Table 1. Minimum concentrations (micromoles) of reconstituted IgG at which Inv(1) was detected. For controls, the isolated light chains and the isolated untreated myeloma proteins were tested for Inv(1). The antigen was detected at 12.5 μmole/ml and 50 μmole/ml in Per and Har light chains, respectively, and at 0.2 and 0.4 μmole in Per and Har isolated myeloma proteins, respectively.

Name	Nature of the myeloma source of heavy chain		Source of kappa chain	
	γ Chain subclass	Light chain subclass	Per	Har
Hug	1	κ	0.2	0.4
Hen	1	κ	0.2	0.2
Per	1	κ	0.2	N.T.†
Har	1	κ	N.T.	0.4
Mean			0.20	0.33
Pac	3	κ	0.1	0.4
Bri	3	λ	0.2	0.2
Mean			0.15	0.30
Has	2	κ	1.7	6.7
Jon	2	κ	0.8	>6.7‡
Cin	2	λ	1.7	>6.7
Nor	2	λ	0.2	0.2
Mean			1.10	>5.08

* Initial concentration of all proteins: 1 mg/ml, that is, 6.7 μmole for IgG and 50 μmole for light chains. † N.T., not tested. ‡ Not detected at initial protein concentration of 6.7 μmole.

tuted proteins are shown in Fig. 1A. All the lines are equally intense and all meet as lines of identity. The proteins were also tested against horse antiserum to whole normal human serum with a kappa light chain, a γ1 heavy chain, and IgG from a normal donor as controls (Figs. 1, B and C). The reconstituted molecules with γ1 and γ2 heavy chains spur over the kappa chain, the reconstituted molecules with γ1 and γ3 heavy chains spur over the isolated γ1 chain and the reconstituted molecules with γ2 and γ3 chains show identity with the control IgG molecule. There is therefore no evidence to suggest that the Nor γ heavy chain combines more readily with the light chain than the Cin (γ2) heavy chain does.

The greater efficacy of the Nor γ2 chain in revealing the Inv(1) antigen on the light chain of the donor is probably not a function of the light chain with which the heavy chain was originally associated, because both Nor and Cin myeloma proteins have λ light chains, yet the latter required at least eight times the molar concentration of the former for the detection of Inv(1). It should be pointed out, however, that we do not know the Oz subtypes (*9*) of these light chains, hence it is possible that the observed difference is associated with the subtype of the light chain present in the molecule from which the heavy chain was obtained.

The data offer further confirmation that the Inv antigens are dependent upon the quaternary structure of the molecule (*4, 5*). It has also been demonstrated that the Gm antigens located on the Fd portion of the heavy chain [Gm(3) and Gm(17)] are dependent upon the quaternary structure of the molecule for their expression (*4, 5*). It seems likely, therefore, that all allotypic antigens in the Fab portion of the IgG molecule depend, at least in part, upon the quaternary structure of the molecule for their expression. The expression of the Gm antigens on the Fc portion of the heavy chain, on the other hand, seems to be independent of the quaternary structure of the molecule (*7*).

Four times as much protein was required to detect Inv(1) in the isolated light chain from myeloma protein Har as was required to detect it in the isolated light chain from myeloma protein Per (Table 1). In parallel with this observation, IgG molecules reconstituted with light chain Har tended to require more protein for the detection of Inv(1) than those IgG molecules formed with light chain Per. This was especially noticeable for the γ2 heavy chains. Nevertheless, γ2 heavy chain Nor was as effective with light chain Har as it was with light chain Per. We have no explanation for this observation, but the surprisingly great efficiency of γ2 heavy chain Nor suggests that the γ2 heavy chains may comprise at least two subtypes, one typified by heavy chain Nor, and a second type corresponding to the remaining three γ2 heavy chains shown in Table 1.

Possibly the difference between the reactions of light chains Per and Har is associated with the kappa chain subtypes based on differences in the variable portion of the molecule (*10*). Unfortunately, we do not have enough of either of these proteins to permit a test of this suggestion.

Our conclusion that γ2 heavy chains are less efficient than γ1 and γ3 heavy chains in enhancing Inv(1) activity seems to contradict that of Litwin and Kunkel (*5*). They concluded that, ". . . heavy chains of any γG type could restore the Inv(1)—Mo antigen. . . ." The contradiction may be more apparent than real, because as they showed, the antigen detected by Mo is markedly different from the antigen detected by Math (Math was called Matt by Litwin and Kunkel).

