tive calcium uptake were studied. Figure 2 shows a typical experiment in which calcium uptake was measured in the same IOV preparation with various free ionized calcium concentrations in the media. The calcium concentration required for half-maximal stimulation of active calcium uptake (K_{Ca}) by using unbuffered calcium is about 40–50 μM, and a saturation of the calcium uptake appears at 300–500 μM calcium. In contrast to this, if the free ionized calcium concentration is adjusted by a calcium-EGTA buffer which contains 500 μM total calcium ($Ca_t = 500$ μM), the value of K_{Ca} obtained is between 0.5 and 0.7 μM, that is about 100 times smaller than in the unbuffered system. At this Ca_t the maximum rate of active calcium uptake equals that observed with unbuffered calcium. If, however, the calcium-EGTA buffer is prepared by using a Ca_t of 50 μM, the K_{Ca} value obtained is about 0.5 μM again, whereas the maximum rate of calcium uptake is smaller than that at 500 μM total calcium, and corresponds to the rate of uptake observed with 50 μM unbuffered calcium in the medium.

In order to analyse the effects of Ca_t in a calcium-EGTA buffer, we determined the rate of active calcium uptake by IOVs at about equal free ionized calcium concentrations (which saturated calcium uptake in calcium-EGTA buffers), but using different total calcium concentrations. As it is shown in the table, the maximum transport rates obtained with calcium-EGTA buffers containing different total calcium levels, are similar to the rates obtained at unbuffered calcium concentrations corresponding to the given Ca_t.

According to the above findings, the rate of active calcium uptake by inside-out vesicles is influenced by both the free ionized and the total calcium concentrations in a calcium-EGTA buffer. Similar data are obtained if the activator protein is added to the medium (figure 3). The maximum rate of active calcium uptake is about doubled by the activator either in the presence of unbuffered calcium or calcium-EGTA buffers containing Ca_t greater than 500 μM. However, if Ca_t in the buffer is smaller than 500 μM, the maximum transport rate depends on the actual value of total calcium (not shown). The values for K_{Ca} in the calcium-EGTA buffers are about 0.5–0.7 μM both in the control and the activator-supplemented vesicles, in contrast to the K_{Ca} of 40 μM and 15 μM obtained with the control and activator-treated vesicles, respectively. The low protein concentration in the experimental systems makes any significant calcium or EGTA binding by the IOVs unlikely, and the high, outwardly directed calcium gradient obtained during calcium uptake suggests a low passive permeability of the vesicle membrane for calcium as well as for EGTA.

The explanation offered by us for the above results is the following: Active calcium transport system in the red cell membrane has at least 2 different binding sites for calcium. One of these sites has high affinity for calcium with a K_{Ca} of about 0.5 μM, whereas the 2nd binding site has a K_{Ca} of 15–50 μM, depending on the presence or absence of the activator protein. Active calcium transport occurs only if both binding sites are occupied by calcium. In order to explain the effects of Ca_t in the calcium-EGTA buffers on the calcium transport rate, we have to suppose that the 2nd binding site accepts calcium also in a chelated form. The calcium-EGTA complex is bound to this site, and in a following step calcium is removed from the chelate and is transported through the membrane. An alternative explanation for the results would be to suppose that the transport enzyme has high affinity for calcium, but higher concentrations of either unbuffered calcium or calcium-EGTA complex are required to alter the conformation of the cell membrane for favouring active calcium transport.

In both explanations it is suggested that the calcium-EGTA complex is recognized by the cell membrane as free calcium. This finding suggests a careful reconsideration of those data in the literature which were obtained by investigating calcium-dependent phenomena in calcium-EGTA buffer systems.

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Studies of the blood of *Ascidia ceratodes*. Total blood cell counts, differential blood cell counts, hematocrit values, seasonal variations, and fluorescent characteristics of blood cells[1]

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Summary. Seasonal, and animal size and weight variations of the blood cells of the vanadium-containing ascidian, *Ascidia ceratodes*, were determined. The fluorescent properties of various cell types were ascertained, and discussed in terms of cell development, phylogenic position of the species, and chemicals in the cells.

