

Bacteriology of Manganese Nodules

I. Bacterial Action on Manganese in Nodule Enrichments

HENRY L. EHRLICH

Department of Biology, Rensselaer Polytechnic Institute, Troy, New York

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ABSTRACT

EHRLICH, H. L. (Rensselaer Polytechnic Institute, Troy, N.Y.). Bacteriology of manganese nodules. I. Bacterial action on manganese in nodule enrichments. *Appl. Microbiol.* **11**:15-19. 1963.—Bacteria, found in manganese nodules from the Atlantic Ocean, enhance the adsorption of Mn^{++} from sea water by crushed manganese nodules in the presence of peptone. When bacterial outgrowth from crushed manganese nodules was experimentally delayed, peptone did not enhance Mn^{++} adsorption by nodular substance, but hindered it in some cases. A mechanism to explain the role of bacteria in enhancing Mn^{++} adsorption by manganese nodules is presented. Oyster shells were shown to adsorb Mn^{++} in the absence of bacteria. Peptone did not enhance the rate of Mn^{++} adsorption. Adsorbed Mn^{++} was not visibly oxidized during experimental observation. These results suggest one way whereby nodule formation may be initiated in the oceans. Some bacteria in the nodules were found to release manganese from them in the presence of glucose and peptone. Bacteria may, therefore, play a role not only in nodule buildup but also in nodule breakdown.

Manganese nodules are mineral concretions, rich in manganese and iron oxides. They were first discovered and described by the Challenger Expedition (Murray, 1891). They appear to be unique to marine environments. They have been found on the bottom of all oceans, except the Mediterranean and the Red Sea, at depths of at least 65 fathoms and as great as 3,125 fathoms. The nodule sizes may range from fractions of a millimeter to 20 cm in diameter. They may contain as much as 63% Mn as MnO_2 . These and other characteristics have been discussed by Dietz (1955).

Almost as soon as manganese nodules were discovered, theories regarding their origin were proposed. Early theories by Murray, Guembel, Buchanan, and Dieulafoy have been discussed by Murray (1891). Although the individual explanations of these workers differed in detail, they all assumed that a primary oxidation of manganous manganese forming MnO_2 occurred in the sea, followed by agglomeration of the MnO_2 into nodules. The process seems to have been visualized by all as purely physico-

chemical. In 1935, a biochemical theory was offered by Dorff (cited by Goldberg, 1961) without attracting much attention from oceanographers. The idea of a biochemical mechanism for nodule genesis was revived by Graham (1959) and Graham and Cooper (1959). Even as late as 1961, doubt prevailed among oceanographers that any kind of biological agent could be directly involved in manganese-nodule formation (Goldberg, 1961).

After personal discussion with J. W. Graham of the Woods Hole Oceanographic Institution in 1960 about the possibility of a biochemical origin of manganese nodules, I examined nodule specimens for the presence of microorganisms in and on them, and for the action by such organisms on manganese. This paper will describe my attempts to demonstrate, by enrichment culture, the presence of bacteria in manganese nodules from the Atlantic Ocean, and a study of the action of these enrichment cultures on manganese dissolved in sea water.

MATERIALS AND METHODS

Collection and storage of manganese nodules. All specimens used in this investigation were kindly furnished by J. W. Graham of the Woods Hole Oceanographic Institution, Woods Hole, Mass. Some were obtained by dredging at different locations in the Atlantic during several oceanographic cruises. In bringing specimens to the surface, no extraordinary precautions were taken with respect to surface contamination. Once raised, they were individually wrapped in polyethylene bags and stored in a deep-freeze. Frozen specimens, packed in dry ice, were sent by air-freight from Woods Hole to Troy, N.Y. Unfrozen specimens were shipped by parcel post, without regard to drying or surface contamination. All specimens used for work in this paper were immediately and separately stored in a deep-freeze, unwrapped or in polyethylene bags, in clean nonsterile beakers.

