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12. Potato tubers were allowed to produce etiolated sprouts at 20° to 25°C. Sprout samples (0.1 to 0.2 g) were homogenized at room temperature in a small conical ground-glass homogenizer with 200 m*M* K_2HPO_4, 10 m*M* sodium diethyldithiocarbamate, and 0.1 percent Triton X-100 (1.5 ml per gram of tissue). Tissue debris was removed by brief low-speed centrifugation, and the sap was used immediately.
13. Portions (3 to 5 μl) of the sap samples to be tested were pipetted either onto treated nitrocellulose membrane (*11*) and then baked for 2 hours at 80°C in a vacuum oven or onto DBM paper backed by two sheets of Whatman 3 MM paper and transferred with 150 m*M* sodium acetate and 850 m*M* acetic acid at 4°C (*14*). The same buffer [40 percent formamide, 0.18*M* NaCl, 10 m*M* sodium cacodylate, 1 m*M* EDTA, 0.1 percent sodium dodecyl sulfate, and yeast transfer RNA (400 μg/ml) at *p*H 7.0] was used before and during hybridization. The reaction before hybridization (16 hours at 42°C in the presence of 1 percent glycine) and the hybridization reaction [24 hours at 55°C in the presence of 10 percent dextran sulfate and ^{32}P-labeled nick-translated pDC-29 recombinant DNA (1 to 2.5 × 10^6 cpm/ml)] were performed essentially as described (*14*). Nick translation in the presence of deoxycytidine [α-^{32}P]triphosphate followed a protocol supplied by the Amersham Corporation and yielded DNA with an initial specific activity of ~ 2 × 10^8 cpm/μg. The ratio of buffer volume to membrane (or paper) area was at least 1 ml per 35 cm^2. Nick-translated DNA was denatured by heating for 2 minutes at 100°C in the presence of 50 percent formamide before addition to the hybridization reaction. Nitrocellulose membranes and DBM paper were washed at room temperature with five changes of 0.36*M* NaCl, 5 m*M* tris-HCl (*p*H 7.5), and 0.1 percent sodium dodecyl sulfate and then with two changes of the same buffer diluted tenfold before autoradiography, which was carried out for 24 to 48 hours at −70°C with Kodak X-Omat film and DuPont Cronex Lightning-Plus intensifying screens.
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Circadian Rhythms of Blood Minerals in Humans

Abstract. *Circadian rhythms of ionized calcium and phosphate concentrations have been demonstrated in human blood. A computer-derived model curve representing the 24-hour fluctuations in ionized calcium cannot be correlated consistently with curves for total calcium or phosphate. Knowledge of these circadian rhythms provides a physiological basis for further understanding the interactions between blood minerals and calcium-regulating hormones.*

Several hormones and minerals undergo daily variations in concentration in human blood (*1–5*). Analysis of deviations from normal circadian patterns may provide a sensitive basis for diagnostic evaluation and therapeutic intervention in many disease states (*2, 3*).

Attempts to define the biological rhythms of calcium and inorganic phosphate (P_i) in mammalian blood have yielded disparate results (*4, 5*). To our knowledge, no one has examined, in any species, 24-hour patterns of blood Ca^{2+}, the physiologically active fraction of total calcium (Ca_T) (*6*). This report establishes the presence of 24-hour patterns for blood Ca^{2+} and serum P_i in humans.

Seven 20- to 30-year-old males in good health and without a history of drug or vitamin use were studied during a 24-hour period. After their informed consent was obtained, they were adapted to study rooms for 12 hours overnight. In the morning an indwelling venous catheter was placed in the antecubital fossa of each subject. To maintain catheter patency for blood sampling every 30 minutes, 650 ml of a solution containing dextrose (0.05 g/ml), sodium chloride (0.04 mEq/ml), and heparin (5 U/ml) was slowly infused throughout the 24-hour period. Meals were offered at 0900, 1200, and 1730 hours; the 24-hour intake of calcium and phosphorus was estimated to be at least 500 and 900 mg, respectively. Activity was limited to movement within the study rooms during the daytime.

Observations at each of the 48 time points were averaged for all subjects to produce the curve shown in Fig. 1A. The U-shaped curve shows that the concentration of Ca^{2+} fell during the day after an initial maximum at 1000 hours, began rising late in the evening, and continued to rise until the concentration measured at 1000 hours was again reached in the early morning. This pattern is seen more clearly after the data are smoothed by using running medians to filter out small fluctuations (Fig. 1B) (*7*). The smoothed data were fitted by a third-order polynomial regression of the variable (Ca^{2+}) against time (*8*). The resulting parameters were used to construct a simple mathematical representation of the curve which preserves all but minor variations in the raw data. This representation, designated the Ca^{2+} model, is shown in Fig. 2A. Pearson correlation coefficients (*9*) between the Ca^{2+} model and the smoothed and raw data are .97 and .92, respectively. The model, therefore, provides an excellent fit to both the raw and smoothed data.

