COMPLEMENT ACTIVE MATERIALS FROM LARVAL TAENIIDS

Dissertation for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY
BRUCE HAMMERBERG

1977

This is to certify that the

thesis entitled

COMPLEMENT ACTIVE MATERIALS
FROM LARVAL TAENIIDS

presented by

Bruce Hammerberg

has been accepted towards fulfillment
of the requirements for

PhD degree in Microbiology & Public Health

J. F. Williams
Major professor

Date 8/8/77

O-7639

ABSTRACT

COMPLEMENT ACTIVE MATERIALS
FROM LARVAL TAENIIDS

By

Bruce Hammerberg

Non-specific complement-fixing activity was found in bladder fluid and cyst fluid of larval stage taeniid cestodes. Larval *T. taeniaeformis* was shown to produce complement active material during *in vitro* maintenance and to shed this substance when incubated in a Tris-HCl buffer containing EDTA. The biological activity of these materials was characterized and physico-chemical analyses of larval extracts was conducted.

The results of the characterization of larval material interactions with complement demonstrated the depletion of hemolytic complement in normal sera of several species, the generation of anaphylatoxin-like activity *in vitro*, the conversion of C3, and the production of vascular permeability changes *in vivo*. Complement fixation appeared to be initiated non-immunologically via both the alternative and classical pathways. Parasite factors given intravenously to rats led to profound depression of circulating complement levels and repeated dosing over a 3 week period did not cause the rats to become refractory to this effect. Circulating levels of hemolytic complement were not altered in infected rats.

Complement-dependent inflammatory responses in the skin of rats were inhibited in animals given intravenous doses of parasite factors.

Physico-chemical analysis of materials extracted from *T. taeniaeformis* larvae was accomplished by using enzymatic digestion, gel-filtration, anion exchange chromatography, ultracentrifugation and gel electrophoresis. The results of these analyses provided evidence of an association between antihemolytic activity and macromolecules containing carbohydrate, protein, sulphate and hexosamine, heterogenous in net charge but resistant to proteolysis and beta elimination reactions, and free of sialic and uronic acids. The anion exchange chromatography fraction with the greatest activity was eluted from diethylaminoethyl cellulose with 0.3 M sodium chloride in 0.01 M Tris-HCl pH 8.0 and when parasites were incubated with a source of $^{35}SO_4^{--}$, radioactivity was incorporated into molecules eluting under identical conditions. Active chromatographic fractions precipitated with protamine sulphate suggesting that they were polyanionic in nature. There was some evidence of dissociation of the active substance on polyacrylamide gels where fast-moving sulphate containing bands were observed which no longer reacted with protamine or complement.

The results are consistent with the possibility that the active substance is a polysulfated proteoglycan. These types of molecules have been detected on the surface of a variety of infectious organisms, and are known to interact with complement non-immunologically. The location of these molecules at host-parasite interfaces may be significant in parasite evasion of immune rejection.

COMPLEMENT ACTIVE MATERIALS

FROM LARVAL TAENIIDS

By

Bruce Hammerberg

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

1977

Dedicated to my wife JoAnn

ACKNOWLEDGEMENTS

I am greatly indebted to Dr. Jeffrey F. Williams for sound and stimulating academic guidance, thorough and accurate evaluations of data resulting from my thesis research, and invaluable assistance in composing manuscripts for publication. His efforts in obtaining financial support for me from the NIH have made it possible for me to complete my work in the shortest period of time possible. Most important of all and sincerely felt is the warm and relaxed personal communication which he encourages.

The advice and encouragement of my guidance committee, Drs. Harold Miller, Richard Patrick, Wayne Smith, and Donald Twohy are gratefully acknowledged.

The camaraderie of those working with me in this laboratory during the last three years has made this experience very enjoyable. For this companionship, I thank all the members of Dr. Williams' laboratory.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LITERATURE REVIEW

Justification for research in experimental taeniiasis is two-fold. Firstly, the experimental cysticercoses provide a convenient model for the cyclozoonotic cestode infections which are of major public health and economic importance in many parts of the world. There is a need for an improved understanding of the immune response in these infections, both from the viewpoint of its potential exploitation in a preventive way and in terms of the development of immunodiagnostic procedures. Secondly, the larval cestodes are able to survive in tissues for prolonged periods and any clarification of the mechanisms which contribute to this phenomenon are relevant to infectious disease processes as a whole.

In recent years there have been ample reviews of the global impact of cysticercosis and hydatidosis (Schenone, 1974; Abdussalam, 1974). These reviews of the current situation reflect the increasing importance of these infections, and the less than impressive progress made in control programs, and emphasize the need for immunological means of inducing resistance. There is ample evidence from field studies of the occurrence of an acquired immunity to taeniiasis (Gemmell and McNamara, 1972), and the evidence derived from studies of immune mechanisms in the laboratory animal models have been extensively discussed by Leid (1973).

Musoke (1975) and Hustead (1976). These reviews point up the paradox of coexistence of a high degree of acquired immunity in animals which harbor persistent infections throughout their lives.

The issue of prolonged survival of parasites in immune hosts and the question of how tissue-dwelling organisms interact with specific and non-specific elements of the immune response have not been reviewed from the perspective of the common problems faced by invasive protozoa, helminths and bacteria. This dissertation deals with substances which have demonstrable interactions with the complement and coagulation cascades and therefore may have a significant role in determining the outcome of host-parasite confrontation. For that reason an attempt is made in the literature review to describe current hypotheses on prolonged survival of infectious organisms. The demonstratable effects of polyanions are discussed and their potential role in the relationship between cells and resistance to immune attack mechanisms is outlined. A comparable role is proposed for molecules on the surface of parasites.

I. IMMUNE AVOIDANCE MECHANISMS

Infectious agents ranging from viruses to metazoan organisms multiply and/or undergo obligatory morphologic development in immunologically competent hosts. In some instances acute disease syndromes occur in which the host immune system is effective in eliminating the organisms but not before significant reproductive or morphological changes have occurred which facilitate the survival of the parasite in a new environment, or permit its transmission to a non-immune host or vector. Propagation of many eukaryotic and protozoan parasites is therefore linked to the delay in the primary immune response and to the ability of the parasite to evolve or divide rapidly. On the other hand, most multicellular infectious organisms, and some protozoan and bacterial organisms, do not develop or divide with sufficient quickness to leave the host before the onset of the primary immune response. In these host-parasite relationships avoidance mechanisms have evolved which result in the evasion of specific immune recognition and/or rejection systems. This characteristic is a hallmark of helminthiases of medical and veterinary importance and the variety of evasive mechanisms which have been described will be reviewed here. There is a wealth of information on evasive mechanisms in the broader context of chronic infectious disease which is very relevant to the study of helminthiasis, and that also will be reviewed.

Currently, much more is known about bacterial products which contribute to avoidance of or interference with the host immune system, than about helminthic or protozoan factors. This is

undoubtedly attributable, in part, to the advantage which bacteriologists have in being able to select and culture specific organisms and strains. In a recent review of bacterial factors which affect host rejection mechanisms Glynn (1972) described substances in the category of bacterial "impedins" which; inhibit local inflammatory responses (Hill, 1968), non-specifically aggregate immunoglobulins and depress phagocytosis (Dossett et al, 1969),provide surface barriers to complement-mediated lysis (Glynn and Howard, 1970), and non-specifically suppress host cell-mediated immunity (Bullock, 1968). There is an abundance of information on bacterial lipopolysaccharides and their function in immune evasion (Glynn, 1972). Comparable data are presently unavailable for non-bacterial parasite avoidance mechanisms, but the current state of knowledge was reviewed in a Ciba Foundation Symposium (1974) and some recent developments have been highlighted by Ogilvie and Wilson (1976).

a. Antigenic variation

Antigenic variation which occurs in protozoan infections of mammals has been investigated by numerous researchers and this work was reviewed recently for *Plasmodium* (Brown, 1974), *Trypanosoma* (Vickerman, 1974), and *Babesia* (Greenwood, 1974). Those antigenic changes occurring on the parasite surface are believed to be elicited by specific antibody binding (Seed, 1977). They may be induced phenotypically when antibody-binding leads to capping and elimination of the former antigens without parasite lysis. Alternatively, antibody binding and subsequent lysis may result in the emergence of genetically distinct parasites which go undetected until the new antigen is recognized by the host (Vickerman, 1974). In the

African trypanosomes the variant antigens are located in the surface coat, which is distinct from the cell membrane. When shed *in vivo* from live trypanosomes, these antigens are found in the circulating blood of infected animals and are also known to be released from the parasite at a pH lower than 7.3 (Allsopp et al, 1971). Removal of variant antigens from *T. brucei* by tryptic digestion reveals a carbohydrate layer, demonstrable by periodic acid-silver staining, external to the plasma membrane (Wright and Hales, 1970).

b. Masking effects

Another mechanism of immune avoidance which involves alteration of surface antigens has been postulated, based on evidence that schistosomes are able to selectively acquire or produce host antigens (Smithers et al, 1969). Clegg (1974) showed with *Schistosoma mansoni* that invading schistosomula lacked host red blood cell antigens three hours after penetration and were susceptible to immune attack; whereas, four days after penetration schistosomula had acquired red blood cell antigens and were able to avoid antibodies from immune serum. Whether or not the acquisition of host antigens is responsible for the prolonged survival of mature schistosomes or is merely coincidental has not yet been established (Dean, 1977). Because the schistosome surface is the site of a dynamic antigen flux and also is the site of immune attack it clearly plays an important role in avoidance of immune rejection (Ogilvie and Wilson, 1976).

c. Immunosuppression

Many parasites which undergo antigenic variation or show host antigen uptake are known to shed soluble antigens into the host circulation. Other protozoa and helminths such as *Plasmodium*

(Wilson et al, 1969), *Babesia* (Zuckerman and Ristic, 1968), *Trypanosoma* (Williamson and Brown, 1964), *Haemonchus* (Stumberg, 1933), and *Trichinella* (Bozicevich and Detre, 1940) are known to release circulating antigens. The observation that host immune systems may be depressed has led to speculation that these antigens may function as blocking agents via antigen-specific immunosuppression of lymphocytes or by non-specific saturation of the reticulo-endothelial system (Wilson, 1974).

More detailed mechanisms of soluble antigen action on immune systems have been proposed but evidence that these function *in vivo* to the advantage of the parasite has yet to be developed. For example there may be antigenic competition at the macrophage-level inhibiting phagocytosis or blocking cooperation between T and B lymphocytes; there could be stimulation of suppressor T cells; binding and subsequent neutralization of high affinity antibodies may occur; and soluble circulating antigen-antibody complex formation has been proposed to block killer cell action against the parasite (Ciba Foundation Symposium, 1974).

Whatever the mechanism, instances of immunosuppression in parasitic infection are now widely recognized. Circumstantial evidence from the comparison of immune competence of normal humans with parasitized subjects indicates a nonspecific depression of antibody response to *Clostridium tetani* and *Salmonella typhi* vaccines during *Plasmodium falciparum* and *Trypanosoma gambiense* infections. Also, *T. gambiense* infection inhibits the response of patients to skin tests for PPD or *Candida* antigen, and reduces susceptibility to skin sensitization with dinitrochlorobenzene (Greenwood, 1974; Wedderburn, 1974). On the whole these results indicate suppression

of humoral immunity during malarial infections, whereas trypanosomiasis may lead to suppression of both humoral and cell-mediated immunity.

In malarial infections in mice (*P. berghei yoelii*), acute stage immunosuppression of the IgG response may be due to an effect of macrophage functioning, such as antigen presentation to T or B cells, but is not apparently attributable to failure of antigen uptake by macrophages (Greenwood, 1974). In acute infections with *Trypanosoma brucei* in mice (Terry et al, 1973; Freeman et al, 1974) the IgG response to sheep red blood cells is drastically reduced and this effect is completely reversible upon elimination of the parasite. In chronic infections both IgM and IgG responses are greatly depressed and there is no recovery of normal responsiveness after clearance of the parasites.

Generalized immunosuppression has been demonstrated by enhanced tumor growth in rats infected with *Nippostronglyus brasiliensis* (Keller and Jones, 1971), and in hamsters infected with *Schistosoma mansoni* or the filarial worm *Dipetalonema viteae* (Capron et al, 1972). Early stage *Trichinella spiralis* infections in mice produced immunosuppression that has been characterized by Faubert and Tanner (1974) and Cypess et al (1973), and which is passively transferable with infected mouse serum. Depression of primary and particularly secondary antibody responses to sheep red blood cells occurs both acutely and chronically in infection with the larval stage of the tapeworm, *Taenia crassiceps* (Good and Miller, 1976).

II. ROLE OF COMPLEMENT IN SPECIFIC AND NON-SPECIFIC IMMUNITY

The mechanisms reviewed thus far for immune avoidance by helminths or protozoa attempt to account for evasion of specific effector mechanisms mediated via antibody production or cell-mediated immunity. Little attention has been given to non-specific resistance mechanisms although it is clear that these are an important component of the host-parasite relationship. Non-specific lytic effects initiated by the alternative pathway of the complement cascade have been proposed as a key factor in host-resistance to certain infectious agents (Pillemer et al, 1956).

Research on this type of immunity has not kept pace with advances in specific reactivity and the phenomena have often been classified as comprising "innate" resistance. However, it has become clear that in some cases the success or failure of an infectious agent may reflect a balance between parasite resistance and host rejection mechanisms which revolve around complement activation at the host-parasite interface. This activation of complement can occur by either antigen specific or non-specific initiators. For most parasites the limiting surface which contacts the host is a membrane which can be shown to be susceptible to lytic effects, and therefore interactions between infectious organisms and this host amplification system will be reviewed in some detail.

a. Complement fixation

In recent reviews by Fearon and Austen (1976) and Fearon et al (1974), a detailed description of the alternate pathway for complement activation and the enzymes involved is provided. These authors also reiterate the evidence for the existence of the

alternate pathway as distinct from the classical pathway. Two of the most critical developments were: a) the purification of a protein, termed properdin, from normal serum by Pensky et al (1968) which was not immunoglobulin, yet was capable, when reacted with certain polysaccharides or particular bacterial, viral, protozoal, or fungal organisms, of activating complement and neutralizing or lysing the organism (Pillemer et al, 1954); b) the discovery of the ability of cobra venom factor to react with non-classical complement proteins to generate complement-consuming activity (Gotze and Muller-Eberhard, 1971).

Although the activation of properdin by a number of complex polysaccharides (e.g. lipopolysaccharide from Gram-negative bacteria, zymosan, dextrans) initiates a pathway for C3 convertase formation and a C3b-dependent amplification loop for more C3 cleavage, this pathway is relatively inefficient in comparison with the classical pathway in C3b generation (Brade et al, 1973). This is true only for the initial C3b generation since activated properdin seems to greatly facilitate cleavage of C3 by the amplification loop once it is initiated (Fearon and Austen, 1977). The zymosan-induced properdin-dependent cleavage of C3 is impaired by diluting the serum (Fearon and Austen, 1976).

The biologically important effects on foreign organisms which are properdin-dependent probably require the generation of a lytic complex composed of the terminal complement sequence C5 through C9 at a surface membrane. This sequence is started by cleavage of C5 to form C5a and C5b. C5b is labile and must rapidly join with C6 either on a membrane or in solution in order to form a $\overline{C567}$ complex which is cytolytic. Enhanced cytolytic activity

of membrance bound $\overline{C567}$ is acquired with binding of C8 and C9 to form $\overline{C56789}$. The alternative pathway enzyme required for cleavage of C5 is formed by C3b binding factor B in the presence of factor $\overline{D}$ to form $\overline{C3B}$ which then cleaves C3 into C3a and binds C3b. This complex, $\overline{C3Bb}$, is able to cleave C5 to C5a and C5b. The limiting factor in alternative pathway generation of C3 convertase activity, $\overline{C3Bb}$, and the subsequent formation of lytic complexes is the initial, properdin-dependent formation of C3b from C3 (Fearon and Austen, 1976). However, after C3b is generated by the inducing action of properdin on factors $\overline{D}$ and B, or after it is generated by classical pathway activation, then properdin markedly enhances the C3b-dependent amplification loop which produces $\overline{C3Bb}$ (Fearon and Austen, 1977). Thus serum which is diluted is impaired in cytolytic activity when C3b generation is restricted to properdin induction; however, cytolytic activity is unimpaired if C3b is provided. Indeed, active properdin (generated by incubation with zymosan) greatly enhances cleavage of C3 when C3b is provided.

Lipopolysaccharide (LPS) and zymosan, which are typically associated with alternative pathway activation, have been shown to utilize Ca^{++}-dependent mechanisms (possibly the classical pathway) which enhance C3 conversion. C3 conversion independent of Ca^{++} was approximately 60-70% of C3 conversion in the presence of both Mg^{++} and Ca^{++} (Snyderman and Pike, 1975). If the generation of $\overline{C3Bb}$ activity is critical to the cytolytic effects on bacteria, viruses, and protozoa which are attributed to the alternative pathway by Pillemer et al (1954), then the limited activation to the classical pathway by materials which may be present on these infectious agents, would greatly enhance $\overline{C3Bb}$ formation by supplying

limited amounts of C3b in the presence of active properdin for a fully active amplification loop.

The non-specific generation of cytolytic activity is typically attributed to the alternative pathway when this activity can be demonstrated without antibody-antigen interaction or in the absence of Ca^{++}, or when early classical complement components are not required or consumed. The specific generation of cytolytic activity as reviewed by Fearon and Austen (1976) requires initiation of the classical pathway by complement-fixing antibody-antigen complexes. The first complement component, C1, binds these aggregates of certain classes of antibody (only IgG1, IgG2, IgG3 and IgM in humans). This binding alters the C1 component so that one of its subcomponents, C1s, is then able to cleave complement proteins C4 and C2 with the subsequent formation of $\overline{C142}$ which is a C3 convertase analogous to $\overline{C3Bb}$. The lytic complex formation mechanism which follows C3 conversion is identical for both the classical and alternative pathways, i.e., C5 cleavage and $\overline{C56789}$ formation.

Though the classical pathway for complement-dependent lysis is usually considered only as a specifically directed lytic activity, this pathway can be activated non-specifically (without antibody-antigen complexes) by other molecular interactions such as binding in the presence or absence of C-reactive protein. Rent et al (1975) demonstrated that when protamine (polycation) and heparin (polysulfated polyanion) in the proper proportion were incubated together in the presence of serum, complement components C1, C4, C2, and C3-9 were consumed. This consumption of complement components was also shown to occur when interactions between polyanions, such as DNA, chondroitin sulfate, and dextran sulfate, and either protamine

sulfate or poly-L-lysine where carried out in the presence of C-reactive protein (CRP) (Claus et al, 1977).

CRP is a serum protein found in increased levels during the acute phase of inflammatory conditions. It was originally detected by its ability to precipitate pneumonococcal C-polysaccharide (Abernethy and Avery, 1941); this activity is now known to be due to a binding capacity for polycations (Siegel et al, 1975). CRP binding with pneumococcal C-polysaccharide or with the choline phosphatides, lecithin and sphingomyelin, initiates the classical pathway (Kaplan and Volanakis, 1974) and CRP in small amounts greatly enhances classical complement consumption by interactions between polyelectrolytes. These reactions are all physiologically feasible in that the amounts of CRP required are similar to those detectable during inflammatory conditions and polyelectrolytes such as heparin, DNA, chrondroitin sulfate and lysozyme commonly occur where cells are being destroyed (Claus et al, 1977).

When polycations are not present during incubation of certain polyanions (heparin, DNA, dextran sulfate, and cellulose sulfate) with serum, the effect is C1 depletion and consumption of C3-9 while C4 and C2 are not consumed (Rent et al, 1975). The consumption of C3-9 by certain polysaccharides and certain polyanionic, heparin-like mucopolysaccharides, including heparin, is believed to be the result of induction of the alternative pathway (Loos et al, 1974).

In summary then, some of the non-specific activators of complement are: 1) many polysaccharides, both neutral and negatively charged, which are able to activate the alternative pathway, 2) certain polyanions, including some polysulfated polysaccharides which are able to suppress C1 activity by enhancing a naturally

occurring inhibitor of C1 (Rent et al, 1976), 3) interacting polyelectrolytes which in certain proportions can activate the entire classical pathway. This activity is greatly enhanced by CRP.

b. The involvement of complement in resistance to parasites

There is ample evidence that lytic effects on protozoan and helminthic parasites do occur but until recently they have not been examined critically in the light of experiences with bacterial organisms. Both non-specific lysis and specific lysis have been described and persistence of organisms in the host probably depends on avoidance of surface complement fixation in some manner. Clearly it is not possible to review this aspect of the whole spectrum of host-parasite relationships, but several important diseases have been investigated in sufficient detail to provide some basis for discussion. The trypanosomes, schistosomes and taeniid cestodes have been selected for this purpose.

In the genus Trypanosoma, circulating parasites have been shown to interact with complement in several ways. These hemoflagellates are vector borne and are responsible for several important disease syndromes in man and animals. In South American trypanosomes there is both an intracellular phase in macrophages and a circulating blood plasma phase. The role of humoral immunity in *T. cruzi* infection (Chagas' disease) has only recently begun to be investigated (Gable, 1970 and Budzko et al, 1975).

Early evidence from the clinical observation that blood stream forms of *T. cruzi* could be isolated from humans and experimental animals with circulating antibodies to the parasite (Romana, 1963; Cerisola et al, 1969; Pizzi, 1957) suggested that it was resistant

to immune lysis. Later in *in vitro* experiments parasites were observed directly and there was no evidence of immune lysis of blood stream forms (Teixeira, 1974). However, other studies on experimental infections in mice showed that if complement was depleted in infected mice parasitemia levels rose more rapidly, survival times were shortened and greater numbers of parasites developed. This was particularly true if complement depletion was performed at the stage of infection when blood stream forms were emerging from macrophages (Budzko et al, 1975). These workers also established that blood stream forms could be lysed *in vitro* in hyperimmune mouse sera as long as complement was present (Budzko et al, 1975). Whether or not the parasite is susceptible to lysis *in vivo* is still debatable. It may be that this parasite in one or all of its forms is able to co-exist with complement and antibody in certain mammalian hosts. This may be supported by the observation of Anziano et al (1972) that culture forms of *T. cruzi* (mainly epimastigotes) are lysed by antibody and complement. The fact that *T. cruzi* is a more acute disease in children than adults and that certain species of animals may be naturally resistant due to non-specific complement-dependent lysis mediated by the alternative pathway (Kierzenbaum et al, 1976) suggests that the complement level is involved in avoidance of immune lysis by the parasite.

In African trypanosomiasis parasite numbers in the blood characteristically fluctuate and this pattern has been correlated with the appearance of new surface antigenic determinants and the development one week later of specific (IgM) antibodies (Seed et al, 1969). Unfortunately there are no reports in which the role of complement has been studied *in vivo* in the humoral immunity

demonstrated in African trypanosomiasis. Immunoglobulins IgM and IgG have been shown to be responsible for passive immunity (Seed and Gam, 1966; Seed, 1972, 1977) and the appearance of new antigenic variants in relapse parasitemias (Luckings, 1974; Takayanagi and Enviguez, 1973). The African trypanosomes are entirely extracellular and regularly occur in the blood stream in high numbers causing a long term chronic disease, and it seems likely that they have an ability to evade immune lysis even in the presence of antibodies to specific or non-variant antigens (Losos and Ikede, 1972).

There is evidence for a lytic effect of serum in schistosomiasis. *Schistosoma mansoni* in the adult form is a small trematode which has been found to exist for more than twenty years in the lumen of the hepatic portal veins of man. It is estimated that more than 200 million people are hosts of this chronic infection. A variety of other *Schistosoma* species infect the blood vessels of man and other mammals. Cercaria released from the snail, the intermediate host, enter the primary host by skin penetration. Cercaria, immediately upon penetration of the primary host, undergo drastic morphological changes, including shedding and replacement of the surface (tegument). The early stage migrating form, prior to the adult sessile stage, is called a schistosomule and it is during this stage within 96 hours of host penetration that the parasite is most likely to be destroyed by the immune response, particularly if it has entered a previously sensitized host (Clegg, 1974). Adult schistosomes of a primary infection or even adult schistosomes transplanted into a sensitized host (Hockley and Smithers, 1970) are resistant to immune rejection though they effectively immunize the host against the invading schistosomule.

Many investigators have postulated that the appearance of host antigens on schistosomules during the early invasive stage is the critical immune evasive mechanism (Clegg, 1974; Smithers, 1972). However, Dean (1977) has shown that this may not be the case and that schistosomules which develop for 96 hours *in vitro* in the absence of host antigens are still capable of immune avoidance.

Indirect evidence from *in vitro* studies indicates that complement is required for the parasiticidal effects of normal immune sera on cercariae and schistosomules of *Schistosoma mansoni*. Standen (1952) and Kagan and Levine (1955) demonstrated that normal sera from a variety of mammals had cercaricidal activity *in vitro* which was heat labile (56°C for 30'). When sera from these animals after hyperimmunization were reacted with cercariae *in vitro* the parasites were immobilized by precipitin formation, but they were not killed. The interaction of cercariae with normal serum with the resulting cercaricidal effects has been shown to be complement-dependent and generated mainly through the alternative pathway (Machado et al, 1975). This interaction of cercariae with normal serum resulted in the generation of anaphylatoxin. Schistosomula which lack the external membranes of cercariae are not killed by normal serum (Capron et al, 1974). However, early stage (24 hour) schistosomula incubated with serum from a naturally or experimentally infected host are killed in the presence of heat labile serum factors (Capron et al, 1974). Later stage (4 days) schistosomula are much more resistant to complement-mediated antibody-dependent lysis (Clegg and Smithers, 1972). Von Lichtenberg et al (1977) suggested that these schistosomula non-specifically activate complement. Adult schistosomes incubated in fresh serum from an infected homologous

host do not suffer damage; however, adult schistosomes incubated in fresh serum containing antibodies for host antigens, which are on the parasite surface, suffer tegumental damage but are not killed unless activated peritoneal macrophages are present (Perez and Terry, 1973).

