THE INTERACTION OF THE VARIANT SURFACE GLYCOPROTEIN OF *TRYPANOSOMA BRUCEI* WITH LIPID MONOLAYERS

by

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Summary

The bloodstream form of Trypanosoma brucei is completely covered by a thick surface coat which is composed of molecules of variant-specific surface glycoproteins (VSGs). An understanding of the mode of attachment of VSG to the plasma membrane is important in the search for new treatments against this parasite.

The use of lipid monolayers with measurement of surface pressure, surface potential and surface radioactivity show that there is a weak interaction between water-soluble VSG and phosphatidyl choline or phosphatidyl ethanolamine, and no interaction with sphingomyelin.

The interaction of VSG with sterols, however, is significantly stronger than for phospholipids as estimated by changes in surface pressure using either force-area curves or film penetration experiments. The order of the VSG-lipid interaction is as follows: Cholesterol>ergosterol>phospholipids>sphingomyelin.

The preferential interaction of VSG with sterols appears to show a certain degree of chemical structure recognition as deduced from studies using cholesterol derivatives, and competition with filipin for cholesterol. Thus substitution of the 3β-OH group of cholesterol by either negatively charged groups or neutral groups inhibit the film penetration, whilst positively charged substituents maintain or enhance the film penetration.

Dissociation of VSG with sodium cholate results in a strong interaction of VSG with phosphatidyl choline and spingomyelin: this does not happen with cholesterol. This suggests that dissociation of VSG into monomers is a necessary pre-requisite for film penetration.

Studies of the VSG-lipid interaction using liposomes support the finding of a weak interaction of VSG with phospholipids as determined with the monolayer technique. It is suggested that net positive charges, or intrinsic proteins, or both, are necessary on the parasite surface to stabilize the binding of VSG to the plasma membrane.

Preface and declaration

This dissertation describes investigations related to the biochemical and biophysical properties of the interaction of the variant specific surface antigen from *Trypanosoma brucei brucei* with various lipids. The experimental work was carried out during the period March 1979 to September 1982 in the Molteno Institute, Medical Research Council, Biochemical Parasitology Unit, University of Cambridge.

I am deeply grateful to my supervisor Dr R.A. Klein for his advice, encouragement, much instruction and helpful discussion, and his patience. I would also like to acknowledge the helpful discussions with, and advice received from Dr M.J. Turner of this Institute, as well as those with Austin Amadife during the writing of this thesis. Technical advice from Esther Chcolun and Judith Angus are also acknowledged with thanks. Many other people, at one time or another, have provided practical help and advice and I should like to thank all of them.

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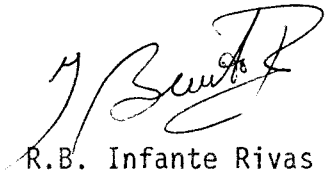
Many of the sterol derivatives used in this thesis were synthesized in this laboratory by Dr R.A. Klein. Details of these syntheses will be published shortly.

Part of this thesis has already been presented, and the abstracts published in proceedings of the following scientific conferences:-

1. Turner, M.J., Infante, R.B. & Klein, R.A. (1980) "Mode of attachment of variant specific antigen to the plasma membrane of Trypanosoma brucei". Proceedings of the Third European Multicolloquium of Parasitology (EMOP): Colloquium C5, 67, (1980).
2. Klein, R.A., Turner, M.J. & Infante, R.B. (1980) "The interaction of variant specific antigen from Trypanosoma brucei with lipid monolayers". Parasitology 81, xxvii.
3. Infante, R.B. & Klein, R.A. (1982) "Structure-activity relationship (SAR) for the interaction of cholesterol with Trypanosoma brucei". Parasitology 85, 11A.

This thesis is all my own work and has not been presented in whole or in part for any other degree or diploma.



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Abbreviation list:

VSG:	variant surface glycoprotein
VSA:	variant surface antigen
cDNA:	complementary deoxyribonucleic acid
SDS:	sodium dodecyl sulphate
BSA:	bovine serum albumin
CHL:	cholesterol
DLPC:	dilauroylphosphatidyl choline
DMPC:	dimyristoylphosphatidyl choline
DPPC:	dipalmitoylphosphatidyl choline
DSPC:	distearoylphosphatidyl choline
DOPC:	dioleoylphosphatidyl choline
PC:	phosphatidyl choline
ERG:	ergosterol
SDS-PAGE:	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SPC:	soybean phosphatidyl choline
EYPC:	egg yolk phosphatidyl choline
SPH:	sphingomyelin
mV:	millivolts
PE:	phosphatidyl ethanolamine
CFP:	capacity for film penetration
π-A:	force-area
$\Delta\pi$-A:	surface potential-area

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CHAPTER 1

AFRICAN TRYPANOSOMIASIS

CHAPTER 1

AFRICAN TRYPANOSOMIASIS

1.1 Introduction

The following section is devoted to a brief description of the basic general biology and pathology of the pathogenic haemoflagellate protozoa known as Trypanosomes. African trypanosomiasis is one of the parasitic diseases prevalent in Africa. The causative agent is a protozoan of the Genus Trypanosoma which infects both man and animals. The disease is transmitted by the tsetse fly (Glossina spp.). When feeding on the blood of the mammalian host the fly introduces the parasite into the bloodstream where it lives and multiplies. Trypanosomes in a later stage invade the central nervous system, and reproduction of the organism in the cerebrospinal fluid causes damage to the brain leading to the coma (this stage gives rise to the common name of 'sleeping sickness'). Trypanosomes survive the action of the host immune system by undergoing antigenic variation (Vickerman, 1978). Sleeping sickness is considered as a major health problem in Africa where about 35 million people and 25 million cattle are exposed to the risk of infection (WHO, 1979). For these reasons this disease was incorporated into the World Health Organization (WHO) Special Programme for Research and Training in Tropical Diseases.

1.2 Classification of trypanosomes

Table 1.1 shows the systematic taxonomy of the Genus Trypanosoma. The trypanosomatids of major importance in human disease include the African trypanosomes of which Trypanosoma brucei is the representative example. The south and central American trypanosomes are typified by T.cruzi and several species of Leishmania. Thus the member of this

Family kinetoplastidae are probably all parasitic with the exception of the free living Bodonidae which are non-parasitic.

Classification of the species of the Genus trypanosoma is complex, thus necessitating further sub-classification. This is due to the relatively few criteria for identifying trypanosome species. For example, the size and shape can be measured. Similarly size and position of nucleus and kinetoplast, development of the undulating membrane and flagellum are morphological parameters which may be determined. Biological critera such as site and stages of the development of parasites in the insect host are also considered. Very important are the clinical symptoms of virulence and epidemiology of trypanosomiasi for classifying the type of trypanosomes.

A summary for the classification of the mammalian trypanosomes, with special emphasis on African species follows:

Section Stercoraria

1. Subgenus Megatrypanum

Trypanosoma (Megatrypanum) theileri: it infects bovids and antelopes.

2. Subgenus Herpetosoma

Trypanosoma (Herpetosoma) lewisi: its common host the laboratory rat. This species is found in some parts of South America. Another important species (non-African) is T.(H.) rangeli which infect man as well as dogs, cats and monkeys is found in South America.

3. Subgenus Schizotrypanum

Trypanosoma (Schizotrypanum) vespertilionis infects rats. A very important non-African species is T.(S.)cruzi, the major causative agent of Chaga's disease or American trypanosomiasis: trypanosomes of this species infect man and other mammals.

4. Subgenus Pycnomonas

Trypanosoma (Pycnomona) suis infects wild and domestic pigs.

The existence of many subgenera of trypanosomes was simplified by the introduction of two major divisions, Stercoraria and Salivaria (Hoare, 1967, see Table 1.1). The characteristic of each group is as follows. Trypanosomes of the Stercoraria undergo a complex cycle of development in the insect-vector. The infective stage (metacyclic form) develops in the hind-gut (posterior station) and is carried with the faeces. When the insect feeds on the blood of the mammalian host, it deposits the infected faeces on the abraded skin. The parasites penetrate actively through this region.

For trypanosomes of the Salivaria the initial stage of its complex life cycle takes place in the insect mid-gut. The parasites migrate to the mouth parts (anterior station) and the infective stages develop in the proboscis or salivary glands. Thus the transmission of parasites to the mammalian host occurs by direct inoculation.

In general citation of species can be done without the subgeneric name but observing always Genus and species, i.e. Trypanosoma (Duttonella) vivax can be abbreviated to T.(D.)vivax or T.vivax.

Stercoraria contain three Subgenera of importance with infectivity to man and animals. These species are less widespread in Africa than those of Salivaria.

In general the Salivarian trypanosomes are confined to Africa; exceptions are T.(T.)evansi, T.(D.)vivax and T.(T.)equiperdum which are also found in Central and South America.

5. Subgenus trypanozoon

This subgenus is considered by Hoare (1970) as the most homogenous group of the Salivarian trypanosomes. The constituent species are

morphologically indistinguishable but can be differentiated by epidemiology, host range and virulence characteristics.

Two groups can be separated by whether they are cyclically, or non-cyclically transmitted. The species which are cyclically transmitted include T.(T.) brucei brucei, T.(T.) brucei rhodesiense and T.(T.) brucei gambiense. The last two are regarded as subspecies of the first. The non-cyclically transmitted species are made up of T.(T.)evansi which is transmitted by direct inoculation by a biting fly (Diptera spp.); T.(T.) equiperdum which is transmitted by direct contact with the mammalian host.

Most of the research on variant antigens has been conducted on T.brucei. T.brucei brucei has been however, more intensively investigated than the other two sub-species. T.b.brucei infects dogs, cats, horses, cattle and other domestic animals. This sub-species infects and adapts easily to laboratory rodents, producing high parasitaemias. Trypanosomes of this sub-species are not infectious to humans probably because they are lysed by high density lipoproteins in human serum (Rifkin, 1978). Parasites of this species infecting the mammalian host are localized mainly in the bloodstream. They can adopt various forms during their infective stages, a process known as pleiomorphism. Two peculiar forms are generally observed, (i) long slender trypanosomes (LS forms, mean lengths: 23-30 μ) with long free flagella, well-developed undulating membranes and an elongated nucleus. This form is present in all the sub-species. (ii) Short stumpy trypanosomes (SS forms, mean lengths: 17-22 μ) usually without free flagella with well developed undulating membranes and rounded nuclei. The frequency of this form fluctuates during the course of the infection and may disappear after prolonged mechanical passage in laboratory animals.

Human African trypanosomiasis (sleeping sickness) is caused by both T.brucei gambiense and T.brucei rhodesiense. The virulence of infectivity can be used to differentiate these sub-species from their presumed ancestral species, T.brucei; otherwise they are morphologically indistinguishable. The distribution of sleeping sickness and its causative agents is restricted to the tropical region lying between 20^{o}N and 20^{o}S of the Equator. Trypanosomiasis produced by T.b.gambiense is endemic throughout the region from West Africa to East Africa. The disease caused by T.b.rhodesiense is restricted to East Africa and is prevalent in Tanzania, Malawi, Zambia, Zimbawe and Mozambique.

Trypanosomes pathogenic to man are transmitted by various species of Glossina spp. in which the parasite undergoes a complex life cycle. Glossina spp. is in general resistant to infection by T.b.gambiense and T.b.rhodesiense (Baker, 1974). In addition to the difference in geographical distribution between these two sub-species of T.brucei, they can be differentiated according to their virulence. Thus the acute symptoms of sleeping sickness are associated with infections due to T.b.rhodesiense whilst a mild and relatively chronic disease is characteristic of T.b.gambiense infections.

1.3 The life cycle of T.b.brucei

The tsetse fly ingests parasites when taking blood from an infected animal. The complex life cycle which starts in the insect can be considered in three stages. First there is the establishment and multiplication within the endoperitrophic cavity of the mid-gut. Secondly the colonization of the ectoperitrophic space and the proventriculus. The third and final stage is the invasion of the salivary glands (Vickerman (1965). In the insect mid-gut the parasites differentiate

into procyclic forms. These procyclic forms are not infectious to the animal host. They lack a surface coat which they develop only after migrating to the insect salivary gland. They differentiate into metacyclic forms. Figure 1.1 shows a diagrammatic representation of the life cycle of T.b.brucei. The metacyclic trypanosomes are infectious to animals, resemble the animal bloodstream form and possess a surface coat which is formed by a variant surface glycoprotein, as will be described in Chapter 2. This is the key antigenic glycoprotein which enables the parasite to evade the host immune system (Le Ray et al., 1978). When an infected fly bites another animal, the parasites present in the saliva are infected into the blood where they rapidly develop into long slender (LS) bloodstream forms. These cells divide rapidly by binary fission, alter their metabolic pathways, and continue to express variant surface glycoproteins. The differences in infectivity accompanying trypanosome pleiomorphism correlate with changes in the mitochondrial metabolism. The short stumpy trypanosome (SS forms) activates the repressed single mitochondrion. These cells actively respire using a cyanide-sensitive electron transport system and they have Krebs cycle enzymes (Vickerman, 1971; Bienen et al., 1981). Thus they become adapted to life in the tsetse fly, where proline and other amino acids replace glucose as the principal source of energy. In contrast, the long slender trypanosomes (LS forms) have completely suppressed many mitochondrial functions. Their mitochondria show very few cristaes and a reduce volume. LS forms are completely deficient in cytochromes and Krebs cycle enzymes, and glycolysis becomes their principal mechanism of ATP-generating (Hill, 1976).

During the course of the infection the LS forms differentiate further into short stumpy bloodstream forms. The non-dividing short

Fig. 1.1

Diagrammatic representation of the life cycle of T.b.brucei. Trypanosome forms possessing a surface coat (outlined by broken lines) can undergo antigenic variation. After Vickerman (1971).

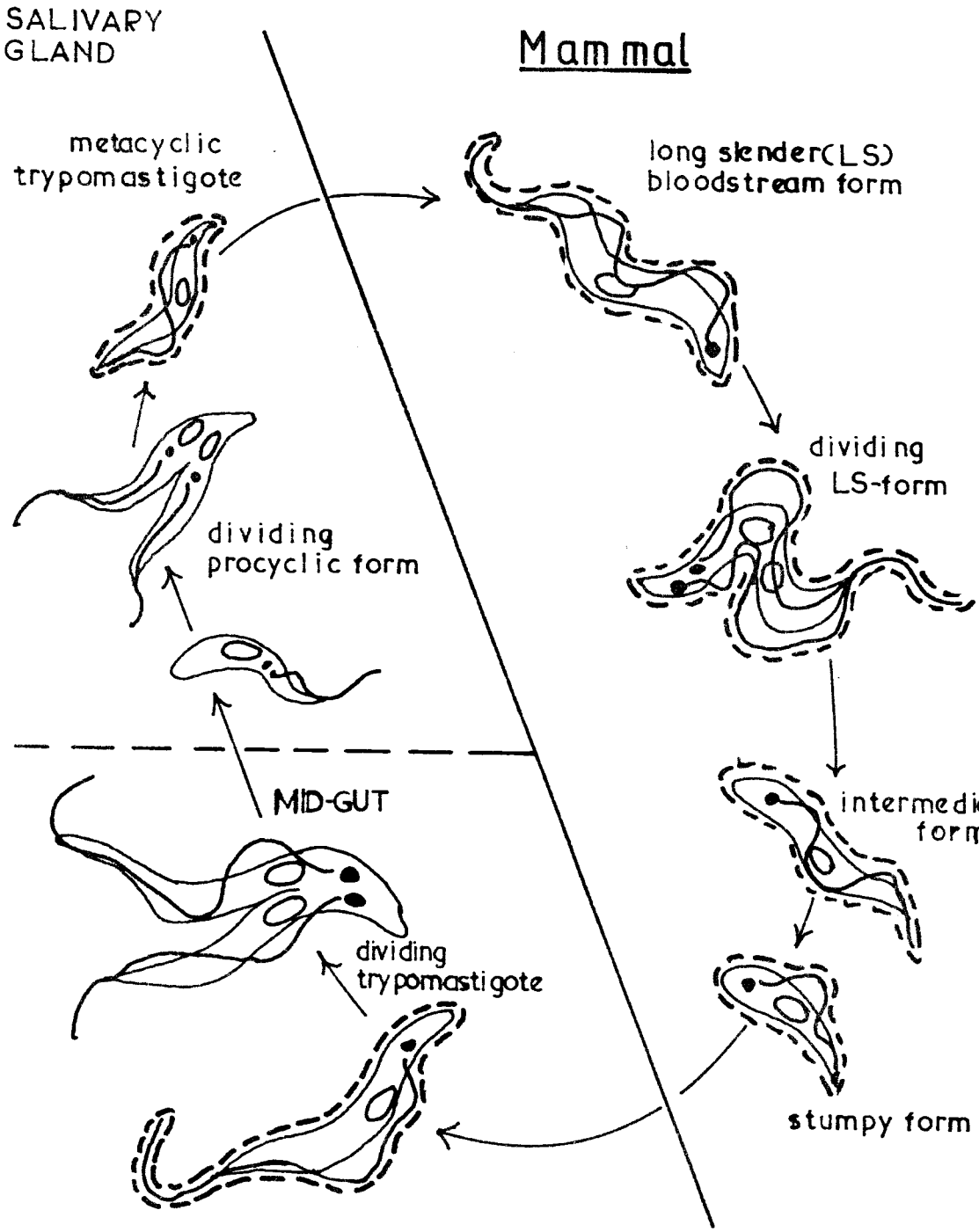
SALIVARY GLAND
Mammal
metacyclic trypomastigote
long slender(LS) bloodstream form
dividing LS-form
dividing procyclic form
MID-GUT
intermedi
form
dividing trypomastigote
stumpy form
Tsetse Fly

FIGURE 1.1

stumpy trypanosome and those of intermediate morphology are only slightly infective to other mammals by syringe transfer, but if ingested by the tsetse fly will initiate the cycle in the vector (Wijers & Willet, 1960; Vickerman, 1965, 1971). The correlation of the trypanosome pleiomorphism with the difference in infectivity is better illustrated by some monomorphic strains of T.b.brucei kept in laboratory animals. These strains, consisting exclusively of long slender trypanosomes, produce very high parasitaemias and usually kill the host within a few days (Ashcroft, 1960). They do not generate a significant number of stumpy forms and they hardly infect the tsetse fly (Fairbain & Culwick, 1947).

