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ISOLATION OF TUNICHROME B-1, A REDUCING BLOOD PIGMENT OF THE SEA SQUIRT, *ASCIDIA NIGRA*[1]

REIMAR C. BRUENING,[2] EUGENE M. OLTZ, JUN FURUKAWA, KOJI NAKANISHI,*

Department of Chemistry, Columbia University, New York, New York 10027

and KENNETH KUSTIN

Department of Chemistry, Brandeis University, Waltham, Massachusetts

ABSTRACT.—The tunicates, or sea squirts, are common marine organisms that selectively accumulate metals such as V, Fe, Mo, Nb, in their blood cells. Despite the more than 70 years of interest in the compounds responsible for this accumulation, their extreme lability has eluded attempts to isolate and characterize them. The isolation and structure ofthe first of these blood pigments tunichrome B-1 from the *Ascidia nigra* is reported.

We discuss herein isolation studies carried out on tunichromes, the bright yellow blood pigments of the tunicate *Ascidia nigra* L. that are involved in the irreversible accumulation of vanadium in these animals. It has been the most difficult isolation carried out in our laboratory because of the crude pigment's great sensitivity to water and oxygen and because of the co-occurrence of several closely related pigments. Moreover, the isolation had to be carried out on the underivatized pigments because they were desired for further studies.

STRUCTURE OF TUNICHROME B-1 (TB-1) (**1**).—The structure of one of the major pigments, tunichrome B-1 (TB-1), can formally be regarded as being constructed of three hydroxy-DOPA units (Figure 1). During the six-year period since we started these studies, approximately 6,000 tunicates have been sacrificed, and it has taken a full five years to be able to obtain finally 0.5 mg of TB-1 in pure form.

ASCIDIA NIGRA.—The tunicates (order *Ascidiacea*), more commonly known as sea squirts, are members of the same phylum as vertebrates (chordata) because the tadpole-

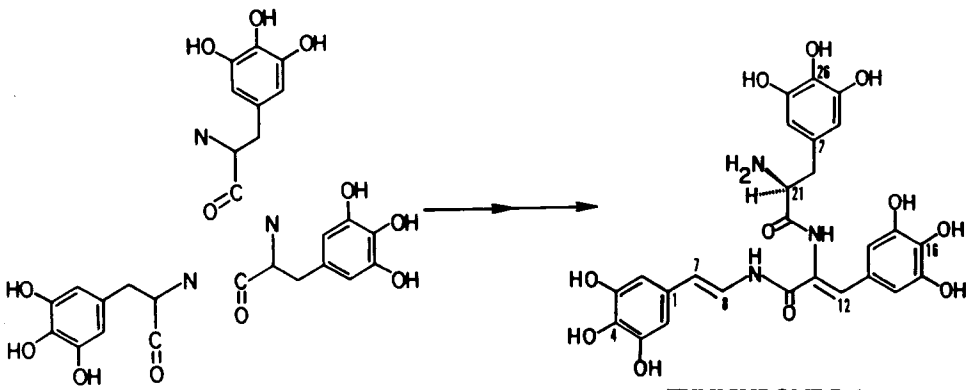

FIGURE 1. Structure of Tunichrome B-1.

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[2]Present address: Department of Chemistry, University of Hawaii at Manoa, 2545 The Mall, Honolulu, Hawaii 96822.

like larvae possess notochords. They are common marine organisms distributed throughout the world. Figure 2 shows the adult form of *A. nigra*, the solitary species investigated in this study; they are about the size of a fist and are covered by a rubber-like, thick outer mantle, the tunic. Tunicates are sessile animals that are attached to the substrate at one end; the other end has two openings, an incurrent oral opening and an excurrent atrial opening. The tunic is made largely of cellulose-like polysaccharides, the black color possibly arising from melanin. In some species, many small individuals are embedded in a common gelatinous matrix to form colonies; such colonial tunicates exist in a wide variety of colorful forms ranging from thin, flat mat-like growths to large lumpy colonies. Some tunicates, especially the orange-red *Halocynthia roretzi* distributed widely in Japan, are treasured as appetizers and eaten raw by some people.