Clearly complement plays a critical role in immune damage or lysis at each stage of development of *Schistosoma mansoni* in mammalian hosts. Each stage avoids immune damage with varying success depending on whether or not the host has been sensitized to the parasite and on the ability of later stages to counter complement-dependent immunity. Recent reports indicate that adult schistosomes are capable of avoiding complement-dependent immunity even without the presence of host antigens on their surfaces (Dean, 1977), though the mechanism of this is unknown.

There is a similarity between early infectious stages of *Schistosoma sp* and the metacestodes of *Taenia taeniaeformis* with respect to complement-mediated immune rejection. *T. taeniaeformis* exists as an adult in the lumen of the small intestine of cats and inhabits the liver tissue of rodents in its larval form. Gravid segments from adult tapeworms, when passed in the feces of cats, contain eggs which upon ingestion by rats become infective embryos in the rat small intestine. These embryos pass through the intestinal epithelium and are subsequently carried to the liver by the portal circulation. Within a few hours of egg ingestion, invasive embryos are found migrating in the liver parenchyma and continue to do so until by 3 days they begin to form spherical cysticerci. The fluid filled cysticercus enlarges from about 15 to 250 μ in diameter at six days post-infection. During this time

the cellular activity around the parasite is complex, but a particularly prominent eosinophil infiltration occurs by about 10 days which continues until about 35 days post-infection. During the second week of infection a layer of fibroblasts begins to surround the cysticercus which becomes a thin fibrous capsule around the late larval stage.

Campbell (1932) demonstrated a passively transferable humoral immunity for *T. taeniaeformis* in rats where serum from an infected rat was effective in killing invasive or developing larvae to a gradually decreasing extent during the first 10 days post-infection. Leid and Williams (1974) found that the passively transferable humoral immunity which developed in rats during the first 4 weeks of infection was due to antibodies of the IgG_{2a} immunoglobulin class. The IgG_{2a} class of immunoglobulins of the rat fixes complement and the identification of complement as the required mediator for the protective effect of immune rat serum was determined by Musoke and Williams (1975) both *in vitro* and *in vivo*. Developing larvae up to four days post-infection were completely susceptible to complement-dependent immunity, but by 10 days parasites were no longer affected. Between 4 and 8 days post-infection the larvae showed intermediate degrees of resistance. This acquisition of resistance to complement-mediated immune destruction during the initial days post-infection closely parallels the chronology of resistance development by schistosomules.

In addition to the similarities of immune avoidance in early invasive stages between *S. mansoni* and *T. taeniaeformis* further parallels can be drawn between adult *S. mansoni* and fully developed larval *T. taeniaeformis* by examining the interaction of these

stages with immune serum and complement. Adult schistosomes experience tegumental coat perturbation but not death when exposed to hyperimmune serum. Similarly Hustead and Williams (1977a,b) have shown that resistant late stage cysticerci incubated in immune rat serum transiently lost membrane permeability control for small proteins such as ribonuclease but remain able to exclude bovine serum albumin. This disruption in functional membrane integrity is rapidly corrected by the parasite within an hour while still in the presence of immune rat serum, during which complement levels in the serum were greatly depleted. Thus both parasites have some ability to compensate for the destructive action of antibody and complement to which they were subjected in *in vitro* experiments, although the mechanisms by which they do so are not clear.

c. Resistance to lysis in living cells

The above discussion of lysis in relation to parasites provides some grounds for believing that organisms surviving in tissues can develop to the point where they may no longer be susceptible to complement attack, and that surface membrane factors may be involved in the avoidance mechanism. In fact, the standard assay for activation of the complement cascade to completion is based on the lysis of aged erythrocytes which are dead cells bounded by a bilaminate membrane. Extrapolation of the mechanism of cytolysis to cells actively metabolizing and capable of membrane repair must be done with caution. There is evidence that in various cell types surface complement-fixation does not necessarily lead to cell lysis or cell death, and the mechanisms whereby this comes about may be important in evasion of immune attack by parasites.

Certain strains of Gram-negative bacteria differ only in their resistance to complement-mediated lysis. Two Gram-negative bacteria, *Salmonella typhimurium* and *Escherichia coli* have been extensively studied for resistance to complement-mediated lysis and for virulence as disease organisms. The non-virulent, serum-sensitive M206 strain of *Salmonella typhimurium* is a rough strain which is identical to the virulent, serum-resistant, C5 strain of *S. typhimurium* except that C5 is smooth. Similarly *S. typhimurium* LT2, *S. minnesota* 218S and *S. enteritidis* 795 are all smooth strains with complete resistance to the bactericidal action of complement, while the parallel rough strains are lysed in serum (Reynolds and Pruul, 1971a,b). *E. coli* which differ only in the amount of lipopolysaccharide (LPS) in their surface coat are either resistant or sensitive to complement lysis (Glynn, 1972). Smooth strains of Gram- negative bacteria carry greater amounts of LPS on their surfaces compared to rough strains. The amount of LPS on the bacterial surface is directly proportional to complement resistance (Glynn, 1972). This LPS is highly active in depleting hemolytic complement levels by initiating the complement cascade (Gewurz et al, 1968).

The deposition of $\overline{C56789}$ and the formation of complement "lesions" on the LPS material extracted from both smooth and rough *Salmonella minnesota* as well as on the surface of both strains of the whole bacterium (Mergenhagen et al, 1968) indicates that the 90Å doughnut-shaped electron-dense rings seen on SRBC membranes after complement-mediated lysis (Borsos et al, 1964) do not necessarily induce lysis of bacteria when formed on the LPS surface coat.

Experiments with mammalian eukaryotic cells have provided the most direct evidence to date that the formation of antibody-induced complement "lesions" at or near the cell membrane does not necessarily lead to cell lysis or death. Cikes (1970a,b; 1971) reported that virus-infected cells were susceptible to complement-mediated, anti-viral antibody lysis only during the stationary phase of growth in culture, but were resistant to lysis during the logarithmic growth phase. These workers suggested that antigenic sites were not exposed during logarithmic growth, but this was shown to be incorrect when Lerner et al (1971) demonstrated that viral antigen was present on the cell surface, accessible to antibody, and able to activate complement in the presence of antibody throughout all cellular growth phases. They confirmed that cytotoxicity was limited to the G_1 phase of cell growth, but all nine complement components were consumed and C3 and C4 were bound to lymphoma cells in the presence of antibody during all phases of cell growth. Further quantitative studies by Cooper et al (1974) demonstrated that equal amounts of C5 and C8 bound to antibody-treated cells in all stages of growth, and that electron dense circular areas, typical of terminal complement "lesions", appeared on the surfaces of cells at all stages of growth when exposed to complement and antiviral antibody.

The ability of cultured cells of mammalian origin to resist lysis in the presence of specific antibody and complement during S phase of growth has been demonstrated for murine tumor lines YCAB (Lerner, 1971), YAC (Cikes, 1972), L1210 (Gotze, 1972) and chinese hamster lung cells (Shipley, 1971). The human lymphoid cell line RPMI8866 was shown by Pellegrino et al (1974) to be

similarly variable in its susceptibility to complement and antisera directed against histocompatibility antigens, even though the quantitation of antigens, bound antibody, and bound C3, C4, and C8 per cell surface area showed no variability during the cell cycle. Pellegrino et al (1974) concluded that differential susceptibility to lysis was due to changes in cell surface properties during the cell cycle, other than those associated with antigenic expression.

III. MECHANISMS OF RESISTANCE TO LYSIS

a. Tissue location

Whether or not growth-related surface properties are involved in resistance to complement by replicating or developing successful tissue-dwelling parasites remains to be seen. There is presently insufficient information on the differential susceptibility to lysis in parasites, comparable to that which has been derived for bacterial and mammalian cell systems. In order to relate these observations to evasion of immunity by organisms in tissues it is relevant that hemolytic complement titres are not identical throughout all host tissues.

Tissue location is certainly an important determinant of survival in bacteria. Complement-sensitive strains of *E. coli* may inhabit the gut lumen, but complement-resistant strains differing only in the amount of K antigen on their surface are able to survive in the circulatory system (Roantree and Rantz, 1960; Roantree and Pappas, 1960). Evidently the effector mechanism is not able to affect lumen-dwelling organisms. Host encapsulating reactions may serve to limit the access of large molecular weight humoral components to tissue-dwelling sessile parasites (Campbell, 1938), and recent work on measles supports this notion. Tissue sites distant from the plasma may be particularly deficient for immune responses requiring the functional alternative pathway, as postulated by Joseph and Oldstone (1975) for virus-infected cells in the central nervous system. Joseph, et al (1975) have demonstrated that lysis of measles virus-infected cells requires specific human IgG antibody and the components of the alternative pathway. Thus dilution of

serum, perhaps in conjunction with other mechanisms which selectively block the alternative pathway, may obviate IgG-antibody lysis of measles virus-infected cells. *Trypanosoma vivax* and *brucei* multiply in the connective tissue and may thereby avoid high blood levels of complement during cell cycle stages which may be more susceptible to lysis.

b. Surface coat characteristics in bacteria

In many cases, however, accessibility of plasma factors is clearly not an issue and parasites flourish even though they confront complement in the blood at many stages in their life cycle. It seems more likely that some surface component or components are crucial in effecting evasion. The background of information on bacterial surface characteristics provides support for this proposal. In the case of the pneumococcus the thick layer of polysaccharide molecules which is outside the limiting cell membrane is thought to restrict the penetration of large host molecules by the highly hydrated state of the saccharide matrices (Glynn, 1972; Apffel and Peters, 1970). Common examples of hydrated saccharide matrices or gelification excluding proteins are Sephadex gel filtration beads and hyaluronic acid exclusion of protein (Ogston and Preston, 1966; Laurent, 1963). In a similar manner, the cell wall of *Mycobacteria sp.* has been proposed to function as a protective inert barrier (Glynn, 1972).

More definitive experiments with serum-resistant Gram-negative bacteria have demonstrated that the lipopolysaccharide surface coat of smooth strains is required for complement resistance (Dlabac, 1968; Sterzl, 1964). Reynolds and Pruul (1971a,b) have shown that

smooth strain *Salmonella* are insensitive to the action of specific antibody and complement in the presence of Mg^{++} or saline but are lysed if monovalent cations or low concentrations of EDTA are substituted or added. The data were interpreted as indicating that divalent cations such as Mg^{++} or Ca^{++} are required for the structural integrity and bridging of the polyanionic molecules composing LPS. Endotoxic LPS of *Salmonella* activates complement and binds terminal complement components (Mergenhagen et al, 1968), yet antibody has been shown to penetrate to the plasma membrane level without lysis in the presence of complement, unless monovalent cations or chelators are present, in which case bacterial lysis occurs (Reynolds et al, 1975). Two mechanisms have been proposed to account for the results of these experiments; activation and decay of complement components at loci distant from critical membrane sites, and inhibition of activated C5 binding by LPS (Reynolds et al, 1975).

c. Surface polyanions and their potential role in resistance in lysis

Unfortunately no direct evidence exists to explain the phenomenon of cell cycle variation in susceptibility to complement-mediated lysis for tumor and transformed cells of mammalian origin. However, a correlation does exist between diminished susceptibility to lysis and the presence of heparan sulfate (sulfated polyanion) on the surface of cultured cells (Kraemer and Tobey, 1972). Heparan sulfate and other polysulfated molecules are known to interact with the complement proteins. Heparan sulfate is synthesized intracellularly and appears in the surface coat of many different mammalian cell types, and premitotic loss of heparan sulfate occurs during the cell cycle of cultured cells (Kraemer and Tobey, 1972; Chiarugi,

1974) and in the regenerating rat liver (Kojima and Koizumi, 1974). The highest levels of heparan sulfate occur on the cell surface during the S phase of the cell cycle which corresponds to the stage at which virus-transformed cells and leukemia cells are most resistant to complement lysis (see section II, c).

More direct evidence that sulfated polyanions or acid mucopolysaccharides play a role in resistance to lysis was obtained when Lippman (1968) treated Moloney-induced tumor cells with various acid mucopolysaccharides prior to injection into allogeneic hosts or before incubation with antisera to H-2 antigens or viral antigens plus complement. In her experiments, enhanced tumor growth and resistance of tumor cells to complement-mediated immune lysis was observed in those cells pretreated with acid mucopolysaccharides (heparin, keratin sulfate and, to a lesser degree, hyaluronic acid).

Chiarugi et al (1974) have speculated that surface heparan sulfate is a control element in regulating cell mitosis and differentiation. Such a role for cell surface heparan sulfate would have important implications in cell-mediated immunity as well as humoral immunity. A limited number of reports on the effects of heparan sulfate upon cell-mediated immunity have been published, and even though this review has been mainly concerned with humoral immunity, these results emphasize the potential consequences of polyanionic polysaccharides on immune reactions. Viklicky et al (1974) showed that long term treatment of recipients of skin allografts (with weak non-H-2 histocompatibility barrier) with small doses of chondroitin sulfate results in 19% successful grafts compared to no successful grafts in the controls. Further studies by Viklicky et al (1975) demonstrated that if chondroitin sulfate or heparin was present in the reaction medium for a complement-

independent cytotoxic reaction the percentage of cytotoxicity was reduced to control levels (non-sensitized cells and killer cells. In this same paper antibody-induced redistribution or capping of H-2 antigens on lymph node cells was found to be greatly accelerated when these cells were pretreated with chondroitin sulfate or heparin. Pokorna (1976) extended this experimental design to show that chondroitin sulfate inhibited PHA-induced stimulation of lymphocytes: 67% of control cells were stimulated compared to 24% of chondroitin sulfate-treated cells.

There is evidence that some other immunologically important events in cells are relatable to surface polyanions. Membrane antigenic modulation by capping, shedding and pinocytosis are thought to be influenced by surface acid mucopolysaccharides through their role in control of Ca^{++} flux (Viklicky et al, 1975; Pokorna, 1976; and Vlodavsky and Sachs, 1977). There is recent evidence for this role in pinocytotic processes (Josefsson, 1975). These observations and other known properties of polyanions provide some grounds for proposing that they are important in determining susceptibility of cells to host attack mechanisms.

The potential for polyanion interaction with complement has been referred to above (see section II, a). The ability of an outer coat material to trigger complement non-specifically at sites distal from the labile membrane could serve to consume complement and maintain local microenvironment titers at low levels. Ramifications of the enhancement of Cl-INH by polyanions in terms of their effects on a variety of components of inflammation have not been investigated (e.g. Cl-INH has effects on plasmin, kallikrein, Hageman factor, Hageman factor fragments and coagulation factor XI (Austen, 1971)). Surface polyanions could contribute to steric

exclusion of macromolecular proteins by matrices of cross-linked polysaccharides (for review see Laurent, 1977). The presence of such a surface molecular seive could permit exclusion of large proteins, such as Clq.

Electrostatic interactions between negatively charged outer coat polysaccharides and host proteins could result in repulsion or binding. Repulsion of other negative charges has been proposed as a means of preventing interaction between maternal cells and the glycosaminoglycan coated surfaces of mammalian trophoblast cells of the embryo and fetus (Calatroni and Ferrante, 1969; Billington, 1975). Polyanion binding of inflammatory mediators such as histamine could neutralize its local effects. Heparin is the natural "counter-ion" for the histamine stored in mast cells (Jaques, 1975).

d. Parasite surface characteristics and evidence for the presence of polyanions

It is clear from the literature reviewed so far that a correlation has been established between resistance to complement cytolysis *in vitro* in tumor and virus-transformed cells from a variety of mammalian tissues and the levels of surface polyanions, and that there is limited evidence that exogenously provided acid mucopolysaccharides confer resistance against host immune rejection. Although comparable experimental work has not been done for parasitic organisms there is developing evidence that many parasites have substantial amounts of polyanionic substances in their surface coats, and partial characterization has been achieved in several cases.

Histochemical studies have shown that both stercorarian

trypanosomes such as *T. lewisi* and *T. cruzi* (Dwyer, 1975 and 1976; DeSouza and Meyer, 1975) and salivarian trypanosomes such as *T. vivax* and *T. brucei* (Allsopp and Njoger, 1974; Wright and Hales, 1970) contain polyanionic polysaccharides either in the surface coat or between the surface coat and the plasma membrane. Employing selective lectins, Dwyer (1976) has found these polysaccharides in *T. lewisi* to contain N-acetyl-D-galactosamine, N-acetyl-D-glucosamine and possibly mannose. In his experiments culture forms of the parasite showed less affinity for each of these lectins than did blood stream forms. Alves and Coli (1975) identified glucosamine, galactose, glucose and mannose in the surface glycoprotein of *T. cruzi*.

In contrast to *T. cruzi* the lectin-binding amino sugars of the blood stream forms of African trypanosomes are not detectable unless a superficial glycoprotein layer is removed by protease digestion. These glycoproteins may constitute the variant or exoantigens which characterize the fluctuating parasitemia of *T. brucei* infections (Allsopp et al, 1971). The fact that exoantigens are released from the surface coat at low pH suggests that there is non-covalent binding to the saccharide coat. This may be similar to the electrostatic binding of glycoproteins to heparin (Laurent, 1977). If a polyanionic polysaccharide layer is interposed between the trypanosome limiting membrane and the highly immunogenic variant antigens (Vickerman, 1974) then the polysaccharide coat may be facilitating an energy conservative flux of proteins without exposing more critical invariant antigens of the membrane to immune attack. Indeed, *T. vivax* with a much less compact surface coat has been shown to adsorb host serum

proteins on to its surface (Desowitz, 1970).

Electron microscopic studies by Vickerman (1974) point to a common feature for *T. brucei*, *T. congolense*, and *T. vivax* involving appearance of a surface coat on infective forms borne by arthropod vectors. In the last stage of development in the insect vector, just prior to infecting a mammalian host, African trypanosomes acquire a surface coat external to the plasma membrane which they maintain as blood stream forms in the mammalian host. This surface coat is known to be lost when blood stream forms enter the insect vector, though no information is available concerning its presence on extravascular phases of *T. brucei*, and *vivax* during replication. Allsopp and Njoger (1974) analyzed surface glycoproteins from blood stream *T. brucei* and detected D-glucosamine, D-galactose and D-mannose. Unfortunately none of the chemical analyses of *Trypanosoma* (of either type) surface carbohydrates included assays for sulfate groups. Thus the electron microscopic visualization of acidic polysaccharides on both types of trypanosomes by ruthenium red (Luft, 1971) and colloidal iron can only be circumstantially confirmed by the chemical evidence for hexosamines and galactose, which are the monosaccharides (along with uronic and iduronic acids) making up acid mucopolysaccharides or glycosaminoglycans (Lindahl et al, 1977).

Another protozoan, *Leishmania donovani*, which encounters two distinct environments in its mammalian host has two distinct surface types. Intracellular forms (amastigotes) have no apparent surface coat material whereas extracellular forms (promastigotes) have densely staining coats of negatively charged polysaccharides. Cultured forms of amastigotes, developing into promastigotes,

acquired this same surface coat within 7 hours (Dwyer et al, 1974).

In reviewing the literature on parasitic helminth surfaces, it is apparent that there has recently developed an awareness of the dynamic state of tegumental structures. In an extensive review of surface ultrastructure and cytochemistry by Lumsden (1975) the concept of an inert surface "cuticle" is dismissed and evidence is presented on the transport of nutrients across the tegument and on the mechanisms of avoidance of host proteolytic enzymes.

The immune susceptible early stage schistosomule and the immune-resistant adult schistosome of *S. mansoni* offer possible insights into the role of surface acidic proteoglycans or muco-polysaccharides. Upon penetration of mammalian skin, cercariae lose a coat of neutral polysaccharides and are left with a very thin layer of acid mucopolysaccharides on their surfaces. The growing schistosomula rapidly increase in surface area by adding multiple layers of plasmalemma which possess acidic staining poly-saccharides (Stein and Lumsden, 1973). This surface structure is continually being replaced in mature schistosomes (Clegg, 1972) and there is recent data to suggest that some surface component enables the adult fluke to inhibit coagulation (Tsang and Damian, 1977). The surface material shed into the host circulation may be the high molecular weight polysaccharide schistosome antigen found in the blood of patients with schistosomiasis (Buck et al, 1975). In a recent paper it has been suggested that non-specific initiation of complement-fixation occurs around schistosomules (Von Lichtenberg et al, 1977).

Larval and adult stages of most cestodes have been shown to be invested with a surface glycocalyx which is distal to the

plasma membrane of the tegumental cells themselves (Lumsden, 1975). There is cytochemical evidence for the elaboration of carbohydrate containing surface material (Oaks and Lumsden, 1971) and histochemical studies have shown that tegument is the site of synthesis of non-glycogen polysaccharides (Lumsden, 1975), some of which have been ultrastructurally located at the tegument surface.

Histochemically the cestode glycocalyx reacts positively with stains for carbohydrate vicinal hydroxyl groups (Lumsden et al, 1970), and there is a high density of anions which Lumsden (1972) interpreted as a reflection of the presence of acidic glycans. Morris and Finnegan (1968) suggested that the surface of tissue-dwelling larval cestodes of *Schistocephalus solidus* contained sulfated acid mucopolysaccharides. Lumsden (1973) considered that carboxyl groups plus inorganic acid residues also probably contributed to the net electronegative charge on the cestode surface. While a variety of roles have been ascribed to cestode surface acidic polysaccharides, including participation in transport systems and enzyme inhibition, no consideration has apparently been given to their potential significance in the avoidance of rejection through any of the mechanisms outlined above. The work reported in this dissertation suggests that this possibility should not be overlooked in the future. Recently reported observations by Kassis and Tanner (1976) and Herd (1976) on the lytic effects of normal serum upon larval and mature cestodes *in vitro* provide impetus for defining those factors which allow parasite survival *in vivo*.

REFERENCES

REFERENCES

Abdussalam, M. 1974. The problem of taeniiasis-cysticercosis. VII InterAmerican Mtg. Ministerial Level Foot and Mouth Dis. Zoonoses Control, W.H.O. Port of Spain, Trinidad (April).

Abernethy, T. J. and Avery, O. T. 1941. The occurrence during acute infections of a protein not normally present in the blood. I. Distribution of the reactive protein in patient's sera and the effect of calcium on the flocculation reaction with C polysaccharide of *Pneumococcus*. J. Exp. Med. 73:173-200.

Allsopp, B. A. and Njoger, A. R. 1974. Monosaccharide composition of the surface glycoprotein antigens of *Trypanosoma brucei*. Parasitology 69:271-281.

Allsopp, B. A., Njoiju, A. R. and Humphryes, K. C. 1971. Nature and location of *Trypanosoma brucei* subgroup exoantigen and its relationship to its antigen. Exp. Parasitol. 29:271-284.

Alves, M. J. and Coli, W. 1975. Glycoproteins from *Trypanosoma cruzi*: partial purification by gel chromatography. FEBS Letters, Amsterdam 52:188-190.

Anziano, D. F., Dalmasso, A. P., Lelchuk, R., Vasquez, C. 1972. Role of complement in immune lysis of *Trypanosoma cruzi*. Infect. Immun. 6:860-864.

Apffel, C. A. and Peters, J. H. 1970. Regulation of antigenic expression. Theor. Biol. 26:47-59.

Austen, K. F. 1971. Chemical mediators of the acute inflammatory response in man. p. 723-744. *In*: Progress in Immunology. First International Congress of Immunology. (B. Amos, ed.) Academic Press, New York and London.

Billington, D. 1975. Organization, ultrastructure and histochemistry of the placenta: immunological considerations. p. 67-85. *In*: Immunobiology of Trophoblast. (R. G. Edwards, C. W. S. Howe, and M. H. Johnson, eds.) Cambridge University Press, Cambridge, MA.

Borros, T., Dourmashkin, R. R. and Humphrey, J. H. 1964. Lesions in erythrocyte membranes caused by immune haemolysis. Nature 202:251-252.

Bozicevich, J. and Detre, L. 1940. Studies on trichinosis. VIII. The antigenic phase of trichinosis. Public Health Rep. 55:683-692.

Brade, V., Lee, G. D., Nicholson, A., Shin, H. S. and Mayer, M. M. 1973. The reactions of zymosan with the properdin system in normal and C4-deficient guinea pig serum. J. Immunol. 111: 1389-1400.

Brown, K. N. 1974. Antigenic variation and immunity to malaria. p. 35-51. *In*: Parasites in the immunized host: mechanisms of survival. Excerpta Medica, North Holland.

Buch, A. A., Davis, A., Deelder, A. M., Enders, B., Faure, M., Grasbeck, R., Haworth, J., Kent, N. H., Marcoullis, G., Senft, A. W., Vicari, G., Voller, A., and Weiss, N. 1975. Parasite Antigens. Bull. W.H.O. 52:237-248.

Budzko, D. B., Pizzimenti, M. C., Kierszenbaum, F. 1975. Effects of complement depletion in experimental Chagas' Disease: immune lysis of virulent blood forms of *Trypanosoma cruzi*. Infection and Immunity 11:86-91.

Bullock, W. E. 1968. Status of immune mechanisms in leprosy. I. Depression of delayed allergic response to skin test antigens. New England Journal of Med. 14:889-898.

Calatroni, A., and N. di Ferrante. 1969. The glycosaminoglycans of human term placenta. Carbohyd. Res. 10:535-548.

Campbell, D. H. 1938a. The specific protective property of serum from rats infected with *Cysticercus crassicollis*. J. Immunol. 35:195-204.

Campbell, D. H. 1938b. Further studies on the "nonabsorbable" protective property in serum from rats injected with *Cysticercus crassicollis*. J. Immunol. 35:465-476.

Capron, A., Capron, M., Dupas, H., Bout, D. and Petitprez, A. 1974. Etude *in vitro* des phenomenes immunologiques dans la schistosomiase humaine et experimentale. I. Etude comparative *in vitro* de l'activite lethale d'immunserums sur les formes immatures et sur les adultes de *Schistosoma mansoni*. Int. J. Parasitol. 4:613-623.

