0273-2289/91/3101—0097$02.20

Partial Purification and Some Properties of a Hemolymph Lectin from *Panstrongylus megistus* (*Hemiptera, Reduviidae*)

YARA DE M. GOMES,*[,1] ANDRÉ F. FURTADO,[1] AND LUANA B. B. COELHO[2]

[1]*Laboratório de Imunologia, Centro de Pesquisas Aggeu Magalhães, FIOCRUZ C. P. 7472, 50730 Recife, PE, Brazil; and* [2]*Departamento de Bioquímica, Centro de Ciencias Biológicas, Universidade Federal de Pernambuco-UFPE, 50730, PE, Brazil*

Received January 28, 1991; Accepted April 22, 1991

ABSTRACT

Hemagglutinating activity was studied in homogenates of three embryonic stages, and in the hemolymph of most instar larvae and in adult insects of *Panstrongylus megistus*, an important Chagas' disease vector in Brazil. A hemolymph lectin from the 5th instar larvae of *P. megistus* was purified through a biospecific adsorption by using formaldehyde-treated erythrocytes. The lectin fraction was desorbed with 0.2*M* D-galactose in 0.15*M* NaCl. The lectin fraction activity was inhibited by L-rhamnose, D-lactose, raffinose, D-galactose, and D-fucose. The electrophoretic pattern to native and acidic proteins resolved lectin fraction in two main bands with lectin activity. These bands were considered as multiple molecular forms or isoforms of *P. megistus* lectin. Under denaturating conditions, isoform 1 showed one band with apparent mol wt (MW) of 64 kDa while isoform 2 was resolved in two bands with MW of 64 and 33 kDa.

Index Entries: *Panstrongylus megistus;* lectin purification; hemolymph lectin; insect.

*Author to whom all correspondence and reprint requests should be addressed.

INTRODUCTION

Lectins are carbohydrate-binding proteins or glycoproteins of non-immune origin, with more than one binding site, which agglutinate cells and/or precipitate glycoconjugates in vitro (*1,2*). Although the in vivo function of lectins has not been elucidated, experimental evidences suggest that these molecules may act as opsonins in primitive immune systems (*3,4*), protect seeds against fungal attack (*5,6*), and mediate symbiosis between host plants and nitrogen-fixing bacteria (*7,8*). Membrane lectins also participate in endocytosis and intracellular translocation mechanisms of glycoproteins (*9*).

The hemolymph and tissues of various insects have demonstrated lectin activity and inhibition studies have been carried out to define the specificity of binding for the agglutinating molecules (*10–12*). However, most of these studies have been done with only one developmental stage, mainly the larval stage (*12–14*). Komano et al. (*15*) purified an induced lectin from the hemolymph of *Sarcophaga peregrina* larvae. This lectin has been studied under ontogenical and phylogenetical viewpoints (*16*).

We have previously shown that hemolymph of fifth instar larvae of *Panstrongylus megistus* presented a natural lectin activity toward vertebrate erythrocytes and that activity toward type O fresh human erythrocytes was inhibited by L-rhamnose, D-lactose, raffinose, D-galactose, and D-fucose. The lectin activity was present in both sexes, and the supernatant of lysed hemocytes did not influence such an activity (*17*). *P. megistus* is a hematophagous hemimetabolous insect, and its biological cycle, from eggs to adults, passes through five larvae instars (Fig. 1). Hemolymph, the insect blood, is the only circulating fluid, and fills the body cavity, or hemocoel. It is separated from the cellular tissues by only a thin, permeable connective tissue membrane and is maintained in circulation by a tubular dorsal heart. In addition, *P. megistus* is the most important vector of Chagas' disease in Brazil, mainly in the Northeastern area.

In the present work we explored the lectin activity in most embryonic and larval stages, as well as in adult insects of *P. megistus*. Also, a lectin was partially purified from the hemolymph of the fifth instar larvae of this insect, and some of its properties were evaluated.

MATERIALS AND METHODS

Insects

Insects were reared according to Furtado (*18*) and obtained from the insectary of the Centro de Pesquisas Aggeu Magalhães, FIOCRUZ, Recife, PE, Brazil.

