Cations in Hemolymph and Alimentary Tract Tissues of Healthy and Milky Diseased European Chafer (*Amphimallon majalis*) Larvae

K. H. STEINKRAUS, C. C. FIELD, M. C. KOCHANSKY, M. E. KAEGBEIN, AND H. TASHIRO

*Cornell University, New York State Agricultural Experiment Station, Geneva, New York 14456*

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A study was made of certain cations present in hemolymph and alimentary tract tissues of healthy and diseased European chafer larvae and the spores of *Bacillus popilliae* collected from diseased hemolymph. The major ions found in the hemolymph, in order of decreasing abundance, were potassium, magnesium, sodium, and calcium. Hemolymph of diseased larvae contained relatively higher concentrations of sodium, potassium, magnesium, iron, and zinc than hemolymph of healthy larvae. Concomitantly, the concentrations of ions were lower in the mid-gut and anterior intestinal tissues of diseased larvae. Only sodium decreased slightly in the diseased tissues of the rectum and rectal sac; other ions remained unchanged or increased. Little or no manganese or copper was detected in the hemolymph or tissues. The major cations of spores of *B. popilliae* were sodium, calcium, and magnesium. Small amounts of potassium, manganese, copper, iron, and zinc were detected in the spores. Based on calcium and dipicolinic acid determinations of the spores, sufficient calcium was found to allow for the formation of calcium dipicolinate in the expected concentrations.

**Cations in hemolymph of beetle larvae.** Although the literature on both the milky disease of beetle larvae and *Bacillus popilliae* is becoming voluminous (7, 11, 19, 24, 30, 32), the basic problem of achieving high-level production of *B. popilliae* spores in vitro similar in numbers and infectivity to those produced in vivo remains unsolved.

It appears that one area which has escaped intensive study and which might hold important clues to the problem of duplicating in vitro life cycles of *B. popilliae* Dutky in vitro is that of trace elements, particularly cations, which are present in larval hemolymph and tissues and which may be intimately related to growth and sporulation of the milky disease bacteria. Thus far, apparently no group involved in intensive study of the milky disease bacteria has studied the trace metals in the hemolymph or tissues of the host or related the levels of cations to the needs of the bacilli.

Ludwig (17) published data on the composition of the hemolymph of the Japanese beetle (*Popillia japonica* Newman) larvae. The data included levels of the following cations reported in mg per 100 ml of hemolymph: calcium, 31.6; magnesium, 47.1; sodium, 46.5; and potassium, 37.2. These data calculated as percent dry weight for comparison are: calcium, 0.39%; magnesium, 0.57%; sodium, 0.57%; and potassium, 0.45%, based on 8.2% solids reported in the hemolymph.

According to Chapman (5), the most abundant cation in insect hemolymph is usually sodium, but in some coleoptera there is very little and it contributes only about 10% of the total osmolar concentration. The absolute concentration of potassium is usually lower than that of sodium, contributing 2 to 10% of the osmolar concentration. The sodium to potassium (Na:K) ratio, however, in individual species, can vary from as high as 40 to less than 1. The concentration of magnesium is generally quite high. Phytophagous beetles generally have Na:K ratios less than 1 because of the high potassium content in plant tissue (4).