Capron, A., Wattie, P., Capron, M. and Lefebre, M. M. 1972. C. R. Hebd. Seances Acad. Sci. Ser D. Sci. Nat. (Paris) 275:719-721.

Cerisola, J. A., Alvarez, M., Lugones, J., Resbosolan, J. B. 1969. Sensibilidad de las reacciones serologicas para el diagnostico de la enfermedad de Chagas. Bol. Chil. Parasitol. 24:2-8.

Chiarugi, V. P., and Vannucchi, S. 1976. Surface heparan sulphate as a control element in eukariotic cells: a working model. J. Theor. Biol. 61:459-475.

Chiarugi, V. P., Vannucchi, S. and Urbano, P. 1974. Exposure of trypsin-removable sulfated polyanions on the surface of normal and virally transformed $BHK^{21/C13}$ cells. Biochem. Biophys. Acta 345:283-293.

Ciba Foundation Symposium 25 (new series). 1974. Parasites in the Immunized Host: mechanisms of survival. Excerpta Medica, North Holland.

Cikes, M. 1970a. Antigenic expression of a murine lymphoma during growth *in vitro*. Nature (London) 225:645-647.

Cikes, M. 1970b. Relationship between growth rate, cell volume, cell cycle kinetic and antigenic properties of cultured murine lymphoma cells. J. Natl. Cancer Inst. 45:979-988.

Cikes, M. and Friberg, S., Jr.. 1971. Expression of H-2 and moloney leukemia virus-determined cell-surface antigens in synchronized cultures of a mouse cell line. Proc. Nat. Acad. Sci. 68:566-569.

Cikes, M., Friberg, S., Jr. and Klein, G. 1972. Quantitative studies of antigen expression in cultured murine lymphoma cells. II. Cell-surface antigens in synchronized cultures. J. Natl. Cancer Inst. 49:1607-1611.

Claus, D. R., Siegel, J., Petras, K., Skor, D., Osmand, A. P. and Gewurz, H. 1977. Complement activation by interaction of polyanions and polycations. III. Complement activation by interaction of multiple polyanions and polycations in the presence of C-reactive protein. J. Immunol. 118:83-87.

Clegg, J. 1972. The schistosome surface in relation to parasitism. Symposium of the British Society for Parasitology 10:23-40.

Clegg, J. A. and Smithers, S. R. 1972. The effects of immune Rhesus Monkey serum on schistosomula of *Schistosoma mansoni* during cultivation *in vitro*. Int. J. Parasitol. 2:79-98.

Clegg, J. A. 1974. Host antigens and the immune response in schistosomiasis. p. 161-183. *In:* Parasites in the immunized host: mechanisms of survival. Excerpta Medica, North Holland.

Cooper, N. R., Polley, M. J. and Oldstone, M. B. A. 1974. Failure of terminal complement components to induce lysis of moloney virus transformed lymphocytes. J. Immunol. 112:866-868.

Cypess, R., Lubiniecki, A. S. and Hammon, W. M. 1973. Immunosuppression and increased susceptibility to Japanese B encephalitis virus in *Trichinella spiralis*-infected mice (37345). Proc. Soc. Exp. Biol. Med. 143:468-473.

Dean, D. A. 1977. Decreased binding of cytotoxic antibody by developing *Schistosoma mansoni*. Evidence for a surface change independent of host antigen adsorption and membrane turnover. J. Parasit. in Press.

De Souza, W. and Meyer, H. 1975. An electron microscopic and cytochemical study of the cell coat of *Trypanosoma cruzi* in tissue cultures. Z. Parasitenk. 46:179-187.

Desowitz, R. S. 1970. *In*: Immunity to Parasitic Animals. vol. 2, p. 551-596.

Dlabac, V. 1968. The sensitivity of smooth and rough mutants of *Salmonella typhimurium* to bactericidal and bacteriolytic action of serum lysozyme and to phagocytosis. Folia Microbiol. 13:439-449.

Dossett, J. H., Kromvall, G., Williams, R. C., Jr., Quil, P. G. 1969. Antiphagocytic effects of staphlococcal protein A. J. Immunol. 103:1405-1410.

Dwyer, D. M., Langreth, S. G. and Dwyer, N. K. 1974. Evidence for a polysaccharide surface coat in the developmental stages of *Leishmania donovani*: a fine structure-cytochemical study. Z. Parasitenk. 43:227-249.

Dwyer, D. M. 1975. Cell surface saccharides of *Trypanosoma lewisi*. I. Polycation-induced cell agglutination and fine structure cyctochemistry. J. Cell Sci. 19:621-644.

Dwyer, D. M. 1976. Cell surface saccharides of *Trypanosoma lewisi*. II. Lectin-mediated agglutination and fine structure cytochemical detection of lectin-binding sites. J. Cell. Sci. 22:1-19.

Faubert, G. M. and Tanner, C. E. 1974. The suppression of sheep rosette-forming cells and the inability of mouse bone marrow cells to reconstitute competence after infection with nematode *Trichinella spiralis*. Immunology 27:501-505.

Fearon, D. T. and Austen, K. F. 1976. The function of properdin in the alternative complement pathway amplification loop: Stabilization of the C3b-dependent C3 convertase. Arthritis Rheum. (abst.)

Fearon, D. T. and Austen, K. F. 1977. The human complement system: biochemistry, biology, and pathobiology.

Fearon, D. T., Ruddy, S. L., Knostman, J. D., Carpenter, C. B. and Austen, K. F. 1974. The functional significance of complement. Advances in Nephrology 4:15-35.

Freeman, J., Hudson, K. M. and Bryner, C. 1974. Immunosuppression in trypanosome infections: attempts to characterize the defects in the response of infected mice to sheep red blood cells. Trans. R. Soc. Trop. Med. Hyg. 68:149.

Gemmell, M. A. and MacNamara, F. N. 1972. Immune response to tissue parasites. II. Cestodes p. 245-272. Immunity to Animal parasites. (Soulsby, E. J. L., ed.) Academic Press, New York.

Gewurz, H., Shin, H. S. and Mergenhagen, S. E. 1968. Interactions of the complement system with endotoxic lipopolysaccharide: consumption of each of the six terminal complement components. J. Exp. Med. 128:1049-1057.

Glynn, A. A., Howard, C. J. 1970. The sensitivity to complement of strains of *Escherichia coli* related to their K antigens. Immunology 18:331-346.

Glynn, A. A. 1972. Bacterial factors inhibiting host defense mechanisms. Symp. Soc. Gen. Microbiol. 22:75-112.

Goble, F. C. 1970. South American trypanosomes p. 597-689. *In*: Immunity to parasitic animals. (J. Jackson, R. Herman, and I. Singer, eds.) Appleton-Century-Crofts, New York.

Good, Anne H., and Miller, Kathleen L. 1976. Depression of the immune response to sheep erythrocytes in mice infected with *Taenia crassiceps* larvae. Infec. Immun. 14:449-456.

Gotze, O. and MullerEberhard, H. J. 1971. The C3-activator system: an alternate pathway of complement activation. J. Exp. Med. 134:90-108.

Gotze, D., Pellegrino, M. A., Ferrone, S. and Reisfeld, R. A. 1972. Expression of H-2 antigens during the growth cycle of cultured tumor cells. Immunol. Commun. 1:533-544.

Greenwood, R. M. 1974. Immunosuppression in malaria and trypanosomiasis. p. 137-159. *In*: Parasites in the immunized host: mechanisms of survival. Excerpta medica, North Holland.

Herd, R. P. 1976. The cestocidal effect of complement in normal and immune sera *in vitro*. Parasitology 72:325-334.

Hill, M. J. 1968. A staphylococcal aggressin. J. Med. Micro. 1:33-43.

Hockley, D. J. and Smithers, S. R. 1970. Damage to adult *Schistosoma mansoni* after transfer to a hyperimmune host. Parasitology 61:95-100.

Hustead, S. T. 1976. Permeability studies on taeniid metacestodes. M.S. degree thesis. Michigan State University, East Lansing, Michigan

Hustead, S. T., and Williams, J. F. 1977a. Permeability studies of taeniid metacestodes. I. Uptake of proteins by larval stages of *Taenia taeniaeformis, Taenia crassiceps* and *Echinococcus granulosus*. J. Parasitol. 63:314-321.

Hustead, S. T. and Williams, J. F. 1977b. Permeability studies of taeniid metacestodes II. Antibody-mediated effects on membrane permeability in larvae of *Taenia taeniaeformis* and *Taenia crassiceps*. J. Parasitol. 63:322-326.

Jaques, L. B. 1975. The mast cell in the light of new knowledge of heparin and sulfated mucopolysaccharides. Gen. Pharmac. 6:235-245.

Josefsson, J. O. 1975. Studies on the mechanisms of induction of pinocytosis in *Amoeba proteus*. Acta Physiol. Scand. Supplement 432:1-65.

Joseph, B. S., Cooper, N. R., and Oldstone, M. B. A. 1975. Immunologic injury of cultured cells infected with measles virus. I. Role of IgG antibody and the alternative complement pathway. J. Exp. Med. 141:761-774.

Joseph, B. S. and Oldstone, M. B. A. 1975. Immunologic injury in measles virus infection. II. Suppresion of immune injury through antigenic modulation. J. Exp. Med. 142:864-876.

Kagan, I. G. and Levine, D. M. 1956. Studies on the serology of schistosomiasis. II. The *in vitro* activity of cercariae of *Schistosoma mansoni* in sera of normal and antigen-injected animals. Parasitology 5:48-58.

Kaplan, M. H. and Volanakis, J. E. 1974. Interaction of C-reactive protein complexes with the complement system. Consumption of human complement associated with the reaction of C-reactive protein with pneumococcal c-polysaccharide and with the choline phosphatides, lecithin and sphinogomyelin. J. Immunol. 112:2135-2147.

Kassis, A. I. and Tanner, C. E. 1976. The role of complement in hydatid disease: *in vitro* studies. Int. J. Parasitol. 6:25-36.

Keller, R. and Jones, V. E. 1971. Role of activated macrophages and antibody in inhibition and enhancement of tumour growth in rats. Lancet 2:847-

Kierzenbaum, F., Ivanyi, J. and Budzko, D. B. 1976. Mechanisms of natural resistance to *Trypanosoma cruzi* infection. Immunology 30:1-6.

Kojima, K. and Koizumi, K. 1974. 11th Inter. Cancer Congress, Florence.

Kraemer, P. M. and Tobey, R. A. 1972. Cell-cycle dependent desquamation and heparan sulfate from the cell surface. J. Cell Biol. 55:713-717.

Laurent, T. C. 1963. The interaction between polysaccharides and other macromolecules. 5. The solubility of proteins in the presence of dextran. Biochem. J. 89:253-259.

Laurent, T. C. 1977. Interaction between proteins and glycosaminoglycans. Fed. Proc. 36:24-27.

Leid, R. W. 1973. Immunological events associated with *Taenia taeniaeformis* infections in the laboratory rat. Ph.D. degree thesis; Michigan State University, East Lansing, Michigan.

Leid, R. W. and Williams, J. F. 1974. Immunological response of the rat to infection with *Taenia taeniaeformis*. I. Immunoglobulin classes involved in passive transfer of resistance. Immunology 27:195-208.

Lerner, R. A., Oldstone, M. B. A. and Cooper, N. R. 1971. Cell cycle-dependent immune lysis of moloney virus-transformed lymphocytes: presence of viral antigen, accessibility to antibody, and complement activation. Proc. Nat. Acad. Sci. 68:2584-2588.

Lindahl, U., Hook, M., Backstrom, G., Jacobsson, I., Riesenfeld, J., Malmstrom, A., Roden, L. and Feingold, D. C. 1977. Structure biosynthesis of heparin-like polysaccharides. Federation Proc. 36:19-23.

Lippman, M. 1968. Transplantation and cytotoxicity changes induced by acid mucopolysaccharides. Nature 219:33-36.

Loos, M., Raepple, E., Hadding, U. and Bitter-Suerman, B. 1974. Interaction of polyanions with the first and the third component of complement. Fed. Proc. (Abst.) 33:775.

Losos, G. J. and Ikede, B. O. 1972. Review of pathology of diseases in domestic and laboratory animals caused by *Trypanosoma congolense, T. Vivax, T. brucei, T. rhodesiense* and *T. gambiense*. Vet. Path. suppl. ad. 9:1-71.

Luckings, A. G. 1974. Immunoglobulin response in zebu cattle infected with *Trypanosoma congolese* and *T. vivax*. Trans. R. Soc. Trop. Med. Hyg. 68:148-149.

Luft, J. 1971. Ruthenium red and violet. II. Fine structural localization in animal tissues. Anatomical Record 171:369-416.

Lumsden, R. 1972. Cytological studies on the absorptive surfaces of cestodes. VI. Cytochemical evaluation of electrostatic charge. J. Parasitol. 58:229-234.

Lumsden, R. 1973. Cytological studies on the absorptive surfaces of cestodes. VII. Evidence for the function of the tegument glycocalyx in cation binding by *Hymenolepis diminuta*. J. Parasitol. 59:1021-1030.

Lumsden, R. D. 1975. Parasitological review. Surface ultrastructure and cytochemistry of parasitic helminths. Expl. Parasitol. 37:267-339.

Lumsden, R., Oaks, J. and Alworth, W. 1970. Cytological studies on the absorptive surfaces of tapeworms. IV. Localization and cytochemical properties of membrane-fixed cation binding sites. J. Parasitol. 56:736-747.

Machado, A. J., Gazzinelli, G., Pellegrino, J. and Dias da Silva, W. 1975. *Schistosoma mansoni*: the role of the complement C3-activating system in the cercaricidal action of normal serum. Experimental Parasitology 38:20-29.

Mergenhagan, S. E., Gewurz, H., Bladen, H. A., Nowotny, A., Kasi, N., and Luderitz, O. 1968. Interactions of the complement system with endotoxins from *Salmonella minnesota* mutant deficient in o-polysaccharide and heptose. J. Immunol. 100:227-229.

Morris, G. and Finnegan, C. 1968. Studies of the differentiating plerocercoid cuticle of *Schistocephalus solidus*. I. The histochemical analysis of cuticle development. Canadian Journal of Zoology 46:115-121.

Musoke, A. J. 1975. Immunologic mechanisms in resistance to experimental infection with *Taenia taeniaeformis*. Ph.D. degree thesis; Michigan State University, East Lansing, Michigan.

Musoke, A. J. and Williams, J. F. 1975. Immunological response of the rat to infection with *Taenia taeniaeformis*. V. The sequence of appearance of protective immunoglobulins and the mechanism of action of $7S\gamma 2_a$ antibodies. Immunol. 29:855-865.

Oaks, J. and Lumsden, R. 1971. Cytological studies on the absorptive surfaces of cestodes. V. Incorporation of carbohydrate containing macromolecules into tegument membranes. J. Parasitol. 57:1256-1268.

Ogilvie, B. M. and Wilson, R. J. M. 1976. Evasion of the immune response by parasites. Br. Med. Bull. 32:177-181.

Ogston, A. G. and Preston, B. N. 1966. The exclusion of protein by hyaluronic acid. Measurement by light scattering. J. Biol. Chem. 241:17-19.

Pellegrino, M. A., Ferrone, S., Cooper, N. R., Dierich, M. P. and Reisfeld, R. A. 1974. Variation in susceptibility of a human lymphoid cell line to immune lysis during the cell cycle. Lack of correlation with antigen density and complement binding. J. Exp. Med. 140:578-590.

Pensky, J., Hinz, C. F., Jr., Todd, E. W., Wedgwood, R. J., Boyer, J. T., and Lepow, I. H. 1968. Properties of highly purified human properdin. J. Immunol. 100:142-158.

Perez, H. and Terry, R. J. 1973. The killing of adult *Schistosoma mansoni in vitro* in the presence of antisera to host antigenic determinants and peritoneal cells. Int. J. Parasitol. 3:499-503.

Pillemer, L., Blum, L., Lepow, I. H., Ross, O. A., Todd, E. W. and Wardlaw, A. C. 1954. The properdin system and immunity. I. Demonstration and isolation of a new serum protein, properdin, and its role in immune phenomena. Science (Washington, D. C.) 120:279-285.

Pillemer, L., Blum, L., Lepow, I. H., Wurz, L. and Todel, E. W. 1956. The properdin system and immunity. III. The zymosan assay of properdin. J. Exp. Med. 103:1-13.

Pizzi, T. 1957. Immunologia de la enfermedad de Chagas. p. 157. Universidad de Chile, Santiago.

Pokorna, Z. 1976. Changes in agglutination of cells with H-2 antibodies or lectins and in stimulation of lymphocytes as induced by certain polymers. Fol. biol. (Praha). 22:151-158.

Rent, R., Ertel, N., Eisenstein, R. and Gewurz, H. 1975. Complement activation by interaction of polyanions and polycations. I. Heparin-protamine induced consumption of complement. J. Immunol. 114:120-124.

Rent, R., Myhrman, R., Fiedel, B. A. and Gewurz, H. 1976. Potentiation of Cl-esterase inhibitor activity by heparin. Clin. Exp. Immunol. 23:264-271.

Reynolds, B. L. and Pruul, H. 1971a. Sensitization of complement-resistant smooth gram-negative bacterial strains. Infect. Immun. 3:365-372.

Reynolds, B. L. and Pruul, H. 1971b. Protective role of smooth lipopolysaccharide in the serum bactericidal reaction. Infect. Immun. 4:764-771.

Reynolds, B. L., Rother, V. A. and Rother, K. O. 1975. Interaction of complement of components with a serum-resistant strain of *Salmonella typhimurium*. Infect. Immun. 11:944-948.

Roantree, R. J., Pappas, N. C. 1960. The survival of strains of enteric bacilli in the blood stream as related to their sensitivity to the bactericidal effect of serum. J. Clin. Invest. 39:82-88.

Roantree, R. J. and Rantz, L. A. 1960. A study of the relationship of the normal bactericidal activity of human serum to bacterial infection. J. Clin. Invest. 39:72-81.

Romana, C. 1963. Enfermedad de Chagas. p. 89. Lopez Librerias Editores S.R.L. Buenos Aires.

Schenone, H. 1974. Cysticercosis as a public health and animal health problem. *In*: VII Int.-Amer. Mtg. at the Ministerial Level on Foot and Mouth Disease and zoonoses control. Trinidad.

Seed, J. R. 1972. Characterization of variant specific antigens. Exp. Parasitol. 31:98-108.

Seed, J. R. 1977. The role of immunoglobulins in immunity to *Trypanosoma brucei gambiense*. Int. J. Parasitol. 7:55-60.

Seed, J. R., Cornille, R., Risby, E. and Gam, A. A. 1969. The presence of agglutinating antibody in the IgM immunoglobulin fraction of rabbit antiserum during experimental trypanosomiasis. Parasitology 59:283-292.

Seed, J. R. and Gam, A. A. 1966. Passive immunity to experimental trypanosomiasis. J. Parasitol. 59:283-292.

Shipley, W. V. 1971. Immune cytolysis in relation to the growth cycle of Chinese hamster cells. Cancer Res. 31:925-929.

Siegel, J., Osmond, A. P., Wilson, M. and Gewurz, H. 1975. Interactions of C-reactive protein with the complement system. II. CRP-mediated consumption of complement by poly-L-lysine polymers and other polycations. J. Exp. Med. 142:709-721.

Smithers, S. R. 1972. Recent advances in the immunology of schistosomiasis. Br. Med. Bull. 28:49-54.

Smithers, S. R., Terry, R. J., and Hockly, D. J. 1969. Host antigens in schistosomiasis. Proc. Roy. Soc. Lond. B Biol. Sci. 171: 483-494.

Snyderman, R. and Pike, M. C. 1975. Interaction of complex polysaccharides with the complement system: Effect of calcium depletion on terminal component consumption. Infect. Immun. 11:273-279.

Standen, O. D. 1952. The *in vitro* effect of normal and immune serum upon the cercariae of *Schistosoma mansoni*. Journal of Helminthology 26:25-42.

Stein, P., and Lumsden, R. 1973. *Schistosoma mansoni*: topochemical features of cercariae, schistosomula and adults. Exp. Parasitol. 33:499-514.

Sterzl, J., Pesak, V., Kostka, J. and Jilek, M. 1964. The relation between the bactericidal activity of complement and the character of the bacterial surfaces. Folia Microbiol. 9:284-298.

Stumberg, J. E. 1933. The detection of proteins of the nematode *Haemonchus contortus,* in the sera of infected sheep and goats. Am. J. Hyg. 18:247-265.

Takayanagi, T. and Enviquez, G. C. 1973. Effects of the IgG and IgM immunoglobulins in *Trypanosoma gambiense* infections in mice. J. Parasitol. 59:644-647.

Teixeira, A. R. L. and Santos-Buch, C. A. 1974. The immunology of experimental Chagas' disease. I. Preparation of *Trypanosoma cruzi* antigens and humoral antibody response to these antigens. J. Immunol. 113:859-865.

Terry, R. J., Freeman, J., Hudson, K. M. and Longstaff, J. A. 1973. Immunoglobulin M production and immunosuppression in trypanosomiasis: a linking hypothesis. Trans. R. Soc. Trop. Med. Hyg. 67:362-371.

Tsang, V. C. W. and Damian, R. 1977. Demonstration of mode of action and an inhibitor for activated Hageman factor (Factor XIIa) of the intrinsic blood coagulation pathway from *Schistosoma mansoni*. Blood 49:619-633.

Vickerman, K. 1974. Antigenic variation in African trypanosomes. p. 53-80. *In*: Parasites in the Immunized Host: mechanisms of survival. Excerpta Medica, North Holland.

Viklicky, V., Pokorna, Z., and Peknicova, J. 1975. The effects of glycosaminoglycans on surface-mediated interactions of lymphoid cells. Fol. biol. (Praha). 21:87-94.

Viklicky, V., Polackova, M., and Astorquiza, M. 1974. The effects of chondroitin sulfate and vitamin A on the survival of skin allografts across weak H-barriers. Fol. biol. (Praha). 20:161-167.

Vlodavsky, I. and Sachs, L. 1977. Difference in the calcium regulation of concanavalin A agglutinability and surface microvilli in normal and transformed cells. Expl. Cell Res. 105:179-189.

Von Lichtenberg, F., Sher, A. and McIntyre, S. 1977. A lung model of schistosome immunity in mice. Amer. J. Path. 87:105-124.

Wedderburn, N. 1974. Immunodepression produced by malarial infection in mice. p. 123-136. *In*: Parasites in the Immunized Host: mechanisms of survival. Excerpta Medica, North Holland.

Williamson, J. and Brown, K. N. 1974. The chemical composition of trypanosomes. III. Antigenic constituents of *brucei* trypanosomes. Exp. Parasitol. 15:44-68.

Wilson, R. J. M. 1974. Soluble antigens as blocking antigens. p. 185-204. *In*: Parasites in the Immunized Host: mechanisms of survival. Excerpta Medica, North Holland.

Wilson, R. J. M., McGregor, I. A. and Wilson, M. E. 1973. The stability and fractionation of malarial antigens from the blood of Africans infected with *Plasmodium falciparum*. Int. J. Parasitol. 3:511-520.

Wright, K. A. and Hales, H. 1970. Cytochemistry of the pellicle of bloodstream forms of *Trypanosoma (Trypanozoon) brucei*. J. Parasitol. 56:671-683.

Zuckerman, A. and Ristic, M. 1968. p. 79-122. *In*: Infectious Blood Diseases of Man and Animals. (D. Weinman and M. Ristic, eds.) Academic Press, New York.

ACTIVATION OF COMPLEMENT BY HYDATID CYST FLUID OF *ECHINOCOCCUS GRANULOSUS*

COMPLEMENT ACTIVATION BY HYDATID FLUID

B. HAMMERBERG AND J. F. WILLIAMS

Department of Microbiology and Public Health

Michigan State University

East Lansing, Michigan 48824

This is journal article No. _____ from the Michigan Agricultural Experiment Station.

ABSTRACT

Factors present in hydatid cyst fluid of *Echinococcus granulosus* were found to interact with complement from several species. This non-immunologic fixation resulted in the depletion of hemolytically active complement from fresh guinea pig serum, the conversion of human C3 to an electrophoretically faster species and the generation of smooth muscle contracting substances, analogous to anaphylatoxins, in normal rat serum *in vitro*. Vascular permeability changes were produced *in vivo* in rats and humans after intradermal inoculation of complement interacting fractions of hydatid fluid. It is suggested that these factors may contribute to the pathogenesis of the shock syndrome which follows intravenous administration of hydatid fluid in normal animals, and to the non-specificity of immunodiagnostic skin tests for hydatid infection in man and animals.

INTRODUCTION

It has been known for many years that the parenteral injection of hydatid cyst fluid in animals results in a striking sequence of pathophysiologic changes leading to profound shock and sometimes death (Giusti and Hug, 1923; Rocha e Silva and Grana, 1945; Tabatabai et al, 1973). A similar sequence of events has been described in human patients in whom cysts of *Echinococcus granulosus* have accidentally ruptured (Dew, 1928; Handjani et al, 1969). Experimental analysis of the shock syndrome in normal animals (Rocha e Silva and Grana, loc. cit; Tabatabai et al, 1973, 1974) has provided a detailed account of the cardiovascular and respiratory responses which occur and of their susceptibility to pharmacologic modification. Nevertheless, the underlying mechanism is not understood and although supportive evidence is lacking, some form of immunologically mediated anaphylaxis is generally postulated to account for the phenomenon. Little consideration has been given to alternate explanations, but an inherent toxicity of substances in hydatid fluid has been suggested recently (Tabatabai et al, 1974).

In the following study we have examined interactions between factors present in hydatid fluid and the host complement system. Our results indicate that these factors can initiate non-immunologic activation of complement in normal serum and the production of anaphylatoxins which are able to contract smooth muscle and effect vascular permeability changes in both rats and humans. We propose that such a mechanism may be involved in the pathogenesis of the hydatid fluid shock syndrome and may also contribute to the development of non-specific reactivity in immunodiagnostic skin tests.

