five synthetic peptides, calculated according to Hopp and Woods[8], was −0.20; +0.16; +0.67; +0.33; +0.56 respectively. The commercial rabbit anti-TPA:B1 antibody (Byk Gulden, Milano) used for the identification of linear determinants in the BrCN:B fragment, was first purified from bovine serum albumin added as protecting agent by chromatography on a Bio Rad Affi Gel-Blue column in 0.02 M K_2HPO_4. The first peak eluted (IgG) corresponded to approx. 1/10 of the total protein amount loaded. This enriched antibody preparation with known protein concentration was then tested in an ELISA on the five synthetic fragments using preimmune rabbit IgG at the same dilutions as a control. The binding of this sample to the five peptides, as well as to 2 synthetic fragments selected from unrelated proteins used as controls, is shown in the figure. Anti-TPA antibodies appeared to react with peptides 4 and 5 only, corresponding to the C-terminal part of the BrCN:B fragment. An enrichment in anti-4 and anti-5 antibodies could be obtained by loading the commercial sample on a 0.5 × 5 cm column packed with an immunoadsorbent prepared by linking Sepharose AH to peptide 4 by aid of glutaraldehyde, and equilibrated with phosphate-saline buffer (PBS); 0.2 M Gly-HCl buffer pH 2.6 eluted a protein peak that was retested, under the same conditions, on the same set of synthetic peptides. The binding of the antibodies to the COOH-terminal fragment was more apparent in this case (fig.).

The antigenic sites of proteins are of two types, structurally: they may comprise residues that are on a continuous segment of the protein chain[9] or are far apart but, due to the folding of the polypeptide chain, come into close spatial proximity (discontinuous sites)[10]. Antibodies to an intact protein may be directed against both site structures[11]. Since commercial anti-TPA antibodies appear to bind large synthetic peptides closely resembling TPA BrCN:B fragment, and recognize an epitope centered around Arg 15[5] while they fail to react with short-sized synthetic peptides that include this residue, this finding strongly suggests that this antigenic determinant is conformational; however, we found that anti-TPA antibodies display an affinity for the C-terminal part of the fragment, where a continuous epitope therefore should be located.

The lack of binding of anti-TPA antibodies to synthetic fragments 1, 2 and 3 might have different alternative explanations: those linear sequences might display low immunogenicity in the intact TPA molecule, and elicit undetectable amounts of antibodies; alternatively, they might assume, in the native protein, a poorly flexible conformation, and elicit antibodies to this precise conformation but unreactive with random-coiled synthetic peptides. Finally, they might be buried or masked in the native molecule. This last possibility might be true for fragments 1–10 that display a negative H.I.[8], but not for the other two peptides that, because of their positive indices, (H.I. +0.16, +0.67, respectively), are probably located on the outside of the TPA molecule: peptides 2, 3, 4 and 5 might be used therefore for rabbit immunization and production of antipeptide antibodies directed to predetermined regions of TPA. However, peptides 2 and 3 share a more than 83% and a 72% homology, respectively, with human 50 K keratin, and it is predictable that antibodies to those peptides would extensively crossreact with these filamentous proteins, and therefore be of limited application. In contrast, peptide 4 (homology to keratin: 53%) and especially 5 (homology: 33%) might generate specific anti-TPA antibodies and be of considerable help in determining TPA amounts levels in sera of suspected cancer patients. Efforts are being made in our laboratory to raise an immune response against peptides 2, 3, 4 and 5, and detect whether the corresponding antipeptide antibodies can be used as specific anti-TPA reagents.

Acknowledgments. This investigation was partly supported by the Associazione Italiano per la Ricerca sul Cancro.

