Effects of the endogenous clock and sleep time on melatonin, insulin, glucose and lipid metabolism

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Abstract

This study was undertaken to determine whether the internal clock contributes to the hormone and metabolic responses following food, in an experiment designed to dissociate internal clock effects from other factors. Nine female subjects participated. They lived indoors for 31 days with normal time cues, including the natural light: darkness cycle. For 7 days they retired to bed from 0000 h to 0800 h. They then underwent a 26-h 'constant routine' (CR) starting at 0800 h, being seated awake in dim light with hourly 88 Kcal drinks. They then lived on an imposed 27-h day (18 h of wakefulness, 9 h allowed for sleep), for a total of 27 days. A second 26-h CR, starting at 2200 h, was completed. During each CR salivary melatonin and plasma glucose, triacylglycerol (TAG), non-essential fatty acids (NEFA), insulin, gastric inhibitory peptide (GIP) and glucagon-like peptide-1 (GLP-1) were measured hourly. Melatonin and body temperature data indicated no shift in the endogenous clock during the 27-h imposed schedule. Postprandial NEFA, GIP and GLP-1 showed no consistent effects. Glucose, TAG and insulin increased during the night in the first CR. There was a significant effect of both the endogenous clock and sleep for glucose and TAG, but not for insulin. These findings may be relevant to the known increased risk of cardiovascular disease amongst shift workers.

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Introduction

Health problems of shift workers include disturbed sleep patterns and poor sleep quality, increased incidence of cardiovascular disease and gastrointestinal disorders. The causes are multifactorial; however a major factor is considered to be the maladaptation of endogenous circadian rhythms to abrupt changes in shift times (Akerstedt 1995). The endogenous clock governing the circadian system normally adapts slowly to imposed phase shifts such as are found in shift work and jet lag and many night shift workers show very little adaptation. To the extent that any physiological function depends on internal circadian timing it will show abnormal responses during the period of adaptation. There is good evidence that the use of strategies to hasten circadian adaptation to night shift results in improved sleep, alertness and performance (Czeisler *et al*. 1990). In contrast, there is a paucity of data concerning the time-dependent hormonal and metabolic responses to food and to what extent they are governed by the circadian clock. Glucose tolerance decreases during the day in normal individuals (Carroll & Nestel 1973, Service *et al*. 1983). The persistence of a diurnal variation of glucose tolerance following a standard meal (Owens *et al*. 1996) or glucose itself (Van Cauter *et al*. 1989) in a constant routine (constant environment, continuously low levels of activity, constant caloric intake and absence of sleep) suggests that at least part of the variation is controlled by the circadian clock. Diurnal variation has also been reported in a number of gastrointestinal hormones including glucose-dependent insulinotropic peptide (GIP), gastrin and motilin but to our knowledge these have not been assessed in a constant routine.

We have recently reported that immediately after a simulated 9-h phase advance, such as might be found in rotating shift work or after Eastward time zone change, postprandial insulin and glucose responses to a standard mixed meal were significantly greater than to the same meal at the same external clock time before the phase shift (Hampton *et al*. 1996). The changes were suggestive of insulin resistance, a risk factor for cardiovascular disease (Reaven 1988). Furthermore, triacylglycerol (TAG) showed delayed postprandial clearance after the phase shift indicating possible lipid intolerance – another risk factor for cardiovascular disease. Thus each time a day worker starts night shift or an adapted night worker starts a day shift they are likely to show hormonal and metabolic abnormalities. In view of the importance of the associated pathology we consider that it is important to investigate the mechanisms involved in order to devise counter measures.

In order to determine to what extent the internal clock contributes to the hormonal and metabolic responses

observed following a meal we have undertaken an experiment designed to dissociate the effects of the internal clock from other factors.

Materials and Methods

Subjects

Nine female undergraduates took part in the study. They were aged 19–20 years and of mean body mass index $20 \cdot 19 \pm 2 \cdot 98$ (range $16 \cdot 42$–$26 \cdot 30$). Ethical permission for the study was obtained from the University of Wales Swansea Ethical Committee in accordance with the guidelines issued by the Royal College of Physicians 1984 and all subjects gave written informed consent. The study took place between 22 March and 21 April 1995.

