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With 5 figures in the text

Antibodies to the glutamate dehydrogenase of *Plasmodium falciparum*

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SUMMARY

Polyclonal antisera raised against *Plasmodium knowlesi* reacted with (1) NADP-specific glutamate dehydrogenase (GLDH) of *P. knowlesi*, (2) GLDH of *P. falciparum* and (3) GLDH of *Proteus spp.* The antisera did not react with NAD(P) GLDH from bovine liver. Polyclonal antisera raised against the GLDH of *Proteus spp.* cross-reacted with GLDH from *P. falciparum.* Monoclonal antibodies (McAbs) obtained from mice immunized with *Proteus* GLDH were either specific for the bacterial enzyme or cross-reacted with *P. falciparum* GLDH. The selected McAbs did not react with GLDH from *P. knowlesi*, P. *chabaudi* or *P. berghei.* The GLDH of *P. falciparum* was shown to be a cytosolic protein (by FAT) with a subunit molecular weight of approximately 49000 Da (by immunoprecipitation) having a predominantly hexameric form (by sucrose density gradient). Implications of the conserved sequences of GLDHs and other enzymes are discussed.

INTRODUCTION

Glutamate dehydrogenase (EC 1 . 4 . 1 . 4) has been demonstrated in several species of malarial parasites (Sherman, 1979). The predominant reaction is the oxidation of glutamate to α-ketoglutarate. This may be of key importance to the plasmodium for the entry of amino acid skeletons into Kreb's tricarboxylic acid cycle and it is the only well-documented source of endogenous NADPH production by the intraerythrocytic stages (Walter, Nordmeyer & Königk, 1974). In every case, the cofactor is NADP and not NAD. In this respect the glutamate dehydrogenase (GLDH) of *Plasmodium spp.* differs from that of vertebrate tissues; the latter enzyme (EC 1 . 4 . 1 . 3) is activated by purine nucleotides, undergoes reversible association and dissociation and can utilize both NAD(H) and NADP(H) as coenzymes (Smith, Austen, Blumenthal & Nyc, 1975).

The asexual stages of malaria parasites can synthesize glutamate *de novo* by fixation of CO_2 (Sherman, 1979). Although human erythrocytes infected with *P. falciparum* remain relatively impermeable to glutamate, there is a large selective increase in permeability for glutamine (Elford, Haynes, Chulay & Wilson, 1985) which can be deaminated to glutamate. Glutamate released by the proteolytic degradation of cytosolic host proteins may also be available to the parasite.

In an earlier brief report (Hempelman & Wilson, 1982) we indicated that monkeys immunized with *P. knowlesi* readily produce antibodies to plasmodial enzymes such as GLDH. We have used these polyclonal antisera to develop a screening test for monoclonal antibodies to GLDH. In addition, we report that from mice immunized with

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GLDH from a bacterial source, *Proteus spp.*, we have isolated monoclonal antibodies which cross-react with the GLDH of *P. falciparum* but not with three other species of *Plasmodium*.

MATERIALS AND METHODS

Parasite preparations

For immunization and radio-isotope labelling purposes, schizonts of 2 clones (D7 and C10) of *P. falciparum* (Hempelmann, Ling & Wilson, 1981) were purified from *in vitro* cultures by centrifugation on a cushion of Percoll (Dluzewski, Ling, Rangachari, Bates & Wilson, 1984). Lysates of packed cells in 25 mM Tris/HCl, pH 7·4, were prepared by rapidly freezing and thawing 3 times, followed by centrifugation at 100000 *g* for 1 h. Similar preparations were also made with *P. knowlesi*, *P. berghei* and *P. chabaudi*. Prior to cultivation, or prior to lysis, all blood preparations were passed through columns of CF-11 to reduce contamination with leucocytes ($\leqslant 0{\cdot}07\,\%$).

For GLDH assays, parasite lysates were prepared as follows: cultures with mature stages of *P. falciparum* (parasitaemias $> 10\,\%$) were washed and the cells lysed by freezing and thawing. A particle-free haemolysate was prepared by centrifugation as described above.

Metabolic labelling

Schizonts of *P. falciparum* were metabolically labelled by incubation for 2–4 h in methionine-free RPMI + 10 % human serum to which was added 100–200 μCi [^{35}S]methionine (Amersham). The labelled cells were repeatedly washed in phosphate-buffered saline (PBS) and lysed by sonication in PBS containing 2 mM phenylmethyl-sulphonylfluoride and 10 μg/ml DNase 1. Lysates were used immediately for immunoprecipitation, or stored at -20 °C.