1 Bjorklund, B., and Bjorklund, V., Int. Arch. Allergy *10* (1957) 153.
2 Bjorklund, B., Bjorklund, V., Wiklund, B., Lundstrom, R., Ekdahl, R. H., Hagbard, L., Kaiser, K., Eklund, G., and Luning, B., Immunological technique for detection of cancer, p. 133. Bonnier, Stockholm 1973.
3 Menendez-Botet, C. J., Oettgen, H. F., Pinsky, C. M., and Schwartz, M. K., Clin. Chem. *24*/6 (1978) 868.
4 Redelius, P., Luning, B., and Bjorklund, B., Acta chem. scand. B*34* (1980) 265.
5 Luning, B., and Nilsson, U., Acta chem. scand. *37* (1983) 731.
6 Merrifield, R. B., J. Am. chem. Soc. *86* (1964) 304.
7 Yajima, H., Ogawa, H., Watanobe, H., Fujii, N., Kurobe, M., and Miyamoto, S., Chem. Pharm. Bull. *23* (2) (1975) 371.
8 Hopp, T. P., and Woods, K. R., Proc. natl Acad. Sci. USA *78* (1981) 3824.
9 Atassi, M. Z., Immunochemistry *12* (1975) 423.
10 Atassi, M. Z., Immunochemistry *15* (1978) 909.
11 Berzofsky, J. A., Buckenmeyer, G. K., Hicks, G., Gurd, F. R. N., Feldmann, R. J., and Minna, J., J. biol. Chem. *257* (1982) 3189.

0014-4754/89/020184-03$1.50 + 0.20/0

Distribution of tunichrome and vanadium in sea squirt blood cells sorted by flow cytometry

E. M. Oltz, S. Pollack[a], T. Delohery[b], M. J. Smith, M. Ojika, S. Lee, K. Kustin[c] and K. Nakanishi

Department of Chemistry, Columbia University, New York (New York 10027, USA), [a] Department of Hematology, Albert Einstein College of Medicine, 1300 Morris Park Ave, Bronx (New York 10461, USA), [b] Department of Genetics, Comprehensive Cancer Center, Institute of Cancer Research, College of Physicians and Surgeons, Columbia University, New York (New York 10032, USA), and [c] Department of Chemistry, Brandeis University, Waltham (Massachusetts 02254, USA)
Received 16 August 1988; accepted 1 November 1988

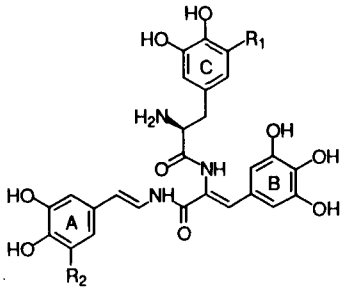
Dedicated to the memory of Father G. Ruggieri, Director, New York Aquarium.

Summary. Specialized blood cells of many tunicates accumulate high concentrations of vanadium and phenolic peptide pigments called tunichromes (TC). In order to determine whether V and TC reside in the same cells, *Ascidia nigra* and *Ascidia ceratodes* blood cell subpopulations were isolated by fluorescence-activated cell sorting (flow cytometry) and chemically analyzed. V was found in the spherical, green/grey signet ring cells, and to a lesser degree in the mulberry-shaped, yellow/green morula cells (MRs), whereas free TC was detected mainly in MRs.
Key words. Fluorescence activated cell sorting (FACS); tunicates; morula cells; signet ring cells; vanadium.

Certain species of sea squirts (tunicates) accumulate vanadium in specialized blood cells [1] to levels approaching ten million times that of seawater [2]. *Ascidia nigra* and *A. ceratodes* are exemplary V accumulators, where more than 90% is present as V(III) [3,4]. We have recently isolated and characterized a family of reducing blood pigments, the tunichromes (fig. 1), from *A nigra* [5] and other species [6]. The (hydroxy-) Dopa moieties of tunichromes provide them with potent chelating properties towards vanadium. Despite extensive studies by numerous groups the biological roles of tunichrome and vanadium, a trace element essential to mammalian survival [7], are yet to be established [8]; much basic chemistry and histochemistry remains unresolved, including the question of which blood cells assimilate and store vanadium. Two seemingly disparate, but mutually compatible proposals have been put forth to explain the existence of oxygen sensitive V(III) in a living organism, namely, an acidic intracellular milieu [9], and/or complexation by the tunichromes [6,10]. Therefore, determining the relative locations of tunichrome and vanadium is crucial to clarifying their chemistry and biological roles. In the following, we have sorted whole blood into cell subpopulations by fluorescence activated cell sorting (FACS) and have chemically analyzed the cell fractions. We wish to report that when appropriate cell selection criteria are used (easily oxidized) free tunichrome and (readily oxidizing) vanadium are detectable in the same cells, thereby creating the potential for this ligand to sequester vanadium *in vivo*. The combination of FACS and microanalysis has provided a powerful probe to histological studies such as these.

