Parasitology (1986), **92**, 75–82
With 3 figures in the text

Metacyclic *Trypanosoma vivax* possess a surface coat

P. R. GARDINER[1], P. WEBSTER[1], L. JENNI[2] *and* S. K. MOLOO[1]
[1]*International Laboratory for Research on Animal Diseases, P.O. Box* 30709, *Nairobi, Kenya*
[2]*Swiss Tropical Institute, Socinstrasse* 57, *CH*-4051 *Basel, Switzerland*

(*Accepted* 20 *May* 1985)

SUMMARY

Coated metacyclics of *Trypanosoma vivax* exist in the hypopharynges of infected tsetse flies and are extruded in low numbers when the flies are induced to probe onto warm slides or into medium. After extensive searching of *T. vivax*-infected proboscides, and resort to a process for the examination of single, extruded, metacyclic trypanosomes, electron microscopic evidence is presented that, contrary to an earlier report, metacyclic *T. vivax* acquire a surface coat before contact with the mammalian host. Since *T. vivax* exhibits antigenic variation, the role of the surface coat in this species is likely to be functionally equivalent to the surface coat of the other tsetse-transmitted trypanosome species, *T. brucei* and *T. congolense*.

INTRODUCTION

The cycle of development that *Trypanosoma* (*Duttonella*) *vivax* Ziemann 1905 undergoes in its tsetse fly vector is confined to the proboscis (Bruce, Hamerton, Bateman & Mackie, 1910; Bruce, Hamerton, Bateman, Mackie & Bruce, 1911). Earlier descriptive work (Fraser & Duke, 1912; Lloyd & Johnson, 1924; Roubaud, 1935; van Hoof, Henrard & Peel, 1937; reviewed in Hoare, 1972) suggested that ingested bloodstream trypomastigotes attach to the inner wall of the food canal of the tsetse proboscis where they differentiate into epimastigote forms (called crithidial forms in the older works). The epimastigote forms divide at their site of attachment giving rise to clusters of trypanosomes (Roubaud, 1935). Vickerman (1973) has described the ultrastructure of these attached trypanosomes in detail. Some of the epimastigotes detach from their original site and enter the hypopharynx where free-swimming metacyclic trypanosomes are seen following differentiation of some of the epimastigotes through intermediate trypomastigote stages. Roubaud (1935) stressed, however, that the appearance of trypanosomes, morphologically resembling metacyclic forms, often preceded the actual acquisition of infectivity of tsetse flies for vertebrate hosts. In *T. brucei* and *T. congolense* the acquisition of infectivity for vertebrate hosts is correlated with the assumption by metacyclic trypanosomes of an electron-dense surface coat (Vickerman, 1969, 1974), the site of the variant-specific surface glycoprotein (Vickerman & Luckins, 1969). All other phenotypes in the vector are uncoated as are the epimastigotes of *T. vivax* (Vickerman, 1973). It was to be expected therefore that metacyclics of *T. vivax* would also possess a surface coat before their injection by the fly into the vertebrate host. However, Tetley, Vickerman & Moloo (1981) failed to find coated trypanosomes upon electron microscopical investigation of *T. vivax*-infected tsetse hypopharynges, and suggested that the attached, uncoated trypomastigotes in the hypopharynx represent the true metacyclic stage of *T. vivax* and hypothesized that the surface coat, prominent on bloodstream forms of *T. vivax*, as it is in the bloodstream forms of *T. brucei* and *T. congolense*, was synthesized after entry into the mammalian host.

Table 1. *An estimate of the number of metacyclic trypanosomes of various* Trypanosoma vivax *stocks extruded in salivary probes by* Glossina morsitans centralis

T. vivax stock	Place of isolation	Maturity of infection (days)	Infection rate in tsetse‡ (%)	Tsetse maintained on	No. positive* for metacyclics (%)	Mean no. of metacyclics/ positive probe (range)
IL 20-E11						
(i)	Zaria, Nigeria	31	40	Rabbit	18/79 (22·8)	4·6 (1–16)
(ii)	Zaria, Nigeria	33	50	Goat (15 days) Rabbit (13 days)	11/82 (13·4)	1·7 (1–4)
IL 1392						
(i)	Zaria, Nigeria	30	90	Goat	23/112 (20·5)	N.D.†
(ii)	Zaria, Nigeria	26	90	Goat	39/171 (22·8)	N.D.
IL 1480	Galana, Kenya	30	100	Cow	65/100 (65)	12·8 (1–65)
IL 2133	Kilifi, Kenya	79	90	Cow	70/84 (83)	50·9 (1–273)
IL 2292	Likoni, Kenya	38	100	Cow	81/100 (81)	22·9 (1–159)

* No. of tsetse extruding metacyclic trypanosomes/no. of tsetse probed.
† N.D., Not done, but only rarely was more than 1 metacyclic present/positive probe.
‡ Derived by determining hypopharynx infection in 10 random flies.

