© Masson, Paris, 1987.

Ann. Parasitol. Hum. Comp.,
1987, 62 n° 5 pp. 418-425.

FRACTIONATION OF MOUSE MALARIOUS BLOOD ACCORDING TO PARASITE DEVELOPMENTAL STAGE, USING A PERCOLL-SORBITOL GRADIENT

H. GINSBURG*, I. LANDAU**, D. BACCAM**, D. MAZIER***

SUMMARY. Asexual intraerythrocytic malarial parasites permeabilize the membrane of their host cell to small monelectrolytes and anions. Since permeabilization increases with parasite maturation, this property has been used previously to fractionate blood infected with *Plasmodium falciparum* and *P. knowlesi* according to the developmental stage of the parasite, using Percoll-sorbitol density gradients. We have extended this method to fractionate mouse blood infected with four species of rodent malaria: *P. chahaudi*, *P. vinckei*, *P. voelii* and *P. berghei*. While the method works in principle in this case, the polyparasitism which characterizes these species prevented explicit separation according to developmental stage. Hence, erythrocytes harbouring several ring-stage parasites appeared in the same fraction which contained cells hosting a single trophozoite, and polyparasitized trophozoites were associated with singly-infected schizont. This observation implies that permeabilization of the host cell membrane results from the integrated metabolic activity of the parasite(s) and is not related to a specific phase of parasite development.

Key-words: Rodent malaria. Cell cycle. Membrane permeability. Percoll sorbitol gradient.

Fractionnement, à l'aide d'un gradient de Percoll-sorbitol, de sang de Souris paludéenne, en fonction du stade parasitaire.

RÉSUMÉ. Les stades érythrocytaires asexués des *Plasmodium* perméabilisent la membrane de leur cellule-hôte aux petites molécules non chargées et aux anions. Du fait de l'augmentation de cette perméabilisation au cours de la maturation du parasite, cette propriété a déjà été utilisée auparavant pour fractionner du sang infecté par *Plasmodium falciparum* et *P. knowlesi*, en utilisant un gradient de Percoll-sorbitol et ceci en fonction du stade de développement du parasite. Nous avons appliqué cette méthode au fractionnement de sang de Souris infectées par 4 espèèes de *Plasmodium* de Rongeurs : *P. chabaudi*, *P. vinckei*, *P. yoelii* et *P. berghei*. Bien que la méthode s'applique en principe dans ce cas, le polyparasitisme des globules rouges, qui caractérise ces espèces, empêche une séparation stricte des parasites en fonction de leur âge. Ainsi, des érythrocytes hébergeant plusieurs anneaux se retrouvent dans la même fraction que des cellules contenant

* *Department of Biological Chemistry, Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem 91904, Israel.*
** *Muséum National d'Histoire Naturelle, Laboratoire des Vers, 61, rue Buffon, F 75321 Paris.*
*** *Département de Parasitologie, Groupe Hospitalier Pitié-Salpêtrière, 47-83, boulevard de l'Hopital, F 75651 Paris Cedex 13.*

Accepté le 11 mars 1987.

Article available at http://www.parasite-journal.org or http://dx.doi.org/10.1051/parasite/1987625418

un trophozoïte unique et des érythrocytes contenant plusieurs trophozoïtes sont associés à des globules contenant un seul schizonte. Ces observations impliquent que la perméabilisation de la membrane de la cellule-hôte résulte de l'activité métabolique globale du ou des parasites et est sans rapport avec une phase évolutive spécifique.

Mots-clés : Paludisme des Rongeurs. Cycle cellulaire. Perméabilité membranaire. Gradient au Percoll-sorbitol.

Introduction

The intraerythrocytic development of malarial parasites is accompanied by substantial alterations in the permselectivity properties of the host cell membrane: The infected red blood cell becomes highly permeable to a variety of carbohydrates, amino acids and anions, which otherwise permeate into the cell at low pace through specialized transport systems, or by simple diffusion, if at all. This phenomenon has been described for different species of parasites and host cells: *Plasmodium lophurae* in duck red blood cells (Sherman and Tanigoshi, 1974, 1975), *P. berghei* in mouse (Homewood and Neame, 1974) and *P. falciparum* grown in human red blood cells in culture (Ginsburg *et al.*, 1983, 1985; Kutner *et al*, 1983, Elford *et al*, 1985). It has been shown recently that the permeabilization of the host cell membrane progresses with parasite maturation, and on this basis, a technique has been devised for the isolation of *P. falciparum*-infected human erythrocytes according to the of developmental stage of the intracellular parasite (Kutner *et al*, 1985).