Although morphological cell classification is often fraught with subtle ambiguities, tunicates appear to possess at least five varieties of circulating blood cells [11]: lymphocytes (LC), stem cells, leucocytes (LU), pigment cells, and vacuolated cells. Three varieties of the latter have been reported: green/grey signet ring cells (SRC) characterized by one large vacuole; green compartment cells (CC) composed of several angular vacuoles; and bright yellow or yellow/green, mulberry-shaped morula cells (MR) composed of several spherical vacuoles that exhibit a pumpkin-colored fluorescence [6]. Traditionally, morula cells have been regarded as the ultimate repository of accumulated metals. When the stored metal is vanadium, the morula cells have been termed vanadocytes, within which the membrane-enveloped vanadophores have been assumed to sequester the metal [12]. As a point of clarification the term vanadocyte should encompass those cells which assimilate vanadium, thereby obviating any reference to a particular stage in cell development. In other words, the cells which begin to assimilate vanadium may be morphologically distinct from their mature counterparts. The inference that MRs are the vanadocytes was originally based on the staining of morula cells by osmic acid. However, direct evidence obtained by X-ray microanalyses [13–15] and density separation of cells [16,17] indicates that much more vanadium is detected in cells identified as signet ring cells. These techniques, although successfully implemented for vanadium analyses, were felt to be less suited to our needs, those of obtaining homogeneous living cell populations for assaying free tunichromes [6] (fig. 1), which are very air-sensitive.

By comparison, flow cytometry sorts individual cells according to event-gating (e.g., fluorescence intensity) in a manner whereby live, homogeneous cell subpopulations can be obtained rapidly. Undesired events such as lysed cells are electronically eliminated prior to fractionation; the subsequent lysis of sorted cells does not permit significant cross-contamination between cell varieties. Moreover, the presence of innately fluorescent tunichrome in diverse ascidians suggested that FACS on fresh blood may elucidate which cell varieties tunichrome and vanadium reside in.



a: An-1, $R_1 = R_2 = OH$

b: An-2, $R_1 = H$, $R_2 = OH$

c: An-3, $R_1 = R_2 = H$

Figure 1. Tunichromes isolated from *Ascidia nigra*. (An-1,2,3) *A. ceratodes* contains only An-1.

Materials and methods. Ascidia nigra and *Ascidia ceratodes* were collected off the coasts of Key Biscayne, FL, and Monterey, CA, respectively, and airfreighted the following day in small containers of seawater. Blood was withdrawn from specimen with a plastic syringe (1–6 ml per individual) and diluted with chilled buffer (0.5 M NaCl, 12.5 mM HEPES (pH 7), 0.5% BSA, 1% mercaptoethanol) to a concentation of 1–4 million cells per ml. Each diluted blood sample was submitted to total and differential cell counts using standard morphological guidelines [11], and filtered through 44-μ nylon mesh to remove cell debris.

A complete description of the FACS methodology used will be published elsewhere [13]. Data acquisitions, analyses and cell sorts were performed on a Becton Dickinson FACS IV equipped with Consort 40 software. A Spectra-Physics argon laser set at the 488-nm line was used as an excitation source. 'Green' fluorescence was collected using a 520-nm long pass in conjunction with a 525-nm band pass filter (50 nm band width) and 'red' fluorescence was collected using a 590-nm long pass in conjunction with a 650-nm wide band filter (60 nm band width). Fluorescence and laser light scatter measurements were made using linear signal processing electronics with 256 channel resolution. Debris was eliminated from cell sample measurements using a forward angle light scatter triggering threshold. Sheath fluid consisted of 0.5 M NaCl buffered with 12.5 mM HEPES to pH 7. Cells were collected in excess buffer in order to minimize impact shock. After photography and differential cell counting, sorted and whole blood cells were pelleted (3000 rpm, 10 min), submerged in liquid nitrogen, lyophilized and stored under argon at −78 °C.

