983b) drew attention to the close synchrony of cell proliferation circadian rhythms in a variety of mouse tissues, but the reports they reviewed made no mention of the sleep and wakefulness patterns in experimental animals.

Results in the present investigation support the sleep dependence of G_2 release and M influx (these G_2 resting cells are possibly those described by Adam and Oswald (1983) as 'primed for mitosis') but not of G_1 release and S influx. It is evident that Adam's hypothesis is dependent on the mitotic event being a measure of increased cell proliferation, but in reality cell proliferation is dependant on flux through all phases of the cell cycle. It is interesting that if the anti-mitotic effect of sleep deprivation in the present investigation was due to the continued activity of hamsters, this would confirm the reports by Bullough (1948a,b) and Alov (1963) of the inverse relationship between exercise and mitosis in mouse tissues.

G_1 and G_2 release would seem to be dependant on a reduction in production and/or effectiveness of G_1 and G_2 chalone. This investigation suggests that G_1 and G_2 chalone are so essentially distinct that their production and/or effectiveness is controlled by different mechanisms. It would appear that:

The G_1 chalone is coupled to the light/dark cycle. This coupling mechanism was evident in the apparent anticipatory reaction whereby G_1 chalone depletion occured a little in advance of the dark/light transition.

The G_2 chalone is strongly coupled to sleep and wakefulness. The strength of the coupling tentatively suggests control for two reasons. Firstly, control hamsters began to sleep a little in advance of the dark/light transition and this was consistent with the

increase in I_m noted at this time. Secondly, when wakefulness was continued at a time when sleep was anticipated, (sleep-deprivation), I_m did not increase to the same extent as in sleeping (control) hamsters. It must be remembered, however, that sleep did not impose absolute control for I_m did rise above the value in natural wakefulness during sleep deprivation.

This model of cell proliferation could be criticized on the grounds that the cell cycle is an intergrated phenomenon and as such it is not compatible with the proposal of 'two compartment' control over the cell cycle, in other words that control is conferred at different stages and coupled to different mechanisms. It is the review of two resting phases in the cell cycle at G_1 and G_2 in a variety of animal tissues (Gelfant, 1977) and the radically different cellular activity in S (Meuller, 1971) and M phases (Tobey et al., 1971) that suggest this two-compartment control over cell cycle maybe a viable proposition. Furthermore, it should be remembered that many physiological variables in the body are so well orchestrated that their synchrony and coupling (Czeisler et al., 1980), evident in their circadian rhythmicity, under normal conditions, enhances overall physiological well-being.

The elegantly controlled investigation carried out by Rechtschaffen et al., (1983) reported the deleterious effect of prolonged sleep deprivation in the rat. The reported pathological changes in tissues and ultimately death of rats may have been a manifestation of the disruption of the circadian system consequent on the absence of sleep. Indeed, the investigations of Minors and Waterhouse (1980) suggested that the stability of circadian rhythms in man were dependent on a regular period of 'anchor sleep'.

The present investigation has added to the information of the deleterious effect of sleep deprivation specifically on the cell cycle in the hamster jejunum. It has shown that the fully enhanced flux of cells into mitosis is dependent on sleep, and has gone some way to promote the belief in the necessity of sleep for adequate cell proliferation and thus tissue restoration. It is equally evident, however, that well integrated progression of cells around the cell

cycle is dependent not only on sleep , but in addition may be dependant on the light/dark cycle which is possibly coupled to the release of cells resting in G_1.

Further investigations are needed before there can be full acceptance of sleep as a time of body restoration, especially in view of the anomalies in the literature concerning peak timing of cell proliferation parameters. For example, in the jejunum of the rat peak proliferative rates have been found to occur in the light period (Scheving et al., 1972; Al-Dewachi et al., 1976) but in the jejunum of the mouse peak proliferative rates have been found to occur in the dark period (Sigdestad et al., 1969; Al-Nafussi and Wright, 1982b). Presented like this, it would appear that a likely explanation for these anomalies is the different species of experimental animal. There is, however, a further important factor which should be considered: the sleep and wakefulness patterns of experimental animals in the different investigations. Unfortunately, although all these authors mention the light/dark period to which animals were entrained they all omit to mention of the predominant timings at which animals were sleeping or waking. It cannot be merely assumed that, being nocturnal animals, both rats and mice were predominantly asleep during the light period as animal house routine can easily disturb the sleeping pattern of experimental animals.

It is to be hoped that investigators assessing circadian rhythms in cell proliferation will now take note of, and indeed make mention of, predominant timing of sleep and wakefulness in experimental animals and not just the light/dark cycle to which animals are entrained.

Possible Strategies for Future Investigations

Cell proliferation assessment in a controlled investigation such as that carried out by Rechtschaffen et al., (1983), where both control and experimental animals receive the same stimuli, but in which stimulus presentations are linked to sleep onset in only the experimental animals.

This type of investigation could be carried out over several days to

establish whether any rebound effect in cell proliferation makes an appearance during a period of prolonged sleep deprivation.

In addition, allowance should be made for calculation of cell cycle time and phase duration in control and sleep deprived hamsters, and in an ideal investigation analysis of circulating levels of both catabolic and anabolic hormones should be made.

CHAPTER 11

THE EFFECT OF SLEEP AND WAKEFULNESS ON THE 24 H VARIATION OF INTRAEPITHELIAL LYMPHOCYTE COUNT IN THE JEJUNUM OF THE MALE SYRIAN HAMSTER (Mesocricetus auratus)

Introduction

Non-epithelial cells constitute approximately 20% of the total cell population in the epithelium of the small intestine (Toner and Ferguson, 1971). They were first described by Weber (1847) and are now recognised as sub-populations of leucocytes: lymphocytes, eosinophils and mast cells (Toner and Ferguson, 1971).

Many tissues contain lymphocytes (Schaffer, 1936). It is their presence in the gut which will be discussed here. The name: intraepithelial lymphocyte is abbreviated to IE lymphocyte (after Ferguson, 1977).

The majority of IE lymphocytes are situated in the basal section of the intestinal epithelium at, or below, the level of enterocyte nuclei (Kelsall, 1946; Darlington and Rogers, 1966; Meader and Landers, 1967). Studies using the electron microscope have shown unequivocably that IE lymphocytes lie between, not inside enterocytes (Toner and Ferguson, 1971) and are thus described as inter-enterocytic (Ferguson, 1977).

IE lymphocytes leave the blood stream via the capillaries in the lamina propria and enter the intestinal epithelium through herniations in the basal lamina (Toner and Ferguson, 1971). Their absence near the brush border of intestinal epithelium indicates that they are not lost into the intestinal lumen. Furthermore, unlike epithelial cells, they do not migrate along the villi but circulate rapidly through the epithelium, returning to the lamina propria (Marsh, 1975b) through basal lamina herniations (Meader and Landers, 1967). The resemblance between IE lymphocytes and intralymphatic cells, suggests that IE lymphocytes, on leaving the intestinal epithelium, directly enter adjacent lymphatics to reach the thoracic duct lymph (Marsh, 1975b).

The lymphocyte population in man and animals is largely composed of T (thymus dependant) and B (bursa dependant or bone marrow derived) lymphocytes (also called T and B cells respectively). In addition small populations of K cells (lineage uncertain) and null cells which lack T or B cell markers (perhaps a stage in lymphocyte differentiation or an entirely distinct cell type) are found (Roitt, 1980).

T and B lymphocytes are heterogeneous populations containing short and long-lived cells which may be small, medium or large in size.

A variety of sizes and forms of IE lymphocytes have been identified (Collan, 1972; Marsh 1975a; Röpke and Everett, 1976). The majority have been identified as T cells. The first evidence of this came from reports of low IE lymphocyte counts in thymus deprived mice (Fichtelius et al., 1968; Ferguson and Parrott, 1972). Confirmation came in later reports of studies utlilizing the advanced techniques now available for definition of lymphocyte sub-populations. Studies using surface cell markers have reported that the majority of IE lymphocytes are T cells in man (Meuwissen et al., 1976) and guinea-pigs (Arnaud-Battandier et al., 1978). Using immunoflourescent techniques together with specific anti-sera to T lymphocytes, Selby (1981) reported that over 95% of IE lymphocytes in the stomach and proximal small intestine of man were T lymphocytes.

IE lymphocytes are, however, not exclusively T cells. In congenitally athymic nude mice, small numbers of IE lymphocytes persist (Rőpke and Everett, 1976; Parrott and de Sousa, 1974). No evidence of B cells within the IE lymphocyte population has been reported (Selby, 1981) which suggests that the non-T IE lymphocytes may be null cells or a differentiated T cell subset which has lost T cell surface markers.

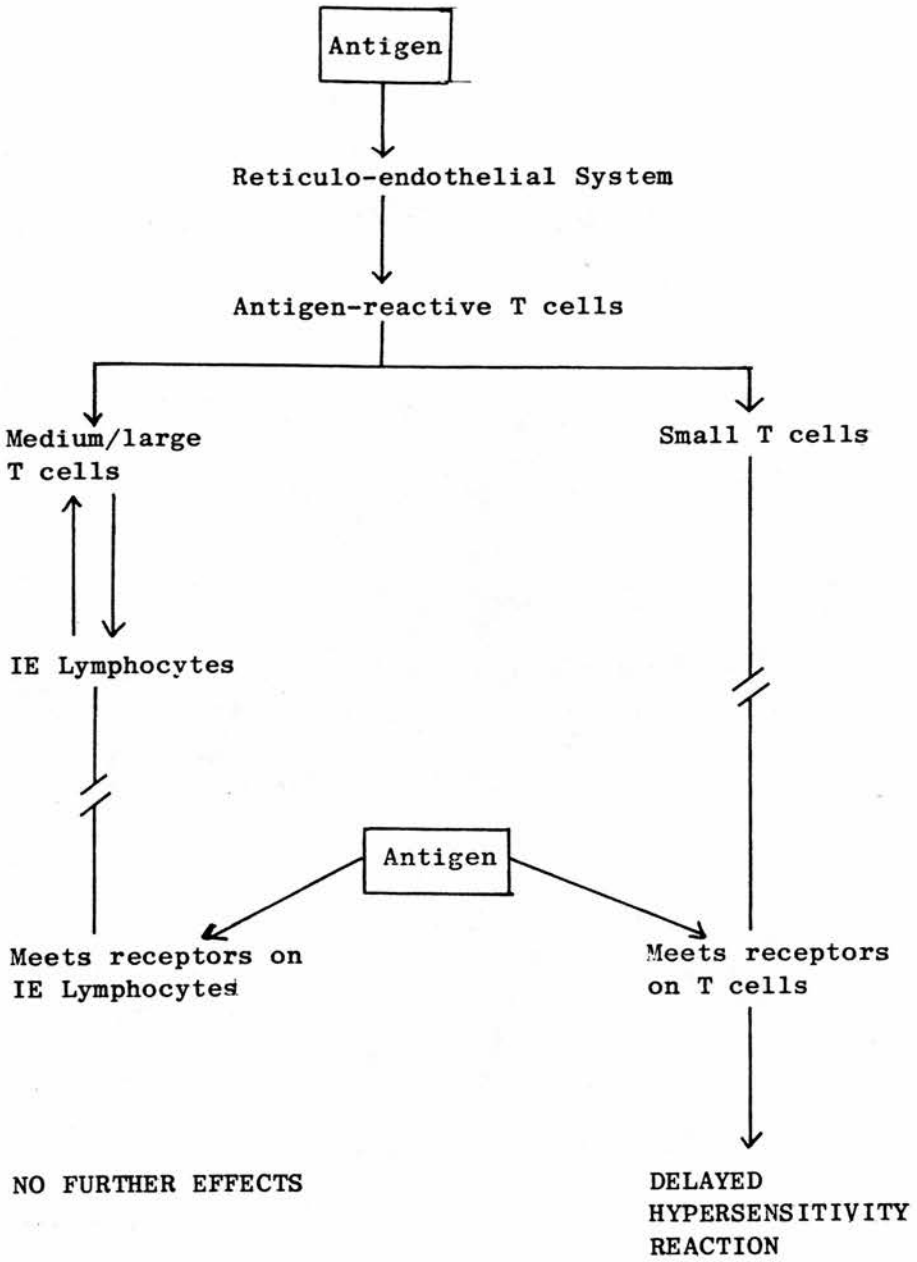
Some uncertainty still surrounds IE lymphocyte function. The belief that they were attracted to the intestinal epithelium by the presence of luminal antigen was refuted by reports of IE lymphocytes in antigen-free isografts (Ferguson and Parrott, 1972) and that antigens in food play little part in influencing IE lymphocyte numbers

(Ferguson, 1976).

It is unlikely that IE lymphocytes alone are markers of cell-mediated immune reactions such as in coeliac disease (Ferguson, 1974) as IE lymphocytes were found to be absent in 20% of rejecting allografts: a particularly severe cell-mediated immune reaction (MacDonald and Ferguson, 1976).

Ferguson (1977) suggested that the T cells of the IE lymphocyte population combine with antigen but have few or no other properties. As such IE lymphocytes could be of great value to the mucosal surfaces (i.e. of the small intestine) by preventing the initiation of, and perhaps blocking, local hypersensitivity reactions (see FIG 11.1). Similarily, Selby (1981) proposed that IE lymphocytes confer immunological tolerance to orally ingested antigens, and modulate the immune response by blocking antigen at the mucosal surfaces.

FIG 11.1



Hypothetical Role of IE Lymphocytes After Antigen Challenge

Reproduced after Ferguson (1977)

Aims of This Investigation

To determine the effect of time of day and sleep and wakefulness on IE lymphocyte numbers in jejunal epithelium of male Syrian hamsters (Mesocricetus auratus).

Reasons for so Doing

1. The proliferation kinetics of IE lymphocytes have been defined (Darlington and Rogers, 1966; Marsh, 1975b; Röpke and Everett, 1976) but there is no report on the kinetic variation with time of day. Sections of tissue obtained for my study on cell population kinetics in the hamster jejunum (Chapter 8) provided the material for a fellow student, Shona Dougherty, to investigate the proliferative indices of IE lymphocytes during 24 h. Shona used tissue obtained from the 3 thymidine-labelled hamsters per time group, and although no significant results were found in the variation of proliferative indices, there was an indication that IE lymphocyte numbers did vary significantly with time of day.

2. There is well documented evidence that the magnitude of the immune response varies with time of day (Pownall and Knapp, 1980). In a study in which I participated, a circadian variation in total lymphocyte count of peripheral blood was reported in man, with a peak at approximately 22.00 h and a trough at approximately 10.00 h (Ritchie et al., 1983); a variation which is inversely related to cortisol levels (Abo et al., 1981; Ritchie et al., 1983). There is an increasing interest in the use of IE lymphocyte counts along with measurement of mucosal architecture to quantify mucosal cell-mediated immune reactions (Mowat and Ferguson, 1982). As such it was prudent that the variation in IE lymphocyte numbers with time of day was fully investigated by carrying out counts in all 12 hamsters per time group (Chapter 8). It was evident that if significant variation in IE lymphocyte numbers was found to occur with time of day, it could greatly affect the viability of counts as a diagnostic procedure and perhaps indicate the most advantageous time at which to minister tailored immunotherapy to manipulate the immunoregulatory cells. In addition, investigation of the relationship between counts and the

sleep/wake cycle may add to the knowledge of the control of movement of lymphocytes to the intraepithelial position.

Methods

Sections of hamster jejunum where the long axis of the crypts and villi were in the plane of section (see Chapter 8) were examined. Areas of tissue were selected where the plane of sectioning was precisely vertical to the epithelium of the villi. Differential counts of nuclei were carried out on the epithelium covering the villi in all hamsters, according to the methods of Ferguson and Murray (1971). All counts were made "blind" by myself in randomly coded slides (Chapter 8).

Using a light microscope, (Leitz Dialux 20 EB, x 2000 magnification) the nuclei of epithelial cells, goblet cells and IE lymphocytes were readily identified (FIG 11.2). The nuclei of IE lymphocytes are basally situated, densely stained and are surrounded by pale cytoplasm. Small nuclear fragments which could not be classified as to cell type were ignored.

In each hamster, the area covered by 500-600 villus epithelial cell nuclei was examined. The resulting counts were expressed as the number of IE lymphocytes per 100 villus epithelial cells (Ferguson and Murray, 1971).

Data Analysis

Results of counts were anaylsed using the BMPD (1982) statistical package.

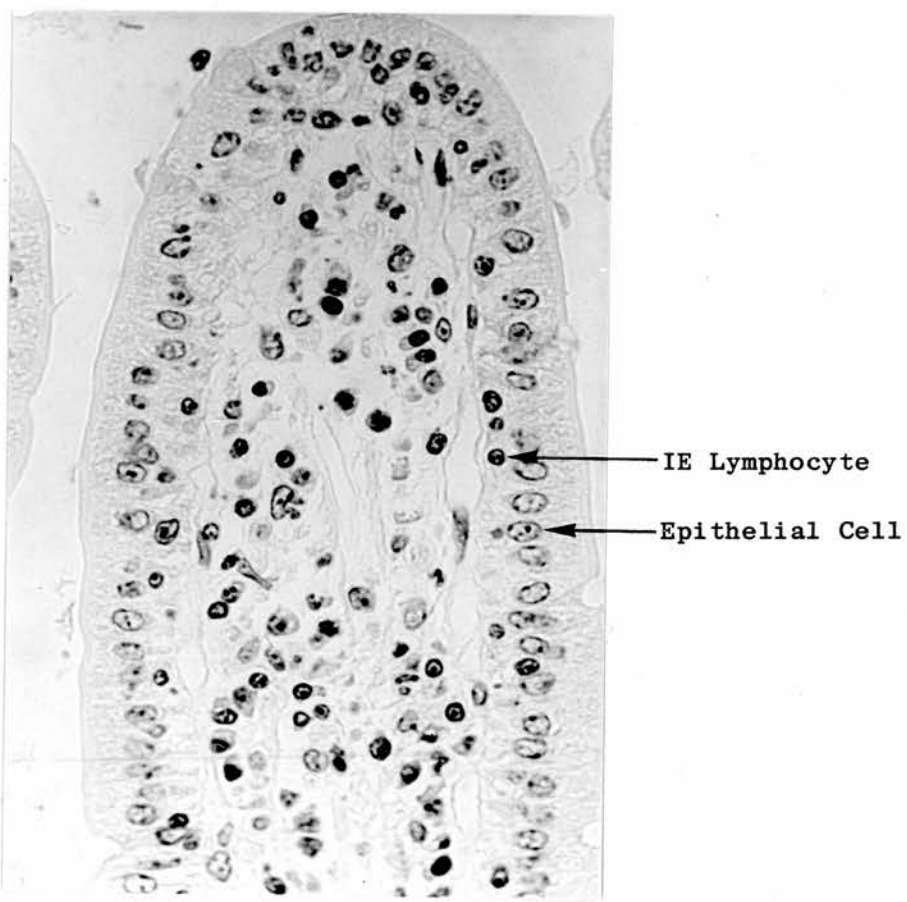
The fit of data to a sine wave and to a rectangular wave, approximated over 24 h, was tested.

ANOVA was used to test for significant variation in counts with time over 24 h, and to assess the effect of sleep deprivation on counts (comparison of light period counts in time matched control and sleep deprived hamsters).

FIG 11.2

Hamster Jejunal Villus Epithelium: the nuclei of IE lymphocytes can be readily distinguished from those of epithelial cells

(Harris's Haematoxylin x 500)



Differences between maximum/minimum counts in control hamsters and control/sleep deprivation counts were analysed using the two-sample t-test.

Results

The IE lymphocyte count during 24 h with sleep and sleep deprivation is given in Appendix 2.1 and means (± SEM) in Table 11.1.

The variation in IE lymphocyte count with time of day in control and sleep deprived hamsters can be seen clearly in FIG 11.3.

Control

Values did not show a significant fit to a sine wave nor a rectangular wave approximated over 24 h, but ANOVA revealed a significant variation in count with time of day ($F = 4.83$, $df = 8,99$, $p<0.001$). Maximum values were evident during the middle of the dark period and minimum values were evident during the middle of the light period. The maximum count ($\bar{X}$ 35.4 ± 1.8 (SE) IE lymphocytes per 100 epithelial cells at 12.00 h) was significantly different from the minimum count ($\bar{X}$ 25.2 ± 1.6 at 24.00 h), ($t = 4.25$, $df = 22$, $p<0.001$).

