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# ACCUMULATION OF HEAVY METALS BY SOME SOLITARY TUNICATES

by

Gordon Patrick Danskin

B.Sc., Simon Fraser University, 1969

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in the Department

of

Biological Sciences

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## Abstract

Spectrophotometric procedures for vanadium and iron, and a gravimetric procedure for vanadium were used to determine the heavy metal content of selected solitary tunicates. The vanadium content of Ciona intestinalis increases linearly as the dry weight of the animal, and can be estimated from a concentration value of ~90 $\mu$g V/g dry weight, independent of the size class of the animal. The importance of this baseline information to studies of the minimum vanadium requirements for the ascidians is discussed.

There is no evidence of seasonal variation in the vanadium concentration of Ciona intestinalis, and the mode and rate of vanadium uptake are considered in light of this observation.

The vanadium concentrations of ten species of local solitary ascidians are reported. It is noted that the occurrence of the metal within the class does not conform to taxonomic groups, but instead occurs sporadically throughout. Implications of this finding for theories of the evolution of the group are examined.

Acknowledgments

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# Introduction

Among the biochemical oddities which distinguish the ascidians from all other animals is their ability to concentrate vanadium in blood cells at levels 100,000 to 1,000,000 times that in seawater (Rummel et al., 1966). Vanadium is present in the sea at 0.3 to 3.0 ppb as an equimolar mixture of $H_2VO_4^{-1}$ and $HVO_4^{-2}$ (Burton, 1966; Kustin et al., 1975; McLeod et al., 1975). Kalk (1963a and 1963b) describes the uptake process as one in which the anion, adsorbed onto pharyngeal mucus, is passed through the cells of the branchial basket to the blood plasma by pinocytosis. Amoebocytes gather the pinocytosed vesicles and undergo an inferred maturation (Kalk, 1963b; Endean, 1955a), becoming morular cells termed vanadocytes (Webb, 1939). Vanadium is present in vacuoles called vanadophores in a form called hemovanadin (Bielig et al., 1966). Although the chemical nature of hemovanadin is not well understood, the metal is thought to occur in a low oxidation state (Bielig et al., 1966; Swinehart et al., 1974; Kustin et al., 1976) as a powerful reducing agent (Bielig et al., 1966). Sulfuric acid (0.4 N) contained in the corpuscles (Kobayashi, 1933 and 1935; Endean, 1955a) prevents spontaneous oxidation of the tervalent ion (Bielig et al., 1966). The organic constituent of the complexed ion appears to be a sulfated basic protein (Bielig et al., 1966) rather than a porphyrin or chain of pyrroles as had been suggested by earlier work (Webb, 1939).

The accumulation process by the blood cells is specific for vanadium (Rummel et al., 1966) and is an active process of limited capacity (Kustin et al., 1975). Passage of the ion across the pharyngeal wall to blood spaces is dependent upon ATPase activity, subject to competitive inhibition (Rummel et al.,1966), most efficient at low concentration, and is self-regulated (Kustin et al., 1975). The accumulation process is

clearly not one of diffusion.

Remarkably little is known about the physiological significance of the vanadium blood pigment. There is general agreement that hemovanadin does not act as a respiratory carrier (Hecht, 1918; Webb, 1939 and 1956; Kovalsky and Rezayeva, 1964; Rezayeva, 1964; Bielig et al., 1966), but it has been suggested that it functions either in fixation of carbon in the ascidian test as tunicin (Henze, 1932 cited in Webb, 1939) or in some other redox reaction as yet unknown (Kovalsky and Rezayeva, 1964; Rezayeva, 1964; Bielig et al., 1966). Endean (1955b and 1961) suggests that vanadocytes synthesize the polysaccharide constituting the fibrous matrix material of the test, but other authors believe that the cells either produce a substance which causes pre-existing fibers to coalesce (Tanaka et al., 1973) or produce a "surface" which causes the fibers to coalesce as regular laminae (Smith, 1970). Vanadium is believed to exist within vanadocytes in a biologically active state, but the metal has yet to be shown to be essential for the animals.

This study was undertaken to determine the seasonal vanadium content of various size classes of the well-studied ascidian Ciona intestinalis (Linnaeus) accumulated under normal conditions in the field. To assess whether vanadium is found only in primitive families of the order Phlebobranchia as earlier study suggests (Webb, 1939) and whether the presence of vanadium can be used to recapitulate the evolution of the class Ascidiacea as several authors believe (Webb, 1939; Millar, 1966; Swinehart et al., 1974) representative solitary tunicates collected from Barkley Sound were analyzed for their vanadium concentration.

## Materials and Methods

### 1. General

Solitary ascidians representing four families were collected intertidally and subtidally at Bamfield, British Columbia (48° 50' N, 125° 8' W) during the spring and summer of 1976. Species were identified by the author based on the work of Van Name (1945) and Kozloff (1974), and included the following: Ascidia ceratodes (Huntsman), Ascidia paratropa (Huntsman), Boltenia villosa (Stimpson), Cnemidocarpa finmarkiensis (Kiaer), Corella willmeriana Herdman, Halocynthia aurantium (Pallas), Halocynthia igaboja Oka, Pyura haustor (Stimpson) and Styela montereyensis (Dall). Only solitary tunicates were used in this study because the test, frequently encrusted with epizoites and sediment, is easily removed. Ciona intestinalis (Linnaeus) was collected subtidally at Venice California (34° 59' N, 118° 28' W) in winter and summer 1976. This particular species was examined for its vanadium content as a function of weight class and season of collection since it is the only species for which an average vanadium concentration based on a large sample size is available (Bielig et al., 1961b).

Animals used for metal assay were squeezed gently to expel extraneous seawater from the branchial chamber. Each specimen was dissected from its test over a weighed scintillation vial or beaker as size demanded in order to prevent loss of blood and fluids. The stomach and intestine were opened to remove gut contents which frequently included sediments. After drying to constant weight at 110°C, specimens were wet ashed in a 2:1 mixture of hot, concentrated nitric acid and sulfuric acid. Digestion was continued until additional small aliquots of the acid mixture failed to produce further nitric oxide, and the digestion solution appeared clear.

The resulting solution was heated until almost dry. Upon cooling, the mixture crystallized, and was redissolved in distilled water for transfer to a volumetric flask. Following dilution to a convenient volume, normally 10.0 ml in the case of specimens analyzed individually, the solution was passed through a glass fiber filter. Aliquots of these solutions were used for determinations of iron and vanadium as described below.

Chemicals used in metal analyses were of reagent grade, and water was double-distilled. New glassware was used whenever possible to avoid contamination, but in any event, all glassware was soaked in 50% nitric acid overnight, washed with six changes of distilled water and dried prior to use. Spectrophotometric determinations were made using a Zeiss PMQ spectrophotometer (±0.0005 absorbance units), and weights were determined on a Mettler H20T balance (±0.00001 g).

The following statistical methods (Sokal and Rohlf, 1969) were used when applicable: linear regression by Model I analysis of variance and tests of the significance of calculated regressions, tests for simultaneous equality of regression coefficients of several lines, calculation of correlation coefficients and tests for the significance of and differences between correlation coefficients, linear regression analysis by Model II analysis of variance, 0.95 confidence levels of calculated regressions, and F-test for comparison of means. F-ratios were considered significant if the probability that the event was a random occurrence was $\leq 0.05$.

## 2. Vanadium Determinations

### a. Evaluation of Colorimetry using Phosphotungstate

Wright and Mellon (1937) describe a spectrophotometric procedure for the determination of vanadium in alloy steel using phosphotungstic acid. Before applying the procedure to the determination of vanadium in asci-

dians, preliminary investigations of the technique were undertaken and are reported below.

In order to determine the wavelength at which Beer's Law is best observed by the yellow phosphotungstovanadate complex, a series of standards employing ammonium vanadate were prepared. The molar ratio of phosphoric acid to sodium tungstate was set at 19:1 as recommended (Wright and Mellon, 1937), and vanadium concentration varied from 0 - 25 μg/10 ml. Absorbance values at 410, 430 and 440 nm were plotted against vanadium concentration in order to choose the wavelength at which absorbance increases linearly with vanadium concentration.

To assess the percentage change in absorbance resulting from variations in the ratio of phosphoric acid (90%) to sodium tungstate (0.5 N), a series of standards containing 25 μg V/10 ml were prepared in which the reagent ratio was varied from 9.9:1 to 33:1. Absorbance was measured at 430 nm, and was plotted against moles of phosphate per mole of tungstate.