1.4 Host-parasite relationship

The gradation of virulence in human trypanosomiasis allows one to distinguish between acute sleeping sickness and the chronic disease. The virulence of the disease is dependent on the relationship between the parasite and the host. An overwhelming parasitaemia may kill the host in a short time whereas an effective antibody response may bring about complete cure (Ormerod, 1970). Since the presence of other mammalian hosts in the same geographical area, and the insect vector, are dependent on forest, water, temperature and various other factors, the influence of these environmental variables on the host-parasite relationship has to be taken in consideration. These may favour the selection of a group from a population of trypanosomes.

The distinction between trypanosomiasis due to T.b.rhodesiense and T.b.gambiense has been described in Section 1.2. It is, however, not always possible to distinguish between these on totally pathological grounds. For instance, the detailed pathological examination and morbid anatomical findings do not conform to a single pattern for each

type but cut across identifiable barriers. As an illustration, perivascular Cuffing (vessels surrounded by lymphocyte cells in the brain) is almost absent in the acute epidemic Rhodesian disease but is very common in the chronic Gambian disease. T.b.rhodesiense described in the Zambesi basin, however, has shown a high density of perivascular cuffing (Koten & de Raadt, 1959; Ormerod & Venkatesan, 1971).

The maximum infective dose for man is about 300-450 metacyclic trypanosomes (Fairbain & Burtt, 1946). A chancre is formed at the site of inoculation on the host's skin after the fly bite. Microscopically, perivascular infiltrates of small round cells are identifiable. The multiplying trypanosomes in the skin thus become a continuous source of antigen (De Raadt, 1974). Thus immunocomplexes may be formed where the antigens are deposited and hypersensitivity with increased vascular permeability results (Gell & Coombs, 1968; Morrison et al., 1979). Trypanosomes from the chancre invade the local lymph vessels and the bloodstream forms may invade a wide range of other tissues. During the third week of the infection high levels of parasitaemia may be detected and trypanosomes can readily be found in fluid taken from the peritoneal, thoracic and pericardial cavities (Morrison et al., 1979). Despite the widespread distribution of the parasites, however, there appears to be some predilection for certain tissues such as the heart, the central nervous system, skeletal muscle and the pituitary gland which are severely damaged (Ikede & Losos, 1972; Morrison et al., 1979; Valli et al., 1979). Anaemia is also a characteristic sign of T.b.brucei infections. The cause is unknown. Herbert & Inglis (1973) suggested that by adsorbing antigen on the erythrocyte surface, these may be haemolysed by immune reactions.

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The production of biologically active substances by either dead or living trypanosomes can infuse red cells. A haemolytic factor has been found; it is a heat-stable protein with a molecular weight of 10000 which has been found to be produced by T.b.brucei and its subspecies (Huan et al., 1978; Morrison et al., 1979). The presence of permeability and inflammatory factors have been demonstrated in association with infections by T.b.gambiense and T.b.congolense (Seed, 1969; Tizard et al., 1979). In addition, lysis of trypanosomes in vitro release active phospholipase A1 which acts on phosphatidyl choline and generates free fatty acids (Tizard et al., 1979). It is proposed that this process occurs in vivo following immune cytolysis. Although the free fatty acids cause haemolysis in vitro, probably its activity in vivo is blocked by serum albumin. Linoleic acid is a potent immunosuppressive agent. Therefore, trypanosomal phospholipase and its products are capable of inducing a number of lesions and may contribute to the pathogenic mechanism in this disease.

Table 1.1 Systematic taxonomy of the Genus Trypanosoma

Phylum	Protozoa	
Subphylum	Sarcomastigophora	
Superclass	Mastigophora	
Class	Zoomastigophora	
Order	Kinetoplastida	
Suborder	Trypanosomatina	
Family	Trypanosomatidae	
Genus	Trypanosoma	
Subgenera	Herpetosoma	Stercoraria†
	Megatrypanum	Stercoraria†
	Duttonella	Salivaria†
	Nannomonas	Salivaria†
	Trypanozoon	Salivaria†
	Pycnomonas	Salivaria†

† Divisions introduced by Hoare (1967).

CHAPTER 2

THE VARIANT SURFACE GLYCOPROTEIN

CHAPTER 2

THE VARIANT SURFACE GLYCOPROTEIN

2.1 Variant surface antigen of Trypanosoma brucei brucei

Bloodstream forms of T.brucei brucei and its sub-species can survive the attacks of the immune response of the mammalian host and establish a chronic infection. The process by which these organisms avoid complete elimination is due to antigenic variation. This mechanism results in the appearance of antigenically distinct populations of trypanosomes in successive waves of parasitaemia (Ritz, 1916; Gray, 1962). For instance, at 5-7 day intervals during the course of infection specific antibodies capable of agglutinating, or lysing these parasites are detectable in the host's serum. Subsequently the parasitaemia falls rapidly and trypanosomes are scarcely seen in the bloodstream. The population of trypanosomes, however, is not completely eliminated and the survivors of this crisis now bearing an altered antigenic type multiply and cause a relapse. This cycle of recrudescense and remission can continue, apparently indefinitely, until the death of the host. The number of antigens which a single trypanosome can produce is unknown, but is large and of the order of 100 (Lourie & O'Connor, 1937; Gray, 1965; Capbern et al., 1977).

Antigenic variation has only been demonstrated in species of salivarian trypanosomes such as T.(T.)brucei brucei and its sub-species T.(T.)evansi, T.(T.)equiperdum; T.(N.)congolense, and T.(D.)vivax. Stercorarian trypanosomes employ a different mechanism for evading the host's immune response. For example T.cruzi adopt an intracellular form to avoid the immune system of the mammalian host.

Excellent detailed discussion of the mechanisms and course of antigenic variations in various species of Salivarian trypanosomes has been given in recent reviews (Turner, 1982; Englund et al., 1982) and only that for T.brucei will be discussed here. This is discussed in the following sections within the context of the molecular structure of VSG and its organization as the trypanosomes surface coat.

2.2 Mechanism of antigenic variation

Bloodstream forms of T.b.brucei are covered by a surface coat which covers the entire plasma membrane of the body and flagellum (Vickerman, 1969). This coat, with a uniform thickness 12-15 nm of electron dense material also found on the infective metacyclic forms from the tsetse salivary glands, but it was absent from the procyclic forms present in the fly midgut. The importance of the cost to the process of antigenic variation was revealed by staining with ferritin-labelled, variant-specific antibodies (Vickerman & Luckins, 1969). Biochemical studies have shown that it is made up of molecules of a unique polypeptide of molecular weight 60-65,000 (Cross, 1975). This polypeptide which is probably the only protein exposed on the outside of the cell surface, carries all the antigenic determinants (Cross & Johnson, 1976).

Experiments using hyperimmune sera raised against purified intact antigens only show recognition of the homologous trypanosome population by agglutination and immunofluoresence reactions (Cross, 1975; Doyle et al., 1980). The cross-reactivity among various antigens purified from different clones was only detectable by radioimmunoassay (Barbet & McGuire, 1978). Thus, ^{125}I-radiolabelled antigen binds to both homologous and heterologous antibodies. Binding of heterologous antibody

by ^{125}I-labelled antigens could be inhibited by any unlabelled antigen tested, whereas homologous interaction of antigen and antibody could only be inhibited by the homologous antigen. It can be concluded that all the antigens share at least a common antigenic determinant. The ineffective inhibition of homologous antibody and antigen binding by heterologous antigens suggests, however, that the number of common determinants is small in comparison to the total number of specific determinants. On the other hand on the surface coat of trypanosomes a very restricted number of antigenic determinants are exposed on the variable surface antigens (Pearson et al., 1981).

The growing investigation of the surface antigens of the salivarian trypanosomes prompted the development of a nomenclature to describe antigenic variation (Lumsden, 1978). Variant antigen type (VAT) refers to trypanosomes expressing a particular antigen; the antigen type usually refers to the specific variant surface glycoprotein (VSG). For example VAT_{221} defined as the clone MITat 1.2 defines the source, the place and the re-clone number. Thus MIT means Molteno Institute Trypanozoon, "at" is antigen type, and 1.2 is the re-clone 2 of serodeme 1 of T.b.brucei (see Doyle et al., 1980 and Section 4.1.4).

Various VATs, all derived from a single trypanosome clone, form a serodeme. It has proved difficult to predict a definite number of VATs within a serodeme. Capbern (1977) determined 101 distinctive VATs after establishing a clone from a single trypanosome of T.(T.)-equiperdum and the repertoire has not been exhausted yet. Although different serodemes usually have different repertoires, related VATs are occasionally found in different serodemes (Vervoort et al., 1981). These VATs are known as isotypes (iso-VATs).

Current studies are centred on two questions. First, how is antigenic variation induced and what is the rôle of the immune response? Second, is the sequence of antigen expression constant within a clone and does reversion to a fixed starting VAT occur during cyclical transmission.

Since the discovery of antigenic variation in T.b.brucei, several hypotheses have been proposed to explain its biological mechanism. Ritz (1916) concluded that antigenic variation was due to the influences of host's antibodies and this was supported by several later observations. Gray (1965) and Vickerman (1969) assumed that the replacement of the surface coat was due to an organized process programmed in the genetic constitution of the cell. De Raad (1974), however, questioned this hypothesis on the grounds that it could not explain why a few individuals in each population succeed in using their genetically fixed device of evading the defence mechanism of the host. He proposed the hypothesis of biological competition amongst different variants within each trypanosome population. More explicitly, trypanosomes of any such variant could multiply or reappear continuously while remaining consistently suppressed in numbers by the more virulent, predominant variant. When the predominant variant is eliminated by the host's antibodies then the next most virulent variant in turn becomes predominant in the next population. A third hypothesis was based entirely on random mutation and selection. According to Watkins (1964), mutation would take place independently of external stimuli.

Doubts have been cast on the hypothesis of mutations as an explanation of antigenic variation by experiments which showed that individual infections initiated by the same trypanosome strain tended to produce recognizable variants in a similar sequence (Gray, 1962). Recent studies

using populations of trypanosomes derived from a single trypanosome show that a clone is capable of expressing a large number of antigens, somewhere between one and several hundreds (Capbern et al., 1977).

The hypothesis based on the influence of the host's antibodies has now been discarded because of the following results. Doyle et al. (1980) provided the first evidence that antigenic variation can occur in the absence of anti-variant antibodies when clones of bloodstream forms of T.b.brucei were isolated and maintained under in vitro culture conditions. Eighteen clones of a variable antigen type 052 were derived and maintained in vitro for periods of up to 60 days. The appearance of one or more variable antigen types was detected in 9 clones over a range of 14 to 46 days after initiation of the clone.

The detection of variant antigens during the early stages of infection, before an immune response has developed, and in immunosuppressed animals also proved that host's antibodies are not inducers of this process (Hajduck & Vickerman, 1981; Jenny, 1977; Van Meirvenne et al., 1975). It is likely that antibody plays a selective rather than an inductive role in antigenic variation. The series of experiments by McNeillage and colleagues (1969) showed that some VATs may differ in virulence according to their proportion in the first relapsed population. Although these results are from non-cloned trypanosomes, later experiments using an unrelapsed clone population of trypanosomes demonstrated its heterogeneity with respect to VAT (Van Meirvenne and co-workers, 1975). Minor VATs (heterotypes) were present in addition to the major VAT (homotype) and once the parasitaemia reached its peak any one heterotype could become the homotype of a subsequent population.

Finally the hypothesis of biological competition amongst different variants is contradicted by the same finding that several variants may

be detected in a trypanosome population obtained from a single cell.

In relation to the question of a possible sequence of antigen expression, early studies by Gray (1965) suggested a strong tendency for variant antigens to appear in the same order in different infections initiated with homologous trypanosome populations (Fig. 2.1). Furthermore if any variant was recycled through the tsetse the whole sequence would start all over again and the basic antigen 'A' always came first. Doyle (1977), however, showed that a homogeneous variant at an intermediate point in the putative gene sequence while changing predominantly to F also gave rise to a proportion of variant D (see Fig. 2.1). In addition the relapsed population from D contained variable numbers of trypanosomes which were unrecognized by antisera to variants D, E or F. This result suggests that antigens are not expressed in predetermined sequence (see Cross, 1978). It is important to mention that a more precise description of trypanosome infections was made possible by the technique of immunofluorescence and complement-dependent immune lysis in conjunction with the availability of mono-specific antisera (Van Meirvenne et al., 1975; Englund, 1982).

