Malaria and the Red Cell, pages 45-61

MEROZOITE PROTEINS SYNTHESIZED IN *P. FALCIPARUM* SCHIZONTS

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INTRODUCTION

The asexual erythrocytic cycle of *Plasmodium* species consists of alternating intraerythrocytic stages (the ring, trophozoite, and schizont morphological forms) and the free extracellular merozoite stage. Therefore, the surface of both the merozoite and the parasitized erythrocyte are targets in the host's immune response and components of both may be useful in the development of an anti-malarial vaccine. While the protein profile of the intraerythrocytic stages has been extensively studied, the merozoite proteins of *P. falciparum* are only beginning to be identified. Previous studies on the merozoite which have used metabolic or covalent radiolabeling techniques (Kilejian, 1980; Perkins, 1982; Perrin and Dayal, 1982; and Reese, unpublished observations) have required the purification of the merozoite from contaminating schizonts and erythrocyte membranes. Because such separation is difficult, the results obtained so far are equivocal. Nevertheless, because of these reports and additional biochemical and immunocytochemical studies (Hall, 1983; Howard and Reese, 1983), a partial picture of the merozoite proteins is beginning to emerge.

We have recently contributed to this information by using an alternative method of identifying metabolically-labeled merozoite proteins (Howard and Reese, 1983). This technique is described here and a characterization of some

of the parasite proteins and glycoproteins is presented. As a part of this we demonstrate that, unlike eucaryotes known to make sialic acid from monosaccharide precursors, P. falciparum synthesizes little or no sialic acid from those precursors.

METHODS

The P. falciparum isolates FVO (Vietnam) and Honduras I (Honduras, Central America) were grown as previously described (Howard and Reese, 1983). Cultures were radiolabeled with [^{35}S]methionine in methionine-free growth medium or with [6-^{3}H]fucose, [6-^{3}H]glucosamine, [2-^{3}H]mannose, or [6-^{3}H]N-acetyl mannosamine (ManNAc) in complete growth medium. After labeling, the cells were hemolyzed in 1mM phenylmethylsulfonyl fluoride/10mM sodium phosphate pH 7.5, the membrane ghosts and parasites sedimented, and aliquots of the ghosts boiled in SDS (sodium dodecyl sulfate) gel sample buffer (Laemmli, 1970) or treated with two-dimensional gel lysis buffer (Howard and Reese, 1983). Samples were analyzed by electrophoresis on 8% polyacrylamide gels in the presence of SDS (SDS-PAGE, see Laemmli, 1970) or by two-dimensional (isoelectric focusing followed by SDS-) PAGE (O'Farrell, 1975) as described elsewhere in detail (Howard and Reese, 1983). ^{14}C-Labeled molecular weight marker proteins (myosin, 200K; β-galactosidase, 116K; phosphorylase b, 97.4K; bovine serum albumin, 69K; γ-globulin, 53K and 22.5K; ovalbumin, 43K; and carbonic anhydrase, 30K) were co-electrophoresed with the samples. Fluorographs were produced (Bonner and Laskey, 1974) on Kodak X-ray film with Dupont intensifying screens.

The radiolabeled schizont-labeled ring stage was prepared as follows: Synchronized cultures of P. falciparum were obtained by sorbitol lysis (Lambros and Vanderberg, 1979) and/or the Physiogel sedimentation technique (Reese et al., 1979). The resulting late stages were freed of remaining ring-stage parasites and most of the uninfected erythrocytes by an additional Physiogel separation or centrifugation through Percoll (62% isoosmotic Percoll, 1000 x g, 20 min). The resulting upper phase containing the late-stage parasites was radiolabeled (mid- to late-schizonts for 1h with [^{35}S]-methionine, or beginning with late trophozoites or early schizonts for 6-12 h with [^{3}H]-glucosamine). After the metabolic labeling was complete an

additional Percoll separation was performed to remove any newly-formed ring stages. Fresh, uninfected erythrocytes (1-2 volumes) were added to the labeled schizonts, and the cultures were incubated for 6-10 h. The resulting schizont-labeled ring-stage parasites were separated from the remaining schizonts by sedimentation through Percoll and prepared for electrophoresis as described above.

