J. Physiol. (1983), **345**, *pp.* 451–467
With 7 text-figures
Printed in Great Britain

DOES 'ANCHOR SLEEP' ENTRAIN CIRCADIAN RHYTHMS? EVIDENCE FROM CONSTANT ROUTINE STUDIES

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(*Received 1 March 1983*)

SUMMARY

1. Experiments have been performed in an isolation unit to investigate the effects of abnormal sleep–waking schedules upon circadian rhythms of renal excretion and deep-body temperature.

2. In confirmation of previous work, nychthemeral rhythms appeared to be 'anchored' to a 24 h period if 4 h sleep was taken regularly each day, even though another 4 h was taken irregularly.

3. The endogenous components were investigated by assessing circadian rhythmicity under constant routine conditions, that is, when rhythmic influences in the environment and sleep–waking pattern had been minimized.

4. Analysis of the constant routine data indicated the presence of a rhythmic component which had been stabilized to a period of 24 h by the 'anchor sleep'. In addition, a delayed component was also present.

5. The starting time of the constant routines produced a direct effect upon the rhythms, which was presumed to result from removing the 'masking' effect that sleep normally exerts upon rhythms. There was some evidence that the relative importance of the masking effect and the delayed component depended upon the variable under consideration.

6. The implications of these findings, in terms of the effects of anchor sleep, the presence of more than one internal clock and the usefulness of constant routines, are discussed.

INTRODUCTION

Living under normal, nychthemeral circumstances, circadian rhythms in humans are synchronized to a period of exactly 24 h. This adjustment is achieved by means of rhythmic cues from the external world (for example: the alternation of light and dark; the sleep–waking cycle; social influences, etc.) known as zeitgebers (Aschoff, 1951; Wever, 1979; Minors & Waterhouse, 1981*a*; Moore-Ede, Sulzman & Fuller, 1982).

Such synchrony is upset by changes in the routine of sleep and wakefulness, as after time-zone transitions and during shift work, and there is believed to be a link between an abnormal phasing of circadian rhythms and what is commonly called 'jet-lag syndrome' or the general malaise experienced by shift workers. Attempts to alleviate

these difficulties have been made either by allowing time for recuperation after a flight (Klein & Wegmann, 1979, 1980), by changing the frequency of shift rotation (Rutenfranz, Knauth & Colquhoun, 1976; Rutenfranz, Colquhoun, Knauth & Ghata, 1977; Winget, Hughes & LaDou, 1978), or by seeking characteristics of the worker that might better suit him to shift work (Patkai, Akerstedt & Pettersson, 1977; Folkard, Monk & Lobban, 1979; Reinberg, Vieux, Ghata, Chaumont & Laporte, 1979; Reinberg, Andlauer & Vieux, 1981; Minors & Waterhouse, 1983).

Recently, interest has been shown also in other abnormal work–rest regimens (likely to be used by naval or other military personnel) in which schedules approximating to 18 h 'days' or irregular days are involved (Colquhoun, Paine & Fort, 1978, 1979; Schaefer, Kerr, Buss & Haus, 1979; Beare, Bondi, Biersner & Naitoh, 1981; Minors & Waterhouse, 1983). The circadian rhythms of subjects undergoing these schedules have in common a component which has a period in excess of 24 h. This would seem to be a 'free-running component' either because regular zeitgebers are missing or because those that are present have a period outside the range of entrainment of the internal clock (see also Aschoff, 1981).

In addition, in experiments performed in an isolation unit, it has been shown that if 4 h of sleep are taken regularly (but the other 4 h continue to be taken irregularly) then rhythms of urinary excretion and deep-body temperature continue to show a period indistinguishable from 24 h. It can be argued that a regular sleep–wake cycle is the intermediary by which many zeitgebers (light–dark; eating–fasting; social influences) exert their effects upon the internal clock and, accordingly, these sleeps have been called 'anchor sleeps' (Minors & Waterhouse, 1981*b*, *c*).

A difficulty of interpretation of these data exists however, since it is known that sleep also exerts a direct, 'masking' effect upon many rhythms, generally decreasing the value for a variable (Aschoff, 1978, 1981; Mills, Minors & Waterhouse, 1978*a*). Thus, the stability observed in the anchor sleep experiments might have resulted from the regular masking effect of the 4-h sleep and the internal clock need not have been synchronized to a 24-h day, but rather have continued to 'free-run'. The further observations that the stabilized rhythms showed a constant relationship to sleep when anchor sleep was taken at different times (Minors & Waterhouse, 1981*b*, *c*) and that rhythms during night work appeared to adjust so that they bore a normal relationship to mid-sleep (Reinberg, Chaumont & Laporte, 1975; Knauth, Rutenfranz, Herrmann & Poeppl, 1978), accord with the view that sleep is an important determinant of the phase of circadian rhythms, but do not yet enable a distinction between 'masking effect' and zeitgeber to be made.

A further difficulty arises in that the size of the direct effect of sleep might be expected to depend upon the variable under consideration (Minors & Waterhouse, 1982). This is clearly seen when human subjects live on non-24-h 'days' (Simpson, Lobban & Halberg, 1970; Mills, Minors & Waterhouse, 1977). Under these conditions, variables manifest two components to their rhythms: an exogenous component with a period equal to that of the artificial day and one which is endogenous and has a free-running period in excess of 24 h. In the isolation unit experiments (Mills *et al.* 1977) urinary flow, urate, calcium and phosphate excretory rhythms showed marked exogenous components, deep-body temperature and urinary potassium excretory rhythms showed a marked endogenous component and urinary sodium and chloride

excretory rhythms were intermediate. An implication of this is that 4-h anchor sleeps will be more likely to produce stability by masking in the cases of urinary flow, urate, etc., than temperature and urinary potassium rhythms.

