The Role of the Agglutinins in the Coelomic Fluid of the Oligochaete Eisenia foetida.

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ABSTRACT.

Pronounced opsonic activity has been demonstrated in the coelomic fluid of the Oligochaete Eisenia foetida. This humoral factor enhances the uptake of particles by Eisenia phagocytes by 94% in the case of S.cerevisiae, 31.5% in E.coli and by 34.6% in B.megaterium.

In order to determine whether agglutinin molecules are responsible for this activity, the 20,000D haemagglutinin was purified. This agglutinin was found to be heat sensitive, (15 min at 60°C destroying activity completely), partially dependent on calcium ions and with an optimal pH range of 5-8. The agglutinin was found to be active towards a range of vertebrate erythrocytes and various fungal and bacterial cells but is not responsible for opsonic activity.

Polyclonal antibodies to this purified agglutinin were raised in rabbits and used in immunocytochemical studies to determine the source of this molecule.

It was found that the cell membranes of Eisenia phagocytes bear agglutinin or agglutinin like molecules and their possible roles in internal defence mechanisms is discussed.

Eisenia coelomocytes were found to release agglutinins in vitro though the site of synthesis of these molecules is yet to be established.

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LIST OF CONTENTS.

LIST OF TABLES.

LIST OF FIGURES

Fig.4.

A) The relationship between the fluorescent intensity and the number of particles present (S.cerevisiae ▲-▲ ;E.coli o-o ;S.aureus ●-● ; Bmegaterium △-△ .)

B) The relationship between the number of phagocytic cells and their protein content as estimated by the Bradford assay. 91

Fig.5.

The uptake of FITC stained microorganisms by Eisenia foetida phagocytes with time as determined by measurement of the fluorescent intensity of the ingested particles (S.cerevisiae -A; S.aureus -B; E.coli -C; B.megaterium -D). 93

Fig.6.

The uptake of opsonized (o-o) and nonopsonized (●-●) FITC stained microorganisms by Eisenia foetida phagocytes with time as determined by measurement of the fluorescent intensity of the ingested particles (S.cerevisiae -A; S.aureus -B; E.coli -C; B.megaterium -D). 95

Fig.7.

The uptake of opsonized (o-o) and nonopsonized (●-●) FITC stained microorganisms by individual Eisenia foetida phagocytes with time. The data were expressed as the average number of ingested particles per cell. Standard deviations

LIST OF PLATES.

Plate 2.

A)SDS PAGE of individual peaks of coelomic fluid from Eisenia foetida separated by gel filtration (Lane 1- molecular weight markers; Lane 2 -crude coelomic fluid; Lane 3 -Peak A; Lane 4 -Peak B; Lane 5 -Peak C; Lane 6 -Peak D).

B)SDS PAGE of peak d from chromatofocusing. Molecular weight markers are also shown.

71

Plate 3.

Scanning electron micrographs, space bars= 10 μm

A) A phagocyte from the coelom of Eisenia foetida, allowed to adhere to the surface of the incubation dish for 10 min prior to fixation.

B) A group of Eisenia foetida phagocytes containing ingested yeast particles. Incubation lasted for 2h prior to fixation, enabling extensive spreading of the phagocytes on the surface of the dish.

85

Plate 4.

A) FITC stained yeast particles viewed under ultra violet light (x1000).

B1 and C1) Several Eisenia foetida phagocytes containing ingested yeast particles (x400).

B2 and C2) The same phagocytes as above, viewed under ultra violet light (x400) after quenching of extracellular yeast particles with methylene blue (B1 corresponds to B2 as

does C1 to C2). 88

Plate 5.

A) An ouchterlony slide showing precipitin lines formed between Eisenia foetida agglutinin antigens and rabbit anti-agglutinin serum. (Well contents: 1 -pure agglutinin,25 μg; 2-coelomic fluid,1/16; 3-pure agglutinin,50μg; 4-coelomic fluid,1/8; 5-coelomic fluid,1/32; 6-undiluted control rabbit serum; 7-undiluted rabbit antiserum).

B) Western blotting and immunolabelling of coelomic fluid proteins from Eisenia foetida with anti-agglutinin Rabbit serum on nitrocellulose paper (Lane 1-25 μg agglutinin; Lane 2-40μl coelomic fluid; Lane 3-50μg pure agglutinin). Arrows indicate positions of labelled bands.

118

Plate 6.

Gold labelling (using 20nm particles) of Eisenia foetida phagocytes with anti-agglutinin rabbit serum linked to Goat anti-Rabbit gold conjugate (A-control phagocyte with primary antibody omitted (x50,000); B,C and D labelled phagocyte (30,000x,45,000x and 90,000x respectively).

131

Plate 7.

SDS PAGE of incubation media from Eisenia foetida coelomocytes (Lane 1-molecular weight markers; Lane 2 -released proteins; Lane 3-control; Lane 4-pure 20,000D agglutinin).

149

ABBREVIATIONS

BSA	Bovine serum albumin
BSM	Bovine submaxillary mucin
DAB	Diaminobenzidine
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(aminoethylether)N,N,N1,N1-tetra-acetic acid.
FITC	Fluorescein isothiocyanate
Gal	Galactose
GalNAc	N-acetyl-D-galactosamine
GluNAc	N-acetyl-D-glucosamine
kD	Kilodalton
KDO	2-keto-3-deoxyoctonate
Lac	Lactose
NANA	N-acetylneuraminic acid
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
SDS	Sodium dodecyl sulphate
Suc	Sucrose
TEMED	N,N,N^1,N^1-Tetramethylene diamine
TRIS	Tris(hydroxymethyl)methylamine

1. INTRODUCTION

1.1. THE IMMUNE SYSTEM.

The ability to distinguish 'self' from 'non-self' is basic to biological defence and the survival of all living organisms, both plant and animal. This defence is directed against threatening micro-invaders, parasites and against mutant cells generated within organisms and capable of becoming neoplastic.

Vertebrate animals respond to the presence of foreign materials in their tissues both by innate and acquired immune mechanisms. Innate (i.e. present at birth) responses are non specific in the sense that they are effective against a wide range of potentially infective agents and include defenses such as mechanical barriers and surface secretions, bactericidal substances of the tissues and body fluids and phagocytosis.

Foreign organisms which overcome or circumvent the innate non specific immune mechanisms come up against the host's second line of defence. Antigens of the invading organisms come into contact with cells of the immune system (macrophages and lymphocytes) which leads to the initiation of the acquired immune response, specific for the inducing antigen. This response takes two forms, humoral and cell mediated, which usually develop in parallel and although

treated separately, are closely linked as both depend on cells of the lymphoid system. The characteristic of humoral immunity is the appearance in the blood of immunoglobulins or antibodies. These combine specifically with the antigen which stimulates their production and can lead to various consequences. For example, the antigen molecules may be clumped, their toxicity may be neutralized, their uptake and subsequent digestion by phagocytes facilitated (opsonization) and cellular antigens that are part of a cell surface such as a red blood cell or bacterium may cause lysis of the cell. In cell mediated immunity, lymphoid cells may be induced, by prior exposure to antigen, to react subsequently, directly with the inducing antigen and bring about cytotoxic effects, as for example, on foreign cells from a graft.

Acquired immunity involves co-operation between three major classes of circulating leukocytes, namely B lymphocytes, T lymphocytes and the highly phagocytic macrophages. The reactions against foreignness involving these cells exhibit a high degree of specificity. Once a response to a particular antigen has occurred, a memory system is established that permits a rapid, more intense and specific response upon re-exposure to that same substance.

It is clear that invertebrates have a well developed non-specific cellular defence mechanism to cope with foreign materials that enter the tissues. The process is

mediated by cells called haemocytes (in the haemocoel) or coelomocytes (in the coelom, as the case may be) that phagocytose the foreign particle and may localize it in a fibrous nodule. This process is quite distinct from the cell mediated immune response of vertebrates as it does not result in the formation of a descendant family or clone of cells with a specificity limited to the inducing agent. However, evidence has been obtained in some invertebrates showing that they are capable of rejecting grafts from unrelated members of the same species. On repeated grafting, accelerated rejection takes place, indicating the development of what could be likened to a secondary immune response (Linthicum, Marks et al, 1977; Lemmi and Cooper,1981; Cooper, 1985).

It appears that, in vertebrates, immunoglobulins are unique evolutionary molecules that are necessary for specificity in immunity. In general, it would seem that the invertebrate humoral response must rely on non-specific factors and these may include agglutinins (or lectins), precipitins, lysins and bactericidins.

1.2. AGGLUTININS.

Agglutinins are a class of proteins which were discovered almost ninety years ago for their ability to clump red blood cells and so they were called haemagglutinins . Because they were first isolated from higher plants,they came to be known as

phytohaemagglutinins. In time, it was noticed that these proteins agglutinate not only erythrocytes but also other kinds of cells including fungi, lymphocytes, spermatozoa and bacteria. It also became clear that agglutinins are present not only in higher plants but also in animals, both vertebrates and invertebrates and in bacteria, algae, fungi, protozoa and viruses (Gold and Balding,1975; Goldstein et al, 1980). In general, most agglutinins can also be described as lectins though there are some exceptions (Fuke and Sugai, 1972; Ryoyama, 1974).

Lectins are carbohydrate binding proteins that agglutinate cells and/or precipitate glycoconjugates through their multiple binding sites causing interconnections between large numbers of cells resulting in agglutination. Particular lectins bind more or less specifically to particular sugar molecules or groups of molecules and it was for this property of selectivity that lectins were named from the Latin, legere, to pick or choose (Sharon,1977).

In general, each molecule of a lectin has two or more regions, perhaps clefts or grooves, each of which fits a complementary molecule of a sugar or several sugar units of an oligosaccharide.[1] Although the carbohydrate binding specificity is ubiquitous for binding by lectins, secondary forces such as hydrophobicity and charge interaction possibly operating through multivalent binding may also stabilize the bond (Olafsen,1986).

1. Sharon,1977.

Individual lectins frequently occur as a group of closely related proteins, designated as isolectins, the synthesis of which is under direct genetic control. In other cases, multiple lectins may occur and these may differ physically in sugar affinity, molecular weight and charge (Lis and Sharon,1986).

There is considerable support, but little solid evidence for the belief that lectins function primarily as recognition molecules (Weir,1980). In plants, two proposed functions of lectins are currently attracting most attention: a) as mediators of symbiosis between plants and micro-organisms and b) in the protection of plants against phytopathogens (Lis and Sharon,1986).

Bacterial surface lectins and lectin like substances seem to be involved in the initiation of infection by mediating bacterial adherence to host cells. The binding of Gram negative bacteria such as Escherichia coli, Salmonella typhi and Vibrio cholerae to mammalian epithelial cells, a prerequisite for colonization or infection of mucosal membranes, is mediated by carbohydrate specific lectin like receptor molecules on the bacterial cell surfaces. Recognition of mating types in E.coli occurs via specific cell envelope proteins (Monroy and Rosati,1979) and recognition and adhesion of gametes in the unicellular algae Chlamydomonas, occurs by means of a lectin-like mating substance (Goodenough and Adair,1979).

In viruses, the first stage of viral infection involves attachment to the host's cell membrane and this occurs in certain species of viruses by means of lectins in the viral protein coat (Laver,1973).

Vertebrate lectins are divided into two classes: a) integral membrane lectins that require detergents for their extraction, for example, mammalian and avian hepatocytes and b) soluble lectins[1]. Membrane lectins are thought to mediate the binding of soluble extracellular and intracellular glycoproteins as well as of cells[2]. Vertebrate hepatocyte lectins participate in the binding and clearance of a variety of circulating serum proteins[3]. In mammals, recognition by the hepatic lectin receptors occurs when the glycoprotein has lost terminal sialic acid residues, exposing penultimate galactose. Binding by the galactosyl-specific lectins is then followed by uptake and subsequent degradation of the proteins by the hepatocytes[4]. Mammalian Kuppfer cells and mononuclear spleen cells are also capable of binding desialated erythrocytes, the process can be inhibited by carbohydrates specific for the red cell membrane (Kolb and Kolb-Backofen,1978).

A common function proposed for the soluble vertebrate lectins is to bind to the complementary glycoconjugates on and around the cells that release them[2].

Lectins are found in all of the approximately thirty

1.Barondes,1984. 2.Lis and Sharon,1986.
3.Stein and Cooper,1982. 4.Ashwell and Morrell,1977.

phyla and the various classes and subclasses of invertebrates (Gold and Balding,1975) and will be discussed below. Since these lectins are ubiquitous within invertebrates, the possibility has been raised that the humoral lectins might be the functional analogs of vertebrate immunoglobulins.[1] This suggestion has also been put forward for plant lectins (Mirelman et al,1975).

However, there are several major differences between immunoglobulins and lectins.[2] The most important is that antibodies are products of the immune system of higher animals in which they are manufactured in response to stimulation by a foreign substance that has entered the body. Lectins, on the other hand, are present as constituent proteins of organisms and predominantly of organisms such as plants that are incapable of an immune response and of forming antibodies. Another difference is that specificity of a range of antibodies is broad, encompassing not only sugar, but also many other classes of compounds such as amino acids, proteins and nucleic acids (although each antibody is of course specific for the antigen that elicits it's formation). No lectin has been found that is specific for compounds other than carbohydrates. A third difference is in chemical structure.[3] All antibodies are structurally similar to one another, whereas the lectins are structurally diverse: examination of the amino acid composition , molecular size and other molecular properties of many lectins shows that they have very little in common except that they are all proteins or

1.Lis and Sharon,1986. 2.Stein and Cooper,1982.
3.Sharon,1977.

glycoproteins (Stein and Cooper,1982).

1.3 INVERTEBRATE AGGLUTININS.

Naturally occurring invertebrate haemagglutinins were first described by Noguchi (1903) in the lobster Homarus americanus and in the Horseshoe crab Limulus polyphemus, and have since been found in almost every species of invertebrate studied, (summary in Table 1, Ratcliffe and Rowley,1984) occurring mainly in the haemolymph or coelom, and in molluscs, in the albumin glands.

Physical and Chemical Properties

Most invertebrate haemagglutinins are proteins or glycoproteins. Only the haemagglutinins from Hemicentrotus pulcherimus[1], Nereis virens[2] and Styela plicata[3] have been shown to be probably non-proteinaceous and therefore do not conform to the strict definition of a lectin.

Most invertebrate lectins are polymers of non-covalently bound subunits of molecular weights between 15,000-20,000 D, though no universal homology has been demonstrated.[4]

Many invertebrate haemagglutinins need divalent cations, in particular, calcium,for their biological activity.[4] These divalent cations are necessary in some cases for stabilization of the molecule and therefore agglutination of the cells. Multiple lectins in the haemolymph of the

1.Ryoyama,1974. 2.Russel et al,1983.
3.Fuke and Sugai,1972. 4.Olafsen,1986.

Table 1.

Physiochemical Properties of Some Invertebrate Haemagglutinins (from Ratcliffe and Rowley, 1984).

TABLE 1
PHYSICOCHEMICAL PROPERTIES OF SOME INVERTEBRATE HEMAGGLUTININS

Group and representative genus/species	Specific name for agglutinin	Basic chemical nature	Molecular weight	Heat sensitivity	Need for divalent cations	Binding specificity	References
Sponges							
Aaptos papillata	*Aaptos* lectin I	Protein ?	21,000, 12,000 (subunits)	ND[a]	ND	GlcNAc[b]	Bretting *et al.* (1976)
	Aaptos lectin II	Protein ?	16,000 (subunits)	ND	ND	GalNAc, NANA	
	Aaptos lectin III	Protein ?	16,000 (subunits)	ND	ND	GalNAc, NANA	
Axinella polypoides	*Axinella* I	Protein	21,000	ND	ND	D-Gal	Bretting and Kabat (1976)
	Axinella II	Protein	15,000	ND	ND	D-Gal	
Geodia cydonium	*Geodia* lectin I	Glycoprotein with four identical subunits	60,000 (15,000 subunits)	ND	ND	Lac GalNAc	Bretting *et al.* (1981)
Annelids							
Lumbricus terrestris	—	Probably proteinaceous but resistant to trypsin	ND	− (100°C)	+ (Ca^{2+},Mg^{2+})	—	Stein *et al.* (1982)
Nereis virens	—	High-MW lipid(?)	ND	− (100°C)	ND	BSM	Russell *et al.* (1983)
		Low-MW protein	ND	− (100°C)	ND	BSM	
Mollusks							
Crassostrea gigas	Gigalin H	Protein	15,000 (subunits)	ND	ND	GlcNAc GalNAc NANA	Hardy *et al.* (1977[c])
	Gigalin E	ND	ND	ND	ND	ND	
Helix pomatia	*Helix pomatia* A (Anti-A_{HP})	Glycoprotein	72,000–79,000	+ (>80°C)	+ (Ca^{2+})	α-Methyl-GalNAc GalNAc GlcNAc	Hammarström and Kabat (1969)
Mytilus edulis		Protein	480,000	+	−	Mucin	Renwrantz (1982); Renwrantz and Stahmer (1983)
Limax flavus	LFH	Proteinaceous	44,000 (2 subunits of 22,000)	ND	ND	NANA	Miller (1982)
Tridacna maxima	Tridacin	Glycoprotein	470,000 (10,000, 20,000, and 40,000 subunits)	ND	+ (Ca^{2+})	GalNAc	Baldo *et al.* (1978)
Merostomates							
Limulus polyphemus	Limulin	Glycoprotein	ca. 400,000	−	+ (Ca^{2+})	GlcNAc NANA	Marchalonis and Edelman (1968); Finstad *et al.* (1972); Roche and Monsigny (1974); Oppenheim *et al.* (1974)
Tachypleus tridentatus	TTA-I	Proteinaceous[d]	—	ND	ND	ND	Shishikura and Sekiguchi (1983)
	TTA-II	Proteinaceous[d]	—	ND	ND	ND	
	TTA-III	Proteinaceous[d]	40,000 (subunits)	ND	ND	ND	
	TTA-IV	Proteinaceous[d]	—	ND	ND	ND	

(continued)

TABLE 1 *(continued)*

Group and representative genus/species	Specific name for agglutinin	Basic chemical nature	Molecular weight	Heat sensitivity	Need for divalent cations	Binding specificity	References
Arachnids							
Centruroides sculpturatus	—	Protein (at least 2 different)	ND	+ (65°C)	+ (Ca^{2+})	NANA *N*-glycolylneuraminic acid	Vasta and Cohen (1982)
Crustaceans							
Cherax destructor	—	Protein	81,000 (subunits 13,500)	+	+ (Ca^{2+})	ND	Unpublished observations reported in Ey and Jenkin (1982)
Homarus americanus	LAg-1	Protein	55,000 (subunits	+ (56°C)	+	NANA	Hall and Rowlands (1974)
	LAg-2	Protein	55,000 (subunits)	+ (56°C)	+	GalNAc	Hartman *et al.* (1978)
Insects							
Periplaneta americana	—	Protein	ND	+ (56°C)	ND	— (?)	Scott (1971a,b); Lackie (1981)
Sarcophaga peregrina	—	Protein	190,000 (subunits of 32,000 and 30,000)	ND	ND	Lac Gal	Komano *et al.* (1980, 1981)
Schistocerca gregaria	—	ND	ND	ND	ND	Suc Fetuin	Lackie (1981)
Teleogryllus commodus	—	Aggregated metalloprotein	>10⁶ (subunits of 31,000 and 53,000)	+ (56°C)	+	GlcNAc GalNAc	Hapner and Jermyn (1981)
Echinoderms							
Asterias forbesi	—	ND	120,000–150,000	+ (65°–70°C)	+	ND	Finstad *et al.* (1972)
Anthocidaris crassispina	—	Proteinaceous	>200,000	+ (70°C)	+ (Ca^{2+})	—	Ryoyama (1974)
Hemicentrotus pulcherrimus	—	Complex carbohydrate	ND	−	+ (Ca^{2+})	—	Ryoyama (1974)
Pseudocentrotus depressus	—	Proteinaceous	>200,000	+ (85°C)	+ (Ca^{2+})	Gal and several others	Ryoyama (1974)
Urochordates							
Botryllioides leachii	HA-1	Proteinaceous	200,000	−	+ (Ca^{2+})	Lac Gal	Schluter *et al.* (1981); Ey and Jenkin (1982)
	HA-2	Globular protein	63,000	−	−	Lac	
Halocynthia pyriformis	—	Proteinaceous	ND	− (50°C)	+ (Ca^{2+})	NANA	Anderson and Good (1975)
Halocynthia roretzi	—	Proteinaceous	41,000 (subunits)	−	+ (Ca^{2+})	Gal	Yokosowa *et al.* (1982)
Styela plicata	—	Polysaccharide or mucopolysaccharide	ND	−	−	ND	Fuke and Sugai (1972)

[a] ND, Not determined.

[b] GlcNAc, *N*-Acetyl-D-glucosamine; GalNAc, *N*-acetyl-D-galactosamine; NANA, *N*-acetylneuraminic acid; Lac, lactose; Gal, galactose; Suc, sucrose; BSM, bovine submaxillary mucin.

ascidian, Botrylloides leachii, showed differing calcium requirements (Schluter et al, 1981). Binding sites with differing calcium requirements within the same molecule have been reported for a tunicate lectin (Form,1979).

Haemagglutination inhibition tests using a range of carbohydrates have shown that many invertebrate agglutinins bind to similar sugars on the surfaces of erythrocytes. Carbohydrates widely found involved include N-acetylgalactosamine , galactose and sialic acids, the latter being a widespread feature of lectin binding in the arthropods.

The serum of many invertebrates contains several different haemagglutinins which react with a limited range of vertebrate erythrocytes. This is particularly demonstrated in the ascidian Botrylloides leachii (Schluter et al, 1981). Here, two haemagglutinins designated HA-1 and HA-2 have been isolated. HA-1 is specific for guinea pig erythrocytes whilst HA-2 agglutinates a wide range of erythrocytes such as pigeon,rabbit, mouse and guinea pig.

A Brief Survey of Invertebrate Lectins (see Table 1)

Sponge lectins may be involved in cellular aggregation of tissues. The lectins from the sponge Axinella polypoides are low molecular weight proteins (21,000 and 15,000 D) with minor differences in specificity, though both react with D-galactose (Bretting and Kabat, 1976).

The molluscs have probably been investigated for the presence of lectins more than any other group of invertebrates. The blood group A specific agglutinin from Helix pomatia was purified from the albumin gland of adult snails (Hammarstrom, 1974). It is a glycoprotein with a molecular weight of 79,000D. Later, this lectin was fractionated into 12 isolectins (Vretblad et al, 1979). It was suggested that heterogeneity of this lectin could either result from synthesis of a number of different lectin molecules or combinations of different polypeptide chains to make molecules of different structures. Other investigated molluscan lectins include those from Helix aspersa (Fountain and Campbell,1984), Biomphalaria glabrata (Bretting et al, 1983) and Crassostrea virginica (Tripp, 1966, 1974).

Studies on the agglutinins of the Crustacea include those of the american oyster, Homarus americanus (Hall and Rowlands,1974) and the crayfish Cherax destructor (McKay and Jenkin, 1970).

Many insect species contain agglutinins, for example, the beetle Allomyrina dichotoma (Umetsu et al, 1984) contains two lectins: Allo A-1 (65,000 D) consisting of two subunits (17,500 and 20,000 D) and Allo A-11 (65,500 D) also with two subunits (19,000 and 20,000 D). They are considered to be isolectins and increased activity was correlated with molt, injury or age. Komano et al (1980),

working with a lectin in the fleshfly Sarcophaga peregrina larvae that agglutinated sheep erythrocytes and was inhibited by galactose and lactose, demonstrated the native molecular weight to be 190,000D consisting of four α (32,000 D) and two β (30,000 D) subunits. The lectin was induced in two different ways: by injury to the larval body wall and during pupation. It was demonstrated that haemolymph from normal larvae contained only the α subunit (Komano et al, 1981). It was suggested that following injury to the body wall, part of the α subunits was converted to β by a protease resulting in the formation of an active $\alpha_2\beta_4$ lectin.

In the case of the Merostomes, the horseshoe crab Limulus polyphemus contains a well described lectin system. The lectin, limulin, first purified and characterized by Marchalonis and Edelman (1968), agglutinates a variety of erythrocytes. It is specific for N-acetylglucosamine and 2-keto-3-deoxyoctonate (KDO) and calcium was required for agglutination. Limulin is a 400,000D glycoprotein consisting of six non-covalently bound subunits (67,000 D) that were further dissociated into 22,500D subunits by 8M urea, i.e. six basic units each composed of three subunits. Other merostome agglutinins that have been investigated include those from the Japanese horseshoe crab Tachypleus tridentatus (Shimizu et al, 1977).

In the Arachnids, multiple lectins have been detected in the scorpion Vaejovis confuscius and in the whip scorpion

Mastigoproctus giganteus (Vasta and Cohen, 1984). The scorpion Hadrurus arizonensus contained lectins that agglutinated a panel of erythrocytes that were heterogeneous in specificity.

Finally, in the Urochordata, tunicates have been surveyed for lectin activity (Ey and Jenkin, 1982; Vasta et al,1982). Halocynthia pyriformis haemolymph contains a polymeric lectin (900,000 D) composed of non-covalently bound subunits of 20,000D. The binding specificity revealed the presence of two different binding sites within the same molecule: one specific for Bovine Submaxillary Mucin (BSM) and the other specific for galactosyl residues. Calcium is required for agglutination of horse but not human erythrocytes (Form,1979).

Agglutinins for a variety of erythrocytes were demonstrated in Botrylloides leachii as mentioned earlier, (Schluter et al, 1981). One, HA-1, is a glycoprotein of molecular weight 138,000D consisting of 27,000D subunits. HA-2 is a globular protein consisting of two non-covalently bound subunits of molecular weights 32,000-33,000D.

So far, the agglutinins characterized have been assayed for their ability to bind to erythrocytes. But several invertebrates have been shown to cause the aggregation of a variety of foreign materials such as bacteria (Bretting et

al,1978), protozoans (Bang,1967; Ingram et al, 1983), sperm (Tyler,1946) and metazoan parasites (Stein and Basch, 1979; Lackie,1981).

Antibacterial agglutinins

Bretting et al (1978) demonstrated the existence of a distinct agglutinin from the sponge Axinella polypoides which reacts with Nitrobacter winogradskyi. There is also an antibacterial agglutinin in the haemolymph of the Horseshoe crab, Limulus polyphemus (Pistole, 1976,1978 and 1982) which has a molecular weight of 200,000D and binds with 2-keto-3-deoxyoctonate, which is present in the outer membrane of many Gram negative bacteria. This antibacterial agglutinin is identical to the previously characterized haemagglutinin Limulin (Rostam-Abadi and Pistole,1982). The agglutinins of Lumbricus terrestris have been shown to have antibacterial activity (Stein and Cooper,1981) and this may also be the case in Eisenia foetida (Vaillier et al,1985).