The extension of this technique to different species of murine malaria parasites developing in mice is presented in this report.

Materials and methods

Four diferent parasite species were tested: *P. yoelii yoelii* 265 BY, *P. berghei berghei* NK 65, *P. chabaudi chabaudi* AS and *P. vinckei petteri* 279 BY. Random bred Swiss albino mice, weighing 20-25 grams (obtained from IFFA-CREDO, France), were inoculated intraperitoneally with 10^6-10^7 parasites and the development of parasitemia was followed by microscopic inspection of Giemsa stained thin blood smears. When parasitemia was higher than 25-30 %, mice were bled into test tubes containing heparin. Blood was washed 3 times in 20 volumes of cold HEPES-buffered saline (HPS), plasma and buffy coat were removed by aspiration. Discontinuous Percoll-sorbitol gradients were prepared as described previously (Kutner *et al*, 1985) except that the Percoll concentration range was 10-40 %. Erythrocytes were suspended at 20 % hematocrit in HPS supplemented with sorbitol, 6 % w/v, and layered on top of the gradient. After 20 min centri-

fugation at 20,000 rpm in a fixed angle rotor, cell-containing layers were removed and washed successively in HPS containing decreasing concentrations of sorbitol. Thin blood smears were prepared from each layer, stained with Giemsa and inspected under the microscope.

Results

The distribution of cells obtained from mice infected with different parasite species in the Percoll-sorbitol gradient is depicted in *figure 1.* Distinct cell layers were observed throughout the gradients, although their position in the gradient varied somewhat with the species.

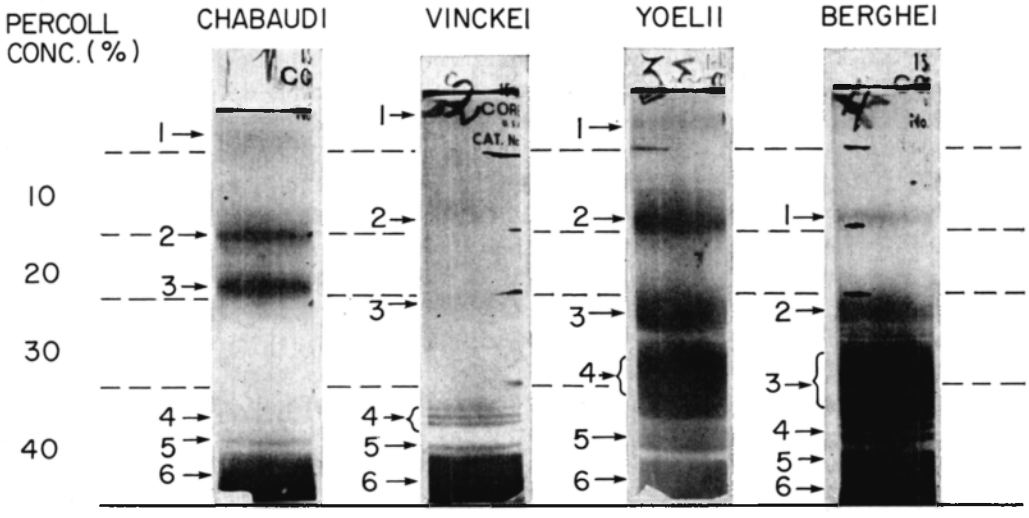


FIG. 1. — Partitioning of cell fractions of parasitized blood in Percoll-sorbitol gradient. Percoll concentrations are shown on the left. Fractions were numbered from top to bottom, at their order of withdrawal from the gradient. See *table I* for morphological description.

The morphological appearence of the parasites in the various layers of the gradient is described in *table I* and pictures of *P. berghei*-infected red blood cells in the different layers, are shown in *figure 2.*

Discussion

The intraerythrocytic development of malaria parasites involves dramatic changes in the structure and composition of the host cell membrane (see Sherman, 1985 for a recent review). One of the most obvious functional consequence of these changes is the permeabilization of the infected red blood cell to anions (Kutner

TABLE I. — Morphological description of erythrocytes infected with different species of parasites, as they appear in fractions isolated from the Percoll-sorbitol gradient. Numbering of layers goes from the top of the gradient to its bottom. Percentiles indicate % parasitemia.

