Bull. Org. mond. Santé }
Bull. Wld Hlth Org. } 1972, **46**, 337-343

A cytoplasmic polyhedrosis virus in midgut cells of *Anopheles stephensi* and in the sporogonic stages of *Plasmodium berghei yoelii*

R. G. BIRD,[1] C. C. DRAPER,[2] & D. S. ELLIS [3]

Although it has been known for some time that nuclear and cytoplasmic polyhedrosis viruses may infect the larval stages of mosquitos capable of transmitting mammalian malaria this paper reports for the first time the presence of a cytoplasmic polyhedrosis virus in adult Anopheles stephensi. *The virus was shown to be present in sporogonic stages of* Plasmodium berghei yoelii *with which the mosquitos were infected. It is possible that other viruses may affect both vector and malaria parasites. Further studies are required of viral infections in mosquitos as a possible method of biological control.*

Reviews of mosquito pathogens by Jenkins (1964) and by Chapman et al. (1970) refer to observations of iridescent viruses and nuclear (NPV) and cytoplasmic (CPV) polyhedrosis viruses, mainly in the larval stages of culicine mosquitos, but not in adult anophelines. Although it is probable that an NPV was responsible for the intranuclear bodies seen by Das Gupta & Ray (1954, 1957) in the midgut of *Anopheles subpictus* larvae and that the tetragonal inclusions noted by Chapman et al. (1970) in *Anopheles crucians* larvae were due to a CPV, no electron microscope evidence of viral infection was obtained in either case.

When difficulty was experienced in obtaining transmission of *Plasmodium berghei yoelii* by *Anopheles stephensi* mosquitos, which had previously been good intermediate hosts for the parasite, a closer examination of the adult vector mosquito was thought necessary. This revealed the occasional presence in the midguts of yellowish opaque patches; when squash preparations of guts containing such lesions were treated with warm Giemsa stain, direct light microscopy showed inclusion bodies suggestive of a virus infection. A preliminary account of an examination of this material by electron microscopy has already appeared (Bird et al., 1969). Here we record in greater detail our identification of a CPV in these adult *An. stephensi* and in developing oocysts and deformed mature sporozoites of the rodent malaria parasite *P. berghei yoelii* within the infected mosquitos.

The colony of *An. stephensi* involved was the progeny of a Delhi strain of the mosquito (STSSDPI) kept at the Malaria Reference Laboratory, Horton Hospital, Epsom, England. It was reared in an insectary of the Ross Institute of Tropical Hygiene, at 25°C and 70% relative humidity, where it had been maintained over a period of 12 years. During this time mosquitos had been used for much other experimental work.

ELECTRON MICROSCOPY METHODS

Direct examination of crushed midgut cells

Midguts with extensive, oedematous, yellowish patches of opacity were selected by light microscopy and their cells ruptured in 0.1 ml of distilled water on a clean slide. Staining was done by the method of Brenner & Horne (1959). Using a fine glass pipette one drop of the cell debris suspension so obtained was mixed on another clean slide with an equal volume of 3% ammonium molybdate in distilled water adjusted to pH 6 with dilute ammonium hydroxide. Smaller drops of the mixture were pipetted immediately on to Formvar and carbon coated " old 400 " Smethurst grids for examination in a GEC/AE1 EM6 electron microscope.

[1] Director, Electron Microscope Laboratory, London School of Hygiene and Tropical Medicine, England.

[2] Senior Lecturer, Ross Institute of Tropical Hygiene and London School of Hygiene and Tropical Medicine.

[3] Assistant Electronmicroscopist, Electron Microscope Laboratory, London School of Hygiene and Tropical Medicine.

The preparation of fixed and stained sections of midgut cells with oocysts of P. berghei yoelii

Following identification under the dissecting microscope, and within 30 sec of removal, some diseased midguts carrying oocysts were fixed with 3% glutaraldehyde in modified Rhodheim & Zetterqvist buffer solution at pH 6.8 for 3 hours. After adequate washing in plain buffer solution, fixation was completed in 1% osmium tetroxide in the same buffer solution. Excess of osmium tetroxide was removed by repeated washing in plain buffer solution before the midguts were dehydrated by graded mixtures of ethanol and distilled water and stained with a 2% potassium tungstophosphate solution in absolute ethanol. The midguts were then soaked in several changes of toluene before being embedded in Araldite.

