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ACKNOWLEDGMENTS

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Abstract

A high concentration of physiologically inactive manganese has been found in the gill tissues of freshwater bivalve molluscs. It has been suggested that this manganese, existing in a non-ionic form, may be in complex with organic material, possibly protein or it might exist as an oxide.

Attempts have been made biochemically to verify this hypothesis. Results obtained *in vitro* do not support the existence of a manganese protein complex. The manganese, when extracted, is in the trivalent state while pyrophosphate is found in the same extract. A manganese pyrophosphate complex *in vivo* would explain the observed properties of the system.

Résumé

Le tissu branchial des mollusques d'eau douce possède une quantité élevée de manganèse métaboliquement inactif. On a suggéré que ce manganèse pourrait être associé à des composés organiques, probablement des protéines, ou encore, pourrait exister sous forme d'oxide.

Des expériences furent faites pour vérifier ces hypothèses. Les résultats "in vitro" ne prouvent pas l'existence d'un complexe protéine-manganèse. L'extraction révèle la présence de manganèse trivalent et de pyrophosphate. Ce complexe pyrophosphate-manganèse expliquerait les résultats obtenus "in vivo".

Introduction

The trace element manganese was recognized as a common constituent of tissues of organisms in the early studies of the nineteenth century biological chemists. The idea that manganese was involved in biological oxidation and reduction processes arose from the discoveries by Griffiths (1892) of 'pinnaglobin', a manganese containing respiratory globulin from the Mollusc *Pinna* and by Bertrand (1897) of a plant enzyme, laccase which required manganese for its functioning. Both of these discoveries were proved wrong (Henze 1926, Suto 1938 for pinnaglobin and Suminokura 1930 for laccase). However interest in studies of manganese in the normal physiology of organisms was aroused.

Addition of manganese stimulates the growth of a wide variety of plants (Bertrand 1912). In 1936, Wilgus *et al* showed that perosis, a characteristic bone defect, was produced in fowl on a diet deficient in manganese. Klein and Ziese (1935) demonstrated *in vitro* that manganese was involved in activation of arginase and in 1936 Edlbacher and Zeller showed that the addition of manganese would induce arginolytic activity in the kidney tissues of the dove. Since then the role of manganese in living organisms, and, in particular, its association with enzyme systems has been extensively investigated.

Manganese can replace magnesium in virtually all systems where magnesium acts as activator of the enzymes which catalyse the transfer of phosphate from adenosine triphosphate to a phosphate acceptor or from a phosphorylated compound to adenosine diphosphate (Vallee 1960).

Manganese can also substitute for the zinc atom in the enzyme leucine aminopeptidase (Spackman et al 1955). Manganese is one of the metallic ions which can function as reactivators in certain inhibited enzyme systems (Coleman et al 1960). Despite the evidence of its importance in enzyme systems, there is little information on its actual mode of functioning. In 1955 Kenten et al showed that manganese undergoes a direct oxidative reaction in a biological system. Hochster et al (1952), found that manganese dioxide may be used as an artificial electron acceptor to reduce cytochrome c, thus replacing cytochrome oxidase. Halperin et al (1954) showed that anaerobic oxidation of cholic acid also involves the oxidation and reduction of manganese.

Some organisms accumulate large amounts of manganese which have no obvious physiological function. In 1907 and 1910, Bradley showed that large amounts of manganese were present in the tissues of fresh water bivalve molluscs of the family Unionidae. Dubuisson et al (1930), working with Anodonta cygnea confirmed his findings. The gills have the highest manganese concentration of all the body tissues. In 1949, Bowen used a radioactive isotope, Mn-56, to follow the metabolic pathway of the manganese. He found that the high concentration of manganese in the gills was physiologically inactive and called it non-metabolized manganese. He observed that little of this manganese was dialysable even over a long period. Using buffers of various pH values, he found that acidic solutions would dissolve the manganese more readily than neutral or alkaline solutions. Incubating homogenized gill tissues with a proteolytic enzyme, trypsin, in neutral phosphate buffer failed to liberate manganese into solution. He concluded that the major portion of the non-metabolized manganese in the gill tissues exists as an inorganic oxide of manganese, probably of high oxidative state.

In 1934, Bureau reported that the manganese in the gill tissues could be stained histochemically. A color complex of manganese is formed with sodium dihydrogen phosphate in the presence of ammonia. The development of color takes at least two days to complete. This led him to postulate that the existence of manganese in non-ionic form, possibly as a manganese-protein complex, might account for its strange behavior.

The intention of my investigation was to examine the manganese-protein complex hypothesis advanced by Bureau,as well as to study Bowen's conclusions. Instead of taking a histochemical approach, attempts have been made to isolate biochemically the manganese complexes or compounds, using a variety of extraction and fractionation procedures. It was hoped that the chemical identity of such complexes or compounds obtained <u>in vitro</u> would indicate the state of combination of the manganese <u>in vivo</u>.

Materials and Methods

A. Experimental animal

Fresh water clams, Elliptio complanatus (Solander),(Clarke 1959) were collected from Britannia Bay in the Ottawa River and kept alive in running tap water without feeding throughout the entire period of investigation. This species is the most abundant of the local Unionidae. After an initial survey of the total manganese content of each tissue, only the gills were used for further studies.

B. Qualitative and quantitative determinations

(1) Wet ashing and total manganese

After removing the shells, the animal was dissected into: gills, mantles, foot, adductor muscles, digestive gland and visceral mass. The wet weight of each tissue was recorded. Organic materials were destroyed by 'wet ashing'. Each tissue was placed in a 125 ml Erlenmyer boiling flask which was warmed over a hot plate. Ten ml of concentrated sulphuric acid was added to dissolve the tissue. Vigorous heating was used with occasional dropwise addition of concentrated nitric acid to the solution. When the solution turned clear, the flask was cooled. About 5 ml of distilled water was added and heating was resumed until white fumes of sulphur trioxide were driven off. The flask was again cooled and the solution transferred to a volumetric flask (100 ml) and made up to the mark with distilled water. Aliquot portions were taken for analysis of manganese.

Determination of manganese was made using a modified version of the method of Sandell (1950). Manganese in solution was oxidized to the colored permanganate ion. To each aliquot of the sample solution, one gram of potassium persulfate was added and the flask was heated until the white fumes of sulfur trioxide were given off. The solution was cooled and 75 ml of distilled water were added. The solution was heated to the boiling point. Three ml of saturated phosphoric acid was added followed by 0.3 gram of potassium periodate. The mixture was maintained at or near 100°C for half an hour to allow complete conversion to permanganate. Then the solution was cooled and diluted to 100 ml with distilled water. The color intensity was measured at 525 mμ in a Beckman DU Spectrophotometer. A blank was prepared by replacing the sample solution with 3 ml of distilled water. A standard curve was prepared using different dilutions of a potassium permanganate solution, standardized volumetrically against arsenic trioxide.

