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The endosymbionts of *Glossina morsitans* and *G. palpalis:* cultivation experiments and some physiological properties

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Summary

Pyruvate, malate, and succinate are the main substrates for bacteroid respiration; oxygen uptake can be inhibited by rotenone and antimycin A, but not by cyanide. The symbionts displayed limited growth and survival for over 80 days in a medium with succinate and pyruvate as main substrates, and supplemented with nucleotides. It was not possible to cultivate the endosymbionts of *G. morsitans* and *G. palpalis* intracellularly in cell cultures of the tsetse fly or of vertebrates. A high attraction between cells and symbionts was observed in these systems; about 10% of all bacteroids were incorporated by the cells but they were lysed and digested within 48 h.

Key words: endosymbionts; mycetome; bacteroids; *Glossina* cell culture; respiratory metabolism; oxygen uptake; *Glossina morsitans; Glossina palpalis.*

Introduction

Cultivation of tsetse midgut mycetome endosymbiotic bacteroids has thus far not been possible (e.g. Reinhardt et al., 1972). Consequently, little is known about their physiology (Nogge, 1976) and systematic position; the same applies to other intracellular bacteroids of insects (Buchner, 1965; Lanham, 1968; Brooks, 1970, 1976; Kurtti, 1974).

We attempted to culture the endosymbionts of *Glossina* intracellularly by using recently established cell culture systems of the tsetse fly (I. Schneider, pers. comm.; Wink, 1977a, b).

In addition the respiratory metabolism of the symbionts was subjected to a preliminary investigation, and these experiments resulted in defining a cell-free medium in which the symbionts could be maintained over extended periods.

Material and methods

Endosymbionts. 20 to 30-day-old pupae of *Glossina m. morsitans* Westwood and *G. p. palpalis* (Robineau-Desvoidy) were surface sterilized (2.5 μg fungizone/ml 70% ethanol for 20 min). The puparium was removed; the pupae were dissected in a tsetse tissue culture medium (GCM; Wink, 1977a, 1979) and their mycetomes excised. Symbionts were released from their mycetocytes by rupturing the mycetome with fine forceps. Cultures were started by inoculating of 10^5 to 10^6 symbionts (= 1 to 2 mycetomes) in polysterene tissue culture flasks (Falcon) containing 2 to 3 ml of culture medium.

Viability of the endosymbionts was indicated by their motility and was further assayed by an acridine orange test (Strugger, 1949): living symbionts displayed a green fluorescence and dead bacteroids a red fluorescence when acridine orange was added at a dilution of 1:200000; acridine orange was dissolved in GCM.

Cell culture. Pupal, haemocyte-like cells of *G. morsitans* and *G. palpalis* were cultured in suspension (GCM with 10% fetal calf serum (FCS; Gibco)). A larval monolayer cell line of *G. morsitans* (established by I. Schneider) was maintained in Schneider's medium or GCM with 15% heat inactivated FCS. Glossina cells were cultured at $25 \pm 0.5°$ C according to Wink (1977a).

Vertebrate monolayer cell cultures, consisting of Human epidermal cells (Hep), Human embryonic lung cells (Hel), or HeLa-cells were grown in Eagle's medium (MEM) with 15% FCS and were kept at 37° C under standard conditions.

Media. All symbiont and *Glossina* media were adjusted to a pH of 6.9 and sterilized through EKS I filters (Seitz) or disposable millipore filters (0.45 μm).

Sterility. Sterile conditions were maintained for all experiments. If bacterial growth was apparent in any culture, a sample was plated on CASO-agar (Merck) and incubated at 25° C. Any microorganisms able to grow under these conditions were considered to be contaminants, even if they resembled symbionts morphologically.

All cultures were observed daily with an inverted microscope (Diavert, Leitz). With these precautions confusion of the symbionts with bacterial contaminants was ruled out (Brooks, 1970).