Induction of Invertebrate Agglutinins

In most invertebrates, agglutinins occur naturally and higher titers cannot be induced by antigenic stimulation. A few studies have however shown a limited success in the stimulation of haemagglutinin activity. Fleshfly larvae, Sarcophaga peregrida, haemagglutinins can be induced by injury to the body wall (Komano et al,1980) and higher levels of haemagglutinins are produced in the Pacific

oyster, Crassostrea gigas, after exposure to bacteria (Hardy et al, 1977). However, this response may not be indicative of a defence reaction as bacteria may be a potential food source for this filter feeding bivalve. A nutritive role of the agglutinin is in part supported by the fact that in vivo exposure of oysters to algal cells also leads to an increase of lectin activity in the haemolymph (Olafsen,1986).

In the earthworm, Lumbricus terrestris, increased titers of haemagglutinins may also be induced by immunization with specific erythrocytes (Cooper et al, 1984). Many of these experiments also showed that haemagglutinins can be induced by a range of substances totally unrelated to the indicator erythrocytes, suggesting a non-specific stimulation of the response.

Agglutinin Synthesis

Agglutinins appear to be synthesized by the fat body in insects (Kubo et al 1984) and the coelomocytes or haemocytes (Amirante,1976; Yeaton 1980, Van der Knaap et al,1981; Stein and Cooper,1988) in other invertebrates.

In the insect Hyalophora cecropia, Yeaton (1980) demonstrated by three different methods that agglutinins are synthesized by haemocytes, probably granulocytes. Radioactively labelled amino acids were incorporated in vitro by haemocytes and secreted as agglutinins.

Fluorescein labelled antisera to haemagglutinins demonstrated the presence of agglutinins in both the granules and the cytoplasm of granulocytes, whilst plasmatocytes (phagocytic cells) were stained over the surface of the cell membrane.

Immunofluorescent labelling of the cockroach Leucophaea maderae haemocytes with anti-lectin serum localized two lectins in and on the cells (Valvassor and Amirante, 1976). Since pretreatment of the cells with cycloheximide inhibits this label, Amirante and Mazzalai (1978) have concluded that lectin synthesis occurs in the haemocytes also.

In the sponge, Goedia cydonium, lectin is synthesized by mucoid cells and is later transported to the surface of choanocytes (Muller,1980).

Agglutinin function.

Many suggestions and much speculation have been advanced on the functions of invertebrate lectins. Possible non-immunoglobulin functions include carbohydrate and calcium transportation (McDade and Tripp, 1967), ingestion of food particles in sea anemones (Gawerky et al, 1974), control of sponge cell reaggregation (Vaith et al, 1979; Muller, 1982; Hildemann, 1974), the utilization of plankton and a recognition role in cell to cell interactions in fertilization (Vacquier and Moy, 1977). Recognition of a host may also be attributed to lectins; a parasitic

protozoan, Plasmodium, bears cell membrane associated lectins which are specific for glycoproteins present on erythrocyte plasma membranes, suggesting that they are responsible for the selection of the particular blood cells they penetrate and infest (Jungery et al,1983).

Another function of agglutinins may be in an involvement in symbiotic relationships (Uhlenbruck and Steinhausen, 1977), either as a mediator as observed between algae and clam (Baldo and Uhlenbruck, 1975) and between intermediate 'guest' nematode and snail (Ratanarat-Brockelman, 1977) or as a regulator. In this latter function, the haemagglutinin, tridacnin, may serve to recognise effete algal symbionts and consequently bring about their aggregation and digestion by haemocytes and other cells.

Suggestions as to immunological modes in which the agglutinins may function include participating in protecting the host from invasion by foreign organisms, perhaps as sugar configeration specific molecules which can agglutinate bacteria, viruses, fungi, sperm and parasites (Bretting et al, 1978; Scott, 1971a; Hunt et al, 1977). Yeaton (1980), showed in the silkmoth Hyalophora cecropia, that substantial amounts of agglutinin are bound to the haemocoelic side of the alimentary canal lining. Parasites penetrating through the gut barrier may hence become coated by the agglutinin and subsequently localized and immobilized enabling haemocytes to deal with them. Agglutinins may also act as perivitelline protectors to

maintain the sterility inside eggs (Pemberton, 1974; Yeaton, 1980).

The possibility that agglutinins function in an immunological role is further substantiated by the observation that invertebrates can discriminate quite specifically between different non-self materials (e.g. Duprat, 1964,1967; Cooper, 1969; Renwrantz et al, 1981; Thomas and Ratcliffe,1982). Evidence is accumulating that agglutinins may be the recognition and receptor molecules involved.

The crayfish Parachaeraps bicarinatus and the gastropod Helix pomatia eliminated different strains of bacteria at different rates (Tyson et al, 1974; Renwrantz et al, 1981). As these clearance patterns could result from varying particle sizes, Helix was injected with different types of erythrocytes, i.e. non-self cells of equal size but differing surface molecules. As human A type cells were cleared twice as fast as B erythrocytes and four times as fast as rabbit erythrocytes, the snail's internal defence system must have recognized different membrane structures of the foreign cells. This assumption was supported by the fact that masking of surface molecules of yeast cells by Helix haemocyanin strongly decreased their elimination rate when compared to clearance rates of untreated or yeast cells which had been coated with Bovine Serum Albumin (BSA),(Renwrantz et al,1981).

The involvement of organs in clearance is comparable to the situation in vertebrates where the liver is primarily responsible for the clearance of carbohydrate containing molecules.[1] Recognition of these compounds and their clearance from the plasma are mediated by carbohydrate specific receptors associated with macrophages and cells of the reticuloendothelial system. The binding of these lectin like receptors to soluble or particulate glycoconjugates is calcium dependent and can be inhibited by specific saccharides (Ashwell and Harford,1982). Cell bound recognition molecules with a carbohydrate binding specificity also occur in invertebrates. The clearance of yeast from the circulation of Helix pomatia is inhibited by GalNac and GluNac but not by fucose (Renwrantz et al,1981).

An immunocytochemical investigation has demonstrated membrane linked agglutinin at the surface of cells lining blood vessels of Lymnaea stagnalis and of the membrane of haemocytes from this gastropod (Van der Knaap et al,1981).

With regard to function of agglutinins, by far the most extensive studies have looked into the role of agglutinins as opsonins. The term opsonin designates those elements in serum or plasma which promote phagocytosis (opsono- I cater for, I prepare victuals for). The phagocytic cells of animals display a high degree of selectivity in what they will or will not ingest. In the vertebrates, overwhelming evidence points to the importance of the immunoglobulin molecules in providing this selectivity. Recent

1.Renwrantz et al,1981.

observations have shown that the phagocytic cells of invertebrates do react efficiently to a variety of foreign particles and that this recognition involves the prior interaction of the foreign material with factors in the haemolymph (Jenkin, 1976).

Many workers who have shown that the body fluids of invertebrates act as opsonins believe that the agglutinins present in these animals are the recognition molecules involved. The evidence in favour of agglutinins acting as recognition molecules in at least some invertebrates has recently become more substantial. Agglutinins are found in the blood, coelomic fluid and reproductive organs of a broad range of invertebrates (Chorney and Cheng, 1980; Ey and Jenkin, 1982). Agglutinins are also present in vertebrates and in addition, some immunoglobulins may have both haemagglutinating and opsonic properties (Ey and Jenkin, 1982). Although the carbohydrate specificity of agglutinins appears to be rather limited, this is because only the carbohydrate with which they have the highest affinity may be reported. In actual fact, this does not reflect their overall affinity since they probably bind to a whole range of carbohydrates (Vasta and Marchalonis, 1983). Even if the specificity of agglutinins is somewhat limited, these substances may be able to recognise an extensive variety of cell surface carbohydrates originating by changes in the isomeric configerations of a relatively limited number of monosaccharides.

Perhaps Tripp (1966) was the first to provide a clue that haemagglutinins may be active as opsonins. He demonstrated that rabbit red cells treated *in vitro* with oyster haemolymph are more readily phagocytosed by oyster haemocytes than non treated cells. Stuart (1968) found that in the octopus, phagocytosis of erythrocytes is dependent on a haemolymph factor. In an effort to determine if oyster haemagglutinin was involved in an immune situation analogous to vertebrates, Acton and Evans (1968) injected different groups of oysters with varying amounts of sheep erythrocytes, but a rise in haemagglutinin titers as a consequence of the injection schedule, was not observed.

Although it had been shown in a number of invertebrates that a factor in the haemolymph was opsonic, no evidence pointed to the agglutinins as being these recognition factors. Later, results of *in vivo* clearance experiments provided strong circumstantial evidence that the agglutinins are opsonic. Pauley et al (1971) showed that the marine gastropod *Aplysia californica* rapidly removed marine bacteria from the haemolymph but failed to clear *Serratia marcescens*. The cleared species are all agglutinated by *A.californica* haemolymph in contrast to the terestrial *S.marcescens* which persists in the haemolymph several weeks post injection.

Other evidence for the function of agglutinins as opsonins is provided by Renwrantz (1979) and Harm and Renwrantz (1980) in their investigations on

immunorecognition in the gastropod Helix pomatia. Using secondary doses of erythrocytes pretreated with either purified snail or sponge agglutinin, they demonstrated that only the former produced clearance rates equal to those of primary doses and was therefore probably functioning as an opsonin. Furthermore, because N-acetylglucosamine (a potent inhibitor of haemagglutination activity in Helix) but not fucose (a non-inhibitor of Helix haemagglutination) also decreased the clearance rates of primary doses of erythrocytes, the agglutinin and the opsonin may be one and the same thing.

The strongest evidence of an opsonic role for invertebrate agglutinins is provided by in vivo and in vitro experiments with purified molecules (Hardy et al, 1977; Harm and Renwrantz, 1980; Renwrantz and Stahmer, 1983). Hardy et al (1977) tested the effect of the purified agglutinin from the Pacific oyster Crassostrea gigas on the phagocytosis of bacteria in vitro. Pretreatment of E.coli and Vibrio anguillarum with the agglutinin or whole haemolymph resulted in an enhanced uptake of the micro-organisms in comparison with the saline incubated controls. Enhancement was as much as 50% and was specific for the Crassostrea agglutinin, since an increased ingestion was not recorded following pretreatment with an agglutinin (Limulin) from the horseshoe crab.

Renwrantz and Stahmer (1983) purified the agglutinin from the haemolymph of the mussel Mytilus edulis and

pretreated yeast cells with a solution of this purified agglutinin or with 50% haemolymph or with Tris buffered saline alone before placing them on haemocyte monolayers for phagocytosis to occur. A high rate of phagocytosis involving in excess of 50% of the haemocytes, was obtained from the haemolymph and agglutinin incubated yeast cells, while only 5% of the haemocytes ingested the saline incubated yeast cells. Phagocytosis rates were thus very similar for the whole haemolymph and the purified agglutinin thus proving the opsonic role of the agglutinin.

If agglutinins do act as recognition molecules, it should be possible to detect these factors or receptors for them at the surfaces of haemocytes or coelomocytes. Renwrantz and Stahmer (1983) used anti-humoral agglutinin antibodies to detect membrane associated agglutinins on the surface of the Mytilus haemocytes. Other molluscan species have also been reported to have cell associated lectin-like molecules (Van der Knaap et al, 1981,1983; Vasta et al, 1982). In the Pond snail Lymnaea stagnalis, the peroxidase-DAB technique revealed the presence of agglutinin at the cell surface and in the cytoplasm of the haemocytes where it may be synthesized (Van der Knaap et al 1981). Detection of cytophilic membrane-integrated agglutinin molecules is not confined to the molluscs but has also been demonstrated for the haemocytes of an insect, Leucophaea maderae (Amirante and Mazzalai, 1978) and a tunicate, Botrylloides leachii (Ey and Jenkin, 1982).

The presence of agglutinin receptors on the haemocyte or coelomocyte surface has been reported in a wide range of invertebrate species by utilizing labelling, rosette formation and inhibition techniques. A variety of receptors for sugars has been demonstrated on the coelomic cell membranes of earthworms (Roch and Valembois, 1978), sipunculids (Gebbinck, 1980), bivalves (Yoshino et al,1979; Cheng et al,1980), gastropods (Sminia et al, 1981; Yoshino, 1981), crustaceans (Gebbinck, 1980) and tunicates (Warr et al, 1977). Since a number of viruses and bacteria bear surface agglutinins, it is possible that micro-organisms can bind to these membrane-integrated receptors. This may explain why, in many cases, uptake of erythrocytes, bacteria and other particles can occur independently of serum factors (Scott,1971a; Tyson and Jenkin,1974).

Agglutinins may bind foreign particles and mediate recognition by one or a combination of three different pathways (Renwrantz 1983):

1) By direct binding of carbohydrate determinants of the foreign particles to membrane bound agglutinins;

2) By indirect binding via humoral agglutinin which links the carbohydrate determinants of the foreign body to opsonin receptors of the haemocytes; obviously the agglutinin must undergo some conformational change once it combines with the micro-organisms which allows it to bind to the haemocyte surface, otherwise normal ciculating agglutinin would interact with the opsonin receptors on the invertebrate cells before binding to the foreign particle

to be ingested;

3) By direct binding of surface agglutinins of foreign particles to carbohydrate determinants on the surface of haemocytes or coelomocytes.

The pathways above are shown diagrammatically in Fig.1.

Because of their ability to agglutinate cells, lectins have lent themselves readily to speculation as to whether they are primitive recognition molecules even though they bear little or no relationship to vertebrate immunoglobulins. With the exception of opsonization in a few invertebrate species, proof of the many potentially vital functions attributed to agglutinins remains elusive. Lectins may simply be a heterogenous class of proteins with little or no structural or functional relationship and it is still an open question whether their <u>in vitro</u> ability to agglutinate cells has any universal biological significance.

Evolution of Agglutinins.

Much effort has been directed at detecting antibody-like substances in invertebrates and as agglutinins may represent the recognition molecules in at least some invertebrates, attempts have been made to show an evolutionary relationship between these molecules (Marchalonis and Warr, 1988).

However the question of homology remains an open one.

Fig.1.

Possible Agglutinin Mediated Binding Reactions Between Microorganisms and Immunocompetent Invertebrate Cells (From Renwrantz,1983).

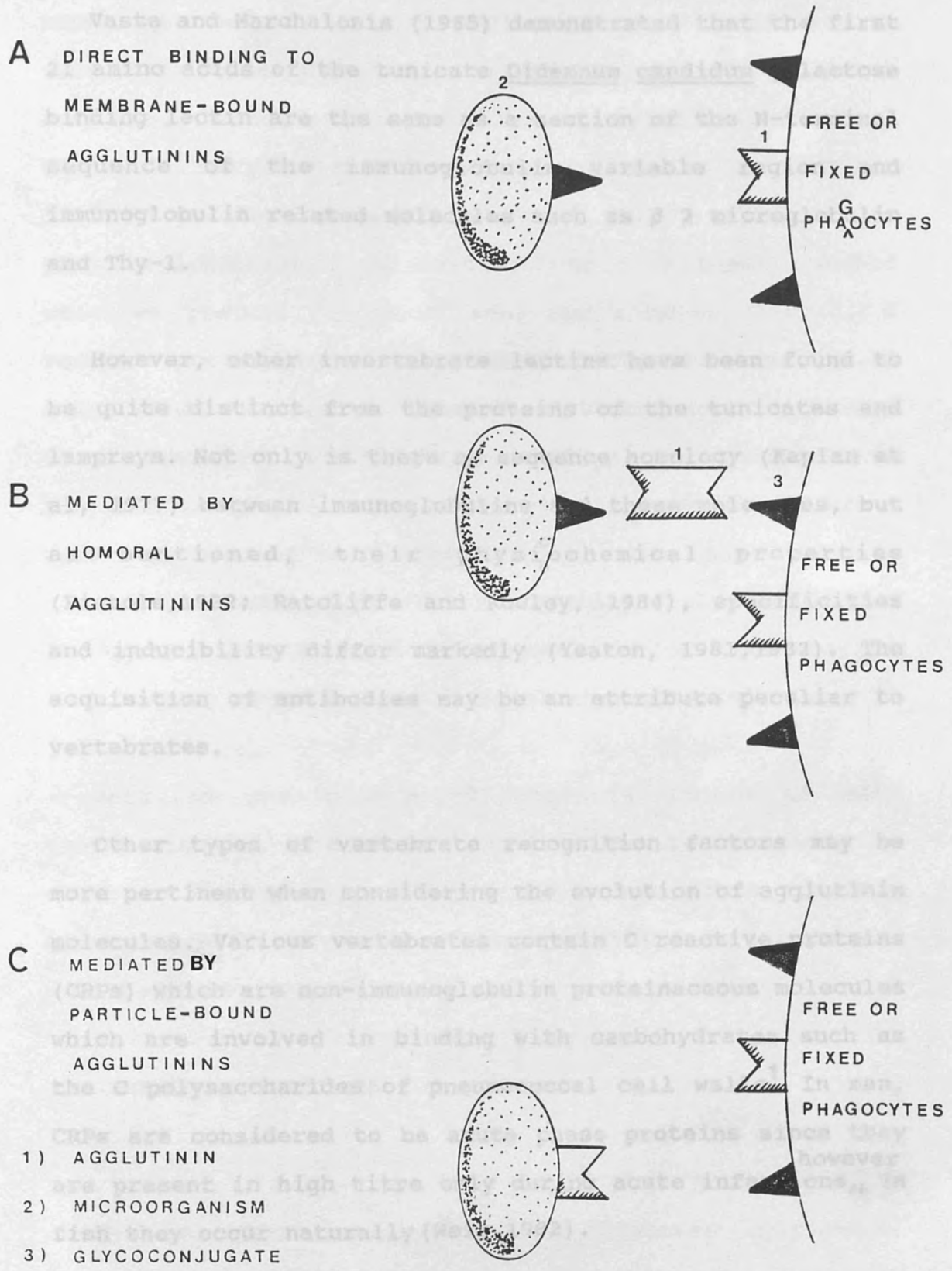

A
DIRECT BINDING TO MEMBRANE-BOUND AGGLUTININS
2
1
FREE OR FIXED PHAGOCYTES
B
MEDIATED BY HOMORAL AGGLUTININS
1
3
FREE OR FIXED PHAGOCYTES
C
MEDIATED BY PARTICLE-BOUND AGGLUTININS
FREE OR FIXED PHAGOCYTES
1) AGGLUTININ
2) MICROORGANISM
3) GLYCOCONJUGATE

FIG.1.

Vasta and Marchalonis (1985) demonstrated that the first 21 amino acids of the tunicate Didemnum candidum galactose binding lectin are the same as a section of the N-terminal sequence of the immunoglobulin variable region and immunoglobulin related molecules such as β 2 microglobulin and Thy-1.

However, other invertebrate lectins have been found to be quite distinct from the proteins of the tunicates and lampreys. Not only is there no sequence homology (Kaplan et al, 1977) between immunoglobulins and these molecules, but as mentioned, their physicochemical properties (Pistole,1982; Ratcliffe and Rowley, 1984), specificities and inducibility differ markedly (Yeaton, 1981,1982). The acquisition of antibodies may be an attribute peculiar to vertebrates.

Other types of vertebrate recognition factors may be more pertinent when considering the evolution of agglutinin molecules. Various vertebrates contain C reactive proteins (CRPs) which are non-immunoglobulin proteinaceous molecules which are involved in binding with carbohydrates such as the C polysaccharides of pneumococcal cell walls.[1] In man, CRPs are considered to be acute phase proteins since they are present in high titre only during acute infections, however in fish they occur naturally (Weir,1982).

Robey and Lui (1981) showed that the agglutinin limulin, from the horseshoe crab Limulus polyphemus, not only has

1.Vasta and Marchalonis,1983.

some sequence homology and serological cross reactivity to vertebrate C reactive proteins but also like this latter molecule, it binds phosphorylcholine. Marchalonis et al (1984) demonstrated that monoclonal antibodies to the TEPC-15 idiotype (the dominant idiotype on BALB/C antibodies to phosphorylcholine) cross reacted with both Limulin and C reactive protein. Sequence analyses between TEPC-15, C reactive protein and Limulin showed that small stretches of amino acids were shared. These results were not interpreted as evidence for evolutionary homology, but rather as a possible example of convergent evolution in which different molecules binding to a common ligand have been forced to use similar residues to form the combining site. Therefore it appears that C reactive proteins do not represent an evolutionary holdover from invertebrate agglutinins.

Detailed physicochemical characterization of both vertebrate and invertebrate agglutinins is necessary in order to determine if they are truly homologous and if a genuine link exists between these molecules in vertebrates and their invertebrate ancestors.

1.4.ANNELID AGGLUTININS.

The annelids consist of three classes; Polychaeta, Oligochaeta and Hirudinea. The polychaetes are primarily marine worms, the oligochaetes are both aquatic and terrestrial and include the familiar earthworms, while

hirudinaeans or leeches are primarily parasitic. Annelid coelomic fluid serves different physical and biological functions; it acts as a hydrostatic skeleton and also as a medium for trophic, excretory and respiratory exchanges.

It has been found that the coelomic fluid of various annelid species possess agglutinins (Du Pasquier and Duprat,1968; Cooper et al, 1974; Gold and Balding, 1975; Garte and Russel, 1976; Roch, 1979; Anderson, 1980; Chateaureynaud-Duprat and Izoard,1977; Stein et al, 1981; Dales,1982; Russel et al, 1983.) and lysins, and although the relationship of these substances to the immune response is for the most part unclear, they are assumed to have immune related functions (Anderson, 1980).

Annelid agglutinins appear to vary from species to species in their molecular structure and biochemical composition (Gold and Balding, 1975; Roch et al, 1981). In most instances they have been tested for activity against erythrocytes and are therefore generally termed haemagglutinins, though in some species, activity has also been demonstrated against bacteria (Roch et al,1981; Stein et al,1981; Valembois et al, 1982).

Polychaete agglutinins

Garte and Russel (1976) reported the isolation, purification and characterization of three agglutinins from the whole body extracts of the polychaetous annelid,

Amphitrite ornata. There are two high molecular weight fractions (100,000 and 54,000D) which are thought to be aggregates of a 30,000D fraction named Amphitritin. Amphitritin is a glycoprotein which contains 13% carbohydrate, consisting of mannose, galactose and sialic acid - a similar composition to oyster and spiny lobster agglutinins (McDade and Tripp, 1967; Acton et al, 1973). Amphitritin is active towards human blood groups A, B and O, and especially so towards rat erythrocytes. Chicken erythrocytes are also agglutinated. None of the saccharides tested inhibited the agglutination of chicken erythrocytes though N-acetylgalactosamine lowered the haemagglutination titre against rat red blood cells. Activity is unaffected at temperatures below 85^{o}C, this heat stability is unusual as most invertebrate lectins are inactive above 70^{o}C.

In 1980, Anderson reported the presence of a haemagglutinin active against rabbit and sheep erythrocytes in the coelomic fluid of Glycera dibranchiata. Freezing and thawing had no effect on the agglutinin, though heating at 56^{o}C for 30 min reduced the activity to 5-10% of the capacity of untreated coelomic fluid. Attempts to alter haemagglutinin activity by prior intracoelomic injections were unsuccessful. Glycera coelomic fluid also contains a haemolysin (Anderson, 1980) and a factor with potent antibacterial properties against Serratia marcescens (Chain and Anderson, 1983a, 1983b).

Of the few studies undertaken on annelid agglutinins

most have searched for activity in the coelomic fluid (with the exception of agglutinin preparations from whole body homogenates). Dales (1982) demonstrated the presence of weak agglutinins in both the coelomic fluid and in the blood of some individual Arenicola marina and Neoamphitrite figulus. Coelomic fluid activity was most noticeable towards rabbit, rat and guinea pig red cells in the former case and rabbit, sheep and horse in the latter. Agglutinating activity in the blood of Arenicola was directed towards human A and B, rat and horse red cells whilst guinea pig cells were not agglutinated. The blood of Neoamphitrite showed strongest haemagglutination toward horse and rabbit cells. These results show that the agglutinating activity of blood and coelomic fluid of both animals are different, both qualitatively and quantitatively (Dales, 1982). In both animals the agglutinating activity was fairly resistant to heat (60°C for 30 min causing a reduction by one or two wells in the case of Arenicola and by two or three wells in Neoamphitrite). Haemagglutination was inhibited to a small extent in Neoamphitrite by the α – methyl and acetylated sugars. The coelomic fluid of Neoamphitrite also contains a lysin.

The coelomic fluid of Nereis virens agglutinates rat, mouse, chicken, guinea pig and Rhesus monkey erythrocytes (Russel et al, 1983). In the same study it was found that there are at least two types of agglutinins; high molecular weight lipids which are inhibited by bovine submaxillary

mucin (BSM), thyroglobulin and transferrin, and low molecular weight proteins which are inhibited by BSM, fetuin and mannan preparations.

Other preliminary studies on polychaete agglutinins include that of Parinello (1981), who reported that fluid drained from crowns of Sabella spallanzanii agglutinate and lyse rabbit red cells. Brown et al (1968) demonstrated the agglutination of human erythrocytes by whole animal extracts of Sabella magnifica and Tyler (1946) reported the agglutination of frog and lizard cells by humoral factors in Chaetopterus variopedatus.

Oligochaete agglutinins

The coelomic fluid of Lumbricus terrestris contains low levels of naturally occurring haemagglutinins, active towards rabbit and rat erythrocytes, and lysins (Cooper et al, 1974; Stein et al, 1981). More recently, bacteriostatic factors have been reported (Kauschke and Mohrig, 1987; Anderson,1988). If worms are injected intracoelomically with specific vertebrate erythrocytes (Stein et al, 1982; Cooper et al,1974), some strains of bacteria (Stein, unpublished) or with certain sugars or other organic substances (Wojdani et al, 1982), agglutinin levels increase significantly within 24 h. In uninjected worms, only one or two agglutinins are usually present in sufficient amounts to be easily detected, while coelomic fluid of injected worms is believed to contain greater

numbers (3 or 4) of different agglutinins (Stein et al, 1981,1982). These induced agglutinins raise the activity by 30-50 fold and as with the naturally occurring agglutinins they react most strongly to rat and rabbit erythrocytes (Stein et al, 1981,1982; Kauschke and Mohrig, 1987).

Activity of the natural agglutinin remains stable after heating at 100^{o}C and after trypsin treatment suggesting that it may be non-proteinaceous. However, treatment with ammonium sulphate precipitated most of the active material and trichloroacetic acid abolished most of the activity, confirming that the naturally occurring agglutinin is probably a protein but one that is highly stable to heat (Stein et al,1982).

Cross reactivity and absorption data indicate a close or possibly identical relationship between Lumbricus agglutinins induced against different erythrocyte types. However, temperature inactivation data suggest that there are three different induced agglutinins; one inactivated between 50-60^{o}C, one inactivated between 80-100^{o}C and one stable to heating at 100^{o}C. These induced agglutinins have molecular weights ranging from approximately 8×10^{3} D to 4×10^{5} D (Stein et al,1981).