MATERIALS AND METHODS

Experimental Animals

Spartan (Spb (SD) Br) rats were purchased from Spartan Research Animals, Haslett, Michigan.

Maintenance of Parasites

Hydatid cysts of *E. granulosus* were originally derived from secondary infections in jirds *(Meriones unguiculatus)* obtained through the generosity of Dr. C. W. Schwabe, University of California, Davis, CA. Organisms were surgically implanted into the peritoneal cavity of rats and allowed to develop for periods of 10 to 15 months before transfer or use (Varela-Diaz et al, 1974).

Hydatid Fluid

Cysts harvested from rats were punctured and the hydatid fluid was collected and concentrated 7X by negative pressure dialysis. Sheep hydatid cyst fluid (SHCF) was collected by one of us (JFW) from viscera obtained at slaughterhouses in Buenos Aires, Argentina. Protoscolices were allowed to sediment and the clear supernatant was removed, lyophilized and stored at -20 C for several years before use in our laboratory.

Fractionation Procedures

After overnight dialysis at 4 C against 0.1 M Tris HCl buffer pH 8.0, 5 ml of concentrated rat hydatid cyst fluid (RHCF) was chromatographed on a Sephadex G-200 column (90 x 2.5 cm) equilibrated with the same buffer. The elution pattern was monitored at 280 nm. Host serum components were removed from RHCF or chromatographic fractions by passage over immunoadsorbent columns of glutaraldehyde

polymerized sheep anti-rat serum or anti-rat Fab (Avrameas and Ternynck, 1969; Leid and Williams, 1974).

Complement Fixation

A slight modification of the procedure described by Kabat and Mayer (1961) was employed for the quantitative estimation of immune hemolysis. Sheep red blood cells (SRBC) were sensitized with anti-serum prepared in rats by the intraperitoneal inoculation of 10^8 washed cells 8 days prior to bleeding.

Quantitation of complement fixation was carried out by measuring the number of CH/50 units remaining in 0.5 ml of normal guinea pig serum after incubation for 1 hr at 37 C with an equal volume of hydatid cyst fluid or chromatographic fractions. One half ml of a suspension of sensitized SRBC (3×10^8) was added to doubling dilutions of the incubation mixture and after an additional 1 hr at 37 C, 2 ml of ice-cold phosphate buffered saline (PBS) pH 7.4 were added to stop the reaction. The samples were then centrifuged at 1000 g for 10 min at 4 C and the degree of hemolysis was determined by spectrophotometric analysis of the supernatant at 541 nm.

C3 Conversion

Immunoelectrophoretic analysis of normal human serum was conducted on 2.5 X 7.5 cm slides coated with 2% agar in 0.1 M barbital buffer pH 8.6 containing 0.001 M EDTA. Electrophoretic separation was continued for 2.5 hr and precipitation bands were developed using monospecific anti-human C3 prepared in sheep. Purified C3 for the production of this reagent was kindly provided by Dr. R. A. Patrick, Department of Microbiology and Public Health, Michigan State University.

Positive control preparations exhibiting complete conversion of C3 were incubated with zymosan for 1 hr at 37 C (Muller-Eberhard and Fjellstrom, 1971).

Anaphylatoxin assay

Anaphylatoxin generation for positive control reagents was accomplished by the incubation of agar with normal rat serum (NRS) for 1 hr at 37 C (Lepow et al, 1969). The assay for the production of smooth muscle contracting substances was performed on segments of atropinized normal guinea pig ileum as described by Dias da Silva et al (1967). Histamine (Sigma Chemical Co., St. Louis, MO) was used as a standard for the detection of smooth muscle responsiveness. Mepyramine maleate (Merck, Sharp and Dohme, Research Laboratories, Rahway, NJ) (0.05 ug) was added to the tissue bath when antihistamine treated preparations were required. Bradykinin was also purchased from Sigma Chemical Co.

Vascular permeability changes in skin

Normal female rats were shaved over the dorsal region and given intravenous (i.v.) doses of 0.5 ml of 1% Coomassie sky blue 10 min prior to the intradermal inoculation of 0.05 ml of samples to be tested. Vascular permeability changes were manifested by the extravasation of dye over the test sites and the reactions were recorded 10 min after inoculation. Histamine (10 ug) was used to provide a positive control. Diphenhydramine HCl, mepyramine maleate or cyproheptadine treatment of rats at 20 mg/kg i.v. 15 min prior to the test was used to inhibit histamine mediated reactions.

Intradermal inoculations of 0.05 ml quantities of hydatid fluid fractions were made in the skin of the forearm of 3 human

volunteers. Maximum diameters of edematous wheals were recorded 10 min later.

RESULTS

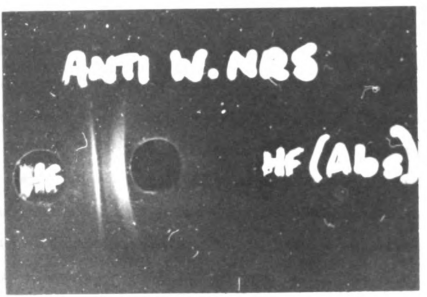
A profound depression of complement levels in normal guinea pig serum (NGPS) resulted from incubation with an equal volume of either RHCF (7X conc.) or SHCF (10X conc.). CH/50 levels in NGPS after incubation with RHCF were consistently reduced to 80 units or less while control preparations incubated with buffer maintained values of 160 units or above. Reductions with SHCF were slightly greater.

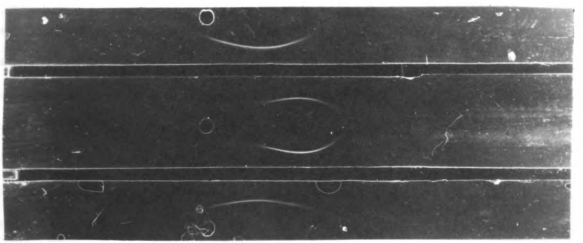
Double immunodiffusion tests showed that RHCF contained several host serum proteins. These were removed by passage over an immunoadsorbent column of sheep anti whole rat serum (Fig. 1). Removal of host components from cyst fluid did not affect its capacity to reduce complement levels in NGPS. After gel filtration of RHCF, the major portion of the antihemolytic activity was present in the initial void volume peak. Rat IgM was removed from the fraction by passage over an immunoadsorbent column of sheep anti-rat Fab. The complement fixing activity of the fraction was retained and this preparation, designated RHCF-1, was used in the biological assays detailed below.

The capacity of RHCF and SHCF to deplete complement levels was associated with their ability to cause the conversion of C3 to an electrophoretically faster migrating species. For this test SHCF or RHCF was incubated with an equal volume of normal human serum (NHS) for 1 hr at 37 C. Control preparations consisted of NHS incubated with PBS or NHS incubated with an equal volume

Figure 1. Ouchterlony analysis of rat hydatid fluid (HF) before and after absorption of host serum proteins (HF Abs). Anti whole rat serum was placed in the center well.

Figure 2. Immunoelectrophoretic analysis of human serum following incubation with either hydatid fluid (top) or zymosan (bottom). Normal serum was used as a control. The troughs contained sheep anti-human C3.


ANTI W. NRS
HF
HF (Abs)



of zymosan (15 mg/ml). After electrophoresis and development with anti-human C3 almost complete conversion was demonstrated for NHS mixed with cyst fluids or zymosan whereas minimal degradation occurred with NHS alone (Fig. 2).

The ability of factors in cyst fluid to generate the production of anaphylatoxins was tested in a bio-assay for smooth muscle contracting substances *in vitro*. Normal rat serum was incubated for 1 hr at 37 C with RHCF-1, or with agar. After centrifugation, 0.05 ml samples of supernatant sera were added to an incubation vessel in which a strip of guinea pig ileum was suspended and attached to a lever-arm recording device. Normal rat serum-agar incubation mixtures are known to result in the production of anaphylatoxins which are characterized by the following criteria: a) they cause contraction of atropinized ileum, b) these contractions are inhibited by pre-treatment of the muscle preparation with antihistamines, and c) repeated application leads to diminishing responsiveness and eventually desensitization (tachyphylaxis). Desensitized preparations retain responsiveness to histamine and bradykinin. In our experiments all these criteria were met for both NRS-agar and NRS-RHCF-1 mixtures (Fig. 3). In addition, the activity generated by cyst fluid factors was shown to be analogous to anaphylatoxins in that tachyphylaxis induced by NRS-agar cross-desensitized to NRS-RHCF-1 and vice versa. Mepyramine maleate pre-treatment of smooth muscle preparations completely abolished responses to histamine, NRS-agar or NRS-RHCF-1, but contractions were still obtained by the addition of 0.05 ug of bradykinin to the bath.

The *in vitro* demonstration of C3 conversion and the production

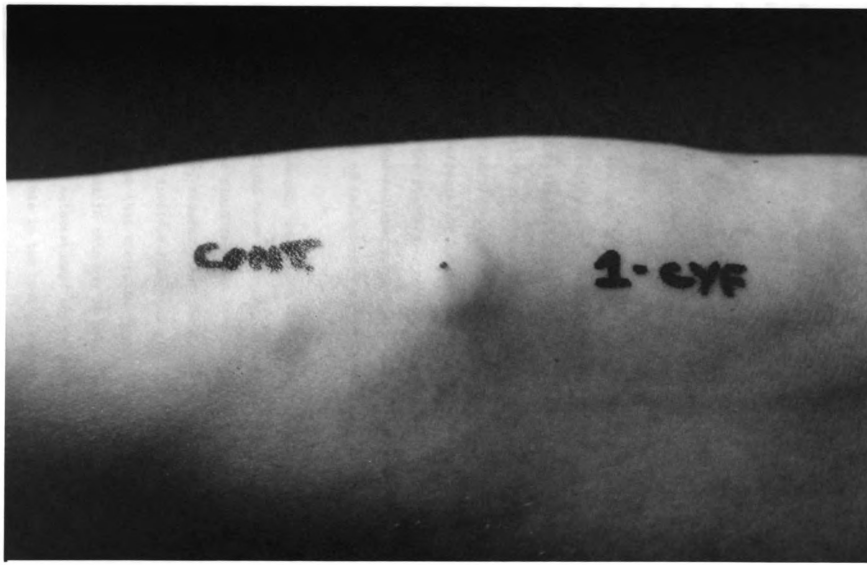
Figure 3. Smooth muscle contraction in response to sequential stimulation. Vertical bars represent height of contraction (mm) registered by guinea pig ileum strip suspended in bath to which were added histamine (open bar), RHCF-1 plus normal rat serum (dark stipple), agar plus normal rat serum (light stipple) or bradykinin (diagonal hatch). The preparation was mephraminized at ↑ .

CONTRACTION (mm)
10
20
STIMULUS
HIST
RHCF
HIST
RHCF
HIST
RHCF
HIST
AGAR
HIST
AGAR
HIST
AGAR
HIST
AGAR
HIST
RHCF
HIST
RHCF
BRADY
HIST
BRADY
RHCF

of smooth muscle contracting substances suggested the possibility that RHCF-1 might cause changes in vascular permeability *in vivo*. Rats prepared by the i.v. inoculation of Coomassie blue dye were given intradermal doses of 0.05 ml of RHCF-1, histamine (10 ug) or buffer diluent. Marked extravasation of dye occurred within 10 min at sites receiving RHCF-1 or histamine. Reactions reached up to 8 mm in diameter 10 min after inoculation and tended to subside rapidly thereafter. Control sites showed only minimal extravasation of dye at the site of needle trauma. Administration of antihistamines prior to the intradermal inoculations completely abolished reactivity to histamine. However, some extravasation of dye persisted at sites inoculated with RHCF-1 although responses to this fraction were substantially reduced.

The ability to produce vascular permeability changes *in vivo* was not restricted to rats. Three normal human volunteers were given inoculations of 0.05 ml of RHCF-1 intradermally in the skin of the forearm. Marked wheal and erythema reactions occurred in all subjects with maximum reactivity evident by 10 min post-inoculation (Fig. 4). In one case diffuse swelling of the site developed over the succeeding hours and this area remained swollen and mildly painful for 2 days.

Fiugre 4. Response to intradermal inoculation of 0.05 ml RHCF-1 (rat hydatid fluid fraction) in normal human subject. On the left (control) 0.05 ml of the buffer diluent was given. The photograph was taken 10 min after inoculation.


CONT
1-CYF

DISCUSSION

These results indicate that factors present in hydatid cyst fluid from rats or sheep are capable of interacting with serum complement from a variety of species. These substances do not appear to be host-derived since removal of rat serum proteins from cyst fluid by immunoadsorption did not reduce the effectiveness of RHCF in depleting complement levels in guinea pig serum. Additional indicators of complement fixation were C3 conversion to an extent comparable to that achieved with zymosan, and the generation of smooth muscle contracting activity similar in its characteristics to the anaphylatoxin produced in rat serum by incubation with agar. It did not appear that the vascular permeability changes which were observed *in vivo* were entirely attributable to anaphylatoxin production since some alterations of permeability were effected in antihistamine-treated rats, and the protracted course of the reaction in one of the human subjects was not typical of histamine-mediated responses. Possibly other pathways leading to the release of inflammatory mediators are activated *in vivo*.

The mechanisms whereby complement activation is initiated by *E. granulosus* cyst fluid have yet to be determined, although recent work with bladder fluid from *Taenia taeniaeformis*, in which comparable factors occur, suggests that the alternate or properdin pathway is predominantly involved (Hammerberg et al, 1976). Sufficient evidence exists to propose that the production of factors capable of interacting with complement in this way is a characteristic of a broad spectrum of taeniid metacestodes, and we have speculated that they play a crucial role in the host-parasite relationship

(Hammerberg et al, unpublished; Hustead and Williams, 1976).

The manifestations of complement activation which we have demonstrated may be of importance in the shock syndrome produced by hydatid fluid injections in normal animals. The respiratory and cardiovascular responses which characterize this syndrome have been investigated by Tabatabai et al (1973, 1974). They showed that pre-treatment with antihistamines abolished responses to intravenously administered cyst fluid in dogs, but was less effective in sheep. Atropinization was without effect in either species and clear evidence of tachyphylaxis appeared when dogs were given repeated sub-lethal doses of SHCF.

Tabatabai et al considered that their findings were probably the result of immunologically mediated histamine release, and that prior sensitization to cross reacting antigens was involved in the induction of the response in normal animals. Reaginic antibodies are produced in both definitive and intermediate hosts which become infected with *E. granulosus* (Williams and Perez-Esandi, 1971; Schantz, 1973; Dessaint et al, 1975) and cross reacting antigens are known to occur in closely related helminths which may parasitize animals and man. While an allergic mechanism may indeed account for the shock phenomenon in suitably sensitized hosts, our observations on the interaction between parasite factors and complement suggest that this also should be considered as a contributing mechanism in the pathogenesis of the syndrome. Likewise, the widespread occurrence of non-specificity in immediate hypersensitivity skin tests for hydatid cyst infection (Varela-Diaz and Coltorti, 1974; Cherubin, 1969) can be explained on the basis of allergic sensitization to cross reacting antigens. However the generation

of anaphylatoxins *in vivo* by cyst fluid components cannot be ignored as a possible element in the production of rapid wheal and flare responses in the skin. Further studies on the pathophysiological effects of these soluble complement-activating factors are presently under way in our laboratory. They do not appear to be related to the complement-fixing activity associated with insoluble calcareous granules described by Kassis and Tanner (1976) although they may prove to have similar roles in the host-parasite relationship.

ACKNOWLEDGEMENTS

This work was supported by U.S.P.H.S. Grant AI-10842. This is journal article no. 7682 from the Michigan Agricultural Experiment Station. We thank Drs. R. A. Patrick and David Liu for helpful discussions and advice during the course of this work.

LITERATURE CITED

AVRAMEAS, S. and TERNYNCK, T. (1969). 'The cross-linking of proteins with glutaraldehyde and its use for the preparation of immunoadsorbents.' Immunochem. *6*:53-66.

CHERUBIN, C. E. (1969). 'Nonspecific reactions to Casoni antigen.' Am. J. Trop. Med. Hyg. *18*:387-390.

DESSAINT, J. P., BOUT, D., WATTRE, P. and CAPRON, A. (1975). 'Quantitative determination of specific IgE antibodies to *Echinococcus granulosus* and IgE levels in sera from patients with hydatid disease.' Immunol. *29*:813-823.

DEW, H. R. (1928). 'Hydatid Disease: Its Pathology, Diagnosis and Treatment.' pp. 332-330. Sydney Australasian Medical Publishing Co.

DIAS DA SILVA, W., EISELE, J. W. and LEPOW, I. H. (1967). 'Complement as a mediator of inflammation. III. Purification of the activity with anaphylatoxin properties generated by interaction of the first four components of complement and its identification as a cleavage product of C3.' J. Exp. Med. *126*:1027-1048.

GIUSTI, L. and HUG, E. (1923). 'Proprietes pharmacodynamiques du liquide hydatique.' Comptes Rendus Seanc Soc. Biol. *88*:344-346.

HAMMERBERG, B., MUSOKE, A. J., HUSTEAD, S. T. and WILLIAMS, J. F. (1976). 'Anticomplementary substances associated with *Taenia taeniaeformis* larvae.' *In*: E. J. Soulsby, ed. Pathophysiology of Helminth Infections. London and New York Academic Press. (In press).

HANDJANI, A. M., FARPOUR, A., MECHANIC, K., HAGHIGHAT, A. and DUTZ, W. (1969). 'Cardiovascular echinococcosis.' Am. J. Surgery *117*:666-670.

HUSTEAD, S. T. and WILLIAMS, J. F. 'Permeability studies on Taenid metacestodes: Antibody mediated effects on membrane permeability in larvae of *Taenia taeniaeformis* and *Taenia crassiceps*.' J. Parasitol. (In press).

KASSIS, A. I. and TANNER, C. E. (1976). 'The role of complement in hydatid disease: *in vitro* studies.' Int. J. Parasitol. *6*:25-36.

KABAT, E. A. and MAYER, M. M. (1961). *In*E. A. Kabat, ed. Experimental Immunochemistry, 2nd ed., Chapt. 4, Charles C. Thomas, Springfield, Illinois.

LEID, R. W. and WILLIAMS, J. F. (1974). 'The immunological response of the rat to infection with *Taenia taeniaeformis*. II. Characterization of reaginic antibody and an allergen associated with the larval stage.' Immunology. *27*:209-225.

LEPOW, I. H., DIAS DA SILVA, W. and PATRICK, R. A. (1969). *In*: Henry A. Movat, ed. Cellular and Humoral Mechanisms in Anaphylaxis and Allergy. Proceedings of the Third International Symposium of the Canadian Scoiety for Immunology, S. Karger AG., Basel and New York, p. 237-252.

MULLER-EBERHARD, H. J. and FJELLSTROM, K. E. (1971). 'Isolation of the anticomplementary protein from cobra venom and its mode of action on C3.' J. Immunol. *107*:1666-1672.

ROCHA E SILVA, M. and GRANA, A. (1945). 'Shock produced in dogs by hydatid fluid.' Am. J. Physiol. *143*:306-313.

SCHANTZ, P. M. (1973). 'Homocytotropic antibody to *Echinococcus* antigen in sheep with homologous and heterologous larval cestode infection.' Am. J. Vet. Res. *34*:1179-1181.

TABATABAI, M., BOROOMAND, K., GETTNER, S. and NAZARIAN, I. (1973). 'Respiratory and cardiovascular responses resulting from intravenous injection of sheep hydatid cyst fluid to dogs.' Exp. Parasitol. *34*:12-21.

VARELA-DIAZ, V. M. and COLTORTI, E. A. (1974). 'Limitaciones de la intradermorreaccion de Casoni en el inmunodiagnostico de la hidatidosis humana.' Boletin de la Oficina Sanitaria Panamericana. *76*:400-405.

VARELA-DIAZ, V. M., WILLIAMS, J. F., COLTORTI, E. A. and WILLIAMS, C. S. F. (1974). 'Survival of cysts of *Echinococcus granulosus* after transplantation into homologous and heterologous hosts.' J. Parasitol. *60*:608-612.

WILLIAMS, J. F. and PEREZ-ESANDI, M. V. (1971). 'Reaginic antibodies in dogs infected with *Echinococcus granulosus*.' Immunology *20*:451-455.

INTERACTION BETWEEN *TAENIA TAENIAEFORMIS* AND THE COMPLEMENT SYSTEM

B. HAMMERBERG AND J. F. WILLIAMS

Department of Microbiology and Public Health

Michigan State University

East Lansing, Michigan 48824

SUMMARY. Factors present in the cystic bladder fluid of metacestodes of *Taenia taeniaeformis* and released by these parasites maintained *in vitro* were shown to interact with the complement system *in vitro* and *in vivo*. This interaction resulted in the depletion of hemolytic complement in normal sera of several species, the generation of anaphylatoxin-like activity *in vitro*, the conversion of C3, and the production of vascular permeability changes *in vivo*. The substances appeared to initiate complement fixation non-immunologically via both the alternative and classical pathways. Intravenous administration of parasite factors in rats led to profound depression of circulating complement levels and the rats did not become refractory to this effect after repeated dosing over a 3 week period. Circulating levels of hemolytic complement were not altered in infected rats over the first 8 weeks of infection. Complement-dependent inflammatory responses in the skin of rats were inhibited in animals given intravenous doses of parasite factors. The possibility is raised that local consumption of complement around the metacestode *in vivo* could contribute to its successful evasion of inflammation and immune rejection during infection.

INTRODUCTION

It has been known for many years that infection of the rat with *Taenia taeniaeformis* results in the development of a specific acquired resistance to challenge infection which is antibody-mediated (1,2). In recent studies it has been shown that protective antibodies produced during the first several weeks of infection are exclusively in the IgG_{2a} immunoglobulin subclass (3,4) and that depletion of complement in challenged recipients of protective antibodies

interferes with the manifestation of resistance (5). Nevertheless, parasites derived from primary exposure to infection continue to survive in the tissues of highly immune rats, and this ability to evade immunologic attack is rapidly acquired during larval development (5,6).

The continued survival of the parasite would appear to depend, at least in part, on successful avoidance of complement-mediated effector mechanisms. In preliminary studies we found that the fluid surrounding larvae of *T. taeniaeformis in situ* in hepatic cysts was able to fix complement in normal serum from rats or guinea pigs (7). We have further investigated this phenomenon and report here on the interaction of larval factors with the complement system. These interactions result in; consumption of complement via both the alternate and classical pathways; conversion of C3 and the generation of anaphylatoxin *in vitro*; the depression of circulating hemolytic complement activity, and alterations in vascular permeability *in vivo*. In an accompanying paper we describe the physico-chemical characteristics of substances extracted from larvae which are responsible for these effects.

MATERIALS AND METHODS

Reagents

Carbowax PEG 20,000 (Union Carbide, Cleveland, Ohio) was purchased from Fisher Scientific Company. Bovine serum albumin (BSA), bradykinin triacetate, histamine dihydrochloride, and cyproheptadine, were obtained from Sigma Chemical Company. Mepyramine maleate was a gift from Merck, Sharp and Dohme Research Laboratory

(Rahway, New Jersey). Diphenhydramine hydrochloride (Benadryl) was purchased from Parke, Davis and Company (Detroit, Michigan).

Cobra venom from *Naja haje* was purchased from Sigma Chemical Company and the active anticomplementary cobra venom factor (CoF) was purified chromatographically by the method described by Ballow and Cochrane (8).

Sheep red blood cells (SRBC) were collected in sterile Alsever's solution and stored at 4°C before use. Antiserum to washed SRBC was prepared in rats by injecting 8 X 10^6 cells intraperitoneally into mature 200 g rats and collecting serum 8 days later. A specific antiserum against purified human complement component C3 was prepared in sheep. C3 was generously supplied by Dr. R. A. Patrick. This reagent produced a single band in double diffusion and immunoelectrophoresis tests with purified C3 and with normal human serum (NHS), and prior incubation of NHS with zymosan resulted in conversion of the band to a more anodally migrating form.

Sources of Parasite Materials

Female rats (Spb [SD] BR) 28 days old were obtained from Spartan Research Animals, Haslett, MI, and each was dosed orally with 500 eggs of *Taenia taeniaeformis*. Eggs were liberated by dissection from gravid tapeworm segments collected from the feces of experimentally infected cats, washed in saline and stored at 4°C in distilled water containing penicillin (1000 i.u/ml), streptomycin (100 µg/ml) and fungizone (2.5 µg/ml). Three to five months later the rats were killed with CO_2 vapor and parasites were dissected from the hepatic cysts (Fig. 1). These larvae were washed 10 times by sedimentation in 10 volumes of distilled water,

and 3 times in sterile 0.15M NaCl before transfer to sterile flasks containing tissue culture medium. Fifty larvae were incubated with 200 ml of Hanks' BME at 37°C and the supernatant fluid collected after 24 hrs. By following this procedure bacterial contamination of the medium was avoided in almost all cultures. Those which became contaminated were discarded. Parasites maintained in this manner *in vitro* retained motility, gross morphologic appearance and infectivity for cats. Supernatant fluids were concentrated 100-fold by dialysis against Carbowax prior to dialysis against distilled water for 48 hr at 4°C. This preparation was termed *in vitro* products (IVP).

Bladder fluids from the cystic parasites were collected by needle puncture of larvae in infected livers at 3-5 months of infection and concentrated 3-fold by Carbowax treatment before dialysis against distilled water for 48 hr. This preparation was termed cyst-fluid (CYF). Bladder fluids were similarily collected from parasites in the livers of rats infected for 20 days. In some experiments larvae freed from the liver by dissection were incubated directly in normal serum samples and the remaining complement activity in the serum assayed after 30 min at 37°C.