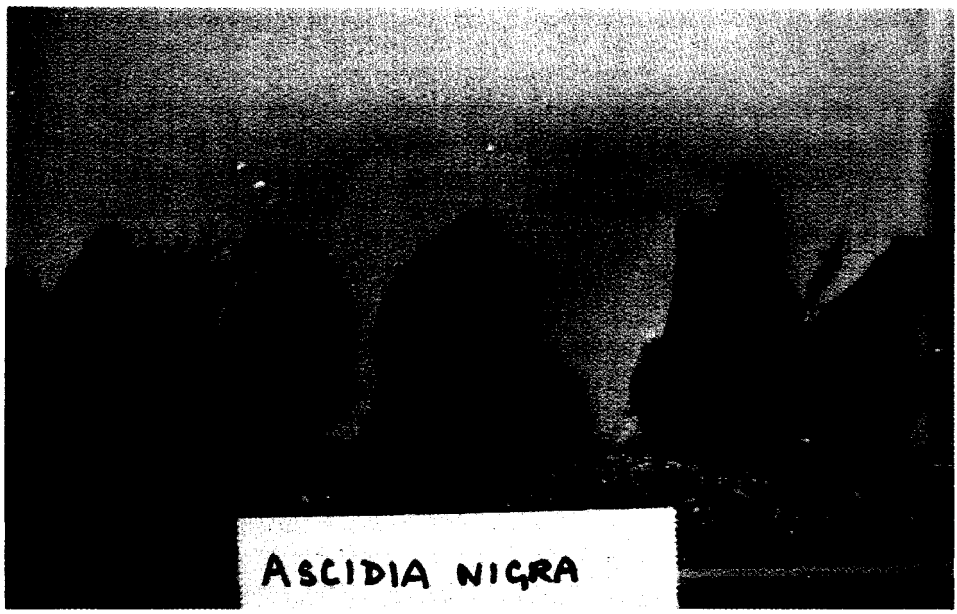

FIGURE 2. *Ascidia nigra*.

CROSS SECTION OF *ASCIDIA NIGRA*, ADULT AND LARVA.—Most of the body is occupied by a large phàrynx or branchial sac that communicates via gill slits with the surrounding chamber called the atrium. The seawater enters the mouth, where the respiratory/feeding current created by the cilia of the pharynx passes the water through the stigmata into the atrial cavity and out through the excurrent siphon (see Figure 3). The circulatory system is reversible, the heart pumping the blood alternately in one direction into the pharynx and then in the reverse direction into the viscera and body.

Tunicates are hermaphroditic. Although colonial tunicates can reproduce asexually by budding, both solitary and colonial species shed sperm cells into the water where they fertilize eggs from other individuals. The resulting gametes develop into tailed, tadpole-like larvae. The free-swimming larval stage is a brief planktonic nonfeeding period that soon undergoes metamorphosis to the sessile adult form upon stimulation with heavy metals, oligosaccharides, peptides, and a variety of other chemicals.

VANADOPHORES.—*The study of tunicate blood pigments.*—Aristotle (1) had already commented on the deeply colored tunics of ascidians as "the most remarkable characteristic of all those (lower marine) animals." The first observation of unusual color changes in ascidian blood dates back to 1847 (2), when it was noticed that the blood exposed to

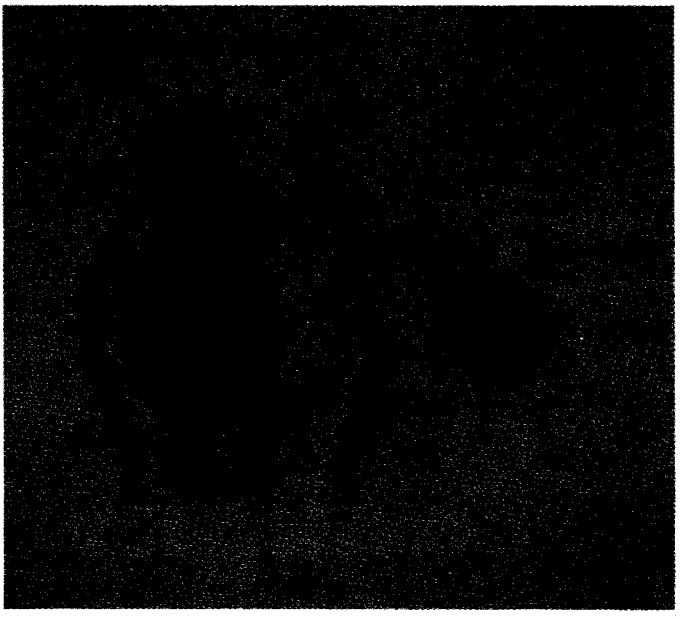

FIGURE 3. Cross Section of *Ascidia nigra*, Adult and Larva.

air turns deep blue within a few minutes. By 1909, Winterstein (3) at the Naples Zoological Station had shown that the ascidian blood neither binds oxygen nor contains oxygen-binding proteins such as hemoglobin or hemocyanin. On the other hand, dat-