Iron as Fe(III) interferes with phosphotungstate determinations of vanadium (Wright and Mellon, 1937). The brown color produced by Fe(III) is reportedly dispelled by boiling the solution briefly (Wright and Mellon, 1937). Since several species of ascidians have been found to accumulate substantial amounts of iron (Endean, 1955a, 1955b and 1955c; Smith, 1970), the effectiveness of boiling as a means of overcoming interference from iron was examined. Blanks were prepared which contained phosphotungstic acid but no vanadium; instead, iron as $FeCl_3 \cdot 6H_2O$ was added in increments from 0 to 120 μg Fe/10 ml The vials containing the solutions were sealed and heated to 100°C in a water bath. After cooling to ambient temperature, the solutions were examined for absorbance at 430 nm.

Because small amounts of seawater are unavoidably introduced into tunicate samples wet ashed as described above, the effect of diverse seawater ions on phosphotungstate determinations was studied. Vanadium-free artificial seawater was prepared with Instant Ocean (Aquarium Systems, Inc., Eastlake Ohio 44094), replacing the manufacturer's trace element solution with one which contained no vanadium but all other traces in appropriate concentration. Two series of standards containing 10 and 25 $\mu$g V/10 ml were prepared in which the volume of distilled water normally used was replaced with increasing proportions of vanadium-free seawater (0 to 59% of total volume). The molar ratio of phosphate to tungstate was set at 19:1; after being boiled, the solutions were examined for absorbance at 430 nm.

Occasionally, the wet oxidation process described above yields a solution with residual yellow color. To correct for this interference, a single specimen of Ciona intestinalis was digested, and half of the resulting solution was treated with activated charcoal. Aliquots of treated and untreated solutions were reacted with phosphotungstic acid, and, for comparison, aliquots of treated and untreated solutions were diluted to equal volume with distilled water rather than with reagents. Absorbance values were read at 430 nm against a blank which contained reagents but no tunicate preparation.

A Beer-Lambert curve for the phosphotungstovanadate complex was prepared using $NH_4VO_3$ as the vanadate standard. The ratio of phosphate to tungstate was set at 19:1, and solutions were boiled briefly in sealed containers. Prior to measuring absorbance at 430 nm, all solutions remained at room temperature for 2 hours to permit maximum development of color (Wright and Mellon, 1937).

In order to determine whether organic or inorganic residues in tuni-

cate digestion solutions either enhance or suppress the development of the yellow phosphotungstovanadate complex, absorbance curves were produced for wet oxidized solutions of Ciona intestinalis and Corella willmeriana to which standard additions ($NH_4VO_3$) were made. The slopes of the resulting curves were tested for equality with the slope of the aqueous standards curve (Sokal and Rohlf, 1969).

b. Vanadium content of Ciona intestinalis

Specimens of Ciona intestinalis collected in January and June 1976, were assayed individually for their vanadium content according to the procedure outlined above. Each digestion solution was divided into 3 portions: two were reacted with phosphotungstic acid and the third was diluted to appropriate volume with distilled water rather than with reagents. Absorbance values obtained were compared to those obtained for aqueous standards.

c. Vanadium concentration of local solitary ascidians

Specimens of local solitary tunicates collected at Bamfield were assayed for vanadium using the above procedure. In general, determinations were made of single whole animals, but in some cases, small specimens of a single species were pooled to produce a single sample.

d. Gravimetric determination of vanadium in Boltenia villosa

The vanadium content of Boltenia villosa was recovered and weighed as $V_2O_5$ by adapting a colorimetric procedure for vanadium described by Talvitie (1953). Vanadium is separated from iron and copper as a vanadium-oxine complex soluble in chloroform. Following removal of solvent, the product is charred, fused, and oxidized to constant weight at 800°C. Percentage recovery of vanadate as vanadium pentoxide was assessed by applying the technique to solutions containing known amount of vanadium and iron. The effectiveness of the separation was examined by repeating

the procedure on a control which contained only iron.

3. Iron Determinations

The iron content of Corella willmeriana was determined using the thiocyanate method of Sandell (1959). Specimens were wet ashed as described above for colorimetric determination of vanadium. Assays were performed in triplicate using the method of standard additions (Christian and Feldman, 1970). Freshly prepared Fe(III) as $FeCl_3 \cdot 6H_2O$ was used as the internal standard, and colorimetric preparations contained freshly prepared thiocyanate diluted to 0.3 M as recommended (Sandell, 1959). Because the red color which develops is transitory, the thiocyanate was added one minute before measuring its absorbance at 480 nm. Determinations were made using a Zeiss PMQ II spectrophotometer. The curve which resulted was extrapolated to the x-axis in order to determine the amount of iron present in the sample.

## Results

### 1. Vanadium Determinations

#### a. Evaluation of colorimetry using phosphotungstate

At 430 nm, the absorbance of the yellow phosphotungstate complex of vanadium increases linearly with concentration of V(V) (Fig. 1). When the ratio of phosphoric acid (90%) to sodium tungstate (0.5 N) varies between 13:1 and 26:1, the resulting change in transmittance is ±1.26% of the value observed for the recommended ratio of 19:1 (Table 1). The absorbance peaks at a reagent ratio of 13:1 and falls off rapidly as the proportion of phosphoric acid decreases (Fig. 2).

The addition of Fe(III) or vanadium-free artificial seawater to phosphotungstate blanks yields a brown color which absorbs at 430 nm, but this color is destroyed by boiling the sample (Tables 2 and 3). Inclusion of increasing proportions of this same artificial seawater in vanadium standards produces no change in the absorbance of the phosphotungstate complex at 430 nm so long as the samples are boiled briefly (Table 4).

The yellow color occasionally noted in wet ashed tunicate preparations absorbs at 430 nm in the absence of colorimetric reagents, but this interference is removed by treatment with activated charcoal (sample 2 and 3, Table 5). When the absorbance of untreated digestion solution diluted to 5 ml is subtracted from the absorbance of a 5 ml solution of untreated material reacted with reagents, (samples 2 and 4, Table 5), one observes the same absorbance due to vanadium (0.005) as when charcoal-treated material is reacted with phosphotungstate (sample 5, Table 5).

The absorbance of phosphotungstate as a function of aqueous vanadate concentration is determined at 430 nm (Fig. 3). Similar curves are prepared in which vanadate standard was added to wet oxidized solutions of *Ciona intestinalis* and *Corella willmeriana* (Fig. 4). The slopes of

FIGURE 1. Absorbance at three wavelengths vs. concentration of phosphotungstovanadate