Effect of Sleep Deprivation

Sleep deprivation significantly altered the variation in IE lymphocyte count over the light period ($F = 26.19$, $df = 1,110$, $p<0.001$). IE lymphocyte count in sleep deprived hamsters was maximum at 24.00 h - the middle of the light period ($\bar{X}$ 38.6 ± 1.5) and was significantly above the count in control hamsters at this time ($\bar{X}$ 25.2 ± 1.6), ($t = 6.05$, $df = 22$, $p<0.001$).

Table 11.1

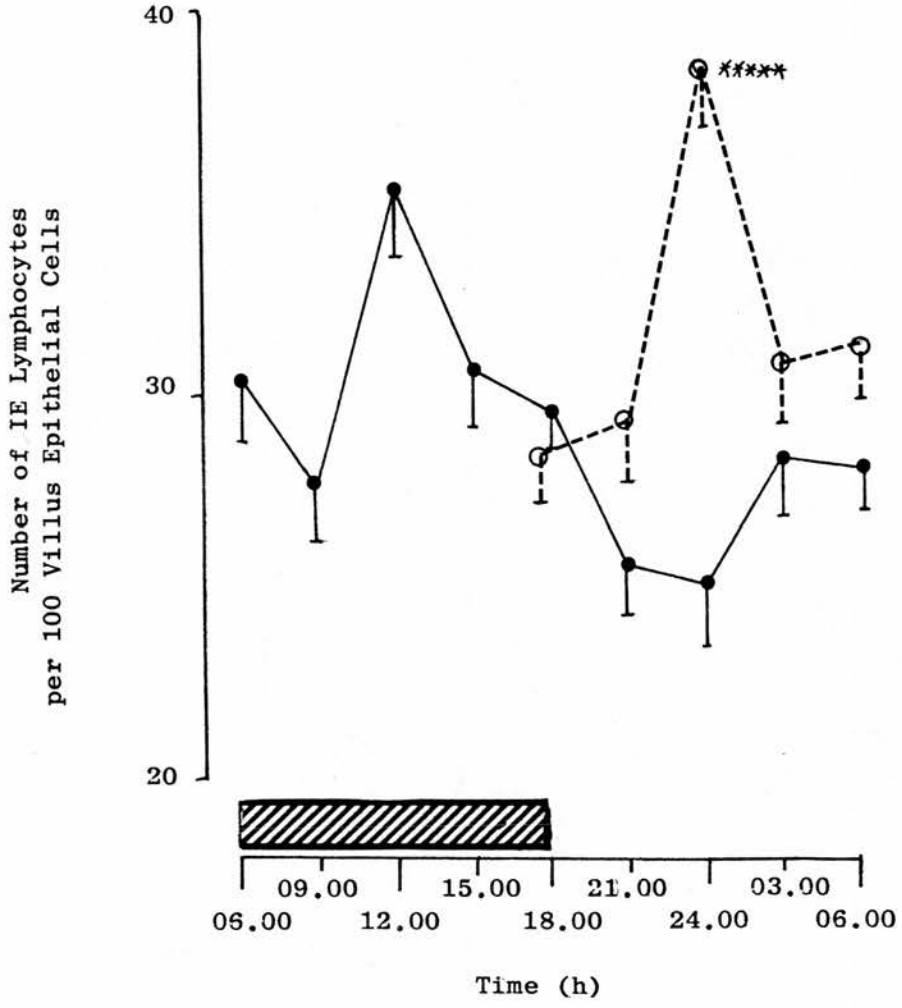
Number of IE Lymphocytes in the Jejunal Epithelium of the Male Syrian Hamster Over a 24 h Period With Sleep or Sleep Deprivation

	Time (h)	Number of IE Lymphocytes Per 100 Villus Epithelial Cells Mean	(S.E.M)
CONTROL	06.00	30.4	(1.6)
	09.00	27.6	(1.4)
	12.00	35.4	(1.8)
	15.00	30.6	(1.4)
	18.00	29.6	(0.9)
	21.00	25.6	(1.3)
	24.00	25.2	(1.6)
	03.00	28.3	(1.4)
	06.00	28.0	(0.9)
SLEEP DEPRIVED	18.00	28.6	(1.4)
	21.00	29.5	(1.7)
	24.00	38.6	(1.5)
	03.00	30.9	(1.5)
	06.00	31.4	(1.4)

FIG 11.3

Changes in IE Lymphocyte Count With Time of Day in the Jejunal Epithelium of Control (●—●) and Sleep Deprived (○---○) Hamsters

Means of 12 hamsters (±S.E.M) are shown at each point



Dark period

Significant difference between control and sleep deprived hamsters using t-tests: ***** $p < 0.001$

Discussion

This investigation has shown that under normal conditions of sleep and wakefulness a significant 24 h variation is evident in the IE lymphocyte count in the jejunum of the male Syrian hamster. The 24 h variation showed an established time of maximum and of minimum, suggesting circadian rhythmicity although as this is the first report of its type, further investigations are needed before this is confirmed.

In control hamsters, maximum counts were evident in the middle of the dark period (hamsters' activity phase) and minimum counts in the middle of the light period (sleep phase). Counts did not drop during the light period when wakefulness was continued (sleep deprivation) which suggests that the rhythm in IE lymphocyte count is dependant upon or closely coupled to the rhythm of sleep and wakefulness rather than the light/dark cycle.

In man (Hoskins et al., 1967) and mice (MacDonald and Ferguson, 1978) raised counts of IE lymphocytes have been reported in association with Giardia infection. As all hamsters used in the investigation were chronically infected with Giardia muris, resulting counts would be expected to be higher than those in an uninfected hamster population. MacDonald and Ferguson (1978) reported an approximate increase of 56% in IE lymphocytes in Giardia infected mice, and associated this with the protozoan infection. It is evident from this investigation that adequate information is needed on IE lymphocyte count in infected and uninfected animals at various time points over 24 h, together with strict control of sleep and wakefulness to fully evaluate the use of IE lymphocytes as a diagnostic tool of this protozoan infection.

It is interesting that distinctly different patterns in IE lymphocyte count over the light period were noted in control and sleep deprived hamsters in the absence of any difference in food intake. This is consistent with the finding that IE lymphocyte count is not directly associated with the prescence of luminal antigen (Ferguson and

Parrott, 1972; Ferguson, 1976)and suggests a more discrete control mechanism whereby the stimulus for an increased movement of IE lymphocytes into their intraepithelial position is associated with sleep and wakefulness, which couples the maximum count with the time of maximum probability of encountering luminal antigen. This would support the association of IE lymphocytes to food tolerance mechanisms by their blocking of local hypersensitivity reactions (Ferguson, 1977; Selby, 1981), such that in healthy animals, the immune responses to ingested food stuffs are harmless (Ferguson and Strobel, 1983).

The majority of IE lymphocytes are reported to be T cells in man (Meuwissen et al., 1976; Selby, 1981) and guinea-pigs (Arnaud-Battandier et al., 1978). Maximum T cell numbers in peripheral blood are evident during the rest (sleep) phase both in man (Abo et al., 1981; Ritchie et al., 1983) and the mouse (Kawate et al., 1981). It is clearly evident that the variation in IE lymphocyte numbers shows the reverse in phasing and may (a) be associated with physiological needs for immune defence in peripheral blood at a time inversely related to these needs in the intra-epithelial region of the gut mucosa, and/or (b) a direct reflection of the different way in which systemic and mucosal immunoregulatory apparatus react to the same antigen (Ferguson and Strobel , 1983).

In addition a second possibility arises. Abo et al., (1981) noted that in man the proportion of lymphocytes with receptors for IgG-FC and those involved in the antibody-dependant cell-mediated cytotoxicity (ADCC) system increased during the day (activity period) and decreased during the night (sleep period) in peripheral blood. The authors suggested that the majority of these cells were null cells. There is unfortunately no way of identifying these markers on the IE lymphocytes counted in the present investigation, the possibility that these cells were the same as those noted by Abo et al., (1981) is only tentatively suggested by their similar phasing over 24 h. The synthesis of IgG is largely governed by antigenic

stimulation and it functions to combat micro-organisms and their toxins. The extracellular killing of target cells coated with IgG antibody is mediated by recognition of the FC site on IgG by K cells bearing the appropriate receptor. As mentioned the exact lineage of K cells is uncertain, and further difficulties in the interpretation of this hypothesis come from the lack of clarification of the physiological significance of FC binding sites on lymphocytes (Roitt, 1980). The ADCC system is an extracellular cytotoxic mechanism whereby surface receptors for the IgG FC region, bind the effector cell to the target cell-which is then killed by an extracellular mechanism. Although studied extensively in vitro, there is little information of the role of ADCC system in vivo, though it would be expected to be of significance where the target is too large for ingestion by phagocytosis i.e. as in parastic infection (Roitt, 1980). This raises the possibility that the nature of the IE lymphocytes in these Giardia infected hamsters was directly associated with the parasitic infection.

Several questions arise from this investigation:

(1) Is there a significant circadian variation in IE lymphocyte count in uninfected hamsters?
(2) Are the majority of IE lymphocytes T cells or are they IgG-FC receptor bearing cells and ADCC implicated cells? And if this is so,
(3) Is the nature of the IE lymphocyte population in this investigation associated with Giardia infection promoting the need for this immune defence mechanism?
(4) If the 24 h variation in IE lymphocytes is mediated by parasitic infection why should there be an increased need for defence during the activity period of hamsters?

Further investigations designed to answer these questions may lead to further clarification of the role of IE lymphocytes and also the effect of Giardia muris on the immune response in the gut.

This investigation has stimulated a number of proposals for the mechanisms controlling the number of IE lymphocytes in the jejunum of

the male Syrian hamster, and has shown how studies of oscillating variables stimulates discussion on their control mechanisms and physiological function. It would follow that further information on a variety of circadian variables will lead to a greater understanding of the finely tuned integrated physiological phenomena in the body. It is to be hoped that further information on IE lymphocyte count variation with time and day and sleep and wakefulness may lead to its greater sensitivity as a diagnostic tool of cell-mediated immune reactions in the gut mucosa.

PART TWO

Human Study

CHAPTER 12

THE EFFECT OF SLEEP DEPRIVATION ON CIRCADIAN VARIATION IN BODY LENGTH MEASURES OF YOUNG AND MIDDLE-AGED MEN

Introduction

Literature documenting the diurnal oscillation in whole body measures spans the last two centuries.

The fourth supplement to Buffon's treatise entitled 'Histoire Naturelle' (1777) carried a report of an investigation carried out by Montbeillard, possibly the first published evidence of a diurnal change in stature. Montbeillard carried out stature measurements on his 17 year-old son and found that stature loss occured during the day and was recovered with a night of rest in the recumbent position. Greater daily loss was noted with prolonged activity.

Later investigators consistently reinforced Montbeillard's findings. Bradford (1883) measured the stature of an adult in both the erect and recumbent position at 07.00 h and 22.00 h. Stature, in both positions, was measured as greatest in the morning and least in the evening. In addition to noting a diurnal change in stature, Curtiss (1898) reported that a correlation was evident between daily stature loss and activity level in 3 young men. Greater daily activity resulted in greater daily stature loss.

Diurnal changes in stature were also reported in two children by Weiner (1896). The mean daily decrease in stature was 16 mm, a small recovery in stature followed a nap and full recovery followed a night of sleep. In addition, Weiner noted that the greatest proportional decrease (53% of total daily loss) occured during the first hour after morning rising.

Unequivocable proof of diurnal changes in stature came from an extensive study of 200 men (Backman, 1924). The mean daily loss of 23.6 mm was recovered during a night of sleep. The greatest proportional loss (29% of total daily loss) occured by one hour after

morning rising.

The generally accepted explanation for diurnal change in stature is that it is due to variations in thickness of intervertebral discs: occuring through compression during upright posture, and expansion during recumbency.

Intervertebral discs are found between the vertebral bodies of cervical, thoracic and lumbar vertebrae and account for approximately 33% of the total spinal column length (Kazarian, 1975).

Each disc is composed of two parts i) an outer fibrous ring - the annulus fibrosus and ii) an inner soft centre of gelatinous tissue - the nucleus pulposus - which has hydrophilic properties (De Pukey, 1935; Duthie and Ferguson, 1973).

The weight borne by the vertebral column increases from the cervical to the lumbar region. Nachemson and Morris (1964) carried out in vivo measurements of intradiscal pressure and found a positive correlation between body weight and disc pressure. Furthermore, the authors reported that pressure on intervertebral discs was greatest in the sitting position, and was reduced by 30% in standing and 50% in reclining positions.

Marked age related changes occur in the nucleus pulposus of intervertebral discs. In a young person the nucleus pulposus is soft and mucoid, with increasing age it becomes dryer and more solid through progressive dehydration. The water content of the nucleus pulposus is 88% at birth, 80% at age 12 and 70% at age 70 (Keyes and Compere, 1932). Also with increasing age, a progressive loss of elasticity in the discs is evident, and gradual invasion of fibrocartilage into the nucleus from the annulus transforms the disc into an almost uniform fibrocartilage in old-age (Maurice-Williams, 1981).

Consistent with age related changes in discs are the age differences noted in diurnal changes in stature. Mean daily stature loss in children was found to be 16 mm by Weiner (1896) and 25 mm by Palmer

(1930). These changes are evidently greater as a proportion of overall stature than the mean daily loss of 23.6 mm occuring in adult man (Backman, 1924). Following measurement of 1216 persons in the age range 5-90 years, De Pukey (1935) concluded that amount of daily loss in stature decreased with increasing age.

Aims of the Investigation

The present investigation was prompted by the lack of recent literature on diurnal changes in stature and was designed with the following aims:-

1) To investigate the relationship between body length and time of day in two age groups, by using accurate modern techniques of measurement.
2) To investigate the relationship between body length and the sleep/wake cycle, by assessing the effect of sleep deprivation on body length measures.

The opportunity was taken to measure male subjects involved in sleep deprivation studies in the Sleep Research Laboratory. The two age groups (young and middle-aged men) participated in two separate sleep deprivation studies, the differing protocols of which demand subdivision of this investigation into subsections:

Study I: The effect of prolonged sleep deprivation on body length measures of young men.

Study II: The 24 h variation in body length measures of middle-aged men with a night of sleep and a night of sleep deprivation.

Methods

Study I

Twelve healthy young men, aged 19-28 years (mean 22), took part and all were resident in the sleep laboratory for 5 days and nights as follows:

Start of study -----► Monday night - baseline night of normal sleep

63 hours of sleep deprivation

- Tuesday day
  night - first sleep deprivation night
- Wednesday day
  night - second sleep deprivation night
- Thursday day
  night - first recovery night

Friday day
night - second recovery night

Saturday morning --------► End of Study

The two recovery nights balanced the two sleep deprived nights so that any rebound effect could be evaluated (Dement, 1960).

On baseline and recovery nights, subjects were in bed from 23.00 h - 08.00 h.

During the study period, subjects did not take part in any strenuous

physical exercise, and all were kept under constant surveillance.

Measurement of Body Lengths

Measurements of stature and vertebral lengths (cervical, thoracic and lumbar) were carried out at 08.00 h and 23.00 h of each day, beginning at 23.00 h on the first day and ending at 08.00 h on the last day of the study. On baseline and recovery nights measurements were carried out immediately before retiring to bed, and immediately following morning rising.

At the time of the first measurement, the following was recorded in each subject:

(a) Weight (kg) in light indoor clothing.
(b) Stature (mm): overall body height.
(c) Head height (mm): measurement from the external auditory meatus to top of head.
(d) Height of C7 (mm): the prominent vertebral spine of which is readily identified by palpation.
(e) Height of lower angle of scapula (mm): identified by palpation.
(f) Height of iliac crest (mm): identified by palpation.
(g) Height of S2 (mm): identified by the permanent dimple in the skin over the point where the iliac crest meets the sacrum.

(Heights specified in (b) and (d)-(g) refer to height from floor)

Using measurements (b) to (g), vertebral lengths were calculated as follows (also see FIG 12.1).

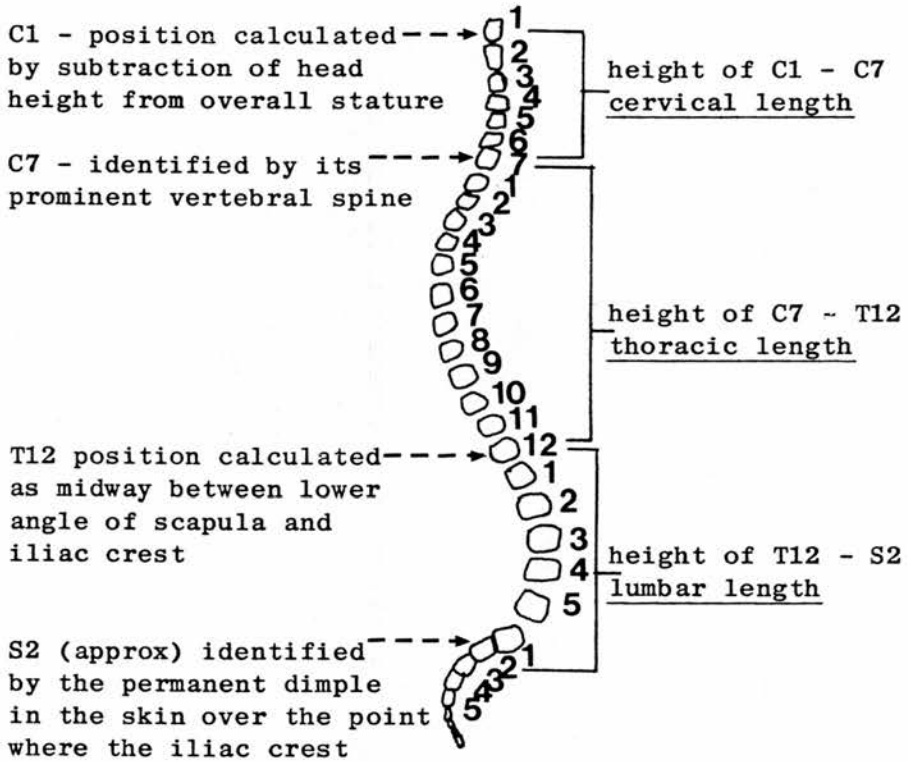
i) Cervical length (C1-C7) : [(b)-(c)]-(d).
ii) Thoracic length (C7-T12): As T12 was not readily identifiable, its position was calculated as mid-way between lower angle of scapula (e) and iliac crest (f):

$$T12 = \frac{(e)-(f)}{2} + (f)$$

iii) Lumbar length (T12-S2). L5 was not readily identifiable so the most satisfactory solution was to measure the level of S2. The

FIG 12.1

Definition and Measurement of Vertebral Lengths



sacrum is a fused bone with no intervertebral discs. The measurement of S2 as the lower limit of lumbar vertebrae thus allowed any change in this region to be attributed to lumbar disc compression.

In view of the indirect identification of T12, and to avoid error arising through possible observer differences in the exact identification of vertebral points at each measurement time, marked elastoplast was placed on each subject's back at initially identified positions of C7, T12 and S2. These marks remained in position for the duration of the study period.

Following initial identifications and measurements, stature and cervical, thoracic and lumbar lengths were recorded at each subsequent measurement time.

Anthropometric Equipment and Subject Position

Head height was measured to the nearest mm using a craniometer. The subject's head was held such that the Frankfort plane (a line from the lower border of the left orbit to the upper margin of the external auditory meatus) was horizontal during measurement (Weiner and Lourie, 1969).

Stature and vertebral lengths were measured to the nearest mm using a Harpenden, digital read-out, wall mounted stadiometer; with an additional extendable rod attached to the head board.

As postural changes can cause large concomitant changes in body lengths measured by this method, great care was taken to position subjects in the correct stance immediately prior to measurement; according to the methods of Weiner and Lourie (1969). Each subject stood in bare feet, with heels together and on the floor, in front of the stadiometer and was instructed to stretch upwards to the fullest extent (aided by gentle traction on the mastoid processes). The subject's back was kept as straight as possible by following the manoeuvre of relaxing shoulders and manipulating posture. As soon as the subject was positioned, the head board of the stadiometer was

brought down so that the bar rested firmly on the subject's head (held with the Frankfort plane horizontal).