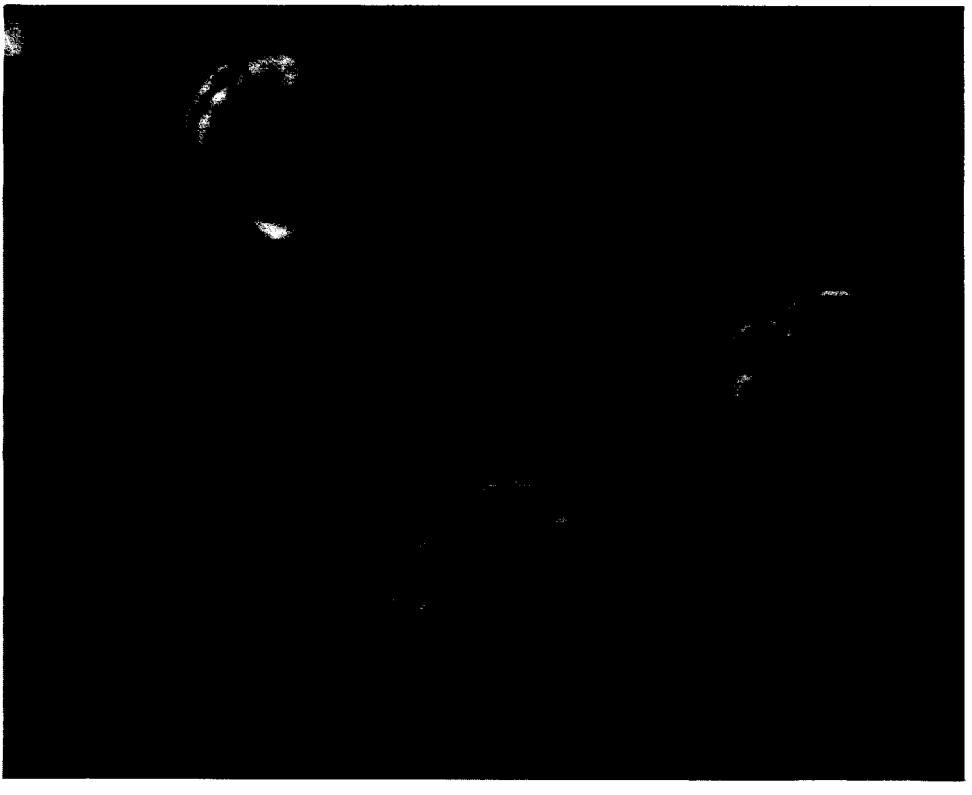

FIGURE 4. Vandophores.

ing back from 1893 (4) until quite recently (5-7), others postulated the presence of oxygen-carrying blood proteins called hemovanadins (5); it is now clearly shown that such proteins do not exist (see below) (8).

In 1911, Henze (also at Naples) found (9) that the blood of *Phallusia (Ascidia) mamillata* contains large quantities of vanadium, the blood pH was below 2 (10), its color in air changed from yellow-green to red-brown to dark blue, and addition of barium ion gave rise to a white precipitate. Thus, the animal is occasionally described erroneously, even in the *Encyclopaedia Britannica*, as living in dilute H_2SO_4. (We now know that the blood pH is neutral and that the precipitate is barium vanadate. See below.) Today, approximately 2,000 tunicate species are known around the world (11), and most of them accumulate metals such as vanadium (12-16), iron (17-22), manganese (23), niobium (24,25), and tantalum (25). No gold-, platinum-, or uranium-collecting species have been found or reared.

A. nigra possesses five types of blood cells. The type of cell involved in vanadium sequestering is the green vanadocyte, 90% of the volume of which consists of vanadophores (26,27), or vacuoles containing vanadium and the tunichromes. The low pH of air-exposed ascidian blood ("Henze's solution") (9) and the white precipitate formed with barium salts (9), which led to the chimera that the blood is 1.8 N H_2SO_4, is due to the fact that earlier workers had titrated free acid after cell lysis (28) or stained intact blood cells with pH indicators (29). The problem with the conclusions drawn from these studies is that some pH indicators may also act as redox indicators and therefore be affected by the strongly reducing intracellular milieu of vanadocytes. However, vital staining with pH indicators can yield useful data in determining intracellular pH if artifacts such as response to electrochemical potential can be eliminated, for example, by comparison of results obtained with several different indicators. Lysed blood cells, indeed, produce a strongly acidic solution, not known to contain vanadic acid, H_3VO_4, which is similar to phosphoric acid in acidity, and forms insoluble $BaVO_4$ with $BaCl_2$. The blood pH problem was solved recently by diffusion measurements of ^{14}C-methylamine into intact vanadocytes (30), and by ^{31}P-nmr measurements and staining of whole cell preparations (31). Both studies showed that the intracellular pH is physiological or only slightly below 7.

The phase diagram of V is extremely complex. It can assume oxidation states from +5 to −1 and can form oligomers and polymers; each oxidation state is accompanied by various hyydrolysis products. When present in low concentrations, as in the ocean (ca. 5×10^{-8} M) (32), V(V) exists as $H_2VO_4^-$ (33). Because of this resemblance to inorganic phosphate, vanadium is an important biochemical probe for sodium-potassium ATPase inhibition (34-36); other than this, very little is known about the significance of this essential trace element (37), the daily requirement for humans being about 0.1 ppm (38).

The evidence for predominance of V(III) (vanadic, green; refer to Figure 4 for colors) in the vanadocytes comes from magnetic susceptibility, polarimetric and potentiometric analyses on vanadocytes and their lysates (39), measurements of the ^1H-nmr chemical shifts of the water signal in live vanadocytes that resemble exactly those from V(III) aquocomplexes (40), and chemical analysis of lysates (41). The presence of tetravalent vanadium (vanadyl, blue) manifests itself in the electron paramagnetic resonance (EPR) spectra of whole blood cells (31), V(IV) being the only EPR-active species at room temperature. Studies by X-ray absorption spectroscopy (EXAFS or extended X-ray absorption fine structure) of living ascidian (*Ascidia ceratodes*) vanadocytes has confirmed that V(III) is indeed the predominant species present; it was further found that V(III) was present in a highly symmetrical coordination environment best explained by six oxygen atoms, and that the V(IV) content is less than 10% (42).