Copper, an essential metal for tyrosinase, is present in the hemolymph of larvae of European chafers (*Amphimallon majalis* [Razoumowsky]) and Japanese beetles (*Popillia japonica* Newman). Milky disease interferes with activity of this enzyme (2).
Relationship of cations to sporulation. Mineral salts are essential for sporulation by a number of bacilli (9). The metallic elements required for sporulation have been adequately reviewed by Murrell (20, 21) and Curran and Evans (10). The following ions are essential for sporulation in at least one species of Bacillus: magnesium in B. mycoides (15); potassium in B. cereus, (12); and manganese in B. subtilis (6, 31). Also calcium, iron, zinc, copper, molybdenum, and cobalt are required for sporulation (21). Among these ions, manganese is one of the most essential. Also, manganese broadens the temperature and pH over which B. coagulans var. thermoacidurenus will sporulate (1). There is a possibility that manganese may function by suppressing substances which inhibit sporulation (10). Normally, spores contain from 2 to 3% calcium and 5 to 15% dipicolinic acid in a 1:1 mol ratio (21). Calcium deficiency appears to limit sporulation and reduces heat resistance of the spores which are produced (13). With B. anthracis, sporulation is poor unless tap water is used as a base for the casein hydrolysate medium and calcium, magnesium, and manganese are added. This indicates the essentiality of certain other ions present in tap water and not present in distilled water (10). By using a chemically defined medium for the same organism, Brewer et al. (3) reported that calcium at 0.005 M increased the yield of spores 4.5 times. Additional iron, although not essential for sporulation, increased spore yield by 2.5 times. Copper, zinc, cadmium, and cobalt at 0.1 to 1.0 ppm were without effect on sporulation. Increasing the amount of manganese did not affect sporulation, but it was hypothesized that enough Mn⁺² was present as a contaminant of other chemicals to satisfy the sporulation requirements. To study the minimal requirements for sporulation of B. megaterium, Kolodziej and Slepecky (16) prepared a sucrose-salts medium with highly purified chemicals and apparatus carefully cleaned of trace contaminants. The ultrapure system allowed them to identify copper and molybdenum as required for the normal sporulation of B. megaterium, along with calcium, manganese, iron, and zinc, although molybdenum could substitute for iron and zinc. They also noted a requirement by B. cereus species for copper and molybdenum to obtain optimal sporulation. Roberts and Baldwin (26) were the first to demonstrate the effect of agar on sporulation of B. subtilis. The effect is partially due to agar containing magnesium, potassium, calcium, manganese, and iron B. Q. Ward (Ph.D. thesis, Univ. of Texas, 1947) found that sporulation of B. thermoacidurenus was increased in proteose-peptone agar by the addition of lithium, magnesium, calcium, iron, zinc, and manganese.

MATERIALS AND METHODS

Hemolymph from healthy and milky diseased third instar European chafer (A. majalis [Razoumowsky]) larvae was collected by heating the larvae to 60°C for 5 min to suppress coagulation of the hemolymph and puncturing the hemocoel with a sterile needle.

Spores of B. popilliae were separated from diseased hemolymph by centrifuging the hemolymph at 15,000 rpm for 5 min. The hemolymph was pipetted off and used in the cation determinations. The pelleted spores and other particulate matter from the hemolymph were then resuspended in 3% sodium lauryl sulfate and heated for 5 min at 60°C to remove contaminating lipids. The spores were subsequently repelleted and washed six times with distilled water. The resulting spores were free of debris when examined through a phase-contrast microscope (x 1,000).

The hemolymph from healthy larvae was centrifuged and the supernatant fluid was collected as described for diseased hemolymph. Thus, the blood cells were removed from the sample, as well as some proteins which might have been denatured by the initial heating of the larvae at 60°C for 5 min and any ions complexed with these proteins and blood cells.

Cations (Na, K, Ca, Mg, Mn, Fe, Zn, Cu) were determined in a Perkin-Elmer atomic absorption spectrophotometer (model 303) by using the method of McBride (18) and later repeated with the following modifications: the samples were dried in a vacuum oven at 70°C until they reached constant weight. Then they were dissolved in a minimum quantity of hot concentrated nitric acid. The samples were not perchlorated since perchlorate treatment did not affect the results.

Dipicolinic acid content of spores was determined by using the method of Janssen et al. (14).

Spore counts were made on appropriate dilutions in an American Optical “Bright-Line” hemocytometer.

Values were calculated as percent element in the dry material. The values for healthy hemolymph were the average of five samples, each being the pooled hemolymph from about 20 larvae. The values for diseased larvae per sample, and two were the pooled average of five samples; however, three samples were the pooled hemolymph or spores from about 15 diseased larvae per sample, and two were the pooled hemolymph of about 60 diseased larvae per sample. The range given with certain data indicate the unexplained variation encountered a few times during this investigation.