Vertebral points were measured in the order C7,T12 and S2; by horizontal alignment of the extendable bar (attached to the stadiometer head board) to each marked point.

All identification procedures, positioning and measurements were carried out by myself to prevent error arising through measurements by different observers (Boyd, 1929).

Study II

Six healthy middle-aged men, aged 44-50 years (mean 47), took part. All were resident in the sleep laboratory for 24 h (08.00 h -08.00 h) on two separate occasions 7 weeks apart. One occasion included a normal night of sleep, and another a night of sleep deprivation. The six subjects were studied in two groups of three, with the sleep and sleep deprivation conditions in balanced order.

Body length measurements were obtained by the methods detailed in Study I, but differed in their timing. Measurements began at 08.00 h when the subjects arrived at the laboratory, and were taken every 3 h thereater until 23.00 h. The final measurement was taken the following morning at 08.00 h; after either a night of sleep or sleep deprivation.

During the study period none of the subjects took part in any strenuous exercise and all were closely supervised throughout the night of sleep deprivation.

When the 24 h period included a night of sleep, subjects were in bed from 23.00 h - 08.00 h.

As in Study I, all identification procedures, positioning and measurements of subjects were carried out by myself.

Data Analysis

Raw measurements of body lengths in young and middle-aged men are presented in Appendix 3.1, and Appendix 3.4 respectively. Because of the large subject-dependant variation in measures, change scores were calculated for analysis.

Change Scores

Are a measure of the increase or decrease in body lengths against the preceeding measurement. In Study I, for example, the stature of subject 1 was 1763 mm at 23.00 h immediately preceeding the baseline night, and was 1780 mm the following morning. The overnight change score was thus +17 mm. In Study II, change scores were calculated over each 3 h period (08.00 h - 23.00 h) and overnight (23.00 - 08.00 h). The change scores in Study I are given in Appendix 3.2 and Study II in Appendix 3.5.

Percentage Change

Percentage change in raw measurements were calculated at each time point as follows:

$$\text{Percentage stature change} = \frac{\text{Stature change score}}{\text{Overall stature*}} \times 100$$

$$\text{Percentage cervical change} = \frac{\text{Cervical change score}}{\text{Cervical length*}} \times 100$$

Percentage thoracic change and percentage lumbar change were calculated in the same way, * Measurements at 08.00 h following baseline night in Study I, and at 08.00 h following a study night of sleep in Study II.

The reason for calculating percentage changes in body lengths was to see whether body lengths exhibited a different pattern over the study period when expressed as percentage change than when expressed as change scores. Excellent linear correlation was evident between

measurements of change calculated by these two different methods and correlation values are shown in Table 12.1. As such, it was not deemed necessary to repeat statistical analysis using percentage changes.

Statistical Package

The BMDP statistical package, developed at the Health Sciences Computing Facility (University of California, Los Angeles) was chosen for its facilities for analysis of variance (ANOVA) on repeated measures of subjects.

a) Calculation of Mean (± SEM).

Change score mean (± SEM) was calculated at each time point (using BMDP).

Cumulative change in body lengths were obtained by successive addition of change scores. The mean (± SEM) of cumulative change was calculated using BMDP. The first measurement of each study period was taken as baseline point, and the cumulative change from baseline was plotted to show the pattern of change in body lengths over each study period.

b) Effect of Time of Day and Sleep Deprivation

Analysis of variance (ANOVA) with repeated measures was carried out on change scores (i) to look for time effects i.e. diurnal change and (ii) treatment effects by comparing time-matched periods where there was sleep with periods when there was sleep deprivation. If significant effects were found, correlated t-tests (2-tailed) were performed between pairs of conditions.

Table 12.1

Correlation Between Percentage Change and Change Scores During Study Periods

(a) Young Men

Measure	r value	df	significance
Stature	0.99	7	$p < 0.001$
Cervical Length	0.78	7	$p < 0.02$
Thoracic Length	0.98	7	$p < 0.001$
Lumbar Length	0.98	7	$p < 0.001$

(b) Middle-aged Men (1) 24 h with a night of sleep

Measure	r value	df	significance
Stature	0.99	4	$p < 0.001$
Cervical Length	0.97	4	$p < 0.01$
Thoracic Length	0.98	4	$p < 0.001$
Lumbar Length	0.99	4	$p < 0.001$

(11) 24 h with sleep deprivation

Measure	r value	df	significance
Stature	0.99	4	$p < 0.001$
Cervical Length	0.91	4	$p < 0.02$
Thoracic Length	0.99	4	$p < 0.001$
Lumbar Length	0.91	4	$p < 0.01$

Calculation of the Relationship Between Change Scores and Overall Stature

The linear correlation between overall stature* and change scores was calculated in Study I and Study II. (*Measured at 08.00 h immediately following baseline night of sleep in Study I, and at 08.00 h following a study night of sleep in Study II).

Calculation of the Relationship Between Change Scores and Body Mass

The linear correlation between body mass (young men: Appendix 3.3, middle-aged men: Appendix 3.6) and change scores was calculated. The index of body mass used was W/H2, where W= weight in kg, H= height in meters (taken as above*). This index, sometimes referred to as Quetelet's Index, gives the most reliable estimate of body mass (Khosla and Lowe, 1967; Keys et al., 1972).

Results

Study I

i) Stature

Change scores (mean ± SEM) and cumulative changes (mean ± SEM) over the study period are shown in Table 12.2.

The change in stature during the study period is shown in FIG 12.2.

ANOVA using change scores over baseline and recovery periods revealed a significant pattern of change with time of day ($F=88.51$, $df=1,11$, $p<0.001$), loss in stature occured during the day, and was regained during nights of sleep.

Sleep deprivation significantly altered the pattern of change with time of day ($F=40.75$, $df=2,22$ $p<0.001$; ANOVA over baseline and sleep deprivation periods), as with sleep deprivation the diurnal change in stature over 24 h noted over the baseline period (when there was sleep) was no longer evident.

Changes occuring during nights of sleep and sleep deprivation were compared by t-tests. During both first and second sleep deprivation nights, change was significantly different from that during a night of baseline sleep ($t = 8.28$ $df = 11$, $p<0.001$ and $t = 6.46$, $df = 11$, $p<0.001$, respectively). Stature decreased during nights of sleep deprivation and increased during nights of sleep.

The change during the first recovery night was not significantly different to that during baseline night of sleep. To see whether the accumulated loss in stature during the sleep deprivation period had been re-gained on this one recovery night of sleep, stature after a baseline night of sleep ($\bar{X}$ 11.2 mm ± 1.9 SE above baseline) was compared with stature after the first recovery night ($\bar{X}$ 15.4 mm ± 1.2 SE) by t-test and no

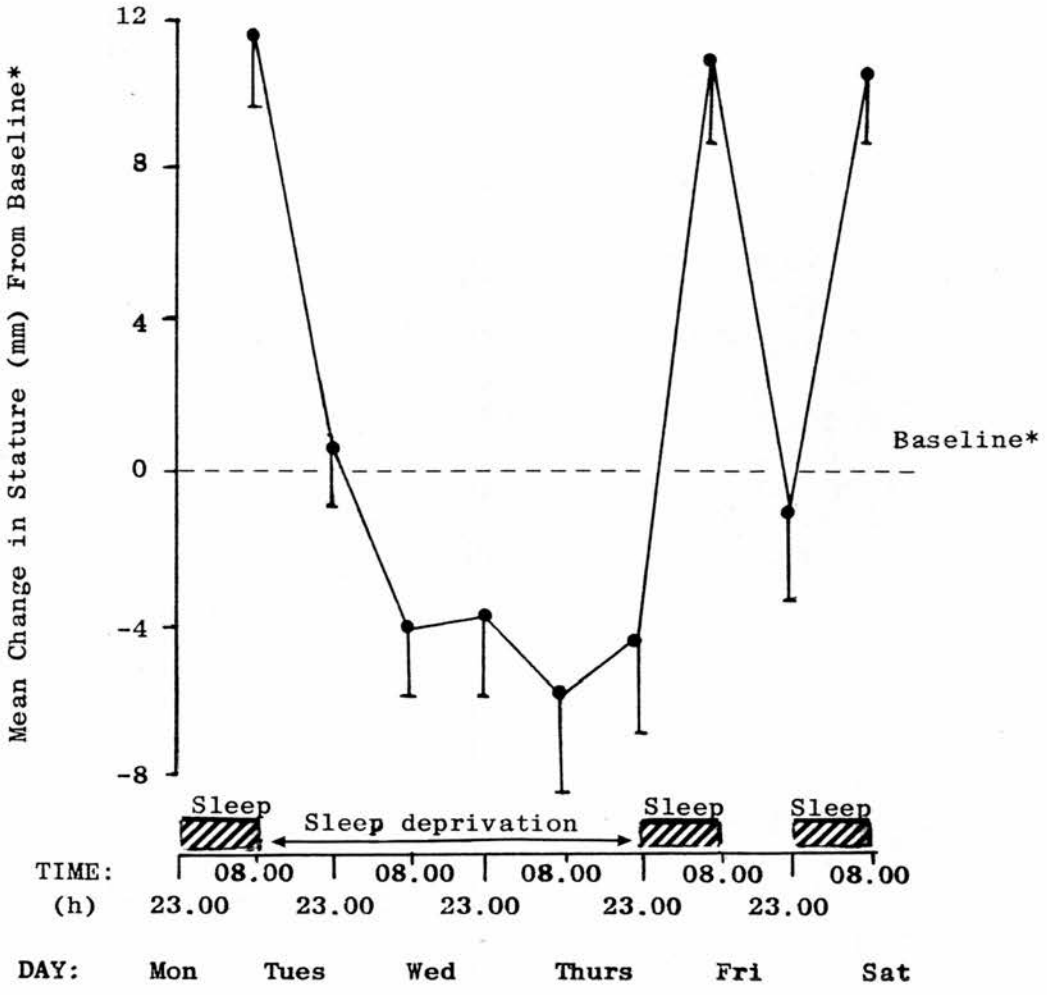
Table 12.2

Change Scores and Cumulative Change in Stature of 12 Young Men

	Day/Period		Change Score (mm) Mean (S.E.M)	Cumulative Change (mm) Mean (S.E.M)
(Sleep).....	Mon	night	+11.2 (1.9)	+11.2 (1.9)
	Tues	day	-10.7 (1.7)	+0.5 (1.6)
		night	-4.6 (1.2)	-4.1 (1.8)
63 h sleep deprivation	Wed	day	+0.2 (1.3)	-3.9 (2.0)
		night	-2.1 (1.2)	-6.0 (2.4)
	Thurs	day	+1.4 (0.9)	-4.6 (2.3)
(Sleep).....		night	+15.4 (1.2)	+10.8 (2.2)
	Fri	day	-11.8 (1.4)	-1.1 (2.3)
(Sleep).....		night	+11.6 (1.3)	+10.5 (1.9)

FIG 12.2

Mean Change in Stature (±S.E.M) of 12 Young Men During 6 Days Including 63 h of Sleep Deprivation.



* Baseline: stature at start of study.

significant difference was found.

ii) Vertebral Lengths

Change scores (mean ± SEM) and cumulative changes (mean ± SEM) over the study period are shown in Table 12.3. The changes in cervical, thoracic and lumbar length over the study period is shown in Fig 12.3.

(a) Cervical Length

There was no significant variation in cervical change with time of day (ANOVA over baseline and recovery nights) and sleep deprivation did not alter the baseline pattern of change (ANOVA).

(b) Thoracic Length

A significant pattern in change with time of day was evident ($F=7.34$, $df=1,11$, $p<0.025$, ANOVA over baseline and recovery periods), length decreased during the day and increased during the nights of sleep. Sleep deprivation did not significantly alter this pattern (ANOVA over baseline and sleep deprivation periods).

(c) Lumbar Length

Change scores exhibited a significant pattern with time of day ($F=15.26$, $df=1,11$, $p<0.01$, ANOVA over baseline and recovery periods). Sleep deprivation did not significantly alter this pattern (ANOVA on baseline and sleep deprivation periods).

ANOVA results are summarized in Table 12.4.

iii) Relationship Between Change Scores and Overall Stature

No significant correlation was evident between overall stature

Table 12.3

Change Scores and Cumulative Change in Vertebral Lengths of 12 Young Men

(a) Cervical Length

	Day/Period		Change Score (mm) Mean	(S.E.M)	Cumulative Change (mm) Mean	(S.E.M)
(Sleep).....	Mon	night	+1.1	(1.5)	+1.1	(1.5)
	Tues	day	-2.8	(1.1)	-1.7	(1.8)
		night	-0.1	(1.5)	-1.8	(1.9)
63 h of sleep deprivation	Wed	day	-2.5	(1.3)	-4.3	(1.6)
		night	-2.2	(1.2)	-6.4	(2.1)
	Thurs	day	+6.1	(2.2)	-0.3	(2.1)
(Sleep).....		night	+3.4	(1.4)	+3.1	(2.2)
	Fri	day	-0.3	(1.5)	+2.8	(2.3)
(Sleep).....		night	-0.7	(1.8)	+2.2	(1.6)

Table 12.3 (Continued)

(b) Thoracic Length

	Day/Period		Change Score (mm) Mean (S.E.M)		Cumulative Change (mm) Mean (S.E.M)	
(Sleep).....	Mon	night	+4.8	(2.6)	+4.8	(2.6)
	Tues	day	-3.6	(2.2)	+1.2	(2.0)
		night	-2.3	(1.7)	-1.1	(1.7)
63 h of sleep deprivation	Wed	day	+0.7	(0.9)	-0.4	(1.6)
		night	+1.0	(1.3)	+0.6	(2.2)
	Thurs	day	-3.0	(1.5)	-2.4	(1.7)
(Sleep).....		night	+7.2	(2.0)	+4.8	(2.2)
	Fri	day	-5.4	(2.2)	-0.6	(2.4)
(Sleep).....		night	+5.6	(2.2)	+4.9	(2.1)

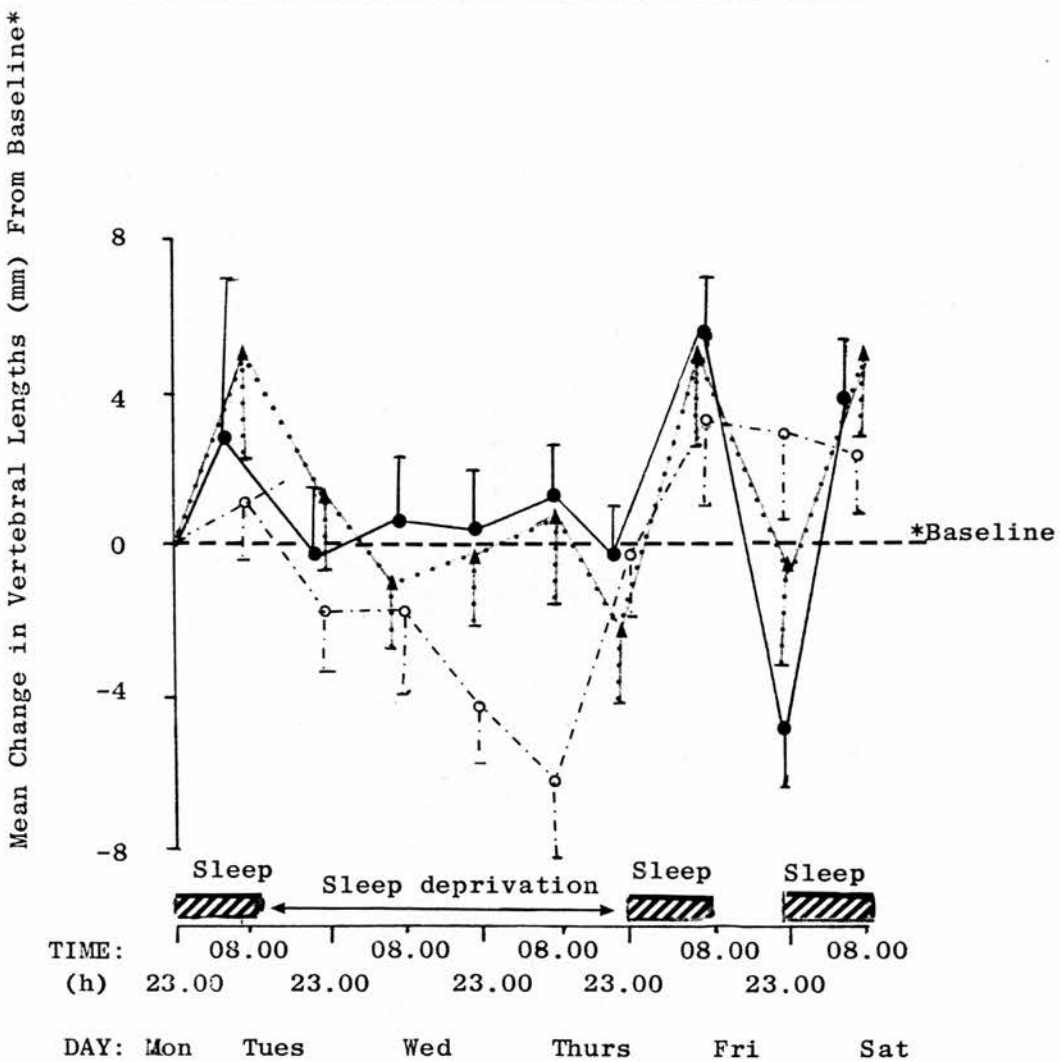
Table 12.3 (Continued)

(c) Lumbar Length

	Day/Period		Change Score (mm)		Cumulative Change (mm)	
			Mean	(S.E.M)	Mean	(S.E.M)
(Sleep).....	Mon	night	+3.9	(2.0)	+3.9	(2.0)
	Tues	day	-4.3	(1.4)	-0.3	(1.7)
		night	+0.7	(0.9)	+0.3	(1.9)
63 h of sleep deprivation	Wed	day	0.0	(0.8)	+0.3	(1.6)
		night	+0.7	(0.7)	+1.0	(1.6)
(Sleep).....	Thurs	day	-1.3	(1.0)	-0.3	(1.2)
		night	+5.8	(1.2)	+5.4	(1.5)
(Sleep).....	Fri	day	-10.3	(1.2)	-5.0	(1.5)
		night	+8.8	(1.7)	+3.8	(1.5)

FIG 12.3

Mean Change in Vertebral Lengths (±S.E.M) of 12 Young Men During 6 Days Including 63 h of Sleep Deprivation.



* Baseline: vertebral lengths at start of study.

(o—o) Cervical length

(▲—▲) Thoracic length

(●—●) Lumbar length

Table 12.4

Analysis of Variance on Measures of 12 Young Men

Measure	(a) Variation with time of day (baseline and recovery periods df=1,11	(b) Effect of sleep deprivation df=2,22
Stature	$F=88.51$, $p<0.001$	$F=40.75$, $p<0.001$
Cervical Length	N S	N S
Thoracic Length	$F=7.34$, $p<0.025$	N S
Lumbar Length	$F=15.26$, $p<0.01$	N S

and change scores in body lengths during either a night of sleep or the period of prolonged sleep deprivation.

iv) Relationship Between Change Scores and Body Mass

No significant correlation was found between body mass and the change scores in body lengths during a night of sleep. Body mass did, however, correlate significantly with change in stature during prolonged sleep deprivation ($r = 0.68$, $df = 10$, $p<0.02$) but did not correlate significantly with changes in vertebral lengths during this period. It was evident that subjects with greater body mass showed greater change in stature over a period of prolonged sleep deprivation.

Study II

In this study of middle-aged men, the only difference between the two occasions of measurement was the night-time treatment (sleep or sleep deprivation). ANOVA was used to test the comparability of day-time changes in body length measured every 3 h (08.00 h - 23.00 h) on the two occasions and no significant difference was found between the recorded changes in stature and cervical, thoracic and lumbar length.

i) Stature

Change scores (mean $\pm$ SEM) and cumulative changes (mean $\pm$ SEM) during 24 h are shown in Table 12.5. Changes in stature over 24 h with sleep and sleep deprivation are shown in Fig 12.4. The greatest decrease in stature occured during the first 6 h of measurement (08.00 h - 14.00 h). During the 24 h period with a night of sleep stature change scores varied significantly with time of day ($F = 8.52$, $df = 5,25$, $p<0.001$). Stature was lost during the day and regained during a night of sleep.