Sections were cut on a Huxley type ultramicrotome and viewed in either a Zeiss EM9 or GEC/AEI EM6 electron microscope.

RESULTS

Negative stained material

Stellate virions, some full and some without the inner core of nucleic acid, were easily distinguished among the many bits of cell debris, outlined by the ammonium molybdate. Virions were seen either singly or in groups (Fig. 1). They appeared to have a basic icosahedron symmetry with at least six short projections at right angles to the facet intersections. The average body diameter of these virions was 54 nm. By using the rotation technique of Markham et al. (1963) the image was enhanced and the surface morphology was seen to resemble the structural model of a CPV as given by Hosaka & Aizawa (1964) (Fig. 3A–3E). The length of the surface projections, approximately 10 nm, when added to the previous body measurement gave an overall diameter of 74 nm.

Other areas of the preparation contained small but characteristic crystalline inclusion bodies, similar to the larger ones seen by light microscopy; some had virions embedded in their substance (Fig. 2).

Fixed and stained sections

In thin sections cut from heavily infected specimens it was not difficult to find cells in which the elaboration of virions was taking place. The nuclei of these cells appeared normal. Virions were confined to the cell cytoplasm and were grouped in association with electron-dense granular areas of what has been described in the literature as viral stroma. Nearby, early small inclusion bodies, often surrounded by clear spaces, were seen displacing cytoplasmic structures (Fig. 4).

In some of the infected cells there were oocysts of *P. berghei yoelii*. The majority of these were vacuolated and contained in their cytoplasm recognizable groups of virions (Fig. 5). Where oocysts were mature virions were present in some of the deformed and vacuolated sporozoites (Fig. 6 and 7).

DISCUSSION

Although intranuclear and cytoplasmic inclusions have been described in the larvae of anopheline mosquitos this is the first report, to our knowledge, giving definite evidence of a CPV in adult anophelines. Several searches by electron microscopy failed to demonstrate the virus in the larvae, which appeared healthy and morphologically normal by light microscopy. Initial experiments to transmit the virus to larvae and adults were unsuccessful, as were attempts to grow the virus in tissue cultures of *An. stephensi* larval cells. We are therefore unable to discuss, at present, either the origin or the method of spread of the virus. It is, perhaps, of interest that infected adult mosquitos sent to another laboratory succeeded in transmitting the virus to a similar colony of *An. stephensi* kept there.

The virus was readily demonstrated over a period of several months in mosquitos in our insectary, but the infection gradually subsided and it has not been possible to demonstrate either inclusions or virions for over 9 months since the last observations were made. This has not been associated with any change in techniques in the insectary.

It is of particular interest that the virus was seen to be multiplying in concomitant developing and mature oocysts of *P. berghei yoelii*, and also to be present in mature deformed sporozoites within the oocyst. However, the relationship between malarial transmission and the presence of this CPV is left in doubt. While it is certainly true to say that the CPV infection was associated with a temporary reduction in transmission and that virions were seen in sections of oocysts and sporozoites, it is known that *P. berghei yoelii* is often liable to cause variable infections in mosquitos. As controlled experiments with the virus could not be carried out, pure coincidence cannot be ruled out.

An earlier demonstration of a different but unidentified virus particle of diameter 20–30 nm in a