Polarographic oxygen determination. Oxygen uptake was measured according to Beechey and Ribbons (1972). The oxygen electrode (Beckman) was maintained at $25 \pm 0.5°$ C and the fluid in the reaction chamber (2 ml) was continuously stirred. The buffer solution (Keeley, 1971) consisted of 30 mmol/l K_2HPO_4, 15 mmol/l KCl, 5 mmol/l $MgCl_2 \cdot 6 H_2O$, 2 mmol/l EDTA, 50 mmol/l Tris and was adjusted to a pH of 6.9.

The isolated mycetomes were gently pulled apart and cleaned of tissue fragments. The symbionts were further released from the mycetocytes by continuous stirring in the reaction chamber. 1 to 3×10^7 bacteroids were employed in each test.

Results

Respiratory metabolism of the endosymbionts

A preliminary study on the respiratory metabolism, employing polarographic oxygen determination, was carried out to gain some information on the nutritional requirements of the bacteroids. Due to technical difficulties, all test suspensions contained symbionts as well as fragments of the host cells. Respiration of the contaminating gut cells, however, could be completely inhibited by addition of cyanide, whereas the oxygen uptake of the symbionts was not affected by CN^-. On account of this property the oxygen uptake of bacteroids in the presence of CN^- was determined in relation to different substrates; see Table 1.

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Table 1. Respiration of the endosymbionts of *G. palpalis*

	Number of experiments	Oxygen uptake nmol $O_2 \cdot h^{-1} \cdot 10^{-7}$ symbionts $\bar{x} \pm$ standard error (SE)
Total respiration of suspension*	12	541.0 ± 96.1
After addition of 5 mmol/l CN^{-**}	12	46.8 ± 6.3
5 mmol/l CN$^-$		
+ pyruvate	5	136.0 ± 50.5
+ succinate	8	97.5 ± 14.4
+ pyruvate/succinate	9	350.7 ± 42.7
+ pyruvate/malate	3	462.0 ± 62.7
+ pyruvate/I.....................	9	129.9 ± 9.5
+ succinate/II	10	129.5 ± 14.3

 I: Substrates of the citric acid cycle and related compounds (glyoxylate, glutamate, proline), except succinate, malate.

 II: Substrates of the glycolytic pathway and related compounds (trehalose, alanine, acetyl CoA, acetate), except pyruvate.

All substrates were applied at a concentration of 5 to 20 mmol/l.

 * High O_2 uptake due to contaminating fragments of gut cells.
 ** Residual activity may be caused by endogeneous substrates.

Table 2. Influence of inhibitors and other effectors on the respiration rate of tsetse endosymbionts. The value of a respiring suspension was set at 100% (previously treated with 5 mmol/l CN$^-$)

Compound	Number of experiments	Resulting O_2-uptake $\bar{x} \pm$ SE (%)
10 mmol/l CN$^-$	4	104.0 ± 9.7
+20 μmol/l rotenone	9	60.2 ± 7.4
+20 μmol/l rotenone +5 μmol/l antimycin A ...	7	12.4 ± 5.4
5 μmol/l CCCP	5	150.5 ± 15.6
2 mmol/l ADP.............................	5	142.8 ± 17.1
10 mmol/l malonate	3	65.6 ± 5.2

Of all the substrates tested, pyruvate and succinate, when given together, promoted a maximal respiration of 580 nmol $O_2 \cdot h^{-1} \cdot 10^{-7}$ symbionts. Succinate could be replaced by malate only.

The oxygen uptake was inhibited by 40% when rotenone was added to the respiring symbionts. It could be reduced to 13% of the normal respiration by further addition of antimycin A (see Table 2).

