sudden death but may only be an indirect consequence of other primary factors (low cardiac output, myocardial damage) that lead to electrical instability.

It is also conceivable that the observed alterations in heartrate variability are due to the fact that the subjects were taking pharmacological agents that could modify heartrate or autonomic regulation, independent of disease. We have no way of testing this hypothesis since we do not have Holter recordings of these patients prior to their treatment with drugs. The effects of pharmacological agents on the heartrate of patients at high risk for sudden death needs to be characterized, but we do not think it is likely that the drugs themselves are responsible for the observed heartrate variability. This is because the subjects were taking a variety of drugs (digitalis, diuretics, vasodilators, antiarrhythmic drugs), and one would have to postulate that any of them could produce the same type of heartrate patterns.

The observation of bifurcation behavior and sustained periodic heartrate dynamics has potential diagnostic and prognostic importance. Conventional diagnostic analysis of ambulatory heartrate data is usually limited to description of the mean heartrate and range, and to counts of the number of and morphology of abnormal (ectopic) beats. Time series and spectral analysis of these same records, however, clearly show that different runs of sinus rhythm are not necessarily equivalent. For example, one subject may show physiological heartrate variability with a broad, 1/f-like spectrum. Another with nearly identical heart rate mean and variance may show oscillations and bifurcations reflecting an instability in cardiovascular control [3 – 5]. Furthermore, apparently erratic ectopic beats may actually follow a periodic bursting pattern that is detectable only with time series analysis of long heartbeat data sets [21, 22], rather than appearing at random time intervals.

Finally, these preliminary observations of complex heartrate phenomena emphasize the need to construct nonlinear mathematical models to provide testable explanations for physiologic heartrate fluctuations. A mechanistic explanation for the 1/f-like heartrate spectrum in healthy subjects as well as the bifurcation behavior and the periodic dynamics associated with perturbations in cardiovascular control parameters seen in high risk patients should be provided by a unified model of the cardiovascular system and its autonomic control.

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## Chloride transport in the freshwater protozoan *Tetrahymena pyriformis*

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*Summary. Tetrahymena* accumulates $^{36}Cl^-$ in both nutrient and salt media. Up to 80% of the initial net $^{36}Cl^-$ influx was inhibited by the anion exchange inhibitor DIDS (4,4'-diisothiocyano-2,2'-stilbene-disulfonic acid). The 'loop' diuretic, bumetanide, had no effect. A DIDS-sensitive anion exchange system is proposed to regulate the cytoplasmic $Cl^-$ concentration in *Tetrahymena*.
*Key words. Tetrahymena;* anion exchange; DIDS; chloride transport; ion concentrations.

*Tetrahymena pyriformis* is a protozoan, which inhabits freshwater environments of widely differing solute composition [1]. Accordingly, it has been found that *Tetrahymena* possesses a number of finely regulated membrane transport mechanisms, which operate to control cellular water and solute content. Extensive studies of the regulation of cell water and of cellular concentrations of $Na^+$ and $K^+$ under various external conditions have previously been published [1–4]. In contrast, only little information exists about the regulation of the anion composition of these cells. The major inorganic anions are orthophosphate and chloride [1, 2] and the bulk of $Cl^-$ has been assumed to be 'passively' distributed across the cell membrane [1, 2]. Studies of $Cl^-$ transport in vertebrate cell systems [5] have shown that 'passive' transport of $Cl^-$ involves one or more of the following transport systems: a) exchange of $Cl^-$ with $Cl^-$ or bicarbonate (anion exchange), b) cotransport of $Cl^-$ with $Na^+$ and/or $K^+$ and c) electrodiffusion through conductive $Cl^-$ channels. The following study was undertaken in order to investigate whether such transport systems also exist in a non-vertebrate cell like *Tetrahymena*. Our results strongly suggest that an anion exchange system is present in *Tetrahymena*.

