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Neutron Activation Analysis of Manganese and Sodium in Bacterial Cells

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The application of neutron activation analysis for mineral determinations in bacteria was investigated. Elements considered here were manganese and sodium. The sporeformer *Bacillus megaterium* ATCC 19213 was utilized. With this method, the manganese and sodium levels of whole and ashed vegetative cells, sporulating cells, and free spores were determined. The culture medium was also analyzed for these two elements. The results indicate that neutron activation analysis is readily applicable to the study of mineral content of bacterial cells, spores, and culture media. The method has been shown to be ideal for the study of incorporation and egression of mineral elements during vegetative growth and secondary metabolism of sporulation.

Neutron activation analysis is one of the most sensitive analytical methods for determining a number of mineral elements in biological materials. It has been used to determine the mineral content in a number of biological tissues and fluids (1, 2, 3, 4, 5). The use of neutron activation analysis (NAA) for mineral determinations in bacterial systems is not documented in the literature even though its sensitivity is suited for trace metal analysis.

The ease of sample preparation, the elemental specificity, and the nondestructive irradiation of samples allowing multiple irradiations and analyses all contribute to the advantages of using NAA as an analytical method for trace metal determinations. The purpose of this paper is to demonstrate the practicality of using NAA as a method for mineral determinations in bacterial systems. The bacterial system utilized in this study was the aerobic sporeforming process. The elements analyzed were manganese and sodium.

MATERIALS AND METHODS

Organism and growth medium. *Bacillus megaterium* ATCC 19213 was utilized throughout this study. The organism was grown in a sucrose-mineral salts medium (SMS) at 30 C. The medium consisted of the following chemicals, per liter: sucrose, 1 g; NaCl, 1 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; KH_2PO_4, 1.2 g; $(NH_4)_2HPO_4$, 5.5 g; $FeSO_4 \cdot 7H_2O$, 10 mg; $ZnSO_4 \cdot 7H_2O$, 10 mg; $MnSO_4 \cdot H_2O$, 7.8 mg; $CaCl_2 \cdot 2H_2O$, 5.6 mg; and $CuSO_4 \cdot 5H_2O$, 0.10 mg; in demineralized distilled water, final *p*H 7.2.

Growth conditions. Stock cultures were maintained on nutrient agar slants. Cells and spores used for analyses were grown by the active culture technique. Cells from a 14-hr slant were used to inoculate a 500-ml Nephelo flask containing 100 ml of SMS medium. The cells were incubated in a New Brunswick psychrotherm rotary shaker at 30 C (250 rev/min). A 5-ml sample of this actively growing culture was transferred to another 500-ml Nephelo culture flask containing 100 ml of the SMS medium. This culture reached mid-log growth in 4 to 5 hr; the entire 100 ml of cells was then used as an inoculum for 8 liters of SMS medium in a 14-liter New Brunswick MicroFerm fermentation jar. Aeration of the medium was constant at 3 liters/min with an agitation rate of 200 rev/min. The transfer of actively growing cells into the fermentation jar provided a degree of cell synchrony through sporulation.

Cell harvesting. Samples of four different stages of growth and sporulation were utilized. When cells reached a desired stage, growth or sporulation was stopped by cooling the growth medium to approximately 4 C by the addition of ice made from demineralized distilled water. The cells were harvested by centrifugation in an RC2-B refrigerated centrifuge. The cells from each centrifuge bottle were washed three times in 200-ml portions of demineralized distilled water. The final wash water (25-ml samples) was analyzed for manganese and sodium by neutron activation analysis. The cells from the final pellet were transferred to acid-cleaned lyophilizing vials, lyophilized, and stored in a freezer until sample preparation for neutron activation analysis.

Determination of growth stages. Growth was monitored by following the increase in optical density by using a Klett-Summerson photoelectric colorimeter (540 nm). Post-logarithmic development and sporulation were followed by dark-phase-contrast microscopy. Nonrefractive rod-shaped cells were designated as vegetative cells, rod-shaped cells containing distinct phase-dark bodies were considered forespores (stage

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III), rod-shaped cells containing distinct phase-bright bodies were considered sporangia (stage IV), and free oval or spherical bright refractile bodies were considered free spores (stage VII).

