Enzymes capable of splitting the β-linkages of N-acetylglucosamine polymers have been reported in several insect tissues, particularly in the gut, where they may have a digestive function[17]. Glycoproteins in plants are known to contain carbohydrate side-chains consisting of N-acetylglucosamine and mannose residues. It is perhaps significant, therefore, that N-acetyl β-D glucosaminidase and α-D mannosidase activity was present in *C. maculatus* larvae which feed on the seeds of the cowpea, *Vigna unguiculata*, which contain large amounts of such glycoproteins[18]. Similar enzymes have been reported in the alimentary tracts of other insect species. For example, in the crop of *Schistocerca gregaria*[19] and the midgut of *Stomoxys calcitrans*[14].

Esterases are ubiquitous non-specific enzymes that catalyse the hydrolysis of ester linkages. Such enzymes have been implicated in a variety of functions in insects. These include lipid digestion, the general metabolism and mobilisation of fats, energy-related fat catabolism in muscles and the synthesis and transport of cuticular wax[20, 21]. In addition, a large number of xenobiotics to which insects are exposed are metabolised by esterases. For example, the presence of carboxyl ester groups in malathion and other insecticides makes these compounds vulnerable to hydrolysis by carboxylesterases[22]. In the present study thin layer polyacrylamide gel electrophoresis revealed the presence of a number of bands exhibiting non-specific esterase activity in homogenates of both larval and adult guts (figure 3). Seven distinct esterase bands were present in the larval preparation (Rf values being 0.27, 0.35, 0.38, 0.45, 0.46, 0.48, 0.54) whilst only four bands were detected in the adult (R_f values being 0.35, 0.38, 0.45, 0.55). Similar changes in gut esterases have been reported during the life cycle of other insects. Clements[20] reported a marked fall in the number of esterases in the pupal stage, as compared to the larval stages, of *Pieris brassicae*. Whilst there was a recovery of most of the late instar larval esterases in the adult *Pieris*, not all were present in detectable amounts. Desnuelle and Savary[23] have pointed out that most of the substrates used for characterising esterase activity can be hydrolysed by proteolytic enzymes such as chymotrypsin and trypsin. Since recent studies have revealed the presence of a proteolytic enzyme in larval gut homogenates of *C. maculatus* which was relatively insensitive to the Kunitz soyabean trypsin inhibitor (Gatehouse, unpublished data) it was decided to examine the effect of the chymotrypsin inhibitor TPCK on the activities of the various esterases present in adult and larval guts of *C. maculatus*. In both stages of the life cycle this agent was without significant effect on esterase activity. This clearly confirms that the bands are almost certainly esterases and not merely side-effects of proteolytic enzymes. Further studies are in progress to determine the inhibition characteristics of these esterases.

Acknowledgments. The authors would like to thank Mr. I. Walton for technical assistance and acknowledge receipt of a Nuffield grant.

1 Booker, R.H., J. Stored Prod. Res. *3* (1967) 1.
2 Podoler, H., and Applebaum, S.W., Biochem. J. *121* (1971) 317.
3 Gatehouse, A.M.R., and Anstee, J.H., Experientia *39* (1983) 1013.
4 Anstee, J.H., and Charnley, A.K., J. Insect Physiol. *23* (1977) 965.
5 Dawson, R.M.C., Elliott, D.C., Elliott, W.H., and Jones, K.M., Data for Biochemical Research. Clarendon Press, Oxford 1969.
6 Gabriel, O., Meth. Enzymol. *22* (1971) 565.
7 Shaw, C.R., and Prasad, R., Biochem. Genet. *4* (1970) 297.
8 Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., J. biol. Chem. *193* (1951) 265.
9 Charnley, A.K., Ph. D. Thesis, University of Durham, Durham, 1975.
10 Evans, W.A.L., and Payne, D.W., J. Insect Physiol. *10* (1964) 657.
11 Droste, H.J., and Zebe, E., J. Insect Physiol. *20* (1974) 1639.
12 Krishna, S.S., and Saxena, K.N., Physiol. Zool. *35* (1962) 66.
13 Price, R.G., and Robinson, D., Comp. Biochem. Physiol. *17* (1966) 129.
14 DeLoach, J.R., and Spates, G.E., Insect Biochem. *14* (1984) 169.
15 Robinson, D., Eur. J. Biochem. Physiol. *12* (1964) 95.
16 Yamamoto, T., and Tonomura, Y., J. Biochem., Tokyo *62* (1967) 558.
17 Gilmour, D., The metabolism of insects. Oliver and Boyd, Edinburgh and London 1965.
18 Khan, Md. R.S., Gatehouse, J.A., and Boulter, D., J. exp. Bot. *31* (1980) 1599.
19 Woollen, J.W., Walker, P.G., and Heyworth, R., Biochem. J. *79* (1961) 294.
20 Clements, A.N., J. Insect Physiol. *13* (1967) 1021.
21 Kapin, M.A., and Ahmad, S., J.N.Y. ent. Soc. *86* (1978) 300.
22 Dauterman, W.C., and Hodgson, E., in: Biochemistry of Insects, p. 541–577. Ed. M. Rockstein. Academic Press, New York 1978.
23 Desnuelle, P., and Savary, P., J. Lipid Res. *4* (1963) 369.