Experimental protocol

The subjects entered a small hall of residence at the University of Swansea in which they were then confined for a total of 31 days. Normal time cues were present throughout the study, including clocks and watches, access to television and radio programmes and the natural light: darkness cycle through windows. For the first 7 days of the study they were required to retire to bed in darkness at 0000 h and were woken at 0800 h the following morning to establish normal circadian rhythmicity. Following this baseline period the subjects underwent a 26-h 'constant routine' (constant routine 1) starting at 0800 h, during which they remained seated (except for visits to the toilet), awake at a desk under dim light conditions (range $0 \cdot 5$–42 lux). Subjects were given liquid test 'meals' of Complan (Beechams, Brentford, Middx, UK) at hourly intervals on the hour throughout the constant routine period. Each 'meal' contained 12 g carbohydrate, $4 \cdot 2$ g protein and $2 \cdot 9$ g fat with an energy content of 384 kJ (total energy intake/24 h=9592 kJ). The total 24-h energy and macronutrient intake was representative of a typical Western diet. At the end of the first constant routine subjects were allowed to sleep for nine hours and were then requested to live on a 27-h day (18 h of wakefulness, 9 h allowed for sleep), but still in the presence of normal time cues. Thus bed times and meal times were delayed by 3 h each day. A total of 17 × 27 h days were completed i.e. just over 19 × 24 h days. The study ended with a second 26-h constant routine as before but starting at 2200 h (constant routine 2). The schedule is shown in Fig. 1. The subjects ate a controlled vegetarian diet throughout the study except during the constant routines. The meals were chosen to be similar in terms of energy and macronutrient content. Subjects' mean daily energy intake was 9287 ± 173 kJ, comprising $49 \cdot 5 \pm 2 \cdot 0\%$ carbohydrate, $38 \cdot 1 \pm 1 \cdot 6\%$ fat and $12 \cdot 4 \pm 2 \cdot 0\%$ protein.

Sampling and recording

Throughout the study subjects wore wrist activity monitors (Gaehwiler actigraphs, Gaehwiler Electronics,
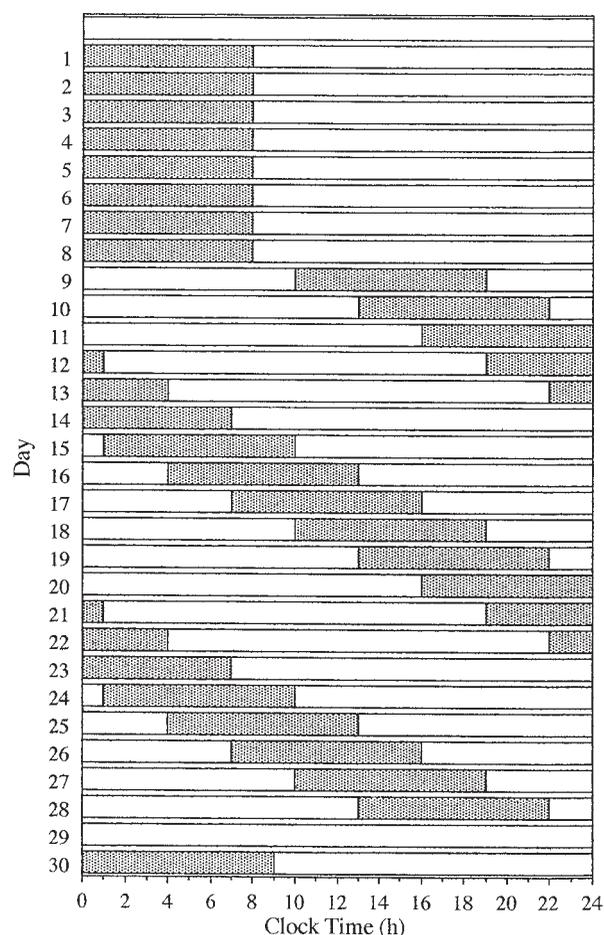

Figure 1 Experimental schedule used to force desynchrony in volunteers. Shaded areas, periods of enforced rest or sleep with lights turned off. Open areas, subjects awake.

