Research Articles

In vivo incorporation of ^{14}C-phenylalanine into ascidian tunichrome

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Abstract. Ascidia ceratodes exposed to ^{14}C-phenylalanine in the surrounding seawater incorporates the radiolabel into newly biosynthesized tunichrome molecules. Radioactivity can be detected in tunichrome extracted from circulating blood cells within one day following initial exposure to the radiolabel; weak activity (≤ 4 μCi/mol tunichrome = 22 nmol phenylalanine/mol tunichrome) is detected in 1 to 10 days; significantly higher amounts of radiolabel (57 μCi/mol tunichrome = 318 nmol phenylalanine/mol tunichrome) appear 20 days after seawater exposure. Therefore, phenylalanine can function as a precursor in the biosynthesis of tunichrome.
Key words. Tunichrome; phenylalanine; ascidian; tunicates; blood pigments.

Tunichrome is a general term for a group of low molecular weight (370–560 dalton) hydroquinonoid pigments found in the blood cells of some members of the Phlebobranch and Stolidobranch ascidians (Phylum Chordata; Subphylum Urochordata)[1, 2, 3]. Tunichromes contain two or three catechol or pyrogallol moieties[2, 3] depending on the species of tunicate (fig. 1; e.g. An-1, An-2 and An-3 are all present in *Ascidia nigra*, whereas only An-1 is present in *A. ceratodes*, Mm-1 and Mm-2 are present in *Molgula manhattensis*)[3]. The phenolic moieties are thought to account for the molecules' affinity for iron and vanadium[4]. Recently, An-1 (fig. 1) has been synthesized de novo in the laboratory in its unstable and unprotected form[5].

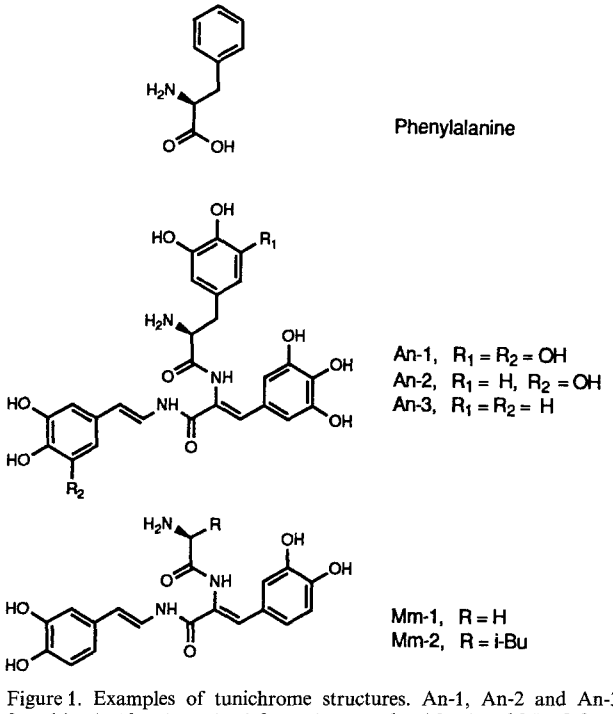

Figure 1. Examples of tunichrome structures. An-1, An-2 and An-3 found in *Ascidia nigra*; An-1 from *A. ceratodes*; Mm-1 and Mm-2 from *Molgula manhattensis*. Phe molecule presented for comparison of structure.

As strongly reducing compounds, tunichromes and other natural products such as catecholamides have recently been novel compounds for biochemical studies[6]. In addition, knowledge about the tunichrome-vanadium complex may help to unravel the age-old question facing both inorganic chemists and biologists of what function vanadium has in tunicate blood cells. To date, however, the study of metal-tunichrome interactions has been entirely limited to in vitro experiments, although our knowledge of even this aspect of metal-tunichrome interactions is scant[3]. In order to understand better both the morphological and physiological relationship between metals and tunichrome in living animals, a procedure for labeling tunichrome in vivo is needed. The present study demonstrates that radiolabeled phenylalanine can be incorporated into tunichrome An-1 found in the circulating blood cells of *A. ceratodes* (Huntsman, 1912).