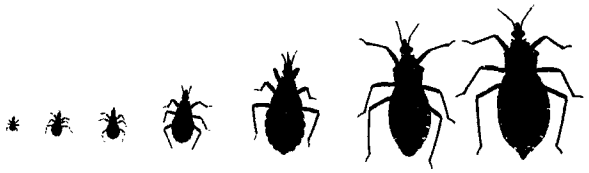


Fig. 1. Biological cycle of *Panstrongylus megistus:* egg; 1st, 2nd, 3rd, 4th, and 5th larval instars; male and female adults.

Homogenate of Eggs and Hemolymph

The eggs used were in the embryonic stages with medial, postmedial, and apical ocular spots, and were selected according to Sales (*19*). Ten eggs of each stage were homogenized in 0.5 mL of 0.15*M* NaCl and centrifuged at 10,000*g* for 10 min. Hemolymph from the 3rd, 4th, and 5th instar larvae and adult insects was collected according to Gomes (*17*) and centrifuged at 10,000*g* to remove the hemocytes. The supernatants were stored at −20°C until the use.

Erythrocytes

Human (ABO-Rh$^+$) and other vertebrates (mouse, sheep, and chicken) erythrocytes were used fresh or formaldehyde-treated by the method of Butler (*20*).

Assay of Lectin Activity

Fresh or formaldehyde-treated erythrocytes were washed four times in 0.15*M* NaCl by centrifugation at 900*g* for 10 min and resuspended in the same solution to give a final 1% (v/v) cell suspension. Assays were carried out in V plates by twofold serial dilutions of 50 μL of hemolymph (78 μg of protein) or 50 μL of purified lectin, lectin fraction, (250 ng of protein) in 0.15*M* NaCl. Results were ready after 45 min and were expressed as the reciprocal of the highest dilution giving positive agglutination or as specific agglutination activity (titer divided by the protein concentration of the hemolymph or lectin fraction). Protein concentration was determined by the method of Lowry et al. (*21*).

Inhibition Tests

The carbohydrates L-rhamnose, lactose, D-galactose, glucose, D-fucose, raffinose, D-xylose, *N*-acetyl-D-mannosamine, *N*-acetyl-D-glucosamine, β-methyl-D-arabinose, and methyl-α-D-mannoside, were used in the inhibition tests. Assays were performed using formaldehyde-treated human erythrocytes, type O. Fifty μL of the hemolymph or the lectin fraction were diluted in 50 μL of 0.15*M* NaCl which contained various

inhibitor concentrations (100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, and 0.19 m*M*). Double dilutions were performed, and after incubation for 30 min at room temperature, the 1% (v/v) cell suspension was added.

Biospecific Adsorption

Type O human formaldehyde treated erythrocytes were used as an affinity adsorbant according to the method of Reitherman (*22*). Hemolymph of fifth instar larvae (1 mL containing 37.5 mg of protein) diluted in 12 mL of 0.15*M* NaCl were mixed with erythrocytes (4 mL) and incubated at room temperature for 30 min under gentle shaking. The cells were washed four times with 0.15*M* NaCl, and after each wash they were centrifuged. The supernatants were tested to lectin activity. The adsorbed proteins were eluted by resuspending very gently the cells with 0.2*M* D-galactose in 0.15*M* NaCl (30% v/v) for 30 min. The supernatant was collected by centrifugation at 5000*g* for 5 min, recentrifuged at 10000*g* for 20 min, exhaustively dialyzed against 0.15*M* NaCl. The proteins were determined by the method of Read and Northcote (*23*).

Polyacrylamide Gel Electrophoresis for Acidic and Basic Proteins Under Nondenaturing Conditions

Slabs consisting of a 3.0% (w/v) acrylamide stacking gel and a 5–15% (w/v) acrylamide separating gel were used with two different buffer systems, according to Davis (*24*) and Reisfeld (*25*). Samples (50 μg) of lectin fraction were applied to two wells in each system and electrophoresis was carried out at 20 mA at room temperature. The basic system gel was stained as described by Reisfeld et al. (*25*), followed by a silver stain (*26*). One well of the acidic proteins was stained with Coomassie blue according to Andrews (*27*). The nonstained bands from Davis' gel were extracted (*27*) and the lectin activity was assayed.