Immunization

Monkeys and rabbits were immunized with merozoites of *P. knowlesi* as described by Hempelmann, Putfarken, Rangachari & Wilson (1986). Balb/c mice were immunized by intraperitoneal inoculation of schizonts of *P. falciparum* purified on Percoll, or with lysates of the parasites (100 μg protein) prepared as described above. Booster inoculations (300 μg protein) without adjuvant were given subcutaneously, or intravenously in the case of mice to be used for hybridoma production. A similar protocol was used for Balb/c mice immunized with *Proteus* GLDH (Sigma).

Immunoelectrophoresis

One-dimensional immunoelectrophoresis in agarose gels was carried out as described by Hempelmann *et al.* (1986). Countercurrent immunoelectrophoresis was performed under similar conditions.

Polyacrylamide gel electrophoresis (PAGE)

Electrophoresis in slab gels under non-dissociating conditions was as described by Hempelmann & Wilson (1980). A stacking gel of 3·6 % acrylamide was used routinely.

PAGE screen for antibody

A 10 μl sample of parasite lysate (or an alternative source of GLDH) was incubated with 20 μl of serum or hybridoma fluid and 10 μl glycerol in 70 μl of 25 mM Tris/HCl, pH 7·4, at 37 °C for 2–3 h. Insoluble material was pelleted by means of a bench Microfuge and the clarified sample was loaded onto a polyacrylamide gel under non-dissociating conditions, as described above. Electrophoresis was at 4 °C overnight.

Glutamate dehydrogenase (GLDH) assay

After electrophoresis, gel slabs were incubated in the following substrate solution for 1–2 h at 37 °C: 50 mM Tris/HCl, pH 8·0, (32 ml), sodium glutamate (80 mg), NADP (8 mg), 3-(4,5-dimethylthiazoy-2)-2,5-diphenyltetrazolium bromide (4 mg), and phenazine methosulphate (0·4 mg). GLDH activity was also quantitated by a spectrophotometric method as described by Schmidt (1974).

Immunodots

The procedure of Horejsi & Hilgert (1983) was adapted as follows. Antibody samples (1 μl) were spotted on scribed nitrocellulose paper (Millipore HA 0·45) and dried at 37 °C. Unreacted sites were blocked by immersion in 3% (w/v) bovine serum albumin (BSA) for 1 h at room temperature. After 'blocking', the paper was placed on a glass surface and drops of parasite lysate were applied to the scribed side. The lysate was spread evenly and retained in place as a thin film by overlaying with a second glass plate. After overnight incubation at 4 °C in a moist box, the paper was removed and washed 4–5 times with Tris-buffered saline (TBS) until haemoglobin could no longer be seen. The nitrocellulose sheet was then incubated with the enzyme substrate solution (see above) for 1–2 h at 37 °C in the dark before finally washing with distilled water. Exposure to bright light was minimized to avoid background staining.

Hybridoma production

NS-1 mouse myeloma HAT-sensitive cells were fused with spleen cells from immunized mice in a ratio of 1 : 5 in the presence of polyethylene glycol (Galfre, Howe, Milstein, Butcher & Howard, 1977). Hybrids were selected by cultivating the cells (10^4–10^5/well) on mouse macrophage feeder cells in RPMI 1640 growth medium, supplemented with 10 μM deoxycytidine, 3 μM glycine, 0·4 μM aminopterin, 30 μM thymidine and 100 μM hypoxanthine. After 2 weeks, the culture supernatant fractions were screened for antibody and the selective agent, aminopterin, was omitted from the medium. Cell suspensions from wells shown to contain anti-GLDH by the primary screening test were cloned on macrophage feeder layers by limiting dilution. Recloned positive cells were subsequently grown in bulk *in vitro* and passaged in Pristane-primed mice to produce ascitic fluid.

Western blotting

Samples were run at 40 mA constant current at room temperature for 4–5 h on vertical slab SDS–PAGE gels (10% acrylamide) using the discontinuous buffer system of Laemmli (1970). After electrophoresis, gels were transferred to 500 ml buffer (25 mM Tris, 192 mM glycine and 20% (v/v) methanol, pH 8·3). Proteins were transferred to

nitrocellulose paper pre-soaked in the same buffer by electrophoresis in a Bio-Rad trans Blot Cell at 55 V constant at 4 °C for 16 h. After blotting, filters were soaked in 5 % (w/v) BSA in Tris-buffered saline (TBS). Antibody was added in BSA/TBS and blots were incubated for a further 1·5 h before washing by gentle agitation in TBS for 1 h at room temperature. This was repeated twice with fresh solution for 30 min washes. The penultimate wash included 0·05 % Triton X-100. Bound antibody was detected by addition of 0·5 μg Protein A iodinated with 5–10 μCi of ^{125}I in BSA/TBS followed by incubation for 2 h. The filter was then washed extensively in several changes of TBS including an overnight wash. Filters with low background counts were dried and autoradiographed with pre-flashed Fuji RX Safety 4 X-ray film.