Sleep deprivation significantly altered the pattern in change with time of day ($F=335.23$, $df\ 1,5$, $p<0.001$). As there was no significant difference between change occuring in the

Table 12.5

Change Scores and Cumulative Change in Stature of 6 Middle-aged Men During 24 h

(I) with a night of sleep (23.00 h-08.00 h)

(II) with sleep deprivation

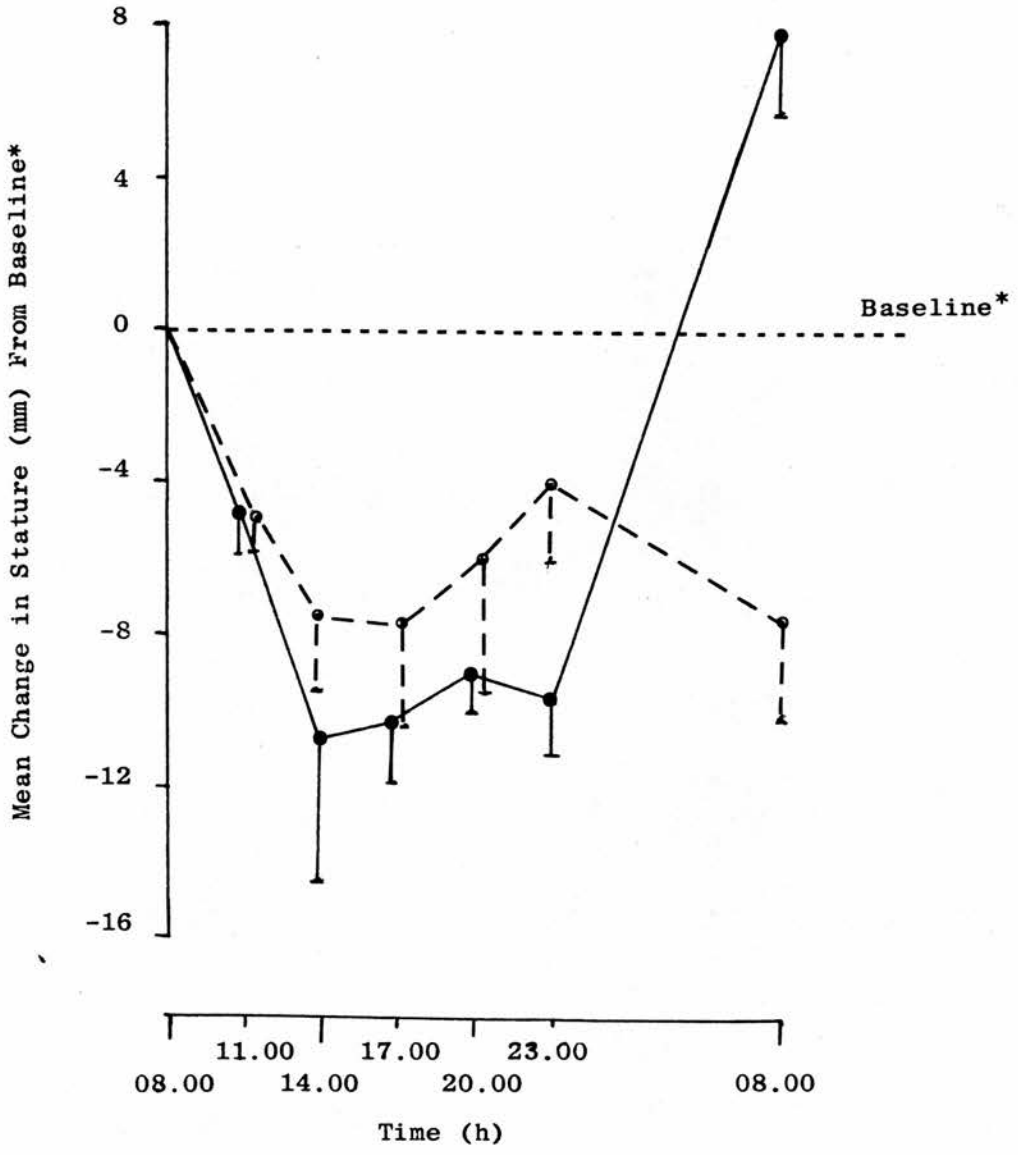
Time (h)		Change Score (mm) Mean	(S.E.M)	Cumulative Change (mm) Mean	(S.E.M)
08.00-11.00	I	-4.8	(1.1)	-4.8	(1.1)
	II	-4.8	(1.5)	-4.8	(1.5)
11.00-14.00	I	-5.8	(4.3)	-10.7	(3.7)
	II	-2.7	(1.7)	-7.5	(2.0)
14.00-17.00	I	+0.3	(4.3)	-10.3	(1.6)
	II	-0.3	(1.3)	-7.8	(2.6)
17.00-20.00	I	+1.3	(1.4)	-9.0	(1.0)
	II	+0.8	(1.0)	-7.0	(2.5)
20.00-23.00	I	-0.7	(1.1)	-9.7	(1.4)
	II	+3.0	(1.1)	-4.0	(2.1)
23.00-08.00	I	+17.5	(1.2)	+7.8	(2.1)
	II	-3.7	(0.8)	-7.7	(2.4)

FIG 12.4

Mean Change in Stature (±S.E.M) of 6 Middle-aged Men During 24 h

(a) with a night of sleep (23.00 h-08.00 h) (●——●)

(b) with sleep deprivation.(o— —o)



*Baseline: stature at start of study

day-time:08.00 h - 23.00 h, on the two occasions, the significantly different 24 h pattern was conferred by the night of sleep deprivation. The overnight changes with sleep and sleep deprivation were found to be significantly different ($t = 21.60$, $df = 5$, $p<0.001$); after a night of sleep stature was significantly above baseline ($t=14.83$, $df = 5$, $p<0.001$) whereas after a night of sleep deprivation stature was significantly below baseline ($t=4.37$, $df=5$, $p<0.01$). The increase in stature noted during a night of sleep did not occur during a night of sleep deprivation.

ii) Vertebral Lengths

Changes scores (mean $\pm$ SEM) and cumulative changes (mean $\pm$ SEM) over 24 h are shown in Table 12.6. Changes in vertebral lengths over 24 h with a night of sleep and sleep deprivation are shown in FIG 12.5 (cervical change), FIG 12.6 (thoracic change), and FIG 12.7 (lumbar change).

By using ANOVA it was evident that cervical, thoracic and lumbar length change scores did not vary significantly with time of day and there was no significant sleep deprivation effect on changes.

iii) Relationship Between Change Scores and Overall Stature

No significant correlation was found between day-time or night-time (sleep or sleep deprivation) change scores in body lengths and overall stature

iv) Relationship Between Change Scores and Body Mass

No significant correlation was found between body mass and the day-time or night-time (sleep or sleep deprivation) change scores in body lengths.

Table 12.6

Change Scores and Cumulative Change in Vertebral Lengths of 6 Middle-aged Men During 24 h

(I) with a night of sleep (23.00 h-08.00 h)

(II) with sleep deprivation

(a) Cervical Length

Time (h)		Change Score (mm)		Cumulative Change (mm)	
		Mean	(S.E.M)	Mean	(S.E.M)
08.00-11.00	I	-0.2	(2.5)	-0.2	(2.5)
	II	-0.2	(2.6)	-0.2	(2.6)
11.00-14.00	I	-4.7	(3.7)	-4.8	(3.1)
	II	-0.7	(2.4)	-0.8	(2.5)
14.00-17.00	I	-0.8	(4.2)	-5.7	(2.3)
	II	-1.8	(1.7)	-2.7	(3.0)
17.00-20.00	I	+2.3	(1.8)	-3.3	(1.9)
	II	+1.3	(1.8)	-1.3	(2.7)
20.00-23.00	I	+0.3	(2.9)	-3.0	(4.1)
	II	+0.7	(1.9)	-0.6	(2.5)
23.00-08.00	I	+5.0	(1.4)	+2.0	(3.8)
	II	-2.2	(0.9)	-2.5	(2.7)

Table 12.6(Continued)

(b) Thoracic Length

Time (h)		Change Score (mm) Mean	(S.E.M)	Cumulative Change (mm) Mean	(S.E.M)
08.00-11.00	I	-5.3	(2.4)	-5.3	(2.4)
	II	-5.2	(3.9)	-5.2	(3.9)
11.00-14.00	I	-1.7	(1.5)	-7.0	(3.7)
	II	+2.3	(3.1)	-2.8	(1.8)
14.00-17.00	I	+3.3	(2.9)	-3.7	(4.8)
	II	-1.7	(3.1)	-4.5	(2.6)
17.00-20.00	I	-1.5	(1.1)	-5.2	(3.7)
	II	-2.3	(1.7)	-6.8	(2.0)
20.00-23.00	I	+2.7	(1.9)	-2.5	(2.5)
	II	+5.7	(1.8)	-1.2	(2.7)
23.00-08.00	I	+1.2	(1.3)	-1.3	(2.6)
	II	+0.5	(1.4)	-0.7	(2.8)

Table 12.6(Continued)

(c) Lumbar Length

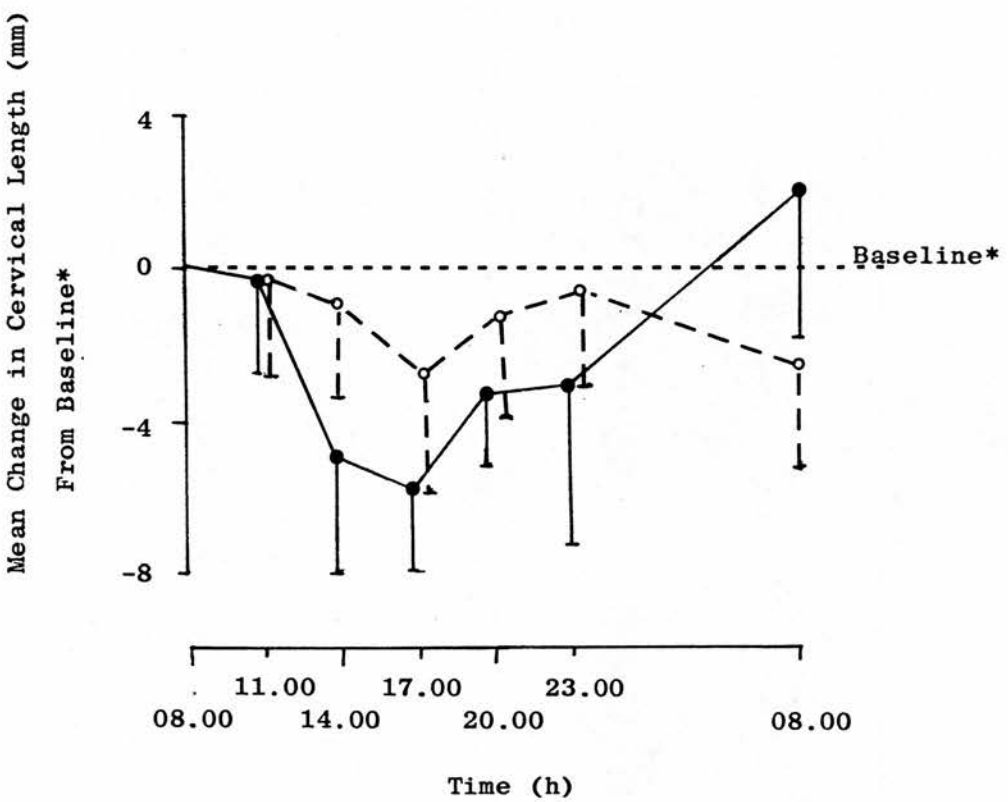
Time (h)		Change Score (mm) Mean	(S.E.M)	Cumulative Change (mm) Mean	(S.E.M)
08.00-11.00	I	-1.3	(3.8)	-1.3	(3.8)
	II	+3.3	(4.0)	+3.3	(4.0)
11.00-14.00	I	-0.3	(1.6)	-1.7	(3.3)
	II	-1.7	(2.2)	+1.7	(4.2)
14.00-17.00	I	-1.8	(1.9)	-3.5	(4.0)
	II	+1.0	(2.7)	+2.7	(4.7)
17.00-20.00	I	-3.0	(2.2)	-6.5	(4.7)
	II	+4.0	(2.4)	+6.7	(5.2)
20.00-23.00	I	+1.3	(2.0)	-5.2	(5.1)
	II	-3.8	(2.5)	+2.8	(4.5)
23.00-08.00	I	+11.7	(3.5)	+6.5	(4.0)
	II	0.0	(2.1)	+2.8	(6.0)

FIG 12.5

Mean Change in Cervical Length (±S.E.M) of 6 Middle-aged Men

During 24 h (a) with a night of sleep (23.00 h-08.00 h)(●—●)

(b) with sleep deprivation. (o— —o)



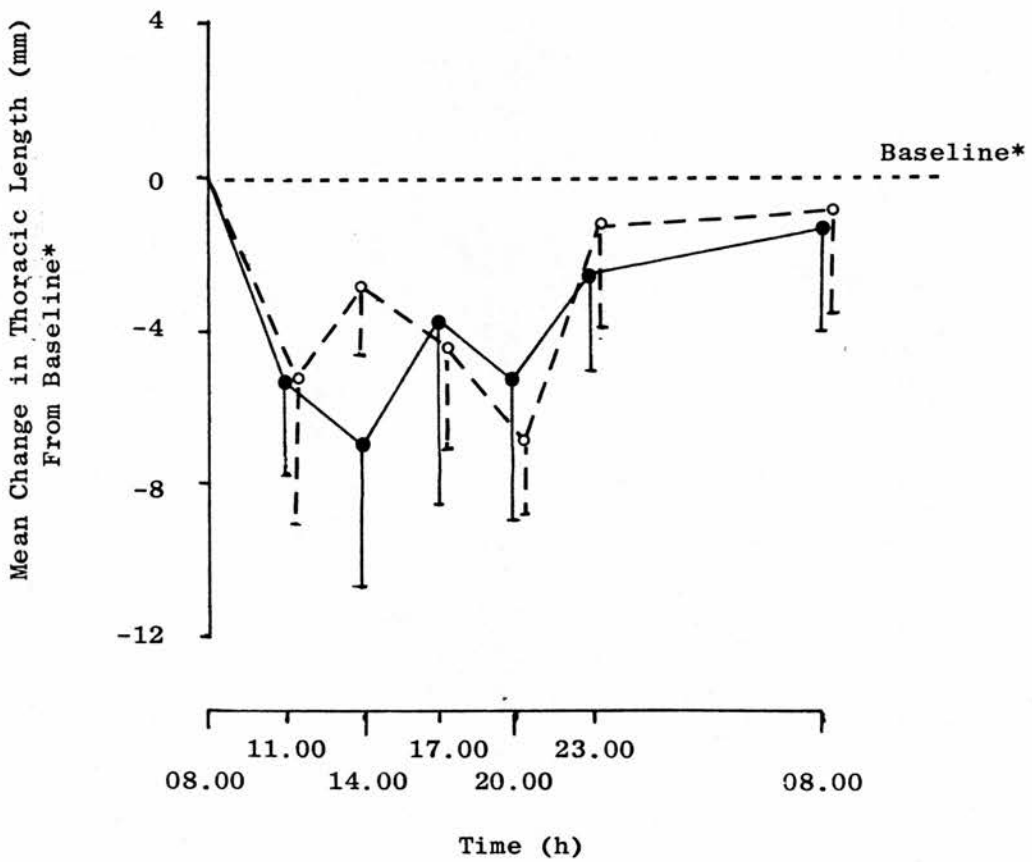
*Baseline: cervical length at start of study

FIG 12.6

Mean Change in Thoracic Length (±S.E.M) of 6 Middle-aged Men During 24 h

(a) with a night of sleep (23.00 h-08.00 h) (●——●)

(b) with sleep deprivation. (o— —o)



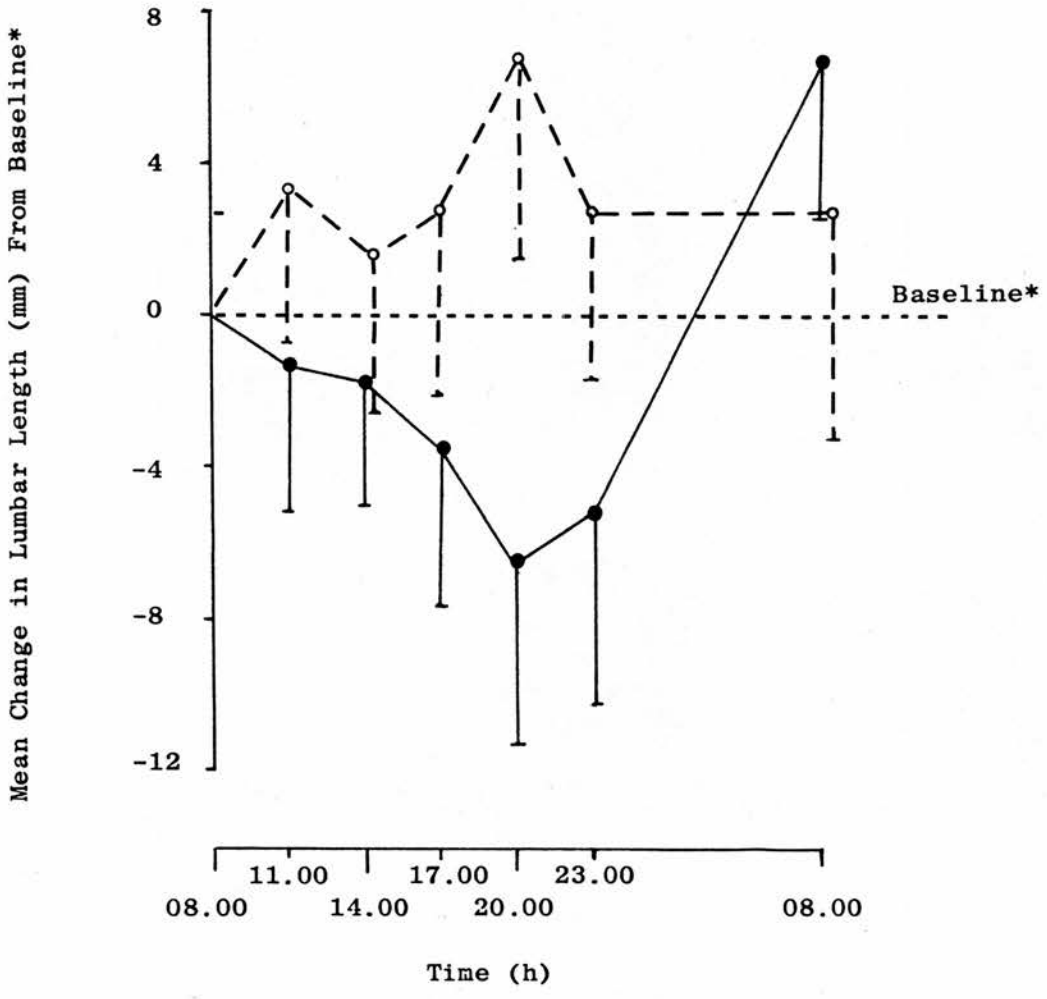
*Baseline: thoracic length at start of study

FIG 12.7

Mean Change in Lumbar Length (±S.E.M) of 6 Middle-aged Men During 24 h

(a) with a night of sleep (23.00 h-08.00 h)(●——●)

(b) with sleep deprivation. (o— —o)



*Baseline: lumbar length at start of study

The Contribution of Vertebral Length Changes

It is evident that during a night of sleep stature increases in both young and middle-aged men. How much of this increase is due to vertebral length increase?

Young men: Vertebral length change (mean of changes during 3 nights of sleep) accounted for 97% of the mean increase in stature (12.3 mm of 12.7 mm).

Middle-aged men: Mean vertebral length change during a night of sleep accounted for all of the mean increase in stature (17.9 mm of 17.5 mm).

Discussion

Stature is lost during the day-time and regained during a night of sleep. This investigation has confirmed the existence of a significant pattern of diurnal change in stature of two age groups of men under normal 24 h conditions (i.e. when the 24 h includes a night of sleep). The findings are consistent with all previous investigations cited, and the more recent investigations of Goode and Theodore (1983) and Reilly et al. (1984) both of which were published after the present investigation was completed.