We therefore undertook to examine *T. vivax* trypanosomes both in the hypopharynx of infected tsetse flies, and those trypanosomes extruded by tsetse flies induced to probe onto warmed slides or into medium. Because of the relative paucity of extruded trypanosomes in fly probes we evolved a technique for single cell electron-microscopy. In this paper we demonstrate that coated metacyclic trypanosomes of *T. vivax* exist in the tsetse vector before contact with the mammalian host.

MATERIALS AND METHODS

T. vivax stocks IL 20-E11 and IL 1392 are derivatives of IL V17 – the stock employed in the previous study (Tetley *et al.* 1981) – originally isolated by Leeflang, Buys & Blotkamp (1976), as Y486 from a steer in Nigeria. *T. vivax* IL 1480 has been syringe passaged through 2 calves since its isolation from a steer at Galana, Kenya. *T. vivax* IL 2133 has been cycled twice through tsetse, once between cattle and once between a steer and a goat, since its isolation from a bovine at Kilifi, Kenya. *T. vivax* IL 2292 has been passaged 5 times in cattle, including 2 tsetse transmissions between cattle, since its isolation from a steer at Likoni, Kenya.

Teneral *Glossina morsitans centralis*, or *G. m. morsitans* in one experiment, from the ILRAD colony were fed on goats for the West African stocks of *T. vivax*, or on cattle in the case of the East African stocks, shortly after the first appearance of trypanosomes in the blood, and then fed daily (except Sundays) as shown in Table 1. Infection rates in the tsetse were estimated after 25 days by counting the number of flies showing hypopharyngeal infections when 10 tsetse were dissected at random. The tsetse were starved for 2 days and then allowed to probe onto individual wells of a warmed (37 °C) Teflon-coated slide. The dried probe was examined without staining by means of a light microscope at a final magnification of 320 × . All metacyclic trypanosomes (i.e. excluding occasional long forms) were counted in each probe.

For more thorough investigation of metacyclic morphology at the light microscopic level, fly probes were Giemsa stained by a slight modification of the method of Gray, Ross, Taylor & Luckins (1984). The modification added was pre-fixation of the probe with phosphate-buffered 2% (v/v) formaldehyde (pH 7·0) for 2–5 min before rinsing in buffer alone and methanol fixation.

For direct electron microscopical examination of an infected hypopharynx the head and proboscis of a *G. m. morsitans* fly was fixed 11 days after feeding on the blood of a goat infected with IL 20-E11. The proboscis was processed in a manner similar to that described (Tetley *et al.* 1981) but the initial fixative [3% (v/v) glutaraldehyde in 200 mM phosphate buffer] contained 2% (w/v) saccharose rather than $CaCl_2$. All reagents were of a grade of purity sufficient for electron microscopy. Ultrathin sections of the embedded proboscis were examined in a Zeiss EM 9 electron microscope.