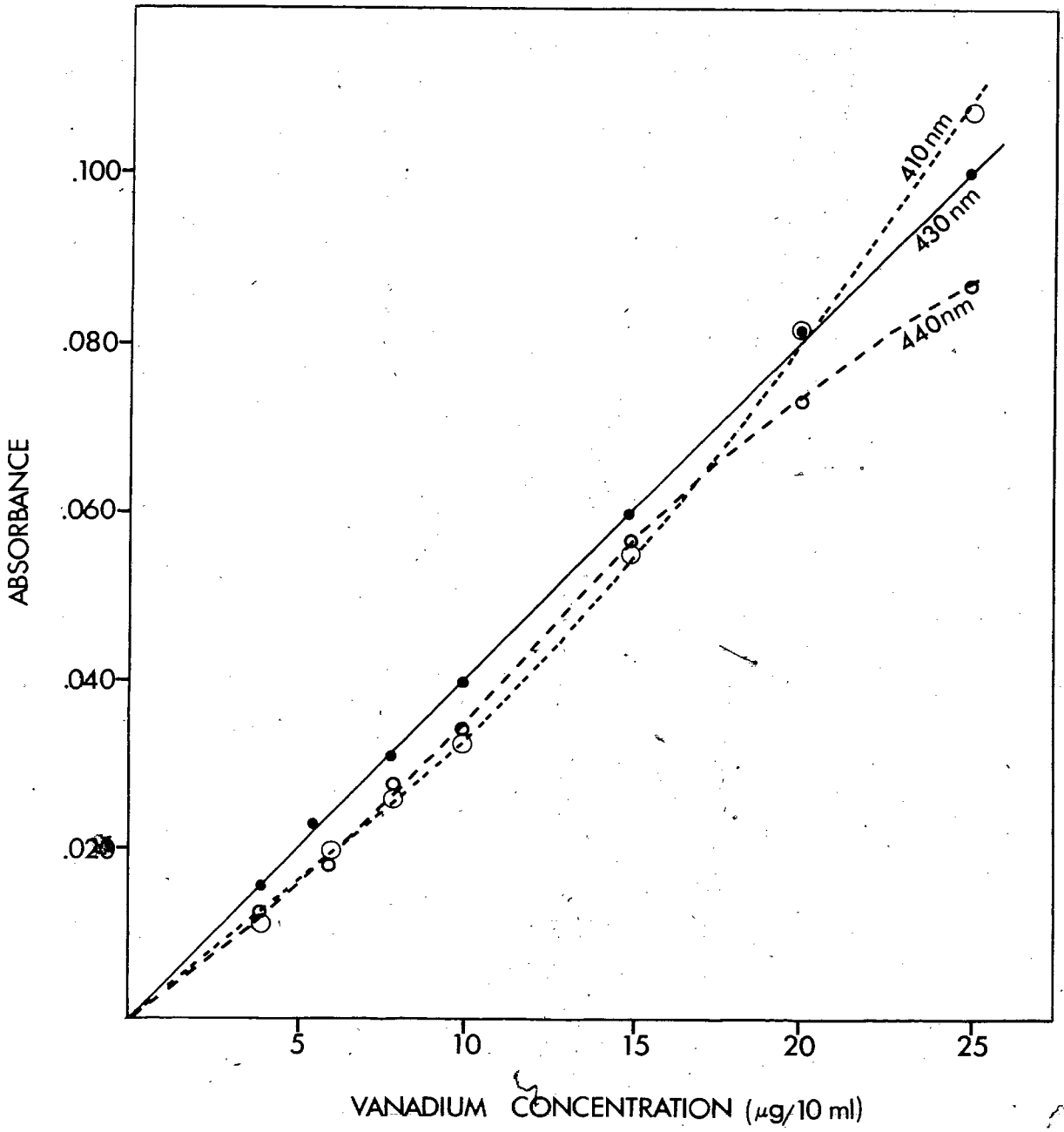

.100
.080
.060
.040
.020
ABSORBANCE
410nm
430nm
440nm
5
10
15
20
25
VANADIUM CONCENTRATION (μg/10 ml)

TABLE 1. Differences observed in the transmittance of phosphotungstovanadate at 430 nm with changes in the ratio of phosphoric acid to sodium tungstate[a]

| No. | 90% $H_3PO_4$ ml | 0.5 N $Na_2WO_4$ ml | Moles $H_3PO_4$ : Moles $Na_2WO_4$ | Transmittance Difference % |
|---|---|---|---|---|
| 1 | 0.15 | 0.5 | 9.9:1 | 2.26 |
| 2 | 0.20 | 0.5 | 13.2:1 | 1.26 |
| 3 | 0.25 | 0.5 | 16.5:1 | 0.63 |
| 4 | 0.30 | 0.5 | 19.0:1 | 0 |
| 5 | 0.35 | 0.5 | 22.7:1 | -0.38 |
| 6 | 0.40 | 0.5 | 26.4:1 | -1.00 |
| 7 | 0.30 | 0.3 | 33.0:1 | -2.26 |

[a]No. 4 taken as standard (Wright and Mellon, 1937). All solutions contain 25 $\mu$g vanadium, 1.00 ml of 50% nitric acid, and distilled water to 10.0 ml. Absorbance is read in a 1.00 cm cell.

FIGURE 2. The effect of the molar ratio of phosphate to tungstate on the absorbance at 430 nm of 25 $\mu$g vanadium as phosphotungstate.

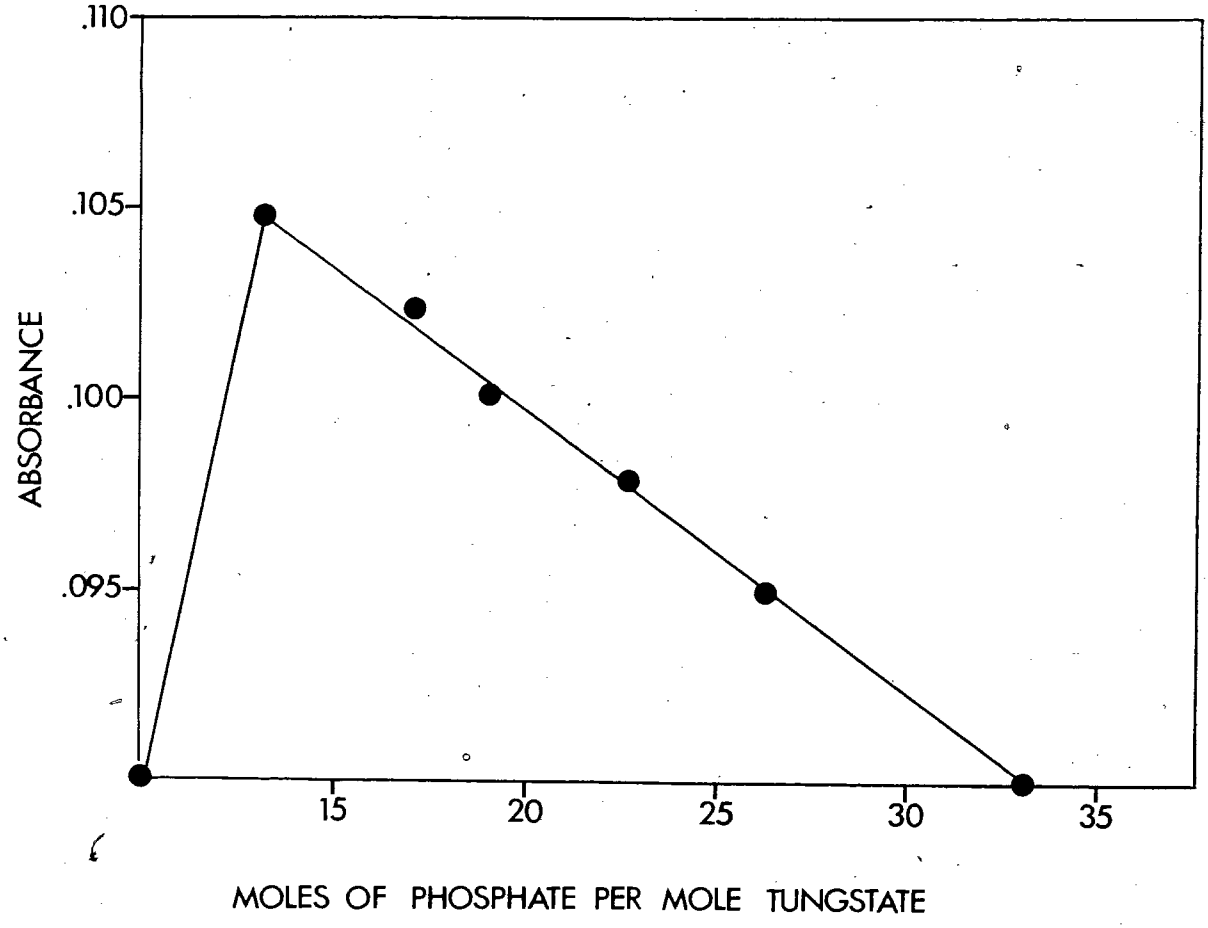

.110
.105
.100
.095
ABSORBANCE
15
20
25
30
35
MOLES OF PHOSPHATE PER MOLE TUNGSTATE

TABLE 2. The effect of boiling on the absorbance of phosphotungstate blanks containing Fe(III)[a]

| No. | Concentration of Fe(III) $\mu$g/10 ml | Absorbance at 430 nm |
|---|---|---|
| 1 | 0 | 0.000 |
| 2 | 10 | 0.000 |
| 3 | 20 | 0.000 |
| 4 | 40 | 0.000 |
| 5 | 60 | 0.001 |
| 6 | 120 | 0.000 |

[a]All solutions contain 1.00 ml 50% nitric acid and distilled water to give a total volume of 10.0 ml. Molar ratio $H_3PO_4$:$Na_2WO_4$ = 19:1. Absorbance values are the mean of 3 determinations.

TABLE 3. The effect of boiling on the absorbance of phosphotungstate blanks containing vanadium-free artificial seawater[a]

| No. | Vanadium-free seawater | Absorbance at 430 nm | |
|---|---|---|---|
| | ml | before boiling | after boiling |
| 1 | 0.0 | 0.000 | 0.000 |
| 2 | 2.0 | 0.004 | 0.000 |
| 3 | 4.0 | 0.007 | 0.000 |
| 4 | 5.7 | 0.010 | 0.000 |

[a]All solutions contain 1.00 ml 50% nitric acid and distilled water to produce a total volume of 10.0 ml. Molar ratio $H_3PO_4$:$Na_2WO_4$ = 19:1. Absorbance values are measured in a 1.00 cm cell, and are the mean of 3 determinations.

TABLE 4. The effect of boiling on the absorbance of phosphotungstovanadate standards containing vanadium-free artificial seawater[a]

| No. | Vanadium standard | Vanadium-free seawater | Absorbance at 430 nm | |
|---|---|---|---|---|
| | | | before boiling | after boiling |
| | $\mu$g V | ml | | |
| 1 | -- | 0.0 | 0.000 | 0.000 |
| 2 | -- | 5.7 | 0.010 | 0.000 |
| 3 | 10 | 0.0 | | 0.036 |
| 4 | 10 | 2.0 | | 0.036 |
| 5 | 10 | 4.0 | | 0.035 |
| 6 | 10 | 5.7 | | 0.035 |
| 7 | 25 | 0.0 | | 0.089 |
| 8 | 25 | 2.0 | | 0.089 |
| 9 | 25 | 4.0 | | 0.090 |
| 10 | 25 | 5.7 | | 0.089 |

[a]All solutions contain 1.00 ml 50% nitric acid and distilled water to produce a total volume of 10.0 ml. Molar ratio $H_3PO_4$:$Na_2WO_4$ = 19:1. Absorbance values are measured in a 1.00 cm cell.