Free tunichrome analysis: Acetic anhydride (1.5 ml) and pyridine (1.5 ml) were added to the lyophilized pellets, and the suspension stirred at room temperature for several hours. Reaction solvents were removed (in vacuo), and the tunichrome peracetate so formed was extracted into CH_2Cl_2 (500 μl). UV spectra were taken in CH_3OH, aliquots of which were analyzed by reversed phase – HPLC: Perkin Elmer HS-3 C_{18}, 0.5 × 3.5 cm, 3 μ column; $CH_3CN/THF/H_2O$ (43:4:53), 0.4 ml/min, 320 nm detection. Peak areas of the tunichrome peracetates were measured using purified specimen as standards (8×10^{-5} M by UV) [6].

V analysis: Methanol was evaporated from aliquots of the above samples, after which 2% HNO_3 was added. Vanadium was analyzed by atomic absorption spectrophotometry using a Varian AA-875 Series machine in the background correction mode.

Results. Four parameters were scrutinized during flow cytometric analyses: the degree of forward and 90° laser light scatter, as well as the intensity of red and green fluorescence. The initial sorting procedure on *A. nigra* blood used linear scale fluorescence gating and resolved diluted whole blood into three distinct cell populations: MR, LC + LU, and SRC + CC (see the contour plot of cell populations depicted

in figure 2). The first population (MRs) emitted high Green and high Red fluorescence (G and R), had a narrow, small size distribution, and exhibited a large 90° light scatter (i.e., non-spherical cell shape). The second population (LCs + LUs) emitted high green but low red fluorescence (G low R), had a wide size distribution and very low 90° scatter (i.e., spherical cell shape). The third population (SRCs + CCs) emitted negligible fluorescence (No), exhibited a medium size distribution and very low 90° scatter. A summary of the differential cell counting data on whole blood and FACS fractions is presented in the accompanying table. Interestingly, centrifuged cell pellets of MRs were yellow (the color of TC), whereas SRCs were dark green initially [the color of V(III) and of TC-V complexes], while LCs were colorless. We wish to note that our preliminary data initially led us to believe that tunichrome and vanadium were located in 'different cell types' [see *A. nigra* (I) in the table]. However, we later recognized that the fluorescence gate settings used might have favored the selection of certain cell subpopulations to the exclusion of others. Specifically, since paramagnetic metallic elements can effectively quench a chromophore's fluorescence, it was possible that the initial fluorescence gate settings did not select for (vanadium-containing) MRs with a diminished degree of fluorescence. On the other hand, chemical detection of tunichrome in cells laden with vanadium would be very difficult due to its ease of oxidation by transition metals. Further resolution of the cell subpopulations was thus warranted.

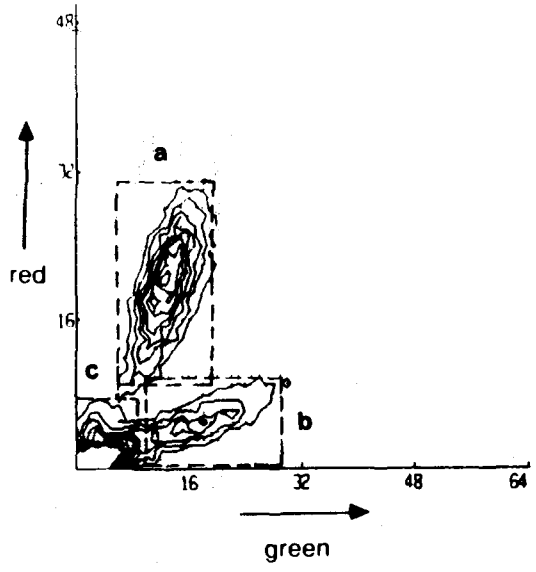


Figure 2. Contour plot analysis of *A. nigra* (I) blood cells using linear scale fluorescence gating. Map represents a slice through the z axis (i.e., detected events; ≅ cell number), and contour levels are incremented by 10 events. The boxed regions highlight the three distinguishable populations, and correspond closely to sorting criteria used. Buffer contained 0.5 M NaCl, 12.5 mM HEPES (pH 7), 0.5% BSA, and 1% mercaptoethanol. (a) MRs, (b) LCs + LUs, and (c) SRCs + CCs (differential cell counts are provided in the table).