Dissection of alimentary tracts from third instar European chafer larvae. Sixteen each of healthy and milky diseased third instar European chafer larvae were selected for removal of their alimentary tracts. They were first washed with distilled water to remove any adhering soil and then dissected under water on a paraffin-activated carbon tray. The alimentary tracts were severed at the esophagus (fore-gut) and rectum. They were separated at the junction of the anterior
intestine (ileum) and rectal sac, and each section was cut open longitudinally. All contents were washed out. All adipose tissue was removed mechanically. Amber crystals (of unknown origin or purpose) attached to the interior wall of the rectal sac were largely removed. All washing and cutting were done under distilled water.

The healthy and diseased alimentary tracts were divided into lots as follows: (i) mid-gut and anterior intestine, and (ii) rectal sac and rectum.

These were dried to constant weight in a vacuum oven at 70 C. They were then dissolved in concentrated nitric acid which was boiled to minimum volume. These samples were then perchlorated and tested for cations as described above. Values are given as the percent element of dry tissue.

RESULTS

Cations in hemolymph. Sodium, potassium, calcium, magnesium, iron, and zinc were all present in relatively higher concentrations in diseased hemolymph than they were in the hemolymph of healthy larvae (Table 1). The amount of potassium was considerably greater than that of sodium, which is to be expected with phytophagous beetle larvae (5) but is in contrast to the results of Ludwig's (17) study of the Japanese beetle. Similar to the report of Chapman (5), the content of magnesium in hemolymph was quite high, much higher than that of calcium. No manganese was found in hemolymph, but a detectable, although small, amount of copper was present.

Cations in tissues of the alimentary tract. The ionic composition of the alimentary tract tissues differed considerably from the hemolymph and varied with the location of the tissue and the health of the larvae (Table 2). Tissues of the mid-gut and anterior intestine of healthy larvae contained a higher concentration of ions than the tissues of the rectal sac and rectum. No manganese or copper was found in any tissue. The concentration of ions decreased in the mid-gut and anterior intestinal tissues of diseased larvae. Sodium decreased slightly in the posterior tissues of diseased larvae; calcium remained the same, and potassium, magnesium, iron, and zinc increased in concentration.

Cations in spores. Spores of B. popilliae

| Table 1. Percent element in hemolymph of healthy and diseased European chafer (A. majalis Razoumowsky) larvae |
|---------------------------------------------------------------|
| **Element** | **Healthy hemolymph** | **Diseased hemolymph** |
| | Dry weight (%) | Dried hemolymph (mol/g) | Dry weight (%) | Dried hemolymph (mol/g) |
| Sodium | 0.51 (0.23-0.92)* | 2.2x10^-4 | 1.02 (0.74-1.12) | 4.4x10^-4 |
| Potassium | 2.24 (1.21-3.02) | 5.7x10^-4 | 3.72 (2.14-5.00) | 9.5x10^-4 |
| Manganese | <0.01 (0-0.01) | <1.8x10^-6 | 0* (0) | <10^-4 |
| Calcium | 0.40 (<0.01-0.70) | 1.0x10^-4 | 0.69 (0.24-1.05) | 1.7x10^-4 |
| Magnesium | 1.70 (0.75-2.50) | 7.1x10^-4 | 2.93 (2.40-3.70) | 1.2x10^-3 |
| Copper | 0.02 (<0.01-0.05) | 3.1x10^-6 | 0.02 (<0.01-0.03) | 3.1x10^-6 |
| Iron | 0.04 (0-0.06) | 7.1x10^-6 | 0.13 (0-0.24) | 2.3x10^-6 |
| Zinc | 0.03 (0.02-0.04) | 4.6x10^-6 | 0.17 (0.03-0.31) | 2.6x10^-6 |

*Values in parentheses indicate the range.