0014-4754/85/091202-04$1.50 + 0.20/0

Malarial parasites complete sporogony in axenic mosquitoes[1]

U.G. Munderloh and T.J. Kurtti

Waksman Institute of Microbiology, Rutgers University, P.O. Box 759, Piscataway (New Jersey 08854, USA), 23 October 1984

Summary. To obtain sporogonic stages of malaria free from microbial contaminants for in vitro studies, *Anopheles stephensi* were reared under sterile conditions using a mosquito cell line as larval food. The adult females, kept in sterile humidified containers and allowed to engorge on parasitemic hamsters, supported the sporogonic development of the rodent malarial parasite *Plasmodium berghei*. In 10 experiments, the proportion of infected mosquitoes varied from 0 to 92%, and the geometric mean number of oocysts per female mosquito from 2.5 to 58.6, with a range of 1 to 548. The average number of salivary gland sporozoites per infected mosquito was determined by direct sporozoite counts in the pooled homogenate of the thoraces of all female mosquitoes. In five experiments, it varied from 2.7×10^3 to 9.0×10^3. The sterile sporozoites, harvested on day 19 or 20 after the infective blood meal, were as infective for rodents as nonsterile ones.

Key words. Anopheles stephensi; Plasmodium berghei; axenic mosquitoes; transmission of malaria; mosquito cell culture.

In the mosquito, the malarial parasite undergoes a series of changes that begins with the formation of gametes and culminates in the production of sporozoites infective for the vertebrate host. These events have not been analyzed experimentally in detail because of the lack of in vitro systems that allow the formation and manipulation of the oocyst and the developing sporozoites within.

Several researchers have reared mosquitoes under sterile conditions[2], but the resulting adults are unable to support complete development of mosquito-born parasites[3]. We have found that axenic *Anopheles stephensi* can transmit rodent malaria caused by *Plasmodium berghei*[4], and we now demonstrate for the first time that malarial parasites complete sporogonic development in axenic mosquitoes and differentiate into mature sporozoites.

Materials and methods. Parasite. Plasmodium berghei (ANKA strain) was cyclically maintained in Syrian golden hamsters *(Mesocricetus auratus)* and *A. stephensi* mosquitoes as described[5].

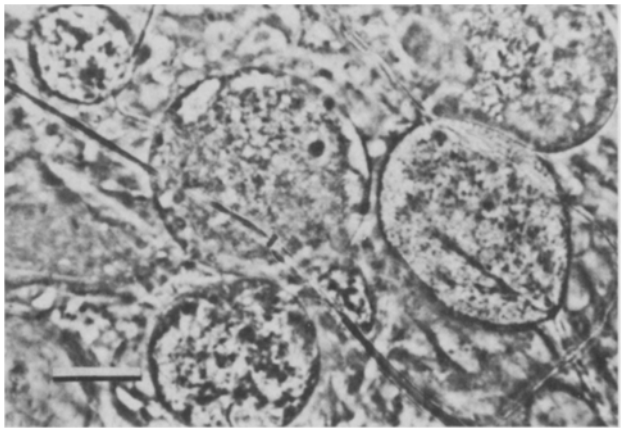
Culture medium. Leibovitz's L-15 medium was modified by the addition of the following compounds: 2.25 mM L-aspartic acid, 2.61 mM L-proline, 3.4 mM L-glutamic acid, 2 mM L-glutamine, 2.05 mM alpha-ketoglutaric acid, 12.43 mM glucose, 33 µM reduced glutathione, 75 µM ascorbic acid, 7.3 µM p-aminobenzoic acid, 0.37 µM vitamin B_{12}, 0.41 µM d-biotin, 8.4 nM $CoCl_2 \cdot 6H_2O$, 8.0 nM $CuSO_4 \cdot 5H_2O$, 95 nM $MnSO_4 \cdot H_2O$, 1.8 µM $FeSO_4 \cdot 7H_2O$, 8.3 nM $NaMoO_4 \cdot 2H_2O$, 12 nM Na_2SeO_3, 70 nM $ZnSO_3 \cdot H_2O$. This medium is referred to as L-15B.

