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CIRCADIAN RHYTHMS IN THE FLOW RATE AND COMPOSITION OF UNSTIMULATED AND STIMULATED HUMAN SUBMANDIBULAR SALIVA

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SUMMARY

1. Nine subjects recorded oral temperature and collected unstimulated submandibular saliva and submandibular saliva stimulated by sour lemon drops at a constant flow rate of 1 ml./min, at about 07.00, 11.00, 14.00, 17.00 and 22.00 h daily for time spans of about 11 days.

2. Least-squares cosine waves were fitted to the data to test for the presence and characteristics of circadian rhythms and the results were subjected to cosinor analysis and the Rayleigh test to assess the statistical significance of any rhythms for the group of subjects as a whole (i.e. whether or not the computed acrophases were randomly distributed throughout the 24 h of the day).

3. Unstimulated submandibular saliva showed circadian rhythms, significant for the group as a whole, in flow rate and in the concentrations of sodium, potassium, magnesium, chloride and inorganic phosphate but not in protein or calcium.

4. Stimulated submandibular saliva showed circadian rhythms, significant for the group as a whole, in sodium, potassium, calcium, magnesium, chloride and inorganic phosphate but not in protein. Oral temperature also showed a significant circadian rhythm.

5. Because of the high amplitude of many of these rhythms, they must be taken into account when establishing the normal range of salivary values, or when salivary composition is to be used as an indicator of systemic disease or to be implicated in the aetiology of oral disease.

INTRODUCTION

In previous studies from this laboratory, statistically validated circadian rhythms have been demonstrated in the flow rate and composition of unstimulated whole saliva (Dawes, 1972), unstimulated parotid saliva

(Dawes & Ong, 1973*a*, *b*) and in the composition of stimulated parotid saliva secreted at a constant flow rate (Dawes, 1972).

Since submandibular saliva has been shown to make the greatest contribution by volume both to unstimulated whole saliva (Schneyer & Levin, 1955) and to stimulated whole saliva at moderate flow rates (Enfors, 1962) it was of interest to study submandibular saliva for evidence of circadian rhythms.

The only previous studies of rhythms in the flow rate or composition of submandibular saliva appear to be those of Ahrens & Lücke (1972) and Ferguson & Fort (1973, 1974). Ahrens & Lücke (1972) actually studied unstimulated mixed submandibular–sublingual saliva rather than pure submandibular saliva and, although their four subjects made six collections each day for several successive days, the saliva was analysed only for calcium. Ferguson & Fort (1973) tested unstimulated submandibular saliva for circadian rhythms in the concentrations of calcium, inorganic phosphate and total phosphate. They collected saliva every four hours beginning at 13.00 h on day 1 and ending at 09.00 h on day 2. After a break of 10 h, a further six collections were made every 4 h. The two sets of data were combined to give analytical values for every odd-numbered hour of one 24 h time span.

Ferguson & Fort (1974) again used a similar but not identical type of collection schedule for unstimulated submandibular saliva which was then analysed for a variety of inorganic and organic components. However, the calcium, inorganic phosphate and total phosphate results from this study appear to be identical with those reported in their 1973 paper. Sine waves of period 24 h were fitted to the data from each subject as well as a complex curve consisting of a sine wave of period 24 h together with its first harmonic. Disadvantages of this sampling schedule were that the sleep of the subjects was disturbed by saliva collections and that the collections extended over only two cycles.

In the present study, both unstimulated and stimulated submandibular saliva were collected over a much larger number of cycles (11) and, to avoid possible confounding effects due to disturbed sleep, no samples were taken during the night. Although Aschoff, Fatranska, Gerecke & Giedke (1974) have shown that the circadian rhythm in rectal temperature is not affected by two 15 min interruptions of sleep, it is possible that longer interruptions (our temperature and saliva sampling sessions each lasted about 35 min) would have affected some of the rhythm parameters. Since the composition of stimulated submandibular saliva is strongly influenced by both flow rate and duration of stimulation (Dawes, 1974*a*), these variables were maintained constant. Thus the experiment provided no information about possible variations in stimulated flow rate with

time of day. Nevertheless, the demonstration and interpretation of circadian variations in the composition of stimulated submandibular saliva were greatly facilitated by the standardized sampling conditions.