*Materials and methods.* *Tetrahymena pyriformis*, strain GL, was grown in a shaking water bath at 28 °C for 24 h (early stationary phase, $0.5–0.8 \times 10^6$ cells/ml) in 250-ml Erlenmeyer flasks containing 50 ml growth medium. Growth medium (PY) was 0.75% proteose peptone and 0.75% yeast extract (both from Difco) supplemented with Kidder's salts [6] and 1% glucose. Prior to experimental treatment, cells in PY were concentrated ($700 \times g$, 1 min) or transferred to an inorganic salt medium (IM) by two washings ($700 \times g$, 1 min). IM had the following composition (mM): $Na^+$:10, $K^+$:9, $Cl^-$:21, $Mg^{2+}$:5, $Ca^{2+}$:1, phosphates:5 and glucose:40 (modified from Hoffmann and Kramhøft [7]). In both cases cells were resuspended to a final cell concentration of $1.5 \times 10^6$ cells/ml. Cell suspensions were left unshaken (approx. 50 ml cell suspension in a 500-ml Erlenmeyer flask) at room temperature for about 1.5 h prior to the experiments, which were conducted at room temperature under continuous shaking. Isotope uptake experiments were performed as follows: $^{36}Cl^-$ (Amersham, approx. 7 kBq/ml) was added at time zero, 1-ml samples were subsequently removed, and the cells separated from the medium by differential density separation according to Ballentine and Burford [8] ($20{,}000 \times g$, 1 min). The separation layer consisted of a mixture of dioctylsebacinate (14.73 weight%) in dibutylphtalate. 100 µl of the supernatant was removed in order to assess medium radioactivity and the remaining supernatant plus the separation layer was then carefully removed. The amount of trapped medium was estimated in separate experiments and constituted $1.4 \pm 0.1$ µl/mg protein ($\pm$SEM, n = 4). Average protein content was $0.86 \pm 0.04$ mg/$10^6$ cells ($\pm$SEM, n = 17). The cell pellet was resuspended and permeabilized in 0.05% Triton X-100. Samples were then deproteinized with perchloric acid (PCA, final concentration 7%), centrifuged ($20{,}000 \times g$, 10 min) and the supernatant subsequently counted for radioactivity. Protein was assessed using the PCA precipitate according to the method of Lowry [9]. $Na^+$, $K^+$ and $Cl^-$ were measured in 5-ml samples after separation of cells from medium essentially as described above ($2000 \times g$, 5 min), $Na^+$ and $K^+$ was estimated by flamephotometry and $Cl^-$ by coulometric titration. Cellular ion concentrations were calculated from parallel measurements of cell concentration and cell wet and dry weight after correction for trapped extracellular medium.

*Results and discussion.* The intracellular concentrations of $K^+$, $Na^+$ and $Cl^-$ in *Tetrahymena pyriformis* under the present experimental conditions are shown in table 1. Like most other cells *Tetrahymena* maintains a high intracellular $K^+$ concentration relative to the media. The cellular $Na^+$ concentration is slightly lower than the medium concentration in both media. From table 1 it appears that the extracellular and intracellular concentrations of $Na^+$ and $K^+$ are the same in IM and PY. In an inorganic medium with the same composition as IM, except that the $Na^+$ concentration was 30 mM instead of 10 mM as used in the present work, intracellular concentrations of $K^+$ and $Na^+$ were previously found to be 55 mM and 15 mM, respectively [7]. Thus, good agreement exists between previous and present results. The ion composition of cells grown in PY has not previously been reported. Table 1 also shows that while the extracellular $Cl^-$ concentration is 1.6-fold higher in IM than in PY, the intracellular concentrations are not significantly different (Student's t-test, 3 independent, paired experiments).

