Possible Mechanisms Responsible for the Reduced Intestinal Flora in Hibernating Leopard Frogs (Rana pipiens)

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Mechanisms and factors that normally control the large intestinal flora were investigated to determine whether changes in these parameters could account for the decreased bacterial concentration and facultative nature of the flora found in hibernating frogs. It appeared that low temperatures and limited nutrients were the main factors responsible for the increase in the bacterial concentration and may also have been responsible for the increase in the proportions of facultative organisms, since no change in the redox potential was seen. The hibernating frogs were extremely sluggish in the removal of India ink particles from the circulatory system by the Kupffer cells of the liver compared with nonhibernating frogs. They were unable to mount an antibody response to bovine serum albumin, but their serum did exhibit killing of Pseudomonas paucimobilis, suggesting opsonization by preformed antibody and complement. The role of these host factors in protecting the hibernating frog against this indigenous flora is discussed.

The large intestinal flora in hibernating frogs is significantly different from the flora in nonhibernators (4) in that a lower concentration of bacteria is present and there is a greater proportion of facultative psychrophiles and facultative anaerobic and aerobic bacteria, especially pseudo monads. The selective pressure of low temperature is probably responsible for the increased proportion of psychrophiles. The reasons for the lower bacterial concentration and increased proportion of facultative organisms are not known, though low temperatures may also be responsible to some degree for the reduction in the bacterial concentration.

Gossling et al. (16) subjected groups of nonhibernating frogs to conditions of cold or fasting to determine the effects of these conditions on the intestinal flora. Fasting for 2 weeks at 25°C had no effect, whereas chilling, either for 1 week at 4°C or for 3 weeks in the course of simulated hibernation, resulted in a decrease in the viable count. However, the viable count should remain low throughout hibernation after this initial response to cold takes place is not known. It might be expected that the psychrophilic bacteria would grow in this environment and accumulate, given the fact that peristalsis in hibernators is about 5% of the rate in nonhibernators (17). Thus, it is not clear whether the reduced bacterial concentration is due to a reduced growth rate secondary to low temperatures or due to other factors. It is also not clear to what extent the increase in intestinal pseudomonads, which are potential pathogens, poses a threat to the health of the hibernating frog during a time when its immunological capacity may be hypoactive (1, 3, 8, 20, 25). There are a number of mechanisms or factors believed to be involved in shaping or controlling the large intestinal flora (13, 29, 32, 37). Some of these were examined in this investigation in an attempt to achieve a better understanding of how the frog interacts with its intestinal flora during the period of hibernation.

MATERIALS AND METHODS

Frogs. All frogs were adult Rana pipiens (NASCO, Ft. Atkinson, Wis.) with a snout-to-vent length of 6.0 to 9.0 cm. Nonhibernating frogs were housed at room temperature in Michigan Environmental Enclosures for Small Animals (Keyco Co., Inc., Peach Bottom, Pa.) at AmpTech, Inc. (Ypsilanti, Mich.). Hibernators were housed in individual plastic containers in a cold room at 1 to 4°C. The containers contained sufficient tap water to allow the frogs to submerge themselves. The tap water was dechlorinated by overnight evaporation and contained 3.2 g of NaCl per liter.

Measurement of redox potential. A platinum wire electrode was constructed by combining a single piece of platinum wire with a wire connection compatible with a model 801 pH meter (Orion Research). The connection was sealed with waterproof epoxy. A calomel electrode (Beckman Instruments, Inc., Fullerton, Calif.) was used as a reference. Standardization of the electrode was accomplished by using a ferrous-ferric solution (26). Measurements of the redox potential in the large intestine were taken either by passing the wire through the anus and into the colon or by making an incision through the abdomen and then inserting the wire into a portion of the large intestine and also the small intestine.

