(range, 3.5 to 6.0 percent; $N = 12$), 88 percent of which is protein. Combined with low protein levels (*5*), the paucity of nonprotein amino acids in Lake Sibaya detrital aggregate accounts for the stunting of the *S. mossambicus* living there.

These results resolve a long-standing problem concerning the trophic structure of aquatic ecosystems. It has never been clear whether animals that feed on detrital aggregate are microphages that obtain their nutrition exclusively from microorganisms, or are true detritivores (*14*). Since living microorganisms contain negligible amounts of free amino acids, nonprotein amino acids that support the rapid growth of *S. mossambicus* must be present in detritus. This establishes detritivory as a valid trophic category, comparable to carnivory and herbivory.

Finally, these findings raise several interesting questions. What factors influence the abundance of nonprotein detrital amino acids in aquatic systems? Are other detritivorous fish similarly dependent on nonprotein amino acids? Do these compounds play a role in the nutrition of invertebrate detritivores? Answers to these questions will help in the culture of *S. mossambicus* and will contribute to our understanding of aquatic ecosystems.

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Babesia bovis: Continuous Cultivation in a Microaerophilous Stationary Phase Culture

Abstract. *The protozoan parasite* Babesia bovis, *a causative agent of bovine babesiosis, has been continuously cultivated in a settled layer of bovine erythrocytes. Lowered oxygen tension within the layer of host erythrocytes results in a darkening of infected cultures and provides a rapid means of evaluating parasite growth. Deprivation of carbon dioxide causes the merozoites to accumulate in the medium rather than invading new erythrocytes. When separated from the culture, these extraerythrocytic parasites retain their infectivity. Parasites produced in vitro are morphologically identical to parasites from the blood of infected cattle and are susceptible to antibabesial drugs.*

Babesiosis is one of the most important tick-borne diseases of domestic animals: at least 1.3×10^9 domestic animals are at risk worldwide (*1*). Human babesiosis caused by parasites from bovine, rodent, and equine hosts is detected with increasing frequency, indicating the zoonotic potential of these organisms (*2*). Exotic babesias (*3*) occasionally infect animals in the United States. The cattle babesia, *Babesia bovis*, is an obligate intraerythrocytic parasite that occurs in the tropics and subtropics, and its pathogenicity in cattle closely resembles that of *Plasmodium falciparum* in humans. The finding that *P. falciparum*, the causative agent of malignant tertian malaria, could be successfully cultured in vitro (*4*) suggested that the bovine parasite could be cultivated under similar conditions. However, attempts to use the Trager-Jensen method for cultivation of *B. bovis* were unsuccessful (*5*). Short-term cultivation of *B. bovis* was recently accomplished in constantly agitated cultures (*5*), and, by lowering the *p*H to 7, this spinner flask method is reported to support continuous growth (*6*). Relatively low parasite yields (*7*) and the large culture volumes required by this technology limit its application.

Table 1. Statistical data for the microaerophilous stationary phase (MASP) culture of *Babesia bovis*.

Days in culture	82.6
Number of subcultures	33
Parasitemia of 2-day-old subculture*	19.9 ± 7.3
Parasitemia of 3-day-old subculture†	26.1 ± 7.1
Maximum parasitemia	38.1 ± 3.0
Cumulative dilution	2.49×10^{26}
Cumulative increase	1.74×10^{27}
1 log increase‡	73.2 hours

*Mean age, 44.5 hours. †Mean age, 68.6 hours. ‡For routine cultivation, this rate was observed; however, when cultures were subjected to a cumulative dilution of 3.15×10^{17}-fold over 31 days, an average of 42.3 hours was required for a 10-fold increase.

Continuous cultivation of *B. bovis* in a settled layer of bovine erythrocytes has now been achieved, with the parasitemias occasionally exceeding 40 percent. To initiate the cultures we obtain defibrinated peripheral blood from intact or splenectomized *B. bovis*-infected cattle when the parasitemias reach 0.1 to 2 percent (*8*). The use of standard anticoagulants in blood collection, for example, heparin, ethylenediaminetetraacetic acid (EDTA), or acid-citrate-dextrose, is detrimental to the parasites. Infected erythrocytes are suspended to a final packed cell volume of 9.1 percent in a medium consisting of 60 percent Medium 199 (*9*) and 40 percent normal bovine serum (NBS) (*10*), supplemented with 15 m*M* of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), 100 μg of streptomycin, and 100 units of penicillin G per milliliter (*11*). The culture suspensions are titrated to *p*H 7 with 1*N* HCl (*12*); portions of the suspensions are then placed in containers in a ratio of 0.62 ml of suspension to 1 cm^2 of culture area. The cultures are incubated at 37° to 38°C under an atmosphere of 5 percent CO_2 and 95 percent humidified air. Every 24 hours the overlying medium is removed and replaced with fresh *Babesia* tissue culture medium No. 4. This medium consists of 40 percent NBS and 60 percent