Materials and methods

Chemicals. L-amino acid oxidase (type I), bovine liver catalase and sodium phenylpyruvate were obtained from Sigma (St. Louis, MO, USA). Tunichrome acetate standard, consisting of a mixture of An-1 acetate and An-2 acetate, was synthesized in the laboratory. Cold L-phenylalanine (Phe), acetic anhydride, pyridine, methylene chloride, iso-propanol (i-PrOH), sodium phosphate, cation-exchange resin (Dowex 50X8-200, strong acid form), High Performance Liquid Chromatography (HPLC)-grade acetonitrile, methanol and tetrahydrofuran (THF), as well as all other miscellaneous chemicals were obtained from Aldrich (Milwaukee, WI, USA).

Experiment A. Two groups of animals, each of which consisted of nine *A. ceratodes* (obtained from Sea Life Supply Co., Sand City, CA, USA), were exposed in separately maintained aquaria to 6.1 ng/mol of Phe in 1000 ml seawater (2:1, cold:radiolabeled Phe, radioactivity = 6.7 nCi/ml; specific activity = 1.1 nCi/ng Phe) for 25 h during which seawater samples (1 ml) from each aquarium were monitored for radioactivity using liquid scintillation counting (LSC; Beckman LS-100C Liquid Scintillation system). A control aquarium was maintained without animals for the same period of time. After 25 h exposure, animals were transferred to clean seawater and maintained for up to 20 days. During this entire experimental period (25 h exposure plus 20 days follow up), seawater was constantly aerated and held at 12–14 °C.

The tunichrome (An-1) content of circulating blood cells was determined by an acetylation procedure[3], providing the more stable tunichrome acetate rather than the reactive natural compound. Three animals were picked randomly from each of the two aquaria after 1, 3, 6, 10 and 20 days following exposure and sacrificed. Blood was collected by cardiac puncture and immediately centrifuged (10 min at 1722 × g) to obtain a blood cell pellet. After discarding the supernatant, 6 ml acetic anhydride and 6 ml pyridine were added to the pellet. The mixture was ultrasonicated and then stirred for two hours at room temperature under aerobic conditions. After evaporating the sample to dryness in vacuo (approximately 2 h), the residue was dissolved in CH_2Cl_2 and its components separated by Thin Layer Chromatography (TLC; Analtech 250 µm precast silica gel plates) using 4% i-PrOH in CH_2Cl_2 as a mobile phase. The tunichrome acetate band was identified by its distinctive pumpkin-colored fluorescence under long wavelength UV light (365 nm). This band was scraped off the TLC plate and eluted from the silica gel with 25% i-PrOH in CH_2Cl_2. The solution was then evaporated to dryness and the purified tunichrome acetate residue was dissolved in 1.5 ml CH_3CN. Subsamples of this solution were used for quantification of An-1 acetate by HPLC and measurement of the incorporated radiolabel by LSC.

Tunichrome acetate concentrations were measured in the 1.5 ml CH_3CN solutions by reversed phase HPLC, using two µBondapak C-18 columns (300 × 3.9 mm) in series, a Waters model M-6000A liquid chromatograph and a Waters model 440 absorbance detector set at 280 nm. The mobile phase (1.5 ml/min flow rate) consisted of CH_3CN, CH_3OH, THF and H_2O (27.2:24.0:1.3:47.5 by volume). A mixture of synthetic An-1 and An-2 acetates (0 to 1.96 mg/ml total tunichrome) was used for standardization since pure An-1 acetate, the only tunichrome present in *A. ceratodes*[3], was unavailable. Considering the similarities in structure (fig. 1), molecular weight, and UV absorption of An-1 and An-2 acetates, it is reasonable to assume that the ratio of peak areas for An-1 acetate and An-2 acetate would be the ratio of their molarities. Using this assumption, tunichrome An-1 molarities were calculated for each sample.

Following HPLC, the remaining CH_3CN subsample solutions were evaporated to dryness in an LSC vial. The dried residues were decolorized with 1.3 ml 30% H_2O_2 in order to eliminate color-induced quench. Samples were then counted on a Beckman LS-7000 Liquid Scintillation System (^{14}C window; 20 min counts; background corrected; less than 2% error) using 10 ml of New England Nuclear Aquasol-2 as a fluor. Known concentrations (0 to 27.5 nM) of ^{14}C-Phe, evaporated to dryness and digested in 1.3-ml aliquots of 30% H_2O_2, were used as standards.