(2) Ionic manganese

A qualitative test of Vogel (1960) for manganous ion in solution was used. In the presence of ammonium sulphide solution, a pink precipitate of manganous sulfide is obtained. On boiling, the manganous sulfide turns green as it becomes less hydrated. Manganous chloride is used as a control. Ethylene-diamine-tetra-acetic acid (EDTA) which interferes with the test was first removed by precipitating it with acetic acid.

(3) Protein

An equal volume of 30% TCA (Trichloroacetic acid) was added to an aliquot of the test solution. The mixture was agitated briefly with a test tube shaker then left to stand for 20 minutes before the precipitate was collected by centrifugation at 20,000 x g for 5 minutes.

The precipitate was then dissolved in 0.1 N NaOH and an aliquot of this solution was taken for protein determination. The biuret method (Berkman et al 1957) was adopted. Crystalline bovine serum albumin was used as a standard.

(4) Phosphates

The acid molybdate-sulphonic method for inorganic ortho-phosphate of King (1932) was used. Inorganic orthophosphate, acidified with perchloric acid, will form an intensely blue colored complex in the presence of ammonium molybdate and amino-naphtholsulphonic acid. Quantitative measurement was made by means of a Beckman DU Spectrophotometer at 660 mμ. Sodium dihydrogen mono-phosphate was used as a standard. Phosphorous compounds separated by paper chromatography were determined as orthophosphate after wet ashing the appropriate spots.

(5) Ultraviolet absorption

Ultraviolet absorption spectra of solutions were measured by means of a Beckman DU Spectrophotometer. Quartz cuvettes of 5 mm light path and 1.5 ml volume were used. In each case, 0.1 ml of the solution diluted to 1.5 ml with distilled water was measured unless otherwise stated. An appropriate blank was used with each sample.

C. Extraction and fractionation procedures

(1) Lipids

The extraction of lipids was done only with gill tissues. Three pairs of gills were finely minced and were treated successively with 100 times their volume of absolute acetone for one hour at 4°C; a 1:1

mixture of acetone and diethyl ether for one hour at 4°C; and pure ether for two hours at room temperature. A magnetic stirrer provided continuous agitation during the extraction. At the end of each extraction the residue was allowed to settle. The supernatants were decanted and saved for manganese determination. Each portion of extract was evaporated to dryness and redissolved in a small portion of ether for 'wet ashing' and subsequent manganese determination.

(2) Using protein solvents

In a typical extraction, 1.5 grams wet weight of gills were homogenized and extracted with 20 ml of an extractant for 24 hours at room temperature. A drop of toluene was added to prevent bacterial growth. The supernatant was separated by centrifugation for 15 minutes at 20,000 x g and was used for salt fractionation.

Fractionation of the extract was done by adding a protein precipitant (e.g. $(NH_4)_2SO_4$) to an aliquot of the supernatant, with stirring provided by a magnetic stirrer, until precipitation occurred. After every precipitation, the precipitate was removed by centrifugation, and addition of precipitant was repeated. The precipitates were tested for protein and manganese.

(3) EDTA (Ethylene-diamine-tetra-acetic acid)

Following the failure of various protein and fat solvents to extract significant amounts of manganese from the gill tissues, another technique was required. Ethylene-diamine-tetra-acetic acid has been widely used in analytical chemistry, particularly for extracting traces of metals and removing metallic ions in solution from biological systems (e.g. enzyme systems). As an extractant, it will bring into solution both individual ions and complexes of metals plus other organic or inorganic groups (West et al 1960). As far as I can determine EDTA has never been described as a solvent for proteins. Most protein solvents, particularly for enzyme extraction, are low salt-concentration buffers or distilled water. That EDTA has not been used as a protein extractant did not rule it out as a potential solvent for a metalloprotein complex. Many proteins form strong complexes with heavy metal ions such as zinc, manganese, barium, calcium, mercury, iron and copper. In 1950, Cohn et al, in a systematic separation of protein components of human plasma, succeeded in bringing insoluble metalloprotein into solution by means of EDTA.

Whole pairs of gills were extracted with 0.48M (10%) EDTA, neutralized to pH 7 with NaOH, for 24 hours at room temperature. The supernatant obtained, after removing the residues, was called the 'crude extract'.

D. Paper chromatography

The solvent systems of Janke et al (1958) was adopted to separate ortho- and pyrophosphates. It contains methanol: isopropanol: ammonia: water (45:35:15:10 v/v). Whatman number 4 paper, 12" x 6", prewashed with 2N hydrochloric acid was used. Single dimension descending chromatograms were run at room temperature for periods of 15 to 30 hours depending on the distance of separation of the elements.

E. Dialysis

18/32 mm Visking dialysis tubing was used. The two ends of the tubing were tied and the dialysis was carried out at room temperature.

One drop of toluene was added to the material to be dialysed to prevent bacterial growth.

F. Radioactive isotope technique

(1) Counting

The isotope used was Mn-54, which emits a gamma ray of 0.84 Mev and has a half life of 314 days (Wilson 1966). It was purchased 'carrier free' in hydrochloric acid solution from New England Nuclear Company, Boston. Solutions for injection were made up in 0.01M neutral sodium acetate buffer shortly before use.

Samples to be counted were placed in half inch diameter glass tubes. A maximum of 5 ml of material was counted in each tube. The counter consisted of a 2 inch diameter sodium iodide well crystal, connected via a preamplification stage to a single channel radiation analyser and scaler (Nuclear Chicago model DS-202, model 1810 and type 8166 respectively). The system was set up to accept all energies above 30 Kev. A standard sample was prepared for each experiment and counted whenever samples were counted. This provided corrections for instrument variations and radioactive decay. Values obtained were an average of several separate counts on the same sample.

(2) Radioautography of chromatograms

Kodak no screen X-ray film, 8" x 10", was used. The procedure for radioautography outlined by Chase (1960) was followed. To protect the film from chemical attack by materials in the chromatogram, a sheet of thin plastic was inserted between the film and the chromatogram. The exposure time was one to two months.

G. Manganic complexes (manganese (111))

(1) Manganipyrophosphate complex

The soluble complex of manganese (111) was prepared according to the method of Kenten et al (1955). Manganous sulfate, when oxidized by manganese dioxide in the presence of 0.1 M sodium pyrophosphate (pH 7), will form a colored manganic complex. This reaction takes 18 hours at room temperature.