Contrary to earlier postulations, tunicates acquire all of the oxygen needed by simple diffusion from seawater filtered through the body and do not have oxygen-carrying proteins. Namely, although metals are present in the blood, reversible oxygen binding has not been detected in *A. nigra* blood (43). Electrochemical measurements clearly show the absence of reversible oxygen binding by the vanadocytes; however, the oxygen is irreversibly bound to a yellow chromogen that coexists with vanadium and shows a uv absorption at 330 nm in the unoxidized form (44).

The color of the well-known yellow-green pigment present in vanadocytes (8,39,40) was shown by Swinehart to be due to this chromogen and not to vanadium (45). It was then found that the pigment could be separated by gel chromatography (44); it is sensitive to oxygen and decomposes in media above pH 3.5; it possess acidic moieties, and it rapidly reduces Fe(III) and V(V). The pigment was named tunichrome (43,46). Tunichrome clearly plays a crucial role in maintaining strongly reducing conditions in the vanadocytes. The vanadophores have an anion-transport system that allows the vanadium oxyanion to enter but prevents the reduced complexed species from leaving (47). Despite the long history of the tunicate blood pigment, all attempts carried out to date have failed in isolating the pigment because of its sensitivity to air and water in exposed blood. As mentioned above, V(III) is green and V(IV) is blue, but this is only at a pH below 3; above pH 3, they form the precipitates V_2O_3 (black) and V_2O_4 (green-black). The blood pH is around neutrality, and yet, reduced vanadium exists here in solution; we believe that the strongly reducing and complexing properties of the tunichromes provide an atmosphere that stabilizes the intracellular V(III).

It is interesting to note that the metal can assume many other colors: vanadous ion V(II) is violet while V(V) can be yellow, orange, or colorless, depending on the polymeric state. Vanadium was therefore named after Vanadis, the goddess of beauty and love, and daughter of the goddess Njord (according to north European mythology) (48).

ISOLATION SCHEME OF TUNICHROMES.—After numerous failures with aqueous systems, we arrived at the scheme shown in Figure 5. The entire process is carried out

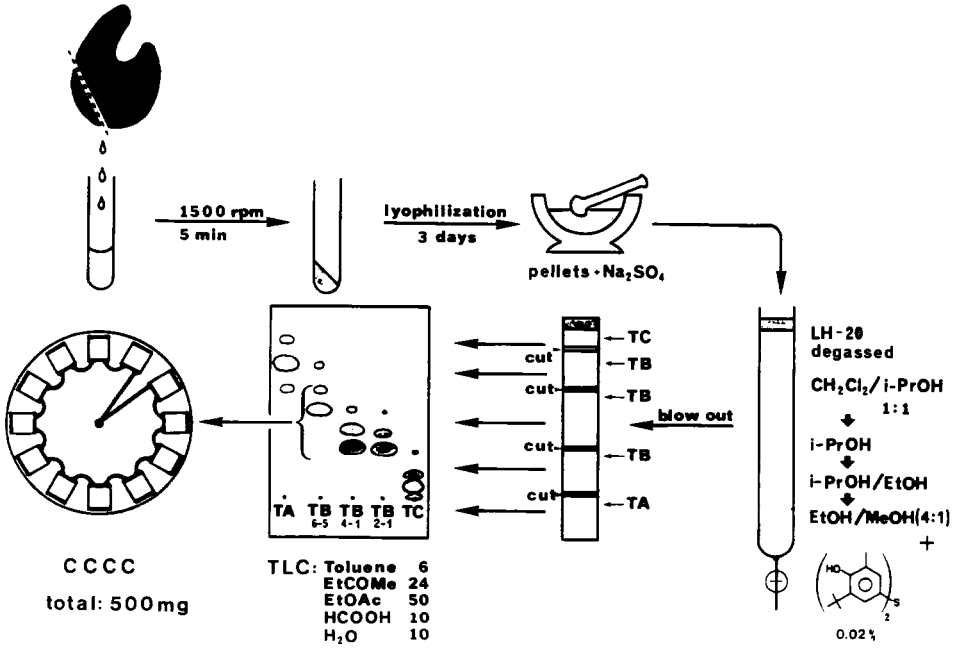

FIGURE 5. Tunichrome Isolation Scheme (entire process under Ar).

under deoxygenated Ar and with exclusion of moisture. It is an unusual isolation in the sense that, besides the extreme air and water sensitivity of the tunichromes, there was no particular assay to monitor the isolation except that we were after very unstable yellow compound(s) absorbing at 330 nm (44). The tunicates were flown in from Florida alive in lots of 1,000, brought in the evening to the laboratory, and immediately processed. Collection of blood from 1,000 animals is a 5-h process carried out by ten people.

a) The heart is cut along the dashed red lines shown in Figure 3, and the blood is squeezed into centrifuge tubes under a current of dry, oxygen-free Ar. Unless prepurified deoxygenated Ar was used, the yellow-green blood would become tinted brown, an indication of irreversible oxidation and decomposition.

b) Aliquots of blood are collected and centrifuged for 5 min at 1500 rpm, to give a pellet composed mainly of yellow-green vanadocytes.

c) The centrifuged pellets are immersed in liquid N_2 to lyse and freeze the cells and then lyophilized for 3 days for complete dehydration. Although the blood medium is water, once the cells are lysed, humidity leads to browning and destruction of the green-yellow color, especially when the tunichrome is still impure.

d) The lyophilized pellet is then ground in a mortar with an equal amount of dry Na_2SO_4 in a glove-bag; the role of the sulfate is to assist in grinding the cells as well as to absorb humidity.