Sample preparation. Four different cell types were prepared for analysis: (i) vegetative cells from three-quarter logarithmic growth, (ii) forespore with over 90% of the cells containing phase-dark bodies, (iii) sporangia with over 90% containing phase-bright bodies, and (iv) free spores with over 98% of phase-bright bodies dissociated from the mother cell. Lyophilized cells (50 mg) were placed into snap-top polyethylene vials obtained from the Ernest F. Fullman Co. After the samples were placed into these vials, they were filled with Bioloid paraffin. This step assured sealed samples as well as decreasing the amount of radioactive argon gas formed during irradiation procedures by eliminating most of the air from the samples.

Ashed sample preparation. Lyophilized cells were ashed by using a Perkin-Elmer Coleman 40 low-temperature R.F. asher. Oxygen gas was used as the oxidizing agent at a flow rate of 12 cc/min. The duration of ashing continued until the weight loss of the sample was negligible after a 15-min ashing period. Ashed samples were prepared for irradiation by adding 25 mg of bacterial ash to the irradiating vials.

Medium analysis. Samples of the growth medium were taken periodically throughout the growth and sporulation process. These samples were evaporated to dryness in an oven (80 C). The dried samples were filled and sealed with paraffin for neutron irradiation.

Sample irradiation. Samples were irradiated at full power for 15 min in a 10-kw swimming pool nuclear reactor with a neutron flux of 2×10^{10} neutrons per sec per cm^2. Samples were thoroughly washed in demineralized distilled water before irradiation. Bacterial samples along with appropriate standards were placed into a polyethylene tube [8 by 1.75 inches (20.3 by 35.9 cm)] which was used to transport the samples to and from the core of the reactor by a pneumatic air system. The system termed the "rabbit" facility allowed immediate access to samples when the period of irradiation was terminated.

Sample counting. The decay of activated samples was monitored by measuring the emission of gamma radiation by using a sodium iodide crystal [3 by 3 inches (7.62 by 7.62 cm)] coupled to a transistorized Packard 400 channel pulse-height analyzer with a typewriter readout. Calibration of this analyzer was carried out by using standard gamma-ray sources over an energy range of 0 to 2 Mev. The use of 100 channels per sample from the available 400 channels allowed four samples to be counted consecutively.

Prior to counting, the samples were allowed to "cool" for 1 hr. This waiting period reduced radiation exposure as well as allowing the decay of ^{27}Mg which has a similar photopeak energy (0.845 Mev) to ^{56}Mn (0.840 Mev). Since the half-life of ^{27}Mg is 9.5 min, essentially all of the ^{27}Mg decays within 1 hr after neutron irradiation and is no longer a significant factor interferring with the counting of ^{56}Mn.

Preparation of standards. Standards containing 1,000 μg of both sodium ion and manganese ion per ml were prepared and diluted to appropriate levels as required. A 1-ml amount of each standard solution was then placed into polyethylene vials, evaporated to dryness, and prepared for neutron irradiation. The simultaneous irradiation of known levels of standards with the cell samples provided a method for quantitating manganese and sodium levels in the unknown cell samples.

RESULTS AND DISCUSSION

Cell sample irradiation. Samples of *B. megaterium* representing vegetative cells from three-quarter log growth (stage I), forespore (stage III), sporangia (stage IV), and free spores (stage VII) were irradiated. The growth curve in Fig. 1 illustrates these stages.

Initial activation analysis of these cell samples revealed one primary photopeak and several less

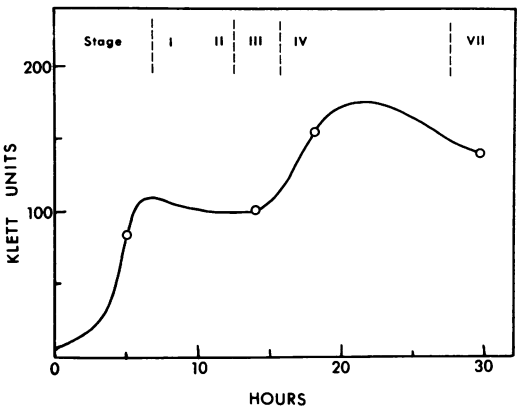

FIG. 1. *Growth curve for Bacillus megaterium. 0 = Time cell samples were obtained for neutron activation analysis.*