Hombrechtikon, Switzerland) and rectal temperature was recorded continuously every 6 min (Squirrel data loggers, Eltek, Cambridge, Cambs, UK). Throughout the study tests of mood and cognition were completed at 2-h intervals whilst subjects were awake. Prior to each constant routine subjects were cannulated in an arm vein under local anaesthetic (lignocaine) and two fasting blood samples (12 ml) were taken. Twelve millilitres blood were then withdrawn at hourly intervals 30 min following ingestion of each Complan drink into lithium–heparin tubes for the measurement of TAG, insulin, GIP and glucagon-like peptide-1 (GLP-1) and into fluoride oxalate tubes for glucose estimation. To prevent GLP-1 and GIP degradation, a protease inhibitor, aprotinin (200 KIU/ml blood) was added to the tubes used to collect these samples. Plasma was separated immediately by centrifugation, aliquoted and stored at -20 °C. At the same time 5 ml saliva was collected into a polypropylene vial, frozen and stored at -20 °C.

Biochemical analyses

The analysis and results for activity and rectal temperature are reported elsewhere (Minors *et al.* 1996). Melatonin was measured in saliva by RIA (English *et al.* 1993), with reagents supplied by Stockgrand Ltd (University of Surrey, Guildford, Surrey, UK). The limit of detection was $1·5 ± 0·2$ pg/ml and the inter- and intra-assay coefficients of variation were less than 10%. Plasma immunoreactive insulin, GIP and GLP-1 levels were measured using established in-house radioimmunoassays (Hampton 1983, Morgan *et al.* 1978, Elliott *et al.* 1993). The sensitivities of the immunoreactive insulin, GIP and GLP-1 assays were 19, 30 and 8 pmol/l respectively. Their inter- and intra-assay coefficients of variation were all less than 10%. Plasma glucose, non-esterified fatty acids (NEFA) (Wako, Neuss, Germany) and TAG (Roche, Basle, Switzerland) were measured by standard automated enzymatic spectrophotometric methods. The interassay coefficients of variation were less than 5% for these assays. For all analyses, samples obtained from each constant routine from each subject were measured in the same assay.

Data and statistical analyses

Data from the fasting blood samples for insulin, glucose, GIP, GLP-1, TAG and NEFA from each leg were evaluated by paired *t*-tests. Postprandial hormone data from each leg were then subjected to repeated measures analysis of variance, factor time, with a Greenhouse–Geisser correction for sphericity, to determine the presence of any variations. The two fasting samples and the first postprandial sample were excluded as subjects were considered not to have reached a steady postprandial state until two hours after the start of the 'meals'.

Melatonin onset was defined as the time when the saliva concentration reached 4 times the limit of detection of the assay, provided that this was followed by two further detectable values, or the time of the value at 2 times the s.d. of the mean baseline where detectable, provided that this was followed by two further equal or greater values. There were no important differences in the timing of melatonin (see Results) indicating that no shift in the endogenous clock had occurred during the 27-h imposed schedule. This observation was also confirmed by the core temperature data (Minors *et al.* 1996). Thus, for each constant routine, internal 'clock' time was constant in relation to real time, whereas length of prior wakefulness and actual sleep time for any given 'real time' were different. This effectively enables a separate estimation of the effects on the variables studied of the endogenous circadian clock (C) and of the displaced sleep/wake cycle as assessed by length of prior wakefulness (W). For example, the first sample of each constant routine (at 0830 h and 2230 h real time respectively) was 0·5 h after wake up, the second (at 0930 and 2330 h real time) was

1·5 h after wake up etc. Thus, using the sampling sequence as the base, W is constant and C is different. Comparison of the data from each constant routine, using sample number as the base, by repeated measures ANOVA using a Greenhouse–Geisser correction for sphericity, was used to determine whether there was a reliable C effect. Subtraction of the data from each leg at each postprandial sampling time after wake up leads to an estimate of the internal clock contribution to the variability for each subject. Having derived the internal clock component for each subject this can then be subtracted from the raw data from each leg to give the W component. The time-dependent changes of glucose, TAG, insulin, GIP and GLP-1 were evaluated in this way and the two components C and W were then subjected to repeated measures analysis of variance, factor time, with a Greenhouse–Geisser correction, to assess the reliability of any effects. In view of the design of the experiment it was necessary to combine adjacent values such that 2-hourly means (referenced to the mid point of the 2-h period) were used for calculation of W in order to normalise the time periods compared.

