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CIRCADIAN RHYTHMS IN HUMAN PAROTID SALIVA FLOW RATE AND COMPOSITION

D. B. FERGUSON, A. FORT, A. L. ELLIOTT and A. J. POTTS

Department of Physiology, Medical School, University of Manchester, Manchester, England

Summary—The circadian variations in flow rate and pH of stimulated parotid saliva and the concentrations of sodium, potassium, calcium, chloride, inorganic and total phosphate, nitrogen, protein, glucose and total carbohydrate have been studied and shown to fit curves derived from a combination of a fundamental sine wave of 24 hr period and its first harmonic. Such curves are reproducible in an individual subject but considerable variation exists between subjects.

INTRODUCTION

STUDIES of the circadian variations in the flow-rate and composition of human saliva are of interest for three reasons. If significant variations occur, these affect the concept of normal values for salivary constituents and impose the need for specification of collection times when reporting results of estimations of salivary variables. Secondly, variations in salivary constituents may affect the initiation or continuation of oral disease and its treatment. Finally, the recognition of circadian rhythms in salivary components would suggest that saliva might prove a convenient fluid in which to study human circadian rhythms in general, since the simple and painless collection of saliva has advantages over both urine collection and blood sampling by venepuncture.

There have been many studies of circadian variations in flow rate and in concentrations of particular ions in saliva. Some of these are reviewed by DAWES (1972). The results of these studies are most conveniently summarized as tables. Analyses have been carried out on stimulated and unstimulated whole saliva (Table 1) and on stimulated parotid saliva (Table 2). Two reports describe variations in stimulated parotid saliva collected with techniques which attempted to maintain constant flow rates (DOGON, AMDUR and BELL, 1971; DAWES, 1972) and there is one report (ÄHRENS and LUCKE, 1972) on variations in unstimulated parotid saliva.

The majority of these studies contain no statistical or mathematical analysis. The criteria for establishment of circadian rhythms enunciated by MILLS (1966) are fulfilled by only a few of these papers. For example, only five papers record observations during the night. Frequency and timing of sampling has been a matter of convenience rather than that best suited to rhythm evaluation. Thus, if rhythms other than a simple sine curve of 24 hr period are possible, observations must cover more than 4 points in the 24. The 4 hr samples of DOGON *et al.* (1968, 1971) and BISSADA

TABLE 1. ANALYSIS OF CIRCADIAN VARIATION IN CON

	Stimulated or not	Times	Night observations	Flow rate peak	pH peak	Na peak
EDDY *et al.* (1933)	Not	hourly	No			
HOLMES (1964)	Not		No	Variable		
PALMAI and BLACKWELL (1965)	Not	2 hourly	Yes	04.00 hr		
PALMAI *et al.* (1967)	Not	2 hourly	Yes	04.00 hr		
DREIZEN *et al.* (1952)	Not					a.m.
GRAD (1954)	Not	Before meals	No			early a.m.
DE TRAVERSE and COQUELET (1952)	Not	6 hourly	No			a.m.
PAWAN (1955)	Not	Not stated	No			a.m.
PRADER *et al.* (1955)	Not	06.00 08.00 10.00 12.00 15.00 18.00 23.00	No			06.00
KRAL, GRAD and HUNZINGER (1959)	Not					a.m.
SCHNEYER *et al.* (1956)	Not	Not sleeping	Yes	Low at night		
KRASNOW (1936)	Not				Evening	
WALKER and SHEPHERD (1935)	Not	Before meals	No			
SHANNON and PRIGMORE (1959a,b)	Stimulated	09.00 (a) (b) 2 hourly 05.00 21.00	No	Evening		a.m.
SHANNON and PRIGMORE (1962)	Stimulated	2 hourly	No	Evening		a.m.
SHANNON (1958)	Stimulated		No	07.00 hr 21.00 hr		Some rhythm
SCHAEFER *et al.* (1967)	Stimulated	6 hourly		Rhythm		Some rhythm
BISSADA and ELMOSTEHY (1971)	Stimulated and not	4 hourly	Yes			Unstimulated 06.00 Stimulated none
DAWES (1972)	Not	07.00 11.00 14.00 17.00 22.00	No	15.26 hr		05.00

TABLE 2. ANALYSIS OF CIRCADIAN VARIATION IN CONSTITUENTS OF PAROTID SALIVA RE-

	Stimulated or not	Times	Night observations	Flow rate peak	pH peak	Na peak	Cl peak
BATES (1962)	Stimulated	09.30 10.30 12.00	No				
SHANNON and SEGRETO (1968a)	Stimulated	08.00 10.00 12.00 14.00 16.00	No	12.00		08.00	08.00
(1968b)	Stimulated	08.00 10.00 12.00 14.00 16.00	No				
(1968c)		08.00 10.00 12.00 14.00 16.00	No				
BISSADA and HAUS (1968)	Stimulated	4 hourly	Yes	18.00		02.00	
FERGUSON *et al.* (1969)	Stimulated	2 hourly	Yes	Early p.m.	None	Early p.m.	Early p.m.
DOGON *et al.* (1971)	Stimulated constant flow	4 hourly	Yes			07.00	07.00
KATZ and SHANNON (1969)	Stimulated	06.00 12.00 18.00 24.00	No				
DAWES (1972)	Stimulated constant flow	07.00 11.00 14.00 17.00 22.00	No			05.00	05.00
ÄHRENS and LUCKE (1972)	Stimulated Unstimulated	07.00 09.00 11.00 13.00 15.00 17.00	No	13.00			
Our observations	Stimulated	2 hourly	Yes	02.00 to 11.00	22.00 to 24.00	01.00 to 07.00	02.00 to 08.00

STITUENTS OF MIXED SALIVA REPORTED BY VARIOUS AUTHORS

Cl peak	K peak	Ca peak	PO_4 peak	Protein peak	Other components	Statistical analysis of rhythms
	None					
	p.m. None 15.00					
	None					
		Evening		Amylase low in a.m.		
a.m.	p.m.					
Some rhythm	15.00 Some rhythm					
Some rhythm	Some rhythm				Uric acid–some rhythm	Curve fitting
	None	Unstim. 10.00 Stimulated 06.00	None			Analysis by cosinor
05.00	None	09.00	21.40	Not significant 08.00	Urea not sig. 24.00	Analysis by cosinor