Computer enhancement of the protein profiles of the [^{3}H]-glucosamine-containing schizont-labeled ring stage was performed at the UC San Diego Visibility Lab with the assistance of B.L. McGlamery. Details have previously been given (Howard and Reese, 1983).

The biosynthesis of sialic acids was studied in [6-^{3}H]glucosamine- and [6-^{3}H]N-acetylmannosamine-labeled cultures. After hemolysis and washing, an aliquot of each suspension was treated with cold 5% trichloroacetic acid (TCA) and the TCA-precipitated proteins were filtered through glass-fiber filters and counted. A larger aliquot of the labeled suspensions was studied for the presence of labeled sialic acids. The suspension was first treated with 0.1N NaOH (45 min, on ice) to eliminate O-acetyl groups which can interfere in the subsequent sialic acid purification steps. The material was then adjusted to 1.6M acetic acid and heated (80°C, 3 h) to release all glycoconjugate- and nucleotide-bound sialic acids (Varki and Diaz, 1983). Authentic radiolabeled sialic acid, [4-^{14}C]N-acetylneuraminic acid (Neu5Ac, 10^{4} cpm) was added to each hydrolysate to monitor subsequent recoveries. The released sialic acids were then purified by ultrafiltration on an Amicon Centrifree micropartition unit, and ion-exchange chromatography on Dowex-50 (H^{+} form) and Dowex 3x4A (formate form). The purified samples were chromatographed on Whatman 3MM paper in butanol:acetic acid:water (4:1:5, upper phase) for 14 h. One cm strips were monitored for ^{3}H and ^{14}C. The amount of ^{3}H co-migrating with the ^{14}C standard was noted, and corrected for the overall recovery of the standard. This approach has been used for the study of sialic acid biosynthesis in mammalian cell lines; the full details and rationale of the method will be published elsewhere (A. Varki, manuscript in preparation).

RESULTS AND DISCUSSION

Partial Protein Composition of the Merozoite

Over twenty proteins are readily apparent in the [^{35}S]-methionine pulse-labeled schizonts of the FVO isolate (Fig. 1, lane 1) and in the subsequent schizont-labeled ring stage (lane 2). Most significantly, the schizont-labeled ring stage appears to be enriched for certain proteins which were synthesized in the pulse-labeled schizonts. Proteins of M_r = 202K, 82K, and 46K are especially prominent. Others of M_r = 185K, 142K, and 136K are also present, but with a relatively lower labeling intensity. In contrast, the ring stage (lane 3) contains a quite different distribution of proteins and the trophozoite stage (lane 4) has proteins which were present in both the ring and schizont stages. In other words proteins such as p202, p82, and p46 appear to be merozoite proteins which were synthesized during schizogony and carried by the merozoite into the following ring stage. This process was clearly selective since some proteins, for example p88, are easily detectable in the schizont stage, but virtually absent from the schizont-labeled ring stage, and other proteins such as p46 are present during the schizont stage, but not readily apparent until the schizont-labeled ring stage due to the presence of many more-intensely labeled proteins.

The pulse-labeled schizonts and the schizont-labeled ring stage were also examined in the Honduras I isolate of *P. falciparum*. Like the schizont-labeled ring stage of the FVO isolate, the schizont-labeled ring-stage of Honduras was enriched for proteins of M_r = 202K, 82K, and 46K. The other FVO proteins (p185, p142, p136) likewise had counterparts of similar M_r in the Honduras isolate (data not shown). Since the FVO and Honduras isolates were obtained from widely separated locations, these results suggest that proteins of similar M_r may be found in most merozoites regardless of their geographic origin.

Identification of Parasite Glycoproteins

Radiolabeled monosaccharides serve as precursors for the oligosaccharide moieties of glycoproteins (Yurchenco *et al.*, 1978). Therefore, in order to identify the