Table 3. Affinity between *Glossina* endosymbionts and various cell types. 48 h prior to the experiments cell cultures were diluted so that the cell colonies were up to 100 μm apart. After inoculation of the symbionts the cultures were left unchanged for 3 h at 25°C, and the distances between a symbiont and 2 neighbouring cells were measured. An index was calculated (A/B) where A always is the shorter distance. The index shows the relative affinity of the cells for the symbionts; a small value indicating high affinity

Cell culture system		A/B frequencies									
		0.0–0.1	–0.2	–0.3	–0.4	–0.5	–0.6	–0.7	–0.8	–0.9	–1.0
Hel		22	9	6	9	4	2	4	0	0	0
Hep		35	7	1	1	7	6	5	0	1	6
HeLa		23	3	3	4	4	2	1	1	0	2
G. morsitans											
– old medium	a*	47	8	7	3	2	3	1	1	0	0
	b*	38	9	7	6	0	1	0	0	0	2
– fresh medium	a	19	18	9	4	4	5	4	3	4	2
	b	19	10	6	1	1	3	1	4	3	7

* a = symbionts of *G. morsitans;* b = symbionts of *G. palpalis*

Culture of the symbionts in cell-free media

As a conclusion of the results obtained above a medium was devised which consisted of 10 mmol/l succinate, 20 mmol/l pyruvate, 1500 mg/l PPLO-broth and the polarographic buffer components. In 12 experiments symbionts were cultured in this medium and they remained viable for over 30 days. When the medium was further supplemented with nucleotides (ADP, CTP, UTP, TMP, GTP) and NAD^+ (1 mmol/l each) the symbionts (3 out of 4 cultures) elongated from 8 μm to over 24 μm within the first 3 weeks, then the cells divided to single units of 5 μm length. They remained viable for over 85 days; but no further growth was observed. Addition of 5 mmol/l ATP stimulated the motility of the symbionts significantly.

In 40 additional experiments (consisting of 120 trials) the *Glossina* cell culture medium was supplemented with different sera, substrates of the glycolytic pathway and citric acid cycle (5 mmol/l each), nucleotides, nucleic acids, coenzymes (1 mmol/l each), ecdysteroids and juvenile hormone I (0.8 μg/ml). No growth was observed in these experiments although the osmotic pressure and pH of all media seemed to be adequate; the symbionts died within the first 96 h of incubation.

Endosymbionts in cell culture systems

In contrast to culture in cell-free media, we attempted to grow isolated bacteroids intracellularly or extracellularly in cell and tissue cultures of *G. morsitans* and *G. palpalis* and of vertebrates (Hel, Hep. HeLa) at 25°C.

The symbionts displayed a similar behaviour in the insect and vertebrate systems: During the time that the bacteroids were still suspended in the medium above the cell monolayers, they often pointed directly towards the cells below. When the symbionts had sunk down to the bottom of the tissue culture flasks, they would slowly (maximum speed 10 μm/min) approach cells or cell colonies which were up to 200 μm away. Bacteroids of 8 to 10 μm length also bent slowly.

After 3 h of incubation, about 60 to 70% of all symbionts were closely associated with the *Glossina* cells and about 30–50% with the vertebrate cells (see Table 3). Other particles or cell fragments did not display this behaviour. The distribution pattern of the symbionts in relation to the cells was significantly ($p < 0.001$) different from random distribution. The number of associated bacteroids could be reduced significantly ($p < 0.01$) to 33% when the *Glossina* cell cultures were washed 3 times with fresh medium before inoculation of the symbionts. This fact indicates some sort of chemotaxis on the part of the bacteroids. The attraction towards *G. morsitans* cells was similar for symbionts of *G. palpalis* and *G. morsitans*. About 10% of the symbionts were incorporated by the cells. Microscopic examination showed that the bacteroids were lysed and were no longer visible 12 to 48 h after incorporation. The symbionts never grew or developed inside the cells or in the surrounding medium (>100 experiments).

Discussion

Endosymbiotic bacteroids are known from a number of blood-sucking insects. The symbionts of *Triatoma* have been cultured successfully in vitro (Bewig and Schwartz, 1965; Macarini and Garcia, 1972). A few authors claim to have grown the obligate intracellular symbionts of the tsetse fly in vitro (Southwood et al., 1975). Their experiments were repeated in this study but could not be confirmed (Wink, 1977a). Limited growth of isolated symbionts was achieved, however, in a cell-free medium which contained pyruvate, succinate, and nucleotides. The importance of nucleotides was supported by the observation that exogeneously supplied ATP stimulated the movement of the symbionts; uptake of ATP is also a known feature of the intracellular *Rickettsiae* (Davis et al., 1970).