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Medium 199 and is supplemented with Hepes and antibiotics and titrated to *p*H 7 as already described. After 48 to 72 hours, subcultures are prepared by diluting old culture 3 to 25 times with medium containing freshly collected uninfected bovine erythrocytes (new culture). The parasites in each subculture multiply for only 3 to 4 days; maximum multiplication rates are achieved when parasitemias are reduced to 0.5 to 1.0 percent at each passage.

For routine maintenance, we use flat-bottom tissue culture microtiter plates. Each well has a diameter of 6.4 mm and receives 0.2 ml of culture; 16 wells are used for each passage. By using a variety of tissue culture containers we have scaled up the cultures more than 750-fold, and as long as the proper culture depth is maintained (0.62 cm), there appears to be no limit to the volume of a single culture.

Parasitemias are determined by counting 500 to 1000 erythrocytes on Giemsa-stained thin blood films from triplicate culture wells. Each well is sampled only once so as not to introduce the variable of erythrocyte removal. The amount of parasite dilution at each subculture is determined by obtaining the packed cell volume of the old culture and the new culture and the parasitemia of the old culture.

Under the described culture conditions, the erythrocytes become dark red to black, indicating a condition of low oxygen tension (deoxyhemoglobin). Resuspension of the cells, addition of fresh oxygenated medium, or lowering of the temperature to 4°C results in reversion to the bright red appearance of oxygenated erythrocytes. The darkening of erythrocytes cannot be attributed to acidic conditions because the *p*H of the culture increases to approximately 7.2 during incubation. Generally, cultures containing less than 1 percent infected erythrocytes fail to darken. These observations indicate that the depletion of oxygen from the settled cell layer results from parasite metabolism. The column of medium above the cells acts as a barrier to oxygen exchange and its depth is crucial to successful cultivation. Reducing the depth to 0.16 cm maintains the cultures in an oxygenated state (bright red) and such cultures cannot be maintained continuously. Moreover, there appears to be a minimum seed density, because cultures that fail to achieve parasitemias great enough to darken the cells within 4 to 5 days begin to produce aberrant and degenerate parasite forms and eventually die out. Thus the culture conditions allow the parasites to become established as a result of their metabolic activity which reduces the oxygen in the environment. The term microaerophilous stationary phase (MASP) culture is used to describe our culture system.

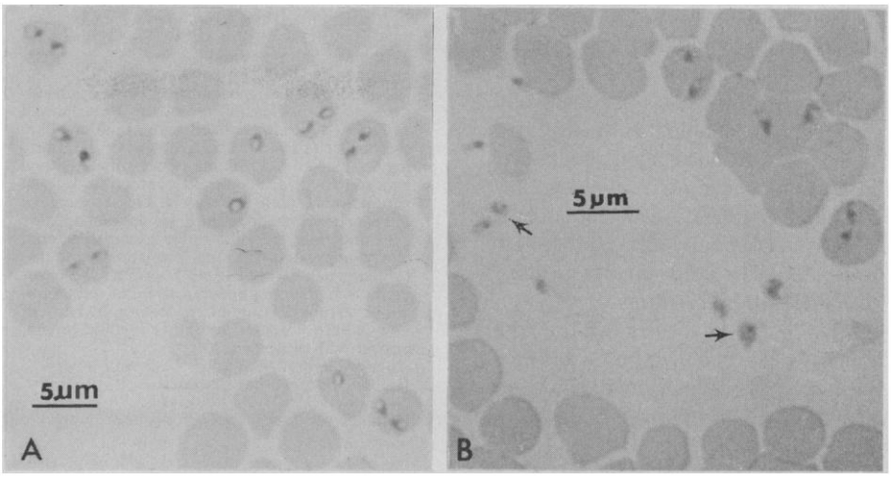

Fig. 1. (A) *Babesia bovis* from a microaerophilous stationary phase (MASP) culture after 105 days of cultivation in vitro. The parasites are morphologically indistinguishable from those observed in the blood of infected animals. This specimen was stained with Giemsa after fixation in Wright's-methanol. (B) Merozoites of *B. bovis* (arrows) that accumulate in MASP cultures incubated under ambient air (CO_2-deprived) for 6 hours. Intraerythrocytic parasites retain their normal morphology. The specimen was prepared as in (A).