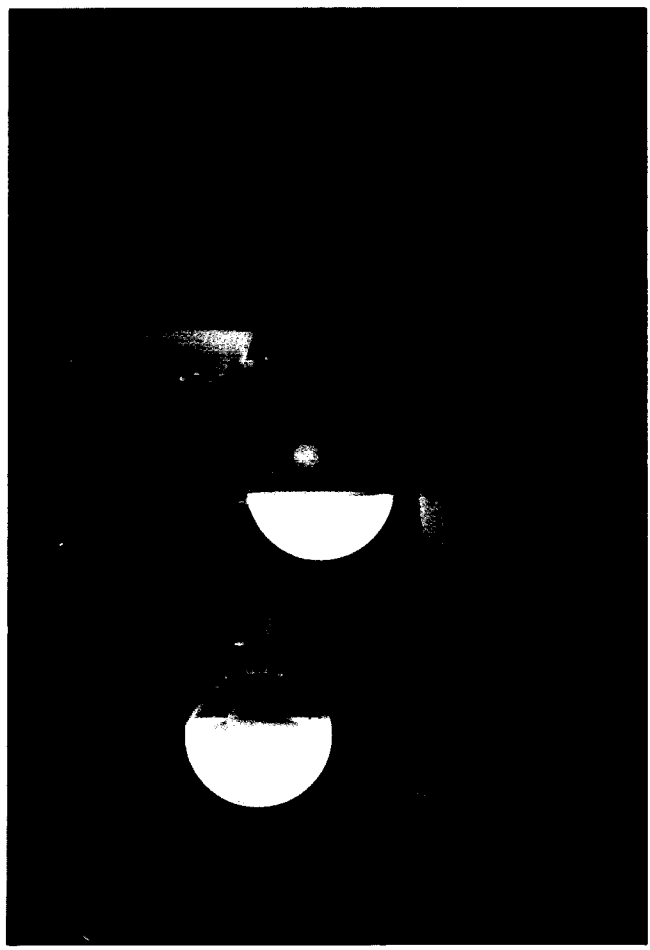

FIGURE 6. Tunichrome Fluorescence.

e) Sephadex LH-20 is pre-swollen in a 1:1 mixture of CH_2Cl_2/i-PrOH, degassed in a sonicator under vacuum, and added to a column, 5×100 cm. All solvents are thoroughly degassed and contain 0.04% 3,5-butyl-4-hydroxy-5-methylphenylsulfide as a radical inhibitor (49). The pulverized mixture is placed on top of the LH-20 column and eluted stepwise as shown in Figure 5; the bright yellow tunichromes are preceded by broad bands of carotenoids (fluorescing in red and purple under uv) and then by pale yellow bands possessing a blue fluorescence. The tunichrome band started moving with EtOH-MeOH (4:1).

f) The tunichromes are not eluted from the column. Instead, when the first yellow band reaches the column end, the entire column content is extruded into a glove bag and sliced into portions according to color intensities: TA (least polar), TB, and TC.

THE TUNICHROME AND PRECEDING FLUORESCENT FRACTION FROM LH-20 COLUMN.—The LH-20 column is shown in Figure 6 under uv light just after the broad carotenoid bands have been eluted. The dark band at the top of the column contains all the detached vanadium and crushed cells. The three flasks containing eluted solutions from a previous run are held against the corresponding bands of the LH-20 column. The top yellow flask contains the tunichrome mixture. The second and third flasks contain highly fluorescent and extremely unstable fractions that started to turn brown after several hours even when stored under Ar at −70° in the dark. It appears that the destabilizing factor(s) are contained in these fractions; once they are separated from the top tunichromes, the latter becomes much less sensitive to air and moisture. Although intriguing, the constituents of the blue fluorescent fraction are unknown so far.

PREPARATION OF CRUDE TA, TB, AND TC FRACTIONS (Figure 7).—The middle segment of the LH-20 column containing mostly TB was extracted with MeOH containing 1% t-butylsulfide (volatile antioxidant). From this MeOH extract, tunichrome B was obtained as a yellow amorphous powder by repeated precipitation with solvents of decreasing polarity. The nature of the white and green precipitates shown in the scheme is as yet unknown. The other segments of the LH-20 column were extracted and processed in the same manner. At this stage, 1,000 tunicates collected in late September 1984, gave 120 mg TA, 1,300 mg TB, and 300 mg TC, which amounts to approximately 1.7 mg of tunichromes per animal; these amounts are strongly season-dependent, as shown by the fact that a batch collected in February 1984, gave only half of the mentioned quantities.