Absorption of Host Serum Proteins from Parasite Material

Antisera against normal rat serum (NRS) or rat immunoglobulin were polymerized by glutaraldehyde treatment, according to the method of Avrameas and Ternynck (9). Polymerized antisera were used to absorb out rat serum proteins which were present in cyst fluid. The effectiveness of absorption was determined by double diffusion in gel tests.

Assay for Hemolytic Complement

Hemolytic complement titers were measured in CH_{50} units following a modification of the procedure of Kabat and Mayer (10). Sheep erythrocytes (SRBC) were sensitized with rat anti-SRBC sera. 3 X 10^8 sensitized cells (EA) in 0.5 ml of veronal buffered saline containing 1.5 X 10^{-4}M Ca^{++} and 5 X 10^{-4} M Mg^{++}, pH 7.4, ionic strength 0.15, were added to 0.5 ml of the complement source. After incubation at 37°C for 1 hr, 2 ml of buffer were added to each tube and the absorbance at 541 nm recorded after centrifugation at 1000 g for 10 min. To assay for complement-fixing activity the inhibitor in distilled water was incubated with equal volumes of either normal guinea pig serum (GPS) or NHS for 60 min at 37°C, and the mixture assayed for hemolytic complement as described above. Comparison with the distilled water control CH_{50} level provided a percent inhibition value. A modification of the above procedure, described by Sakamoto and Nishioka (11), was used when measuring rat hemolytic complement titers; in this modification dilutions of rat serum plus EA were incubated at 22°C for 2 hr instead of 37°C for 1 hr.

Quantitation of C3 Activation Via Alteranate vs. Classical Pathway

EAC $\overline{142}$ were prepared by the method described by Leon (12) in which 90 ml of EA at 3.0 X 10^8 cells/ml were incubated with 22.5 ml of a 1:10 dilution of NHS previously incubated with zymosan (Sigma) at a concentration of 15 mg/ml for 60 min at 37°C. The incubation time of EA with zymosan-treated serum was variable depending upon optimal EAC$\overline{142}$ formation. These cells were used immediately after being washed in cold triethanolamine-buffered saline (TBS), pH 7.5 containing 1.5 X 10^{-4} M Ca^{++} and 5 X 10^{-4} M Mg^{++}. The EAC $\overline{142}$

Figure 1. Liver of rat infected with cystic larvae of *Taenia taeniaeformis* (above). Larvae dissected from within the host capsule (below). The fluid filled bladder is indicated by the arrow.

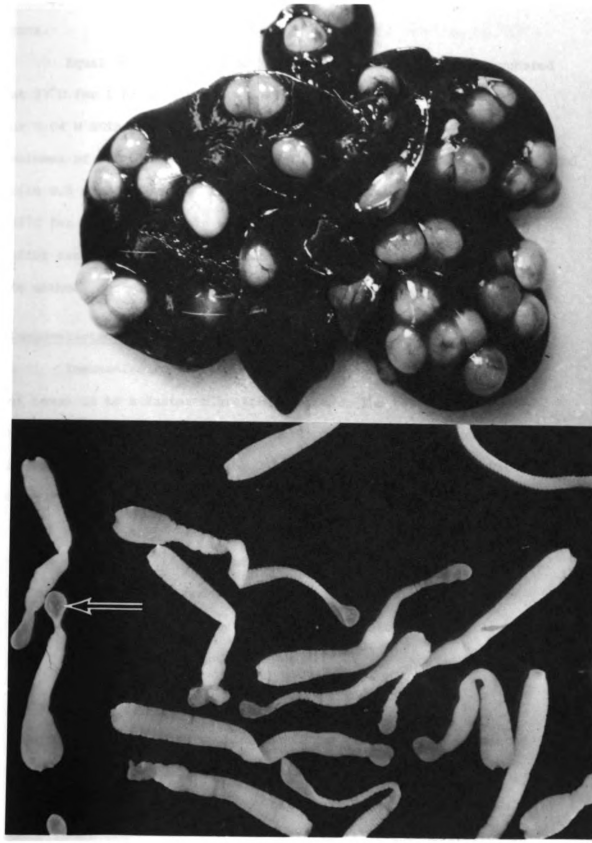


Figure 1

were lysed in dilutions of guinea pig serum (GPS) containing 0.04 M EDTA.

Equal volumes of CYF or IVP or distilled water were incubated at 37°C for 1 hr with GPS containing no chelators, 0.04 M EDTA, or 0.04 M EGTA plus 0.04 M $MgCl_2$. Serial dilutions in 2.5 ml volumes of TBS plus 0.04 M EDTA of the incubated solution were combined with 0.5 ml of 5.0 X 10^8 EAC $\overline{142}$/ml before incubation again at 37°C for 1 hr. Hemolysis was quantitated by absorbance at 541 nm after centrifugation. CH_{50} units per ml were calculated according to methods described by Kabat and Mayer (10).

Immunoelectrophoretic Analysis

Immunoelectrophoresis was employed to detect conversion of human C3 to a faster migrating species. The test was conducted using microscope slides coated with 2% agarose in a veronal buffer pH 8.6 and current applied for 2 1/2 hrs at 15 mamp/slide. Troughs were filled with monospecific anti-human C3. Positive conversion slides were obtained by incubation of human serum with an equal volume of zymosan (1 mg/ml) at 37°C for 1 hr before electrophoresis. The influence of Ca^{++} and Mg^{++} chelation was determined by prior treatment of electrophoresed samples with either 0.01 M EDTA or 0.01 M EGTA plus 0.05 M $MgCl_2$.

Assay for Anaphylatoxin Activity

Normal rat serum samples incubated with an equal volume of CYF or IVP at 37°C for 1 hr were assayed for the presence of anaphylatoxin using the bioassay procedure described by Dias Da Silva et al (13). This involved measurement of the deflection in

millimeters of a balanced lever arm attached to a 1 cm segment of guinea pig ileum suspended in a bath of Tyrode's solution at 37°C. Deflections were registered after additions to the bath of 50 ng of histamine, 150 ng bradykinin, or 0.05 ml of NRS incubated with either CYF, IVP or noble agar. The criterion for the production of anaphylatoxin in NRS was the generation of tachyphylaxis and cross densensitization of the ileum with repeated application of NSR-agar, NRS-CYF or NRS-IVP samples. Ileum preparations were treated with 50 ngm of the antihistamine (mepyramine maleate) in order to distinguish between contractile effects mediated by histamine and those due to bradykinin.

Assay for Localized Vascular Permeability Changes

Four groups of four rats were shaved over the back and each rat received intradermal injections of 0.05 ml of a solution containing 1 µg/ml histamine, CYF, IVP, or PBS followed immediately by intravenous inoculation of 0.5 ml of a 1% solution of Coomassie Brilliant blue. One hr before skin testing three of the groups received one of the following antihistamines: diphenylhydramine HCl at 20 mg/kg, mepyramine maleate at 20 mg/kg, or cyproheptadine at 20 mg/kg. These dosages of antihistamines were chosen since they prevented any detectable bluing at histamine injection sites. The influence of systemic complement depletion on the skin bluing reactivity of each preparation was determined by the intravenous administration of chromatographically purified CoF prior to skin sensitization. Total hemolytic complement levels were determined in rats prepared in this way and found to be consistently less than 5% of normal values.

Passive Cutaneous Anaphylaxis

Complement-dependent, histamine-mediated passive cutaneous anaphylaxis (PCA) reactions were generated in normal adult rats by intradermal injection of serum containing IgG_{2a} antibodies from rats sensitized to bovine serum albumin (BSA) (14), followed 2 hrs later by intravenous administration of 0.5 ml of BSA (1 mg/ml) containing 1% Coomassie Brilliant blue. Diameters of blued areas in the skin were compared. Solutions to be assayed for complement depleting activity were given intravenously 30 min prior to BSA challenge. Positive controls for skin bluing in complement-depleted rats were established by intradermal administration of 0.05 ml of solutions of histamine (1 μg/ml).

RESULTS

The addition of CYF or IVP to normal serum of rats, guinea pigs or humans in the proportions described above consistently resulted in depression of total hemolytic complement levels (measured in CH_{50} units) (Table 1). Preincubation of EA in parasite factors followed by washing in buffer, resulted in no change in their susceptibility to complement-mediated lysis. Rabbit antiserum against whole normal rat serum (anti-NRS) and sheep anti-rat Fab produced several precipitin bands when examined in double diffusion tests against CYF, but none against IVP. Absorption of CYF with polymerized rabbit anti-NRS removed all detectable activity with these antisera. However, the capacity of absorbed CYF to deplete complement levels in normal serum samples was unaffected. Absorbed CYF was used in the experiments described below. Cyst fluid aspirated in small amounts from cysts 20 days after infection was found to inhibit

TABLE I

Percent depression of total hemolytic complement levels

Source of parasite material	Serum source Rat	Guinea Pig	Human
CYF	70	60	50
IVP	75	70	60

complement to the same extent as cyst fluid from later stages of development. A single live 2 month-old parasite incubated in 1 ml of NRS resulted in a decline of total hemolytic complement to less than 5% of the normal value in 30 mins.

The results of CYF or IVP incubation with guinea pig serum in the presence of 0.04 M EDTA or 0.04 M EGTA plus 0.04 M $MgCl_2$ are shown in Table 2. Complement depletion was reduced by 37% in the EGTA-$MgCl_2$ chelated preparations, but was inhibited almost totally in EDTA-treated mixtures.

After incubation of CYF or IVP with equal volumes of normal human serum for 1 hr at 37°C, the mixtures were subjected to immunoelectrophoretic analysis using sheep anti-human C3. Control samples of serum were incubated with either buffer or activated zymosan. The results are shown in Fig. 2. Conversion of C3 was evident in the NHS-parasite factor mixtures, but no detectable change occurred in the control serum. Conversion was seen in the zymosan-serum mixture. When EDTA was added to each incubation mixture at a concentration of 0.01 M, no conversion was observed; however, 0.01 M EGTA plus 0.05 M $MgCl_2$ did not block completely the conversion of C3 by parasite factors, and had no effect on zymosan conversion.

Incubation of CYF or IVP with NRS at 37°C for 60 min resulted in the generation of smooth muscle-contracting activity. The addition of 0.05 ml of this mixture to the isolated ileum preparation produced a greater degree of contraction than that obtained with 50 ng of histamine (Fig. 3). Contractile activity was also generated by the incubation of NRS with agar. After repeated application of the NRS-agar mixture the ileum became refractory to NRS-CYF or NRS-IVP, but

TABLE II

Effect of differential cation chelation on C3 consumption by products of *T. taeniaeformis*

Complement source	Percent consumption
GPS + IVP	75
GPS + IVP + EDTA	2
GPS + IVP + EGTA + Mg^{++}	47

Figure 2. Immunoelectrophoretic analysis of human serum following incubation with either CYF (top) or zymosan (bottom). Normal serum was used as a control. The troughs contained sheep anti-human C3.

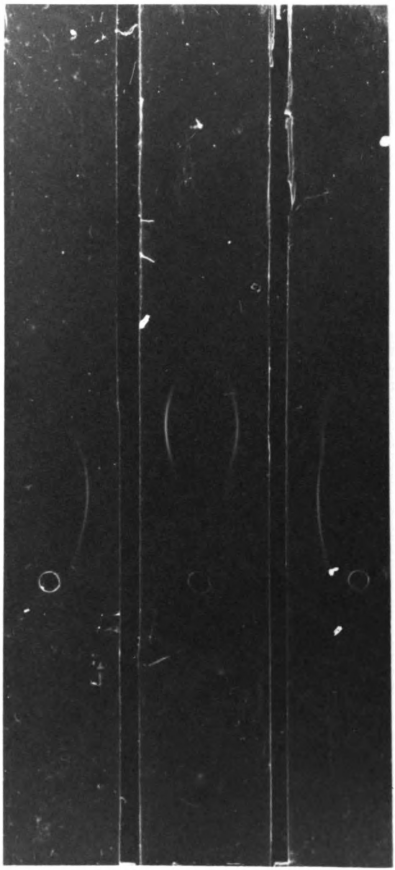


Figure 2

Figure 3. Smooth muscle contraction in response to sequential stimulation. Vertical bars represent height of contraction (mm) registered by guinea pig ileum strip suspended in bath to which were added histamine (Stippled bar), CYF plus normal rat serum (dark stripe) or agar plus normal rat serum (light stripe).

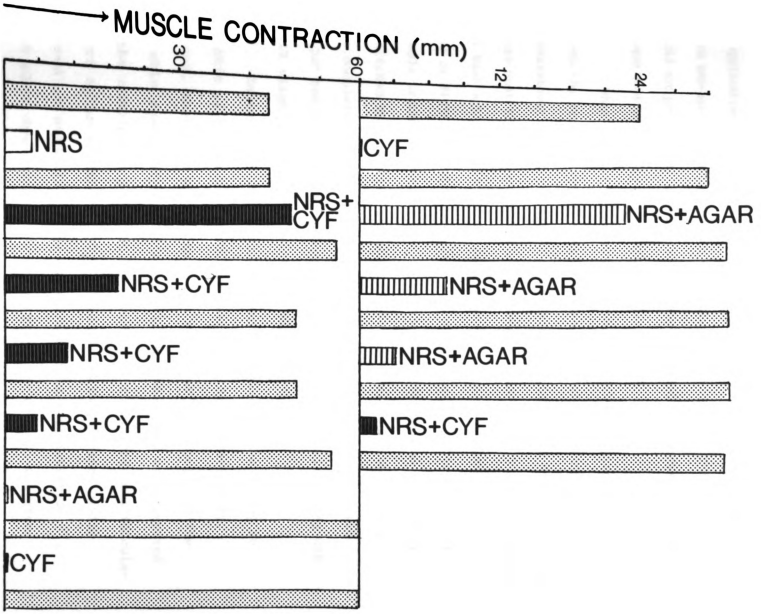

MUSCLE CONTRACTION (mm)
30
60
12
24
NRS
CYF
NRS+
CYF
NRS+AGAR
NRS+CYF
NRS+AGAR
NRS+CYF
NRS+AGAR
NRS+CYF
NRS+AGAR
CYF

Figure 3

remained responsive to the addition of 50 ng histamine. In the reciprocal experiment tachyphylaxis was produced by the repeated application of NRS-CYF and the preparation was also refractory to NRS-agar but responsive to histamine: NRS, CYF or IVP alone did not produce any contraction. Mepyraminized ileum preparations were responsive only to bradykinin (150 ng).

When CYF or IVP preparations were inoculated intradermally into the backs of normal rats, intense local bluing occurred after intravenous administration of Coomassie Brilliant blue dye. Reactions were most intense by 10 min and reached a diameter of 8 mm, comparable to that achieved with 50 ng histamine. Control inoculations of PBS or distilled water produced only minor bluing at the point of needle trauma. Pretreatment of rats with antihistamine completely abolished the reaction at sites of histamine inoculation but did not completely ablate skin bluing due to CYF. Intravenous doses of CoF resulted in a high degree of inhibition, but skin bluing was still slightly visible at sites inoculated with CYF.

Intradermal inoculations of CoF produced intense bluing in the skin of normal rats which had been given dye intravenously. However, when rats were given 0.5 ml of CYF or IVP along with the dye, skin reactivity to CoF was abolished. When rats were prepared for the complement-dependent PCA reaction by the intradermal inoculation of serum containing IgG_{2a}-antibodies to BSA, intense bluing occurred at sensitized sites after intravenous administration of BSA plus dye 2 hrs later. When CYF or IVP (0.5 ml) was given along with the BSA-dye mixture the PCA reaction was completely abolished. However, these rats remained responsive to intradermal

inoculation of 50 ng histamine.

In an attempt to determine if large numbers of parasites developing in the liver of a rat could depress circulating complement, CH_{50} levels were measured serially during the course of an infection. Six 28 day old rats were bled from the orbital plexus prior to infection with 1000 eggs of *T. taeniaeformis* and then bled at 7 day intervals thereafter for 60 days. Six uninfected rats of the same age and from the same litter were also bled. The results are shown in Fig. 4. Both groups showed fluctuating levels with an upward trend in circulating hemolytic complement levels over the period of study but no differences were apparent. More than 100 parasites were present in the liver of each infected rat at necropsy.

Intravenous administration of either CYF or IVP to normal rats resulted in a fall in circulating hemolytic complement levels. One half ml of a preparation of CYF which was able to deplete hemolytic complement *in vitro* by 60% caused a reduction of circulating complement *in vivo* to 15-20% of normal (pre-inoculation) level within 30 min. Restoration of normal levels occurred in 24 hr. When rats were given the same dose intraperitoneally the decline was much less pronounced and levels fell by only 50%, returning to normal in about 8 hr. In order to determine whether rats became refractory to the complement-depleting effects of CYF, 5 rats were given intraperitoneal inoculations of 0.5 ml CYF every 8 hrs for 6 days. On the seventh day intravenous administration of 0.5 ml CYF produced a decline in CH_{50} levels to approximately 50% of normal values. Injections of CYF were continued at weekly intervals for 3 weeks in these same rats and after the third dose hemolytic complement was shown to

Figure 4. Serial determinations of serum complement (CH_{50}) levels of rats dosed with 1000 eggs of *Taenia taeniaeformis* at 28 days of age. Control rats (0-0) were uninfected littermates.

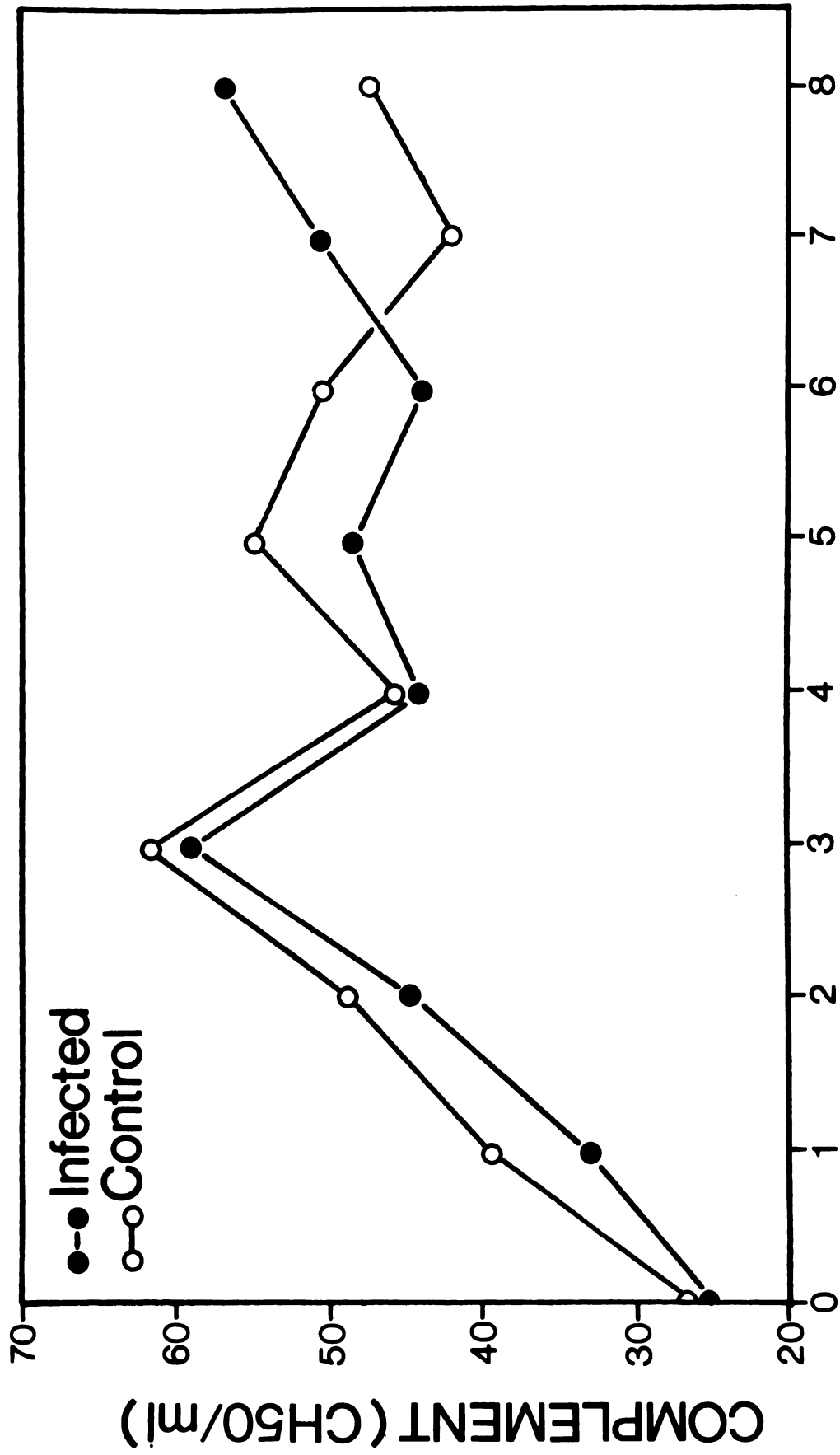

COMPLEMENT (CH50/ml)
70
60
50
40
30
20
0
1
2
3
4
5
6
7
8
WEEKS
Infected
Control

Figure 4

have fallen by 50% of the pre-inoculation level in 30 minutes. Serum samples from these rats were tested in double diffusion against CYF but no precipitin bands were detectable.

DISCUSSION

These experiments indicate that factors present in the cyst fluid of *T. taeniaeformis* as early as 20 days after infection, and released by live worms maintained *in vitro*, are able to interact in a non-immunologic manner with the complement system. Consumption of hemolytic complement occurred in serum of normal rats, the natural host, and in normal guinea pig and human sera. The reaction did not appear to be dependent on the presence of immune complexes in the parasite cyst fluid, since immuno-absorption effectively removed detectable immunoglobulins but did not affect the complement depleting activity of this material, and immunoglobulins were not detectable in the *in vitro* culture supernatants. Consumption of complement was inhibited by EDTA but was only partially (37%) inhibited by selective chelation of Ca^{++} suggesting that the reaction could proceed to some extent via the alternate pathway (15,16). Similarily, the conversion of C3 to an electrophoretically faster migrating species was only partially inhibited under conditions which permit the activation of complement via the alternate pathway (17).

Smooth muscle-contracting substances generated by the incubation of parasite materials with NRS appeared to be analogous to anaphylatoxins since reciprocal cross-tachyphylaxis occurred with NRS-agar preparations, the activity was inhibited by antihistamine, and responsiveness to bradykinin was unaffected. However, *in vivo* vascular permeability changes in rats differed from those due to anaphylatoxins in that substantial but not complete inhibition was seen in both antihistamine-treated and in complement-depleted rats. This finding suggests that parasite materials may influence permeability through

mechanisms other than those involving the complement system. Our recent finding that substances present in cyst fluid interact with the intrinsic coagulation pathway may be of significance in this regard.[1]

Parasite materials were evidently able to deplete complement levels *in vivo* resulting in profound reduction in CH_{50} titers and in the ablation of skin-bluing produced by CoF or by complement-dependent antigen-antibody-mediated cutaneous inflammation. The latter finding is particularly important in view of the observation of Leid and Williams (18) that IgG_{2a} antibodies to *T. taeniaeformis* were ineffective in PCA reactions. In their experiments rats sensitized intradermally with IgG_{2a} failed to show skin-bluing upon intravenous challenge with blue dye plus a crude antigenic extract of *T. taeniaeformis*, whereas IgE-mediated reactions were consistently provoked with this extract. They suggested that IgG_{2a} antibodies produced in response to infection might differ from those classically stimulated by parenteral inoculation of antigens. In retrospect, however, it seems likely that the crude extract inoculations were causing rapid depletion of complement and hence inhibiting the IgG_{2a}-mediated PCA, but permitting the complement-independent IgE-mediated PCA reactions (14) to proceed normally.

The pattern of *in vivo* complement-depletion produced by inoculation of parasite materials differed from that observed with CoF treatment. Repeated exposure did not appear to lead to a refractory state, comparable to that which occurs in 5-6 days when rats are given multiple doses of CoF (5). The onset of refractoriness

Footnote 1. Manuscript submitted for publication.

is generally attributed to immune elimination following recognition of antigenic determinants in the active venom protein (19). Rats which had received 20 inoculations of CYF over a 4 week period still suffered acute, though less profound, reductions in circulating complement shortly after treatment, and no precipitating antibodies were detected in their sera. This suggests that the complement interacting substances have very limited antigenicity in the rat. It may be that the recently reported anticomplementary activity of pentosan-poly-sulfoester in humans is more closely analogous to the activity of larval substances (20).

In spite of the efficacy of the parasite materials in depleting complement *in vivo* after inoculation, there was no evidence of lowered levels in the serum of rats during the first two months of infection. This could be attributable to; the occurrence of little or no release of complement-interacting materials by the parasite *in vivo*; effects of released factors being exerted only at the local level around the cyst; or systemic effects on circulating complement being counteracted by homeostatic mechanisms which tend to compensate and restore normal levels. In view of the fact that hemolytic complement is not detectable in the plasma-protein rich fluid surrounding the parasite *in situ* (7) we believe that release probably does occur *in vivo*, but possibly at a rate insufficient to alter systemic levels. Experimental quantitation of release *in vivo* may be achieved if specific markers can be incorporated into the active molecules, once they have been characterized.[2]

The significance, if any, of these complement-fixing substances

Footnote 2. Manuscript submitted for publication.

in the host-parasite relationship cannot be assessed at this time. The susceptibility of taeniid metacestodes to antibody-mediated attack in the presence of heat-labile serum factors(5), and the finding that complement depletion *in vivo* interferes with the efficacy of passive immunization (5), indicate that prolonged survival in the tissues might involve interference with effector mechanisms which depend on complement. Indeed, under some circumstances complement alone appears to be able to have a deleterious effect on taeniid parasites (21). Local consumption of complement in the soluble phase by factors released by these parasites might provide a defensive mechanism for evasion of either non-specific or specific attack. Similar mechanisms have been proposed for the survival of bacteria. However, such a reaction would be expected to lead to the generation of anaphylatoxic activity which, in turn, would result in cellular infiltration and local vascular changes. In serial studies of the histopathologic response during primary infection there was a very marked infiltration of eosinophils around hepatic parasites, which reached peak levels during the fifth week, but then subsided (22). After several months of infection, some eosinophils were still present in the connective tissue of the local host reaction around the worms but there was no evidence of an acute inflammatory process which one might anticipate if local complement fixation were taking place. In this study, anaphylatoxic activity was not detectable in cyst fluid samples applied directly to the smooth muscle preparation. It is possible that endogenous enzymatic deactivation of anaphylatoxins may lead to reduced inflammatory activity, particularly if they are produced but then

deactivated at some distance from the blood vasculature of the connective tissue capsule. It is also possible that proteolytic enzymes released by the parasites contribute to degradation of biologically active complement fragments. There is histochemical evidence of release of proteases by *T. taeniaeformis in situ* (23) and the enzymatic activities of *in vitro* parasite products could be examined experimentally to explore this hypothesis.