Species	Layer	Description
P. chabaudi	1	Swollen merozoites and debris of aged trophozoites.
	2	Aged schizonts; Polyparasitized trophozoites; 100 %.
	3	Aged and polyparasitized trophozoites; Schizonts; Male gametocytes; 100 %.
	4	Polyparasitized trophozoites; 100 %.
	5	Young polyparasitized trophozoites; Aged trophozoites; Female gametocytes; Few uninfected reticulocytes; 100 %.
	6	Few rings; Mostly aged and some young trophozoites; 20 %.
P. vinckei	1	Cellular debris.
	2	Free merozoites.
	3	Mostly mature and some aged schizonts; Host cell membrane damaged; 70-80 %.
	4	Few rings; Aged trophozoites and immature schizonts; Some reticulocytes; 80 %.
	5	Mostly young and aged trophozoites; Polyparasitized younger stages; Some merozoites—probably immediately after invasion; 30 %.
	6	Rings; 5-10 %.
P. voelii	1	Debris of ghost and damaged parasites.
	2	Mature schizonts with abnormal morphology—no separation into merozoites; 90 %.
	3	Highly polyparasitized trophozoites (> 10/cell); Large schizonts; Young gametocytes; Many reticulocytes; 85 %.
	4	Polyparasitized trophozoites (5-9/cell); Mature and aged schizonts; Many mature gametocytes; 90 %.
	5	Aged trophozoites (2-4/cell); Many young schizonts; Many mature gametocytes; 90 %.
	6	Young and aged trophozoites; Few young schizonts; 50 %.
P. berghei	1	Free parasites and cellular debris.
	2	Highly polyparasitized trophozoites (8-9/retic.); ~ 20 % mature schizonts in mature large and small erythrocytes (in small—8 nuclei/cell; in large—20); Trophozoites in reticulocytes are devoid of pigment; 95 %.
	3	Polyparasitized reticulocytes (4-8/cell); Aged trophozoites and few young schizonts; 100 %.
	4	Aged trophozoites; Polyparasitized reticulocytes (3-4/cell with younger trophs.); 95 %.
	5	Aged trophozoites; Very few polyparasitized reticulocytes (2/cell); 80 %.
	6	Rings and young trophozoites; 5-10 %.

et al, 1982, 1983) and to nonelectrolytes (Homewood and Neame, 1972; Sherman and Tanigoshi, 1974, 1975; Ginsburg *et al*, 1983, 1985; Elford *et al*, 1985). Permeabilization results probably from structural defects induced in the host cell mem-

brane (Ginsburg and Stein, 1987) due to the insertion of parasite-derived polypeptides (Howard, 1982), and is seemingly vital to the normal development of the parasite (Ginsburg, 1987), since blocking of the new transport pathways impairs parasite growth (Cabantchik *et al*, 1983; Kutner *et al*, 1987).

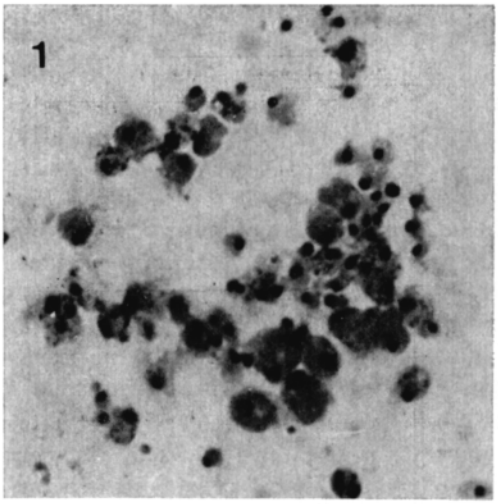
The distribution of infected cells in the various layers of the Percoll-sorbitol gradient has been shown to depend on the extent of permeabilization of the host cell membrane (Kutner *et al*, 1985), which, in turn, depends on the stage of parasite development. Although these phenomena were investigated in *P. falciparum*, the present work indicates that they can be extended to murine species. In all four species investigated, red blood cells containing the more mature parasites were detected in the less dense layers of the gradient, suggesting that these cells are more permeable to sorbitol. However, the general phenomenon of polyparasitism in murine malaria species (Garnham, 1970), resulted here in a distribution which was apparently different from that observed in *P. falciparum*, where each gradient layer contained only one distinct developmental stage. Thus, erythrocytes harboring 2-3parasites at the ring stage appeared in the same gradient layer with cells containing one trophozoite and cells containing 2-3 trophozoites co-sedimented with cells harboring one mature schizont, and so on. It is therefore suggested that permeabilization of the host cell membrane resulted from the integral metabolic activity of the intraerythrocytic parasites, i. e., that cells containing a larger number of parasites were more permeabilized.