Divalent cations were required for full activity of both induced and naturally occurring agglutinins (Cooper et al,1974).

Lumbricus agglutinins show lectin-like properties in that they bind carbohydrates. Although they are not inhibited by any of the simple sugars normally found on eukaryotic cell surfaces (Stein and Cooper,1983a), various glycoproteins and polysaccharides have varying inhibition activities, and have been found to affect naturally occurring and induced agglutinins differently. Naturally occurring agglutinins are inhibited most strongly by BSM, thyroglobulin, mucin and fetuin, whereas induced agglutinins are inhibited by BSM, thyroglobulin, hyaluronic acid and fetuin. One sugar, the bacterially derived monosaccharide KDO was specifically inhibitory to both naturally occurring and induced agglutinins (Stein and Cooper,1983b). This may suggest that bacterial surfaces are the primary target for Lumbricus agglutinins. The fact that coelomic fluid agglutinates several species of bacteria, reacting with three of the seven strains isolated from Lumbricus, as well as with stock cultures of E.coli and A.hydrophila may further support this suggestion (Stein and Cooper, 1981).

The effects of coelomic fluid on phagocytosis of yeast cells by Lumbricus coelomocytes were studied in vitro, examining both whole coelomocyte populations and specific coelomocyte types (Stein and Cooper, 1981). It was found that the cells described as 'neutrophils' showed enhanced phagocytosis in diluted coelomic fluid or after pre-opsonising yeast with coelomic fluid. Measurements of phagocytosis in whole coelomocyte populations showed little

evidence of opsonization by coelomic fluid, probably because of the relatively small numbers of 'neutrophils' (<20% of coelomocyte population- Stein et al, 1977) found in the coelomic fluid compared with 'basophils' and granular cells. It has yet to be determined whether these coelomic fluid factors facilitating opsonization are in fact the agglutinin molecules.

It has been shown that Lumbricus coelomocytes cultured in defined media release low amounts of haemagglutinins. In rosette formation studies coelomocytes were cultured with rabbit erythrocytes. It was found that addition of cycloheximide to the culture medium did not reduce the number or size of rosettes, suggesting that rosette formation resulted from the release of presynthesized agglutinin rather than by de novo synthesis (Cooper et al,1984; Stein and Cooper, 1988). Although it would seem that agglutinins are stored in the coelomocytes, it remains to be determined whether these cells actually synthesize the molecules or if they are transported to them from other sites.

1.5. Eisenia foetida

The coelomic fluid of the Lumbricid Eisenia foetida andrei possesses a strong haemolytic activity (Du Pasquier and Duprat, 1968) which has been well characterized (Roch et al, 1979; Roch, 1979; Roch and Davant, 1982; Vaillier et al, 1985) and has been termed the Eisenia foetida andrei

Factor or EFAF (Roch et al,1981). The EFAF is able to lyse various vertebrate erythrocytes (Du Pasquier et Duprat, 1968) and to inhibit the growth of certain bacteria. This bacteriostatic activity can be demonstrated against bacteria which are pathogenic for the earthworms (Lassegues et al, 1986; Vaillier et al, 1985; Valembois et al, 1982).

In polyacrylamide gel electrophoresis (PAGE), the coelomic fluid is seen to consist of 18 different protein components. EFAF appears as two lipoproteins with molecular weights of 45,000D and 40,000D. In isoelectric focusing, EFAF migrates as four different molecules referred to as isoforms and characterized by their isoelectric points which range from 5.9 to 6.3. All of the animals possessed either two or three isoforms and among all of the population tested, only six different patterns were found (Roch, 1979; Roch et al, 1981; Roch et al, 1986).

More recently, Kauschke and Mohrig (1987) demonstrated the existence of at least twelve haemolytic proteins in pooled coelomic fluid whilst individual worms have between two to eight haemolytic bands.

The two haemolytic proteins are not only bacteriostatic but they have been found to participate in the constitution of a clot network (Valembois et al,1988). It has been proposed that these clottable coelomic fluid proteins are released through the dorsal pores and spread over the outer

surface of the body wall where they polymerize to form a clot network which acts as a filter trapping bacteria.

The presence of a strong haemolytic activity in the coelomic fluid of Eisenia foetida prevents the investigation of agglutinins using fresh erythrocytes. To overcome this, erythrocytes are stabilized by incubation with glutaraldehyde. Although glutaraldehyde is capable of interacting with amino acids, it does not cause spontaneous agglutination of the erythrocytes. Using such stabilized erythrocytes, the presence of haemagglutinating activity was reported in both the coelomic fluid and the cocoon albumin of E.foetida (Roch, 1979). The coelomic fluid agglutininin is active towards sheep, human A, B and O, rabbit, rat, badger, chicken and frog red blood cells, whilst that in the cocoon albumen is active at much lower levels towards sheep, rabbit and rat erythrocytes.

Cenini (1983) tested individual worms for agglutinating activity and found that it was only present in 39% of the animals studied. Activity was lost after treating the coelomic fluid at 56^{o}C for 30 min.

Chromatographic techniques demonstrated that agglutinin activity was due to four different molecules (Roch et al, 1984) of molecular weights 11,500, 20,000, 35,000 and 40,000 D. The 40,000 D molecule is capable of both haemolysis and haemagglutination. The 35,000 D molecule is a subunit of a 175,000 D protein, and both it and the

20,000 D molecule have been reported to have bacteriostatic activities (Valembois et al, 1986).

So far, no sugar has been found that is capable of inhibiting agglutinin activity and so it remains to be determined whether these Eisenia molecules are true lectins or not.

This study involves an investigation into the nature, origin and function of agglutinins in the coelomic fluid of Eisenia foetida. The object of the study is to determine the roles, if any, that these substances play in internal defence reactions, and if opsonizing activity is found to be present, whether these agglutinin molecules are involved.

2. PURIFICATION, CHARACTERIZATION AND ASSAY OF THE EISENIA FOETIDA AGGLUTININS.

2.1. INTRODUCTION.

The discovery of the presence of an agglutinin or any other molecule of interest in an organism is normally followed by a study of it's physicochemical properties in order to gain an insight into the nature of the molecule before further investigations are undertaken in an attempt to determine the full structure and function of it.

Although the presence of four haemagglutinins has been reported in Eisenia foetida (Roch et al,1984), no physicochemical studies have been reported which involve purified molecules. Studies on crude coelomic fluid may reveal very general properties of the haemagglutinins, but any differences between the molecules may be masked by the combined actions of all four haemagglutinins. Isolation of one or more of these molecules is therefore necessary in order to enable further investigations into the importance of these agglutinins and to determine whether, in fact, they can be designated to the lectin category. Purification was carried out in a similar fashion to that previously reported by Roch et al (1984), whose main interest was in the haemolytic system, employing gel filtration followed by

chromatofocusing.

2.2. MATERIALS AND METHODS.

2.2.1.Extraction of coelomic fluid.

Each worm was washed with tap water and blotted dry with paper towels. The coelomic fluid was harvested by electrical stimulation of each animal (5V) and collected directly into plastic 10ml centrifuge tubes. The fluid from each worm could be kept separate or pooled. After 10 min. centrifugation at 11,000g the cell free coelomic fluid was either used directly or stored at -70°C until required. Each worm yielded an average of 20 μl of fluid. With this technique, using a low voltage, disruption of the blood system was avoided and the yellow liquid obtained was free of blood protein.

2.2.2.Haemagglutination Assays

Quantitative haemagglutination tests were carried out in U well microtitre plates. The first well normally contained 200μl of coelomic fluid or the test solution, 100 μl of which was then mixed with an equal volume of phosphate buffered saline (PBS), pH 7.2 , in the next well. In this way, the test solution was serially diluted with a microdiluter through 12 wells. A control well contained PBS only. Erythrocytes from various vertebrates (though

normally sheep unless stated otherwise) ,stabilized in glutaraldehyde to prevent lysis, were added to all wells (25 μl at 5 x 10^6 cells ml^{-1}). The plates were then covered and horizontally rotated for 1h. After standing for at least 4h , the plates could be read. A negative result showed the button of erythrocytes, at the bottom of the well, flowing over the course of 30 min. In a positive result, erythrocytes which had clumped around the bottom and sides of the wells remained clumped and did not flow. The titre was expressed as the reciprocal of the highest dilution displaying activity.

The effect of coelomic fluid on bacterial and fungal cells was also assessed. 25 μl of suspensions of Saccharomyces cerevisiae, Bacillus megaterium, Escherichia coli and Staphylococcus aureus, at a concentration of 1 x 10^6 cells ml^{-1}, were added to 100 μl volumes of coelomic fluid and allowed to stand for one hour.

2.2.3.Agglutinin activity in individual worms.

Most work involved the use of pooled coelomic fluid. But it was also determined whether all or only some animals possess agglutinin molecules. Coelomic fluid was extracted from each of fifty worms of similar size and stored in separate microfuge tubes. After centrifugation, each extract was made up to 100μl with PBS and 25μl of sheep

erythrocytes (5 x 10^6 cells ml^{-1}) added. Control tubes contained PBS only.

2.2.4.Gel filtration

This technique separates proteins according to their molecular size. The gel filtration media used was Sephadex G100 (Pharmacia) which has a molecular weight range of 4,000-150,000 D. It is supplied as a dry powder and so was allowed to swell in two litres of distilled water for 5 h at 90°C. After cooling, the media was degassed and poured into a glass column (0.02 x 1.2m) using a filter funnel. Care was taken to ensure that no air bubbles were allowed to form. The column was then packed and equilibrated using 0.05M Tris-HCl, 0.5M NaCl, pH 8.0 buffer and this was allowed to pass through overnight. Column calibration was carried out using 50mg of each of the following: Bovine serum albumin (BSA), 67,000 D; Ovalbumin, 45,000 D; Carbonic anhydrase, 30,000 D; Trypsin, 23,800 D and Lysozyme, 14,300 D, to determine the molecular weight of each fraction. For coelomic fluid separation, a 10ml volume of the sample was applied to the column and eluted with the running buffer using a flow rate of 40ml h^{-1}. 3ml samples were collected over 12 h. The protein profile was monitored at 280nm and recorded. All chromatographic techniques were carried out at 4°C.

2.2.5.Chromatofocusing

This technique separates proteins according to their isoelectric points. The separation took place in a small column (0.9 x 27 cm) filled with 10 ml of PBE 94 Gel Exchanger (Sepharose 6B-Pharmacia) previously equilibrated with the appropriate starting buffer and degassed. The column was equilibrated with starting buffer for 3h (at 50 ml h^{-1}). The sample of interest, one fraction from gel filtration, which if necessary had been concentrated by ultrafiltration, using a cellulose triacetate filter with a 10,000 D cutoff, to 10 ml and dialyzed against starting buffer, was applied, by first running 5 ml of the eluant buffer, PB 74 (Pharmacia) which had previously been diluted 1:8 with distilled water and adjusted to the appropriate pH with 1M HCl, followed by applying the sample and then switching back to the eluant. In this way, the sample proteins were kept close to the physiological pH of the worm coelomic fluid. 1 ml samples were collected over a six hour period (at a flow rate of 25 ml h^{-1}). The pH and protein profiles were recorded simultaneously.

2.2.6.Ammonium sulphate precipitation

This procedure was used to remove the polybuffer from the proteins following chromatofocusing. Solid ammonium sulphate was added to the relevant fractions to a

concentration of 100% saturation (697g l^{-1}) and allowed to stand for two hours at 4°C with continuous stirring. The precipitate was collected by centrifugation at 15,000g for 1h. The pellet was redissolved in a small quantity of PBS, pH 7.2. and dialyzed in the same overnight to remove any ammonium sulphate. The samples could then be tested for activity, -with addition of 10mM calcium if required.

2.2.7.Protein assay

Protein content was determined with the Bradford assay (1976) using Bovine serum albumin (BSA) as the standard.

2.2.8.Polyacrylamide gel electrophoresis

PAGE was used to visualize the coelomic fluid proteins and to determine their apparent molecular weights. This technique is described in section 6.2.5.

2.2.9.Heat stability of Eisenia haemagglutinins

200 µl fractions of either pooled coelomic fluid or purified haemagglutinin (containing approximately 50 µg of protein) were treated to various temperatures; room temperature (20°C), 30°C, 40°C, 50°C, 60°C, 80°C and 100°C for 15 min. After this time, any haemagglutinin activity was determined using the haemagglutination assay (section

2.2.2) with sheep erythrocytes.

2.2.10.Metal Requirement of Eisenia Haemagglutinins

1 ml aliquots of coelomic fluid or purified haemagglutinin (containing approximately 250 µg protein) were demetalized by successive overnight dialysis against a 100 fold volume of Tris buffered saline, pH 7.2 containing 0.1M Ethylenediaminetetracetic acid (EDTA) and then the same volume of Tris buffered saline. Haemagglutinating activity was assayed for, after addition of, and 30 min. incubation in, one of various metal ion containing solutions such as zinc chloride , magnesium chloride or calcium chloride, dissolved in Tris buffered saline to a concentration of 10mM. Control aliquots were dialyzed against Tris buffered saline without EDTA or they did not receive the addition of divalent cations.

2.2.11.pH stability of Eisenia haemagglutinins

Samples of coelomic fluid or purified haemagglutinin were dialyzed for 24 h at 4°C against two changes of Tris buffered saline which had been adjusted to various pH values ranging from 2 to 10. Haemagglutination assays were performed on these samples. Unused samples, at each pH, were dialyzed against Tris buffered saline at pH 7.2 to determine whether the activity was reversible.

2.2.12. Carbohydrate Specificities of *Eisenia* Haemagglutinins.

The specificity of the haemagglutinins was determined by performing assays in the presence of a variety of sugars each at a concentration of 0.1M in PBS and including the following: L-fucose, D-galactose, D-mannose, N-acetyl-D-glucosamine, N-acetylneuraminic acid, N-acetyl-D-galactosamine, D-glucoronic acid, D-glucosamine, lactose, D-xylose, L-arabinose, maltose, L-mannose, L-rhamnose, methyl a D-glucopyranoside, L-xylose and 2-keto-3-deoxyoctonate. After serial two fold dilutions had been made of the sample, an equal volume (100 μl) of the sugar solution was added and allowed to stand for 30 min. 25 μl of sheep red blood cells were then added as in section 2.2.2. Wells containing 100 μl of PBS instead of sugar solutions served as controls.

2.3. RESULTS.

2.3.1. Haemagglutination assays

It was found that all of the vertebrate erythrocytes tested were agglutinated to varying extents when compared to the controls (see Plate 1). The strongest activity was seen with rat and sheep erythrocytes (1/256, Lanes A and C

Plate 1.

Haemagglutination Titre Plate Showing the Reactions of Agglutinins from the Coelomic Fluid Of *Eisenia foetida* with various Vertebrate Erythrocytes (Lane A -Rat; Lane C -Sheep; Lane E -Human B; Lane G -Horse; Lanes B,D,F and H are Controls Containing PBS Buffer and no Coelomic Fluid).

PLATE 1.

respectively) then human B cells (1/64, Lane E) and finally horse erythrocytes (1/16 Lane G).

All bacterial and yeast cells were strongly clumped after one hour in either coelomic fluid or purified agglutinin.

2.3.2.Agglutinin activity in individual worms

All worms tested showed positive results for haemagglutination of sheep red blood cells.

2.3.3.Gel filtration.

A volume of 10 ml of undiluted coelomic fluid previously centrifuged at 11,000g was applied to the column. The elution profile was monitored at 280nm and this revealed the presence of six major fractions labelled A-F (figure 2). The molecular weights of the fractions were determined by comparing the elution profiles of the fractions with the calibration curve and were as follows: Fraction A, 87,000D; fraction B, 40,000D; fraction C, 25,000D; fraction D, 16,000D and fraction E, the proteins in which, fall below the separating properties of the gel but are known to fall below 10,000D. The molecular weight estimates were very approximate as each fraction contains many different proteins as revealed by electrophoretic

Fig.2.

Elution Profile of Eisenia foetida Coelomic Fluid Separated in Gel Filtration on Sephadex G100 (o-o). Haemagglutinating activity of Separated Peaks are also shown (---).

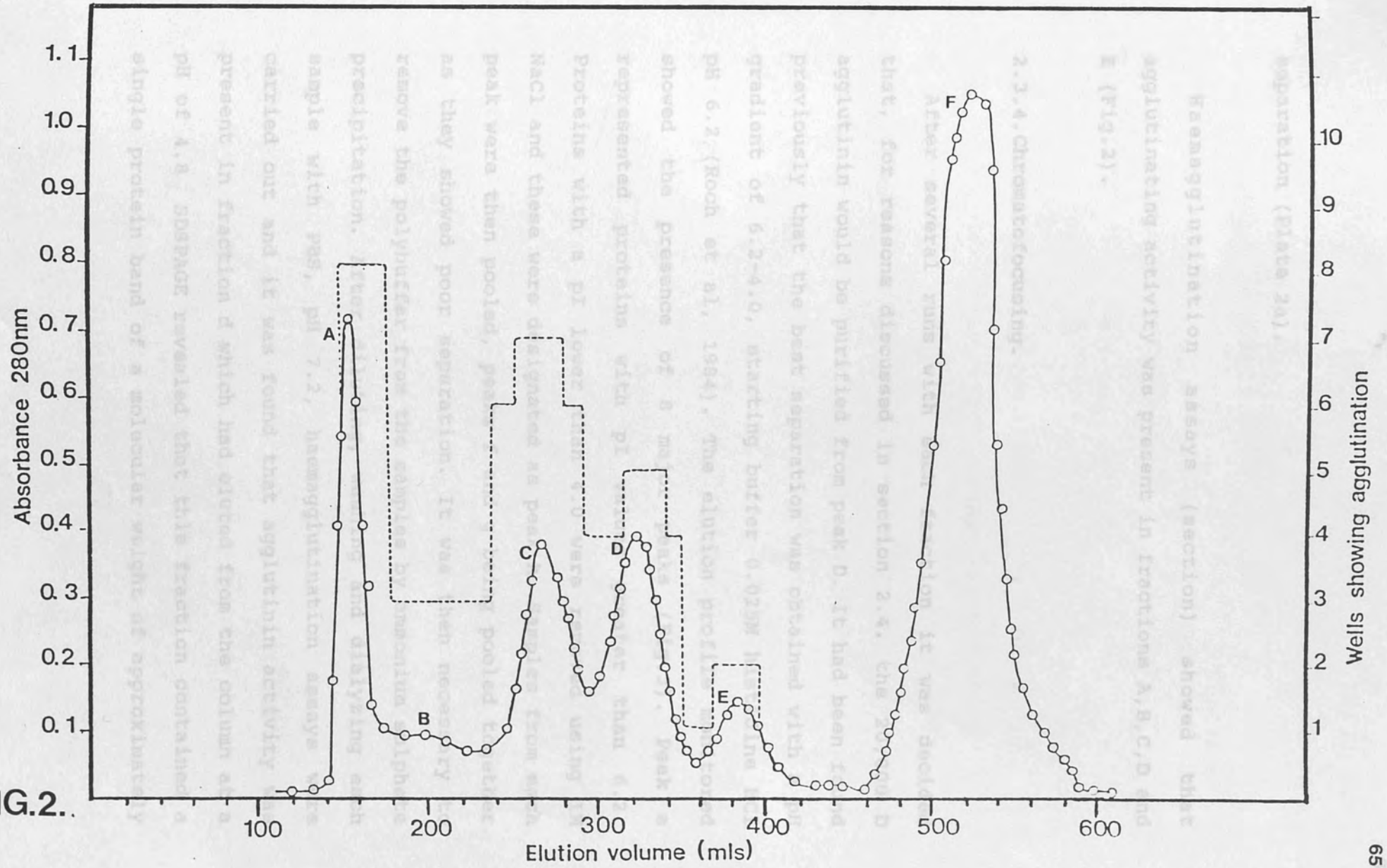

Absorbance 280nm
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
A
B
C
D
E
F
100
200
300
400
500
600
Elution volume (mls)
Wells showing agglutination
10
9
8
7
6
5
4
3
2
1

FIG.2.

separation (Plate 2a).

Haemagglutination assays (section) showed that agglutinating activity was present in fractions A,B,C,D and E (Fig.2).

2.3.4.Chromatofocusing.

After several runs with each fraction it was decided that, for reasons discussed in section 2.4. the 20,000 D agglutinin would be purified from peak D. It had been found previously that the best separation was obtained with a pH gradient of 6.2-4.0, starting buffer 0.025M histidine HCl pH 6.2 (Roch et al, 1984). The elution profile monitored showed the presence of 8 major peaks (Fig.3). Peak a represented proteins with pI values greater than 6.2. Proteins with a pI lower than 4.0 were removed using 1M NaCl and these were designated as peak h. Samples from each peak were then pooled, peaks f and g being pooled together as they showed poor separation. It was then necessary to remove the polybuffer from the samples by ammonium sulphate precipitation. After diluting, washing and dialyzing each sample with PBS, pH 7.2, haemagglutination assays were carried out and it was found that agglutinin activity was present in fraction d which had eluted from the column at a pH of 4.8 SDSPAGE revealed that this fraction contained a single protein band of a molecular weight of approximately

Fig.3.

Elution Profile of Eisenia foetida Peak D from Gel Filtration Separated in Chromatofocusing (o-o). The Dotted Line Corresponds to the pH Gradient Measured in Fractions Leaving the Column. The Arrow Indicates the Addition of 1M NaCl to the Elution Buffer to Regenerate the Column.

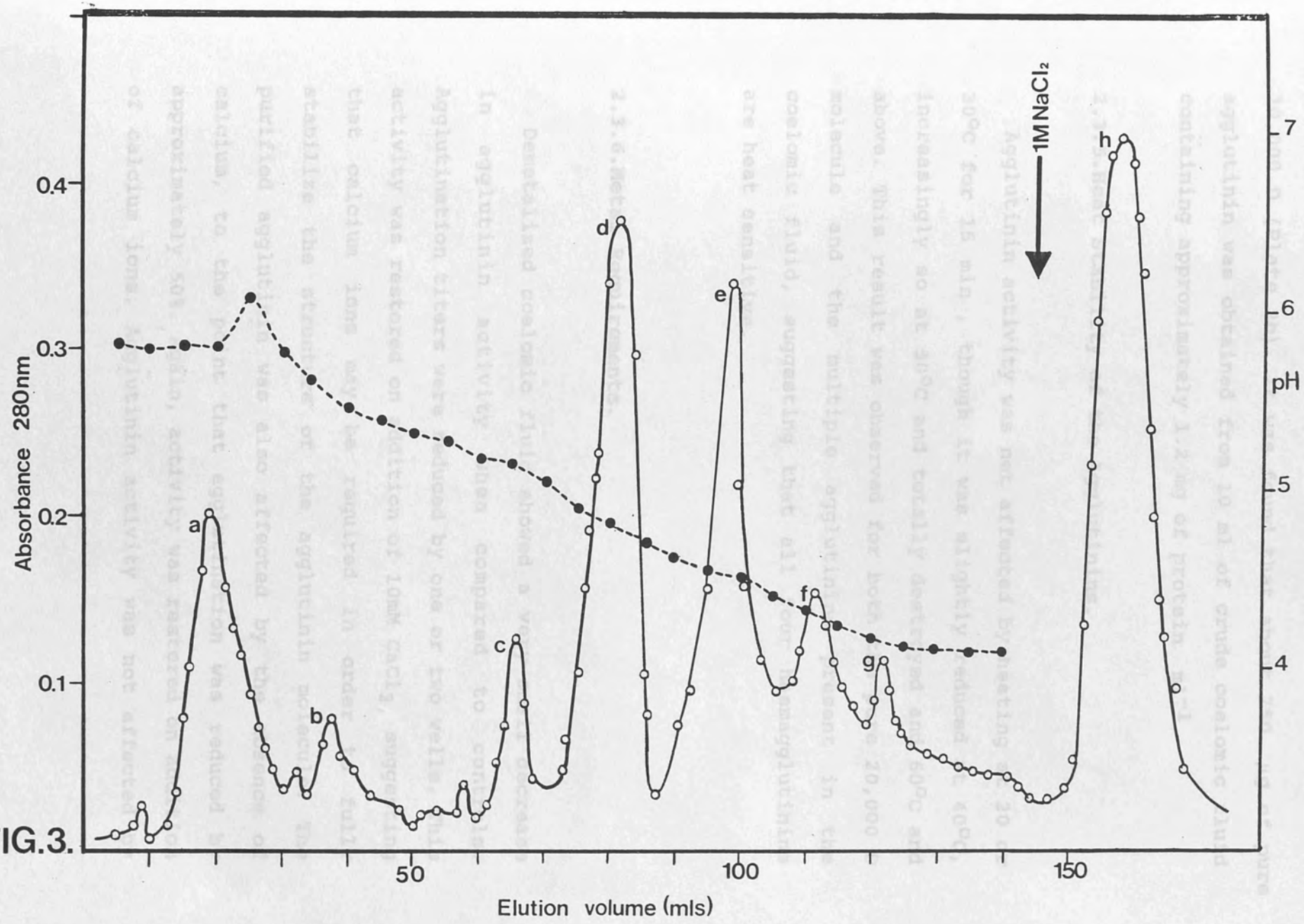

Absorbance 280nm
0.4
0.3
0.2
0.1
a
b
c
d
e
f
g
h
1M NaCl₂
7
6
5
4
pH
50
100
150
Elution volume (mls)

FIG.3.

20,000 D (Plate 2b). It was found that about 750 μg of pure agglutinin was obtained from 10 ml of crude coelomic fluid containing approximately 1.2 mg of protein ml^{-1}.

2.3.5.Heat Stability of the Agglutinins.

Agglutinin activity was not affected by heating at 20 or 30^{o}C for 15 min , though it was slightly reduced at 40^{o}C, increasingly so at 50^{o}C and totally destroyed and 60^{o}C and above. This result was observed for both the pure 20,000 D molecule and the multiple agglutinins present in the coelomic fluid, suggesting that all four haemagglutinins are heat sensitive.

2.3.6.Metal Requirements.

Demetalized coelomic fluid showed a very small decrease in agglutinin activity when compared to controls. Agglutination titers were reduced by one or two wells. This activity was restored on addition of 10mM $CaCl_2$, suggesting that calcium ions may be required in order to fully stabilize the structure of the agglutinin molecule. The purified agglutinin was also affected by the absence of calcium, to the point that agglutination was reduced by approximately 50%. Again, activity was restored on addition of calcium ions. Agglutinin activity was not affected by

Plate 2.