Although the maximal changes in the model appear relatively small (0.30 mg/dl), they are based on intersample variations that are many times greater than the error of measurement for individual mineral concentrations. Changes in Ca_T similar in magnitude to those reported here for the Ca^{2+} have been associated with significant alterations in the concentration of circulating parathyroid hormone (*5*). The oscillations in Ca^{2+} cannot be attributed to fluctuations in the *p*H of the blood samples, since the range of blood *p*H was extremely narrow. Moreover, the constancy of the Ca^{2+} circadian rhythm, as well as the one for serum phosphate described below, was evident in four studies carried out for as long as 40 hours. We conclude that these circadian oscillations in Ca^{2+} are of physiological significance.

The data obtained for serum P_i appear less "noisy" than those for Ca^{2+}; the shape of the P_i curve is readily apparent without median smoothing (Fig. 1C). The circadian pattern of serum P_i concentration consists of a minimum at about 1100 hours followed by two peaks, the largest occurring between 0200 and 0400 hours. We did not observe oscillations in serum P_i associated with meals. An eighth-order polynomial of time was fitted to these circadian variations; the resulting curve is shown in Fig. 2B. The correlation between the raw data and the model is .98 (*10*).

Previous studies have reported a single peak in P_i concentration during the night, when the concentration of Ca_T is at its daily low (*5*). Our data reveal two clearly separable peaks with maximum concentrations in the evening and early morning. Changes in serum P_i of this magnitude have been associated with physiological alterations in the biosynthesis of parathyroid hormone (*11*) and 1,25-dihydroxyvitamin D_3 (*12*).

Total calcium (Fig. 1, D and E) follows a circadian pattern quite different from that of Ca^{2+}; the correlation between

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Ca_T and Ca^{2+} concentrations was significant ($P < .05$) in only two of the subjects studied. Total calcium predominately mirrors diurnal changes in the concentration of serum protein ($r = .61$, $P < .01$) (*5*); in addition, we observed an early-morning decrease in Ca_T (Fig. 1, D and E) which corresponds closely to a reduction in serum protein in subjects who have sufficient daytime activity. Thus, measurements of Ca^{2+} may be of considerable importance for investigating the hormonal and ionic regulation of mineral metabolism in humans.

At least three different types of abnormal Ca^{2+} and P_i patterns are possible: (i) the normal circadian rhythms may be absent completely (hypoparathyroid children treated with single daily doses of 1,25-dihydroxyvitamin D_3 have normal amounts of blood calcium but do not follow the Ca^{2+} curve) (*13*); (ii) the normal Ca^{2+} and P_i circadian rhythms may be nonsynchronous with clock time, although the normal shape of the circadian pattern is retained (*14*); or (iii) the amplitude of the model curves may be altered,

