

Zeitschrift: Acta Tropica
Herausgeber: Schweizerisches Tropeninstitut (Basel)
Band: 44 (1987)
Heft: 3

Artikel: The effect of "Trypanosoma brucei" infection on the localization of salivary gland cholinesterase in "Glossina morsitans morsitans"
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DOI: <https://doi.org/10.5169/seals-313857>

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The effect of *Trypanosoma brucei* infection on the localization of salivary gland cholinesterase in *Glossina morsitans morsitans*

T. K. GOLDER, N. Y. PATEL, N. DARJI

Summary

When salivary glands of the tsetse fly, *Glossina morsitans morsitans*, are stained for cholinesterase (ChE) activity, a net-like pattern of reaction product is observed surrounding each epithelial cell of the gland's secretory region. Glands infected with *Trypanosoma brucei brucei* show a progressive reduction in this ChE activity as the parasites develop. When the infection is mature, ChE is rarely detected in the epithelial layer but appears in the lumen of gland. The luminal ChE responds to substrates and inhibitors in the same manner as the epithelium-associated enzyme and appears to have leaked from the epithelium due to cellular damage in epithelium of the infected gland. The possible effect of glandular damage on feeding behaviour and state of health is discussed.

Key words: tsetse; trypanosome; salivary gland; cholinesterase.

Introduction

Although the tsetse fly has been known to be the vector of trypanosomiasis for more than 80 years, studies showing adverse effects of trypanosomes on tsetse have only recently been reported. Changes in feeding behaviour, including increased voracity and probing activity (Jenni et al., 1980; Roberts, 1981) have been reported as well as blockage of mechanoreceptors in the labrum causing reduced flow rate in the food canal (Livesey et al., 1980). The reported changes in feeding behaviour have not been universally accepted (Moloo, 1982), nevertheless Ryan (1984) reported that trypanosome-infected flies from a natural population were in a poorer nutritional state than noninfected flies. He attributed this nutritional state to possible impaired feeding ability or loss of energy due to energy sources being metabolized by trypanosomes. Based on

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theoretical considerations, Bursell (1980) reckoned that the daily loss of energy due to the trypanosome burden would be considerable. Trypanosome-infected flies have also been shown to be more sensitive to endosulfan and a natural pyrethrum extract (Golder et al., 1982, 1984) and to show dramatic changes in biochemical properties of the salivary secretions (Patel et al., 1982). Morphological alterations of the gland have been described as well. Burt (1942) described the infected gland as having a chalky appearance or sometimes brown to black in very old infections (Burt, 1950). Hecker and Molloo (1980) reported some minor morphometric changes of midgut cells in infected flies. Infected salivary glands, when examined with the electron microscope show several abnormalities and cellular damage (Otieno, personal communication). We have observed that normal salivary glands when dissected out and put in saline, display a sinuous motility. This motility is absent in heavily infected glands.

In a previous study (Golder and Patel, 1982) we characterized and described the distribution of the enzyme cholinesterase (ChE) in noninfected salivary glands. The loss of motility, depletion of many normal salivary constituents and general moribund appearances of infected glands prompted us to look at the ChE distribution in trypanosome-infected glands at various stages of infection.

Materials and Methods

Newly emerged male *G. m. morsitans*, Westwood, reared in the ICIPE insectary, were fed on albino white rats at peak parasitemia. The rats had been injected intraperitoneally with *T. b. brucei* (EATRO, 1969), isolated from a hyena in Tanzania in 1970 and subsequently cloned in our laboratory. Subsequent to the infective feeds, flies were placed in individual 2"×1" plastic holding tubes closed at the ends with a fine nylon netting. The flies were fed daily on rabbits (except Sunday). One week after the infected blood meal, and every day thereafter they were induced to salivate on warmed microscope slides. Saliva obtained was stained with Giemsa's stain and examined for parasites. At various times after the first detection of parasites, salivary glands were removed and stained for ChE activity. Freshly dissected glands were fixed in 10% formalin (v/v) and stained for ChE by a modification (Golder et al., 1977, method A) of a commonly used copper-thiocholine method (Koelle and Friedenwald, 1949). To ascertain that the reaction product seen was the result of ChE activity, various inhibitors were added to the incubation medium: tetraisopropyl pyrophosphoramidate ([iso-OMPA] Sigma Chemical Company, St. Louis, Mo.) at 10^{-5} M; 1,5-bis-(4-allyldimethyl-ammonium-phenyl)pentan-3-one dibromide ([BW284C51], Burroughs-Wellcome, Nairobi) at 5×10^{-5} M or physostigmine sulfate ([eserine sulfate] Sigma) at 10^{-6} M. The substrate was acetylthiocholine iodide ([ATC] Sigma) at 0.5 mg/ml. Some glands were incubated without substrate to detect nonspecific binding of capture ions.