Fluorescent antibody test

Thin blood films were prepared from triple-washed cultures of red cells containing late trophozoites of *P. falciparum*. Air-dried films were fixed by immersion for 5 min in cold acetone. Hybridoma culture supernatant fractions were applied to the slides for 30 min at 37 °C before washing and reacting with fluoresceinated anti-mouse IgG (Miles Yeda).

Sucrose density gradient

Step-wise gradients (4 ml) were prepared with 10–40 % sucrose in 0·175 M veronal buffer, pH 8·6, in Beckman Polyallomer tubes. Parasite lysate (0·1 ml) was layered on the top of the gradient and fractionated by centrifugation at 35000 r.p.m. for 18 h at 4 °C in a Beckman SW55Ti swingout rotor. The linearity of the resulting gradient was confirmed by refractometry. Fractions were collected by means of a tube-piercing apparatus. Proteins run in parallel as molecular weight markers were thyroglobulin (660000), IgG (155000) and ovalbumin (43500).

RESULTS

Polyclonal antisera from rabbits and monkeys

Electrophoresis of soluble extracts of schizonts in agarose gels containing a monkey antiserum resulted in immunoprecipitation of the GLDH of *P. knowlesi* as a single slow-moving band. A precipitin band corresponding to the enzyme activity was visible when the gel was stained with Coomassie Blue. By means of countercurrent electrophoresis it was shown that the optimal amount of antiserum for the precipitation of approximately 10 mU of GLDH activity was 5–20 μl. A semi-preparative immunoprecipitate of GLDH was obtained by countercurrent electrophoresis of 1 ml of schizont extract (20 mg protein) and 100 μl of antiserum (Fig. 1 A).

Antisera from monkeys or rabbits immunized with purified merozoites of *P. knowlesi* cross-reacted with the GLDH of *P. falciparum* and formed insoluble complexes. In accordance with the lack of species specificity, the antibodies failed to distinguish between two isozyme types of GLDH in cloned lines of *P. falciparum*. Titration of antibody–enzyme mixtures by non-dissociating PAGE demonstrated a transition from fully complexed enzyme to the presence of soluble immune complexes and residual free enzyme on dilution of the antiserum (Fig. 1 B).