Isolation of *Babesia* in MASP cultures from infected cattle has been accomplished on four occasions and in all instances the growth rates during the initial culture resembled the rates in cultures passaged several times. This suggests that a period of adaptation or selection does not occur. The statistical data for one such isolate (see Table 1) are typical of all four isolates. After several months in vitro, culture-derived *Babesia* appear identical to forms seen in bovine blood (Fig. 1A).

The unusual requirement for a relatively high serum concentration in the culture medium is well documented (Table 2). Even serum concentrations that fail to support continuous growth, for example, 80 percent, permitted 15-fold increases in growth during the first 48 hours. This indicates that caution should be exercised in evaluating the effects of altering the culture conditions during short-term studies.

The color change of infected cultures from bright red (no infection) to dark red and black (heavy infection) offers a simple means by which growth can be

Table 2. Growth of *B. bovis* in MASP cultures with various serum concentrations. The percentage increase in growth is compared to that obtained with 40 percent serum. Serum or erythrocytes (when required for subcultures) were collected on the day used and the results of three sequential subcultures are reported.

Percentage of serum	Initial percentage parasitemia (IP)	Final percentage parasitemia (FP)	Cumulative increase (CI)*	Morphology	Percentage increase in growth
		Experiment 1			
20	0.5	0.3	41	Degenerate	1
30	0.5	9.9	1451	Good	37
40	0.5	22.8	3958	Good	100
40 (frozen)†	0.5	26.4	4092	Good	103
		Experiment 2			
40	0.8	16.9	3177	Good	100
50	0.8	14.4	2689	Good	85
60	0.8	1.5	412	Degenerate	13
80	0.8	0.2	45	Degenerate	1
100	0.8	< 0.2		Degenerate	

*CI = (FP/IP) CD, when CD (cumulative dilution) is the factor by which the erythrocytes present at the start of the experiment are diluted with fresh erythrocytes during subcultures. †Serum stored at −20°C for 2 to 4 weeks was used in preparation of medium and normal culture. As with other groups, freshly collected erythrocytes were used as host cells.

evaluated. For example, addition of various concentrations of the antibabesial drug 1,3-bis(*p*-amidinophenyl)triazene-bis(*N*-acetylglycinate)-diaceturate (*13*) to cultures with initial parasitemias of 0.3 to 0.5 percent enables us to titrate the drug's action, because a lethal concentration (3.5×10^{-6} g/ml) prevents culture darkening. A gradation of color change is observed between lethal and nonlethal dosages and this color change is closely correlated with parasitemias.

Extracellular babesias (merozoites) are rarely observed in cultures during the exponential phase of growth, indicating that almost all the parasites are able to rapidly infect new cells. However, when cultures are removed from a CO_2 incubator to an incubator containing ambient air (CO_2-deprived), merozoites accumulate in the medium (Fig. 1B). These merozoites can be separated from the erythrocytes by differential centrifugation (*14*) and they remain infectious for several hours.

Examination of the physiological, biochemical, and antigenic properties of *B. bovis* has been hampered by the absence of a method for cultivating it in vitro. The MASP culture system described here will facilitate investigations of this, and perhaps other related pathogens. Parasite antigens generated in vitro in the absence of host immune and metabolic elements and heretofore unobtainable from infected animals should now become available. A study of isolated infectious merozoites made possible by this culture method may provide valuable insight into the characteristics of merozoites of other hemoprotozoa, for example, *Plasmodium* sp., the cause of human malaria.

