Nutritional Significance of Symbiotic Bacteria in Two Species of Hemoflagellates

Abstract. *Symbiote-free strains of* Blastocrithidia culicis *and* Crithidia oncopelti, *obtained by chloramphenicol treatment, were compared nutritionally with normal, symbiote-containing strains. The symbiotic bacteria spare the flagellates requirements for exogenous hemin and for other nutritional factors present in liver extract.*

Endosymbiotic microorganisms occur regularly within the cells of many different species of invertebrates (*1*). Since their numbers are strictly limited and since they are transmitted from generation to generation, often by specialized mechanisms, they appear to have become integrated into the physiology of their hosts. It has even been suggested that mitochondria might have evolved from endosymbiotic bacteria associated with a primitive eukaryote cell (*2*). The endosymbiotes are generally considered to serve some function useful to their host, but this has been demonstrated in relatively few instances. The intracellular microorganisms in a number of insect species have been shown to provide their host with essential growth factors, thereby enabling the insect to live on diets that would otherwise be inadequate (*3*).

Among the protozoa, the best-known endosymbiotes are kappa and related killer particles of *Paramecium* and other ciliates (*4*). Their significance to their hosts is not clear however, although here again one type of symbiote, lambda, has been found to render its host not dependent on an exogenous supply of folic acid (*5*). We here present evidence for the nutritional role of symbiotic bacteria in two species of hemoflagellates.

Among insect trypanosomatid flagellates, *Blastocrithidia culicis* and *Crithidia oncopelti* possess intracellular, self-reproducing entities known as "diplosomes" and "bipolar bodies" (*6*). In an unpublished study, Brueske (*7*) reported having cured *B. culicis* of its diplosomes by using chloramphenicol. He suggests that they are bacterial endosymbiotes nutritionally beneficial to their hosts. A similar suggestion had been made earlier for the bipolar bodies of *C. oncopelti*. Evidence for this was based on ultrastructure, DNA base composition, penicillin sensitivity, and enzyme biochemistry of bipolar bodies (*8*), as well as nutritional study of the flagellates and of a cured strain (*9*). However, biochemical evidence for the presence of a bacterium-specific enzyme in the bipolar bodies proved to be unreliable (*10*), and their sensitivity to penicillin could not be substantiated (*11*). Moreover, the origin of the alleged cured strain became questionable, as it appeared to resemble *Crithidia fasciculata* more than *C. oncopelti* in DNA base composition and in serological characteristics (*12*). Thus, it was questioned whether bipolar bodies are true bacterial endosymbiotes having nutritional significance in the flagellates (*12*). Recently, the bacterial nature of bipolar bodies was indicated by the finding of 70*S* ribosomes in them and inhibition of their protein synthesis by chloramphenicol (*13*). We have studied the diplosomes of *B. culicis* and bipolar bodies of *C. oncopelti* by electron microscopy, by antibiotic "therapy" to obtain cured strains, and by comparing the growth of normal and cured strains in various media.