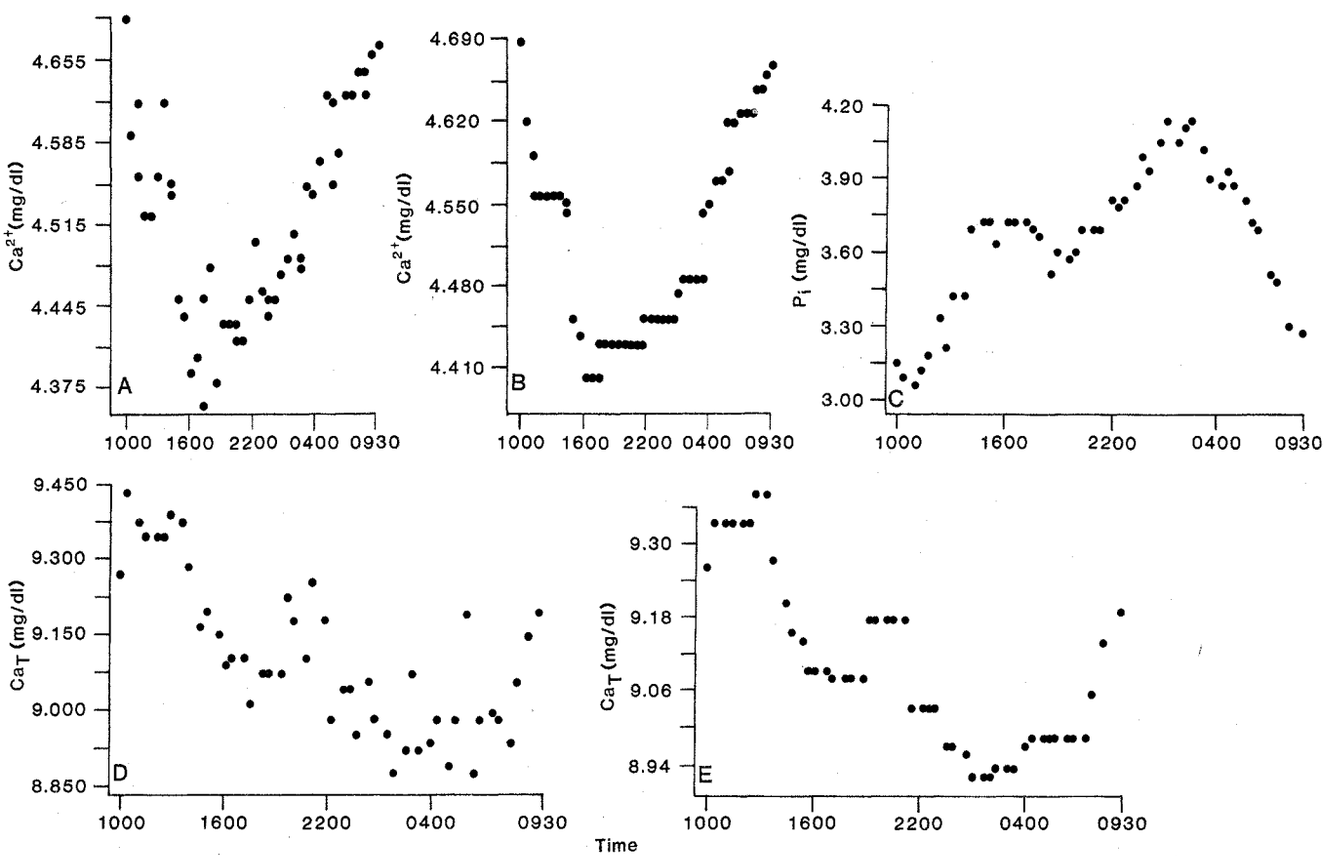

Fig. 1 (above). Curves showing circadian patterns in the concentration of blood Ca^{2+}, serum P_i, and serum Ca_T in the seven subjects. (A) Raw Ca^{2+} data; (B) smoothed Ca^{2+} data; (C) raw P_i data; (D) raw Ca_T data; (E) smoothed Ca_T data. Blood samples were drawn every 30 minutes. At least 8 ml was drawn first into a reservoir syringe to clear the tubing of 1.5 ml of intravenous fluids, preventing dilution of the blood samples. Samples to be analyzed for Ca_T, P_i, and total proteins were allowed to clot and then were centrifuged; the serum thus obtained was frozen for later analysis (*15*). For Ca^{2+}, 1.50-ml samples were collected anaerobically every 30 minutes in 3-ml syringes containing 75 μl of sodium heparin (1000 U/ml; Organon) to prevent clotting. The samples were immediately capped and placed in shaved ice for 1 hour. These techniques ensured a very narrow venous *p*H range of 7.357 ± 0.008 (mean ± standard deviation); and we could not demonstrate any sporadic or systematic changes in venous *p*H associated with meals. The first injection (500 μl) into an ionized calcium analyzer (Nova 2, Nova Biomedical) was used to prime the membrane. The instrument readings obtained for the second and third injections were averaged to yield the Ca^{2+} concentration. Variabilities (± 3 standard deviations) for replicate measurements of Ca^{2+}, Ca_T, and P_i were 0.05, 0.18, and 0.08 mg/dl, respectively. These values were obtained by an analysis of variance for duplicate measurements of blood minerals in all subjects and were expressed as the 99 percent confidence limits (*9*).