ANALYTICAL HPLC ON TB AND DIODE ARRAY DETECTION.—The TB fraction

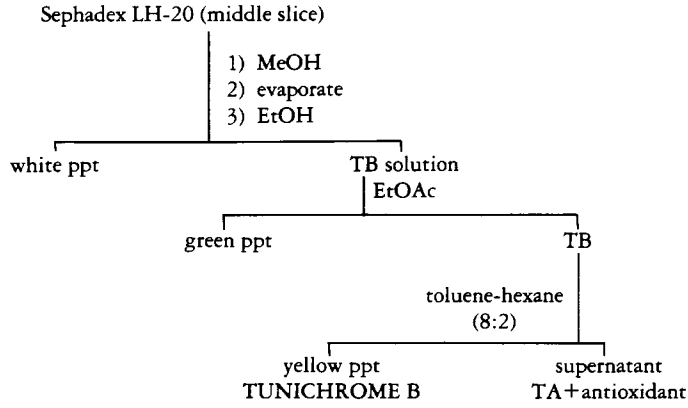

FIGURE 7. Preparation Scheme of Crude TA, TB, and TC fractions.

separated on high pressure tlc into three major yellow bands with orange fluorescence (see Figure 5). However, analytical hplc of TB with diode array detection (50) showed that each of the 3 tlc spots consisted of two compounds. The hplc trace of the major most polar TB spot is shown in Figure 8.

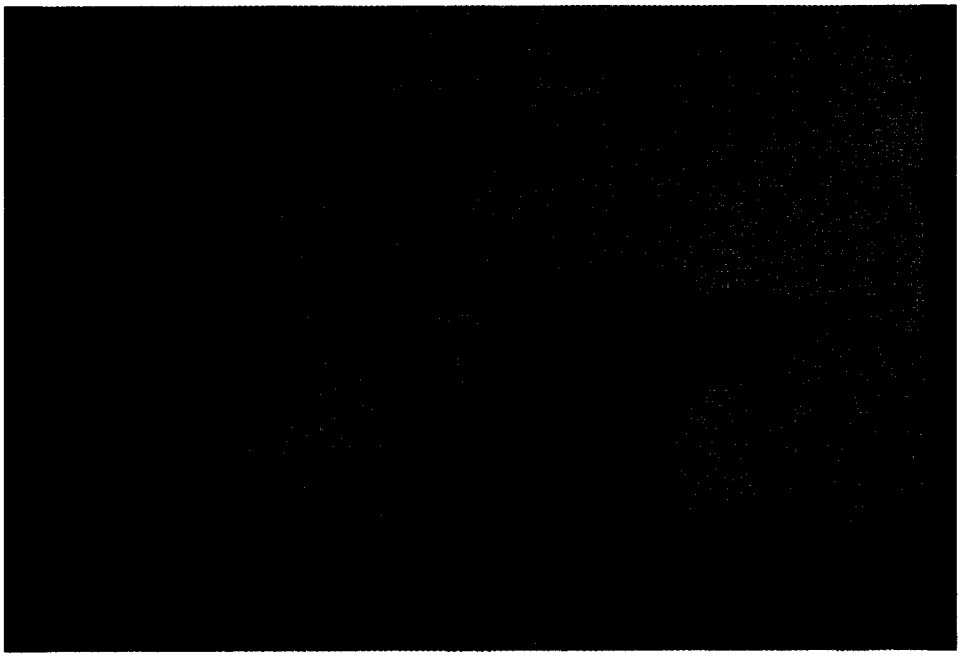

FIGURE 8. High Pressure Liquid Chromatography Separation Isogram of Tunichromes TB 1 and TB 2 (white<0.05; black>0.05; cyan>0.15; blue>0.25; green+0.35; red+0.55; pink+0.65).

The diode array detector scans continuously from 190 nm to 370 nm; in the isogram shown in Figure 8, the absorptions appear vertically and are plotted from 190 nm (bottom of vertical axis) to 370 nm (top) against the retention times on the abscissa. TB has strong absorptions at 210 and 340 nm (see Figure 10). In the isogram, a minor forepeak absorbing at 210 and 340 nm is seen to elute before the major fraction (therefore, more polar); because it absorbs at 210/340 nm, it must be yet another tunichrome; moreover, the symmetric shape suggests that it consists of a single component. In contrast, the asymmetric shape of the peaks around 16 min elution time shows that there are clearly two (TB-1 and TB-2), and possibly three, compounds with tunichrome absorptions.

CENTRIFUGAL COUNTER-CURRENT CHROMATOGRAPHY (CCCC OR C^4) OF TB FRACTION (Figure 9) .—The tunichromes readily decompose an hplc and hence could not be separated on a scale larger than analytical. Luckily, a prototype CCCC instrument (51) became available in our laboratory, and it was only through this chromatographic method that further semiprep scale purification of tunichromes was achieved.

This system is in principle similar to droplet counter-current chromatography (DCCC) (52) but uses centrifugal force instead of gravity. Because it is a liquid-liquid distribution method, it is basically nondestructive; further, it is rapid and can be scaled up readily to gram quantities, and the operation can be switched during separation from "normal phase" to "reversed phase." The theoretical plate number is in the range of N=500-2000. For the tunichrome separation, the following unusually complex two-phase system was found to be optimal: i-AmOH/n-BuOH/n-PrOH/ H_2O/HCOOH/t-butyl sulfide (32:48:40:120:1:4), upper phase=mobile phase, 2.4 ml/min, 1000 rpm.