Results

The salivary melatonin profiles plotted as a function of clock time and of time after waking are shown in Fig. 2 for each constant routine. Inspection suggests that the overall timing of melatonin production with respect to external clock time was unchanged albeit with a small change in duration of secretion. Mean onset and offset times for leg one were 21 h 46 min ± 45 min and 8 h 45 min ± 53 min, and for leg two were 21 h 35 min ± 1 h 23 min and 8 h 56 min ± 30 min respectively, indicating that no shift in the endogenous clock had occurred during the 27-h imposed schedule.

Mean fasting levels of hormones and metabolites are shown in Table 1. There were no significant differences in fasting glucose, insulin, TAG, NEFA, GIP or GLP-1 levels between the constant routines for the two fasting samples.

The plasma profiles for glucose, insulin and TAG, plotted as a function of clock time and of time after waking are shown in Figs 3, 4 and 5. Two subjects with blocked cannulae provided insufficient samples for this initial analysis. In each case, plotted as a function of time after waking, the differences between the legs appear as the contribution of the internal clock component. Plotted as a function of external clock time the differences appear as the contribution of displaced sleep (and external time cues) to the variability between the study legs. All three variables show an increase during the night around 2200 h to 0600 h on constant routine 1, with a minor daytime peak in the case of glucose and TAG and a larger daytime peak in the case of insulin. The profiles for constant
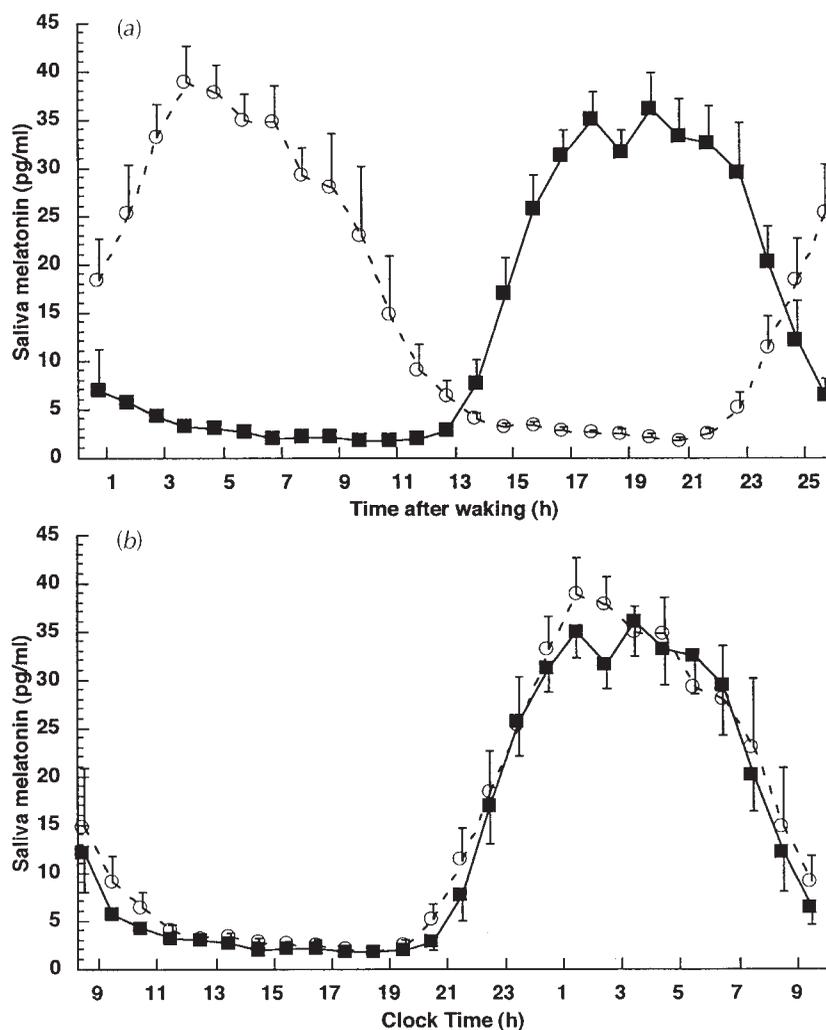
Figure 2 Salivary melatonin concentrations in healthy volunteers during the two constant routines (*a*) plotted as a function of time after waking and (*b*) postprandial samples plotted as a function of clock time. Values are means ± S.E.M., *n*=9. ■, first constant routine; ○, second constant routine.