Rearing of gnotobiotic larvae. The term 'gnotobiotic' was chosen to describe the larvae, because they were reared in association with a moscquito cell line. Gnotobiotic *A. stephensi* larvae were reared from surface sterilized eggs according to a modification of a procedure outlined earlier[6]. The mosquito cell line RU-TAE 12 V that served as larval food was grown in medium L-15B supplemented with 20% heat-inactivated fetal bovine serum (FBS), 10% tryptose phosphate broth, 0.038% $NaHCO_3$, 50 IU penicillin/ml and 50 µg streptomycin/ml. The desired number of mosquito cell cultures (usually 5) was disrupted by pipetting and 100 mM glucose added to each culture. Approximately 100 first instar larvae were introduced into a mosquito cell culture vessel. During subsequent feedings, the larval culture fluids were replaced by equal volumes of mosquito cell suspensions supplemented with glucose, and samples were incubated at 37 °C for 10 days to confirm sterility.

Infection of adult axenic mosquitoes. Fifty to 100 pupae were collected in a 3-cm diameter glass Petri dish with 3 ml sterile spring water inside an autoclaved 1-liter beaker lined with mosquito netting. The latter also contained a cotton-plugged U-tube, and was covered with aluminum foil. Just before the introduction of pupae, the U-tube was filled with sterile 10% sucrose in an aqueous 10^{-4} M hypoxanthine solution, and the beakers placed into desiccator jars (containing ambient air and 100 ml sterile distilled water to provide humidity) at 28 °C. Twenty-four hours before the first infective blood meal, the mosquitoes were shifted to 18 °C, and on day 3 after emergence, they were allowed to engorge on a hamster, cleaned with 95% ethanol, in which 1 to 2% of the erythrocytes harbored mature gametocytes. The feeding was performed in the sterile environment of a laminar flow hood. The mosquitoes were transferred to 20 °C 2 days later, where they remained for another 16–18 days. The female mosquitoes were aseptically removed from the beakers by aspiration and anesthetized with sterile CO_2. The thoraces were collected in ice cold L-15B medium with 50% heat-inactivated FBS. The abdomens were reserved in cold medium, the midguts removed into a drop of Hanks' balanced salt solution, and evaluated for the presence of oocysts. The thoraces were homogenized in a glass tissue grinder and centrifuged for 5 min at 100 × g at 4 °C. Sporozoites in the supernate were counted in a hemocytometer, and the average number per infected mosquito calculated. In three experiments, the number of infective sporozoites harvested was estimated from the duration of the prepatent period (P) according to the method of Warhurst and Folwell[7]. The standard curve for evaluating sporozoites was determined as follows: three groups of four hamsters each were injected intracardially with three dilutions of non sterile salivary gland sporozoites, prepared as outlined for axenic mosquitoes.

Results. In the present study, larval growth and development was similar to that reported earlier[6], but with the additional glucose, pupation started already on day 6 after hatching and proceeded at a faster rate than when it was omitted. Thus, by day 10, over 70% of the larvae had pupated, but onyl 56% in the absence of additional glucose. Ninety-five percent, or more, of the larvae ultimately pupated, and over 95% of the pupae emerged as adults. In 10 experiments, an average of 76.3% (range: 50–97.1%) female, and 24% (range: 0–46.4%) male mosquitoes survived 16 to 18 days after the blood meal.