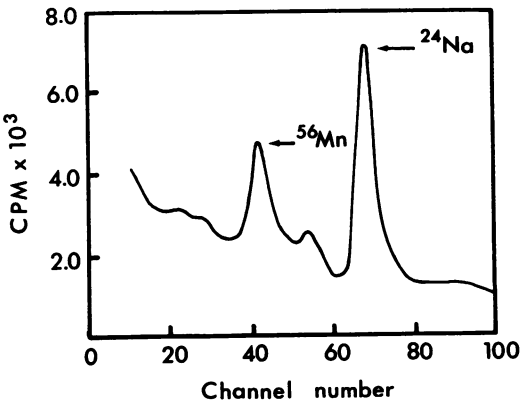

FIG. 2. *Gamma-ray spectrum showing the 0.84-Mev photopeak of ^{56}Mn and the 1.37-Mev photopeak of ^{24}Na.*

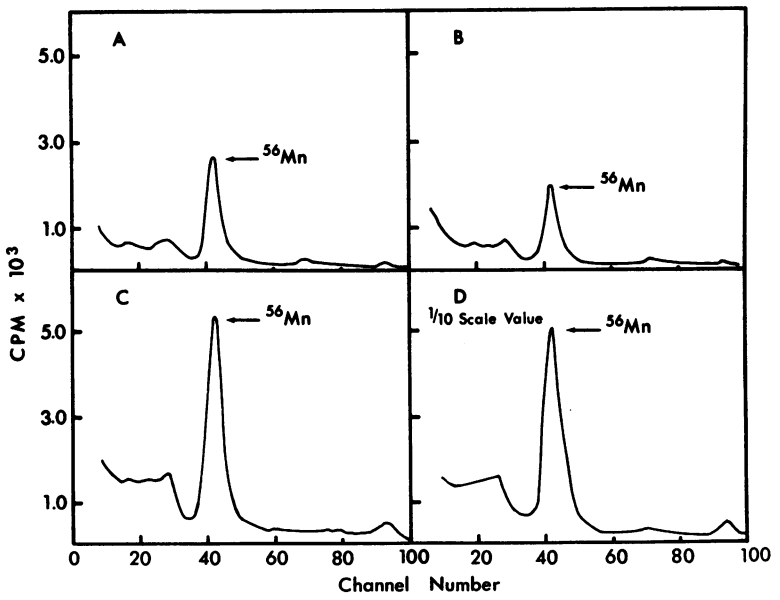

FIG. 3. *One-minute gamma-ray spectra of four different stages of Bacillus megaterium cells 1 hr after a 15-min neutron irradiation.*

distinct peaks. The primary photopeak was identified as ^{56}Mn by its gamma photopeak energies, half-life determinations, and comparison with a known manganese standard (Fig. 2). Spectra of the four different cell samples are represented in Fig. 3. The vegetative cell sample (A) clearly shows the ^{56}Mn gamma photopeak at channel 42 (0.84 Mev). The spectrum of the forespore sample (B) represents the same photopeak but with a slight decrease in the manganese level. The manganese level in the sporangium sample (C) shows a significant increase over the preceeding two samples, whereas the free spore sample (D) shows a further increase in the manganese level. It is evident from these data that the manganese levels between vegetative cell and spore sample are significantly increased. The lyophilized vegetative cells contained 63.2 μg of manganese per g of cells, whereas the forespore, sporangia, and free spore samples contained 55.8, 158.0, and 1,215 μg of manganese per g of cells, respectively.

Irradiation of ashed samples. Lyophilized cells ashed in the Perkin-Elmer low-temperature R.F. asher were irradiated for 15 min. Figure 4 shows 1-min spectra of the ashed samples representing the four different growth stages. A comparison of the ashed samples with nonashed samples reveals a significantly higher mineral content in the ashed samples. These results were based on the higher percentage of oxidizable organics present in the vegetative cells. The weight loss of vegetative cells during ashing showed 86% being oxidized with only 14% remaining as ash. The spore samples lost only 62% to oxidation, whereas 38% remained as ash. These figures indicated that 178 mg of lyophilized vegetative cells was required to give 25 mg of ash, whereas only 56 mg of spores was required to give 25 mg of ash. It is evident from these figures that the overall increase in the manganese levels would be decreased significantly between the vegetative and spore ashed samples.