Table 1 The effect of clock time on fasting hormone and metabolite concentrations. Plasma concentrations in subjects at the beginning of each constant routine day are shown. Values are means ± S.E.M., *n*=7

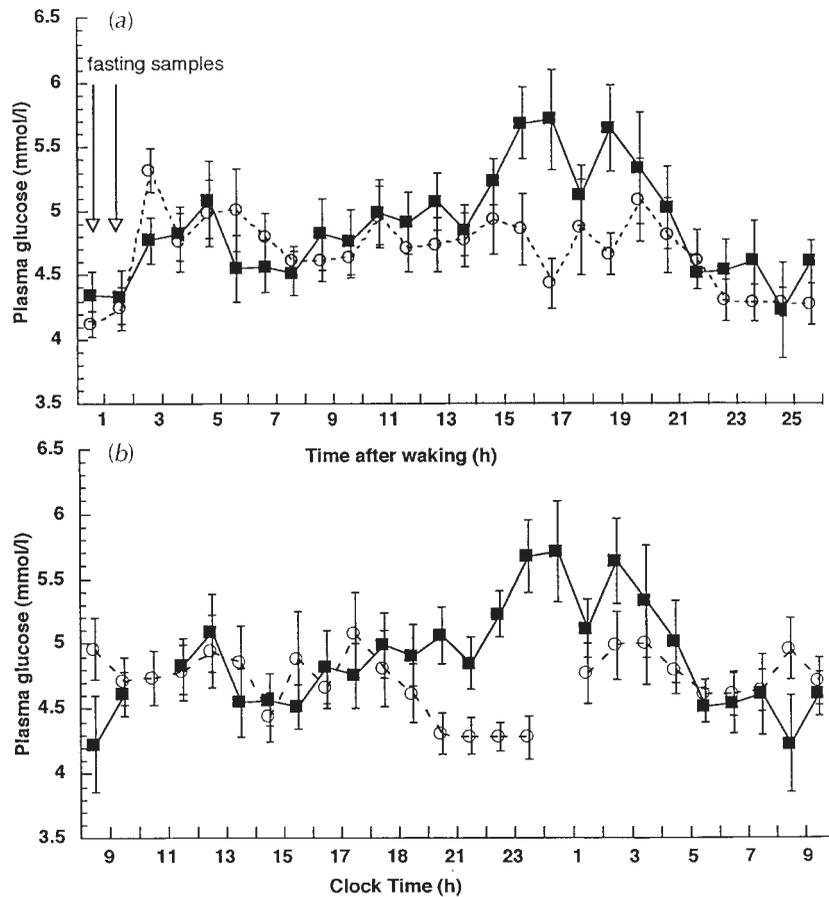|	Glucose (mmol/l)	TAG (mmol/l)	NEFA (mmol/l)	Insulin (pmol/l)	GIP (pmol/l)	GLP-1 (pmol/l)
Constant routine 1 (0830 h)	4·4 ± 0·2	1·3 ± 0·1	0·4 ± 0·1	77 ± 15	34·6 ± 9·4	16·1 ± 2·6
Constant routine 2 (2230 h)	4·2 ± 0·1	1·2 ± 0·1	0·4 ± 0·1	91 ± 16	19·6 ± 3·8	13·4 ± 2·5

Figure 3 Plasma glucose concentrations in healthy volunteers during the two constant routines (a) plotted as a function of time after waking and (b) postprandial samples plotted as a function of clock time. Values are means ± S.E.M., n=7. ■, first constant routine; ○, second constant routine.

routine 2 after living on a 27-h day suggest that the major and minor peaks in the case of glucose and TAG have become more comparable, with a reduction in the major peak and an increase in the minor peak. This is also apparent when the data are plotted as a function of time after wake up.

Repeated measures analysis of variance with respect to time for each constant routine showed significant differences for TAG and glucose on the first constant routine ($P<0.05$) with a trend towards differences for insulin ($P=0.09$). Significant variation with respect to time was found for plasma TAG on the second constant routine ($P<0.05$). Subsequent differentiation of the C and W components as described above necessitated a complete set of samples from each subject; one further subject was eliminated from the analysis due to missing samples, leaving a total of 6 subjects. Subtraction of leg 1 from leg 2 as time after waking provided an estimate of C.