Metabolic activities of intestinal bacteria. Frogs were sacrificed and brought into the anaerobic chamber (85% N₂, 10% CO₂, and 5% H₂). The large intestine was excised, and the contents were expelled. The intestinal lining was homogenized (Tekmar Co., Cincinnati, Ohio) for 20 s in 10 ml of reduced transport fluid (39) without EDTA, and 1.0 ml of the homogenate was added to tubes already containing one of the following substrates. The substrates, suspended in 1.5 ml of reduced transport fluid, included glucose (0.45 g), hog gastric mucin (0.01 g; Sigma Chemical Co., St. Louis, Mo.), hyaluronic acid (0.01 g; Sigma), Casamino acids (0.01 g; Difco Laboratories, Detroit, Mich.), alpha-acid glycoprotein (0.005 g; Sigma), peptone (0.1 g; Difco), N-acetylneuraminic acid (0.01 g), and sodium taurocholate (0.005 g; Sigma). The tubes were incubated at 37°C, and after 3 h, the tubes were assayed for CO₂ production.
acid (0.005 g; Sigma), and chondroitin sulfate (0.01 g; Sigma). A control tube contained only the homogenate and an additional 1.5 ml of reduced transport fluid without a substrate. The homogenates were incubated anaerobically for 24 h at 25°C (nonhibernators) and for 48 h at 4°C (hibernators).

After incubation of the homogenates, the bacteria and tissue particles were pelleted by centrifugation (8,000 x g), and the supernatant was removed for gas-liquid chromatography analysis. Samples were tested for the production of volatile and nonvolatile products (2) by using a gas chromograph (model 3700; Varian, Palo Alto, Calif.). A portion of the homogenate was plated on Schaedler agar (for a total count determination; BBL Microbiology Systems, Cockeysville, Md.) and on Schaedler agar containing metronidazole (to select for facultative anaerobes). Plates were incubated at both 25 and 4°C to determine the proportion of psychrophiles.

**Hibernation-specific inhibition.** The livers and intestines of hibernating and nonhibernating frogs were excised from the animals and homogenized for 20 s in phosphate buffer (pH 7.2). The homogenates were sequentially filtered through filters (Whatman, Inc., Madstone, England, and Millipore Corp., Bedford, Mass.) of decreasing pore size until a sterile liquid remained. A liver filtrate from hibernators was separated by isoelectric focusing over a period of 71 h. Voltage was 300 V at less than 10 mA. Ampholine (third-fourths of the 1% solution in the dense gradient solution) with a pH range of 2 to 9 was used. Filtrate (4 ml) was added to the less dense gradient solution. Fractions were collected, dialyzed for 72 h against Sorenson phosphate buffer (pH 7.2), and assayed for their ability to inhibit bacterial growth.

Inhibition of bacterial growth by the homogenates was tested by using a spiral plater (Spiral Systems Inc., Cincinnati, Ohio). A constant amount of the homogenate was plated in a spiral pattern on a Schaedler agar plate. The plate was rotated 180°, and between the first spiral a decreasing concentration of a bacterial suspension was placed in a spiral pattern. The plates were incubated anaerobically at 25°C for 5 days or at 4°C for 7 days, defined as a decreased bacterial colony count or smaller colony sizes compared with results from a plate inoculated with the bacteria and a saline control.

Eight species of bacteria were tested, including five *Clostridium* strains and one *Bacteroides* strain that were isolated at 25°C from nonhibernators and a *Pseudomonas* strain and a group D alpha *Streptococcus* strain that were isolated at 4°C from hibernators. These bacteria were chosen because they were predominant in nonhibernators and hibernators.

**Antibody synthesis.** Blood was collected from about 30 frogs, pooled, and allowed to clot, and the serum was collected. Serum proteins were precipitated in a 50% ammonium sulfate solution, and after centrifugation the pellet was suspended in a 40% ammonium sulfate solution. This suspension was repelleted by centrifugation, and the pellet was suspended in a small amount of phosphate-buffered saline and dialyzed for 4 days at 4°C. The serum proteins were chromatographed through a DEAE-Sepharose column equilibrated with a 0.02 M phosphate buffer and eluted with increasing concentrations (0.1 and 0.4 M) of potassium phosphate. Three peaks with *A* 280 as a function of the elution gradient were separated, and the fractions within each peak were pooled, dialyzed against 0.01 M phosphate buffer, and lyophilized.

Distilled water (4 ml) was added to each of the lyophilized pools (A, B, and C) and to 0.5 ml of whole frog serum, and an emulsion was made with 1 ml of complete Freund adjuvant and 1 ml of each suspension. Rabbits were injected intramuscularly with 0.5 ml of the various suspension-complete Freund adjuvant mixtures in each hind leg. The rabbits were boosted 1 month later by the same procedure. Two weeks after the booster, the rabbits were bled and the serum was examined for anti-frog immunoglobulin by immunoelectrophoresis. Serum from the rabbits immunized with pool A or whole serum showed the best activity against frog immunoglobulin as judged by comparison with previous immunoelectrophoresis studies with frog serum (14, 18). These rabbits were boosted again with the respective frog immunoglobulin or serum preparations and bled 3 weeks later.