Subsequent fractionations using logarithmic scale fluorescence gating, covering over four decades of fluorescence intensity, greatly enhanced resolution and revealed the presence of bright and dim morula cell subpopulations (fig. 3), and provided fractions of SRCs predominantly [*A. nigra* (II) and (III) in the table]. Representative photographs of fractionated cells are shown in figure 5. Comparable resolution of cell subpopulations was not obtained for *A. ceratodes* blood preparations, which incidentally, possessed negligible numbers of LCs + LUs (fig. 4) and lower levels of detectable vanadium [*A. ceratodes* (I) and (II) in the table]. Representative samples of sorted fractions were subsequently analyzed for free (not complexed to a metal ion) tunichromes [6] and for vanadium. In both species nearly all of the detected free tunichromes were in the MRs while most of the vanadium was in SRCs. Smaller, variable quantities of vanadium was also detected in certain MR subpopulations, depending on the fluorescence gate setting used.

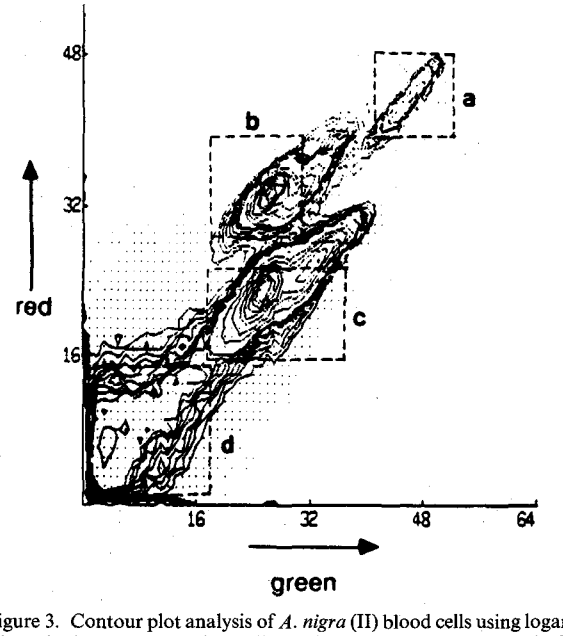


Figure 3. Contour plot analysis of *A. nigra* (II) blood cells using logarithmic scale fluorescence gating. All the conditions are the same as in fig. 2 except buffer contained 0.52 M NaCl, 12.5 mM HEPES (pH 7), 10% fetal calf serum, 10 mM L-cysteine. Gating of parameters distinguished four populations: (a) bright MRs, (b) dim MRs, (c) LCs + LUs, and (d) SRCs.

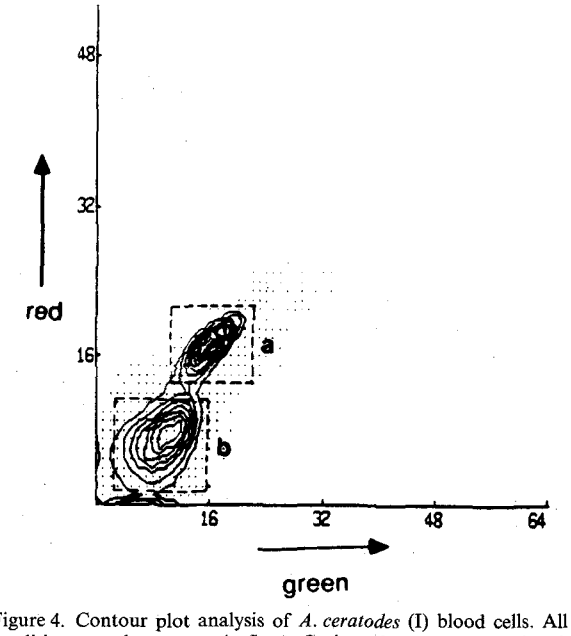
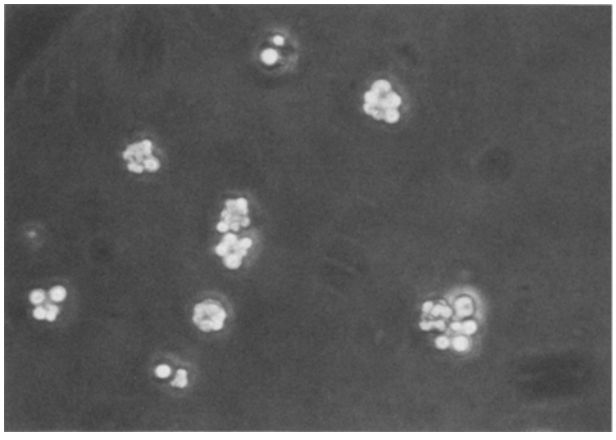
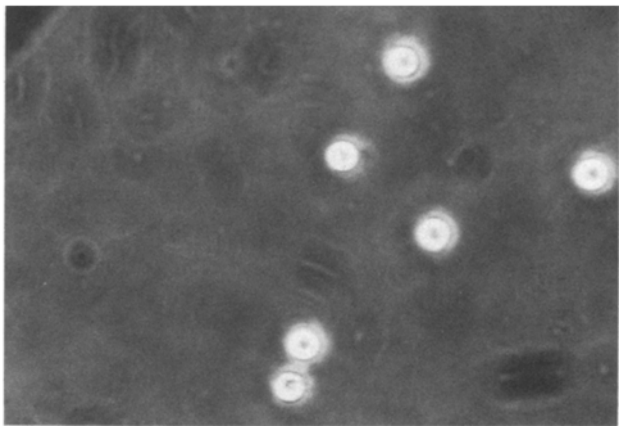