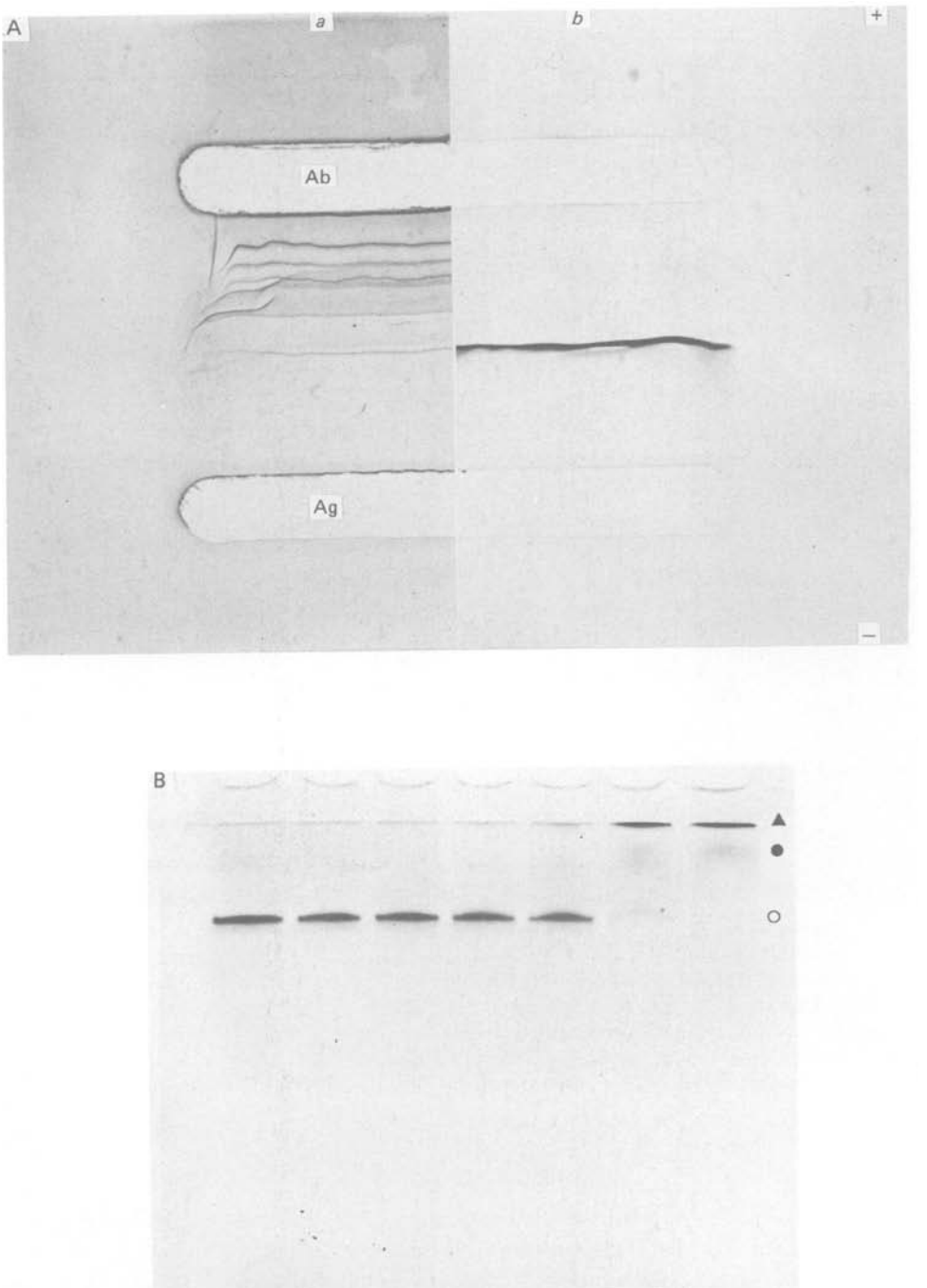

Fig. 1. (A) Preparative counter-current immunoprecipitation of GLDH from *Plasmodium knowlesi*. Ag, schizont extract; Ab, monkey antiserum. Electrophoresis was carried out at 200 V for 20 h at 4 °C. (*a*) Protein stain showing immunoprecipitates. (*b*) GLDH activity. (B) Titration of enzyme–antibody mixtures (1/20 to 1/2000) by non-dissociating PAGE to demonstrate the formation of insoluble immune complexes (▲), soluble immune complexes (●), as well as residual or free GLDH (○). The polyclonal antiserum was raised against *P. knowlesi* and was tested here with the GLDH of *P. falciparum*.