Rabbit immunoglobulin was purified by passage through a staphyloccocal protein A column and then eluted with glycine-HCl. The fractions containing immunoglobulin were pooled, neutralized with Tris buffer, and dialyzed in phosphate-buffered saline. Alkaline phosphatase was conjugated to the immunoglobulin by glutaraldehyde treatment (22, 28).

Prebleeds were taken from hibernating and nonhibernating frogs by the method of Baranowski-Smith and Smith (5). The frogs were immunized with 0.1 ml of a 50 mg/ml solution of BSA injected into the dorsal lymph sac and 0.1 ml of BSA in complete Freund adjuvant (1:1) injected intramuscularly into each thigh of the frog. Frogs were boosted 1 month later. Postbleeds were taken 1 and 5 weeks after the booster.

The serum from the frogs was tested for antibody to BSA by enzyme-linked immunosorbent assay (22, 28). Frog serum samples were serially diluted from 1:50 to 1:6,400, and 0.1 ml of each dilution was added to the appropriate wells. Cat anti-BSA serum was used to standardize color development. *A* 405 was read.

**Serum killing assay.** The ability of serum to kill bacteria was tested by mixing bacteria with frog serum and, after incubation, comparing the viable count with that of a control incubated without serum. The bacteria used included a strain of *Pseudomonas paucimobilis* isolated from the lymph of a sick frog, a rough strain of *Salmonella typhimurium* (rfa69) with 0.1% polysaccharide, an attenuated strain of *Salmonella typhimurium* (SL1344) and a mixture of bacteria present in the intestinal contents. *P. paucimobilis* had previously been isolated from the tissues of frogs with red-leg, and thus it was of interest to know whether it was susceptible to killing by frog serum. The *S. typhimurium* was used because it was thought that it would be susceptible to serum killing and could serve as a positive control for the procedure.

Serum tubes contained 30 μl of serum, 15 μl of Veronal buffer (Winthrop Laboratories) with 0.1% gelatin, and 5 μl of a bacterial suspension (104 CFU/ml). Incubation was for 2.5 h at 37°C and 4 h at 4°C within a candle jar so as to increase the CO2 concentration. After incubation, serial 1:10 dilutions of the suspensions were made and 20 μl of each dilution was plated on Schaedler agar. Inhibition was recorded as at least a 75% reduction in the viable count on plates containing the serum samples compared with plates inoculated with the control samples.

**Carbon particle clearance.** The abilities of the nonhibernating and hibernating frogs to clear particulates from systemic circulation were tested by India ink injection followed by tissue sectioning. India ink in 2% gelatin was formulated to give a carbon concentration of 3.0 mg/ml. Of this suspension, 0.5 ml was administered to the frogs by cardiac puncture so as to give a dosage of approximately 10 mg/100 g of body weight (7). At this dosage in mice, 95% of the carbon is phagocytized by the reticuloendothelial cells of the
livers or spleens. After 15 min or 12 h, portions of the livers and spleens were removed and fixed overnight in neutral buffered Formalin, and tissue sections were cut from paraffin-embedded blocks. The sections were stained with hematoxylin and eosin and observed by light microscopy for the presence of carbon particles within the Kupffer cells of the liver and large reticular cells of the spleen. A semi-quantitative measure of carbon uptake was made by viewing 20 high-power fields (magnification, ×450) from each of three hibernating and three nonhibernating frogs and calculating the average number of carbon particles per field. The quantitation was done blindly by enumerating carbon particle-containing cells and matching results with the origins of the tissues afterwards.