TABLE 5. Corrections for colored residues present in a wet-ashed solution of Ciona intestinalis reacted with phosphotungstate [a]

| No. | Untreated digestion solution ml | Charcoal-treated digestion solution ml | Moles $H_3PO_4$: Moles $Na_2WO_4$ | Absorbance at 430 nm |
|---|---|---|---|---|
| 1 | --- | --- | 19:1 | 0.000 |
| 2 | 1.0 | --- | ---- | 0.005 |
| 3 | --- | 1.0 | ---- | 0.000 |
| 4 | 1.0 | --- | 19:1 | 0.025 |
| 5 | --- | 1.0 | 19:1 | 0.020 |

[a]Nos. 2 and 3 contained only distilled water to produce a total volume of 5.0 ml. Nos. 1, 4, and 5 contained phosphotungstate reagent, 0.5 ml 50% nitric acid and distilled water to produce a volume of 5.0 ml. All samples were boiled prior to measurement of absorbance at 430 nm in a 1.00 cm cell.

FIGURE 3. Absorbance vs. concentration of aqueous vanadate standard reacted with phosphotungstic acid. The limit of detection is approximately 0.25$\mu$g vanadium per 10 ml colorimetric preparation.

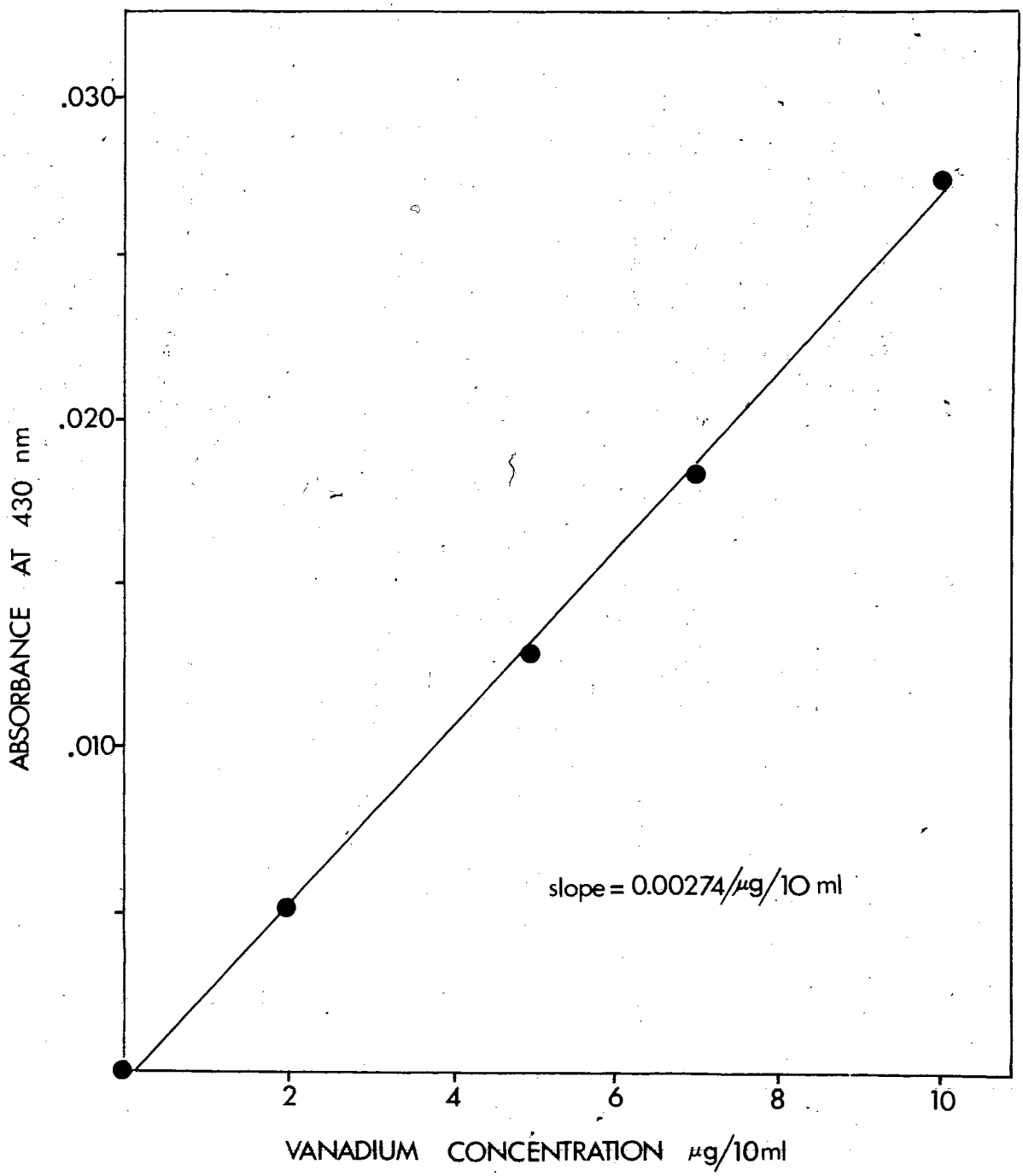
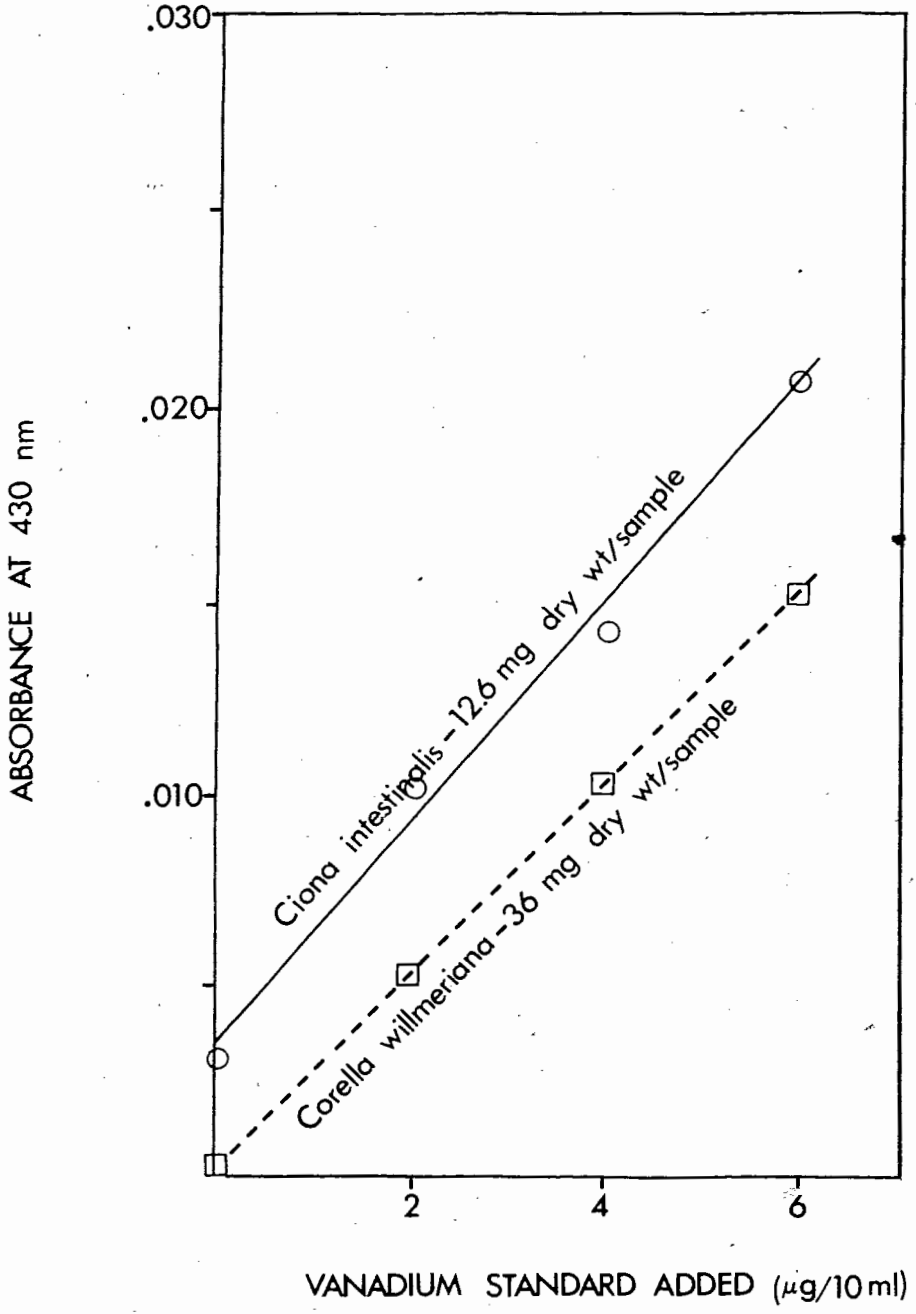

.030
.020
.010
ABSORBANCE AT 430 nm
slope = 0.00274/µg/10 ml
2
4
6
8
10
VANADIUM CONCENTRATION µg/10ml

FIGURE 4. Standard additions of vanadium as vanadate to digestion solutions of *Ciona intestinalis* and *Corella willmeriana*. Preparations are reacted with phosphotungstic acid.


