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Plasmodium Species: Flow Cytometry and Microfluorometry Assessments of DNA Content and Synthesis

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JANSE, C. J., VAN VIANEN, P. H., TANKE, H. J., MONS, B., PONNUDURAI, T., AND OVERDULVE, J. P. 1987. *Plasmodium* species: Flow cytometry and microfluorometry assessments of DNA content and synthesis. *Experimental Parasitology* 63, 88–94. Fluorescence intensities were established by flow cytometry of different erythrocytic stages of *Plasmodium berghei* after staining of their DNA with Hoechst-33258 or Hoechst-33342. Parasites were obtained from highly synchronized infections or *in vitro* cultures. Most fluorescence measurements were performed using a low cost, clinical flow cytometer, equipped with a mercury arc lamp. Cells infected with *P. berghei* could be readily distinguished from uninfected cells on the basis of Hoechst–DNA fluorescence and single, double, and triple ring infected cells were separated clearly. The relative fluorescence intensities of different developmental stages (merozoites, ringforms, trophozoites, schizonts, and gametocytes) corresponded closely to the relative DNA contents of these stages as measured by microfluorometry. Flow cytometry appeared to be a sensitive and rapid method to measure DNA synthesis during asexual development; a C_{50} value of 5 μM of aphidicolin, a specific inhibitor of DNA synthesis, was established. Vital staining of parasites in culture was possible with both Hoechst dyes. After removal of Hoechst-33258, normal *in vitro* development of the stained parasites was observed. After Hoechst staining, the haploid ringforms of *P. vivax* showed slightly less fluorescence (15%) than ringforms of *P. berghei* and *P. falciparum*. No differences in fluorescence intensity were observed, however, by direct microfluorometry after Feulgen-pararosaniline staining, indicating that all three species have the same DNA content.

INDEX DESCRIPTORS AND ABBREVIATIONS: *Plasmodium berghei; Plasmodium falciparum; Plasmodium vivax;* Protozoa, parasitic; Malaria, rodent, human; Flow cytometry; Dyes, Hoechst-33258 and 33342; Deoxyribonucleic acid (DNA) synthesis; Aphidicolin; Base pairs (bp); Phosphate buffered saline (PBS); After infection (ai).

INTRODUCTION

Flow cytometry of malaria parasites has appeared to be a sensitive and rapid method to detect, count, and sort cells, infected with *Plasmodium* spp., after staining of their DNA with fluorescent dyes (Howard and Battye 1979; Brown *et al.* 1980; Saul *et al.* 1982; Myler *et al.* 1982; Whaun *et al.* 1983; Franklin *et al.* 1986). However, the use of this method to establish DNA contents of different stages and the rate of DNA synthesis in parasite populations has been hampered by asynchronicity of infections, presence of multiple infected host cells, and lack of knowledge concerning the process of DNA synthesis in malaria parasites.

Recently, we were able to determine the exact time of DNA synthesis during asexual and sexual development of *P. berghei,* using direct microfluorometry of

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individual Feulgen-pararosaniline stained parasites (Janse *et al.* 1986). Ringforms and developing trophozoites during the first 15 hr of development are haploid. During schizogony, starting at 18 hr after invasion of the host erythrocyte, the haploid amount of DNA is multipled 16–20 times within 4–6 hr. At the same time, limited DNA synthesis also occurs in maturing gametocytes up to 40–70% of the haploid amount.

The purpose of the present study was to compare flow cytometry and microfluorometry in assessing (1) DNA synthesis during intraerythrocytic development and (2) genome sizes of different malaria parasites.

The dyes Hoechst-33258 and 33342 (Loewe and Urbanietz 1974; Latt and Stetten 1976; Cesarone *et al.* 1979) were chosen for staining since these readily enter fixed and living cells in suspension (Arndt-Jovin and Jovin 1977) and have been reported not to be toxic for *P. berghei* (Howard and Battye 1979).

The present work shows that results obtained by flow cytometry of Hoechst stained cells fit very well with those obtained by microfluorometry in studying DNA synthesis during intraerythrocytic development but is less suitable for comparative genome size measurements.