PORTED BY VARIOUS AUTHORS, AND A COMPARISON WITH RESULTS REPORTED IN THIS PAPER

K peak	Ca peak	PO_4 peak	Protein peak amylase	N peak	Carbo-hydrate peak	Other components	Statistical analysis of rhythms
			12.00				None
14.00	10.00	10.00 14.00					None
			Amylase 10.00		Glucose 10.00 16.00	Acid phosphatase 10.00	None
			Protein 10.00	10.00		Uric acid 10.00 Urea 16.00 non-protein N 16.00	None
None	None	10.00				I no peak	Analysis by cosinor
Early p.m.			24.00			17-OH steroids early p.m.	Fourier analysis
15.00	None: high 15.00 to 23.00					Mg no peak: high 19.00 Thiocyanate high 11.00, 19.00	None
						17 OH steroids 06.00	None
17.20	19.00	11.20	15.40			Urea 22.20	Analysis by cosinor
	Low at 09.00						None
14.00 to 17.00	01.00 to 07.00	06.00 to 11.00	15.00 to 20.00	15.00 to 20.00	13.00 to 18.00	Total P 08.00–11.00 Glucose 09.00–11.00 and 12.00–15.00	Fourier analysis

and HAUS (1968) are therefore the minimum number acceptable and observations every 1 or 2 hr, as carried out by PALMAI and BLACKWELL (1965) and such as we have used (FERGUSON, ELLIOTT and POTTS, 1969) would be preferable. The period of the rhythms has been established only by BISSADA and HAUS (1968), FERGUSON *et al.* (1969), BISSADA and ELMOSTEHY (1971) and DAWES (1972).

It is usual to attempt to fit sine (or cosine) curves to data suspected of showing circadian variation although other forms exist. These curves are appropriate to data which show a rhythmical variation without abrupt changes owing to external stimuli. The majority of endogenous, and many exogenous, circadian rhythms conform approximately to sine curves (CONROY and MILLS, 1970).

Previous workers using statistical analysis on salivary rhythms (BISSADA and HAUS, 1968; BISSADA and ELMOSTEHY, 1971; DAWES, 1972) have all used the cosinor analysis of HALBERG *et al.* (1965) to fit a cosine curve of 24 hr period to their data. DAWES (1972) did consider curves of 20–28 hr period but no workers have attempted to fit curves of shorter period or in which the maximum and minimum were asymmetrically placed. Most studies have concentrated on a few variables and have not attempted to look for relationships between rhythms—indeed, this is almost impossible without statistical analysis.

In addition to Mills' criteria, it is desirable that observations of more than one 24-hr cycle be obtained. However, it is usually accepted that if a regular waveform can be fitted to observations over 24 hr, and that similar waveforms are shown by a number of subjects, the presence of a circadian rhythm is established.

In the present work, we have attempted to follow circadian rhythms in parotid saliva by collecting it under standardized conditions at intervals of 2 hr throughout the 24 hr and to analyse these statistically by Fourier analysis. We have compared 12 salivary variables simultaneously and have examined the fit of composite sine curves in which the maximum to minimum time interval might be other than 12 hr.

MATERIALS AND METHODS

Subjects

The subjects before and after the experiments led normal lives, usually sleeping between the hours of 24.00 and 07.00 hr and having three main meals daily at approximately 08.00–09.00 hr, 12.00–14.00 hr and 18.00–20.00 hr. One male subject took part in preliminary experiments. In the first major group of experiments 17 subjects participated, eight male and nine female. The second experiment included three male subjects and the third group included seven subjects from the previous groups, four male and three female, and one female subject who had not previously participated.

Sequences of collections

In the preliminary experiment which has been reported elsewhere (FERGUSON *et al.*, 1969), the subject collected saliva at different times over periods of 8–46 hr during 14 days. Several observations were recorded for each even-numbered hour.

In the main experiment, the subjects were asked to report to the laboratory for saliva collections at appropriate times between 09.00 and 21.00 hr and to ensure that meals were always taken directly after a saliva collection. From 21.00 hr onwards, they remained in the building and went to bed at their usual times in rooms near the laboratory. They were awakened for saliva collections and given breakfast after the last collection of the nocturnal hours. Most subjects slept from 24.00 to 07.00 hr but a few remained awake. Oral temperature was taken and saliva collected every 2 hr for 24 hr starting at 11.00 hr. This regime caused little change in their normal timetable as described above.

In one subject, a "practice effect" was observed with salivary flow-rate increasing throughout the experiment. Although preliminary experiments had established that a 2-hr interval between collections was usually sufficient to avoid serial dependency of samples, the results from a few subjects might have been affected by possible fatigue of salivation. Two other experimental designs were therefore used.

In the first, three subjects collected saliva at 2-hourly intervals for 36 hr instead of 24 hr, but otherwise followed the same sampling sequence as before.

Secondly, eight subjects, including seven of the previous group of 17, then took part in an experiment with 4-hourly collections replacing the previous 2-hourly. The experiment was begun at 21.00 hr on Day 1, sampling 4 hourly until 17.00 hr on Day 2. Then a 6-hr break was made between 17.00 and 23.00 hr and 4-hourly sampling continued until 19.00 hr on Day 3. At the end of the experiment every odd-numbered hour had been sampled covering a period of 46 hr. These subjects slept between 23.00 and 07.00 hr except when awakened for collections. Meals were again taken only immediately after the appropriate collections. This modified experimental design reduced both general fatigue, any possible salivary gland fatigue, and the effect of meals upon subsequent collections, a possible factor mentioned by DAWES (1973).

Collection of saliva

Parotid saliva was collected by a modified Lashley cannula (LASHLEY, 1916) and led by polyethylene tubing to a graduated tube standing in ice. Flow was stimulated by sucking "Spangles, acid-drops" renewed every 3 min. This technique was chosen in preference to the constant flow technique used by DOGON *et al.* (1968, 1971) so that flow-rate itself could be studied and the concentrations of salivary components could be assessed under physiological conditions rather than the somewhat artificial conditions resulting from attempts to control flow rate. Collection was continued until 8 ml (5 ml for the estimation of 17-hydroxycorticosteroids and 3 ml for the other estimations) had been collected and the time between the first appearance of saliva in the collecting tube and the end of collection noted. Although the 17-hydroxycorticosteroid assay was discontinued before the end of the experiment because of its technical difficulty, the volume of 8 ml was retained for consistency. Many workers discard the first portion of saliva collected to eliminate variations in composition of the first few drops, but this precaution was not taken in these experiments since such variation was calculated to be negligible in a total volume of 8 ml.