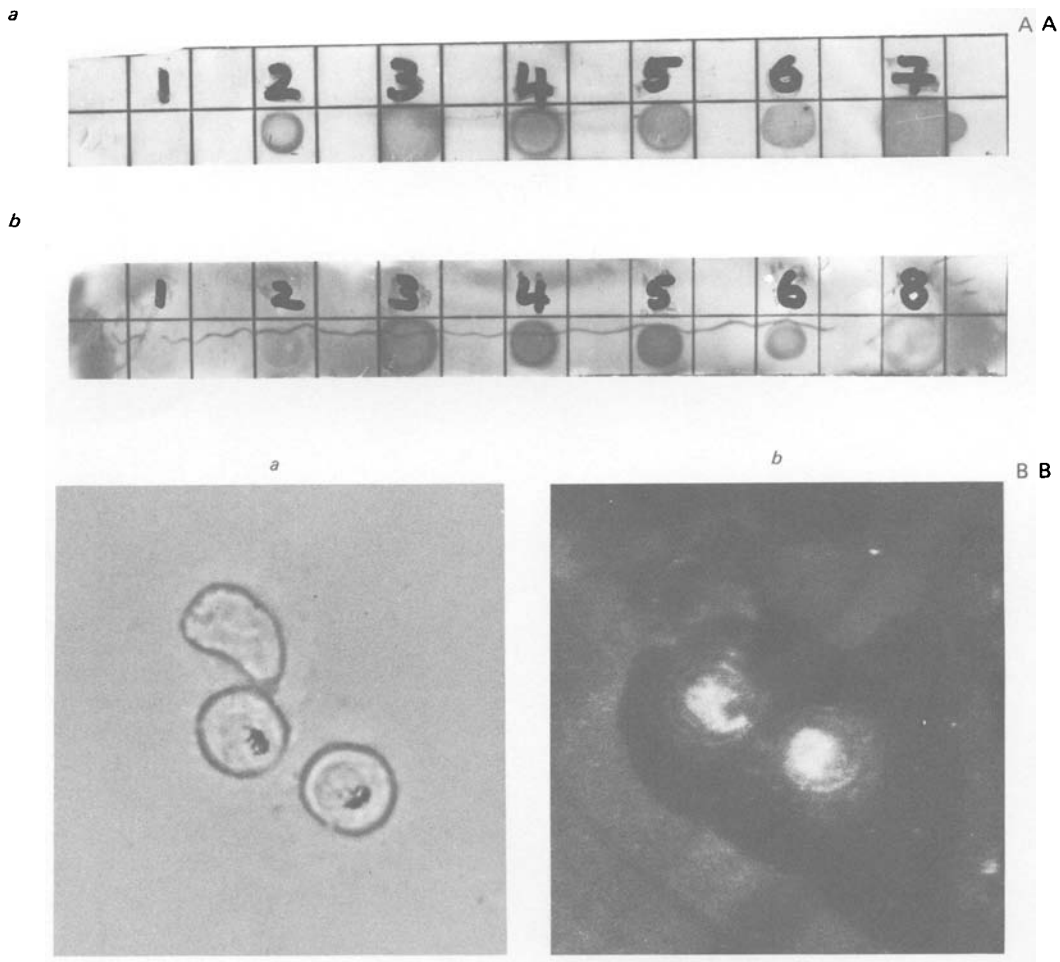

Fig. 2. (A) Immunodot assay for antibody to GLDH. Nitrocellulose paper was pre-treated with normal serum diluted 1/10 (1): mouse anti-*Plasmodium falciparum*, 1/20 (2); mouse anti-*Proteus* GLDH-neat (3 and 7) and 1/10 (4), 1/20 (5), 1/50 (6), or pre-immunization serum (8). Bound antibody then was reacted with GLDH from *Proteus* spp. (*a*) or *P. falciparum* lysate (*b*). (B) Fluorescent antibody test with trophozoites of *P. falciparum* and GLDH cross-reactive monoclonal antibody (A5). The same field containing 2 infected and 1 uninfected erythrocyte is shown under bright field illumination (*a*) or ultra-violet (*b*).

Polyclonal antisera in mice

Not all Balb/c mice immunized with schizonts of *P. falciparum* produced antibody that was detectable by the PAGE method. Such mice also failed to react after booster immunizations with partially purified preparations of parasite GLDH prepared on columns of Procion Red (not shown). This unexpected result frustrated our initial attempts to make murine monoclonal antibodies to the parasite enzyme. The serum from one mouse that had produced antibody, but from which we failed to isolate a relevant hybridoma, was used to explore ways of improving the screening and immunization procedures.