Subsequent subtraction of the C component from the raw data expressed as real time provided an estimate of W. The reliability of C and W was then assessed by repeated measures analysis of variance. The results are shown in Table 2. There was a significant effect of both the endogenous clock, C, and of the displaced sleep/wake cycle, W, for glucose and TAG but not for insulin.

Plasma NEFA levels were suppressed following feeding. They were, however, very variable and no significant differences in postprandial levels were found either with respect to time or between the two constant routines. Postprandial plasma GLP-1 data were also very variable and showed no consistent rise following feeding, or difference between the two constant routines. Plasma GIP levels rose following feeding. No significant differences in postprandial levels were found either with respect to time or between the two constant routines. These data are not shown and were not analysed further.
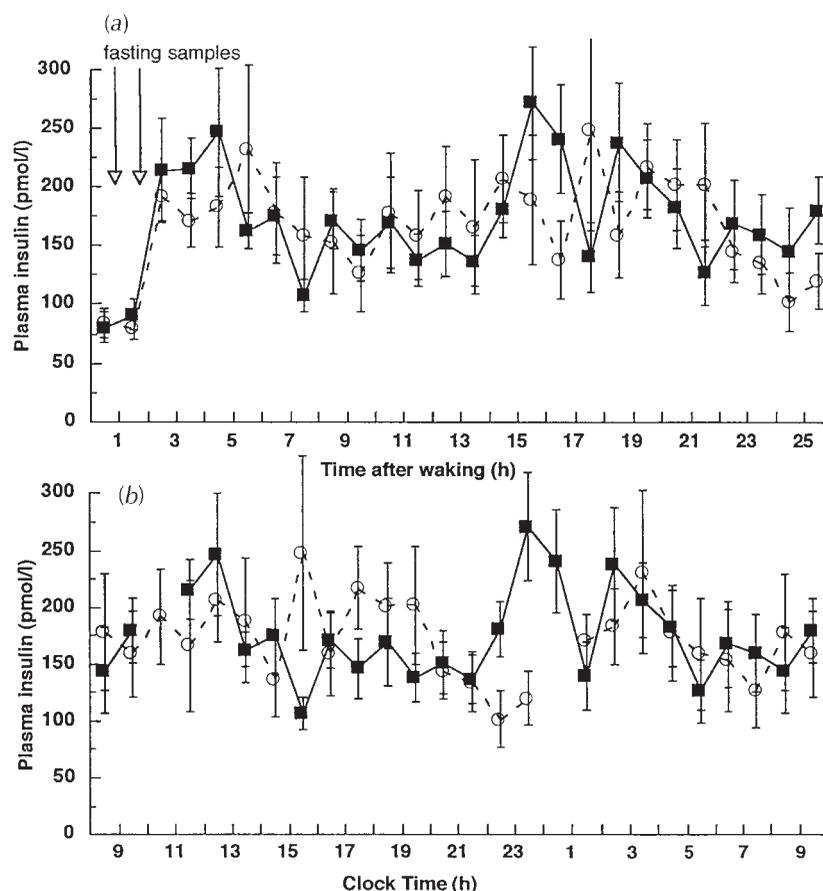
Figure 4 Plasma insulin concentrations in healthy volunteers during the two constant routines (*a*) plotted as a function of time after waking and (*b*) postprandial samples plotted as a function of clock time. Values are means ± S.E.M., *n*=7. ■, first constant routine; ○, second constant routine.

Discussion

Melatonin is considered to be the best marker rhythm for the circadian clock (Arendt 1995). Individual melatonin profiles are highly reproducible and are less subject to masking factors than previously used marker rhythms such as core temperature and cortisol. The major masking factor for melatonin is sufficiently bright light with a minor effect of posture. If light and posture are controlled, salivary or plasma melatonin provides an accurate estimate of circadian phase position. The present data indicate that living on a 27-h day with access to all normal time cues does not shift the melatonin rhythm. The contribution of sleep timing in these circumstances is clearly negligible. As melatonin, and indeed core temperature, did not shift we can be certain that the endogenous clock was not affected by the imposed 27-h day and remained in the same phase position with respect to external clock time. During the first constant routine the contribution of the internal clock and of external factors such as time after wake up were

presumed to be additive as subjects were in phase with the normal day. Similarly, during the second constant routine the contribution of the internal clock and external factors were presumed to be dissociated due to the displacement of the sleep–wake cycle, a situation commonly encountered by shift workers.