Blood cells from ascidians, invertebrates in the phylum Chordata, are unique in many respects[2]. The predominant cell in the blood of vanadium-containing ascidians, the vanadocyte, contains significant levels of vanadium and sulfuric acid. The oxidation state of the vanadium contained in the vanadocytes of the species *Ascidia ceratodes*, the subject of this paper, is plus 3[3].

In preparation for studies of the chemical constituents of the various blood cell types, it was necessary to determine both the normal frequency for each blood cell type and to locate a suitable probe for the study of chemical changes within the cells. To this end total blood cell counts, differential blood cell counts, hematocrit values, seasonal variations in blood cell counts, and the fluorescent characteristics of various blood cells for the solitary ascidian *A. ceratodes* were determined.

Materials and methods. Samples of *A. ceratodes* used in this study were collected from 3 sites in Bodega Bay, California.

No variations were noted between samples from these divergent locations. Total and differential cell counts of selected individuals were obtained by initial cardiac puncture removal of a fixed volume of blood via plastic syringe. A portion of this sample of blood was diluted by addition of 3% NaCl solution buffered to pH 6.8 with 10^{-3} M phosphate. The diluting solution also contained 10^{-2} M dithiothreitol, DTT, and 0.1% neutral red stain. After 15 min at 4 °C, samples of the mixture were placed in a Spencer Bright-Line hemocytometer for counting. A minimum of 25 fields (10–20 cells/field) were counted per sample. Bulk fluorescent measurements were accomplished with a Farrand spectrofluorimeter fitted with a microcell adapter. Individual cells were studied with the aid of a Zeiss model standard RA fluorescent microscope. Individual cell fluorescent spectra were recorded with the aid of St. B. Westrate using a Nanospec/10S computerized microscopectrophotometer system mounted on an Olympus Vanox research fluorescent microscope using the blue-violet excitation band.

Results and discussion. The general cell types previously found to be associated with members of the genus *Ascidia* have been described elsewhere[4–7]. The percentages of the 6 most common cell types were studied: vanadocytes, compartment cells, signet ring cells, vacuolated unstained cells, unstained cells and orange pigment cells. Over 10 animals were studied each month during the period January to June 1973. Figures 1 and 2 detail the average and range observed for each monthly sample. The average value is represented as a circle and the range of individual values observed each month is shown by the line bisecting each circle. In addition to the percentage of each cell type, the total number of cells and the hematocrit values (May and June only) are also shown. The average total cell density observed in this study of *A. ceratodes*, 77,300 cells/mm^3 (range 47,400–144,500 cells/mm^3), is slightly higher than values seen for measurements on members of the genus *Ascidia*: *A. nigra*, 53,300[8] and 53,800[9]; *Phallusia mamillata*, 68,000[10] and 64,000[11]; and *A. ceratodes*, 64,000[12]. The observed percentage of vanadocytes, 53%, agrees with previous work.