METHODS

Collection of submandibular saliva

A collection device similar to that described by Truelove, Bixler & Merritt (1967) was made for each subject after construction of a model of the floor of the mouth. This device allows collection of pure submandibular saliva from both the right and left glands simultaneously.

Unstimulated submandibular saliva was collected for 5 min after the dead space of the cannula had filled completely and a few drops of saliva had been expelled.

With the cannula still in place, submandibular saliva was then stimulated by sour lemon drops at a constant flow rate of 1·0 ml./min using the negative feed-back technique described by Dawes (1967). Briefly, saliva was collected into a graduated centrifuge tube positioned in front of a mirror. By observation the subject was able to calculate the flow rate with the aid of a stopwatch and regulate the degree of sucking on the sour lemon drop to maintain a constant flow rate. Since the composition of submandibular saliva changes markedly during the first few minutes of stimulation (Dawes, 1974*a*), only the 5·0 ml. collected during the 6–10th min of stimulation was refrigerated and retained for analysis.

Saliva was collected five times daily from nine young adults (two female) at arbitrary but convenient times of about 07.00, 11.00, 14.00, 17.00 and 22.00 h. Most of the collections at about 07.00, 17.00 and 22.00 h were done in the subjects' homes. Meals were consumed immediately after the collections at 07.00, 11.00 and 17.00 h to reduce any possible serial dependency of sampling (Dawes & Chebib, 1972). Sleeping hours were from about 23.00 to 06.30 h. Oral temperature was recorded with a clinical thermometer immediately before the saliva collections and this time was also recorded as the sampling time for both the unstimulated and stimulated submandibular saliva.

The subjects collected for 11 successive days, from the Monday of one week to the first sample on the Friday of the following week. For one subject, only the data collected over the first 5 days were used, as on the seventh day she developed a gastro-intestinal infection with fever which distorted some of her rhythms. The subjects had a variety of racial origins: two Anglo-Saxon, two Chinese, two Japanese, two Jewish and one Indian.

Analytical techniques

The saliva was analysed for sodium, potassium, calcium and magnesium by atomic absorption spectroscopy (Dawes, 1967, 1969), chloride by a coloumetric method (Cotlove, Trantham & Bowman, 1958), protein by a colorimetric method (Lowry, Rosebrough, Farr & Randall, 1951) and inorganic phosphate by a colorimetric technique (Chen, Toribara & Warner, 1956) as modified for saliva by Dawes (1969). Preliminary studies showed that when the saliva was refrigerated, but not frozen, for 3 days, there was no change in the measured concentrations of the above components. With longer duration, and also with freezing and thawing, the protein tended to coagulate and precipitate, together with some calcium and phosphate, making removal of a homogeneous aliquot almost impossible. Thus, except at the weekend, most samples of saliva were analysed for protein, calcium and phosphate on the day following collection.

Statistical analyses

A least-squares cosine wave was fitted to each time series of data with the aid of a computer as outlined by Halberg, Engeli, Hamburger & Hillman (1965). A cosine wave is described by the equation

$$f(t) = M + A.\cos\left(\frac{2\pi t}{\tau} + \phi\right),$$

where $f(t)$ = the expected value of the function at time t,
M = the mesor or rhythm-adjusted mean level (Halberg, 1973),
A = amplitude of rhythm,
τ = trial period under study,
ϕ = phase angle of acrophase.

In checking for circadian rhythms, values of τ at intervals of 0.1 h between 20 and 28 h are tested by the computer and for each value of τ the values of M, A and ϕ are simultaneously adjusted to give the best-fitting cosine wave. The computer program also calculates the standard errors of these three parameters and the percentage of the total variance accounted for by the rhythm.