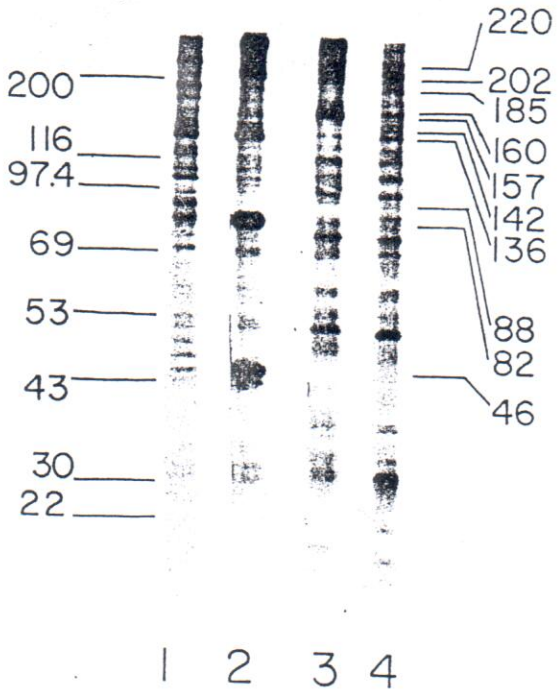

Figure 1. Proteins synthesized by the asexual stages of the P. falciparum erythrocytic cycle (FVO isolate). Schizonts, pulse-labeled with [^{35}S]methionine (lane 1), and the schizont-labeled ring stage (lane 2) were prepared as described in the Methods section. Synchronous ring-stage (lane 3) and trophozoite-stage parasites (lane 4) were labeled with [^{35}S]methionine (2h). The proteins were separated by SDS-PAGE and fluorography performed (lane 1, 3h; lanes 2 and 3, 2d; lane 4, 7h). The apparent Mr of the parasite proteins (right margin) and the molecular weights of the marker proteins (left margin) are shown. Reprinted by permission of Molec. Biochem. Parasitol.

glycoproteins of P. falciparum we radiolabeled schizont-stage parasites with [6-^{3}H]fucose, [6-^{3}H]glucosamine, and [2-^{3}H]mannose. [^{3}H]Fucose failed to radiolabel any glycoproteins (Fig. 2, lane 1). In contrast glycoproteins

of similar M_r's were labeled with both mannose (lane 2) and glucosamine (lane 3), although [^{3}H]glucosamine appeared to label each band more intensely than did the [^{3}H]mannose. Thus, neither monosaccharide appears to label additional glycoproteins that the other fails to label. Since [^{3}H]glucosamine labeled the parasite proteins more intensely than did [^{3}H]mannose, the former sugar was generally used to radiolabel the glycoproteins.

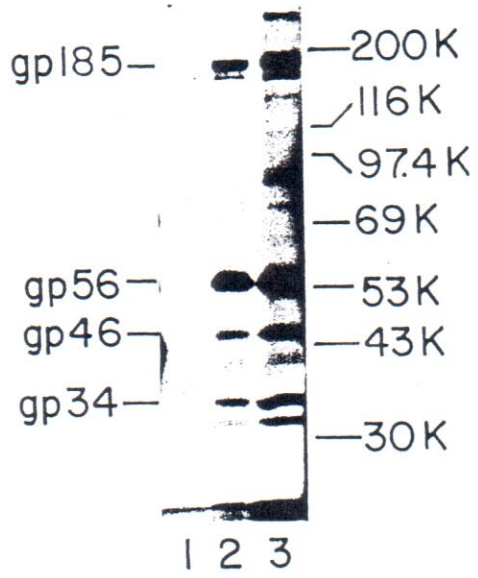

Figure 2. Glycoproteins labeled with [6-^{3}H]fucose, [6-^{3}H]glucosamine, and [2-^{3}H]mannose. Equal volumes of schizont-stage parasites were radiolabeled with 50 μCi/ml of the radiolabeled sugars (21 h), then electrophoresed (SDS-PAGE) and fluorographed for 12 d. Parasite glycoproteins (M_r) are labeled on the left and molecular weights of the ^{14}C-marker proteins are shown on the right.

Schizont (FVO) glycoproteins which had been radiolabeled with [^{3}H]glucosamine were co-electrophoresed with [^{35}S]methionine-labeled proteins from the schizont and schizont-labeled ring stage (Fig.3). Of the 13 or more macromolecules which are labeled with [^{3}H]glucosamine in

this experiment, those of M_r=185K, 56K, 46K, 40K, and 34K are the most prominent. Gp185 and gp56 appear the best labeled of these. Although [^{35}S]methionine-labeled proteins of equivalent M_r are observed for gp185, gp46, and gp40, the gp56 and gp34 are generally not well labeled with [^{35}S]methionine. Nevertheless, all of the glycosylated molecules were sensitive to pronase digestion, and, therefore, all appear to be glycoproteins.