Preparation of nodules for experimentation. Because of the lack of asepsis in collecting and storing the nodule specimens, all or portions of them were surface-sterilized by boiling completely submersed in distilled water for 30 min in a 600-ml beaker, covered with a watch glass. A control experiment demonstrated that 1.0×10^8 *Bacillus subtilis* spores in 150 ml of distilled water were inactivated in less than 5 min by this procedure. During boiling of nodule fragments, a rapid evolution of gas, probably air,

was observed, indicating a high degree of porosity of the material. After boiling, the water was removed by decantation. The lump of nodule was then transferred to an autoclaved porcelain mortar (outer diam, 150 mm) in an inoculating hood, which had been presterilized for at least 30 min by six General Electric germicidal lamps. The lump was pulverized by pounding in the mortar with a sterile pestle. The pulverized material was transferred to a sterile 125-ml Erlenmeyer flask, using an alcohol-flamed, stainless-steel spatula.

Bacterial enrichments from crushed manganese nodule in sea water. To determine whether bacteria that might be present in manganese nodules could cause Mn^{++} to be incorporated into nodular material, equal amounts (1 to 2 g) of aseptically crushed nodule substance were weighed into pairs of sterile, 125-ml Erlenmeyer flasks. To one of the flasks, 18 ml of autoclaved, natural sea water and 2 ml of sterile 0.2 M $MnSO_4$ solution were added. To the other flask, 18 ml of autoclaved, sterile, natural sea water and 2 ml of sterile 0.2 M $MnSO_4$ solution containing 1% peptone were added. The contents of each flask were mixed, particulate matter allowed to settle, and 0.2 ml of the supernatant fluid analyzed in duplicate for manganese (immediately and after daily intervals). At the same time that Mn was assayed, loopfuls of supernatant fluid were examined in wet mounts for the presence of bacteria by dark M phase-contrast microscopy (American Optical Co., Buffalo, N.Y.). Incubation of enrichments took place at 25 ± 1 C.

Effect of glucose on nodule enrichments. To follow the effect of glucose on the manganese level in the supernatant fluids of nodule enrichments, equal amounts (1 to 2 g) of aseptically crushed nodular material were weighed into pairs of 125-ml Erlenmeyer flasks. To one of a pair of flasks, 18 ml of sterile natural sea water, 1 ml of sterile 0.2 M $MnSO_4$ solution, and 1 ml of sterile 10% glucose solution were added. To the other flask, 18 ml of sterile natural sea water, 1 ml of sterile 0.2 M $MnSO_4$ containing 1% peptone, and 1 ml of sterile 10% glucose solution were added. The content of each flask was mixed and allowed to settle. The supernatant fluid was assayed chemically for Mn and examined for bacteria by phase-contrast microscopy of wet mounts (immediately and at daily intervals). Incubation of the enrichments was at 25 ± 1 C.

Mn^{++} adsorption by oyster shells. Oyster shells, picked from the beach at Wellfleet, Mass., were washed under tap water and broken in a mortar with a pestle into pieces of about 1 to 2 cm². In one experiment, 8.0 g were weighed into each of two 125-ml Erlenmeyer flasks. To one of the flasks, 20 ml of a solution (pH 3.76) containing 0.02 M $MnSO_4$ and 3% NaCl were added. To the other flask a solution (pH 5.75) of 0.02 M $MnSO_4$, 0.1% peptone, and 3% NaCl was added. Immediately and at 1-hr intervals, 0.2 ml of supernatant fluid in each flask was assayed in duplicate for Mn^{++} . The flasks were kept at

room temperature throughout this experiment. In a second experiment, two 125-ml Erlenmeyer flasks were set up as in the previous experiment, but each contained 5 g of crushed oyster shell, and the solution in each was adjusted to an initial pH of 6.31. The Mn^{++} in the supernatant fluid in each flask was assayed immediately, on the second day, and on the fourth day. The flasks were held at 4 C in a refrigerator during this time to keep down bacterial growth. No bacteria were seen in wet mounts of the supernatant fluid at any time in any of the flasks in either experiment.