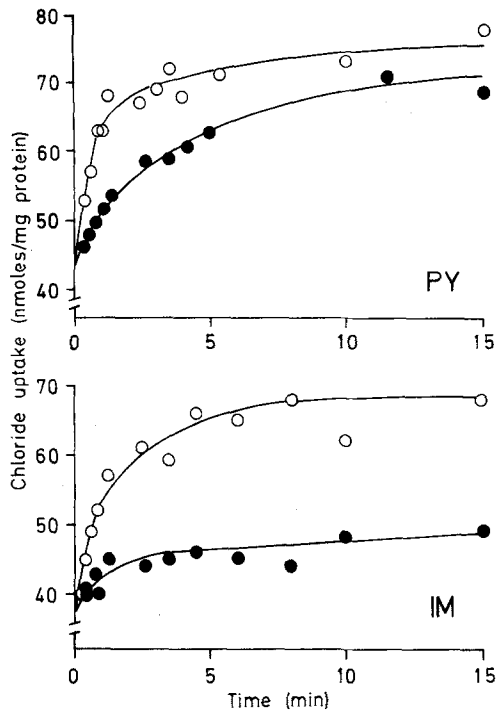
Table 1. Ion concentrations in media and cells of *Tetrahymena*

| | Medium (mM) | | Cells (mM) | |
|---|---|---|---|---|
| | PY | IM | PY | IM |
| $K^+$ | $10.6 \pm 0.4$ (n = 5) | 9 | $60.3 \pm 2.6$ (n = 3) | $59.7 \pm 1.8$ (n = 3) |
| $Na^+$ | $11.1 \pm 0.2$ (n = 5) | 10 | $5.6 \pm 0.3$ (n = 3) | $6.3 \pm 0.3$ (n = 3) |
| $Cl^-$ | $12.9 \pm 0.9$ (n = 5) | 21 | $8.3 \pm 0.7$ (n = 3) | $11.0 \pm 2.0$ (n = 3) |

PY: Proteose peptone, Yeast extract medium; IM: Inorganic Medium. Values are given as average $\pm$SEM of n independent experiments.

Previously, an intracellular $Cl^-$ concentration of about 9 mM was measured in two chemically defined growth media with $Cl^-$ concentrations of 5 mM and 27 mM, respectively [10]. These observations are consistent with earlier data from Dunham and Child [4] who found that the intracellular $Cl^-$ concentration is maintained almost constant over a wide range of extracellular $Cl^-$ concentrations [2, 4]. Assuming that $Cl^-$ is passively distributed, the Nernst equilibrium potential for $Cl^-$ may be calculated at $-16.6$ mV and $-11.3$ mV in IM and PY, respectively, using the values for $Cl^-$ shown in table 1. A resting membrane potential of $-24$ mV and $-30$ mV was previously reported in *Tetrahymena vorax* [11] and *Paramecium* [2], respectively. The Nernst equilibrium potential for $K^+$ can be calculated, using the values for $K^+$ distribution from table 1, at $-45$ mV and $-49$ mV in IM and PY, respectively. If the actual membrane potential is anywhere near the $K^+$ equilibrium potential, these combined observations indicate that some regulation of the intracellular $Cl^-$ concentration occurs.

The figure shows the net uptake of $^{36}Cl^-$ by *Tetrahymena* as a function of time in nutrient medium (PY, top panel) and inorganic medium (IM, bottom panel). $^{36}Cl^-$ uptake is initially very rapid in both media and isotope equilibrium is attained after about 2 and 5 min in PY and IM, respectively. Furthermore, the amount of $Cl^-$ taken up is about the same in the two media. In several vertebrate cell systems it has been demonstrated that $Cl^-$ transport under steady state (i.e. unperturbed) conditions is dominated by a $Cl^-/Cl^-$ or $Cl^-/HCO_3^-$-exchange system [5]. If $Cl^-$ transport in *Tetrahymena* is mediated by such an anion exchange system it should be inhibitable with DIDS (4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid), which is widely used as inhibitor of anion exchange systems [12]. As shown in the figure this is, indeed, the case. In PY medium (upper panel) the initial rate of $^{36}Cl^-$ uptake is reduced by DIDS (60 µM) to about 50% of the control rate. In IM (lower panel) the uptake is inhibited by about 80%. In one experiment $^{36}Cl^-$ uptake by DIDS treated cells in IM was followed for 1 h after addition of the isotope. It was found that the amount of $^{36}Cl^-$ taken up by the DIDS treated cells had almost reached the control equilibrium level at this time point. Table 2 summarizes data from two experiments in PY and 5 in IM. DIDS seems to be




