THE ATTACHMENT OF ENCEPHALOMYOCARDITIS VIRUS TO KREBS II ASCITES TUMOR CELLS

SARAH LYN SHEPPARD

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THE ATTACHMENT OF ENCEPHALOMYOCARDITIS VIRUS TO KREBS II ASCITES TUMOUR CELLS

by

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Abstract

Attachment, the first stage of viral infection, was investigated using Encephalomyocarditis (EMC) virus and Krebs II ascites tumour cells (Krebs cells). The initial stage of the research was concerned with the Krebs cell surface, which was found to consist of a plasma membrane and a glycocalyx. Plasma membranes from Krebs cells, the purity of which was determined by marker enzyme assays, electron microscopy and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), contained about 20 coomassie blue staining components (proteins) and one periodic acid Schiff's (PAS) staining component (glycoprotein) of apparent molecular weight 225,000 daltons as revealed by SDS-PAGE. Three of the major proteins were labelled when Krebs plasma membranes were iodinated but the glycoprotein component was not labelled. SDS-PAGE of the glycocalyx was shown to influence cell surface activities since it partially blocked the iodination of whole cells and the action of neuraminidase on the cell surface.

The Krebs cell receptor for EMC virus was not found to be a glycocalyx component because attachment, growth and haemagglutination of EMC virus were unaffected by the presence or absence of the glycocalyx. EMC virus attached equally well to whole Krebs cells, isolated plasma membranes and human erythrocytes. Neuraminidase or trypsin treatment of Krebs cells or plasma membranes reduced EMC virus attachment and infectivity suggesting that the virus was attaching to a sialoglycoprotein component.

Glycoprotein preparations from Krebs cells and plasma membranes examined by SDS-PAGE contained a single PAS positive component of apparent molecular weight 215,000 daltons which is very similar to that of the membrane PAS component. These preparations inhibited virus attachment, infectivity and haemagglutination, and inhibition was reduced by treating the preparations with trypsin or neuraminidase. It is, therefore, probable that EMC virus attaches to the sialoglycoprotein component of the Krebs plasma membrane.

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Introduction

1. Background

Viruses, one of the smallest types of organisms, are entirely dependent on other living organisms to support their growth and replication (70). The beginning of virology research was in 1899 when Beyerinck showed the transmission of disease in bacteria-free filtrates (70) and further research has been made possible by technological advances throughout this century.

Virus replication can be considered in the following sequence of events: attachment, penetration, uncoating, biosynthesis, maturation and the release of new virions. Virus infections may be cytocidal, resulting in the host cell's death, non-cytocidal when the host cell survives despite viral replication occurring, or infections may cause transformation of the host cell. Attachment, the first stage of any viral infection may be defined as the initial specific interaction and joining of the virus and cell.

In order for attachment to occur a virion component must interact with a cellular receptor thus fixing the virion and host cell together to enable infection to take place. There may be secondary functions associated with attachment such as stimulating cell surface molecules to cluster into patches which may bind virus more firmly or participate in penetration.

Internal structures directing virus penetration could be stimulated by the attachment of the virion to the host cell surface. Attachment may play a secondary role in promoting cell transformation. It is thought, for example, that the virus-cell surface interaction may select a subpopulation of thymic cells with murine leukemia virus (MLV) receptors and these cells also comprise the transformable cells in the population (78). In this case, MLV attachment may trigger host cell transformation.

The mechanism and specificity of attachment varies with different virus types. In poxviruses, for example, which become attached to susceptible and resistant cells at the same rate (2), maintainance of the virions in a stable bound state on the cell surface is thought to be due to electrostatic forces (3). The host range of avian RNA tumour viruses is dependent on specific components of the viral envelope and the host cell surface receptors (28, 120). Attachment may improve the efficiency of virus replication by being sufficiently specific to permit viruses to attach to susceptible host cells only. Less specific attachment may still be of importance in that refractory cells to which a virus can attach may act as vectors carrying the virus to a susceptible cell. For instance, Rous sarcoma virus can attach to resistant cells which may then act as viral vectors (120). Several viruses can attach to erythrocytes causing haemagglutination, such as influenza virus (47) but erythrocytes cannot act as host cells. As circulatory cells erythrocytes would make very

very good vectors for carrying viruses to susceptible tissues. Attachment is, therefore, the first important stage of a viral infection and may influence further events during infection.

2. Purpose of research

The attachment of Encephalomyocarditis (EMC) virus to Krebs II ascites tumour cells (Krebs cells) was investigated in this project. The main emphasis was placed on the cellular receptor for EMC virus on Krebs cells rather than the attachment component of the virus. Although only one virus and host cell type were used this research has much broader implications.

Many vaccines are now available against viruses but there is essentially still no cure for viral infections. As more knowledge accumulates about attachment the ability to cope with viral infections improves. If attachment is more thoroughly understood the process may be blocked thus preventing infection. Unfortunately, when the symptoms of a viral infection are observed and a diagnosis has been made at least one cycle of attachment has occurred and already many virions are being produced. The blocking of attachment would be useful as a preventative measure, similar to vaccination or in controlling infections that may recur. Since antibodies against viral surface components are generally effective in neutralizing virions there is great potential for the production of safer and more efficient vaccines by inducing antibodies against the viral components involved in

attachment.

For this investigation, Krebs cells, which are cancerous and lethal to host mice were used. Information discovered about these cells during attachment studies will help the progress of tumour cell research.

3. Description of organisms used

1) EMC virus

For this study a picornavirus, EMC virus, belonging to the Cardiovirus genus was used. Originally EMC virus was isolated from monkeys in Florida and Africa (53) but it is highly infectious for rodents. As its name suggests the wild strain of EMC virus infects the heart muscle and central nervous system in mice (53).

The picornaviruses are small, icosahedral viruses approximately 30 nm in diameter. They are relatively simple animal viruses with a single stranded RNA genome. The capsid is made up of four proteins VP1, 2, 3 and 4 (94) which are sometimes referred to as α, β, γ and δ. EMC virus has been crystallized (32) and no carbohydrate or lipid has been found in the virus particles (14). It is not yet known which protein or combination of proteins is the virion component involved in attachment. EMC virus agglutinates erythrocytes from several animal species (4) which has been a useful tool in attachment studies.

ii) Krebs II ascites tumour cells

The K_2 variant of EMC virus which was used for this work can grow in Krebs II ascites tumour cells (99). This cell line is derived from the Krebs II solid carcinoma which arose spontaneously in the inguinal region of a hybrid male mouse, and was maintained by subcutaneous transfers into similar mice (117). A haemorrhagic exudate was observed in these mice associated with the carcinoma. The exudate was injected into more mice and those mice producing exudate rather than solid tumours were selected. After several cycles of injections of exudate from selected mice ascites tumours rather than solid tumours developed in the mice. This ascitic tumour line was designated the Krebs II ascites tumour cell line and was found to have several similar characteristics to the well established Ehrlich ascites tumour cell line (56). The frequency of non-tumourous cells, the median survival time of animals inoculated with cells and the DNA and RNA content are all very similar between the two cell types (56). Krebs II ascites tumour cells can be grown in the peritoneal cavity of mice. The K_2 variant of EMC virus may then be grown in suspensions of washed Krebs cells in culture flasks or as plaques on solid medium (99).

4. Review of cell surface structure

At the cellular level, the first barrier to viral infection is the host cell surface. Since the cellular receptor of virus

attachment is the main focus of this research cell surface structure is reviewed briefly here. Little has been published about the Krebs cell surface but plasma membranes have been isolated from Ehrlich ascites tumour cells, which are very similar to Krebs cells, and some of the plasma membrane enzymes have been characterized (36).

In some, if not all cells, there are two principle components of the cell surface, the plasma membrane and the glycocalyx. Many cells including Ehrlich cells have a layer of components external to the plasma membrane (96). Intestinal cells have an outer "fuzzy layer", (20) whilst kidney cells have an outer layer brown as a brush border (101). In other cells, such as Ehrlich cells, this outer layer consists of proteins and glycoproteins (96) which can be easily removed in contrast to the fuzzy layer of intestinal cells or brush borders. The term glycocalyx, which means "sweet husk" (7) has been used to describe these extramembraneous components of cells and will be used as such in this text.

The plasma membrane itself consists largely of lipids and proteins. Other components such as ions are present in small quantities. Sugars form an integral part of glycolipids and glycoproteins of the cell membrane. Water molecules are associated with the membrane although they are not bound to it. The major lipid components of the membrane are cholesterol and phospholipids (121). These maintain the bilayer structure of the membrane

which has proteins distributed randomly throughout. The membrane proteins can move around but the lipids maintain a fairly stable, liquid crystalline state. There is constant exchange and movement of molecules within the membrane although its overall integrity remains unaltered. This is the fluid mosaic model of membrane structure which is now generally accepted (106, 107).

Cellular receptors for viruses would be expected to be located on the cell surface but it is necessary to determine whether the receptor is a glycocalyx or plasma membrane component. Within the cell surface, which molecules are potentially capable of acting as viral receptors? Cell surface components such as cholesterol, which is common in high quantities in the same form, are not likely to provide the specificity observed in the attachment mechanisms of many viruses. Components of the cell surface which have a wide range of chemical structures would have the potential for specificity necessary in a receptor.

The plasma membrane proteins are very diverse, ranging in size and frequency. Some are glycosylated which gives them greater diversity owing to the potential variability of the oligosaccharide chains. Glycoproteins consist of a polypeptide backbone linked to branching carbohydrate side chains through N or O-glycosidic bonds. These side chains are generally linked to the part of the polypeptide on the extracellular side of the membrane. They comprise a combination of at least nine sugars (52), D-glucose, D-galactose and D-mannose which are stereoisomers, L-fucose,

L-arabinose and D-xylose which are also steroisomers but have one less oxygen atom than the glucose group and finally the amino sugars, N-acetyl glucosamine, N-acetyl galactosamine and sialic acid. The sialic acids are derivatives of neuraminic acid including N-acetyl neuraminic acid (NANA) (9).

Since the glycolipids contain carbohydrates they are also very diverse in structure. Most of the glycolipids are sphingolipids such as cerebrosides which contain only neutral sugars, or the gangliosides which always contain sialic acid in addition to other sugars (34). Gangliosides have been demonstrated to be receptors for tetanus (116) and cholera toxins (25) and for interferon (8).

The cell surface can be altered by treatment with physical, chemical and biological agents. After transformation, for example, tumour cells have altered surface and receptor activities (104). The glycocalyx of cells also changes after transformation (100, 105). Virus proximity affects the cell membrane (58). HeLa cells, Krebs II ascites tumour cells and fowl erythrocytes lose potassium when Newcastle disease virus (NDV) or Sendai virus absorb to them and haemolysis occurs in the fowl erythrocytes (58).

Receptors for viruses on cells would be expected to perform other important functions to warrant their presence on the cell surface. The erythrocytes receptor for influenza virus, glycophorin, is the MN blood group determinant (50). Receptors for Semliki forest virus on mouse or human cells have been identified

as the mammalian (mouse or human) histocompatability antigens (HLA, HLB, H2K and H2D) (46). There is some evidence that the subgroup B of avian leukosis sarcoma viruses utilizes the blood isoantigen R1 as a cellular receptor (21). Reovirus agglutinates type A human erythrocytes more easily than types O or B and tentative suggestions have been made that the isoantigens of the ABO alleles may participate in reovirus attachment (12). The plasma membrane plays a prominant role in several cellular functions such as regulation of metabolism and growth. It also contains receptors for inorganic transport systems, hormones and metabolites. Some molecules in the plasma membrane monitor dangers in the environment and act as communication lines between the cell and its external environment (121). Any of these membrane components might serve as virus receptors on the cell surface.

5. Virus attachment

i) General review of virus attachment.

Research on the attachment of most viruses to cells is still in its early stages. The effect of different substances and environmental conditions have been investigated giving indications of the type of virus-cell surface interactions taking place. At the molecular level very little is known about attachment, particularly to host cells. Owing to the simplicity of its membrane, research with erythrocyte receptors for viruses has

been more productive.

The work on the influenza virus receptor on red blood cells is probably the most complete at the present time. Virus receptor substance for influenza virus was isolated from human erythrocytes (50) and is inactivated by removing carbon atoms from NANA residues (112) or by removing the whole residue (51). Sialic acid containing glycoproteins, known as α or Francis inhibitors, compete with virus receptor substance for virus particles thus reducing the binding of virus to the virus receptor substance (37). Most of the human erythrocyte surface NANA was found to be present in the virus receptor substance which was calculated to be of molecular weight 31,000 (115). This receptor substance, serving as the human erythrocyte receptor for influenza types A and B viruses, has been identified as glycophorin A (54).

Other viruses such as polyoma virus and most of the paramyxoviruses show similar attachment characteristics to the influenza viruses in that sialic acid is required and attachment is inhibited by Francis inhibitors. Exceptions within the paramyxoviridae are Sendai virus which may utilize a ganglioside receptor (122) and the Morbillivirus genus where the removal of sialic acid has no effect on attachment. The similarity between influenza and polyoma virus attachment is quite strong because influenza virus can use polyoma virus receptor sites on erythrocytes (82) but there are fewer polyoma receptor sites than influenza

receptor sites (82).

Adenovirus 7 receptors on monkey erythrocytes have been solubilized and partially purified (84). This neuraminidase insensitive receptor is of 44,000 molecular weight and quite distinct from glycophorin, having no inhibitory effect on influenza virus attachment (84).

Currently most advances have been made with viruses that attach to glycoprotein receptors but there is evidence that Sindbis virus may bind to lipid receptors on erythrocytes (81). The binding of radioactively labelled Sindbis virus to liposomal model membranes made from a mixture of erythrocyte phospholipids was greatly diminished when either cholesterol or phosphatidylethanolamine were omitted. The authors suggest that these two components are important in a bilayer configuration for specific virus binding (81).

Having briefly discussed some of the more important achievements in studies on the attachment of viruses to cells, the following section is a more detailed review of the current knowledge concerning picornaviral attachment.

ii) The attachment of picornaviruses to cells

EMC virus, which is used in this study is a picornavirus. There is considerable knowledge concerning the attachment of picornaviruses to cells but little is known about attachment at the molecular level.

Many of the Picornaviridae attach to a small range of host

cell types. Wild type human picornaviruses, for example, generally only replicate in primate cells (89). In addition to primate cells coxsackie B viruses can attach to mouse cells since they are pathogenic for new born mice (60). Even in fetal mouse tissue no receptors were found for coxsackie A viruses (15) but limited replication of coxsackie A viruses does occur in primary mouse fetal cells (40). HeLa cells possess receptors for coxsackie B viruses but non-permissive established mouse L cells do not have receptors (24). Coxsackie A9 virus infects primary human and monkey cells in tissue culture but does not infect HeLa cells or other established cell lines which lack receptors (79). Attachment shows considerable specificity in that the loss of receptors results in a lack of permissiveness of cells (45, 110, 118). The loss of human chromosomes from mouse-human hybrid cells is correlated with the loss of poliovirus receptors and thus host cell permissiveness (118). Human cells transformed by Simian virus 40 (SV40) show increased resistance to poliovirus (44). Some picornaviruses, however, share the same cellular receptors, that is, "receptor families" exist within the Picornaviridae. Coxsackie B viruses may share a receptor on HeLa cells, which is distinct from poliovirus, coxsackie A virus and echovirus receptors (65). Polioviruses 1, 2 and 3 share the same receptor (22). Different degrees of specificity of attachment are demonstrated at the cellular level by looking at haemagglutinating picornaviruses. Echoviruses, coxsackieviruses and some unclassified

enteroviruses agglutinate human or primate erythrocytes only (88) whereas cardioviruses (4) and some rhinoviruses (110) can agglutinate erythrocytes from a wider range of species.

Various groups of picornaviruses show different physical characteristics of attachment. For example, the number of coxsackie B3 viruses attaching to HeLa cells did not increase linearly with increasing quantities of virus (22). Saturation of host cells by various picornaviruses occurs at different levels (66) but this is not due to spacial limitation because 10^4 picornaviruses,, the saturation level of some species has been calculated to cover only about 1% of the surface of the host cell (66). This suggests that virions are attaching to specific sites which have become saturated.

Changes in the extracellular environment such as pH, ionic strength and temperature influence the attachment of picornaviruses. Most enteroviruses have a pH optimum broadly in the neutral range but echovirus 7 attaches to erythrocytes with a sharp pH optimum between pH5 and pH6 (89) and attachment of coxsackie virus B4 shows a sharp optimum between pH 3.0 and pH 3.5. Although several of the coxsackie B viruses share receptors they have different pH optima for attachment (23). Poliovirus shows a broad pH optimum for attachment between pH 4.5 and pH 8.5 (23), despite the virion conformation being considerably altered in the range pH 4.0 to pH 7.0 (73).

The ionic strength of the extracellular medium influences

attachment. In isotonic sucrose the attachment of poliovirus 1 is poor but it is improved on the addition of sodium chloride (49). Divalent cations have no effect on poliovirus attachment (49) but enhance the attachment of coxsackievirus A9 (79) and human rhinovirus 2 (66). Attachment of both of the latter viruses is inhibited by EDTA (66, 79).

Attachment of some picornaviruses is temperature sensitive possibly indicating metabolic involvement on the part of the cell. The attachment of rhinoviruses 2 and 14 and polioviruses I and 2 shows an increase by a factor of 2 to 3 per 10°C within the temperature range of 10°C to 37°C (67). Poliovirus 1 and echovirus 7 each form a reversible attachment at 0°C where infectious virions are eluted on dilution of the cell-virus complexes. (89). Certain picornaviruses which attach to erythrocytes at 4°C elute from them in a non-enzymatic fashion at 37°C (55, 66, 91). High temperatures can inactivate cellular receptors for viruses such as the HeLa cell receptor for poliovirus which is inactivated at 56°C (89). HeLa cell receptors for two types of coxsackie B viruses were, however, viable at this temperature (93).

Several substances have been shown to inhibit the attachment of different picornaviruses to cells. Attachment of poliovirus (6) and some EMC virus variants (64) is inhibited by dextran sulphate which is a negatively charged polymer. Attachment of five echovirus types was prevented by N-acetyl glucosamine (102).

Monosaccharides can also interfere with the attachment of EMC virus (59) and various enteroviruses (61) to erythrocytes. Anticellular sera inhibit the attachment of poliovirus (49, 92) and some types of coxsackie A virus (43) and echoviruses (43, 92).

Picornaviral receptors on host cells can be inactivated by treatment with certain enzymes. Poliovirus receptors on cells are inactivated by trypsin treatment (49, 80, 89, 93, 123) but are less sensitive to chymotrypsin. Cellular or solubilized erythrocyte receptors for coxsackie B3 virus and echovirus are more sensitive to chymotrypsin than trypsin (89, 123). Under certain conditions poliovirus attachment to HeLa cells is enhanced by trypsin or chymotrypsin treatment possibly due to the clumping of membrane components into patches (123). Erythrocyte and host cell receptors for cardioviruses are sensitive to neuraminidase (57), EMC virus receptors on L cells being less sensitive to neuraminidase than those on HeLa cells (57). Francis inhibitors and glycophorin also inhibit haemagglutination of cardioviruses (30, 74).

Considerable preliminary experiments on picornaviral attachment have been carried out but the molecular mechanism of attachment can only be speculated upon. In the case of EMC virus experiments with neuraminidase and Francis inhibitors suggest that the cellular receptor may be a glycoprotein. The results are not, however, conclusive and this project takes a closer look at the actual receptor molecule on the cell surface involved in

EMC virus attachment.

6. Methods used in studying viral attachment.

The above review of viral attachment demonstrates that different approaches have been used to study attachment. The ultimate goal of attachment research is to discover which molecule, or part thereof, on the cell surface interacts with the viral component and to determine the nature of the interaction between them. To achieve this goal the effect of physical, chemical and biological agents on attachment can be studied and individual cellular receptors for viruses may be isolated and studied. To obtain a full picture of the attachment mechanisms of viruses it is, of course, necessary to employ both types of approach and these are discussed below.

i) Physical parameters of attachment.

Temperature, pH, ionic strength and virion concentration are all physical factors which can affect attachment of viruses. The attachment of viruses that bind to cells in a manner dependent on the pH or ionic strength of the extracellular environment may involve some type of ionic interaction between the virus and cell. Some viruses, for example, echovirus 7, (89) have specific pH optima. NDV attachment which is poor in isotonic sucrose is considerably improved when sodium chloride is used (98). These viruses may be attracted to cells by their surface charge.