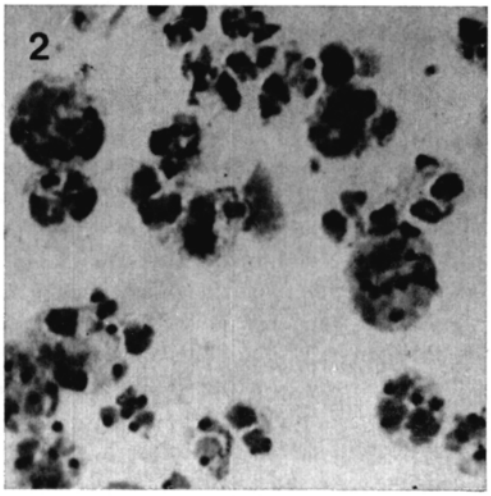
While this conclusion is true in its broadest sense, there were distinct differences in the partitioning of cells infected with the various parasite species in the gradient. Since this diversity may bear on the onset of permeabilization and on systemic effects on non infected erythrocytes, it deserves special attention:

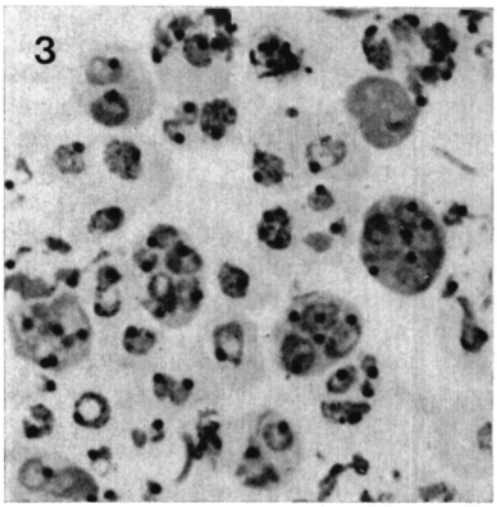
P. chabaudi: This species, unlike the others investigated in the present work, develops synchronously and most parasites were at the trophozoite stage (mice were bled in late morning). Since trophozoites were detected in the most dense layer together with non infected cells, it is very probable that permeabilization starts at or just after this stage. Partitioning of infected cells in the gradient depended almost exclusively on the number of parasites per host cell and on trophozoite age. Most interestingly, layers 3 and 5 also contained gametocytes, the first mostly female and the second mostly male gametocytes. Thus, this method could be used for the differential investigation of these cells in the future.

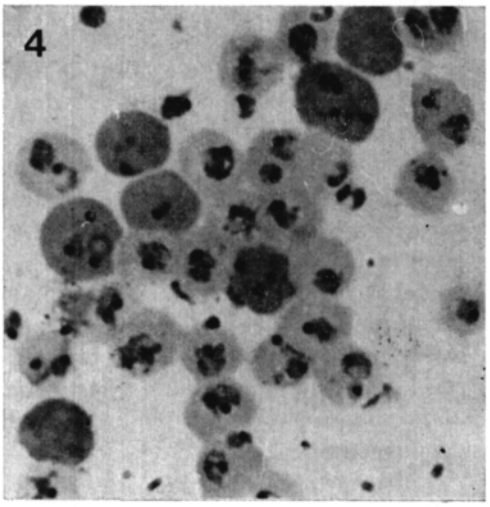
P. vinckei: Enhanced permeability starts to develop at the ring stage but cells polyparasitized with rings can be seen also in the less dense layers. In mature stages, the host cell membrane was usually damaged, implying that it is more fragile compared to cells infected with other species. Alternatively, these infected cells

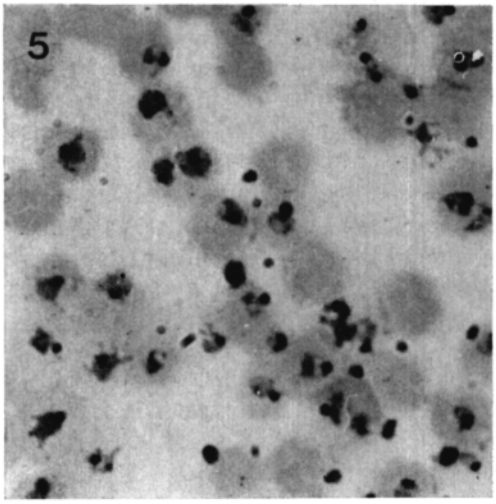
FIG. 2. — Morphological appearance of *P. berghei*-infected cells in the different layers of the Percoll-sorbitol gradient. Numbering of fractions refers to the designation in *figure 1*.