The significance of pyruvate, malate and succinate as main metabolic substrates was confirmed in the study on the symbiont respiratory metabolism. It is not clear, however, why these substrates are only active when given simultaneously. Some substrates of the glycolytic pathway, the citric acid cycle, and related compounds did not promote respiration significantly. It cannot be excluded that these compounds were not transported across the bacteroidal membranes; in symbionts of the cockroach these metabolic pathways seem to be present (Laudani et al., 1974).

Some enzymes of the respiratory chain seem to be present in tsetse symbionts as indicated by the action of respiratory inhibitors (Table 2): NADH-dehydrogenase, succinate dehydrogenase (SDH), and cytochromes b and c. The presence of SDH is further supported by the inhibitory action of malonate; this enzyme was reported for cockroach symbionts (Brooks, 1970; Laudani et al., 1974). Cytochrome oxidase seems to be insensitive to cyanide, a phenomenon known in some bacteria and higher plants (Solomos 1977).

Oxidative phosphorylation could not be demonstrated with certainty; uncouplers as carbonylcyanide-m-chlorophenyl hydrazone (CCCP) did not exhibit a significant effect and the acceptor control ratios were as low as 1.4 (see Table 2); on the other hand oxidative phosphorylation can be uncoupled by tris, the buffer used in these assays.

In >100 experiments we tried to cultivate insect endosymbionts intracellularly in cell culture systems; only very limited success has been achieved so far, as is true for other workers (Schwemmler and Vago, 1970; Schwemmler, 1973; Kurtti, 1974). Even though close association between isolated symbionts and cells was soon established and 10% of the symbionts were incorporated by the cells, no growth could be detected; on the contrary all symbionts seemed to be lysed by the cells. Digestion of incorporated microorganisms is known in macrophages and insect haemocytes (Ratcliffe and Rowley 1974). Even established cell lines seem to produce a chitinase, which exhibits lysozymic activity (Landureau and Grellet, 1975). A similar control mechanism may be present in the tsetse cells, too; lysozyme was found to lyse the tsetse endosymbionts in vitro (Wink, 1977a).

The only possibility to grow symbionts intracellularly might be in mycetocyte cultures; these cells do not possess this cellular defence mechanism. Unfortunately, it was not possible in this study to propagate *Glossina* mycetocytes in vitro. The failure of tsetse symbionts to grow in vertebrate cells indicates, that they are not closely related to *Rickettsiae*, since the latter would have grown under these conditions (Suitor, 1964).

The data of this study suggest, that the symbionts of the tsetse fly are highly adapted intracellular organisms. They seem to depend on metabolic substrates and nucleotides provided by the host cells and in return synthesize essential substances for the tsetse fly, such as vitamins (Nogge, 1975, 1976).

1 Beechey R. B., Ribbons D. W.: Oxygen electrode measurements. In: Methods of microbiology, ed. by L. M. Norris and D. W. Ribbons, vol. 6 B, p. 25–53. Academic Press, London/New York 1972.

2 Brooks M. A.: Comments on the classification of intracellular symbiotes of cockroaches and a description of the species. J. Invert. Path. *16*, 249–258 (1970).

3 Brooks M. A.: Application of insect tissue culture to an elucidation of parasite transmission. In: Invertebrate tissue culture, ed. by K. Maramorosch, p. 181–199. Academic Press, London/New York 1976.

4 Bewig F., Schwartz W.: Untersuchungen über die Symbiose von Tieren mit Pilzen und Bakterien. VII. Über die Physiologie der Symbiose bei einigen blutsaugenden Insekten. Arch. Mikrobiol. *24*, 174–208 (1956).