Temperature can affect attachment, which as previously stated,

may be indicative of metabolic involvement on the part of the cell. The Poxviridae (26) and the cardioviruses (59) attach to cells with the same efficiency at 4°C and 37°C but others, for example, murine leukemia virus (27) attach to cells in a temperature dependent manner, 37°C being the optimum. Temperature dependency could indicate the involvement of lipids in attachment since the fluidity of the membrane lipids shows a marked increase at specific transition temperature (121).

By varying the number of virus particles with respect to the number of host cells it is possible to construct saturation curves for viral attachment. The kinetics of virus binding can be examined in this way. If saturation of the cell surface occurs before it is completely covered with virions it is a good indication of a limited number of specific binding sites existing for virus attachment.

ii) Chemical inhibitors

Many chemicals have been used to determine the nature of cellular receptors for viruses. Some act as inhibitors and a few have been found to enhance attachment. Formaldehyde, which reacts with free amino groups has been shown to inactivate receptors for several haemagglutinating enteroviruses (62). Periodate reacts with terminal serine and threonine residues at pH7 and may thus block receptors that contain these amino acids (18). Sodium borohydride reacts with and blocks disulphide bonds in addition to carbonyl groups (63). Careful periodate oxidation at pH 4.0 followed by sodium borohydride reduction removes carbon

atoms 8 and 9, or 7 from NANA residues which, for example, inactivated the glycoprotein receptor for influenza virus (112). Dextran sulphate, a synthetic acid polysaccharide inhibits Japanese encephalitis virus (86) and herpes virus (114) attachment, possibly by altering the charge on the cell surface. Carbohydrates can be potent inhibitors of virus attachment. Monosaccharides interfere with the haemagglutination of enteroviruses (61) and D-mannose and similarly related sugars inhibit haemagglutination of Japanese encephalitis virus (85). Reovirus haemagglutination is inhibited by N acetyl D glucosamine (38). By studying the effect of chemical inhibitors on attachment it is possible to begin to elucidate the nature of the interaction between virions and cells.

iii) The effect of proteases and glycosidases on attachment.

Proteolytic enzymes have commonly been used for attachment studies. Cells can be treated with 1% trypsin, chymotrypsin or subtilisin at 37°C and still remain viable (90, 123). Sensitivity to proteolytic enzymes does not necessarily mean that a cellular receptor is a protein because trypsin, for example, causes considerable changes in membrane conformation in addition to cleaving proteins (109). Under certain circumstances trypsin and chymotrypsin enhance attachment of viruses, for instance, adenovirus type 2 (90). This may be caused by a change in cell surface charge or by the removal of glycocalyx components which may expose additional receptor sites. Trypsin and chymotrypsin

treatment of cells inhibits the attachment of many viruses including herpes simplex virus (10) and human rhinovirus (66). Proteolytic enzymes may cause inhibition of attachment not only by removing peptides but also by releasing glycopeptides containing carbohydrate moeities which may be involved in attachment.

Some cellular receptors for viruses are inactivated by glycosidases. Neuraminidase, originally known as receptor destroying enzyme releases NANA and other sialic acids from many glycoproteins and glycolipids. Many viruses require sialic acid for attachment and neuraminidase treatment of cell surfaces inhibits the attachment of these viruses. There are also other specific glycosidases at the researcher's disposal to remove specific sugars from lipids and proteins. Lipases may be used to inactivate any lipid moeities of cellular receptors for viruses.

iv) Comparative studies and the use of biological agents.

Useful information pertaining to attachment can be obtained by comparing the attachment of related viruses to different host cells. The red blood cell has been greatly used because of its relatively simple structure and the ease of membrane preparation (76). Attachment can be assayed easily by haemagglutination with erythrocytes although attachment of viruses to host cells may not involve the same type of mechanism. Comparison of erythrocytes and host cell receptors may reveal common features in the receptors that are particularly important in the attachment interaction. As described for picornaviruses, receptor families exist amongst

related viruses. Different viruses may attach to the same receptor on a host cell and the same virus may attach to a range of receptors on cells. Analagous to the erythrocyte - host cell situation, if chemical structures for different cellular receptors for the same virus are known, any identical or similar segments in the molecules may be the site of attachment. Similarly investigating common features of receptors for the same virus on host cells of different species could considerably increase our knowledge of viral attachment. Antibodies against virus can interfere with attachment to host cells but neutralized NDV can still attach to host cells (97). Poliovirus neutralized with a 7S antibody can attach to cells but when neutralized with 19S antibody cannot (72). Antiviral and antireceptor antibodies may be useful hosts in viral attachment research.

v) Isolation of cellular receptors for viruses.

The cellular aspect of viral attachment can be investigated using whole cells, isolated plasma membranes or solubilized receptors. Glycoprotein receptors may be isolated using phenol, pyrimidine or butanol extraction (50). Lipid receptors may be isolated using chloroform-methanol extraction (122) or other methods. The effect of enzymes and chemical substances on the activity of solubilized receptors can be tested in the same way as on receptors in situ. Isolated receptors can be tested for inhibitory properties against haemagglutination and attachment. Once the receptor is isolated it can be characterized

more easily than in situ on the cell surface. Some viral receptors also serve as lectin receptors, for example, the erythrocyte receptor for influenza virus is also a wheat germ agglutinin receptor (75). This factor can be utilized in the preparation of purified receptor by affinity chromatography. To elucidate the biochemical nature of any virus–cell surface interaction it is essential to isolate the receptor and characterize it.

7. Experimental strategy

i). The Krebs cell surface

The first part of the research was concerned with the Krebs cell surface, about which, little information was available. Initially a method was devised for the isolation and purification of Krebs cell plasma membranes. The purity of the membranes was assessed using marker enzyme assays and by electron microscopy.

The plasma membranes were then examined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using stains for proteins and glycoproteins. Attempts were made to radioactively label the cell surface with ^{125}I. There was the possibility that the EMC virus receptor might be labelled but even though it was not labelled, iodinated membranes were useful in other experiments.

The plasma membrane by itself does not comprise the complete Krebs cell surface. A glycocalyx, containing proteins, was isolated

from the Krebs cell surface and characterized by SDS-PAGE using stains for proteins and glycoproteins. With some insight into the composition and structure of the Krebs cell surface it was possible to continue with the second stage of the research which was to look at EMC virus attachment to the Krebs cell surface.

ii) The attachment of EMC virus to Krebs cells.

Initially several experiments were performed which demonstrated that the EMC virus receptor is located in the plasma membrane rather than the glycocalyx. The attachment of radioactively labelled EMC virus and the growth of EMC virus, using Krebs cells with and without the glycocalyx was examined. Isolated glycocalyx fractions were tested for their ability to inhibit EMC virus haemagglutination, that is, to find out if a glycocalyx component was competing with erythrocytes in virus binding.

Having ascertained the location of the EMC virus receptor on Krebs cells, experiments were devised to determine the nature of this receptor. Since the receptor is an integral part of the plasma membrane both purified plasma membranes and whole cells were used in attachment experiments. The use of radioactively labelled virus was a convenient method for measuring attachment but the attachment of infectious virions was measured using non-radioactive EMC virus, and by quantitating in terms of plaque forming units. There is evidence that the erythrocyte receptor for EMC virus is a glycoprotein (30) and therefore emphasis was placed on the effect

of neuraminidase and trypsin treatment of cells on EMC virus attachment.

The effect of trypsin and neuraminidase treatment of cells on EMC virus attachment implicated a sialoglycoprotein as the EMC virus receptor on Krebs cells. The next stage of the research, therefore, was concerned with a solubilized glycoprotein fraction which was obtained from Krebs cells. This glycoprotein fraction had receptor properties which were revealed by testing for its ability to inhibit the attachment, infectivity and haemagglutination of EMC virus. The glycoprotein preparation's susceptibility to neuraminidase, trypsin and other treatments was used in its characterization. Finally SDS-PAGE was used to examine the glycoprotein fraction and to estimate its molecular weight and other properties compared with that of the proteins and glycoproteins found in the Krebs plasma membrane.

Materials and Methods

Growth and Harvesting of Krebs ascites tumour cells

Materials

Phosphate buffered saline, (PBS), (29) was prepared from solutions A and B. Solution A contained 40g NaCl, 1g KCl, 5.75 g Na_2HPO_4 and 1g KH_2PO_4 in 4 litres of distilled water. Solution B contained 0.5 g of dried $CaCl_2$, 0.5 g $MgCl_2$ $6H_2O$ and distilled water to a volume of 1 litre. These solutions were sterilized separately by autoclaving at 121°C for 20 minutes. After sterilization the solutions were combined and 500,000 units of penicillin and 1 mega-unit of streptomycin were added. The pH which should be pH 7.3, was checked with a Beckman digital pH meter. Calcium and magnesium free PBS (Ca^{++} - Mg^{++} free PBS), was prepared identically to PBS except that solution B was omitted and solution A was made up to 5 litres. Trypan blue was a 0.1% solution in PBS, filtered through Whatman number 1 paper.

Method

Krebs ascites tumour cells were grown in genetically heterogenous, albino mice of approximately 30g in weight. Either 0.1 ml of a washed cell suspension or 0.2 ml of a frozen cell suspension each containing 10^8 cells/ml was injected intraperitoneally into each mouse. The cells were harvested aseptically into $CA^{++}Mg^{++}$ free PBS 7 to 8 days after injection. The cells were washed once in $Ca^{++}Mg^{++}$ free PBS by sedimenting at 500 to 1000 rpm for 5 minutes and resuspending them in PBS. The cells were washed at least twice more in PBS until all the erythrocytes were removed. Washed,

packed cells were resuspended in an equal volume of PBS and a sample was counted by diluting firstly, 10 fold in PBS and secondly, a further 10 fold in 0.1% trypan blue. This sample was counted in a haemocytometer. The percentage of "dead", trypan blue staining, cells was calculated and the concentration of viable cells adjusted to 10^8 cells/ml. Up to 10% of the cells staining with trypan blue was considered acceptable for use.

Growth of EMC virus (99)

Materials

The Earles saline growth medium contained:

100 ml of 10X concentration of Earle's saline.

50 ml of 44% sodium bicarbonate gassed with carbon dioxide for about half an hour immediately prior to use.

10 ml of inactivated horse serum.

Sterile distilled water to a volume of 1 litre.

0.1% trypan blue in PBS, filtered through Whatman number one paper, was used for counting cells.

Method

The Earle's saline growth medium was warmed to 37°C. Krebs cells, at a concentration of 10^8 cells/ml were infected with virus at a multiplicity of infection of 3. The multiplicity of infection is the total number of plaque forming units of virus per total number of viable cells. After maintaining the virus-cell mixture at 4°C for 30 minutes the cell suspension was diluted to 10^7 cells/ml with the previously warmed Earle's saline medium and distributed in Erlenmeyer flasks such that the culture occupied 10% of the flask's

volume. The flasks were sealed with screw caps and swirled at 75 revs/min at 37°C. An uninfected control was prepared in a similar way. After overnight incubation, 0.1 ml of well-mixed culture was diluted with 0.9 ml of 0.1% trypan blue. When the percentage of stained cells was 80% or more of the total cell number in the control, which should contain not more than 10% stained cells, virus growth was considered complete. If incomplete, the cultures were incubated further and counts repeated at 1 hour intervals until 80% of the cells were stained. Preparations at this stage were termed "crude" virus. To grow radioactively labelled virus, ^{3}H amino-acids or ^{3}H adenosine were added to the cell cultures to give a final concentration of 5μCi per ml of culture, three hours after incubation had begun. Virus was stored at -15^{o} to -20^{o} until used.

Purification of EMC virus

Materials

pp8 NaCl buffer was prepared by dissolving 178.43 g $Na_4P_2O_7.10H_2O$, 34.83g K_2HPO_4 and 11.69g NaCl in water, adjusting the pH to pH8.0 with concentrated H_3PO_4 and making the volume up to 2l with distilled water.

2.5 M phosphate solution contained 435.45g K_2HPO_4 per litre, adjusted to pH7.5 with concentrated H_3PO_4. If necessary, this solution was filtered through Whatman number 1 paper.

The organic solvent was a mixture of one volume of butoxyethanol and two volumes of ethoxyethanol.

0.5 M phosphate buffer was made up of two solutions: 70.98 g Na_2HPO_4 per litre of distilled water and 69.0g $NaH_2PO_4.H_2O$ per litre of

distilled water, which were mixed together to give a solution of pH8.0.

NaCl-PO_4 buffer consisted of 4 ml of 0.5 M phosphate buffer and 10 ml of 1.0 M NaCl made up to 100 ml with distilled water.

Ribonuclease 100mg/ml in water, of activity 2,700 units/mg (Worthington Biochemical Corporation).

Trypsin 2.5 mg/ml in water, of activity 8575 BAEE units/mg solid. (Sigma Co.).

Method

Crude virus, 1-2 l in volume, was clarified by centrifugation at 10,000 g in 250 ml centrifuge bottles for 15 minutes in the cold to remove cellular debris, including mitochondria. The supernatant fluid containing the virus was transferred to a large beaker standing in ice on a magnetic stirrer. When the virus fluid was at 0°C, cold 10% acetic acid was added rapidly with stirring to bring it to pH4.8. Precipitation was allowed to occur for 15 minutes at 0°C. The precipitate was collected by centrifugation at 1000g for 10 minutes in the cold and the supernatant fluid discarded. The pellets were each resuspended in 5 ml of pp8-NaCl and then combined. The centrifuge bottles were rinsed out with pp8 NaCl until the total volume of virus suspension was 40ml. The suspension was homogenized with a Tissumizer (Tekmar Co, Super Dispax tissumizer). At this stage virus may be stored overnight at 4°C or for longer at -20°C. To one volume of homogenized virus in a 250ml measuring cylinder, one volume of 2.5M phosphate and 0.8 volumes of organic solvent were added. The cylinder was inverted 20 times to mix the contents which were then transferred to 150ml glass bottles and centrifuged at 4,000 g for 5 minutes in the cold. Using a Pasteur pipette, the upper and lower layers were completely removed by aspiration.

leaving the virus which was in the form of a precipitate at the interface. These interfaces were combined and resuspended in about 30ml of pp8 NaCl in two 30ml corex centrifuge tubes using a tissumizer. The suspension was clarified at 10,000g for 15 minutes in the cold. The supernatant fluid containing virus was stored in the cold whilst the precipitate was washed by two successive homogenizations and centrifugations using about 30ml of pp8 NaCl each time. The supernatants were combined and as soon as possible centrifuged at 105,000g in a Spinco SW40Ti rotor for 90 minutes. The supernatant fluid was discarded and the virus pellets covered with 0.5ml pp8 NaCl. The pellets were left at 4°C for 2 hours or overnight to soften. The pellets were treated with 0.1ml of RNase at 37°C for 30 minutes followed by 0.1ml of trypsin at 37°C for 30 minutes. The pellets were resuspended with a Pasteur pipette and combined in a 15ml corex centrifuge tube. The suspension was clarified by centrifugation at 10,000g for 10 minutes. The supernatant fluid was transferred to clean SW40Ti tubes whilst the pellet was washed with ppNaCl used to rinse the tubes from its first ultracentrifugation. The pellet was washed in this way and centrifuged at 10,000g for 10 minutes twice. The washings were added to the supernatant fluid and centrifuged for 90 minutes in a Spinco SW40Ti at 105,000g. The tubes were drained well and 1ml of NaCl-PO_4 was added to each of the virus pellets which were left to soften for at least 2 hours or overnight at 4°C. The virus pellets were resuspended 1 ml of NaCl-PO_4 buffer and 10 ml was diluted in 1 ml of NaCl-PO_4 to estimate the virus concentration; 100 µg/ml virus has an extinction of 0.77 at 260 nm wavelength (13). The virus was stored at -20°C.

Haemagglutination assay (H.A.)

Materials

Phosphate buffered saline - glucose-gelatin (PBS-g-g) contained 50% PBS, 50% of 4.5% glucose and 5% of a 1% solution of gelatin.

Method

Sheep or human blood was washed in PBS-g-g by centrifugation for 5 minutes at about 3000 rpm in a graduated conical centrifuge tube until there was no sign of haemolysis. A 0.2% suspension of red blood cells was made by diluting 100 µl of washed, packed cells to 50 ml with PBS-g-g. V-shaped micro-titer plates (Dynatech Laboratories) were placed on damp paper towels to reduce the static electricity and then used for the assay. One drop, (50 µl) of PBS-g-g was added to each cup on the plate. One diluter full (50 µl) of virus was added to the first cup in the row, mixed and removed from the cup. It was then transferred to the next cup and the procedure repeated until the virus is diluted to the last cup in the row when the diluter was removed full, dried, flamed and cooled in water. The procedure was repeated in an adjacent row using virus of known HA titre and a row was prepared with diluent alone. One drop (50 µl) of 0.2% red blood cell suspension was added to each cup. The tray was covered and kept for at least 5 hours at 4°C before reading. The endpoint is the highest cup showing a trace of haemagglutination. The number of HA units per ml is the dilution of the virus at the endpoint divided by 50 µl, the volume of the diluter.

Inhibition of haemagglutination (HAI)

Materials: PBS-g-g as described for HA

Method

If a particular substance was a receptor for virus attachment it would be expected to compete with the erythrocytes in the HA in virus binding. There would, therefore, be less available virus to cause haemagglutination which would be inhibited. This technique can thus be used to assay for putative solubilized cellular receptors for viruses. The assay was prepared in a similar way as for HA with PBS-g-g added to each cup and virus diluted up the HA plate. Using 50µl diluters the potential receptor was diluted at a 90° angle across the plates producing a gradation of inhibitor concentration and virus concentration at right angles across the same plate. In the first cup, which is diluted in two directions, twice as much virus is added, that is 100µl of virus suspension and no PBS-g-g. On each plate two control rows contained virus and no inhibitor to determine the virus' HA titre and two control rows contained just the inhibitor to check for non-specific haemagglutination. A 0.2% suspension of red blood cells was added to each cup and the plate incubated at 4°C for at least 5 hours. If the substance tested was an inhibitor of haemagglutination, a gradation in HA titre was seen across the tray, the highest HA titre being seen at the lowest concentration of inhibitor. Maximum HA titre would occur in the virus control with no inhibitor. An example of the results which may be expected is shown in Fig. 1. Plate A shows the HA pattern using a substance which was not an inhibitor of HA so that the titres of the test rows were virtually identical to that of the virus control HA. Plate B shows the HA pattern in the presence of an inhibitor, the HA titres of the

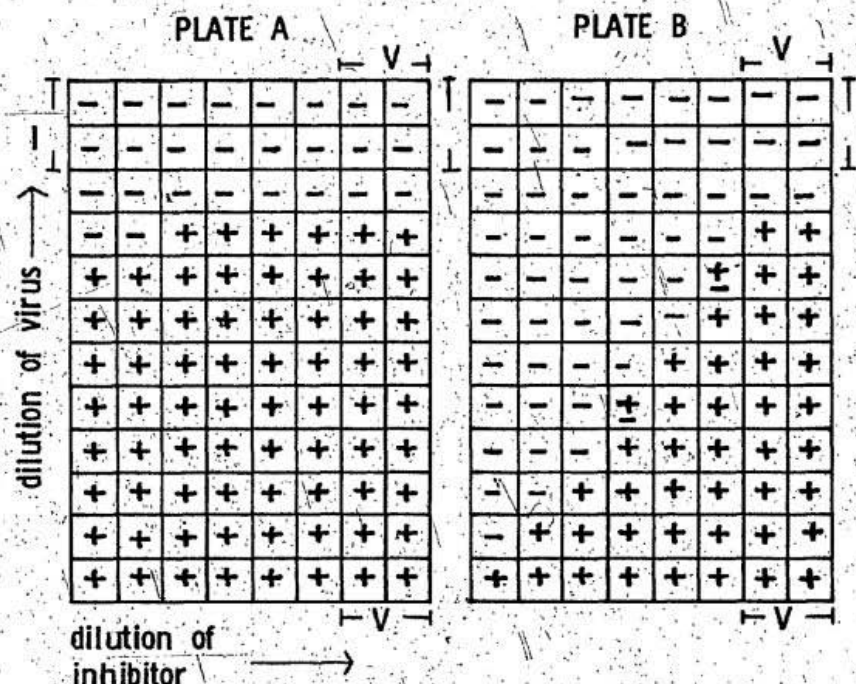


Figure 1. Inhibition of haemagglutination. Plate A, an example of a poor inhibitor, shows very little inhibition of haemagglutination. Plate B, however, is an example of a good inhibitor and shows considerable inhibition of haemagglutination. The potential inhibitor is diluted across the tray and the virus is diluted up the tray. I- represents the inhibitor control, i.e. no virus is present, V- represents the virus control, i.e. no inhibitor is present, +represents haemagglutination, - represents no haemagglutination and $\pm$ represents a trace of haemagglutination.

test rows being reduced compared with virus control to an extent depending upon inhibitor concentration. Inhibition was quantified by determining the number of cups that were not showing HA but that are within the range of haemagglutination of the virus control. Plate A (Fig. .1) shows 2 cups of inhibition out of 54 cups which is the maximum number of cups which could be inhibited. Plate B shows 30 cups of inhibition out of a possible 54 cups. From these results the percentage inhibition was 2.16% and 55.5% for plates A and B respectively and this was mostly the way in which HAI results were expressed. If an inhibitor was treated in such a way as to reduce its inhibitory capability the results were expressed as the percentage of inhibition relative to the percentage inhibition in an untreated control.