Measurement of oral temperature

Oral temperature was measured before and after each collection as an indication of other circadian variations peculiar to the individual (HALBERG *et al.*, 1969). On arrival in the laboratory, the subjects opened a folder containing their record cards and numbered psychological tests. They placed a clinical thermometer beneath the tongue and carried out two consecutive 1 min tests. The thermometer was removed and the temperature read. This procedure was repeated twice whilst other tests were completed. If the readings differed by 0·1°C or more, readings continued at 2 min intervals until two consecutive readings were in agreement. After the saliva collection, readings were again taken at 2 min intervals until a steady reading was obtained. The mean of the readings taken before and after saliva collection was calculated. The laboratory was used frequently for experiments on circadian rhythms and maintained a fairly constant temperature both day and night.

Analytical methods

The pH was measured immediately in a small aliquot and the remainder of the saliva stored in a refrigerator and analysed within 24 hr. Sodium and potassium were measured by flame photometry in the EEL clinical flame photometer, chloride by potentiometric titration on the EEL chloride meter, inorganic phosphate by the method of KUTTNER and COHEN (1927), protein by the biuret method of ROSENTHAL and CUNDIFF (1956) and total carbohydrate, total phosphate, nitrogen and calcium by the micromethods of SILVERMAN and KLEINBERG (1967).

Treatment of data

The data obtained for each constituent from each individual were subjected to Fourier analysis, a mathematical technique of fitting sine curves and combinations of harmonics to the data. The closeness of fit of the curves to the data and the relative importance of the harmonics were evaluated by the analysis of variance developed by HARTLEY (1949). As the subjects were leading normal lives,

it was assumed that the fundamental period of any cyclic variation would be 24 hr. However, as a normal life pattern concentrates food intake, and therefore saliva secretion, into the waking period of the 24 hr it was thought that the peak-trough interval might vary from 12 hr. We considered therefore the fit of the fundamental sine wave to the data (period 24 hr, peak-trough interval 12 hr), the harmonics of this sine curve with periods 12, 8, 6 and 4·8 hr, and the waves resulting from progressive addition of these harmonics to the simple fundamental curve. This last procedure allows for an asymmetrical variation. Preliminary experiments showed that a combination of the fundamental with the first harmonic fitted most sets of data significantly without being so complex as to be difficult to interpret. Only small improvements in fit were obtained with the further addition of harmonics.

Correlation coefficients between each pair of variables were calculated from the data for each individual subject in case such factors as flow-rate might be significantly related to other variables.

Where fatigue or practice effects were thought to occur, a correction was applied by plotting the "best fit" straight line to the data and then expressing the data as percentage deviations from this line. As this procedure had little effect on the final curves, it was discontinued. However, it did demonstrate that fatigue did not affect results in the experiment of 36 hr duration.

Considerable variation in times of maxima and minima for different subjects was found and so these were plotted as a frequency diagram. The times at which the frequency of maxima and minima differed significantly (χ^2 test; $p < 0{\cdot}01$) from a random distribution were taken as the times of maxima and minima for the population studied.

A similar procedure was followed to investigate relationships between rhythms, by plotting the number of subjects with given time intervals (-11 hr up to $+12$ hr) between maxima or minima for different components. Again, a relationship between maxima or minima of the components was considered to be present when the frequency of a given time interval was found to differ significantly (χ^2 test; $p < 0{\cdot}01$) from a random distribution.

RESULTS

Table 3 shows the mean and standard deviation of each variable for all estimations from all subjects. These are within normally accepted ranges.

TABLE 3. MEANS AND STANDARD DEVIATIONS FOR ALL VARIABLES STUDIED

Component	Mean	Standard deviation	No. of estimations
Oral temperature (°C)	36·93	0·41	352
Flow rate (ml/min)	1·22	0·72	349
pH	7·57	0·37*	349
Sodium (mEq/l)	42·6	22·6	352
Potassium (mEq/l)	26·5	8·8	352
Calcium (mM)	1·26	0·88	336
Chloride (mEq/l)	32·00	17·85	351
Phosphate (mM) (inorganic)	3·28	2·60	349
Total phosphate (mM)	5·60	2·57	353
Protein (mg/100 ml)	229	179	354
Total nitrogen (mg/100 ml)	19·4	32·7	341
Carbohydrate (mg/100 ml)	30	48	353
Glucose (mg/100 ml)	0·9	1·1	353

* pH units: not calculated as (H^+).

Table 4 shows the number of subjects to whose data a sine curve could be fitted at differing levels of significance. The combination of the 24-hr fundamental curve with a first harmonic provided a significant fit in 288 sets of data from a total of 364 and 202 of these curves reached the $p < 0{\cdot}01$ level of significance. Very few (9) sets of

TABLE 4. NUMBERS OF SUBJECTS TO WHOSE DATA SINE CURVES COULD BE FITTED AT THREE LEVELS OF SIGNIFICANCE

Component	Fundamental sine curve period 24 hr			First harmonic period 12 hr			Combined first harmonic and fundamental			No sig. fit to sine curves
	0·05	$p <$ 0·01	0·001	0·05	$p <$ 0·01	0·001	0·05	$p <$ 0·01	0·001	
Oral temperature	16	8	4	2	1	1	26	25	16	2
Flow rate	9	4	2	1	0	0	21	16	6	7
pH	6	2	0	7	3	0	23	15	10	5
Sodium	13	7	3	0	0	0	27	21	11	1
Potassium	10	6	3	2	0	0	25	13	7	3
Calcium	7	3	0	1	0	0	16	12	5	12
Chloride	11	7	3	5	2	0	26	20	11	2
Inorganic phosphate	4	2	1	3	1	0	20	10	4	8
Total phosphate	7	5	1	3	1	0	19	13	7	9
Protein	11	7	3	3	0	0	22	19	11	6
Total nitrogen	11	3	0	1	0	0	22	14	7	6
Carbohydrate	5	0	0	2	1	1	17	9	4	11
Glucose	5	2	0	4	0	0	21	15	5	7

data gave a significantly close fit to a first harmonic curve alone (i.e. to a sine wave of 12-hr period). Only 115 sets of data fitted a fundamental curve at a significance of $p < 0{\cdot}05$. Variables to which curves could be fitted most consistently in the 28 sets of data were: oral temperature (26 out of 28) sodium (27 out of 28) chloride (26 out of 28) and potassium (25 out of 28). Total carbohydrate showed only 17 significant curves and calcium only 16. However, 7 of the 16 calcium curves were significant at $0{\cdot}001 < p < 0{\cdot}01$ and 5 at $p < 0{\cdot}001$. Figures 1–13 show the curves fitted for variables in one of the six subjects to whose data curves could be fitted for all variables. One of these subjects repeated the experiment and again showed significant circadian variation in all variables. The example chosen is the best of the sets obtained from the original experimental design but is not as good as any of the four sets obtained on the final one. The individual curves are not necessarily the best examples for a given variable and the times of peaks may not be typical of the majority of subjects.