Flow cytometry. Approximately 50 to 100 μl of blood was collected from a frog, and the leukocytes were separated by Ficoll-Hypaque sedimentation. The upper layer containing neutrophils was removed and centrifuged, and the pellet was suspended in phosphate-buffered saline-glucose-gelatin buffer. Smears were made of peripheral blood and the leukocyte suspension, Wright stained, and examined for the presence of mature neutrophils. The neutrophils were treated with 2‘,7‘-dichloro-fluorescin diacetate (DCFH-DA; Eastman Kodak Co., Rochester, N.Y.), and the fluorescence of resting phagocytic cells and cells stimulated with phorbol myristate acetate (Consolidated Midland, Brewster, N.J.) was measured by flow cytometry (10, 34).

RESULTS

Redox potential. The measurements of the redox potential in the intestines of frogs showed no differences between nonhibernating and hibernating frogs (Table 1). The measurements in the small intestines were significantly higher than the measurements in the large intestines for both nonhibernators and hibernators. The differences indicate that the lower redox potential in the large intestine was due to the metabolic activity of the resident bacteria. Readings within the large intestine did not differ whether the electrode was inserted through the anus or into the colon after an abdominal incision.

Metabolic activities of intestinal bacteria. The abilities of the intestinal bacteria to metabolize various substrates were evaluated by adding each substrate to resting cell suspensions. The preliminary results from nonhibernators indicated that glucose was utilized occasionally but that sugars commonly found in glycoproteins, such as galactose and fucose, were not. Other substrates utilized occasionally included chondroitin sulfate, peptone, N-acetyleneuraminic acid, and the amino acids aspartate and glutamate. A component of some insect exoskeletons, chitin, and a plant product, pectin, were not utilized by the large intestinal flora. The latter substances are not digested by the frog and may be present within the large intestine (30, 33). The bacteria from the nonhibernators utilized hyaluronic acid, Casamino acids, hog gastric mucin, and bovine alpha-acid glycoprotein in the majority of cases.

Glucose, mucin, alpha-acid glycoprotein, hyaluronic acid, and Casamino acids were evaluated further in both nonhibernators and hibernators. The bacterial suspensions from the nonhibernators utilized these substrates in the majority of cases, with the exception of glucose (Table 2). However, the bacteria from the hibernators degraded only the mucin at both 4 and 25°C, indicating that metabolism occurred at hibernation temperatures (Table 3). Succinic acid was a prominent end product formed by the hibernators, whereas it was not detected in suspensions of the flora of nonhibernators. The bacteriology of these suspensions was comparable to that of the flora reported previously for nonhibernators

<table>
<thead>
<tr>
<th>End product type</th>
<th>Avg mmol of product/bacterium from:</th>
<th>Avg mmol of product/bacterium from:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mucin, 25°C (4/4)*</td>
<td>Glycoprotein (3/4)</td>
</tr>
<tr>
<td>Propionic</td>
<td>2.2 × 10^-7 (4)*</td>
<td>8.5 × 10^-8 (2)</td>
</tr>
<tr>
<td>Succinic</td>
<td>4.9 × 10^-8 (3)</td>
<td>1.1 × 10^-7 (4)</td>
</tr>
<tr>
<td>Fumaric</td>
<td>4.6 × 10^-7 (1)</td>
<td>5.1 × 10^-7 (3)</td>
</tr>
<tr>
<td>Oxalacetic</td>
<td>8.9 × 10^-7 (1)</td>
<td></td>
</tr>
<tr>
<td>Isobutyric</td>
<td>1.1 × 10^-8 (1)</td>
<td></td>
</tr>
<tr>
<td>Butyric</td>
<td>4.9 × 10^-8 (2)</td>
<td></td>
</tr>
<tr>
<td>Isovaleric</td>
<td>1.2 × 10^-8 (2)</td>
<td></td>
</tr>
<tr>
<td>Isocaproic</td>
<td>9.8 × 10^-9 (1)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>(1)</td>
<td>2 Peaks (1)</td>
</tr>
</tbody>
</table>

* Fractions in parentheses are number of positive frogs per number tested. A positive result was the detection of acid or alcohol end product from the given substrate. Of four frogs tested with glucose, none was positive.

* Numbers in parentheses are number of frogs which contributed to the average.