When inhibitors were used, glands were preincubated in buffered copper sulfate solution plus inhibitor for 20 min in the absence of substrate. Following incubation with substrate and inhibitor, glands were washed in 0.1 M phosphate buffer (pH 6.0) then immersed in a 3% aqueous solution (w/v) of potassium ferricyanide. Stained glands were again rinsed in the buffer, mounted in glycerol and examined immediately. Samples from noninfected flies were stained in a similar manner at ages ranging from one day old nonfed to 49 days old.

To determine if the luminal ChE activity behaved as a typical insect ChE with respect to hydrolyzing a wide range of substrates, a few glands were dissected from flies showing old, mature infections (i.e. greater than two weeks from first sign of parasites) and stained using an esterase

Table 1

Age ^a	Number	Epithelial stain			Luminal stain		
		Normal	Broken	Absent	Absent	Light	Heavy
<i>Noninfected</i>							
Teneral	25	25	0	0	25	0	0
1-14 days	7	7	0	0	7	0	0
15-49 days	10	10	0	0	10	0	0
Total	42	42	0	0	42	0	0
<i>Infected</i>							
7 days	18	1	15	2	2	15	1
7-14 days	7	0	13	4	1	4	2
14-25 days	5	0	1	4	0	3	2
Total	30	1	19	10	3	22	5

^a For the noninfected group, age refers to the actual age of the fly, for the infected group, age refers to the age of the infection (time from first detection of parasites in the saliva to the time of dissection and staining for ChE).

method of Whitmore and Gilbert (1974). Glands were incubated in a solution containing 0.1% (w/v) B-naphthyl acetate and 0.5% (w/v) fast blue B in a 0.1 M phosphate buffer, pH 6,7, for 30 min at 37°C, buffer rinsed, mounted in glycerol and examined immediately.

Results

The salivary glands of *G. m. morsitans* are paired, unbranched, tubular organs. They are lined by a simple epithelium which varies in height between the secretory, absorptive and duct regions. The secretory region is covered externally by a single layer of striated muscle. Between the muscle layer and the epithelium is a prominent basal lamina. Electron micrographs (Sequeira, 1971) have shown a number of nerve axons in the basal lamina which, morphologically, appear as cholinergic.

The localization and characterization of normal (i.e. noninfected) glands has been published (Golder and Patel, 1982) and is shown in Fig. 1. The reaction product of ChE activity surrounds the epithelial cells of the secretory region. Reaction product is absent from the lumen of the gland and from the epithelium of the absorptive and duct regions. The normal localization of reaction product gives the noninfected gland a continuous, net-like appearance. Table 1 shows that of the 42 noninfected flies examined, all had the normal staining pattern. Our previous study (Golder and Patel, 1982) showed that this normal ChE distribution did not vary with age, sex or feeding history. When trypanosomes invade the gland, the ChE distribution changes. These changes are: a breaking