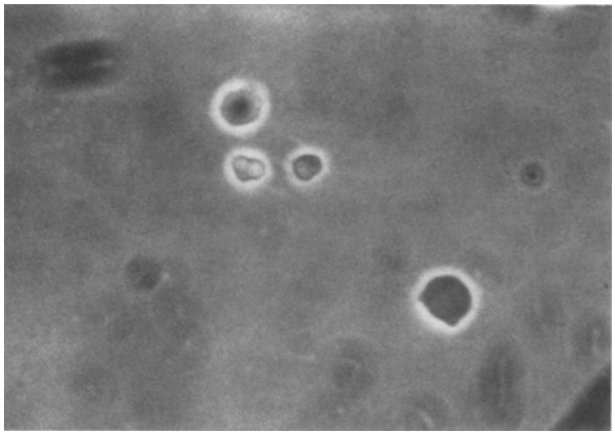
Figure 4. Contour plot analysis of *A. ceratodes* (I) blood cells. All the conditions are the same as in fig. 3. Gating of parameters distinguished only two populations: (a) MRs and (b) SRCs (see the table).



a



b



c

Figure 5. Photographs (400 × magnification) of FACS-sorted *A. nigra* (II) blood cells (fig. 2): (*a*) dim MRs, (*b*) LCs + LUs, and (*c*) SRCs.

Discussion. A comparison of the contour plots in figures 2 and 3 illustrates that the resolution of cell subpopulations hinges on the sensitivity of fluorescence detection, as well as on the blood preparation. For example, so-called bright MRs were not always present and may, in part, reflect the physiological state of the blood preparation. As noted by other investigators, the relative proportions of the various cell types fluctuated from animal to animal. In addition, the correlation between fluorescence and 'morphological cell type' was not strict, although readily identifiable MRs were usually very fluorescent, while apparent SRCs were usually non-fluorescent; the distinction between CCs and other cell types was not always clearcut, however. Moreover, cell specimens are somewhat labile, thereby compounding the difficulty of cell classification and tunichrome quantitation. The table reveals that 50 to 70% of the vanadium present in whole blood can be accounted for. It is possible that the vanadium unaccounted for resides in the completely non-fluorescent cell population at a higher concentration than in the collected SRCs (i.e., at the origin of the contour plots);

FACS separated Ascidian blood cells: sorted populations, tunichrome and vanadium assays. All of the tunichrome and vanadium found in whole blood is not accounted for in the assays on sorted cells.