The daily loss in height of young men (during baseline and recovery periods) was rather less than the amount of change noted in young men in other investigations. For example, daily loss of $\bar{X}$ 18.2 mm in 3 young men (Curtiss, 1898); $\bar{X}$ 23.6 mm in 200 young men (Backman, 1924); $\bar{X}$ 19.3 mm in 8 young men (Reilly et al., 1984). It is interesting that the authors noted these changes in 'normally active' subjects and suggests that the smaller daily decrease noted in the present investigation may be due to reduced activity. The correlation between daily stature loss and activity has been suggested by Montbeillard (1777) and Curtiss (1898). In the present investigation strenuous physical exercise was prohibited in the young men due to the specific nature of my colleagues' sleep deprivation studies. This, together with the need for subjects to remain within the close environs of the sleep research laboratory, led to activity levels considerably lower than could be considered the norm in young men.

The rapid decrease in stature during the first few hours of rising noted in Study II is consistent with all previous investigations where measurements were carried out at intervals over the day-time period, (Weiner, 1896; Backman, 1924; Reilly et al., 1984). This rapid decrease may be the direct result of a sudden increase in pressure on intervertebral discs on assumption of upright posture.

In Study II, stature measured at 08.00 h after a night of sleep was above the baseline level set at 08.00 h at the start of the study. This apparent discrepancy possibly arose because subjects slept at

home and then travelled to the sleep laboratory for the start of the study. By the time the first measurement was carried out, stature may have decreased significantly; for it is during the first hour following morning rising that the greatest proportional decrease in stature is noted (Weiner, 1896; Backman, 1924).

In view of the more accurate methods of stature measurement now available to investigators, the similarities evident in results of investigations spanning the years 1777-1984 gives enormous credit to the accuracy of the earlier work.

The present investigation has added to the knowledge about diurnal change in stature by showing that this change is eliminated during sleep deprivation and is thus dependent on recumbency, or sleep, for rhythmicity. It is not possible to state unequivocably that diurnal change in stature is dependent on the sleep/wake cycle for the reason that during sleep deprivation subjects were not recumbent. Reilly et al. (1984) suggested the existence of an endogenous rhythm in stature that is reinforced, or perhaps masked, by changes in posture and spinal loading during day-time and night-time. De Pukey (1935) mentioned a personal attempt to clarify the importance of sleep versus recumbency relating to diurnal change in stature. Daily measurements were carried out on patients confined to hospital beds, there were, however, no conclusive results as it was found that illness and post-operative pain caused muscle rigidity which prevented accurate measurement.

Tentative support for the sleep dependence of diurnal change in stature comes from a number of investigations. Weiner (1896), Goode and Theodore (1983) noted only a partial return in stature during a rest, but full recovery during a night of sleep. Duthie and Ferguson (1973) reported that in cadavers and anaesthetised patients with fully relaxed muscles, the pressure on intervertebral discs was lower than in resting but awake subjects. When a subject is awake the spinal muscle tone produces considerable compressive force on the discs, and muscle tone is only lost during REM sleep (Pompeiano, 1967).

As spinal load, causing disc compression, increases from cervical to lumbar regions, I tried to evaluate change in stature in terms of change in cervical, thoracic and lumbar lengths. It was evident, however, that these vertebral lengths did not follow a significant pattern of change and this may be due to precision of measurement being less in smaller body measures. Boyd (1929) reported that stature measurement had the lowest degree of inherent error, but that measurements of shorter bodily dimensions carried a larger variability, for the inherent error in their measurement was proportionately greater.

I have purposefully avoided direct comparison of the diurnal change in body lengths of the two age groups. De Pukey (1935) found daily oscillation in body length to be greatest in the group aged 10-20 years, and least in the group aged over 50 years. Unfortunately, no further information on this interesting phenomenon can be gained from the present investigation owing to the differing protocols of Study I and Study II.

In summary, this investigation has confirmed the existence of a significant pattern of diurnal change in stature in men of two age groups, a pattern which is eliminated during sleep deprivation. During prolonged sleep deprivation, subjects with greater body mass exhibited greater stature loss, this was possibly due to greater compression of intervertebral discs (Nachemson and Morris, 1964). Furthermore, only one night of sleep is required for full recovery in stature following a period of prolonged sleep deprivation. Results of this investigation, together with previous works, suggest the recovery during night-time is because muscle tone falls in sleep (Pompeiano, 1967).

If subject samples and protocols had been designed expressly for the purpose of this investigation, I would have increased the number of middle-aged subjects and studied the two age groups of men over immediately comparable times and treatments. This would enable direct comparison of the two age groups and lead to more conclusive discussion.

Whether it is sleep or mere recumbency that is the more important factor for full recovery of stature remains to be proven. In addition, further assessment of change in vertebral lengths may provide experimental evidence of disc compression during upright posture.

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INDEX TO APPENDICES

Appendix 1.1

Food Intake Per Cage of 6 Hamsters over 3 Hourly Periods During 24 h in Control and Sleep Deprived Hamsters

	Period of Time (h)	Food Intake (g) *	Food Intake (g) **
Control	06.00 - 09.00	8.1	6.3
	09.00 - 12.00	11.3	13.2
	12.00 - 15.00	7.4	6.6
	15.00 - 18.00	5.4	5.7
	18.00 - 21.00	3.7	4.3
	21.00 - 24.00	2.0	2.3
	24.00 - 03.00	1.1	1.4
	03.00 - 06.00	2.6	2.9
Sleep Deprivation	18.00 - 21.00	4.0	3.7
	21.00 - 24.00	2.6	3.1
	24.00 - 03.00	1.7	1.5
	03.00 - 06.00	2.4	3.0

* and ** cages of 6 hamsters (see time-table FIG 8.1)

Appendix 1.2

Counts of Labelled Crypt Cells in the Jejunum of the Male Syrian Hamster During 24 h

(Counts carried out in 30 crypts in 3 hamsters per time group)

(a) With Sleep (Control Hamsters)

Time (h)	Labelled Cells	Total Cells
06.00	320	1285
	287	1376
	267	1132
09.00	274	1236
	297	1251
	164	1187
12.00	236	1086
	178	1050
	152	1221
15.00	160	1139
	148	1095
	136	1060
18.00	271	1348
	358	1273
	295	1247
21.00	238	1138
	248	1107
	300	1275
24.00	311	1254
	351	1238
	232	950
03.00	182	1157
	339	1096
	255	1285
06.00	252	1174
	378	1381
	329	1158

Appendix 1.2 (Continued)

(b) With Sleep Deprivation

Time (h)	Labelled Cells	Total Cells
18.00	296	1089
	336	1156
	255	1112
21.00	231	1222
	277	1126
	260	1214
24.00	282	1150
	317	1219
	276	1227
03.00	122	1248
	51	1225
	235	1199
06.00	77	1162
	246	1157
	127	1113

Appendix 1.3

Counts of Mitotic Crypt Cells in the Jejunum of the Male Syrian Hamster During 24 h

(Counts carried out in 30 crypts in the first 3 hamsters and in 60 crypts in the remaining 9 hamsters per time group)

(a) With Sleep (Control Hamsters)

Time (h)	Mitotic Cells	Total Cells	Time (h)	Mitotic Cells	Total Cells
06.00	101	1278	15.00	97	1265
	58	1317		95	1219
	49	1223		92	1088
	95	2368		71	2457
	122	2466		127	2454
	118	2420		107	2102
	141	2514		107	2572
	74	2354		90	2086
	124	2247		115	2180
	111	2541		85	2204
	105	2594		52	1786
	102	2329		82	1915
09.00	43	1117	18.00	68	1114
	88	1086		83	1144
	49	1119		84	1156
	81	2344		233	2087
	91	2256		173	2136
	109	2392		147	2055
	79	2330		251	2302
	136	2589		234	2204
	79	2273		176	2352
	79	2341		151	1781
	125	2610		228	2510
	103	2580		138	2196
12.00	54	1052	21.00	110	1097
	61	1015		93	1066
	77	1230		86	1109
	123	2591		212	2625
	94	2465		278	2580
	89	2240		195	2469
	91	2439		263	2616
	91	2163		190	2260
	210	2956		224	2425
	107	2040		220	2356
	104	2120		233	2653
	112	2532		234	2379

Appendix 1.3 (Continued)

(a) With Sleep (Control Hamsters) Continued

Time (h)	Mitotic Cells	Total Cells
24.00	149	1182
	153	1264
	133	1257
	157	2359
	147	2440
	162	2616
	136	2364
	168	2217
	148	2495
	136	2530
	126	2279
	151	2517
03.00	95	1276
	146	1222
	141	1148
	157	2178
	140	2367
	115	2360
	174	2310
	113	2431
	144	2431
	217	2460
	141	2460
	115	2400
06.00	56	1194
	113	1422
	70	1273
	93	2459
	109	2541
	143	2596
	65	2347
	69	2160
	175	2893
	84	2229
	116	2538
	94	2487

Appendix 1.3 (Continued)

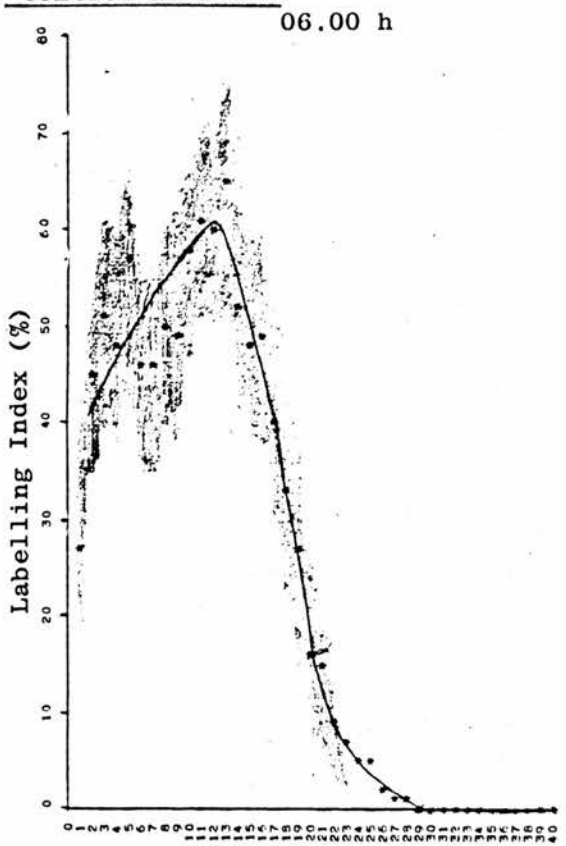
(b) With Sleep Deprivation

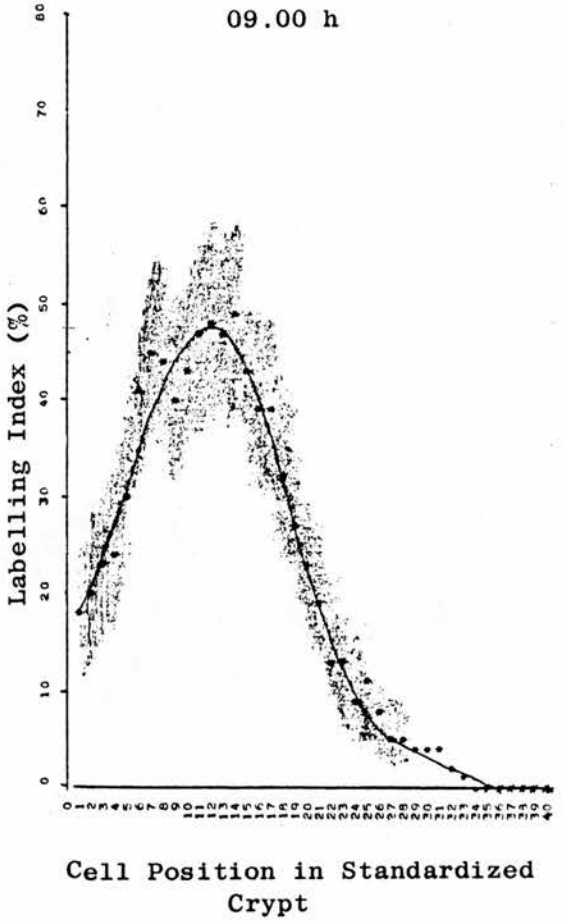
Time (h)	Mitotic Cells	Total Cells	Time (h)	Mitotic Cells	Total Cells
18.00	69	1011	03.00	53	1044
	82	977		84	1009
	69	967		76	1090
	177	2908		113	2415
	114	2431		99	2378
	126	2621		123	2768
	155	2629		95	2479
	140	2451		167	2666
	83	3396		164	2890
	132	2552		78	2084
	91	2423		141	2432
	145	2563		143	2443
21.00	80	1158	06.00	112	1209
	94	1130		112	1169
	113	1185		86	1185
	112	2523		129	2605
	181	2565		114	2533
	132	2177		118	2383
	200	2591		100	2497
	149	2801		157	2592
	150	2264		158	2605
	173	2608		101	2445
	89	2172		107	2255
	91	2651		113	2514
24.00	59	1066			
	59	1068			
	53	882			
	132	2868			
	156	2583			
	86	2393			
	113	2713			
	152	2536			
	139	2793			
	153	2529			
	164	2576			
	96	2491			

Appendix 1.4

Distribution of Labelled Cells in the Hamster Jejunal Crypt During 24 h (data from 3 hamsters)

(a) Control Hamsters

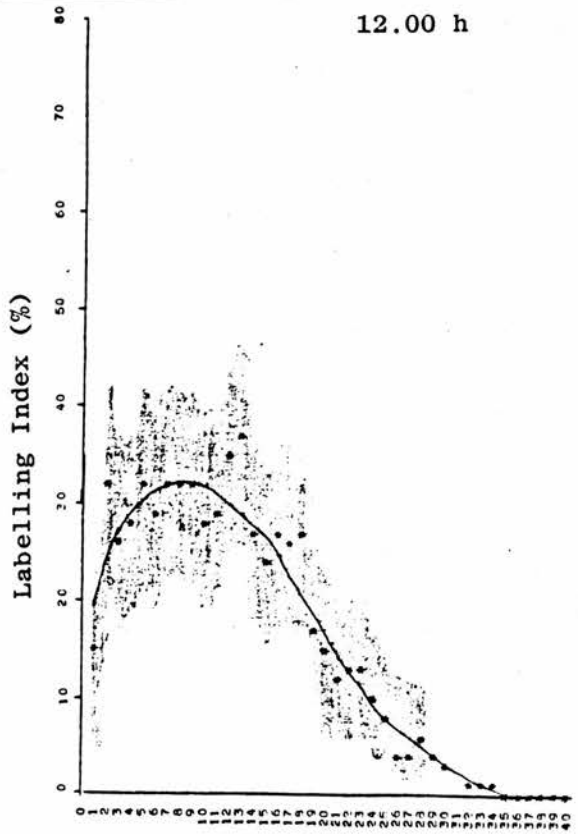


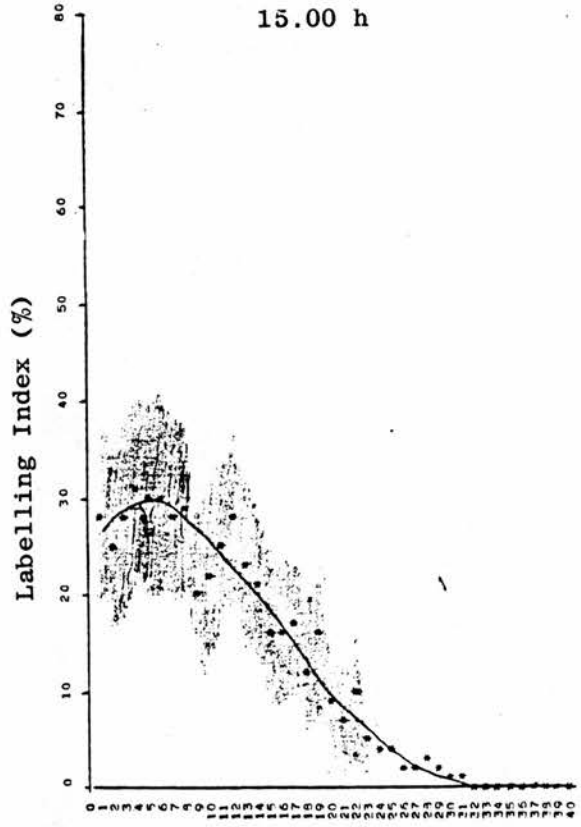


Shaded areas indicate 95% confidence limits

Appendix 1.4 (Continued)

(a) Control Hamsters Continued

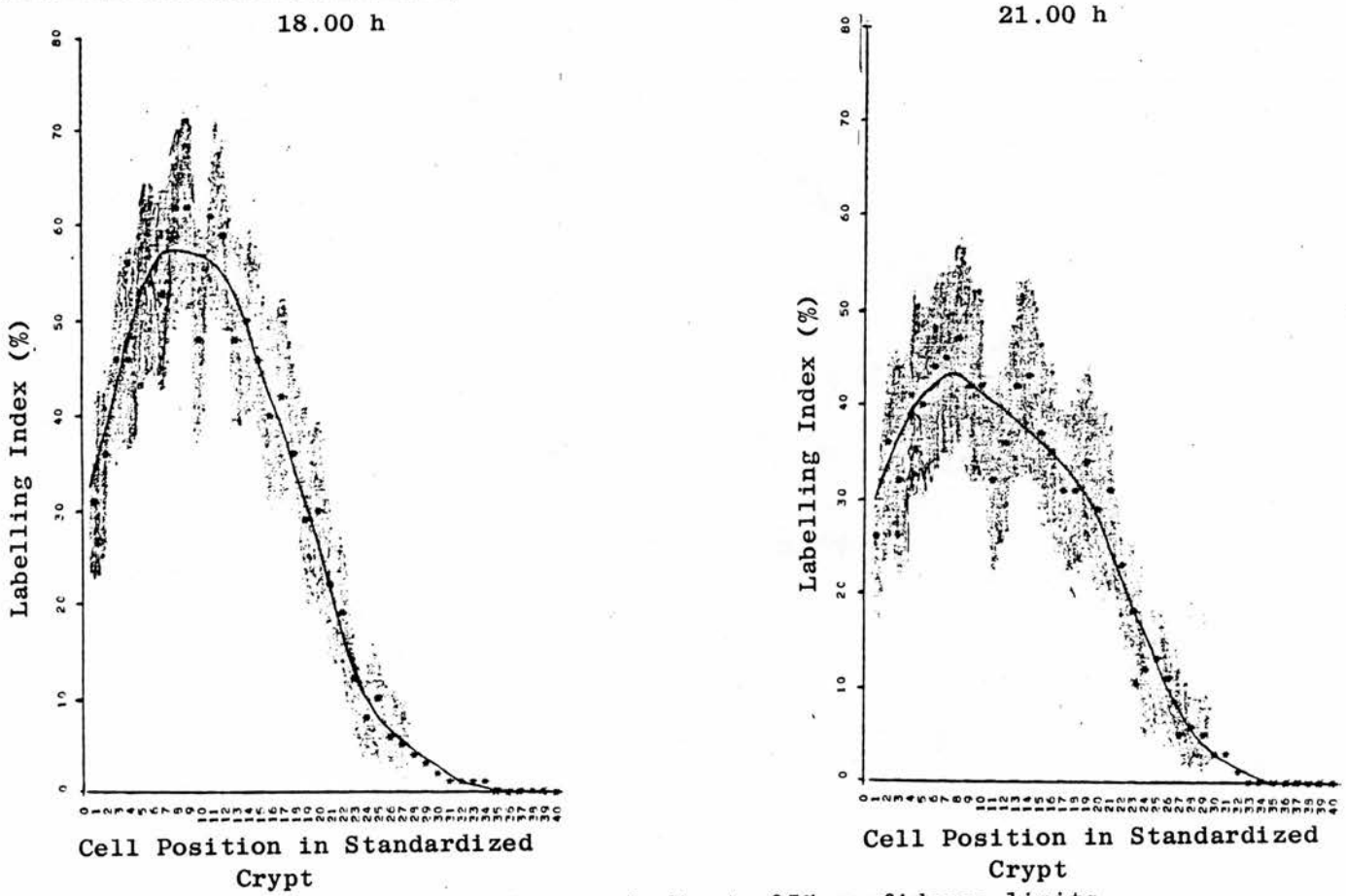




Shaded areas indicate 95% confidence limits

Appendix 1.4 (Continued)