For electron microscopy of extruded metacyclics, 40–50 infected tsetse flies were allowed to probe through a silicone membrane into approximately 1·5 ml of medium contained in a test-tube. The medium contained HEPES-buffered Modified Eagle's Medium with 20% (v/v) foetal calf serum, 1% (v/v) non-essential amino acids, 1% (v/v) glutamine (all from Gibco Ltd., Paisley, Scotland) and 5 μg/ml gentamycin sulphate. The probed medium was fixed with an equal volume of a fixative containing 5% (v/v) glutaraldehyde, 2·8% (w/v) formaldehyde and 0·28% (w/v) picric acid in 63 mM phosphate buffer, pH 7·4, and then centrifuged at 1300 ***g*** for 10 min. Most of the supernatant fraction was discarded whilst the bottom 0·2 ml was retained and gently mixed by pipetting up and down. Glass cover-slips, pre-coated with a film of 0·1% (w/v) gelatin and 0·5% (w/v) agarose which had been allowed to dry, were trapped between a glass slide and the filter paper of a Cytospin (Shandon Southern, Cheshire, England) and pre-spun for 2 min at 1000 r.p.m. (speed setting 7) with approximately 0·1 ml distilled water to rehydrate the gelatin/agarose film. The fixed samples were then spun similarly onto the same cover-slips and the cover-slips rapidly removed and flooded with further fixative for 5–10 min. Taking care to keep them moist at all times the cover-slips were then subjected to the following processing. They were washed by constant replacement of 100 mM phosphate buffer, pH 7·4, for 5 min followed by a 1 min rinse in 0·1 M sodium cacodylate buffer, pH 7·4. A 10 min fixation in cacodylate buffered 1% (w/v) osmium tetroxide was followed by constant rinsing for 5 min in cacodylate buffer. Washing for 1 min in 0·05 M sodium maleate, pH 5·4, preceded staining for 10 min in 1% (v/v) uranyl acetate in the same buffer. After a further rinse for 5 min in sodium maleate the gelatin/agarose films were dehydrated by 1 min step-wise processing through graded alcohols. Two rinses of 1 min each in propylene oxide preceded incubation for 10 min in a 1:1 mixture of propylene oxide/resin mixture before overnight embedment in resin mixture (1:1 mixture of Epon 812/Araldite 6005, Ernest F. Fullam, Schenectady, N.Y., USA) at 60 °C. After polymerization the resin was peeled away with the help of a scalpel blade to expose the back (i.e. not the gelatin/agarose film side) of the cover-slip. The cover-slip was then broken away after plunging the resin block into liquid nitrogen and allowing the embedded cover-slip to come to room temperature. The resin blocks were scanned with the aid of a light microscope using a 32× long working distance objective lens. The position of individual trypanosomes was marked by scratching the resin with a syringe needle. Marked areas were removed, mounted on resin stubs with sealing wax, trimmed and then aligned to a diamond knife edge with the aid of a backlighted knife stage and block holder (Sunkay Laboratories, Tokyo, Japan) on a Sorvall MT-2B ultramicrotome (Dupont, Wilmington, DE, USA). All sections were collected on carbon-coated, single slot grids (2 × 1 mm), stained with

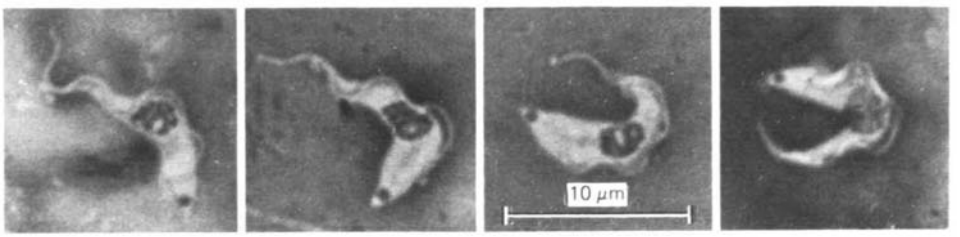


Fig. 1. Light micrograph of Giemsa-stained trypanosomes (*Trypanosoma vivax* IL 1480) extruded in the salivary probe of a *Glossina morsitans centralis* fly. Four typical metacyclic trypanosomes are shown. The acid treatment extracts cytoplasmic organelles but reveals the outline morphology, kinetoplast and nucleus in detail.

uranyl acetate and lead citrate and viewed in a Zeiss EM 10A electron microscope operating at 60 kV. Trypanosome profiles were detected approximately 20–30 sections into the block, depending upon the thickness of the gelatin layer.