Plaque assay (99)

Materials

Earle's saline, 2x concentration was made from the following three solutions.

Solution A contained 13.6g NaCl, 0.8g KCl, 0.4g $MgSO_4.7H_2O$ made up to a volume of 700 ml with distilled water.

Solution B contained 0.316g $NaH_2PO_4.2H_2O$ and 4.0g glucose made up to 200 mls with distilled water.

Solution C contained 0.4g $CaCl_2$ (dried) made up to 100ml with distilled water. The solutions were autoclaved separately at 121°C for 20 minutes. Solutions A and B were mixed and solution C was added with vigorous mixing. The solution was filtered through Whatman number 54 paper if a precipitate formed. Penicillin (200,000 units) and

streptomycin (400,000 units) were added to the solution.

Stock solution of Pardee's buffer (16) was made by dissolving 6g of thiourea (thiocarbamide) in 1630 ml glass distilled water before adding 2400 ml of diethanolamine (dihydroxy-diethylamine). To the mixture was added 5g of activated charcoal and after 15 min to allow for decolourization, the mixture was filtered slowly through Whatman number 54 filter paper until about 2% was collected. This filterate was returned to the original vessel and filtered again. The stock solution was stored in a screw capped bottle at 37°C.

Pardee's buffer for use was prepared by mixing 400 ml of Pardee's stock solution with 200 ml of 6M HCl. To this was added 120g of potassium bicarbonate previously crushed to a powder and the mixture was stirred in a stoppered bottle until all the potassium bicarbonate had dissolved. This solution was also stored at 37°C in a stoppered bottle.

2% agar in water

4.4% sodium bicarbonate with carbon dioxide bubbled through for at least 30 minutes immediately prior to use. Neutral red was a 1:100 dilution in water of 1% stock solution.

Method

Agar medium was prepared by mixing 50ml of 2X Earle's saline and 50ml of melted 2% agar, both at 44°C in a water-bath, with 5ml of sodium bicarbonate. The agar medium was distributed in 5ml quantities to 6cm diameter plastic petri dishes to set. To 0.7 ml samples of washed Krebs cells containing 10^8 cells/ml, 0.1 ml of 10^{-4} to 10^{-6} dilutions of virus was added. Each tube containing this mixture was transferred to the 44°C waterbath, 1 ml of

agar added with mixing and the contents of the tube were immediately poured on to the surface of previously prepared agar surface in a petri dish. Each dilution was plated out, at least in duplicate but preferably in triplicate. The plates were incubated at 37°C in a plastic box sealed by standing it in a shallow tray of water and containing Pardee's buffer in a large glass petri dish to give an atmosphere 5% in carbon dioxide. After 2 days incubation, 5 ml neutral red was added to each and staining allowed to proceed for one hour. Plaques appeared as yellow-orange spots on a pinkish red background.

Infectious centre assay

Materials

The materials used were those described for the plaque assay.

Method

This assay is carried out in a similar way to the plaque assay except that the number of infected cells is determined rather than the plaque forming units of virus. 50µl "crude" EMC virus (see p. 26) was mixed with 0.5 ml of 10^8 Krebs cells/ml and incubated at 4°C for 30 minutes. The cells were then washed three times by centrifuging them at 1000 rpm for five minutes and resuspending in PBS to remove unattached virions. Instead of a virus dilution series, which is used in plaque assays, a dilution series of infected cells was prepared and 10^4, 10^3 and 10^2 concentrations of infected cells were plated out in the way described for the plaque assay. The plates were incubated and plaque numbers recorded as described for the plaque assay.

Preparation of Krebs plasma membranes.

Materials

0.125M sucrose – 0.075 M KCl solution

1.5M sucrose

0.75M sucrose - 0.5M KCl solution

0.01M tris HCl pH8.0

30% sucrose in 0.01M tris HCl pH8.0

45% sucrose in 0.01M tris HCl pH8.0

Method

The technique used was a modification of Aitkinson's method for preparing Hela cell plasma membranes (1). Approximately 20 ml of 10^8 Krebs cells/ml were centrifuged at 50-150g (500-1000 rpm) for 5 minutes and resuspended in 0.125M sucrose-0.075M KCl solution before centrifuging again at 1500g (3,000 rpm) for 5 minutes. The packed cell volume was noted and the cells resuspended in 4.5 volumes of ice cold distilled water, followed by recentrifugation at 1500g for 3 minutes. The volume of water absorbed by the cells, that is the increase in packed cell volume, was measured. Ten times the original packed cell volume, minus the volume already absorbed, of ice cold distilled water was added and the cells were allowed to swell on ice for five minutes. Sufficient 1.5M sucrose was added to the cell suspension to give a final concentration of 0.1M sucrose. The cells were left to stand on ice for a further five minutes before being disrupted with seven strokes in a Potter Elvehjem tissue grinder with a teflon pestle. To stabilize the free nuclei 0.75M sucrose - 0.5M KCl solution was immediately added to give a concentration of 0.1M potassium chloride. The resulting homogenate was centrifuged

at about 400g for one minute to collect whole cells and nuclei which were washed twice in 0.01M tris-HCl, pH8.0. The combined supernatant and washes were centrifuged at 600g for 12 minutes to collect the crude plasma membrane fraction. This pellet was resuspended in a small volume of 0.01M tris-buffer, pH8.0 and layered on to discontinuous 30-45% sucrose (in 0.01M tris buffer pH8.0) gradients made by layering 15 ml of 30% sucrose on 5 ml of 45% sucrose in a 30 ml corex centrifuge tube. The gradients were centrifuged at 9,000g for 15 minutes. The plasma membranes, which formed a creamy band at the 30-45% sucrose interface, were collected, diluted in 0.01M tris buffer, pH8.0 and centrifuged at 9,000g for 5 minutes to wash away the sucrose. The sucrose gradient step was repeated and followed by at least two washes of the membranes in 0.01M tris buffer pH8.0 to remove sucrose. Purified plasma membranes were stored frozen at -15°C. The concentration of plasma membranes was estimated by protein determination.

Preparation of human erythrocyte membranes

Materials

Isotonic buffer consisted of 174.0g of NaCl and 14:2g of Na_2HPO_4 made up to 20ℓ with distilled water.

Hypotonic buffer consisted of 14.2g of Na_2HPO_4 made up to 20ℓ with distilled water.

Both of these solutions were adjusted to pH8.0 using 85% phosphoric acid.

Method

The anticoagulant was removed from the human erythrocytes by mixing

10 ml packed volume of red blood cells with a small volume of isotonic buffer and by centrifuging at 3,000 rpm (1500g) in a bench centrifuge. The cells were washed a second time before being lysed by resuspension in a large volume of hypotonic buffer. Contaminating haemaglobin was removed by several washes consisting of resuspension of the membranes in hypotonic buffer and centrifugation at 7,700g for 20 minutes. When the membrane pellet was free from red coloration the membranes were concentrated into a small volume by centrifuging at 12,000g for 20 minutes. Erythrocyte membranes were stored frozen at -15°C. Again the membrane concentration was estimated by determination of protein.

Protein determination

Protein determinations were done using the method of Lowry (69).

Materials

Solution A 2% Na_2CO_3 - 0.02% NaK tartrate in 0.1M NaOH

Solution B 0.5% $CuSO_4$ in water.

Solution C Solutions A and B mixed in the ratio 50:1.

Solution D 2M Folin reagent freshly diluted in a 50:50 ratio with water

Solution E Bovine serum albumin solutions, 100-500µg as standard solutions.

Method

Samples of 20-50 µl of plasma membrane suspensions in 0.01M tris pH buffer pH8.0 (in the case of Krebs plasma membranes) were made up to 0.2 ml. To each of duplicate membrane samples 1 ml of freshly

prepared solution C was added. After standing at room temperature for ten minutes, 0.1 ml of solution D was added and the colour allowed to develop for 30 minutes before reading at 660 nm wavelengths. Standard solutions, E and a water blank were assayed in the same way to give a standard curve from which unknown values could be read.

Sialic acid determination

The thiobarbituric acid method of Warren (119) was used to determine sialic acid content.

Materials

0.2M sulphuric acid

Solution A was 0.2M sodium - m - periodate in 9M phosphoric acid.

Solution B was 10% sodium arsenite in 0.5M sodium sulphate and 0.1M sulphuric acid.

Solution C consisted of 0.6% thiobarbituric acid in 0.5M sodium sulphate.

Cyclohexanone

Method

This method detects only free sialic acid which was released from 0.1 ml of plasma membrane suspension by hydrolyzing with an equal volume of 0.2M H_2SO_4 at $80^{o}C$ for 1 hour. To each sample, 0.1 ml of solution A was added and the samples were shaken and allowed to stand at room temperature for 20 minutes before adding 1.0 ml of solution B. The yellow-brown colour formed disappeared after shaking and 2.5 ml of solution C was added. The samples were mixed

and heated in a boiling waterbath for 15 minutes to develop the colour which was extracted by shaking with 1.5 ml of cyclohexanone and centrifuging to separate the two layers. The absorbance of the top layer was read at 549 nm wavelength. To obtain a standard curve known concentrations of N-acetyl neuraminic acid (NANA) ranging from 10-40 µg/ml were also assayed.

Phosphorus assay (17)

Materials

Phosphate stock solution - 0.01M KH_2PO_4

Solution A was 10M H_2SO_4

Solution B was 2.5% ammonium molybdate

Solution C was phosphorus reducing agent, that is one envelope of 1-amino-2naphthol-4 sulphuric acid dry mixture in 100 mls of hot distilled water, filtered when cool. (Fisher Scientific Co).

Method

Standard solutions were made by diluting the phosphate stock solution in the ratios of 1:100 and 1:50 with distilled water. Duplicate 2 ml samples of standards and water blanks were assayed together with unknown which were diluted to 2 ml so that they were within the range of the standards. To these samples, 0.1 ml of solution A was added followed by 0.2 ml of solution B, and then 0.1 ml of solution C with mixing after each addition. The colour was allowed to develop for at least 10 minutes before reading the absorbance at 660 nm wavelength.

Enzyme assays

The following enzymes have been reported to be markers for different cell organelles in some cell types. Magnesium stimulated $Na^{+}K^{+}$ dependent adenosine triphosphate (ATPase) (48), 5' nucleotidase (103) and alkaline phosphatase (33) are plasma membrane markers. Reduced nicotinamide-adenine dinucleotide (NADH) oxidoreductase (19) and glucose-6-phosphatase (113) are endoplasmic reticulum markers and succinic dehydrogenase (42) is a mitochondrial marker enzyme. Various cell fractions were examined for those enzymes to monitor purity.

Mg^{++} stimulated, $Na^{+}K^{+}$ dependent ATPase (48)

Materials

The Na^{+} series solution consisted of:

0.4 ml of 0.25M NaCl-0.00138M $MgSO_4$

0.4 ml of the Na^{+} salt of ATP (freshly prepared)

0.15 ml of 0.0625M tris HCl pH8.6 - 3.12×10^{-4} M NaEDTA

The $Na^{+}K^{+}$ series solution consisted of:

0.4 ml of 0.25M NaCl-0.0125M KCl-0.00138M $MgSO_4$

0.4 ml of the Na salt of ATP (freshly prepared)

0.15 ml of 0.0625M tris HCl pH8.6 - 3.12×10^{-4} M NaEDTA.

10% trichlorocicetic acid in water (TCA)

Reagents for phosphate determination

Method

To both the Na^{+} and the $Na^{+}K^{+}$ series solutions 50 µl of plasma membrane suspension was added. After incubation at 37°C for 30 minutes the reaction was stopped by the addition of 1 ml of cold TCA.

The samples were centrifuged to remove any precipitate and the phosphate concentration in the supernatant fluid determined. The activity of the enzyme was defined in terms of number of μ moles of phosphate released per hour per mg of protein.

5' Nucleotidase

The method of Bodansky and Schwartz (103) was used.

Materials

The reaction mixture contained the following substances.

10 μl of 0.1M KCl

50 μl of 0.1M MgCl

100 μl of 50 mM adenosine monophosphate (AMP) in

0.1M tris HCl pH8.6

0.55 ml of 0.1M tris HCl pH8.4

10% TCA.

Reagents for phosphorous determination

Method

The above mixture was freshly prepared and 0.1 ml of membrane suspension was added. The mixture was incubated at 37°C for 15 minutes, at which time, the reaction was stopped by the addition of 1 ml of cold TCA. Any precipitate was removed by centrifugation prior to phosphorous determination of the samples. The enzyme activity was expressed as the number of μmoles of phosphorous released per hour per mg of protein.

Glucose-6-phosphatase (113)

Materials

0.1M citrate buffer pH6.5 was made by acidification of sodium citrate with 1M citric acid.

0.001M glucose-6-phosphatase

10% TCA

reagents for phosphorous determination

Method

To 0.2 ml of citrate buffer were added 0.1 ml of glucose-6-phosphate and 0.1 ml of membrane suspension.. This reaction mixture was incubated at 37°C for 30 minutes when the reaction was stopped with 1ml of cold TCA. Any precipitate formed was removed by centrifugation and the supernatants were made up to 2 ml with distilled water for phosphorous determination. The enzyme activity was expressed in number of μ moles of phosphorous released per hour per mg protein.

NADH oxidoreductase (19)

Materials

The reaction mixture contained the following:

0.4 ml of 0.02M tris-HCl pH7.4

0.4 ml of NADH (2 mg/10 ml in 0.02 M tris HCl pH 7.4)

0.2 ml of 0.0033M potassium ferricyanide

Method

The above reagents were combined in a 1 ml cuvette and 50 μl (4-20 μg protein) of membrane suspension was added. The solution was quickly mixed by inversion and readings of absorbance at 340 nm wavelength were taken at 15 second intervals over a 2-5 minute period against a blank containing all of the above except potassium ferricyanide. Generally, the decrease in absorbance at 340 nm is linear against time until about 80% of the NADH is oxidized. The extinction coefficient is 6.22×10^3 litre $mole^{-1} cm^{-1}$ and the specific enzyme activity is the micromoles of NADH oxidized per hour per mg of protein.

Alkaline phosphatase (33)

Materials

The following comprise the reaction mixture:

0.5 ml of 1M $MgCl_2$

0.4 ml of 0.5M tris-HCl, pH 10.0

0.1 ml of 100mM *p*-nitrophenyl phosphate

0.75 M sodium carbonate

Method

The first three solutions were combined with 50 µl of membrane suspension (0.02-0.20 mg protein) and incubated at 37°C for 30 minutes. The addition of 1 ml of 0.75M sodium carbonate stopped the reaction and the resultant precipitate was removed by centrifugation. The absorbance at 420 nm wavelength of the supernatant fluid was read against the blank containing the same solutions but no membrane suspension. The specific activity defined as µmoles of nitrophenol consumed per hour per mg of protein was calculated using the extinction coefficient of 1M nitrophenol = 1.69×10^4 OD 420.

Succinic dehydrogenase

The reduction of indophenol was used to detect succinic dehydrogenase activity (42)

Materials

The following solutions were combined in a cuvette:

100 µl of 10X concentration of Ca^{++} Mg^{++} free PBS, ie 10 µl moles of phosphate at pH 7.3)

50 µl of bovine serum albumin at 10 mg /ml concentration

50 µl of 40 mM KCN

50 µl of 0.2M sodium succinate

50 µl of 0.4 mg/ml indophenol.

Method

To the above solutions were added 100 µl of membrane suspension and the mixture was made up to 1 ml with distilled water. The contents of the cuvette were mixed by inversion and the absorbance at 600 nm wavelength was recorded every 30 seconds against a blank which gave a maximum reading and which contained all of the above solutions minus the succinate. The change in absorbance divided by 19.1 gives the µ moles of indophenol reduced. The specific enzyme activity was expressed as the µ moles of indophenol reduced per hour per mg of protein.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The method of Fairbanks et al (31) was used to carry out SDS-PAGE.

Materials

Con Ac Bis stock solution contained 40g of acrylamide and 1.5g bis made up to 100 ml with distilled water.

Electrophoresis buffer 10x concentration was made by combining 40 ml 1.0M tris, 10 ml 2.0M sodium acetate, 10 ml 0.2M EDTA, acetic acid to give pH 7.4 and water to a volume of 100 ml.

Electrophoresis buffer for use contained 10% of 10x concentration electrophoresis buffer and 1% SDS in water.

Gel Solution; 1.4 ml of Con Ac Bis stock solution, 1.0 ml 10% SDS (w/w), 5.1 ml distilled water, 1.0 ml 1.5% ammonium persulphate (w/v) and 0.5 ml of 0.5% Temed (w/v) was mixed to give a solution 5.6% in

acrylamide and 10 mls in volume which is sufficient for 4 gels.

Gel overlay contained 0.1% SDS, 0.15% ammonium persulphate and 0.05% Temed in water.

Degradation buffer contained 1% SDS, 5-10% sucrose, 10 mls tris HCl pH 8.0, 1mM EDTA pH8.0 and 4mM dithiothreitol (DTT) in water.

Tracking dye was a 0.1% of pyronin Y in water.

Coomassie blue dye was a 0.25% solution in methanol and acetic acid.

Periodic acid Schiff's (PAS) reagent was prepared by dissolving 2.5g of basic fuchsin in 500 ml of distilled water, then adding 5g of sodium metabisulphite and 50 ml of 1.0M HCL. The solution was stirred for several hours and then decolorized with approximately 2g of activated charcoal.

Method

The gels were made by adding 2.5 ml freshly prepared gel solution to stoppered glass tubes of internal diameter of 6mm. The gel solution was covered with gel overlay until polymerization occurred which takes approximately 45 minutes. The gel overlay was then carefully removed and replaced with electrophoresis buffer, and the gels were left overnight before use. Suspensions of purified Kreba plasma membranes, Krebs cell fractions or purified erythrocyte membranes of 0.5 - 40 mg protein/ml in a volume not greater than 100 µl were prepared for electrophoresis by incubating with an equal volume of degradation buffer. Before layering the samples on gels, 5-10 µl of tracking dye was added to each sample. Electrophoresis buffer was used to fill the chamber so that the tops of the gels were covered. Electrophoresis was carried out using 20 volts at 8 m amps per tube which contained a gel. In most cases,

electrophoresis was carried out for 2 hours, by which time the tracking dye had reached within 1 cm of the bottom of each gel. The gels were then removed from the tubes and placed in a mixture of 25% isopropyl alcohol and 10% acetic acid in water, in 250 ml conical flasks and swirled at 37°C overnight to remove the SDS. Two different staining techniques were employed. Coomassie blue stain was used to detect proteins by covering each gel with coomassie blue stain in a 15 ml culture tube for three hours. The stain was removed and retained for further use whilst the gels were placed back in the flasks and destained in 10% acetic acid and 5% methanol in water overnight. This solution was changed several times during destaining. For the PAS staining procedure the gels were kept in the flasks, after SDS removal, and incubated in 0.5% periodic acid for 2 hours at 37°C with swirling followed by 0.5% sodium arsenite in 5% acetic acid for 1 hour and then two periods of 20 minutes in 0.1% arsenite in 5% acetic acid. Finally the gels were incubated in 100% acetic acid for 10 minutes. The gels were transferred to 15 ml tissue culture tubes and covered with PAS stain. PAS staining was allowed to take place in the dark, overnight. PAS stained gels were destained in 0.1M H_2SO_4-0.1% sodium metabisulphite until the rinse solution failed to turn pink with the addition of formaldehyde. Both PAS and coomassie blue stained gels were scanned in a Gilford 250 spectrophotometer at 550 nm wavelength. PAS stained gels, in particular, were scanned as soon as possible because the stain was unstable and faded within a few days.