Manganese analysis. Manganese in the supernatant fluid of enrichments was determined by a persulfate oxidation method adapted from Sandell (1959). A 0.2-ml amount of the solution to be analyzed, 8.8 ml of distilled water, 1 ml of special reagent, and 0.1 g of sodium persulfate were added to test tubes (25 by 200 mm). The special reagent consisted of 22.5 g of mercuric sulfate, 120 ml of concentrated HNO_3 , 60 ml of distilled water, 60 ml of syrupy 85% H_3PO_4 , 60 ml of distilled water, and 10.5 mg of $AgNO_3$, added in that order. The tubes containing the assay mixture were held in a boiling-water bath for 6 min. Further additions of 0.1 g of sodium persulfate were made to each tube after 2 and 4 min of boiling. On completion of boiling, the tubes were immediately plunged into cold water. After cooling to room temperature, color development was read in matched Coleman cuvettes (19 by 105 mm) against a reagent blank in a Coleman Junior spectrophotometer (model 6A) at 545 μ . Although Beer's law was followed over a range of 0 to 20 μ moles of Mn per ml, results from the assays were interpreted from a standard curve. In general, this method proved to be reproducible to within 0.16 μ mole of Mn per ml. The presence of 0.1% peptone or 1.0% glucose, or both, did not affect the accuracy of the assay.

RESULTS AND DISCUSSION

In a typical experiment in which crushed manganese nodule material was overlaid with sea water, containing 0.02 M $MnSO_4$ in one case and 0.02 M $MnSO_4$ and 0.1% peptone in the other, a rapid disappearance of Mn^{++} from the supernatant fluid was noted. Typical results during a period of 14 days are shown in Fig. 1A. The initial pH, measured with a Beckman model G pH meter, in the flask without peptone was 5.85, and in the flask with peptone 6.99. The final pH's in the respective flasks were 7.08 and 7.48. When the supernatant fluid in each flask was removed by pipette and replaced with fresh, sterile solution of the same composition as before, results as shown in Fig. 1B were obtained during 14 days of observation. The final pH's in this case were 6.05 without peptone and 6.40 with peptone. A second replacement of supernatant fluids in the flasks gave results in Fig. 1C, and a third replacement gave the results in Fig. 1D. The final pH's in the second replacement were 6.52 without peptone and 6.70 with peptone. The final pH's in the third replacement

were 6.18 without peptone and 6.49 with peptone. In the absence of peptone, no bacteria were seen in wet mounts of supernatant fluids of the initial enrichment or of the three replacements. In the presence of peptone, on the other hand, a large number of bacteria were found in the supernatant fluid on the second day of the initial enrichment and thereafter through the three replacements. These results indicate that the presence of peptone enhances the rate of disappearance of Mn^{++} from the supernatant fluid, and suggest that the bacteria developing at the expense of the peptone are the cause of this phenomenon. Support for this interpretation is lent by experiments in which the nodular material in the Erlenmeyer flasks was autoclaved at 15 lb/in² for 20 min. In initial enrichment cultures in such experiments, no bacteria developed, and peptone did not enhance the rate of Mn^{++} disappearance from sea water. Indeed, peptone slowed the rate of Mn^{++} disappearance in most instances. In one of these experiments, after replacing the supernatant fluids of the respective flasks with appropriate fresh solution, bacteria were noted in the supernatant fluid after 6 days of incubation in the flask with added peptone. The appearance of bacteria was accompanied by a simultaneous increase in the rate of drop in Mn^{++} concentration in that flask. Qualitatively similar results were obtained in some other such experiments. It

was found that nodular material could not be completely sterilized, even after 40 min of autoclaving at 15 lb/in².