RESULTS

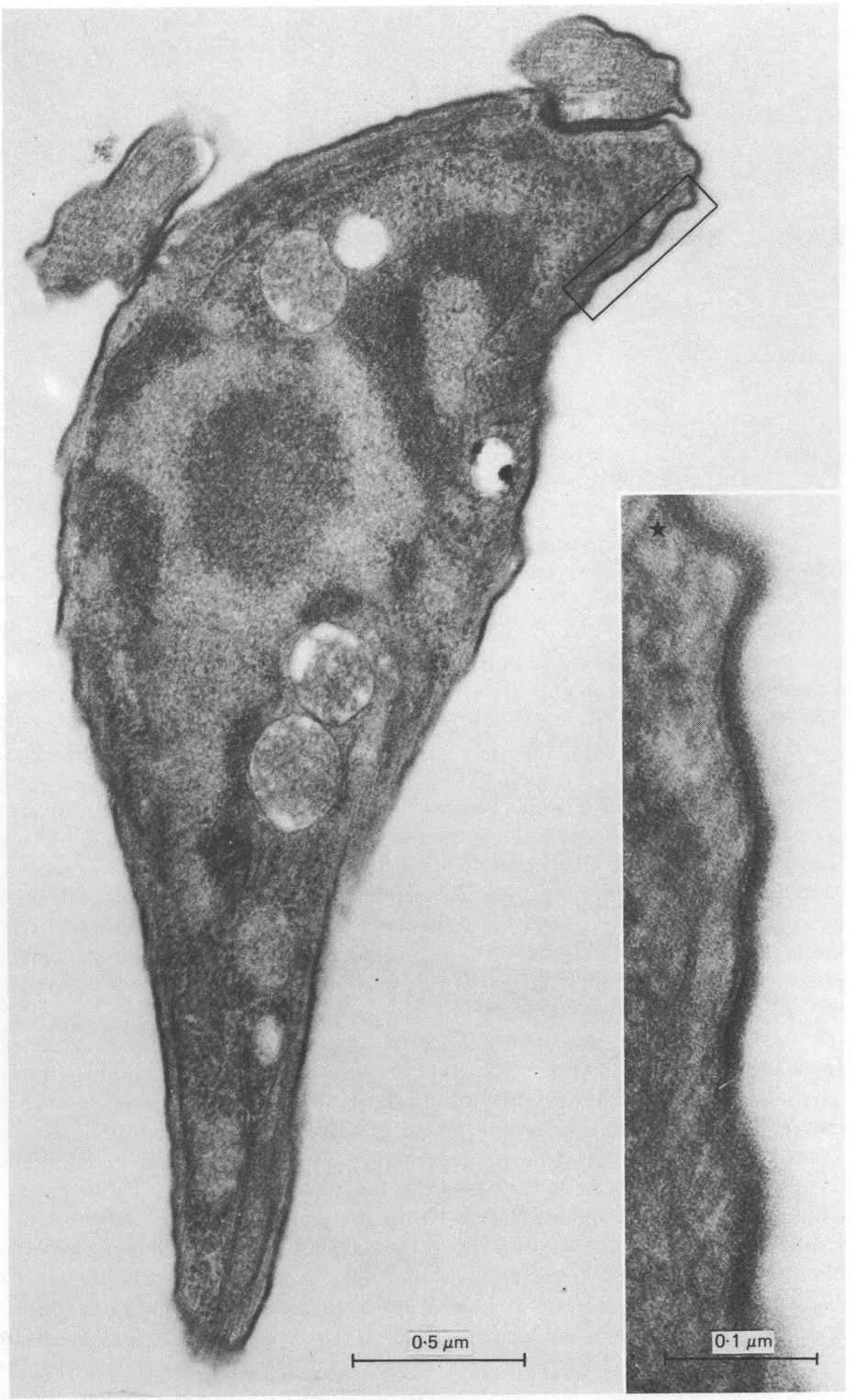
The number of metacyclic trypanosomes of the *T. vivax* stocks employed in this study which are extruded by *G. m. centralis* in salivary probes under experimental conditions is low, the naturally rodent-infective stock from Nigeria being poorer in this regard than the East African stocks (Table 1). Giemsa-stained metacyclics of IL 1480 (Fig. 1) show the large kinetoplast typical of *T. vivax* located terminally. The body of the trypanosome appears broad posteriorly, tapering sharply anteriorly of the nucleus. The undulating membrane is well developed and a pronounced free flagellum is characteristically present. This morphology corresponds closely with the drawings of Hoare (1970, form *o* in fig. 2.1; and 1972, form *k* in fig. 86) of 'metatrypanosomes in the hypopharynx'. The more slender forms depicted by Hoare (1970, form *n* in fig. 2.1; and 1972 forms *i* and *j* in fig. 86) were only rarely noted in this study.