One way in which the endogenous component can be studied with less interference is to attempt to minimize exogenous rhythmicity. This can be achieved by requiring subjects to stay awake in surroundings of constant temperature, lighting, humidity and social contact for 24 h and to take regular, identical snacks throughout. This regimen is called a 'constant routine' and its usefulness in assessing changes of the endogenous component has already been tested after simulated time-zone transition (Mills, Minors & Waterhouse, 1978*b*), 21-h 'days' (Minors & Waterhouse, 1981*d*) and has been referred to in connexion with shift work (Akerstedt, 1979).

In summary, the aims of the present study are: (1) to investigate again if taking 4-h sleeps regularly can appear to stabilize circadian rhythms; (2) to assess, by means of constant routines, what happens to the endogenous components during such anchor sleep schedules; and (3) to establish whether such results depend upon the relative importance of endogenous and exogenous components in the variables under consideration.

METHODS

Experiments were performed on presumed healthy human subjects in an isolation unit fully described elsewhere (Elliott, Mills, Minors & Waterhouse, 1972). Subjects of the same sex were studied in groups of three or four, each group being denoted by a pair of letters and individuals within a group by a number. Further details of subjects are shown in Table 1.

TABLE 1. Details of subjects and experimental protocols

				Start of constant routines		
Group	No.	Sex	Age	1st	2nd	Time of anchor sleep
BQ	4	M	18–19	04.00	04.00	No anchor sleep
AX	4	F	18–20	04.00	04.00	24.00–04.00
AZ	4	M	19–20	04.00	04.00	24.00–04.00
BB	4	M	19–21	04.00	04.00	20.00–24.00
BC	4	F	18–22	04.00	04.00	20.00–24.00
BH	4	M	18–19	04.00	24.00	20.00–24.00
BI	4	M	18–20	04.00	24.00	20.00–24.00
BN	3	F	19–22	08.00	24.00	20.00–24.00
BR	4	M	19–20	08.00	24.00	20.00–24.00

Experiments of the same design were performed at different times of the year so that there was no systematic effect due to any annual variations.

Five experimental protocols were used and are shown in Fig. 1. The first (group BQ, Fig. 1*A*) was a control experiment in which subjects slept and ate at normal times (sleep 24.00–08.00) except on two occasions, in the middle and at the end of the experiment when this routine was interrupted for the constant routine (Mills *et al.* 1978*b*). Each started at 04.00 and lasted 24 h; subjects remained awake and sedentary throughout and took an identical snack each hour, the composition of which resulted in a normal 24-h intake of sodium (200 mmol), chloride (200 mmol) and potassium (80 mmol). Following the first constant routine, subjects were allowed to sleep 04.00–12.00 to recuperate sleep loss but slept thereafter 24.00–08.00 until the second constant routine.

All other protocols were divided into two phases: an initial control phase during which subjects slept at a conventional time (24.00–08.00) followed by an experimental phase during which sleep

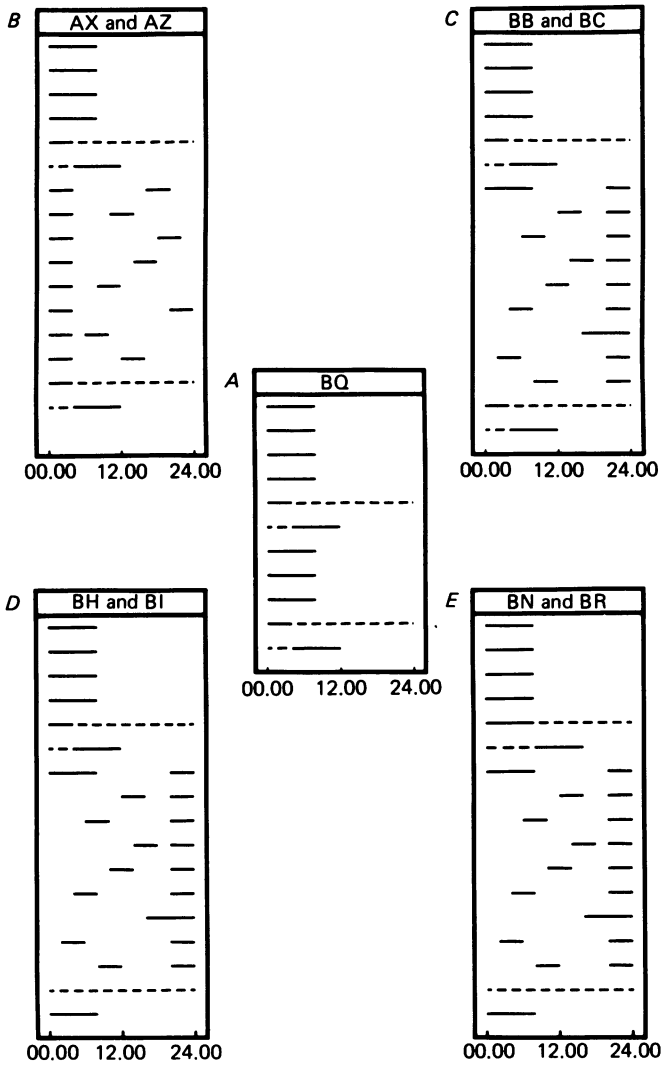

Fig. 1. Experimental schedules. Each day extends across the Figure and successive days are shown below each other. ——, time spent asleep in the dark. ------, constant routine. *A*, group BQ; *B*, groups AX and AZ; *C*, groups BB and BC; *D*, groups BH and BI; *E*, groups BN and BR.

was taken in two 4-h periods; one of these sleeps was taken at the same time each day (referred to as anchor sleep) and the other at irregular times. Throughout both phases, however, both the timing and composition of meals were kept as near as possible to conventional values. During the control phase and again at the end of the experimental phase subjects underwent constant routines as described for group BQ. The experimental protocols for the other groups differed in the times of the anchor sleep period and the start of the constant routine during both the control phase and the experimental phase. The protocols are shown in Fig. 1*B*, groups AX and AZ; 1*C*, groups BB and BC; 1*D*, groups BH and BI; and 1*E*, groups BN and BR, and are detailed in Table 1.