.030
.020
.010
ABSORBANCE AT 430 nm
Ciona intestinalis - 12.6 mg dry wt/sample
Corella willmeriana - 36 mg dry wt/sample
2
4
6
VANADIUM STANDARD ADDED (μg/10 ml)

these two curves are compared simultaneously with the slope of the curve for aqueous standards (Table 6). There is no significant difference between the slopes of the three lines.

b. Vanadium content of Ciona intestinalis

There is a positive correlation between vanadium content and dry weight of the ascidian Ciona intestinalis (Table 7). Based on two samples of 32, collected in January and June 1976, the calculated correlation coefficients (r) are +0.94 and +0.84 respectively. The probability that either sample is derived from a population where ($\rho$) is 0.0 is less than 0.005 (Table 7) and the difference between the observed correlation coefficients is not significant at the 0.95 level.

The metal content shows a linear dependence upon weight (Figs. 5 and 6) and regression coefficients (b') which have the units of concentration ($\mu$g V/g dry weight) are calculated for the two samples individually (Table 8). The difference between the regression coefficients is not significant at the 0.95 level and a regression coefficient is calculated for pooled data (Fig. 7 and Table 8). As a second estimate of vanadium concentration, mean values are calculated for each sample from raw data, and are compared with F-test (Table 9). Based on these two estimates of the vanadium concentration of Ciona intestinalis, the metal content of an individual animal is given by:

$$V_C \simeq 90 W_C$$

where $V_C$ is the vanadium content in $\mu$g and $W_C$ is the dry weight of the specimen in g. This same value was obtained independently above (Table 6).

The mean vanadium concentration of winter and summer samples of Ciona intestinalis collected in the same location, are not significantly different at the 0.95 confidence level (Table 9).

TABLE 6. Comparison of the slope of the absorbance curve for aqueous vanadium standards to the slopes obtained for standard additions to *Ciona intestinalis* and *Corella willmeriana*

| Sample[a] | Regression coefficient[d] $\mu g^{-1}$ vanadium | Intercept |
|---|---|---|
| Aqueous standards | $2.74 \times 10^{-3}$ | $-3.4 \times 10^{-4}$ |
| *Ciona intestinalis*[b] | $2.81 \times 10^{-3}$ | $34.7 \times 10^{-4}$ [e] |
| *Corella willmeriana*[c] | $2.48 \times 10^{-3}$ | $3.0 \times 10^{-4}$ [f] |

[a]All samples for colorimetric determination contain 0.8 ml phosphotungstic acid (moles $H_3PO_4$:moles $Na_2WO_4$ = 19:1), 1.0 ml 50% nitric acid and distilled water to produce a volume of 10.0 ml. Solutions are boiled prior to determination of absorbance at 430 nm in 1.00 cm cells.

[b]Each sample contains 12.6 mg dry weight plus additions of 0, 2, 4, and 6 $\mu$g vanadium as ammonium vanadate.

[c]Each sample contains 36 mg dry weight, and additions of 0, 2, 4, and 6 $\mu$g vanadium as ammonium vanadate.

[d]The 3 regression coefficients are not significantly different at the 0.95 and 0.99 confidence levels ($F_S = 2.786 < F_{0.01}[2,7]$).

[e]By extrapolation to the X-axis, the vanadium present in the 12.6 mg sample alone is 1.2 $\mu$g (90 ppm).

[f]Vanadium content is not detectable.

TABLE 7. Correlation of the vanadium content and the dry weight of *Ciona intestinalis*[m]

| Sample | Date of collection | Correlation coefficient (r) |
|---|---|---|
| 1 | Jan. 1976 | +0.94 (n=32) |
| 2 | June 1976 | +0.84 (n=32) |

[m]The probability that either sample is derived from a population where ($\rho$) = $0 \ll 0.005$.

FIGURE 5. Vanadium content vs. dry weight of Ciona intestinalis collected January 1976.

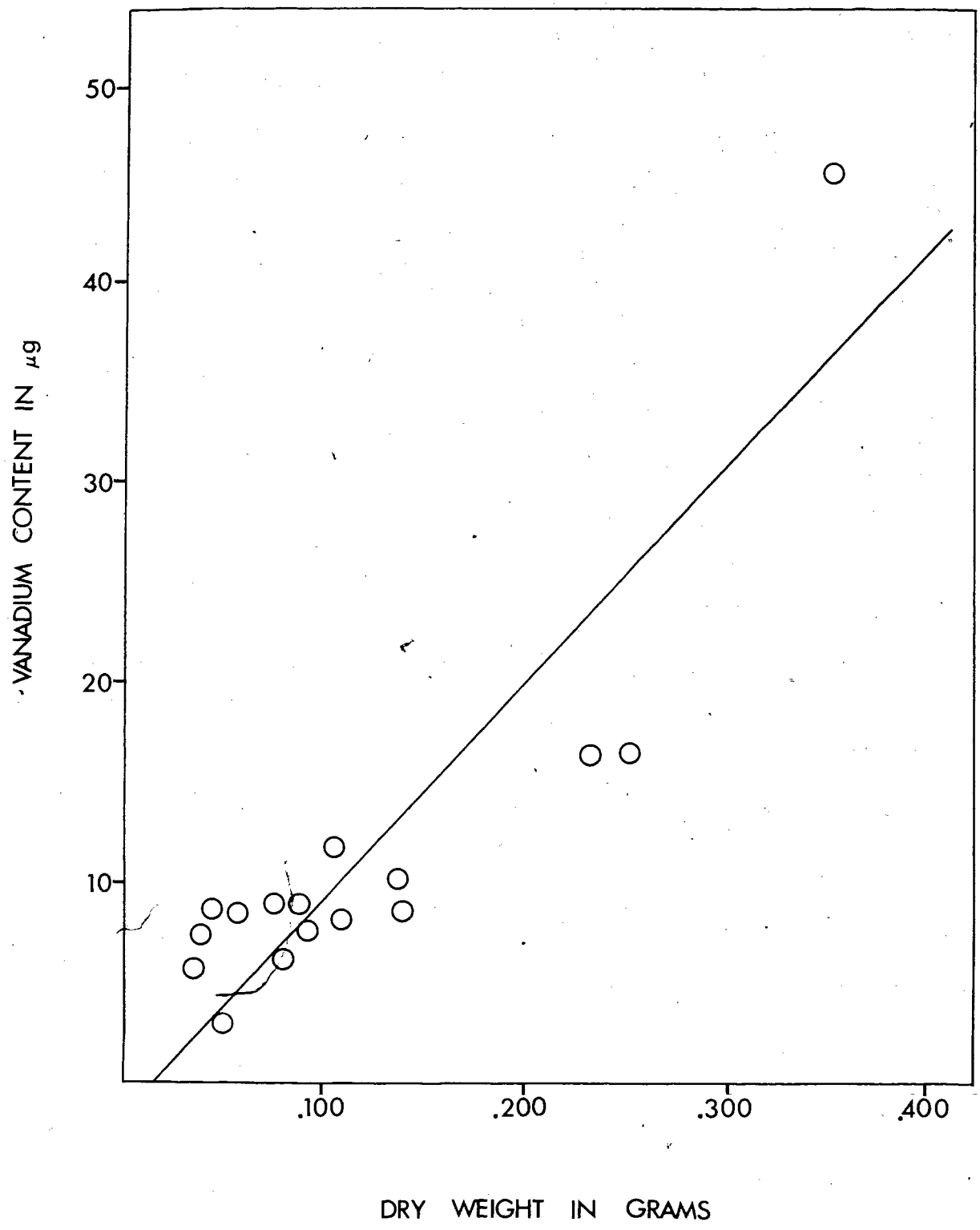

50
40
30
20
10
VANADIUM CONTENT IN μg
.100
.200
.300
.400
DRY WEIGHT IN GRAMS

FIGURE 6. Vanadium content vs. dry weight of Ciona intestinalis collected June 1976.