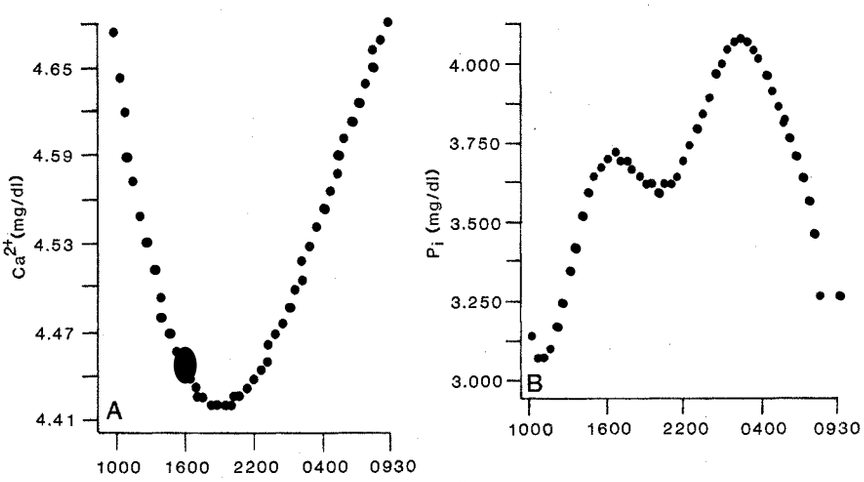

Fig. 2 (right). Computer-derived models of circadian fluctuations in Ca^{2+} (A) and P_i (B) concentrations.

though the normal patterns remain intact. This has been observed in hypocalcemic hypoparathyroid children who daily are treated with two suboptimal doses of 1,25-dihydroxyvitamin D_3 (*13*).

Our data establish the presence of circadian patterns of Ca^{2+} and P_i in healthy, young adult males. Models of these patterns provide a physiological basis for future studies of blood minerals and calcium-regulating hormones in other healthy populations and at specific stages of metabolic bone disease.

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Brain Acetylcholine Synthesis Declines with Senescence

Abstract. *The synthesis of whole brain acetylcholine is reduced in two strains (C57BL and BALB/c) of senescent mice. The incorporation of [U-^{14}C]glucose into acetylcholine decreased in both strains by 40 ± 4 percent in 10-month-old mice and by 58 ± 9 percent in 30-month-old mice compared with mice 3 months old. The incorporation of [$^2H_4$]choline into acetylcholine declined 60 and 73 percent in 10- and 30-month-old mice, respectively. Deficits in the cholinergic system may contribute to brain dysfunctions that complicate senescence.*

Senescence is a complicated process characterized by many morphological and chemical alterations in the brain. Cognitive function declines with aging in humans and other animals (*1*). The biochemical mechanism of this decrease is unknown, however. Several studies suggest that impaired cholinergic function may be an important factor in the production of geriatric memory deficits. Pharmacological manipulation of the cholinergic system in aged animals or in humans (*1–3*) implies an age-related decrement in cholinergic function. Neurophysiological studies also demonstrate these reductions (*4*, *5*). Cholinergic receptors, choline acetyltransferase, and acetylcholinesterase activities in the brain are reduced with aging and further depressed with Alzheimer-type dementia (*6*, *6a*, *7*, *8*). Some of these changes are controversial. For example, Davies and Verth (*7*) found no decrease in muscarinic receptors with aging in humans. Furthermore, the decline may be specific to discrete brain regions, since Morin and Wasterlain (*8*) observed a decrease in receptors in the striatum and cerebellum but not in the hippocampus, hypothalamus, or amygdala. Similar disputes exist over the decline in choline acetyltransferase and acetylcholinesterase.

Brain oxidative metabolism decreases with senescence in brain slices, homogenates, mitochondria, and in vivo (*9*, *9a*). In aged patients, decrements in cerebral oxygen consumption correlate with the degree of dementia, but in normal elderly subjects, inconsistent changes are reported (*10*). In vitro, reduced oxygen utilization produces a proportional inhibition in acetylcholine synthesis even though less than 1 percent of the oxidized precursor becomes acetylcholine

Table 1. Lactate, acetylcholine, and choline brain concentrations (in nanomoles per milligram of protein) in senescent mice. Values are means ± standard errors of the means. Lactate was determined fluorometrically. Acetylcholine and choline were separated and their concentrations measured by gas chromatography–mass spectrometry. Protein was determined by the biuret reaction with bovine serum albumin as the standard (*14*).

Strain	Age (months)		
	3 (N = 11)	10 (N = 11)	30 (N = 11)
	Lactate		
BALB/c	9.47 ± 0.62	11.59 ± 0.99	12.92 ± 1.18*
C57BL	10.80 ± 0.73	11.61 ± 0.80	27.08 ± 4.60†
	Acetylcholine		
BALB/c	0.26 ± 0.02	0.26 ± 0.01	0.22 ± 0.01
C57BL	0.33 ± 0.02	0.32 ± 0.02	0.29 ± 0.02
	Choline		
BALB/c	0.45 ± 0.04	0.46 ± 0.07	0.45 ± 0.07
C57BL	0.42 ± 0.06	0.42 ± 0.07	0.42 ± 0.08

*Significantly different ($P < .05$) from 3-month-old mice (analysis of variance with least significant difference multiple comparison test) (*15*). †Significantly different ($P < .05$) from both 3-month-old and 10-month-old mice.

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