Specimen	Fractionated sample	% MR	% LC + LU	% SRC	TC-Ac[a]	corr. TC-Ac[b]	V[a]	corr. V[b]
A. nigra (I)	Whole blood	44	28	28	200		54	
	MR	92	2	6	400	***>400***	5	0
	LC + LU	0	95.5	4.5	0	0	0	0
	SRC + CC	0	6	94	0	0	109	***116***
A. nigra (II)	Whole blood	24	41	35	16.7		93	
	Bright MR	80	10	10	6.3	7.9	7.8	0
	Dim MR	86	0	14	95.5	***111***	31.8	***15.7***
	LC + LU	0	87	13	0.03	0.03	3.3	0
	SRC	0	7	93	0	0	120	***129***
A. nigra (III)	Whole blood	33	33	33	35.4		49.1	
	Dim MR	98	0	2	55.5	***56.6***	8.4	*7*
	LC + LU	4	96	0	0.08	0.06	1.8	1.9
	SRC	0	4	96	0.09	0.09	72.8	***75.8***
A. ceratodes (I)	Whole blood	57	–	43	1.43		12.6	
	MR	91	–	9	0.19	***0.2***	2.1	***1.8***
	SRC	1	–	99	0	0	5.1	***5.2***
A. ceratodes (II)	Whole blood	55	–	45	91.4		59.6	
	MR	92	–	8	116.4	***126.5***	1.3	***1.0***
	SRC	2	–	98	5.1	3.2	3.3	***3.4***

[a] nmol/10^6 cells (≅ FACS events collected); [b] values corrected for cell inhomogenities as determined by differential cell counting, nmol/10^6 cells. Important numerals are bold-italicized.

these cells are predominantly SRCs also, but cannot be selected electronically due to the total absence of fluorescence. In spite of the difficulties encountered with these relatively unstable cells our studies confirm and extend the findings previously reported [14-18]; vanadium is not strictly distributed according to morphological cell type. The trends in cellular vanadium distribution are similar in the two species studied. Interpreting the tunichrome distribution is more difficult, however. As mentioned above, failure to detect free tunichrome in signet ring cells by the peracetylation assay [6] does not necessarily signify that tunichrome is absent; it may be present, but complexed to a metal ion, or further oxidized. The finding [6] that SRCs contain an uncharacterized pigment (UV of 0.1 N aq. HCl solution: 275 nm sh, 340 nm sh) which is strikingly similar to a reconstituted tunichrome-vanadium complex lends credence to this interpretation. Failure of such cells to fluoresce also does not preclude the presence of tunichrome; paramagnetic metallic elements such as vanadium can effectively quench a chromophore's fluorescence. Therefore, the free tunichrome distribution does not necessarily reflect the true distribution of tunichrome, free, complexed, or further oxidized; in essence, the tunichrome assay available is semi-quantitative and improved detection methods for the air-sensitive tunichromes is warranted.

Our results do not formally rule out the interpretation that tunichrome is functionally unrelated to vanadium accumulation; its excellent reducing and ligating properties being simply fortuitous. The crux of the issue at hand is detecting and characterizing a putative metal complex of extreme lability. An alternative interpretation is that vanadocyte maturation depends on vanadium assimilation which, in turn, occurs at the expense of tunichrome oxidation. Here, the distribution of tunichromes and vanadium renews the question of tunicate blood cell development. Since SRCs contain more vanadium than MRs, it seems more plausible that MRs give rise to SRCs, provided the putative developmental pathway is tied to vanadium assimilation. In any event, it is clear that the term 'vanadocyte' can no longer be ascribed to one morphological cell type.

The recent total synthesis of unprotected tunichrome [19] will undoubtedly contribute to clarifying the role of tunichromes. The fact that free tunichromes comprise 50% of the dry weight of the abundant MRs in *A. nigra* underscores their importance [6]. Morula cells are considered to play an important physiological role, for they appear to be involved in immunological responses and possibly in the production/organization of tunicin fibers [20]. As catecholic amino acids, the tunichromes could function as chemical crosslinking agents in a manner analogous to the sclerotization of insect cuticle [21, 22].

Finally, FACS on tunicate blood [23] could make a substantial contribution toward resolving many pressing hematological issues [3, 23, 24], such as the controversy over 'vanadocyte' pH [9, 25, 26]. Elucidation of the respective roles of tunichrome and vanadium in tunicates could also lend insights into related topics, such as natural transplant rejections and the function of vanadium in higher organisms.