The ashed samples were recounted 24 hr after the initial 15-min neutron irradiation. Five-minute counts of each sample were taken and the spectra are shown in Fig. 5. The vegetative cell sample (A) still shows the ^{56}Mn photopeak at 0.84 Mev, but it is extremely small. The most prominent photopeak at this time is the ^{24}Na with a photopeak energy of 1.37 Mev as confirmed by half-life determinations and comparison with sodium standards (Fig. 2). The forespore stage sample (B) shows decreased levels in both the manganese and sodium photopeaks when compared to the vegetative cell sample, whereas the sporangia sample (C) reveals an increased manganese level with a further decrease in the sodium level. Examination of the spore sample (D) indicates that manganese is still the primary photopeak 24 hr after the initial irradiation. The level of sodium indicates a further decline in the spore sample. This sequence of cell samples shows that manganese ion accumulates in the cells during

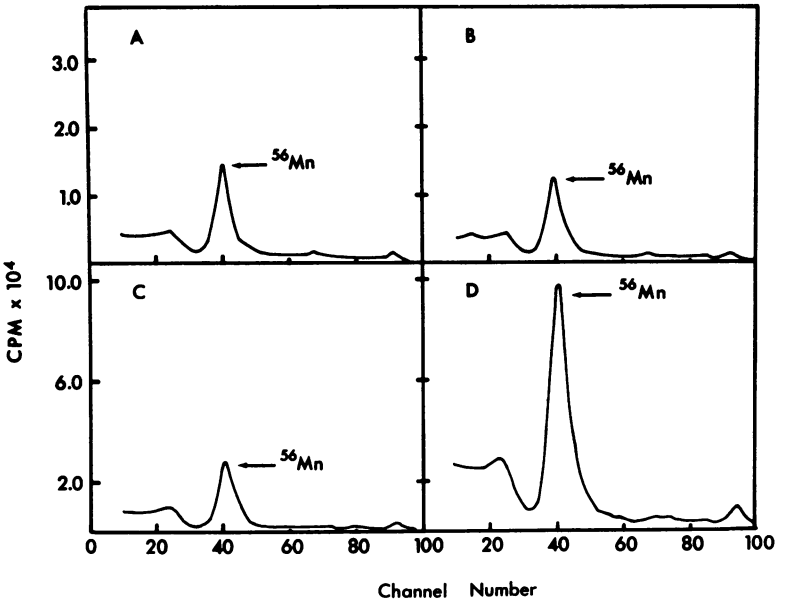

FIG. 4. *One-minute gamma-ray spectra of four different stages of ashed Bacillus megaterium cells 1 hr after a 15-min neutron irradiation.*

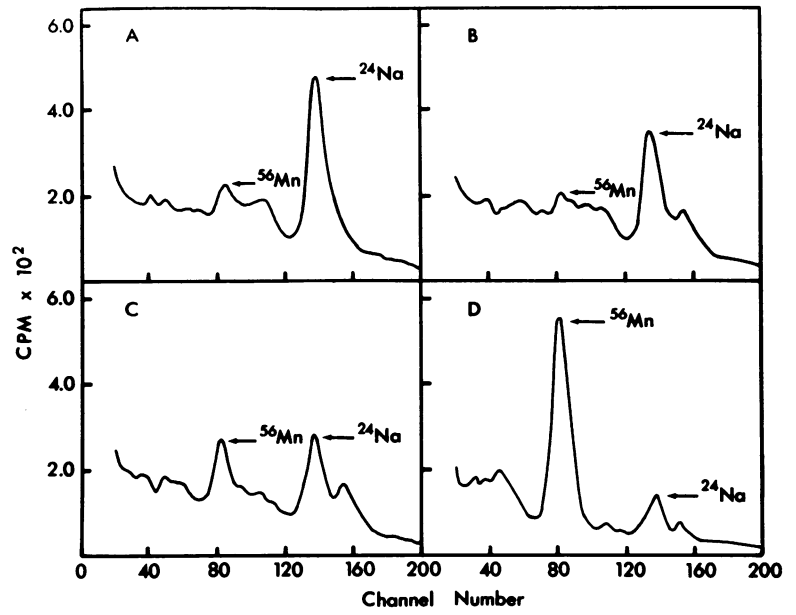

FIG. 5. *Five-minute gamma-ray spectra of four different stages of ashed Bacillus megaterium cells 24 hr after a 15-min neutron irradiation.*

sporulation. Conversely, the sodium ion content of the cells decreases during sporulation.