MATERIALS AND METHODS

Synchronized infections of the ANKA-strain of *Plasmodium berghei* in Wistar rats and short term *in vitro* cultures of this parasite were obtained as described elsewhere (Mons *et al.* 1985; Janse *et al.* 1984, 1986), except that cultured mature schizonts were separated by Nycodenz gradient centrifugation (Nyegaard & Co., Torshov, Norway). A detailed description of parasite morphology during synchronous development is given by Mons *et al.* (1985).

P. berghei merozoites, obtained from purified mature schizonts, were separated from (infected) erythrocytes essentially as described by Dennis *et al.* (1975).

P. berghei gametocytes, obtained from a synchronized infection at 28 hr ai, were purified by Nycodenz gradient centrifugation: infected blood was layered on top of a 48% (v/v) mixture of Nycodenz and culture medium (described by Janse *et al.* 1984) and centrifuged at 350*g* for 25 min at 4 C. Cells from the interfase, about 80% gametocytes, were collected.

P. vivax ringforms were obtained from a Dutch patient with a parasitemia of 0.3%, 7 months after his return from Nepal. *P. falciparum* ringforms (isolate NF54) were obtained from a synchronous culture established as described by Ponnudurai *et al.* (1986). Differential parasite counts in Giemsa stained bloodfilms were made in 5×10^3 erythrocytes.

For microfluorometry, thin bloodfilms were prepared, fixed, hydrolyzed, and stained with Feulgen-pararosaniline (SO_2) (Chroma, Stuttgart, West Germany). In each experiment, 50 stained parasites were measured as described earlier (Cornelissen *et al.* 1984; Janse *et al.* 1986). For flow cytometry, cells were either fixed with 0.25% glutaraldehyde in PBS (2×10^8 cells/ml) for 10 min at 4 C, washed twice with PBS, stained with 2 μM Hoechst-33258 (Janssen Chimica, Beerse, Belgium) in PBS (2×10^8 cells/ml) for 1 hr at 37 C in the dark, and analyzed within 1 hr, or living cells were stained with Hoechst-33258 or 33342 by adding them to *in vitro* cultures of *P. berghei* at 6 hr ai at a final concentration of 10 μM. After 1.5–2 hr of incubation at 37 C in the dark, the medium was removed and the cells were resuspended in PBS at 4 C and analyzed within 1 hr. To test their viability after Hoechst staining and incubation in PBS, about 10^6 stained parasites were intraperitoneally injected into Swiss mice (20–25 g) or incubated *in vitro* under standard conditions for 20 hr after two washings with prewarmed (37 C) culture medium.

Hoechst stained cells were analyzed with a FACS Analyzer and a FACS IV (Becton, Dickinson, Mountain View, CA, U.S.A.). The FACS Analyzer was equipped with a mercury arc lamp (USHIO, Japan) and excitation was realized using a bandpass 360 and an SP 375 filter. An SP 375 was used as a dichroic mirror. The blue Hoechst fluorescence was selected with a bandpass 490 and two LP 400 filters. By gating on cell volume, erythrocytes and leukocytes were electronically selected for analysis, thus eliminating platelets, free parasites, and cellular debris. Fluorescence intensity measurements (linear or logarithmic gain setting) were collected for 50,000 cells per sample. Fluorescence histograms, divided into 256 channels, were processed and analyzed using the standard BD Consort 30 software. Cell sorting is not feasible with the FACS Analyzer. The FACS IV was operated with a high power argon ion laser with a laser emission of 20 mW in the ultraviolet light (351 nm/363 nm). Low angle light scatter and fluorescence signals were simultaneously detected for each cell. By electronic gating, erythrocytes were selected on the basis of their forward light scatter. Further details of operating conditions have been described by Howard and Battye (1979).

Fluorescence intensity measurements were collected for 50,000 cells per sample, and cells of interest

were selected electronically and sorted with a speed of 1000 cells/sec.

Erythrocytic ringforms contain the haploid amount of DNA (Janse *et al.* 1986) and were, therefore, used as the reference value in flow cytometry and direct microfluorometry. Since fluorescence values of Hoechst stained erythrocytes, infected with a single ringform, showed a narrow symmetrical frequency distribution (see Results), the peak value, after subtraction of the mean "background" fluorescence of uninfected erythrocytes, is taken to represent the mean fluorescence value of ringforms (1C = haploid value). Normally the background fluorescence was less than 10% the value of cells infected with ringforms.