(2) Manganese (111)-EDTA complex

A complex of EDTA with trivalent manganese was prepared according to Yoshino et al (1961). When manganese dioxide, suspended in distilled water, is vigorously mixed with 2 gram of ethylene-diamine-tetra-acetic acid (free acid form) by means of a magnetic stirrer at room temperature, a purple red soluble complex of manganese (111)-EDTA is formed. The reaction is completed within half an hour. Insoluble manganese dioxide left over is removed by filtering. The filtrate had a pH of 2.7.

Results

1. Total manganese content of tissues

Table 1 (p. 12) shows the manganese content of the tissues of *Elliptio complanatus*. As expected, the gills contain the highest concentration of manganese. The manganese in the gills represents about 75-80 per cent of the total manganese in a typical animal.

Local winter conditions interfere with collection of specimens for 6 months of the year, hence the experimental animals were maintained in the laboratory for long periods. A group of 14 animals kept for 7 days was compared with a group of 20 animals kept for 6 months. The differences in tissue manganese concentrations were not significant even at the 90% level, when compared using the 't' test (Bailey 1959).

2. Dialysis of minced gill tissues

Gill tissues were homogenized with small portions of distilled water and were dialysed against 600 ml of various solvent media at room temperature. After the period of dialysis the dialysis media were evaporated almost to dryness, then these solutions and the whole contents of the dialysis bags were wet ashed and total manganese determined. The results (Table 2, p. 13) show that most of the manganese did not pass through the dialysis bag.

3. Extraction from homogenized gill tissues

Table 3 (p. 14) shows qualitatively the amount of manganese extracted with a number of solutions in which protein was soluble.

Table 1

Manganese in tissues of E. complanatus six months after collection. Twenty animals used.

Organs	mean weight of tissue/ animal in grams	mean content of manganese/ animal in mg	mean content of manganese/gram tissue in mg with 95% confidence range
Gill	1.75	3.76	2.17 ± 0.343
Mantle	1.53	0.75	0.47 ± 0.098
Adductor muscle	1.23	0.03	0.02 ± 0.012
Foot	2.47	0.17	0.06 ± 0.025
Visceral mass	0.83	0.06	0.08 ± 0.033
Digestive gland	0.57	0.03	0.04 ± 0.084

Table 2

Effect of 28 hours dialysis on the manganese in homogenized gill tissues

Wet weight gill tissues in gram	medium of dialysis	Mn inside dialysis bag (mg)	Mn outside dialysis bag (mg)	Total Mn recovered mg
1.200	distilled water	1.63	0.10	1.73
1.109	0.9M NaCl	0.97	0.07	1.04
1.208	0.9M KCl	1.98	0.08	2.06
1.146	0.9M $MgCl_2$	1.82	0.07	1.89

Table 3

Manganese content of homogenized gill tissue extracts, following extraction for 24 hours at room temperature.

Solvents	distilled water	5% NaCl	5% KCl	5% $MgCl_2$	0.05N Na_2HPO_4	0.05N HCl	1N H_2SO_4
Manganese content	*	**	*	*	*	**	***

*	very small amount
**	small amount
***	moderate amount

Aliquots of each extract were precipitated by the addition of protein precipitants: sulfates of ammonium, sodium, magnesium or zinc. It was found that all the precipitates obtained gave a positive test for protein but none contained detectable manganese.

The amount of manganese extracted with 1 N H_2SO_4 was 40% of the total manganese in the tissues. When whole gill tissues were used instead of homogenized tissues, 1% of the manganese was extracted with 1N H_2SO_4 and none was extracted by the other solutions.

4. Lipid extraction

The initial extraction of minced gill tissues with acetone removed most of the pigments and fatty materials from the gill tissues. The extract was brownish in color. The subsequent two extractions with a mixture of acetone and ether and ether alone removed the last trace of brownish colored pigments from the gill tissues. None of these extracts contained manganese.

5. EDTA extract of whole gill tissues

(a) Efficiency of EDTA as extracting solvent

A preliminary study showed that 10% EDTA at pH 7 would extract much more manganese from the tissues than would 1N H_2SO_4. Subsequently, it was found that all of the manganese could be extracted from whole gill tissues as well as from homogenized ones. The effectiveness of neutral 10% EDTA as an extractant is shown in figure 1 (p. 16). Aliquots of each EDTA solution were taken periodically and analysed. At alkaline pH, EDTA loses its effectiveness.