In order to determine whether or not, for a given component, the acrophases from the different subjects were randomly distributed throughout the 24 h (or 360°), the acrophases of the fitted cosine waves of period 24.0 h for each subject were analysed by the Rayleigh test, as described by Batschelet (1965). In addition, as a further test of statistical significance of a group rhythm, results for each component were subjected to cosinor analysis as described by Halberg, Tong & Johnson (1967). This latter computer program derives mean, weighted amplitude and phase estimates together with 95% confidence limits (as an error ellipse) and plots the results, using polar co-ordinates, directly on to microfilm. The rhythm is only statistically significant for the group when the error ellipse does not overlap the pole.

RESULTS

The results for oral temperature and for unstimulated submandibular salivary flow rate and composition are summarized in Table 1, whilst Table 2 summarizes the results for the composition of stimulated submandibular saliva. The values for which standard errors are given are the arithmetic means of the individual values from the nine subjects and, for the values for A and ϕ, are not the weighted amplitude and phase estimates derived from the cosinor analysis.

The Rayleigh and cosinor tests for group significance of the various rhythms showed good agreement except for the rhythm in the magnesium concentration in stimulated submandibular saliva. For this component, the subject with the highest amplitude rhythm had an acrophase furthest away from the group mean acrophase. As the cosinor analysis gives greater weight to the individual rhythms of higher amplitude, this perhaps explains why the cosinor analysis showed a non-significant group rhythm whereas the Rayleigh test, which uses only the acrophases, showed a group rhythm significant at the 95% confidence level.

TABLE 1. Circadian rhythm parameters for oral temperature and for the flow rate and composition of unstimulated submandibular saliva

	No. of subjects studied	No. with a sig. rhythm	Best-fitting period (τ) (h) ± S.E.	C.V. (%) ± S.E.	Mean level (M) ± S.E.	Amplitude (A) ± S.E.	A/M (%) ± S.E.	Acrophase (ϕ) in h and min ± S.E.	Group sig. by Rayleigh test	Group sig. by cosinor
Oral temp. (°C)	9	9	24·0 ± 0·1	52·1 ± 6·1	36·65 ± 0·07	0·32 ± 0·03	0·58 ± 0·07	17·54 ± 0·35	**	**
Flow rate (ml./min)	9	8	24·1 ± 0·2	33·9 ± 7·2	0·31 ± 0·03	0·10 ± 0·02	32·1 ± 4·3	15·41 ± 1·10	*	*
Protein (mg %)	9	8	23·9 ± 0·2	25·8 ± 4·3	111·6 ± 10·6	15·8 ± 2·0	14·3 ± 1·2	20·36 ± 1·26	n.s.	n.s.
Sodium (mM)	9	9	24·1 ± 0·1	36·4 ± 3·7	4·18 ± 0·98	3·03 ± 0·84	69·9 ± 14·6	04·46 ± 0·31	**	*
Potassium (mM)	9	4	24·0 ± 0·5	14·8 ± 3·3	14·0 ± 0·8	0·92 ± 0·09	6·6 ± 0·6	15·39 ± 0·34	**	**
Calcium (mM)	9	6	24·2 ± 0·3	17·0 ± 4·5	1·62 ± 0·11	0·08 ± 0·01	5·0 ± 0·8	20·34 ± 1·58	n.s.	n.s.
Magnesium (μM)	9	9	23·9 ± 0·1	34·7 ± 5·2	72·4 ± 5·0	17·7 ± 2·0	24·6 ± 2·4	04·29 ± 0·52	**	**
Chloride (mM)	9	9	24·1 ± 0·1	49·3 ± 3·3	11·5 ± 0·5	3·6 ± 0·6	31·1 ± 13·0	05·50 ± 0·26	**	**
Inorganic P (mM)	9	8	23·8 ± 0·3	21·2 ± 4·5	3·79 ± 0·41	0·49 ± 0·10	12·8 ± 1·8	18·36 ± 0·24	**	**

C.V. (%) = percentage variance (the percentage of the total variance accounted for by the fitted cosine wave).
* = $P < 0·05$; ** = $P < 0·01$; n.s. = not significant.
The values for C.V. (%), M, A, ϕ, the Rayleigh test and the cosinor analysis are for the best-fitting cosine wave of period 24.0 h.