Both species of flagellates were routinely maintained at 27°C in Grace's insect tissue culture medium or Trager's chemically defined medium (*14*). For electron microscopy, flagellates were fixed by glutaraldehyde followed by osmium tetroxide or by Kellenberger's standard fixation, and embedded in Epon. In thin sections, diplosomes and bipolar bodies had electron-lucid zones containing fibrillar material. The fibers measured approximately 3 to 5 nm thick, resembling DNA filaments in the nuclear plasma of conventional bacteria (*15*). To obtain a cured strain of *B. culicis*, flagellates were inoculated into a blood broth modified after Wallace (*16*), to which 800 μg of chloramphenicol (Sigma) per milliliter was added. In this medium, growth of flagellates was very limited initially, but resumed 10 to 20 days after inoculation in most cultures. Flagellates then reached a maximal population of 10×10^6 to 20×10^6 cells per milliliter, and thenceforth could be transferred continuously in the blood medium with or without chloramphenicol. After several transfers, flagellates became predominantly slender active forms that grew better (25×10^6 to 30×10^6 cells per milliliter) and formed a thin pellicle. To study the effect of chloramphenicol on the diplosomes of *B. culicis*, flagellates were processed for electron microscopy after different intervals in chloramphenicol medium. Nuclear zones of diplosomes became increasingly expanded—a phenomenon comparable to the effect of chloramphenicol on *Escherichia coli* (*17*). As the treatment proceeded further, diplosomes became degenerate. After about 16 days in the chloramphenicol medium, no diplosomes could be recognized in the flagellates, which corresponded to the cured strain. A cured strain of *C. oncopelti* was similarly obtained after three transfers of the flagellates in the chloramphenicol medium during a period of 36 days or more. The cured strains of *B. culicis* and *C. oncopelti* have been transferred weekly for more than 6 months in blood broth and appear to be stable. The absence of diplosomes and bipolar bodies in the flagellates of cured strains was frequently checked and confirmed by light microscope observation after Tween-80–Giemsa treatment and by electron microscopy.

Cured strains of *B. culicis* and *C. oncopelti* became nutritionally fastidious; they did not grow as well in blood broth as the normal strains and failed to grow in Grace's or Trager's medium, which supported the growth of normal strains (Table 1). This provided another criterion for the absence of diplosomes and bipolar bodies in the cured strains and also indicated their nutritional value to the flagellates. To explore this nutritional relationship further, cured strains were tested for growth in Trager's medium supple-

Table 1. Growth of normal and "cured" strains of *B. culicis* and *C. oncopelti* in various media. Each figure represents the average count, in millions per milliliter, of at least five tests. Counts were done at late log-phase. Dash, no test; No, no growth.

Media*	*B. culicis*		*C. oncopelti*	
	Normal	"Cured"	Normal	"Cured"
BB	168	27	79	13
GM	122	No	67	No
TM	140	No	65	No
TM + L	—	20	—	20
TM + L + BL	—	27	—	22
TM − H	130	No	65	No
TM − H + L	—	No	—	No

* BB, blood broth consisting of one part of each of following components: GM, neopeptone broth (5.5 percent), and BL (rabbit blood lysate, 1:6 in distilled water); GM, Grace's insect tissue culture medium with 10 percent fetal bovine serum; TM, Trager's chemically defined medium; TM + L, TM containing 0.25 percent aqueous liver extract (1:20, Nutritional Biochemicals Corp.); TM + L + BL, TM + L plus 10 percent rabbit blood lysate; TM − H, TM without hemin; TM − H + L, TM − H plus 0.25 percent aqueous liver extract.

mented with various nutritives. Any growth resulting from the addition of a particular material would imply that it contained essential nutrient or nutrients normally supplied to the flagellates by diplosomes or bipolar bodies. For each test, 2 ml of desired medium was inoculated with 2×10^6 flagellates, and growth, if any, was determined by hemocytometer count at late-log phase. Growth was considered positive only when flagellates continued to grow successfully for at least five transfers in the same medium. Each medium was tested at least twice in duplicate. Among various additives tested, only aqueous liver extract (1 : 20, Nutritional Biochemicals Corp.) at a concentration of 0.25 percent in Trager's medium (TM + L) stimulated the growth of cured strains (Table 1). A higher concentration (over 1 percent) of liver extract was inhibitory. Cured strains did not grow in Trager's medium enriched with fetal bovine serum, whole blood, or blood lysate of rabbit. However, the presence of these materials in TM + L seemed to improve or stabilize their growth in that thin pellicles were often formed and the cell count increased slightly (Table 1).