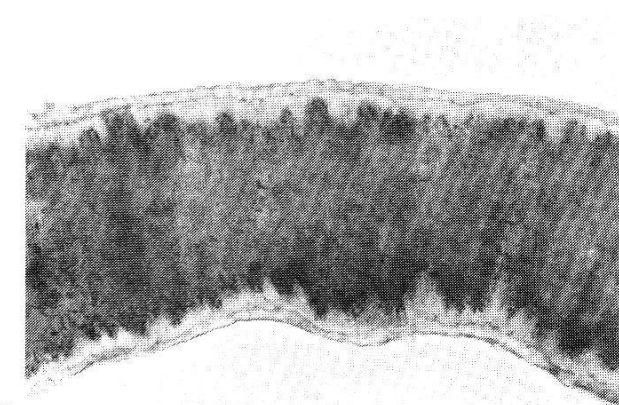
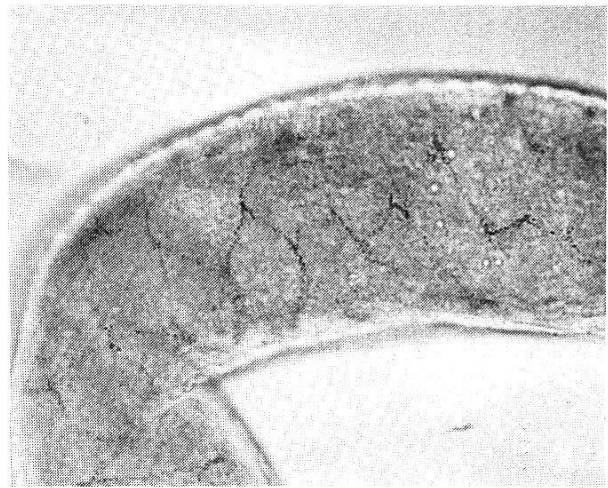
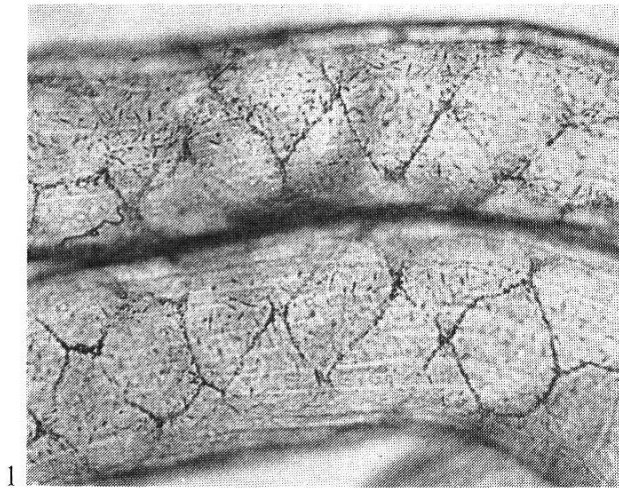


Fig. 1. Salivary gland of *G. morsitans* showing normal net-like distribution of ChE activity surrounding the epithelial cells of the secretory region of the gland. Magnification, 290 \times .

Fig. 2. Salivary gland of *G. morsitans* from a fly which had immature forms of *Trypanosoma brucei* in its saliva. Notice the broken appearance of the net-like pattern of ChE reaction product. Magnification, 290 \times .

Fig. 3. Salivary gland of *G. morsitans* which had metacyclic forms of *T. brucei* in its saliva. Note the heavy deposition of ChE reaction product in the lumen. Magnification, 290 \times .

up of the network (Fig. 2) and finally, the disappearance of epithelial stain, with a concomitant accumulation of luminal stain (Fig. 3). The time course and magnitude of these changes are somewhat variable as can be seen in Table 1. With regard to the infected glands, the epithelial stain was disrupted or absent in 96.7% of the flies examined ($n = 30$). The only instance of a normal stain was in a very young infection. The trend showed increased tendency towards total disappearance in older infections. A similar trend occurs in the appearance of reaction product in the lumen, only 10% of the infected flies show absence of luminal stain (the normal condition). The remaining 90% show luminal reaction product which stains lightly in early infections tending towards heavier in older infections. As is the case with the epithelial stain, the luminal stain is present when iso-OMPA (a selective inhibitor of nonspecific ChE) is used but

absent when incubated in the presence of eserine sulfate (an inhibitor of all ChEs) or BW284C51 (an inhibitor of acetylcholinesterase). In addition, epithelial stain is detected when B-naphthyl acetate and fast blue B are used in the incubation mixture to stain noninfected glands. Infected glands show no epithelial stain but do show luminal stain when this esterase method is used.

Discussion

The results of this study show that the ChE activity normally found in the epithelium of the secretory region of the gland disappears after infection of *G. m. morsitans* salivary glands by *T. b. brucei*. As the epithelium-associated activity diminishes, ChE activity becomes detectable in the lumen. Previous work (Golder and Patel, 1982) has shown the epithelium-associated ChE activity to be the result of a typical insect ChE. The present study shows that the luminal enzyme found in infected glands is similar to the epithelium associated enzyme in its reactions to inhibitors and substrate hydrolysis. The luminal enzyme's extreme sensitivity to eserine sulfate is typical of ChEs and shows that the reaction product is not the result of an arylesterase or carboxyesterase (aliesterase). The absence of reaction product in the presence of BW284C51 (a specific inhibitor of acetylcholinesterase, AChE) and presence of reaction product when incubated with iso-OMPA (a specific inhibitor of nonspecific cholinesterase) suggests that the enzyme is a true ChE. That the luminal enzyme will also hydrolyse B-naphthyl acetate is in agreement with the well established fact that insect ChEs do hydrolyse a wide range of substrates (Wood et al., 1979). The epithelium-associated enzyme also hydrolyzes B-naphthyl acetate (Golder and Patel, 1982), as well as the luminal enzyme.