Throughout all experiments subjects micturated every 2 h during the hours of wakefulness (hourly on the constant routines) and collected a sample on waking which encompassed each sleep period. Subjects collected all urine passed and noted its volume and the time of micturation. An

aliquot was then refrigerated for subsequent analysis by AutoAnalyzer II for sodium, potassium, chloride, creatinine, phosphate, calcium and urate. The rate of excretion of each of these was calculated for each collection period, the rates of creatinine excretion being used to correct for bladder-emptying errors as described by Longson & Mills (1953).

In addition, throughout each experiment the subjects' rectal temperature was measured using a thermistor probe inserted 10 cm beyond the external anal sphincter. For groups AX, AZ, BB and BC this temperature was measured hourly by the subjects during the hours of wakefulness and monitored continuously while they were asleep. For the other groups, the rectal temperature was measured every 2 min throughout using a Thermolog recording system (see Halberg, Fanning, Halberg, Cornelissen, Wilson, Griffiths & Simpson, 1981, for details).

Circadian rhythms were sought by the fitting of cosine curves to the data for each variable. For the temperature data the single cosinor method (Nelson, Tong, Lee & Halberg, 1979) was used and for the urinary data the method of Fort & Mills (1970). To determine the period of any rhythm, a spectrum of cosine curves with periods from 22–27 h in increments of 0·2 h was fitted to each variable. The most appropriate period was assessed as that of the fitted cosine curve which minimized the residual error. For all groups this spectral analysis was applied to the data obtained during the 96 h before the second constant routine. In addition, 24-h cosine curves were fitted to each successive day between the two constant routines to assess the daily acrophase (time of maximum). In all cases a fitted cosine curve was accepted only if $P < 0{\cdot}05$ that the amplitude was zero.

The data from the two constant routines were analysed by cross-correlating the data. Such cross-correlation was performed by introducing a series of phase lags (incrementing by 1 h) to the first constant routine data and mixing each in any proportion (0:10–10:0) with all other phase lags (0–23 h) of the same data. The mixture so achieved which, when cross-correlated with the second constant routine data, yielded the highest correlation coefficient was accepted as describing the phase changes in the control rhythm which had occurred during the experimental phase.

Other statistical analyses will be described as they arise in the following section.

RESULTS

When the daily acrophases of each rhythm during the experimental phase were inspected it was found that the acrophase was similar day by day. A typical example is shown in Fig. 2. This suggests that the 4-h anchor sleep was sufficient to maintain rhythms synchronized to 24 h. To test this more formally, the period of each rhythm during the last 96 h of the experimental phase was considered further. The mean period of all the rhythms was determined and tested for a significant difference from 24 h (Student's *t* test). For this analysis the data from groups AX and AZ were analysed separately from the other groups since their time of anchor sleep differed. In addition, the data were subdivided: temperature and urinary potassium; urinary sodium and chloride; and urinary water, phosphate, calcium and water being considered as three separate groups. This subdivision has already been mentioned in the Introduction and its rationale will be described in the Discussion. The mean periods of the rhythms for all variables and for the different subdivisions during the last 96 h of the experimental phase are shown in Table 2. Also included in this Table are the mean periods of rhythms for the control group (BQ) who slept at conventional times throughout. It can be seen that in no case was the mean period significantly different from 24 h at the 5% level, indicating that, with anchor sleeps, the rhythms remained synchronized to 24 h.

This analysis, however, does not indicate whether the phase of the rhythms was the same as that when subjects slept for conventional times. To test for any phase shift of the rhythms, the acrophases of each rhythm during the control phase and

last 96 h of the experimental phase were assessed by fitting a 24-h cosine curve to each phase. These acrophases were compared (paired t test) to test for significant departures from 0. Table 2 indicates the mean phase differences thus calculated. As would be expected, it can be seen that for the control group (BQ) there was no significant phase shift. For the other groups a small mean phase advance of rhythms was found when all variables were considered. This was most marked, however, for the rhythms in excretion of water, phosphate, calcium and water and the phase shifts determined for urinary potassium and rectal temperature were not significantly different from 0.

