CIRCADIAN CHANGES IN MEMBRANE PROPERTIES OF HUMAN RED BLOOD CELLS IN VITRO, AS MEASURED BY A MEMBRANE PROBE

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1. Introduction

In a previous paper we have reported the existence of circadian and semi-circadian rhythms in enzyme activity in in vitro suspensions of human red blood cells. Since these cells are enucleated and lack the machinery for protein synthesis, the oscillations in enzyme activities, although endogenously controlled, were not dependent on DNA replication, gene transcription or protein synthesis. Furthermore, evidence was presented which indicated that the changes in enzyme activity were governed in some manner by the membrane.

Recently a membrane model for the circadian clock was proposed by Njus et al. [2]. In their model the activity of the clock is associated with changes in membrane properties arising from a feedback between ion transport proteins and ion concentrations, specifically K ion.

To test this model, the concentration of potassium ion in the external medium was measured by means of flame photometry. Also, measurements of the relative membrane potential were performed using a modification of the method of Hoffman and Laris [3] with the cyanine dye di $OC_5(3)$. As was shown by Hoffman and Laris [3] and Sims et al. [4], the relative fluorescence intensity from the membrane probe is a function of membrane potential. Upon depolarization of the membrane there is a release of dye from the blood cells and an increase in fluorescence intensity and upon hyperpolarization there is an uptake of dye and a decrease in fluorescence intensity.

2. Materials and methods

In our studies human red blood cells were isolated as previously described [1] from blood drawn at 8 a.m. from healthy donors. The cells were suspended in 9 volumes of isotonic saline (153 mM NaCl) and incubated at 35°C in pyrex bottles in a shaking bath. At 2-h intervals duplicate aliquotes were removed, one being frozen for later measurement of enzyme activity, the other being used immediately in the fluorescence assay with the dye di $OC_5(3)$.

The fluorescence measurements were made at 23°C using a standard 3 ml fluorescence cuvette in a specially constructed fluorimeter. The large (50 mm) endon photomultiplier, (EMI 9635) which was positioned at right angles to the excitation beam (λ = 436 nm), was blocked to pass only wavelengths above 510 nm. The excitation source was a 200 W high pressure mercury lamp (Osram HBO200). The beam was passed through a 5% $CuSO_4$ heat filter (5 cm), a series of neutral density filters and a Schott 435 narrow band interference filter as well as a Schott SPK20 Hg filter combination. Light intensity

at the cuvette surface was less than 1000 ergs/cm^2 sec. The cyanine dye di $OC_5(3)$ at a final concentration of 2×10^{-6} M was added to a potassium-free medium (153 mM NaCl) and the fluorescence intensity brought to 100 units. Blood samples were taken at various times during the incubation period and immediately added to the cuvette (final blood concentration, 0.33%) and the fluorescence level measured. The enzymatic activities of acetyl choline esterase and glucose 6-phosphate dehydrogenase were determined as previously described [1].

Potassium was measured by the method of flame photometry with an Eppendorf Flame Photometer using a propane–air flame. At two-hour intervals, samples were taken and blood cells were removed by centrifugation. The resultant supernatant was aspirated directly into the flame for potassium determination. Standard additions of potassium ion were added to the samples as internal standards to compensate for any change in viscosity.

3. Results

As can be seen in fig.1, there is no cycling of potassium ion. There is instead a constant increase in the concentration of potassium ion in the external medium.

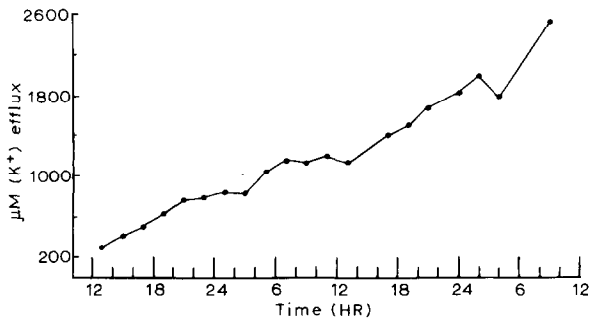

Fig.1. The efflux of potassium ion into the external medium as measured by flame photometry.

The membrane properties of the red blood cells as measured with the membrane probe were found to exhibit a circadian rhythm (fig.2). The relative fluorescence increases from 19 p.m. to 23 p.m. exhibiting a broad peak between midnight (24) and 4 a.m., followed by a decrease in fluorescence around 5 a.m. A second slightly smaller peak with a two-hour phase advance is seen on the second night.