TABLE 2. Circadian rhythm parameters for the composition of sour-lemon-drop-stimulated submandibular saliva (1 ml./min)

	No. of subjects studied	No. with a sig. rhythm	Best-fitting period (τ) (h) ± S.E.	C.V. (%) ± S.E.	Mean level (M) ± S.E.	Amplitude (A) ± S.E.	A/M (%) ± S.E.	Acrophase (ϕ) in h and min ± S.E.	Group sig. by Rayleigh test	Group sig. by cosinor
Protein (mg %)	9	5	24·1 ± 0·4	16·5 ± 6·1	148·4 ± 23·1	14·7 ± 3·0	10·3 ± 1·8	19·24 ± 1·32	n.s.	n.s.
Sodium (mM)	9	6	24·7 ± 0·3	28·8 ± 6·8	18·8 ± 3·4	4·9 ± 1·1	26·2 ± 4·1	05·23 ± 0·43	**	**
Potassium (mM)	9	9	24·0 ± 0·1	50·7 ± 6·1	12·8 ± 0·8	1·84 ± 0·11	14·6 ± 0·7	15·51 ± 0·20	**	**
Calcium (mM)	9	6	23·8 ± 1·3	15·8 ± 3·8	1·81 ± 0·08	0·07 ± 0·01	4·2 ± 0·7	00·27 ± 0·44	**	**
Magnesium (μM)	9	5	24·2 ± 0·3	12·0 ± 2·4	34·7 ± 2·2	2·81 ± 0·51	8·0 ± 1·4	21·38 ± 1·16	*	n.s.
Chloride (mM)	9	8	24·0 ± 0·2	39·8 ± 6·5	16·1 ± 2·2	4·1 ± 0·9	23·5 ± 3·1	05·52 ± 0·56	**	**
Inorganic P (mM)	9	7	24·4 ± 0·2	27·6 ± 5·5	2·22 ± 0·27	0·28 ± 0·04	12·7 ± 1·1	19·32 ± 1·02	**	**

C.V. (%) = percentage circadian variance (the percentage of the total variance accounted for by the fitted cosine wave).
* = $P < 0.05$; ** = $P < 0.01$; n.s. = not significant.

The values for C.V. (%), M, A, ϕ, the Rayleigh test and the cosinor analysis are for the best-fitting cosine wave of period 24.0 h.

Figs. 1 and 2 illustrate the best-fitting 24 h cosine waves for those parameters of unstimulated and stimulated submandibular saliva which showed rhythms significant for the group as a whole.

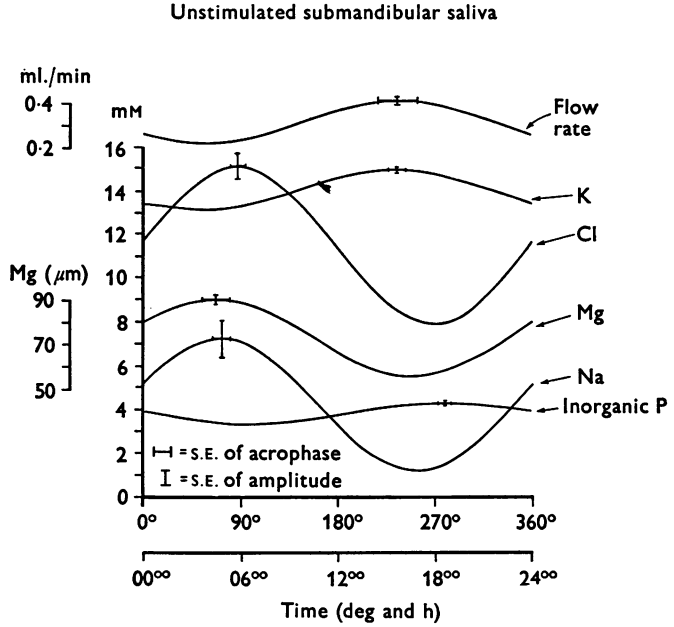

Fig. 1. The best-fitting cosine waves of period 24 h for the circadian rhythms in the flow rate and concentrations of sodium, potassium, magnesium, chloride and inorganic phosphate in unstimulated submandibular saliva.