Experiment B. The second series of experiments was conducted to determine whether Phe decomposes in seawater in either the presence or absence of animals. Since the low concentrations of Phe used in Experiment A (6.1 ng/ml seawater) were below the detection limit of the HPLC method employed (approximately 15 ng/ml seawater; described below), Experiment B was conducted at a thousand-fold higher Phe concentration (7.0 µg/ml of Phe in 1000 ml seawater; 4663:1, cold:radiolabeled Phe; radioactivity = 5.0 nCi/ml; specific activity = 0.71 nCi/µg Phe). Seawater was continuously aerated at 12–14 °C and sampled at approximately hourly intervals for radioactivity (1-ml samples) and at approximately 2-h intervals for Phe concentrations ([Phe]; 2-ml samples) over a 30-h period. A second aquarium was prepared identically, except that it contained five *A. ceratodes*.

Seawater[Phe]was determined using a modification of the method described by Lecavalier et al.[7] whereby Phe was enzymatically converted to phenylpyruvate, which was then directly measured by reversed phase HPLC. Phenylpyruvate was separated on a 5-µm Hypersil ODS column (250 × 4.6 mm), using a Dionex Bio-LC HPLC with variable wavelength UV detector set at 214 nm. The mobile phase (1.0 ml/min flow rate) consisted of 0.2 M sodium phosphate (pH 7.0) and acetonitrile (90:10 v/v). Recoveries of known Phe solutions averaged 96% with a detection limit of approximately 15 ng/ml seawater.

Results

Experiment A was designed to optimize the detection of radiolabel incorporated into tunichrome. It utilized low concentrations of total seawater Phe (6.1 ng/ml) but a high specific activity of labeled Phe (1.1 nCi/ng). Therefore, any Phe that was taken up by the animals would have a high specific activity, and any Phe that was newly incorporated into tunichrome should contain a high concentration of radioactive label. The design of Experiment A was limited, however, in that it was not possible to determine whether the Phe in the surrounding seawater remained intact or was degraded prior to the uptake process. Consequently, Experiment B was designed to utilize a high concentration of total Phe (7000 ng/ml) in order to investigate the fate of Phe in seawater directly. Approximately the same amount of radioactively labeled

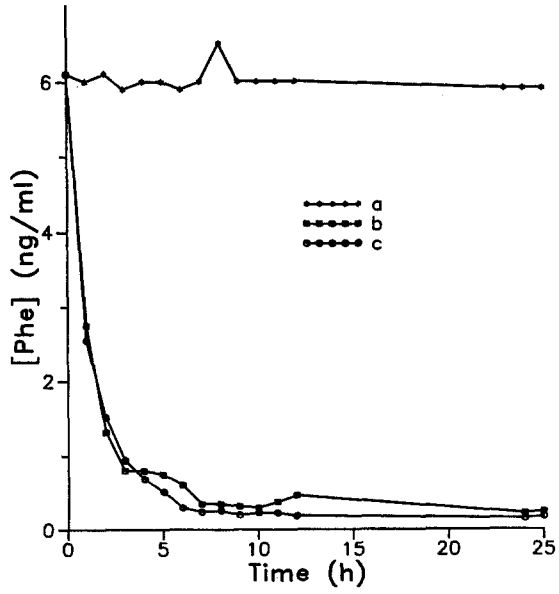

Figure 2. Phe concentrations, determined from radioactivity counts from 1-ml seawater samples (assuming no degradation of Phe) during 25-h exposures (Experiment A). Data shown for each of the experimental groups (lines b and c) plus a control aquarium (line a).

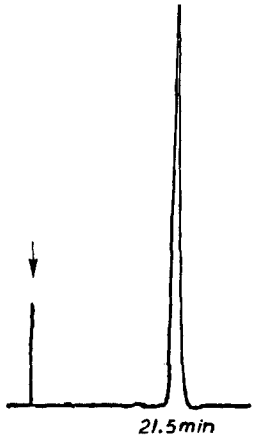

Figure 3. Reversed Phase HPLC chromatogram of An-1. Arrow indicates injection of sample. After acetylated blood cell components were separated by TLC, the pumpkin-fluorescing band was scraped from the silica plate, eluted from the solid phase with 25% i-PrOH and CH_2Cl_2, and checked for purity by reverse phase HPLC. The TLC separation yielded essentially pure An-1.