The table summarizes the results obtained when axenic *A. stephensi* were fed on *P. berghei*-infected hamsters. The figure shows the appearance of oocysts in the midgut of an axenic mosquito. To compare the infectivity of sporozoites from conventionally reared and gnotobiotic *A. stephensi*, known numbers of sterile thoracic sporozoites were injected into hamsters as outlined. The mean P per group and the log of the number of sporozoites, sterile as well as nonsterile, inoculated per animal were combined to calculate the linear regression: $Y = -0.85$, $X = +8.32$, where Y is P and X the log number of sporozoites



Midgut of female axenic *Anopheles stephensi* mosquito infected with the rodent malarial parasite, *Plasmodium berghei*. Note varying degree of oocyst development. Day 19 after infection. Phase contrast image of living material. Bar represents 20 µm.

Infection of axenic *Anopheles stephensi* by *Plasmodium berghei*

Experiment	% survival of female mosquitoes	No. infected female mosquitoes (%)	Geometric mean ± SD of the number of oocysts per infected mosquito (range)	No. of salivary gland sporozoites per infected mosquito[a]	Infectivity for golden hamsters[b]
I	73.1	0 (0)	0	0	–
II	95.7	2 (9.1)	2.5 (2,3)[d]	4.6×10^2 [c]	+
III	91.3	7 (33.3)	not determined	23[c]	+
IV	64.1	23 (92.0)	22.7 ± 2.5 (4–88)	not determined	not determined
V	50.0	18 (78.3)	29.3 ± 3.9 (3–328)	6.6×10^3	+
VI	97.1	10 (31.3)	3.4 ± 2.4 (1–44)	2.1×10^2 [c]	+
VII	89.7	7 (28.0)	11.3 ± 2.2 (4–42)	6.2×10^3	+
VIII	71.9	34 (79.5)	15.7 ± 3.8 (1–155)	6.3×10^3	+
IX	50.0	17 (68.0)	58.6 ± 4.7 (1–548)	9.0×10^3	+
X	80.0	11 (47.8)	19.4 ± 3.4 (1–64)	2.7×10^3	+

[a] Sporozoites were counted in a hemocytometer; [b] 3–4-week-old golden hamsters were inoculated intracardially; [c] estimate; see ref. 7 and text; [d] average of two values.

injected per hamster. All hamsters became infected and in those inoculated with the same number of sporozoites the prepatent period did not vary by more than ± 8 h. The infectivity of sterile sporozoites did not differ appreciably from that of nonsterile ones (correlation coefficient: 0.99).

Discussion. Several years ago, there was considerable research interest in the cultivation of the sporogonic stages of *Plasmodium* spp.[8]. With the exception of the in vitro formation of ookinetes of *P. berghei* from rodent blood[9], attempts to cultivate further developed mosquito stages of malaria of mammals have been frustrated, firstly, by the large numbers of fungi and bacteria associated with the digestive tract of mosquitoes, and, secondly, by the unavailability of culture systems that support extended parasite development in vitro. Recently, we have cultured guts and abdomens individually in antibiotic-free medium for up to 10 days (unpublished). Incidents of contamination with either fungi or bacteria were neither observed in these cultures nor in the rearing containers of the experiments described here. The samples taken from larval cultures equally remained sterile. In later experiments, contamination of adult cages was sometimes experienced when it was necessary to offer two consecutive infective blood meals. This was evident from the condition of the sucrose-hypoxanthine solution in the U-tube, and the filter paper. This problem can be alleviated by the use of autoclaved membrane feeders equipped with surface-sterilized membranes, rather than hamster. Keeping the mosquitoes at the lower temperature shortly before and after the infective blood meals resulted in a higher proportion of infected females. Presumably, this treatment reduced the activity of the proteolytic enzymes in the midgut[10], thus allowing a greater number of ookinetes to develop into oocysts. This contention is supported by the work of Gass[11, 12] and Gass and Yeates[13] who demonstrated that malarial ookinetes can be subject to considerable damage by digestive enzymes in the mosquito midgut. Even with high levels of infection, mosquito mortality was usually lower than in the conventionally reared colony where up to 90% of the females may not survive the 18 days required for complete sporogony. We attribute this to the lack of microbes which in nonsterile mosquitoes can aggrevate the delicate condition of heavily infected females[14]. We feel that the wide variations in the percentage of infected female mosquitoes are due to the problems of handling material in a newly developed system. The modification of Leibovitz's L-15 medium described here readily supports the growth of a wide variety of mammalian and invertebrate cells in vitro (Munderloh and Kurtti, unpublished). In conjunction with mosquito cells, it permits the rearing of axenic *A. stephensi* that are efficient vectors of rodent malaria, and are likely to transmit other species as well.