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TABLE 1. Redox potential measured with a platinum wire

<table>
<thead>
<tr>
<th>Group</th>
<th>Large intestine</th>
<th>Small intestine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonhibernators</td>
<td>275 ± 35 (13)</td>
<td>77 ± 21 (9)</td>
</tr>
<tr>
<td>Hibernators</td>
<td>265 ± 29 (25)</td>
<td>71 ± 23 (21)</td>
</tr>
</tbody>
</table>

* Values are in millivolts ± the standard error of the mean. Numbers in parentheses indicate the number of animals contributing to the data. The measurements taken in the large intestine are significantly different from the measurements taken in the small intestine as determined by a Kruskal-Wallis test, P < 0.05.
and hibernators in that anaerobes dominated in the nonhibernators and facultative bacteria accounted for about half the flora in the hibernators (4).

**Hibernation-specific inhibition.** Efforts to detect a hibernation-specific inhibitor of bacterial growth were negative. Filters of the intestine or liver, serum, and fractions from an isoelectric focusing of a liver filtrate were unable to inhibit bacterial growth on agar plates of any of the eight strains tested, as evaluated by a decrease in colony number or size.

**Antibody synthesis.** Both nonhibernators and hibernators were immunized with BSA and tested for the presence of serum antibodies to BSA by an enzyme-linked immunosor- bent assay. Postbleeds from nonhibernators gave a signifi-
cantly higher response to BSA than prebleeds (Fig. 1A), but postbleeds from hibernators were not significantly different from the prebleeds (Fig. 1B). Figure 1 combines the results of duplicate trials on each frog. Even on an individual basis no hibernating serum appeared to contain a significant concentration of anti-BSA antibody. Two different post-
bleeds were taken at 1 and 5 weeks after the second booster to test for the possibility that the kinetics of the appearance of antibody is slower in hibernators than in nonhibernators. However, no differences were seen between postbleeds 1 and 2 in either nonhibernators or hibernators.

**Serum killing assay.** Antibody synthesized before hibernation may still be circulating and used as needed during the period at low temperatures. If this were true then it might be possible to detect the presence of antibody by a serum killing assay. At 37°C, serum samples from both nonhibernators and hibernators were able to decrease the viable counts of *P. paucimobilis* (Table 4). *P. paucimobilis* was also tested at hibernation temperatures, but at 4°C, serum from only one hibernator showed a sizable colony reduction and one serum was borderline. Neither of the nonhibernator serum samples showed killing at 4°C. Serum from one hibernator acted against a rough strain of *S. typhimurium*, and serum from one nonhibernator reduced the viable count of the intestinal contents.

**Carbon particle clearance.** Upon injection of the India ink into nonhibernators, it was evident that the carbon particles had circulated almost immediately, since the liver became visibly darker. In hibernators, in which the heartbeat averaged only 6 beats per min (compared with 48 beats per min in a nonhibernator), the darkening of the liver was not as evident after 15 min. At 15 min after injection, most of the carbon appeared to be associated with erythrocytes within blood vessels. Some phagocytosis by Kupffer cells was evident in nonhibernators but not in sections from hibern-
tors (Table 5). Sections of the spleens of both nonhibernators and hibernators showed little in the way of carbon accumu-
lation.

In liver sections from a hibernator taken 12 h after carbon particle injection, carbon could still be seen within blood vessels, indicating that clearance was extremely slow. Some examples of phagocytosis were evident, but these were not as numerous as the abundant carbon observed within Kupffer cells in nonhibernators 12 h after injection. A similar pattern was observed in the spleen 12 h after carbon injec-
tion.

**Flow cytometry.** Wright stains of leukocytes isolated from frog blood revealed few mature polymorphonuclear leukocytes. Most of the leukocytes appeared to be mononuclear and possibly were immature forms of polymorpho-
uclear leukocytes. These cells did not show any fluorescence indicative of a respiratory burst when measured in the flow cyto-
meter. Leukocytes from both nonhibernators and hiber-
nators were tested in a resting state and after stimulation with phorboxy myristate acetate.