In conformity with previous findings on the effect of flow rate on the composition of submandibular saliva (Dawes, 1974*a*), the rhythm-adjusted mean concentrations of protein, sodium, calcium and chloride were higher in stimulated than in unstimulated saliva whilst potassium, magnesium and phosphate showed the reverse relation.

DISCUSSION

Halberg, Reinhardt, Bartter, Delea, Gordon, Reinberg, Ghata, Halhuber, Hofmann, Günther, Knapp, Pena & Garcia Sainz (1969) have noted the value of recording body temperature as an internal standard in studies of human circadian rhythms. In the present study the mean acrophase for oral temperature (17.54 h ± 35 min) was about an hour later than that for eight subjects who collected unstimulated whole saliva and stimulated parotid saliva (temperature acrophase at 16.41 h ± 16 min)

(Dawes, 1972). However, only one subject was common to the two experiments. This relatively close agreement in temperature acrophase suggests that it is valid to compare results from the different groups of subjects. Acrophases of circadian rhythms in the flow rate and composition of different types of saliva, together with an indication of the group significance of the rhythms, are brought together in Table 3.

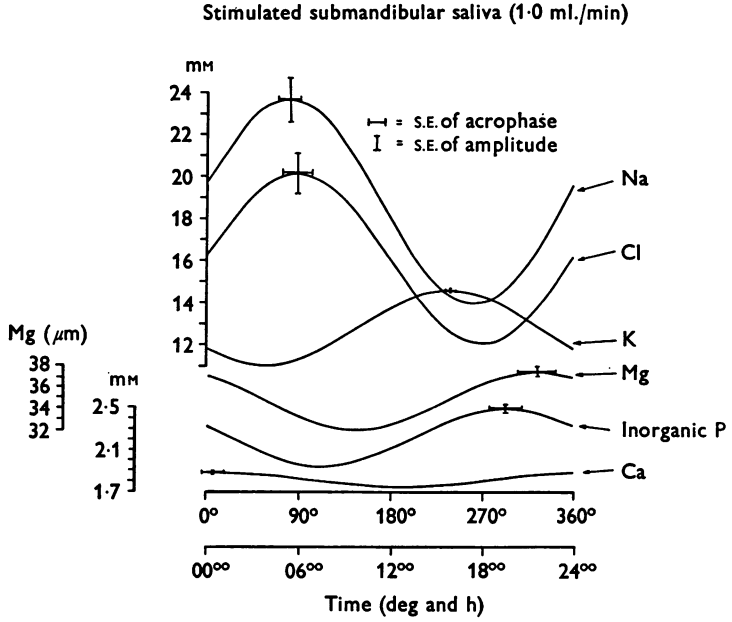

Fig. 2. The best-fitting cosine waves of period 24 h for the circadian rhythms in the concentrations of sodium, potassium, calcium, magnesium, chloride and inorganic phosphate in stimulated submandibular saliva at a flow rate of 1·0 ml./min.

As far as unstimulated flow rate is concerned, there was excellent agreement in the acrophases for parotid, submandibular and whole saliva with peak values occurring at about 15.30 h. It should perhaps be emphasized that during sleep, salivary flow rate falls virtually to zero, which has important implications in preventive dentistry, as discussed previously (Dawes, 1972). Ahrens & Lücke (1972) noted peak submandibular (and parotid) flow rates at 13.00 h, but as no samples were taken between 13.00 and 17.00 h, their results are not inconsistent with an acrophase in the middle of the afternoon. Ferguson & Fort (1974) present their results on each component of unstimulated submandibular saliva as a histogram of maximum and minimum values from each subject. Application of the Rayleigh test to their results did not reveal a significant flow-rate rhythm