In the normal phase (the first constant routine) plasma glucose levels are significantly higher at night. This is in agreement with previous reports of impaired glucose tolerance following oral glucose (Aparicio *et al.* 1974), bolus or continuous intravenous glucose infusion (Van Cauter *et al.* 1989, Lee *et al.* 1992) or meals (Hampton 1983, Van Cauter *et al.* 1992) at night. These effects are due to a relative insulin resistance at night, causing decreased insulin-mediated peripheral glucose uptake, and are inversely correlated with the diurnal variation in circulating cortisol (Van Cauter *et al.* 1992). Dissociation of the internal clock and sleep in the second constant routine leads to a dampening of the peak response to glucose and the appearance of two comparable peaks, suggesting a
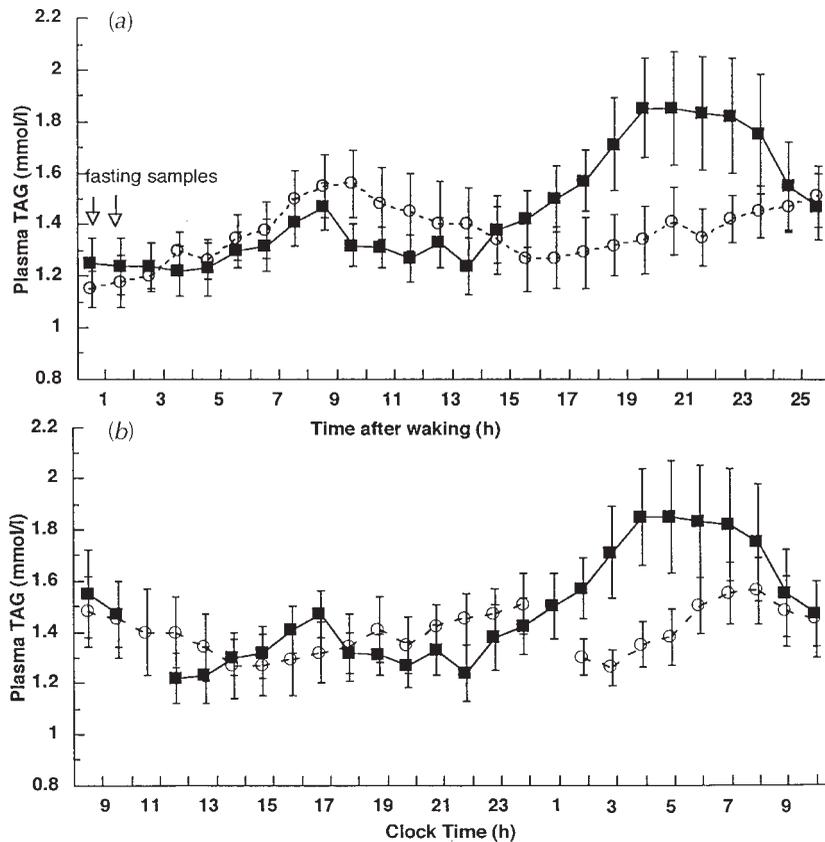
Figure 5 Plasma TAG concentrations in healthy volunteers during the two constant routines (a) plotted as a function of time after waking and (b) postprandial samples plotted as a function of clock time. Values are means ± S.E.M., *n*=7. ■, first constant routine; ○, second constant routine.

'spreading' of glucose intolerance such that it occurs at two periods of the day. Sleep has been shown to increase circulating glucose levels following constant i.v. glucose infusion, due to decreased glucose utilisation, although diurnal variations in glucose have been shown to persist in the absence of sleep (Scheen *et al.* 1996). Diminished brain utilisation (which is insulin independent) has been shown in one study to account for approximately two thirds of

Table 2 Reliability of the endogenous circadian effects due to 'clock time' (the 'C' component) and those due to length of prior wakefulness (the 'W' component) for circulating glucose, TAG and insulin. (See text for details)

	C component (probability of significant effect)	W component (probability of significant effect)
Glucose	0·036	0·040
Insulin	0·099	0·055
TAG	0·0074	0·046

this effect; peripheral glucose utilisation accounted for the remainder (Boyle *et al.* 1994). The present study has shown for the first time that the length of wakefulness also has an effect on glucose tolerance, independent of any circadian rhythm or sleep itself.