The results of electron microscopy of single, extruded metacyclic *T. vivax* demonstrate that they possess a dense surface coat around the body and flagellum of the trypanosome (Figs. 2 and 3A) before contact with the mammalian host. Further, profiles of coated trypanosomes are present within the hypopharynx of *T. vivax*-infected tsetse flies (Fig. 3B). The observation of uncoated trypanosomes (Fig. 3C) in the same salivary probes as coated trypanosomes, verifies that the surface coat of extruded metacyclic trypanosomes is not an artefact of the specimen preparation technique employed. These findings are at variance with an earlier report (Tetley *et al.* 1981) but confirm that the maturation of *T. vivax* in the insect vector culminates in a coated metacyclic trypanosome as is the case with *T. brucei* and *T. congolense*.

DISCUSSION

Before the present study was undertaken there was good circumstantial evidence that the forms of *T. vivax* injected by tsetse into mammals must possess a surface coat since goats immunized with, and producing antibody to, an M_r 83 K surface membrane

Fig. 2. Transmission electron micrograph of a section of an extruded metacyclic of *Trypanosoma vivax* IL 2292 showing a surface coat over the body and flagellum of the trypanosome. The inset (*) reveals the surface coat in the area enclosed by the box.



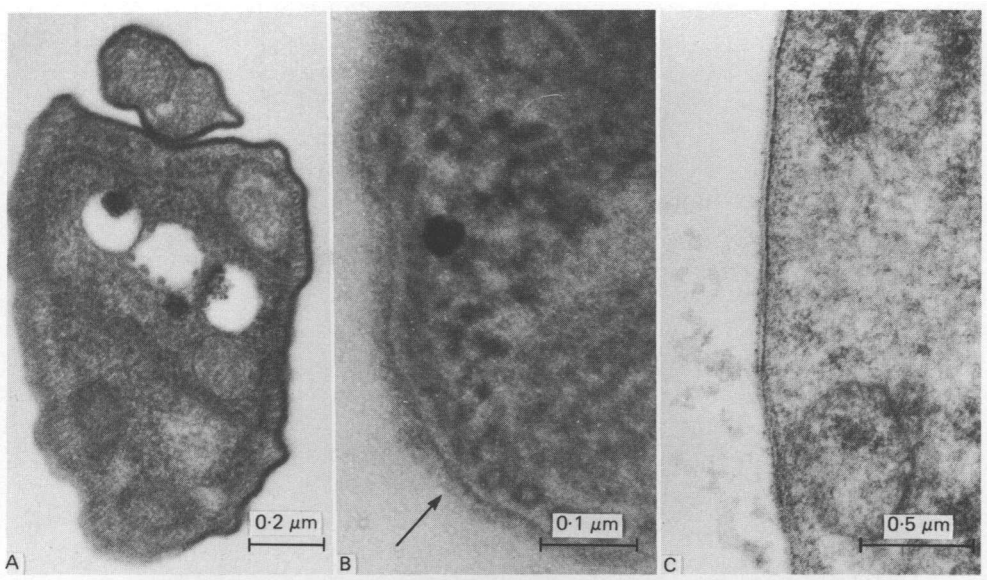


Fig. 3. Transmission electron micrographs of sections of *Trypanosoma vivax*. A. Extruded metacyclic of *T. vivax* IL 2292 clearly showing a surface coat in the parts of the section cut perpendicular to the surface membrane. B. Part of a section of coated metacyclic trypanosome of *T. vivax* IL 20-E11 in the hypopharynx of *Glossina morsitans morsitans*. The arrow indicates the thickness of the surface coat. C. An extruded trypomastigote of *T. vivax* IL 2292 from the same sample as the trypanosomes depicted in Figs 2 and 3A. The trypanosome lacks a surface coat but shows small, surface membrane accretions thought to represent an artefact of the phosphate-buffered fixative.

protein common to *T. vivax*, *T. congolense* and *T. rhodesiense* were completely susceptible to *T. vivax* infection upon tsetse challenge (Rovis, Musoke & Moloo, 1984). If metacyclic *T. vivax* lacked a surface coat (Tetley *et al.* 1981), thus exposing the M_r 83 K membrane protein, some degree of protection would have been expected to have been afforded the immunized goats. Similarly, the susceptibility of livestock to homologous and heterologous rechallenge by tsetse fly (Moloo & Emery, unpublished; Gardiner, unpublished), would be difficult to explain if the infecting trypanosomes presented invariant, cell membrane antigens to the host animal.