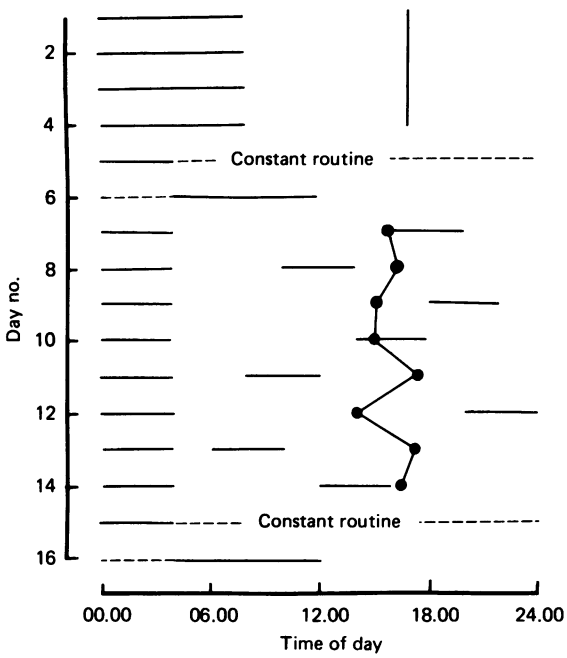

Fig. 2. Acrophases of the temperature rhythm in subject AZ1. Successive days are plotted from above down. ——, time in bed; ●, acrophases of the temperature rhythm over successive days. The vertical bar spanning days 1–4 indicates acrophase of the rhythm during the control days.

The results thus far have confirmed our results from previous experiments (Minors & Waterhouse, 1981*b*, *c*) namely that the 4-h anchor sleep seems capable of keeping rhythms synchronized to a period of 24 h though the phase of the rhythm is determined by the time over which the anchor sleep is taken. However, since rhythms are directly influenced by external factors (in particular, sleep markedly affects many rhythms (Mills *et al.* 1978*a*)) as well as the body's internal clock, it is not clear whether this synchronization of rhythms is a masking effect of these external factors or reflects a true entrainment of the body's internal clock.

During the constant routines, external factors are minimized and thus a comparison of rhythms from two such routines would give a better estimate of any differences in rhythms resulting from changes of the endogenous clock. Fig. 3 shows a

representative example of the urinary potassium data from the two constant routines of subject AZ2. It can be seen that during both constant routines the times at which potassium excretion reaches a maximum and a minimum are similar. However, high rates of excretion are more protracted in the second constant routine (following the experimental phase) than in the first (at the end of the control phase). A difference between the two constant routines has been tested more formally by cross-correlating the twenty-four data points from the second constant routine with a mixture derived

TABLE 2. Mean periods of rhythms (h) ± 1 S.E. of the mean during the last 96 h of the experimental phase (A). Mean phase difference between rhythm during control and the last 96 h of the experimental phase (B). Positive phase difference indicates experimental rhythm phase-advanced from control

	Groups					
	AX, AZ		BB, BC, BH, BI, BN, BR		BQ	
	A	B	A	B	A	B
Temperature and urinary potassium	24·04 ± 0·24 (13)	0·02 ± 0·46 (15)	24·03 ± 0·12 (42)	−0·68 ± 0·41 (31)	23·78 ± 0·28 (8)	0·22 ± 0·20 (8)
Urinary sodium and chloride	23·89 ± 0·28 (14)	0·67 ± 0·71 (14)	24·11 ± 0·21 (39)	1·36 ± 0·54 (20)*	23·58 ± 0·32 (8)	0·13 ± 0·44 (7)
Urinary water, phosphate, calcium and urate	24·25 ± 0·27 (22)	1·00 ± 0·31 (24)*	24·26 ± 0·15 (81)	2·08 ± 0·33 (48)*	24·27 ± 0·29 (15)	−0·33 ± 0·34 (14)
All rhythms	24·09 ± 0·16 (49)	0·64 ± 0·27 (53)*	24·16 ± 0·09 (162)	1·07 ± 0·26 (99)*	23·96 ± 0·18 (31)	0·07 ± 0·21 (29)

The numbers in parentheses refer to the number of rhythms.
* $P < 0·05$ that difference equals 0.

from the first as described in Methods. Such an analysis of the data shown in Fig. 3 yielded the highest correlation coefficient when the data from the second constant routine were correlated with the data from the first modified as follows: six parts of unshifted data mixed with four parts of the same data phase-delayed by 5 h. This analysis was performed on all variables for groups AX and AZ and the results have been combined by considering the two proportions in the mixture with the highest correlation coefficient derived as described above, Fig. 4. Thus the example of Fig. 3 would contribute '6' to the 'zero shift' entry and '4' to the '5 h delayed' entry of Fig. 4. When the data from all subjects are considered, it can be seen that mixtures so derived rarely contained phase advances; thus there was no rhythm best described by a mixture containing a component advanced by 6 h. By contrast, a frequent finding was that the 'best-fitting' mixture contained a component which was unshifted (and hence similar to the nychthemeral result) together with a component that was phase-delayed by about 4 h. A similar result was obtained when the data from each variable were displayed separately.

This splitting of rhythms into two components was not an artifact produced, in some way, by the stay in the isolation unit since it was not found in group BQ who spent a similar duration in the isolation unit but took no split sleeps. When the same analysis was applied to this group, then a component delayed by 4 h was absent, the majority of rhythms being best described by an unshifted component (Fig. 5).