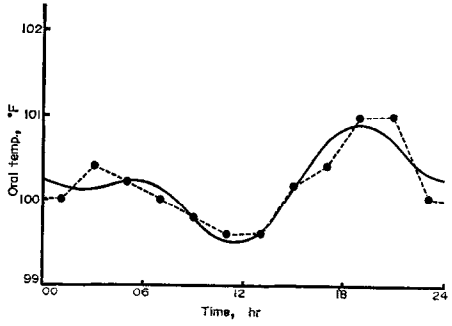


FIG. 1.

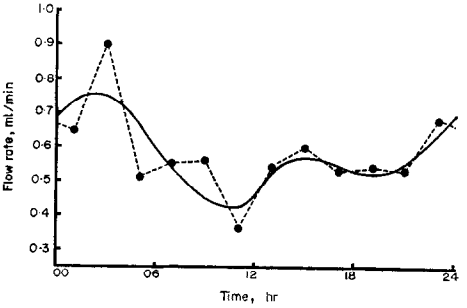


FIG. 2.

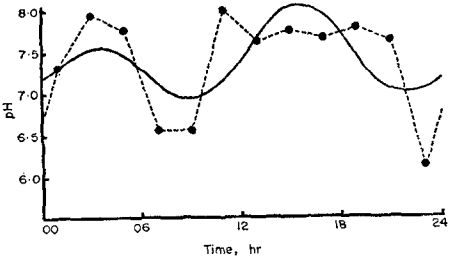
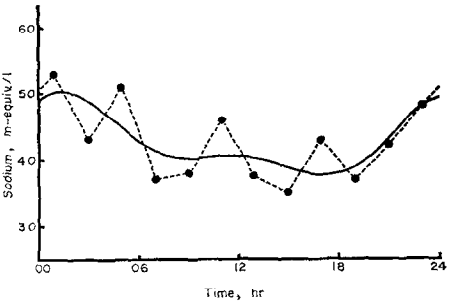


FIG. 3.



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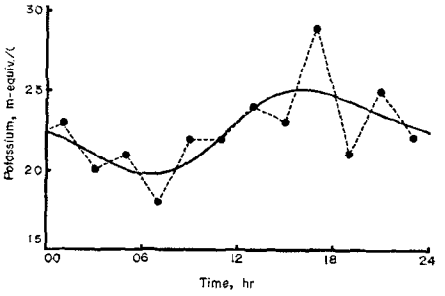

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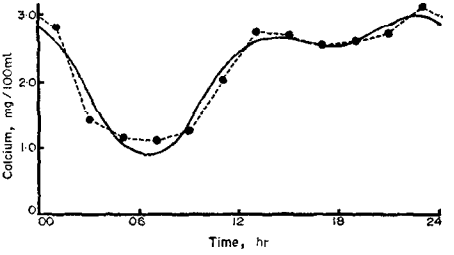

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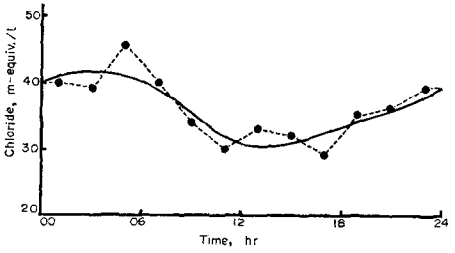

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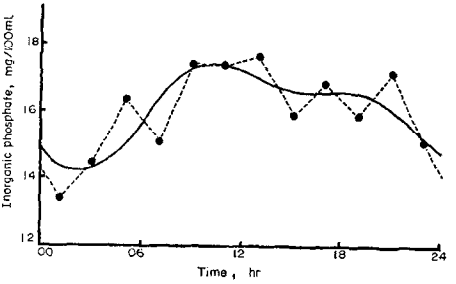

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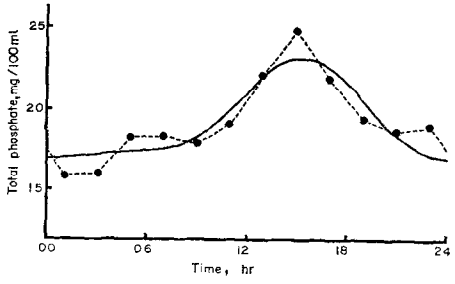
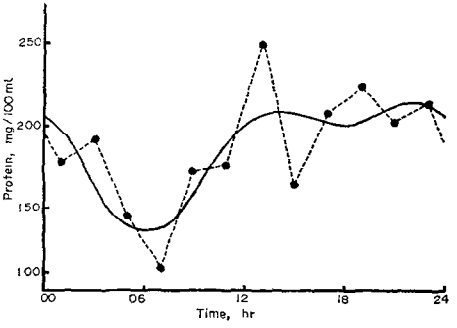


FIG. 9.



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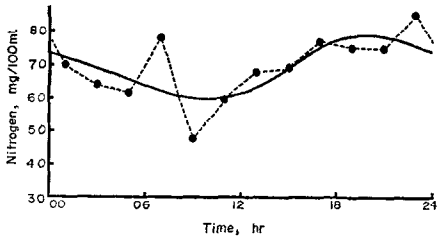
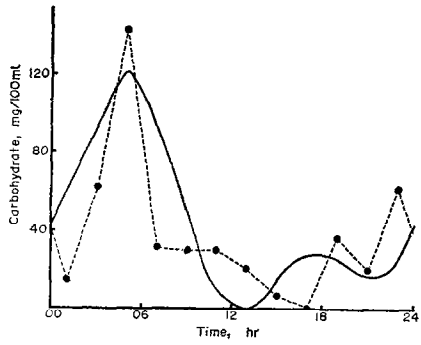


FIG. 11.



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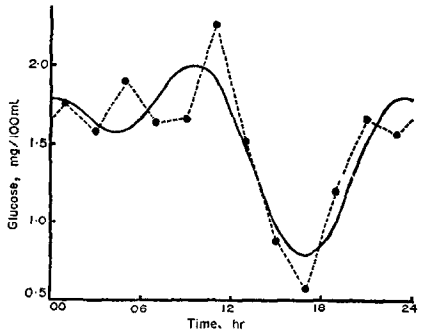

FIG. 13.