Phe (5.0 nCi/ml) was added to the seawater as in Experiment A (6.7 nCi/ml), but a much higher concentration of unlabeled Phe was present. The specific activity was a thousand-fold lower (0.71 nCi/µg) than that used in Experiment A (1.1 nCi/ng). Since any Phe that was taken up and used in the synthesis of tunichrome in Experiment B would have a low radioactivity, it was not useful to extract and analyze tunichrome in this second series of experiments.

Figure 2 shows the result of Phe uptake by animals in Experiment A plotted as[Phe]in seawater against time. Total Phe concentrations (radiolabeled plus cold Phe, $[Phe]_T$) were calculated from radioactivity measurements of each sample, assuming no decomposition. The $[Phe]_T$ showed a rapid decline followed by a slower, nonzero leveling off. More than 50% of the label was taken up in the first hour of the experiment. The majority of uptake by the animals occurred during the first three hours of exposure.

Once inside the animals, subsequent incorporation of the radiolabel was analyzed in tunichrome extracted from the tunicate blood cells and converted to tunichrome An-1 acetate. TLC separations revealed over nine variously-colored bands extracted from the blood cell lysates. All nine of these bands, including the pumpkin-colored fluorescing tunichrome band, contained detectable radioactivity. The majority of the radioactivity was in the first band just above the origin of the TLC spot. Subsequent analysis of the pumpkin-colored fluorescing band by reverse phase HPLC revealed only a single (major) peak corresponding to the An-1 peak of the synthetic tunichrome standard (fig. 3).

All of the radioactivity associated with the TLC pumpkin-colored fluorescing band could therefore be attributed to newly synthesized tunichrome. Since we could determine both the An-1 acetate concentration in a CH_3CN solution and its radioactivity in the samples, we were able to determine the amount of Phe that was incorporated into the tunichrome pool, based on our standard curve of radioactivity vs nmoles Phe. Incorporated Phe was normalized per mole of measured tunichrome and used for analysis of the uptake dynamics (table). The measured amount of tunichrome (1.0–3.4 mM) is comparable to that reported for tunichrome in *Ascidia nigra*[1,2]. Incorporation was detectable in tunichrome An-1 on days 1, 3, 6 and 10 and measured radioactivity was more than 5 times greater than background at day 20. As shown in the table, while only 22 nmoles Phe were incorporated per mole of tunichrome on day 1; 318 nmoles Phe/mole tunichrome were measured on day 20.

The result of Experiment B, in which $[Phe]_T$ was much higher than in Experiment A to allow direct Phe analysis by HPLC, is shown in figure 4. In the absence of animals both LSC and Phe analysis (curves a and b) indicated that Phe did not decompose in seawater during the time interval used for animal exposures in Experiment A (25 h). In the presence of animals, declining curves c and d showed Phe uptake (even at these high seawater Phe

Combined results of tunichrome analysis and radioactive Phe uptake. The initial concentration of total Phe added was 37.2 nM.

Sample (d)	Tunichrome ($nM \times 10^{-6}$)	Incorporated tunichrome ($nM \times 10^{+2}$)	TC_T (nmol Phe/mol tunichrome)
1	1.75	3.89	22
3	3.37	4.71	14
6	1.97	7.40	38
10	1.72	7.80	45
20	1.66	52.73	318