The change in visual appearance of infected glands from translucent to chalky (Burt, 1942) might be attributed to the presence of the parasites causing a change in optical properties. In older infections when the gland turns brown to blackish (Burt, 1950), the change in appearance is more likely to be the result of cellular damage. A variety of morphological abnormalities have been observed in the epithelial cells of trypanosome-infected glands (Otieno, personal communication). These include the disappearance of rough endoplasmic reticulum, corrugation and infolding of the basal lamina, vacuolization, and penetration of the trypanosomes into and between epithelial cells. Considering this type of damage to the epithelium and the time-course of the change in ChE distribution, plus the fact that the luminal ChE behaves like the epithelium-associated enzyme with regard to inhibitors and substrate hydrolysis, we are drawn to the conclusion that the enzyme leaks into the lumen from the damaged epithelium. Unfortunately the function of salivary gland cholinesterase in *G. m. morsitans* is unknown and, as such, it is difficult to speculate on what effect the ChE abnormality has on the normal function of the gland. Because of the vast distribution and discrete localization of the enzyme in the secretory region only,

one must assume that the enzyme plays an integral role in salivary gland physiology; because of the loss of motility, necrotic appearance, cellular damage and shift in ChE distribution of trypanosome-infected glands, one must assume that the glands do not function properly. Indeed, it has been shown that salivary secretions of glands with mature infections of *T. brucei* are depleted of many of their normal constituents (Patel et al., 1982). Undoubtedly the developing trypanosomes utilize some components of the saliva but loss of rough endoplasmic reticulum in many of the secretory epithelial cells suggests that the glands are also secreting less.

Altered salivary gland function would almost certainly be expected to affect normal feeding behaviour. Jenni et al. (1980) reported that flies with salivary gland infections showed increased probing activity and fed more voraciously than noninfected flies. This change was attributed to trypanosomes blocking mechanoreceptors in the labrum, altering the sensation of feeding, and causing changes in fluid mechanics more distally in the food canal, making it more difficult to obtain a blood meal (Livesey et al., 1980). The relative contribution of impaired salivary gland function, loss of feeding sensation, and changes in fluid mechanics, to altered feeding behaviour are difficult to assess. Nevertheless, it is reasonable to expect that abnormal salivary gland function might play a contributory role in the observed changes in feeding behaviour. Impaired feeding ability has been suggested as one explanation for the increase in feeding activity, lowered fat reserves and residual bloodmeal in infected flies from a natural population (Ryan, 1984).

It has been hypothesized that *G. m. morsitans* infected with *T. brucei* are weakened by the infection and are thus in a poorer state of health than noninfected flies, and it was demonstrated that infected flies were significantly more susceptible to endosulfan (Golder et al., 1982) and a natural pyrethrum extract (Golder et al., 1984). One might expect that such weakened flies would show decreased longevity. This issue is controversial. Duke (1928) in an analysis of his and other people's data collected at Entebbe, Uganda, concluded that trypanosome infection had no effect on the longevity of *G. palpalis*. Baker and Robertson (1957) held the view that female tsetse flies with *T. b. rhodesiense* infections had a tendency to live longer than uninfected flies but a statistical analysis of their data showed the difference to be insignificant. It is difficult to predict the effect of trypanosome infection on longevity of flies based on laboratory experiments.

Interest in the effects of trypanosome infection on tsetse flies has been rekindled in the last few years after decades of belief that no harm is done to infected flies. This belief is no longer tenable in the light of the works discussed in this paper. Still, very few physiological and behavioural parameters of infected flies have been examined. As more information on the biology of infected flies is obtained we may have to alter some of our thinking on the epidemiology of African trypanosomiasis.

Acknowledgments

We gratefully acknowledge the technical assistance of Mr. E. Mpanga. We thank Dr. G. H. M. Mulela and Dr. Hodson of Wellcome Kenya Ltd. for the generous gift of the BW284C51. We also thank Dr. L. H. Otieno for his helpful comments and the Director of ICIPE, Professor T. R. Odhiambo for permission to publish this paper. This work was funded by grants from the International Atomic Energy Agency, Vienna, Austria.

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