Amount was below the limit of detection of the spectrophotometer.

| Table 2. Percent element in the alimentary tract tissues of healthy and milky diseased third instar European chafer larvae |
|---------------------------------------------------------------|
| **Element** | **Rectal sac and rectum** | **Mid-gut and anterior intestine** |
| | Healthy | Diseased | Healthy | Diseased |
| Sodium | 3.01 | 2.82 | 5.95 | 2.60 |
| Potassium | 0.85 | 1.03 | 1.08 | 0.29 |
| Manganese | <0.01 | <0.01 | <0.01 | <0.01 |
| Calcium | 0.19 | 0.20 | 0.50 | 0.25 |
| Magnesium | 0.14 | 0.44 | 1.20 | 0.11 |
| Copper | <0.01 | <0.01 | <0.01 | <0.01 |
| Iron | 0.09 | 0.32 | 0.50 | 0.20 |
| Zinc | 0.24 | 0.52 | 0.64 | 0.25 |
formed in vivo contained concentrations of potassium, manganese, calcium, magnesium, copper, iron, and zinc (Table 3) similar to other aerobic bacilli spores, as reported by Murrell and Warth (22). The concentration of sodium was higher than the range formerly reported (22). Calcium was concentrated to about five times above the level present in vegetative cells grown in the growth medium of Steinkraus and Provvidenti (29). This has been observed in other bacillus species and is partially attributed to a complex formed by calcium with dipicolinic acid (DPA) in a 1:1 mol ratio (21). Our determination of DPA in B. popilliae spores showed about 1.9% of the dry weight as DPA, higher than the 0.4% reported by St. Julian et al. (27), and "small amount" indicated by Rhodes (25).

**Cations in media.** In tryptone, yeast extract, and brain-heart infusion, sodium was the only ion determined whose concentration in the medium was higher than its concentration in diseased hemolymph (Table 4). Therefore, except for sodium, the concentration of the ions could be adjusted in media to correspond to the concentration in the hemolymph. A medium previously used for the growth of B. popilliae (29) contained less sodium than the other media tested but had a high potassium content, mainly due to the addition of potassium phosphate as a buffering agent. All the media tested were deficient in manganese and relatively low in calcium, both known to be essential for sporulation. The addition of various ions at the proper concentrations and use of tris(hydroxymethyl)aminomethane as the buffering agent provided a growth medium similar to hemolymph in its concentration of the ions determined during this investigation. The rate of growth of B. popilliae in this medium was not affected by the addition of ions; however, the maximum cell population (as colony-forming units) was lower in the presence of the additional ions. After several weeks at 30 C, no spores had been formed by cells grown in this ion-enriched medium.

**DISCUSSION**

The quantitation of certain ions in the hemolymph and alimentary tract tissues provides a knowledge of not only the concentration of ions suitable for sporulation in the hemolymph but also the effect which milky disease has on the concentration of ions in the hemolymph and alimentary tract tissues. Interestingly, the increase in the concentration of ions in the hemolymph accompanied a decrease in the concentration in the mid-gut and anterior intestinal tissues (Tables 1 and 2). Copper and manganese, which were only in minute amounts, were the only ions which did not show this trend. Although the decrease in the tissues did vary greatly from the amount of ion which increased in the hemolymph, the results suggested that B. popilliae may affect the permeability of mid-gut and anterior intestinal tissues. Since the rectal sac and rectum taken from diseased larvae lost only sodium (Table 2), general cell leakage appears to be localized in the anterior intestinal tissues of the alimentary tract.

**Table 3. Percent element in spores of B. popilliae**

| Element     | Dry weight (%) | Dried spores (mol/g) |
|-------------|----------------|----------------------|
| Sodium      | 0.38 (0.09-0.96) | 1.7 × 10^-4         |
| Potassium   | 0.06 (0.03-0.09)  | 1.5 × 10^-4         |
| Manganese   | 0.02 (0.00-0.06)  | 3.6 × 10^-4         |
| Calcium     | 1.35 (0.20-1.71)  | 3.4 × 10^-4         |
| Magnesium   | 0.86 (0.50-1.55)  | 3.5 × 10^-4         |
| Copper      | 0.03 (0.03-0.06)  | 4.7 × 10^-4         |
| Iron        | 0.05 (0.0-0.11)   | 8.9 × 10^-4         |
| Zinc        | 0.08 (0.05-0.10)  | 1.2 × 10^-4         |