**DISCUSSION**

One of the possibilities that could explain the increased proportion of facultative anaerobes in the large intestines of hibernating frogs is that there may have been a decrease in bacterial metabolism and a subsequent rise in the redox potential, which would be detrimental to the survival of obligate anaerobes. When measured, however, the redox potentials within the large intestines of nonhibernators and hibernators were similar. The actual values were higher than the ~520 to ~575 mV readings found in the cecal contents of rats and mice (24). In the rodents the electrodes were implanted and the readings were decreased maximally in the first 10 to 20 min but required 1 to 2 h before stabilizing. In our experiment, electrodes had to be held in place by hand. The readings leveled out after about 10 min but might have slowly decreased if the electrode had remained longer within the intestine. The pH in the frog large intestine was higher than the pH in the rats or mice. In two equal systems the *E*ₘ reading will be lower in the one with a higher pH. Therefore, pH differences alone cannot account for the higher readings observed in the frogs. Temperature effects in relation to the standard error of the mean have been reported to be minimal (23). Likewise, taking measurements in the animals in an aerobic environment, as opposed to taking measurements anaerobically within a glove box or anaerobic chamber, has been shown to have no effect on the results (24). It can be concluded that the redox potentials within the large intestines of nonhibernators and hibernators were significantly negative and that the changes in the redox potential were not responsible for the increased proportions of facultative anaerobes in hibernators.

Frogs normally feed on crickets, and thus the intestinal flora would be accustomed to a protein-rich diet, which would account for the ability of the suspensions of the intestinal flora to utilize substrates, such as amino acids and glycoproteins, as opposed to simple carbohydrates, such as galactose, fucose, or glucose. These results were not surprising given the high diversity of individual bacterial isolates from nonhibernators did not utilize glycerol (24) and those that did were mainly enterobacteria. Few of the anaerobic isolates, whether *Bacteroides* or *Clostridium* spp., were saccharolytic, a finding which differs markedly from results obtained with mammals, in which the large proportion of *Bacteroides* species in the colon are saccharolytic (27, 31).

Mucin, along with cell sloughings and remnants of dead bacteria, would be an expected source of nutrients for the intestinal bacteria during hibernation. The fact that these bacteria metabolized hog gastric mucin even at 4°C provides evidence that the flora in hibernators is capable of growing. The utilization of mucin is consistent with the finding that ruminal, cecal, and colonic bacteria in mammals and birds can degrade complex plant polysaccharides and mucin oligosaccharides (27). The similarity of hog gastric mucin to frog colonic mucin is not known, though it is likely that since the intestinal bacteria utilized hog gastric mucin in vitro they would be capable of utilizing frog colonic mucin in vivo.

The metabolic experiments suggested a significantly slower pace of sustenance during hibernation, but other methods for reducing the bacterial population could not be discounted. A hypothesis was put forth suggesting that perhaps hibernating frogs synthesize a compound(s) that is
nonhibernators

Hibernators

ABSORBANCE

Dilution

Prebleed

Postbleed 1

Postbleed 2

400 800 1600 3200 6400

FIG. 1. Average absorbance (plus or minus the standard error of the mean) for each dilution in an enzyme-linked immunosorbent assay for frog anti-BSA antibody. The prebleed was taken before immunization. A booster immunization followed the primary immunization by 2 weeks. Postbleed 1 was 1 month after the boost, and postbleed 2 was 2 months after the boost. An asterisk indicates postbleed value significantly higher than prebleed value (P < 0.05, determined by paired t test). (A) Nonhibernators (n = 6); (B) hibernators (n = 5).

inhibitory to a wide range of bacterial species within the large intestine and thereby keep this population in check. This theoretical compound(s) was sought for in filtrates of the large intestine and liver. The large intestine was chosen because it would be the site of action of the substance, and the liver was chosen because compounds from the liver, such as bile acids, are secreted into the intestine. No inhibition was seen with any filtrate, liver fraction, or frog serum of any of the bacterial species that were tested. The lack of any observed inhibition in these trials does not preclude the possibility that an inhibitory substance exists, such as the powerful, wide-spectrum, inhibitory substance recently isolated from the skin of the African clawed frog, *Xenopus laevis*, by Zasloff (41). Antibacterial peptides in *R.*
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A colony reduction of at least 75% relative to the control was considered positive for serum killing.

**TABLE 4. Results of bacterial killing by frog serum**

<table>
<thead>
<tr>
<th>Serum source</th>
<th>Assay temp (°C)</th>
<th>No. positive*/*no. tested:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonhibernator</td>
<td>37</td>
<td>S. typhimurium: 0/5, P. paucimobilis: 7/7, Contents of large intestine: 1/5</td>
</tr>
<tr>
<td>Hibernator</td>
<td>37</td>
<td>S. typhimurium: 1/3, P. paucimobilis: 3/3, Contents of large intestine: 0/3</td>
</tr>
</tbody>
</table>

*p A colony reduction of at least 75% relative to the control was considered positive for serum killing.

pipiens would have been missed in our study if they were destroyed by endogenous proteases.