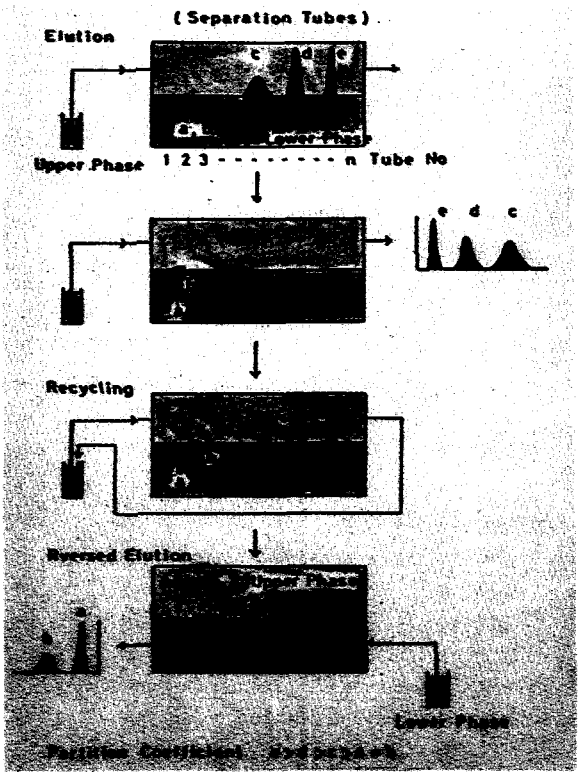

FIGURE 9. Elution, Recycling, and Reversed Elution via CCCC.

In the normal phase mode, the organic layer of a biphasic solvent system is used as mobile phase, whereas in reversed phase mode, the mobile phase is the aqueous layer.

When 200 mg of crude TB mixture was applied with the mentioned solvent system in the normal phase mode, the least polar TB-5/6 mixture, ca. 30 mg, was eluted at 3-4 h, followed by TB-3/4 mixture, ca. 60 mg, 5-6 h. After 7 h, the phases were switched, allowing the most polar pair TB-1/2, ca. 30 mg, to elute as a sharp peak. (See Figure 9.)

TUNICHROME B-1.—The CCCC peak consisting of the most polar pair TB-1/2 was now submitted to preparative hplc despite the loss (known to be ca. 90%) in material upon one passage, because this was the only high resolution method available. The hplc was run on two ODS columns connected in series using an acetonitrile/formate-buffer solvent system. It was found that adding small amounts of methoxyethanol as a stationary phase modifier influenced the elution pattern in a subtle manner. Shaving of the first peak shown in Figure 10 gave pure TB-1 for the first time (after five years), the yield being 500 μg from 5 mg of TB-1/2; this would correspond to a yield of 18 mg of TB-1 from 1,000 tunicates. Purified TB-1 is much more stable than an impure sample; an aqueous solution can be kept in the air for several hours without noticeable browning. However, it is still susceptible to decomposition upon warming, extended exposure to air, or treatment with weak base.

STRUCTURAL STUDIES.—The structure of TB-1 was determined by spectroscopic studies and chemical conversions (53). Reaction of TB-1 with CH_2N_2 gave the nonamethyl ether that was still unstable; the instability could be due to an inter- or intramolecular Michael addition of the primary amino group to the 11,12-ene moiety. Acid hydrolysis of the methyl ether yielded trimethoxyphenylalanine (compared with a synthetic *dl*-specimen), the absolute configuration of which was determined by the

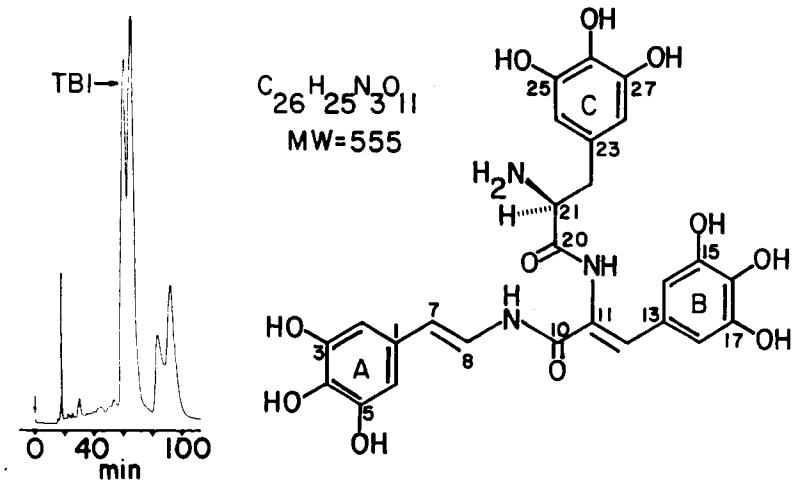

FIGURE 10. Tunichrome B-1; *Ascidia nigra* L. {ir (KBr): 3700-2400 (br), 1705, 1648, 1612, 1518, 1460; uv (MeOH): 340 (ϵ=19,600), 285(sh), 245(sh), 210 (ϵ=68,000); cd (MeOH): 340 ($\Delta\epsilon$=−3.3), 281 ($\Delta\epsilon$=+1.2), 243 ($\Delta\epsilon$=−2.5)}.