TABLE 3. Acrophases of various salivary rhythms
(means ± S.E. in h and min with phase reference of midnight)

	Whole saliva	Parotid saliva		Submandibular saliva	
	Unstimulated†	Unstimulated‡	Stimulated†	Unstimulated	Stimulated
Flow rate	15·26 ± 0·45**	15·36 ± 0·20**		15·41 ± 1·10*	
Protein	07·58 ± 2·26 n.s.	18·05 ± 0·22**	15·54 ± 0·27**	20·36 ± 1·26 n.s.	19·24 ± 1·32 n.s.
Sodium	04·40 ± 0·10*	04·47 ± 0·22**	05·03 ± 0·37**	04·26 ± 0·31**	05·23 ± 0·43**
Potassium	14·01 ± 2·58 n.s.	03·40 ± 0·41**	17·22 ± 0·38**	15·39 ± 0·34**	15·51 ± 0·20**
Calcium	09·09 ± 2·14 n.s.	20·31 ± 1·28 n.s.	19·12 ± 0·55*	20·34 ± 1·58 n.s.	00·27 ± 0·44**
Magnesium		02·59 ± 0·57**		04·29 ± 0·52**	15·38 ± 1·16*
Chloride	04·56 ± 0·26**	04·28 ± 1·14*	05·06 ± 0·32*	05·50 ± 0·26**	05·52 ± 0·56**
Inorg. P	21·42 ± 1·53 n.s.	00·58 ± 1·03**	11·17 ± 2·19 n.s.	18·36 ± 0·24**	19·32 ± 1·02**

* = group rhythm significant at $P < 0·05$.
** = group rhythm significant at $P < 0·01$.
n.s. = group rhythm not significant.
† Dawes (1972).
‡ Dawes & Ong (1973*a*, *b*).

on a group basis, probably because the small number of cycles studied in each of their subjects greatly limits the accuracy with which the acrophase can be estimated unless the rhythm accounts for a high percentage of the total variance.

Although the majority of our subjects showed a significant rhythm in the protein content of both unstimulated and stimulated submandibular saliva, the amplitudes were relatively low and there was a wide spread in acrophases (Tables 1 and 2) with the result that the rhythms were not significant on a group basis. This perhaps explains why unstimulated whole saliva did not show a significant group rhythm in protein content (Table 3) even though parotid saliva shows a protein rhythm of high amplitude.

The most consistent rhythm in salivary composition appears to be that in the sodium concentration, and for all types of saliva studied the acrophase was consistently around 05.00 h (Table 3). The data of Ferguson & Fort (1974) also yield a similar acrophase, and the rhythm is most probably due to the known rhythm in aldosterone which itself is associated with the postural changes of the sleep–wake cycle, as discussed previously (Dawes, 1972, 1974*b*). Plasma aldosterone concentration is known to peak about noon (Wesson, 1964) and aldosterone has been shown to increase sodium reabsorption from, and potassium secretion into saliva (Blair-West, Coghlan, Denton & Wright, 1967). One subject showed extremely low sodium concentrations in most samples of unstimulated saliva (as low as 0·2 mM) although the potassium level was about average. Since a low sodium concentration may be due to primary aldosteronism (Blair-West *et al.* 1967), a 24 h urine collection was made and blood was removed at 14.55 h, 4 days after conclusion of the saliva collection span. Urine analysis for electrolytes and plasma analysis for electrolytes, aldosterone and renin (courtesy of Dr J. K. McKenzie) revealed no abnormality, and hence the reason for the low salivary sodium concentration remains uncertain.

Chloride also shows a consistent rhythm in all types of saliva studied (Table 3) with an acrophase closely corresponding to that of the sodium rhythm. This result is expected since chloride tends to be passively distributed across salivary ductal epithelium (Schneyer, Young & Schneyer, 1972) and its concentration is partially dependent on the degree of sodium reabsorption. In contrast, application of the Rayleigh test to the data of Ferguson & Fort (1974) did not reveal a significant chloride rhythm.