A) SDS PAGE of Individual Peaks of Coelomic Fluid from Eisenia foetida Separated by Gel Filtration (Lane 1 -molecular weight markers; Lane 2 -crude coelomic fluid; Lane 3 -Peak A; Lane 4 -Peak B; Lane 5 -Peak C; Lane 6 -Peak D). B) SDS PAGE of Peak d from Chromatofocusing. Molecular weight markers are also shown.

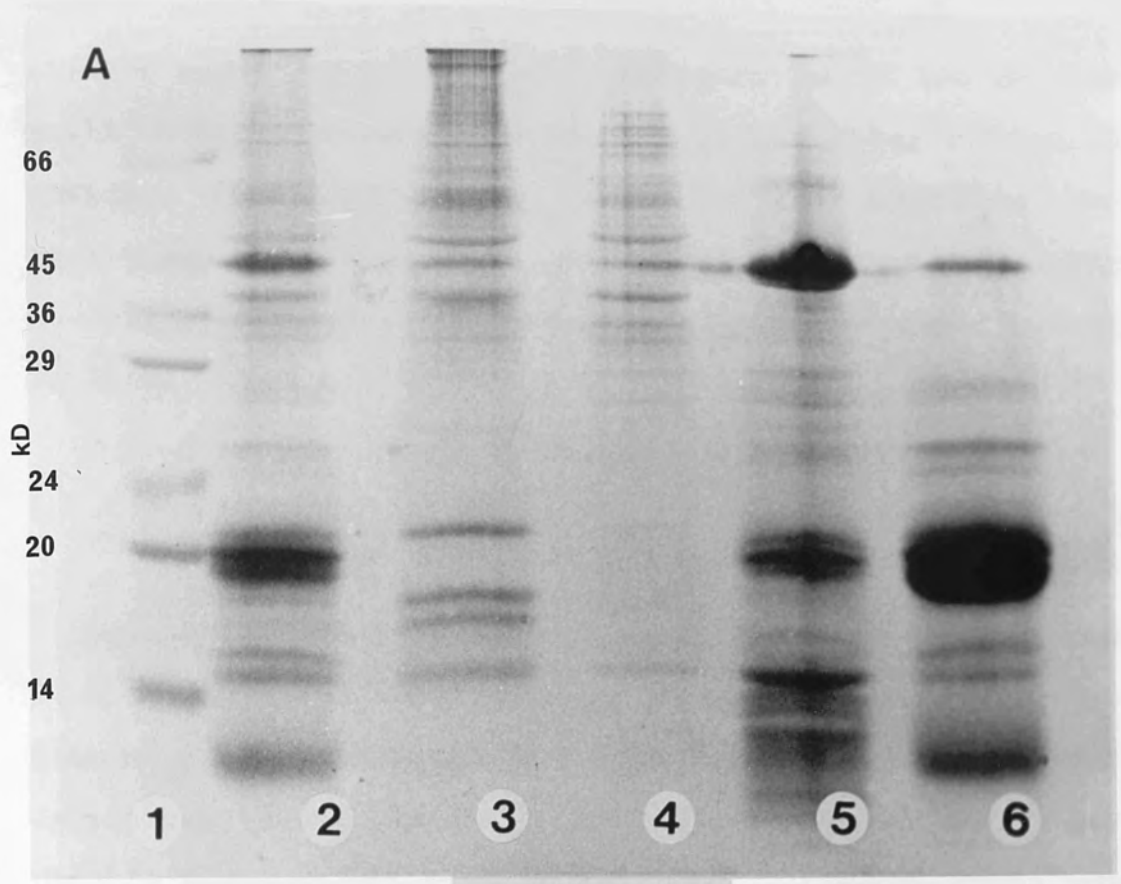
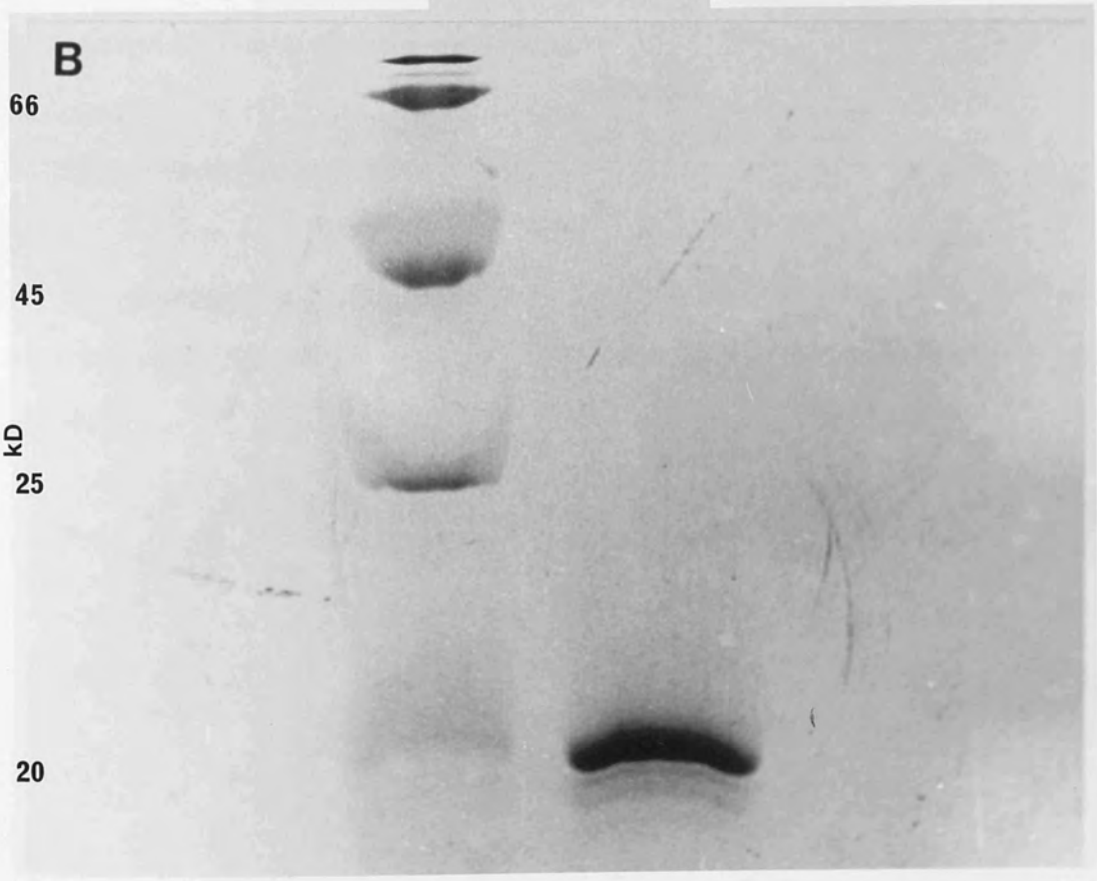


PLATE 2.



zinc or magnesium ions up to a concentration of 100 mM. The small decrease observed in the agglutinin titer of pooled coelomic fluid may solely be due to the effect on the 20,000 D molecule, if so it would appear that the other three agglutinins do not exhibit a requirement for divalent cations.

2.3.7.pH Stability

Agglutinin activity of both pooled coelomic fluid and pure material was unaffected by pH values between 5.0-8.0 though a sharp cut off occurred below and above these respective values. Activity was slightly higher at pH 6.0 than at 5.0, 7.0 and 8.0. In both cases, full activity was restored on redialysis of those agglutinins which had been at pH values 4.0, 9.0 and 10.0, though pH values of 2.0 and 3.0 irreversibly destroyed activity.

2.3.8.Carbohydrate Specificity.

No carbohydrate tested was found to inhibit or reduce agglutinin activity in pooled coelomic fluid or purified material.

2.4.DISCUSSION

All erythrocytes were agglutinated to varying extents by pooled coelomic fluid. Valembois et al,(1983) also showed that rabbit, badger and frog erythrocytes are agglutinated by coelomic fluid.

The effect of coelomic fluid from individual worms was examined in order to determine whether all or only some worms possess the agglutinin molecules. Sheep red cells were used as the test erythrocytes and positive results were found in all cases. Cenini (1983), using rabbit erythrocytes, also tested individual worms for agglutinating activity and found that only 39% of the animals used were positive, which would suggest that at least two agglutinins are involved, one present in all of the worms and responsible for sheep cell agglutination and one which is only found in less than half of the worms and able to agglutinate rabbit erythrocytes. The presence of agglutinins with different specificities has been demonstrated in several invertebrates such as Botrylloides leachii (Schluter et al, 1981); the haemagglutinin HA-1 is specific for guinea pig erythrocytes whilst HA-2 agglutinates a wide range of vertebrate red cells.

Although the presence of four haemagglutinins was demonstrated by Roch et al, (1984), the present study only succeeded in revealing three proteins capable of agglutinating activity; those of molecular weights 20,000;

40,000 and 32,000 D. The 11,500 D molecule, which had been previously found in fraction B from gel filtration, was detected after chromatofocusing on SDSPAGE gels, but no agglutinin activity was observed. Several possibilities may account for this. The molecule observed on gels was not the agglutinin previously reported, the agglutinin activity was lost due to the separation techniques (e.g the effect of Polybuffer), the agglutinin may not have been specific for the erythrocytes used in the assay or finally, agglutinin activity may be weak and much more of the material was required for visualization of agglutinin activity. The 32,000 D agglutinin obtained from chromatofocusing fraction A was present only in trace amounts and on occasions not detected on the UV protein profile at all. Although this agglutinin had a high activity, purification was time consuming due to the small amount available and difficulty in localizing the protein peak. Both the 20,000 D agglutinin and the 40,000 D agglutinin eluted off at pI values of 6.3 and 4.8 respectively. SDSPAGE showed that the 40,000 D molecule comprised three closely associated protein bands whilst the 20,000 D molecule appeared as one band only. As the latter appeared to be less contaminated by other proteins (as shown by silver staining the gel:section 6.2.6.) and gave the highest yield it was decided that purification of the 20,000 D molecule would be scaled up for antibody production and other physicochemical tests.

It was found that the purified agglutinin had properties very similar to the combined properties of all four agglutinins in the coelomic fluid. It is heat sensitive, the activity being destroyed at 60°C for 15 min. indicating a proteinaceous nature. It was also found to be fairly tolerant to changes in pH, activity being unaffected over the range from 5.0-8.0. It would appear that divalent cation requirements of the agglutinins may differ, the 20,000 D agglutinin being partially destabilized in the absence of calcium. More recently it has been found that this haemagglutinin is a protein rich in aspartic acid and glutamic acid (Roch et al 1986).

The 20,000D agglutinin appears to have similar properties to other invertebrate agglutinins so far studied. However, it has not yet been determined whether this agglutinin can be classed as a lectin as a sugar specificity has not yet been revealed. As can be seen from Table 1, this is also the case for a number of other invertebrate agglutinins. Attempts to find carbohydrates able to bind with Eisenia agglutinins were unsuccessful in another study (Valembois et al, 1983) though more recently it was reported that the bacterially derived KDO was found to block activity (Roch, personal communication), although this has not been shown in this study and so the target for agglutinin binding may remain obscure.

3.THE OCCURRENCE OF AN OPSONIN IN THE COELOMIC FLUID OF EISENIA FOETIDA.

3.1.INTRODUCTION.

It is clear that invertebrates are capable of resisting infection and can remove from their body fluids, a variety of living and non-living particles. It seems that this removal and the destruction of parasites is a function of the phagocytic cells (Stauber,1950; Tripp,1958,1960).

The universality and importance of the phagocyte was first emphasized by Metchnikoff in 1893. Since then, invertebrate phagocytes have been given a multitude of names, including amoebocytes, granulocytes, macrophages, monocytes, plasmatocytes and granular cells (Ratcliff and Rowley, 1979). Phagocytosis is the most common of the cellular immune mechanisms and forms the first line of defence once the physicochemical barriers have been breached. The involvement of phagocytes in these responses is known from studies on cephalopods (Stuart,1968), bivalves (Tripp,1958,1960; Feng,1965; Reade and Reade 1972,1976) and gastropods (Tripp,1961; Brown and Brown,1965; Brown 1967; Pauley et al,1971).

Studies on the recognition and phagocytosis of foreign material by phagocytic cells from invertebrates, strongly

favour the idea that the recognition mechanism is based on a system of serum proteins analogous to immunoglobulin molecules of vertebrates. Most *in vitro* studies on the uptake of particles by haemocytes or coelomocytes from invertebrates indicate that recognition is facilitated by factors free in the haemolymph (Jenkin,1976). In certain species of invertebrates, these recognition factors may be associated with the membrane of the phagocytic cell (Van der Knaap et al,1981,1983; Vasta et al,1982).

Some investigations have suggested that the haemagglutinins present in the sera from invertebrates function as the recognition units, enabling the phagocytic cells to recognize foreign particles, though only two studies have proved this to be the case by using purified haemagglutinin molecules (Renwrantz and Stahmer, 1983; Hardy et al,1977).

An investigation was carried out to determine whether the coelomic fluid of *Eisenia foetida* contains any factor or factors which enhance phagocytic uptake by the coelomocytes. The coelomocytes of *Eisenia* can be divided into two broad categories: eleocytes and phagocytes. The eleocytes range in size from 15-30 μm whilst the phagocytes are generally smaller with an average diameter of 10 μm. These cell types are present in equal numbers in pooled coelomic fluid and on extraction both populations adhere to

plastic or glass surfaces. Separation of these cell types has not been successful (see section 6.2.)and therefore, after ensuring that the eleocytes were not phagocytic, the numbers of phagocytes present was, in all cases, assumed to be half of the coelomocyte count.

3.2.MATERIALS AND METHODS.

3.2.1.Preparation of Coelomocyte Monolayers

Individual worms were washed in tap water, dried and placed in a small beaker containing 20 ml of cell culture medium (Medium 199, Sigma). Coelomic fluid, containing coelomocytes, was harvested by electrical stimulation (5V). Extraction continued until the coelomocyte count reached the required count (as determined using a haemocytometer). 3 ml aliquots of the cell suspension were placed onto 5 cm wide plastic petri dishes and allowed to stand at room temperature for 90 min. During this time, the coelomocytes spread out and adhered to the plastic surface of the petri dish. Any non-adherent cells and traces of coelomic fluid were then removed by rinsing the plates several times with cell culture media.

3.2.2.Preparation of Micro-organisms.

Stock cultures of Bacillus megaterium, Escherichia coli,

and Staphylococcus aureus were subcultured into nutrient broth and incubated at 37°C for 24 h. The bacteria were then harvested by centrifugation, washed three times in PBS, pH 7.2, and washed once with distilled water. Bakers Yeast (Saccharomyces cerevisiae) was cultured overnight in 1M sucrose solution and harvested and washed as above. All organisms were then heat killed (121°C for 15 min.) and lyophilized.

3.2.3.Preparation of Fluorescent Particles.

Fluorescent micro-organisms were prepared as described by Oda and Maeda (1986). Briefly the method is as follows: 20 mg of lyophilized bacteria or yeast was suspended in 50 ml of 0.1M carbonate-bicarbonate buffer, pH 9.0, containing 0.9% saline at 4°C. To this solution, 10 mg of fluorescein isothiocyanate (FITC) was added. After mixing for 30 min at 4°C, the cells were washed at least three times with cold PBS and once with distilled water before being lyophilized. The supernatant of the last centrifugation was found to be uncontaminated by free fluorescent dye.

3.2.4.Opsonization.

Either pooled coelomic fluid, pure agglutinin or individual peaks from gel filtration of coelomic fluid were used as possible sources of opsonin. Known numbers of

fluorescent particles (normally 1 x 10^7 yeast cells and 1 x 10^8 bacterial cells ml^{-1}) were pre-incubated in 200 μl of the possible opsonin solution (the pure agglutinin solution containing 150 μg of protein, the G100 peaks adjusted to 500 μg protein). Incubation was carried out at 37°C for 90 min. the particles were then washed three times with PBS to remove any unbound material before addition to the coelomocyte monolayers.

3.2.5.Initial Observations of Phagocytosis.

a) Light microscopy.

Unstained freeze dried yeast cells were made up to 3 ml with cell culture media, to give a concentration of $1x10^8$ cells ml^{-1} and added to petri dishes containing coelomocyte monolayers. After a 2h incubation period at room temperature, the dishes were washed three times to remove non-phagocytosed yeast cells. 1 ml of medium was added to prevent the coelomocytes from drying out and the dishes were then observed under the light microscope.

b) Scanning electron microscopy.

Coelomocyte monolayers were fixed in 5 ml of 4% paraformaldehyde, 0.5% glutaraldehyde in sodium cacodylate buffer (prepared as in section 5.2.2.) for 1 h and rinsed with PBS. The petri dish was then cut into squares measuring 1 cm x 1 cm. The cells on the plastic squares

were serially dehydrated as in section 5.2.4. Critical point drying followed by sputter coating completed the cell preparation. Observations were then made using the scanning electron microscope (Cambridge S100).

3.2.6.Measurements of Phagocytosis.

A) The Relationship Between the Fluorescent Intensity and the Number of Particles.

The fluorescent particles used in this procedure were suspended in PBS. The suspension was vigorously agitated by sonication to ensure that the particles were completely dispersed, after which the number of particles was counted directly using a haemocytometer. For the determination of fluorescent intensity of the particles by fluorescence spectrophotometry, known numbers of particles were suspended in 20 mM Tris-HCl buffer, pH 8.5, containing 0.2% sodium dodecyl sulphate (SDS) and read at an excitation wavelength of 490 nm and an emission wavelength of 520 nm.

B) The relationship Between the Number of Cells Present and the Protein Content.

To quantify the adherent cell number, the protein content in an aliquot of solubilized solution, containing a known number of cells, was determined using the Bradford estimation (section 2.2.7.) and compared to a standard curve of cell numbers against absorption at 595 nm. The extent of phagocytosis could then be expressed in terms of

numbers of particles per cell.

C) The Measurement of Ingestion of Fluorescent Particles by Phagocytic Cells

Fluorescein labelled particle suspensions,(either unincubated in opsonin or sensitized as in section 3.2.4.) in 3 ml of cell culture media, were added to adherent phagocytic cells (3 x 10^5 cells per dish). The final particle to phagocytic cell ratios were as follows: yeast, 50:1; bacteria, 500:1. These ratios of particles to cells were used in an attempt to produce maximal rates of phagocytosis in preliminary experiments, after which the ratios could be varied. After incubation at 20^oC for varying time intervals, the phagocytic cells were washed three times with Eisenia media by aspiration to remove any non-cell associated particles. Cells were then solubilized by addition of 1 ml of 25 mM Tris-HCl buffer, pH 8.5, containing 0.2% SDS. The solubilized solution was then transferred to a cuvette and made up to 3 ml with PBS , for measurement of the fluorescent intensity using a fluorescent spectrophotometer with an excitation wavelength of 490 nm and an emission wavelength of 520 nm. The number of phagocytosed particles was determined from a standard curve of the relationship between the fluorescent intensity and the number of particles present.

3.2.7. The Effect of Temperature on Phagocytosis.

Coelomocytes were incubated with yeast particles for 4h at 4°C, or at 37°C.

3.2.8.Sugar Inhibition of Opsonic Activity

0.5M solutions of the following sugars were prepared: D-glucose, L-fucose, N-acetyl-D-glucosamine, D-mannose, D-galactose, N-acetylneuraminic acid and N-acetyl-galactosamine. 200 µl of each solution was added to 200 µl of coelomic fluid and incubated at 37°C for one hour. Known numbers of fluorescent yeast cells were added and incubation continued for a further 90 min. The yeast cells were then added to Eisenia coelomocytes as described as above and phagocytic rates were later measured.

3.3.RESULTS.

3.3.1.Initial Observations of Phagocytosis.

From both light and scanning electron microscopy it would appear that Eisenia phagocytes (one cell shown in Plate 3a) readily ingest a variety of micro-organisms. Plate 3b shows the result of exposing large numbers of yeast cells to phagocytes. After 2h incubation, the yeast

Plate 3.

Scanning Electron Micrographs, Space Bars= 10 μm

A) A Phagocyte from the Coelom of Eisenia foetida, Allowed to Adhere to the Surface of the Incubation Dish for 10 min Prior to Fixation.

B) A Group of Eisenia foetida Phagocytes Containing Ingested Yeast Particles. Incubation lasted for 2h prior to Fixation, Enabling Extensive Spreading of the Phagocytes on the Surface of the Dish. Arrow indicates one yeast cell.

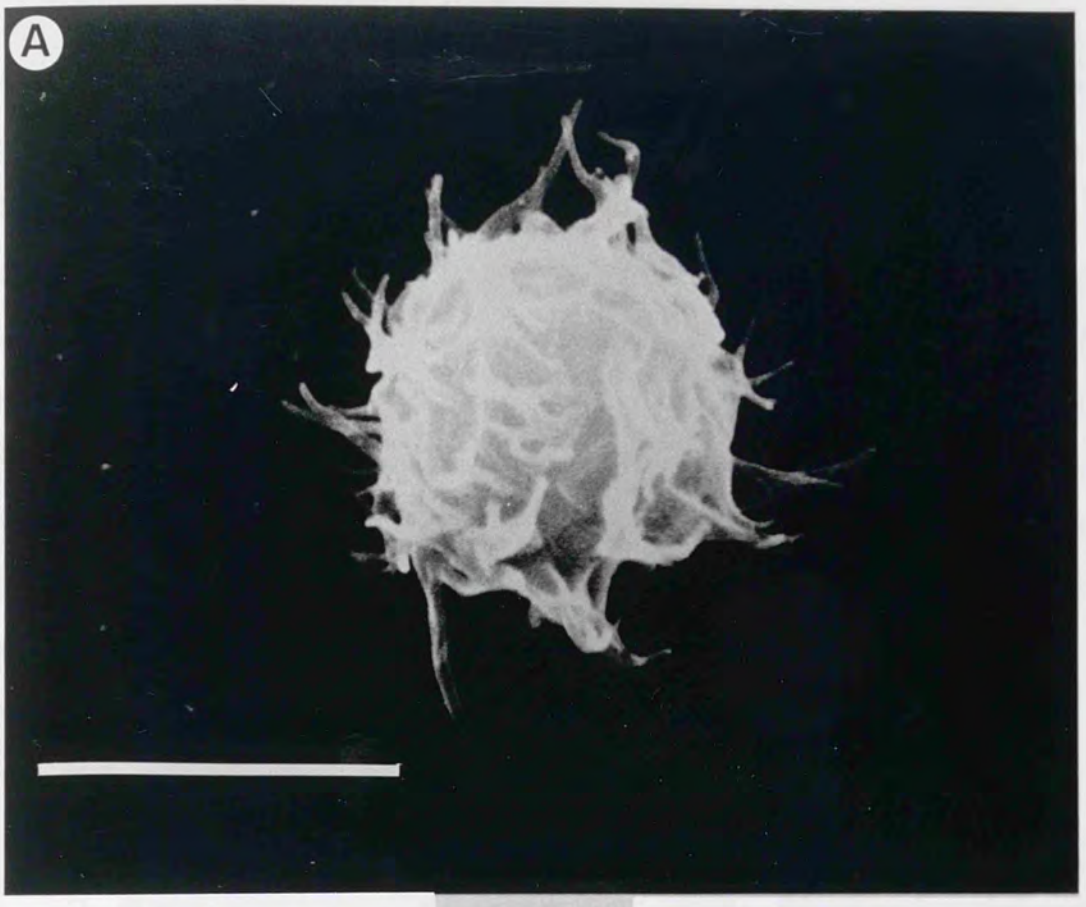
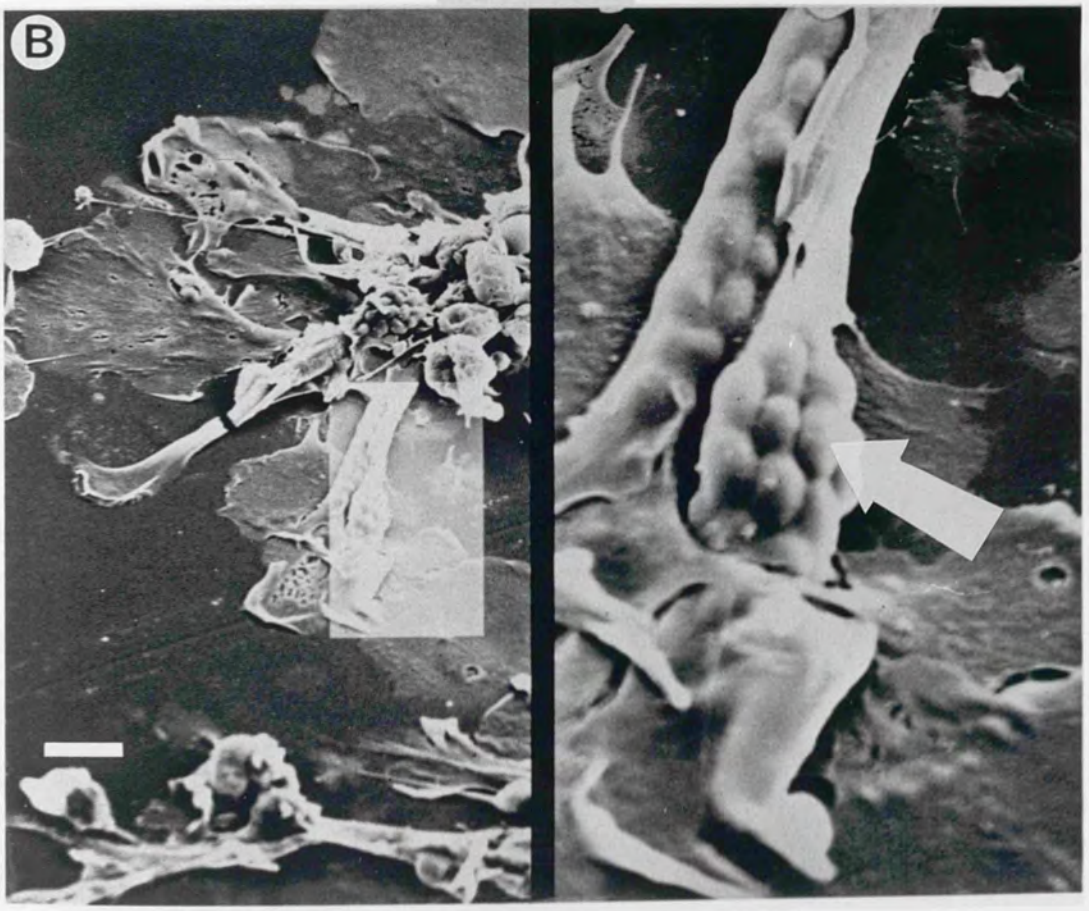


PLATE 3.



cells were either adhering to phagocyte cell membranes or had been ingested. When using the light microscope it was difficult to determine whether the yeast cells were inside the phagocytes or on the cell surface, the scanning electron micrographs would appear to confirm that all of the yeast cells were in fact ingested, (Plate 3b) though it is possible that any cells that were adhering to the phagocyte membrane could have been removed in the preparative steps. A differential fluorescent quenching technique was employed to confirm that the yeast cells had in fact been phagocytosed. Methylene blue (at a final concentration of $0.1\%_{\Lambda}^{w:v}$) was added to coelomocytes which had been incubated with yeast cells. Extracellular or surface bound organisms adsorbed methylene blue, which resulted in the quenching of their fluorescence. Such quenched organisms were readily distinguishable from brightly fluorescent intracellular organisms by fluorescent microscopy. As shown in Plate 4 (B2 and C2), almost all cell-associated organisms were not quenched by methylene blue which indicates that the number of truly phagocytosed organisms was determined in the assay method used.