In recent work we and others (21,24,25) have detected complement interacting substances in a variety of taeniid cestodes. Whatever the role of these substances may be during infection, their presence provides some basis for interpreting several other phenomena associated with these organisms. In particular the high proportion of normal people who show skin reactivity to taeniid extracts (26) could be due to the non-immunologic generation of vascular permeability factors. Also the anaphylactic-type shock syndrome which can be provoked in non-infected normal animals by the intravenous administration of taeniid extracts might be due to systemic effects of massive complement fixation (27). Identification of the nature of these factors may therefore be relevant in context of the clinical diagnosis of taeniiases as well as in basic investigations on host-parasite relationships and evasion of rejection. Details of the physico-chemical characterization of these substances in *T. taeniaeformis* are presented in an accompanying paper.[2]

ACKNOWLEDGEMENTS

This work was supported by NIH Grant AI-10842. We are indebted to Dr. A. J. Musoke for helpful advice in several aspects of this study.

This is Journal Article No. _____ from the Michigan Experimental Station.

REFERENCES

1. Campbell, D. H. 1938. Further studies on the "nonabsorbable" property in serum from rats infected with *Cysticercus crassicollis*. J. Immunol. 35:465-476.

2. Miller, H. M. 1932. Further studies on immunity to a metazoan parasite, *Cysticercus fasciolaris*. J. Prev. Med. 6:37-46.

3. Leid, R. W., and J. F. Williams. 1974. Immunological response of the rat to infection with *Taenia taeniaeformis*. I. Immunoglobulin classes involved in passive transfer of resistance. Immunology 27:195.

4. Musoke, A. J., and J. F. Williams. 1975. Immunological response of the rat to infection with *Taenia taeniaeformis*. III. Protective antibody response to implanted parasites. Int. J. Parasitol. 6:265-269.

5. Musoke, A. J., and J. F. Williams. 1975. Immunological response of the rat to infection with *Taenia taeniaeformis*. V. The sequence of appearance of protective immunoglobulins and the mechanism of action of $7S\gamma 2_a$ antibodies. Immunology 29:855-865.

6. Campbell, D. H. 1938. The specific protective property of serum from rats infected with *Cysticercus crassicollis*. J. Immunol. 35:195-204.

7. Hammerberg, B., A. J. Musoke, S. T. Hustead, and J. F. Williams. 1975. Anticomplementary substances associated with taeniid metacestodes. *In* Pathophysiology of Helminth infections. Ed. E. J. L. Soulsby. Academic Press, 233-240.

8. Ballow, M., and C. G. Cochrane. 1969. Two anti-complementary factors in cobra venom: hemolysis of guinea pig erythrocytes by one of them. J. Immunol. 103:944-952.

9. Avrameas, S., and T. Ternynck. 1969. The cross-linking of proteins with glutaraldehyde and its use for the preparation of immunoadsorbents. Immunochemistry 6:53-59.

10. Kabat, E. A., and M. M. Mayer. 1961. *In* Experimental Immunochemistry. 2nd edition. Charles C. Thomas, Springfield, Illinois, p. 149.

11. Sakamoto, M., and K. Nishioka. 1975. Studies on rat complement. I. Immune adherence and immune hemolysis activity of rat serum. Jap. J. Exp. Med. 45:183-189.

12. Leon, M. A. 1957. Quantitative studies on the properdin complement system. II. Kinetics of the reaction between properdin and zymosan. J. Exp. Med. 105:403-415.

13. Dias Da Silva, W., J. W. Eisele, and I. H. Lepow. 1967. Complement as a mediator of inflammation. III. Purification of the activity with anaphylatoxin properties generated by interaction of the first four components of complement and its identification as a cleavage product of C3. J. Exp. Med. 126:1027-1048.

14. Stechschulte, D. J., R. P. Orange, and K. F. Austen. 1970. Two immunochemically distinct homologous antibodies capable of mediating immediate hypersensitivity reactions in the rat, mouse and guinea-pig. Excerpta Medica, Inter. Congress Series No. 232, Proc. VII Inter. Congress of allergology, 245-254.

15. Snyderman, R., and M. C. Pike. 1975. Interaction of complex polysaccharides with the complement system: Effect of calcium depletion on terminal component consumption. Infection and Immunity 11:273-279.

16. Bryant, R. E., and D. E. Jenkins. 1968. Calcium requirements for complement-dependent hemolytic reactions. J. Immunol. 101:664-668.

17. Muller-Eberhard, H. J., and K. E. Fjellstrom. 1971. Isolation of the anticomplementary protein from cobra venom and its mode of action on C3. J. Immunol. 107:1666-1672.

18. Leid, R. W., and J. F. Williams. 1974. Immunological response of the rat to infection with *Taenia taeniaeformis*. II. Characterization of reaginic antibody and an allergen associated with the larval stage. Immunology 27:209-225.

19. Pryjma, J., and J. H. Humphrey. 1975. Prolonged C3 depletion by cobra venom factor in thymus-deprived mice and its implication for the role of C3 as an essential second signal for B-cell triggering. Immunology 28:569-580.

20. Berthoux, F. C., A. M. Freyria, P. Y. Zech, and J. Traeger. 1977. Activite anticomplementaire d'un polyanion: le polyester sulfurique de pentosan. I. Etude *in vitro* et assai *in vivo* dans les glomerulonephrites humaines. Pathologie. Biologie. 25:33-36.

21. Kassis, A. I., and C. E. Tanner. 1976. The role of complement in hydatid disease: *In vitro* studies. Int. J. Parasit. 6:25-35.

22. Ansari, A., and J. F. Williams. 1976. The eosinophilic response of the rat to infection with *Taenia taeniaeformis*. J. Parasitol. 62:728-736.

23. Lewert, R. M., and C. L. Lee. 1957. The collegenase-like enzymes of skin-penetrating helminths. Am. J. Trop. Med. Hyg. 6:473-479.

24. Hammerberg, B., A. J. Musoke, and J. F. Williams. 1977. Activation of complement by hydatid cyst fluid of *E. granulosus*. J. Parasitol. 63:327-331.

25. Herd, R. P. 1976. The cestocidal effect of complement in normal and immune sera *in vitro*. Parasitology 72:325-334.

26. Varela-Diaz, V. M., and E. A. Coltorti. 1974. Limitaciones de la intra-dermorreaccion de Casoni en el inmunodiagnostico de la hidatidosis humana. Boletin de la Oficina Sanitaria Panamericana. 76:400-405.

27. Tabatabai, M., K. Boroomand, S. Gettner, and I. Nazarian. 1973. Respiratory and cardiovascular responses resulting from intravenous injection of sheep hydatid fluid to dogs. Exp. Parasitol. 34:12-21.

PHYSICO-CHEMICAL CHARACTERIZATION OF COMPLEMENT-INTERACTING FACTORS FROM *TAENIA TAENIAEFORMIS*

BRUCE HAMMERBERG AND JEFFREY F. WILLIAMS

Department of Microbiology and Public Health

Michigan State University

East Lansing, Michigan 48824

Short Title: Complement interacting factors of *T. taeniaeformis*

ABSTRACT

Complement-fixing activity was washed from the surface of metacestodes of *Taenia taeniaeformis* and characterized physico-chemically using enzymatic digestion, gel-filtration, anion exchange chromatography, ultracentrifugation and gel electrophoresis. The results provided evidence of an association between antihemolytic activity and macromolecules containing carbohydrate, protein, sulphate and hexosamine, heterogenous in net negative charge but resistant to proteolysis and β elimination reactions, and free of sialic and uronic acids. The most active fraction was eluted from DEAE-cellulose with 0.3 M NaCl in 0.01 M Tris-HCl pH 8.0, and when parasites were incubated with a source of $^{35}SO_4^{--}$, radioactivity was incorporated into molecules eluting under identical conditions. Active chromatographic fractions precipitated with protamine sulphate, suggesting that they were polyanionic in nature. There was some evidence of dissociation of the active substance on polyacrylamide gels where fast-moving sulphate-containing bands were observed which no longer reacted with protamine or complement.

The results are consistent with the possibility that the active substance is a polysulfated proteoglycan. Molecules of this nature have been detected on the surface of a variety of infectious organisms, and are known to interact with complement non-immunologically. Their location at host-parasite interfaces may have strategic significance in evasion of immune rejection and this possibility is discussed in relation to current understanding of immunity to *T. taeniaeformis*.

INTRODUCTION

In a previous report we showed that substances in the cyst fluid of metacestodes of *Taenia taeniaeformis* and released into the surrounding medium *in vitro* could apparently initiate complement fixation non-immunologically via both the classical and alternate pathways.[1] These findings are comparable to results obtained with certain bacterial lipopolysaccharides (1,2). We have therefore pursued the physico-chemical characterization of parasite factors in order to determine if they might be structurally analogous to the bacterial products, and our results are presented here.

Maximal complement-depleting activity was consistently associated with a fraction containing a highly sulfated polysaccharide or proteoglycan. Recently it has been shown that molecules of this nature may interact with complement in a variety of ways (3,4,5,6), and their occurrence at bacterial surfaces has been considered of primary importance in the host-parasite relationship (7,8). It is suggested their presence at the surface of tissue-dwelling metacestodes might also be significant in the avoidance of host rejection mechanisms.

MATERIALS AND METHODS

Reagents

Bovine serum albumin (BSA), glucosamine, glucose, N-acetyl neuraminic acid, papain insolubilized on carboxymethylcellulose, chondroitinase ABC, chondro-4-sulfatase, chondro-6-sulfatase, β-glucuronidase, neuraminidase, β-galactosidase, and deoxyribonuclease

Footnote 1. Manuscript submitted for publication.

were purchased from Sigma Chemical Company, Saint Louis, Missouri. Ethylenediamine tetra-acetic acid disodium salt (EDTA), polyethylene glycol, 20,000 (Carbowax), and pepsin were purchased from Fisher Scientific Company, Fair Lawn, New Jersey. Anthrone reagent and cetylpyridinium chloride monohydrate were purchased from Aldrich Chemical Company, Inc., Milwaukee, Wisconsin. Trypsin (1:250) was obtained from Difco Laboratories, Detroit, Michigan. $H_2{}^{35}SO_4$ was obtained from New England Nuclear, Boston, Massachusetts. Granular (pre-swollen) anion exchanger, Whatman DE-52, was purchased from Reeve Angel, Clifton, New Jersey. Sephadex G-200 was purchased from Pharmacia Fine Chemicals, Inc., Piscataway, New Jersey. Sepharose 6-B was purchased from Bio Rad Laboratories, Richmond, California.

Preparation of Larval Extracts and *in vitro* Products

Larvae of *Taenia taeniaeformis* (3-5 months old) were recovered from the livers of rats as described previously. They were washed 10 times with 10 volumes of sterile 0.15M NaCl and then incubated in 0.1 M Tris-HCl buffer, pH 8.0, containing 0.02 M EDTA at 37°C for 30 min. Four volumes of the Tris-HCl-EDTA buffer were used for every volume of packed larvae. After incubation, $MgCl_2$ was added to the extract solution to make it 0.05 M, before dialysis against distilled water and 15-20 fold concentration by Carbowax. Final exhaustive dialysis against distilled H_2O was carried out to remove any Carbowax which had entered the dialysis sac. This product will be referred to as Tris-EDTA extract (T.E.). The medium in which larvae had been maintained at a concentration of 50 larvae/200 ml for 24 hrs was concentrated 100 fold, and then dialysed against distilled water for 48 hrs; this product will be referred to as

in vitro products (IVP).

Radio-labelled IVP was obtained by adding 6 mC of $H_2{}^{35}SO_4$ to a culture flask in which 50 larvae were maintained in 200 ml of medium for 24 hrs. Labelled material was counted in a Packard liquid scintillation counter (Packard Instrument Co., Downer's Grove, Illinois.).

Assays

Protein concentrations were determined by the method described by Lowry et al (9) using BSA as a standard. Neutral sugars were assayed using the Anthrone reagent and glucose as a standard (10). Amino sugars were detected by the micro-technique developed by Gatt and Berman (11) and concentrations were determined by comparison with a glucosamine standard. Sialic acid was assayed by the technique described by Aminoff (12), with a neuraminic acid standard. Uronic acid concentrations in unknown samples were measured using the method of Dische (13).

Labile SO_4^{--} determinations were conducted by a modification of the method described by Saito et al (14). One tenth ml of 0.2 N HCl was added to 0.4 ml of the sample in an ampoule which was then sealed and heated at 100°C for 2.5 hrs. The seal was broken and 0.1 ml of 0.2 N NaOH was added, followed by 2.0 ml of 0.5% cetylpyridinium in 0.3 N HCl to precipitate polysaccharides. After 10 min at 37°C 2.1 ml of $BaCl_2$ gel in 0.3 N HCl was added to 0.9 ml of the supernatant and the optical density at 530 nm was measured 10 min later. When K_2SO_4 was used as a standard, the optical density was linearly related to concentration over the range 5-80 µg/ml.

Enzymatic Digestion

For the tryptic digests 1 mg of trypsin 1:250 was added to each milliter of T.E. containing 10 mg of protein in 0.2 M $NaHCO_3$, pH 8.0. The mixture was incubated at 37°C for 48 hrs, with 0.1% sodium azide added to prevent microbial growth. Trypsin was removed after dialysis by precipitation with 10% trichloracetic acid (TCA). Peptic digestion was also carried out for 48 hrs at 37°C using 5 mg of pepsin for each milliter of T.E. containing 10 mg of protein in 0.1 M acetate buffer, pH 4.5. Pepsin was removed by molecular sieving on Sephadex G-200 or by precipitation with 10% TCA. Papain (50 mg), insolublilzed on carboxymethyl cellulose, was incubated for 24 hrs at 37°C with one milliter of T.E., (containing 10 mg/ml protein) in 0.1 M sodium phosphate buffer, pH 7.0, and 0.1 M l-cysteine plus 0.002 M EDTA. The papain was removed by centrifugation and the digest dialysed against distilled H_2O. In a similar manner, protease from *Streptomyces griseus* covalently bound to carboxymethyl cellulose was added to T.E. in 0.1 M Tris-HCl, pH 7.3. Ten mg of the protease preparation was incubated with 1 milliter of T.E. containing 2 mg of protein for 24 hrs at 37°C. Protease was removed by centrifugation and the digest dialysed against distilled H_2O.

One unit of chondroitinase ABC, 1 unit of chondro-4-sulfatase, or 1 unit of chondro-6-sulfatase was used to digest 1 milliter of T.E. containing 1 mg of amino sugar. These enzymes in the amounts used and under the conditions of the hemolytic complement assay in control mixtures had no apparent effect on CH_{50} levels in normal guinea-pig serum. Therefore, they were not removed prior to assaying for residual T.E. activity in the hemolytic assay. For this same

reason the antihemolytic activity in 1 milliter of T.E. containing 2 mg of neutral sugar was assayed in the presence of β-glucuronidase, neuraminidase, β-galactosidase, or deoxyribonuclease, after incubation with these enzymes. Bacterial β-glucuronidase (100 Sigma units of Type II) was incubated with 1 milliter of T.E. in phosphate buffer, pH 6.8, for 24 hrs at 37°C. One unit of neuraminidase (Type VE), purified from *Clostridium perfringens*, was incubated with one milliter of T.E. in 0.1 M acetate buffer, pH 5.0, for 24 hrs at 37°C. Eight units of deoxyribonuclease II from bovine spleen were incubated with one milliter of T.E. in 0.1 M acetate buffer, pH 4.6 for 16 hrs at 37°C.

Complement Assays

The procedures for assay of anaphylatoxin and total hemolytic complement levels were described previously[1].

Column Chromatography

Anion-exchange chromatography was carried out using DE-52, equilibrated with 0.01 M Tris-HCl, pH 8.0. Stepwise elution of fractions was accomplished with NaCl at concentrations from 0.1 M to 2 M (15).

Molecular sieving using either Sephadex G-200 or Sepharose 6-B was conducted in columns 100 cm x 2.5 cm and in 0.1 M Tris-HCl buffer, pH 8.0 plus 0.1% sodium azide. In one instance the G-200 Sephadex column was equilibrated with 2 M NaCl in the Tris buffer.

Protamine Precipitation in Agarose Gel

Acid-cleaned microscope slides were coated with 3 ml of 1.5% agarose in veronal buffer pH 7.4. Wells (2 mm) were punched and

filled with solutions of protamine sulfate at 10 mg/ml or with T.E. preparations or chromotographic fractions. Precipitin bands formed overnight at 22°C.

Polyacrylamide Gel Disc Electrophoresis

Disc electrophoresis was performed by the methods of Ornstein and Davis (16). A 5% running gel was prepared in glass tubes (0.5 cm x 10 cm), in Tri-HCl buffer, pH 8.8. A spacer gel of 4% cyanogum gel in Tris-HCl buffer pH 7.9 was layered on top to a depth of 1 cm, and 20 µl of the sample in 5% sucrose was added immediately before electrophoresis. The reservoir buffer was Tris-glycine, pH 8.8, and a current of 2 mamp per tube was applied for 3 hr.

After electrophoresis, gels were stained with either 0.1% Amido Black for proteins or with 1% Toludine blue for acid mucopolysaccharide, and destained in 2% acetic acid. Gel slices (2 mm) were prepared after electrophoresis of the $^{35}SO_4^{--}$ labelled sample, and the radioactivity in each segment counted.

RESULTS

Effect of Enzymatic Digestions and Chemical Hydrolysis on T.E. Activity

T.E. preparations were active in hemolytic complement inhibition and anaphylatoxin generation tests when assayed as described previously for parasite cyst fluid[1]. When T.E. was made 10% TCA at 4°C a precipitate formed which was removed by centrifugation at 10,000 g for 30 min. After neutralization with NaOH and dialysis overnight, the supernatant contained approximately 80% of the original antihemolytic activity.

Incubation of T.E. with pepsin, trypsin, papain, or protease of

Streptomyces griseus origin under optimal conditions of temperature and pH did not reduce its activity. However, each digestion resulted in a reduction in protein content from 1/3 to 1/4 of the original level. Similarly, incubations of T.E. in chondroitinase ABC, glucuronidase, β-galactosidase, chondrosulfatases 4 or 6, neuraminidase, or deoxyribonuclease were carried out with no detectable loss of antihemolytic activity.

Alkaline hydrolysis with 0.5 M NaOH at 60°C for 16 hrs had no effect on T.E., but acid hydrolysis with 0.1 N HCl at 60°C for 5 hrs eliminated 85% of the antihemolytic activity (Fig. 1).

Molecular Sieving and Preparative Ultracentrifugation Studies

The elution profiles of T.E. and IVP, in Sepharose 6 B columns are shown in Figure 2. The majority of material detectable as protein or neutral sugar was present in the void volume. The void volume peak also contained most of the antihemolytic activity. When either T.E. or IVP were subjected to ultracentrifugation at 10^6 g for 5 hrs activity was concentrated in the sedimented material with lesser amounts in the supernatant. In the presence of 2 M NaCl, most of the T.E. activity was still excluded from G-200 Sephadex resin, with only a small amount retarded in its elution (Fig. 3).

When T.E. was digested with pepsin for 48 hrs most of the activity still eluted from Sepharose 6-B in the void volume (Fig. 4). This elution pattern was not changed when the pepsin-digested T.E. was treated with 1 M NaOH at 60°C for 1 hr, (Fig. 4).

Anion Exchange Chromatography

T.E. was applied to a column of DEAE cellulose in 0.01 M

Figure 1. Effect of acid hydrolysis of T.E. on inhibitory activity in a complement-mediated hemolytic assay. T.E. was incubated with 0.1 N HCl at 60°C, and activity was assayed in samples withdrawn hourly.

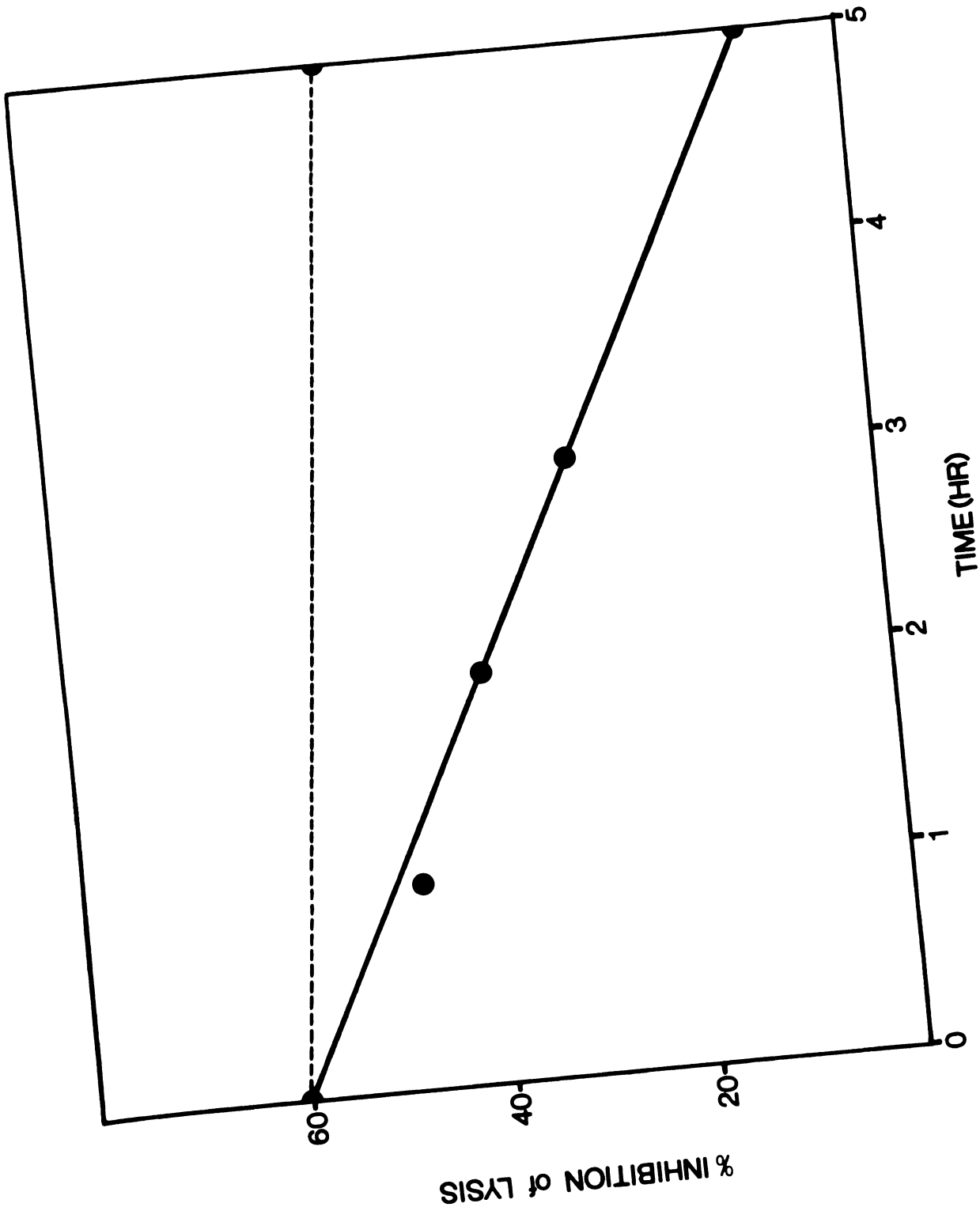


Figure 1

Figure 2. Protein (OD 280 nm) and neutral sugar (OD 620 nm) elution profiles of IVP (——) and T.E. (---) from a Sepharose 6-B column equilibrated with 0.1 M Tris-HCl buffer pH 8.0. The IVP sample contained 4 mg of protein and 2 mg of neutral sugar. The T.E. sample contained 12 mg of protein and 20 mg of neutral sugar.