SDS-PAGE of radioactively labelled samples was also carried out. These gels were pulverized, after electrophoresis, dividing the gel into

1 mm fractions lengthwise. To each vial containing one fraction 1 ml of 1% SDS was added with vigorous mixing followed by 10 ml of Aquasol 2 (New England Nuclear). After standing overnight the samples were counted in a liquid scintillation counter (Beckman LS8100).

Radioactive labelling of the Krebs cell surface with ^{125}I.

Materials

The following reagents were combined and used to iodinate the Krebs cell surface.

25 µl of ^{125}I (50 µCi) sodium iodide (carrier free), New England Nuclear

100 µl of 1% Beta D glucose

5 µl of a 1:1000 dilution of lactoperoxidase (Sigma Chemical Co.) 0.061 units of activity/ml.

5 µl of 1:1000 dilution of glucose oxidase (Sigma Chemical Co.) from Aspergillus niger containing a final concentration of 1.4 units of activity/ml.

Also required for iodination were:

0.01 mM sodium thiosulphate

Aquasol 2 (New England Nuclear) liquid scintillation fluid.

Method

The ^{125}I, glucose, lactoperoxidase and glucose oxidase were combined with 3 ml of 10^8 Krebs cells/ml and incubated at room temperature for 30 minutes unless otherwise stated. The reaction was stopped with an equal volume of 0.01M sodium thiosulphate. The iodinated Krebs cells were disrupted and Krebs plasma membranes were prepared. Samples

were counted in 10 ml of Aquasol 2 using the combined ^{3}H and ^{14}C windows of a liquid scintillation counter.

Iodination of Krebs plasma membranes

Materials

The following reagents were combined:

25 µl of ^{125}I (50 µCi)

250 µl of 1% Beta D-glucose

5 µl of 1:1000 dilution of lactoperoxidase with the same activity as that described for labelling whole cells.

5 µl of 1:1000 dilution of glucose oxidase with the same activity as that described for labelling whole cells.

The following reagents were also used:

0.01M sodium thiosulphate

Aquasol 2 scintillation fluid

Method

The ^{125}I, glucose, lactoperoxidase and glucose oxidase were combined with 0.5 ml of plasma membrane suspension, containing 1–2 mg protein, and made up to 1 ml with 0.01M tris-HCl, pH8.0. The samples were incubated at 37°C with gentle agitation for 1 hour unless otherwise stated. The reaction was stopped with an equal volume of 0.01M sodium thiosulphate. Free radioactive iodine was removed by washing the membranes three times in 0.01M tris-HCl, pH8.0 with centrifugation at 600g for 12 minutes. The radioactivity in the washes and membranes was monitored using the liquid scintillation system previously described for radiolabelling whole cells.

Wheat germ agglutinin (WGA) Chromatography of ^{125}I labelled Krebs plasma membranes.

Materials

0.5% Triton-borate buffer contained 0.5% Triton X-100 in 0.056 M sodium tetraborate adjusted to pH8.0 with boric acid.

N-acetyl glucosamine solution contained 2.2% N-acetyl glucosamine in 0.5% Triton X-100 and 0.056M sodium borate buffer, pH8.0.

WGA Sepharose

Method

Approximately 0.2 ml of a membrane suspension was stirred gently with 14 ml of 0.5% Triton-borate buffer for 30 minutes at 4°C. Undissolved material was removed by centrifugation for 1 hour at 25000g and about 6 ml of the supernatant fluid was transferred to a lectin-affinity column of bed volume 1.5 x 12 cm pre-equilibrated with 0.5% Triton-borate buffer. The column was washed with Triton-borate buffer until the radioactivity of the eluate was reduced to background level. Material was released from the WGA-sepharose column by washing with N-acetyl glucosamine solution. Samples of the 1 ml fractions collected were counted in 10 ml Aquasol-2 in a liquid scintillation counter. The fractions comprising the radioactive peak were combined, dialysed to remove the N-acetyl glucosamine and lyophilized. The lyophilized samples were examined by sodium dodecyl sulphate

polyacrylamide gel electrophoresis.

Removal and isolation of the glycocalyx of Krebs cells

Method

This procedure was described by Rittenhouse et al (95) for Ehrlich ascites tumour cells. Krebs cells were harvested from mice individually and only those cell suspensions relatively free of erythrocytes were selected so that three washes was sufficient to produce cream-coloured cell suspension. The cell concentration was adjusted to 10^8 cells/ml and the suspension was incubated at $4^{\circ}C$ for 1 hour with gentle swirling every fifteen minutes. The cells were then centrifuged at 50-150g for 5 minutes and the supernatant recentrifuged at 48,000g for 1 hour twice. Any sediment formed was discarded. This supernatant is the crude glycocalyx fraction which may be further concentrated by centrifuging at 100,000g for 24 hours. The gelatinous pellet obtained is termed the cell coat particle and the supernatant is the cell coat fraction which contains a higher proportion of low molecular weight components than the cell coat particle.

Isolation of glycoproteins from whole Krebs cells and Krebs crude and purified plasma membranes (16, 75)

Method

Whole Krebs cells, frozen or fresh, at a concentration of 10^8 cells/ml in PBS, crude or pure plasma membrane preparations in 0.01M tris HCl pH8.0 were used for lithium diiodosalicylate-phenol extraction. A volume of LIS was added equal to the volume of cell

or membrane suspension and the mixture stirred at room temperature for 20 minutes. Twice the volume of the original cell or membrane suspension of cold distilled water was added and the mixture was stirred for a further 20 minutes whilst kept in an ice bath. The mixture was then centrifuged at 100,000g for one hour. The resultant pellet was discarded but the supernatant was mixed with an equal volume of 50% phenol solution for twenty minutes on ice. The suspension was centrifuged at 4,000g for 1 hour. Two layers formed and the top aqueous layer was removed by aspiration. This layer contained the glycoproteins and was dialysed against distilled water for about 2 days at 4°C with frequent changes of water. The material was then lyophilized and since experiments showed that the biological activity was retained best in this form glycoprotein fractions were stored lyophilized at this stage of their preparation. The material was further purified by three ethanol extractions performed by incubating the freeze-dried solid in ethanol at -15°C for 1 to 2 hours followed by centrifugation at about 3000g for 5 minutes each time. The material was dissolved in the minimum amount of distilled water possible and dialysed overnight to remove any alcohol present. The solubilized glycoprotein preparation was then ready for use.

Enzymes used during experiments

Some enzymes were used for several different experiments and their enzyme activities are listed below:

Trypsin 8575 BAEE units/mg solid (Sigma Co.)

Lima Bean Trypsin inhibitor - 1 mg inhibits 3.5 mg trypsin

(Worthington Biochemical Corporation)

Deoxyribonuclease, DNAse, 3000 units/mg (Worthington Biochemical Corporation)

Ribonuclease, RNAse, 2700 units/mg (Worthington Biochemical Corporation)

Neuraminidase 500 units/ml activity, derived from Vibrio cholerae (Calbiochem-Behring Corporation)

Commonly used experimental techniques

Washing Krebs cells

Krebs cells were washed in PBS by centrifuging at about 1,000 rpm (150g) and resuspending in fresh PBS.

Washing red blood cells

Red blood cells were washed in PBS by centrifuging at about 3,000 rpm (1500g) and resuspending in fresh PBS.

Monitoring radioactive samples

Samples of 1 ml or not more than 2 ml in volume were counted by mixing with 10 ml of Aquasol 2 (New England Nuclear) and counting in a liquid scintillation counter. Samples of red blood cells of not more than 1 ml in volume were pretreated with 0.7 ml of 30% H_2O_2 to bleach them, mixed with 10 ml of Aquasol 2 and left overnight before counting in a Beckman LS8100 liquid scintillation counter to ensure that chemical interference was at a minimum.

Results

Part 1. The Krebs ascites tumour cell surface.

I Krebs cell plasma membranes

a) Protein and sialic acid content.

All purified plasma membranes suspensions prepared from Krebs cells were assayed for their protein and sialic acid content. In addition to providing information about the composition of the plasma membrane, protein and sialic acid determinations were used to express membrane concentrations, since concentration could not be measured by counting because putified membranes readily formed clumps that were impossible to disperse and some membranes were fragmented. The protein content of membrane preparations varied with the number of cells initially used, 10 to 30 ml of 10^8 cells/ml, but gave 1 to 2 ml of membrane suspension within the concentration range of 2 to 5 mg protein/ml. Variable recoveries and the failure to obtain homogeneous membrane suspensions probably contributed to the variation in the protein concentration of membrane suspensions. The sialic acid content also differed between membrane preparations but the mean ratio of sialic acid to protein was found to be 13.6 µg sialic/mg protein with a standard error of ± 2.2 for 15 determinations. The yield of membrane preparations would never be 100%, that is, one membrane ghost per cell, because some of the cells remained unbroken, a few of the membrane ghosts fragmented and several of

the membranes would be lost during purification in order to minimize, absolutely, the contamination of the final pure membrane preparation.

b) Cellular enzyme assays

Certain enzymes have been found to be markers for different cell organelles in some cell types. Krebs cell fractions isolated during the preparation of membranes, including purified plasma membranes were examined for their enzyme content to assess any contamination by other cell components. The following cell fractions were considered. Homogenate, consisting of disrupted whole cells; the crude plasma membrane fraction, which was the pellet from centrifuging the homogenate at 600g for 12 minutes; mitochondria and microsomes, the supernatant after centrifuging the homogenate at 600g for 12 minutes; and purified plasma membranes comprising the washed material harvested from the 30-45% sucrose interface of the second sucrose gradient.

All of these fractions were assayed for alkaline phosphatase (33), 5' nucleotidase (103) and Mg^{++} stimulated Na^+, K^+ dependent adenosine triphosphatase (ATPase) (48), each of which has been found in the plasma membrane of some cell types. If present, these enzymes should be concentrated in the purified plasma membrane fraction of Krebs cells. All fractions were also tested for glucose-6-phosphatase (113) and NADH oxidoreductase (19) which are microsomal marker enzymes and succinic dehydrogenase which is a mitochondrial marker enzyme (42). The results given in

Enzyme	Specific activities of enzymes in: (moles/hr/mg protein)			
	Homogenate	Crude Membranes	Mitochondria and Microsomes	Purified Membranes
Alkaline Phosphatase	493.8	577.1	N.D.	773.3
5'Nucleotidase	0.736	3.17	2.70	3.80
Na^+, K^+ dependent ATPase	0.027	0.0481	N.D.	0.122
Succinic dehydrogenase	0.00107	N.D.	N.D.	0.000776
Glucose-6-phosphatase	0	0	0.676	0
NADH oxidoreductase	N.D.	13.59	N.D.	1.176

Table 1. Enzyme assays of fractionated Krebs cells. Samples were taken during the preparation of Krebs plasma membranes and assayed for marker enzymes. The specific activity is the amount of substrate in moles, used in 1 hour per mg protein present in the sample. In some cases, the enzyme activity could not be determined (N.D.) because turbidity in the samples interfered with absorbance readings. The results given in the table are from one particular cell sample used to prepare membranes but are a good representation of the distribution of these enzymes in Krebs cells.

table 1 are a representative example of the distribution of these enzymes in a particular sample of fractionated Krebs cells.

Alkaline phosphatase, 5' nucleotidase and Na^+K^+ dependent ATPase were concentrated in the purified plasma membrane fraction as expected. The activity of the ATPase was, however, low in all cell fractions. Succinic dehydrogenase was present in the pure membrane fraction but the concentration was lower than in the homogenate indicating that there may be slight mitochondrial contamination of purified membranes. Glucose-6-phosphatase was found only in the mitochondrial-microsomal fraction at low levels and was not detected even in the homogenate. A small amount of NADH oxidoreductase was found in the purified plasma membranes compared to the crude plasma membranes indicating that there was slight contamination of the purified membranes with microsomes.

e) Electron microscopy of Krebs plasma membranes

Krebs plasma membranes were examined under the electron microscope to look for impurities. A drop of freshly prepared purified plasma membrane suspension was placed in a petri dish and a 400 mesh "Formar", carbon coated grid was floated on top of the drop for 1 minute. The grid was dried with filter paper and stained for half a minute with 2% phosphotungstic acid at pH 6.5. Negatively stained grids were examined in a Philips EM300 electron microscope at an operating voltage of 80 Kv.

Figure 2 shows groups of whole membranes, aggregates of membranes and small vesicles of membrane fragments but no nuclei,

Figure 2. Electron micrograph of Krebs plasma membranes. Clumps of membranes, whole and fragmented membranes can be seen in this photograph which is magnified 41,500 times.

represents 1 μm

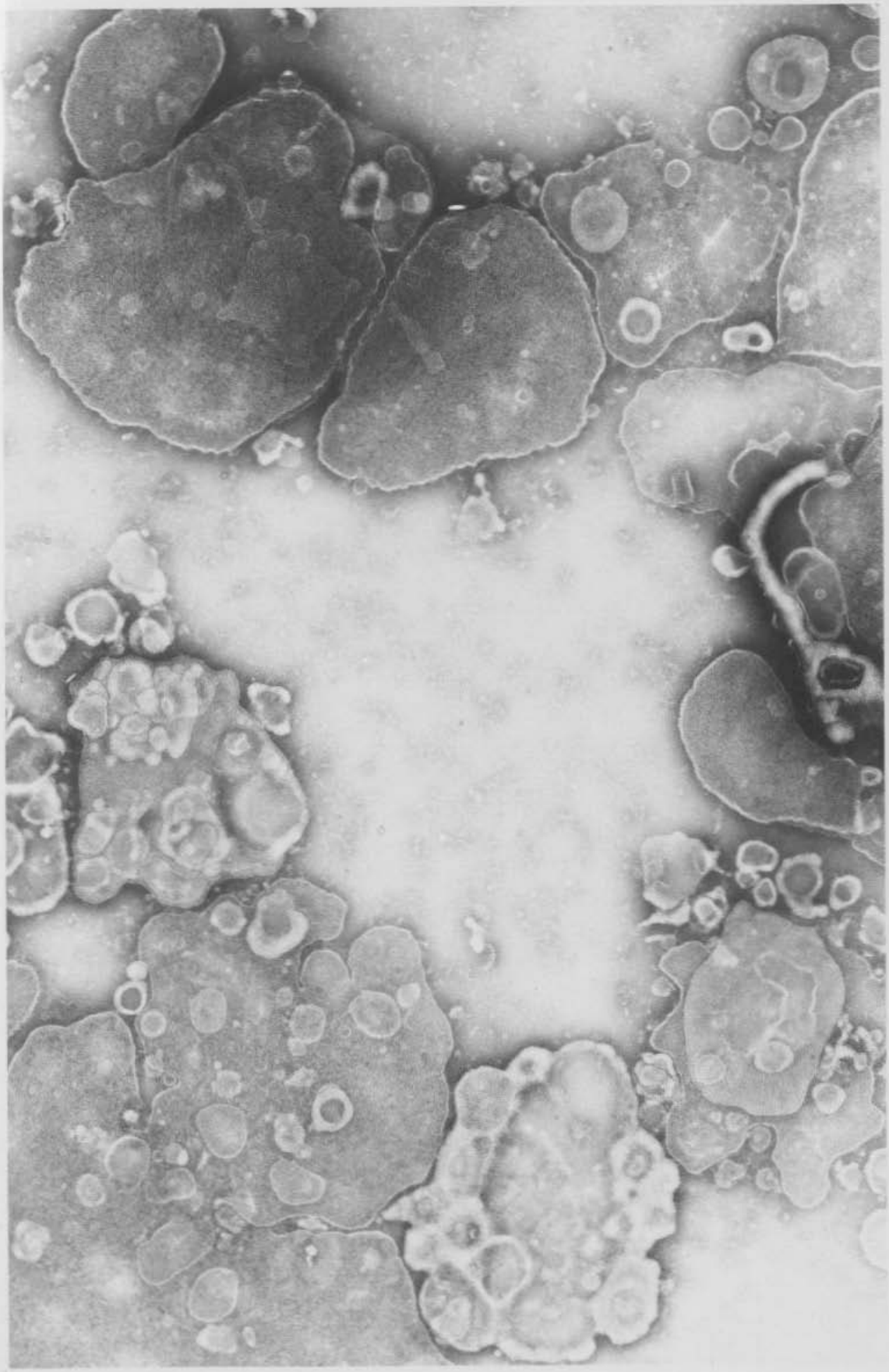


Figure 3. Electron micrograph of Krebs plasma membranes. Three whole membrane ghosts and several membrane fragments can be seen in this photograph which is magnified 41,500 times.

represents 1 μm

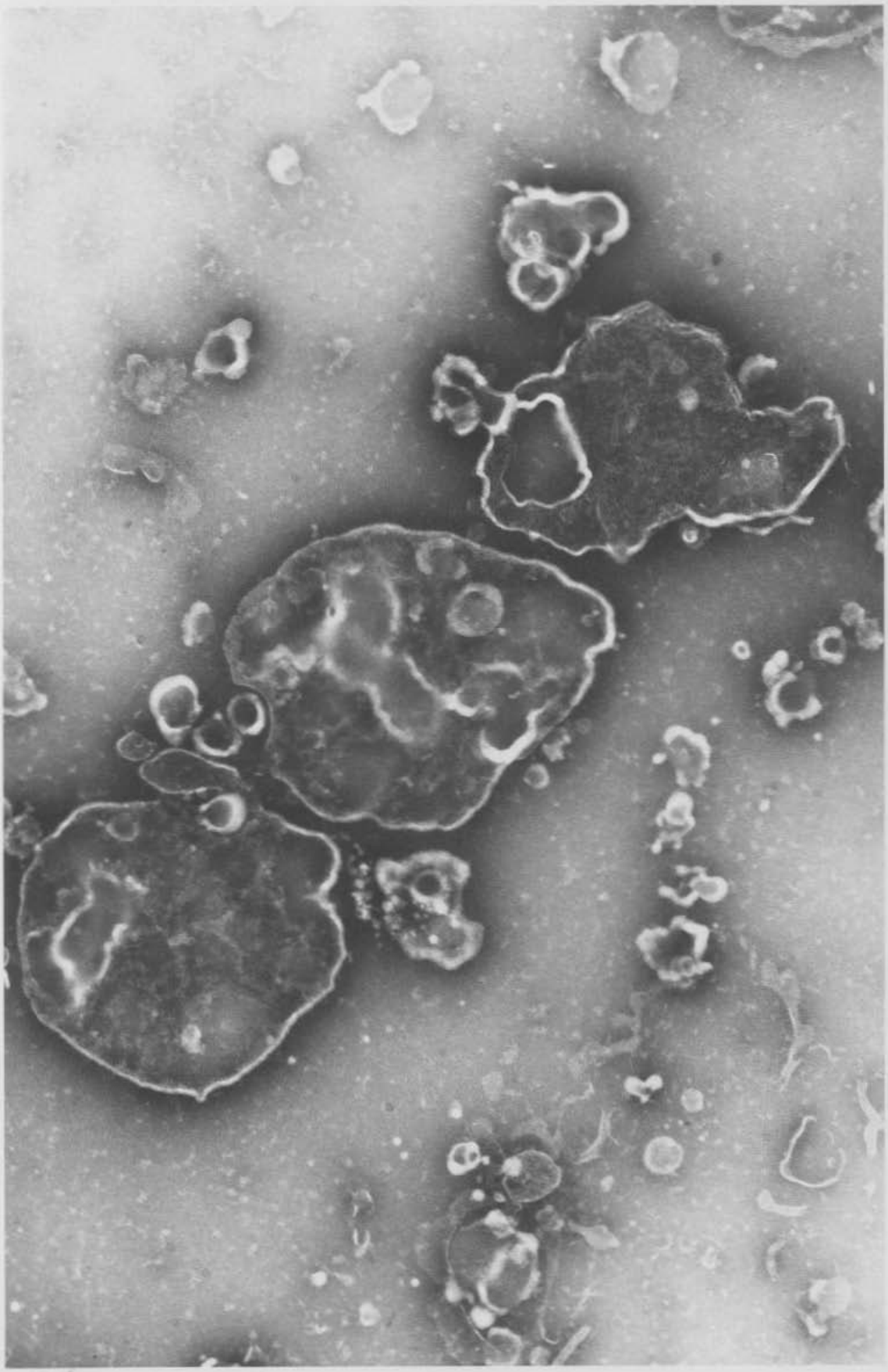
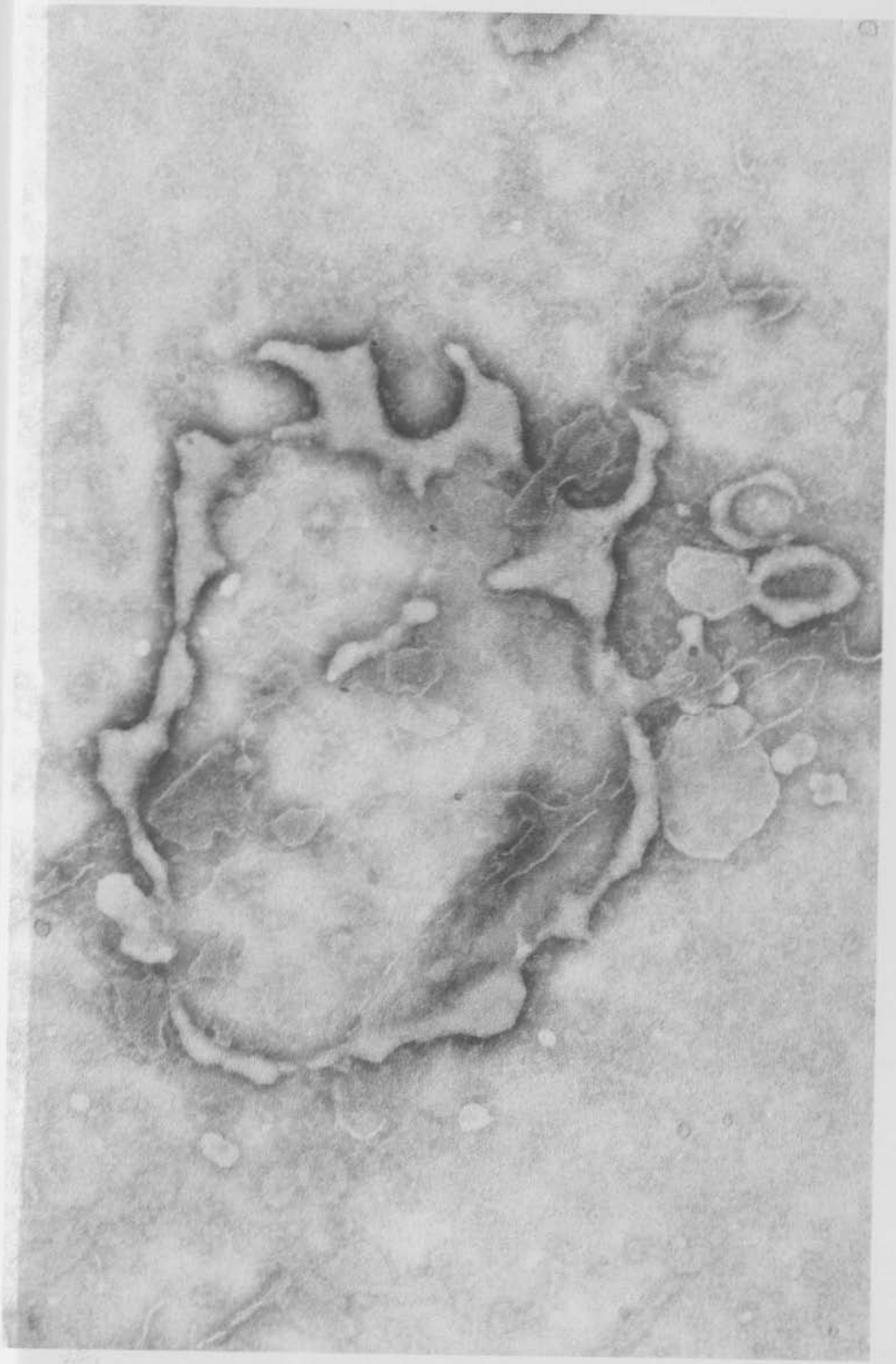