Acknowledgment. We thank Prof. R. Bruening, Drs B. Horenstein, and W. Robinson for discussion, the late Dr G. Ruggieri for coordinating shipments of *A. nigra*, and Prof. C. Cantor for allowing access to the FACS instrument. E. M. Oltz was the 1985–1986 American Chemical Society/Eli Lilly Fellow. These studies were supported by NIH grant AI 10187 (KN) and by NSF grant DCB-8500309 (KK).

1 Henze, M., Hoppe Seyler's Z. Physiol. Chem. *72* (1911) 494.
2 Macara, I. G., McLeod, G. C., and Kustin, K., Comp. Biochem. Physiol. *63 B* (1979) 299.
3 Frank, P., Hedman, B., Carlson, R. M. K., Tyson, T. A., Roe, A. L., and Hodgson, K. O., Biochemistry *26* (1987) 4975.
4 Lee, S., Kustin, K., Robinson, W., Frankel, R., and Spartalian, K., J. inorg. Biochem. *33* (1988) 183.
5 Bruening, R. C., Oltz, E. M., Frukawa, J., Nakanishi, K., and Kustin, K., J. nat. Prod. *49* (1986) 193.
6 Oltz, E. M., Bruening, R. C., Smith, M. J., Kustin, K., and Nakanishi, K., J. Am. chem. Soc. *110* (1988) 6162.
7 It has been estimated that humans require an intake of ~2 mg V per day: Underwood, E. J., in: Trace Elements in Human and Animal Nutrition. Academic Press, London 1971.
8 (a) Intact V complexes of unknown function have been isolated from fungi. Kanai, F., et al., J. Antibiot. *38* (1984) 31, and Kneifel, H., and Bayer, E., J. Am. chem. Soc. *108* (1986) 3075. (b) two V metalloenzymes have also been described for lower organisms; a bacterial nitrogenase, Robson, R. L., et al., Nature *322* (1986) 388, and a sea weed bromoperoxidase, DeBoer, E., VanKooyk, Y., Tromp, M. G. M., Plat, H., and Wever, R., Biochim. biophys. Acta *869* (1986) 48.
9 Frank, P., Carlson, R. M. K., and Hodgson, K. O., Inorg. Chem. *27* (1988) 118
10 Macara, I. G., McLeod, G. C., and Kustin, K., Biochem. J. *181* (1979) 457.
11 Wright, R. K., in: Invertebrate Blood Cells, vol. 2, p. 566. Eds N. A. Ratcliffe and A. F. Rowley, Academic Press, New York 1981.
12 Goodbody, I., Adv. mar. Biol. *12* (1974) 1.
13 Delohery, T., and Smith, M. J., in preparation.
14 Botte, L., Scippa, S., and deVincentiis, M., Experentia *35* (1979) 1228.
15 Rowley, A. F., J. mar. Biol. Ass. U. K. *62* (1982) 607.
16 Pirie, B. J. S., and Bell, M. V., J. exp. mar. Biol. Ecol. *74* (1984) 187.
17 Kustin, K., Levine, D. S., McLeod, G. C., and Curby, W. A., Biol. Bull. *150* (1976) 426.
18 Michibata, H., Hirata, J., Vesaka, K., Takahura, N., and Sakurai, H., J. exp. Zool. *244* (1987) 33.
19 Horenstein, B., and Nakanishi, K., submitted for publication.
20 Chaga, O. Yu., Tsitologiya *22* (1980) 619.
21 Sugumaran, M., Bioorg. Chem. *15* (1987) 194.
22 Schaffer, J., Kramer, K. J., Garbow, J. R., Jacob, G. S., Stejskal, E. O., Hopkins, T. L., and Speirs, R. D., Science *235* (1987) 1200.
23 Schlumpberger, J. A., Weissman, I. L., and Scofield, V. L., J. exp. Zool. *229* (1984) 401.
24 Hawkins, C. J., James, G. A., Parry, D. L., Swinehart, J. H., and Wood, A. L., Comp. Biochem. Physiol. *76 B* (1983) 556.
25 Agudelo, M. I., Kustin, K., and McLeod, G. C., Comp. Biochem. Physiol. *75 A* (1983) 211.
26 Hawkins, C. J., James, G. A., Parry, D. L., Swinehart, J. H., and Wood, A. L., Comp. Biochem. Physiol., *76 B* (1983) 559.

0014-4754/89/020186-05$1.50 + 0.20/0