Polyacrylamide Gel Electrophoresis Under Denatured and Reduced Conditions

Electrophoresis on SDS polyacrylamide slab gel was carried out by the method of Laemmli (*28*) using a 3.5% (w/v) acrylamide stacking gel and a 5–15% (w/v) acrylamide separating gel. The proteins eluted from Davis' gel (3 μg) were freeze-dryed, dissolved in sample buffer containing (or not containing) β-mercaptoethanol, and heated for 5 min at 100°C. Samples were submitted to electrophoresis at 20 mA and the gel was silver-stained by the method of Morrissey (*26*). For apparent mol wt (MW) determination, the gel was calibrated with albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate-dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20,1 kDa), and α-lacto-albumin (14,2 kDa).

Table 1
Lectin Activity
During the Biological Cycle of *Panstrongylus megistus*[a]

Stages		Activity, titer^{-1}
Embryo	medial[b]	—
	postmedial[b]	—
	apical[b]	—
Larva	3rd	512
	4th	512
	5th	1,024
Adult		512

[a]Using type O formaldehyde-treated human erythrocytes.
[b]Egg ocular spots.

Effects of Temperature on Stability

The lectin fraction (aliquots of 300 mL) was submitted to 27, 37, 47, 57, 67, 77, and 87°C for 10, 30, and 60 min, and then tested to lectin activity with formaldehyde-treated human erythrocytes, type O. The effect of freezing at −20°C and thawing was also evaluated.

RESULTS

Lectin Activity of *Panstrongylus megistus*

No lectin activity was detected in the different embryonic stages of *P. megistus* when formaldehyde-treated (type O) or fresh (ABO) human and vertebrate (mouse, sheep, and chicken) erythrocytes were used; titers of 512^{-1}, 512^{-1}, 1024^{-1}, and 512^{-1} were obtained in the hemolymph of 3rd, 4th, and 5th instar larvae and adult insects, respectively (Table 1). This lectin activity was inhibited by L-rhamnose, L-lactose, raffinose, D-galactose and D-fucose in the hemolymph of the different instar larvae and adult insects (Table 2). *N*-acetyl-D-mannosamine, *N*-acetyl-D-glucosamine, β-methyl-D-arabinose, methyl-α-mannoside, L-fucose, D-xylose, L-sorbose, maltose, and glucose, were uneffective.

Biospecific Adsorption of the Hemolymph Lectin

When hemolymph of the fifth instar larvae was incubated to type O human formaldehyde-treated erythrocytes, the lectin activity was completely removed from the obtained supernatant. After elution, the desorbed lectin fraction showed titers of activity to formaldehyde-treated human erythrocytes of types O (16^{-1}), A (4^{-1}), B (8^{-1}), and AB (16^{-1}). The

Table 2
Inhibition by Carbohydrates
of Agglutination of Formaldehyde-Treated Human Erythrocytes
Type O, in Hemolymph of Larvae and Adults of *Panstrongylus megistus*

	Minimal inhibitory concentration, m*M*			
	Instars			
Inhibitors	3rd	4th	5th	Adult
L-rhamnose	0.78	0.78	0.78	0.78
D-lactose	6.25	6.25	6.25	12.5
Raffinose	12.5	12.5	6.25	12.5
D-galactose	12.5	12.5	12.5	12.5
D-frucose	25.0	12.5	25.0	25.0

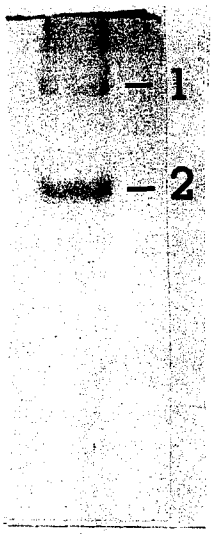


Fig. 2. Electrophoretic pattern of the lectin fraction under nondenaturing conditions. 1. isoform 1; 2. isoform 2.

recovered protein (50 μg) showed a specific lectin activity of 3000 (titer^{-1}/mg protein·mL^{-1}) and corresponded to 25% of the adsorbed activity.

Properties of the Hemolymph Lectin

No basic protein bands were detected in the lectin fraction; however, the electrophoretic pattern from Davis' gel showed two main bands, termed 1 and 2 (Fig. 2). After gel elution, these bands showed lectin activity to fresh human erythrocytes, type O, and were considered as distinct molecular forms, or isoforms from the *P. megistus* lectin. Band 1 was termed isoform 1 (I1) and band 2, isoform 2 (I2).