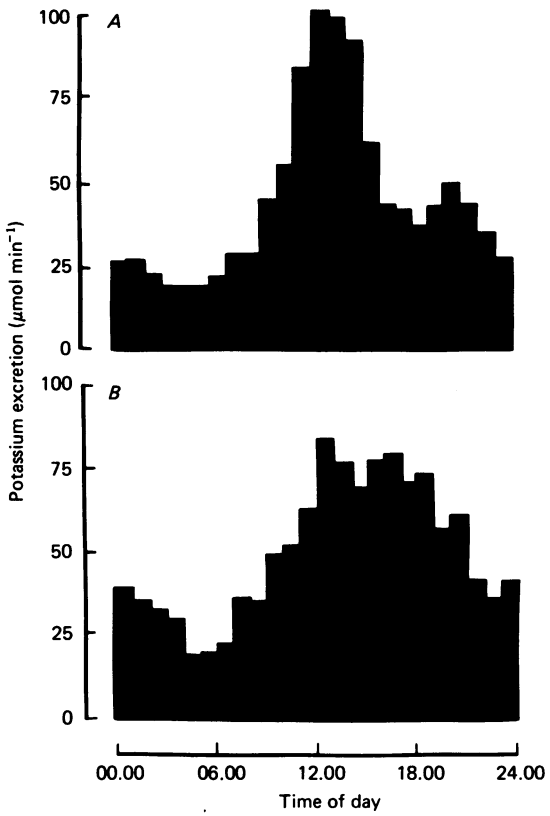

Fig. 3. Urinary potassium excretion rates in subject AZ2 during the two constant routines. *A*, during the control constant routine; *B*, during the constant routine following the days when anchor sleep had been taken. Cross-correlation of these yielded the highest correlation coefficient when the data from the second constant routine were cross-correlated with those from the first modified as follows: six parts of unshifted control data mixed with four parts of the same data phase-delayed by 5 h.

Thus the observation of two components in the data of groups AX and AZ required other explanations. The unshifted component might be explained by at least some component of the human circadian system being entrained by the anchor sleeps. An alternative or additional explanation, however, is that it might be a masking effect due to the two constant routines beginning at the same time (04.00). As regards the other delayed component, the explanation here too might be ambiguous. Thus, possibly the anchor sleeps were unable to entrain a clock controlling these rhythms and so the component determined by it free-ran with a period in excess of 24 h. Alternatively there might be some effect due to an interaction between the anchor sleep time and the timing of the constant routines.

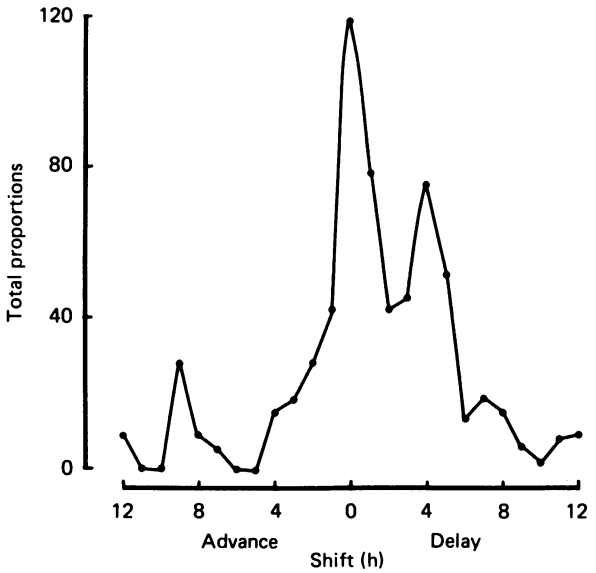

Fig. 4. Proportions and phase shifts of the data from the control constant routine which yielded the highest correlation coefficient when compared with the second constant routine (for further details see text). The Figure shows summed proportions for all rhythms measured in groups AX and AZ.

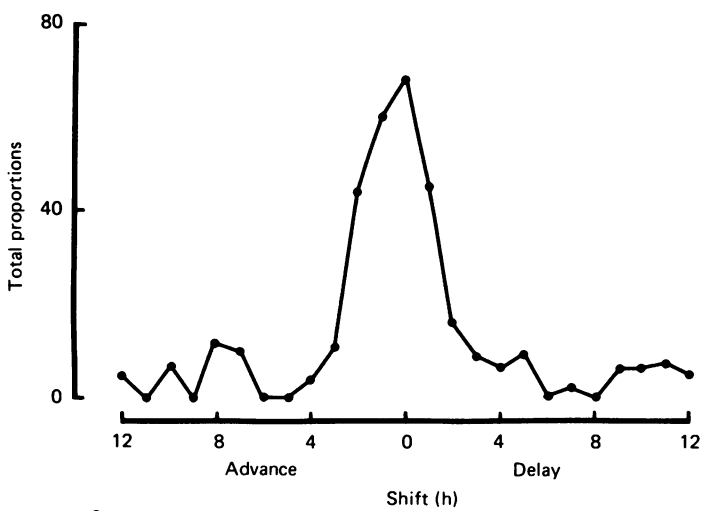

Fig. 5. The same conditions as given in the legend to Fig. 4 for group BQ.

To try to establish which of these options were appropriate, the other groups, for whom the times of anchor sleeps and the starting times of the constant routines were varied, were studied (Table 1). For those other groups, the time of anchor sleep was changed but for groups BB and BC the time of constant routines was unaltered (Fig. 1*C*). Previous work had shown (Minors & Waterhouse, 1981*b*, *c*) that taking anchor sleep at times coincident with, or later than, normal sleep times resulted in unchanged or phase-delayed rhythms at least measured under nychthemeral circumstances. Since such a delay would not enable a distinction to be made in the present

TABLE 3. Summary of expected phase-shifts from the effects of the starting time of the constant routines, the anchor sleep times and unentrained rhythm

	Constant routine			Anchor sleep		
Group	1st start	2nd start	Masking effect	Time	If rhythm entrained*	If rhythm unentrained
BQ	04.00	04.00	0 shift	Control	No change	Later
AX, AZ	04.00	04.00	0 shift	24.00–04.00	0, 2, 4 h earlier	Later
BB, BC	04.00	04.00	0 shift	20.00–24.00	4, 6, 8 h earlier	Later
BH, BI	04.00	24.00	4 h earlier	20.00–24.00	4, 6, 8 h earlier	Later
BN, BR	08.00	24.00	8 h earlier	20.00–24.00	4, 6, 8 h earlier	Later

* Whether effect due to sleep onset, mid-sleep or waking, respectively.