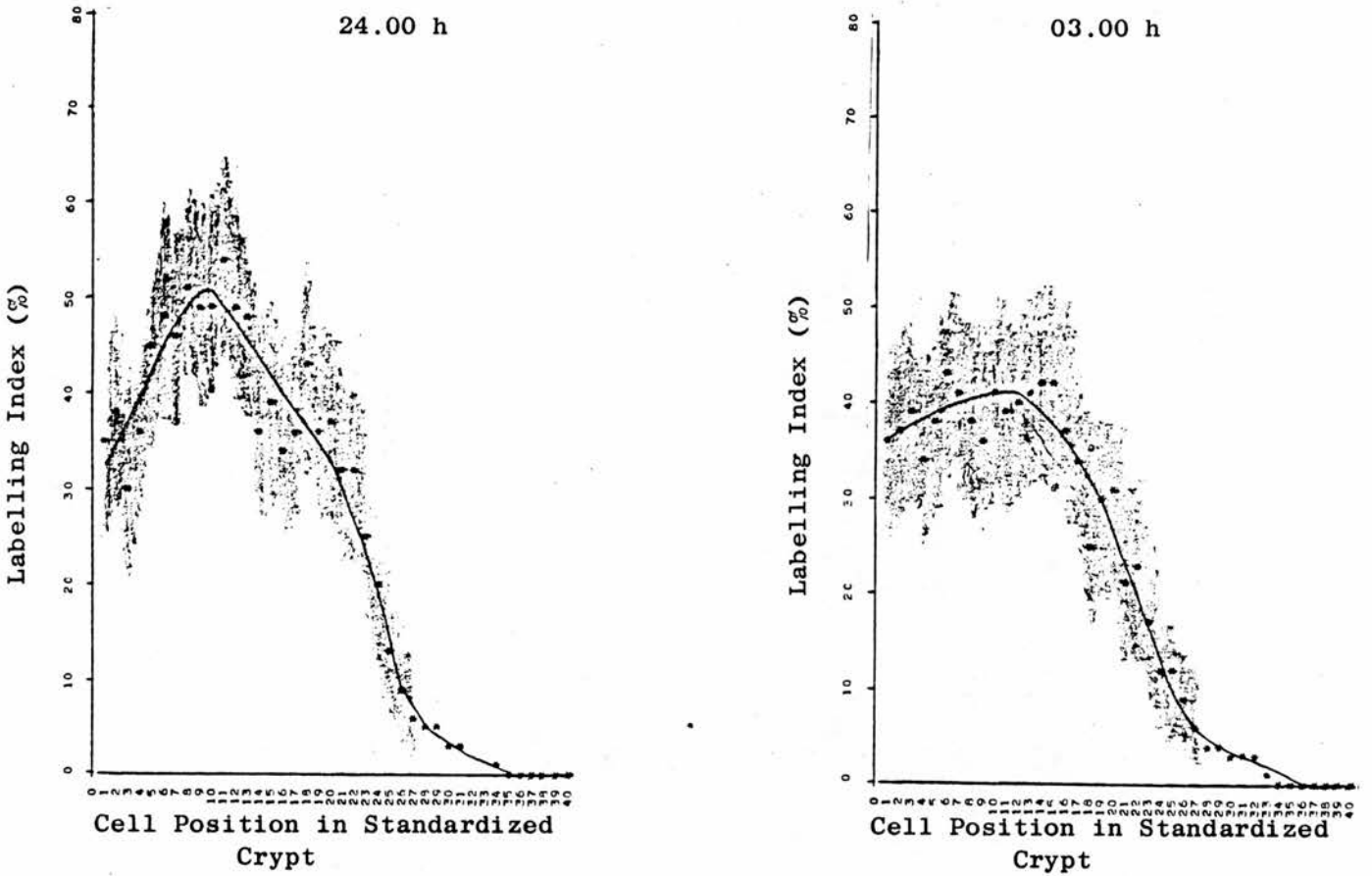
(a) Control Hamsters Continued



Shaded areas indicate 95% confidence limits

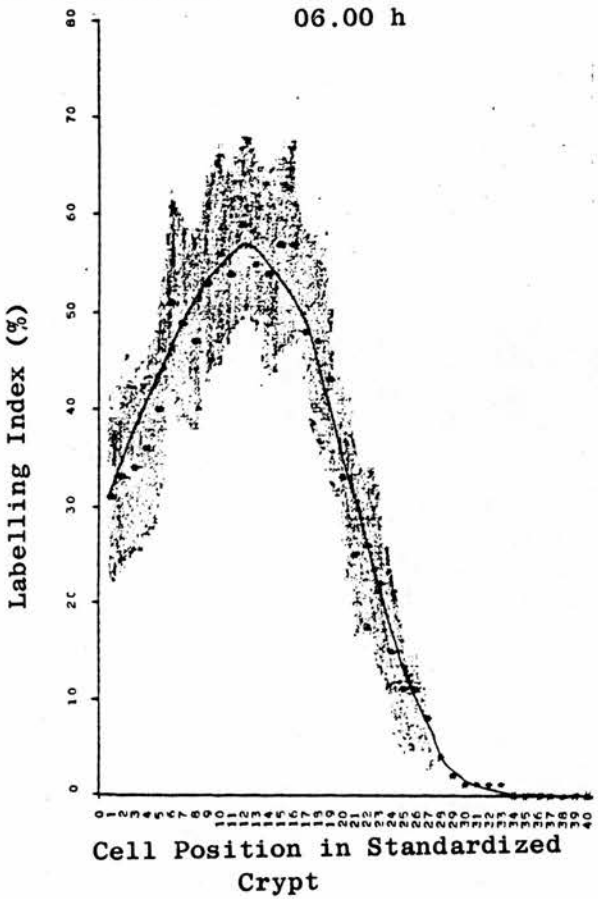
Appendix 1.4 (Continued)

(a) Control Hamsters Continued



Appendix 1.4 (Continued)

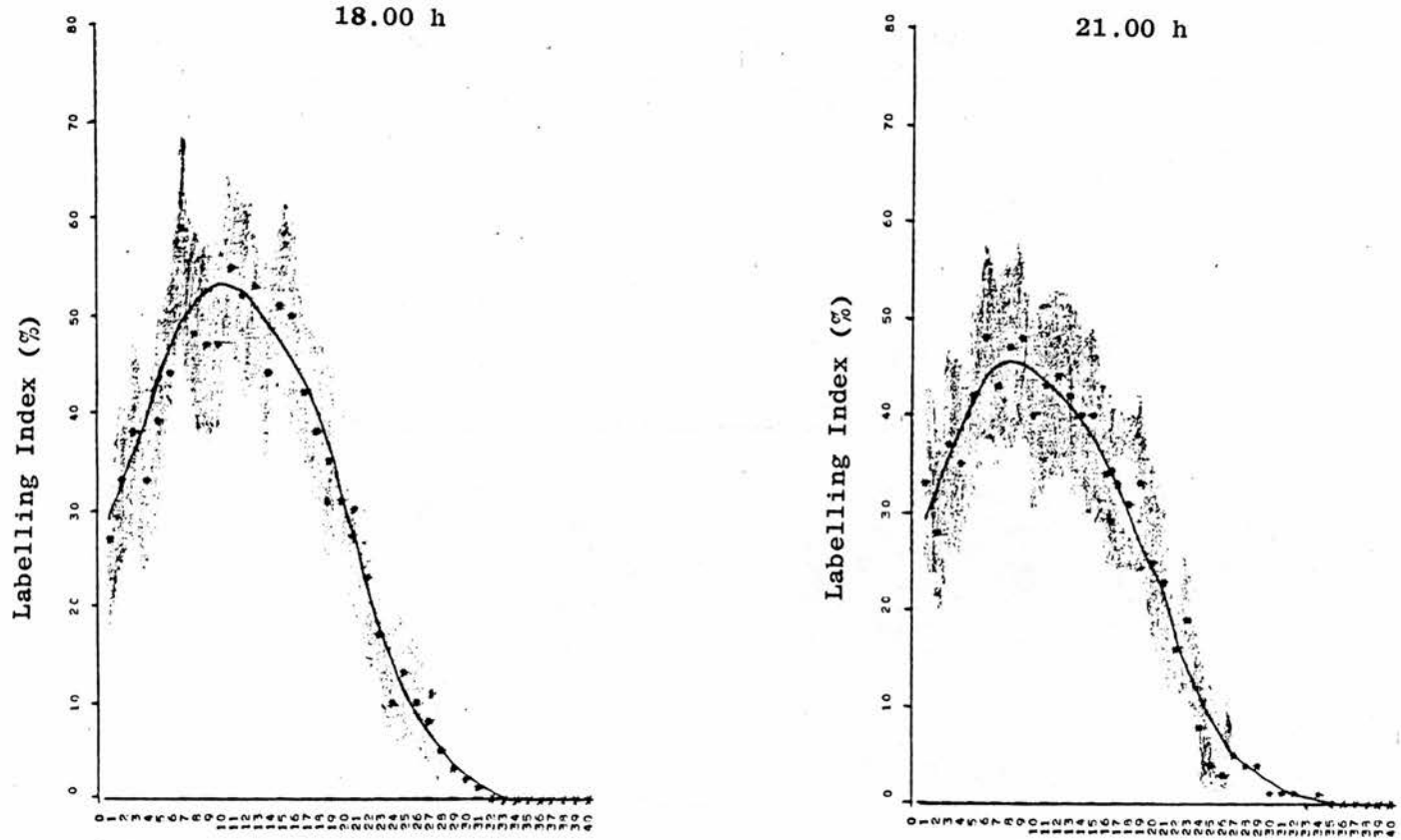
(a) Control Hamsters Continued



Shaded area indicates 95% confidence limits

Appendix 1.4 (Continued)

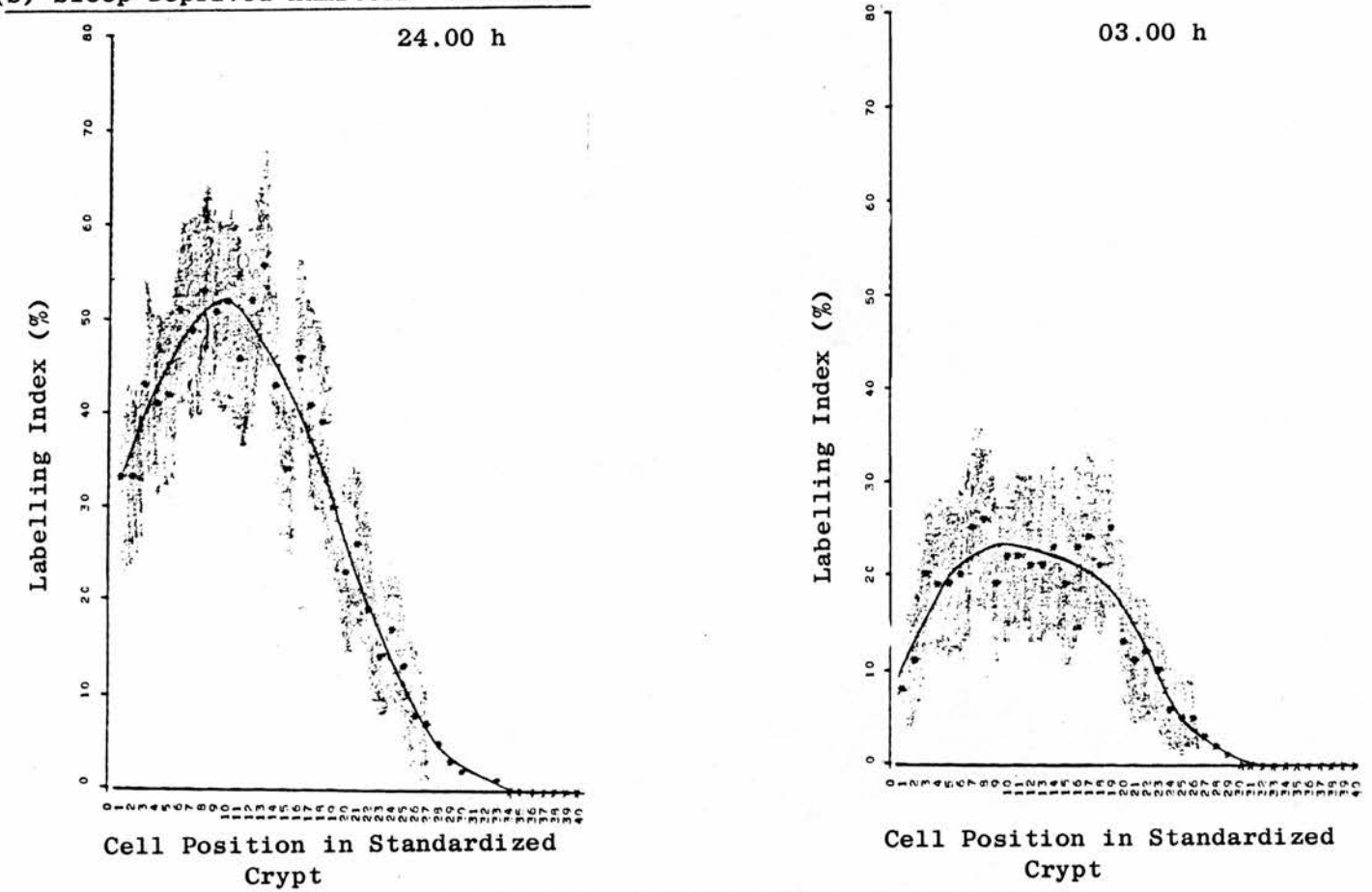
(b) Sleep Deprived Hamsters



Shaded areas indicate 95% confidence limits

Appendix 1.4 (Continued)

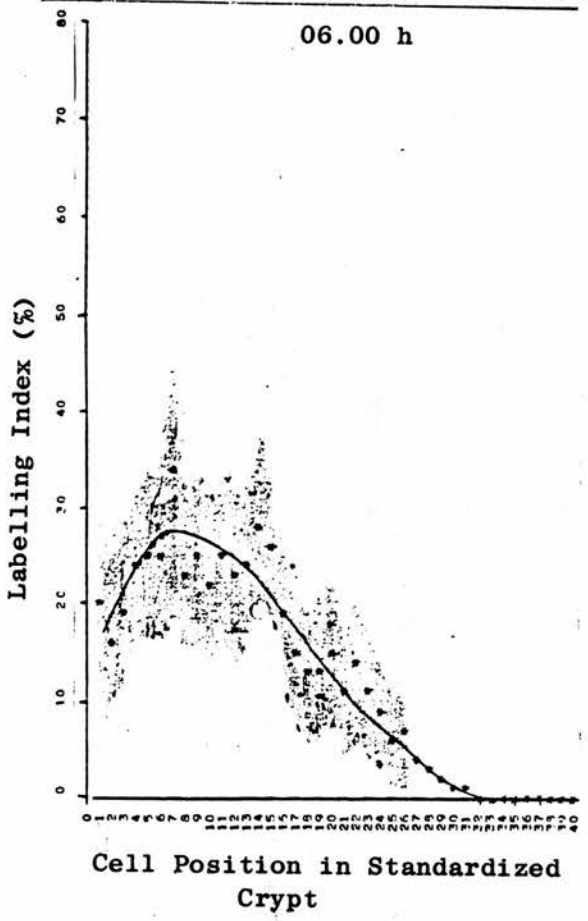
(b) Sleep Deprived Hamsters Continued



Shaded areas indicate 95% confidence limits

Appendix 1.4 (Continued)

(b) Sleep Deprived Hamsters Continued

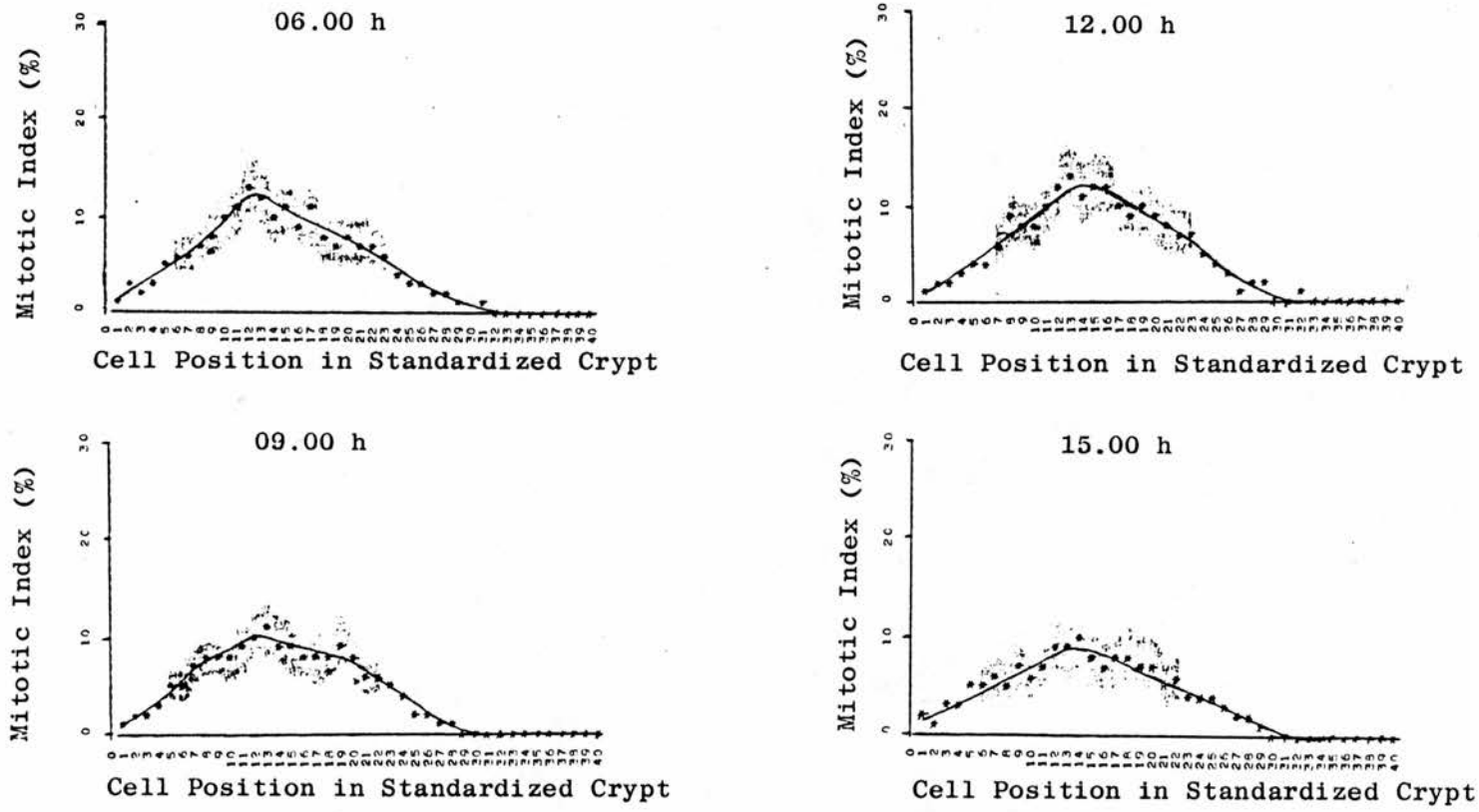


Shaded area indicates 95% confidence limits

Appendix 1.5

Distribution of Mitotic Cells in the Hamster Jejunal Crypt During 24 h (data from 9 hamsters)