That the surface coat of metacyclic *T. vivax* has previously been so difficult to demonstrate is no doubt due to the paucity of these forms both in the hypopharynx of infected tsetse and in salivary probes, and perhaps to the technical aspects of fixation (phosphate-buffered fixatives give better preservation of the surface coat of *T. vivax* than similar fixatives buffered in cacodylate – Professor S. Ito, Harvard University, personal communication). The numbers of metacyclic trypanosomes given in Table 1 can only be an estimate, since the extent of the probe on the glass slide inevitably differs from fly to fly, but from the data presented the West African stock which was employed in a previous investigation with negative results (Tetley *et al.* 1981) would seem the least suitable material to examine. Nevertheless metacyclics of this stock also possess a surface coat (Fig. 3B). Other variables which might increase the number of metacyclic *T. vivax* produced by tsetse flies, such as the passage history of the stock, the species of animal on which the tsetse are maintained, the maturity of infection in the tsetse or the tsetse species itself, have not been critically examined in this study. If, however,

the figures given here for metacyclic numbers are representative of the biology of *T. vivax* in the field, the bite of *T. vivax*-infected tsetse will deposit fewer metacyclics in the skin of the vertebrate host than do flies infected with *T. congolense* or *T. brucei*. It seems probable also that a mixture of coated and uncoated trypanosomes will be introduced into the skin of an animal bitten by *T. vivax*-infected tsetse, since both were found in salivary probes by electron microscopy, and larger, presumably epimastigote forms, were occasionally noted in some salivary probes at the light microscope level. That uncoated *trypomastigote* forms are extruded by tsetse flies as well as coated metacyclic trypanosomes suggests that a process of maturation may be occurring in the trypomastigotes in the hypopharynx of the tsetse fly (Roubaud, 1935). This presumably involves reduction in the tenacity of flagellar attachment, as well as the generation of a surface coat and, perhaps, subtle modulations in the morphology of these hypopharyngeal forms to yield the final metacyclic trypanosome depicted in Fig. 1. Nothing is known of the biochemistry of this differentiation but Roubaud's (1935) observation that there is a delay in the assumption of infectivity after trypanosome invasion of the hypopharynx, and Tetley *et al.*'s (1981) finding that trypomastigotes in the proboscis are unaffected by fresh guinea pig serum (uncoated trypanosomes from 25 °C cultures of the same stock of *T. vivax* are both agglutinated and lysed by fresh guinea pig serum – Hirumi & Gardiner, unpublished observations), together suggest that generation of the surface coat in *T. vivax* may be a gradual process and that cell surface changes in trypomastigote forms occur in advance of a demonstrable surface coat on mature metacyclic trypanosomes.

T. vivax demonstrates antigenic variation (reviewed by Gray & Luckins, 1976; De Gee, Shah & Doyle, 1979, 1980; Murray & Clarkson, 1982; Barry & Gathuo, 1984) and undergoes a cyclical development in the tsetse fly in which it first loses and then reacquires a surface coat. This life-cycle is thus functionally comparable with that of the two other major tsetse-transmitted species, *T. brucei* and *T. congolense*.

Note added by Mr L. Tetley and Professor K. Vickerman:

We happily concede that metacyclic *Trypanosoma vivax* does possess a surface coat and that the choice of material on which we based our 1981 paper was unfortunate, as indicated by Dr Gardiner and colleagues. Our recent detailed study of metacyclic differentiation in *T. brucei* (Tetley & Vickerman, 1985) makes it clear that the attached uncoated *T. vivax* trypomastigotes that we described from the tsetse hypopharynx correspond to the pre-metacyclic trypomastigotes of *T. brucei*. The latter acquire a surface coat to become 'nascent metacyclics' while still attached to the tsetse salivary epithelium, and are then released as mature metacyclics.

The authors would like to thank Ms Ravi Thatthi and staff members of the ILRAD Tsetse Vector Laboratory for technical assistance, and Dr Maureen Gray for bringing the method of staining trypanosomes in salivary probes to our attention in advance of publication. This is ILRAD publication number 336.

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Printed in Great Britain