An immunodot test proved to be less cumbersome than the PAGE procedure for the

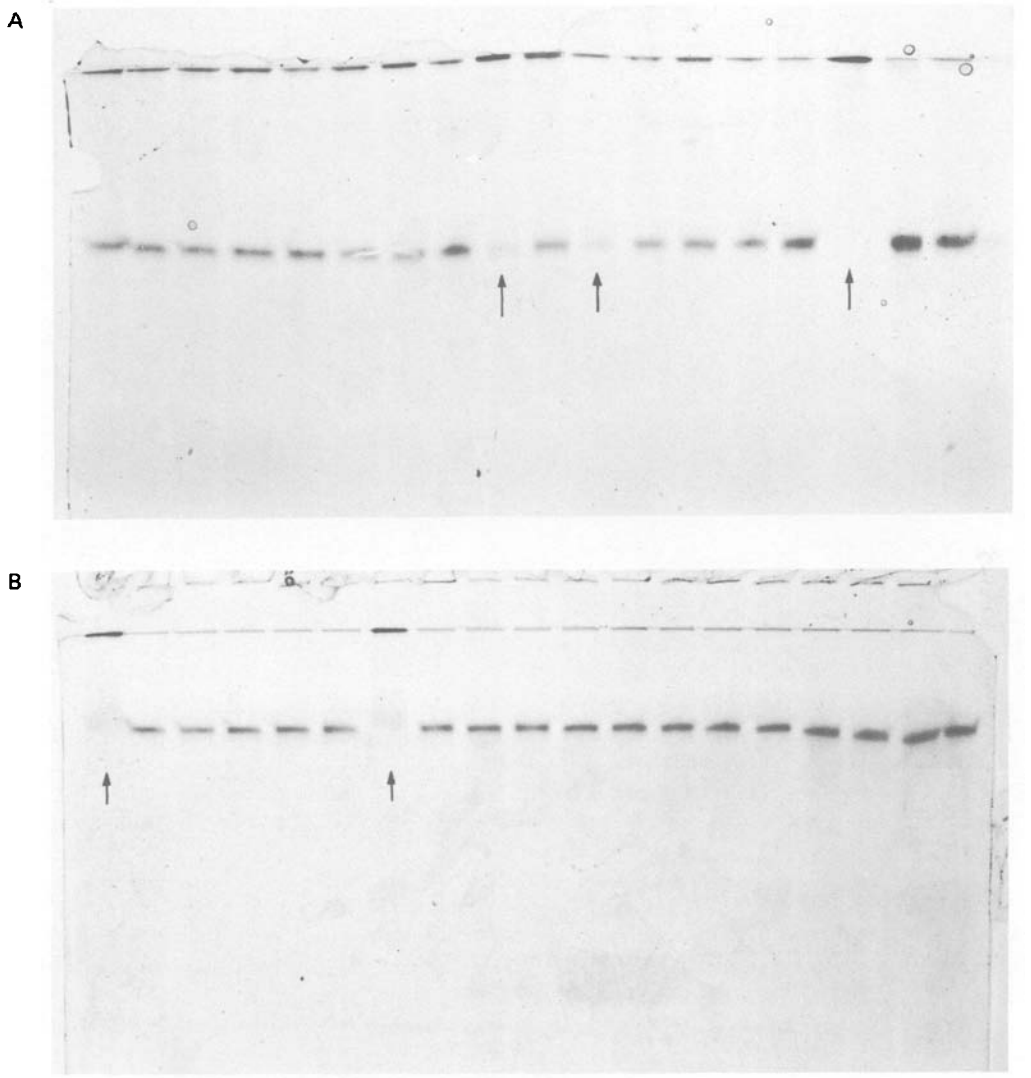

Fig. 3. (A) Culture supernatants (20 μl) containing individual hybridoma fusion products were screened by PAGE after mixing with 10 μl of a 1/5000 dilution of *Proteus* GLDH. Weak reactions were detected in tracks 9 and 11. The enzyme was totally complexed by the antibody in track 16. Controls with normal mouse serum and RPMI were tested in tracks 17 and 18, respectively. Track 16 contains a monoclonal that was recloned and designated A5. (B) Culture supernatant fractions were screened as in (A) but using a lysate of *Plasmodium falciparum* as the source of GLDH. Both the positive supernatant fractions (tracks 1 and 7), corresponding to recloned monoclonals A5 and A4, were also reactive with *Proteus* GLDH.

detection of antibody but was less sensitive. Exploratory tests with immunodots revealed that monkey and mouse polyclonal antisera to plasmodial GLDH also cross-reacted with the bacterial enzyme from *Proteus* spp., but not with bovine GLDH (not shown). These results suggested that heterologous GLDH might serve as an

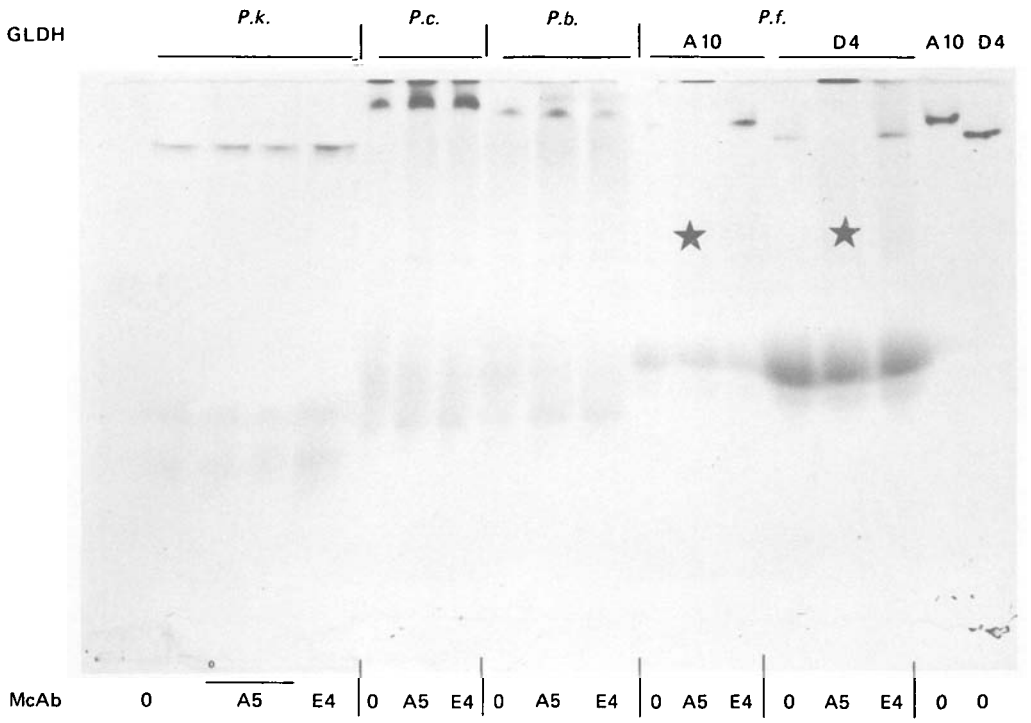

Fig. 4. Monoclonal antibodies specific for the GLDH of *Proteus* (E4) or cross-reactive with *Proteus* and *Plasmodium falciparum* (A5) were screened with parasite lysates from four species of malarial parasites: *P. knowlesi* (tracks 1–4), *P. chabaudi* (tracks 5–7), *P. berghei* (tracks 8–10), *P. falciparum* – clone A10 (tracks 11–13 and track 17), and *P. falciparum* – clone D4 (tracks 14–16, track 18). Controls without antibody are indicated (○). Specific absorption of GLDH was confined to *P. falciparum* (*).

alternative immunogen for the plasmodial enzyme. This prediction was borne out when sera from mice immunized with *Proteus* GLDH were found to produce antibodies cross-reactive with *P. falciparum* GLDH (Fig. 2A).