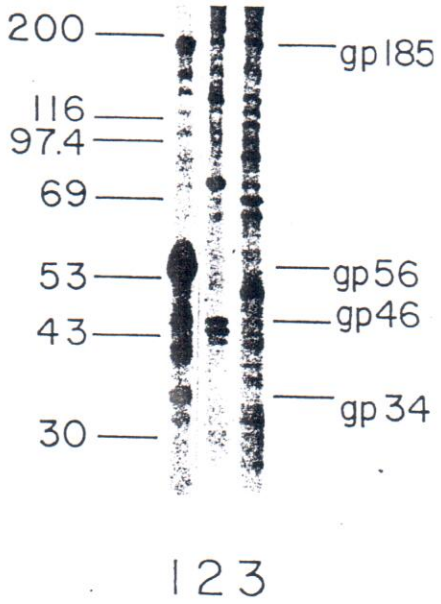

Figure 3. Comparison of *P. falciparum* proteins and glycoproteins (FVO isolate). Glycoproteins of synchronous schizont-stage parasites were biosynthetically labeled (6 h) with [^{3}H]glucosamine (lane 1). [^{35}S]Methionine-labeled schizont proteins (lane 3) and the resulting schizont-labeled ring-stage proteins (lane 2) were resolved by SDS-PAGE and then fluorographed. Molecular weights of the marker proteins and M_r of the parasite glycoproteins are indicated. Reprinted by permission of *Molec. Biochem. Parasitol.*

The proteins of [^{35}S]methionine-labeled schizonts, the schizont-labeled ring stage, and [^{3}H]glucosamine-labeled schizonts were separated by two-dimensional PAGE (Fig. 4) to determine whether p185 and p46 are truly glycoproteins. The schizont protein p185 (panel A, arrowhead) was focused at about the same isoelectric point (pH 6.7-7.0) as was gp185 (panel C), further suggesting that they are identical. P46 seems to be composed of the multiple polypeptides p46a-e (at least four in the schizont and five in the schizont-labeled ring stage), the most dominant of which is p46a (pI=5.5-6.0). Although only one gp46 (panel C) has so far been detected, it too focuses with a pI=5.5-6.0. Thus, at least

one of the p46 polypeptides appears to be a glycoprotein; whether or not p46b-e are also glycosylated must await further analysis.

Since p185 and p46 appear to be glycoproteins and the methionine-labeled molecules were shown (Fig. 1) to be present in the merozoite and schizont-labeled ring stages, it appeared likely that the merozoite contains

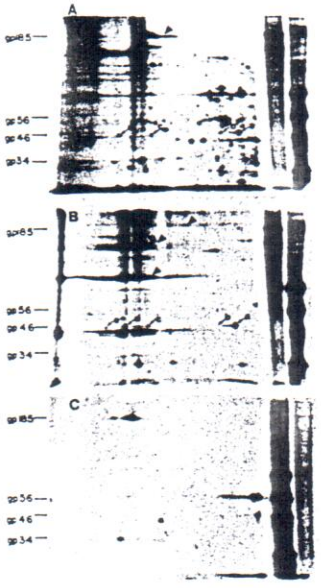

Figure 4. Proteins and glycoproteins resolved by two-dimensional polyacrylamide gel electrophoresis. Proteins of the [^{35}S]-methionine-labeled schizonts (panel A), the resulting schizont-labeled ring stage (panel B), and [^{3}H]glucosamine-labeled schizonts (panel C) were separated by isoelectric focusing (basic end at left) and SDS-PAGE. Panels A and B contain equal numbers of TCA-precipitable cpm. The symbols: panel A, large arrowhead, p185, and the small arrows, the four 46K polypeptides p46a-c,e; panel B, the large arrowheads, p202, the p142-p136 region, and p82, and the small arrows, the five 46K polypeptides p46a-e. ^{14}C-Labeled molecular weight markers (200K, 69K, 53K, 43K, 30K; two right lanes) and [^{3}H]glucosamine-labeled proteins (left margin) were co-electrophoresed in the second dimension with the focused proteins. Reprinted by permission of Molec. Biochem. Parasitol.