Considerable variation is noted in total and differential cell counts from one animal to another. This observation is common to all studies of ascidian blood thus far reported. Many animals (11/month) having a wide range of weight (9–37 g) and length (5–9 cm) were examined and no correlation was observed between the physical dimensions of the animal and either the total cell population or the percentage of different cell types. While there are some possible seasonal trends within the average values of unstained cells (figure 2), no compelling evidence is available to suggest seasonal behavior as an explanation of individual cell count variation. Furthermore, it can be seen that by utilization of reasonably large numbers of animals (> 10), individual variations in blood cell population can be averaged. There also appears to be no correlation between cell variation and larval production from *A. ceratodes*. Dr J. Standing at Bodega Marine Laboratory, University of California, in a private communication, has indicated that the peak month for larval production from *A. ceratodes* is June. It would appear the variability in total and differential cell populations between individual specimens is a normal observation and not an artifact of analysis technique or seasonal change. Fluorescent spectra observed for whole blood from *A. ceratodes* and for cells lysed in 1.0 N H_2SO_4 and in buffers ranging from pH 1.23 to 4.75 have maxima at 525 and 470 nm respectively. These spectra reflect the effects of extended exposure to the exciting line (see later discussion). The florescent spectra observed for individual cell types are summarized below. The vanadocyte, initially possesses a yellow-orange fluorescence confined exclusively to the vacuoles (vanadophores) within each vanadocyte. The outer membrane and cell nucleus possess no fluorescent qualities. Clear demarcation lines can be observed between the vanadophores within the vanadocyte in fresh blood samples. However, after several min of exposure, an area in the center of the vanadocyte, the cell nucleus, previously showing no fluorescence, begins to fluoresce yellow-orange. At the same time, the demarcation lines between the vanadophores vanish. The initial fluorescent spectrum obtained for the vanadocyte indicates 2 maxima: the first at 525±5 nm and the second at 590±5 nm. A weak shoulder is infrequently observed at 470–480 nm. Upon irradiation for several min, the intensity at 590 nm fades, while that at 525 m (seen in whole blood) increases.

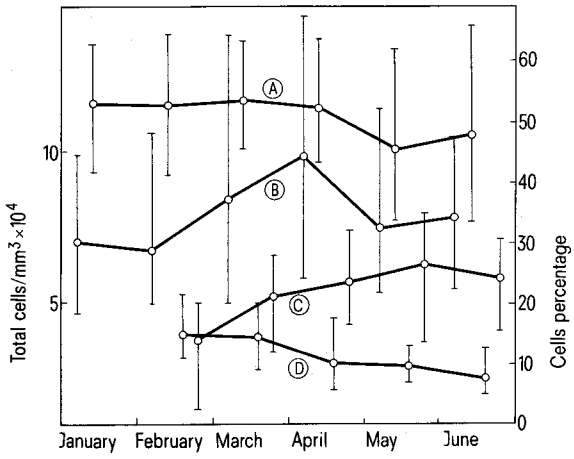

Fig. 1. Monthly variations of vanadocytes (A), total cells (B), signet ring cells (C) and compartment cells (D) observed for *Ascidia ceratodes*. Total cells, (B), refers to left-hand ordinate and (A), (C), and (D) refer to the right-hand ordinate.

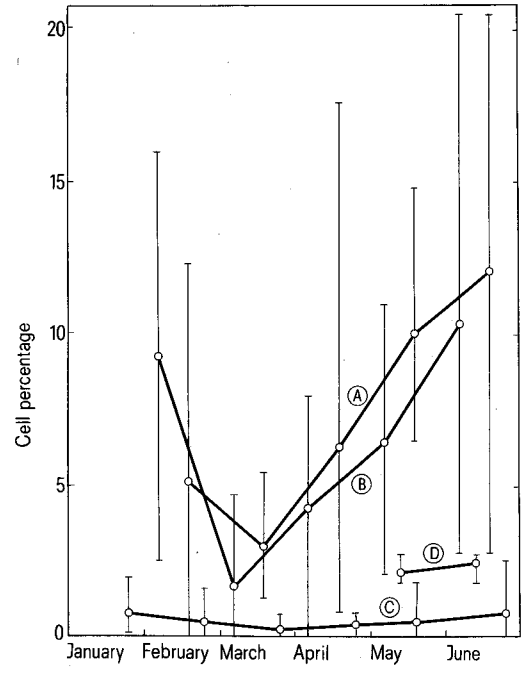

Fig. 2. Monthly variations observed for vacuolated unstained (A), unstained (B), and orange pigment (C) cells, and hematocrit values (D) in *Ascidia ceratodes*.