Production of monoclonal antisera (McAb)

Hybridomas were prepared from the spleen cells of a mouse immunized with *Proteus* GLDH. Of 125 wells containing hybridomas which were screened by the PAGE procedure, 2 wells contained antibody to the *Proteus* enzyme and cross-reacted with *P. falciparum* GLDH, and 2 wells contained antibody to the *Proteus* enzyme only (Fig. 3). Hybridomas of both types were cloned by limiting dilution and were propagated as ascites in mice. The immunoglobulin subtypes were both IgG_1.

Specificity of McAb to plasmodial GLDH

McAb A5 that cross-reacted with the GLDH of *Proteus* spp. and *P. falciparum*, as well as McAb E4 that reacted with the *Proteus* enzyme only, were tested against cell lysates from three other species of *Plasmodium*; namely, *P. knowlesi*, *P. chabaudi* and *P. berghei*. None of the GLDH enzymes in these species reacted with the McAb antibody to the enzyme from *P. falciparum* (Fig. 4).

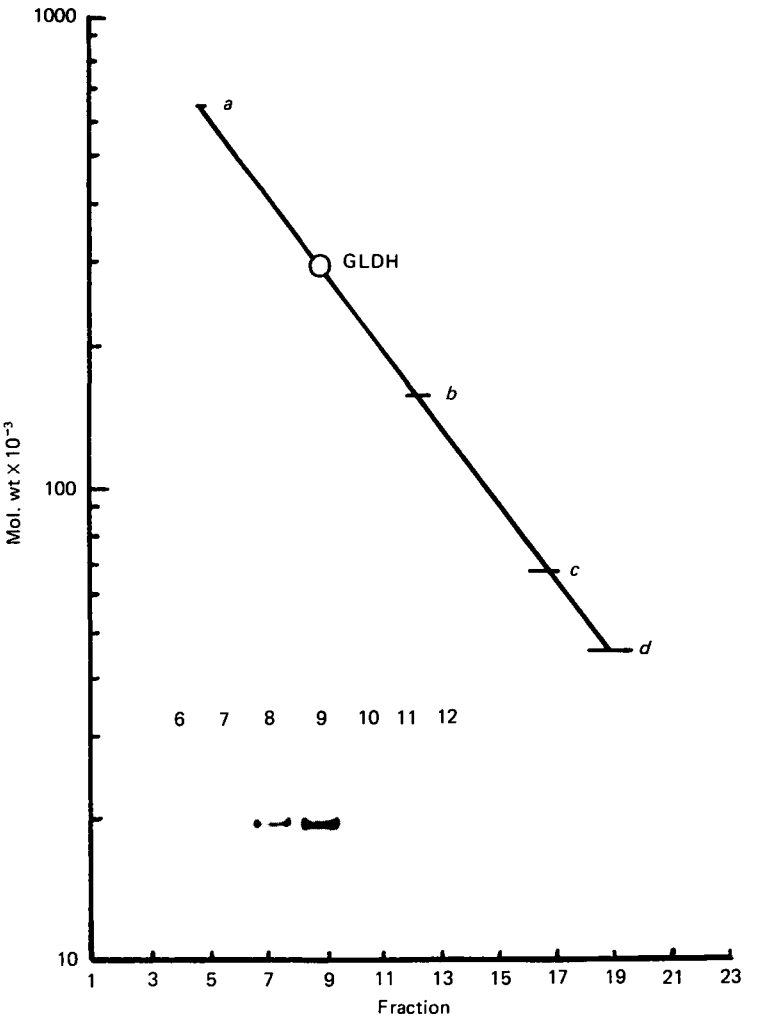

Fig. 5. Molecular weight determination of *Plasmodium falciparum* GLDH by sucrose density gradient (10–40%) centrifugation. The inset shows the active fractions. Molecular weight markers are as follows: (*a*) thyroglobulin (660000); (*b*) immunoglobulin G (160000); (*c*) haemoglobin (64500); (*d*) ovalbumin (43500).

Intracellular localization of GLDH

Fluorescent antibody tests with acetone-fixed smears of trophozoites of *P. falciparum* and McAb A5 showed that the enzyme was located in the parasite's cytoplasm (Fig. 2B). The presence or absence of the enzyme in the parasite's mitochrondrion-like organelles could not be distinguished in this analysis.