Furthermore the discovery that metacyclic forms are not of a single antigenic type, but instead are heterogeneous, makes the sequence shown in Fig. 2.1 no longer representative of the real process (Le Ray et al., 1978; Vickerman, 1978; Vickerman et al., 1980). These authors found three important properties of metacyclic forms. First, the metacyclics are of heterogenous antigenic types. Although it is not yet known how many different metacyclic VATs are in the salivary glands of the fly, three different VATs make up about 40% of the metacyclics. Second, a comparison of metacyclics from different serodemes revealed that each has a distinct VAT repertoire. Finally, the relative proportion of the

Figure 2·1

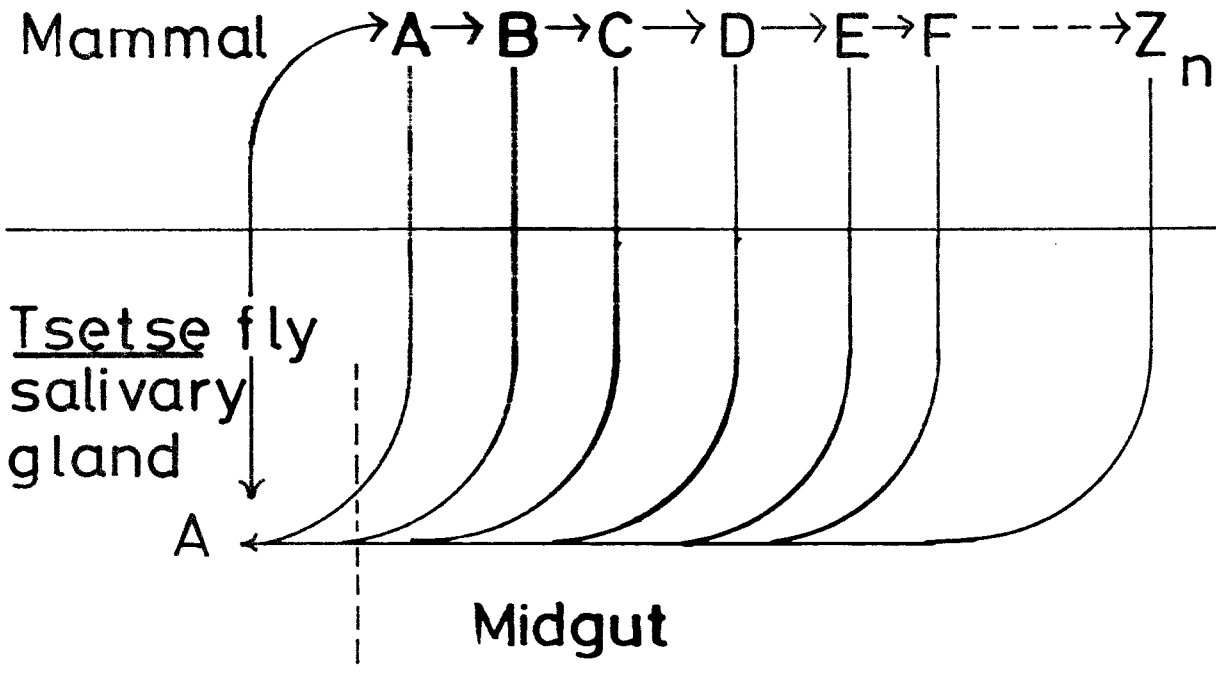


Fig. 2.1

Sequential expression of variant antigens in the bloodstream form. Variant antigens are absent from midgut or *in vitro* culture (25°C) stages. After Gray (1965).

three metacyclic VATs always was the same independent of the VAT used to infect the tsetse (Hajduk et al., 1981). Despite the non-existence of a basic antigen type these results suggest that a characteristic repertoire of metacyclic VATs exist. Ten days after a tsetse bite, VAT heterogeneity is detected in the first parasitaemia. These VATs are the same as the infecting metacyclics and begin to disappear about 5 days after infection (Barry et al., 1979; Englund et al., 1982). At this stage the use of parasite clones has been extremely useful in the study of the appearance of VATs. The parasitaemia after injecting a single trypanosome consists of a single major VAT (the homotype) which is approximately 99% of the population (Cross, 1975). This has simplified the preparation of monospecific antisera and the isolation of pure VSG before any heterotype reaches significant proportions.

2.3 Possible role of genetic mechanisms in antigenic variation

During the last three years the use of modern methods of recombinant DNA technology in analysing the genetic basis of antigenic variation has provided important information. Two possible genetic mechanisms have been considered. First, the rearrangement of chromosomal sequences could create new VSG genes. This mechanism has been observed for the coding of immunoglobulins. Second, the presence of genes for all of the VSGs in the repertoire are located within the trypanosome's chromosome (Hoeijmakers, 1980). Only one of these genes is expressed at a time. Thus by suppressing the expression of one gene and activating that of another one would create antigenic variation.

Most of these studies rely on the production of VSG-specific complementary DNA (cDNA) clones, and their subsequent use to probe the structure of the genome. A detailed description of the methodology of the preparation of cDNA clones and hybridization techniques is beyond

the scope of this thesis. It can be summarised, however, as follows: cDNA is synthesized from a template of poly-adenylated mRNA purified from trypancsomes using the enzyme "reverse transcriptase". The cDNA is transformed to double stranded cDNA and inserted into a plasmid cloning vector contained in a bacteria. Colonies of bacteria are screened for the presence of variant specific sequences. Bacteria grown on mitrocellulose filters are lysed in situ under conditions in which DNA is bound to the filter. Radioactive probes prepared by synthesizing cDNA from mRNA of distinct variants are hybridized to replica filters. Colony lysates containing sequences common to various variants will hybridize to them (see Turner, 1982). Those containing variant specific sequences will hybridize only to the probe containing homologus mRNA. A cDNA probe sometimes hybridized to different sized genomic restriction fragments in different variants, indicating that a particular VSG gene may be located at different chromosomal sites in different VATs. This implies that genomic rearrangements could be responsible for antigenic variation (Williams et al., 1979; Hoeijmakers, 1980; Pays et al., 1981).

2.4 Variant surface glycoprotein

Early studies of antigenic variation showed that soluble antigens (exo-antigens) in the serum of rats infected with T.b.brucei exhibited properties similar to those of the variant antigens (Weitz, 1963). Vickerman & Luckins (1969) demonstrated by the use of variant specific ferritin-labelled antibody that variant-specific antigens were present in the surface coat of the trypanosome. Allsopp et al. (1971) provided the first evidence that these exo-antigens were identical to the variant-specific antigens found in the surface coat. The purification

of variant antigens, however, was only partially achieved using a combination of fractionation with ammonium sulphate and DEAE-Sephadex column chromatography (Seed, 1974). A 68-fold increase in biological activity was obtained with this method. Due to the presence of other protein contaminants from the host, however, it could not be said unequivocally that the precipitating antigen and the VATs in the surface coat were the same. It was Cross (1975) who first purified and characterized VATs from the trypanosome surface coat rather than from exo-antigens. Two types of experimental approach were used to identify the surface components of clones of T.b.brucei. First labelling of outside surface proteins with a radioactively labelled reagent was used to identify the protein component of the surface coat. Formylmethioninesulphone-methylphosphate (FMSMP) was the reagent mainly used, since it has the property of reacting with amino group forming a covalent linkage, and, under known conditions, does not penetrate the cell membrane. The second approach was to investigate which proteins were modified or destroyed following treatment of living trypanosomes with proteolytic enzymes where the surface coat was removed, but without disrupting the cell.

The procedure for purification and characterization of soluble surface glycoproteins is summarised below. Details can be found in Section 4.

A volume of $\sim$ 60 ml of 2.5 x 10^9 trypanosome per ml, at pH 7.6, was homogenized at high-speed in the presence of small glass beads at 4^{o}C. The homogenate was centrifuged at 15000 x g_{av} for 15 min and the supernatant centrifuged for 2 h at 165000 x g_{av} to sediment ribosomal particles. The proteins contained in this latter supernatant solution were desalted on a column of Sephadex G25 equilibrated with

10 mM sodium phosphate pH 8.0, and then separated on a DEAE-cellulose column equilibrated with the same buffer. The material eluted as an unretarded sharp peak was collected and dialysed against distilled water for 16 h before isoelectric focusing (1000 V for 44-64 h; pH range from 3 to 10). Finally the eluate was collected in 3 ml fractions with monitoring of the absorbance (280 nm), pH and radioactivity.

The peak of radiolabelled [^{35}S]FMSMP-protein was correlated with that of absorbance at 280 nm. Analysis of amino acid and sugar residues, as well as determination of molecular weight by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) led to the conclusion that this purified polypeptide was a glycoprotein of approximately mol. wt. 65000, containing around 600 amino acid and 20 monosaccharide residues. Furthermore, material released from the cell surface by trypsin or pronase treatment was structurally related to the corresponding purified FMSMP-labelled glycoprotein. This result demonstrated that this glycoprotein is the main, and probably the sole constituent of the trypanosome surface coat.

Immunochemical evidence for the identity of the purified cell surface polypeptide was obtained from experiments in which mice were immunized with purified glycoprotein and challenged with a small number of infective trypanosomes of homologous or heterologous type; the results indicated a complete protection against challenge by the homologous but not the heterologous variant (Cross, 1975).

2.4.1 Structure of variant surface glycoprotein

VSGs purified from seven clones of T.b.brucei showed different isoelectric points and different amino acid composition. Since these VSGs could be differentiated immunologically this is interpreted as a

reflexion of the variation in amino acid sequence (Cross, 1978). Bridgen et al. (1976) reported the N-terminal amino acid sequence of four VSGs isolated during a 3 week period from a rabbit infected with a clone of T.b.brucei (Table 2.1). These sequences show no homology within the first 30 amino acids suggesting that the antigenic diversity is primarily due to the variation in amino acid sequence. Whether this assumption may be extended to the rest of the sequence ($\sim$ 95%) is yet to be demonstrated.

As discussed by Turner (1982), on the basis of amino acid composition and fingerprint analyses, the unrelated VSGs might contain as much as 50% sequence homology scattered in small segments throughout the length of the molecule. The technique of tryptic mapping could hardly detect such homologies. Cross & Johnson (1976) confirmed the extreme structural variation using tryptic maps, originally reported by Le Page (1968).

Tryptic digestion of VSGs, however, resulted in an N-terminal fragment of molecular weight 40,000 to 50,000, and a C-terminal fragment usually in the range of 10,000 to 17,000 molecular weight (Johnson & Cross, 1979). These fragments are assumed to form two different structural domains with VSG. In comparison to the C-terminal regions the N-terminal regions of the native glycoproteins are resistant to proteolytic cleavage.

Holder and colleagues (1980) isolated tryptic and pronase glycopeptides from VSGs obtained from five variants of a single clone of T.b.brucei. The internal tryptic glycopeptides, containing only mannose and glucosamine in the carbohydrate moiety, were non-cross reacting in radioimmunoassay tests. The second group which included the C-terminal amino acid, containing galactose as well as mannose and

glucosamine, showed considerable size variation in the carbohydrate chain, and showed cross-reactivity in the radioimmunoassay tests. Amino acid sequences showed short homology regions amongst peptides of this second group in agreement with the cross-reacting tests. Interestingly examination of the complementary cDNA clone to one of these, the VSG_{117} peptide, confirmed the amino acid sequence as well as the structural sequence of the glycosylation site. This site has been found in all the C-terminal and internal VSG glycopeptides (Cross et al., 1980). Moreover the cDNA sequence predicts a C-terminal extension of 23 amino acids terminating in a hydrophobic tail of 15 amino acids (Fig. 2.2). This hydrophobic tail could penetrate the hydrophobic site of the lipid bilayers to anchor to the protein in the membrane (Holder et al., 1980; Cross et al., 1980).

Inspection of cDNA sequences for other VSGs suggests that the C-terminal hydrophobic tails may be a common structure on the initial translation product of those VSGs (Matthyssens et al., 1981; Rice-Ficht et al., 1981; Boothroyd et al., 1982).

In general the carbohydrate content varies from 7 to 17% in different VSGs (Johnson & Cross, 1977). Detailed analysis showed the presence of four sugars namely mannose, galactose, glucose and N-acetylglucosamine (Holder & Cross, 1981; Duvillier et al., 1983). The oligosaccharide was attached directly to the C-terminal residues. Depending on the VSG, this residue was serine or aspartic acid (Holder & Cross, 1981). Results of cDNA sequencing identified the aspartic acid site as the glycosylation site (Boothroyd et al., 1980). This latter differs from the established triplet Asx-X-Ser/thr, which is used as the signal sequence for glycosylation of asparagine through linkage with N-acetyl-glucosamine (see Turner, 1982).

Fig. 2.2

A partial DNA sequence of VSG_{117} and the predicted protein sequence representing the C-terminal region of the protein.

Figure 2·2

```
TGG GAA AAT AAT GCT TGC AAA GAT TCC TCT ATT CTA GTA ACC AAG AAA
Trp Glu  Asn Asn Ala Cys Lys Asp Ser Ser Ile Leu Val Thr Lys Lys

TTC GCC CTC ACC GTG GTT TCT GCT GCA TTT GTG GCC TTG CTT TTT TÂA
Phe Ala  Leu Thr Val Val Ser Ala Ala Phe Val Ala Leu Leu Phe
```

Barbet & McGuire (1978) showed that purified VSGs have cross-reaction determinants detectable only by radioimmunoassay but which were not demonstrable when intact trypanosomes were used. Barbet et al. (1979) reported that treatment of VSGs with trypsin did not inhibit cross-reaction; however, treatment with periodate for 24 hours at $4^{o}C$ abolished cross-reaction without affecting the homologous reaction. Periodate cleaves carbon-carbon bonds of 1,2 cis diols, commonly found in carbohydrates. The removal of carbohydrate was verified by the inability of the periodate-treated antigen to bind concanavalin A. It was inferred therefore that the antigenic cross-reacting site of VSG contained a carbohydrate. A definitive demonstration that carbohydrate was responsible for the cross-reaction came from Holder & Cross (1981). As discussed above C-terminal glycopeptides were prepared from VSG by enzymatic treatment with trypsin and pronase. Glycopeptides containing mannose and galactose acted as effective inhibitors of heterologous cross-reaction.

2.5 Organization of VSG in the surface coat

Early work demonstrated the presence of glucosamine, mannose and galactose in the fraction of specific variant antigen with a sedimentation coefficient of 45 (Allsopp et al., 1971; Wright & Hales, 1970). These authors also presented results from cytochemical staining techniques showing the presence of carbohydrates in the pellicle of bloodstream form of T.brucei. The use of lectin-binding techniques showed firstly that radiolabelled ConA did not bind to the trypanosome surface unless it was trypsinized; and secondly that the binding site of lectins was located close to the plasma membrane (Steiger, 1975; Cross & Johnson, 1976; Seed & Brindley, 1976). VSGs are susceptible

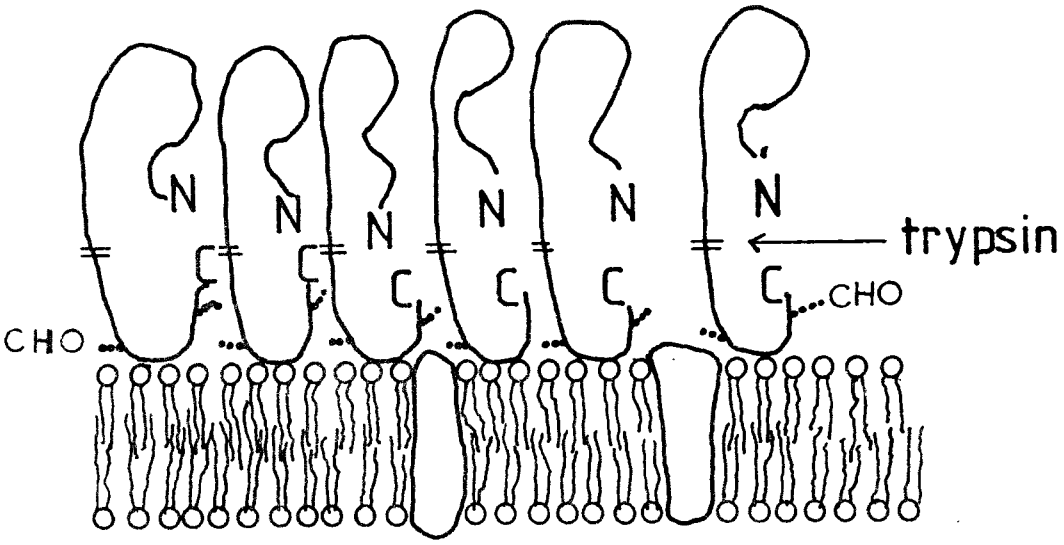
to proteolytic cleavage and this property was used to distinguish two distinct domains. Johnson & Cross (1979) using short trypsinization (5 minutes) cleaved the molecule of VSG into a large N-terminal fragment (48000 to 55000 molecular weight) and a shorter C-terminal fragment (10000 to 17000 molecular weight). As was discussed above the C-terminus contains the carbohydrate moieties which confer the cross-reacting properties observed for various VSGs (Barbet & McGuire (1978). The difference between these two domains was clearly established using competitive inhibition of immunoprecipitation. Fragments from the N-terminus could not inhibit immunoprecipitation due to the C-terminal fragments (Cross, 1979). The N-terminal region, which is highly variable in amino acid sequence, and which probably contains the antigenic determinants detectable on the living parasites, may be located at the exterior of the surface coat (Bridgen & Cross, 1976). Experiments using monoclonal antibodies showed high specificity for the immunizing antigen determined by radioimmunoassay and immunofluorescence. When tested against the surface of living trypanosomes, however, only 2 out of 10 of these monoclonal antibodies bound to the cell surface (Pearson et al., 1981). The implication of these results is that antigenic sites unique to each variant surface antigen are not necessarily exposed on the exterior of the surface coat. The presence of unexposed sites on the N-terminus of the VSG was revealed by immunofluorescence on acetone-fixed trypanosomes (Pearson et al., 1981; Lyon et al., 1981). Two interpretations relating to the unexposed sites can be advanced; (i) the packing of the molecules of VSGs prevent the expression of the antigenic site; and (ii) during the process of purification the conformational structure of the N-terminus

of VSG is altered (Turner, 1982). The available experimental evidence, however, does not conclusively support any of these propositions. That the surface coat has a tight packing is inferred from experiments in which concanavalin A could not reach the carbohydrate moiety unless the parasite was previously trypinized (Cross, 1979). Similar results were observed for specific antibodies against the C-terminus of VSG. Cross & Johnson (1976) using the results described above proposed a model with the C-terminus located at the membrane boundary with the surface coat, and the N-terminus oriented to the exterior of the surface coat (Fig. 2.3). Studies of amino acid composition of the C-terminal domain showed a polar component suggesting that an electrostatic charge may be one of the mechanisms of attachment to the plasma membrane lipids. These experiments involved the cleavage of five purified VSGs into large N-terminal and smaller C-terminal fragments, and the determination of the isoelectric points of these fragments. Variant 099 produced fragments with molecular weights of 48000 (f48), 40000 (f40) and 17000 (f17). Fragment f48 had an N-terminal amino acid sequence identical with that of the native VSG, and the sum of the amino acid composition of f(48) + f(17) corresponded to that of the intact VSG. f(17) contains the C-terminus of the molecule. Fragment f(40) was assumed to be a degradation product of f(48). The isoelectric points of the fragments f(48), f(40) and f(17) were 8.66, 8.20 and 5.67 respectively, whereas that of native VSG_{099} was 6.1. The discovery that loss of the surface coat makes these parasites carry a strong negative charge, led to the proposition that ionic interactions between lysine residues in the C-terminus of the VSG and a negatively charged receptor possibly a glycolipid, hold the VSG to the membrane (Cross, 1978). Fig. 2.3 shows two groups of carbohydrates attached to the molecule of VSG; one of them is at the C-terminus of the VSG and is

Fig. 2.3

Model for the arrangement of variant antigens at the surface of T.b.brucei. The C-terminus containing the two groups of carbohydrates (CHO) is located at the membrane boundary with the surface coat. After Cross & Johson (1976).