RESULTS

Erythrocytes infected with one, two, and three *Plasmodium berghei* ringforms could be differentiated after Hoechst-33258 staining, with both the FACS Analyzer (Fig. 1A) and the FACS IV (Fig. 1B), and were clearly separated from uninfected cells on Hoechst–DNA fluorescence intensity. Double and triple infected cells showed fluorescence intensities of about two (mean of 2.05, 4 experiments) and three times (mean of 2.98, 4 experiments), respectively, the fluorescence value of single infected cells and microscopic examination showed that mainly single (89%) and double (82%) infected cells were sorted from the first and second peak, respectively (Fig. 1B). Percentages of infected cells, as determined by flow cytometry, corresponded closely with percentages established in Giemsa stained bloodfilms (data not shown).

The fluorescence intensity of free merozoites (mean ± SEM (*n*): 0.96C ± 0.0004 (40,000)) was comparable to that of intraerythrocytic ringforms. Only a slight increase of fluorescence intensity (±10%) was observed during development of ringforms (4 hr ai) into large trophozoites (16 hr ai) (Fig. 2A,B). From the time the first schizonts appeared (18 hr ai), the percentage of cells with fluorescence values of

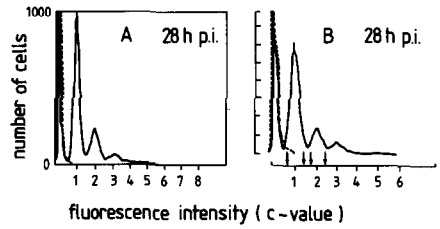

FIG. 1. Frequency distributions of fluorescence values of infected erythrocytes present in a synchronous *Plasmodium berghei* infection at 28 hr after infection (ai) (4 hr after reinvasion started), obtained with a FACS Analyzer (A) equipped with a mercury arc lamp and a FACS IV (B) equipped with a high-power argon ion laser, both after Hoechst-33258 staining. At 28 hr ai, only ringforms (single, double, and triple infected erythrocytes) and gametocytes (less than 1%) were present. The fluorescence channels selected for sorting (0.7–1.3C and 1.7–2.3C) are indicated. C-values were calculated as in Fig. 2. Dotted lines show the background fluorescence of uninfected cells at 0 hr ai.

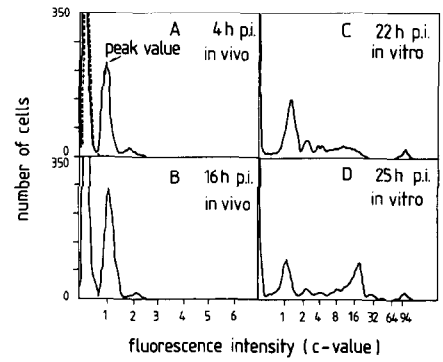

FIG. 2. Frequency distributions of fluorescence values of erythrocytes infected with *Plasmodium berghei* at 4, 16, 22, and 25 hr ai, as measured by a FACS Analyzer after Hoechst-33258 staining. At 4 hr ai, *in vitro* cultures were started with tail blood from a rat in which only ringforms (single and double infected erythrocytes) were present. During trophozoite development, from 4 hr ai (A) until 16 hr ai (B), parasites enlarge but do not synthesize DNA. At 25 hr ai (D), most parasites were schizonts with 8–24 nuclei in the *in vitro* cultures and only 2% of the erythrocytes contained reinvaded ringforms. The "background" fluorescence of uninfected cells was established just before intravenous inoculation of purified schizonts (see dotted line in A). The haploid (1C) value is defined as the difference between the peak fluorescence value of singly infected erythrocytes (=first peak at 4 hr ai) and the mean background fluorescence. Cells with values higher than 64C were leukocytes with a mean peak value of 115C (8 experiments SD: 7.4). (A,B) Linear gain setting; (C,D) logarithmic gain setting.

over two times the haploid value increased (Fig. 2C) and *in vitro,* where schizonts hardly release their merozoites in the absence of shearing forces (Mons *et al.* 1985), the culture contained a high percentage of cells with fluorescence values of about 16C at 25 hr ai (Fig. 2D). Purified mature micro- and macrogametocytes showed fluorescence values between 1 and 2C (mean ± SEM (*n*): 1.4C ± 0.002 (36,000)), confirming the results obtained with microfluorometry of Feulgen-pararosaniline stained gametocytes (Janse *et al.* 1986).