Figure 4. Electron micrograph of a Krebs plasma membrane ghost.

An individual Krebs plasma membrane ghost which resembles a flattened, folded sack-like structure can be seen in this photograph which is magnified 83,000 times.

represents 1 μm



mitochondria or ribosomes can be seen. Figure 3 shows three whole membrane ghosts and several pieces of fragmented membrane. Figure 4 shows an individual whole membrane ghost resembling a burst balloon, with folds around the edge where the membrane has collapsed. Even in fields of view not photographed no other cell organelles than the plasma membrane could be distinguished indicating that the preparation was highly purified.

d) 1. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of Krebs cell fractions.

The Krebs cell fractions prepared for marker enzyme assays, including purified membranes were examined by SDS-PAGE after the following treatments. The homogenate was too viscous to run successfully on a gel and, therefore, the whole cells and nuclei were removed first by centrifuging about 150g for 1 minute. The mitochondrial-microsomal fraction was further separated into the mitochondrial fraction, sedimented at 15,000 g for 15 minutes and the microsomal fraction sedimented at 105,000 g for 60 minutes. All of these cell fractions including the final microsomal supernatant fluid were electrophoresed for 2 hours on gels which were then stained with coomassie blue to reveal proteins.

Many components were present in gels of all cell fractions (figs. 5, 6). The homogenate, mitochondrial and microsomal samples contained many components covering a wide range of molecular weights but the final microsomal supernatant fluid contained mainly medium and low molecular weight components. There was more protein at the origin of the gels of the homogenate, microsomes and purified membranes,

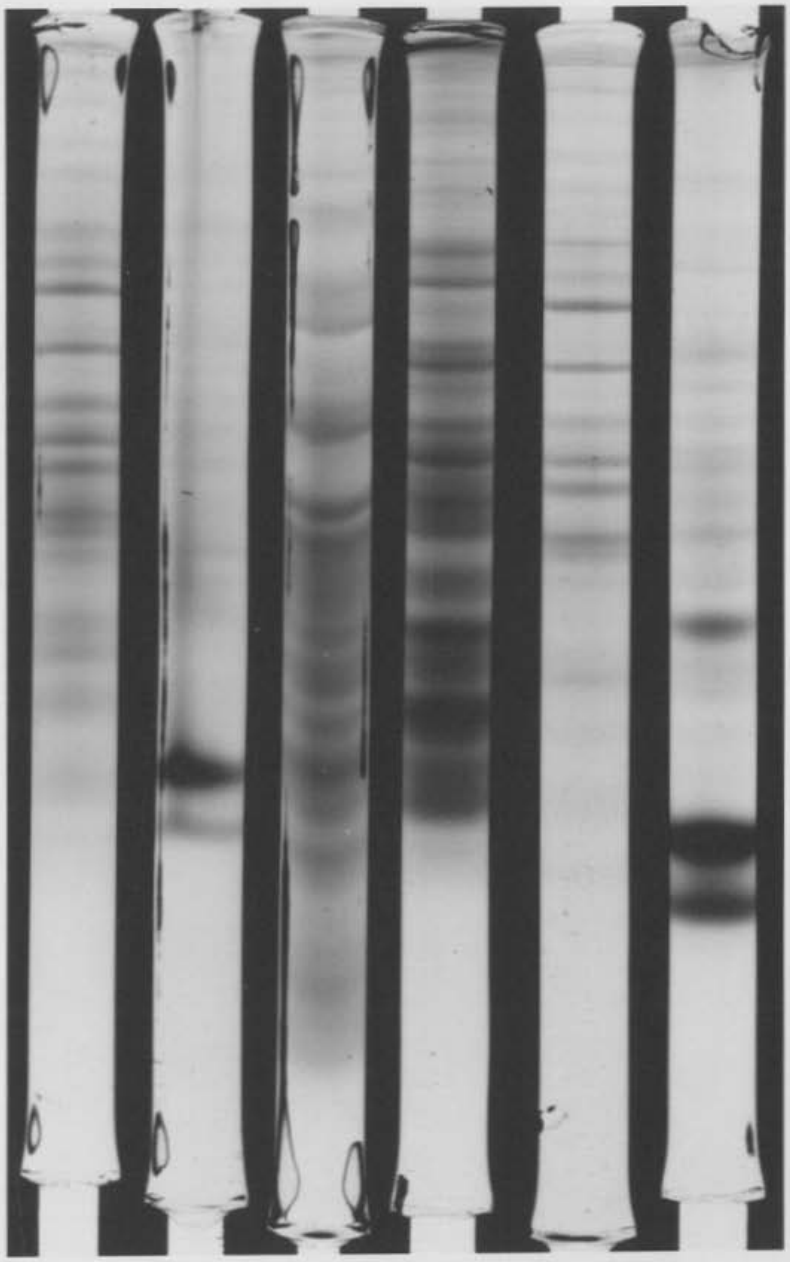


Figure 5. SDS-PAGE of Krebs cell fractions. The gels, stained with coomassie blue and electrophoresed for 2 hours, are of the following; A- cell homogenate with the whole cells and nuclei removed, B- crude plasma membranes, C- mitochondria, D- microsomes, E- soluble cell material and F- purified plasma membranes.

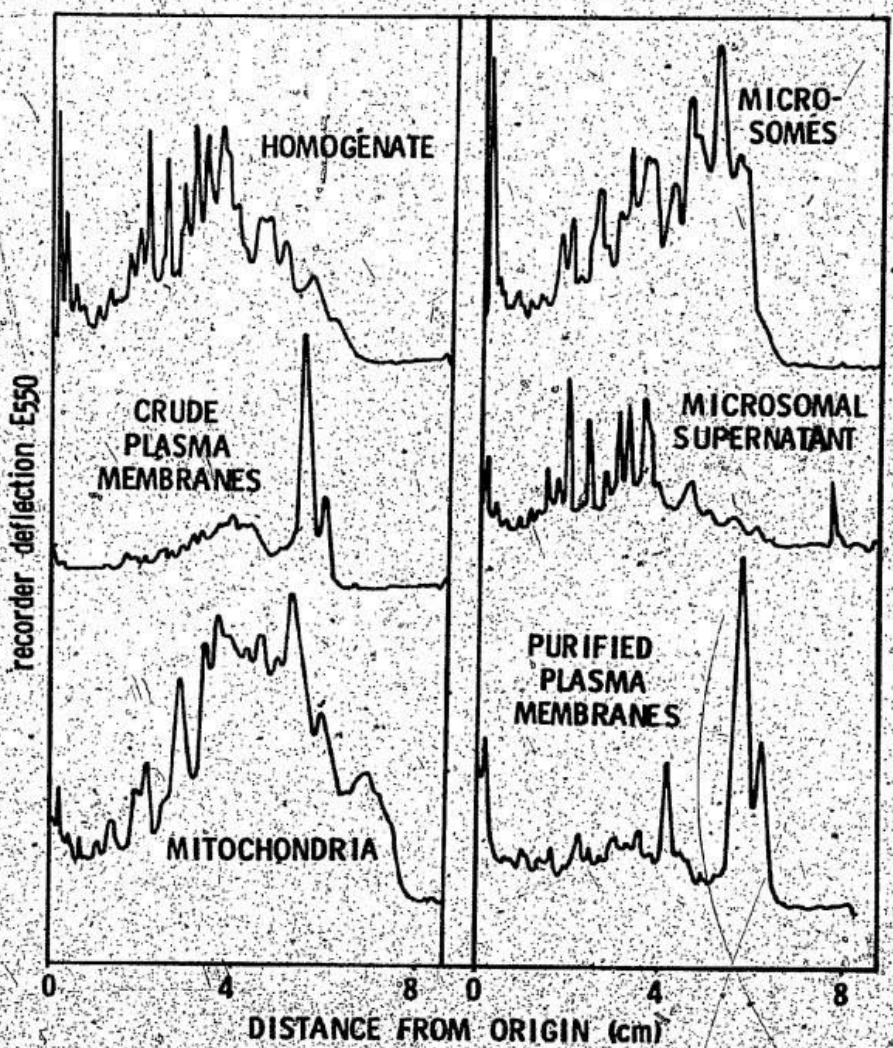


Figure 6. SDS-PAGE scans of Krebs cell fractions. These scans are profiles of coomassie blue stained gels which had been electrophoresed for 2 hours.

possibly due to aggregation of lower molecular weight components. Since, however, the same amount of protein was placed on each gel any protein concentrated in one fraction would have a larger peak in that profile and may be missing in the profile of another fraction where its concentration is low in comparison to other components.

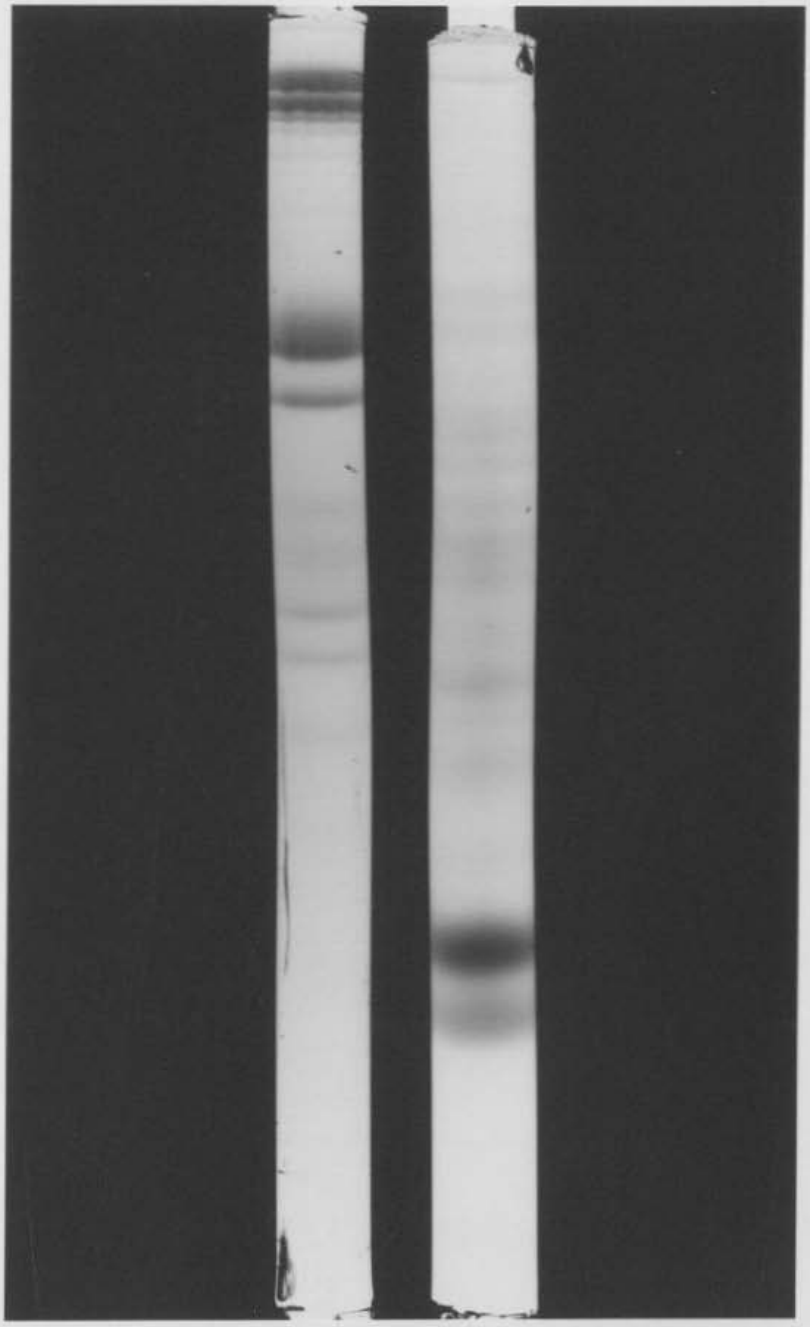
The electrophoresis profile of plasma membranes and particularly of purified plasma membranes showed distinctly different patterns compared with other cell fractions. Components present in other fractions were very faint in the purified plasma membrane sample indicating that there was little contamination with these other fractions.

d) ii. SDS-PAGE of purified plasma membranes

Purified plasma membranes, electrophoresed on gels for two hours, gave characteristic, consistently observed patterns of bands whether coomassie blue stain for detecting proteins or periodic acid Schiff's (PAS) reagent for glycoproteins were used. For comparison purposes human erythrocyte membranes were occasionally analysed on parallel gels.

After two hour electrophoresis (fig. 7, 8) at least 20 coomassie blue stained bands were observed, three of which were heavily stained. In contrast, only one band was observed after PAS staining. Since this single PAS stained component migrated a very short way into the gel during the two hour period, electrophoresis was also carried out for eighteen hours again staining with both coomassie blue and PAS stains.

A faint band was now seen in the high molecular weight region



A B

Figure 7. SDS-PAGE of purified plasma membranes. The gels, stained with coomassie blue and electrophoresed for 2 hours, are of the following; A- human erythrocyte membranes and B- Krebs cell plasma membranes.

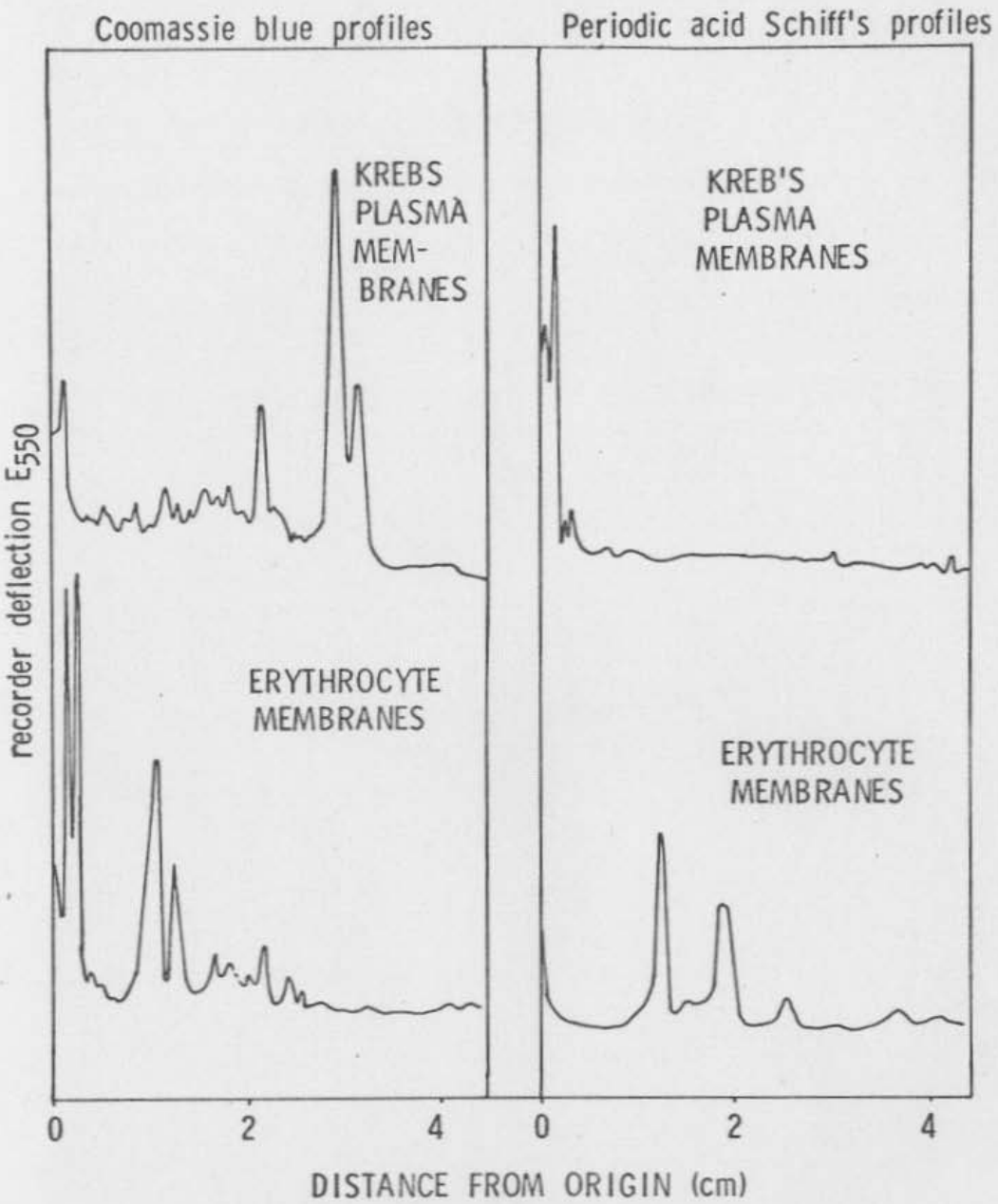


Figure 8. SDS-PAGE scans of purified membranes. Plasma membranes prepared from human erythrocytes and Krebs cells were electrophoresed for 2 hours and stained with coomassie blue to detect proteins and PAS reagent to detect glycoproteins. The scans of these gels are shown.