It would be of interest to test the same reconstituted molecules with antibody Math and Mo. Unfortunately, we do not have any antibody Mo at our disposal.

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References and Notes

1. C. Ropartz, L. Rivat, and P.-Y. Rousseau, *Proc. Congr. Int. Soc. Blood Transfusion 9th*, Mexico, 455 (1962); C. Ropartz, P.-Y. Rousseau, L. Rivat, J. Lenoir, *Rev. Franç. Études Clin. Biol.* **6**, 374 (1961).
2. E. C. Franklin, H. Fudenberg, M. Meltzer, D. R. Stanworth, *Proc. Nat. Acad. Sci. U.S.* **48**, 914 (1962); M. Harboe, C. K. Osterland, H. G. Kunkel, *Science* **136**, 979 (1962).
3. W. D. Terry, J. L. Fahey, A. G. Steinberg, *J. Exp. Med.* **122**, 1087 (1965).
4. S. H. Polmar and A. G. Steinberg, *Science* **145**, 928 (1964); A. G. Steinberg, *Cold Spring Harbor Symp. Quant. Biol.* **29**, 449 (1964); S. D. Litwin and H. G. Kunkel, *Nature* **210**, 866 (1966).
5. S. D. Litwin and H. G. Kunkel, *J. Immunol.* **99**, 603 (1967).
6. A. G. Steinberg, *Progr. Med. Gen.* **2**, 1 (1962).
7. S. H. Polmar and A. G. Steinberg, *Biochem. Genet.* **1**, 117 (1967).
8. Unpublished data of Steinberg and Ropartz quoted in W. A. Muir and A. G. Steinberg, *Seminars in Hematol.* **4**, 156 (1967).
9. D. Ein and J. L. Fahey, *Science* **156**, 947 (1967); E. Appella and D. Ein, *Proc. Nat. Acad. Sci. U.S.* **57**, 1449 (1967); D. Ein, *ibid.* **60**, 982 (1968); C. Milstein, B. Frangione, J. R. L. Pink, *Cold Spring Harbor Symp. Quant. Biol.* **32**, 31 (1967).
10. C. Milstein, *Nature* **216**, 330 (1967); H. D. Niall and P. Edman, *ibid.*, p. 262.
11. Horse antiserum to whole normal human serum (lot T-1654) was obtained from Mann Laboratories, New York.
12. We thank Drs. P. Bennett, J. T. Boyer, G. A. Spengler, L. G. Surhland, and W. D. Terry for supplying the serum samples from the myeloma patients; Dr. Terry for antiserums to determine the H chain subtypes and for confirming our typing of these chains, and Dr. C. Ropartz for reagents to determine GM(8). Our study was supported in part by NIH grant Gm 7412. I.R. is supported by funds from the Pan American Union and the Comision Nacional de Energia Nuclear, Mexico.

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Dihydroorotic Acid Dehydrogenase: Introduction into Erythrocyte by the Malaria Parasite

Abstract. *Mature, circulating mammalian erythrocytes lack dihydroorotic acid dehydrogenase activity. However, we have detected activity, which appears to reside in an enzyme, in malaria parasites purified from mammalian blood.*

Dihydroorotic acid dehydrogenase (DAD, E.C. 1.3.3.1) is one of the enzymes in the biosynthetic pathway leading to uridine-5′-monophosphate, and it catalyzes the reversible conversion of dihydroorotic acid to orotic acid. The

enzyme is present in a variety of microorganisms (*1, 2*), in human leukocytes (*3*), and in cultured human fibroblasts (*4*), but it is absent from mature human erythrocytes (*3*). The enzyme is also absent from mature rabbit erythrocytes, but can be recovered from rabbit hemolyzates containing reticulocytes (*5*). In our experiments we studied whether the malaria parasite introduces DAD activity into the erythrocytes it infects.

The DAD activity was assayed as previously described (*4*). Hemoglobin was measured by Drabkin's method (*6*) and total cell protein by the method of Lowry (*7*). Erythrocytes (of all species) from both infected and control animals were processed as follows. Whole blood, collected in a heparinized syringe, was centrifuged at 1500*g* for 15 minutes. The plasma, the buffy coat, and the erythrocytes immediately below the buffy coat to a depth of 3 mm, were aspirated from the centrifuge tube and discarded. The erythrocytes were then washed three times in Saline G (*8*)—a buffered isotonic saline solution. The red cells were suspended in 0.01*M* potassium phosphate buffer (*p*H 7.0), and disrupted by four 30-second pulses of sonic energy (*9*). The extract so obtained was centrifuged at 1500*g* for 10 minutes, and the supernatant (or a dilution of it) was then used for the estimation of DAD activity and of hemoglobin concentration.