Previous studies had documented a hypofunctioning immune system in hibernators (1, 3, 8, 20, 25), either because of hibernation itself or because of the low temperature. It was possible, though, that the agglutination assays used in these studies were not sensitive enough to detect a small, but possibly sufficient, specific antibody response in hibernators or that naturally hibernating frogs may be immunologically responsive, whereas nonhibernators subject to low temperatures to simulate hibernation were not. Nonetheless, even the sensitive enzyme-linked immunosorbent assay failed to detect a positive antibody response to the BSA immunogen in hibernators. These results support the idea that hibernating frogs are incapable of mounting even a small humoral immune response. One possibility that was not tested, though, was that perhaps a particulate antigen like a bacterial suspension could elicit an antibody response in contrast to a soluble antigen like BSA.

The possibility existed that antibody formed before the frog entered hibernation could function in opsonization and complement-mediated lysis and that this could provide the hibernating frog with some degree of protection from bacterial invasion. Complement levels had been shown to decrease during hibernation (19), but any complement carried over into hibernation should be able to function at low temperatures (11, 36). Results from a serum killing assay implied that hibernating serum contained enough antibody and complement to kill certain bacteria or levels of antibody that could function in opsonization. The reduction in viable counts at 37°C was believed to be due to killing of bacteria and not clumping, since no reduction was seen at 4°C in most samples. However, heat-inactivated serum was not tested to ensure that clumping did not occur.

It was somewhat surprising that all frogs tested had activity against *P. paucimobilis*. Few, if any, *Pseudomonas* species were isolated from nonhibernators, whereas *Pseudomonas* species make up 25% or more of the flora in hibernators. It is possible that a nonhibernating frog may encounter *Pseudomonas* strains in its aquatic or terrestrial environment and that upon occasional ingestion or infection a specific immune response is generated. Alternatively, in addition, it is possible that *Pseudomonas* species within the intestine during the winter months prime the immune cells of the gut-associated lymphoid tissue, which can then synthesize antibody after the temperature rises in the spring. Antibody synthesis continues during the summer, and a circulating titer remains as the frog enters hibernation the following year. The latter alternative has a basis of support in observations that frogs immunized at low temperatures can synthesize antibody without an additional booster immunization when warmed (9, 12). It was also surprising that *S. typhimurium* was not more susceptible to serum. This organism was chosen to be a positive control because of a lipopolysaccharide core defect (40). The reason(s) for its resistance to killing by frog serum was unclear.

There have been conflicting reports about whether phagocytic capabilities in the frog are affected by temperature. It was reported both that low temperatures do not adversely affect phagocytosis (2) and that phagocytosis of erythrocytes ceases in the winter and resumes in the spring (15). We found that a small amount of carbon particles were slowly taken up by the Kupffer cells in the hibernating animals, but it was quite clear that to quantitative differences in carbon clearance between nonhibernators and hibernators were substantial. Such differences in clearance rates could be expected, given the difference in heart rates. But even after 12 h, substantially more carbon was taken up in nonhibernators than in hibernators. Phagocytosis thus occurs in the hibernator and is indicative of another means of defense for the frog, but clearance of particles from the circulatory system is substantially reduced.

Upon activation, a phagocytic cell undergoes a respiratory burst which can be measured by flow cytometry (35). Flow cytometry should have revealed a more quantitative difference between phagocytes from nonhibernators and hibernators than the India ink experiments. However, the majority of leukocytes isolated from both nonhibernators and hibernators appeared mononuclear and gave no oxidative response that would be detected in the flow cytometer when stimulated by phorbol myristate acetate.

The cumulative results suggest that low temperatures and lack of exogenous intake may be the foremost factors in the selection of the hibernating intestinal flora and the reduced bacterial concentration. Changes in other common mechanisms or factors operating to control the intestinal flora might be expected, if anything, to have resulted in an increased bacterial concentration. The hibernating frog retains some capacity for phagocytosis and some preformed antibody, which may help protect the frog from systemic infection with *Pseudomonas* species, which may become a large proportion of the intestinal flora during hibernation.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**