3.2.2.Measurements of Phagocytosis.

A) The Relationship Between the Fluorescent Intensity and the Number of Particles

The fluorescent intensity of the yeast and bacteria

Plate 4.

A) FITC Stained Yeast Particles Viewed Under Ultra Violet Light (x1000).

B1) and C1) Several Eisenia foetida Phagocytes Containing Ingested Yeast Particles (x400).

B2) and C2) The Same Phagocytes as Above, Viewed under Ultra Violet Light (x400) after Quenching of Extracellular Yeast Particles with Methylene Blue (B1 corresponds to B2 as does C1 to C2).

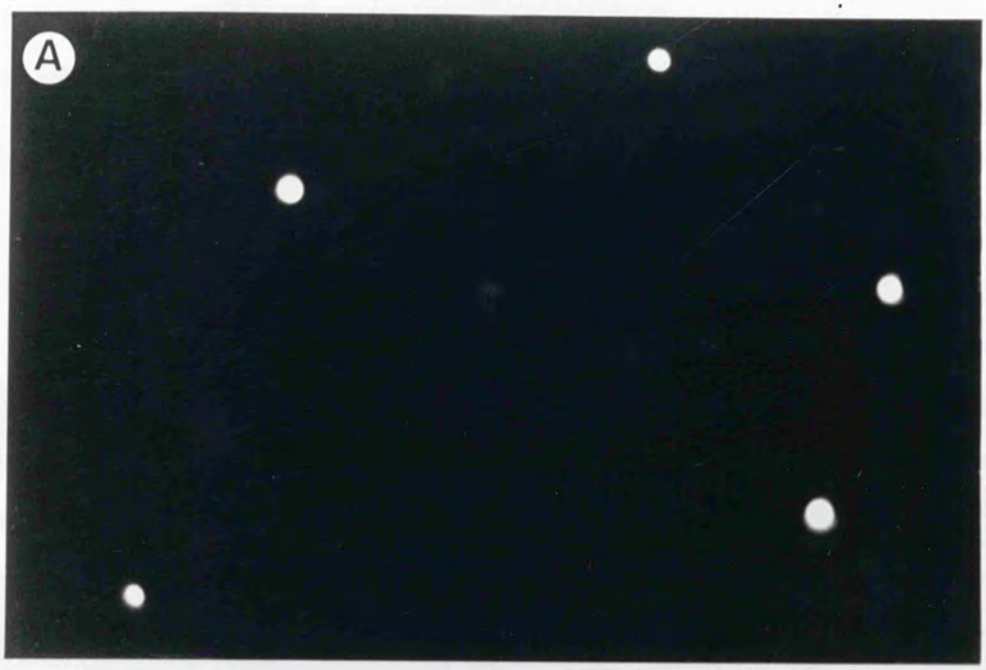
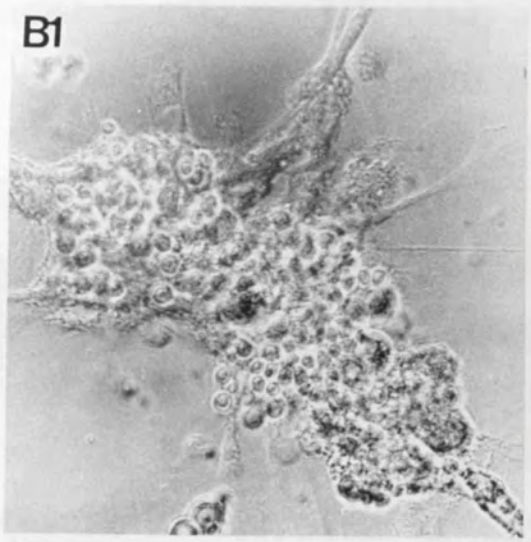
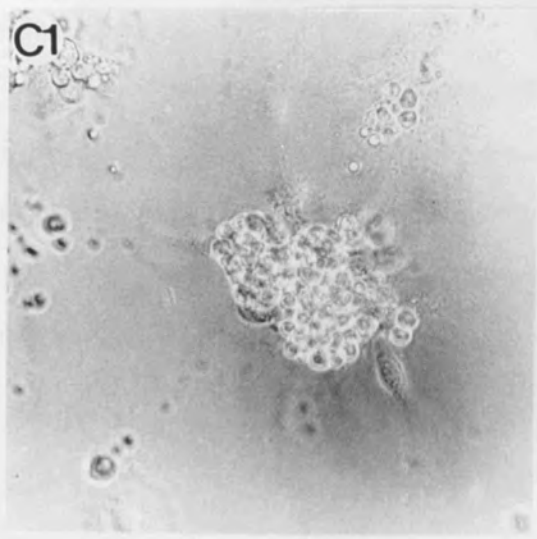


PLATE 4.





particles was directly proportional to their concentration. The standard curves (Fig.4a) enabled the estimation of particle number from fluorescent intensity measurements.

B) The Relationship Between the Number of Cells and Their Protein Content.

A standard curve (Fig.4b) of cell numbers against absorption at 595 nm enabled the estimation of cell numbers from solubilized cell suspensions using the Bradford Assay.

C) Kinetics of Phagocytosis of Fluorescent Particles.

The time course of uptake of fluorescent particles was examined at room temperature.

Yeast cells.

The rate of ingestion of yeast cells was essentially linear with time up to 90 min and then reached a plateau after 120 min (Fig.5a). The numbers of phagocytosed particles and coelomocytes present were determined from the standard curve and from this the number of particles taken up by each phagocyte could be determined. As can be seen in Fig.7a, it appears that each phagocyte ingested an average of 3.4 yeast cells over the course of 90 min reaching a maximum of 4 yeast particles after 120 min.

Bacteria.

The course of phagocytosis of E.coli, S.aureus and B.megaterium was followed over 3h. As with the yeast cells, the rate of ingestion of the bacteria rose fairly rapidly in all cases, reaching a maximum after 90 min and then reaching a plateau after 120 min (Fig.5;b,c,and d).

Fig.4.

A) The Relationship Between the Fluorescent Intensity and the Number of Particles Present (S.cerevisiae ▲—▲ ; E.coli ○–○ ; S.aureus ●–● ; B.megaterium △–△ .)

B) The Relationship Between the Number of Phagocytic Cells and Their Protein Content as Estimated by the Bradford Assay.

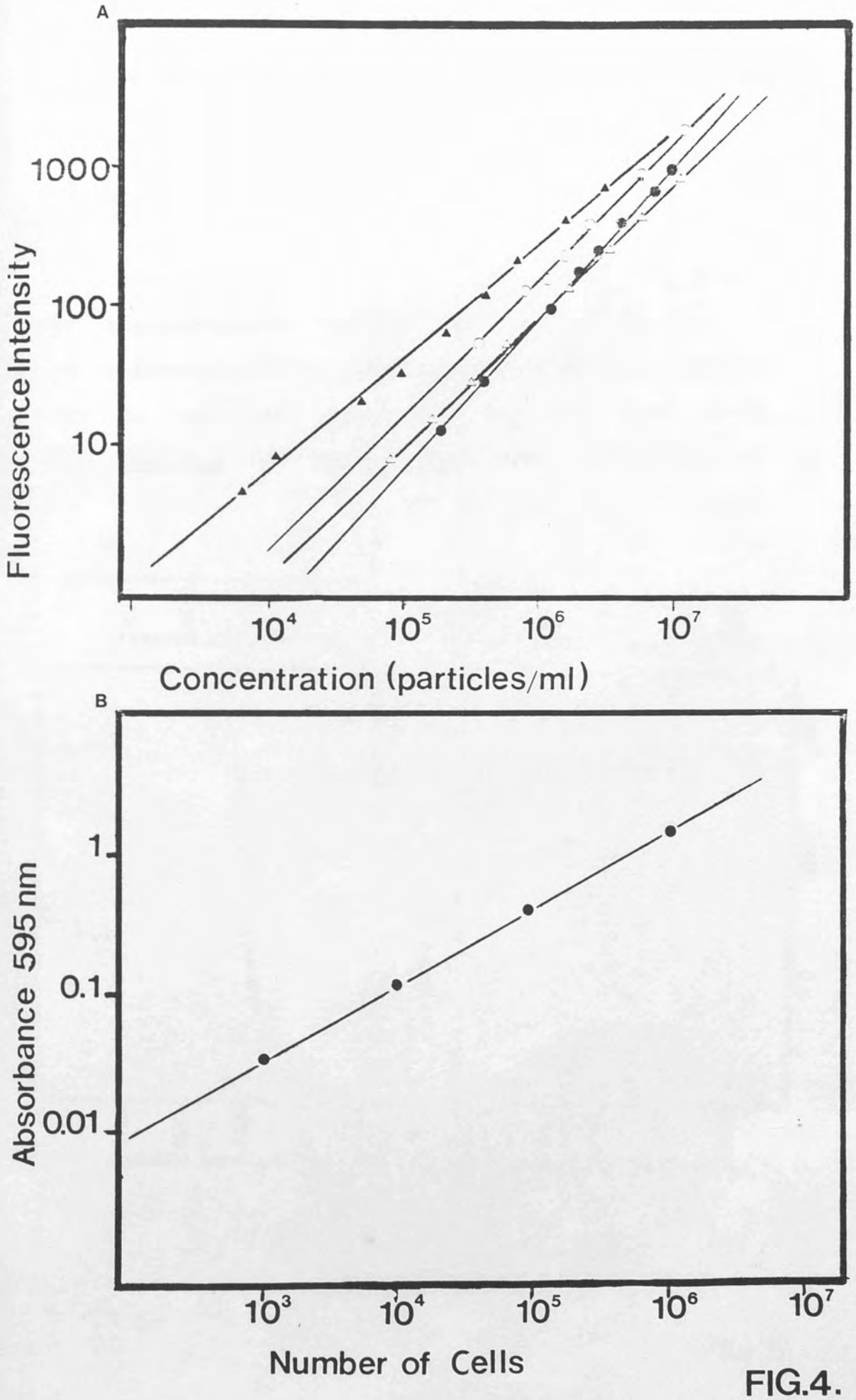

A
1000
100
10
Fluorescence Intensity
10^4
10^5
10^6
10^7
Concentration (particles/ml)
B
1
0.1
0.01
Absorbance 595 nm
10^3
10^4
10^5
10^6
10^7
Number of Cells

FIG.4.

Fig.5.

The Uptake of FITC stained Microorganisms by Eisenia foetida Phagocytes with Time as Determined by Measurement of the Fluoresent Intensity of the Ingested Particles (S.cerevisiae -A; S.aureus -B; E.coli -C; B.megaterium -D).

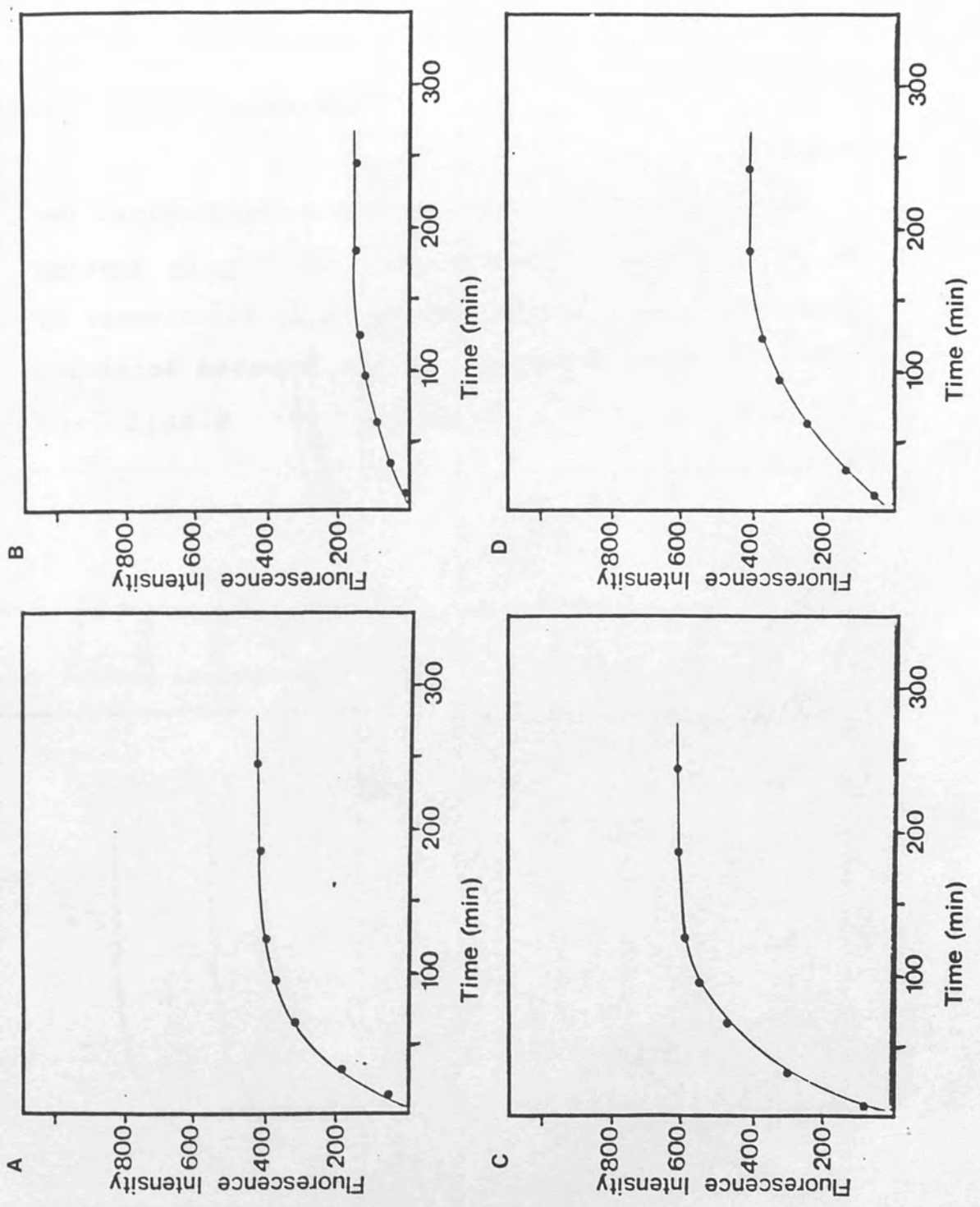

A
B
C
D
Fluorescence Intensity
Time (min)
200
400
600
800
100
200
300

FIG.5.

Fig.6.

The Uptake of Opsonized (o-o) and Nonopsonized (●-●) FITC Stained Microorganisms by Eisenia foetida Phagocytes with Time as Determined by Measurement of the Fluorescent Intensity of the Ingested Particles (S.cerevisiae -A; S.aureus -B; E.coli -C; B.megaterium -D).

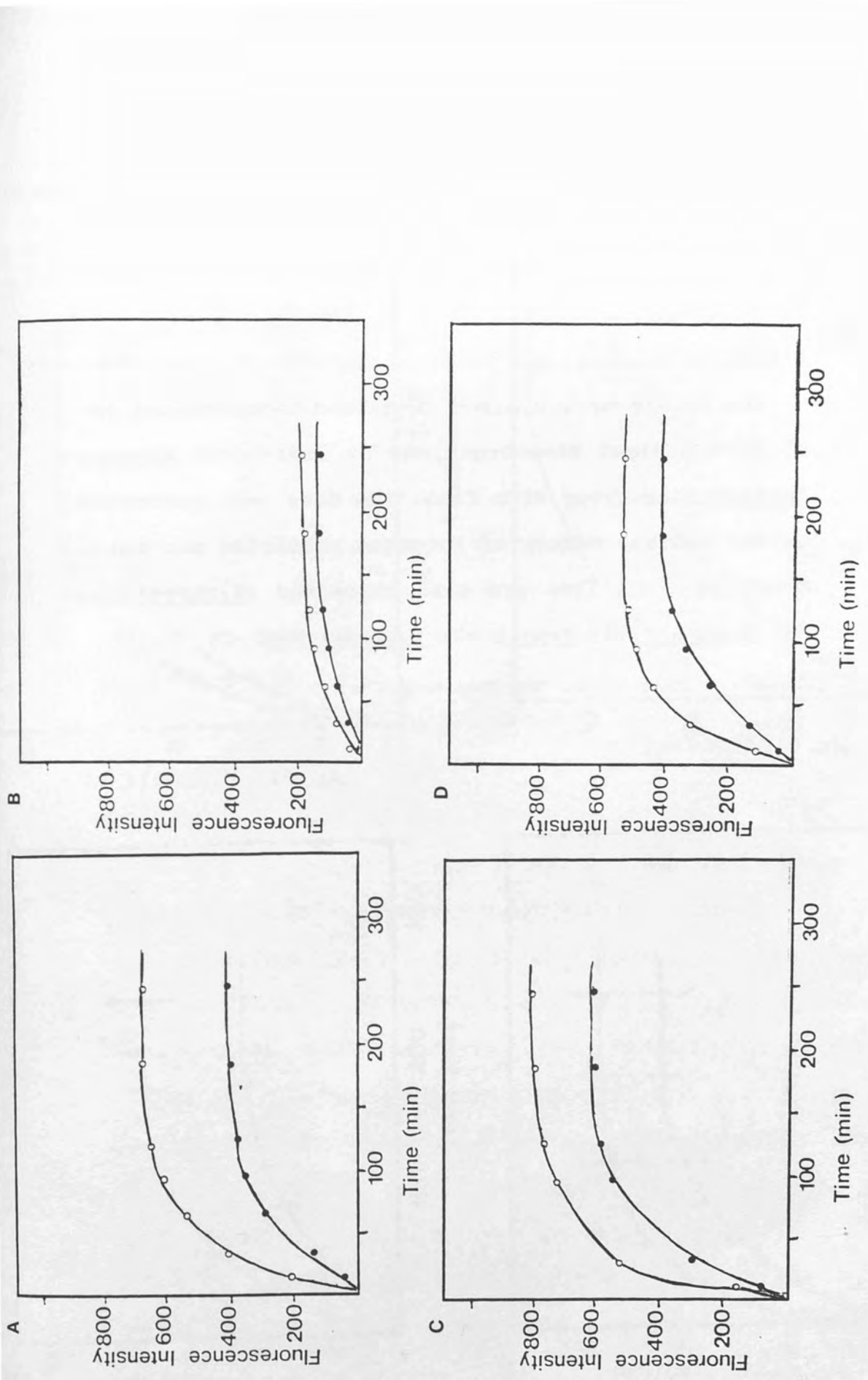

A
B
C
D
Fluorescence Intensity
Time (min)
200
400
600
800
100
200
300

FIG.6.

Fig.7.

The Uptake of Opsonized (o-o) and Nonopsonized (●-●) FITC Stained Microorganisms by individual Eisenia foetida phagocytes with Time. The data were expressed as the average number of ingested particles per cell. Standard Deviations are also expressed (S.cerevisiae -A; S.aureus -B; E.coli -C; B.megaterium -D).

Number of determinations per data point = 4.

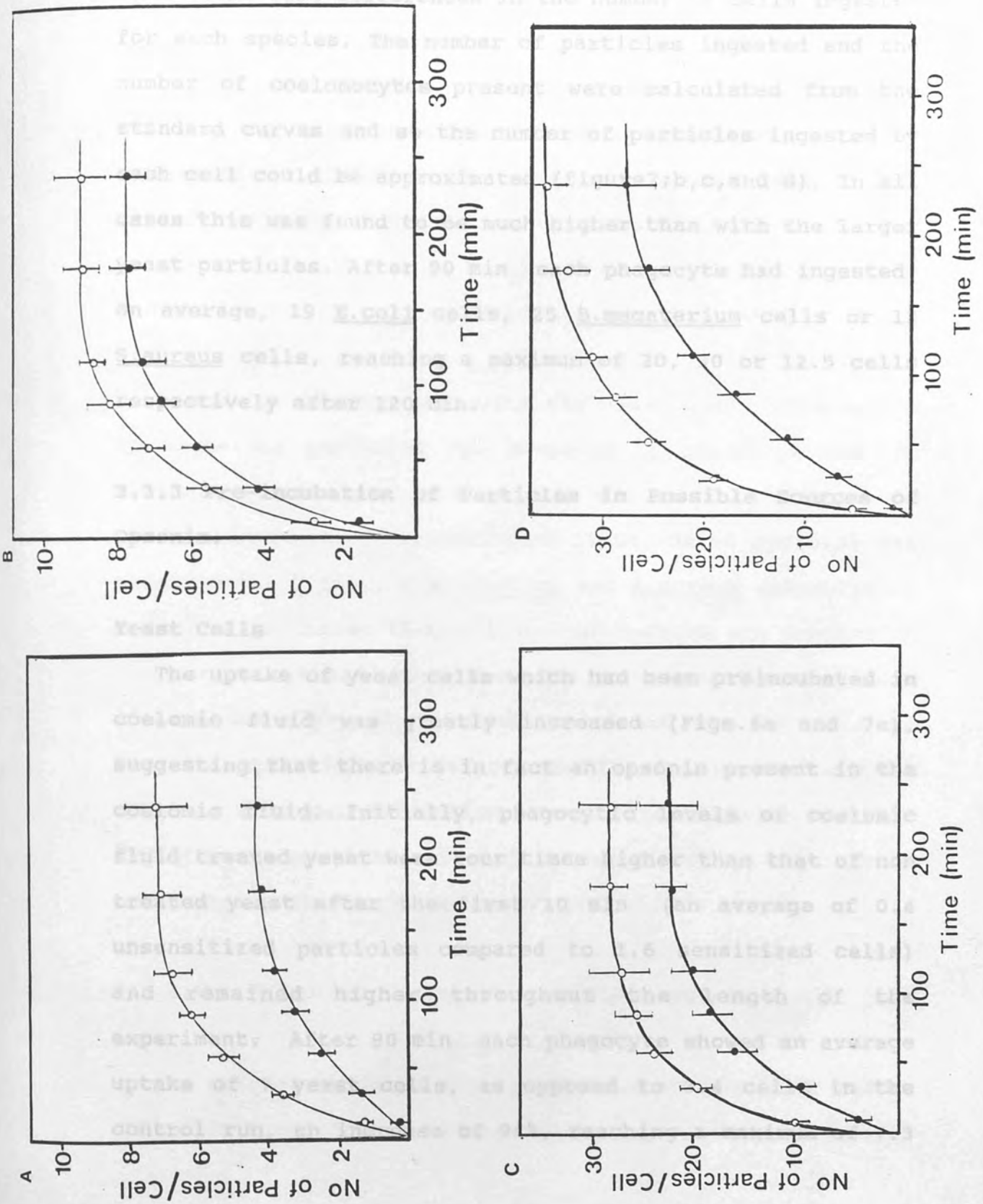


FIG.7.

However, from fluorescence measurements, it was obvious that there were differences in the number of cells ingested for each species. The number of particles ingested and the number of coelomocytes present were calculated from the standard curves and so the number of particles ingested by each cell could be approximated (figure7;b,c,and d). In all cases this was found to be much higher than with the larger yeast particles. After 90 min each phagocyte had ingested, on average, 19 E.coli cells, 25 B.megaterium cells or 12 S.aureus cells, reaching a maximum of 20, 30 or 12.5 cells respectively after 120 min.

3.3.3 Pre-incubation of Particles in Possible Sources of Opsonin.

Yeast Cells

The uptake of yeast cells which had been preincubated in coelomic fluid was greatly increased (Figs.6a and 7a), suggesting that there is in fact an opsonin present in the coelomic fluid. Initially, phagocytic levels of coelomic fluid treated yeast were four times higher than that of non treated yeast after the first 10 min (an average of 0.4 unsensitized particles compared to 1.6 sensitized cells) and remained higher throughout the length of the experiment. After 90 min each phagocyte showed an average uptake of 7 yeast cells, as opposed to 3.4 cells in the control run, an increase of 94%, reaching a maximum of 7.3

cells after 4h.

Pre-incubation of yeast cells in purified agglutinin, with or without calcium, or in fractions obtained from gel filtration of coelomic fluid, in a number of different combinations, showed no effect on the extent of phagocytosis when compared to the controls.

Bacteria.

Again, as was the case with the yeast cells, the uptake of bacterial particles was enhanced by pre-incubation in coelomic fluid though not to such a high degree. After 90 min an increase in ingestion of 31.5%, 34.6% and 8.3% was observed for E.coli, B.megaterium and S.aureus respectively and again indicates that a factor or factors are present in the coelomic fluid which are able to enhance phagocytosis (Figs.6 and 7;b,c,d). Preincubation of the bacteria in purified agglutinin, with or without calcium, or fractions obtained from gel filtration of coelomic fluid in a number of different combinations produced no effect on the rate of phagocytosis when compared to controls.

3.2.4.The Effect of Temperature on Phagocytosis.

The rate of phagocytosis was studied at both 37^{o}C and at 4^{o}C. From Fig.8 it can be seen that phagocytosis is greatly

Fig.8.

The Rate of Phagocytosis of FITC Stained S.cerevisiae Particles by Eisenia foetida Phagocytes at Different Temperatures (room temperature 20°C ○–○; 4°C ●–●; 37°C ▲–▲).