Uptake of $^{36}Cl^-$ by *Tetrahymena* as a function of time in PY medium (upper panel) and IM (lower panel). DIDS (Sigma, final concentration 60 μM) was added from an aqueous stock solution about 1 min prior to the initiation of the uptake experiments. The figure is representative of two experiments in PY and 5 in IM. $^{36}Cl^-$ uptake is given as nmol $Cl^-$/mg protein, calculated from known values of the specific activity of $^{36}Cl^-$ in the medium (see Methods) and cellular protein content. The values were not corrected for trapped extracellular medium. However, the radioactivity estimated from extrapolation of the uptake curve to time zero agrees well with the expected value arising from contamination of the pellet with trapped medium (see Methods). Controls, ○; cells in DIDS: ●.

Table 2. Rates of $^{36}Cl^-$ uptake by *Tetrahymena* in the absence and presence of an inhibitor of anion exchange

| | PY | IM |
|---|---|---|
| Control | 1.00 | 1.00 |
| DIDS (60 μM) | 0.51; 0.49 | 0.22 ± 0.03 |

The initial rates of $^{36}Cl^-$ uptake were calculated by linear regression of the uptake measured during the first 1.5 min after addition of the isotope (see also fig. 1). The rates are given relative to the uptake rates calculated from parallel controls. The figures in PY are rates obtained in two separate experiments. The figure in IM is average ± SEM of 5 experiments. PY: Proteose peptone, Yeast extract medium. IM: Inorganic Medium.

a more potent inhibitor of $Cl^-$ transport in IM than in PY in spite of the fact that the concentration of DIDS used was the same in both cases. There are two main differences between the two experimental systems: a) PY contains peptides, the amino groups of which might bind DIDS thereby reducing the free (inhibitory) concentration, and b) food vacuoles are formed by cells in PY but not in IM [13]. Both of these circumstances may contribute to the observed apparent different sensitivity to DIDS. It is, however, clear that a major part of the $Cl^-$ transport in *Tetrahymena* is sensitive to the anion exchange inhibitor DIDS. The fact that $Cl^-$ is taken up only slightly faster in PY but to about the same extent as in IM suggests that $Cl^-$ transport is not dependent on the formation of food vacuoles. However, the possibility that formation of food vacuoles is, indeed, responsible for the slightly faster rate of $^{36}Cl^-$ uptake seen in PY cannot be excluded. It follows, though, that the $Cl^-$ transport sites primarily must be located in the pellicular and ciliary membranes.

As mentioned above, $Cl^-$ transport may also be due to a cotransport of $Cl^-$ with $Na^+$ and/or $K^+$. Evidence for an ethacrynic acid-sensitive $Na^+$ transport (i.e. presumably cotransport) in *Tetrahymena* has previously been reported [7]. If $Cl^-$ is transported by a cotransport system, this should be sensitive to the 'loop' diuretic, bumetanide, a known inhibitor of cotransport systems [14]. Bumetanide had, however, no effect on the rate of $^{36}Cl^-$ uptake in *Tetrahymena* (data not shown). It follows that the residual $^{36}Cl^-$ uptake (about 20% of the initial influx in IM), which is insensitive to DIDS, most likely represents the function of $Cl^-$ channels. This possibility has, however, not yet been investigated. The above results strongly suggest that under steady state conditions the intracellular $Cl^-$ concentration in *Tetrahymena* is largely controlled by the operation of an anion exchange system. Consequently, this ciliate shows a remarkable resemblance to vertebrate cells with respect to mechanisms involved in the control of cellular $Cl^-$.

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