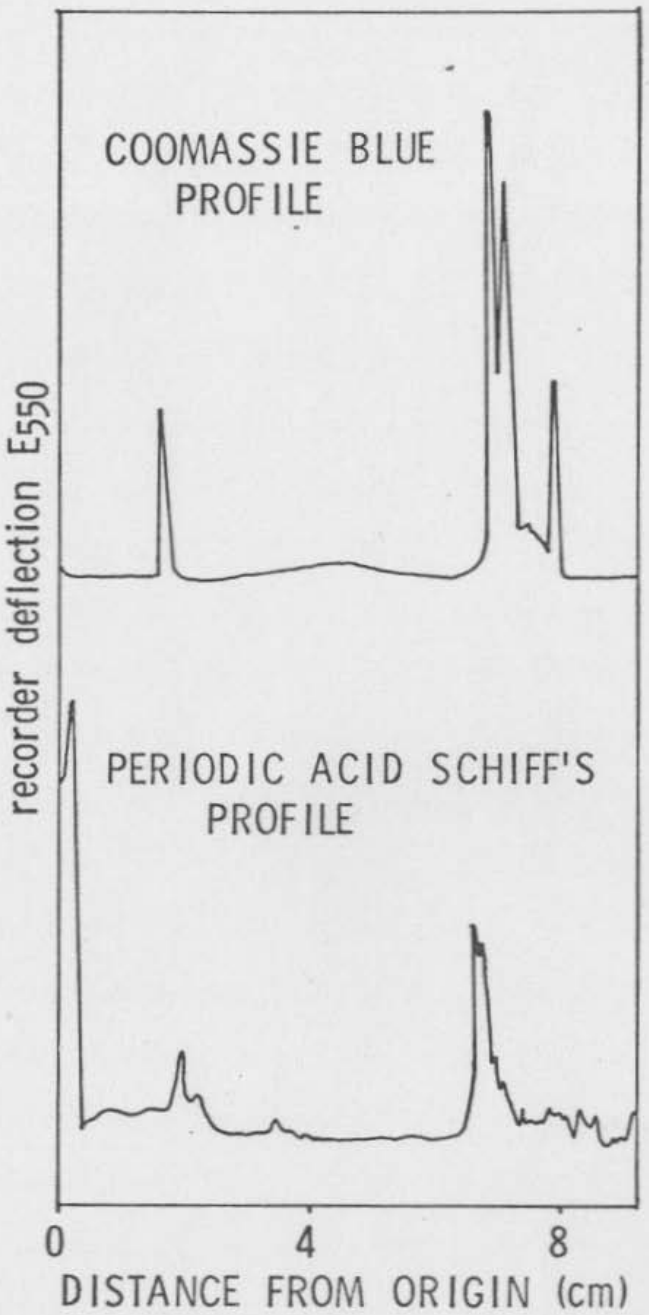


Figure 9. SDS-PAGE of purified Krebs plasma membranes. These scans are of gels electrophoresed for 18 hours and stained as shown.

of the gel with two darker bands very close together in the lower molecular weight region of the gel (fig. 9). The PAS stained gel run for 18 hours had a very faint band near the origin and a more intense band with a shoulder near the bottom or lower molecular weight region of the gel, (fig. 9). On the top of the gel there was PAS staining material which had not been able to enter the gel probably due to aggregation.

e) Krebs cell surface labelling with ^{125}I.

i) Iodination of whole cells with 50 µCi ^{125}I.

An attempt was made to introduce iodine, ^{125}I, into cell surface components to aid in their identification and to give a convenient tool for further analysis. Labelling was performed in the presence of glucose oxidase and lactoperoxidase. After incubating Krebs cells with ^{125}I for 0.5, 1 and 2 hours they were washed and then fractionated. Of the total incorporated cpm most was found in the mitochondrial-microsomal-soluble cell material fraction with only 0.028% after 0.5 and 1 hour's incubation and 0.035% after 2 hours of the total radioactivity added becoming incorporated into the plasma membrane (table 2). Of the radioactivity bound to cells only 1.6%, 2.7% and 4.5% of the radioactivity was bound to the plasma membrane after incubation for 0.5, 1 and 2 hours respectively. Labelling for longer time periods increased the amount of radioactivity incorporated into plasma membranes only slightly.

ii) Iodination of Krebs cells with 100 µCi ^{125}I.

In an attempt to improve the amount of radioactivity in purified plasma membranes, labelling of whole cells was repeated

Sample	Radioactivity (counts per minute, cpm) incorporated during an exposure time, in hours, to ^{125}I of:		
	0.5	1	2
unattached radioactivity	1616007	1665804	1480850
homogenate	21708	15642	11760
whole cells and nuclei	4662	4929	6316
crude membranes	463	441	490
mitochondria and microsomes and soluble cell material	15885	9900	7108
purified plasma membranes	350	427	530

Table 2. Iodination of Krebs cells with 50 μCi ^{125}I. Krebs cells were iodinated with 50 μCi ^{125}I, the reaction being catalyzed by lactoperoxidase, and the cells were then fractionated. Samples (100μl) were monitored for radioactivity during membrane preparation and the total radioactivity present at each stage of membrane preparation is shown above.

Sample	Total radioactivity (cpm)
homogenate	32022
whole cells and nuclei	10172
crude membranes	5914
mitochondria, microsomes and soluble cell material	19558
purified plasma membranes	410

Table 3. Iodination of Krebs cells with 100 μCi ^{125}I. Krebs cells were iodinated with 100 μCi ^{125}I, the reaction being catalyzed by lactoperoxidase, and the cells were then fractionated. Samples were monitored for radioactivity during membrane preparation and the total radioactivity present at each stage of membrane preparation is shown above.

using 100 µCi ^{125}I and exposing the cells to the radioactive label for one hour. The cells were washed to remove unattached ^{125}I and fractionated to obtain purified plasma membranes.

Again most of the incorporated radioactive label (60%) was found in the mitochondrial fraction (table 3). There was an improvement in the incorporation of radioactive label into purified plasma membranes which contained 1.3% of that incorporated into the homogenate.

iii) Iodination of purified Krebs plasma membranes for different time periods.

Since attempts to label whole cells were unsuccessful, in that an insignificant amount of radioactivity was incorporated into the plasma membrane, it was decided to determine if isolated plasma membranes could be labelled giving better ^{125}I incorporation. Purified plasma membranes, 0.5 ml of a suspension of 4.35 mg protein/ml were iodinated for 0.5, 1 and 2 hours each with 50 µCi ^{125}I. The membranes were washed and their radioactive content determined.

Labelling isolated plasma membranes considerably increased the amount of radioactivity incorporated into membranes (table 4) as compared with using whole cells (tables 2, 3). Incorporation of ^{125}I was improved by labelling for 1 hour rather than 0.5 hours but longer periods of exposure to ^{125}I did not increase incorporation further (table 4).

iv) Iodination of Krebs plasma membranes by exposing them to different quantities of ^{125}I.

In order to try to increase the amount of ^{125}I incorporated

Time of exposure to ^{125}I in hours	Radioactivity (cpm) bound to membranes	Specific radioactivity (cpm/μg membrane protein
0.5	31524	18.1
1	111502	63.9
2	106837	61.3

Table 4. Iodination of isolated Krebs plasma membranes with 50 μCi ^{125}I. Krebs plasma membranes were iodinated with 50 μCi ^{125}I for different time periods, and the reaction was catalyzed with lactoperoxidase. Unattached radioactivity was removed by washing before monitoring the total radioactivity in the membrane samples.

Amount of label added	Radioactivity (cpm) bound to membranes	Specific radioactivity cpm/μg membrane protein
50 μCi in the presence of lactoperoxidase	194,550	1,995
50 μCi in the absence of lactoperoxidase	114,925	1,179
200 μCi in the presence of lactoperoxidase	1,366,600	14,016

Table 5. Iodination of Krebs plasma membranes with different amounts of ^{125}I. Lactoperoxidase catalyzed iodination of isolated Krebs plasma membranes was carried out using 50 μCi and 200 μCi of ^{125}I. As a control a sample of isolated plasma membranes were exposed to ^{125}I in the absence of lactoperoxidase. All the samples were exposed to ^{125}I for 1 hour.

into membranes more ^{125}I was used during labelling. The membranes used for this experiment contained 1.95 mg protein/ml. Each of two 0.5 ml membrane samples with all the necessary enzymes present were labelled with 50 µCi and 200 µCi of ^{125}I. As a control an identical sample was prepared with 50 µCi ^{125}I but with the enzyme lactoperoxidase absent. All of the samples were iodinated for one hour and the membranes were washed before radioactive counting.

The incorporation of label per mg of membrane protein was improved about six fold by labelling with 200 µCi rather than 50 µCi (table 5). The control sample with lactoperoxidase absent contained some label but as will be shown in the next section this was probably ^{125}I that would possibly have been removed by further washing.

v) SDS-PAGE of iodinated Krebs plasma membranes

The purified plasma membrane samples iodinated with 50 µCi and 200 µCi of ^{125}I, together with the control membranes iodinated with 50 µCi of ^{125}I in the absence of lactoperoxidase were analysed in duplicate by 2 hour SDS-PAGE. One of each duplicate gel was stained with coomassie blue and the other used for radioactive counting.

The coomassie blue stained gels showed the characteristic pattern of protein bands for Krebs plasma membranes (fig. 7, 8), whilst the radioactivity profiles were much simpler (fig. 10). The radioactive peaks had similar mobilities to the heavily stained bands on the coomassie blue stained profiles. There were no radioactive peaks on the gel of the sample iodinated in the

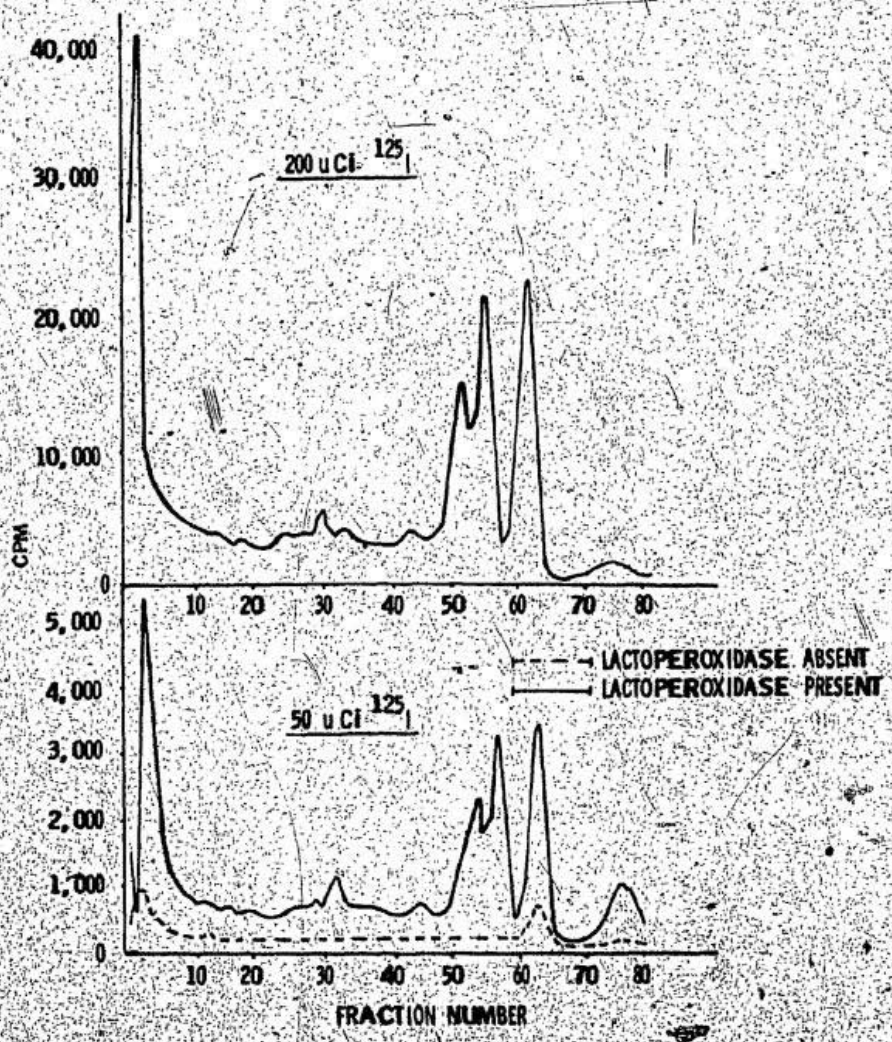


Figure 10. Radioactive profiles of SDS-PAGE of iodinated Krebs plasma membranes. Krebs plasma membranes were iodinated with 50μCi and 200μCi ^{125}I in the presence of lactoperoxidase and as a control, with 50μCi ^{125}I in the absence of lactoperoxidase. Samples of these membranes were electrophoresed for 2 hours and the radioactivity in pulverized 1mm slices of the gels was monitored.

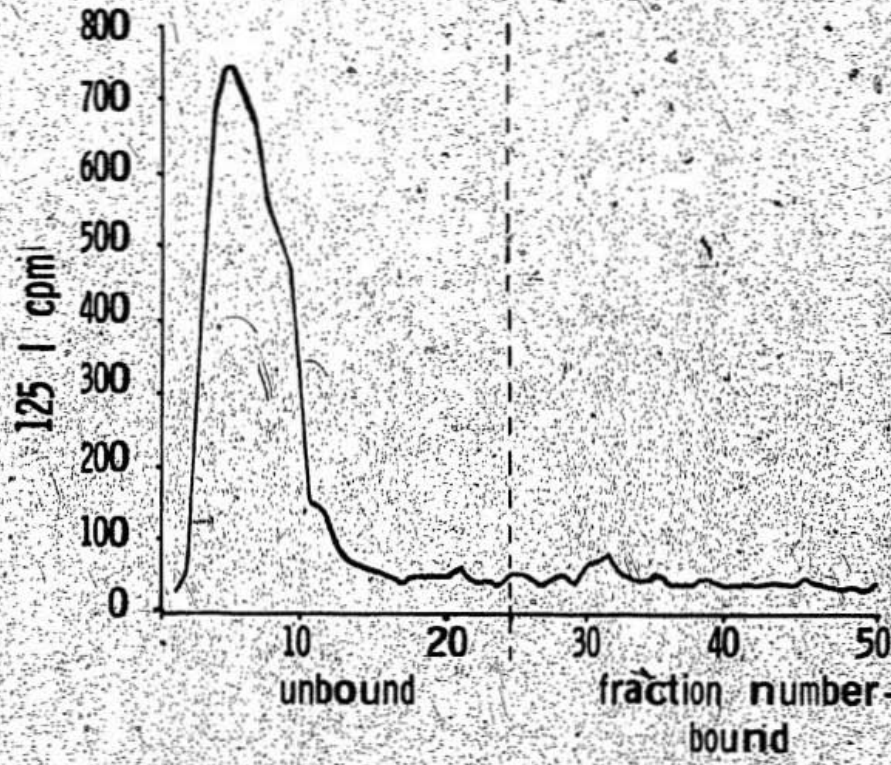


Figure 11. Affinity Chromatography of solubilized Krebs plasma membranes on WGA-sepharose. Iodinated Krebs plasma membranes were solubilized with Triton X100 and chromatographed on a WGA-sepharose column. Bound material was released with N-acetyl glucosamine. Fractions were collected from the column and monitored for radioactivity.

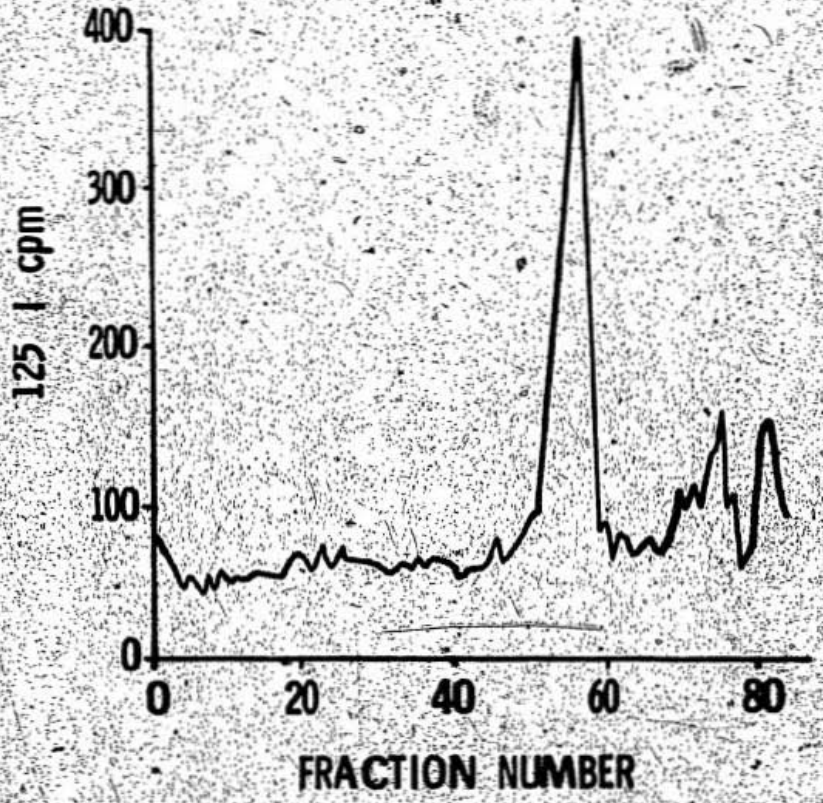


Figure 12. SDS-PAGE of the unbound fractions from the WGA-sepharose column. Unbound ^{125}I labelled material from the WGA-sepharose column was electrophoresed for 2 hours and the radioactivity in 1mm diameter pulverized fractions of the gel was monitored.

absence of lactoperoxidase (fig. 10) indicating that the radioactivity present was not incorporated into macromolecules and was probably unreacted ^{125}I which would have been removed by extensive washing.

vi) Chromatography of iodinated Krebs plasma membrane components on wheat germ agglutinin (WGA) sepharose.

Influenza (75) and EMC virus (87) receptors on human erythrocytes are also WGA receptors. It is possible that a WGA receptor, if present on the Krebs cell may also serve as the EMC virus receptor. To test whether or not Krebs plasma membranes have a WGA receptor which may have been labelled by ^{125}I, iodinated Krebs plasma membranes were solubilized in Triton X100 and chromatographed on a WGA Sepharose affinity column. This column had previously been shown to bind glycophorin.

Close to 100% of the recovered radioactivity was eluted in the unbound fraction (fig. 11). No radioactivity was eluted with N-acetyl-glucosamine which displaces any material specifically bound to the column thus causing the elution of any bound radioactivity.

The unbound fraction was examined by SDS-PAGE. The profile of the unbound fraction (fig. 12) revealed two less major peaks but several more small low molecular weight peaks than the original profile (fig. 10). This suggests that degradation of one or more components had occurred.

II. Krebs cell glycocalyx

a) Release of proteins from Krebs cells.