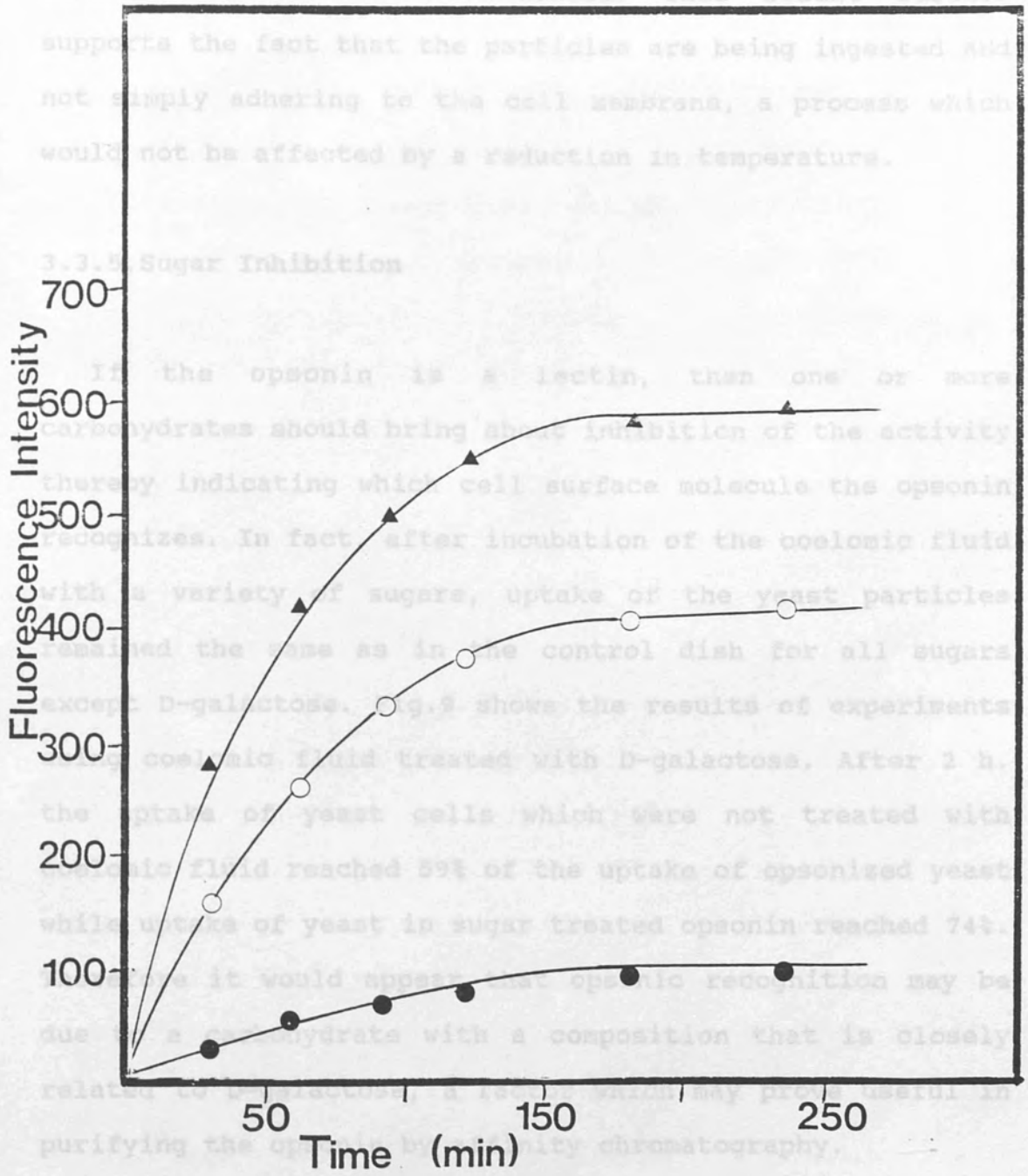

Fluorescence Intensity
700
600
500
400
300
200
100
50
150
250
Time (min)

FIG.8.

reduced at 4°C when compared to room temperature whilst it was increased at 37°C. It appears that phagocytosis of these particles is temperature dependent as would be expected of an active mechanism. This result further supports the fact that the particles are being ingested and not simply adhering to the cell membrane, a process which would not be affected by a reduction in temperature.

3.3.5. Sugar Inhibition

If the opsonin is a lectin, then one or more carbohydrates should bring about inhibition of the activity thereby indicating which cell surface molecule the opsonin recognizes. In fact, after incubation of the coelomic fluid with a variety of sugars, uptake of the yeast particles remained the same as in the control dish for all sugars except D-galactose. Fig.9 shows the results of experiments using coelomic fluid treated with D-galactose. After 2 h. the uptake of yeast cells which were not treated with coelomic fluid reached 59% of the uptake of opsonized yeast while uptake of yeast in sugar treated opsonin reached 74%. Therefore it would appear that opsonic recognition may be due to a carbohydrate with a composition that is closely related to D-galactose, a factor which may prove useful in purifying the opsonin by affinity chromatography.

Fig.9.

Phagocytosis of Fluorescent S.cerevisiae Particles by Eisenia foetida Phagocytes (nonopsonized ●-●; opsonized o-o; opsonized in D-galactose treated coelomic fluid ▲-▲).

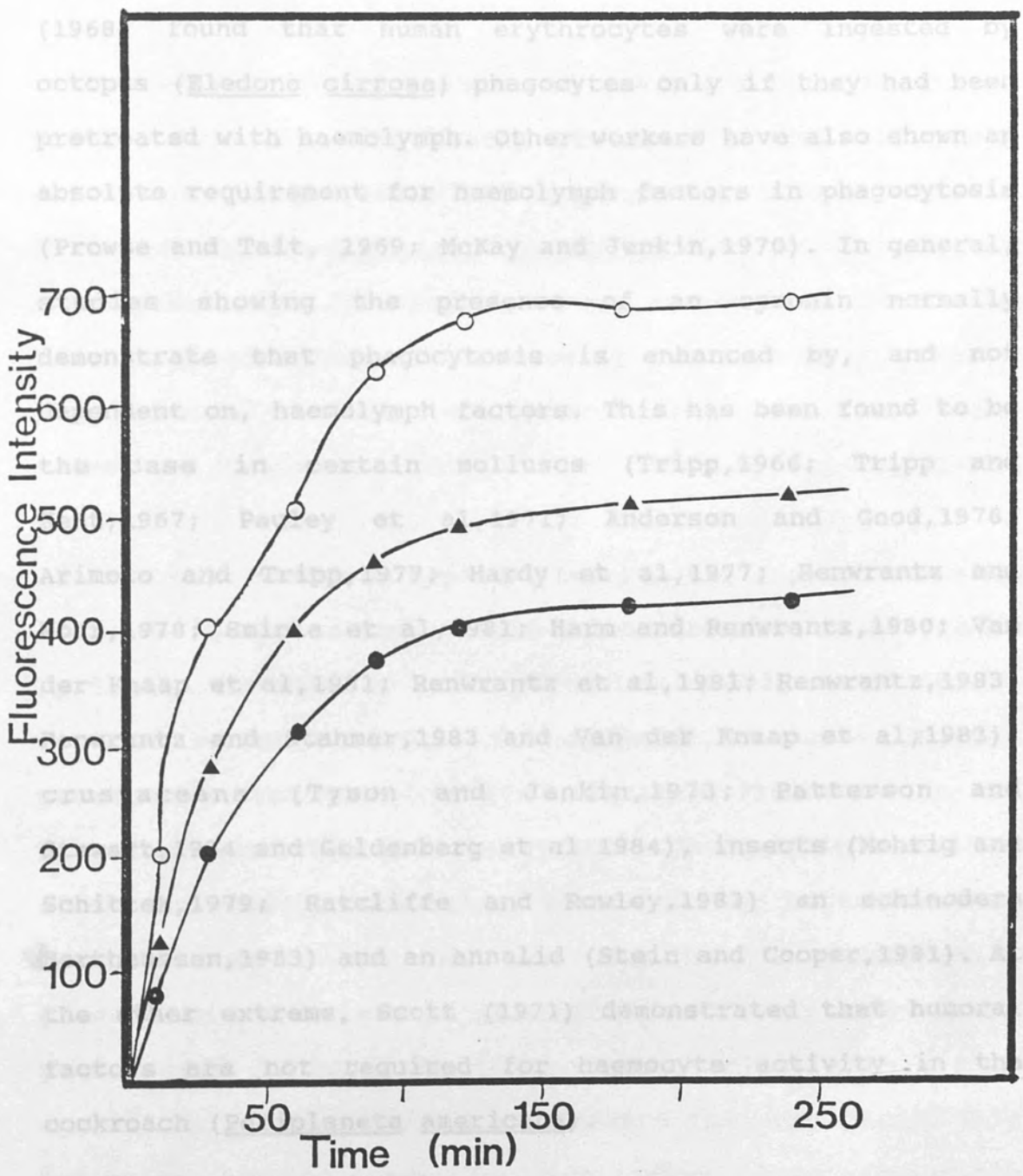

Fluorescence Intensity
700
600
500
400
300
200
100
50
150
250
Time (min)

FIG.9.

3.4.DISCUSSION

The relative importance of opsonization in the phagocytic process varies among different invertebrates studied. Stuart (1968) found that human erythrocytes were ingested by octopus (Eledone cirrosa) phagocytes only if they had been pretreated with haemolymph. Other workers have also shown an absolute requirement for haemolymph factors in phagocytosis (Prowse and Tait, 1969; McKay and Jenkin,1970). In general, studies showing the presence of an opsonin normally demonstrate that phagocytosis is enhanced by, and not dependent on, haemolymph factors. This has been found to be the case in certain molluscs (Tripp,1966; Tripp and Kent,1967; Pauley et al,1971; Anderson and Good,1976; Arimoto and Tripp,1977; Hardy et al,1977; Renwrantz and Mohr,1978; Sminia et al,1981; Harm and Renwrantz,1980; Van der Knaap et al,1981; Renwrantz et al,1981; Renwrantz,1983; Renwrantz and Stahmer,1983 and Van der Knaap et al,1983), crustaceans (Tyson and Jenkin,1973; Patterson and Stewart,1974 and Goldenberg et al 1984), insects (Mohrig and Schittek,1979; Ratcliffe and Rowley,1983) an echinoderm (Bertheussen,1983) and an annelid (Stein and Cooper,1981). At the other extreme, Scott (1971) demonstrated that humoral factors are not required for haemocyte activity in the cockroach (Periplaneta americana).

Although many workers believe that the agglutinin molecules are responsible for opsonization, only in two studies, in Mytilus edulis (Renwrantz and Stahmer,1983) and in Crassostrea gigas (Hardy et al,1977) has this been shown to be the case.

The extent of enhancement of phagocytosis also varies widely, from over 50% (Hardy et al,1977) down to barely detectable values (Stein and Cooper,1981). However, in the latter study of Lumbricus terrestris it was found that phagocytes named 'neutrophils' were enhanced by 24-25% by humoral factors but as these cells comprise only a small proportion of the entire coelomocyte population, the overall effect was very small.

From this study it appears that Eisenia foetida contains a factor in the coelomic fluid which increases phagocytosis by 94% in the case of yeast particles, and by 8-35% in the case of bacterial cells. The effector molecules of this pronounced activity remain elusive. Preincubation of particles with the purified 20,000 D agglutinin had no effect on phagocytosis rates, though it may be argued that opsonic activity has been lost due to the purification procedures. However, agglutinating activity was still present and it would therefore appear unlikely that this agglutinin and the opsonin are one and the same thing. This may also be the case for the other three agglutinin

molecules present in the coelomic fluid for preincubation with various gel filtration fractions, which exhibit agglutinating activity, did not enhance phagocytosis rates. A further observation may indicate that the agglutinins are not responsible for opsonization; the enhancement of phagocytosis was partially inhibited by D-galactose, whereas this sugar has no effect on haemagglutination titers.

Other possibilities exist: a polymer of two or more different molecules separated in the purification procedure may be required for opsonization, or a chain of events involving different molecules, again separated by gel filtration, may bring this enhancement of phagocytosis about. To determine whether either of these possibilities was the case, the gel filtration fractions were pooled in various combinations. It was found that there was no enhancement of of phagocytosis rates. In fact, this remained so when all peaks were combined and concentrated. Therefore it would appear that opsonic activity was irreversibly lost on chromatographic separation of the coelomic fluid. It may be argued that the enhancement of phagocytic activity was simply due to ionic influences of the coelomic fluid and not due to a specific molecule but this is highly unlikely as all sensitized particles were rinsed well to remove any traces of coelomic fluid and also, it is unlikely that D-galactose, and not any sugar in general, would specifically inhibit activity brought about by ionic influences.

The uptake of particles by Eisenia phagocytes was found to be variable. After 120 min each phagocyte, on average, ingested 4 yeast particles, 12.5 S.aureus cells, 20 E.coli cells or 30 B.megaterium cells without sensitization with coelomic fluid, and rising to 7, 14, 25, or 35 cells respectively after incubation in coelomic fluid. Therefore it would appear that Eisenia phagocytes may recognize differences in surface properties of foreign cells and thus the phagocytes ingested different strains of bacteria at different rates.

These differences may result from varying particle sizes especially so in the case of the much larger yeast particles, which presumably having a greater surface area would require a larger number of recognition molecules. However in the case of the smaller bacterial cells , it would appear that the phagocytes are capable of recognizing and distinguishing between the bacteria. These differences in phagocytic susceptibility may explain the course of bacterial infection. Namely, S.aureus is a pathogen whilst E.coli and B.megaterium are not, thus the vulnerability of the host to the bacterial infection may in part be determined at the level of phagocytosis.

It would appear that the opsonin in Eisenia foetida may not be an agglutinin molecule, or if it is, it is probably

acting in combination with another factor in the coelomic fluid. Recently it was suggested that the phagocytes of Lumbricus terrestris have receptors on the membrane which recognize the vertebrate opsonins IgG and complement C3 fragments (Laulan et al,1988). Vertebrate C3b appears to be responsible for an opsonizing effect of erythrocytes by echinoid phagocytes (Bertheussen,1983). It may be that another possible candidate for an opsonin in some invertebrates is emerging as well as the agglutinin molecules. Considering the diversity of invertebrates it would perhaps not be surprising to find a diversity in the nature of their recognition molecules.

4. ANTIBODY PRODUCTION

4.1. INTRODUCTION.

The extreme specificity shown by individual antibodies for the corresponding antigen makes them ideal tools for the study of proteins and other substances. An attempt was made to produce rabbit polyclonal antibodies to the purified haemagglutinin in order to carry out subsequent experiments which would indicate where in the animal these haemagglutinins occur and where they may be synthesized.

4.2.MATERIALS AND METHODS.

4.2.1.Dose and Form of Antigen.

200 μg of purified haemagglutinin in 500 μl of PBS pH 7.2 was thoroughly mixed with an equal volume of the appropriate adjuvant (the adjuvant allows a slow prolonged release of antigen in a highly aggregated form together with immune reactive substances) until the emulsion that formed was thick and creamy. For primary injections, Freund's complete adjuvant (a water in oil emulsion in which heat killed and dried bacteria -Myobacterium tuberculosis) are suspended in the oil phase) was used, whereas booster injections employed Freund's incomplete adjuvant (in which the bacteria are omitted to prevent

possible hypersensitivity reactions).

4.2.2.Bleeding and Collection of Serum.

A three month old male rabbit, initially weighing 2.3 kg, of the New Zealand White variety was used. The animal was held firmly and one of the ears rubbed to induce vasodilation. A small transverse cut was made in the marginal ear vein and the required volume of blood collected. Serum was separated from cells as rapidly as possible to prevent contamination from cell proteins or the degradation of any antibodies by proteolytic enzymes released by lysis of the cells. The blood was allowed to clot at room temperature for 1h. The serum was then recovered by centrifugation for 30 min at 2500g at 4°C and stored at -70°C.

4.2.3.Immunization Protocol

Although rabbits are variable in their immune responsiveness between individuals, only one animal was immunized due to the shortage of pure antigen.

Initially, the rabbit was bled, 10 ml of blood was collected, the control serum recovered and stored at -70°C. On day 1, 1 ml of the primary emulsion, containing 200 μg of pure antigen, was injected subcutaneously over 4-5 sites

around the neck and hind quarters. The rabbit was given booster injections on days 30, 44 and 58. 10 ml of blood was collected on day 72 and the serum was tested for the presence of antibodies.

4.2.4.Analysis of Serum for the Presence of Polyclonal Antibodies.

1) Double Immunodiffusion- The Ouchterlony Technique.

Grease and dirt were removed from a glass slide (65 x 25 mm) by rinsing it in successive changes of distilled water, ethanol, ethanol:ether (1:1v/v) and finally ether. A thin coat of molten 1% agarose gel (made up in 0.05M barbital buffer, pH 8.2) was brushed onto the surface of the slide and dried in an oven for 30 min. This precoating procedure enables the final layer of agarose to stick tightly to the slides. After placing on a levelling table, 2.7 ml of molten agarose was poured onto the slide and allowed to spread evenly over the glass surface. Once the gel had solidified, a hexagonal pattern of holes was punched into it using a gel puncher. The plugs of agar were removed from the wells using a pasteur pipette attached to a vacuum line.

Dilutions of the antiserum, control serum and antigens were made up in 0.05M barbital buffer. These dilutions

were; neat, 1/2, 1/4, 1/8, 1/16 and 1/32. 5µl of neat antiserum was pipetted into the centre well of the hexagon using a Hamilton syringe. Known dilutions of the antigens were placed in order of concentration in the surrounding wells. This was repeated with control serum.

Diffusion was allowed to take place over 48 h at room temperature in a covered petri dish containing moistened filter paper to prevent desiccation of the agarose gel. The slide was periodically examined for precipitin formation which would be observed as white light scattering lines, best viewed with a strong sideways illumination and a black background. The slide was then washed to remove non-precipitating antibody and antigen and then dried and stained for protein as discussed below.

Washing, drying and staining

The slide was sandwiched between two sheets of wet filter paper covered on each side by a glass plate. Pressure was applied, to squash the gel, for 15 min. The slide was washed in 0.9% NaCl until fully reswollen (1 h), pressed, washed and pressed again. After washing in distilled water, a final press was carried out and the slide dried at 60^{o}C.

Staining of the gel was carried out using 5.0g Coomassie brilliant blue G in 450 ml of acetic acid and 450 ml

distilled water. The slide was immersed in this solution for 10 min , then rinsed in destaining solution (12.5% isopropanol: 7.5% acetic acid). The destaining was monitored and as soon as the background became faint and the precipitin arcs clear, the slide was removed and allowed to dry.

2) Western Blotting and Immunochemical Labelling.

SDSPAGE gels were set up and allowed to run as in section **6.2.5**

Samples for electrophoresis included coelomic fluid, control rabbit serum and purified antigen. All samples were run in duplicate in wells at either side of the gel.Each gel was carefully removed from the running apparatus and rinsed in transfer buffer (Tris glycine, pH 8.3, containing 20% methanol) for 3 minutes. The western blotting was carried out as performed by Vaessen et al (1981) with slight modification. A biorad transfer blot apparatus was used and a current of 55V was applied for 4.5 h , after which time the proteins would have migrated from the gel onto the nitrocellulose sheet.

When protein transfer was complete, the gel was stained with Coomassie blue and destained as in section 6.2.5. to ensure that no protein remained on the gel and therefore transfer had been successful. The nitrocellulose sheet was

carefully taken out of the apparatus and washed twice in Tris HCl saline, pH 7.4 One half of the sheet was stained for protein in 0.1% amido black for 2-3 min. before destaining with acetic acid, methanol and distilled water (1:6:13). This step was also carried out to ensure that protein transfer had been successful and the position of the various protein bands could be visualized.

The other half of the nitrocellulose sheet was saturated in 1% BSA for 1h. to prevent non-specific binding of the antibody. Excess BSA was removed by several rinses in tris-HCl saline, pH 7.4 or control serum. The nitrocellulose sheet was then incubated in 25 ml of various dilutions of the antibody (1:100, 1:500 and 1:1000) in Tris HCl saline, pH 7.4 for two hours with gentle stirring, after which it was left for a further 16 h. at room temperature.

Excess antibody and serum were removed by three washings in Tris saline, pH 7.4, containing 0.05% Tween 20 followed by another three washings in Tris saline only (each wash taking 10 min.).

The nitrocellulose sheet was next incubated for 2h. in antirabbit goat serum coupled to horseradish peroxidase (50 μl into 25 ml buffer) at room temperature. The excess antibody was washed off as before.

Finally, the nitrocellulose sheet was treated with 36 ml of substrate solution prepared as follows: 18 mg of 4-chloro-1-naphthol was dissolved in 6 ml of methanol and this was gently poured into 30 ml of Tris HCl saline and mixed well. 12 μl of hydrogen peroxide (H_2O_2) was added to the above solution and after gentle stirring for a few seconds, the entire solution was carefully poured onto the nitrocellulose sheet and observed for the appearance of purple bands. The sheet was then washed in Tris HCl and allowed to dry.

4.3.RESULTS.

From Plate 5a it can be seen that precipitation arcs were formed, suggesting the presence of antibodies to the antigen in the rabbit serum. The most defined arcs were obtained using undiluted serum in the middle wells and the surrounding wells either containing coelomic fluid to a dilution of 1 in 8 or 1 in 16 or purified haemagglutinin to a concentration of 50 μg 100 μl^{-1}.

Precipitation arcs formed between the middle well and wells 1 to 5 and no arcs formed in the control wells,6. Furthermore, the immunoprecipitin lines fused, indicating immunochemical identity of the coelomic fluid and antigens. This reaction of identity is of course expected, since the coelomic fluid contains the haemagglutinin used as the

Plate 5.

A) An Ouchterlony Slide Showing Precipitin Lines Formed Between Eisenia foetida Agglutinin Antigens and Rabbit Anti-Agglutinin Serum (Well contents: 1- pure agglutinin,25 μg; 2 -coelomic fluid,1/16; 3 -pure agglutinin,50 μg; 4 -coelomic fluid,1/8; 5 -coelomic fluid,1/32; 6 -undiluted control rabbit serum; 7- undiluted rabbit antiserum).

B) Western Blotting and Immunolabelling of Coelomic Fluid Proteins from Eisenia Foetida with Anti-Agglutinin Rabbit Serum on Nitrocellulose Paper (Lane 1 -25 μg agglutinin; Lane 2 -40 μl coelomic fluid; Lane 3 -50 μg pure agglutinin). Arrows indicate the positions of labelled bands.

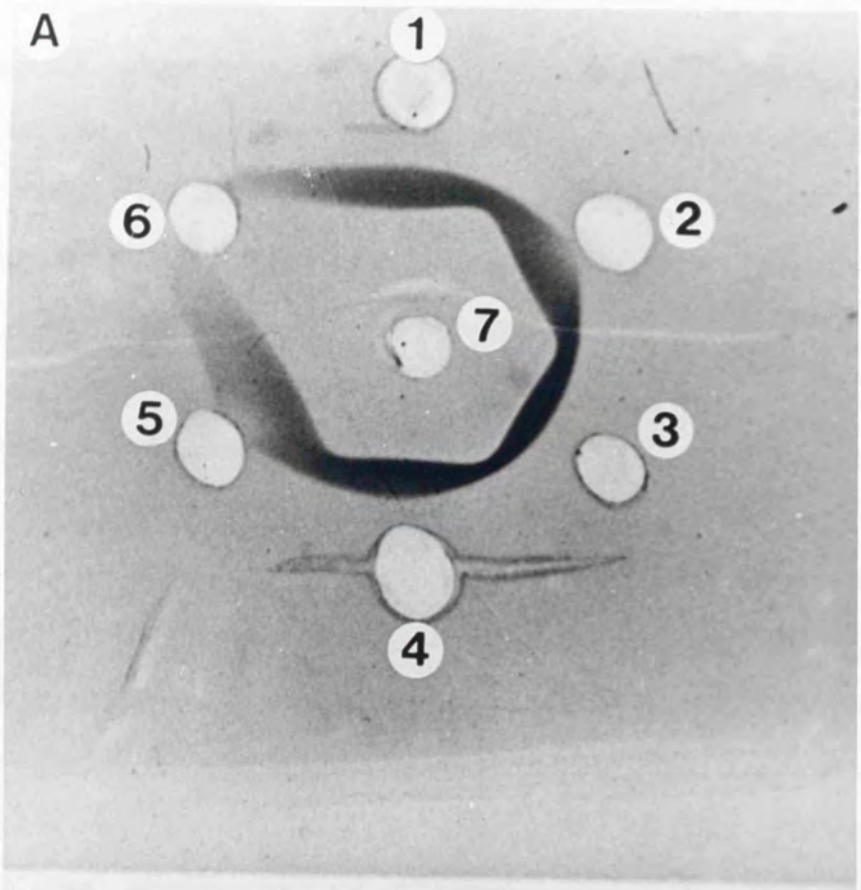
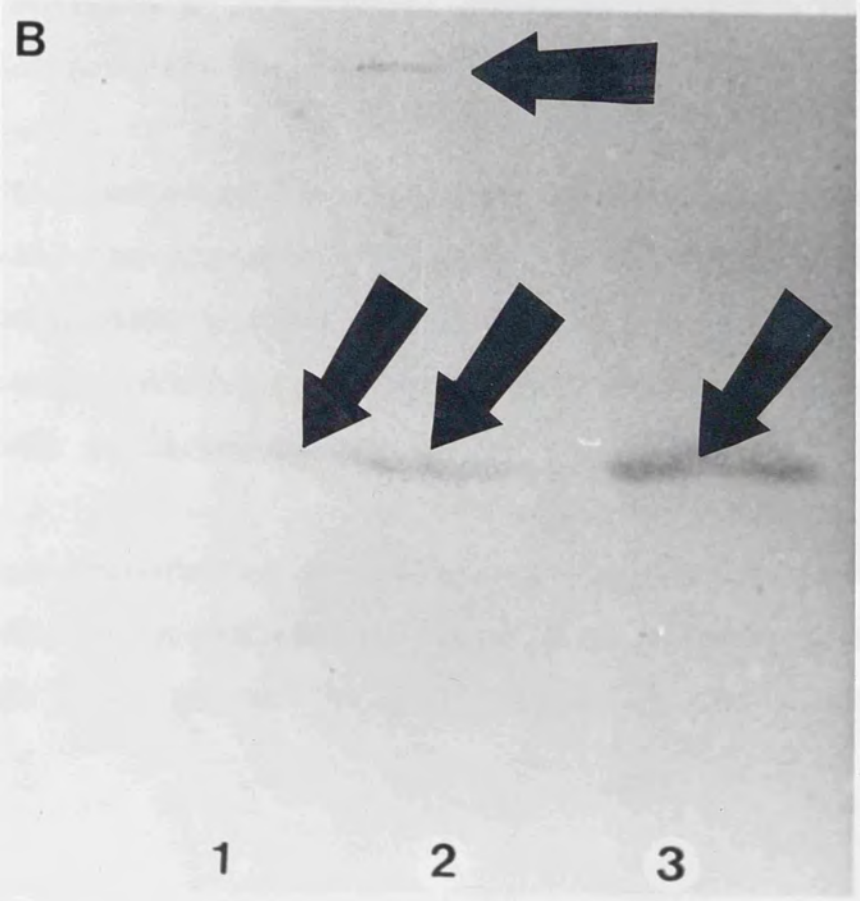


PLATE 5.



immunogen. Although the result indicates the presence of antibodies, this test is crude in so far as determining the specificity of the antiserum and so a more sensitive system using SDSPAGE, western blotting and immunochemical labelling was used.

Immunochemical labelling

SDSPAGE was carried out using the following samples; 40 µl of crude coelomic fluid, 50 µg or 25µg of pure haemagglutinin in 40 µl of PBS buffer, pH, 7.2, and 10 µl of molecular weight marker solution (SDS-7, Sigma). Transfer of proteins from the gel to the nitrocellulose sheet was confirmed by staining half of the sheet with amido black and this revealed all of the bands expected. Back staining of the SDSPAGE gel with Coomassie blue showed that no proteins remained.