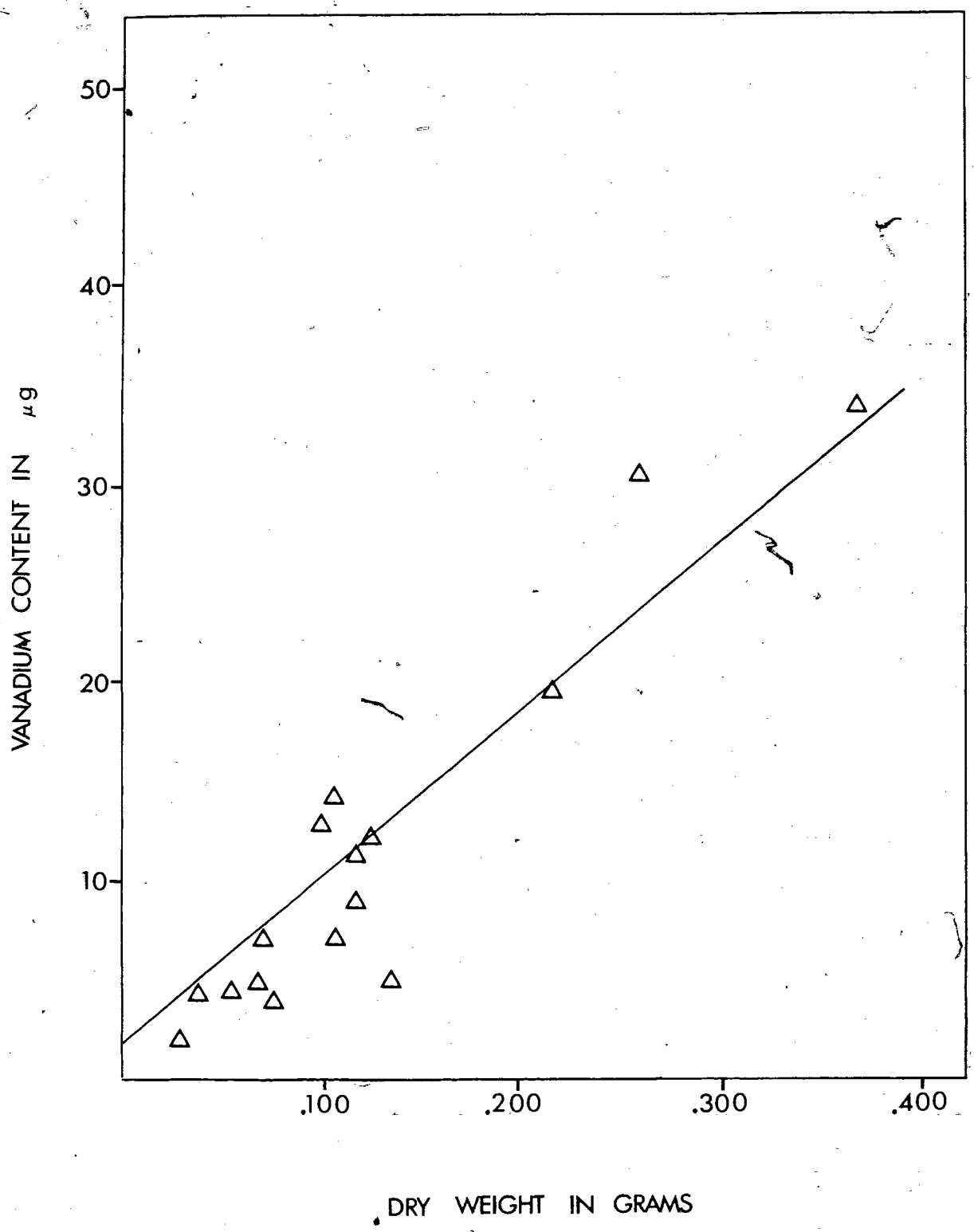

50
40
30
20
10
VANADIUM CONTENT IN µg
.100
.200
.300
.400
DRY WEIGHT IN GRAMS

FIGURE 7. Vanadium content vs. dry weight of Ciona intestinalis. January and June data are pooled. 95% confidence limits of Y values are calculated on X.

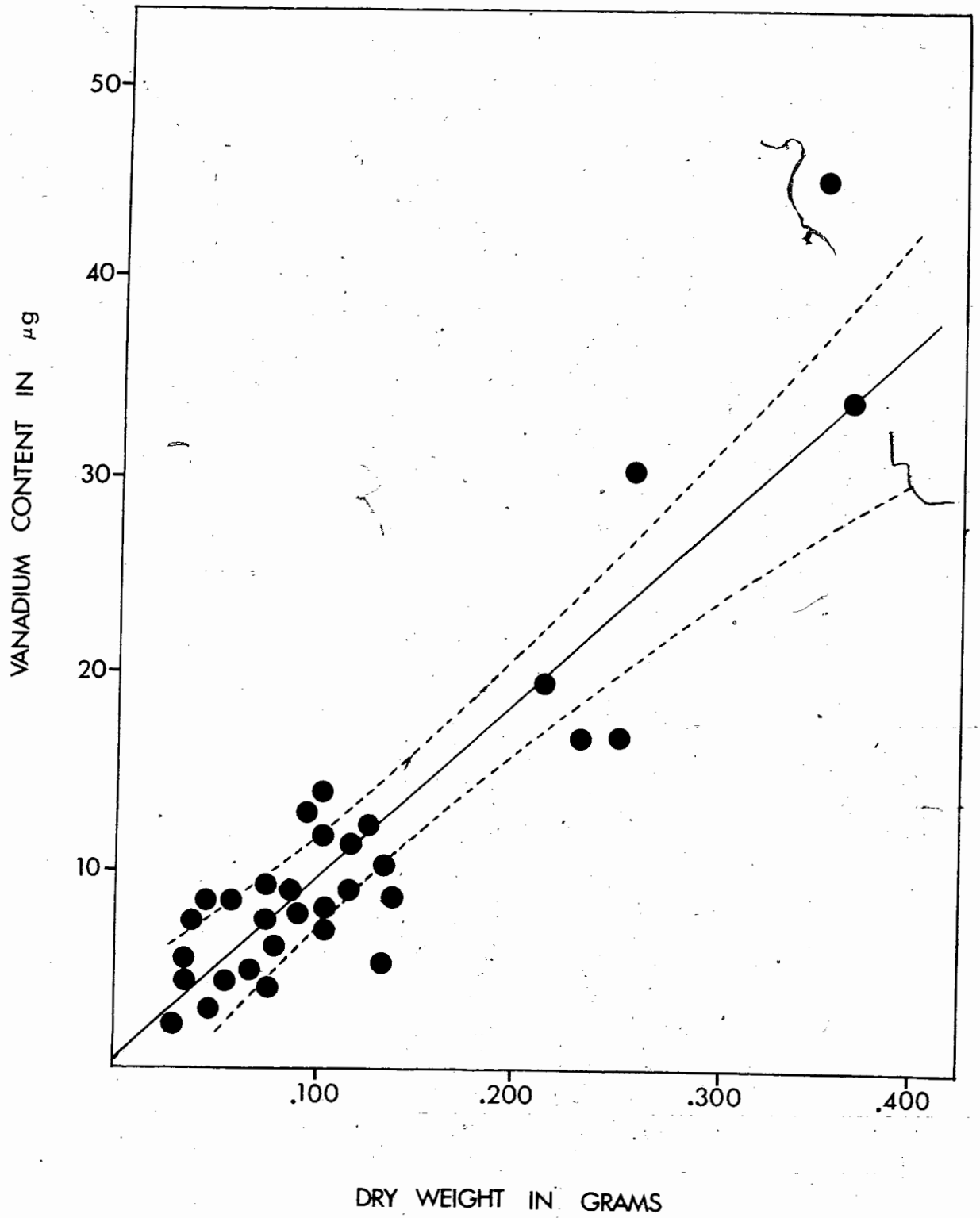

50
40
30
20
10
VANADIUM CONTENT IN μg
.100
.200
.300
.400
DRY WEIGHT IN GRAMS

TABLE 8. Vanadium content of Ciona intestinalis determined by linear regression analysis[a]

| Sample | Regression Coefficient ($b_n'$) ($\mu$g V/g dry weight) | Intercept ($a_n'$) ($\mu$g V) |
|---|---|---|
| Jan. 1976 | 102.1 | -1.5 |
| June 1976 | 83.4 | 1.7 |
| pooled data | 90.3 | 0.3 |

[a]The regression coefficient ($b_1'$) falls within the 0.95 confidence level of ($b_2'$) and vice versa.