After a short exposure period, compartment cells begin to show a blue fluorescence with a maximum at 470–480 nm. Again the fluorescence is associated with inner membranes, but does not fade significantly with time. Under excitation the entire orange pigment cell shows a stable fluorescence at 520±5 nm. No fine structure is observed within the cell at 1200-fold magnification.

The fluorescent properties of various blood cell types can be used to explore several problems: cell development, phylogenic positions of various species and probing the chemical processes occurring within the cell and at the vacuolar membrane. It has been suggested from an examination of the cell types in the blood of *P. mamillata,* a species closely related to *A. ceratodes,* that the vacuolized compartment cell evolves into the vanadocyte, and the orange pigment cell is unrelated to the vanadocyte[10]. This study suggests that the 3 types of cells in *A. ceratodes* are related. Vanadocytes show an interconversion of the 590 and 525 nm bands upon irradiation, suggesting a chemical interrelation of the compounds these bands represent. The presence of the 525–590 nm system suggests an evolutionary relationship between the vanadocyte and the orange pigment cells which have a fluorescent band at 520 nm. Note that the true maximum of the vanadocyte 525 nm band falls at a shorter wavelength; approximately 520 nm. In some vanadocytes a 470-480 nm band occurs suggesting a tenuous relationship with the compartment cell which has such a band after short irradiation.

With respect to phylogenic position, some agreement is observed in the fluorescent properties of the comparment cells observed in the bloods of *A. ceratodes* and *P. mamillata*[13], however, not between vanadocytes of the 2 species. This observation is surprising as both *P. mammillata* and *A. ceratodes* belong to the same family (Ascidiidae), and have nearly identical blood cell morphologies. The blood of another member of the family Ascidiidae, *A. nigra,* is reported to possess no visible fluorescence upon UV-irradiation[9].

It has been suggested that the difference in behavior noted between vanadocytes of *Ciona intestinalis* (fluorescent) and *P. mamillata* (not fluorescent) is due to the quenching effect of highly-concentrated vanadium(III) in the latter relative to the former[13]. This chemical explanation is not supported by the results of this study, since *A. ceratodes* vanadocytes have approximately the same level of vanadium[3] as those of *P. mammilata* and fluoresce.

Some aspects of the compounds responsible for the fluorescent behavior can also be discussed. The orange pigment cells have been suggested as depositories for purines and related compounds[14], and purines, pteridines and their derivatives have been reported in the ascidians *Microcosmus polymorphus*[15] and *Ascidiella aspersa*[16]. The UV-spectral and fluorescent properties of bloodcell extracts of *A. ceratodes* are consistent with this view.

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Computer-aided biochemical system analysis in open systems with environment simulation

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Summary. A computer-aided arrangement is described which allows kinetic and regulative studies with enzymes, organelles and cells in an open system. This is demonstrated with some simple examples.

Organisms and their subunits are open systems. Their steady state properties can only be recognized by measurement of input and output. The character of a flowing equilibrium is visualized by applying external perturbation and monitoring the subsequent return to the equilibrium[1,2]. In this way the typical time-function of a regulative system is realized. Due to the complexity of metabolic systems the application of computers is indispensable. Simulation of environmental factors is performed by programming the computer to a number of time functions governing the addition of chemical substances, which interfere with the system on various levels of biochemical regulation.

Methods. To begin with a simple case, we take as an example an enzyme or several enzymes intercorrelated by reaction (figure 1). The enzymatic solution is circulated through the reaction chamber (RC) of a measuring unit (e.g. the cuvette of a photometer) and a microfilter (e.g. hollow fibre type laid in a vacuum chamber VC) by means of a peristaltic pump (P_2). Particles of low molecular weight (including the reaction products) are filtered through the microfilter by means of a vacuum (VAC) suction pump. The loss in volume is restored with substrate by another peristaltic pump (P_1). Volume regulation is performed by a level indicator (LI) and a magnetic valve (MV) which con-