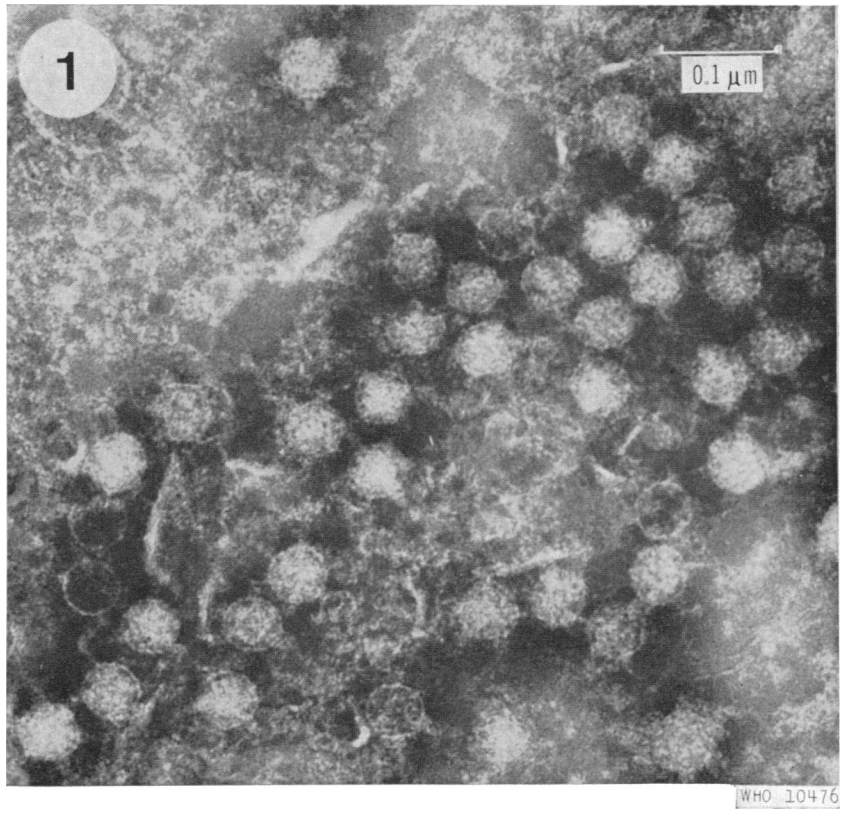

Fig. 1. A group of virions, some full, some empty, among the cell debris of crushed and negatively stained midgut cells (×160 000).

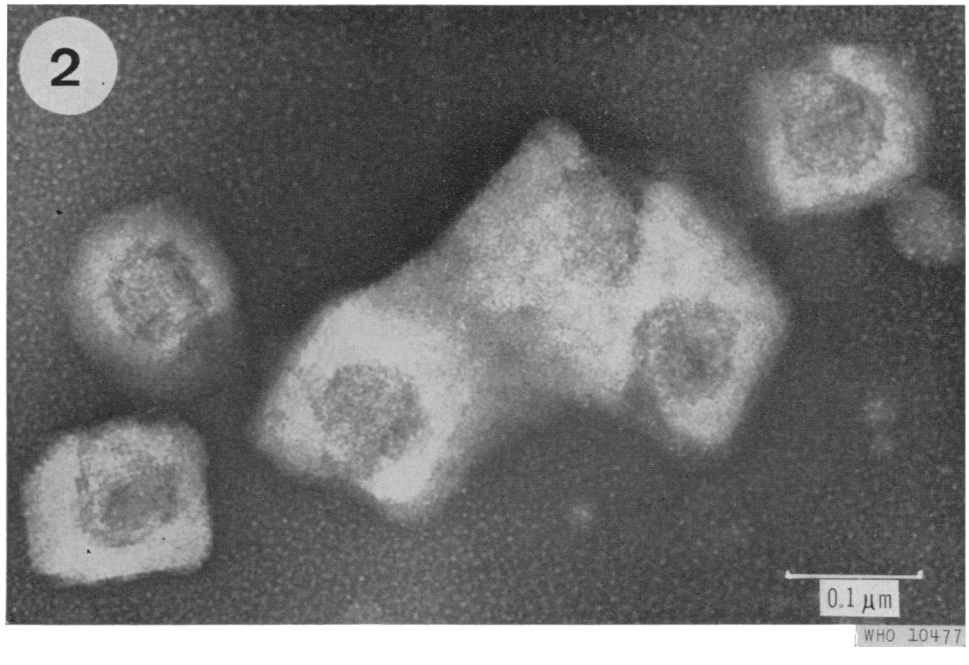

Fig. 2. Small crystalline inclusion bodies with central virions (×189 000).