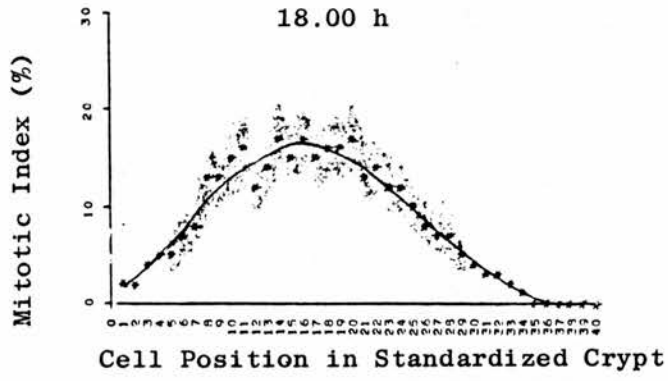
(a) Control Hamsters

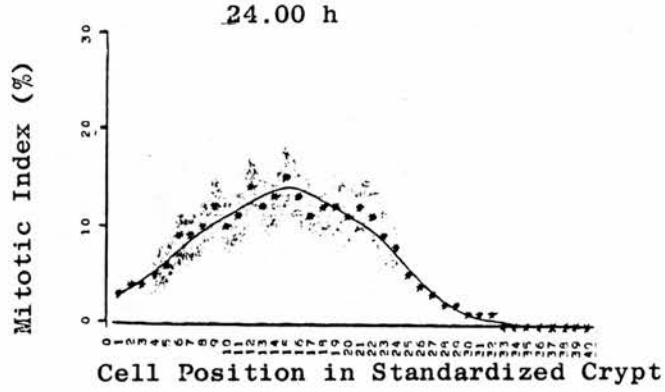


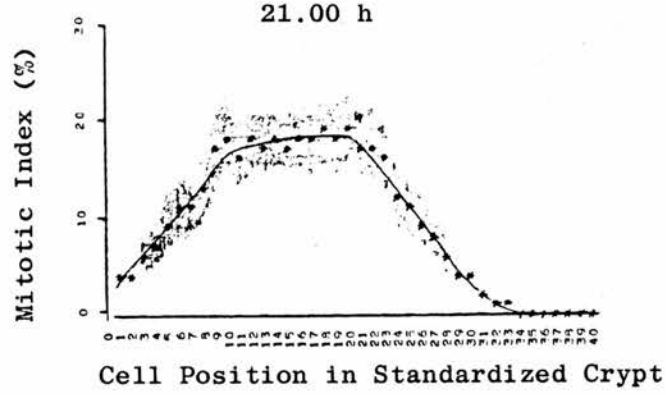
Shaded areas indicate 95% confidence limits

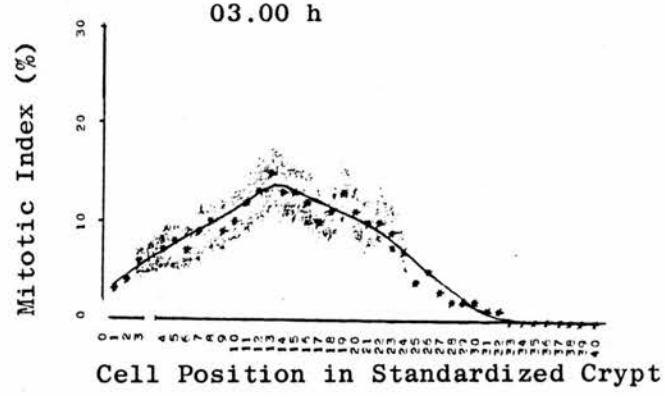
Appendix 1.5 (Continued)

(a) Control Hamsters Continued





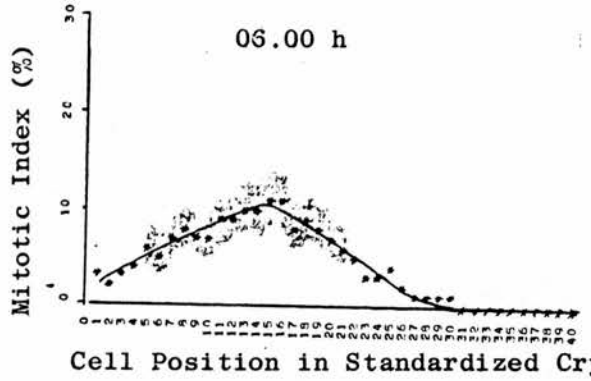




Shaded areas indicate 95% confidence limits

Appendix 1.5 (Continued)

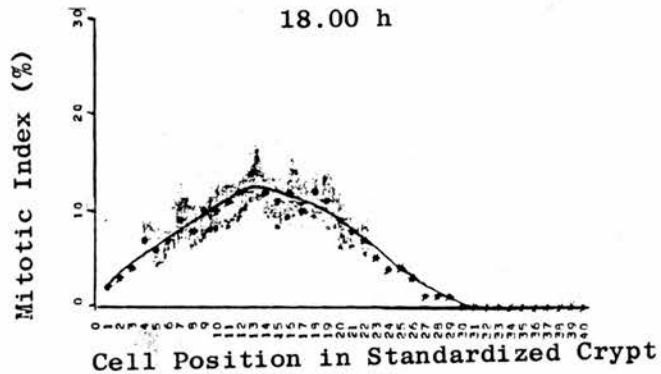
(a) Control Hamsters Continued

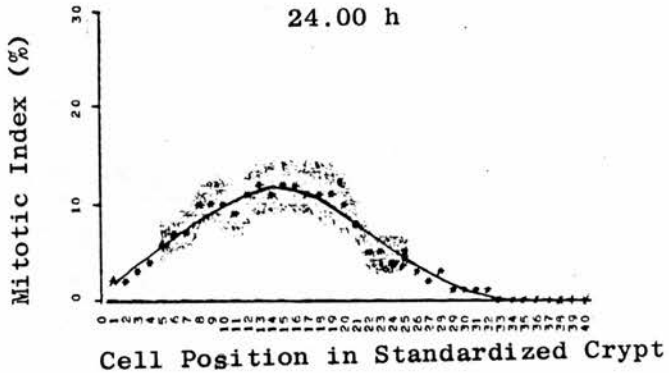


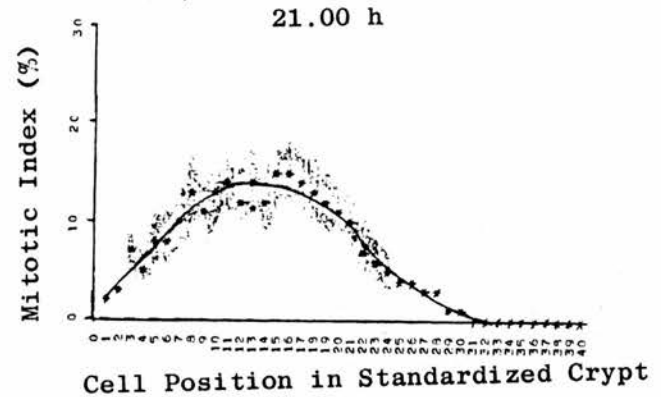
Shaded area indicates 95% confidence limits

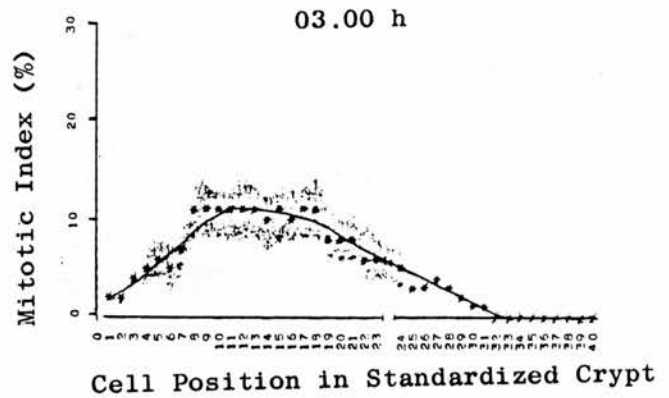
Appendix 1.5 (Continued)

(b) Sleep Deprived Hamsters





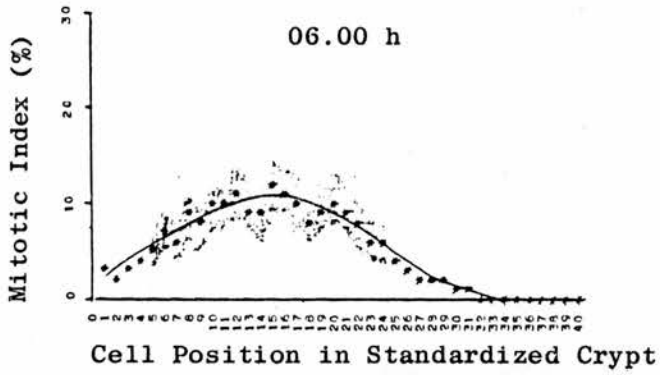




Shaded areas indicate 95% confidence limits

Appendix 1.5 (Continued)

(b) Sleep Deprived Hamsters Continued



Shaded area indicates 95% confidence limits

Appendix 2.1

IE Lymphocyte Count in the Jejunum of the Male Syrian Hamster During 24 h

(Counts in 12 hamsters per time group)

(a) With Sleep (Control Hamsters)

Time (h)	IE Lymphocytes	Villus Epithelial Cells	IE Lymphocytes Per 100 Villus Epithelial Cells
06.00	185	529	35.0
	129	537	24.0
	127	510	24.9
	122	520	23.5
	158	518	30.5
	144	515	28.0
	137	521	26.3
	196	516	38.0
	150	519	28.9
	170	519	32.8
	210	509	41.3
	165	520	31.7
09.00	130	506	25.7
	105	518	20.3
	155	532	29.1
	168	540	31.1
	130	511	25.4
	130	533	24.4
	195	530	36.8
	147	510	28.8
	188	542	34.7
	138	523	26.4
	115	519	22.2
	138	515	26.8
12.00	140	510	27.5
	160	530	30.2
	175	518	33.8
	182	544	33.5
	266	521	51.1
	154	500	30.8
	211	518	40.7
	197	553	35.6
	182	518	35.1
	161	551	29.2
	213	541	39.4
	191	505	37.8

Appendix 2.1 (Continued)

(a) With Sleep (Control Hamsters) Continued

Time (h)	IE Lymphocytes	Villus Epithelial Cells	IE Lymphocytes Per 100 Villus Epithelial Cells
15.00	153	534	28.7
	139	513	27.1
	153	511	29.9
	190	534	35.6
	149	516	28.9
	149	541	27.5
	164	525	31.2
	129	535	24.1
	130	520	25.0
	198	513	38.6
	169	511	33.1
	204	538	37.9
18.00	153	501	30.5
	126	530	23.8
	142	510	27.8
	172	515	33.4
	154	548	28.1
	165	548	30.1
	173	538	32.2
	149	500	29.8
	176	512	34.4
	142	512	27.7
	172	549	31.3
	134	504	26.6
21.00	116	513	22.6
	120	510	23.5
	148	562	26.3
	166	528	31.4
	126	508	24.8
	117	519	22.5
	156	545	28.6
	133	587	22.7
	146	514	28.4
	110	544	20.2
	177	503	35.2
	112	526	21.3

Appendix 2.1 (Continued)

(a) With Sleep (control Hamsters) Continued

Time (h)	IE Lymphocytes	Villus Epithelial Cells	IE Lymphocytes Per 100 Villus Epithelial Cells
24.00	110	533	20.6
	104	532	19.5
	153	518	29.5
	161	502	32.1
	103	546	18.9
	112	504	22.2
	106	513	20.7
	170	529	32.1
	108	511	21.1
	126	548	23.0
	148	525	28.2
	171	504	33.9
03.00	113	522	21.6
	152	504	30.2
	140	512	27.3
	154	549	28.1
	155	531	29.2
	103	519	19.8
	156	536	29.1
	171	527	32.4
	190	503	37.8
	143	535	26.7
	145	559	25.9
	169	532	31.8
06.00	127	542	23.4
	158	508	31.1
	123	508	24.2
	135	531	25.4
	174	567	30.7
	152	514	29.6
	164	525	31.2
	156	532	29.3
	160	525	30.5
	148	521	28.4
	124	531	23.4
	149	529	28.2

Appendix 2.1 (Continued)

(b) With Sleep Deprivation

Time (h)	IE Lymphocytes	Villus Epithelial Cells	IE Lymphocytes Per 100 Villus Epithelial Cells
18.00	134	509	26.3
	152	509	29.9
	130	518	25.1
	127	500	25.4
	149	589	25.3
	170	535	31.8
	109	503	21.7
	136	500	27.2
	132	514	25.7
	161	516	31.2
	172	500	34.4
	199	510	39.0
21.00	123	508	24.2
	128	521	24.6
	183	536	34.1
	162	524	30.9
	177	548	32.3
	221	548	40.3
	178	528	33.7
	136	506	26.9
	113	503	22.5
	186	513	36.3
	141	542	26.0
	113	501	22.6
24.00	201	540	37.2
	167	505	33.1
	174	511	34.1
	211	519	40.7
	237	549	43.2
	187	547	34.2
	276	549	50.2
	223	518	43.1
	191	545	35.0
	226	537	42.1
	180	517	34.8
	206	514	35.0

Appendix 2.1 (Continued)

(b) With Sleep Deprivation Continued

Time (h)	IE Lymphocytes	Villus Epithelial Cells	IE Lymphocytes Per 100 Villus Epithelial Cells
03.00	141	503	28.0
	160	524	30.1
	141	507	27.8
	152	500	30.4
	200	519	38.5
	165	505	32.7
	181	509	35.6
	172	549	31.3
	111	548	20.3
	213	546	39.0
	148	550	26.9
	165	539	30.6
06.00	134	509	26.3
	152	509	29.9
	130	518	25.1
	168	522	32.2
	212	506	41.9
	132	512	25.8
	176	524	33.6
	181	527	34.3
	192	582	33.0
	166	506	32.8
	153	557	27.5
	177	518	34.2

Appendix 3.1

Raw Measurements (mm) of 12 Young Men Over the Study Period

(a) Stature

	Day:	Mon	Tues		Wed		Thurs		Fri		Sat
Subject	Time: (h)	23.00	08.00	23.00	08.00	23.00	08.00	23.00	08.00	23.00	08.00
1		1763	1780	1761	1763	1755	1756	1758	1777	1765	1778
2		1651	1655	1656	1650	1653	1652	1654	1664	1649	1663
3		1721	1733	1722	1717	1711	1717	1719	1733	1722	1729
4		1863	1872	1860	1856	1855	1848	1849	1871	1847	1869
5		1758	1769	1751	1754	1756	1750	1754	1775	1757	1766
6		1742	1747	1741	1730	1734	1726	1727	1743	1736	1744
7		1820	1827	1819	1815	1818	1817	1817	1829	1820	1828
8		1907	1919	1904	1895	1896	1891	1891	1909	1900	1912
9		1855	1857	1848	1841	1843	1842	1843	1853	1845	1859
10		1834	1847	1843	1837	1845	1845	1840	1856	1847	1852
11		1739	1756	1744	1743	1740	1741	1741	1756	1745	1757
12		1777	1802	1787	1780	1777	1773	1782	1794	1785	1800

Appendix 3.1 (Continued)

(b) Cervical Length

Subject	Day : Mon	Tues		Wed		Thurs		Fri		Sat
	Time : 23.00 (h)	08.00	23.00	08.00	23.00	08.00	23.00	08.00	23.00	08.00
1	115	118	117	116	110	110	115	119	126	114
2	129	123	122	118	123	126	127	123	121	127
3	122	122	116	124	123	120	130	139	128	126
4	149	156	150	154	154	149	154	152	149	157
5	153	148	142	152	147	143	150	158	151	155
6	121	118	117	115	111	110	108	116	116	116
7	162	158	160	154	159	158	158	165	162	166
8	138	138	127	125	124	121	124	129	131	132
9	136	135	138	132	128	129	138	132	138	137
10	108	116	113	113	110	110	115	121	124	114
11	119	125	124	126	120	120	123	126	128	121
12	115	123	121	117	107	94	121	124	127	128

Appendix 3.1 (Continued)

(c) Thoracic Length

Subject	Day :	Mon	Tues		Wed		Thurs		Fri		Sat
	Time: (h)	23.00	08.00	23.00	08.00	23.00	08.00	23.00	08.00	23.00	08.00
1		304	320	300	302	302	302	303	313	298	319
2		273	282	269	278	272	271	272	285	280	284
3		276	287	289	279	277	282	277	281	282	279
4		329	338	338	324	325	324	318	342	325	329
5		282	280	284	284	287	286	285	284	285	285
6		309	303	310	306	310	311	307	316	304	314
7		323	312	311	310	309	309	309	309	305	310
8		325	334	332	330	330	323	329	335	333	335
9		322	322	320	318	322	330	318	322	316	329
10		320	316	314	311	317	316	312	316	319	322
11		308	319	313	309	308	308	308	318	304	318
12		308	323	313	315	315	324	312	315	320	314

Appendix 3.1 (Continued)

(d) Lumbar Length

Subject	Day :	Mon	Tues		Wed		Thurs		Fri		Sat
	Time (h) :	23.00	08.00	23.00	08.00	23.00	08.00	23.00	08.00	23.00	08.00
1		159	157	159	160	157	158	158	162	155	163
2		174	178	177	180	177	177	177	179	173	177
3		164	163	154	155	155	152	156	163	147	163
4		182	181	181	187	186	189	186	185	177	185
5		159	179	169	168	168	167	164	172	155	175
6		176	185	180	175	178	178	174	178	175	179
7		162	170	161	160	158	161	163	172	160	160
8		214	216	208	204	210	215	209	217	204	211
9		156	151	156	159	161	157	161	163	153	160
10		171	182	178	180	177	179	172	185	172	181
11		163	163	154	157	157	158	158	160	151	168
12		167	169	166	166	167	168	165	176	166	171

Appendix 3.2

Change Scores (mm) in 12 Young Men Over the Study Period

(a) Stature

Day:	Mon	Tues		Wed		Thurs		Fri	
Subject Time (h):	night 23.-08.	day 08.-23.	night 23.-08.	day 08.-23.	night 23.-08.	day 08.-23.	night 23.-08.	day 08.-23.	night 23.-08.00
1	+17	-19	+2	-8	+1	+2	+19	-12	+13
2	+4	+1	-6	+3	-1	+2	+10	-15	+14
3	+12	-11	-5	-6	+6	+2	+14	-11	+7
4	+9	-12	-4	-1	-7	+1	+22	-24	+22
5	+11	-18	+3	+2	-6	+4	+21	-18	+9
6	+5	-6	-11	+4	-8	+1	+16	-7	+8
7	+7	-8	-4	+3	-1	0	+12	-9	+8
8	+12	-15	-9	+1	-5	0	+18	-9	+12
9	+2	-9	-7	+2	-1	+1	+10	-8	+14
10	+13	-4	-6	+8	0	-5	+16	-9	+5
11	+17	-12	-1	-3	+1	0	+15	-11	+12
12	+25	-15	-7	-3	-4	+9	+12	-9	+15

Appendix 3.2 (Continued)

(b) Cervical Length

Day	Mon	Tues		Wed		Thurs		Fri	
Subject Time (h)	night 23.-08.	day 08.-23.	night 23.-08.	day 08.-23.	night 23.-08.	day 08.-23.	night 23.-08.	day 08.-23.	night 23.-08.00
1	+3	-1	-1	-6	0	+5	+4	+7	-12
2	-6	-1	-4	+5	+3	+1	-4	-2	+6
3	0	-6	+8	-1	-3	+10	+9	-11	-2
4	+7	-6	+4	0	-5	+5	-2	-3	+8
5	-5	-6	+10	-5	-4	+7	+8	-7	+4
6	-3	-1	-2	-4	-1	-2	+8	0	0
7	-4	+2	-6	+5	-1	0	+7	-3	+4
8	0	-11	-2	-1	-3	+3	+5	+2	+1
9	-1	+3	-6	-4	+1	+9	-6	+6	-1
10	+8	-3	0	-3	0	+5	+6	+3	-10
11	+6	-1	+2	-6	0	+3	+3	+2	-7
12	+8	-2	-4	-10	-13	+27	+3	+3	+1

Appendix 3.2 (Continued)