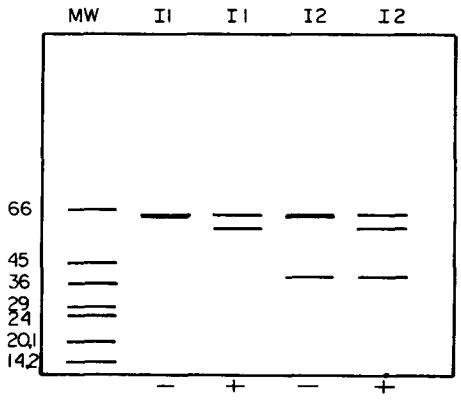


Fig. 3. Schematic representation of SDS-polyacrylamide gel electrophoresis from isoforms 1 and 2. (+) with β-mercaptoethanol; (−) without β-mercaptoethanol.

The isoforms showed a distinct electrophoretic pattern on polyacrylamide SDS-gel (Fig. 3). Under denaturing conditions, I1 showed one band (named alpha), with MW of 64 kDa. In the presence of the reductant agent this band was maintained and one additional 50 kDa band was visualized. The I2, under denaturing conditions, was resolved in two bands, named beta 1 and beta 2, of 64 and 33 kDa, respectively. Denaturing and reducing conditions showed a similar behavior to beta 1 and alpha 1 bands, with polypeptides of MW 64 and 50 kDa, respectively. The beta 2 band did not alter with β-mercaptoethanol.

Treatments of 10 or 30 min of heating did not affect the lectin fraction activity. The lectin activity presented a titer of 16^{-1} and remained stable even when treated at 27°C for 60 min. Titers of 8^{-1} were obtained after heating from 37 up to 77°C. The lectin activity was completely lost at 87°C (Fig. 4). Repeated freezing and thawing did not affect the lectin activity.

Similarly to the activity in the hemolymph, the lectin fraction activity was inhibited by L-rhamnose, D-lactose, raffinose, D-galactose, and D-fucose (Table 3). Rhamnose was the most potent inhibitor (0.19 m*M*). The other carbohydrates tested did not have any effect on the activity of the purified lectin.

DISCUSSION

Previously, we have shown that the hemolymph of the fifth instar larvae of *P. megistus* demonstrated a natural lectin activity for human (ABO) and other vertebrate erythrocytes (*17*). In this paper, we found that this activity was present in larvae and adult insects, but it was absent in the embryonic stages studied. The hemolymph lectin of *P. megistus*

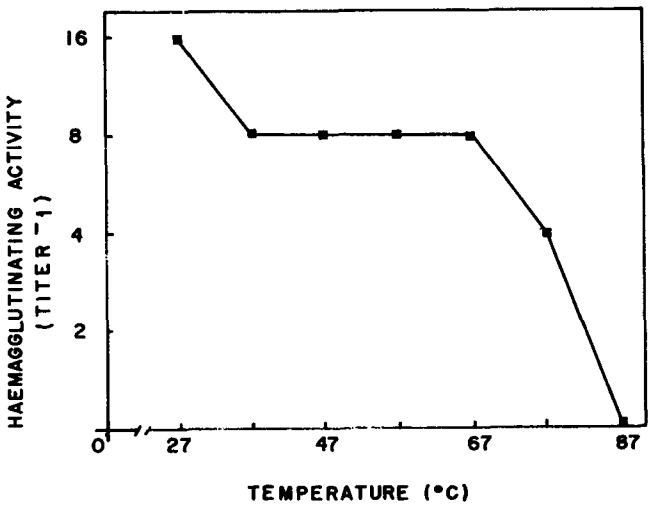


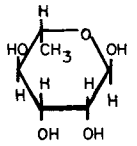
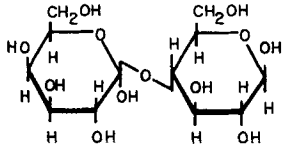
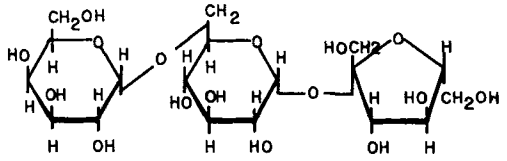
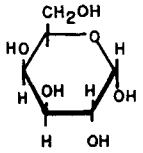
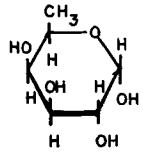
Fig. 4. Effect of heating on hemagglutinating activity of the lectin fraction. Samples were heated for 60 min.

behaved differently from that detected in the holometabolous *Sarcophaga peregrina* whose normal larvae did not present lectin activity, but on injury of the body wall, it was promptly induced in the hemolymph (*15*). Takahashi et al. (*29*) showed that no gene for this lectin was expressed in the 3rd instar larvae. However, the expression of this gene was observed in pupal stage during normal development.