Using flow cytometry, the rate of DNA synthesis during schizogony was determined in the presence of different concentrations of aphidicolin, a specific inhibitor of DNA polymerase-α (Ikegami *et al.* 1978). DNA synthesis was inhibited progressively with increasing drug concentrations and a C_{50} value of about 5 μ*M* was established (Fig. 3). Living parasites—ringforms, trophozoites, and schizonts—could be stained with Hoechst-33258 as well as with Hoechst-33342. The fluorescence intensity of infected cells was dependent on the dye concentration in the culture medium and on staining time (data not shown) as was found earlier for the former dye (Howard and Battye 1979). Mice showed a normal infection pattern after inoculation of Hoechst-33258 and 33342 stained parasites and died within 7–14 days ai as did the control mice (6 animals/group). *In vitro,* Hoechst-33258 stained ringforms/young trophozoites developed normally after removal of the dye. As in control cultures, 60–80% of the parasites were mature schizonts at 18 hr after staining with 12–24 merozoites (3 experiments) capable of invasion. However, no development of ringforms was observed after Hoechst-33342 staining, even with a lower dye concentration (5 μ*M*), shorter staining time (1 hr), and more frequent replacement of the culture medium after staining. We have, at present, no satisfactory explanation why Hoechst-33342 stained parasites did not develop in culture while giving rise to normal infections in mice.

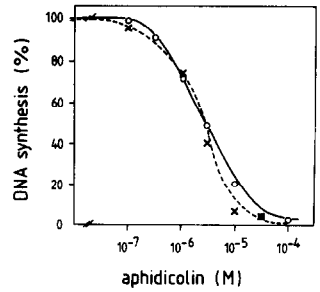

FIG. 3. Inhibition of DNA synthesis in asexual *Plasmodium berghei* parasites by aphidicolin, as measured by flow cytometry (FACS Analyzer) after Hoechst-33258 staining. Ringforms (4 hr after infection, ai) were incubated for 18 hr under standard culture conditions with various aphidicolin concentrations. At 0 and 18 hr of incubation, the fluorescence intensity of infected cells was determined. At 0 hr, infected cells had fluorescence values between 0.5 and 2.5C with a mean of 1.2C (single and double ring infected cells). At 18 hr, 70% of parasites in the controls had fluorescence values higher than 2.5C with a mean of 10.1C, while 30% had values between 0.5 and 2.5C (mean 1.3), thus had not yet started DNA synthesis. At this time, about 26% of the parasites in the controls were still uninucleated. In all wells, parasitemia was constant during the culture period (7.1–7.3%). The amount of DNA synthesized in each well is defined as the difference between the mean fluorescence value of infected cells at 18 hr (which is corrected for the mean value of parasites that did not start DNA synthesis at 18 hr in controls) and the mean fluorescence value at 0 hr. o Flow cytometry; x, direct microfluorometry (Janse *et al.* 1986), for comparison.

Erythrocytes infected with ringforms of *P. vivax* and *P. falciparum* could be distinguished from uninfected cells by flow cytometry after Hoechst-33258 staining, and fluorescence values showed a narrow symmetrical frequency distribution. *P. falciparum* had the same mean fluorescence intensity as *P. berghei,* whereas *P. vivax* showed a slightly lower value (Table I). The three different *Plasmodium* species showed no significant differences in fluorescence intensity after Feulgen-pararosaniline staining (Table I).

TABLE 1
Fluorescence Intensity

	Feulgen-pararosaniline mean ± SEM (*n*)	Hoechst-33258 mean ± SEM (*n*)
P. berghei	100 ± 0.7 (50)	100 ± 0.15 (2500)
P. falciparum	101 ± 0.7 (50)	102 ± 0.09 (7200)
P. vivax	105 ± 1.3 (30)	85 ± 0.5 (200)

Note. Mean fluorescence value of ringforms of *Plasmodium berghei, P. falciparum,* and *P. vivax* determined by flow cytometry (FACS Analyzer) after Hoechst-33258 staining and by direct microfluorometry after Feulgen-pararosaniline staining. The mean fluorescence value of *P. berghei* ringforms is used as reference value and set at 100.