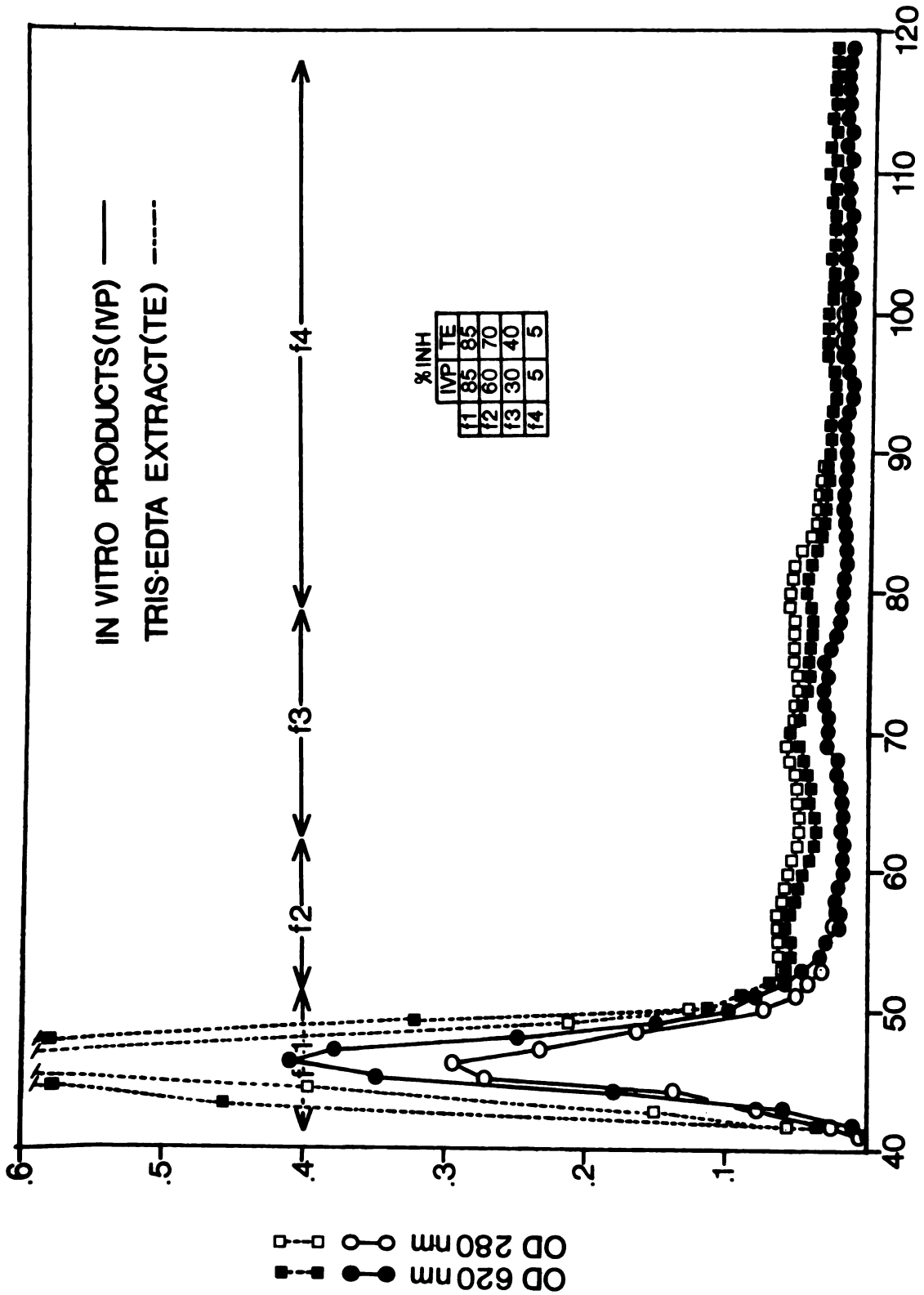

IN VITRO PRODUCTS(IVP) ——
TRIS·EDTA EXTRACT(TE) ----
% INH
IVP TE
f1 85 85
f2 60 70
f3 30 40
f4 5 5
f1
f2
f3
f4
.6
.5
.4
.3
.2
.1
40
50
60
70
80
90
100
110
120
TUBE Nº
OD 620nm
OD 280nm

Figure 2

Figure 3. Protein (OD 280 nm) and neutral sugar (OD 620 nm) elution profile of T.E. from a Sephadex G-200 column equilibrated with 2 M NaCl in 0.1 M Tris-HCl, pH 8.0. The T.E. sample contained 10 mg of protein and 12 mg of neutral sugar.

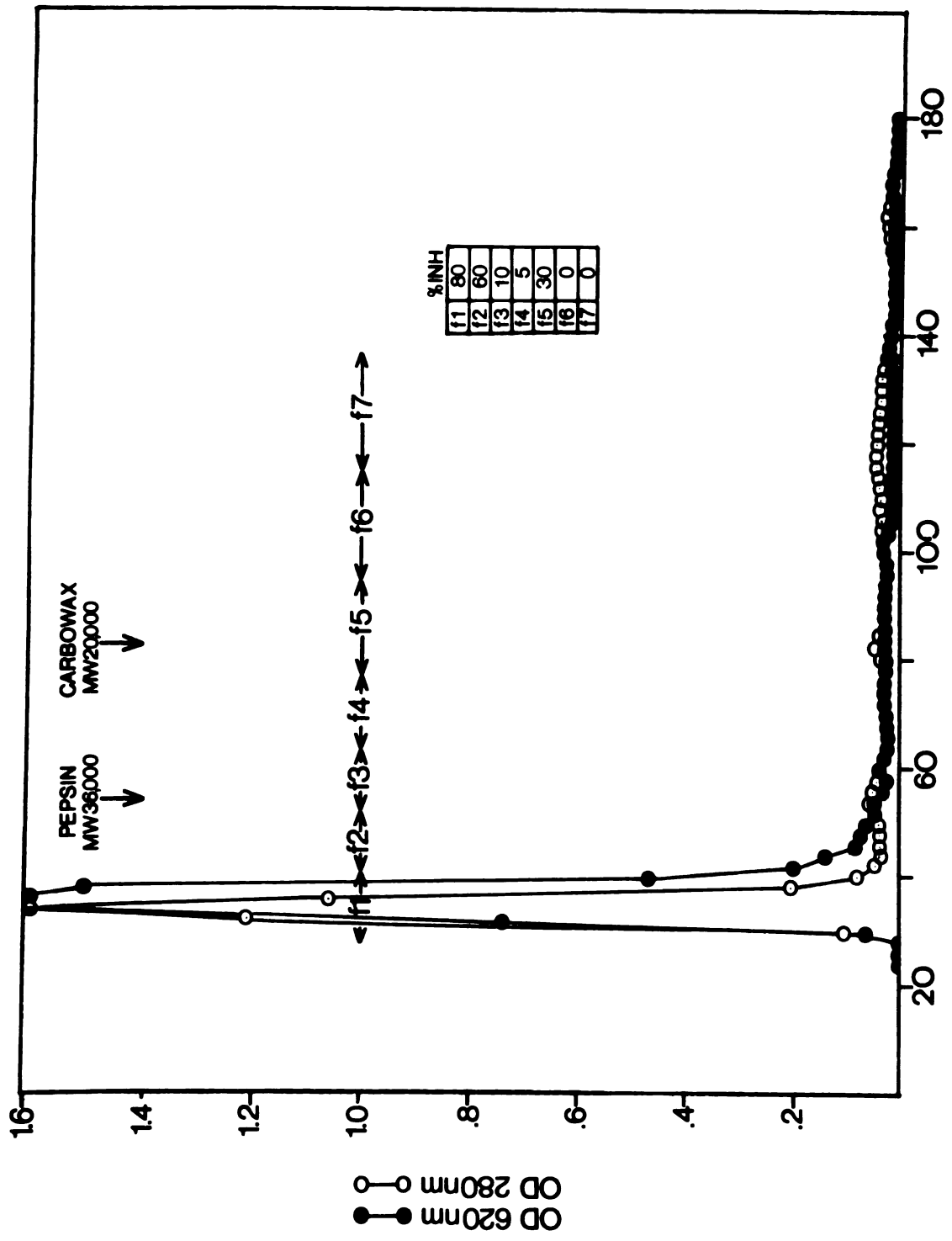

OD 620nm
OD 280nm
TUBE Nº
PEPSIN MW36000
CARBOWAX MW20000
f1
f2
f3
f4
f5
f6
f7
%INH
f1 80
f2 60
f3 10
f4 5
f5 30
f6 0
f7 0

Figure 3

Figure 4. Protein (OD 280 nm) and neutral sugar (OD 620 nm) elution profile of pepsin-digested (——) and pepsin-digested plus NaOH-treated (---) T.E. from a Sepharose 6 B column equilibrated with 0.1 M Tris-HCl buffer, pH 8.0. Prior to treatment the sample contained 8 mg of protein and 11 mg of neutral sugar. Protein elution in the NaOH-treated sample was identical, except that there was no peak corresponding to pepsin (tubes 85-120).

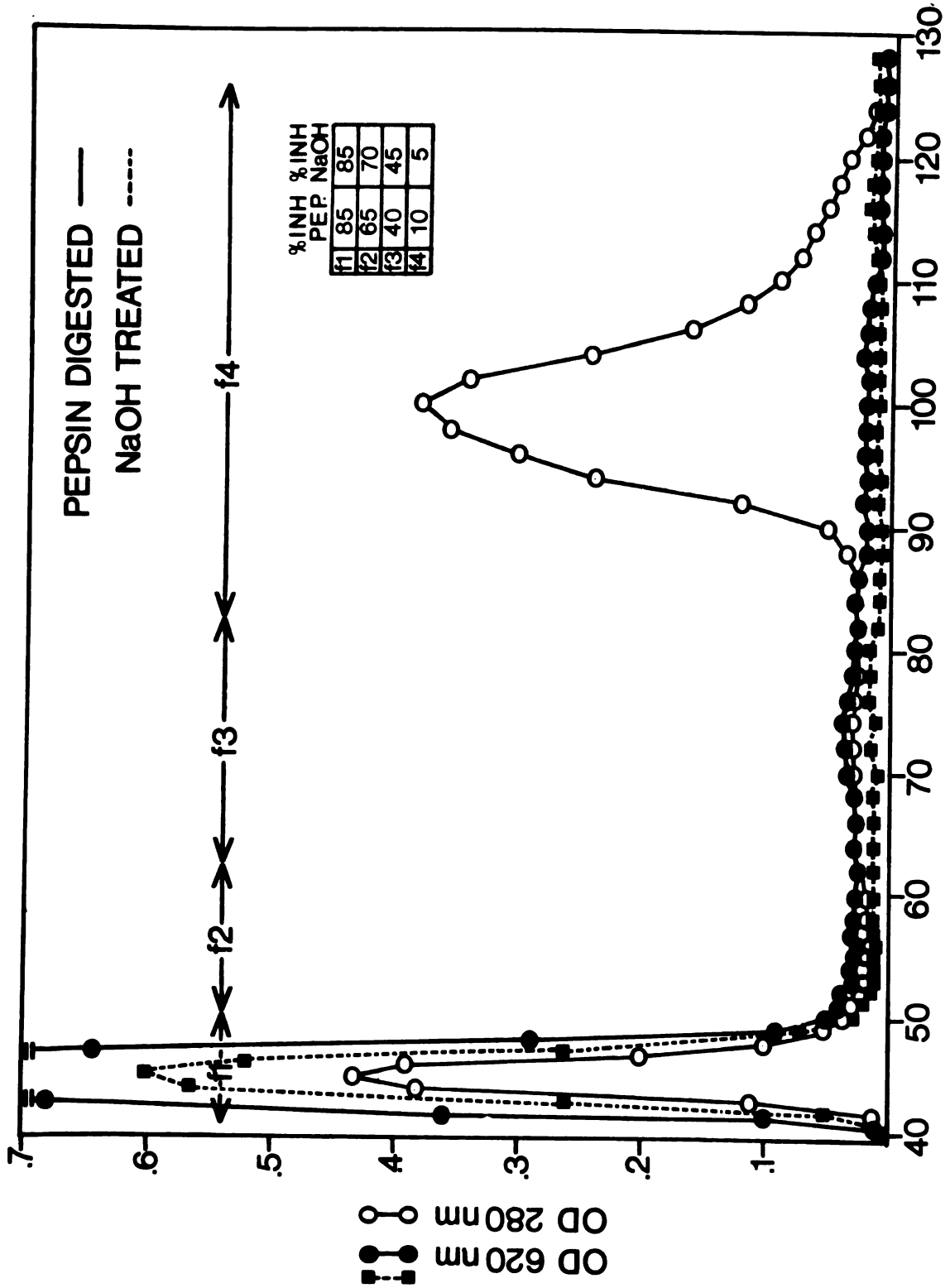

PEPSIN DIGESTED
NaOH TREATED
%INH PEP.
%INH NaOH
f1 85 85
f2 65 70
f3 40 45
f4 10 5
f1
f2
f3
f4
OD 620nm
OD 280nm
TUBE Nº
.7
.6
.5
.4
.3
.2
.1
40
50
60
70
80
90
100
110
120
130

Figure 4

Tris-HCl, pH 8.0, and subsequently eluted with stepwise additions of increasing concentrations of NaCl, (Fig. 5). The fraction eluting with 0.3 M NaCl had the highest activity in the hemolytic complement assay and contained the highest concentrations of amino-sugars and sulfate ions. Neither the T.E. applied to the column nor any of the concentrated pooled fractions eluted from the DEAE contained any detectable sialic acid or uronic acid. T.E. was then digested with pepsin and treated with 10% TCA to remove the pepsin and other proteins before chromatography on DEAE. The elution profile was not appreciably different from undigested TE, nor were there any marked alterations in the quantitative analysis of each fraction (Fig. 5).

In order to verify that the sulfate-containing material in the 0.3 M NaCl peak was of parasite origin, larvae were incubated with $H_2{}^{35}SO_4$ for 24 hrs and the non-dialysable labelled material produced was chromatographed on DEAE as above, (Fig. 6). Counts of ^{35}S were highest in the 0.3 M NaCl fraction, coinciding again with the peak of antihemolytic activity.

Since the elution pattern on DEAE was not affected by proteolytic digestion or TCA precipitation, an attempt was made to assay for the effects of alkaline hydrolysis on the elution of the 0.3 M NaCl fraction. Glycoproteins with glycosidic linkages between sugar moieties and amino acids are susceptible to β-elimination reactions in the presence of 0.5 M NaOH for 24 hrs at 22°C (17). T.E. was therefore digested with pepsin and then treated with 10% TCA before DEAE chromatography. The 0.3 M NaCl peak was then subjected to alkaline hydrolysis. The total sugar content of the fraction was reduced but the protein content and antihemolytic activity were not

Figure 5. Comparison of the protein (OD 280 nm) and neutral sugar (OD 620 nm) elution profiles of T.E. before (A) and after (B) digestion with pepsin. Elution of the sample from DEAE cellulose was accomplished by stepwise addition of increasing concentrations of NaCl. The sample in A contained 6.0 mg of protein and 10 mg of neutral sugar. The sample in B after pepsin digestion and treatment with 10% TCA contained 2.5 mg of protein and 7.0 mg of neutral sugar. Pooled fractions were concentrated to 3 ml. ND indicates "none detectable."

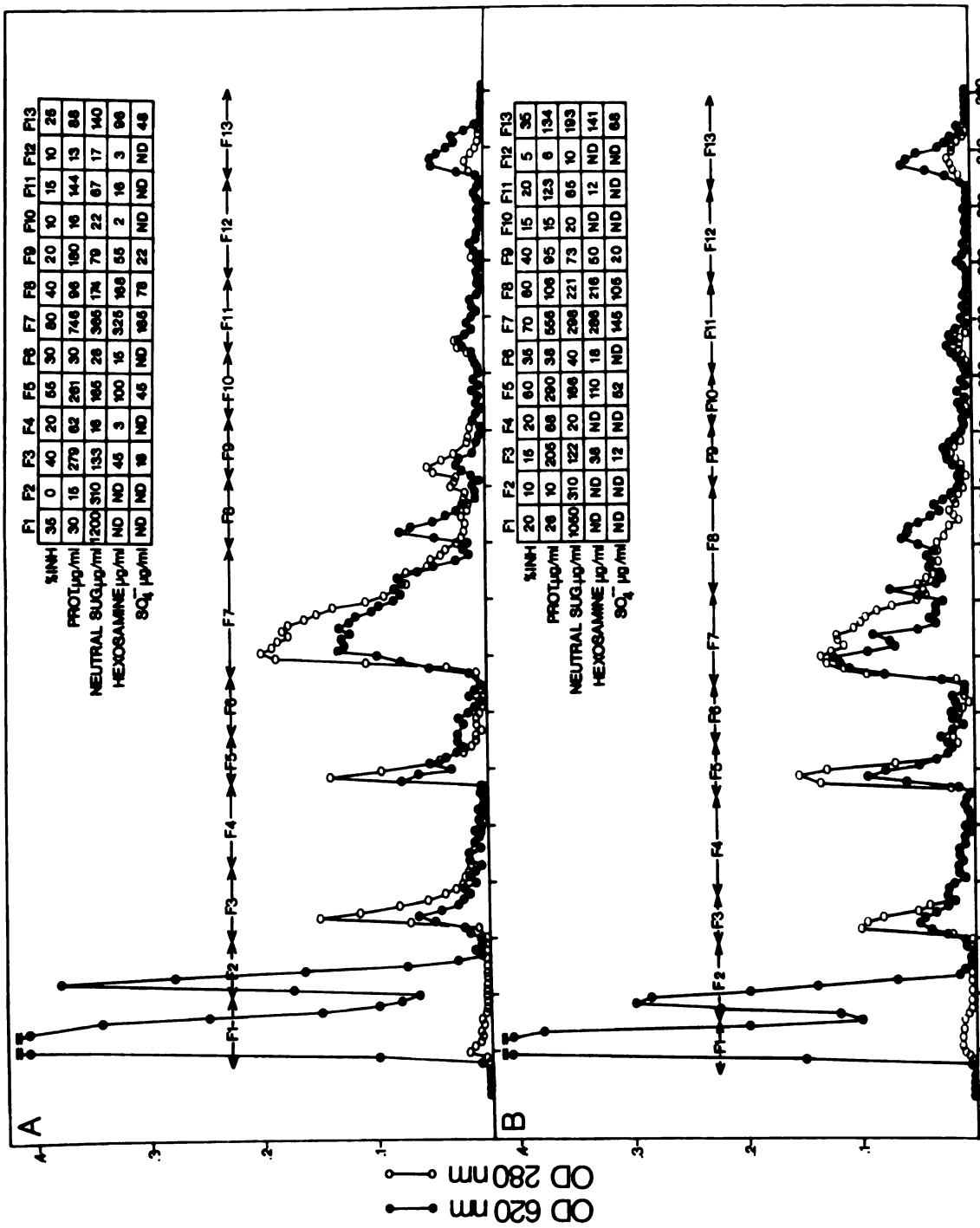

A
B
F1 F2 F3 F4 F5 F6 F7 F8 F9 F10 F11 F12 F13
%INH 35 0 40 20 55 30 80 40 20 10 15 10 26
PROT µg/ml 30 15 279 62 261 30 745 96 180 16 144 13 88
NEUTRAL SUG µg/ml 1200 310 133 16 165 28 395 174 79 22 67 17 140
HEXOSAMINE µg/ml ND ND 45 3 100 15 325 105 55 2 16 3 96
SO_4^- µg/ml ND ND 18 ND 45 ND 185 78 22 ND ND ND 48
F1 F2 F3 F4 F5 F6 F7 F8 F9 F10 F11 F12 F13
%INH 20 10 15 20 60 35 70 60 40 15 20 5 35
PROT µg/ml 28 10 205 68 290 38 556 106 95 15 123 6 134
NEUTRAL SUG µg/ml 1080 310 122 20 186 40 208 221 73 20 65 10 193
HEXOSAMINE µg/ml ND ND 38 ND 110 18 286 216 50 ND 12 ND 141
SO_4^- µg/ml ND ND 12 ND 82 ND 145 105 20 ND ND ND 68
OD 620 nm
OD 280 nm
0.1M
0.2M
0.3M
TUBE NO
0.4M
0.5M
0.7M
2M

Figure 5

Figure 6. Relationship of protein (OD 280 nm) and neutral sugar (OD 620 nm) to cpm from $^{35}SO_4^{--}$ and complement-inhibitory activity in fractions of IVP eluted from DEAE cellulose. The IVP sample contained 2 mg of protein, 1 mg of neutral sugar and 4,000 cpm of $^{35}SO_4^{--}$. Pooled fractions were concentrated to one ml.

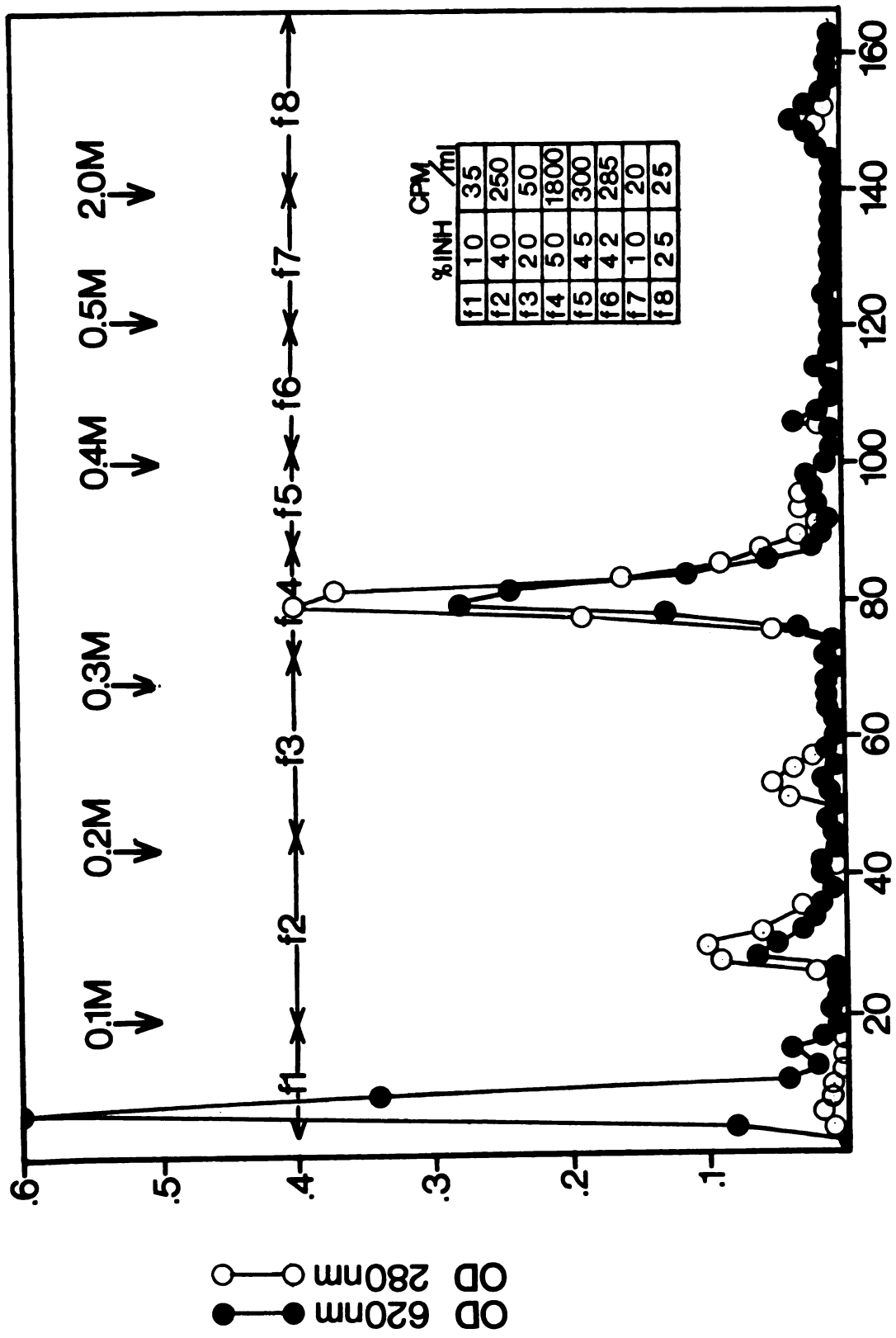

OD 620nm
OD 280nm
.6
.5
.4
.3
.2
.1
20
40
60
80
100
120
140
160
TUBE Nº
0.1M
0.2M
0.3M
0.4M
0.5M
2.0M
f1
f2
f3
f4
f5
f6
f7
f8
CPM/ml
%INH
f1 10 35
f2 40 250
f3 20 50
f4 50 1800
f5 45 300
f6 42 285
f7 10 20
f8 25 25

Figure 6

changed. Antihemolytic activity and the majority of the non-dialysable protein and neutral sugar eluted in 0.3 M NaCl fraction after rechromatrography on DEAE.

Protamine Precipitation

When T.E. and fractions of T.E. from DEAE chromatography were allowed to diffuse in agarose gels against protamine, distinct precipitin bands formed. Only T.E. and the 0.2 M and 0.3 M NaCl eluates formed bands, and the 0.3 M NaCl fraction formed a more dense band closer to the protamine well (Fig. 7). These two chromatographic fractions still precipitated with protamine in double diffusion gels after they had been treated with 0.5 M NaOH at 60°C for 12 hrs.

Polyacrylamide Gel Disc Electrophoresis of T.E. Extracts

After polyacrylamide gel disc electrophoresis of T.E., T.E. digested by pepsin, or T.E. incubated with 1 M NaOH (60 min at 60°C) all gels showed the same number and location of bands detectable with Amido Black or toluidine blue, (Fig. 8). Heavy protein-staining bands occurred in all samples at the origin of the gel and at the level of the tracking dye (bromcresol blue), and a lightly-staining diffuse area was present in the upper 2 cm of the running gel. Bands staining with toluidine blue appeared at the origin of the gel, at the tracking dye site and at two areas beyond the tracking dye. Again a lightly stained area was present in the upper 2 cm. When IVP labelled with $^{35}SO_4^{--}$ was subjected to electrophoresis, counts were concentrated at the level of the tracking dye (Fig. 9).

Protamine-precipitating activity eluted from the gel segments

Figure 7. Precipitation bands formed when protamine sulfate (10 mg/ml), in the center well (P), initial wash (W), 0.1 M, 0.2 M, 0.3 M, 0.4 M, and 2 M NaCl eluates from DEAE chromatography of T.E. before (on the right) and after alkaline hydrolysis (on the left) were allowed to diffuse in agarose gels.