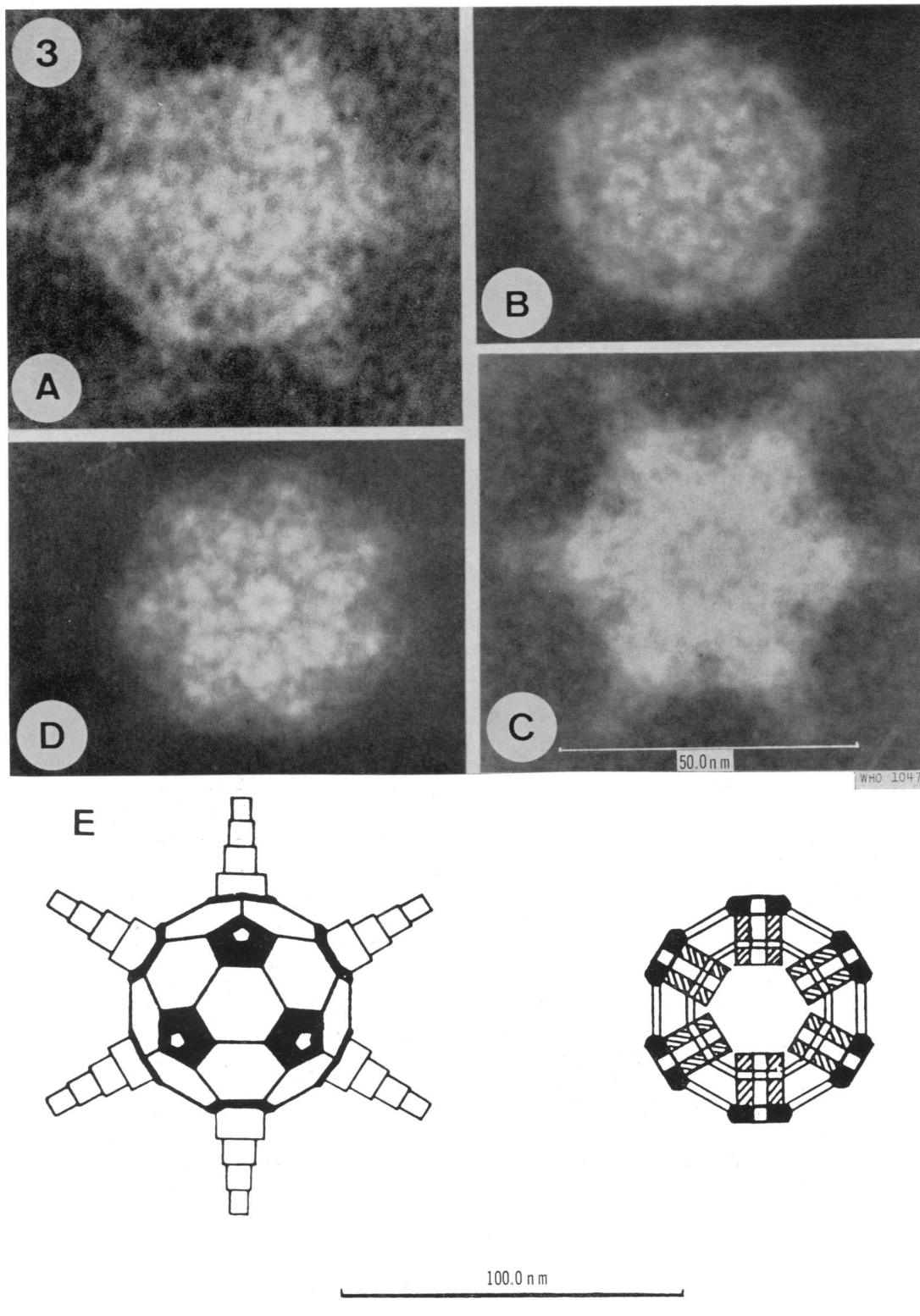

Fig. 3. Surface morphology of a selected virion—an icosahedron with surface projections. The electron micrographs show the structure as revealed by the rotation technique of Markham et al. (1963). (A) virion chosen, (B) image of same virion after 5 rotations, (C) after 6 rotations, and (D) after 7 rotations—all ×560 000. Diagram (E) represents a structural model of a CPV and is reproduced by permission from Hosaka & Aizawa (1964) (Copyright by Academic Press, Inc.).