Circulating insulin levels follow a similar pattern to the plasma glucose levels: they tended to be higher at night in the first constant routine but the differences did not achieve statistical significance. This is in agreement with previous workers who have found an inconsistent rise in plasma insulin levels at night, despite the raised plasma glucose levels (Shapiro *et al.* 1988, Van Cauter *et al.* 1992). This failure to observe a compensatory rise in plasma insulin in response to the raised glucose has been shown to be partially due to a diminished sensitivity of the pancreatic B cell to glucose (Lee *et al.* 1992) and also to an increase in the rate of insulin clearance from the plasma at night (Van Cauter *et al.* 1992). Measurement of insulin secretion rates using individual C-peptide kinetics (Van Cauter *et al.* 1992) would be of value in future work.

The gastrointestinal peptides, GIP and GLP–1, stimulate insulin secretion via the enteroinsular axis (Morgan 1992). Their secretion rates are influenced both by the rate of gastric emptying and absorption of nutrients. Previous studies have shown a circadian rhythm in the gastric emptying rate of solid meals, with decreased emptying rates at night (Goo *et al.* 1987). The present study failed to demonstrate consistent differences in post-prandial GIP and GLP-1 levels on either of the constant routines. This indicates that differences in gastric emptying and absorption rates are unlikely to affect carbohydrate and lipid tolerance in the present study, and that these gastrointestinal hormones are not responsible for the observed variations in circulating insulin.

This report details for the first time the striking increase of plasma TAG at night and its dissociation into two significant components related respectively to the internal clock and to time after wake up. There have been few studies of the diurnal variation in circulating lipid levels and the factors which might influence them. Diurnal changes in plasma TAG not under constant routine conditions have been shown (Romon *et al.* 1997); the magnitude of these changes correlates with fasting TAG levels (Fainaru *et al.* 1994). Expression of the alpha-subtype hepatic peroxisome proliferator-activated receptor shows a diurnal rhythm which parallels corticosterone in the rat (Lemberger *et al.* 1996). This receptor indirectly influences TAG hydrolysis via regulation of apoprotein CIII synthesis and may, therefore, affect circulating TAG levels. A circadian rhythm of low density lipoprotein (LDL) receptor activity has been demonstrated which is influenced by cortisol, but not mediated by it (Balasubramaniam *et al.* 1994). Melatonin itself has been shown to inhibit LDL receptor activity and cholesterol synthesis in human mononuclear leucocytes (Müller-Wieland *et al.* 1994); preliminary work in our laboratory (Chapman 1997) indicates that melatonin also influences lipoprotein lipase (LPL) activity, a key regulatory enzyme in circulating TAG clearance, in adipose tissue. LPL is activated by insulin; a lower nocturnal insulin sensitivity could, therefore, be associated with lower LPL activity and relatively impaired plasma TAG clearance. Insulin also suppresses very low density lipoprotein (VLDL) secretion by the liver (Sparks & Sparks 1993); nocturnal insulin resistance could, therefore, also be associated with higher circulating TAG levels of hepatic origin.

Both insulin resistance and elevated circulating postprandial TAG concentrations are independent risk factors for cardiovascular disease (Reaven 1988, Sethi *et al.* 1993). There are several reasons why elevated postprandial TAG may confer increased cardiovascular disease risk. Chylomicron and VLDL remnant lipoproteins, derived from TAG–rich chylomicrons and VLDL, have been shown to be atherogenic, and raised plasma TAG pre-disposes towards the formation of small dense LDL, a lipoprotein subfraction whose elevation is also established as a risk factor for cardiovascular disease (Griffin *et al.* 1994). Our findings have important implications for shift workers, who may be eating at night during unadapted night shifts when a pattern of relative glucose and lipid intolerance is found. Shift workers have increased plasma TAG levels independent of energy and nutrient intake (Romon *et al.* 1992). They also have a higher risk of developing cardiovascular disease than day-time workers (Kawachi *et al.* 1996, Knutsson 1989). A growing part of the population in industrialised countries has irregular or displaced working hours, working during the early morning, late evening or night. The development of dietary or shift scheduling strategies and the timing of meals relative to sleep in order to minimise insulin insensitivity and postprandial lipid intolerance would, therefore, be desirable.

Acknowledgements

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