Subunit size of plasmodial GLDH

Metabolically labelled [^{35}S]methionine-GLDH was extracted from a lysate of red cells infected with *P. falciparum* and immunoprecipitated with McAb A5. By means of SDS–PAGE and fluorography the enzyme subunit was detected as a single band with a molecular weight of about 49000 Da ($\pm$ 10000 in 3 determinations). Parasite extracts

fractionated on sucrose gradients (10–40%) yielded a single peak of GLDH with maximal enzymic activity corresponding to a globular protein of about 290000 Da (Fig. 5). These data are consistent with an enzyme consisting of 6 subunits.

DISCUSSION

Our study confirms that GLDH from malaria parasites has structural similarities with the NADP-GLDHs from other lower eukaryotes and prokaryotes. The monoclonal antibody A 5 that reacted with both the parasite and bacterial enzymes did not cross-react with the NAD(P)-GLDH of bovine origin (EC 1 . 4 . 1 . 3) – for a report on a monoclonal antibody to the bovine enzyme see Martinez-Ramon & Renau-Piqueras (1984). This pattern of cross-reactivity would be expected because of the high degree of sequence homology shown by parts of the prokaryotic (*Escherichia coli*) and lower eukaryotic (*Neurospora crassa*) NADP-GLDH enzymes on the one hand, and the NAD(P)-GLDHs of higher eukaryotes on the other (Mattaj, McPherson & Wooton, 1982). We were surprised, however, to find that our McAb A5 discriminated between the GLDHs of different malarial species. This can be taken as additional evidence that the antibody is directed towards an epitope distinct from the highly conserved substrate and co-enzyme binding sites. Other studies have found that complexes of GLDH and antibody retain activity, but antibody can interfere with the action of allosteric modifiers due to conformational changes (Fahien, Steinman & McCann, 1966).

The GLDHs of animal cells are in mitochondria, probably in the matrix, whereas in fungal cells the enzyme is in the soluble phase of the cytosol (Smith *et al.* 1975). This is also the case with *P. falciparum*. The mitochondrial enzyme may be a late acquisition in evolution and coincides with the expression of an enzyme that is not specific in its requirements for NAD or NADP. *Neurospora crassa* contains distinct GLDHs for NADP and NAD, the former being biosynthetic and the latter degradative. The structure of the two enzymes is determined by two unlinked genes and the two proteins are antigenically unrelated. The isolation of modified forms of the enzyme produced by mutant strains has permitted the identification of residues important for the maintenance of normal activity (Brett, Chambers, Holder, Fincham & Wootton, 1976).

Molecular studies on various GLDHs have proceeded rapidly since the elucidation of the primary structures of some of these enzymes (Smith *et al.* 1975). Bovine liver GLDH is predominantly in a form of 330000 Da which contains 6 identical subunits. This homohexameric form is the smallest enzymatically active unit. Our study confirms earlier findings (Walter *et al.* 1974) that the NADP–GLDHs of *Plasmodium* spp. have smaller subunits, like those of other lower eukaryotes.

As has been mentioned, the amino acid compositions of GLDHs from different organisms are strikingly similar, indeed they are distinct from the composition of most other dehydrogenases with a high ratio of arginine to lysine. The bovine and chicken GLDHs are remarkably conserved with only 27 differences in the 500 amino acid residues which each subunit possesses in common (Smith *et al.* 1975). Comparison of the sequence of the NADP-dependent enzyme of *Neurospora* with those of the vertebrate enzymes reveals that a significant homology exists, particularly within the first 200 residues of the chain. The homology with the remainder of the sequence is less striking. Although the polypeptide chain of the *Neurospora* enzyme is shorter than the bovine enzyme by 48 residues, the insertion of a small number of gaps confers considerable homology. All these proteins contain a uniquely reactive lysine residue in the part of the sequence with the greatest homology (Mattaj *et al.* 1982). The bulk of the conserved

residues must be critical for conformation rather than catalysis. Consequently, it is not surprising that antibodies to these enzymes are generally cross-reactive (Roberts & Pateman, 1964).

An intriguing possibility that derives from our study is that genes for highly conserved enzymes from other lower eukaryotes may serve as direct probes for the corresponding genes of malaria parasites. To some extent this has already been borne out by the comparative studies of McCutchan, Dame, Miller & Barnwell (1984). They found that heterologous probes to the enzymes dihydrofolate reductase (mouse) and thymidylate kinase (yeast), hybridize to restriction digest fragments of DNA from various species of malaria parasites. Such an approach might open the way for sequence studies or chromosomal assignment (Kemp, Corcoran, Coppel, Stahl, Bianco, Brown & Anders, 1985) of the genes for the glycolytic enzymes which have long been used as genetic markers in *Plasmodium* (Walliker, 1983) as well as in other protozoan parasites.

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