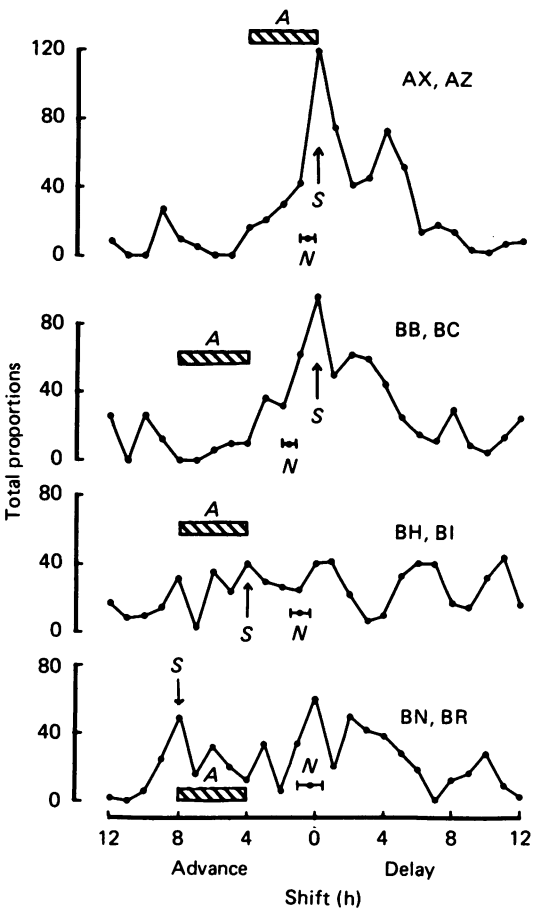

Fig. 6. The same conditions as given in the legend to Fig. 4 for groups AX and AZ, BB, and BC, BH and BI, BN and BR. The hatched bars labelled *A* indicate phase-shifts expected if the phase-shift was due to a shift of sleep times during the anchor sleep; *S*, phase-shift expected for direct effect of the waking time at the start of the two constant routines (for further details, see Table 3). *N*, mean shift of acrophase from control period (±1 S.E. of the mean) observed during last 96 h when anchor sleeps were being taken.

experiment between an entrained or a free-running rhythm, an earlier time for anchor sleep (20.00–24.00) was chosen.

In the other experiments the times of starting the two constant routines were made different (groups BH and BI, Fig. 1 *D*) or both constant routines were started at the same time as during the immediately preceding days (groups BN and BR, Fig. 1 *E*).

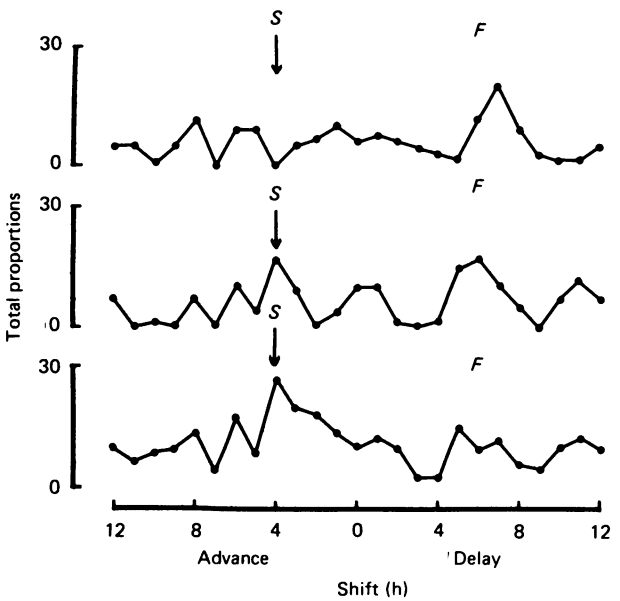

Fig. 7. The conditions as given in the legend to Fig. 4 for groups BH and BI. Results are shown separately for: urinary potassium excretion and body temperature, top; urinary sodium and chloride excretions, middle; urinary water, phosphate, calcium and urate excretions, bottom. *S*, phase-shift predicted from direct effect of waking time. *F*, phase-delay expected if rhythms were unentrained and free-running.

By these last two protocols the effects of the starting time in constant routines could be investigated. A summary of these aspects of the protocols, together with the effect being investigated, is given in Table 3.

The data from the constant routines in these experiments were cross-correlated and the results treated as described previously. The results from such an analysis applied to all variables (together with AX, AZ for comparison) are shown in Fig. 6. On this Figure are shown also the phase changes: (1) predicted for the masking effect of the constant routines, *S*; (2) that had been found nychthemerally, *N*; and (3) that would have been predicted if they had been determined wholly by the time of anchor sleep, *A*.

The following general findings emerge: (1) a component (*S*) close to that due to a masking effect of the constant routines was generally present; (2) an effect directly attributable to a shift produced by the anchor sleep, (*A*) was not clearly seen; (3) a component close to that observed nychthemerally, (*N*), was generally seen; and (4) a delayed component was regularly seen. Furthermore, if, for any protocol, different

groups of variables were considered separately, then the relative size of (1) and (4) above depended upon the group under consideration. A typical example of this is shown in Fig. 7.