FIGS. 1–13. Concentrations of parotid salivary constituents, parotid salivary flow rate and oral temperature plotted against time. The dashed line joins experimental observations and the solid line is a plot of the best-fitting sine curve derived from the sum of the fundamental and the first harmonic obtained from a Fourier analysis. All observations from one subject.

1. Oral temperature
2. Flow rate
3. pH
4. Sodium
5. Potassium
6. Calcium
7. Chloride
8. Inorganic phosphate
9. Total phosphate
10. Protein
11. Nitrogen
12. Carbohydrate
13. Glucose.

Figure 14 is a frequency diagram showing the times at which the subjects attained maximal and minimal values for each of the variables studied. Oral temperature showed maxima between 18.00 and 23.00 hr with minima between 03.00 and 09.00 hr in 21 subjects. Minimal flow rates of stimulated parotid saliva occurred between 24.00 and 04.00 hr with a second group between 14.00 and 17.00 hr, but maximal flow rates occurred mainly between 02.00 and 14.00 with the larger grouping between 06.00 and 09.00 hr. However, four subjects showed their highest flow rates in late afternoon between 17.00 and 19.00 hr. Sodium concentrations and chloride concentrations, as

shown by the correlation data, had peak and trough values at approximately the same times, the majority of subjects showing maximum sodium concentrations between 01.00 and 07.00 hr, maximum chloride concentrations between 02.00 and 08.00 hr with the sodium minimum concentrations between 16.00 and 20.00 hr and chloride between 14.00 and 19.00 hr. It is noticeable that no subject showed a maximum sodium concentration between 15.00 and 23.00 hr or maximum chloride concentration between 12.00 and 23.00 hr.

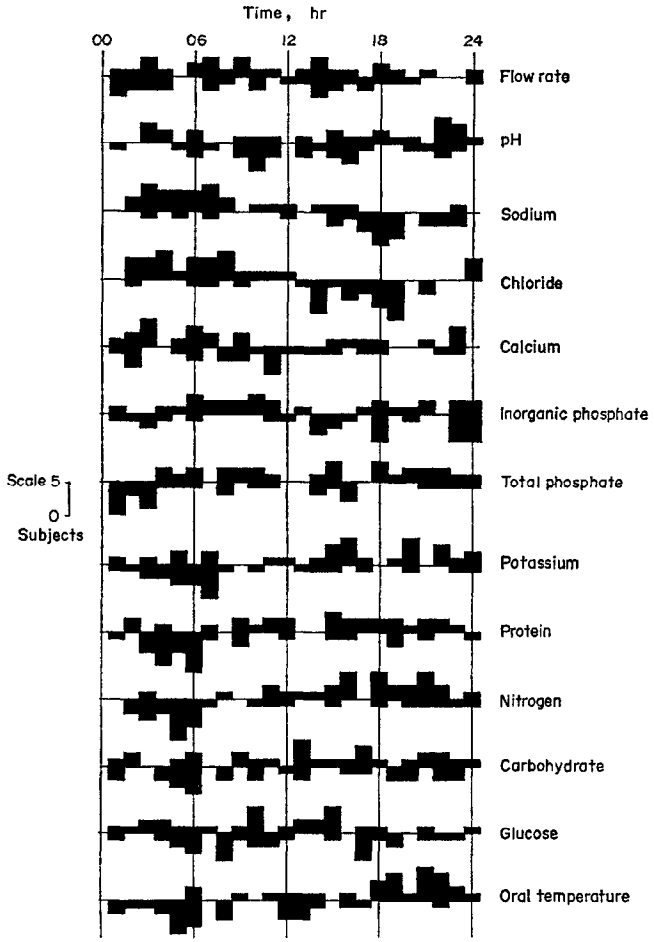

FIG. 14. Histogram showing times of maximum and minimum concentrations of parotid salivary components in 28 subjects. Blocks above the line represent maxima, below the line minima.

Potassium concentration maxima were found between 14.00 and 17.00 hr and between 19.00 and 01.00 hr. The minima were grouped much more clearly, between 02.00 and 08.00 hr.

The pH of parotid saliva was lowest between 08.00 and 15.00 hr in the majority of subjects but maxima appear between 22.00 and 24.00 hr.

Calcium concentrations were lowest between 08.00 and 11.00 hr in a third of the subjects. The major group of maxima is between 01.00 and 07.00 hr.

Inorganic phosphate concentrations show maxima from 06.00 to 11.00 hr, minima from 23.00 to 03.00 hr. Total phosphate shows a similar pattern but the night minima from 23.00 to 03.00 hr are much more obvious and the morning maxima from 08.00 to 11.00 hr less marked than the corresponding groups for inorganic phosphate.

Protein and nitrogen concentrations have minima between 02.00 and 07.00 hr, but much more diffuse maxima between 15.00 and 20.00 hr.

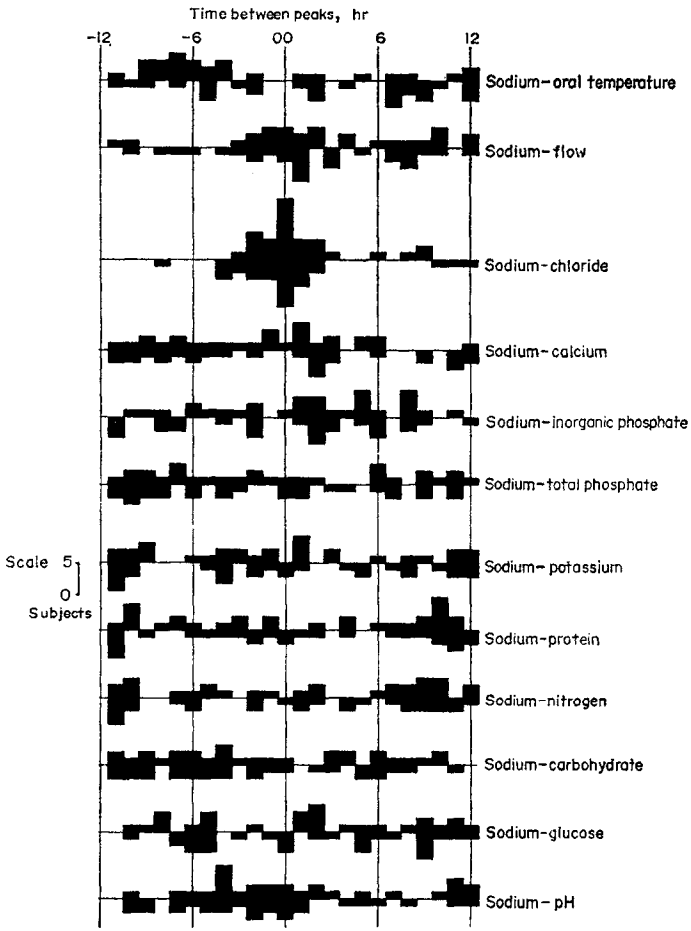

FIG. 15. Histogram showing the relationship of maximum (above line) and minimum (below line) parotid saliva sodium concentrations to the corresponding peaks for other constituents in 28 subjects. A minus sign indicates that the sodium concentration peak is later than the peak of the other component.