Although the growth factor in liver extract for the cured strains has not been characterized, its use with Trager's medium (which normally contains hemin) has made possible a comparison of the hemin requirement of normal and cured strains. The defined medium from which hemin was omitted (TM − H) was tested for growth of the normal strains and, supplemented with 0.25 percent liver extract (TM − H + L), for that of the cured strains (Table 1). The results showed clearly that normal strains of both species do not require hemin. On the other hand, strains free from endosymbiotes have a hemin requirement that is not supplied adequately by the liver extract. These symbiote-free strains thus resemble in their hemin requirement other species of trypanosomatids that normally do not have endosymbiotes, such as *C. fasciculata* and *Leishmania tarentolae* (*18*).

Our findings confirm that diplosomes of *B. culicis* and bipolar bodies of *C. oncopelti* are bacterial endosymbiotes. Furthermore, they indicate that these endosymbiotes supply their hosts with hemin and other essential nutrients.

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Cyclic Adenosine Monophosphate: Selective Increase in Caudate Nucleus after Administration of L-Dopa

Abstract. *Treatment with the dopamine precursor* L-*dopa produced a significant accumulation of adenosine 3′,5′-monophosphate (cyclic AMP) in the caudate nucleus of the rat. In contrast, there was no change in the amount of cyclic AMP in the cerebellum. Accumulation of cyclic AMP in the caudate nucleus after administration of* L-*dopa was prevented by prior treatment with the decarboxylase inhibitor RO 4-4602. These observations and those in other laboratories support the assumption that dopamine formed from* L-*dopa selectively activates striatal adenylate cyclase. The in vivo activation of adenylate cyclase after treatment with* L-*dopa may be a useful model for studying neurological and psychiatric disorders that are thought to involve the dopaminergic system of the brain.*

A dopamine-sensitive adenylate cyclase has been identified in the sympathetic ganglia (*1*), the retina (*2*), and the caudate nucleus (*3*). These in vitro studies and a variety of supporting evidence led to the hypothesis that this enzyme represents a "dopamine-receptor" and that adenosine 3′,5′-monophosphate (cyclic AMP) may be implicated in synaptic transmission in the central nervous system (*3*).

We report here that when L-dopa is

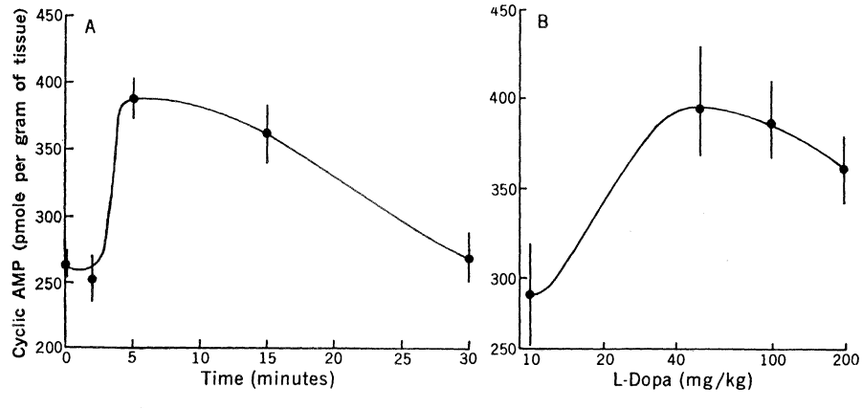

Fig. 1. (A) Concentration of cyclic AMP in the caudate nucleus of the rat after injection of L-dopa (100 mg/kg, intraperitoneally). Rats were killed in a microwave oven, and cyclic AMP was assayed by a protein-binding procedure (*7*). Data are means ± standard errors of mean (S.E.M.) for four to nine determinations. Cyclic AMP concentrations were significantly different ($P < .01$) from control values 5 and 15 minutes after L-dopa treatment. (B) Concentration of cyclic AMP in the caudate nucleus of the rat after various doses of L-dopa. Animals were killed 5 minutes after the injection; data are as in (A). Cyclic AMP concentrations were significantly different ($P < .01$) from control values at L-dopa doses of 50, 100, and 200 mg/kg.