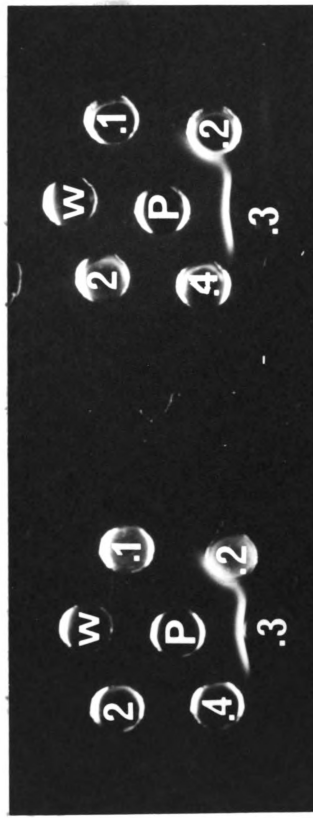


Figure 7

Figure 8. Polyacrylamide disc gel electrophoresis band pattern typical of T.E. or T.E. after alkaline hydrolysis or pepsin digestion. The gel on the left has been stained with Amido Black, the gel on the right with toludine blue, and the arrow marks the extent of tracking dye migration.

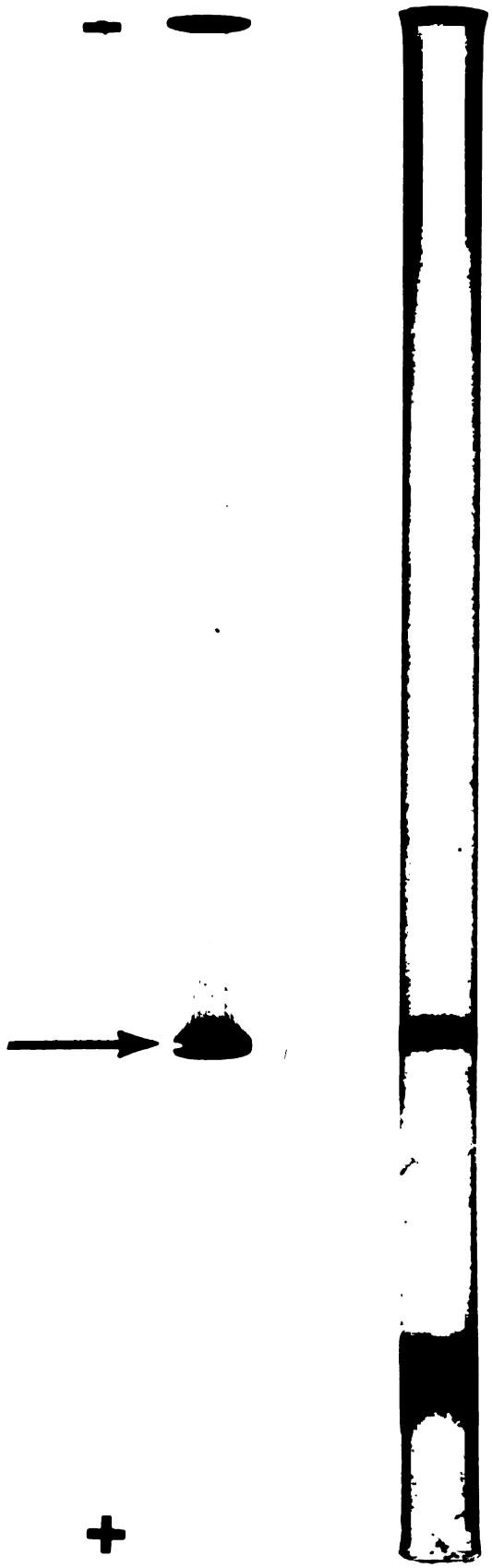


Figure 8

Figure 9. Polyacrylamide gel electrophoresis of IVP labelled with $^{35}SO_4^{--}$. Samples were run for 3 hrs at 2 mamp/tube. Tracking dye was bromocresol blue.

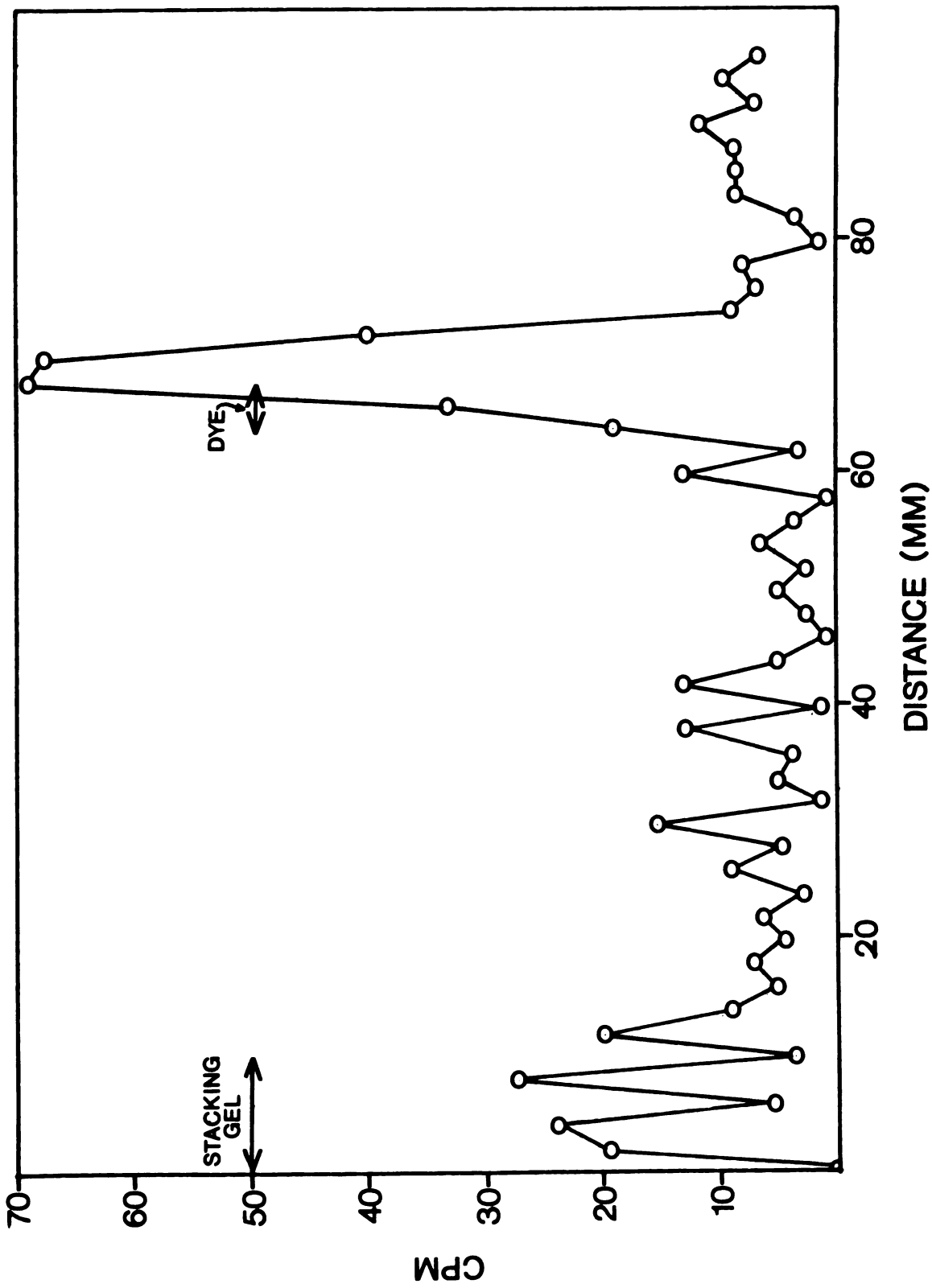


Figure 9

was restricted to materials in the upper 2 cm, and no precipitation occurred with the section containing the fast-moving bands. Anti-hemolytic activity also corresponded with the upper 2 cm section, and no inhibition was detected in the fast-moving band area.

DISCUSSION

Surface material extracted from fully developed larvae of *T. taeniaeformis* with 0.02 M EDTA in 0.1 M Tris-HCl interacted with complement in a similar manner to cyst fluid removed from the parasite bladders[1]. This extraction procedure has been used traditionally to collect endotoxin from gram-negative bacteria (18), and its effectiveness provided us a means of standardizing the preparation of parasite factors for further physico-chemical characterization. Other substances may be present within cyst fluid which interact with complement so that this characterization was necessarily incomplete.

The resistance of antihemolytic reactivity to 10% TCA treatment and to a variety of proteolytic enzymatic digestions suggested that the factors responsible were either non-proteinaceous or that the protein moiety was peculiarly resistant, perhaps by virtue of its association with other components. The latter seemed likely since even after proteolysis and 10% TCA treatment the T.E. supernatant still contained 40% of the original protein content. The insusceptibility of T.E. to several other enzymatic digestions indicated that antihemolytic activity could not be attributed to molecular structures closely related to chondroitin sulfate, hyaluronic acid, β galactoside-linked saccharides, DNA, or sialic acid. Glycosidic linkages susceptible to β-elimination by alkaline hydrolysis did not appear to be involved in the antihemolytic effect since activity was retained after exposure to 0.5 M NaOH at 22°C for 24 hrs. However, there was a striking reduction in antihemolytic activity following hydrolysis in 0.1 N HCl at 60°C. This observation was

consistent with the possible involvement of sulphate groups linked to the nitrogen of amino-sugars, since these bonds are known to be disrupted by mild acid hydrolysis (19).

Preparative ultracentrifugation and gel filtration of T.E. before and after digestion with pepsin or alkaline hydrolysis indicated that the majority of the active material had retained a macromolecular structure. The fact that this remained so after alkaline hydrolysis may be indicative of bonding between polysaccharide side chains (20). Anion exchange chromatography of T.E. and $^{35}SO_4^{--}$ labeled IVP demonstrated that the antihemolytic activity was heterogeneous with respect to net molecular charge, as were molecules containing SO_4^{--} and hexosamine. The radioactivity detectable in IVP after incubation of larvae with $H_2{}^{35}SO_4$ demonstrated that the SO_4^{--} containing materials were of parasite origin and not host-derived contaminants. There was a marked association between antihemolytic activity, SO_4^{--} level and the 0.3 M NaCl eluate from DEAE. The activity was pepsin-resistant and insusceptible to alkaline hydrolysis, and the net molecular charge did not seem to be affected by these treatments because when rechromatographed the fraction eluted under identical conditions, even though the total neutral sugar content had been reduced considerably by hydrolysis.

In sum these observations provide evidence of an association of the anticomplementary activity with macromolecules containing carbohydrate, protein, sulphate and hexosamine, heterogenous in net molecular charge but resistant to proteolysis and β-elimination reactions and free of sialic and uronic acids. The fact that antihemolytic chromatographic fractions, rich in sulphate, precipitated

with protamine suggested that they may indeed be polyanionic, since protamine is a polycationic protein capable of binding polyanionic structures (4). In preliminary experiments (unpublished observations) we found that prior incubation of T.E. with protamine decreased antihemolytic activity, and similar results have been reported by Rent et al (5) with protamine-heparin mixtures. These conclusions appear to be at odds with the results of polyacrylamide disc gel electrophoresis where most of the protein-staining material and sulfate groups migrated rapidly, suggesting that they were not part of a large molecule. This happened whether the T.E. was used untreated, or after protease digestion or alkaline hydrolysis. The protein-staining band at the tracking dye did not correspond exactly with the toludine blue-stained, sulfate-containing material. However, sections of the gel containing fast-moving bands showed no antihemolytic activity and did not contain protamine-precipitating materials. Both these activities were restricted to slow moving molecules in the initial part of the running gel. Possibly non-covalent bonds (eg. electrostatic) were maintaining a polymeric macromolecular structure which was stable at pH 8.0 (21), yet dissociable under conditions of electrophoresis.

On gas-liquid chromatographic analysis of the most active DEAE fraction (0.3 M NaCl) only two neutral sugars were detected; galactose and glucose were present in a molar ratio of 5:1, and a hexosamine, probably glucosamine, was present in amounts equimolar to galactose[2].

Our physico-chemical evidence suggesting that anticomplementary

Footnote 2. Manuscript submitted for publication.

activity is associated with a sulfated polysaccharide or proteoglycan, is also consistent with the fact that complement interaction similar to that described for T.E., IVP, and CYF[1] can occur with polysulfated molecules such as heparin (3). Also important in relation to host inflammatory reactions is the recently described activity of certain polyanions in enhancing the function of Cl-inhibitor (Cl-INH) (6). We have not determined if substances of parasite origin are able to influence Cl-esterase or Hageman factor activity by Cl-INH enhancement, but we have recently found that T.E. will inhibit the intrinsic pathway of co-agulation[2]. These results are particularly interesting in the light of previous histochemical and electron microscopic studies in which acid mucopolysaccharides have been demonstrated at the surface of a variety of metacestodes (22,23). In electron microscopic studies we have shown acid-colloidal iron staining at the surface of larvae of *T. taeniaeformis* (24). This may be attributable to the occurrence of a surface polyanion in this parasite, and it is tempting to speculate that the complement-interacting factors contribute to this layer.

Many other tissue-dwelling parasitic organisms have been shown to have high densities of negative charges associated with their outer surface coats, and these are generally believed to be polysaccharide in nature. Examples of this can be found among the helminths [schistosomes (22)], protozoa [*Trypanosoma* (25,26), *Leishmania* (27,28) and amoebae, (29)] and certain strains of bacteria [*Escherichia coli* and *Salmonella typhi* (7)]. In addition, the surface of trophoblast cells of mammalian embryo and fetal origin carries a negatively charged component with an affinity for

acid-colloidal iron which is believed to be a polysaccharide or glycosaminoglycan (30,31). While most conclusions on the characteristics of cell surface constituents of parasites have been drawn from histochemical studies and lectin binding experiments, in the case of endotoxin of *E. coli* and *Salmonella* origin a molecular definition of the polyanionic K and Vi antigens has existed for some time (7). Increasing evidence that polyanions can affect host amplification systems such as the classical and alternate pathways of complement fixation and the coagulation cascade (3,4,5,6,32) suggests that their role on the surface of infectious organisms may be important in host-parasite relationships.

There is ample evidence that the presence or absence of complement-fixing factors on the surface of gram-negative bacteria is the crucial determinant of resistance or susceptibility of the organism to serum. Reynolds and Pruul have shown that surface lipopolysaccharides of serum resistant *S. typhi* buffer complement consumption in such a way that the cell remains intact (8,33,34). In a variety of other cell systems important functions have been ascribed to surface acidic polysaccharides, including adsorption of other molecules and ions which might then be internalized (35,36), and the inhibition of intestinal enzymes so that membranes might be protected against digestion (35,37). It has also been postulated that the surface glycosaminoglycans of trophoblast cells are involved in the immunological inertness of the mammalian embryo (30,31,38). Support for this proposal can be drawn from recent work in which a correlation has been observed between surface polysulfated glycosaminoglycan levels on transformed cell lines and their

susceptibility to antibody-mediated complement-dependent lysis. Highest amounts of surface heparan sulfates were present during the pre-mitotic phase of the cell cycle (39,21) at which time the cells resisted immunologic lysis, even though antibody binding and complement fixation continued to occur (40,41,42). Direct evidence of inhibition of complement-dependent lysis by surface acid mucopolysaccharides has recently been developed by Lippman (43).

In the host-parasite system which we have studied, an antibody-mediated immunity develops which is dependent on complement for effective protection against early larval stages (44) yet larvae rapidly become insusceptible to this immune mechanism (45). A protective function for the complement-reactive material in the larval surface coat can be postulated, analogous to that proposed by Reynolds, Rother, and Rother (34) for surface lipopolysaccharide in serum-resistant strains of *Salmonella*. It may be that the lethal effect on early stage larvae is due to the ability of the complement-fixing antibodies to concentrate critical amounts of complement at vital membrane foci. With further development the larvae become resistant to complement-fixing antibodies, possibly due to local depletion of complement or inhibition of antibody-binding. Both of these activities have been described for polyanionic polysaccharides (7).

The recently reported susceptibility of cestodes to a lytic effect produced by normal serum complement *in vitro* (46,47) could be due to overwhelming local complement concentrations with fixation to surface factors which, under *in vivo* conditions, are either released or placed in a position to consume complement away from

the membrane. Further sequential studies on the quantitative aspects of production and release of complement-fixing factors during development of *T. taeniaeformis* may provide a means to examine this phenomonen experimentally, and to clarify the role of surface polyanions in the successful evasion of rejection by this parasite.

ACKNOWLEDGEMENTS

This work was supported by USPHS grant AID 10842. This is journal article No. from the Michigan Agriculture Experiment Station. We are indebted to Miss Sharon Garland for technical assistance in many aspects of this study.

LITERATURE CITED

1. Lichtenstein, L. M., H. Gewurz, N. F. Adkinson Jr., H. S. Shin, and S. E. Mergenhagen. 1969. Interactions of the complement system with endotoxic lipopolysaccharide: the generation of an anaphylatoxin. Immunology 16:327-336.

2. Gewurz, H., H. S. Shin, and S. E. Mergenhagen. 1968. Interactions of the complement system with endotoxic lipopolysaccharide: consumption of each of the six terminal complement components. J. Exp. Med. 128:1049-1057.

3. Loos, M., E. Raepple, U. Hadding, and D. Bitter-Suerman. 1974. Interaction of polyanions with the first and the third component of complement. Fed. Proc. (Abst.) 33:775.

4. Fiedel, B. A., R. Rent, R. Myhrman, and H. Gewurz. 1976. Complement activation by interaction of polyanions and polycations. II. Precipitation and role of IgG, Cl_q, and Cl-INH during heparin-protamine-induced consumption of complement. Immunology 30:161-169.

5. Rent, R., N. Ertel, R. Eisenstein, and H. Gewurz. 1975. Complement activation by interaction of polyanions and polycations. I. Heparin-protamine induced consumption of complement. J. Immunol. 114:120-124.

6. Rent, R., R. Myhrman, B. A. Fiedel, and H. Gewurz. 1976. Potentiation of Cl-esterase inhibitor activity by heparin. Clin. Exp. Immunol. 23:264-271.

7. Glynn, A. A. 1972. Bacterial factors inhibiting host defense mechanisms. Symp. Soc. Gen. Microbiol. 22:75-112.

8. Reynolds, B. L., and H. Pruul. 1971. Protective role of smooth lipopolysaccharide in the serum bactericidal reaction. Infection and Immunity 4:764-771.

9. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275.

10. Oettgen, H. F., R. A. Binaghi, and B. Benacerraf. 1965. Hexose content of guinea pig $_1$ and $_2$ immunoglobulins. Proc. Soc. Exp. Biol. Med. 118:336-342.

11. Gatt, R., and E. R. Berman. 1965. A rapid procedure for the estimation of amino sugars on a micro scale. Acta. Biochim. Polon. 12:167-171.

12. Aminoff, D. 1961. Methods for the quantitative estimation of N-acetyl-neuraminic acid and their application to hydrolysates of sialomucoids. Biochem. J. 81:384-392.

13. Dische, Z. 1947. A new specific color reaction of hexuronic acids. J. Biol. Chem. 167:189-198.

14. Saito, H., T. Yamagata, and S. Suzuki. 1968. Enzymatic methods for the determinations of small quantities of isomeric chrondroitin sulfates. Biol. Chem. 243:1536-1542.

15. Inoue, Sadako. 1965. Isolation of new polysaccharide sulphates from *Charonia lampas*. Biochim. Biophys. Acta 101:16-25.

16. Ornstein, L., and B. Davis. 1964. Theory of disc electrophoresis. Parts 1 and II. Distillation Products Industries, Rochester, NY.

17. Downs, F., and W. Pigman. 1969. Preparation of glycopeptides from bovine submaxillary mucin by chemical degradation. Biochem. 8:1760-1766.

18. Leive, L., V. K. Shovlin, and S. E. Mergenhagen. 1968. Physical, chemical, and immunological properties of lipopolysaccharide released from *Escherichia coli* by ethylenediaminetetraacetate. J. Biol. Chem. 243:6384-6391.

19. Dietrich, C. P., and H. B. Nader. 1974. Fractionation and properties of four heparitin sulfates from beef lung tissue: Isolation and partial characterization of a homogeneous species of heparitin sulfate. Biochem. Biophys. Acta 343:34-44.

20. Chiarugi, V. P., and S. Vannucchi. 1976. Surface heparan sulphate as a control element in eukariotic cells: a working model. J. Theor. Biol. 61:459-475.

21. Chiarugi, V. P., S. Vannucchi, and P. Urbano. 1974. Exposure of trypsin-removable sulfated polyanions on the surface of normal and virally transformed BHK21/Cl3 cells. Biochim. Biophys. Acta 345:283-293.

22. Lumsden, R. D. 1975. Parasitological review. Surface ultrastructure and cytochemistry of parasitic helminths. Expl. Parasitol. 37:267-339.

23. Waitz, J. 1963. Histochemical studies of the cestode *Hydatigena taeniaeformis* Batsch, 1786. J. Parasitol. 50:271-277.

24. Hammerberg, B., J. Picone, and J. F. Williams. 1976. Complement reactive products from larval *Taenia taeniaeformis*. 57th Annual meeting of the Conference of Research Workers in Animal Disease. p. 24. (Abstract).

25. Vickerman, K. 1974. Antigenic variation in African trypanosomes. *In* Parasites in the Immunized Host: Mechanisms of Survival. Ciba Foundation Symposium 25 (new series). Associated Scientific Publishers, Amsterdam. p. 53-70.

26. Dwyer, D. M. 1976. Cell surface saccharides of *Trypanosoma lewisi*. II. Lectin-mediated agglutination and fine structure cytochemical detection of lectin-binding sites. J. Cell. Sci. 22:1-19.

27. Dwyer, D. M. 1977. *Leishmania donovani*: surface membrane carbohydrates of promastigotes. Exp. Parasitol. 41:341-358.

28. Dwyer, D. M., S. G. Langreth, and N. K. Dwyer. 1974. Evidence for a polysaccharide surface coat in the developmental stages of *Leishmania donovani*: a fine structure-cytochemical study. Z. Parasitenk. 43:227-249.

29. Pinto da Silva, P., A. Martinez-Palomo, and A. Gonzalez-Robles. 1975. Membrane structure and surface coat of *Entamoeba histolytica*. Topochemistry and dynamics of the cell surface: cap formation and micro exudate. J. Cell. Biol. 64:538-550.

30. Calatroni, A., and N. DiFerrante. 1969. The glycosaminoglycans of human term placenta. Carbohydrate Res. 10:535-548.

31. Billington, D. 1975. Organization, ultrastructure and histochemistry of the placenta: immunological considerations. *In* Immunobiology of Trophoblasts. Edited by R. G. Edwards, C. W. S. Howe, and M. H. Johnson, Cambridge University Press, Cambridge, MA. p. 67-85.

32. Claus, D. R., J. Siegel, K. Petras, D. Skor, A. P. Osmand, and H. Gewurz. 1977. Complement activation by interaction of polyanions and polycations. III. Complement activation by interaction of multiple polyanions and polycations in the presence of C-reactive protein. J. Immunol. 118:83-87.

33. Reynolds, B. L., and H. Pruul. 1971. Sensitization of complement-resistant smooth gram-negative bacterial strains. Infect. Immun. 3:365-372.

34. Reynolds, B. L., V. A. Rother, and K. O. Rother. 1975. Interaction of complement components with a serum-resistant strain of *Salmonella typhimurium*. Infect. Immun. 11:944-948.

35. Revel, J. P., and S. Ito. 1967. The surface components of cells. *In* The Specificity of Cell Surfaces. Edited by B. Davis and L. Warren, Prentice-Hall, Englewood Cliffs, NJ. p. 211-234.

36. Josefsson, J. O. 1975. Studies on the mechanism of induction of pinocytosis in *Amoeba proteus*. Acta Physiol. Scand. Supplement 432:1-65.

37. Oaks, J., and R. Lumsden. 1971. Cytological studies on the absorptive surfaces of cestodes. V. Incorporation of carbohydrate containing macromolecules into tegument membranes. J. Parasitol. 57:1256-1268.

38. Borland, R., W. Y. Lake, and D. Wilson. 1975. Immunological privilege resulting from endocrine activity of trophoblast *in vivo*. *In* Immunobiology of trophoblasts. Edited by R. G. Edwards, C. W. S. Howe, and M. H. Johnson, Cambridge University Press, Cambridge, MA. p. 157-169.

39. Kraemer, P. M., and R. A. Tobey. 1972. Cell-cycle dependent desquamation of heparan sulfate from the cell surface. J. Cell. Biol. 55:713-717.

40. Lerner, R. A., M. B. A. Oldstone, and N. R. Cooper. 1971. Cell cycle-dependent immune lysis of Moloney virus-transformed lymphocytes: presence of viral antigen, accessibility to antibody, and complement activation. Proc. Nat. Acad. Sci. 68:2584-2588.

41. Pellegrino, M. A., S. Ferrone, N. R. Cooper, M. P. Dierich, and R. A. Reisfeld. 1974. Variation in susceptibility of a human lymphoid cell line to immune lysis during the cell cycle. Lack of correlation with antigen density and complement binding. J. Exp. Med. 140:578-590.

42. Cooper, N. R., M. J. Polley, and M. B. A. Oldstone. 1974. Failure of terminal complement components to induce lysis of Moloney virus transformed lymphocytes. J. Immunol. 112:866-868.

43. Lippman, M. 1968. Transplantation and cytotoxicity changes induced by acid mucopolysaccharides. Nature 219:33-36.

44. Musoke, A. J., and J. F. Williams. 1975. Immunological response of the rat to infection with *Taenia taeniaeformis*. V. The sequence of appearance of protective immunoglobulins and the mechanism of action of 7S 2_a antibodies. Immunology 29:855-865.

45. Campbell, D. H. 1938. Further studies on the "nonabsorbable" protective property in serum from rats infected with *Cysticercus crassicollis*. J. Immunol. 35:465-476.

46. Kassis, A. I., and C. E. Tanner. 1976. The role of complement in hydatid disease: *in vitro* studies. Int. J. Parasitol. 6:25-36.

47. Herd, R. P. 1976. The cestocidal effect of complement in normal and immune sera *in vitro*. Parasitology 72:325-334.