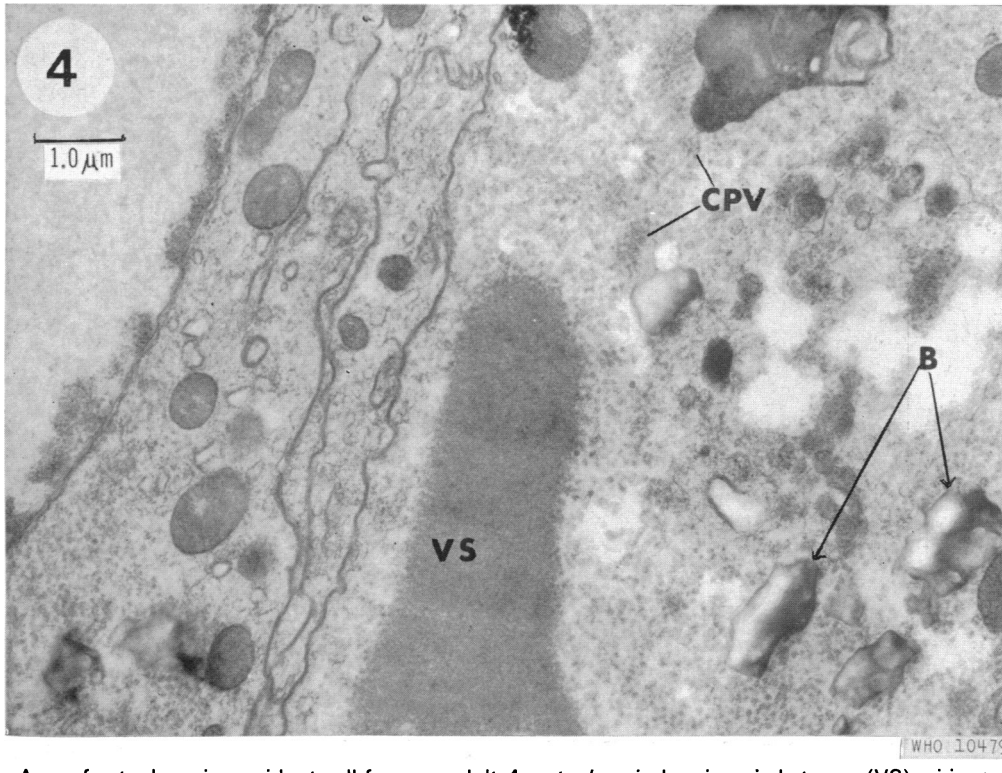

Fig. 4. Area of cytoplasm in a midgut cell from an adult *An. stephensi,* showing viral stroma (VS), virions (CPV), and crystalline inclusion bodies (B) (×12 000).