**Table 4. Cations in several laboratory media**

| Element     | Dry weight (%) | Tryptone (Difco) | Yeast extract (Difco) | Brain-heart infusion (Difco) | Growth medium a |
|-------------|----------------|------------------|-----------------------|----------------------------|-----------------|
| Sodium      | 3.10           | 2.73             | 12.50                 | 1.65                       |
| Potassium   | 0.24           | 2.85             | 1.20                  | 7.49                       |
| Manganese   | <0.01          | <0.01            | <0.01                 | <0.01                      |
| Calcium     | 0.03           | 0.02             | 0.01                  | 0.01                       |
| Magnesium   | 0.03           | 0.24             | 0.01                  | 0.06                       |
| Copper      | <0.01          | <0.01            | <0.01                 | <0.01                      |
| Iron        | 0.01           | 0.02             | 0.02                  | 0.01                       |
| Zinc        | 0.04           | 0.05             | 0.03                  | 0.01                       |

* Medium of Steinkraus and Provvidenti (29) minus activated carbon, with glucose as the only sugar.
Although *B. popilliae* may affect cell permeability, its effect is not like that of *B. thuringiensis*. In the gut the parasporal bodies of *B. thuringiensis* spores are degraded to toxic products which rapidly and severely change the permeability of the anterior intestine in some very susceptible hosts (23). Spores of *B. popilliae* show no degradation of the parasporal body or germination in the mid-gut of the European chafer, but the cells eventually invade the tissues and spread into the hemolymph (C. M. Splittstoesser, H. Tashiro, S. L. Lin, K. H. Steinkraus, and B. J. Fiori. J. Invertebr. Pathol., in press). During the penetration of bacteria into the hemolymph, some degradation of host cells occurs, although no hyaluronidase, proteinase, or other hydrolytic enzymes commonly involved in virulence have been found in extracts of *B. popilliae* (Field and Steinkraus, unpublished data). Such tissue damage would cause the release of ions into the hemolymph. Since the hemolymph and tissues were collected from either healthy or diseased larvae in advanced stages of infection with no intermediate samples taken, the time of onset of the changes in the ion concentration of diseased hemolymph and tissues is not known. Certainly, the survival for a week or longer of a host harboring a massive infection of *B. popilliae* indicates that the pathogen causes very minor tissue damage and noncritical changes in the vital processes of the larvae.

Although spores of *B. popilliae* contained concentrations of ions similar to other bacilli (Table 3), the percentages of the various elements cannot be directly compared with the average of other spores because of an intact sporangium which surrounds the spore and parasporal body and the unique medium necessary for sporulation of the organism. The distribution of the elements in the sporangium has not been determined, and thus, the percentages given are based on the dry weight of the entire spore, including sporangium, and not of the free spore. Slepecky (8, 28) showed that the composition of the medium affects the concentration of ions in the spores. Therefore, *B. popilliae* spores formed in the rich medium provided by the hemolymph might differ greatly in their ionic concentration from other bacilli spores or even *B. popilliae* spores formed in vitro. Likewise, a meaningful comparison cannot be made between the concentration of an ion, such as calcium, in spores of *B. popilliae* formed in hemolymph and vegetative cells grown in vitro.

Manganese is considered essential for sporulation of bacilli and is accumulated above the level in vegetative cells (8). The hemolymph sample collected from diseased larvae contained no detectable manganese. The lack of this ion in the hemolymph may be due to the ion complexing with denatured protein or blood cells and being removed from the sample upon centrifuging (see Materials and Methods). During the sporulation of *B. popilliae* these complexed manganese ions may be available for incorporation by the cells and may thus provide sufficient manganese for the development of spores. Even though the spores contained little manganese, they were resistant to heating at 90 C for 20 min.

The heat resistance of *B. popilliae* spores is partially attributed to the amount of DPA in the spore. Although DPA is only 1.9% of the total dry weight of the *B. popilliae* spore, this amount may not be less than the DPA content of spores of other aerobic bacilli (22) since the weight of the *B. popilliae* spore is increased by the presence of a parasporal body and intact sporangium. The average dry weight of a *B. popilliae* spore is $3.8 \times 10^{-12}$ g, which is more than both the values Murrell and Warth (22) recorded for spores of several Bacillus species and the $6.7 \times 10^{-12}$ g per spore of *B. subtilis* strain 168 which we determined. Furthermore, the average DPA content of *B. popilliae* spores and spores of *B. subtilis* was approximately $7 \times 10^{-14}$ g per spore, which amounts to $4.3 \times 10^{-16}$ mol per spore. According to the data of Murrell and Warth (22), this amount of DPA is expected for a spore with the weight of *B. subtilis*.