TABLE 9. Mean vanadium concentration of Ciona intestinalis[a]

| Sample | Mean |
|---|---|
| Jan. 1976 | 82.1 (n=16) |
| June 1976 | 101.9 (n=16) |
| pooled data | 92.0 (n=32) |

[a]The difference between the means is not significant at the 0.95 confidence level: $F_S = 0.00718 \ll F_{.05}[1,14]$.

c. Vanadium concentration of local solitary ascidians

The vanadium concentration of ten species of solitary tunicates are reported (Table 10). The results are based on spectrophotometric determinations with phosphotungstic acid, and concentrations are expressed as parts per million (ppm) dry weight. Vanadium was found in Ascidia ceratodes, Ascidia paratropa, Boltenia villosa, Chelyosoma productum, Halocynthia igaboja, and Styela montereyensis, but was not detected in Cnemidocarpa finmarkiensis, Corella willmeriana, Halocynthia aurantium, or Pyura haustor.

d. Gravimetric determination of vanadium in Boltenia villosa

The gravimetric determination of vanadium described above is effective in separating vanadium from iron, and the recovery of vanadium is 89% of theoretical yield (Table 11). A sample of Boltenia villosa (0.025 g dry weight) was found to contain 520 ppm vanadium (13 $\mu$g). The same sample, determined spectrophotometrically with phosphotungstate (Table 10) contains 750 ppm vanadium.

2. Iron Determinations

Corella willmeriana, which contains no detectable vanadium (Fig. 4 and Table 10) was found to contain 430 ppm iron (Fig. 8) using colorimetry with thiocyanate.

TABLE 10. Vanadium concentration of local solitary ascidians[a]

| Specimen | Number of animals pooled | Vanadium concentration ppm dry weight | | | | |
|---|---|---|---|---|---|---|
| Order Phlebobranchia | | | | | | |
| Family Ascidiidae | | | | | | |
| Ascidia paratropa | (1) | 3750[b] | 850[c] | | | |
| Ascidia ceratodes | (2) | 1700[b] | 2250[c] | | | |
| Ascidia ceratodes | (1) | 4500 | 1300[d] | | | |
| Family Corellidae | | | | | | |
| Corella willmeriana | (2) | n.d. | n.d. | n.d. | n.d. | n.d. |
| Corella willmeriana | (10) | n.d. | | | | |
| Chelyosoma productum | (8) | 800 | | | | |
| Order Stolidobranchia | | | | | | |
| Family Pyuridae | | | | | | |
| Pyura haustor | (1) | n.d. | | | | |
| Boltenia villosa | (5) | 750 | | | | |
| Halocynthia igaboja | (1) | 175 | | | | |
| Halocynthia aurantium | (1) | n.d.[b] | | | | |
| Family Styelidae | | | | | | |
| Styela montereyensis | (2) | 40 | 36 | 0[e] | | |
| Cnemidocarpa finmarkiensis | (1) | n.d. | | | | |

[a]Spectrophotometric determination using phosphotungstic acid

[b]Blood and body fluids only

[c]Tissues with fluids drained

[d]Swinehart et al., 1974. Based on weight of animal plus test

[e]Goldberg et al., 1951. Based on weight of animal plus test

n.d. not detectable (<0.5 $\mu$g V/10 ml phosphotungstate preparation)

TABLE 11. Vanadium concentration of Boltenia villosa determined gravimetrically and spectrophotometrically

| Sample | Vanadium recovered | Vanadium concentration ppm dry weight |
|---|---|---|
| 25 $\mu$g V, 500 $\mu$g Fe | 23 $\mu$g (89%) | |
| 500 $\mu$g Fe | n.d. | |
| Boltenia villosa[a] | 13 $\mu$g[b] | 520, (750[c]) |

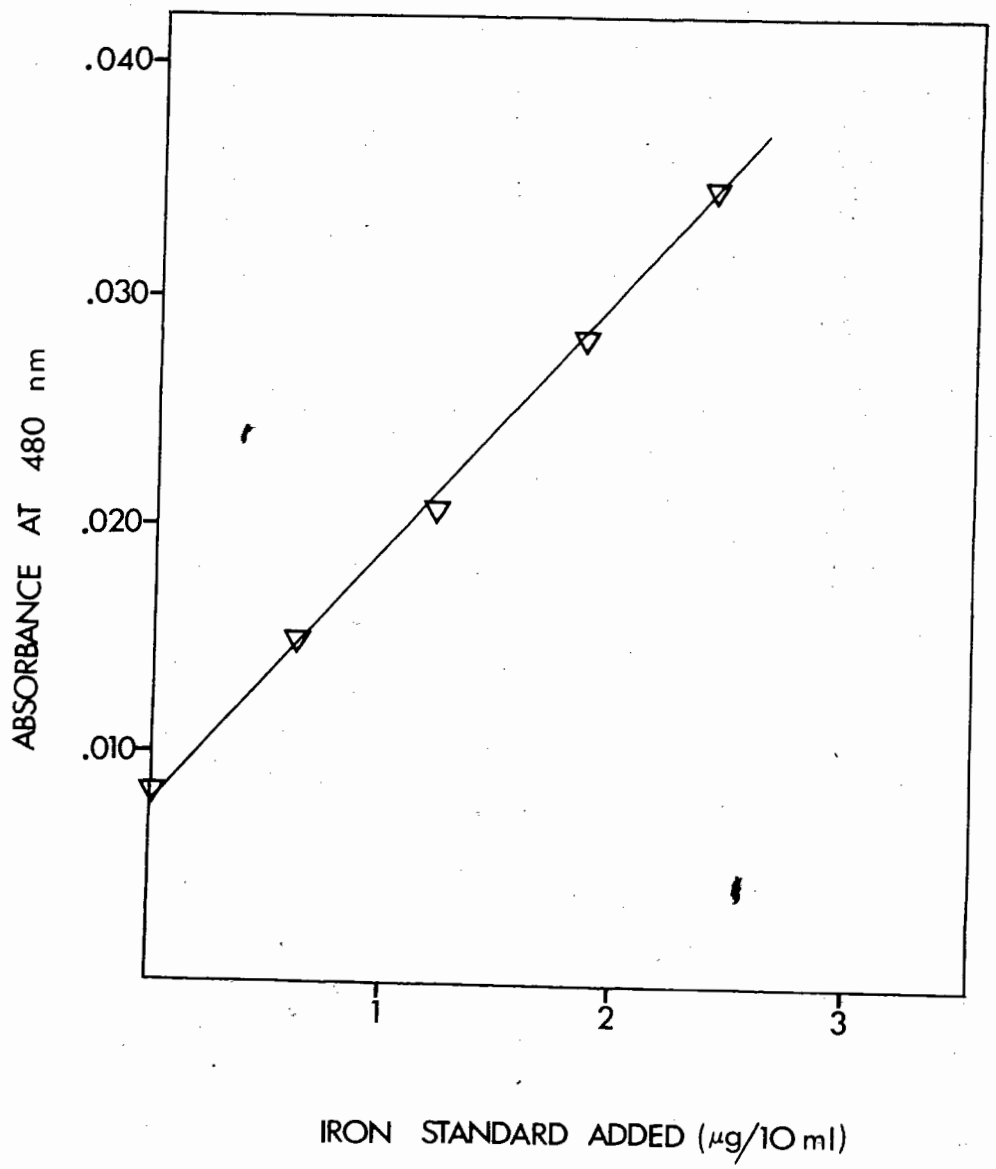
[a] 0.025 g dry weight

[b] assuming 89% recovery

[c] determined colorimetrically (Table 10)

n.d. no detectable product obtained

FIGURE 8. Standard additions of iron as ferric chloride to digestion solution of Corella willmeriana. Preparations are reacted with thiocyanate.


.040
.030
.020
.010
ABSORBANCE AT 480 nm
1
2
3
IRON STANDARD ADDED (µg/10 ml)

## Discussion

Since the accumulation of vanadium by the tunicates was first reported (Henze, 1911), a variety of analytical procedures have been used to determine vanadium levels in various species. Among these techniques is carbon arc spectrography (Azéma and Pied, 1930), which was subsequently shown to give erroneous results because of vanadium impurities in the graphite electrodes (Webb, 1939). Bertrand (1942) recommended the use of cupferron as a colorimetric reagent for assaying vanadium in tunicates and has reported copious data using this procedure (Bertrand, 1950). In preliminary examinations of the cupferron technique, the author found that reproducibility is virtually impossible to achieve for a variety of reasons. The volatility of the solvent used to extract the colored vanadium complex introduces error due to solvent loss. Suspended water droplets cause clouding to the solution, and attempts to dry the organic phase with drying agents such as calcium chloride introduce fine, suspended particles. Filtration to remove the calcium chloride only exacerbates the loss of solvent. In addition, it was observed that upon standing, the reagent and solvent alone develop an interfering, colored substance. Furthermore, cupferron is not specific for vanadium, but reacts with iron as well. Therefore, it is entirely possible that determinations of vanadium reported in the past (Bertrand, 1950) are in part determinations of iron, since it was relatively recently that iron was first detected in tunicates (Endean, 1955a).