Figure 2.3



the external group responsible for heterologous cross-reactions. The location of the second glycosylation site is known for only two antigens. VSG shows this oligosaccharide located 50 amino acid residues from the C-terminus. For a VSG isolated from a clone of T.equiperdum, the attachment site of the mannose-rich oligosaccharide devoid of galactose was located in position 57 of the N-terminus (Boothroyd et al., 1980; Duvillier et al., 1983). Cross & Johnson (1976) suggested that the outer layer of carbohydrate seen by Wright & Hales (1970) might be found only in VSG containing relatively large amounts of carbohydrate. Other possibilities are that this outer layer represents the second glycosylation site and that the surface coat is formed by a bilayer of VSG (Turner, 1982).

The organization of VSG in the surface coat constitutes a structure more complex than that of a single monolayer of VSG. This observation arises from the finding that VSG in solution exists mainly as dimers with a low proportion of trimers and digomers (Auffret & Turner, 1981). The cross-linking of VSG in solution with bifunctional reagents such as dimethyl-suberimidate (DMS) shows that five of eight VSGs gave values above 80% cross-linking. This reagent causes cross-linking by coupling lysine residues which are close to each other (approximately 10Å). The products obtained after this treatment gave diffuse bands on SDS-PAGE, but the molecular weight range observed is consistent with the formation of dimers.

Treatment with sodium metaperiodate followed by reduction of the Schiff base with borohydride cross links carbohydrates with the ε-amino group of lysine. VSG in solution treated with these reagents produced dimers in low yield (25%). The C-terminal glycopeptide did not, however, show any detectable cross-linking. VSG in solution containing sodium cholate when treated with DMS also failed to show any cross-linking.

Table 2.1 N-terminal amino acid sequence of five variant antigens from T.brucei

Variant									10										20
99	thr.	ans.	asn.	his.	gly.	leu.	lys.	leu.	gln.	lys.	ala.	glu.	ala.	ile.	cys.	lys.	met.	cys.	lys.	glu.
49	ala.	lys.	glu.	ala.	leu.	glu.	tyr.	lys.	thr.	trp.	thr.	asn.	his.	cys.	gly.	leu.	ala.	ala.	thr.	leu.
48	thr.	asp.	lys.	gly.	ala.	ile.	lys.	phe.	glu.	thr.	trp.	glu.	pro.	leu.	gln.	leu.	leu.	thr.	gln.	asp.
55	ala.	glu.	ala.	lys.	ser.	asp.	thr.	ala.	ser.	gly.	ser.	val.	asn.	ser.	pro.	gln.	thr.	glu.	ala.	thr.
221	ala.	ala.	glu.	lys.	gly.	phe.	lys.	gln.	ala.	phe.	trp.	gln.	pro.	leu.	cys.	gln.	val.	ser.	glu.	glu.

																				30
	arg.	lys.	val.	ala.	thr.	gly.	val.	leu.	thr.	lys.	leu.	lys.	ser.	his.	ile.						
	phe.	gly.	asn.	leu.	tyr.	asn.	lys.	ala.	lys.	-.	-.	asn.	leu.	asp.							
	tyr.	-.	ala.	gln.	leu.	ala.	lys.	thr.	leu.	gln.	arg.	ala.	leu.	asp.							
	leu.	asp.	asx.	glx.	pro.	Lys.	gly.	ala.	leu.	phe.	thr.	leu.	glx.	ala.	ala.	(ala could be ser)					

Residues underlined are uncertain assignments (From Bridgen et al., 1976; and Johnson & Cross, 1979).

The presence of dimers on the cell surface, however, has not been shown. The observation of trimers and tetramers of VSG in untreated cells on polyacrylamide gels, may indicate a weak interaction between VSG molecules. Such an interaction is less stable than the association of VSGs as dimers in solution (Strickler & Patton, 1982).

2.5.1 Mode of attachment of VSG to cell surface membrane

The mode of attachment of VSG to the surface of trypanosome is still an open question. The bloodstream form of trypanosome is very motile and possesses rapid flagellar and body movements. Depletion of glucose, however, or under conditions in which the accumulation of pyruvate lowers the pH of the medium (to approximately 5) leads to partial or complete release of the surface coat. Disruption of cells by mechanical or osmotic treatment in the absence of detergent results in the release of about 80% of the VSG in a water soluble form (Cross, 1975).

Five possibilities for the mode of linkage between VSG and the plasma membrane can be considered. First, a hydrophobic peptide of VSG is inserted into the hydrophobic site of the lipid bilayer. Second, partial penetration of the outside monolayer of lipid by small hydrophobic peptides of VSG could occur. Third, an electrostatic interaction of VSG with the polar head groups of phospholipids could take place. Fourthly VSG could be covalently bound to a lipid molecule; and fifthly, few VSGs could be attached by hydrophobic peptides to the lipid membrane and hold the water soluble form of VSG by protein-protein interaction.

The first possibility is supported by the discovery of a hydrophobic tail in VSGs as predicted by cDNA sequence analysis (Mattysens

et al., 1981; Rice-Ficht et al., 1981; Boothroyd et al., 1982). The presence of a hydrophobic tail is characteristic of many integral membrane proteins e.g. glycophorin and cytochrome b5 (Tomita et al., 1978; Ozols & Gerard, 1977). Moreover, other strong evidence is provided by the identification of a membrane form of VSG (mfVSG) which behaves as an integral protein. This mfVSG differs both biochemically and immunochemically from the water-soluble form of VSG (de Almeida & Turner, 1983). mfVSG is enzymatically transformed into the water soluble VSG, but under conditions in which this modification does not take place, purified mfVSG has been shown to have amphiphilic properties and behaves as an integral protein as shown by the charge-shift electrophoresis.

The nature of the difference between water-soluble VSG and mfVSG is at present unknown. These results suggest the possibility of the presence of a hydrophobic C-terminus in mfVSG. Studies on the biosynthesis of VSG using pulse-labelling experiments suggest, however, that this hydrophobic tail is processed before VSG is expressed at the plasma membrane (McConnell et al., 1982). VSG would then be released by the action of a protease.

The second possibility for hydrophobic peptides of VSG being partially inserted into the outside monolayer would require short fragments of hydrophobic amino acid residues. The amino acid sequence of VSG_{117} shows a C-terminus with a low proportion of hydrophobic residues, rich in lysine and polar residues. There are, however, eight half-cystine residues involved in disulphide bonds with each other (Allen et al., 1982). Whether this tertiary structure of the C-terminus would provide 'loops' able to interact with the lipid bilayer has yet to be demonstrated. Experiments with apoproteins

suggests, however, that short sequences of polar and non-polar amino acid residues are not indispensable requisiter for strong protein-lipid interaction. The presence of amphipatic α-helices with polar and non-polar faces provides an appropriate mechanism, since the polar residues may interact with the polar groups of phospholipids whilst the non-polar side could bind to the hydrophobic region of the phospholipid bilayer (Segrest et al., 1977; Osborne et al., 1977). Figure 2.4 shows the amino acid sequence for the C-terminus of VSG_{117} represented in the model suggested by Fukushima et al. (1981). There are regions very rich in polar amino acid residues whilst hydrophobic regions are non-existent in this C-terminus (Allen et al., 1982).

The third possibility that due to electrostatic interaction, charged groups of the C-terminus would bind opposite charges in the polar groups of phospholipids is based on a model proposed by Cross (1978). A combination between the second and third possibilities cannot be excluded especially bearing in mind the limited information available concerning the C-terminus. The isoelectric points for two C-terminal glycopeptides suggest that these domains carry a net negative charge; and the amino acid sequences do not show any strikingly charged faces (Johnson & Cross, 1979; Cross et al., 1980; Boothroyd et al., 1980, 1982; Allen et al., 1982). The fourth possibility of VSG bound covalently to a lipid molecule is not supported experimentally. Gas chromatographic analysis of saponified VSG_{117} revealed no detectable fatty acids (Holder & Overath, unpublished).

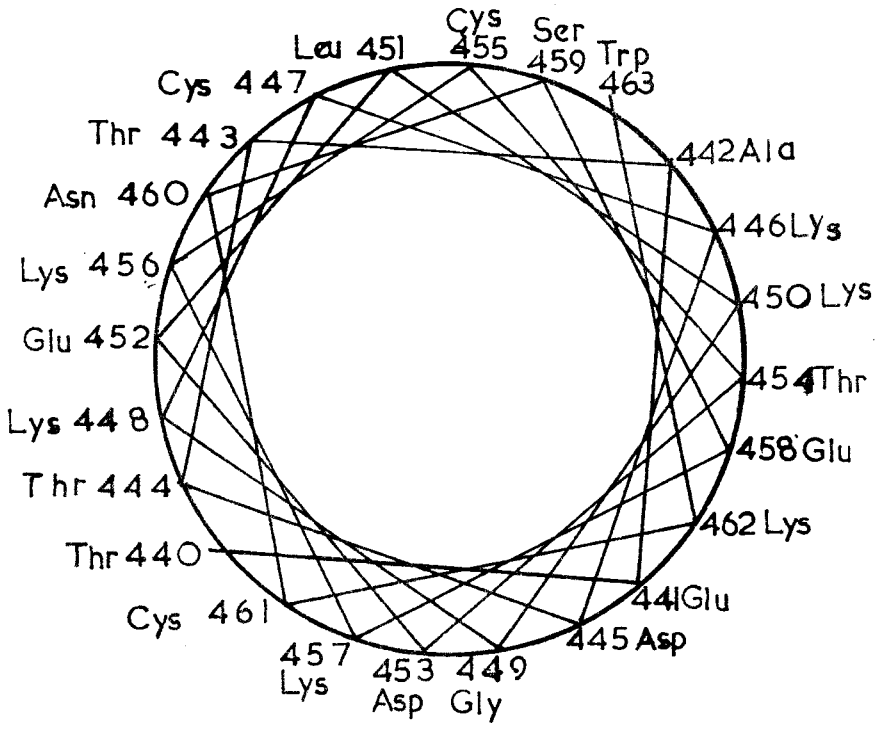
The fifth possibility could involve various forms of VSG. The existence of two types of VSG which differ in their possession of the cross-reacting determinant has been demonstrated by SDS-gel electrophoresis and charge-shift electrophoresis (Rovis et al., 1978; Richard

et al., 1981; de Almeida & Turner, 1983). In general a water-soluble VSG form is released from T.b.brucei and T.b.congolense by homogenization; and a second form of VSG behaving as an integral protein is released by detergent. Conceivably both types of VSG may be attached to the plasma membrane by different membrane-binding mechanisms. The VSG form released by detergent may have part of its C-terminus embedded in the lipid matrix, and be bound to water-soluble VSGs by non-covalent forces.

Fig. 2.4

Model for location of hydrophobic and polar regions. A partial C-terminal amino acid sequence of VSG_{117} is projected axially assuming an α-helix form using the model of Fukushima (1981). The amino acid sequence is that reported by Allen et al. (1982).

FIG. 2.4


Cys 455
Ser 459
Trp 463
442 Ala
446 Lys
450 Lys
454 Thr
458 Glu
462 Lys
441 Glu
445 Asp
449 Gly
453 Asp
457 Lys
461 Cys
440 Thr
444 Thr
448 Lys
452 Glu
456 Lys
460 Asn
443 Thr
447 Cys
451 Leu

CHAPTER 3

LIPID-PROTEIN INTERACTION

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3.1 Interaction of protein and lipid

The essential barrier to diffusion of small molecular weight substances at the cell surface is the plasma membrane. This can be found alone (erythrocytes) or forming a more complex cell-surface structure (intestinal epithelial cell) depending on the type of cell. In general membranes at the cell surface and within the cell are formed of both proteins and lipids. The relationship between protein and lipid varies according to the type of membrane and to some extent depends on the procedure chosen for its isolation. For erythrocyte membranes this ratio is 1:0.75 (Asworth & Green, 1966; Nelson, 1967), 1:3 for myelin membrane (Demel et al., 1973) and 1:0.3 for the purple membrane from Halobacterium halobium (Oesterhelt & Stoeckenius, 1971).

3.2 Membrane proteins

Membrane proteins are divided into two operational categories: peripheral or extrinsic, and integral or intrinsic (Singer & Nicolson, 1972). Peripheral proteins are those which can be removed by relatively gentle procedures, such as sonication or by changing the ionic strength of the medium. Those proteins which can only be removed by more drastic methods such as the use of detergents, organic solvents or chaotropic agents are often classified as integral proteins (Singer, 1974). This classification does not imply a molecular description of how these proteins are arranged in the membranes. Some proteins extend across the bilayer, for example glycophorin in the erythrocyte membrane contains an α-helical hydrophobic segment of 23 amino acid residues which span the lipid bilayer (Tomita & Marchesi, 1975). Some of the lipid-protein

association are schematically represented in Fig. 3.1. Proteins mediate most of the biochemical functions that are performed by membranes. These functional aspects include active and facilitated transport of small molecules through the membrane, enzymic activities, binding of hormones to the cell surface and the transduction of chemical signals across the membrane (Singer, 1971; Zwaal, 1978).

3.3 Membrane lipids

Membrane lipids are structurally organized in the form of a bilayer. This provides the structural framework of the membrane (Danielli & Dawson, 1956). The lipid fraction of most mammalian plasma membranes are mainly composed of phospholipids, cholesterol and to a smaller extent of glycolipids, usually gangliosides. Phosphatidyl choline and sphingomyelin account for a substantial fraction of the total membrane phospholipids, being 50-60% in membranes of erythrocytes (van Deenen & de Gier, 1974; Nelson, 1967), platelets (Marcus et al., 1969; Chap et al., 1977) and liver plasma membrane (Dorling & Le Page, 1973). These lipids are amphipatic molecules, having a hydrophilic (polar) and a hydrophobic (non-polar) end. In simple dispersions of membrane lipids in the presence of excess water the lipid molecules form closed structures of bilayer, burying their tails and leaving the polar groups exposed to water (Dervichian, 1964). Nuclear magnetic resonance (NMR) and electron spin resonance (ESR) techniques has been used to measure the motion of individual lipid molecules or their different parts (Chapman, 1973). Lateral diffusion of a lipid is rapid each molecule exchanging with its neighbour in the order of 10^{-7} seconds (Brulet & McConnell, 1975). In contrast, neighbouring exchange across the membrane takes about 11 days to accomplish (Kornberg & McConnell, 1971; Johnson et al., 1975).