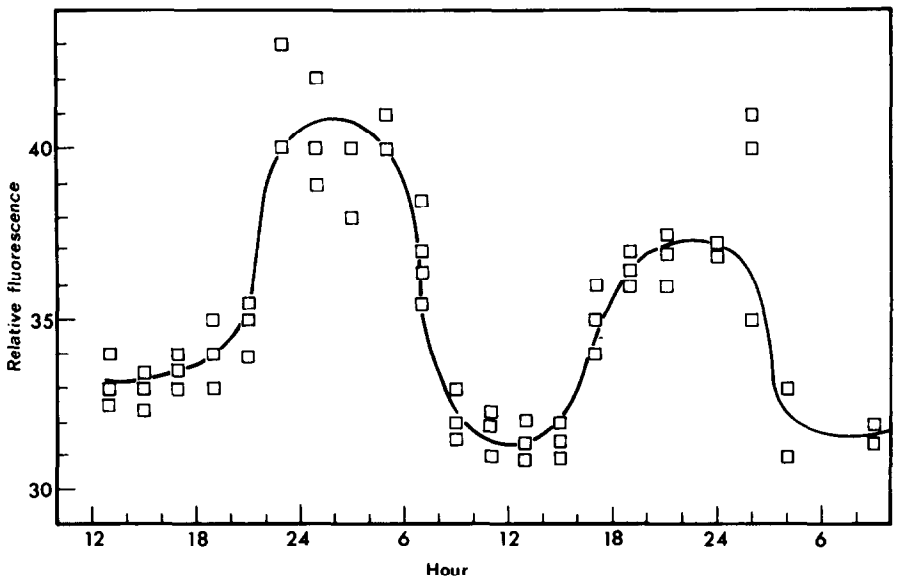

Fig.2. Circadian changes in membrane properties of human red blood cells cultured in vitro as measured with the fluorescent probe di $OC_5(3)$. The blood concentration was 0.33% in a medium containing 153 mM NaCl and 2 μM di $OC_5(3)$. For details of measurement see text.

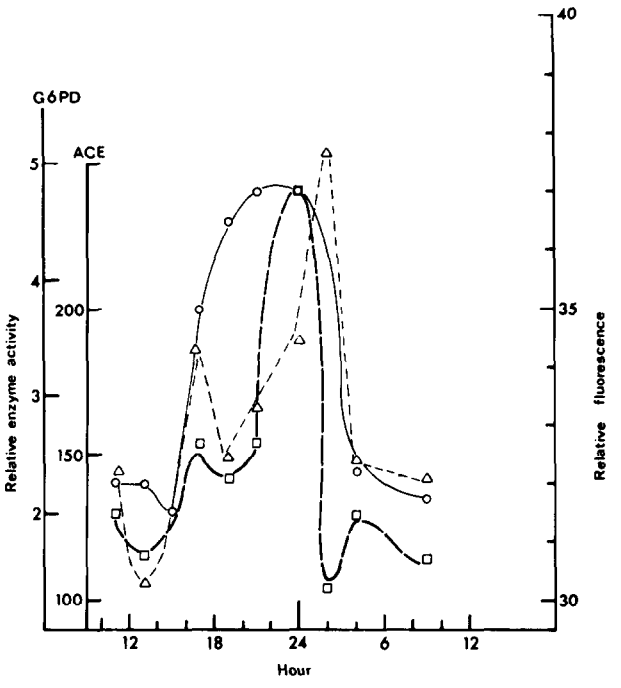

Fig.3. The correlation between changes in membrane properties as measured with the fluorescent probe and the activities of the enzymes acetyl choline esterase and glucose 6-phosphate dehydrogenase. Fluorescence was measured as described in fig.2. Enzyme assays were performed with hemolysates prepared from sedimented frozen cells. The assay for glucose 6-phosphate dehydrogenase was based upon the reduction of NADP as determined spectrophotometrically at 340 mM [6]. The activity of cholinesterase was measured by the hydrolysis of acetyl thiocholine iodide according to the method of Ellman et al. [5].

When the enzymatic activities of acetyl cholinesterase, an enzyme which exhibits a circadian rhythm, and of glucose 6-phosphate dehydrogenase, an enzyme which exhibits a semi-circadian rhythm, were examined, suggestive correlations were found between their activities and the membrane properties as measured by the probe di $OC_5(3)$ (fig.3). Acetyl cholinesterase activity correlated well with the rhythmic changes in the membrane properties while the peaks in activity of glucose 6-phosphate dehydrogenase correlated well with the inflections occurring around either side of the fluorescence peak.

According to the interpretation of Hoffman and Laris [3] and Sims et al. [4] the changes in fluorescence intensity which we have measured should be indicative of changes in membrane potential. Although the relative fluorescence intensity most certainly measures to some extent membrane potential, as indicated by the hyperpolarization effect of valinomycin, we propose that the cyclic fluctuations in relative fluorescence intensity which are reported here are not due to changes in membrane potential but rather to cyclic changes in internal binding sites on the membrane.

Recent measurements on the binding capacity of the dye di $OC_5(3)$ to red blood cells (osmotically shocked before the measurement) sampled over a 24 h period revealed a circadian rhythm in binding capacity similar to that seen in fig.2. Furthermore, at variance with the prediction of the model of Njus et al. [2] no cyclic changes in extracellular potassium ion concentration were found in measurements made with cultured red blood cells which did exhibit a circadian rhythm in membrane properties as measured with the membrane probe.

It is concluded that the circadian rhythms in both enzyme activities and in membrane properties as revealed by the membrane probe are strongly correlated, and that the changes in membrane properties may be fundamentally associated with the time telling mechanism of the clock.

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