TABLE 5. NUMBER OF SUBJECTS WITH STATISTICALLY SIGNIFICANT ($p < 0.05$) CORRELATION COEFFICIENTS BETWEEN PAIRS OF VARIABLES. TOTAL NUMBER OF SETS OF OBSERVATIONS —28

	Flow rate		pH		Sodium		Potassium		Chloride		Calcium		Inorganic phosphate		Total phosphate		Protein		Nitrogen		Carbohydrate		Glucose	
	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−
Oral temperature	1	0	2	0	2	3	4	0	0	5	1	2	0	2	0	1	4	0	1	0	1	1	0	
Glucose	1	2	2	0	2	2	0	3	2	1	1	1	0	2	0	3	1	2	2	0	12	0		
Carbohydrate	0	2	0	2	0	1	1	1	1	3	1	1	0	0	4	1	5	0	3	0				
Nitrogen	1	0	2	1	1	0	3	1	1	3	1	2	1	1	1	1	20	0						
Protein	0	1	2	0	1	2	6	1	0	3	1	1	1	0	2	0								
Total phosphate	1	1	0	0	0	1	2	1	0	2	2	2	5	0										
Inorganic phosphate	1	2	0	0	1	2	0	2	2	1	1	0												
Calcium	2	1	0	1	1	0	2	0	3	0														
Chloride	8	2	0	1	21	0	0	3																
Potassium	1	2	2	1	4	2																		
Sodium	9	1	1	0																				
pH	5	1																						

TABLE 6. NUMBER OF HOURS DIFFERENCE BETWEEN MODAL TIMES FOR MAXIMA AND MINIMA BETWEEN PAIRS OF VARIABLES

	Flow rate	pH	Sodium	Potassium	Chloride	Calcium	Inorganic phosphate	Total phosphate	Protein	Nitrogen	Carbohydrate	Glucose
Oral temperature	12	+2½	+6	+3*	+7½		12*	−10½	−8½			
Glucose	−6½**		+6½		+7½	12*	−6½*		−7½*	+8½	−½**	
Carbohydrate		+4½*	+5½	+2½		+5½	−6	−5½	−2½*	−1½		
Nitrogen			−9*	+½**	12**	+4½*		+3½	+½**			
Protein			12**	+2**	−9½**	+3½**	12	−2				
Total phosphate				+6½*	−6		−½**					
Inorganic phosphate	+1½	−6	−3½		−5*							
Calcium		−½*	−1½		+5½							
Chloride	−½**	−4*	+½**	+11**								
Potassium	12**		+11½*									
Sodium	−½**	−2½*										
pH	−1											

A plus sign indicates that the component in the column has maxima and minima later than the component in the row. The number of subjects exhibiting this time difference was significantly different from a random distribution at the $p < 0.05$ level in all the figures given. ($p < 0.01$ if one asterisk, $p < 0.001$ if two).

Glucose concentration maxima might be expected to occur in association with meal times so the two main time groups 09.00–11.00 hr and 12.00–15.00 hr are not unexpected, although no evening group is seen. The minima, however, are more difficult to group—most of them lie between 04.00 and 08.00 hr. Total carbohydrate is less easy to predict since it arises mostly from glycoprotein rather than from glucose. Clearly, variations in the glucose concentration (mean value of 0·9 mg/100 ml) will have little effect on the total carbohydrate concentration with a mean value of 30 mg/100 ml. Again, the maxima are grouped between 13.00 and 18.00 hr whilst minima this time are found in the early morning between 04.00 and 06.00 hr.

Table 5 shows the number of subjects for whom statistically significant ($p < 0{\cdot}05$) correlation coefficients were obtained for each pair of variables studied. The most significant associations were between the concentrations of sodium and chloride (21 subjects), of protein and nitrogen (20 subjects) and of glucose and carbohydrate (12 subjects). These last two are expected associations. Although there is much convincing evidence for associations between flow-rate and concentrations of some of the constituents, under the strong stimulation conditions of the experiment in which high rates of flow were induced, this was seen only for sodium (19 subjects) and chloride (8 subjects).

The sequence of maxima and minima and the time relationships between variables were difficult to evaluate because of the great individual variation. Relationships were sought, therefore, by plotting frequency diagrams, examples of which are shown in Fig. 15. This shows how the times of sodium concentration maximum and minimum (calculated from the Fourier analysis curves) were related to the times of maxima and minima of other variables. Each unit on the block diagram above the line represents one subject for whom the time of maximum of sodium concentration and the time of maximum of the other variable differed by the given number of hours. When the sodium concentration maximum appeared up to 11·5 hr before the maximum of the other variable the time interval was given a positive sign and, when the maximum of the other variable preceded the sodium concentration maximum, it was given a negative sign. The units below the line similarly represent intervals between minima. All the frequency diagrams were analysed and the occurrence of specific time intervals between maxima and minima tested by a χ^2 test. As observations were taken every 2 hr, we tested the frequency of occurrence of intervals in 4 hr sections advancing by steps of 1 hr. The probability of a time difference between maxima (or minima) exceeding a chance distribution was therefore calculated and this enabled us to draw up a table (Table 6) of the modal time differences; these being those times at which the number of subjects showing a particular time difference exceeded the greatest number expected to do so by chance once in every 20 times. Table 6, then, shows the temporal relationships between calculated maxima and minima for each pair of variables where there was statistically significant agreement between subjects. A plus sign indicates that the variable in the column reached maximum and minimum values during the 12 hr following the time at which the variable in the row reached a similar maximum or minimum. A minus sign indicates that the column variable preceded the row variable by up to 11·5 hr. Since observations were at intervals of 2 hr, differ-

ences of up to 2 hr indicate a close relationship between the two variables. Differences of 10–12 hr indicate that the constituents are out of phase only if the fundamental sine curve is the major component of the circadian variation. Reference to Table 4 shows that the fundamental was the major component in most curves. In the few curves in which the first harmonic exerted a dominant influence, the relationship of maxima and minima to those of other constituents were similar to those observed in other subjects when the difference between maxima was 0–2 hr. Variables whose peak concentrations were 10–12 hr apart in other subjects were approximately 6 hr out of phase when one variable had a curve in which the first harmonic was a major component. Thus, if we take oral temperature as an indication of standard bodily rhythms, flow rate and both inorganic and total phosphate may be regarded as having rhythms completely out of phase with the bodily rhythm, showing maxima when oral temperature is low and vice versa.