In the purification of parasites from infected hemolyzates, the red cells were separated from plasma and buffy coat, as described above, and washed three times in a buffered, isotonic glucose solution (*10*). The parasites were then released by saponin lysis of the erythrocytes (*10*) and centrifuged at 1500*g*. Microscopic examination of the sediment revealed no blood cells; the parasites were readily visualized. The parasites were then washed three times in a solution of 0.01*M* potassium phosphate (*p*H 7.0). They were then suspended in this solution and disrupted by sonic energy, by the same method described above for erythrocytes.

The procedure used, in experiments on the dialyzability of the DAD activity recovered from parasites, is given elsewhere (*11*), except that in the present experiments the dialysis bath was a solution containing 0.01*M* tris(hydroxymethyl)amino methane (tris) buffer (at *p*H 7.4) and 0.86 percent sodium chloride.

Several collections of pooled whole blood were obtained from Carworth CF-1 mice, infected with *Plasmodium berghei* (*12*). In these animals, microscopy revealed that between 30 and 50 percent of the erythrocytes were parasitized. Bloods pooled from approximately 20 animals were used for each experiment. A seed stock of whole blood, from Wistar rats infected with *P. vinckei* (*13*), was injected intravenously into young, uninfected Wistar rats. When between 30 and 80 percent of the erythrocytes were parasitized (usually within 5 days of infection), the rats were heparinized and exsanguinated under general anesthesia. Bloods from 5 to 15 rats were used for each experiment.

Table 1. Specific dihydroorotic acid dehydrogenase activity of hemolyzates from control and infected mice and of purified malaria parasites (*Plasmodium berghei*).

Sample	Specific DAD activity*
Mouse hemolyzates	
Control	0.06†
Infected (50%)	.85
Malaria parasites	
Experiment 1	13.60
Experiment 2	14.40
Experiment 3	8.30

* Nanomoles of orotic acid synthesized per hour per milligram of hemoglobin (hemolyzates) or milligram of protein (parasites). † This level of activity is at the lower limit of the sensitivity of the assay, and is not significantly different from zero.

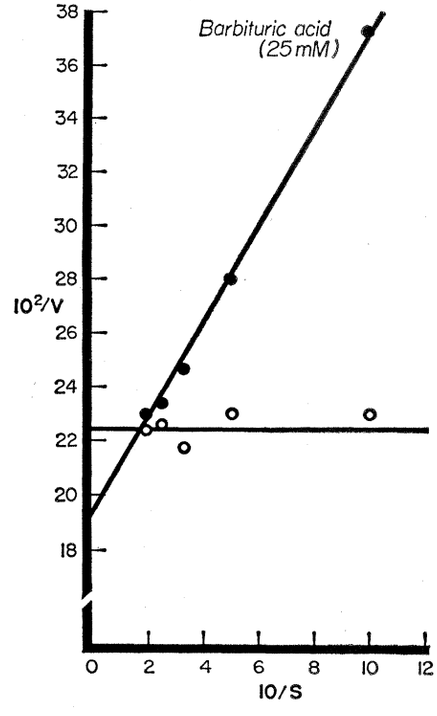

Fig. 1. Inhibition of the dihydroorotic acid dehydrogenase activity recovered from *Plasmodium vinckei* by barbituric acid. Closed circles, points obtained from reaction mixture to which barbituric acid was added; open circles, points obtained from control reaction mixtures, which contained no barbituric acid; *S*, substrate concentration, expressed as micromoles of dihydroorotic acid per milliliter of reaction mixture; *V*, reaction velocity, expressed as millimicromoles of orotic acid synthesized per milligram of parasite protein per hour.