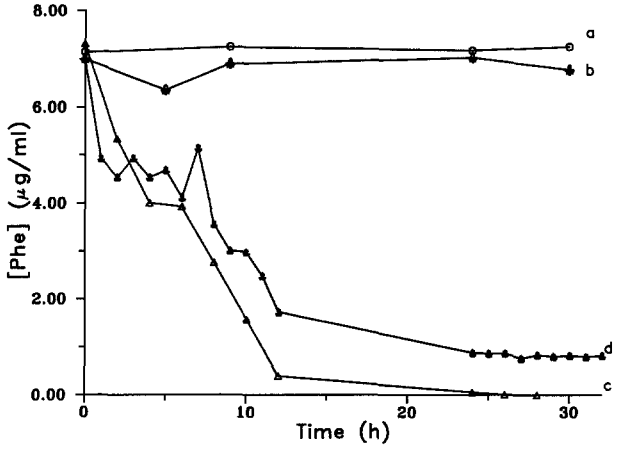

Figure 4. Phe concentration changes in seawater (Experiment B). *a*, *b* in the absence of animals; *c*, *d* in the presence of animals. Phe concentrations for *a* and *c* were measured directly (HPLC analysis). Concentrations for *b* and *d* were calculated from liquid scintillation counts of 1-ml seawater samples (assuming no degradation of Phe).

concentrations). However, calculated [Phe]in seawater (based on radioactivity counts and assuming no decomposition; curve d) was usually higher than that obtained by direct Phe analysis (curve c). This observation suggested that slight decomposition did occur. The experiment also showed that radioactivity in seawater leveled off toward the end of exposure to a non-zero value (curve d) while the directly measured [Phe]leveled off to zero (curve c). Since the relative error in Phe analysis was only $\pm 3\%$, we believe this difference is significant, and estimate that approximately 13% of the Phe could have decomposed. Since the radioactivity in the seawater-containing animals remained relatively constant throughout the remainder of the exposure period (12–32 h), it is apparent that little of this degraded Phe product was absorbed by the animals. Since the maximum difference between curves c and d was 18% (during the 6–12 h period), and the difference during the final 24–32 h period was 13%, the maximum amount of Phe degradation product that the tunicates could have taken up was 5%.

Experiment B was done at a much higher [Phe] than Experiment A and fewer animals were used. The difference can be seen by comparing figure 2, b and c with figure 4, d. In Experiment A (fig. 2), the radioactivity dropped to a stable level in 6–7 h after animal exposure while it took more than 13 h for the radioactivity to drop in Experiment B (fig. 4, d). The rate of radioactivity decline in Experiment A was greater than in Experiment B. This effect could be due to saturation of Phe binding sites in Experiment B. Regardless of the concentration difference, the uptake dynamics of Phe in seawater in Experiment A appears to be the same as that in Experiment B. The conclusion drawn for ^{14}C-Phe uptake in Experiment B should apply to Experiment A: the majority of the radiolabel taken up from seawater by the tunicates was ^{14}C-Phe rather than a degradation product of Phe. Therefore, any radiolabel incorporated into tunichrome originated as the ^{14}C-Phe that was taken up.

Discussion

Seawater [Phe] was measured using a modification of the method proposed by Lecavalier et al.[7] in that phenylpyruvate was extracted from the aqueous layer using 6.5 ml methylene chloride three times instead of once in order to improve the extraction efficiency. In addition, the standard curve we obtained was linear (over the 0 to 44.4 µM range) as opposed to the curves that Lecavalier et al. presented, which were best described by second degree polynomial equations. Lecavalier et al. ascribed the non-linearity of their standard curves to detector response characteristics. Our Phe recovery after Phe to phenylpyruvate conversion and extraction (96%) was greater than that reported by Lecavalier et al. (30%)[7].

We have considered two models to estimate the rate of tunichrome production based on ^{14}C-Phe uptake dynamics. In a zero-order model, the rate determining step is availability of a reaction site, which is independent of[^{14}C-Phe]. In a first-order model, the rate determining step is the reaction of radiolabel with an intermediate compound whose concentration is much larger than that of ^{14}C-Phe, which leads to a rate dependent on[^{14}C-Phe]. Let the total (hot and cold) amount (nmol) of tunichrome produced from Phe per specimen relative to the amount of tunichrome (mol) extracted per specimen at any time, t, during the labeling experiment be TC_T (table, last column) and let the total (hot and cold) concentration of Phe (nmol Phe/mol tunichrome) initially present per specimen at time $t = 0$ be Phe_{T0}. Then, the time-dependent formation of tunichrome is given by equations (1 a) and (1 b) for the zero- and first-order models, respectively.