Sodium and chloride rhythms were significantly related and in phase with flow-rate, potassium rhythm being significantly out of phase. As would be expected, protein rhythm and nitrogen rhythm are in phase with each other, as are glucose and carbohydrate and inorganic and total phosphate. Two main groups of observations emerge, one group with rhythms approximately in phase with flow-rate and the other group approximately in phase with oral temperature and completely out of phase with the first group. In the first group are sodium, chloride, pH and calcium and in the second group potassium, protein and nitrogen. Carbohydrate may also fit into the second group. The phosphate concentrations do not conform to either pattern.

The difference between the maximum and minimum values of the fitted curve combining the 24 hr fundamental with its first harmonic is shown in Fig. 7 as a percentage of the mean value for each component and thus indicates the variation in estimation likely to be due to observations at different times of day. The variation in pH has been expressed in pH units and not calculated as hydrogen ion concentration. Variation is least for potassium and for inorganic phosphate and greatest for glucose and carbohydrate.

TABLE 7. SIGNIFICANCE OF FIT OF CALCULATED CURVES AND TIMES OF MAXIMUM AND MINIMUM OF CURVES FOR EACH COMPONENT IN ONE OF THE SEVEN SUBJECTS STUDIED ON TWO SEPARATE OCCASIONS

	Experiment I			Experiment II		
Component	Sig.	Time of max	Time of min	Sig.	Time of max	Time of min
Oral temperature	$p < 0{\cdot}001$	22	05	$p < 0{\cdot}001$	19	05
Flow rate	n.s.	01	18	$p < 0{\cdot}01$	23	06/18
pH	n.s.	03	17	$p < 0{\cdot}01$	03	13
Sodium	$p < 0{\cdot}01$	02	17	$p < 0{\cdot}001$	04	15
Potassium	$p < 0{\cdot}001$	16	06	$p < 0{\cdot}001$	15	05
Calcium	n.s.	17	10	$p < 0{\cdot}001$	13	05
Chloride	$p < 0{\cdot}05$	03	17	$p < 0{\cdot}01$	02	17
Inorganic phosphate	$p < 0{\cdot}001$	10	23	$p < 0{\cdot}05$	09	18
Total phosphate	$p < 0{\cdot}001$	11	23	$p < 0{\cdot}001$	10	01
Protein	$p < 0{\cdot}001$	12	04	$p < 0{\cdot}001$	11	04
Total nitrogen	$p < 0{\cdot}05$	15	22/09	$p < 0{\cdot}001$	12	05
Carbohydrate	n.s.	18	12	$p < 0{\cdot}01$	17	05
Glucose	$p < 0{\cdot}01$	04	11	$p < 0{\cdot}001$	02	08

Where a strong first harmonic component is present this has been indicated by a second value for that maximum or minimum. Calcium concentration curves in this subject were very flat.

For the seven subjects who returned to perform the 46 hr experiment, comparisons were made between the results obtained under the two schedules. They showed differences in the mean values obtained but the phasing of the circadian rhythms were very similar [χ^2 test, $p < 0{\cdot}001$]. Table 7 gives a comparison of the two sets of observations carried out on one of these subjects. Other subjects compared similarly. Of the 13 maximum times and 13 minimum times, 17 agreed within 2 hr. Only three times (all minima) differed by more than 4 hr. Two of these were values from curves which did not fit the data significantly. The single subject previously studied also showed similar patterns from day to day.

DISCUSSION

As was explained earlier, most circadian rhythms in man can be described by simple sine (or cosine) waves: that is, variables show one maximum and one minimum separated by 12 hr and a smooth variation occurs between these points. A closely fitting sine wave of 24 hr period returns to approximately the same value every 24 hr and this suggests that the cycle demonstrated is part of a series of similar cycles. Other curves such as square wave and "saw tooth" curves have been identified with circadian variation but, unless there is cause to suspect abrupt changes in a variable, such as might result from exogenous factors, sine curve analysis would be the first method used to look for cyclic variation. If sine curves fit the data this is strong presumptive evidence of a rhythm. The presence of a circadian rhythm is usually confirmed by either a transverse method, examining a number of subjects over a single cycle to see whether all show a similar type of variation, or a longitudinal method, examining the consistency of cycles in single subjects. We have used both these methods in these studies.

Most of the variations of the parameters studied may be described by a sine curve of 24 hr period combined with its first harmonic. This is true of the temperature as well as the salivary rhythms, but is more marked with the saliva. One explanation of this is that the normal secretion of stimulated saliva occurs during the waking period of the day and the combined curve can permit distortion of the fundamental sine wave to allow intervals between maximum and minimum other than of 12 hr. Previous reports of circadian variations in saliva (Tables 1 and 2) have not, with the exceptions of those of BISSADA and HAUS (1968), DOGON *et al.* (1968, 1971) and DAWES (1972), included any statistical analysis of the variations observed. BISSADA and HAUS (1968) and DAWES (1972) fitted sine curves of 20–28 hr period but did not attempt to find others. Only sodium and phosphate concentration curves fitted significantly in the 10 subjects of BISSADA and HAUS (1968) but DAWES (1972) found significant curves for protein, sodium, potassium, calcium, and chloride concentrations in parotid saliva. Not all of his subjects showed significant variations—only three out of eight subjects had a significant calcium rhythm. DOGON *et al.* (1971) merely tested the significance of differences between day and night values without attempting to fit curves to their data. Only chloride and thiocyanate concentrations showed significant variation by their method of analysis. We have re-examined their

data, using our methods of statistical analysis and although their 4-hr sampling pattern gives only six points in 24 hr, we found that a combined 24 hr period fundamental and first harmonic sine curve fitted their data for chloride, thiocyanate, potassium and calcium concentrations.