Fig. 3.1

Schematic representation of proteins interacting with a phospholipid bilayer. The apolar areas of proteins and lipids are represented by heavier lining as compared to the polar areas. The lipid-protein associations such as those occurring between opposing charges of proteins and lipids or by bridging negative charges via a bivalent cation (Ca^{2+}) are shown. After Zwaal (1978).

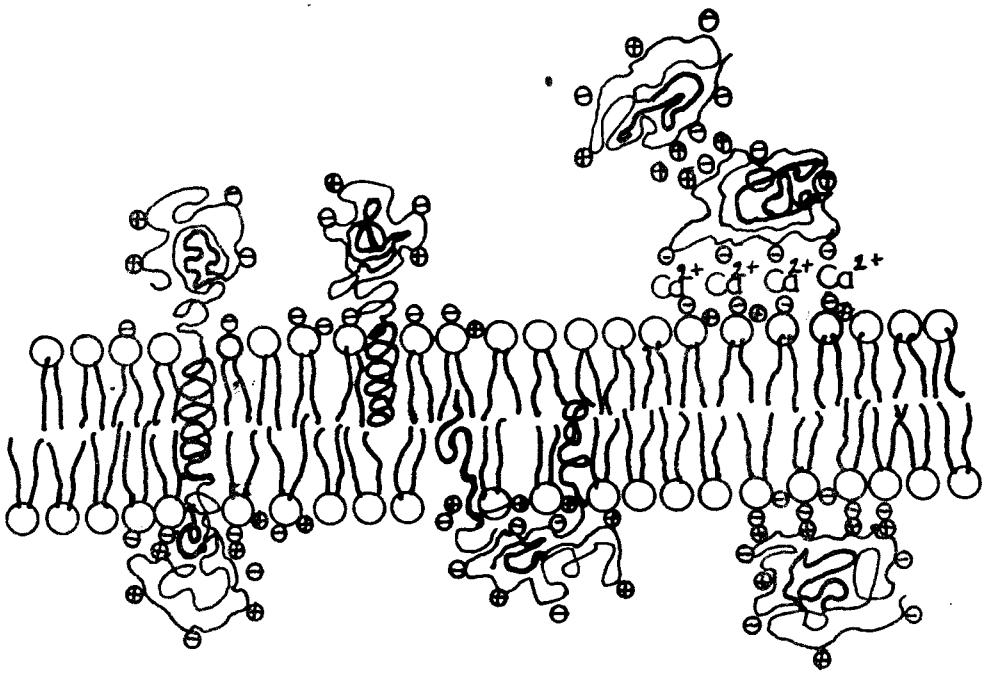


FIG. 3.1

Investigations with natural phospholipids have failed to detect the operation of a flip-flop mechanism in these systems (Rothman & Davidowicz, 1975; Tsai & Lenard, 1975). There is evidence, however, that it operates in synthetic analogues of phospholipids (McNamee & McConnell, 1973). Taken together, the above observations expose an apparent pitfall in extrapolating results from synthetic analogues to cover events taking place in an authentic membrane lipid system. That the mechanism is not observed in membrane lipids can be attributed in part to the high activation energy required to bring the polar group through the hydrocarbon core of the membrane bilayer (Singer & Nicolson, 1972). Another possibility is that in a natural membrane, trans-membrane movements can take place without the involvement of a flip-flop mechanism. Such a movement of phosphatidylcholine has been reported in whole erythrocytes (Renooij et al., 1974). Thus the half-time for the loss of radioisotopic asymmetry is only a few hours in these membranes. A similar mechanism has been found to occur very rapidly in growing bacterial membranes. In this system radioisotopic asymmetry introduced biosynthetically during growth is dissipated in about three minutes (Rothman & Kennedy, 1977).

From these studies, the question has been raised whether a trans-membrane protein might be involved. Such protein could provide a suitable intramembranous surface along which the polar head group of the phospholipid could be translocated through the hydrophobic domain of the membrane (Bretscher, 1973; Rothman & Lenard, 1977). The relation-ship between these mechanisms and lipid asymmetry is not discussed here. It has been extensively reviewed by Rothman & Lenard (1977) and Singer (1974). Lipid asymmetry is, however, briefly described. A non-random distribution of phospholipids between the outside and the inside of the

erythrocyte membrane was first proposed by Bretscher (1972) based on the observation that the relatively non-permeant label formyl-methioninesulphone methylphosphate (FMMP) reacted much more readily with phosphatidyl ethanolamine and phosphatidyl serine in red cell ghosts than in whole cells. He proposed that the phosphatidyl ethanolamine and phosphatidyl serine are predominantly located in the cytoplasmic monolayer, whereas sphingomyelin, phosphatidyl choline and glycolipids are found mainly in the external monolayer of human erythrocyte (Bretscher, 1972, 1973). Several objections that were initially raised to this conclusion were satisfactorily answered by subsequent work by other investigators (Whiteley & Berg, 1974; Bloj & Zilversmit, 1976).

Studies using highly purified phospholipases also favour the view that an asymmetric phospholipid distribution occurs in the erythrocyte membrane (Colley et al., 1973; Verkleij et al., 1973). In addition to lipid class asymmetry there also appears to be an unequal distribution of the acyl chains; this has been observed in cholinergic synaptic vesicles (Michaelson et al., 1983).

The fluidity of membrane lipids appears to correlate with biological functions such as sugar transport across the membrane. The plot of the logarithm of the rate of sugar transport versus the reciprocal of absolute temperature (Arrhenius plots) resulted in two changes in slope detected at 32.3^{o}C and 38^{o}C. The changes in the temperature melting of the membrane lipids were measured by electron spin resonance (ESR) introducing a spin label (2,2,6,6-tetramethylpiperidine-1-oxyl, TEMPO) in the cell membrane. An Arrhenius plot of the ESR parameter versus 1/T^{o}K resulted in two breaks in slope at 30.7^{o}C and 37.7^{o}C. Linden et al. (1973) has correlated these Arrhenius plots and suggested

that the transport rate behaves differently for a membrane that is "fluid", "solid" or "fluid + solid". The main objection to this conclusion comes from models for Arrhenius-type temperature dependence (see Klein, 1982). This model shows that the transition temperature does not always coincide with the break in the slope and depends upon the way in which the process partitions between the gel and liquid-crystalline phase. This is in agreement with the model proposed by Thilo et al. (1977). These authors proposed that for triphasic responses to be observed, accurate data over an appropriately large temperature range must be obtained. When the concept of Arrhenius plots is applied to biological systems, difficulties arise in extending the temperature range sufficiently. For example a broad transition temperature gives an Arrhenius plot having the form of a smooth curve which may be interpreted as two straight lines (Silvius & McElhaney, 1980; Klein, 1982). The temperature range in biological systems is restricted to around $40^{o}K$ by protein denaturation which occurs at high temperatures and by loss of activity at low temperatures.

The presence of intrinsic proteins within lipid bilayer structures has, however, been found to perturb the lipid environment. Various terms which have been used to describe this perturbed lipid such as: boundary-layer lipid (Jost et al., 1973); "halo" lipid (Träuble & Overath, 1973; Stier & Sackmann, 1973); and annulus lipid (Warren et al., 1975). It is assumed that a microclomain of lipid surrounding the hydrophobic intrinsic protein exists and separates the protein from the adjacent fluid bilayer regions. On the other hand Hesketh et al. (1976) using data derived from studies on the Ca^{2+} transport ATPase of the sarcoplasmic reticulum, observed that there is a single rigid phospholipid shell and no cholesterol around this protein. Hence these authors

argue that the rate of exchange between annular lipid and bulk lipid is slow even when the lipid matrix is fluid.

Extensive work has been done on rhodopsin and the boundary layer lipid effects associated with this protein. ESR was used to test the effects of adding rhodopsin to spin-labelled fatty acids (Pontus & Delmelle, 1975). These studies produced evidence for a rigid boundary layer around this hydrophobic protein. Hong & Hubbel (1972) using rhodopsin reincorporated into spin-labelled phospholipids found that the motions of the hydrocarbon chains was dependent on the lipid: protein ratio. Increasing the relative amount of protein brought about a decrease in the freedom of motion of the spin-labelled phospholipid.

Davoust et al. (1980) have carried out further studies on the mobility of the lipid chains which are in direct contact with rhodopsin. The use of spin-labelled rhodopsin reconstituted into phosphatidyl choline liposomes showed an ESR spectrum very similar to that obtained from a spin-labelled fatty acid diffusing freely in the lipid bilayer, i.e. a high degree of disorder is observed; spin-labelled fatty acid covalently linked to rhodopsin via a SH-group was used in these experiments (Davoust et al., 1979). The spectra of spin-labelled rhodopsin showed the presence of two components, one mobile and the other immobile, at least over the temperature range 10 to 20°C. Two explanations have been proposed for the presence of the immobile component. First is the possible formation of an immobilized boundary layer surrounding isolated proteins; the theoretical basis for this model exists in the literature (Owicki et al., 1978). Second, the immobilized component can reflect protein-protein contact, and is therefore due to labelled lipid chains trapped between clustered proteins. Similar models have been proposed for gramicidin A in lipid vesicles (Chapman et al., 1977).

Similar studies using fluorescent probes indicate that the motion of the probe molecule becomes inhibited as the protein concentration increases (Gómez-Fernández et al., 1979). This is taken to indicate that the fluidity of the lipid decreases.

Phospholipids do not undergo a direct transition on heating from gel to liquid crystalline. Various intermediate states between a crystal and a liquid are found to exist, and these have been designated mesomorphic or liquid crystalline or anisotropic liquid (see Williams & Chapman, 1972). The lipid hydrocarbon chains are converted from a relatively rigid, extended, largely all-trans conformation in the gel state to a more disordered state where the characteristics are a number of gauche conformation and largely increased rates of intra- and inter-molecular motions (Levine, 1972; Luzzati & Husson, 1962).

The variation of excess specific heat with temperature for the gel to liquid crystalline state, first order endothermic process can be determined by differential scanning calorimetry (Ladbrooke & Chapman, 1969). The phase transition temperature, usually denoted Tm or Tc, is that temperature at which the excess specific heat reaches a maximum.

The phase transition temperature of a phospholipid system is markedly dependent on the chain length and degree of saturation of the hydrocarbon side chains as well as on the nature of the polar head group. Thus a high transition temperature is found for phospholipids with long hydrocarbon chains as for example in dipalmitoyl phosphatidyl choline (DPPC, C16:0/C16:0) which shows a sharp transition temperature at 41^{o}C. Dilauroylphosphatidyl choline(DLPC, C12:0/C12:0) which has short acyl chains exhibits a transition at -1.8^{o}C (Mabrey & Sturtevant, 1976). The introduction of cis-double bonds into long hydrocarbon chains of phospholipids leads to a lowered of the transition temperature.

The nature of the polar head group is also important in determining the thermotropic behaviour of phospholipids containing identical fatty acyl chains (Williams & Chapman, 1972). Studies of various disaturated phosphatidyl ethanolamine (PE) derivatives using differential scanning calorimetry (DSC) has revealed transition temperatures which are always greater than for the corresponding disaturated phosphatidyl choline (PC) (Simon et al., 1975; Vaughan & Keough, 1974; Wilkinson & Nagle, 1981).

Lipid bilayers made up of a binary mixture of phospholipids exhibit phase diagrams which can range from those indicating nearly ideal mixing both in the liquid and gel state to those that are non-ideal and indicate immiscibility in the gel state (Phillips et al., 1970; Mabrey & Sturtevant, 1976). The disaturated PC derivatives dimysistoyl phosphatidyl choline (DMPC, C14:0/C14:0) and DPPC, which differ by only two carbons in the length of their acyl chains, exhibit almost ideal behaviour in binary phospholipid-water dispersions (Mabrey & Sturtevant, 1978). With a difference of four carbons in the chain length, isothermal melting of the shorter-chain PC is not detected. When the difference is six carbons however, the compounds show monotectic behaviour. This is observed with dilauroyl phosphatidylcholine and distearoyl phosphatidylcholine (DSPC, C18:0/C18:0).

A region of pronounced lateral phase separation of this binary mixture is detected by differential scanning calorimetry (Opden Kamp et al., 1975; Mabrey & Sturtevant, 1976; Phillips et al., 1970; van Dijck et al., 1977). Monotectic behaviour is also observed for the system dioleoyl phosphatidylcholine (DOPC, C18:1/C18:1), DMPC, DPPC and DSPC, thus indicating that di-cis-mono-unsaturated PC and disaturated PCs are largely immiscible in the gel state. Therefore it can be con-

cluded that in the liquid crystalline phase, binary mixtures of two different PCs can result in a homogeneous packing throughout the monolayer or bilayer and that lateral phase separation occurs when the temperature is gradually lowered. The component with the higher transition temperature begins to crystallize and clusters of like lipid molecules in the gel and in liquid-crystalline phases may occur (McElhaney, 1982; Phillips, 1972; Chapman et al., 1974; van Dijck et al., 1977). The effect of polar groups in phase separation is illustrated by binary mixtures of DMPC and DMPE which show non-ideal behaviour. The binary mixtures PC-PE although seem miscible in the liquid-crystalline phase, the solid curves of their phase diagrams showed a minimum at about 20 mol % implying gel state immiscibility (Chapman et al., 1974; Blume & Ackerman, 1974; Lee, 1977).

The occurrence of cholesterol and related sterols in biological membranes has attracted the attention of many investigators as to the role of cholesterol in controlling the fluidity of biological membranes (Demel & De Kruyff, 1976; Demel et al., 1972). The physicochemical state of phospholipids determines the interaction with cholesterol. When it is in the liquid-crystalline state cholesterol decreases the chain mobility, and also the mean molecular area; this is known as the condensing effect of cholesterol (Muller-Landau & Cadenhead, 1979). Conversely in the gel state cholesterol increases the fluidity of the hydrocarbon chains (liquifying effect) (Keough et al., 1973).

The packing of cholesterol and the hydrocarbon chains of phospholipids has been studied by direct measurement of surface pressures at the air-water interface. Some controversy, however, exists around the possible involvement of hydrogen bonding between the 3-β-hydroxyl group

(3-β-OH) of the sterol and the carbonyl oxygen or the phosphate group of phospholipids (Green et al., 1973; Brockerhoff, 1974). An extensive study on the structural properties of phospholipid and cholesterol molecules as well as the motional effects of cholesterol on its analogue on phospholipid bilayers support the preferential bonding between the 3-β-OH group of cholesterol and the carbony oxygen of the acyl chains in phospholipids (Huang, 1976). Differential scanning calorimetry studies have shown that when ideal mixing of PC species occurs cholesterol interacts randomly with them, but when the mixture of phospholipids show monotectic bahaviour cholesterol interacts preferentially with the species with the lowest transition temperature (de Kruyff et al., 1974). Although it has been established that the presence of increasing concentrations of cholesterol broadened the gel to liquid-crystalline phase transition of phospholipid and reduced the enthalpy of the transition (ΔH_{cal}), there is disagreement on the direction of the shift in the transition temperature and on the concentration of cholesterol necessary to completely abolish the transition. The sharp peak of the excess heat capacity versus temperature at the transition temperature of DPPC shifts slightly to lower temperatures as cholesterol is added and ΔH_{cal} decreases linearly with increasing cholesterol content. The initial value of ΔH_{cal} (8.5 Kcal/mol) falls to a minimum at about 20 mol % cholesterol, it may be interpreted as if cholesterol is not homogeneously distributed, see below. As then cholesterol content is increased above 20 mol % the ΔH_{cal} of the broad peak initially increases and then decreases with increasing cholesterol content, reaching close to zero at about 50 mol % cholesterol. Similar results have been reported for PE and spingomyelin (SPH) mixed with cholesterol (Mabrey et al., 1978; McElhaney, 1982). The appearance of both a sharp

and a broad transition at concentrations of cholesterol below 20 mol % is interpreted as a lateral phase separation into cholesterol-free and cholesterol-enriched domains (Snyder & Freyre, 1980).