$$TC_T = k_0 t \quad (1\,a)$$

$$TC_T = Phe_{T0}[1 - \exp(-k_1 t)] \quad (1\,b)$$

where k_0 and k_1 are zero- and first-order rate constants. Since little radioactivity accumulates in the tunichrome band before day 20, i.e., 52.7×10^{-2} nM out of 37.2 nM (see table), and then only a relatively small amount is incorporated in relation to the total amount of tunichrome present, the exponential term in equation (1 b) can also be approximated by a polynomial series truncated after the first term. Therefore, $\exp(-k_1 t) \approx 1 - 0.9664(k_1 t)$ for $k_1 t \ll \ln 2$[8], and the first-order term takes the form of equation (1 c) which is indistinguishable from equation (1 a).

$$c_T = 0.9664\, Phe_{T0}\, k_1\, t \quad (1\,c)$$

Plots of equation (1 b) utilizing such parameters resembled straight lines more than exponentials as predicted by equation (1 c).

Regression analysis of equation (1 a) converged on a constant rate of tunichrome formation corresponding to

$k_0 = (16 \pm 4)$ nmol Phe/mol tunichrome d^{-1}. Since the amount of tunichrome in the circulating blood cells of *A. ceratodes* has been shown to be roughly 1–3 mM, this rate would appear to be inadequate to account for all the native biosynthesized tunichrome. We note, however, that the radiolabel was observed to be present in several TLC bands in addition to the band identified with pure tunichrome An-1. We do not know if these other bands are either precursors or degradation products of tunichrome. For example, Phe is probably not the only tunichrome precursor, and tunichrome synthesis need not occur exclusively in circulating blood cells. It is therefore probable that the models used in the dynamical analysis are oversimplified and do not account for the presence of two or more competing processes in the mechanism.

These results confirm our preliminary experiments in which the radiolabel from both ^{14}C-Phe and ^{14}C-tyrosine was shown to be incorporated into *A. ceratodes* tunichrome using autoradiographic procedures. Identical TLC procedures were utilized previously but, instead of extracting tunichrome from TLC plates and measuring tunichrome concentrations by HPLC as we have done here, the TLC plates were mated with X-ray film to afford a qualitative detection of radiolabel. In the autoradiography experiments weak radioactivity was observed in the tunichrome TLC band 3 days post-exposure, while more activity was recorded for both the 5-day and 10-day groups of animals. Labeled Phe was incorporated into tunichrome in both seawater-exposed *A. ceratodes* (as in the present experiments) as well as in *A. ceratodes* injected with the label.

The major finding of these ^{14}C-Phe uptake studies is that for *A. ceratodes* L-phenylalanine is a precursor of endogenously synthesized (biosynthesized) tunichrome. The incorporation of ^{14}C-Phe within 24 h of exposure indicates that new tunichrome is rapidly synthesized by one or more types of tunicate blood cells. Free tunichrome has been identified primarily in the morula cells of *A. ceratodes*[9]. It is hypothesized that vanadium-bound tunichrome is present in the signet ring cells of this species. Since these two blood cell types probably could not have developed from the lymphocyte-like precursor cells that are released from the hemopoietic tissues in the short period of time our experiments covered[10], we conclude that ongoing tunichrome synthesis occurs in fully differentiated blood cells.

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Dynorphin-degrading cysteine protease is highly specific for paired arginine residues

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Abstract. The cleavage of dynorphin and three analogs containing paired basic residues by several proteases was investigated. The cysteine protease of neuroblastoma cells cleaved only the bond between Arg-Arg residues. Submandibular arginylendopeptidase, however, cleaved bonds between both Arg-Arg and Arg-Lys residues, and pancreatic trypsin at the carboxyl sides of both arginine and lysine residues. This shows that the cysteine protease is highly specific for paired arginine residues.
Key words. Neuropeptide; dynorphin; paired basic residues; neuropeptidase; arginylendopeptidase.

It has been believed that endopeptidases bound to the cell surface membrane play essential roles in the termination of the biological actions of neuropeptides[1]. For example, endopeptidase-24. 11 (enkephalinase, EC 3.4.24. 11), together with an aminopeptidase, is the principal enzyme in the case of the degradation of enkephalin[2,3].

In the course of studies on the proteases functioning in the degradation of dynorphin, an endogenous Leu-