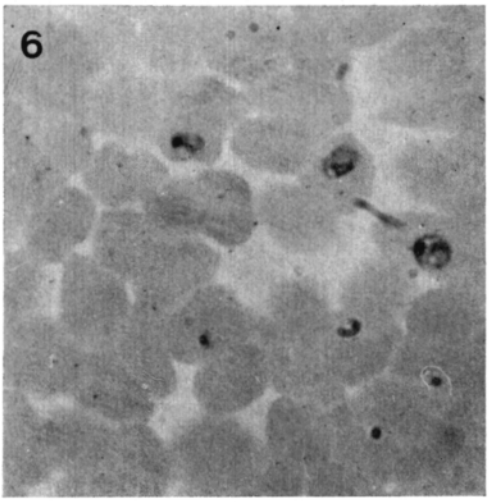

1


2


3


4


5


6

may be substantially more permeable and thus became damaged during the post-gradient washings. Furthermore, the parasitemia in most layers was relatively low, implying that non infected cells were also permeabilized. It has been recently demonstrated that some of the polypeptides produced by the parasite are « secreted » extracellularly (Wilson, 1980 ; Jepsen and Andersen, 1981, Dasilva *et al.*, 1983 for example). Such molecules should be sufficiently amphipatic to partition into the host cell membrane. As a result they could cause structural imperfections with consequent permeabilization, both in the host cell and in non infected cells. Since this phenomenon was typical of *P. vinckei*, it is not unlikely that the nature of the polypeptide of this species is somewhat different from that of other species and deserves further experimentation.

P. yoelli: Permabilization of the host cell membrane develops at the late trophozoite stage. Thereafter it strongly depends on the number of parasites per cell, both at the trophozoite and the schizont stages. There is an indication for the presence of a sub-population of abnormal parasites which fail to develop and mature. Erythrocytes containing young gametocytes, i. e., where the host cytosol is still visible, are more swollen than those harboring mature gametocytes. This observation would be compatible with the decrease of the host cytoplasmic volume which occurs upon maturation of the gametocyte, or if the host cell membrane becomes totally permeabilized, e. g., also to cations.

P. berghei: Permeabilization occurs at the young trophozoite stage. It increases with parasite maturation, mostly from trophozoite to schizont. This observation points to a physiological difference between this species and *P. yoelii*, which is morphologically very similar (Garnham, 1970). There is a distinct change in the permeability of host reticulocytes as a function of parasite number. In fact, only reticulocytes were found to be polyparasitized. It seems as if parasite development accelerates the maturation of host reticulocytes inasmuch as polyparasitized cells harboring trophozoites are deeply stained while those hosting schizonts are as pale as normocytes.

In conclusion, the Percoll-sorbitol gradient which has been developed using cultured *P. falciparum* and seems applicable to *P. knowlesi*, *P. cynomology* and *P. fragile* (Aley *et al*, 1986), is much less effective for the fractionation of mouse blood infected with murine parasite species, probably due to their characteristic polyparasitism. However, since it is easier to isolate host cell membranes from erythrocytes infected with murine species (Heidrich *et al*, 1979), investigation of the composition of such membranes derived from different fractions obtained by this method, could elucidate the nature of the permeabilizing factors and their mode of action: The abundance of these factors in the host cell membrane should not depend on parasite age, but on the position of the infected cell in the Percoll-sorbitol gradient.

ACKNOWLEDGMENT. — This work was supported by the WHO/World Bank/UNDP Special Programme for Research and Training in Tropical Diseases.