5 Buchner P.: Endosymbiosis of animals with plant microorganism. Wiley & Sons, New York 1965.

6 Davis B., Dulbecco R., Eisen H., Ginsberg H., Wood W.: Microbiology. Harper & Row, New York/Evanston/London 1970.

7 Keeley L.-L.: Endocrine effects on the biochemical properties of fat body mitochondria from the cockroach, *Blaberus discoidalis.* J. Insect Physiol. *17*, 1501–1515 (1971).

8 Kurtti T. J.: The development of insect tissue culture systems for studying intracellular symbiotes. Ph.D.-Thesis, Minnesota. In: Dissertation Abstracts *35* (1974).

9 Landureau J. C., Grellet P.: Obtention de lignées permanentes d'hémocytes de Blatte. Caractéristique physiologique et ultrastructurale. J. Insect. Physiol. *21*, 137–151 (1975).

10 Lanham U. N.: The Blochman bodies: hereditary of intracellular symbionts of insects. Biol. Rev. *43*, 269–286 (1968).

11 Laudani U., Frizzi G. P., Roggi C., Montani A.: The function of endosymbiotic bacteria of Blattoidea. Experientia (Basel) *30*, 882–883 (1974).

12 Macarini J. D., Garcia E. S.: A synthetic medium for *Nocardia rhodnii,* a monoxenic symbiont of *Rhodnius prolixus.* Ann. Acad. bras. *44*, 604–605 (1972).

13 Nogge G.: Zur Bedeutung der Endosymbionten für Tsetsefliegen *Glossina morsitans* Westwood. Verh. dtsch. zool. Ges. *159* (1975).

14 Nogge G.: Sterility in tsetse flies *(Glossina morsitans)* Westwood caused by loss of symbionts. Experientia (Basel) *32*, 995 (1976).

15 Ratcliffe N. A., Rowley A. F.: In vitro phagocytosis of bacteria by insect blood cells. Nature (Lond.) *252*, 391–392 (1974).

16 Reinhardt N. A., Steiger R., Hecker H.: Ultrastructural study of the midgut mycetome bacteroids of the tsetse flies *Glossina morsitans, G. fuscipes,* and *G. palpalis* (Diptera. Brachycera). Acta trop. (Basel) *29*, 280–288 (1972).

17 Schwemmler W.: In vitro Vermehrung intrazellulärer Zikadensymbionten und Reinfektion aposymbiontischer Mycetocytenkulturen. Cytobios *8*, 63–73 (1973).

18 Schwemmler W., Vago C.: Infection expérimentale avec des bactéries symbiotiques des cultures de cellules et d'organes de l'Homoptère, *Euscelis plebejus* Fall. C.R. Acad. Sci. Paris *270*, 1644–1647 (1970).

19 Solomos T.: Cyanide-resistant respiration in higher plants. Ann. Rev. Plant Physiol. *28*, 279–297 (1977).

20 Southwood T. R. E., Khalaf S., Sinden R. E.: The microorganisms of the tsetse flies. Acta trop. (Basel) *32*, 259–266 (1975).

21 Strugger S.: Fluoreszenzmikroskopie und Mikrobiologie. Hannover 1949.

22 Suitor E. C.: Propagation of *Rickettsiella popilliae* (Dutky & Gooden) Philip and *R. melolonthae* (Krieg) Philip in cell cultures. J. Invert. Path. *6*, 31–40 (1964).

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23 Wink M.: Entwicklung von Zell- und Gewebekulturen bei Tsetsefliegen, *G. m. morsitans* und *G. g. palpalis*, und Versuch der in vitro Kultur ihrer bacteroiden Endosymbionten sowie von *Trypanosoma theileri*. Dipl. Thesis, University of Bonn 1977a.

24 Wink M.: Hormonal growth stimulation and inhibition of pupal cells of the tsetse fly *Glossina m. morsitans*, in vitro. Naturwissenschaften *64*, 226 (1977b).

25 Wink M.: *Trypanosoma theileri:* in vitro cultivation in tsetse fly and vertebrate cell culture systems. Int. J. Parasitology (in press, 1979).