methoxydiphenylfluorenone method for α-amino acids (54). The peracetate (decaacetate) fished out from a mixture of 30 acetylation products was stable and gave the molecular formula by hrms. Quantitative ozonolysis of the decaacetate afforded two moles of triacetyl-gallaldehyde, thus establishing the peripheral structural components. Finally, the central portion of the TB-1 structure was determined by nOe studies with the decaacetate. The structure of TB-1 thus derived contains hydroxylated phenyl

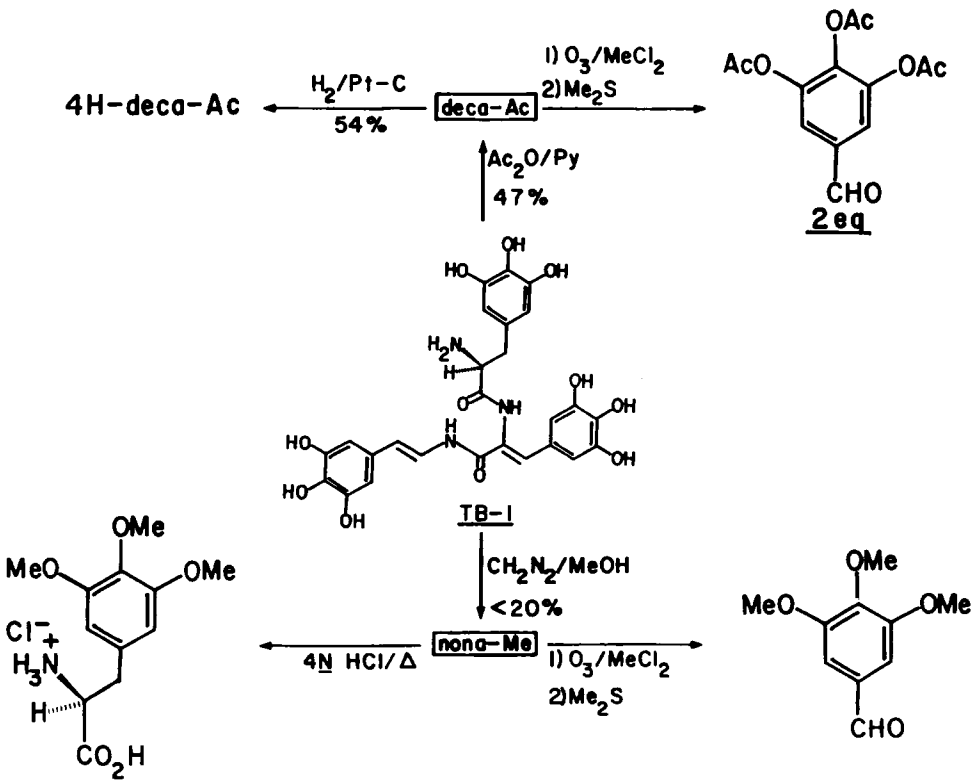

FIGURE 11. Structural Study.

enamide and dehydro-5-hydroxy-DOPA moieties such as those found in clionamide (55) and the celenamides (56).

The structure of TB-1, a member of the tunichrome group of blood pigments that has eluded characterization for years, has been determined (see Figure 11). But the mode of action or biochemical role of tunichromes or vanadium is still obscure. It is known that vanadium accumulates within the vacuoles of the vanadocytes by a trapping mechanism. Pentavalent V in the form of $H_2VO_4^-$ enters the vacuoles through an anionic channel and is eventually concentrated by 10^6-10^7fold (47); the concentration of vanadium can be as high as 0.15 M (44) to 1 M (42), depending on the species. Upon entering the vacuole, the vanadium is immediately reduced to V(IV) and then to V(III), the final ratio of V(IV) and V(III) being 1:9. We believe that at least the first reduction, and possibly the second, is carried out by the tunichromes; however, but the second reductant is not known: Could it be the tunichromes or could it be the unstable fluorescent fraction(s) shown in Figure 6? The resulting cation cannot leave the cell now because it is positively charged and/or bound in a tunichrome complex, neither of which are accepted by the anionic channel. Clarification of the functions of vanadocytes and the biological/biochemical function of tunichromes should yield much insight into the role and fate of trace elements in animals.

As structure determination of molecules becomes increasingly routine, the role played by isolation becomes increasingly important. Modern spectroscopic methods have made it feasible to deal with minute quantities of material or very unstable compounds, and, in many cases, such compounds are essential in daily maintenance of life. However, no structural studies can be carried out unless the factor is isolated in a pure state. Isolation and purification of bioactive factors are thus the first obstacles to be overcome; it frequently is the dividing point between successful or unsuccessful studies of further structure-based investigations on mode of actions, and so on. However, the present tunichrome case also shows that structure determination is merely the very first step in trying to understand mechanisms in life on a structural basis. The mode of action study is far more difficult and challenging but is not an impossible task with the techniques becoming available to natural-products chemistry.

ACKNOWLEDGMENT

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