The disappearance of Mn^{++} from the supernatant fluid is best explained on the basis of its adsorption on the crushed nodular material. We can eliminate the possibility that the drop in Mn^{++} concentration in the supernatant fluid is due to its precipitation as $Mn(OH)_2$, since no evidence of formation of a white precipitate in any of the enrichments was noted. We can also eliminate the possibility that the dissolved Mn^{++} is oxidized to MnO_2 , which then precipitates, because no browning of the supernatant fluid was noted in any of the enrichments. To explain the stimulatory effect of bacteria on Mn^{++} disappearance from the supernatant fluid, the following is postulated. The bacteria that grow in the presence of peptone promote oxidation of adsorbed Mn^{++} to MnO_2 . This new MnO_2 then adsorbs more Mn^{++} . Bacteria now oxidize this additionally adsorbed Mn^{++} to MnO_2 , and the cycle is repeated. Mn^{++} adsorption by MnO_2 was clearly demonstrated by Geloso (1927). Adsorbed Mn^{++} probably autoxidizes, but more slowly than with the help of bacteria. This would explain the results in Fig. 1D in which in the absence of peptone very little Mn^{++} is adsorbed, whereas in the presence of peptone a significant amount disappears from the supernatant fluid during the experiment. The observations on enrichments with autoclaved nodular material have shown that peptone by itself is not the cause of accelerated Mn^{++} adsorption. Whether the bacteria promote Mn^{++} oxidation enzymatically or indirectly by raising the pH of the medium remains to be established with certainty. Since the final pH's in the enrichments were always at least 0.5 units below 8.0, an enzymatic mechanism of oxidation seems likely. Spontaneous oxidation of Mn^{++} does not proceed rapidly below pH 8.0 (Leeper, 1947). Pure cultures of bacteria isolated from nodules were not observed to oxidize Mn^{++} in sea water-peptone broth in the absence of nodular material, but many of the cultures did promote Mn^{++} oxidation in peptone- Mn^{++} agar, using an adaptation of the method of Leeper and Swaby (1940). The mechanism of Mn adsorption and oxidation leading to nodule formation is attractive because it explains the *raison d'être* of nodules and, furthermore, accounts for their continued growth, at least in respect to their Mn component.

Although the over-all results of oft-repeated enrichment experiments have been the same, certain differences in details have been noted. For instance, there seems to be a difference in the ability of nodules to adsorb Mn^{++} . Table 1 lists the results for four different nodule specimens when portions of them were exposed to 0.02 M $MnSO_4$ in sea water in the absence of peptone. Nodule RD48-A266 was most efficient in adsorption. Nodule 1 and nodule 2, though less active than nodule RD48-A266, were about equally efficient. Nodule PD48-A260 was the poorest adsorber of the four. These differences are probably attributable to variations in chemical composition and structure of the nodules. Another variation in detail was the effect of

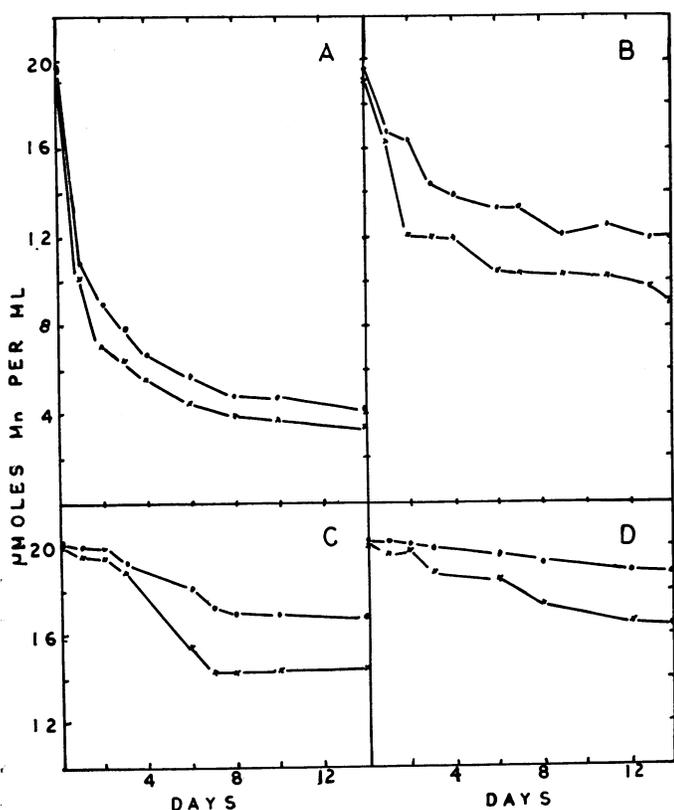


FIG. 1. Rates of Mn^{++} disappearance from sea water in the presence of crushed nodular substance. Closed circles represent experiment without peptone. Crosses represent experiment with peptone.

storage, in the freezer or the refrigerator, of some kinds of crushed nodular materials on time elapsed before bacterial outgrowth in culture enrichments was noted (Table 2). Long-term refrigeration of whole nodules was not observed to have this effect.