Cholesterol may preferentially associate with a class of lipid when added to a mixture of phospholipids exhibiting gel phase immiscibility. Cholesterol interacted preferentially with the lower-melting lipid of a binary monotectic mixture of phospholipids as indicated by a decrease in ΔH_{cal} and a broadening of the phase transition of only one component (Mabrey et al., 1978); van Dijck et al., 1976). In other binary systems, however, cholesterol exhibited a preference for a particular phospholipid whether or not that lipid was the lower or higher-melting component. The order of preference of cholesterol was SPH>>PS≃PG>PC>>PE (Demel et al., 1977; Barenholtz, 1980; McElhaney, 1982; Yeagle et al., 1982).

3.4 Lipid specificity

Studies of some proteins requiring particular lipids in order to function, suggest specific recognition between protein and lipid molecules. Most of the proteins used were membrane enzymes (Hilden & Hokin, 1976; Gennis & Strominger, 1976; Vick & Capaldi, 1977). For example the Na^+/K^+-ATPases isolated from dogfish shark rectal salt gland, when reconstituted in phospholipid liposomes show the Na^+/K^+ transport activity fully restored (Hilden & Hokin, 1976). These enzyme preparations were infused and contained bound phospholipid and cholesterol in an apparent bilayer arrangement (Jorgensen, 1975). Complete removal of the lipids by treatment with deoxycholate inactivates the enzyme; subsequent recombination with specific phospholipids such as the acidic phospholipids, phosphatidyl serine or phosphatidyl glycerol re-activates its function (Dahl & Hokin, 1974).

C_{55}-isoprenoid alcohol phosphokinase was also found to have a preferential affinity for cardiolipin and phosphatidyl glycerol, but the lack of purity of the enzyme preparation did not allow further conclusions (Gennis & Stroninger, 1976).

There are few examples in which polar groups of phospholipids are related to enzymic activity. In these they act as 'allosteric effectors' by enhancing ligand binding to substrate or coenzyme, thereby increasing the enzyme activity (Grover et al., 1975). β-hydroxybutyrate dehydrogenase (BDH) and phosphatidyl choline provide an example where the cofactor, NADH, is not bound except in the presence of the lipid (Gazzotti et al., 1974). Phosphoenolpyruvate phosphotransferase from bacterial membranes requires phosphatidyl glycerol for optimal activity (Kundig & Roseman, 1971). Nevertheless, this lipid specificity is questioned by results in which structurally unrelated detergents were substituted for the different phospholipids. For example C_{55}-isoprenoid alcohol phosphokinase and pyruvate oxidase can be fully activated by detergents (Cunningham & Hager, 1971; Gennis & Jonas, 1977; Dean & Tanford, 1977).

There are a few non-enzymatic proteins which are known to show specific affinity for some lipids. Studies on the recombination of apo A-I of high density lipoprotein (HDL) with binary mixtures of phospholipids have demonstrated this protein to have a high affinity for DMPC (Swaney, 1980). Using film penetration experiments it has been shown that the myelin basic protein A_1 interacts strongly with cerebroside sulphate in preference to phosphatidyl serine, phosphatidyl ethanolamine and cholesterol (London et al., 1974). Similarly the band-3 protein, an integral protein of the human erythrocyte membrane involved in anion transport, showed a stronger interaction with cholesterol than with various phospholipids (Klappauf & Schubert, 1977;

Bretscher, 1971). The lipid affinity by non-enzymatic proteins can also be measured by other physical methods such as differential scanning calorimetry, ESR, NMR and fluorescence depolarization, which may detect conformational changes in the membrane (Chapman et al., 1979; McElhaney, 1982; Devaux, 1982).

3.5 The lipid monolayer model

Most of the lipids of structural importance in biological systems are amphiphilic molecules and contain a polar and a non-polar side. Lipids are usually soluble as monomers in water at very low concentrations. An increase in the lipid concentration induces the formation of lipid associates called micelles which remain dispersed in water. The concentration above which micelles are formed is called the critical micellar concentration (CMC). The CMC of phospholipids is extremely small, for example, DPPC in water has a CMC of 4.7×10^{-10}M (Smith & Tanford, 1972).

This general property of lipids put restrictions on the biochemical studies of lipid-protein interactions. Three main types of model system are generally used: lipid monolayers (Davies & Rideal, 1963; Gaines, 1964); liposomes (Bangham, 1968) and planar lipid bilayers (Mueller & Rudin, 1962.

3.5.1 Spread lipid films

It was as early as 1891 that a proper description of spread films at the air-water interface was first reported (Davies & Rideal, 1963). In 1913, however, Sir William Hardy proposed that monolayers are formed by molecules containing a hydrophobic and a hydrophilic side, and also that at the air-water interface the molecules may be oriented with the polar side buried in the water surface, whilst the hydrophobic part

would be orientated towards the air phase. Langmuir (1917) working on fatty acid monolayers gave experimental support to this hypothesis. He was the first to make an analysis of monolayer properties in terms of molecular structure and molecular orientation. Using a fatty acid of various chain lengths he arrived at an identical limiting area by compressing each monolayer at the air-water interface. From these results it was concluded that the molecules of the different fatty acids were identically oriented with respect to the surface.

3.5.2 General principles

Organic substances which are insoluble in water, spread on the water surface to form insoluble monolayers. Their structure and stability is governed by the balance of the forces of attraction and repulsion between the film-forming molecules, as well as betweem these molecules and the subphase molecules.

The thermodynamic definition of surface tension for a pure liquid is

$$\gamma = \left(\frac{\partial F}{\partial A}\right)_{T,\, V,\, n_i} \qquad 3.1$$

where F is the Helmholtz free energy of the system; A is the area of the surface; n_i the number of moles of component i which are kept constant. For a one component system γ is the isothermal reversible work done in extending the interface by unit area at constant volume (V) and n_i (Adamson, 1967).

The variation of surface tension with composition is formally described by the Gibbs adsorption equation which shows that positive adsorption decreases the surface tension (Section 5.4.1). The surface pressure or spreading pressure (π) is defined as

$$\pi = \gamma_o - \gamma \qquad 3.2$$

where γ_o is the surface tension when no adsorption has occurred and γ when adsorption has occurred (Gaines, 1966). π can be considered as a two-dimensional pressure exerted by the absorbed molecules in the surface plane. The units of surface pressure are the same as for γ, i.e. mN/m.

Surface tension can be measured using various methods such as the capillary rise method, the drop volume method and various modifications of the Wilhelmy plate method (Wilhelmy, 1863; Harkins & Brown, 1919; Tate, 1864; Davies & Rideal, 1963; Gaines, 1966). The Wilhelmy plate is the method used in determining surface pressure of lipid monolayers described in this thesis. Originally the plate was detached from the liquid surface to measure the weight of the meniscus of liquid adsorbed, what follows is a modification (see Adamson, 1967). Essentially a thin plate of glass, mica or platinum is suspended from one arm of a balance and is partially immersed in the liquid surface. A platinum rod can also be used (Slowinski & Masterton, 1961). The force necessary to maintain the plate, or a platinum wire, immersed to a constant depth is determined. It is important to establish that the contact angle of the liquid with the plate is zero (diagram 4.3b), then

$$\gamma \text{ Cos } \theta = \frac{\omega_{total} - (\omega_{plate \text{ or } rod} - b)}{P} \times 980.59 \qquad 3.3$$

where ω_{total} is the weight of the plate or rod partially immersed in the liquid surface, ω_{plate} or rod is the weight before immersing, b is the buoyancy correction for the immersed portion, p is the perimeter of the plate or rod and 980.59 is the gravitational acceleration. The units of γ are dynes cm^{-1} or mNm^{-1}.

3.5.3 Monolayer state

In monolayer of simple compounds there are three major film states which can be clearly recognised. These are gaseous or vapour state film, the expanded film and condensed film. The various other nomenclatures describing these film states have been compiled by Gaines (1966) and are indicated in Table 3.1.

(i) In the gaseous monolayer state the molecules are assumed to be floating widely separated on a molecular scale on the subphase surface so that intermolecular forces are negligible. Such monolayers are characterized by a surface pressure which approaches zero asymptotically, as the surface area is increased; also the surface potential is invariable throughout all the film surface (Fig. 3.2; Gaines, 1966; Gershfeld, 1974).

The interpretation of the curve of the surface pressure (π) versus area per molecule (A) follows the theory of the behaviour of ideal gases but considered in only two dimensions. The total kinetic energy (kT) which is assumed to produce the surface pressure, ignoring the liquid subphase, is represented by an ideal two dimensional gas equation

$$\pi A = kT \qquad 3.4$$

where k is the Boltzmann constant (1.3866×10^{-23} JK^{-1}).

(ii) Expanded monolayers

Expanded lipid monolayers show characteristic force-area (π-A) isotherms with considerable curvature (Fig. 3.2). These films show an apparent cooperative interaction as indicated by their π-A isotherms which extrapolates to zero at very large area per molecule. At low surface pressure the film is less orientated and probably all the molecules all lie flat on the liquid surface (Davies & Rideal, 1963). The surface potential does not show large random fluctuations, thus indicating

Table 3.1 Monolayer states

	Gaseous	Expanded		Condensed		
Adam[a]	Gaseous	Vapor expanded	Liquid expanded	Transition	Close-packed heads	Condensed	Close-packed chains
Harkins[b]	G (Gaseous)		L_1 Liquid expanded	I (Intermediate)	L_2 Liquid condensed	LS Super liquid	S (CS)[c] (Solid
Dervichian[d]	Gaseous		Liquid	Expanded mesomorphous	Mesomorphous		Solid

a N.K. Adam, Physics and Chemistry of Surfaces, pp. 39-55.

b W.D. Harkins, Physical Chemistry of Surface Films, pp. 106-117.

c Suggested by S. Ställberg-Stenhagen and E. Stenhagen, Nature, 156, 239 (1945).

d D.G. Dervichian, J. Chem. Phys., 7, 931 (1939).

(From Gaines, 1966).

a homogenous monolayer (Figs. 5.1 and 5.8). The hydrocarbon chains of the film-forming molecules cannot pack closely and therefore promote the formation of an expanded monolayer. The presence of a second polar group with a strong affinity for the water surface or a double bond has a tendency to disrupt the packing.

The influence of temperature on the state of lipid monolayers was detected early on in monolayers of myristic acid (Adam & Jessop, 1926). π-A curves above 25^{o}C are typical of the expanded type, while at 2.5^{o}C the π-A curves are characteristic of a condensed state. π-A curves determined in the region of the phase transition temperature show intermediate states. These experiments show the close relation between the monolayer state and the lipid physical state (Adam, 1968). Similar experiments carried out with phospholipids, however, have shown that in addition to the hydrocarbon chains, the polar head-groups have a rôle in determining the structure of the monolayer state (Phillips & Chapman, 1968). It was found that the molecules in a condensed film of phosphatidyl ethanolamine (PE) are much more closely packed than those in the equivalent phosphatidyl choline monolayer. Recent studies have shown that insoluble fatty acid monolayers should exhibit eight different types of π-A isotherm (Baret et al., 1982). A model involving only the interaction of polar head groups in different arrangements has been proposed to explain these different types of isotherm (Baret et al., 1982). Phospholipids or fatty acids containing saturated hydrocarbon chains larger than 14 carbons form condensed monolayers at room temperature, whereas those containing shorter chains exhibit liquid-expanded behaviour. The idea of a random, liquid-like arrangement of the flexible hydrophobic chains accounts qualitatively for the behaviour of liquid expanded films (Gaines, 1966; Phillips, 1972; Lundquist, 1978).

(iii) Condensed monolayers

Strong forces of attraction between hydrophobic regions cause the formation of 'floating islands' at areas per molecule larger than the limiting surface area for compounds such as stearic acid and sterols. The characteristic π-A isotherms of these compounds suggest that the hydrophobic regions are oriented at right angles with respect to the water surface at all surface pressures (Demel et al., 1976).

Surface potential is another parameter often measured in studying monolayer states. Changes in surface potential (ΔV) for a fixed surface area provides information about the homogeneity of a lipid film. For an un-ionized monolayer the ionic composition of the subphase can be varied without affecting the general properties of the film (Gaines, 1966). On the other hand, for an ionized monolayer changes in the subphase composition will affect the contribution of the ionic double layer potential (Gouy, 1910; Chapman, 1913). Surface potentials are further discussed in Section 5.4.6.

3.6 Mixed monolayers of phospholipids and cholesterol

The condensing effect of cholesterol on the structure of phospholipid membranes was one of the earliest observed characteristics of this molecule (Leathes, 1925). Recent monolayer studies have shown that the physical state of the pure phospholipid is important for this condensing effect. Expanded films of disaturated phospholipids when mixed with cholesterol show a reduction in mean molecular area occupied by the phospholipid (Demel & De Kruyff, 1976). Phosphatidyl choline species with transition temperatures below -20^{o}C do not show the condensing effect of cholesterol (Müller-Landau, 1979). The condensing effect is dependent on the chain length and degree of unsaturation of the fatty acid constituents. The 3β-OH group on the sterol nucleus appears to be

important for this effect (Huang, 1976). Studies using other methods have shown that cholesterol increases the packing in phosphatidyl choline by restricting the thermal movement of the fatty acid chains (see Section 3.1). This agrees with the observed reduction in bilayer permeability both in expanded model and natural systems (Papahadjopoulos et al., 1973; Demel et al., 1977).

3.7 Lipid-protein interactions in monolayers

Several methods for studying the interaction of lipids and proteins in monolayers have been described. The most used technique, however, is that of film penetration (Colacicco, 1970; Phillips & Spark, 1980). Briefly, a lipid film is formed at a defined initial surface pressure; the protein is then injected into the subphase and the increase in film surface pressure measured as a function of time. The penetration of the lipid film by radio-labelled protein can also be monitored as an increase in surface radioactivity (London et al., 1974).

Combined monolayer experiments at constant surface area and constant surface pressure have been used to determine the mode of interaction of snake venom cardiotoxins with lipids (Bougis et al., 1981). When the galactosyl transferase system of Salmonella typhimurium was reconstituted in monomolecular films of phosphatidyl ethanolamine at the air-water interface, enzymatic assay of its galactosyl acceptor activity indicated that the arrangement of this system in the monolayer was similar to its arrangement in natural membranes (Romeo et al., 1970); furthermore, spreading vesicle suspensions on a water surface using a modification of Trurmit method showed the formation of monolayers (Trurnit, 1960; Verger & Pattus, 1982). Enzymatic activity associated with these vesicles was shown to be present also in their respective derived monolayers. The advantage of this method is that it is thus possible to

study the surface properties of intrinsic proteins in the presence of the appropriate native lipids.

The rôle of lateral surface pressure in the regulation of biological membrane activity is very well illustrated using membrane-associated enzymes. Enzymes which are bound to the surface of the membrane but also partially embedded in the hydrophobic region of the lipid bilayer, show activities which are independent of surface pressure (Verger & Pattus, 1982). An example of such behaviour is given by intestinal aminopeptidase. This enzyme has been found to be attached to the plasma membrane by a short hydrophobic peptide with the active site presumably external to the membrane (Pattus et al., 1976). On the other hand, enzymes completely embedded in the lipid matrix or with their active sites located in the hydrophobic region, show surface pressure-dependent activities. Pattus et al. (1981) showed that the activity of acetyl cholinesterase from human erythrocyte membranes is dependent on surface pressure. Its enzymatic activity increases as the surface pressure is increased.