glycoproteins. This was examined directly by isolating the schizont-labeled ring stage after labeling late trophozoite through schizont stages with [^{3}H]glucosamine (see the Methods section). However, the fluorographic images of the

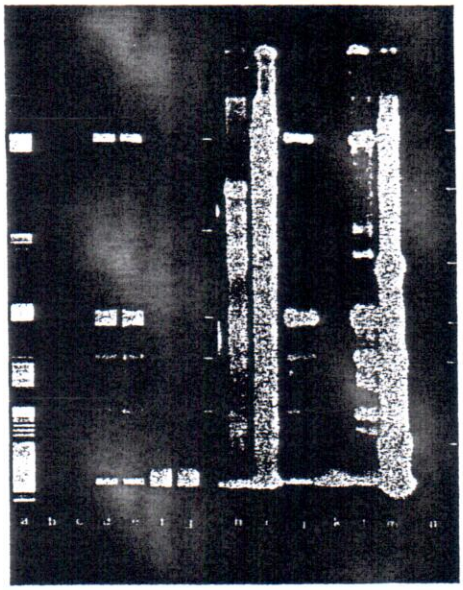

Figure 5. Glycoproteins of the trophozoite through schizont and the schizont-labeled ring-stages with computer enhancement of the fluorographic images. Proteins of the [^{3}H]glucosamine-labeled trophozoites-schizonts (see Methods section) and the resulting schizont-labeled ring stage were separated by SDS-PAGE and fluorographed. The fluorograph was optically scanned (for full details see Howard and Reese, 1983), and the lanes (h-n) enhanced, where lane h, i, and m contain ^{14}C-molecular weight marker proteins of 116K, 200K, and 69K, 53K, 43K, 30K (see right margin); lanes j and k, [^{3}H]glucosamine-labeled schizont and schizont-labeled ring-stage proteins, respectively; lane l, schizonts from a separate experiment; and lane n, no proteins applied to the SDS gel (to serve as background). Lanes j, k, and n were electronically extracted (shown as lanes e,g,c, respectively), averaged, and enhanced to identical extents (lanes d,f,b, respectively). Lane a is a further enhancement of lane f. Some of the glycoproteins of the schizont (gp185, gp88, gp56, gp46, gp34) are indicated by marks between lanes g and h. Reprinted by permission, Molec. Biochem. Parasitol.

schizont-labeled ring-stage glycoproteins (resolved by SDS-PAGE) were weak. For this reason a computer enhancing technique was used to make the fluorographic images clearer and give them added contrast (Fig. 5). The original fluorograph was intensified and is shown in lanes h-n, where lane j contains the [^{3}H]glucosamine-labeled schizont proteins and lane k, the proteins of the schizont-labeled rings. To enhance the images, they were extracted, divided into horizontal sectors which were averaged, and electronically given greater contrast. The results are shown in lanes e and f, respectively (for further details see Howard and Reese, 1983). Lanes e and f have equivalent amounts of contrast, and hence reflect the relative intensity of the radiolabeled protein bands; lane a is a further enhancement of lane f, the schizont-labeled ring stage. In three similar experiments the schizont-labeled ring stage was observed to contain gp185, gp56, gp46, gp40, gp34, as well as what is probably gp88 and one or two other polypeptides. Thus, the merozoite appears to contain many of the glycoproteins synthesized during schizogony.

Apparent absence of sialic acid synthesis by *P. falciparum*

In the past direct chemical assays have suggested that the malarial parasite contains sialic acid (Seed *et al.*, 1974). However, it is quite possible that this represented contamination by red blood cell membranes which are rich in sialoglycoproteins. The immediate precursor of sialic acid, with no known exception, is N-acetylmannosamine (ManNAc) which in turn is derived from cellular glucosamine pools (Schauer, 1982). In order to directly look for biosynthesis of sialic acids in *P. falciparum*, we metabolically labeled different stages of the parasite with [6-^{3}H]ManNAc and [6-^{3}H]glucosamine. In higher eucaryotic cells, 40% or more of the [^{3}H]ManNAc and 8-35% of [^{3}H]glucosamine label is found incorporated into sialic acid (Yurchenco *et al.*, 1978). Our results will be compared with these values.