Since many manganese nodules collected over the years have been reported to have been formed around the teeth of sharks, ear bone of whales, pumice, etc., tests were performed to determine whether calcareous matter, such as oyster shells, could serve as the focus of Mn deposition. The results of two such tests are shown in Table 3. It is seen that, in both types of experiments, Mn^{++} is adsorbed by the oyster shells, and that, in neither experiment, did peptone stimulate Mn^{++} adsorption. The much slower rate of adsorption of Mn^{++} when the initial pH of the supernatant fluids was adjusted to 6.31 may have been due to pH or temperature of incubation, or both. Since the shell material had not discolored at the end of the adsorption period, it was inferred that the Mn^{++} had not oxidized. To test this inference, the shells of one such experiment were washed free of supernatant fluid with distilled water and

then soaked in 1 N NaOH with shaking. The shell material browned almost at once by this treatment owing to autoxidation of the adsorbed Mn^{++} . A control sample of shell material, to which no Mn^{++} had been experimentally adsorbed, did not discolor by shaking in 1 N NaOH. These results thus suggest a possible way by which manganese nodules may be initiated in the oceans.

Lest these findings suggest that the only role of bacteria in manganese nodules is their participation in nodule genesis, experiments are reported which show that bacteria can remove Mn from nodules. Figure 2 summarizes results of an experiment in which glucose and $MnSO_4$ were present in sterile sea water overlying nodular material, freshly crushed under aseptic conditions, in each of two flasks. One of the two flasks also had peptone added. Bacteria appeared in both flasks on the second day of incubation. They persisted throughout the rest of the experiment. The flask without added peptone contained predominantly cocci, whereas the flask with added peptone contained pre-

TABLE 1. Variation in capacity among different manganese nodules to adsorb Mn^{++}

Item	Nodule specimens			
	PD48-A260 ^a	1 ^b	2 ^b	RD48-A266 ^c
Fiduciary limits ^d	2.94 ± 1.16	4.95 ± 0.72	5.00 ± 0.64	7.49 ± 0.97
No. of tests	3	5	5	5

^a Visible presence of carbonate in nodule structure.

^b Botryoidal nodule structure.

^c Layered nodule structure.

^d Units in μ moles of Mn per ml per g of nodule adsorbed in 1 day. Calculated on the basis of a 95% confidence interval.

TABLE 2. Time required for bacterial outgrowth from crushed nodular substance stored for different lengths of time

Nodule specimen	Length of storage of crushed nodule substance	Time lapse before bacterial outgrowth
	days	days
1	0	2
1	13	2
1	29	2
1	36	>12 ^a
1	44	>6 ^b
2	0 ^c	1
2	3	2
2	6 ^c	2
2	10	>15 ^d
2	14	>17 ^d
2	17	>21 ^d

^a During second replacement of supernatant fluid.

^b Inoculated after sixth day, during initial enrichment.

^c Stored in refrigerator; all other samples stored in freezer.

^d During first replacement of supernatant fluid.

TABLE 3. Mn^{++} adsorption by crushed oyster shell^a

Time	Temp	Unadsorbed Mn^{++} (μ moles/ml)	
		Without peptone	With 0.1% peptone
		Initial pH 5.85	Initial pH 6.99
0 hr	Room temp	19.30	19.70
1 hr		17.40	17.80
2 hr		15.90	15.90
3 hr		16.00	15.90
4 hr		15.90	15.70
		Initial pH 6.31	Initial pH 6.31
0 days	4 C	19.90	19.95
1 day		17.80	18.90
4 days		14.15	17.40

^a No bacteria seen in contents of any flask at any time.