The specific activity for DAD was measured in hemolyzates from mice infected with *P. berghei* and from uninfected, control mice of the same strain (Table 1). As can be seen, the hemolyzates from infected animals had about ten times the specific activity of the control hemolyzates. Unfortunately, however, this result in itself is ambiguous. The DAD activity measured might reflect only a reticulocytosis associated with the infection. To rule this possibility out, the parasites were purified by saponin lysis of infected hemolyzates collected from different sets of animal donors, in three separate experiments (Table 1). The purified parasites had about 200 times the specific activity of the control hemolyzate, and about 20 times the specific activity of the infected one. Similar data have been obtained for infected and control hemolyzates of Wistar rats with *P. vinckei*.

To determine whether the catalytic activity resides in an enzyme, *P. vinckei* was purified by saponin lysis from hemolyzates obtained after the Wistar rats were infected. The species of parasite and host were changed in these experiments in order to obtain larger quantities of parasite protein. The parasite extracts, in three separate experiments, had specific activities of between 5 and 6 [nanomoles of orotic acid formed per hour per milligram of parasite protein (*14*)]. All the catalytic activity in the parasite extract was destroyed by a 10-minute incubation at 100°C. Over 70 percent of the activity persisted after dialyzing the extract for 72 hours at 4°C. Figure 1 shows two further features of the DAD activity recovered from the lysed parasites: (i) the reciprocal of reaction velocity is a linear function of the reciprocal of substrate concentration; (ii) catalytic activity is competitively inhibited by barbituric acid—a known inhibitor of the purified bacterial enzyme (*1*) and of the DAD activity detected in extracts of human diploid cell strains (*5*). These observations, collectively, are consistent with the view that the catalytic activity recovered from the parasite resides in an enzyme.

Our experiments show that the mature, circulating erythrocyte of both rat and mouse do not have significant catalytic activity for dihydroorotic acid dehydrogenase. Activity can, however,

be recovered from *P. berghei* and *P. vinckei*, obtained from mouse and rat erythrocytes, respectively. At least in the case of *P. vinckei*, the activity appears to reside in an enzyme.

These observations suggest the possibility that specific inhibitors of DAD may sometimes have antimalarial activity. The absence of DAD from the mature erythrocyte presumably means that the function subserved by the enzyme cannot be supplied by the host cell. Hence, if an inhibitor blocks the parasite enzyme, the parasite is not likely to escape by obtaining the product of the reaction or of the pathway from the metabolic activity of the infected cell. The effectiveness of inhibitors of DAD as antimalarials is, however, likely to depend also on several other considerations. For one thing, species of *Plasmodium* which have a stable tissue phase or which (like *P. berghei*) preferentially infect reticulocytes (*15*) might well be resistant to such therapy, although it is by no means clear that the level of DAD activity in the reticulocyte is sufficient for the needs of the parasite. Infections with parasites, such as *P. vinckei* or *P. falciparum*, should in theory respond better. Second, of course, the inhibitors of DAD must be specific in two senses. They must not have other biological actions which are toxic to the host animal, and they must inhibit the parasite enzyme to a greater extent than they inhibit the enzyme present in the nucleated cells of the host. Third, the parasite must not be able to trap either the product of the pathway (as uracil or uridine) or orotic acid in amounts sufficient to enable it to mature. Both the normal plasma concentrations of these compounds, and the quantities required for nutrition of the parasite, are not presently known. It is, of course, feasible to inquire systematically into each of these questions, but it is probably more logical to determine directly whether inhibitors of DAD have antimalarial activity. Barbituric acid and 2,4-dihydroxy-6-methylpyrimidine are known to inhibit DAD (*1*). Other inhibitors could presumably be synthesized.

Several tissues, in addition to blood, have deficiencies for enzymes in the synthetic sequence leading to uridine-5′-monophosphate (*16*). Some tissues are also known to be deficient in the sequence leading to cystine (*17*). Finally, the mature erythrocytes of both man and rabbit are unable to perform all the steps in the *de novo* synthesis of inosine-5′-monophosphate (*18*). Perhaps as more organs and pathways are studied, it will be discovered that many mammalian tissues have incomplete sequences with respect to one or another of the small molecules universally found in cells. These enzyme deficiencies, presumably due to cellular differentiation, may occasionally be useful in the diagnosis of infection with intracellular parasites. They may also help in a few instances in devising newer approaches to the treatment of the infection.