The lectin activity during the evolutive cycle of insects has been poorly investigated. According to Takahashi et al. (*16*), the lectin of *S. peregrina* seems to be necessary at a specific embryonic stage. This lectin was also detected in 8-d-old embryos (*16*); a significant amount of lectin was present in 8–10-d-old embryos, but after this embryonic stage the lectin decreased rapidly. Bernheirmer (*30*) showed that embryo homogenates and the hemolymph of adult insects of *Hyalophora cecropia* did not agglutinate human erythrocytes. However, such activity was found in embryo homogenates of the same species when rabbit erythrocytes were used (*31*). This fact was not observed with homogenates from embryonic stages of *P. megistus* when formaldehyde-treated or fresh erythrocytes from human (ABO) or other vertebrates (mouse, sheep, and chicken) were used in the agglutination assays. The failure to detect lectin activity in the embryonic stages of *P. megistus* could be related to a low concentration of this component, or it may reflect the presence of a controlled gene.

Since the hemolymph of the 5th instar larvae of *P. megistus* gave the highest lectin activities, it was used to purify the lectin. The lectin fraction obtained showed activity to fresh or formaldehyde-treated human erythrocytes (ABO) and the carbohydrate inhibition was similar to the results obtained in the hemolymph of the studied instar larvae and adult insects.

Table 3
Inhibition by Carbohydrates of Agglutination of Formaldehyde-Treated Human Erythrocytes Type 0, with *Panstrongylus megistus* Lectin Fraction

Inhibitor	Haworth projection	Minimal inhibitory concentration, m*M*
L-Rhamnose		0,19
D-Lactose		0,39
Raffinose		1,5
D-Galactose		3,1
D-Fucose		6,25

The lectin fraction was inhibited by mono- and olygosaccharides but among the monosaccharides, L-rhamnose was the most potent inhibitor. According to Mäkelä (32), the hydroxyl group at C-4 is critically involved in binding lectins, since lectins that recognize mannose and glucose did not interact with galactose and vice-versa. The protein–carbohydrate interaction of *P. megistus* lectin may be related to the hydroxyl group at C-4, which is similar to inhibitor carbohydrates. The noninhibitor carbohydrates differ mainly in these positions, except for L-sorbose and methyl-β-D-arabinose that have furanose rings. The predominant interactions in protein–carbohydrate complexes are hydrogen bonds (33). The L-rhamnose specificity

of *P. megistus* lectin may be the result of the presence of neighboring hydroxyl groups, which could promote the best interaction in the lectin site. The potential presence of isoforms or isolectins could also explain a distinct inhibition pattern.

Contrary to observation made on the *S. peregrina* lectin that demonstrated two subunits not linked by disulfide bonds (*15*), the isoforms of *P. megistus* lectin showed a common subunit that was reduced to two polypeptide chains.

Most insect lectins are heat-labile in the range of 50–70°C (*31*). Then *Periplaneta americana* lectin was denatured at 56°C for 30 min (*13*). Surprisingly, an appreciable activity still remained after heating *S. peregrina* lectin at 80°C for 5 min. The *P. megistus* lectin lost its activity when heated at 87°C for 1 h. Many proteins that are stable at high temperatures contain metallic ions and/or disulfide bonds (*34*).

Similar to the lectins from *Rhodnius prolixus* (*10*), *Schistocerca gregaria* and *P. americana* (*36*), in preliminary studies, the lectin fraction agglutinated *Trypanosoma cruzi* (Y strain) and *Leishmania donovani chagasi* (IMP strain). In the latter parasite, the phenomenon was more prominent. D-galactose effectively inhibited both trypanosomatids. Thus, the isoforms or isolectins of *P. megistus* lectin could be a tool in the characterization of subtle features of cell surfaces.