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7. In contrast to the spinner flask method (*6*) which will support parasite multiplication of 6.4×10^5 times over a 32-day period, increases of 1×10^{18} times are achieved by the method described here over a similar time span.
8. A Mexican isolate of *B. bovis* was used.
9. With Hanks salts (Gibco).
10. Freshly collected bovine blood obtained from mature *Bos taurus* cows is defibrinated by shaking with glass beads and then centrifuged at 1000*g* for 10 minutes at 4°C. The serum may be used immediately, stored for up to 3 days at 4°C, or frozen at −20°C for later use.
11. We have used two procedures for preparing cultures. Erythrocytes and serum may be separated as outlined (*10*) and mixed with Medium 199 in the proper ratios. Alternatively, after determining the packed cell volume of defibrinated blood and adjusting for its serum content, additional serum and Medium 199 may be added to achieve the proper proportions. *Babesia* are damaged by centrifugation (they show, for example, altered morphology and staining properties, and retarded growth for 24 to 48 hours) so the latter method is recommended for initial isolation. However, there is no difference in the ability of new culture prepared by either method to support growth of parasites.
12. An initial *p*H range of 6.9 to 7.2 is satisfactory.
13. Berenil; Farbwerke Hoechst Ag., Frankfurt, Germany.
14. Cultures deprived of CO_2 for 4 to 6 hours are decanted into 50-ml conical centrifuge tubes and sedimented at 400*g* for 5 minutes at 4°C. The top 30 ml is removed and sedimented at 750*g* for 10 minutes. This supernatant is removed and again sedimented at 750*g* for 10 minutes. Centrifugation of the final supernatant at 1000*g* for 20 minutes produces a pellet containing free merozoites and some erythrocyte stroma. These merozoites may be used to infect normal MASP cultures that have been in a CO_2 incubator for at least 1 hour.
15. When infectious merozoites were added to new cultures that had been incubated for 1 hour under CO_2, most of them invaded normal erythrocytes within 1 hour after inoculation, and infection was completed within 3 hours. Merozoites stored in new cultures at 4°C for 16.6 hours under air retained 20 percent of their initial infectivity.
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Mebendazole Therapy of Parenteral Trichinellosis

Abstract. *Mebendazole was highly effective against the helminth parasite* Trichinella spiralis *in mice subjected to a 3-day course of treatment during the invasive and encystment phases of experimental trichinellosis. When treatment began either 2 or 4 weeks after the mice were inoculated with parasites, the number of larvae developing in the host musculature was greatly reduced by twice-daily oral administration of 3.125, 6.25, or 12.5 milligrams of mebendazole per kilogram of body weight.*

Recent reports have demonstrated the chemotherapeutic effectiveness of a benzimidazole anthelmintic, mebendazole, in the treatment of *Trichinella spiralis* infection in rats and mice (*1*–*7*). Because mebendazole (methyl 5-benzoyl-2-benzimidazolecarbamate) is used for the treatment of other helminthoses in man, and because it is highly active against enteral and parenteral forms of *Trichinella* in laboratory animals, this compound is one of great potential utility in the treatment of human trichinellosis. However, there have been comparatively few studies of the susceptibility of *T. spiralis* to mebendazole treatment during the invasive phase of experimental trichinellosis in mice (that is, when the second generation larvae, shed by adults in the gut, have penetrated the muscle cells and when they are in various early stages of encapsulation within muscle fibers) or of the efficacy of dosage regimens similar to those used in man.

Table 1. Number of *T. spiralis* larvae recovered from the musculature of control mice and mice treated by stomach tube with mebendazole twice daily for three consecutive days (days 14 to 16) during the invasive phase of infection.

Larvae per mouse at mebendazole dose (mg/kg)			
0.0	3.125	6.25	12.5
Individual counts			
3,200	*	0	*
11,000	1,200	200	0
11,142	1,320	800	660
11,600	1,400	1,400	1,000
12,100	1,760	2,280	1,200
12,540	3,520	3,200	1,400
13,250	5,850	3,500	1,600
23,000	6,400	6,250	2,600
Mean			
12,229	3,064†	2,204†	1,209†
Efficacy (%)	75	82	90

*Mouse dead before scheduled necropsy. †$P < .05$.

Evidence for a lethal effect of mebendazole on *T. spiralis* during the invasive phase in mice was obtained recently (*7*) by administering mebendazole (50 mg/kg) by stomach tube once daily for five consecutive days (days 14 to 18) of infection. This dosage regimen significantly reduced by 96 percent the number of larvae subsequently recovered from the host musculature on day 45 after inoculation. Although this dosage was extremely high relative to other anthelmintic applications of the drug, the results clearly indicated that the first larval stage of *T. spiralis* was vulnerable to mebendazole treatment. It was then reasoned that a comparable level of drug activity against *Trichinella* might be obtained with a much lower unit dosage of mebendazole if the anthelmintic were administered in a series of divided daily oral doses during the invasive phase (and presumably the encystment phase as well) of infection. In this way, very small quantities of the drug would continuously be available to the parasite via the host bloodstream and tissue fluids and the total dosage required to achieve efficacy

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