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References and Notes

1. H. C. Friedmann and B. Vennesland, *J. Biol. Chem.* **233**, 1398 (1958).
2. J. R. Beckwith, A. B. Pardee, R. Austrian, F. Jacob, *J. Mol. Biol.* **5**, 618 (1962); F. Lacroute, *J. Bacteriol.* **95**, 824 (1968).
3. L. H. Smith and F. A. Baker, *J. Clin. Invest.* **38**, 798 (1959).
4. K. D. Wuu and R. S. Krooth, *Science* **160**, 539 (1968).
5. M. Lotz and L. H. Smith, Jr., *Blood* **19**, 593 (1962).
6. W. H. Crosby, J. I. Munn, F. W. Furth, *U.S. Armed Forces Med. J.* **5**, 693 (1954).
7. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
8. T. T. Puck, S. J. Cieciura, A. Robinson, *J. Exp. Med.* **108**, 945 (1958).
9. R. S. Krooth, R. R. Howell, H. B. Hamilton, *ibid.* **115**, 313 (1962).
10. M. R. Bovarnick, A. Lindsay, L. Hellerman, *J. Biol. Chem.* **163**, 523 (1946).
11. Y. Pan and R. S. Krooth, *J. Cell. Physiol.* **71**, 151 (1968).
12. These bloods were a gift from Dr. P. Thompson of the Parke-Davis Co., Ann Arbor, Michigan.
13. The seed stock was provided by Dr. G. Brewer of the University of Michigan Medical School.
14. Uninfected hemolyzates treated in the same fashion yielded solutions with only 1/20th the protein concentration of those obtained from infected hemolyzates; these solutions had no measurable DAD activity.
15. P. F. Russel, L. S. West, R. D. Manwell, G. MacDonald, *Practical Malariology* (Oxford Univ. Press, London, 1963), p. 110.
16. N. Kretchmer, R. Hurwitz, N. Raiha, *Biol. Neonatorum* **9**, 187 (1966); K. D. Wuu and R. S. Krooth, unpublished observations; R. S. Krooth, in *Comparative Mammalian Cytogenetics*, K. Benirschke, Ed. (Springer-Verlag, New York, in press).
17. S. H. Mudd, J. F. Finkelstein, F. Irreverre, L. Laster, *J. Biol. Chem.* **240**, 4382 (1965).
18. B. A. Lowy, M. K. Williams, I. M. London, *ibid.* **237**, 1622 (1962).
19. Supported by program project grant NIH-1-PO1-GM15419-02. We thank Dr. G. Brewer of the Department of Human Genetics, University of Michigan Medical School, and Dr. P. Thompson of the Parke-Davis Co., Ann Arbor, Michigan, for their assistance.
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Stability of Asymmetric Phospholipid Membranes

Abstract. *Bilayers (black films) composed of phosphatidylserine are unstable under conditions of asymmetric distribution of calcium or hydrogen ions with respect to the membrane. Addition of calcium ions to the solution (100 millimolar sodium chloride, pH 7.0) on one side only, produces lowering of the direct-current resistance and results in breaking of the membrane. However, with calcium ions on both sides the membranes are stable and show very high electrical resistance.*

Phospholipid membranes in the form of bilayer films or as liquid-crystalline vesicles are currently being used as experimental models for certain aspects of biological membrane transport phenomena (*1*). Such model membranes are promising tools for studying, in isolation, certain molecular events relating to specific membrane function. In most work on bilayers, either neutral phospholipids, such as phosphatidylcholine (PC), or mixtures of phospholipids and other polar lipids of natural origin have been used (*2*). Recent studies on the surface properties of acidic phospholipids (*3*) indicate that these compounds would present an advantage for investigation of electrical excitation, where the binding of ions on fixed charges might play an important role.

Studies with liquid-crystalline vesicles have shown that acidic phospholipids can form stable membranes of low permeability to ions (*4*). Moreover, small concentrations of Ca^{2+} produce a large increase in the diffusion rate of trapped K^+ from vesicles composed of phosphatidylserine (PS) and phosphatidic acid (PA). This increase seemed at first to be contrary to the known effect of Ca^{2+} in decreasing the permeability of nerve cells (*5*). However, recent experiments with bilayer membranes composed of PS indicate that, when Ca^{2+} is present on both sides of the membranes, the resistance is higher than that of the same bilayers without Ca^{2+} (*6*).

These two observations, suggesting two contrasting effects for Ca^{2+}, constituted the starting point for the present study. Our results show that the asymmetric distribution of fixed charges and counter-ions on the two opposing sides of phospholipid bilayers have a profound effect on their stability and electrical properties.