DISCUSSION

The results showed that, during the last 4 days of nychthemeral conditions, the average period of the cosine curves fitted to all the data did not differ significantly from 24 h. This indicates that the 4-h sleeps taken at the same time each day had apparently 'anchored' the rhythms to a period indistinguishable from 24 h as has been found previously (Minors & Waterhouse, 1981*b*, *c*). On that occasion anchor sleeps had been taken at or later than normal sleep times; on this occasion, sleeps taken earlier than normal also (from 20.00–24.00) have been used. It seems from this that the ability of a regular 4-h sleep to stabilize rhythms to a 24-h period during nychthemeral conditions does not depend upon the time at which the regular sleep is taken. However, it must be pointed out that the standard errors of the mean are such that they imply that any rhythms free-running with a period close to 24 h could not be distinguished from stabilized rhythms (Table 2).

When the acrophases of the rhythms were considered, then the shift produced by the 20.00–24.00 anchor sleep seems to depend in part upon the variables under consideration. Urinary water, calcium, phosphate and urate excretion showed advances of up to 2·5 h, which suggests that they are considerably affected by the sleep–wake times and less by meal-times (the timing of which did not change). Further, if the data from all experiments with 20.00–24.00 anchor sleep were pooled (groups BB, BC, BH, BI, BN and BR) and the constituents water, urate, calcium and phosphate were considered separately, then phase advances as high as $3{\cdot}28 \pm 0{\cdot}75$ h (no. of rhythms = 13) for calcium were measured. Such results argue for the importance of the sleep–wake and light–dark cycles, rather than that of feeding–fasting, as potential zeitgebers in man (Goetz, Bishop, Halberg, Sothern, Brunning, Senske, Greenberg, Minors, Stoney, Smith, Rosen, Cressey, Haus & Afelbaum, 1976; Aschoff, 1978; Graeber, Gatty, Halberg & Levine, 1978; Wever, 1979; Minors & Waterhouse, 1981*a*; Moore-Ede *et al.* 1982). By contrast, the acrophases of deep-body temperature and urinary potassium, sodium and chloride excretion changed less, or even became delayed. One explanation of this will be given later.

The main undertaking of the present study was to ascertain if the adjustment of rhythm to a 24-h period by the regular 4-h anchor sleeps was one that affected the internal clock (by entrainment) or was exerting a direct effect upon rhythms ('masking'). The constant routines were designed to minimize the effects of rhythmic external changes thereby allowing the endogenous component to be studied more closely.

A comparison of the data from the two constant routines of the first experiment (groups AX and AZ) indicated that there were two components, one which had not shifted and a second which was about 4 h later in the second constant routine (Fig. 4). It was unclear how these two components were to be interpreted. Thus the unshifted component might have been evidence than an internal clock had been stabilized by the anchor sleep. Alternatively this component might have resulted from

the constant routines in some way since they both started at the same time (04.00); presumably the process of waking up would remove any pre-existing effect of sleep and so the two times of waking would form one component of any cross-correlation between two constant routines. The delayed component might have been produced by an unentrained clock or by some other feature of the experimental design; thus fatigue has been reported to delay the phase of certain circadian rhythms and subjects starting the second constant routine might have been more fatigued than at the onset of the first (for example, Wever, 1979, fig. 110; Aschoff, 1981, fig. 2).

TABLE 4. Mean shifts (h) ± 1 S.E. of the mean of acrophases between the control constant routine and nychthemeral controls (sleeping 24.00–08.00). Positive values indicate an advance in acrophase during the constant routine when compared with living nychthemerally

Groups	Temperature and urinary potassium	Urinary sodium and chloride	Urinary water, phosphate, calcium and urate	All rhythms
AX, AZ	1·48 ± 0·54 (14)*	0·53 ± 0·86 (12)	2·78 ± 0·46 (25)*	1·89 ± 0·36 (51)*
BB, BC	1·04 ± 0·61 (15)	3·01 ± 0·55 (15)*	3·54 ± 0·61 (27)*	2·74 ± 0·38 (57)*
BH, BI	0·45 ± 0·52 (15)	2·85 ± 0·42 (14)*	3·44 ± 0·42 (25)*	2·46 ± 0·31 (54)*
BQ	1·68 ± 0·63 (8)*	2·97 ± 0·68 (7)*	2·32 ± 1·03 (12)*	2·30 ± 0·52 (27)*
All	1·08 ± 0·29 (52)*	2·34 ± 0·34 (48)*	3·13 ± 0·29 (89)*	2·37 ± 0·19 (189)*

The numbers in parentheses refer to the number of rhythms.
* $P < 0·05$ that difference equals 0.

Results from group BQ eliminated some of these possibilities since it resulted in only one component when the two constant routines were compared and this was essentially unshifted (Fig. 5).

A possible explanation of the components in groups AX and AZ was attempted by repeating the experiment but with different times for the two constant routines and for the anchor sleep. Some of the factors which limited the changes that could usefully be made have already been mentioned. Additionally, in order to minimize the inconvenience of fatigue, constant routines began after a sleep of at least 4 h (Fig. 1).

The results observed in Figs. 6 and 7 enable the following inferences to be drawn.

(1) *A component due to the direct effect of the waking time for the constant routines was present.* Again, this argues for the importance of a direct masking effect of sleep. It can be assessed also by comparing the acrophases of cosine curves fitted to control nychthemeral data and the first constant routine (Table 4). In all of the groups shown the first constant routine began at 04.00, 4 h earlier than the normal rising time. The Table shows that rhythms were advanced by a similar amount, at least for some of the constituents that are known to be more exogenously determined.

It is quite possible to argue that the effect due to the constant routines is not masking but evidence of a rapidly entrained oscillator. Such a position would render

very fine the distinction between masking and a rapidly entrained oscillator and would not be useful in trying to understand the apparent phase stability observed nychthemerally.