Mn-54 was also used to check the extraction method. About 1 uc

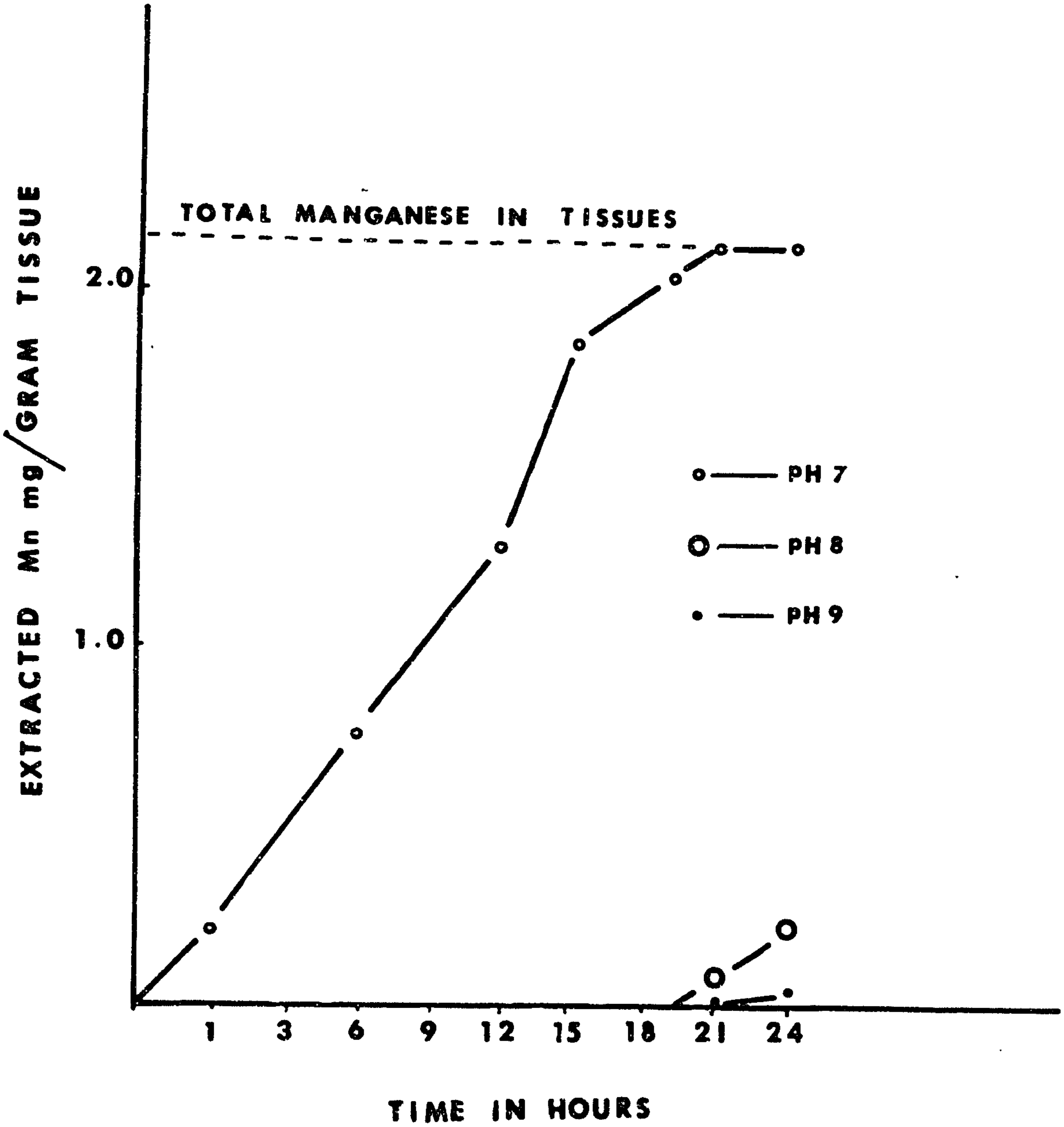

Fig. 1 PH EFFECT ON THE EXTRACTION OF MANGANESE FROM WHOLE GILL TISSUES WITH 10% EDTA

of Mn-54 in 0.1 ml of pH 7 acetate buffer was injected into the visceral mass of each experimental animal via the muscular foot. About 7% of this Mn-54 became permanently bound in the gills. A sample of radioactive gill tissue was counted before and after 10% EDTA (pH 7) extraction:

counts per minute before extraction	-	18,832
counts per minute after extraction	-	963
Efficiency of extraction with 10% EDTA	-	94.8%

After precipitating the EDTA in the crude extract with acetic acid, no manganous ion could be detected in the supernatant. The presence of manganese in the supernatant could be detected only after wet ashing by the method designed for total manganese.

(b) Protein precipitation

The addition of sulfates of ammonium, sodium, magnesium and zinc to the crude EDTA extract did not produce any precipitate. When the extract was acidified to pH 4 with acetic or hydrochloric acid, a precipitate formed even when the above salts were not used. The precipitate obtained gave a positive test for protein with the biuret method, but a negative test for manganese.

Ten ml of crude extract from a Mn-54 treated gill tissues was counted before and after precipitation with 10 ml of 30% TCA (trichloroacetic acid):

counts per minute before precipitation	-	5,236
counts per minute after precipitation	-	4,923

This difference is probably within the limits of experimental error e.g. difficulty in complete washing of radioactivity from the precipitate or loss of solution during transfering processes.

(c) Ultraviolet absorption

Ultraviolet absorption spectra of the following solutions were recorded: pH 7 crude extract, protein free crude extract (protein precipitated at pH 4), and alkaline crude extract (re-adjusting the protein free crude extract to pH 9 with NaOH). It was found that the absorption maxima for the extract at the three pH's were at 280 mµ (figure 2 p. 19).

(d) Phosphates

Inorganic phosphates from the protein-free crude extract were separated by paper chromatography (p. 8). It was found that inorganic ortho- and pyrophosphates were the two main phosphates in the crude extract (plate 1, p. 20). Quantitative analysis gave a ratio of 4 orthophosphate groups to 1 pyro-phosphate. The pyrophosphate could be precipitated from the crude extract by zinc acetate crystals at pH 4 (West <u>et al</u> 1960). It was also found that the removal of pyrophosphate did not change the maximal ultraviolet absorption of the crude extract.

Protein was precipitated from the crude extract of radioactive gill tissues using 30% TCA, and zinc acetate crystals were used to precipitate the pyrophosphate. The Mn-54 activity was counted before and after these precipitations:

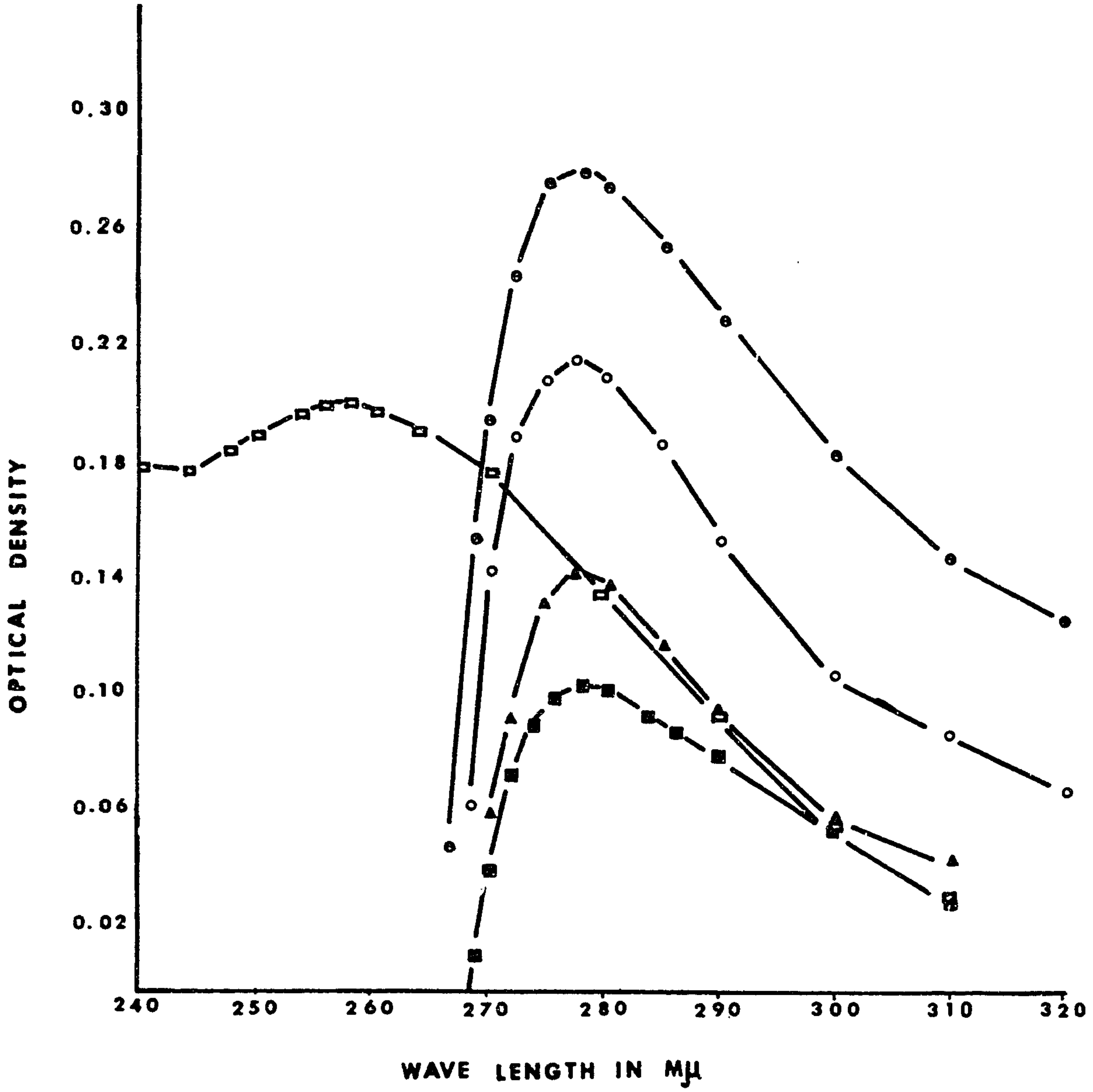

Fig. 2 LIGHT ABSORPTION SPECTRA OF CRUDE EXTRACT

(●—PH4 , ○— PH7, △ — PH9)

AND

0.1 M MANGANIPYROPHOSPHATE AT pH 7

(□—WITHOUT EDTA, ▣—WITH 0.05 ml EDTA)

Plate 1. Paper chromatography of crude extract

PPO_4 - 0.1 M sodium pyrophosphate
$MnPO_4$ - 0.1 M manganipyrophosphate
W.G.T.E. - whole gill tissue extract (crude extract)
PO_4 - 0.1 M sodium dihydrogen monophosphate
EDTA - 10% neutral ethylene-diamine-tetraacetic acid

With the exception of crude extract, 0.05 ml of 10% EDTA was included in the other spots before the run

Solvent system :	methanol :	isopropanol :	ammonia :	water
	45	35	15	10

Running time: 30 hours


PO4.
WHITE.
LOWPO4.
PPO4.
EDTA.

counts per minute of protein-free crude extract before pyrophosphate precipitation - 4,923

counts per minute of protein-free crude extract after pyrophosphate precipitation - 4,146

The difference in counts is too great to be explained by experimental error. A tentative conclusion is that some of the manganese is in complex with pyrophosphate. It had been shown that trivalent manganese will form a complex with pyrophosphate (Kenten _et_ _al_ 1955). The difference in counts would be expected to be minimal had the manganese existed as divalent ions which would be displaced by zinc (Irving _et_ _al_ 1953).

6. Model systems in comparison with the crude extract

(a) Ultraviolet absorption spectrum of manganipyrophosphate

The colored complex of manganipyrophosphate exhibits a maximum ultraviolet absorption at 258 mμ. In the presence of EDTA, the absorption maximum is shifted to 279 mμ as shown in figure 2,(p. 19)

(b) Ultraviolet absorption of manganese (111)-EDTA complex

The trivalent manganese (111)-EDTA complex formed at pH 2.7 was adjusted to pH 4, pH 7 and pH 9 with dilute NaOH and its ultraviolet spectrum was recorded. It was found that the ultraviolet absorption spectra of the manganese(111)-EDTA (figure 3, p. 22) are similar to those of the crude extract and that of the manganipyrophosphate in the presence of EDTA (figure 2, p. 19).

(c) Dialysis of crude extract

When the crude extract (pH 7) was dialysed against two changes of 600 ml of distilled water at room temperature for 24 hours, the