Both unstimulated and stimulated submandibular saliva showed statistically significant potassium rhythms with closely similar acrophases at about 15.40 h. The data of Ferguson & Fort (1974) reveal a similar acro-

phase and significant rhythm by the Rayleigh test. In a previous study on unstimulated parotid saliva (Dawes & Ong, 1973*b*), a significant potassium rhythm was detected but with an acrophase at about 03.40 h. It was suggested that this was caused primarily by the rhythm in flow rate – since the potassium content of unstimulated parotid saliva is strongly inversely related to flow rate – and that this effect overrode the expected aldosterone effect. In submandibular saliva the potassium concentration is much less dependent on flow rate (Dawes, 1974*a*) and thus the effect due to the aldosterone rhythm can assert itself on unstimulated submandibular saliva to produce an acrophase similar to that in stimulated submandibular and parotid saliva. Presumably the discrepancy in acrophases for the potassium rhythms in unstimulated parotid and submandibular saliva accounts for our inability to detect a significant rhythm in the potassium content of unstimulated whole saliva (Table 3).

The results for calcium showed a significant rhythm of very low amplitude in stimulated submandibular saliva but no rhythm in the unstimulated secretion. Since a calcium rhythm could not previously be detected in unstimulated parotid saliva this also explains why no rhythm could be demonstrated in unstimulated whole saliva (Table 3). In contrast, Ferguson & Fort (1974) did find a rhythm in the calcium content of unstimulated submandibular saliva. However, the mean level of calcium (0·81 mM), as determined by their colorimetric technique, was only about half of that reported by other workers (e.g. Lear & Grøn, 1968; Dawes, 1974*a*) who used atomic absorption spectrophotometry. In addition, Dawes (1974*a*) collected samples at about 08.00 h, a time close to that when minimum calcium concentrations were found by Ferguson & Fort (1974). The acrophase of the calcium rhythm in stimulated submandibular saliva (at about 00.30 h) is consistent with the results obtained by Ahrens & Lücke (1972) who found peak calcium concentrations in the first and last samples taken during the day. The mechanism for such a rhythm remains uncertain.

The present results showed significant group rhythms in the magnesium content of both unstimulated and stimulated submandibular saliva. The acrophase for magnesium in unstimulated submandibular saliva was fairly similar to that found previously for unstimulated parotid saliva (Table 3) but was widely out of phase with that for stimulated submandibular saliva. Again, the mechanism responsible for these rhythms is uncertain, although a flow-rate effect could conceivably account for the timing of the acrophase in unstimulated submandibular saliva as the magnesium concentration is known to be inversely related to flow rate (Lear & Grøn, 1968; Dawes, 1974*a*).

Significant rhythms were detected in the inorganic phosphate content

of both unstimulated and stimulated submandibular saliva with very similar acrophases in the early evening. Although a phosphate rhythm in unstimulated parotid saliva has previously been detected (Table 3), the acrophase was many hours out of phase with that for submandibular saliva, which probably accounts for our inability to detect a phosphate rhythm in unstimulated whole saliva (Table 3). The acrophases of the submandibular phosphate rhythms are also fairly close to that which may be calculated for unstimulated submandibular saliva from the data presented by Ferguson & Fort (1974). Although the acrophases for the phosphate rhythms in submandibular saliva are similar to that for the rhythm in plasma phosphate (Conroy & Mills, 1970), it is doubtful whether the plasma rhythm is sufficient to account for the salivary rhythm, as the salivary phosphate level has been found to be relatively independent of an increase in plasma phosphate concentration induced by phosphate infusion (Bates, 1960) or a reduction induced by haemodialysis (Freeman, Shannon & Easterling, 1965).

In Tables 1 and 2 the amplitude of the various rhythms is expressed as a percentage of the mesor and these values should be doubled to obtain the range encountered throughout a 24 h period. Since many of the rhythms show a very high amplitude, such a source of variation should be taken into account in interpreting published values for normal salivary composition (e.g. Shannon, 1973) or in the use of saliva as an indicator of systemic disease (Wotman & Mandel, 1973), particularly where sodium analyses are involved.

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