The amount of calcium per spore of *B. popilliae* is $1.3 \times 10^{-15}$ mol per spore. Therefore, only about one-third of the calcium can be complexed with the $4.3 \times 10^{-16}$ mol per spore of DPA in the *B. popilliae* spore. Whether the calcium is complexed with other compounds of the spores, sporangium, or parasporal body is not known.

Although the ion-enriched medium does not support sporulation, it is more like hemolymph in its ion balance than other reported media. Certainly other undetermined ions in the hemolymph and gut tissues might be necessary for sporulation of *B. popilliae*. Upon the addition to this medium of other ions and nutrients plus the proper environment, sporulation of *B. popilliae* in vitro might become a reality.

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LITERATURE CITED

1. Amaha, M., Z. J. Ordal, and A. Touba. 1956. Sporulation requirements of *Bacillus coagulans* var. *thermoacidurans* in complex media. J. Bacteriol. 72:34-41.
2. Beard, R. L. 1945. Studies on the milky disease of Japanese beetle larvae. Conn. Agr. Exp. Sta. New Haven Bull. 491.
17. Janssen, Ludwig, Kolodziej, Knaysi, Curran, H.R., and F.R. Evans. Grelet, Foster, Charney, J., Costilow, Crosby, Curran, Bacillus Inst. which spores. Colorimetric constituants mineraux durumileu. Bacteriol. requirements for bacterial growth. Zool. mesophilic spores. Applied Bacteriol. J. of the insects: structure and function. American Elsevier Publishing Co., Inc., New York.

16. Charney, J., W. P. Fisher, and C. P. Hegarty. 1961. Manganese as an essential element for sporulation in the genus Bacillus. J. Bacteriol. 62:145-148.

15. Costilow, R. N., and W. H. Coulter. 1971. Physiological studies of an oligosporogenous strain of Bacillus popilliae. Appl. Microbiol. 22:1076-1084.

14. Crosby, W. H., R. A. Greene, and R. A. Slepecky. 1971. The relationship of metal content to dormancy, germination and sporulation of Bacillus megaterium. p. 143-160. In A. N. Barker, G. W. Gould, and J. Wolf (ed.), Spores research 1971. Academic Press Inc., London.

13. Curran, H. R. 1957. The mineral requirements for sporulation. In H. O. Halvorson (ed.), Spores. American Institute of Biological Science Publication 5. Washington, D. C.

12. Curran, H. R., and F. R. Evans. 1964. The influence of iron or manganese upon the formation of spores by mesophilic aerobes in fluid organic media. J. Bacteriol. 67:489-497.

11. Fleming, W. E. 1968. Biological control of the Japanese beetle. Agricultural Research Service, U.S.D.A. Tech. Bull. 1383.

10. Foster, J. W., and F. Heiligman. 1949. Mineral deficiencies in complex organic media as limiting factors in sporulation of aerobic bacilli. J. Bacteriol. 57:613-615.

9. Grelet, N. 1952. Le determinisme de la sporulation de Bacillus megaterium. II. L'effet de la penurie des constituant des mineraux du milieu synthetique. Ann. Inst. Pasteur. 82:66-77.

8. Janssen, F. W., A. J. Lund, and L. E. Anderson. 1958. Colorimetric assay for dipicolinic acid in bacterial spores. Science 127:26-27.

7. Knaysi, G. 1945. A study of some environmental factors which control endospore formation by a strain of Bacillus mycoides. J. Bacteriol. 49:473-493.

6. Kolodziej, B. J., and R. A. Slepecky. 1964. Trace metal requirements for sporulation of Bacillus megaterium. J. Bacteriol. 88:821-830.

5. Ludwig, D. 1951. Composition of the blood of Japanese beetle (Popillia japonica Newman) larvae. Physiol. Zool. 24:329-334.