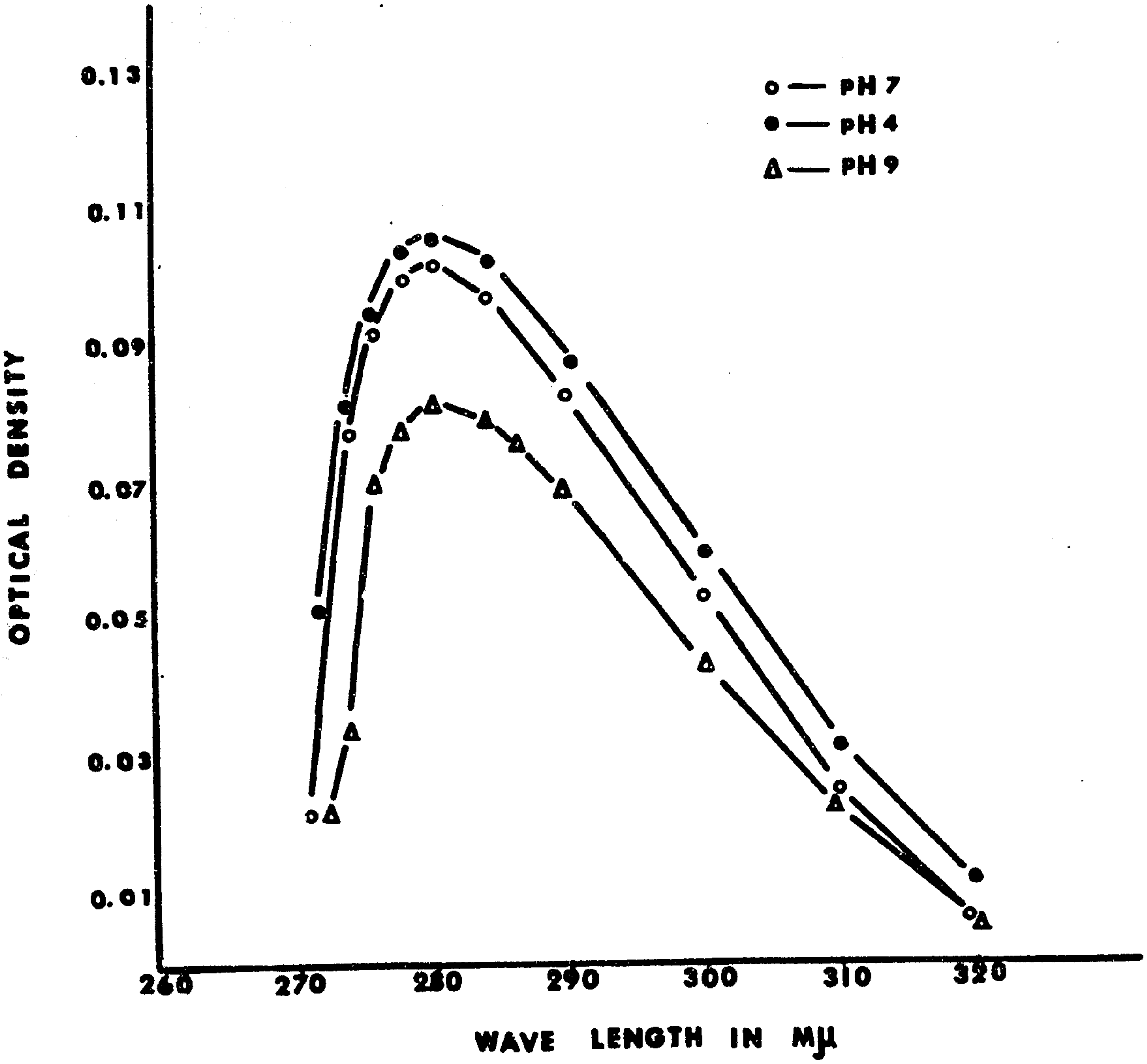

Fig.3 LIGHT ABSORPTION SPECTRA OF MANGANESE(III)-EDTA AT PH 4, PH 7 AND PH 9

manganese was found in the dialysis solution outside of the dialysis bag. This solution gave a negative test for manganous ion.

A solution of manganipyrophosphate was prepared with Mn-54 and divided into two portions. They were dialysed against distilled water as above mentioned.

One portion (sample A) had the following composition:

0.5 ml radioactive manganipyrophosphate pH 7
4.0 ml of 0.1M sodium pyrophosphate pH 7

another portion (sample B) had the following composition:

0.5 ml radioactive manganipyrophosphate pH 7
1.0 ml 10% EDTA at pH 7
3.0 ml of 0.1M sodium pyrophosphate pH 7

After being dialysed, the solutions inside the dialysis bags were counted:

		before dialysis	after dialysis
Sample A	-	26,395 cpm	23,153 cpm
Sample B	-	26,434 cpm	743 cpm

The difference in counts of sample A before and after dialysis is not significant and is, in part, due to changes in counting geometry of the counting solution volume i.e. increase in volume. While the Mn-54 activity in the manganipyrophosphate was found not dialysable, the presence of EDTA renders it dialysable as shown in sample B. The effect of EDTA may be causing the breakdown of the larger molecular weight molecule of manganipyrophosphate (mol. wt. 582.76) or a polymer of manganipyrophosphates (mol. wt. $>$ 10,000) into smaller molecular weight molecules of pyrophosphate (mol. wt. 186.94) and manganic ion

(54.93 mol. wt.) respectively. The manganic ion may then form a complex with EDTA (mol. wt. 342).

(d) Paper chromatography of the radioactive crude extract and subsequent radioautographic study

One ml of radioactive crude extract was concentrated to 0.5 ml and spotted on 8" x 12" Whatman number 4 paper. This solution gave roughly 500 cpm. A volume of 0.1 ml of radioactive manganipyrophosphate, with 0.05 ml of 10% EDTA added, was spotted along with the crude extract. This solution gave about 5,000 cpm. A control of 0.1 M sodium pyrophosphate (0.1 ml) was also included in the run. The chromatogram was subsequently radioautographed.

Plates 2a and 2b show the results (p. 25 and p. 26). The Rf values of both radioactive spots are the same. Mn-54 activity was found not associated with pyrophosphates or orthophosphate. Instead it was found migrating along with the EDTA spots.

To show further that trivalent manganese has a greater preference to form a complex with EDTA than with pyrophosphate, the following experiment was performed. Two 0.1 ml samples of radioactive manganipyrophosphate, about 5,000 cpm each, were spotted on chromatographic paper. In one spot, 0.05 ml of 10% EDTA was added and the paper was chromatographed and radioautographed. Plate 3a and 3b show the results (p. 27 and p. 28). It was found that the Mn-54 activity remained on the starting line at the spot where no EDTA was added and migrated along with the EDTA spot in the case where EDTA was added.

Plate 2a. Paper chromatography of radioactive crude extract and radioactive manganipyrophosphate

Solvent system methanol : isopropanol : ammonia : water

	methanol	:	isopropanol	:	ammonia	:	water
Solvent system	45		35		15		10

Running time: 15 hours

$MnPPO_4$ - manganipyrophosphate (0.1 M)
$MnPPO_4$* - radioactive manganipyrophosphate (0.1 M)
Ext. - crude extract of radioactive gill tissues with 10% EDTA pH 7