In view of the uncertainty of data derived from either spectrographic or cupferron determinations, a need was perceived for further investigation of the vanadium levels in ascidians, using a technique for which reliability could be demonstrated explicitly. Several workers

report success using phosphotungstic acid (Wright and Mellon, 1937) as a colorimetric reagent for vanadium in tunicates (Webb, 1939; Ciereszko et al., 1963; Swinehart et al., 1974), and therefore this technique was assessed for its sensitivity and specificity. Optimum experimental conditions were determined and are reported below.

Because the absorbance of the yellow phosphotungstovanadate complex measured at 430 nm observes Beer's law (Fig. 1), the vanadium content of unknowns can be inferred by comparison to known standards. Since the intensity of color developed is relatively insensitive to large changes in the molar ratio of reagents used (Table 1), small errors in the volume of reagents used will not affect the measured absorbance values. As long as experimental solutions containing solubilized tunicate and phosphotungstate reagent are boiled briefly, the iron content of tunicates (Endean, 1955a, 1955b and 1955c; Smith, 1970; Swinehart et al., 1974) does not interfere with vanadium determinations (Table 2). Similarly, the unavoidable inclusion of small volumes of seawater with tunicate specimens produces no interference as long as the solutions are heated to 100°C (Tables 3 and 4). Absorbance due to organic residues in digestion solutions can be corrected for by subtracting the absorbance of the preparation, suitably diluted, from the absorbance of the preparation reacted with phosphotungstate (Table 5). Because aqueous phosphotungstovanadate standards (Fig. 3) yield an absorbance curve having the same slope (Table 6) as tunicate preparations to which vanadate standards are added (Fig. 4), there is no enhancement or suppression of the colorimetric reaction due to matrix substances (Christian and Feldman, 1970) introduced as ascidian tissue. Consequently, it can be shown mathematically that comparison of an experimental preparation to aqueous standards yields the same results as does standard additions, and therefore values obtained are absolute rather than

relative determinations. Regarding sensitivity of the determinations, an absorbance value of 0.0005 corresponds to a vanadium concentration of 0.25 μg V as phosphotungstovanadate/10 ml (Fig 3). From these preliminary investigations, it was concluded that assays for vanadium with phosphotungstic acid were specific, sensitive, and suffered no interferences that could not be corrected for.

Prior to this investigation, no one has explored the possibility of a relationship between vanadium content and size of the ascidian accumulating the metal. It is observed in this study that the vanadium content of Ciona intestinalis shows a linear dependence upon the dry weight of the animal minus test (Fig.7;Tables 7 and 8), and that the content of an individual is estimated by the equation above. The proportionality constant in this equation, a concentration term equal to 90 μg V/g dry weight is in agreement with previous studies of pooled specimens which were found to contain 100 μg V/g dry weight (Goldberg et al., 1951; Rummel et al., 1966). With this concentration value available and known to be applicable regardless of size class, it is possible to estimate the vanadium content of individual specimens of Ciona intestinalis living under natural conditions. Therefore in laboratory studies in which the animal is cultured in artificial seawater depleted in vanadium, it would be possible to ascertain whether the ascidian is still capable of accumulating the metal to normal levels, and if not, whether any type of deficiency symptoms are manifested. Such studies would assist in determining whether vanadium is an essential element for those species which accumulate it to such high levels. Rats require traces of vanadium for normal growth (Milne, 1971), but whether ascidians which accumulate the metal actually require it, remains to be demonstrated.

In the past it has been noted that smaller weight classes take up

radiovanadium faster than do larger classes (Bielig et al., 1961a and 1961b). At first this observation might suggest that smaller animals should therefore contain proportionately more vanadium than larger ones, resulting in a non-linear relationship between metal content and dry weight. However, it is known that the ascidians "turn over" rather than store all vanadium taken up during their lifespan (Kustin et al., 1975). Therefore, the relationship observed in this study would imply that the smaller animals have a faster turnover rate than do larger ones in order to account for the linearity observed.

Bertrand (1950) and Levine (1962) have speculated that vanadium is preconcentrated by the plankton upon which the ascidians feed and that seasonal differences in the composition of plankton may result in changes in the vanadium levels of the tunicates as well. However, when Ciona intestinalis was sampled in the same location in summer and in winter, there was no significant difference in the metal concentration (Tables 8 and 9). This lack of seasonality is perhaps not surprising since vanadium uptake is regulated by the organism rather than by its availability in the medium (Kustin et al., 1975; McLeod et al., 1975). Not until the supply of vanadium fell so far below its normal level of 0.3 to 3.0 ppb in the sea (Burton, 1966; McLeod et al., 1975) that availability rather than efficiency of uptake and assimilation became limiting, could seasonal change in vanadium supply affect the concentration levels observed in the ascidians.

Regarding the distribution of vanadium-concentrating species within class Ascidiacea, Webb(1939) concluded that the assemblage of families in which vanadium occurs, corresponds to the order Phlebobranchia, and he predicted that phlebobranchs for which no data were available would contain vanadium as well. As a means of evaluating this hypothesis, local solitary ascidians were collected and analyzed for their vanadium concentrations

(Table 10). This study reports for the first time a member of the order Phlebobranchia, Corella willmeriana,which contains no detectable vanadium; therefore, the taxonomic position of a particular species is unreliable in predicting whether it accumulates vanadium. Prior to this investigation, the lowest vanadium concentration found in a phlebobranch was 100 ppm in Ciona intestinalis (Goldberg et al., 1951; Rummel et al., 1966).

According to Webb (1939), the ancestral ascidian which gave rise to the present day orders Stolidobranchia and Aplousobranchia lost the ability to accumulate vanadium, a facility which he considered to be primitive. Nevertheless, in this study, the stolidobranch Boltenia villosa was found to contain 520 - 750 ppm vanadium (Table 11). Comparable high levels previously reported are restricted to two families of the order Phlebobranchia, the Ascidiidae and Perophoridae (Goodbody, 1974). In the past, relatively low vanadium concentrations have been reported in two stolidobranchs: Distomus varialosus (Gartner) with 131 ppm (Bertrand, 1950) and Molgula manhattensis (DeKay) with 100 ppm (Carlisle, 1958). The reliability of Bertrand's (1942) work is questionable as discussed above, while more recent studies of M. manhattensis report less than 20 ppm vanadium (Swinehart et al., 1974). Boltenia villosa appears singular as a stolidobranch which contains a substantial vanadium concentration.

Webb (1939), concluding that vanadium accumulation was restricted to the order Phlebobranchia, considered it unlikely that cells as specialized as vanadocytes would have arisen more than once within the group. However, it has been shown that vanadium accumulation occurs in some aplousobranchs (Goldberg et al., 1951; Levine, 1962; Ciereszko et al., 1963; Swinehart et al., 1974) and consequently Millar (1966), in his modern evolutionary treatment of the ascidians, affords a position of systematic isolation to certain aplousobranch genera such as Euherdmania, partially on the

strength of their ability to concentrate vanadium. The adaptation to different metals or to different oxidation states of vanadium (Endean, 1955; Kokubu and Hidaka, 1965; Smith, 1970; Swinehart et al., 1974; Kustin et al., 1976) has led Swinehart et al. (1974) to hypothesize that vanadium concentrations, and more specifically, vanadium oxidation states follow taxonomic patterns.

The observation that the phlebobranch, Corella willmeriana, contains no detectable vanadium while the stolidobranch Boltenia villosa does, contradicts the putative theories proposed by Webb (1939) and Swinehart et al. (1974). Rather than being restricted to taxonomic groups, it would appear that the ability to accumulate vanadium has arisen (or alternately, has been lost) a number of times within the class Ascidiacea, and that within families there is no consistency in the distribution of vanadium (Table 10). Levine (1962) has suggested that this switch from one metal to another may simply be the result of relatively small genetic changes.

From the scattered distribution of a variety of metals and oxidation states of these metals, it appears that the metal component of ascidian blood pigments cannot be used to reconstruct the evolution of the class.

## Summary

1. Colorimetry with phosphotungstic acid is shown to be a specific and sensitive procedure for the determination of microgram quantities of vanadium in the ascidians.

2. There is a linear relationship between the vanadium content and the dry weight of Ciona intestinalis, and this relationship allows estimation of vanadium contents of individuals from the field.

3. There is no evidence of seasonal differences in the vanadium concentration of Ciona intestinalis.

4. The ability to accumulate vanadium occurs sporadically throughout the class Ascidiacea, and consequently the presence or absence of the metal cannot be used to reconstruct the evolution of the group.

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# CURRICULUM VITAE

| | |
|---|---|
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