Discussion

Recently, we determined the DNA content of asexual and sexual developmental stages of *Plasmodium berghei* by microfluorometry of individual Feulgen-pararosaniline stained parasites (Janse *et al.* 1986, and Introduction). This staining method has been used widely in quantitative measurements of DNA, based on the well-established assumption of a linear relationship between the amount of fluorescence and the original DNA content of stained nuclei (Duijndam and Van Duijn 1975; Prenna *et al.* 1974; Van Prooijen-Knegt *et al.* 1980). Comparison of data obtained by microfluorometry with results presented in this paper shows that the fluorescence intensities of Hoechst stained *P. berghei* parasites correspond with the relative DNA contents of these parasites. The slight increase of fluorescence intensity observed during the first 16 hr of trophozoite development, during which no DNA synthesis is expected, most probably is due to enlargement of the parasite cytoplasm, since we observed a faint fluorescence of the cytoplasm under the fluorescence microscope. From 16 hr ai onward, the fluorescence intensity of asexual parasites increased to about 16 times the haploid value in mature schizonts.

Flow cytometry is an adequate method to study DNA synthesis inhibition by aphidicolin; a similar C_{50} value of about 5 μM was established as earlier found by microfluorometry (Janse *et al.* 1986).

In short, flow cytometry proved to be a sensitive and very rapid method to determine the onset and rate of DNA synthesis during schizogony. A disadvantage, however, is that parasites which have started DNA synthesis to two or three times the haploid amount cannot be distinguished from double and triple infected cells by the FACS, and it is evident that some details of the process of DNA synthesis during schizogony and gametocytogenesis, as published earlier (Janse *et al.* 1986), can only be determined by microfluorometry of individual cells. On the other hand, vital staining and separation of cells based on their different DNA content would make it feasible to select living cells with altered growth properties, e.g., aphidicolin resistant cells or temperature sensitive mutants (Arndt-Jovin and Jovin 1977).

By direct microfluorometry of Feulgen-pararosaniline stained parasites, we found no significant differences in fluorescence intensities among the haploid stages of *P. berghei, P. vivax,* and *P. falciparum,* indicating that these species have the same DNA content. By comparing Feulgen-pararosaniline stained sporozoites and metaphase human chromosome 21, this haploid genome size has been estimated in *P. berghei* as 2.5×10^7 bp (Cornelissen *et al.* 1984). After Hoechst staining, we observed no difference between the fluorescence intensities of *P. falciparum* and *P. berghei;* however, *P. vivax* showed a small but significantly lower value. This difference may

be due to the preference of Hoechst dyes to bind to A-T rich regions of the DNA (Latt and Wohlleb 1975). *P. falciparum* and *P. berghei* have the same low G-C content (18%) in contrast to the DNA of *P. vivax* which consists of a low and a high G-C content component (18 and 30%, respectively) (McCutchan *et al.* 1984). As a result, we may expect a lower affinity of Hoechst dyes for *Plasmodium vivax* DNA.

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REFERENCES

ARNDT-JOVIN, D. J., AND JOVIN, T. M. 1977. Analysis and sorting of living cells according to DNA content. *Journal of Histochemistry and Cytochemistry* **25**, 585–589.

BROWN, G. V., BATTYE, F. L., AND HOWARD, R. J. 1980. Separation of stages of *Plasmodium falciparum* infected cells by means of a fluorescence activated cell sorter. *American Journal of Tropical Medicine and Hygiene* **29**, 1147–1149.

CESARONE, C. F., BOLOGNESI, C., AND SANTI, L. 1979. Improved microfluorometric DNA determination in biological material using 33258 Hoechst. *Analytical Biochemistry* **100**, 188–197.

CORNELISSEN, A. W. C. A., OVERDULVE, J. P., AND VAN DER PLOEG, M. 1984. Determination of nuclear DNA of five Eucoccidian parasites, *Isospora* (*Toxoplasma*) *gondii, Sarcocystis cruzi, Eimeria tenella, E. acervulina* and *Plasmodium berghei,* with special reference to gametogenesis and meiosis in *I.* (*T.*) *gondii. Parasitology* **88**, 531–553.

DENNIS, E. D., MITCHELL, G. H., BUTCHER, G. A., AND COHEN, S. 1975. *In vitro* isolation of *Plasmodium knowlesi* merozoites using polycarbonate sieves. *Parasitology* **71**, 475–481.