(2) *The presence of a delayed component.* This seems to admit no explanation other than the presence of a free-running, unentrained component.

It is possible to make an estimate of the period of the free-running component under the present circumstances. Thus, the two constant routines were separated by 8 days and the delay observed was 4–6 h. This suggests a period of 24·5–24·8 h, a value slightly less than that observed in the much larger series of time-free experiments of Wever (1979). Strictly, the two sets of results are hardly comparable, of course, but one explanation of the present result would be that the anchor sleep exerted some effect upon the internal clock so adjusting its period from the free-running value towards 24 h. Near the limits of entrainment of an oscillator, similar results have been observed or predicted by others (Wever, 1979; Kronauer, Czeisler, Pilato, Moore-Ede & Weitzman, 1982; Moore-Ede *et al.* 1982).

(3) *The absence of a component attributable to a full shift due to the anchor sleep.* This was not unexpected since such a component was not seen nychthemerally. Nevertheless it confirms the conclusions drawn then that a substantial advance in phase of all rhythms was not found.

The inability of the anchor sleep to advance markedly the phase of the endogenous component might result in part from the necessity to place the regular sleep earlier rather than later than normal. Thus, experiments with real or simulated time-zone transitions have indicated that adjustment to an eastward shift (requiring phase advance) is more difficult than that to one in a westward direction (Aschoff, Hoffmann, Pohl & Wever, 1975; Aschoff, 1978; Klein & Wegmann, 1979). The effectiveness of the anchor sleep as a potential zeitgeber presumably could have been increased by 'strengthening' it in some way. One means might have been to lengthen sleep somewhat since 8 h sleep does, of course, stabilize the rhythms to a 24-h period, see group BQ; another would have been to have accentuated and shifted with anchor sleep those factors that are believed to act as zeitgebers, for example social influences (Klein & Wegmann, 1979).

Nevertheless the anchor sleep did exert some effect, namely

(4) *The presence of a component shifted by an amount similar to that observed nychthemerally.* This is evidence in favour of the view that the nychthemeral acrophase was not an artifact but due to a component that had been entrained by the anchor sleep. Thus, if two components were present the acrophase would have been computed to lie between them, but it is then an artifact and does not represent a real oscillator. In the present experiment the earlier component could have been a direct effect of anchor sleep and the later a free-running component (see Minors & Waterhouse, 1981*a*, fig. A 2).

Quite why the endogenous component should be entrained at the observed phase angle (rather than by amounts indicated in Table 3) is unclear, but at the limits of entraiment of an oscillator, not only its period but also its phase can become rather unpredictable (Wever, 1979; Kronauer *et al.* 1982; Moore-Ede *et al.* 1982). Conclusions (2) and (4) taken together imply the presence of more than one endogenous component (or clock), one partially entrained by the anchor sleep and the other much

less so, if at all. This is not a new concept, of course, but has not to our knowledge been demonstrated this way before.

A fifth inference that can be drawn is that, even though in principle all the variables behaved similarly, in detail there were differences. Observation of Fig. 7 indicates that the constant routine (*S*) and free-running (*F*) components were not equally marked for all constituents. Indeed the variables have been grouped for the purposes of analyses with urinary water, phosphate, calcium and urate excretion in one group, urinary sodium and chloride in another and urinary potassium and deep-body temperature in a third. These subdivisions were based upon previous experiments (Simpson *et al.* 1970; Mills *et al.* 1977; Minors & Waterhouse, 1982) which indicated that the first group was affected mainly by exogenous influences, the last by endogenous factors and that sodium and chloride were intermediate in these respects. The present constant routine data confirm such a division, the endogenous (free-running) component being more marked for potassium and temperature, the exogenous (masking) effect of the constant routine being greatest for the water–phosphate–calcium–urate group and the components with sodium and chloride being intermediate in size.

Such a division might explain the nychthemeral result described earlier (Table 2). Thus, if it is accepted that one of the effects of anchor sleep is a direct masking effect and that one of the endogenous components tends to free-run, then the apparent phase change observed nychthemerally will depend upon not only the partially entrained endogenous component but also the relative strengths of the exogenous and endogenous components in the variable under consideration: 'exogenous' variables (flow, etc.) will appear to be phase advanced, 'endogenous' variables (temperature, etc.) will appear phase delayed and sodium and chloride will again show an intermediate result.

Once again, the worth of constant routines in investigating circadian rhythms and the effect of masking influences seems to have been established. Earlier work established that adjustment of the endogenous component to imposed time-zone shifts (Mills *et al.* 1978*b*) or non-24-h days (Simpson *et al.* 1970; Mills *et al.* 1977) could be misinterpreted if measured under nychthemeral conditions. (Indeed the process of 'entrainment by partition' (Aschoff, 1978) seems far more common if measured under conditions that minimize exogenous influences.) In the present study similar misleading inferences about the efficacy of anchor sleep could be made if a nychthemeral protocol only was used. It seems noteworthy that studies of the adaptation of circadian rhythms to shift work have been made whilst the alternation of sleep and wakefulness has been present and the process of adjustment has often been related to mid-sleep or mid-work (Reinberg *et al.* 1975; Knauth *et al.* 1978). The effect of exogenous influences in such conditions has been accepted, of course, but inferences about the internal clock have necessarily been indirect. The potential value of constant routines to the assessment of the adjustment of the internal clock in shift work has been described by Akerstedt (1979); we too believe the problem merits further study.

Our thanks are due to: the M.R.C. for Project Grant No. G978/1126/N to cover expenses for the experiments; to A. Yates and S. Valvona for technical assistance; and to the volunteers themselves.

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