The outcome of representative labeling experiments is shown in Table 1. Much less [^{3}H]ManNAc than [^{3}H]glucosamine was incorporated into TCA-precipitable radioactivity under identical conditions of labeling. In both cases, when the hemolysed, washed cells were heated with acetic acid under conditions that cause maximum release of sialic acids, an excess of radioactivity appeared suggesting the presence of

TABLE 1

ANALYSIS FOR THE BIOSYNTHESIS OF BOUND OR FREE SIALIC ACIDS IN METABOLICALLY-LABELED P. FALCIPARUM

LABEL[a] & STAGES	TOTAL TCA-PRECIPITABLE ^3H, cpm (X 10^{-4})	TOTAL ^3H RELEASED BY ACID (X 10^{-4})	^3H cpm CO-PURIFYING AND CO-MIGRATING WITH [^{14}C] STD.[b]		
			TOTAL cpm	AS % OF TCA-PPTABLE cpm[c]	AS % OF ACID-RELEASED cpm[c]
1.[6-^3H]$GlcNH_2$ RING---> TROPH	9.0	33.4	172	0.58%	0.16%
2.[6-^3H]$GlcNH_2$ TROPH---> SCHIZONT	180.0	950	2532	0.44%	0.08%
3.[6-^3H]ManNAc TROPH---> SCHIZONT	2.63	4.00	24	0.18%	0.12%

a All incubations were carried out with 125 Ci/ml of medium for 15h (sample 1) or 11h (samples 2 and 3). Samples 2 and 3 were identical aliquots of the same cell suspension. $GlcNH_2$, glucosamine.

b 10,000 cpm of authentic [4-^{14}C]Neu5Ac was added to each sample to monitor recoveries. The released sialic acids were purified and subjected to paper chromatography as described in the text. The chromatogram was cut into 1 cm strips and counted for ^3H and ^{14}C.

c Values corrected for recovery of ^{14}C.

trapped low-molecular weight compounds that were not TCA-precipitable. Because we were interested in knowing whether the parasite has the capacity to convert these precursors into sialic acid, regardless of its ability to incorporate them into glycoproteins, we analysed the total radiolabeled parasite, rather than the macromolecular materials alone. As shown in Table 1, we found that, relative to the total label, 0.12% of the [^{3}H]ManNAc-derived label and 0.16% or less of the [^{3}H]glucosamine-derived label co-purified and co-migrated with authentic [^{14}C]Neu5Ac. The percentages are very low even when compared to the TCA-precipitable radioactivity (see Table 1). These data suggest that the parasites themselves synthesize little or no sialic acid. Sialic acid is also absent from plants and even from the relatively complex lower invertebrates such as crustaceans and insects (Schauer, 1982). Nevertheless, the epimastigote, but not the other stages of Trypanosoma cruzi, has been found to contain sialic acids (Pereira et al., 1980). It is equally possible that stages of P. falciparum other than those of the asexual erythrocytic cycle can synthesize sialic acids. In addition, it is possibile that the parasite can re-utilize host erythrocyte sialic acids.

The oligosaccharides found in eucaryotic glycoproteins fall into two major categories: those that are N-linked (to asparagine) and those that are O-linked (to serine or threonine). The monosaccharide compositions of these are distinctly different. The N-linked oligosaccharides can be further subdivided into the high-mannose, complex, and hybrid types, where the complex and hybrid varieties (composed of sialic acid, galactose, N-acetylglucosamine, mannose, and fucose) are derived from processing of the high-mannose chains which are made of mannose and N-acetylglucosamine (Kornfeld, 1980). These specific types of oligosaccharides are synthesized after the radiolabeled monosaccharide precursors are converted into nucleotide-sugars via well-established pathways and the extent of incorporation has been studied in many different systems (Yurchenco et al., 1978). For example, between 65-100% of incorporated [2-^{3}H]mannose is incorporated unchanged (almost exclusively in N-linked oligosaccharides) and the remainder is converted to [^{3}H]fucose. Ninety-100% of [^{3}H]fucose incorporated into glycoproteins remains as fucose, and this sugar is present mainly in the complex type of N-linked oligosaccharides. Mannose and fucose are rarely incorporated into the O-linked oligosaccharides. [6-^{3}H]Glucosamine