1 Supported in part by Public Health Service research grant AI 18345 from the National Institute of Allergy and Infectious Diseases, by a grant from the Agency of International Development DSPE-5542-G-SS-3042-00, and by a Charles and Johanna Busch award.

2 Boorman, J., Nature, Lond. *213* (1967) 197; Rosales-Ronquillo, M.C., Simons, R.W., and Silverman, P.H., Ann. ent. Soc. Am. *66* (1973) 949; Hamilton, D.R., and Bradley R.E., Sr, J. Invert. Pathol. *30* (1977) 318.
3 Sneller, V.-P., and Dadd, R.H., Exp. Parasit. *51* (1981) 169.
4 Munderloh, U.G., and Kurtti, T.J., part of this work was presented as a poster at the 2nd International Conference on Malaria and Babesiosis, Annecy, France, September 1983.
5 Vanderberg, J.P., Nussenzweig, R.S., and Most, H., J. Parasit. *54* (1968) 1009.
6 Munderloh, U.G., Kurtti, T.J., and Maramorosch, K., J. Parasit. *68* (1982) 1085.
7 Warhurst, D.C., and Folwell, R.O., Ann. trop. Med. Parasit. *62* (1968) 349.
8 Schneider, I., and Vanderberg, J.P., in: Malaria, vol. 2, p. 235. Ed. J.P. Kreier. Academic press, New York 1980.
9 Weiss, M.M., and Vanderberg, J.P., J. Parasit. *63* (1977) 932.
10 Clements, A.N., The Physiology of Mosquitoes, p. 154. Pergamon Press, New York 1963.
11 Gass, R.F., Acta trop. *34* (1977) 127.
12 Gass, R.F., Acta trop. *36* (1979) 323.
13 Gass, R.F., and Yeates, R.A., Acta trop. *36* (1979) 243.
14 Maier, W.A., Z. ParasitKde *41* (1973) 11.

0014-4754/85/091205-03$1.50 + 0.20/0

A simple method for counting nuclei in the preimplantation mouse embryo[1]

K.M. Ebert, R.E. Hammer and V.E. Papaioannou

Tufts University School of Medicine, 136 Harrison Avenue, Boston (Massachusetts 02111, USA), and Laboratory of Reproductive Physiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia (Pennsylvania 19104, USA), 23 October 1984

Summary. An easy and rapid method of counting the number of cells in the preimplantation mouse embryo is described. The procedure increases the speed with which large numbers of embryos can be processed using a simple squash technique. Cell numbers are determined by exposing the embryos to the fluorescent DNA-binding dye, Hoechst 33258, removing the zona pellucida and simply squashing the embryo and counting the number of fluorescent nuclei. An increase in fluorescent intensity and maintenance of nuclear conformation of the squashed preparations are greatly improved by the use of the non-ionic detergent Triton X-100. Viability of dye-treated fertilized one-cell and blastocyst stage embryos is maintained at least up to day 13 of pregnancy following transfer of the embryos to the uteri of pseudopregnant recipients. Additional uses for this staining technique are discussed.
Key words. Morula; blastocyst; nuclei; vital stain; Hoechst 33258.

The most generally used technique for counting blastomeres in the preimplantation embryo is the air-drying method of Tarkowski[2] or various modifications of this method as outlined by Kinsey[3]. More recently an improved method for chromosomal preparations from preimplantation mammalian embryos was reported by Dyban[4]. The common features of these methodologies are 1) they were primarily designed as chromosomal preparations, 2) they involve fixing, spreading and staining of the embryos, and 3) they require considerable experience for consistent high quality preparations.

For cell counting, the present method using a fluorescent DNA-binding dye has several advantages over earlier techniques.

1) Embryos are rapidly processed since no exposure to hypotonic solution is required.

2) Embryos are treated in the same medium used for culture and are therefore easily handled.