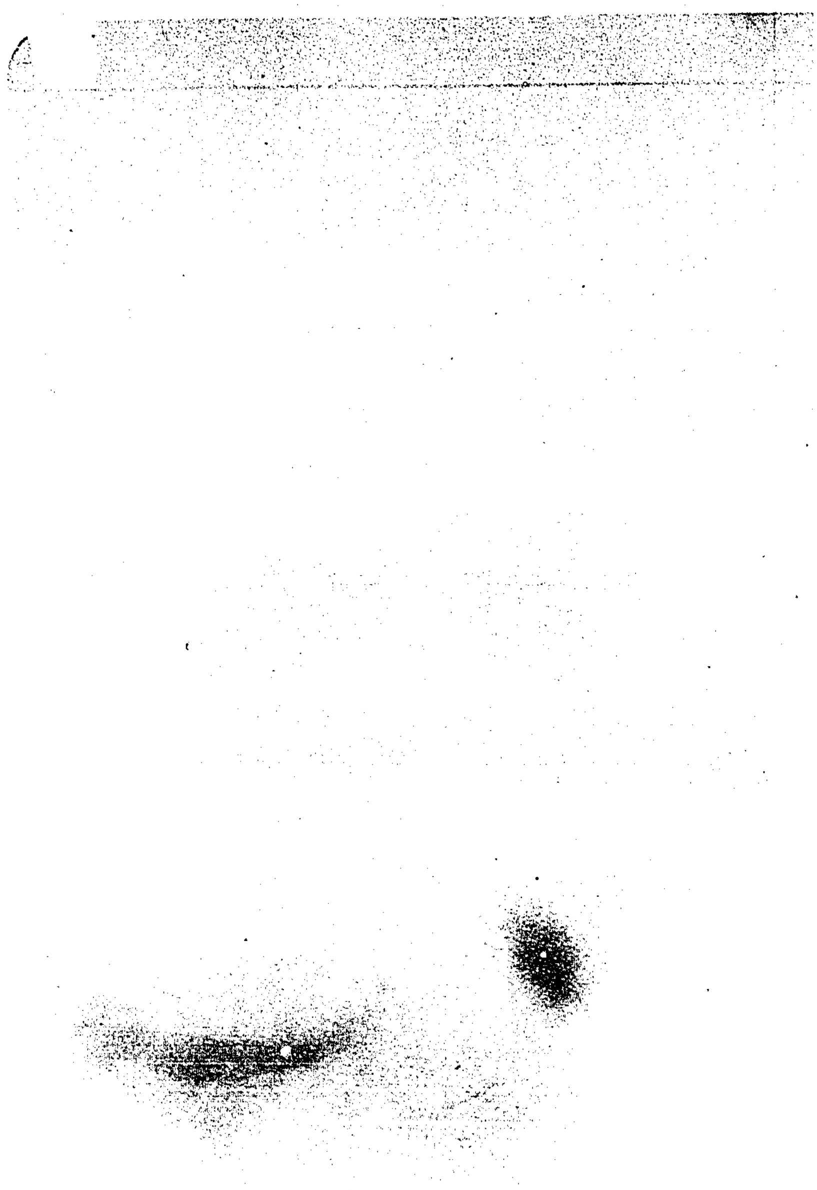

Plate 2b. Radioautography of chromatogram of plate 2a.

Film: Kodak no screen X-ray film

Exposure time: 2 months

Activity: crude extract (500 cpm)
manganipyrophosphate (5000 cpm)

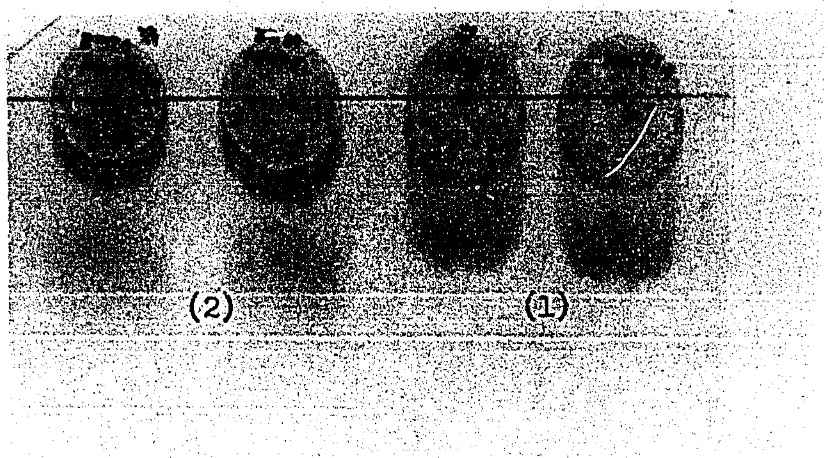

Plate 3a. Paper chromatography of radioactive manganipyrophosphate
(1) without EDTA
(2) with EDTA

solvent system : methanol : isopropanol : ammonia : water
45 35 15 10

Running time: 15 hours

$MnPPO_4$ - manganipyrophosphate (0.1 M)
$MnPPO_4$* - radioactive manganipyrophosphate (0.1 M)

Group (2) with 0.05 ml 0.48 M EDTA added.

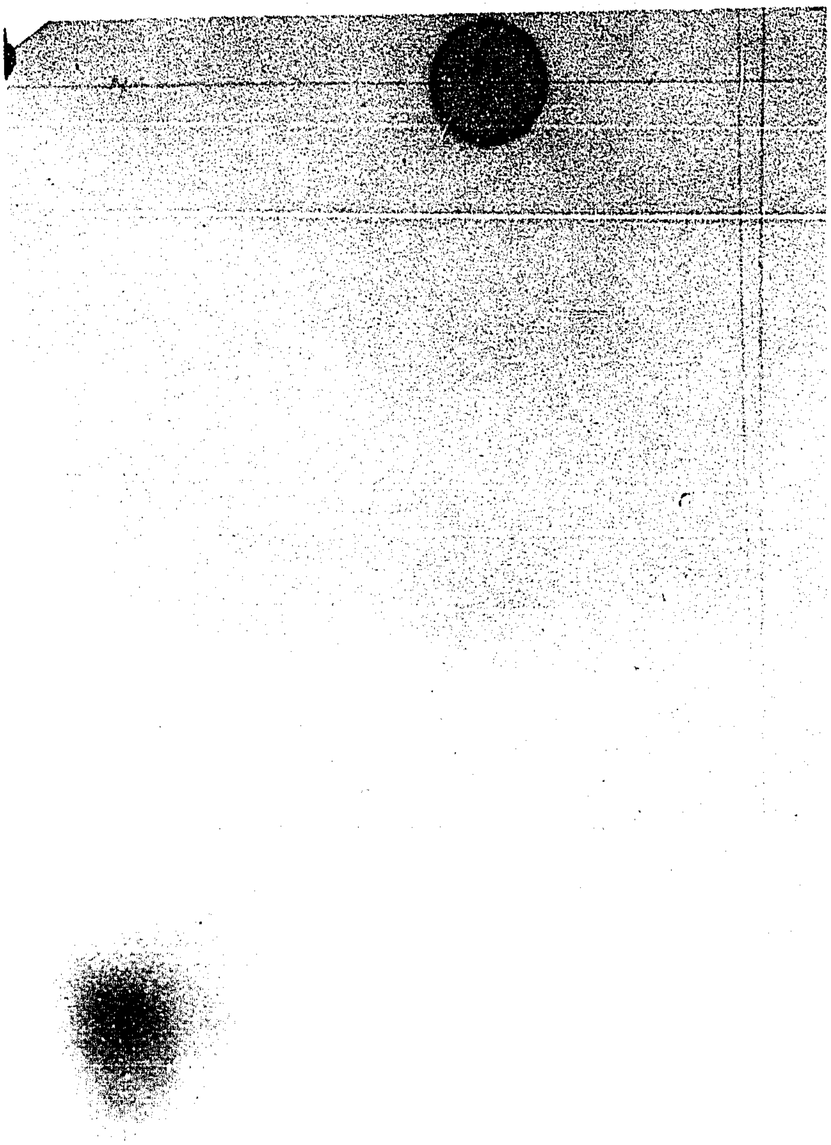

Plate 3b. Radioautography of chromatogram of plate 3a.