DUIJNDAM, W. A. L., AND VAN DUIJN, P. 1975. The influence of chromatin compactness on the stoichiometry of the Feulgen-Schiff procedure studied in model films. II Investigations on films containing condensed or swollen chicken erythrocyte nuclei. *Journal of Histochemistry and Cytochemistry* **23**, 891–900.

FRANKLIN, R. M., BRUN, R., AND GRIEDER, A. 1986. Microscopic and flow cytophotometric analysis of parasitaemia in cultures of *Plasmodium falciparum* vitally stained with Hoechst 33342—application to studies of antimalarial agents. *Zeitschrift für Parasitenkunde* **72**, 201–212.

HOWARD, R. J., AND BATTYE, F. L. 1979. *Plasmodium berghei* infected red cells sorted according to DNA content. *Parasitology* **78**, 263–270.

IKEGAMI, S., TAGUCHI, T., OHASHI, M., OGURO, M., NAGONO, A., AND MANO, Y. 1978. Aphidicolin prevents mitotic cell division by interfering with the activity of DNA polymerase-α. *Nature* (*London*) **275**, 458–460.

JANSE, C. J., VAN DER KLOOSTER, P. F. J., VAN DER KAAY, H. J., VAN DER PLOEG, M., AND OVERDULVE, J. P. 1986. DNA synthesis in *Plasmodium berghei* during asexual and sexual development. *Molecular and Biochemical Parasitology* **20**, 173–182.

JANSE, C. J., MONS, B., CROON, J. J. A. B., AND VAN DER KAAY, H. J. 1984. Long-term *in vitro* cultures of *Plasmodium berghei* and preliminary observations on gametocytogenesis. *International Journal for Parasitology* **14**, 317–320.

LATT, S. A., AND STETTEN, G. 1976. Spectral studies on 33258 Hoechst and related bisbenzimidazole dyes useful for fluorescent detection of deoxyribonucleic acid synthesis. *Journal of Histochemistry and Cytochemistry* **24**, 24–33.

LATT S. A., AND WOHLLEB, J. C. 1975. Optical studies of the interaction of 33258 Hoechst with DNA, chromatin and metaphase chromosomes. *Chromosoma* **52**, 297–316.

LOEWE, H., AND URBANIETZ, J. 1974. Basisch substituierte 2,6-Bis-benzimidazolderivate, eine neue chemotherapeutisch aktive körperklasse. *Arzneimittelforschung* **24**, 1927–1933.

MCCUTCHAN, T. F., DAME, J. B., MILLER, L. H., AND BARNWELL, J. 1984. Evolutionary relatedness of *Plasmodium* species as determined by the structure of DNA. *Science* **225**, 808–811.

MONS, B., JANSE, C. J., BOORSMA, E. G., AND VAN DER KAAY, H. J. 1985. Synchronized erythrocytic schizogony and gametocytogenesis of *Plasmodium berghei in vivo* and *in vitro. Parasitology* **91**, 423–430.

MYLER, P., SAUL, A., MARGAN, T., AND KIDSON, C. 1982. An automated assay of merozoite invasion of erythrocytes using highly synchronized *Plasmodium falciparum* cultures. *Australian Journal of Experimental Biology and Medical Science* **60**, 83–89.

PONNUDURAI, T., LENSEN, A. H. W., MEIS, J. F. G. M., AND MEUWISSEN, J. H. E. TH. 1986. Synchronization of *Plasmodium falciparum* gametocytes using an automated suspension culture system. *Parasitology* **93**, 263–274.

PRENNA, G., LEIVA, S., AND MAZZINI, G. 1974.

Quantitation of DNA by cytofluorometry of the conventional Feulgen reaction. *Histochemical Journal* **6**, 467–489.

SAUL, A., MYLER, P., MANGAN, T., AND KIDSON, C. 1982. *Plasmodium falciparum:* Automated assay of erythrocyte invasion using flow cytofluorometry. *Experimental Parasitology* **54**, 64–71.

VAN PROOIJEN-KNEGT, A. C., REDI, C. A., AND VAN DER PLOEG, M. 1980. Quantitative aspects of the cytochemical Feulgen-DNA procedure studied on model systems and cell nuclei. *Histochemistry* **69**, 1–17.

WHAUN, J. M., RITTERSHAUS, C., AND IP, S. H. C. 1983. Rapid identification and detection of parasitized human red cells by automated flow cytometry. *Cytometry* **4**, 117–122.