REFERENCES

1. ALEY S. B., SHERWOOD J. A., MARSH K., EIDELMAN O., HOWARD R. J. : Identification of isolate-specific proteins on sorbitol-enriched *Plasmodium falciparum* infected erythrocytes from Gambian patients. *Parasitology*, 1986, *92*, 511-525.
2. CABANTCHIK Z. I., KUTNER S., KRUGLIAK M., GINSBURG H. : Anion transport inhibitors as suppressors of *Plasmodium falciparum* growth in *in vtiro* cultures. *Molec. Pharmacol.*, 1983, *23*, 92-99.
3. DASILVA L. R., LOCHE M., DAYAL R., PERRIN L. H. : *Plasmodium falciparum* polypeptides released during in vitro cultivation. *WHO Bull.*, 1983, *61*, 105-112.
4. GARNHAM P. C. C. : Malaria parasites and other haemosporidia. *Blackwell Scientific Publications*, Oxford, 1970.
5. GINSBURG H. : How and why does the malarial parasite permeabilize its host cell membrane? *In:* Biomembranes and Disease (G. Benga, J. Tager, Eds.). *Springer Verlag*, Berlin, 1987 (in press).
6. GINSBURG H., KRUGLIAK M., EIDELMAN O., CABANTCHIK Z. I. : New permeability pathways induced in membranes of *Plasmodium falciparum* infected erythrocytes. *Molec. Biochem. Parasitol.*, 1983, *8*, 177-190.
7. GINSBURG H., KUTNER S., KRUGLIAK M., CABANTCHIK Z. I. : Characterization of permeation pathways appearing in the host membrane of *Plasmodium falciparum* infected red blood cells. *Molec. Biochem. Parasitol.*, 1985, *14*, 313-322.
8. GINSBURG H., STEIN W. D. : Biophysical analysis of novel transport pathways induced in red blood cell membranes. *J. Membr. Biol.*, 1987, *96*, 1-10.
9. HEIDRICH H.-G., RUSSMANN L., BAYER B., JUNG A. : Free-flow electrophoresis for the separation of malaria infected and uninfected mouse erythrocytes and for the isolation of free parasites *(Plasmodium vinckei)*. A new rapid technique for the liberation of malaria parasites from their host cells. *Z. Parasitenk.*, 1979, *58*, 151-159.
10. HOMEWOOD C. A., NEAME K. D. : Malaria and the permeability of the host erythrocyte. *Nature*, 1974, *252*, 718-719.
11. HOWARD R. J. : Alterations in the surface membrane of red blood cells during malaria. *Immunol. Rev.*, 1982, *61*, 67-107.
12. JEPSEN S., ANDERSEN B. J. : Immunoabsorbent isolation of antigens from culture medium of *in vitro* cultivated *Plasmodium falciparum. Acta Pathol. Microbiol. Scand.*, 1981, *C89*, 99-103.
13. KUTNER S., BARUCH D., GINSBURG H., CABANTCHIK Z. I. : Alterations in membrane permeability of malaria-infected human erythrocytes are related to the growth stage of the parasite. *Biochim. Biophys. Acta*, 1982, *687*, 113-117.
14. KUTNER S., GINSBURG H., CABANTCHIK Z. I. : Permselectivity changes in malaria *(Plasmodium falciparum)* infected human red blood cell membranes *J. Cell. Physiol.*, 1983, *114*, 245-251.
15. KUTNER S., BREUER W. V., GINSBURG H., ALEY S. B., CABANTCHIK Z. I. : Characterization of permeation pathways in the plasma membrane of human erythrocytes infected with early stages of *Plasmodium falciparum:* Association with parasite development. *J. Cell. Physiol.*, 1985, *125*, 521-527.
16. KUTNER S., BREUER W. V., GINSBURG H., CABANTCHIK Z. I. : On the mode of action of phlorizin as an antimalarial agent in *in vitro* cultures of *Plasmodium falciparum. Biochem. Pharmacol.*, 1986, *36*, 123-129.
17. NEAME K. D., HOMEWOOD C. A. : Alterations in the permeability of mouse erythrocytes infected with the malaria parasite *Plasmodium berghei. Int. Parasitol.*, 1975, *5*, 537-540.
18. SHERMAN I. W. : Membrane structure and function of malaria parasites and infected erythrocyte. *Parasitology*, 1985, *91*, 606-645.
19. SHERMAN I. W., TANIGOSHI L. : Incorporation of [14C]-amino acids by malaria *(Plasmodium lophurae)*. VI. Changes in the kinetic constants of amino acid transport during infection. *Exp. Parasitol.*, 1974a, *35*, 369-373.
20. SHERMAN I. W., TANIGOSHI L. : Glucose transport in the malaria *(Plasmodium lophurae)* infected erythrocytes. *J. Protozool.*, 1974b, *21*, 603-607.
21. SHERWOOD J. A., SPITALNIK S. L., ALEY S. B., QUAKYI I. A., HOWARD R. J. : *Plasmodium falciparum* and *P. knowlesi:* initial identification and characterization of malaria synthesized glycolipids. *Exp. Parasitol.*, 1986, *62*, 127-141.
22. WILSON R. J. M. : Serotyping *Plasmodium falciparum* malaria with S-antigens. *Nature*, 1980, *284*, 451-452.