ACKNOWLEDGMENTS

To Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq) and Coordenação de Aperfeicoamento de Pessoal de Ensino Superior (CAPES), Brazil.

REFERENCES

1. Goldstein, I., Hughes, R. C., Monsigny, M., Osawa, T., and Sharon, N. (1980), *Nature* **285,** 66.
2. Barondes, S. H. (1988), *TIBS* **13,** 480–482.
3. Arimoto, R. and Tripp, M. R. (1977), *Invert. Pathol.* **30,** 406–413.
4. Knapp, W. P. W., Boerrigter-Barendsen, L. H., Hoeven, D. S. P., and Sminia, T. (1981), *Cell Tiss. Res.* **219,** 291–296.
5. Mirelma, D., Galun, E., Sharon, N., and Lotan, R. (1975), *Nature* **256,** 414–416.
6. Janzen, D. H., Juster, H. B., and Liener, R. I. E. (1976), *Science* **192,** 795–796.
7. Hamblin, J. and Kent, S. P. (1973), *Nature New Biol.* **245,** 28–30.
8. Bohlool, B. B. and Schmidt, E. L. (1974), *Science* **185,** 279–271.
9. Ashwell, G. and Harford, J. (1982), *Ann. Rev. Biochem.* **51,** 532–554.
10. Pereira, M. E. A., Andrade, A. F. B., and Ribeiro, J. M. C. (1981), *Science* **211,** 597–600.

11. Ibrahim, E. A. R., Ingram, G. A., and Molyneux, D. H. (1984), *Trop. Parasitol.* **35,** 795–796.
12. Wallbanks, K. R., Ingram, G. A., and Molyneux, D. H. (1986), *Trop. Parasitol.* **37,** 409–413.
13. Scott, M. T. (1971), *Transplantation* **11,** 73–80.
14. Jurenka, R., Manfredi, K., and Hapner, D. (1982), *J. Insect Physiol.* **28,** 177–181.
15. Komano, H., Mizumo, D., and Natori, S. (1980), *J. Biol. Chem.* **255,** 2919–2924.
16. Takahashi, H., Komano, H., and Shunji, N. (1986), *J. Insect Physiol.* **32,** 771–779.
17. Gomes, Y. M., Furtado, A. F., and Carvalho, A. B. (1988), *Mem. Inst. Oswaldo Cruz* **83,** 509–512.
18. Furtado, A. F. (1978), *Rev. Bras. Biol.* **1,** 67–76.
19. Sales, M., Carvalho, C. R., and Novaes, P. M. (1984), *Rev. Bras. Genetica* **4,** 619–628.
20. Butler, W. T. J. (1963), *J. Immunol.* **90,** 663–671.
21. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem.* **193,** 263–275.
22. Reitherman, R. W., Rosen, S. D., and Barondes, S. H. (1974), *Nature* **248,** 599, 600.
23. Read, S. M. and Northcote, D. H. (1981), *Anal. Biochem.* **116,** 53–64.
24. Davis, B. J. (1964), *Ann. NY Acad. Sci.* **121,** 404–427.
25. Reisfeld, R. A., Lewis, U. J., and Williams, D. E. (1962), *Nature* **195,** 281–283.
26. Morissey, J. H. (1981), *Anal. Biochem.* **117,** 307–310.
27. Andrews, A. T. (1981), *Electrophoresis: Theory, Techniques, and Biochemical and Clinical applications,* Clarendon, Oxford, 128–148.
28. Laemmli, U. K. (1970), *Nature* **227,** 680–685.
29. Takahashi, H., Komano, H., Kawaguchi, N., Kitamura, N., Nakanishi, S., and Natori, S. (1985), *J. Biol. Chem.* **260,** 12228–12223.
30. Bernheirmer, A. W. (1952), *Science* **115,** 150, 151.
31. Yeaton, R. L. W. (1980), PhD thesis, University of Pennsylvania, Philadelphia, PA, 229 pp.
32. Makela, O. (1957), *Ann. Med. Exp. Biol. Fenn.* **35,** Suppl. II.
33. Quiocho, F. A. (1986), *Ann. Rev. Biochem.* **55,** 287–315.
34. Ingram, G. A., East, J., and Molyneux, D. H. (1984), *Parasitology* **89,** 435–451.