Our analysis of the salivary components shows that there is considerable individual variation both in the extent of circadian variation and in the phasing of the curves that are demonstrable. Thus no significant sinusoidal rhythm of calcium concentration can be fitted to the data from 12 subjects but for 12 others the fitted curve is highly significant [$p < 0{\cdot}01$]; and potassium concentrations were maximal in four subjects between 05.00 and 07.00 hr although this was the modal time for minimum values in the other subjects.

Circadian rhythms have been demonstrated here for all the variables studied. Similar compound sine curves could be fitted to the data for any one variable in at least half of the subjects although there was variation in the times of maxima and minima. The seven subjects who were studied in two different experimental regimes showed similar curves and similar times of maxima and minima on the 2 occasions. The subject illustrated in Table 7 is typical of these.

The modal times for maxima and minima for the sodium, potassium, calcium, inorganic phosphate and chloride concentrations are similar to those reported by SHANNON and SEGRETO (1968a,b,c), BISSADA and HAUS (1968), DOGON *et al.* (1971) and DAWES (1972). Since Shannon and Segreto did not analyse samples later than 16.00 hr or earlier than 08.00 hr they could not observe variations occurring during the night. Bissada and Haus did not find rhythms in potassium and calcium concentrations whilst Dogon *et al.*, as previously discussed, found no significant difference between day and night values for calcium. The mean amplitude of the potassium curves is very small and might make analysis of this rhythm difficult. Calcium rhythms in stimulated parotid saliva were not universally found in this study. Unfortunately, the pooling of results and reporting only of mean values by Bissada and Haus makes it impossible to know how much individual variation in rhythms they found.

Although the relationships between components are mainly the predictable ones —sodium and chloride, inorganic and total phosphate, protein and nitrogen, glucose and carbohydrate—the organic components tend to have maxima between 12.00 and 20.00 hr whilst flow rate, sodium, calcium and chloride concentrations usually have their maxima between 02.00 and 06.00 hr. The curves illustrated for one subject in Figs. 1–13 do not conform to this pattern in carbohydrate and glucose concentrations. Studies of unstimulated salivary flow-rate (SCHNEYER *et al.*, 1956) show that, during sleep, parotid flow-rate ceases or becomes very low. In the experiments described here, nine of the 28 subjects showed minimum rates of flow when roused from sleep but other subjects showed very high rates of flow in response to stimulation at these times.

Sodium, chloride, calcium, phosphate and hydrogen ion concentrations in saliva are known to be dependent upon flow-rate and the times of maximum and minimum of sodium and chloride were closely associated with those of flow-rate in these circadian studies. Calcium concentration, however, showed more affinity to pH variation

than to flow-rate variation. The association of similar phasing of sodium and chloride concentration rhythms with flow-rate in our experiments cannot be fully explained by their dependence on flow-rate. The magnitude of the variations was greater than would be predicted from flow-rate variations and they did not occur in parallel. Only one-third of the subjects showed statistically significant positive correlation coefficients between sodium and chloride concentrations and flow-rate; one and two subjects respectively showed significant negative correlations (Table 5). For calcium concentration, three subjects showed significant correlations of which one was negative. However, the correlation coefficient between sodium and chloride concentrations was positive for all subjects and significant for 21, the greatest number of significant correlations between any pair of variables. The association of sodium concentration with chloride concentration is more consistent than that of either of them with flow-rate, which suggests that changes in sodium and chloride concentrations are not necessarily due to changes in flow-rate but that changes in concentrations of these two ions are related.

The influence of mealtimes was largely eliminated by the procedure of collection immediately before meals. However, the variations in glucose and total carbohydrate concentrations were possibly due to the intake of food. No peaks occurred after dinner but after breakfast and lunch the values obtained for these components were very high in comparison with those at other times.

The circadian variations in parotid saliva components cannot be explained as resulting from plasma variation since all the components studied, except phosphate, show only small amplitude rhythms in plasma (WESSON, 1964). The phosphate rhythm demonstrated in parotid saliva is completely out of phase with the rhythms in plasma. PAWAN (1955) suggested that mixed saliva circadian rhythms in sodium and potassium concentrations were related to plasma aldosterone and BLAIR-WEST *et al.* (1967) have shown in the sheep that aldosterone is the main hormone governing the output of sodium in parotid saliva. The rhythms described for parotid sodium concentrations here would be consistent with those given for plasma aldosterone by WESSON (1964). A major rhythm in sodium concentration would be expected to affect the concentration of chloride, the major anion present in saliva, and so a similar rhythm to that of sodium was not surprising.

It is clear from the magnitude of the variations in concentration of the components described that it is necessary to specify time of collection of saliva when giving any figures for concentrations of these components. The practice of many research workers of collecting saliva early in the morning to obtain standard values may ensure reproducibility for an individual saliva at that time but not between salivas of individuals with differing rhythms. Some components—sodium, chloride, potassium, nitrogen and protein—will in most subjects be subject to maximum rate of change of concentration during the period 08.00–10.00 hr since this time is midway between the times of their maximum and minimum concentrations.

Variations in pH, calcium, phosphate and protein may be important in oral health and the effect of these circadian variations may be to impose some kind of rhythm on susceptibility to, or on the progress of, oral disease.

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Résumé—Les variations circadiennes de la vitesse de sécrétion et du pH de salive parotidienne stimulée et les concentrations en sodium, potassium, calcium, chlore, phosphate inorganique et total, azote, protéine, glucose et hydrate de carbone total ont été étudiées et correspondent à des courbes dérivées d'une combinaison d'une courbe sinusoide fondamentale, pendant 24 hr et sa première harmonique. De telles courbes sont similaires pour un seul sujet, mais des variations considérables s'observent d'un sujet à l'autre.

Zusammenfassung—Die circadian Variationen der Sekretionsgeschwindigkeit und des pH vom Speichel der stimulierten Ohrspeicheldrüse, sowie die Konzentrationne an Natrium, Kalium, Kalzium, Chlor, inorganischem und gesamtem Phosphat, Stickstoff, Protein, Dextrose und totalem Kohlenhydrat wurden erforscht. Sie entsprechen Kurven, die aus einer Zusammensetzung einer fundamentalen Sinuskurve abgeleitet sind, während 24 Stunden und ihrer ersten Schwingung. Solche Kurven sind ähnlich bei ein und derselben Versuchsperson; erhebliche Schwankungen jedoch werden von einer zur anderen Versuchsperson beobachtet.

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