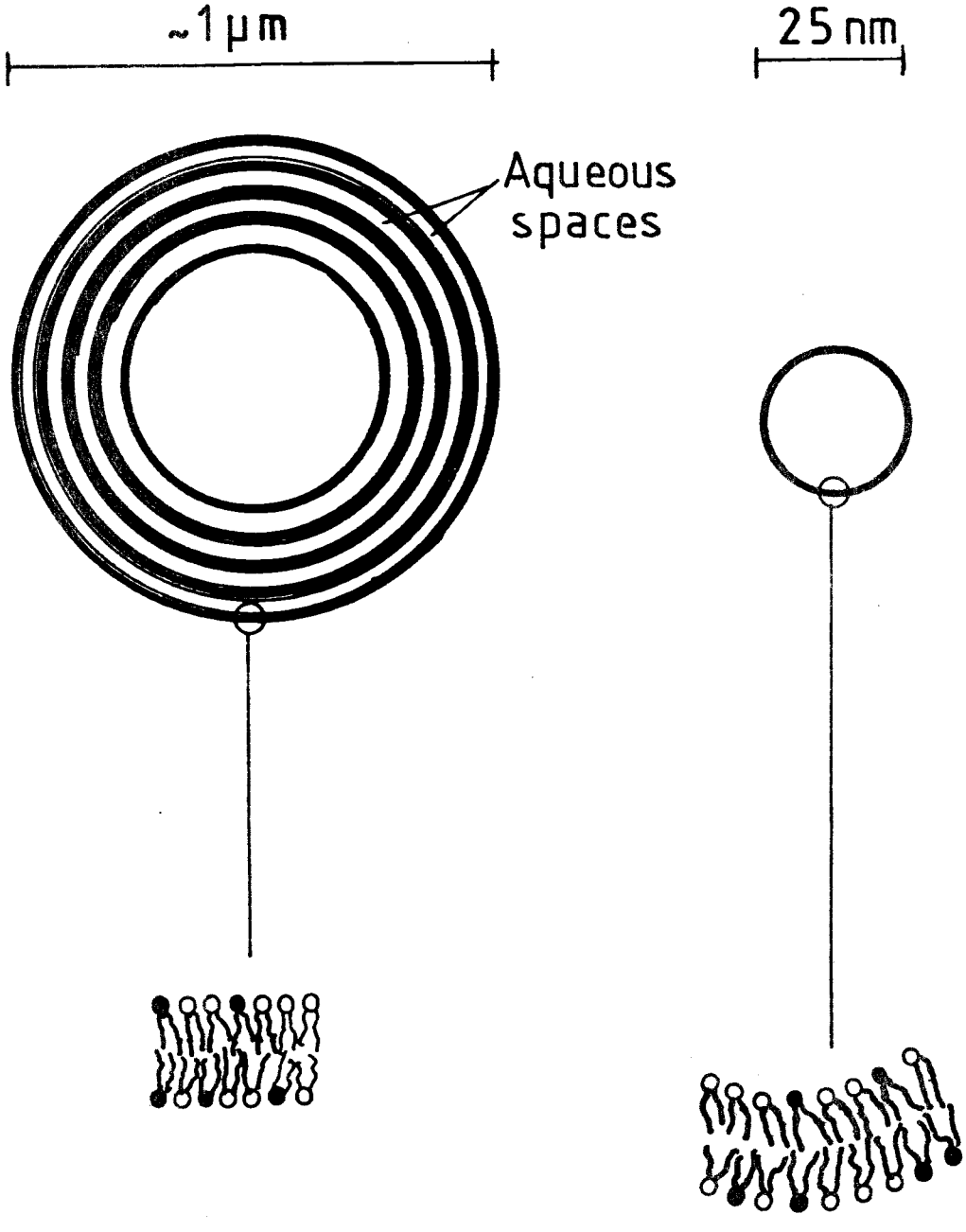
3.8 Study of lipid-protein interaction using liposomes

The amphipatic nature of phospholipids makes them form concentric multilamellar bilayer structures when dispersed in an aqueous modium (Bangham et al., 1965). Sonication (ultrasonic irradiation) has been used as a means of reducing the size of very large multi-lamellar liposomes to small unilamellar liposomes (Huang, 1969). Figure 3.2 shows a diagrammatic representation of a multi-lamellar liposome which can be as large as 1 μm in diameter, and an uni-lamellar liposome with the smallest diameter of 25 nm reported by Huang (1969). Uni-lamellar liposomes or single bilayer vesicles of similar size can also be prepared by either injecting an ethanolic solution of phospholipids into

Fig. 3.2

Schematic representation of multilamellar and unilamellar liposomes. Enlarged view indicates that liposome lipid bilayers can be asymmetric with respect to the distribution of phospholipid species per monolayer. (Sections 3.3 & 3.8).

FIG. 3.2



water, or from phospholipid-cholate micelles followed by removing the detergent by gel filtration (Batzri & Korn, 1973; Brunner et al., 1976).

Phospholipids below their phase transition temperatures cannot form liposomes. Only in the liquid-crystalline state do they form closed bilayer structures (De Gier et al., 1968). The polar group is also important in the formation of liposomes. For example phosphatidyl ethanolamine differentiates from phosphatidyl choline in that above its transition temperature forms non-closed structures, and therefore cannot sequester water (Papahadjopoulos & Miller, 1967). The ability to entrap solutes has made liposomes a widely used model in various fields (Scarpa & de Gier, 1971; Gregoriadis et al., 1971). In their interaction with proteins it is necessary, however, to distinguish between protein entrapment and protein associated with liposome bilayers (see Tyrrel et al., 1976; Poste et al., 1976). Most of the proteins reported to have been entrapped in liposomes are enzymes. The previously accepted criterion of entrapment based on the suppression of enzymatic activity which is only relieved by the addition of a detergent is no longer considered a rigorous proof of entrapment (Sessa & Weissmann, 1970). Enzyme latency could be brought about by adsorption of the enzyme to the lipid bilayer, with a consequent masking of activity (Tyrrel et al., 1976). Various methods are, however, available for detecting the incorporation of proteins into lipid bilayers. Most of them are based on the perturbing effect of proteins on the lipid bilayer causing any of the following effects: (a) changes in the average bilayer fluidity as measured with ESR or fluorescence probes; (b) changes in the phase transition temperature and width of the transition as measured by differential scanning calorimetry; (c) changes in vesicle permeability to small molecules such as Na^+ and glucose (see Section 3.2; Chapman & Morrison, 1966; Demel et al., 1968; Papahadjopoulos

et al., 1972; McElhaney, 1982).

3.9 Scope of this thesis

In the following study the monolayer technique is used extensively to investigate the interaction of variant surface glycoprotein with various lipids. Surface pressure, surface potential and surface radioactivity are the main parameters used to measure that interaction. VSG is a membrane protein, but only scant and indirect information on its mode of attachment to the lipid membrane is available in the literature. An understanding of the mechanism is important in the search for new treatments against Trypanosoma brucei. It is expected that such a mechanism of attachment to the membrane lipid should be independent of the mechanism of antigenic variation. Monolayers of pure lipids were used to determine whether VSG had a preferential interaction with any particular lipid. A strong interaction was detected with cholesterol. As a result studies using mixed monolayers of phospholipid and cholesterol were carried out to establish whether this could enhance the penetration of phospholipid films by VSG. The water soluble form of the antigen forms dimers in aqueous solution, and therefore the detergent sodium cholate was used to dissociate it into monomers. This detergent is, however, surface active and to remove it from the monolayer system an exhaustive washing procedure was developed. The specificity of VSG for cholesterol was further investigated using cholesterol and cholestanol derivatives, as well as polyene antibiotics of known specificity for cholesterol. Finally the liposome model was also used to study the VSG-lipid interaction.

CHAPTER FOUR

MATERIALS AND METHODS

CHAPTER 4

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4.1 History of the trypanosome strain, clones and purification of VSG

Trypanosome brucei, strain 427 (S 427) was isolated in 1960 from an early sheep infection in Uganda (Cunningham & Vickerman, 1962) and maintained by regular passage in mice at the Lister Institute (London) from 1961 to 1967 when samples were taken and stored (10% glycerol) in liquid nitrogen. A frozen sample (the stabilate) was subsequently transferred to the Nuffield Institute of Comparative Medicine in London, and finally to the Molteno Institute, Cambridge (Cross & Manning, 1973). This strain gives essentially monomorphic infections (slender forms) in rats and mice. Being, therefore, a practical example of morphologic changes induced by a prolonged syringe-passaged through mice. Similar behaviour of a strain of T.(T.) rhodesiense, which becomes monomorphic after being syringe-passaged for 9¼ years, was described by Ashcroft (1960). By contrast S42, a strain closely related to S427 and isolated in Tanzania by Dr J.R. Baker in 1966 (Taylor & Cross, 1977), is a pleomorphic.

4.1.1 Derivation of trypanosome clones

Clone 052 was derived from S427 by Cross (1975), as follows: infected blood was diluted with a buffer containing 5 mM KCl, 80 mM NaCl, 1 mM $MgSO_4$, 20 mM Na_2HPO_4, 2 mM NaH_2PO_4, 10 mM glucose, containing 1% bovine serum albumin (trypanosome dilution buffer; TDB) to give a suspension with a parasite density of 2×10^3 trypanosomes/ml. Small aliquots (0.6 µl) of this suspension were deposited onto a microscope slide and examined by dark-field illumination. Droplets

containing only one trypanosome were drawn up into a fine capillary tube; the capillary transferred to a syringe containing 0.3 ml of buffer and the parasite inoculated intraperitoneally into a mouse. After three days 0.5 - 1.0 ml of blood collected from the infected mouse was injected into a second mouse. This procedure was repeated until the last mouse (usually the third mouse) developed a high parasitaemia (3.5×10^8 trypanosomes/ml) within 2-3 days. Finally, the blood collected was mixed with an equal volume of tissue-culture medium 199 containing 0.5% BSA and 7.5% glycerol (Taylor, 1977). The mixture was kept in capillaries which were heat sealed before being slowly cooled (about $1^{o}C$ per minute) and then stored in liquid nitrogen ($-196^{o}C$). Other clones were prepared similarly by sampling at approximately 8-day intervals from an infected rabbit. When clone 052/reclone 1 or 2 was used to infect normal mice, trypanosomes carrying a new variable antigen type (VAT) appeared in the first relapse population. One of them was cloned and labelled VAT 221 (Doyle et al., 1980); VAT 121 was derived from S427/reclone 1. These two kind of antigens are extensively used in studies described in this thesis. Because of the occurrence of antigenic variation and the increasing number of workers using various antigenic types, Lumsden & Herbert (1975) proposed a nomenclature aimed at avoiding confusion with the clone classification. Originally it was intended to have the pedigrees and inter-relationships of materials from a known source, i.e. T.(T.)brucei stabilate TREU 164 (TREU = Trypanosomiasis Research Edinburgh University); TREU 164 is a material originating from an infected fly (Glossina pallidipes) fed with bovine blood in Uganda in 1960. One stabilate TREU 164 was stocked in the World Health Organisation cryobank at the London School of Hygiene and Tropical Medicine,

its stabilate prefix is LUMP. The histories presented are of antigen types - the clone populations isolated that were, successively, found to be antigenically distinct from all those previously identified. Those antigen-types are designated LUMP 63 (ETat 1), ETat = Edinburgh Trypanozoon antigenic type.

This nomenclature provides some practical advantages such as a clone derived from ETat 1 is a close descendent of ETat 2. Or in the case of being recloned, i.e. MITat 1.2 (Molteno Institute Trypanozoon antigenic type) corresponds to the VAT 221 isolated from clone 052 stock S427 by Doyle et al. (1980); the passage history of these clones are also given in this reference.. The antigens VAT 121 and VAT 151 were also obtained from reclones 052 and have basically identical immunoprecipitation (M.J. Turner, personal communication) and are identified as MITat 1.6.

4.1.2 Growth and harvesting of trypanosomes

Growth, harvesting and purification of variant surface glycoprotein has been described by Cross (1975). The procedures can be summarised as follows. Stabilate material, kept in liquid nitrogen, was allowed to equilibrate to room temperature and the parasite density adjusted by dilution to approximately 1×10^6 trypanosomes/ml. Two mice were inoculated intraperitoneally with one ml each; after three days blood was taken and used to infect a group of rats. A parasitaemia of 10^9 trypanosomes/ml after 72 hrs was generally achieved with an inoculum of 5×10^6 trypanosomes. Rats were anaesthetized with ether and bled through the aorta using citrate as anticoagulant. The infected blood was centrifuged at 1500 g_{av} for 10 min, and the upper phase (serum) replaced by TDB in which the trypanosome

layer was resuspended. The trypanosome suspension was centrifuged again, resuspended in fresh separation buffer pH 8.0 (44 mM NaCl, 57 mM Na_2HPO_4, 3 mM Na H_2PO_4, 10 mM glucose; SB) and passed through a column of DEAE-cellulose (Lanham, 1968) on which the contaminant erythrocytes were retained and trypanosomes passed through. These were collected and checked under light microscopy. All these (and subsequent) procedures were carried out at 4°C, unless stated.

4.1.3 Purification of VSG

The harvested trypanosomes were resuspended at 10^9 trypanosomes/ml in 80 ml breaking buffer pH 7.6 (50 mM Tris, 50 mM KCl, 5 mM $(CH_3COO)_2$ Mg) containing in addition the protease inhibitor 1 mM phenylmethylsulphoxide fluoride (PMSF). Cells were broken by high-speed homogenization for 5 min (MSE homogenizer) in the presence of small glass beads (Chance, Ballotini No. 12). The homogenate was centrifuged at 15000 g_{av} for 15 min, the pellet was resuspended and centrifuged. The two 15000 g_{av} supernatant fluids were combined and centrifuged for 2 hrs at 165000 g_{av}. The soluble proteins of the high-g_{av} supernatant fraction was first dialysed in dialysis tubes (Dialysis tubing-visking size 1-8/32", Medical International, London) against 1 lt of 10 mM buffer phosphate, pH 8.0 for approximately 16 hrs, then, applied to a DEAE-cellulose column (at room temperature) previously equilibrated with the same buffer. Eluant was monitored at 280 nm (LKU Uvicord III Spectrophotometer); the peak of material passing unretarded (i.e. at the void volume) was collected and dialysed against deionised water (16 hrs). The dialysed material was subjected to isoelectric focusing over a range from pH 3 to pH 10 (1000 V; in 44-64 hrs, 440 ml LKB column). The gradient was eluted in 3 mls frac-

tions; the protein concentration was estimated continuously at 280 nm and the pH of every fifth fraction measured at room temperature. The pooled fractions were dialysed overnight against distilled water and concentrated by ultrafiltration using a 10,000 molecular weight cut-off membrane (PM-10, Amicon, Lexington, Mass., USA) fixed in a magnetically stirred ultrafiltration apparatus, Amicon Model 52. The high flow was effected with nitrogen gas at a constant pressure of ∿3 Bar (45 p.s.i.). The purified antigen is designated by the clone from which it is derived, i.e. MITat 1.2, yields the variant surface glycoprotein 221 (VSG 221). The purified antigen was dissolved in water and the concentration adjusted to 5 mg per ml (deionized and distilled water), and then stored frozen in liquid nitrogen.

4.2 Polyacrylamide gel electrophoresis

4.2.1 Sodium dodecyl sulphate polyacrylamide slab gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemli (1970).

The following stock solutions were prepared:

a) 30% acrylamide gel solution (30% A/MB-A) containing 30 g monomeric acrylamide and 0.8 g methylenebisacrylamide, made up to 100 ml with distilled water.

b) Solution L TRIS-SDS pH 8.8 (1.5 M TRIS/HCl, 0.4% SDS)
18.17 g TRIS[tris(hydroxymethyl)aminomethane]
24.3 ml 1 M HCl
0.4 g SDS, made up to 100 ml with distilled water.

c) Solution U TRIS-SDS pH 6.8 (0.5 TRIS/HCl, 0.4% SDS)
6.06 g TRIS
0.4 g SDS

Adjust with HCl for pH 6.8, made up to 100 ml.

d) TRIS/glycine reservoir buffer x 10 concentrate, pH 8.4.

30 g TRIS

144 g glycine

10 g SDS, made up to 1 lt.

e) Sample buffer, containing

10 ml glycerol

10 g SDS

12.5 ml U solution tris-SDS

0.0025 g bromophenol blue, made up to 100 ml.

f) Gel solutions of acrylamide/M-bisacrylamide were prepared as follows:

	Percentage of acrylamide solutions (w/v)	
	7%	20%
Solution L: TRIS-SDS	5	5 (ml)
30% A/MB-A	4.33	13.32 (ml)
Sucrose	-	3 (g)
*15% ammonium persulphate (15% APS)	30	30 (µl)
*N,N,N^1,N^1-tetramethyl-ethylenediamine (TEMED)	5	5 (µl)
Distilled water	10.64	1.65 (ml)
Final volume	20	20 (ml)

The acrylamide gel solution was degassed under reduced pressure before the addition of ammonium persulphate and TEMED, and stored at -20^{o}C in the dark.