(c) Thoracic Length

Day	Mon	Tues		Wed		Thurs		Fri	
Subject Time (h):	night 23.-08.	day 08.-23.	night 23.-08.	day 08.-23.	night 23.-08	day 08.-23.	might 23.-08.	day 08.-23.	night 23.-08.00
1	+16	-20	+2	0	0	+1	+10	-15	+21
2	+9	-13	+9	-6	-1	+1	+13	-5	+4
3	+11	+2	-10	-2	+5	-5	+4	+1	-3
4	+9	0	-14	+1	-1	-6	+24	-17	+4
5	-2	+4	0	+3	-1	-1	-1	+1	0
6	-6	+7	-4	+4	+1	-4	+9	-12	+10
7	-11	-1	-1	-1	0	0	0	-4	+5
8	+9	-2	-2	0	-7	+6	+6	-2	+2
9	0	-2	-2	+4	+8	-12	+4	-6	+13
10	-4	-2	-3	+6	-1	-4	+4	+3	+3
11	+11	-6	-4	-1	0	0	+10	-14	+14
12	+15	-10	+2	0	+9	-12	+3	+5	-6

Appendix 3.2 (Continued)

(d) Lumbar Length

Day:	Mon	Tues		Wed		Thurs		Fri	
Subject Time (h):	night 23.-08.	day 08.-23.	night 23.-08.	day 08.-23.	night 23.-08.	day 08.-23.	night 23.-08.	day 08.-23.	night 23.-08.00
1	-2	+2	+1	-3	+1	0	+4	-7	+8
2	+4	-1	+3	-3	0	0	+2	-6	+4
3	-1	-9	+1	0	-3	+4	+7	-16	+16
4	-1	0	+6	-1	+3	-3	-1	-8	+8
5	+20	-10	-1	0	-1	-3	+8	-17	+20
6	+9	-5	-5	+3	0	-4	+4	-3	+4
7	+8	-9	-1	-2	+3	+2	+9	-12	0
8	+2	-8	-4	+6	+5	-6	+8	-13	+7
9	-5	+5	+3	+2	-4	+4	+2	-10	+7
10	+11	-4	+2	-3	+2	-7	+13	-13	+9
11	0	-9	+3	0	+1	0	+2	-9	+17
12	+2	-3	0	+1	+1	-3	+11	-10	+5

Appendix 3.3

Height, Weight and Body Mass (Quetelet's Index) of 12 Young Men

Subject	* Height (m)	** Weight (kg)	*** Body Mass
1	1.780	75	23.7
2	1.655	53	19.3
3	1.733	63	21.0
4	1.872	85	24.3
5	1.769	77	24.6
6	1.747	66	21.6
7	1.827	77	23.1
8	1.919	90	24.4
9	1.857	76	22.0
10	1.847	61	17.9
11	1.756	56	18.2
12	1.802	76	23.4

* Height: as measured at 08.00 h on Tues morning following baseline night of sleep

** Weight: as recorded at 23.00 h on Monday at start of study

*** Body Mass: Quetelet's Index $\frac{W}{H^2}$

Appendix 3.4

Raw Measurements (mm) of 6 Middle-aged Men During 24 h:

I With a Night of Sleep

II With a Night of Sleep Deprivation

(a) Stature

Subject		Time (h)						
		08.00	11.00	14.00	17.00	20.00	23.00	08.00
1	I	1712	1711	1707	1704	1700	1704	1723
	II	1713	1711	1709	1704	1708	1710	1707
2	I	1802	1796	1789	1789	1794	1791	1805
	II	1815	1811	1801	1801	1801	1808	1804
3	I	1737	1730	1728	1724	1728	1726	1746
	II	1734	1730	1732	1734	1731	1730	1730
4	I	1802	1797	1797	1797	1796	1796	1817
	II	1801	1800	1797	1801	1803	1806	1802
5	I	1691	1683	1687	1676	1679	1676	1692
	II	1701	1690	1690	1688	1688	1691	1685
6	I	1783	1781	1755	1775	1776	1776	1791
	II	1785	1778	1775	1774	1776	1780	1775

Appendix 3.4 (Continued)

(b) Cervical Length

Subject		Time (h) 08.00	11.00	14.00	17.00	20.00	23.00	08.00
1	I	267	270	268	261	261	267	268
	II	261	260	266	262	266	264	263
2	I	269	260	255	256	259	257	264
	II	253	259	251	256	250	257	253
3	I	242	248	241	234	244	245	252
	II	241	240	246	246	245	246	247
4	I	255	252	256	252	254	254	255
	II	254	253	250	249	251	254	251
5	I	236	232	235	228	230	221	230
	II	246	235	235	231	233	235	234
6	I	263	269	248	267	264	260	265
	II	265	272	267	260	267	260	255

Appendix 3.4 (Continued)

(c) Thoracic Length

Subject		Time (h)						
		08.00	11.00	14.00	17.00	20.00	23.00	08.00
1	I	325	321	319	333	329	330	330
	II	332	336	333	333	327	335	338
2	I	353	349	349	343	342	348	349
	II	388	386	390	381	385	388	390
3	I	289	288	292	299	295	292	290
	II	287	286	284	272	271	278	282
4	I	343	341	339	343	340	338	337
	II	343	333	333	337	335	333	331
5	I	350	346	343	340	340	349	356
	II	320	320	318	317	315	324	319
6	I	324	307	300	304	307	312	314
	II	324	302	319	327	320	329	330

Appendix 3.4 (Continued)

(d) Lumbar Length

Subject		Time (h) 08.00	11.00	14.00	17.00	20.00	23.00	08.00
1	I	165	151	156	146	148	143	161
	II	117	116	111	110	123	115	110
2	I	151	149	144	148	139	149	151
	II	119	112	114	109	111	113	114
3	I	135	125	127	127	118	118	135
	II	164	164	164	177	182	171	166
4	I	105	107	105	103	98	100	122
	II	105	127	123	123	125	127	136
5	I	90	100	102	101	102	101	110
	II	120	124	130	126	133	124	125
6	I	126	132	128	126	128	130	132
	II	132	134	125	128	123	124	123

Appendix 3.5

Change Scores (mm) of 6 Middle-aged Men During 24 h:

I With a Night of Sleep

II With a Night of Sleep Deprivation

(a) Stature

Subject		Time (h) 08.-11.	11.-14.	14.-17.	17.-20.	20.-23.	23.-08.00
1	I	-1	-4	-3	-4	+4	+19
	II	-2	-2	-5	+4	+2	-3
2	I	-6	-7	0	+5	-3	+14
	II	-4	-10	0	0	+7	-4
3	I	-7	-2	-4	+4	-2	+20
	II	-4	+2	+2	-3	-1	0
4	I	-5	0	0	-1	0	+21
	II	-1	-3	+4	+2	+3	-4
5	I	-8	+4	-11	+3	-3	+16
	II	-11	0	-2	0	+3	-6
6	I	-2	-26	+20	+1	0	+15
	II	-7	-3	-1	+2	+4	-5

Appendix 3.5 (Continued)

(b) Cervical Length

Subject		Time (h) 08.-11.	11.-14.	14.-17.	17.-20.	20.-23.	23.-08.00
1	I	+3	-2	-7	0	+6	+1
	II	-1	+6	-4	+4	-2	-1
2	I	-9	-5	+1	+3	-2	+7
	II	+6	-8	+5	-6	+7	-4
3	I	+6	-7	-7	+10	+11	+7
	II	-1	+6	0	-1	+1	+1
4	I	-3	+4	-4	+2	0	+1
	II	-1	-3	-1	+2	+3	-3
5	I	-4	+3	-7	+2	-9	+9
	II	-11	0	-4	+2	+2	-1
6	I	+6	-21	+19	-3	-4	+5
	II	+7	-5	-7	+7	-7	-5

Appendix 3.5 (Continued)

(c) Thoracic Length

Subject		Time (h) 08.-11.	11.-14.	14.-17.	17.-20.	20.-23.	23.-08.00
1	I	-4	-2	+14	-4	+1	0
	II	+4	-3	0	-6	+8	+3
2	I	-4	0	-6	-1	+6	+1
	II	-2	+4	-9	+4	+3	+2
3	I	-1	+4	+7	-4	-3	-2
	II	-1	-2	-12	-1	+7	+4
4	I	-2	-2	+4	-3	-2	-1
	II	-10	0	+4	-2	-2	-2
5	I	-4	-3	-3	0	+9	+7
	II	0	-2	-1	-2	+9	-5
6	I	-17	-7	+4	+3	+5	+2
	II	-22	+17	+8	-7	+9	+1

Appendix 3.5 (Continued)

(d) Lumbar Length

Subject		Time (h) 08.-11.	11.-14.	14.-17.	17.-20.	20.-23.	23.-08.00
1	I	-14	+5	-10	+2	-5	+18
	II	-1	-5	-1	+13	-8	-5
2	I	-2	-5	+4	-9	+10	+2
	II	-7	+2	-5	+2	+2	+1
3	I	-10	+2	0	-9	0	+17
	II	0	0	+13	+5	-11	-5
4	I	+2	-2	-2	-5	+2	+22
	II	+22	-4	0	+2	+2	+9
5	I	+10	+2	-1	+1	-1	+9
	II	+4	+6	-4	+7	-9	+1
6	I	+6	-4	-2	+2	+2	+2
	II	+2	-9	+3	-5	+1	-1

Appendix 3.6

Height, Weight and Body Mass (Quetelet's Index) of 6 Middle-aged Men

Subject	* Height (m)	** Weight (kg)	*** Body Mass
1	1.723	78	26.3
2	1.805	64	19.6
3	1.746	67	22.0
4	1.817	77	23.3
5	1.692	83	29.0
6	1.791	75	23.4

* Height: as measured at 08.00 h immediately following a night of sleep

** Weight: as recorded at start of study

*** Body Mass: Quetelet's Index $\frac{W}{H^2}$

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Circadian variation of lymphocyte subpopulations: a study with monoclonal antibodies

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Abstract

Use of monoclonal antibodies to identify subpopulations of circulating lymphocytes in healthy adults showed pronounced circadian variations in total T cells, the two major T cell subsets, and HLA-DR$^+$ lymphocytes. When the results for the T cell subsets were expressed as a ratio (helper:suppressor) no significant rhythmic variation was observed. Lymphocytes bearing a surface antigen identified by the HNK-1 antibody (a population containing the natural killer and antibody dependent killer activity) did not show significant rhythmic variation.

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There was an inverse relation between plasma cortisol concentration and numbers of T and B cells.

These observations have therapeutic implications and should be considered in the course of immunological monitoring.

Introduction

Circadian variation of biological phenomena is often ignored by both doctors and scientists. In this report we describe wide circadian variation of some of the lymphocyte subsets, which play a part in the development and regulation of the immune response. This study was made possible by the development of a range of well characterised monoclonal antibodies, which in conjunction with flow cytometry permit rapid, precise enumeration of functionally distinct lymphocyte subpopulations.[1 2] As a consequence of these technological developments lymphocyte subpopulations are under intensive study in a wide variety of diseases. For example, alterations of the relative proportions of the T "helper" and T "suppressor/cytotoxic" populations in peripheral blood of recipients of renal allografts correlate with rejection episodes.[3] Since importance is being attached to relatively small changes in the ratio of T helpers to suppressors we considered it important to establish a baseline by investigating physiological variation of the T cell subsets and other subpopulations with a role in the immune response.

TABLE 1—*Panel of monoclonal antibodies used*

Monoclonal antibody	Cell population identified
Anti-Leu-1, Anti-Leu-4	All T cells
Anti-Leu-3a	Helper subset of T cells
Anti-Leu-2a, Anti-Leu-2b	Suppressor/cytotoxic subset of T cells
Anti-HLA-DR	B cells and activated T cells (monocytes*)
HNK-1 (Anti-Leu-7)	NK and K cells

* Monocytes were excluded from analysis using 90° light scatter variables (see text).

Methods

Peripheral blood samples were obtained at intervals of three hours

from 10 healthy volunteers (mean age 51, range 45-58). Within the constraints of the blood sampling the subjects performed normal daily activities. They slept in a sleep research laboratory, of which they all had previous experience.

Total and differential white cell counts were performed on each blood sample. Plasma was separated and stored frozen for subsequent analysis of cortisol concentrations. Mononuclear cells were isolated on Ficoll Hypaque and then reacted with a panel of monoclonal antibodies to lymphocyte surface antigens (table I). Mouse monoclonal antibodies were either conjugated with fluorescein isothiocyanate direct or were visualised with a second, antimouse antibody conjugated with fluorescein isothiocyanate. All analyses were performed on unfixed cell preparations with a fluorescence activated cell sorter (FACS IV, Becton Dickinson FACS Systems, Sunnyvale, California). Ten thousand cells were analysed for each antibody, the number of fluorescent cells being expressed as a percentage of the total lymphocytes. Non-lymphocytic cells contaminating the preparations were excluded from analysis using scatter gates set on the 90° light scatter profile.[3a] The proportions of cells that were fluorescent on analysis with the fluorescence activated cell sorter were used in conjunction with the total and differential white cell counts to calculate absolute numbers of cells in each subpopulation.

Statistical analyses of the percentages and absolute numbers of cells were performed using the methods of Nelson *et al*,[4] which entailed fitting sine curves to the data from individual subjects and testing whether the variables of these curves showed consistent patterns of circadian variation.

Results

Variations of all the lymphocyte subpopulations were noted over 24 hours of study. The variations were significant ($p < 0{\cdot}05$) for absolute numbers of Leu-1$^+$, Leu-4$^+$ (total T cells), Leu-3a$^+$ (T helper subset), Leu-2a$^+$, Leu-2b$^+$ (T suppressor/cytotoxic subset), and HLA-DR$^+$ (B cells and activated T cells) lymphocytes. In contrast, HNK-1$^+$ cells did not exhibit significant circadian variation. Figure 1 shows the variation in the Leu-3a$^+$ subset of cells, with peak levels occurring at night. When expressed as percentages of the total number of lymphocytes Leu-3a$^+$ and HNK-1$^+$ cells showed significant variation.

Plasma cortisol concentration showed the expected large amplitude rhythm and varied inversely with the numbers of T and B cells (fig 2).

The ratio of T helper to suppressor cells (Leu-3a$^+$:Leu-2b$^+$) showed only minor variations over time and did not exhibit significant rhythmic variation.

Table II summarises the data for each subset.

Discussion

There is now considerable evidence that the magnitude of the immune response varies with the time of day.[5] The findings of this study may in part explain the variations by demonstrating changes in the number of circulating cells available to encounter and process antigen.

Circadian variation of total lymphocytes,[6] surface Ig^+ cells,[7] and cells forming rosettes with sheep erythrocytes[8] has been reported previously. Our use of monoclonal antibodies in

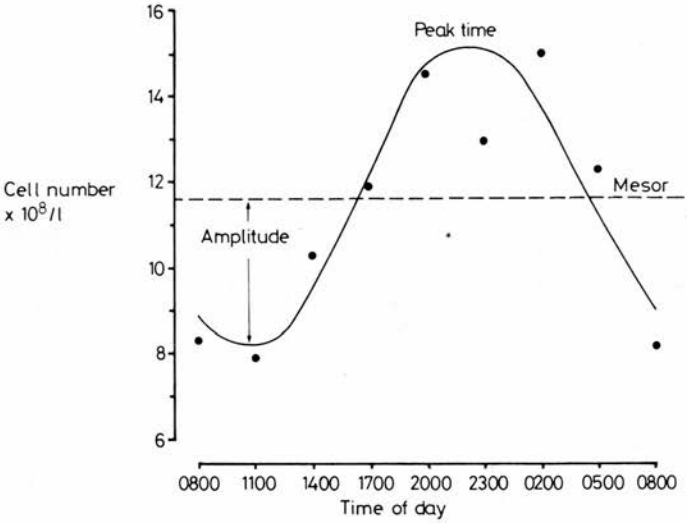

FIG 1—Circadian variation of Leu-3a$^+$ cells (helper subset of T cells). (Each point represents mean value in 10 subjects.) Mesor 11·66; peak time 2232; circadian variation (2 × amplitude/mesor) = 59%.

conjunction with flow cytometry permitted further subclassification of the lymphocyte population and greater precision in enumeration. It would have been impossible to analyse this number of samples and to count the cells in each sample by light or fluorescent microscopy. The development[9] and characterisation[10–12] of the HNK-1 monoclonal antibody enabled us to identify cells with NK and K cell function by surface labelling, without the necessity for time consuming functional assays.

The variation of numbers of circulating B and T cells points to sizable shifts of cells into and out of the circulation. The

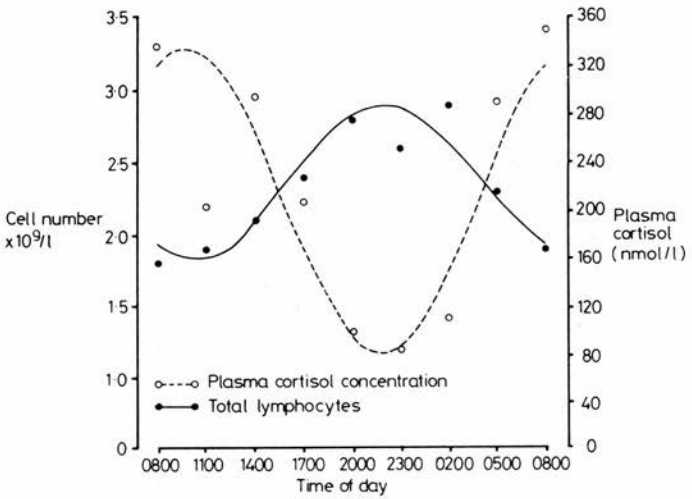

FIG 2—Comparison of circadian variations of total lymphocytes and plasma cortisol concentration. (Each point represents mean value in 10 subjects.)
Conversion: SI to traditional units—Cortisol: 1 nmol/l ≈ 36·3 ng/100 ml.

TABLE II—*Circadian variation of lymphocyte subpopulations contrasted with plasma cortisol variation (data derived from best fitting sine curves)*

	Mesor	Amplitude	Peak time	Circadian variation (%)	Significance of circadian rhythm
			Results expressed as absolute number of cells $\times 10^9/l$		
Leu-4⁺	16·0	4·3	2222	54	$p<0.05$
Leu-3a⁺	11·7	3·4	2232	59	$p<0.001$
Leu-2b⁺	4·3	1·0	2156	47	$p<0.05$
HLA-DR⁺	3·5	0·9	2305	53	$p<0.05$
HNK-1⁺	2·5				NS
			Results expressed as percentage of total lymphocytes		
Leu-4⁺	66·2				NS
Leu-3a⁺	47·4	5·1	2339	22	$p<0.05$
Leu-2b⁺	17·9				NS
HLA-DR⁺	14·5				NS
HNK-1⁺	11·0	2·8	1145	51	$p<0.01$
			Other results		
Helper: suppressor ratio	2·6				NS
Plasma cortisol (nmol/l)	208	126	0948	121	$p<0.01$

Conversion: SI to traditional units—Cortisol: 1 nmol/l ≈ 36·3 ng/100 ml.

traffic of cells through other lymphoid tissue, including bone marrow, may allow for contact between antigen primed regulatory T cells and B cells. The lack of variation of HNK-1$^+$ cells correlates with the fact that only 0·7% of nucleated bone marrow cells have this phenotype.[10] It also correlates with the finding that in lymphoid tissues HNK-1$^+$ cells are predominantly located within germinal centres,[12] which are not regarded as part of the lymphocyte recirculation pathway.[13]

The striking inverse relation of the numbers of circulating T and B cells and plasma cortisol concentration, in conjunction with the reported T lymphopenia in patients with Cushing's syndrome[14] and transient lymphopenia after single doses of exogenous corticosteroid,[15 16] suggest a causal interrelation.

The results have certain clinical implications. For example, would the efficiency of antilymphocyte chemotherapy be improved if administration was timed to produce peak plasma concentrations when the maximum number of cells are in the circulation? Does rejection occur earlier if allografts are transplanted at night, when the number of T cells in the recipients' circulation are at their peak? With respect to the last question, circadian variation in the rejection of rat renal allografts has been described,[17] and the longest surviving grafts were those inserted when the numbers of circulating lymphocytes were at their trough. Sound data relating time of operation to onset of rejection, or graft survival, in man are lacking, although circadian variation in episodes of renal allograft rejection has been suggested.[18]

We have shown that in any attempt at immunological monitoring account must be taken of circadian variation of lymphocyte subpopulations as a source of variance in results. Conversely, we found the ratio of helper to suppressor cells to be fairly stable in our healthy subjects.

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ADDENDUM—Six of the subjects were studied over a further 24 hour period, during which they were deprived of sleep. There were no significant differences in any of the rhythmic variations over this period when compared with the first set of data for these six subjects.

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