After immunochemical treatment, the nitrocellulose sheet revealed the presence of positive bands (Plate 5b). The 1 in 100 antiserum dilution gave clear results whilst higher dilutions produced very faint bands, this suggests that the antibody yield was quite low.

Lane 1 contained 25 µg of pure agglutinin, lane 2 40 µl coelomic fluid and lane 3, 50 µg of pure haemagglutinin. It can be seen (Plate 5b) that lanes 1 and 3 showed the

presence of a dense single band which corresponds to the 20,000 D agglutinin (the molecular weight was estimated by comparing the lanes to those on the other half of the nitrocellulose sheet which had been stained with amido black and included the molecular weight markers).Lane 2, which contained coelomic fluid also produced a positive reaction in the area expected. However, another very faint band also became evident, corresponding to a protein of an approximate molecular weight of 40,000 D and very likely to be the 40,000 D molecular weight agglutinin. Incubation in control serum produced no visible bands, suggesting that the previous results were in fact positive for specific antibody binding.

4.4.DISCUSSION.

It would appear that the raising of polyclonal antibodies to the 20,000 D agglutinin had been successful, but the appearance of the second band in lane 2 may suggest one of two things:

1) The purified haemagglutinin was contaminated with the 40,000 D molecule and so antibodies to both proteins were raised, or

2) A partial homology may exist between the 20,000 D and 40,000 D molecules and the raised polyclonal antibodies recognize a shared antigenic site.

The second explanation would appear to be more likely for several reasons: in the first instance, the 20,000 D molecule has a pI value of 4.8 whereas the 40,000 D molecule has a pI value of 6.2. Chromatofocusing produces a very high resolution and it is therefore very unlikely that any of the 40,000 D molecule eluted off at a very much lower pH. Secondly, SDSPAGE of the purified agglutinin revealed the presence of only one band and this purity was confirmed with the silver staining technique (section 6.2.6.) which will detect down to 0.1 μg of protein. As no other bands appeared after this procedure, there would have to be less than 0.1 μg of contaminating protein present, if any at all, meaning that the 40,000 D protein would be exceptionally antigenic in order to stimulate antibody production and this is unlikely. It would therefore appear that a relationship exists between the 20,000 D agglutinin and the 40,000 D molecule which probably corresponds to the 40,000 D agglutinin.

Elution of proteins purified by SDSPAGE was considered as an alternative procedure for isolating the agglutinins for antibody production. However, various problems arose, including the difficulty of obtaining enough protein, and the possibility of the antigenicity of the SDS and Coomassie blue (Granger and Lazarides, 1980) contained in the excised gel band.

5. LOCALIZATION OF THE PURIFIED HAEMAGGLUTININ ON COELOMOCYTES USING IMMUNOCYTOCHEMICAL TECHNIQUES.

5.1. INTRODUCTION.

Opsonic properties ascribed to lectins have been the subject of controversy because haemocytes or coelomocytes can recognize foreign particles, cells or macromolecules independent of the presence of serum lectins, although in most cases, previous exposure of the foreign material to serum factors enhances the rate of phagocytosis (Jenkin, 1976). From Chapter 2 it can be seen that this is also the case in Eisenia foetida, where control coelomocytes are able to phagocytose a range of foreign particles independent of coelomic fluid, though preincubation results in enhanced activity.

This situation indicates that, although humoral factors, shown to be agglutinins in some cases, might facilitate this recognition and enhance the response, haemocytes or coelomocytes may display agglutinin molecules on the cell membrane and they may interact with foreign materials independent of or in conjunction with humoral factors.

An attempt was made to uncover some of the events in the mechanism of recognition in phagocytosis in Eisenia by

determining whether agglutinins or agglutinin receptors are present on coelomocyte membranes by utilizing the polyclonal antibody to the 20,000 D agglutinin.

5.2.MATERIALS AND METHODS.

5.2.1.Preparation of monolayers for scanning electron microscopy (SEM).

Coelomocytes from three worms were extracted (as in section 3.2.1.) directly into 1 ml of PBS , pH 7.2.100µl drops of this cell suspension were allowed to settle out on 19 mm glass coverslips. After 30 min , non-adherent cells were removed by rinsing the coverslips in 0.1M sodium cacodylate buffer (NaCacod.) pH 7.2. Each coverslip was then immersed in fixative (4% paraformaldehyde, 0.5% glutaraldehyde in NaCacod.) for 1h.

5.2.2.Preparation of Fixative.

2.0g of paraformaldehyde and 25mg of calcium chloride were added to 20 ml of distilled water and heated to 80°C in a fume cupboard. 4 drops of 1M NaOH were added and the solution stirred until it became clear. After cooling to room temperature, 1 ml of 25% glutaraldehyde was added. The volume was then made up to 50 ml using 0.2M NaCacod. pH 7.2.

5.2.3. Immunolabelling of Cell Monolayers.

The coverslips were rinsed in NaCacod. buffer, pH 7.2, containing 1% bovine serum albumen (BSA) to remove any excess fixative and prevent non-specific binding by the antibody. Three washes were carried out, each taking 10 min. The underside and peripheral edges of the coverslips were gently dried in order to prevent any incubation medium from leaking over the edge.

Dilutions of the rabbit antiserum, containing the primary antibody, were prepared at 1:10, 1:100 and 1:1000 in NaCacod. buffer. Each coverslip was incubated at 4^{o}C overnight with 100 μl of a known dilution of the primary antibody. Coverslips were then placed in humidity chambers to prevent desiccation of the coelomocytes.

The excess primary antibody was removed in three washings of the NaCacod. buffer, again each wash taking 10 min. The secondary antibody (goat anti-rabbit IgG conjugated to 20 nm gold particles- Biocell Research Laboratories) was prepared by diluting 20 μl with 180 μl of buffer and spinning at 2700 g for 20 min at 4^{o}C to remove any gold particle aggregates.

50 μl of secondary antibody was overlaid on each

coverslip and incubation took place at room temperature for 1 h with gentle rocking of the dishes containing the coverslips.

Three more washings in buffer removed the excess secondary antibody. The antigen antibody gold conjugate complex was then fixed by placing the coverslip in 2.5% glutaraldehyde in PBS, pH 7.2 for 30 min.

5.2.4.Dehydration.

This was carried out by passing the coverslips through increasing concentrations of alcohol: 30%, 50%, 70%, 95% and finally absolute alcohol. Each of the first four steps took 10 min , the final one in absolute alcohol was carried out twice, each rinse taking 20 min.

5.2.5.Final Stages.

After critical point drying, the coverslips were mounted onto stubs, sputter coated with gold and palladium for 1 min to enhance the conductivity of the samples and then examined in a Cambridge S100 stereoscan electron microscope linked to a AN 1085 EDX analyzer for the detection of gold particles.

5.2.6.Preparation of Coelomocytes for Transmission Electron Microscopy.

Coelomocytes from three worms were extracted (as in section 3.2.1.) into 1 ml of NaCacod. buffer, pH 7.2. The cells were washed three times in this buffer, each rinse being removed by centrifugation at 250 g for 10 min. After the final wash, 1 ml of fixative (4% paraformaldehyde, 0.5% glutaraldehyde in NaCacod. buffer, prepared as in section 5.2.2.) was added and the suspension was gently rotated for 1 h.

5.2.7.Immunolabelling of Coelomocytes.

The cell suspensions were spun down and incubated in primary and secondary antibody as in section 5.2.3. using the same time parameters. Cells were rinsed in NaCacod. and 1% BSA., all centrifugations being carried out for 10 min. at 250 g. The complex was fixed as before, and the cells rinsed in buffer to remove excess fixative.

5.2.8.Alginate Entrapment of Cells.

The cells were placed in alginate beads which provided a support medium to enable further procedures to be carried out.

5% sodium alginate was prepared by slowly adding 1 g of powder to 20 ml of distilled water, stirring continuously. The medium was then stirred for an additional 20 min to allow the alginate to swell.

The coelomocytes were spun down, the supernatant discarded and 1.5 ml of alginate added. The cells were resuspended in the alginate with gentle stirring and passing up and down a 1 ml pipette. The suspension was transferred to a 5 ml syringe fitted with a 25 G needle and extruded in a bead like form into 30 ml of 0.3M calcium chloride solution in NaCacod. The beads were allowed to set for 30 min. at room temperature, after which, they were washed three times in distilled water.

The beads were dehydrated by passing them through alcohol as in section 5.2.4.

5.2.9.Embedding.

The transition from the alcohol stage to the embedding material took place via propylene oxide. The beads were placed in propylene oxide and gently stirred for 10 min. After overnight stirring in a 50:50 mixture of propylene oxide and araldite resin they were stirred briefly in fresh resin and polymerization was carried out at 60^{o}C for 22 h.

5.2.10.Grid Preparation.

Sections were prepared to a thickness of 60-90 nm, as judged by interference colours (gold or silvery gold) using freshly made glass knives, and placed onto uncoated nickel grids (300 mesh). The grids were stained with 2% uranyl acetate for 20 min (Dawes 1971) and, after rinsing with distilled water, with lead citrate for 2-5 min (Watson 1958). A final wash with distilled water was followed by allowing the grids to dry. The sections were examined and photographed in a Hitachi H600 electron microscope operated at 75 kV.

5.2.11.Controls.

In all immunolabelling procedures various controls were set up; the primary antibody was omitted, control rabbit serum was added instead of antiserum, or the primary antibody was incubated with an excess of purified agglutinin to block antibody activity.

5.2.12.Alternative Procedures.

In transmission microscopy, 5 nm gold conjugate was used as well as 20nm gold.

Coverslips with monolayers of cells were placed on top

of tubes containing araldite resin. After polymerization, the coverslips were removed by fracturing off in liquid nitrogen, leaving a monolayer of cells embedded at the surface of the resin. This was an alternative method to trapping the cells in alginate beads.

Immunolabelling was also carried out after cutting sections of cells to determine if intracellular agglutinins were present. Grids carrying cell sections were simply immersed in the required solutions.

5.3.RESULTS.

5.3.1.Scanning Electron Microscopy.

Although coelomocytes were clearly observed, the presence of gold was not detected by the analyzer and obviously, the limiting resolution and magnification would make it very difficult to visualize particles with a diameter of 20 nm.

5.3.2.Transmission Electron Microscopy.

This was used to actually visualize any gold particles that were present. The gold conjugate becomes obvious at magnifications greater than 30,000 as dense, black, regular spheres.

Plate 6.

Gold Labelling (using 20nm particles) of Eisenia foetida Phagocytes with Anti-Agglutinin Rabbit Serum Linked to Goat Anti-Rabbit Gold Conjugate (A -control phagocyte with primary antibody omitted (x50,000); B,C and D labelled phagocyte (30,000x,45,000x and 90,000x respectively).

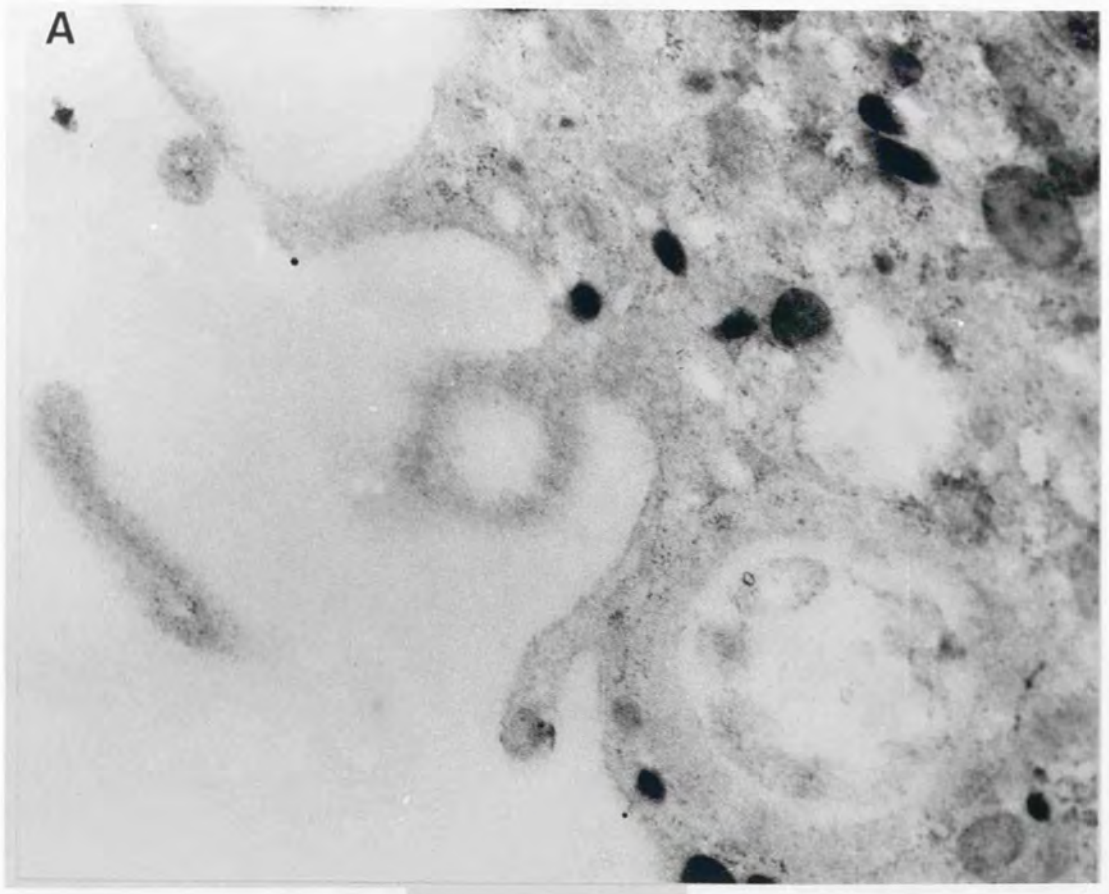
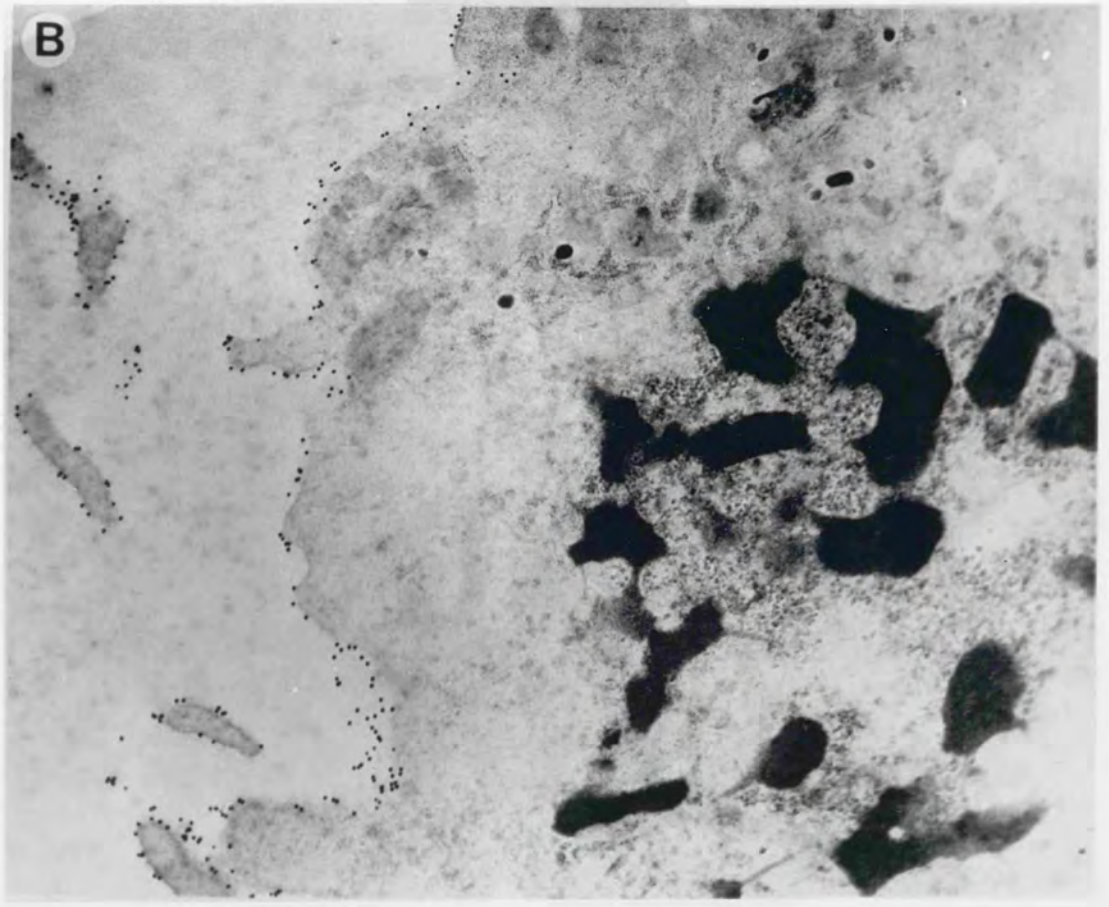
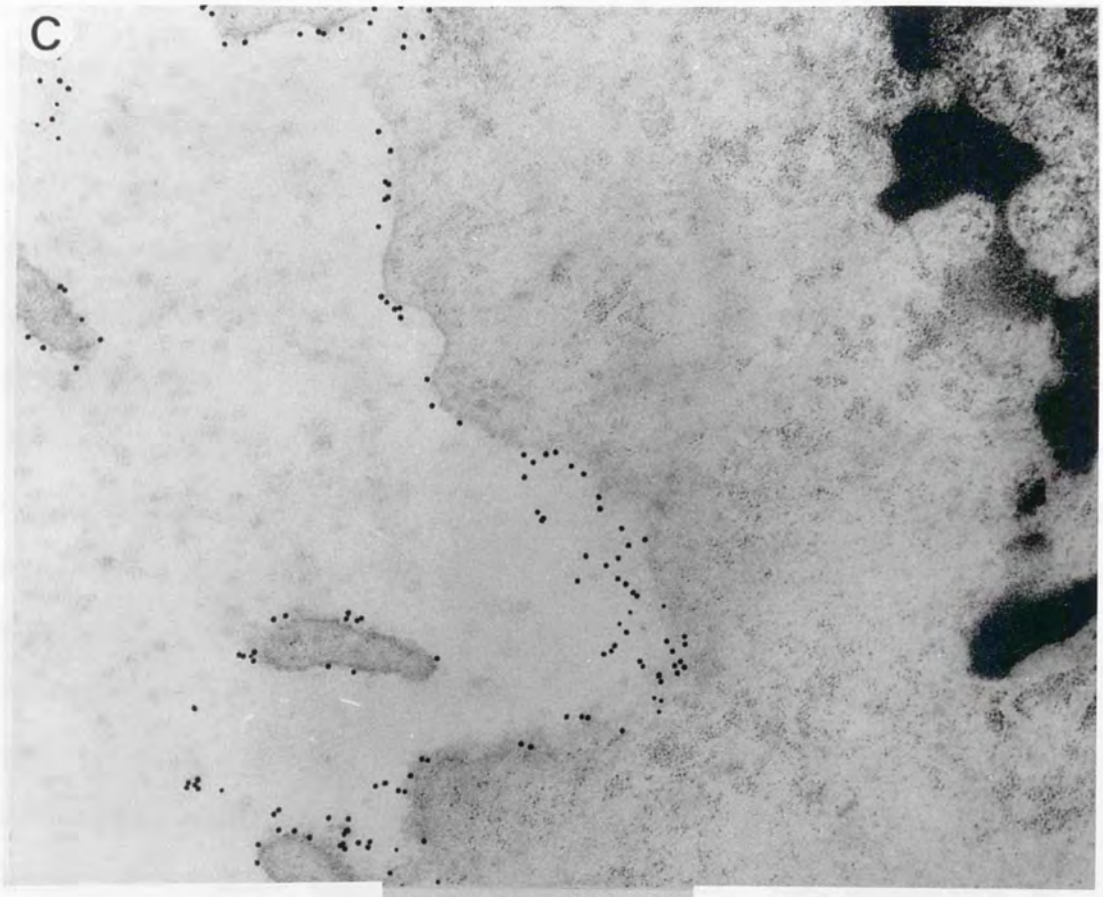
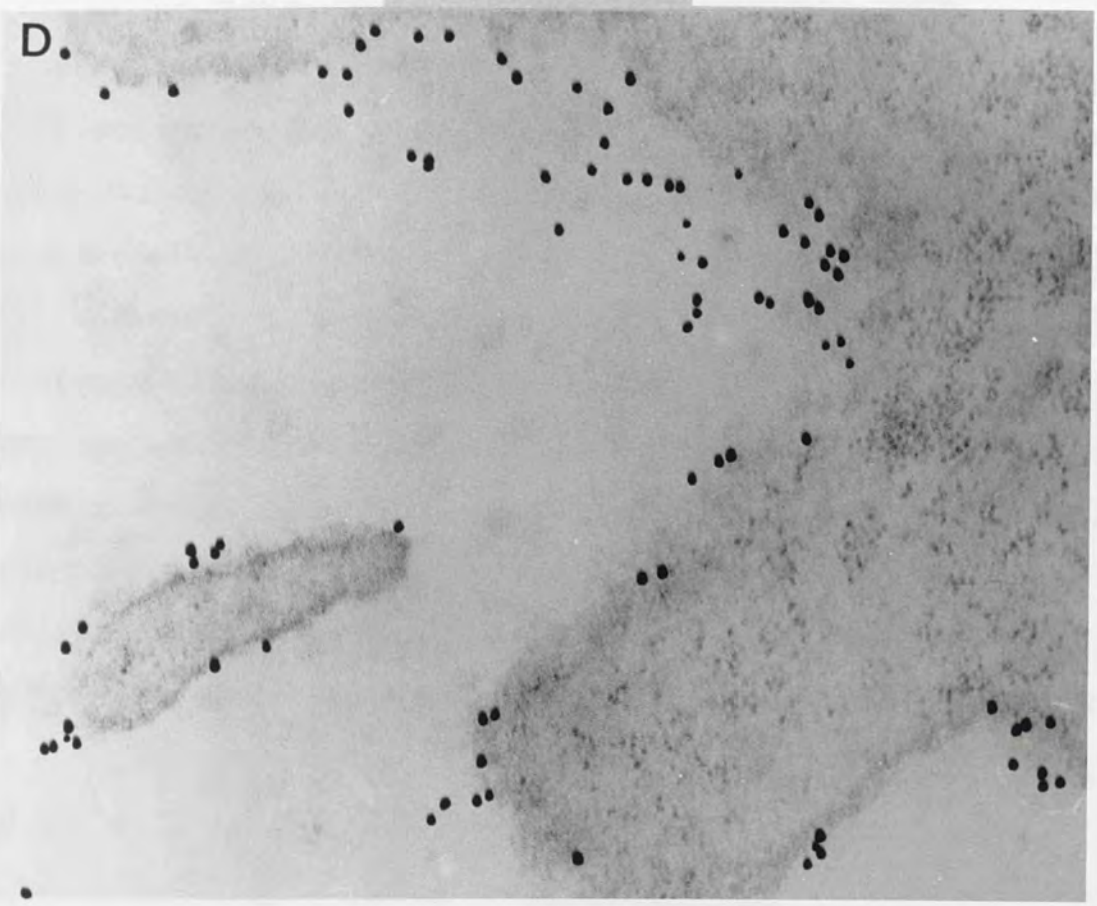


PLATE 6...





... PLATE 6.



Positive results were obtained for the presence of agglutinins or agglutinin like molecules on the phagocyte membranes (Plate 6;b,c,d). It can be seen that the gold particles appear to be fairly evenly distributed on the cell membrane. Control cells which had been treated with control serum or the omission of primary antibody (Plate 6a) showed very few gold particles, suggesting that antibody binding to the coelomocytes was specific for agglutinin or agglutinin like molecules. Blocking of the primary antibody with purified agglutinin present in excess was also used as a control as well as examining the eleocytes present. In both cases, very few gold particles were observed, again indicating that the positive gold labelling was specific for agglutinin like molecules.

Although it would appear that agglutinin or agglutinin like molecules are present on the cell membranes, attempts to localize agglutinin molecules intracellularly were not successful. It would appear that the method used resulted in a loss of antigenicity, probably due to the more thorough fixation that was required. In order to localize intracellular molecules, it was necessary to cut sections of the cells before immunolabelling. This involved fixing the cells and dehydrating and embedding them, before addition of the antibody, all of which may have resulted in a loss of antigenicity. Indeed, no labelling occurred even

at the cell membrane and as this had previously been shown to be positive in cells that had only been lightly fixed before immunolabelling, it would suggest that a problem with the method had occurred. Alternative embedding materials were tried, such as Lowicryl K4M, which are designed to preserve antigenicity with no success.

The use of freeze fractured coverslips on resin allowed immediate access to cell monolayers for cutting sections once the coverslip had been removed. Although the alginate bead method also gave good results, it was often very time consuming to locate cells within the bead when cutting sections, especially if the cell concentration was a little low.

5.4.DISCUSSION.

Since agglutinins or agglutinin like molecules are present on the surface of Eisenia coelomocytes, and that these molecules in a humoral form are capable of agglutinating a range of bacteria and a yeast, if would seem plausible that involvement of membrane lectins in non-self recognition as a first step in eliciting phagocytosis would be possible. Indeed this has been put forward as the role of membrane bound agglutinins in a variety of cells in the most complex vertebrate immune systems (Lis and Sharon, 1986). In certain protozoa, such as Acanthamoeba, membrane

lectins are believed to participate in the recognition of particles to be phagocytosed . The protozoan Plasmodium bears cell membrane associated lectins which are specific for glycopeptides present on erythrocyte plasma membranes, suggesting that they are responsible for the selection of the particular blood group cells they penetrate and infest (Jungery et al, 1983). Renwrantz and Stahmer (1983) detected a haemocyte membrane-bound lectin in the mussel, Mytilus edulis, that cross reacted with antibodies made against a serum lectin, and hypothesized that humoral as well as cell bound lectins are involved in the attachment of yeast cells to Mytilus haemocytes and their subsequent phagocytosis. Amirante and Mazzalai (1978) showed that FITC labelled rabbit antibodies raised against a humoral lectin from the cockroach, Leucophoea maderae, binds to it's haemocytes in patterns that correlate with morphologically different cell populations (α and β cells) and subpopulations (small and large haemocytes). Vasta et al (1984), again using immunocytofluorescence demonstrated the presence of a lectin on the cell membrane of the haemocytes in the oyster Crassostrea virginica. Antisera raised against a serum lectin blocked the binding of haemocyte microsomes to protease treated vertebrate erythrocytes, thus confirming that the haemocyte membrane lectin is serologically related to the serum lectin. More recently, Parrinello and Arizza (1988) have demonstrated the presence of membrane lectins on the membranes of 34% of the

haemocytes of the tunicate Ascidia malaca using rabbit antiserum isolated against serum lectins.