* 15% APS was made up freshly and was added in combination with TEMED just before adding the solutions to the gradient former.

g) Upper stacking solution containing 3% acrylamide was prepared as follows:

Solution U: TRIS-SDS	2.5 ml
30% A/MB/A	1 ml
15% APS	30 µl
TEMED	15 µl
Distilled water	6.5 ml
Final volume	10 ml

The same precautions about storage and addition of 15% APS and TEMED as outlined above were observed.

4.2.2 Preparation of slab gel (acrylamide gradient) of SDS-PAGE

Solutions of 7% and 20% A/MB-A were mixed in a Pharmacia gradient former and pumped into a glass gel-mould 0.15 x 20 x 15 cm. Then a small volume of solution L TRIS-SDS (4 times diluted) was layered onto the surface of the gel solution to provide a flat bed and to exclude air; the polymerisation took about 2 hours at room temperature (21 ± 2°C). Once the gel had solidified, the buffer was removed and the upper stacking gel solution added. A comb was immediately inserted as a template to form the sample wells; polymerization of the stacking gel took about 30 minutes.

4.2.3 Samples for electrophoretic analysis

The 7%/30% gradient acrylamide gels have a sensitive range of between 0.5 and 5 µg protein. Samples were mixed with an equal volume of sample-buffer containing 5% 2-mercaptoethanol (2-ME) and boiled for 3 min. Samples and protein markers were placed in selected wells using a Hamilton syringe (Bonaduz, Switzerland).

Loaded wells were set up in an electrophoresis apparatus with the upper and lower reservoir filled with TRIS/glycine reservoir buffer (diluted 10 times). The loaded gels were run at constant voltage (150 V: 16 hours). The molecular weight marker sample contained bovine serum albumin (BSA) MW 68,000, egg albumin MW 45,000, carbonic anhydrase MW 29,000 and lysozyme MW 14,000. Protein bands were identified after staining the gel for 1 hr at 37°C with 0.1% (w/v) Coomassie brilliant blue in methanol:water:acetic acid 45:45:10 (v/v), followed by destaining with methanol:water:acetic acid 20:73:7 (v/v).

4.3 Analytical determinations

4.3.1 Protein determination

Protein concentrations were measured by the method of Lowry et al. (1951) with the modification introduced by Colacicco (1969) to improve the sensitivity range to between 2 and 12 µg. BSA dissolved in distilled water was used as the standard.

4.3.2 Phospholipid concentration

Lipid phosphate was determined as inorganic phosphate following the ascorbate-molybdate method described by Ames (1966); after oxidation with $Mg(NO_3)_2$ the formation of a phosphomolybdate complex with ammonium molybdate and then reduction of this complex with ascorbic acid gives the high sensitivity as described below. Solutions used were as follows:

a) 10% (w/v) ascorbic acid in distilled water (made up freshly).

b) 0.42% (w/v) ammonium molybdate, $(NH_4)_6\ MO_7\ O_{24}.4H_2O$ in 0.5 M H_2SO_4.

c) 10% (w/v) $Mg(NO_3)_2.6\ H_2O$ in ethanol A.R.

d) 5 mM KH_2PO_4 aqueous solution (fresh).

e) The ammonium molybdate/ascorbic acid mixture was prepared by mixing thoroughly 1 volume 10% ascorbic acid with 6 volumes 0.42% ammonium molybdate, made up freshly.

Sample treatment as follows: 50 µl 10% $Mg(NO_3)_2$ (solution 'C') were added to each phospholipid sample including the phosphate standard, heated for 10 min at 100°C and then heated strongly in a flame until all the material was oxidized to ash. All the samples were allowed to cool and then 0.3 ml 1M HCl was added to each treated sample and heated at 100°C for 15 min in a test-tube with a glass-marble on top. After cooling, 0.7 ml of the ammonium molybdate/ascorbic acid mixture was added to each sample, mixed thoroughly and incubated at 45°C for 30 min. Finally absorbance was read at 820 nm. The range of sensitivity was between 5 and 50 nmole 'P'.

4.3.3 Cholesterol estimation

Cholesterol concentration were measured by the method of Kates (1972) as modified by Punel & Morris (1973). Solutions used were as follows:

a) Ferric chloride stock solution containing 8.4 g anhydrous $FeCl_3$ in 100 ml of glacial acetic acid. This reagent may be stored in brown glass-stoppered bottle for up to three months at room temperature.

b) $FeCl_3$ working solution.

The stock solution (a) was diluted 1/100 with glacial acetic acid.

c) Cholesterol standard, 1 mg/ml in glacial acetic acid (purity checked by TLC as described in 4.6.4).

d) Samples in organic solvents, such as chloroform, were first subjected to suction at the water pump and then treated with a stream of nitrogen gas in order to remove oxygen. 1.5 ml of working solution (b) was added to each sample containing an unknown amount of cholesterol and to dilutions of cholesterol standard, (solution C) in long test tubes; thoroughly mixed; allowed to stand for 10 min; and then 1 ml of concentrated sulphuric acid added. After thorough mixing, the reaction was allowed to develop in darkness at room temperature for 45 minutes. The optical density was determined at 560 nm and the sensitivity range found to be between 10 and 70 µg cholesterol.

4.3.4 Radioimmunoassay

Radioimmunoassay (RIA) technique was set up to measure small quantitites of VSG. This method uses the property of antibodies in adsorbing to solid plastic surfaces but maintaining accessible the antigen-combining sites (Chard, 1978). The following solutions were used:

a) Phosphate buffer saline pH 7.2 (PBS)

	g/L	M
NaCl	8.76	0.15
NaH_2PO_4	0.243	1.76×10^{-3}
Na_2HPO_4	0.6198	4.36×10^{-3}
NaN_3	0.002	3×10^{-3}

b) Blocking buffer, pH 7.2.

0.2% (w/v) BSA in PBS

0.1% (w/v) NaN_3

Vinyl microtitration plates with 96 wells were used as the solid phase. Antiserum-VSG 151 had been highly purified passing it through an affinity chromatographic column, i.e., by adsorbing on other different VSGs previously bound to cyanogen bromide activated Sepharose-4B by Cordingley & Turner (1980). This antiserum-VSG 151 was diluted 1/100 in PBS, 30 µl were delivered to each well and left for 2 hours at room temperature in a humidified chamber. The plate was washed 6X with PBS to remove free antiserum and 100 µl of blocking buffer containing BSA added to saturate any available surface sites. After two hours at room temperature the free BSA was washed off with PBS. Three wells without antiserum but with blocking buffer were used as controls for non-specific adsorption. The coated plastic tubes may be stored at -20°C for 2 weeks in presence of the blocking buffer.

Standard samples were purified VSG 151; the starting solution was adjusted to 1 mg glycoprotein/ml. It was first serially diluted 1/10 three times in blocking buffer. After the third dilution, it was diluted 1 in 2 a further seven times (final dilution). Samples were diluted in blocking buffer according to their expected concentration. Triplicate aliquots of 10 µl from samples and standard were delivered into the previously dried, coated plate. The control wells (non-adsorbed antisera) were filled with 10 µl blocking buffer. 10 µl of radioactive antigen [^{125}I]VSG 151 with an approximate activity of 10,000 cpm/10 µl were added last. Specific binding was allowed to occur at room temperature under a humidified atmosphere for 2 hours. Then the plate was washed with PBS six times to remove free antigen,

dried and each well cut off and counted in a gamma counter (Packard Instrument Co. Inc., Downers Grove, Ill., USA).

4.4 Labelling of VSG

Radioactive iodination of VSG was carried out according to the method of Hunter & Greenwood (1962).

Solutions were prepared as follows:

a) 0.2 M phosphate buffer pH 7.2 (PB).

i) 0.2 M Na_2HPO_4 28.392 g/l

ii) 0.2 M Na H_2PO_4 31.2 g/l.

These solutions (i and ii) were mixed volume to volume for 500 ml to pH 7.2.

b) PBS, pH 7.2 as described in section 4.3.4.

c) Chloramine T 2 mg/ml in PB (made up freshly)

d) Sodium metabisulfite (MBS) 2 mg/ml in PB (fresh)

e) 0.1% (w/v) BSA in PBS pH 7.2.

f) 1 ml of 50% (v/v) glycerol mixed with 1 ml 0.05% (w/v) bromophenol blue in distilled water.

g) VSG 151 5 mg/ml in distilled water.

h) Sodium Iodide [^{125}I] in dilute NaOH solution, pH 7.11, free from reducing agents, specific activity 15.6 mCi/µg of iodine (100 mCi/ml).

i) Sephadex G25 column, 35 x 0.7 cm, equilibrated with 0.2 M PB pH 7.2.

Procedure:

10 µl VSG 151, 40 µl 0.2 M PH pH 7.2, 10 µl of the radioactive Iodine solution and 10 µl of chloramine T solution were added in this order to a single 1 ml plastic tube and allowed to react for exactly 1 min at room temperature. The reaction was stopped by adding 20 µl sodium metabisulfite. Previously excess buffer had been removed from the top

of the Sephadex column and 20 µl of glycerol-bromophenol blue added. 100 µl 0.1% BSA were then added to the reaction mixture which was layered as to the top of the column. All the procedures were carried out in a fume cupboard. In general, ten fractions of 1 ml each, were collected and their radioactivity checked approximately with a [^{125}I] probe type 5-44-scintillation meter (Mini-Instrument, Burham-on-Crouch, Essex, U.K.). Two fractions containing the highest radioactivity, i.e., 5-6 ml (the void volume) were selected and subjected to the next analysis.

. Trichloracetic acid (TCA) precipitation: 10 µl of labelled antigen was added to a solution containing 0.5 ml 0.2% (w/v) BSA and 0.1 ml 1% (w/v) SDS, mixed thoroughly and then 0.4 ml of a 25% (w/v) TCA solution added to give a 10% final TCA concentration. This solution was allowed to stand overnight at +4^{o}C and then centrifuged and washed with 10% TCA in distilled water. The pellet was retained for use below.

I. Binding to antiserum VSG 151: 20 µl of a 50% suspension (w/v) of antiserum VSG 151 coupled to Sepharose 4-B was mixed with a volume (~5 µl) of labelled VSG 151 containing approximately 10^5 cpm in a final volume 200 µl with PBS. This was incubated at 37^{o}C for 30 min, left overnight at +4^{o}C, centrifuged and washed, the pellets were retained. Radioactivity in each incubation sample, before centrifuging, was measured as well as in each pellet (I and II) using a Gamma counter.

II. Molecular weight was verified by SDS-PAGE according to the procedure of McConnell et al. (1981). Labelling of VSG for monolayer experiments was carried out as described above except that the addition of BSA and bromophenol blue was omitted.

4.5 Immunodiffusion test

Double diffusion tests (Ouchterlony & Nilsson, 1973), were carried out in 1% (w/v) agarose in PBS pH 7.2. Holes with a diameter of 4 mm were punched in the gel in the form of a 'six-shooter' pattern, in which six wells are placed circumferentially around a well. The antiserum was poured into the central well after placing the samples in the other wells (15-20 µl of sample or antiserum in each well). The agarose-plate was then incubated in a humid atmosphere overnight at room temperature. Precipitation bands were fixed with PBS for 6 hours and washed with several changes of distilled water for 24 hours. The gel was dried with absorbent paper and stained with Coomassie brilliant blue for 1 min at room temperature, other procedures as described in section 4.2.3.

4.6 Lipid solutions

4.6.1 Phospholipids

The following phospholipids were used dissolved in chloroform (C)/ methanol (M) 2:1 unless stated. Phosphatidylcholine from egg yolk (EYPC) and / phosphatidylcholine from soybean (SPC); synthetics materials, di-palmitoylphosphatidylcholine (DPPC), di-oleoyl phosphatidylcholine (DOPC), di-stearoylphosphatidylcholine (DSPC), di-lauroylphosphatidyl-choline (DLPC), di-myristoylphosphatidylcholine (DMPC). Sphingomyelin (SPH) and phosphatidylethanolamine both from egg yolk. Final concentrations were either 1 mM or 2 mM as stated in results. All of these phospholipids were obtained from the Sigma Chemical Co. (Poole, Dorset), and the purity checked by TLC before use. Solvent System (see Section 4.6.4).

4.6.2 Sterols

Cholesterol (CHL), cholest-5-en-3β-ol (M.Pt. 147-148°C); cholestanol, 5α-cholestan-3β-ol (142-143°C); ergosterol (ERG), cholest-5,7.22-trien, 24 methyl-3β-ol; cholesterol acetate, cholest-5-en-3-acetate; cholesteryl chloride; cholesteryl oleate, β-sitosterol and cholest-5-en-24-ethyl-3β-ol, all of these sterols were obtained from the Sigma Chemical Co. These sterols were dissolved in C:M 2:1 as described for phospholipids. All reagents and solvents were of A.R. grade. Lipid solutions were stored at -20°C under nitrogen to prevent degradation (Klein, 1970a), and purity checked by t.l.c.

4.6.3 Sterol derivatives not commercially available were synthesised by R.A. Klein (unpublished). These were cholesteryl phosphatidyl choline, cholest-5-en-3β-phosphoryl choline; cholesteryl phosphate, cholest-5-en-3β-phosphate; cholesteryl di-phenylphosphate, cholest-5-en-3β-di phenyl phosphate; 3-acetamido cholestane, 5α-cholestan-3β-acetamido; 3-acetamido cholestene, cholest-5-en-3β-acetamido; amino cholestene, cholest-5-en-3β-amino; 5α-cholestan-3β-amino and 3-trimethyl ammonium cholestene, cholest-5-en-3β-trimethyl ammonium. The purity was checked by t.l.c. (chloroform:methanol:water 65:25:4 (v/v)) and stained for identification of the different groups as described in section 4.6.4. The substituted group in the 3-position was verified by infrared spectroscopy as a K Br (1% w/w) pellet using on a Perkin-Elmer 157G infrared spectrophotometer.

4.6.4 Thin-layer chromatography

Thin-layer chromatograph (TLC) was carried out according to Kates (1975, p. 428). After activating the silica gel H plates 20 x 20 cm (Uniplate, Analtec Inc, Luton, England, U.K.) at 110-120°C for 1 hour, a pencilled line was drawn on the silica gel surface approximately 2 cm from the bottom edge, and samples applied along the

line with a Hamilton microsyringe. The plates were developed in chloroform:methanol:water 65:25:4 (v/v) for phospholipids and sterols.

Identification of chemical groups was done as follows:

a) Primary amino groups.

The plates were dried by evaporation and stained with 0.2% (w/v) ninhydrin in acetone:water 1:1 (v/v), by spraying. The plate was heated to 105^{o}C for 4-10 min; the appearance of a blue or purple colour indicated the presence of a primary amino group.

b) Phosphate group

Molybdenum blue reagent was prepared as described by Dittmer & Lester (1964), procedure modified by Vaskovsky & Kosteky (1968). The dried plate was sprayed with the molybdenum blue reagent and sample containing phosphate was detected as blue spots on a white background within a few minutes without heating.

c) Organic material

Staining for organic material was done in parallel with that for phosphate groups. In this case, however, 50% (v/v) sulphuric acid in water was sprayed on plates previously treated with the molybdenum blue reagent, and the plates heated to 150^{o}C for 20 minutes. The presence of organic material was shown by black spots.

4.7 Radiochemicals

The following radiochemicals were obtained from Amersham International Ltd. (Amersham, Buckinghamshire).

i) [Carboxyl-^{14}C]cholic acid, sodium salt, specific activity 52 mCi/mmol (1.92 GPq ^{14}C/mmol) 99% pure.

ii) [1α,2α(n)-^{3}H] cholesterol, specific activity 54 Ci/mmol (37 MBq/mg), 99% pure.

iii) [4-^{14}C]cholesterol, specific activity 58.4 mCi/mmol (2.15 GBq ^{14}C/mmol), 99% pure.