^{125}I labelling of the plasma membrane when present on whole cells

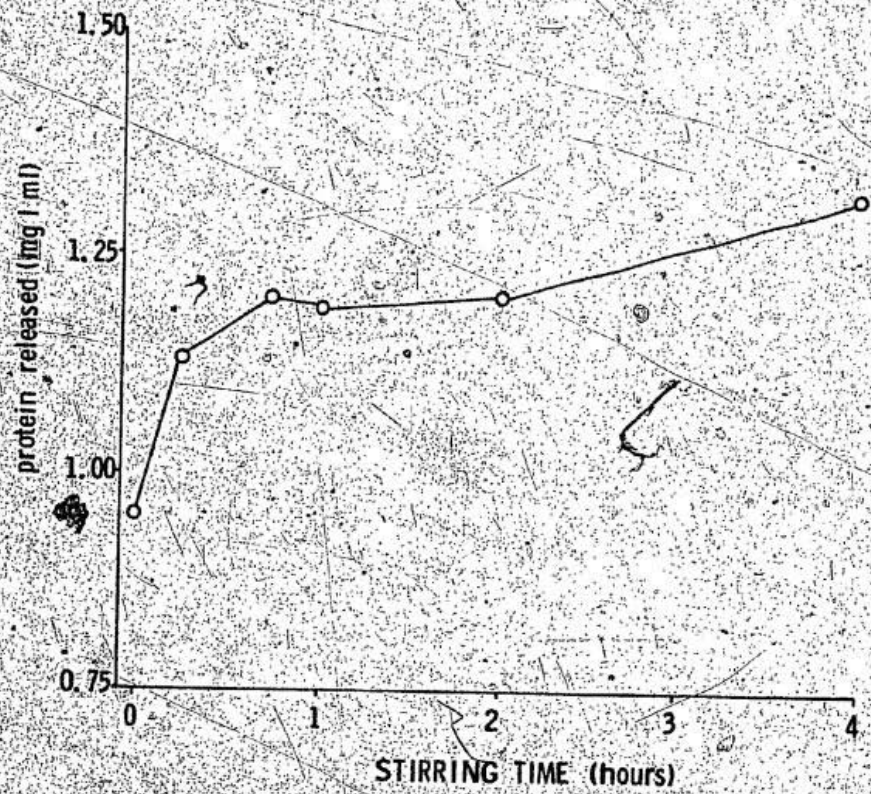


Figure 13. The release of protein from Krebs cells. Suspensions of Krebs cells were stirred gently for different time periods at 4°C and the amount of protein released into the cell suspension was monitored after the cells had been removed by centrifugation.

was considerably less successful than labelling purified plasma membranes. Ehrlich ascites tumour cells, which are very similar to Krebs cells, have been reported to have a glycocalyx (96). It was, therefore, considered pertinent to determine whether or not there was material on Krebs cells such as a glycocalyx which may block the iodination of plasma membranes on whole cells but not on purified plasma membranes since it would most probably be removed during the purification procedure. To release material from the Krebs cell surface, the cells were stirred at 4°C and the supernatant fluid, after the removal of stirred cells was assayed for protein, as described by Rittenhouse et al (96) who released the glycocalyx from Ehrlich cells.

Even at the beginning of the experiment there was some protein in the supernatant fluid (fig. 13). Cells stirred for longer periods of time released more protein, reaching a plateau by 45 minutes. Based on this result, in subsequent experiments to study the glycocalyx, cells were stirred for 1 hour which was more than enough time to liberate the material which was considered to comprise the glycocalyx.

b) Properties of the Krebs glycocalyx

Material in the Ehrlich cell glycocalyx can be sub-divided into the cell coat fraction and the cell coat particle by centrifugation (95). The crude Krebs glycocalyx was separated into two similar fractions to the Ehrlich cell glycocalyx. The amount of protein in each fraction varied but from 5 to 20 ml of 10^8 cells/ml samples within the range of 0.3 to 1.0 mg protein/ml in a total volume of

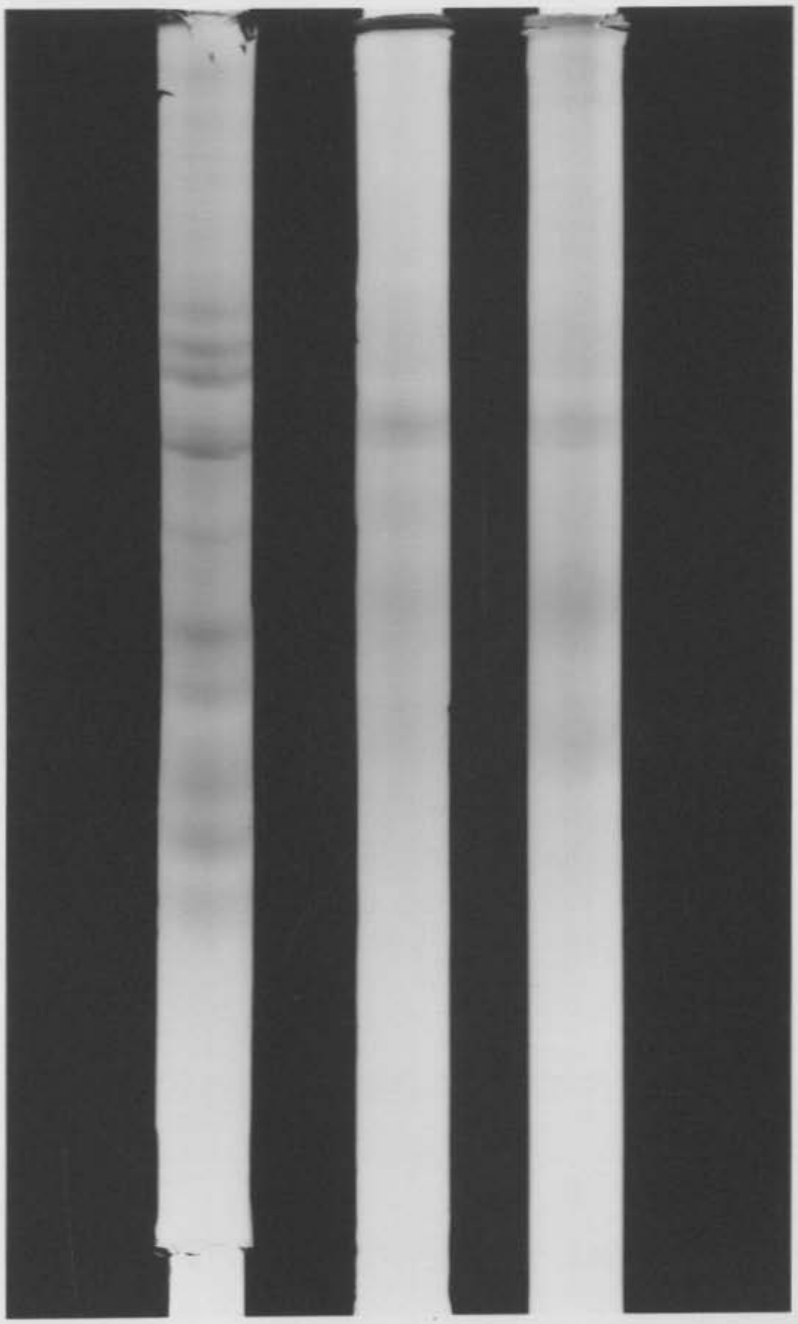


Figure 14. SDS-PAGE of Krebs glycocalyx fractions. The gels, stained with coomassie blue and electrophoresed for 2 hours, are of the following; A- crude glycocalyx, B- cell coat fraction and C- cell coat particle.

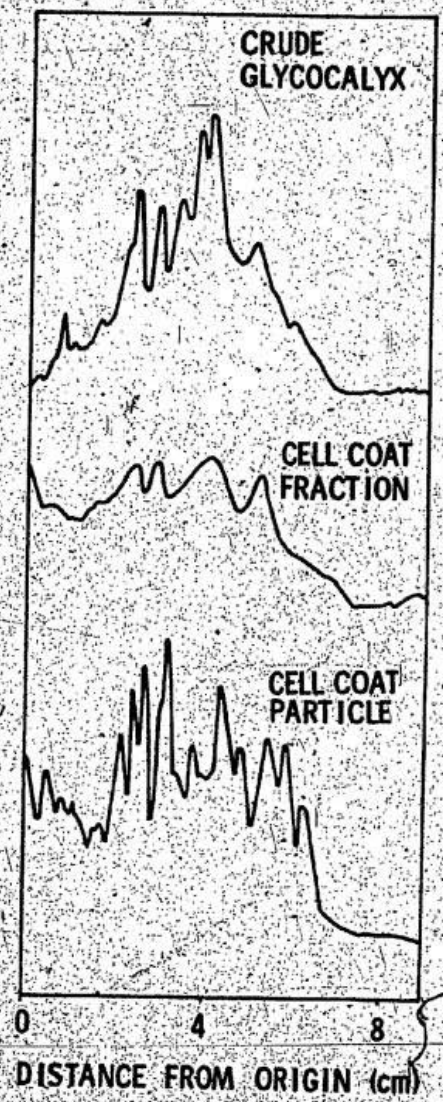


Figure 15. SDS-PAGE scans of the Krebs glycocalyx. These scans, from gels electrophoresed for 2 hours and stained with coomassie blue are of samples of crude glycocalyx, cell coat fraction and cell coat particle.

5 to 15 ml were produced for both the crude glycocalyx and the cell coat fraction. The cell coat particle contained 0.7 to 2.0 mg protein/ml in a total volume of 2 to 3 ml. Rittenhouse et al (95) detected no sialic acid in glycocalyx fractions from Ehrlich cells and none was detected in any of the Krebs glycocalyx fractions either.

The crude glycocalyx, cell coat fraction and cell coat particle of Krebs cells were examined by SDS-PAGE and stained with coomassie blue and PAS stains. Several bands were seen on the coomassie blue stained gels but no PAS staining components were observed (figs. 14, 15). It is interesting to note that several of the crude glycocalyx components which stained with coomassie blue had corresponding mobilities to similarly stained components in the Krebs cell homogenate and microsomal supernatant fluid (figs. 5, 6).

c) <u>The effect of the Krebs glycocalyx on the action of neuraminidase on the Krebs cell surface.</u>

Neuraminidase can be used to remove sialic acid from external cell membrane components. To determine whether the glycocalyx shields any of the surface sialic acid residues normal and glycocalyx depleted cells were treated with neuraminidase as follows. Samples of 10^8 cells with and without a glycocalyx were made up to a volume of 1 ml with PBS and incubated with 10 units of neuraminidase for 0.5, 1, 2 and 4 hours at 37°C. Control cells with the glycocalyx removed were not treated with neuraminidase but were incubated for the same time periods. After incubation all the samples were

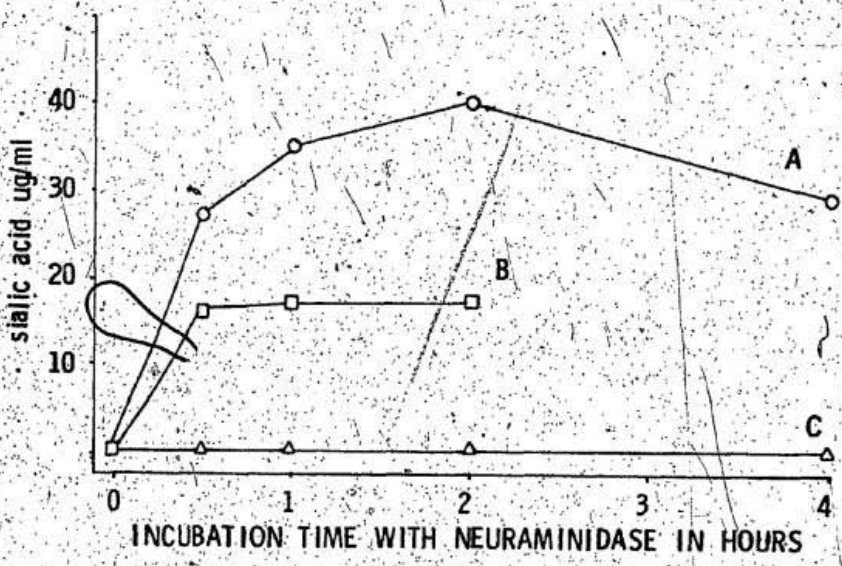


Figure 16. The effect of the Krebs cell glycocalyx on the action of neuraminidase on the Krebs cell surface. The glycocalyx was removed from cell sample A but was intact in sample B. Both of these samples were treated with 10 units of neuraminidase and a third sample C, was the control with the glycocalyx absent and not treated with neuraminidase.

centrifuged to sediment the cells and the supernatant fluids assayed for their sialic acid content. It was necessary to treat the supernatants with an equal volume of cold 10% trichloroacetic acid (TCA) to precipitate macromolecules which interfered with the sialic acid assay. In control experiments no sialic acid was detected in the crude glycocalyx even after treatment with 10% TCA.

Neuraminidase releases sialic acid from cells whether or not the glycocalyx is present. Approximately twice as much, however, is released from cells lacking a glycocalyx which suggests that half of the sialic acid is protected by the glycocalyx (fig. 16). The maximum amount of sialic acid released was after two hours' treatment. After 4 hours treatment it is possible that glycocalyx components are being regenerated and the neuraminidase activity is declining thus explaining the drop in sialic acid release. No sialic acid was released from cells that were not treated with neuraminidase after the removal of the glycocalyx.

Part 2. The EMC virus receptor on Krebs cells.

L. The surface location of the Krebs cell receptor for EMC virus.

Since the Krebs cell surface has been shown to be composed of the plasma membrane and the glycocalyx, the first important stage in elucidating the EMC virus - Krebs cell surface attachment mechanism is to determine the location of the EMC virus receptor on the Krebs cell surface. If the cellular receptor for EMC virus is a glycocalyx component attachment to and growth of EMC virus in cells without a glycocalyx would be impaired. The following

experiments were carried out to try and determine the location of the Krebs cell receptor for EMC virus.

a) Attachment of ^{3}H labelled EMC virus to washed and unwashed Krebs cells and Krebs cells with the glycocalyx removed.

Attachment of ^{3}H labelled EMC virus to Krebs cells with and without the glycocalyx was measured to determine the role of the glycocalyx in attachment. Since it was possible that some glycocalyx components were removed by washing the cells after harvesting, attachment to cells harvested without washing was also measured. Samples of 5×10^{7} unwashed and washed cells with and without the glycocalyx were each incubated at 4°C for 30 minutes with 30 µl of ^{3}H adenosine labelled EMC virus (4.7×10^{6} cpm/ml, 1.6 mg virus/ml). Unattached radioactivity was removed by washing and counting at the same time as cell bound radioactivity.

There was no difference in the percentage of radioactivity bound to the Krebs cells with or without the glycocalyx (table 6). Unwashed cells bound fractionally more radioactivity which may have been due to the presence of a few erythrocytes in the suspension which also bind ^{3}H labelled virus.

b) The effect of releasing cell surface components over a two hour period on ^{3}H labelled EMC virus attachment.

Removal of the glycocalyx does not have any effect on EMC virus attachment. To confirm this result and explore the situation further the effect of time on the release of cell surface components and on ^{3}H labelled EMC virus attachment was tested. Three 3 ml samples containing 10^{8} washed Krebs cells/ml were treated as follows.

Cell Treatment	Unattached radioactivity (cpm)	Radioactivity bound to cells cpm	Percentage of added radio-activity bound to cell
Glycocalyx removed	43,125	38,426	47.1
Glycocalyx present	36,550	32,163	46.8
Unwashed cells	30,701	33,892	52.5

Table 6. The role of the glycocalyx in the attachment of EMC virus to Krebs cells. One sample of Krebs cells was washed and stirred at 4°C for 1 hour to remove the glycocalyx. A second sample of cells was washed after harvesting but the glycocalyx was left intact. A third cell sample was used without washing after harvesting from mice. Each of these samples of 5×10^7 Krebs cell was incubated at 4°C with radioactively labelled EMC virus. Attachment was measured by monitoring the amount of radioactivity bound to cells after washing off unattached virus. The figures given are the mean values of duplicate readings.

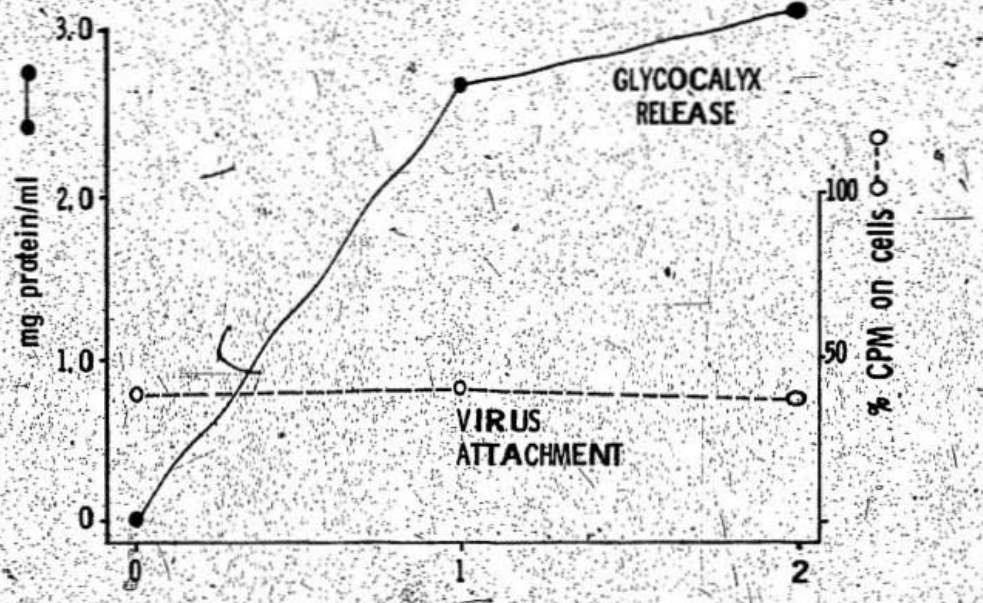


Figure 17. The attachment of EMC virus during the removal of the glycocalyx. The glycocalyx was released by stirring gently at 4°C. The release of material was monitored by assaying for protein in cell supernatent fluids. EMC virus attachment was measured by monitoring the amount of radioactive EMC virus attaching to cells.

The supernatant fluid from the last wash of sample 1 was assayed for protein. Sample 1 was then tested immediately for virus binding by maintaining at 4°C in the presence of 20 µl of ^{3}H adenosine labelled EMC virus (4.7×10^{6} cpm/ml 1.6 mg virus/ml). After 30 minutes the cells were collected by centrifugation and radioactivity in the supernatant fluid and associated with the cells was measured. Meanwhile samples 2 and 3 were shaken gently in 25 ml flasks at 4°C for 1 and 2 hours respectively to remove the glycocalyx. At the end of the treatment samples 2 and 3 were centrifuged and the cells were resuspended in an equal volume of PBS. The supernatant fluids were assayed for protein. Resuspended samples 2 and 3 were incubated at 4°C for 30 minutes with ^{3}H adenosine labelled EMC virus. After this time the unattached radioactivity which was removed by washing was counted as well as the cell bound radioactivity.

Attachment of ^{3}H labelled virus remained constant despite the marked release of protein from the cell surface during the first hour of incubation and further smaller release of protein during the second hour of stirring (fig. 17). More protein was released during this experiment than in the initial release of the glycocalyx (fig. 17) probably because in the latter experiment stirring consisted of swirling once every fifteen minutes whereas in this experiment stirring, whilst it was very gentle, was continuous.

c) <u>The effect of glycocalyx removal on the growth of EMC virus in Krebs cells.</u>

The glycocalyx is not involved in the attachment of virus

Cell Sample	Mean number of cells* Unstained	Stained	Percentage stained	Percent cell survival	HAU per ml
uninfected control	77.3 ± 4.2	8.3 ± 0.48	4.0	80.5	<40
glycocalyx present	2.5 ± 0.96	28.0 ± 2.2	91.8	30.5	1280
glycocalyx absent	4.5 ± 0.65	80.3 ± 0.95	87.1	34.8	1280

Table 7. The role of the glycocalyx in the growth of EMC virus in Krebs cells. A sample of Krebs cells were treated to remove the glycocalyx and a further cell sample remained intact. EMC virus was grown in suspension cultures of these cell samples. A control sample of intact cells uninfected were incubated at 37°C overnight in the same way as the virus infected cell samples. The growth of EMC virus was monitored by cell killing, that is the number of cells stained with trypan blue and the cell concentration compared to the original concentration and also by haemagglutination assays. The number of cells was that number in 0.1 mm^3 of cell suspension and was the mean of 4 determinations. Standard errors are given for cell numbers in the table.

* The volume in the haemocytometer used for counting cells.

particles to Krebs cells. Only a small proportion of a given EMC virus suspension comprises infectious particles (13). In experiments pertaining to the attachment of radioactively labelled virus to cells the infectious virions are only a small group and if they behave differently during attachment it would be masked by the large number of non-infectious virions present. By looking at the effect of glycocalyx removal on virus growth, effectively only the attachment of infectious virions is being measured.

EMC virus was grown in suspension cultures of 10^8 Krebs cells with and without a glycocalyx. Virus growth was monitored by HA titration and by cell killing, as indicated by the uptake of trypan blue. A control consisted of 10^8 uninfected Krebs cells with an intact glycocalyx.

There was very little difference in the percentage of cells stained and no difference in the HA titre between the infected cells with and without the glycocalyx, (table 7). The removal of the glycocalyx, therefore, has no effect on the growth of EMC virus in Krebs cells.

d) Inhibition of haemagglutination by Krebs glycocalyx fractions.