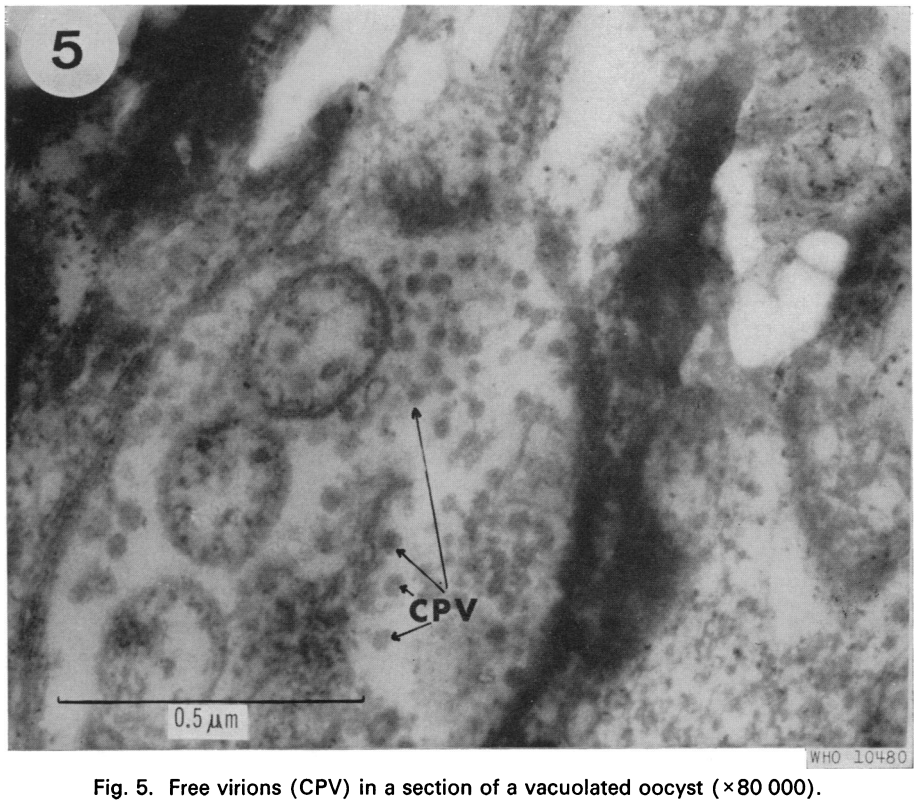

Fig. 5. Free virions (CPV) in a section of a vacuolated oocyst (×80 000).

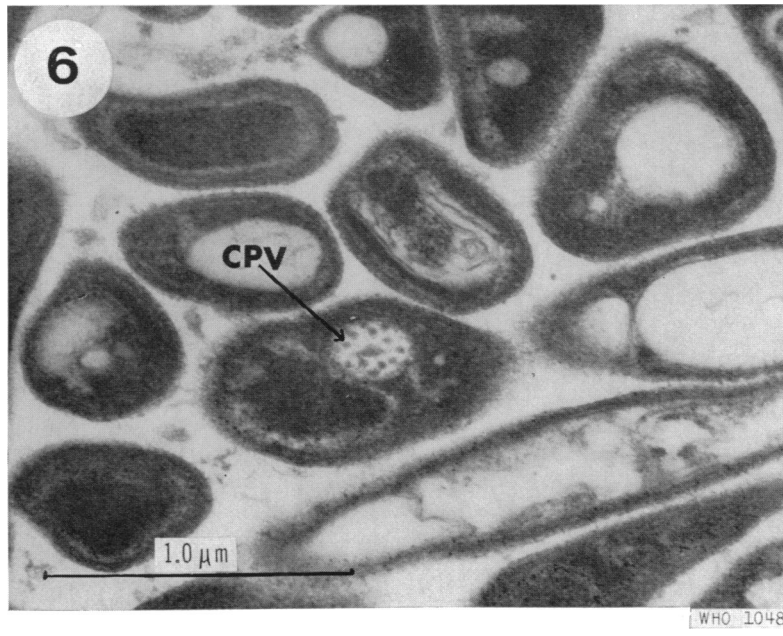

Fig. 6. Deformed and vacuolated sporozoites containing CPV in longitudinal section (×40 000).

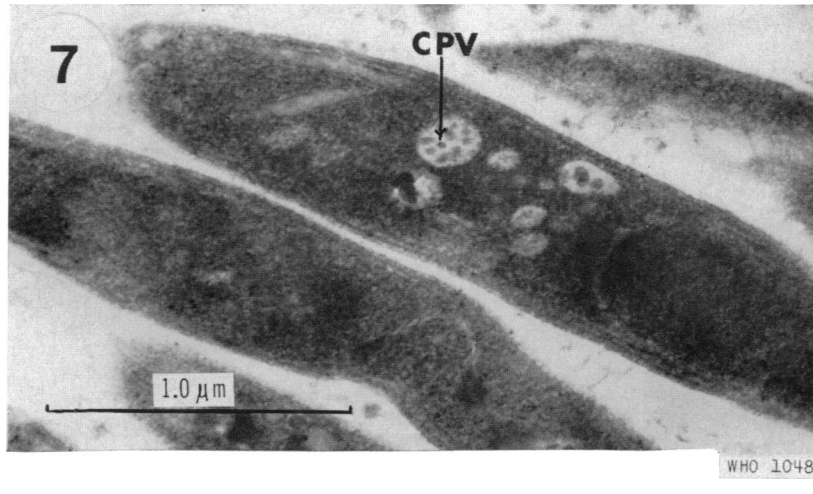

Fig. 7. Deformed and vacuolated sporozoites containing CPV in transverse section (×40 000).

colony of *An. stephensi*, also being used in rodent malaria transmission experiments (Bird et al. 1969), would suggest that a CPV is not the only virion that might affect the sporogonic stages in the life cycle of a mammalian malaria parasite. Bertram et al. (1964) carried out experiments involving mosquitos infected with both *Plasmodium gallinaceum* and an arthropod-borne virus, but their results were inconclusive. With the possibility of using viral infections in vectors as a method of biological control, the need to continue studies on the interaction between concomitant viral and protozoal infections in arthropod vectors, as well as in other situations, is obvious.