Media sample analysis. Analysis of the SMS growth medium was made by irradiating 25 ml of the evaporated cell-free medium in the polyethylene vials. Samples of media were taken throughout growth and sporulation. They were prepared for neutron irradiation as previously described.

Figure 6 illustrates the per cent of free manganese remaining in the medium at various times during cell growth. The manganese level decreased rapidly during logarithmic vegetative cell growth (32% of original level), increased during late-log and post-log growth (55% of initial level), and then decreased to 7% during spore formation.

Figure 7(A) represents a 1-min spectrum of the SMS medium before inoculation. The ^{56}Mn gamma photopeak at channel 42 (0.84 Mev), the ^{24}Na photopeak at channel 68 (1.37 Mev), and the ^{38}Cl gamma photopeak at channel 81 (1.61 Mev) are all evident in this spectrum. Figure 7(B) represents a SMS medium sample 30 hr after inoculation when sporulation was complete. This spectrum clearly shows the ^{24}Na and ^{38}Cl gamma photopeaks, but the ^{56}Mn gamma photopeak has nearly disappeared, indicating almost complete incorporation of the available manganese into the spores.

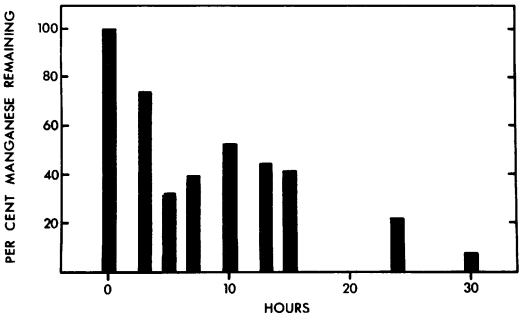

FIG. 6. *Per cent of manganese in growth medium at various times throughout growth and sporulation of Bacillus megaterium.*

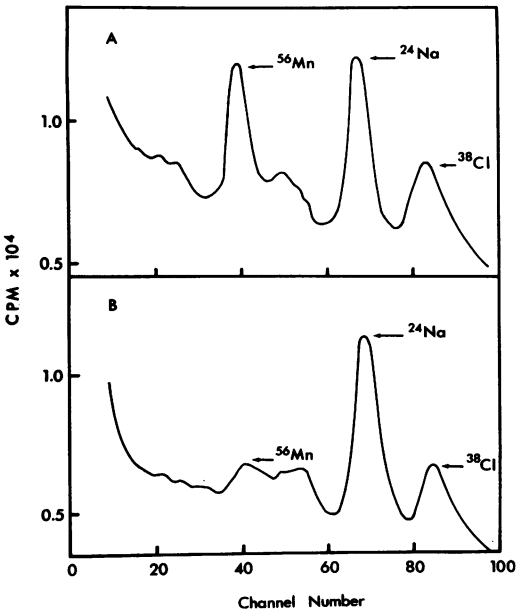

FIG. 7. *One-minute gamma ray spectra of media samples 0 hr (A) and 30 hr (B) postinoculation.*

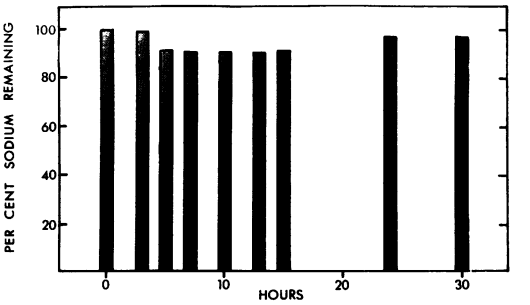

FIG. 8. *Per cent sodium remaining in the growth medium during various times throughout growth and sporulation of Bacillus megaterium.*

Figure 8 represents the results of sodium determinations in the sequence of media samples. The levels of sodium decreased during vegetative cell growth, leveled off during post-log growth, and then increased during sporulation. The final sodium level after sporulation reached 98% of the original concentration present in the medium at the time of inoculation.

It is evident from these data that neutron activation analysis is an excellent method for the determination of both manganese and sodium levels in bacterial cells. The method is also applicable to elemental analysis of the culture medium; hence, incorporation and egression of mineral elements can readily be followed. The bacterial sporulation system lended itself ideally to this study since the manganese content of vegetative cells and spores of a number of sporeformers have been reported previously by using other analytical approaches. These latter data contributed a comparative base. Presently, we are extending these studies to other mineral elements.

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