In Eisenia, the gold label attached to all phagocytes, and although these cells have been divided into subpopulations (Roch,1980), there appears to be no difference in their possession of agglutinin or agglutinin like molecules on the cell membrane. This was also shown to be the case in Ascidia malaca where the haemocytes possessing membrane lectins were of at least three different types (Parrinello and Arizza,1988)

From Chapter 2, it can be seen that the purified 20,000 D haemagglutinin is unable to enhance phagocytosis, though another factor or a combination of molecules will bring this about. From the results of immunolabelling it would seem unlikely that agglutinin receptors for the 20,000 D agglutinin molecule are present on coelomocyte membranes. Therefore, it would appear increasingly unlikely that the humoral molecule is responsible for the opsonic activity in the coelomic fluid (see general discussion-section 7). However, the presence of this agglutinin molecule on the surface of these coelomocytes indicates that recognition of foreign particles may involve the direct binding of carbohydrate determinants of the foreign particles to membrane bound agglutinins.

In general, the recognition of foreigness by membrane bound agglutinins which have so far been discovered, remains the most favoured proposal. Other suggestions are few, but include the involvement of agglutinins in attachment of respiratory pigments onto cell surfaces thereby facilitating their transport across the membrane*. Such agglutinins could also be involved in molecular linkage of various protein subunit structures (Chorney and Cheng, 1980).

6. *IN VITRO* RELEASE OF AGGLUTININATING MOLECULES BY *EISENIA* COELOMOCYTES.

6.1.INTRODUCTION.

As discussed in section 1, it is generally believed that agglutinins are synthesized and released by the coelomocytes in the coelom (Stein and Cooper,1988) or the haemocytes in the haemocoel (Amirante 1976; Yeaton,1980; Van der Knaap et al,1981). As previously shown, the phagocytes of *Eisenia foetida* bear agglutinin like molecules on the cell membrane but attempts to localize agglutinins within the cells was unsuccessful, perhaps due to a loss of antigenicity of these molecules in the preparative steps (section 5). An attempt was made to determine whether *Eisenia* coelomocytes are responsible for agglutinin production and or their release using radioactively labelled amino acids.

6.2.MATERIALS AND METHODS.

6.2.1. Separation of Cell Populations.

Coelomocytes were extracted, by electrical stimulation of the worms, into 3 ml of cold PBS, pH 7.2 until the cell number reached approximately 1 x 10^6 ml^{-1}. The buffer contained 5 mM EGTA to prevent clumping of the cells. The

coelomocytes were spun at various speeds, ranging from 10-100 g for 10 min and the supernatants examined for phagocyte and eleocyte numbers. Attempts were also made to separate the cells using Percoll gradients and employing techniques which relied on the apparent fragility of the eleocytes in order to obtain pure phagocyte suspensions, such as treatment with detergents, sugar solutions and sonication.

6.2.2.Examination of Culture Media for the Presence of Agglutinin Molecules.

Coelomocyte monolayers were prepared (as in section 3.2.1.) and incubated in 3 ml of Holtfreter buffer pH 7.2 (containing 5.4 g NaCl, 0.15 g KCl and 0.3 g NaHCO3 and 1.47 g $CaCl_2$ l^{-1}. Unless stated otherwise, this buffer was used throughout this section. After standing for 9 h at room temperature, the medium was drawn off from several petri dishes, pooled and concentrated by an Amicon filter (as in section 2.2.5) and then tested for haemagglutinating activity using sheep red blood cells as in section 2.2.2. The media was also examined for the presence of proteins using SDS PAGE (6.2.7) The viability of the cells was tested by staining them with 0.02% toluidine blue for 10 min. Healthy cells prevent uptake of the stain and remain clear whilst dead cells stain deep blue. Control dishes contained cycloheximide at a concentration of 10 μg ml^{-1}.

6.2.3. Incorporation of Radiolabel into Coelomocyte Monolayers.

Coelomocyte monolayers were prepared as in section 3.2.1., each petri dish containing 1 ml of the cell suspension at a concentration of 5 x 10^6 cells ml^{-1}. The suspension was allowed to settle as a small drop and was not spread evenly over the entire surface of the dish.

After 30 min., the adherent cells were quickly rinsed and 1 ml of one of the following solutions was added: 1 ml of buffer, 1 ml of buffer containing 100 μCi of ^{35}-S methionine (15 μg,Amersham - supplier) or 500 μl of buffer ,500 μl of coelomic fluid and 100 μCi of ^{35}-S methionine. All three mixtures had a duplicate control solution which contained cycloheximide (10 μg ml^{-1}). The dishes were then left at room temperature for 3 h after which time the cells were rinsed with fresh medium and incubated for a further 6 h in the same medium as initially used except that the radiolabel was replaced with 10 mM methionine which acted as a chaser.

On completion, incubation media plus one wash were drawn off and retained. The viability of the cells was tested using toluidine blue.

6.2.5. Removal of Unincorporated Label.

Although the cells were washed well after incubation in radiolabel this would not be sufficient to remove all excess counts and so this step was carried out after the full incubation period by washing the retained media through a Sep-Pak C18 cartridge (Waters Associates) with increasing concentrations of methanol. Initially the Sep-Pak cartridge was activated by the addition of 3 ml of methanol. After washing this through with 10 ml of distilled water the sample was loaded and any materials released by the cells were washed through the column using 3 ml of methanol initially at a 10% dilution and progressively becoming more concentrated in 10% steps until 100% concentration was reached. Any free label would wash through almost immediately though any larger materials released by the cells would appear much later. 3 ml washings were collected separately and scintillation counts were carried out using 1 ml of each sample.

6.2.4. Scintillation Counting.

Each of the 1 ml samples was mixed well with 7 ml of scintillation fluid (Beckman Readysafe) and counting was carried out for 5 minutes using a liquid scintillation counter (LKB 1219).

6.2.5.Polyacrylamide Gel Electrophoresis (PAGE)

PAGE was used throughout the study to determine the purity, distribution and apparent molecular weights of various samples. The inclusion of SDS makes it possible to separate polypeptides on the basis of their molecular weights.

Proteins treated in the presence of SDS and a reducing agent such as 2-mercaptoethanol (to reduce disulphide linkages) are effectively denatured to form negatively charged, rod shaped, SDS polypeptide complexes. The amount of SDS bound per unit mass of protein has been found in most cases to be constant and the bound SDS apparently masks the original charge of the protein. Hence, during electrophoresis all the SDS polypeptide complexes migrate towards the positive pole, with about the same charge to mass ratio, migration of the SDS complexes depending almost exclusively on their size, the polymerized acrylamide acting as a sieve.

In this study, SDS PAGE was performed as described by Laemmli (1970) with slight modification.

Preparation of Protein Samples for Electrophoresis.

It was often necessary to concentrate protein samples

too dilute for immediate electrophoretic analysis. The sample proteins were precipitated with an equal volume of trichloroacetic acid (TCA) in ice for 30 min. This was followed by centrifugation at 10,000 g for 5 min. Repeated washing of the proteins using ethanol:ether (1:1 v/v) removed the TCA. TCA precipitates were then heated for 3 min. at 100^{o}C in the presence of 0.4 M Tris-HCl buffer pH 6.8 containing 1.25% SDS and 2% 2-mercaptoethanol. The samples were cooled and a drop of glycerol containing bromophenol blue was added. The samples were now ready for electrophoresis.

Preparation of Gels.

Unless stated otherwise, the stacking and separating gels were 5% and 12% respectively and were prepared from a stock solution of 30% w/v acrylamide and 0.8% w/v N,N-methylenebisacrylamide . The final concentrations in the separating gel solutions were as follows: 12% acrylamide, 0.1% N,N-methylenebisacrylamide, 0.1% w/v SDS , 0.03% v/v Temed (N,N,N,N,-tetramethylethylenediamine), 0.03% w/v ammonium persulphate and 0.4M Tris-HCl buffer pH 8.8. The stacking gel solution contained 5% acrylamide, 0.1% N,N-methylenebisacrylamide, 0.1% w/v SDS , 0.05% v/v Temed, 0.05% w/v ammonium persulphate and 0.04M Tris-HCl buffer pH 6.8.

30 ml of the separating gel was poured into the slab gel casts (200 x 160 mm), care being taken to prevent the formation of air bubbles. A few drops of water were gently overlaid on the gel mixture before polymerization of the separating gel to give a smooth interface. 10 ml of the stacking gel was poured on top of the polymerized separating gel after draining off the water. Gels were used within a few hours of polymerization. Slabs were loaded into the electrophoresis tank (Bio-Rad, Protean 11 Slab Cell) and this was filled with reservoir buffer which consisted of 0.38M glycine, 0.01% SDS and 0.05M Tris-HCl pH 6.8, pH 8.3. The protein samples were applied to the gel, the maximum volume being 100 μl per well. Electrophoresis was performed at 25 mA until the samples reached the separating gel, after which the current was increased to 50 mA. The gels were removed when the tracker dye, bromophenol blue reached to within 1 cm of the end of the slab. The gels were stained as below.

Staining and Destaining.

Gels were placed directly into staining solution containing 0.2% w/v Coomassie Brilliant Blue R250 in methanol, acetic acid and distilled water (50:40:10). The gels were left in the stain for 1 h after which they were placed in destaining solution: distilled water, acetic acid and methanol (65:5:30). Repeated washings were carried out

until the protein bands became obvious against a clear background.

Molecular Weight Markers.

Protein standards were obtained in kit forms from Sigma (SDS-7) and consisted of bovine serum albumin (66,000 D), ovalbumin (45,000 D), glyceraldehyde 3- phosphate dehydrogenase (36,000 D), carbonic anhydrase (29,000 D), trypsinogen (24,000 D), trypsin inhibitor (20,100 D) and a lactalbumin (14,200 D). A second kit (BDH-Electran,44223) consisted of ovotransferrin (78,000 D), bovine serum albumin (66,000 D), ovalbumin (45,000 D), chymotrypsinogen (25,700 D), myoglobin (17,200 D) and cytochrome c (12,300 D).

6.2.6.Silver Staining.

Gels which had already been stained with Coomassie Blue could also be stained with a much more sensitive silver stain (Amersham silver stain kit) though gels were no longer suitable for photography after this procedure.

6.2.7. Autoradiography of SDS PAGE gels.

SDS PAGE gels were dried down onto a piece of filter paper using a gel slab drier (Pharmacia GSD-4), each gel

taking approximately 2 h. The dried gel was then placed into an X-ray cassette (Kodak) and overlaid with Kodak X-omat film (200 x 250 mm). The sealed cassette was left for three days and the film then developed.

6.3.RESULTS.

6.3.1.Separation of Cell Populations.

Attempts to separate the phagocytes from the eleocytes using centrifugation met with little success. It appears that the cells have very similar densities. Although the eleocytes sometimes appear to be more fragile than the phagocytes their responses to various agents such as detergents and sonication were similar to those of the phagocytes. It was therefore decided that the coelomocyte population would be examined as a whole and any results would have to be considered as an effect of both types of cells and not simply due to the phagocytes.

6.3.2.Examination of the Culture Media for the Presence of Agglutinin Molecules.

It was found that 100 μl of the incubation media, on 10 fold concentration from 30 ml to 3 ml, gave low haemagglutination titers for sheep red blood cells, ranging from 4-16. SDS PAGE was performed on 1 ml samples of the

media (after TCA concentration) to determine the molecules responsible for this activity. It was found that the media contained many proteins which are normally contained in coelomic fluid (Plate 7), the most obvious bands appearing in the regions corresponding to the 20,000 D agglutinin and the 45,000 D haemolysin (Lane 2). A large number of faint bands were also present and these appeared to be, for the most part, proteins with molecular weights greater than 45,000 D. Control media from cells treated in cycloheximide gave no bands suggesting that the results observed were not due to incomplete washing off of coelomic fluid from the cells (Lane 3). It would therefore appear that the agglutinin and several other proteins are released from the coelomocytes (both the phagocytes and eleocytes) and since cycloheximide inhibits protein synthesis it may be that these cells are responsible for the manufacture as well as the release of these molecules.

6.3.3.Viability of the Cells After Incubation.

Random counts of sets of fifty cells were performed under the light microscope. It was found that no more than 5% of the cells took up the toluidine blue stain, and so the extent of the survival of these cells during the experiment was good. Viability of cells was not affected by cycloheximide.

Plate 7.

SDS PAGE of Incubation Media from Eisenia foetida coelomocytes (Lane 1 -molecular weight markers; lane 2 -released proteins; Lane 3 -control; Lane 4 -pure 20,000 D agglutinin).

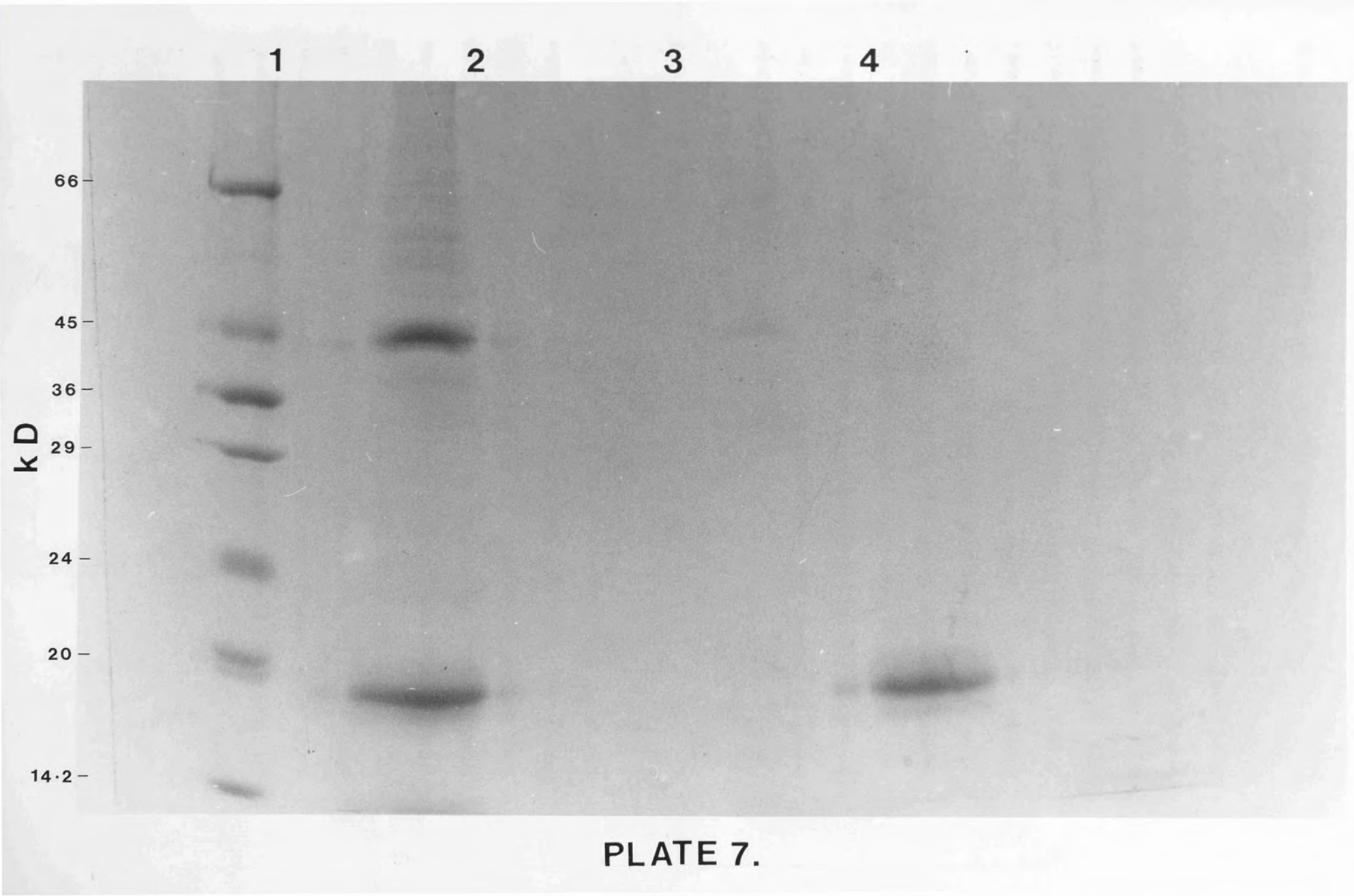


PLATE 7.

6.3.4. Production of Radiolabelled Proteins.

Results of the scintillation counting of the fractions obtained from the Sep-Pak are shown in Fig. 10. The first peak is likely to correspond to excess radiolabelled ^{35}-S methionine as SDS PAGE did not reveal the presence of any protein bands. SDS PAGE of the other smaller peaks showed the presence of two close and very faint bands corresponding to approximate molecular weights of just above 66,000. Silver staining did not show the occurrence of any other proteins. Radiography of the gels did not reveal the presence of any incorporated radiolabel anywhere on the gel and so it is unlikely that any proteins were synthesized by the coelomocytes.

6.4. DISCUSSION.

It would appear that Eisenia coelomocytes are able to secrete small but measurable amounts of various proteins, including the 20,000 D agglutinin. The detected agglutinin activity is unlikely to result from ruptured coelomocytes as cells are shown for the most part to remain viable throughout the incubation period, also, the control cells do not reveal the presence of any proteins on SDS PAGE gels. Lieppe and Renwrantz (1988) have shown that the haemocytes of Mytilus edulis also release a variety of substances in a concentration detected by Coomassie Blue

Fig.10.

Scintillation Count of Samples of Eisenia foetida Coelomocyte Incubation Media Eluted From Sep-Pak Cartridges.

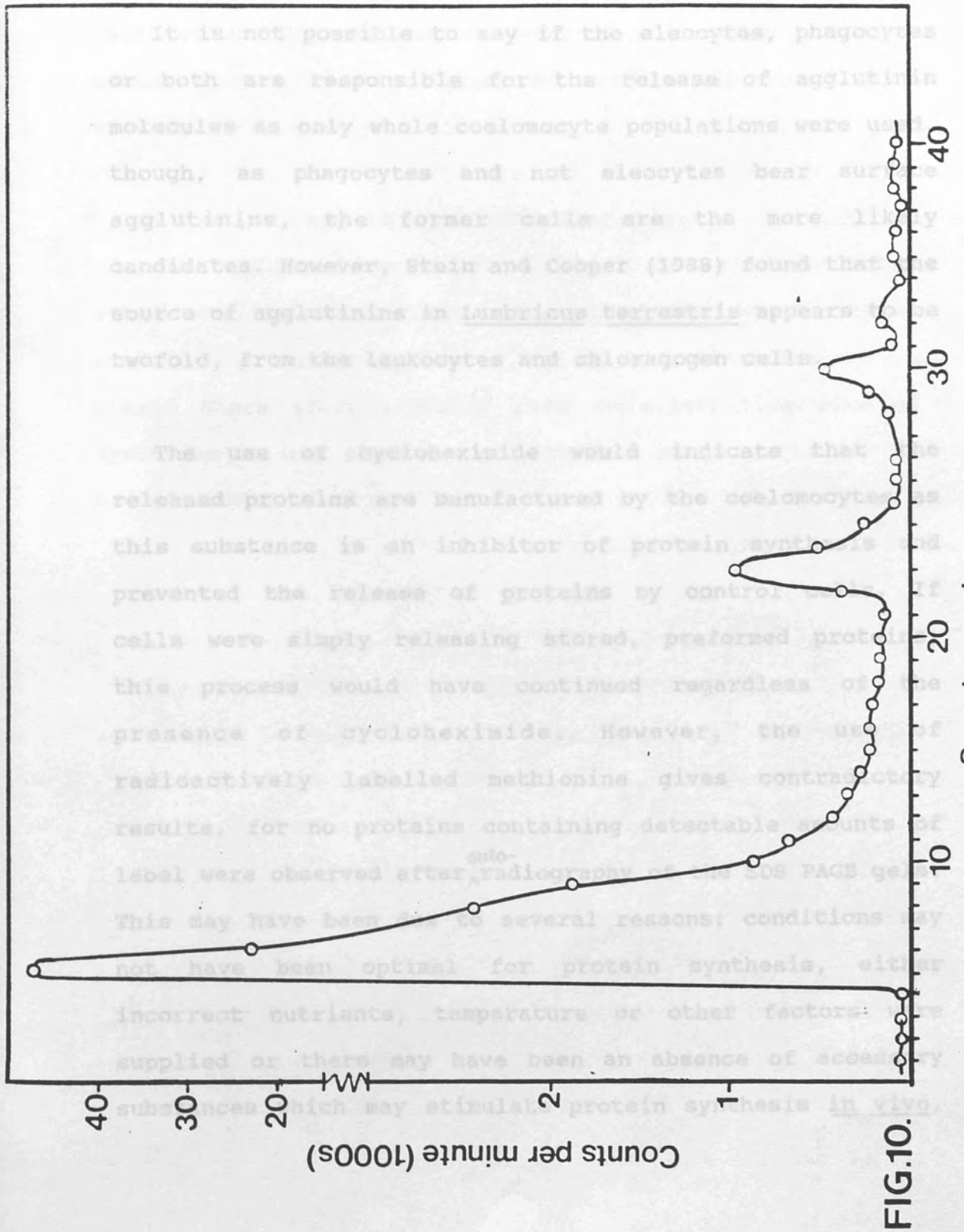


FIG.10.

staining of PAGE gels. The biochemical nature of these molecules still remains to be characterized.

It is not possible to say if the eleocytes, phagocytes or both are responsible for the release of agglutinin molecules as only whole coelomocyte populations were used, though, as phagocytes and not eleocytes bear surface agglutinins, the former cells are the more likely candidates. However, Stein and Cooper (1988) found that the source of agglutinins in Lumbricus terrestris appears to be twofold, from the leukocytes and chloragogen cells.

The use of cycloheximide would indicate that the released proteins are manufactured by the coelomocytes as this substance is an inhibitor of protein synthesis and prevented the release of proteins by control cells. If cells were simply releasing stored, preformed proteins, this process would have continued regardless of the presence of cycloheximide. However, the use of radioactively labelled methionine gives contradictory results, for no proteins containing detectable amounts of label were observed after autoradiography of the SDS PAGE gels. This may have been due to several reasons: conditions may not have been optimal for protein synthesis, either incorrect nutrients, temperature or other factors were supplied or there may have been an absence of accessory substances which may stimulate protein synthesis in vivo,

or, the released proteins may contain quantities of methionine which are so small as to be undetectable by the techniques used. This is unlikely as many proteins were released by the coelomocytes, some of which were likely to contain reasonable quantities of methionine, though none was shown to be radiolabelled.

So far, it may be concluded that Eisenia coelomocytes contain agglutinating molecules which are released along with a variety of other molecules in culture. Whether these proteins, particularly the 20,000 D agglutinin are merely stored there after transfer from an alternative site of synthesis or are actually manufactured on site remains to be determined.

7.GENERAL DISCUSSION.

This study has demonstrated the existence of an opsonin in the coelomic fluid of Eisenia foetida which is responsible for a pronounced increase in phagocytosis of yeast and bacterial cells by coelomic phagocytes. Although it has been shown in a number of invertebrates that a factor in the haemolymph is opsonic, with the exception of two studies, the recognition molecules involved have not been elucidated, though there is much support for the involvement of agglutinin molecules in this process.

As discussed in section 1, and proposed by Renwrantz (1983), recognition of foreign particles may be mediated by agglutinins in a combination of one or more different pathways:

1) By direct binding of surface agglutinins of foreign particles to carbohydrates on the surface of haemocytes or coelomocytes. This proposal is feasible as many microorganisms are known to possess surface agglutinin molecules. This does not explain how the process of opsonization can occur.

2) By humoral agglutinins which act as opsonins and link the carbohydrate determinants of the foreign body to opsonin receptors on the haemocytes or coelomocytes.

Although opsonic activity and humoral agglutinins are present in Eisenia, this study has shown that the 20,000 D agglutinin at least, is unlikely to be involved in this opsonic process.

3) By direct binding of foreign particles to membrane bound agglutinins. This may be feasible as agglutinin molecules have been demonstrated on the membranes of phagocytes of Eisenia foetida and the same molecules are capable of agglutinating microorganisms. Such a proposal may account for the fact that Eisenia phagocytes are also capable of ingesting particles in the absence of coelomic fluid opsonins. In vivo secretion of humoral agglutinins in the coelom may clump foreign particles and localize them for ingestion by the phagocytes.

However, although recognition of foreigness may occur as in (3) above, the phagocytes have not been shown to be responsible for agglutinin synthesis despite the observation that they, or the eleocytes, secrete the 20,000 D agglutinin. It remains to be determined whether the agglutinins are synthesized by or transported to the coelomocytes. It has been shown that the eleocytes are capable of forming secretory rosettes with vertebrate erythrocytes (Lassegues et al,1986), suggesting that they release haemagglutinins. Therefore the possibility exists that these cells are the site of synthesis of the agglutinin molecules which are then transported to the

phagocytes. In the insect Hyalophora cecropia, Yeaton (1980) demonstrated that granulocytes synthesize agglutinins while immunochemical staining of the phagocytic plasmatocytes demonstrated agglutinins only on the cell membrane. In Lumbricus terrestris, Cooper and Stein (1988) found that the source of agglutinins appears to be twofold, from the leukocytes and the chloragogen cells and this may also be the case in Eisenia foetida.

However, the presence of agglutinin molecules within the phagocytes and on the cell membrane may simply be the result of ingestion of old or damaged eleocytes by the phagocytes and the agglutinins may have no direct relationship to defence. As eleocytes do not show 20,000 D agglutinins on their surface it is perhaps unlikely that they are the site of synthesis, or even secretion, of these molecules. The observation that eleocytes in Eisenia form secretory rosettes with vertebrate erythrocytes could be explained by the possible release of agglutinins other than the 20,000D molecule.

It can be seen that, in Eisenia, several questions remain unanswered. Two obvious ones demanding attention : 1) Which cells are responsible for agglutinin synthesis, 2) Do different cells manufacture different agglutinins? Radiolabelling, perhaps with different isotopes, may solve these problems. Furthermore, although opsonic activity was

found to be present, the molecules involved remain to be characterized and the biological significance of this activity, particularly as phagocytosis can occur independently of humoral factors, determined.

Because of their ability to agglutinate cells, lectins have lent themselves readily to speculation as to whether they are primitive recognition molecules operative in defence. There are very few well substantiated cases where lectins have been demonstrated to function as recognition molecules and to play a part in internal defence mechanisms. Also, the importance of these molecules in this role under natural conditions remains uncertain. Considering the diversity of invertebrates it would perhaps not be surprising to find a diversity in their recognition molecules.

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