Film : Kodak no screen X-ray film

Exposure time : 2 months

Activity : Manganipyrophosphate (5000 cpm).

Discussion and Conclusion

Quantitative analysis of manganese in the gill tissues of Elliptio complanatus confirms other workers' findings (Bradley 1907 and Bowen 1949) that the highest concentration of manganese is in the gill tissues. The manganese content in the gills represents 75-80% of the total manganese in a typical animal. The isotope study suggests that the ionic Mn-54 initially incorporated must have been converted into some stable form of manganese, accounting for the constant level of radioactivity maintained in the gills. An average of 7% of the injected Mn-54 became bound to the gills and remained as such without decrease in activity after 91 days in non-radioactive water. The fact that the manganese of homogenized gill tissues was not 'dialysable' tends to confirm that it is in a stable complex. About 4% of the manganese was found in solution outside the dialysis bag when homogenized gill tissues were dialysed. The mono- or divalent ions sodium, potassium and magnesium did not induce appreciable exchange of the manganese.

Only a small amount of manganese is extracted by the various solutions which will dissolve protein material from homogenized gill tissues. Differential salt fractionation of these solutions showed that manganese is not associated with protein. The majority of the manganese in the gill tissues had not gone into solution during extraction. This is contrary to what one would expect had the manganese been in complex with protein. This argument also serves to dispel the idea that the manganese might be in complex with nucleic acid since the solvents used are nucleic acid solvents.

Results from the lipid solvent study indicate the absence of a complex of manganese with lipid or fatty material.

The use of 10% EDTA of neutral pH as an extractant in preparing a crude extract of gill tissue was prompted by the preliminary finding that it could extract much more manganese than other solutions from whole and homogenized gill tissues. The crude extract contains protein material which was found not to be in complex with manganese. Paper chromatographic studies identified two major phosphate groups in the protein free extract: ortho- and pyro- phosphates. The presence of inorganic pyrophosphate suggests that manganese in the crude extract might be in the form of a pyrophosphate complex. It has been shown that trivalent manganese ions are not stable but form stable complexes with complexing anions such as pyrophosphate. Manganipyrophosphate has the empirical formula $[Mn(H_2P_2O_7)_3]^{3}$ (Day et al 1960). The soluble manganipyrophosphate exhibits ultraviolet absorption maximally at 258 $m\mu$ (Kenten et al 1955). It was found that the manganipyrophosphate is not readily dialysable (p. 23). If the manganese in the gill tissues exists in some form of pyrophosphate, it would account for the small amount of dialysable manganese in homogenized gill tissues. Wazer (1958) showed that manganipyrophosphate complex found in living tissues is extremely insoluble in aqueous solution, including a great variety of salt solutions. If all the manganese exists as a manganipyrophosphate complex in the crude extract, this could explain the negative manganous test obtained.

The spectrophotometric studies of the crude extract do not indicate a manganipyrophosphate complex in the extract. At neutral pH, the crude extract absorbs ultraviolet light maximally at 279 $m\mu$ while manganipyrophosphate has a peak at 258 mμ. In the presence of EDTA, however, this absorption maximum is shifted to 279 mμ. This shifting suggests a structural change of the manganipyrophosphate. The change might be due to the dissociation of the manganipyrophosphate complex or due to the formation of a new complex of EDTA and manganese. It has been shown that trivalent manganese ions form a more stable complex with EDTA than they do with other ions (West et al 1960). The precipitation of pyrophosphate from the radioactive gill extract shows that the pyrophosphate in the crude extract is not in complex with manganese in the presence of EDTA.

Ultraviolet absorption spectra of a soluble complex of manganese (111)-EDTA are shown in figure 3 (p. 22). They are similar to those of the crude extract and the manganipyrophosphate, in the presence of EDTA. If the manganese from the gill tissues, when extracted, was in the form of manganese(111)-EDTA complex, it could account for the readily dialysable manganese in the crude extract. The fact that the manganese in the manganipyrophosphate is rendered dialysable in the presence of EDTA (p. 23) tends to confirm the above speculation. The fact that manganipyrophosphate is not dialysable suggests that it forms a complex of high molecular weight ($>$10,000). Paper chromatography of the crude extract and subsequent radioautography show that the manganese in the crude extract is in the form of manganese (111)-EDTA.

The idea that the manganese-EDTA complex in the crude extract might be a complex of divalent manganese instead of trivalent manganese is ruled out by the facts that firstly divalent manganese forms a complex with EDTA only in an alkaline medium (Přibil et al 1949) and secondly the light absorption maxima of EDTA complexes of divalent metallic ions lie in the range 220 mμ to 240 mμ (West et al 1960). The light absorption maxima of a manganese (11)-EDTA preparation, using manganese chloride, lay in the range 220 mμ to 250 mμ. The light absorption maxima of the crude extract is also not due to EDTA alone since the ultraviolet absorption maxima of EDTA lay in the range 250 mμ to 270 mμ (West et al 1960).

The trivalent state of the manganese in the gill tissues could explain the metabolically inactive nature of the metal as reported by Bowen (1949). In the trivalent state, the manganese could be in complex with inorganic pyrophosphate, forming an extremely insoluble manganipyrophosphate complex in vivo. It has been established from studies of soil nutrient elements required by plants that divalent manganese in excess of the plant's need is converted to higher oxides of manganese and extremely insoluble complexes of pyrophosphate (Bower et al 1940, Wain et al 1943, Heintze 1947 and Heintze and Mann 1949). Dubuisson et al (1930) and Bowen (1949) concluded that the non-metabolized manganese in the gills was in the form of physiologically inert inorganic oxides of higher valency. Its accumulation was due to the inability of the animal to develop an effective excretory mechanism to get rid of the manganese.

From direct experimental evidence presented in this investigation and from indirect evidence of the work cited above, it is concluded that the manganese, when extracted, is in the form of a manganese(111)-EDTA complex and by deduction is probably in the form of an inorganic pyorphosphate complex in vivo. The manganese-protein hypothesis is not valid.

It is felt that a combination of histochemical and radioautographic techniques to locate pyrophosphate and manganese would help reveal the exact nature of the manganese in the gill tissues *in vivo*. A study of the enzyme systems in these tissues may shed light on the physiological function of the manganese.

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