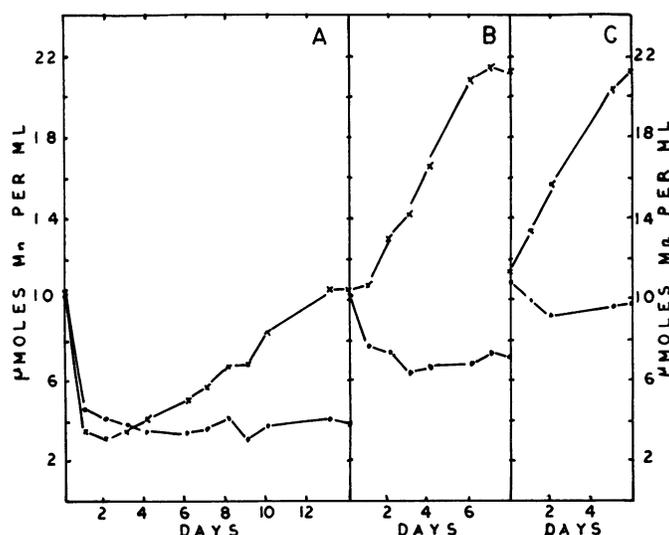


FIG. 2. Effect of glucose on Mn^{++} disappearance from sea water in the presence of crushed nodular substance. Closed circles represent experiment without peptone. Crosses represent experiment with peptone. Glucose was present in both experiments.

dominantly rods. The Mn concentration in the supernatant fluid dropped in both flasks initially, but rose almost as soon as bacteria appeared in the flask with added peptone (Fig. 2A). The Mn concentration did not rise significantly in the flask without peptone. The pH after 14 days in the flask without peptone was 6.35 and in the flask with peptone 6.95. When the supernatant fluids in the flasks were replaced with appropriate fresh solutions and incubated, the Mn^{++} concentration in the supernatant fluid of the flask without added peptone was again seen to drop, whereas the Mn^{++} concentration in the flask with added peptone was seen to rise without any prior drop (Fig. 2B and 2C). The final pH after 14 days in this first replacement of supernatant fluids was 6.15 without added peptone and 6.65 with added peptone. These results indicate that once appropriate bacteria are established in the presence of glucose and peptone they prevent significant adsorption of Mn^{++} on the nodular material and, indeed, release some of the Mn of the nodule substance. When peptone is absent, the bacteria can not prevent Mn^{++} adsorption and do not cause Mn release from the nodule, despite the presence of glucose. The nitrogen source for growth of bacteria in the presence of glucose but the absence of peptone in sea water with added $MnSO_4$ must be the organic nitrogen in nodules (Graham and Cooper, 1959). Since a morphological difference is noted between bacteria in the enrichment in which Mn^{++} is adsorbed and the one in which Mn is released, it is logical to infer that different organisms, both present in the nodule, are involved.

The question now arises as to the abundance of bacteria in the nodules. In preliminary experiments, platings of suspensions of some surface-sterile crushed nodular material on a suitable sea-water agar medium indicate the presence of at least 10^4 viable organisms per g. This number is probably a large underestimate because of the surface-sterilization prior to sampling, and because of difficulty encountered in separating bacteria from the nodular material. The bacterial types found include *Achromobacter*, *Arthrobacter*, *Bacillus*, *Brevibacterium*, *Vibrio*, *Staphylococcus*, an unidentified coccus, and an unidentified rod.

Large-scale bacterial action on manganese is not novel to bacteriology. Mann and Quastel (1946) demonstrated clearly microbial manganese oxidation in soil. They also showed microbial manganese reduction in the presence of glucose. Other workers (Bromfield and Skerman, 1950; Thiel, 1925) observed bacterial and fungal Mn oxidation under laboratory conditions. Most of the organisms employed in these studies derived from soil, but Thiel (1925) also obtained Mn-oxidizing bacteria from marine mud. It remains to be determined whether the mechanism of Mn

incorporation into, or removal from, nodules actually occurs in the sea. The average Mn concentration in the ocean has been reported by Sverdrup, Johnson and Fleming (1942) to be 0.001 to 0.01 ppm, but the possibility of locally high concentrations of Mn may exist. The depth at which some nodules have been found implies great hydrostatic pressures and temperatures around 4 C. This may affect biological participation in nodule growth. Obviously, conditions in the laboratory experiments described in this paper are very different from those in the oceans.

Nothing has been said here about iron incorporation into nodules. This will be a major concern of future work in this laboratory.

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