is incorporated unchanged into both types of N-linked oligosaccharides and up to 30% of this sugar is converted to [6-^{3}H]N-acetylgalactosamine, an essential component of the O-linked oligosaccharides. As stated above, a portion can also be converted into sialic acids which are the outermost residues on O-linked oligosaccharides and on complex N-linked oligosaccharides of mammalian glycoproteins. Thus, the ability of the parasite to incorporate different radiolabeled sugars into glycoproteins should provide some information about the types of oligosaccharides present. It has previously been shown that three different protozoa (Crithidia fasciculata, Tetrahymena pyriformis, and Trypanosoma cruzi) have N-linked oligosaccharides (Parodi et al., 1981; Keenan et al., 1975; and Parodi and Cazzulo, 1982). Therefore, it would not be surprising if P. falciparum also has this type of oligosaccharide.

P. falciparum failed to incorporate [^{3}H]fucose. This and the apparent absence of sialic acid biosynthesis suggest (but do not prove) the absence of complex types of N-linked oligosaccharides. The enzymes endo-β-N-acetylglucosaminidase F and H (endo F and endo H) are capable of cleaving N-linked oligosaccharides from glycoproteins (Elder and Alexander, 1982; Tarentino and Maley, 1974). Unpublished observations (R.F. Howard) indicate that both endo F and endo H release oligosaccharides from [^{3}H]glucosamine-labeled parasite proteins. These data suggest that P. falciparum contains N-linked glycoproteins. Further experiments are necessary to ascertain whether O-linked oligosaccharides are present and what types of N-linked oligosaccharides are synthesized.

In summary, the schizont-labeled ring stage was isolated to identify those merozoite proteins and glycoproteins synthesized during schizogony. A number of proteins, including p202, p185, p142, p136, p82, and p46 were identified as merozoite proteins. The protein compositions of two different isolates of P. falciparum were similar. The merozoite contains glycoproteins of M_r=185K, 56K, 46K, 40K, and 34K; p185 and one of the multiple polypeptides of p46 were shown to be glycosylated. P. falciparum in its asexual erythrocytic cycle synthesizes little or no sialic acid by pathways used by other organisms which produce sialic acids. This and other data suggest that P. falciparum synthesizes N-linked oligosaccharides.

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DISCUSSION

Dr. Ockenhouse: Randy, have you tried to precipitate with immune sera any of the proteins from the schizont-labelled ring stage? If so, which bands precipitate?

Dr. Howard: Most of the reviews report immune monkey serum, and some pooled human immune serum. All the glycoproteins that I talked about with molecular weights of 185, 88, 56, 46, 34,000 plus some small fragments around 40,000 or 30,000 are precipitated by immune sera from monkeys, and most of them are also precipitated by human antisera.

Dr. Ockenhouse: I think it is conceivable, though, that not all the merozoite proteins which you have shown actually in the ring stage are all the merozoite proteins. I think that as the merozoite penetrates the outer coat might be sloughed off, and some of those proteins might be missing in your assay system.

Dr. Howard. Exactly.

Dr. Wood: Is your 46,000 acidic protein a phosphoprotein like the one we have described in *P. berghei*?

Dr. Howard: That is a good question. Certainly I know there are acidic phosphoproteins with that molecular weight, but in *falciparum* they seem to be very poorly phosphorylated, unlike in *berghei*.

Dr. Meshnick: Have you or anyone else tried tunicamycin?

Dr. Howard: It does not seem to work. Once I showed in unpublished results that you can release many of these oligosaccharides from the proteins with endo F and endo H, so those data suggest that they are N-linked. Tunicamycin does not seem to inhibit the glycosylation of any protein. The absence of fucose labelling might suggest that they are a high mannose type of oligosaccharide.

Dr. Olson: Tunicamycin also does not affect growth over long periods of time in culture. Despite massive doses, parasites continue to multiply as rapidly as in untreated samples. I also have a question. You do a one hour pulse when you label them. How old are those parasites?

Dr. Howard: I would say they are in the 3 to 6 n schizont stage.