POSTSCRIPT

After this paper was submitted for publication, midgut sections of *An. stephensi* mosquitos, kindly supplied by Professor W. Peters from the stock at the Liverpool School of Tropical Medicine, infected with a CPV and *Plasmodium vivax*, were examined. When negatively stained material was observed the CPV appeared to be the same as the CPV described above, but the virus-like particles seen in the abnormal oocysts had an average diameter of 30 nm. Similar particles were also seen within deformed sporozoites.

ACKNOWLEDGEMENTS

We are glad to acknowledge helpful advice given by Dr T. W. Tinsley, Insect Pathology Unit, University of Oxford, and the assistance of Miss C. Upton and Miss J. Dorrell with the preparatory techniques and of Mr D. Day with the electron micrograph printing. This work was supported in part by a financial grant from the World Health Organization.

RÉSUMÉ

MISE EN ÉVIDENCE D'UN VIRUS DE POLYÉDRIE CYTOPLASMIQUE DANS LES CELLULES DE L'INTESTIN MOYEN D'*ANOPHELES STEPHENSI* ET DANS LES STADES SPOROGONIQUES DE *PLASMODIUM BERGHEI YOELII*

Grâce à la microscopie électronique, on a identifié un virus de polyédrie cytoplasmique dans les cellules de l'intestin moyen d'*Anopheles stephensi* adultes appartenant à une colonie maintenue en insectarium à l'Institut Ross d'hygiène tropicale depuis 12 ans.

Après coloration négative, le virus se présente sous la forme de particules d'un diamètre moyen de 54 nm, à symétrie icosaédrique. Des excroissances d'une longueur approximative de 10 nm sont disposées à angle droit à l'intersection des faces.

Sur des coupes minces d'intestins infectés par *Plasmodium berghei yoelii*, les noyaux cellulaires apparaissent normaux. Les virions sont répartis par groupes dans le cytoplasme qui contient également des zones granuleuses de stroma viral opaque aux électrons et de petites inclusions cristallines. Dans certaines cellules infectées, on décèle la présence d'oocystes de *P. berghei yoelii*, en majorité dégénérés et renfermant dans leur cytoplasme des amas de virions. Dans certains oocystes mûrs, des virions sont visibles dans les sporozoïtes déformés et vacuolisés.

On ne peut exclure l'éventualité d'une réduction de la transmission du paludisme comme conséquence de l'infection virale du vecteur. Sur le plan de la lutte biologique, l'interaction entre infections concomitantes par un protozoaire et par un virus chez un même vecteur présente un intérêt certain et justifie de nouvelles recherches.

REFERENCES

Bird, R. G., Draper, C. C., Garnham, P. C. C., Pudney, M., Shute, P. G. & Varma, M. G. R. (1969) *Trans. roy. Soc. trop. Med. Hyg.*, **64**, 28

Bertram, D. S., Varma, M. G. R. & Baker, J. R. (1964) *Bull. Wld Hlth Org.*, **31**, 679

Brenner, S. & Horne, R. W. (1959) *Biochim. biophys. Acta (Amst.)*, **24**, 103

Chapman, H. C., Clark, T. B. & Peterson, J. J. (1970) *Misc. Publ. Ent. Soc. Amer.*, **7**, 134

Das Gupta, B. & Ray, H. N. (1954) *Bull. Calcutta Sch. trop. Med.*, **2**, 57

Das Gupta, B. & Ray, H. N. (1957) *Parasitology*, **47**, 194

Hosaka, Y. & Aizawa, K. (1964) *J. insect. Path.*, **6**, 53

Jenkins, D. W. (1964) *Bull. Wld Hlth Org.*, **30** (Suppl.)

Markham, R., Frey, S. & Hill, J. G. (1963) *Virology*, **20**, 88