If a glycocalyx component was a receptor for EMC virus it would be expected to interfere with EMC virus haemagglutination by competing with erythrocytes to bind virus. The following fractions, obtained during glycocalyx preparation, were tested for inhibition of haemagglutination; the initial cell supernatant (3,000 g), the 48,000 g pellet, the crude glycocalyx (48,000 g supernatant fluid), the cell coat fraction and cell coat particle.

The initial cell supernatant showed no inhibition of haemagglutination since the HA titre of the virus was the same in the presence and absence of these fractions. The cell coat fraction and cell coat particle showed an HA titre of 640 HAU/ml as compared to the virus control of 1,280 HAU/ml. This difference, however, only represents one cup of HA and is probably not significant since the assay is only 50% accurate.

These results suggest that glycocalyx components do not inhibit EMC virus haemagglutination and are probably not involved in EMC virus attachment to Krebs cells.

II. The interaction between EMC virus and whole Krebs cells or plasma membranes.

Having determined that the receptor for EMC virus on Krebs cells is a plasma membrane component rather than a glycocalyx component it was possible to examine more closely the attachment of EMC virus to Krebs cells and to isolated Krebs plasma membranes. Attachment was investigated using radioactively labelled virus. As previously described, not all virus particles in a given suspension are infectious and in the case of EMC virus only one particle in 250 is apparently infectious after purification. Thus, in looking at attachment many of the particles attaching may be non-infectious and may attach less specifically or by a different mechanism than infectious virions. Parallel experiments were therefore done looking at infectivity in addition to attachment of EMC virus.

a) The attachment of ^{3}H labelled EMC virus to Krebs cells.

i) The effect of time of exposure to virions and cell concentration on the attachment of ^{3}H labelled EMC virus to Krebs cells.

To investigate the optimum conditions for attachment, two physical parameters were varied. Firstly, the time exposure of cells to virus and secondly, the cell concentration. Samples of 1 ml volume containing 5×10^{7} and 2.5×10^{7} washed Krebs cells per ml were incubated at 4°C with 5 µl of ^{3}H adenosine labelled EMC virus (4.7×10^{6} cpm/ml, 1.0 mg virus/ml) for varying time periods. Unattached radioactivity was collected by washing and was counted together with the radioactivity associated with cells (tables 8, 9).

Attachment was complete within 30 minutes of exposure to ^{3}H labelled virus. Only about 33% of the total radioactivity added adsorbed to the cells at either concentration. After 15 minutes exposure to virus at a concentration of 2.5×10^{7} cells/ml, attachment was only 50% of the maximum virus adsorption whereas at a cell concentration of 5×10^{7} cells/ml maximum attachment was achieved in this time. This difference could have arisen because, with fewer cells present, there is less chance of a virus particle coming into contact with a cellular receptor. Between 30 and 90 minutes exposure to virus there was very little difference in the radioactivity attached at either concentration. The variation in the total ^{3}H labelled virus recovered may be due to difficulty in adding 5 µl of virus accurately which possibly accounts for some of the smaller differences observed in the percentage of

Time of exposure to ^{3}H labelled EMC virus in minutes	Unattached radioactivity (cpm)	Cell bound radioactivity (cpm)	Percentage of radioactivity bound to cells
15	9,053	2,041	18.4
30	6,579	3,806	36.6
60	8,483	2,559	30.2
90	5,976	3,366	36.0

Table 8. The effect of time of exposure to virus on the attachment of ^{3}H labelled EMC virus to Krebs cells at a concentration of 2.5 x 10^{7} cells/ml. Krebs cells, at this concentration, in 1 ml samples were exposed to ^{3}H labelled EMC virus for the time periods shown. Attachment was measured by monitoring the amount of radioactivity bound to cells. The unattached virus was removed by washing.

Time of exposure to ^{3}H labelled EMC virus in minutes	Unattached radioactivity (cpm)	Cell bound radioactivity (cpm)	Percentage of radioactivity bound to cells
15	7,735	2,914	27.4
30	6,817	3,053	30.9
60	6,997	2,900	29.3
90	8,936	3,434	27.8

Table 9. The effect of time of exposure to virus on the attachment of ^{3}H labelled EMC virus to Krebs cells at a concentration of 5×10^{7} cells/ml. Krebs cells, at this concentration, in 1 ml samples were exposed to ^{3}H labelled EMC virus for the time periods shown. Attachment was measured by monitoring the amount of radioactivity bound to cells. The unattached virus was removed by washing.

radioactivity bound to cells.

ii) Comparison of the attachment of ^{3}H labelled EMC virus to Krebs cells and human erythrocytes.

EMC virus can attach to erythrocytes in addition to Krebs cells (4). Attachment of EMC virus to Krebs cells and erythrocytes was compared. An investigation was also made into whether or not virus particles that had been exposed to Krebs cells but failed to attach were able to attach to fresh Krebs cells or erythrocytes.

A 1 ml sample of Krebs cells containing 10^{8} cells was incubated at 4°C for 30 minutes with 2 µl of ^{3}H adenosine labelled EMC virus (4.7×10^{6} cpm/ml, 1.6 mg virus/ml). The sample was centrifuged to give cell 1 and supernatant 1 preparations. After removing a 100 µl sample for radioactive counting, supernatant 1 was mixed with another 1 ml Krebs cells and incubated at 4°C for 30 minutes. This sample was centrifuged to give cell 2 and supernatant 2 preparations. Again, after taking a 100 µl aliquot for radioactive counting, supernatant 2 was mixed with 1 ml of a human erythrocyte suspension containing 20 µl packed volume of cells, and then incubated for 30 minutes at 4°C. This sample was centrifuged to collect cell 3 preparation, erythrocytes in this case, and supernatant fluid 3. To test how well erythrocytes bind virus not previously exposed to Krebs cells, 2 µl of ^{3}H adenosine labelled EMC virus was added to 20 µl packed erythrocytes in 1 ml PBS. This control sample was incubated at 4°C for 30 minutes after which the cells were collected by centrifugation. In all cases, collected cells were washed three times before counting to remove unattached counts.

Samples	Unattached radioactivity (cpm)	Cell bound radioactivity (cpm)	Percentage of radioactivity bound to cells of: Original	added
1st incubation with Krebs cells	4,016	4,252	51.3	-
2nd incubation with Krebs cells	1,603	1,070	4.1	17.5
3rd incubation with human erythrocytes	1,023	794	3.4	19.0
Human erythrocyte control	2,452	2,735	52.9	-

Table 10. Comparison of the attachment of ^{3}H labelled EMC virus to Krebs cells and erythrocytes. Krebs cells, 10^{8} cells, were incubated at 4°C ^{3}H labelled EMC virus. The virus which did not attach to these cells was incubated for a further 30 minutes with a fresh sample of Krebs cells. Any of this virus which did not attach was incubated with human erythrocytes. The amount of radioactive virus bound to cells after each incubation and that which did not attach was measured. The percentage of the original virus added and the virus added to cells before each incubation period, which attached to the cells was calculated. The results are the mean values of duplicate readings. For comparison a control was carried out using human erythrocytes and virus that had not previously been in contact with cells.

All the supernatant fluid samples and washes were counted and results expressed in terms of the total unattached radioactivity. The cell bound radioactivity was also monitored.

There was virtually no difference in the percentage of radioactivity bound to Krebs cells or the erythrocyte control (table 10). Of nearly 50% of the radioactivity which did not bind in the first incubation period, only a small percentage bound on subsequent exposure to fresh Krebs cells or to erythrocytes. The unadsorbed virus, thus remains unadsorbed possibly because about 50% of the radioactivity was no longer present as complete virions or the presence of Krebs cells inactivates virus in some way.

b) The attachment of ^{3}H labelled EMC virus to Krebs plasma membranes.

It has been shown in this study that EMC virus can attach to whole Krebs cells. As a further step towards characterizing the cellular receptor for EMC virus it was necessary to determine whether the plasma membrane alone can bind virus. Owing to the aggregation of purified plasma membranes it was not possible to count individual membrane ghosts so, instead, membrane concentration was expressed as mg membrane protein.

1) Comparison of the attachment of ^{3}H labelled EMC virus to Krebs plasma membranes and erythrocytes.

The purpose of this experiment was to determine whether Krebs plasma membranes could bind virus, and if so, what percentage bound and whether the unbound virus failed to subsequently bind to erythrocytes due to inactivation as was possibly the case when virus interacted with whole Krebs cells.

Sample	Unattached radioactivity (cpm)	Cell bound radioactivity (cpm)	Percentage of radioactivity on cells of:	
			Original	added
1st incubation with Krebs cells	7,512	7,502	49.95	-
2nd incubation with erythrocytes	2,929	105	0.71	3.5

Table 11. Comparison of the attachment of ^{3}H labelled EMC virus to Krebs plasma membranes and erythrocytes. Krebs plasma membranes, 2.2 mg membrane protein, were mixed with ^{3}H labelled EMC virus and incubated at 4°C for 30 minutes. The virus which did not attach to the membranes was mixed with a human erythrocyte suspension. The amount of radioactive virus attaching to the membranes and cells was measured and the percentage attachment of the original virus added and that added at the second incubation were calculated. The figures given are the mean of duplicate readings.

Duplicate 0.5 ml samples of Krebs plasma membranes (4.4 mg protein/ml), were each placed in microfuge tubes, 3 µl of ^{3}H adenosine labelled EMC virus (4.7×10^{6} cpm/ml, 1.6 mg virus/ml) was added to each and the samples were incubated at 4°C for 30 minutes. The membranes were pelleted by centrifuging for 1 minute in a microfuge to give membrane preparation 1 and supernatant fluid 1. A 50 µl aliquot of supernatant fluid 1 was retained for radioactive counting and the remaining 0.45 ml was incubated at 4°C for 30 minutes with 20 ml packed human erythrocytes in 1 ml PBS-glucose-gelatin solution. The erythrocytes were then pelleted by centrifugation and washed twice more as was membrane preparation 1. Unattached radioactivity comprised the supernatant fluids and the washes from the membrane and erythrocyte samples. These samples were counted for radioactivity along with the erythrocytes and membranes.

Approximately 50% of the added radioactivity attached to Krebs plasma membranes which was comparable to that attaching to whole cells (table 10). Only a very small percentage of the radioactivity which did not attach was able to subsequently attach to erythrocytes, again comparable to the situation observed using whole Krebs cells (table 10).

ii) Analysis of ^{3}H labelled EMC virus on sucrose gradients after mixing with Krebs plasma membranes.

To test if association with Krebs cells or plasma membranes destroyed the ability of unattached virus to adsorb to fresh Krebs cells, membranes or erythrocytes by affecting the integrity of the

virus, unadsorbed virus was examined by centrifugation on sucrose density gradients, as follows. To a 0.5 ml sample of Krebs plasma membranes (4.4 mg protein/ml) 3 µl of ^{3}H adenosine labelled EMC virus (4.7×10^{6} cpm/ml, 1.6 mg virus/ml) was added. This membrane sample was incubated at 4°C for 30 minutes along with a control consisting of 3 µl of ^{3}H labelled EMC virus diluted to 0.2 ml with PBS. The Krebs plasma membranes were pelleted by centrifuging for one minute in a microfuge. The radioactivity in a 0.3 ml aliquot of the supernatent fluid was measured as was that in the pelleted membranes and in two successive washes of the membranes. The remaining 0.2 ml of the initial membrane supernatant fluid and the whole of the virus control sample were analysed in 0.1 M NaCl-0.02H PO_4, pH 8.0, on linear 10-30% sucrose gradients which were centrifuged at 234,000 g (50,000 rpm) for 30 minutes in a spinco SW50.1 rotor. Five drop fractions were collected from the gradients and prior to radioactive counting these fractions were diluted to 1 ml with water. The distribution of radioactivity within the sucrose gradients is shown in fig. 18.

The virus control and the virus which had been exposed to Krebs plasma membranes both showed similar radioactive profiles throughout the gradients, demonstrating that interaction with membranes had not caused breakdown of virus. If anything, virus that had been mixed with Krebs plasma membranes sedimented ahead of control virus probably due to experimental variation. It is, however, possible that a cellular component had become attached to the virions causing them to sediment faster and preventing them

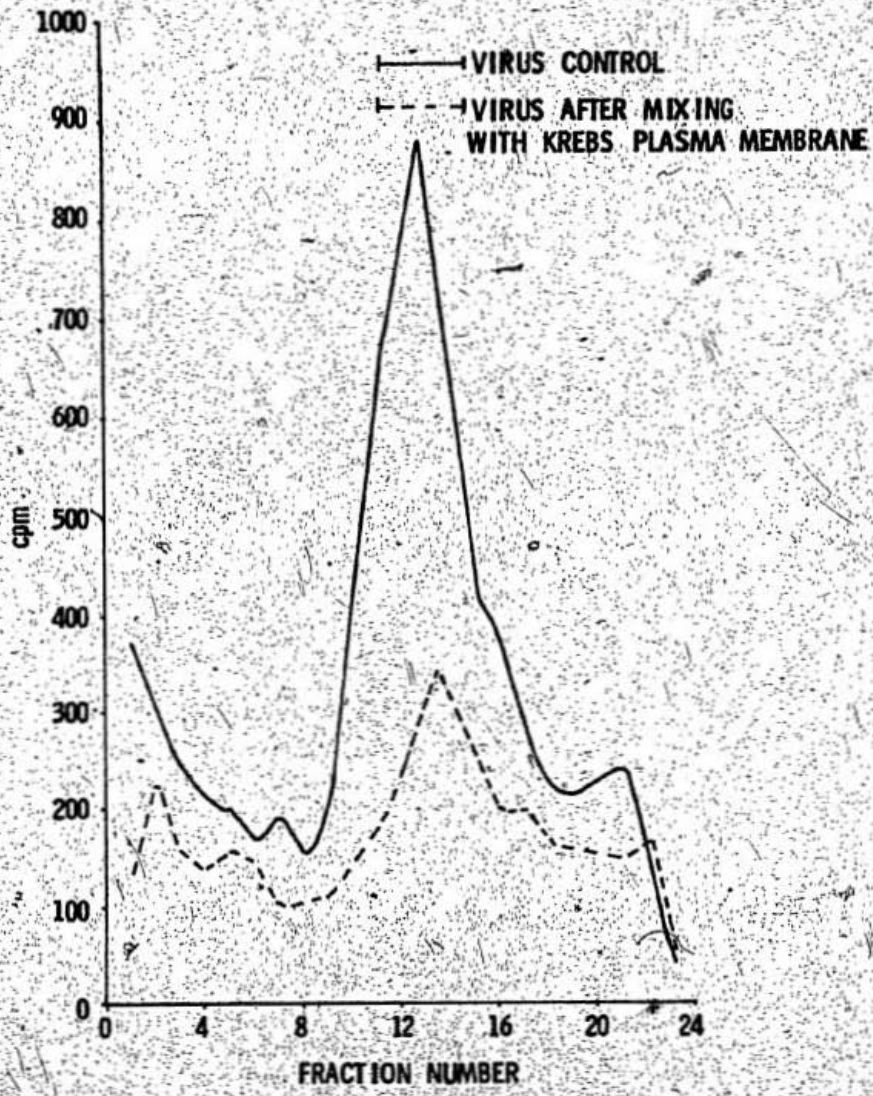


Figure 18. Analysis of EMC virus on sucrose gradients. Control ^{3}H labelled EMC virus and virus that had previously been mixed with Krebs plasma membranes was examined on 10-30 % sucrose gradients. The radioactive profiles of each series of 5 drop fractions are shown.

from attaching to other cells or membranes.

c) The effect of trypsin treatment on the attachment of EMC virus to Krebs cells.

Trypsin is a proteolytic enzyme and if the receptor for EMC virus on Krebs cells is a protein, trypsin treatment of the cell surface might be expected to reduce both attachment and infectivity. This possibility was investigated in the following ways.

i) The effect of trypsin treatment on the attachment of ^{3}H labelled EMC virus to Krebs cells.

A sample of 10 ml of Krebs cells at a concentration of 10^{8} cells/ml was treated with 20 µl (30,000 units) of deoxyribonuclease (DNAse) to prevent clumping of the cells during trypsin treatment.* The following samples were prepared.

1. Trypsin treated; consisted of 10^{8} Krebs cells incubated at 37°C for 30 minutes with 17 BAEE units of trypsin. The reaction was stopped with 100 µl of 1% lima bean trypsin inhibitor.

2. Lima bean trypsin inhibitor treated; consisted of 10^{8} cells treated with 100 ml of 1% lima bean trypsin inhibitor.

3. Untreated control; consisted of 10^{8} Krebs cells incubated at 37°C for 30 minutes.

Each sample was then incubated at 4°C for 30 minutes with 50 µl of ^{3}H amino acid labelled EMC virus (5×10^{5} cpm/ml, 1.6 mg virus/ml). Unattached virus was removed by washing the cells three times and the radioactivity in the unattached virus and cell samples was counted

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* Thank you to Dr. H.B. Younghusband for this suggestion.

Sample	Unattached radioactivity (cpm)	Cell bound radioactivity (cpm)	Percentage of radioactivity on cells
Untreated cell control	10,899	5,517	33.6
trypsin inhibitor treated cells	11,414	5,725	34.5
trypsin treated cells	23,779	929	3.8

Table 12. The effect of trypsin on the attachment of EMC virus to Krebs cells. A sample of Krebs cells was treated with trypsin. A second sample was treated with the trypsin inhibitor only, used to stop the reaction with trypsin and a third sample remained untreated. All of these cell samples were incubated at 4°C with ^{3}H labelled EMC virus for 30 minutes and the unattached and cell bound radioactivity was measured. The results shown are the mean values of duplicate readings.

(table 12).

About 34% of the added radioactivity attached to both the untreated and trypsin inhibitor treated cell samples. Only about 10% of this radioactivity was able to attach to the trypsin treated cells indicating that the receptor for EMC virus on Krebs cells may be a protein.

ii) The effect of trypsin treatment of cells on the infectivity of EMC virus.

Since trypsin treatment of Krebs cells inhibited the attachment of ^{3}H labelled EMC virus, it was decided to measure the effect of trypsin treatment of cells on the attachment of infectious EMC virions in particular.

Trypsin, 17 BAEE units, was added to 5×10^{7} Krebs cells in 1 ml of $Ca^{++}Mg^{++}$ free PBS. The sample was incubated at 37°C for 30 minutes after which the reaction was stopped with 100 µl of 1% lima bean trypsin inhibitor. The clumping of cells due to trypsin, which was overcome by DNAse treatment in the previous experiment, was prevented in this experiment by the use of $Ca^{++}Mg^{++}$ free PBS, lower concentrations of cell suspensions and vortexing after the addition trypsin. Two controls were prepared; firstly one with 100 µl of 1% trypsin inhibitor and secondly one untreated sample. Each of the controls and the trypsin treated cell suspension were used for infectious centre assays (table 13).

There was only about 10% the number of plaques in the trypsin treated sample as compared to the untreated control. The presence of trypsin inhibitor appeared to enhance infectivity.

Sample	Mean number of plaques at 10^4 cells/ml	Percentage of cells infected	Percent infectivity relative to the untreated control
Untreated cell control	49	4.9	100
Trypsin inhibitor treated cells	61	6.1	124
Trypsin treated cells	4.5	0.45	9.2

Table 13. The effect of trypsin treatment of Krebs cells on the infectivity of EMC virus. Krebs cell samples were treated with trypsin, trypsin inhibitor only or left untreated and then used for infectious centre assays. The results shown above are the mean values of duplicate readings.

These results show that the attachment of infectious virions is markedly reduced by trypsin treatment of Krebs cells.

d) The effect of neuraminidase on EMC virus attachment and infectivity.

Neuraminidase removes sialic acid residues from glycoproteins and glycolipids. Since the attachment of EMC virus to erythrocytes is reduced by treatment of the cells with neuraminidase (57), it was important to determine whether or not sialic acid plays a role in the attachment of EMC virus to Krebs cells.

i) The effect of neuraminidase treatment of Krebs plasma membranes on the attachment of ^{3}H labelled EMC virus.

Duplicate samples of 0.5 ml of Krebs plasma membrane suspension (6.4 mg protein/ml) and of 20 µl of washed packed sheep erythrocytes in 0.5 ml of PBS were each mixed with 10 units of neuraminidase and incubated for 1 hour at 37°C. The erythrocytes and membranes were then collected by centrifuging for 30 seconds in a microfuge and the pellets were washed twice more to remove the neuraminidase. To each resuspended membrane and erythrocyte sample 3 µl of ^{3}H adenosine labelled EMC virus (4.7×10^{6} cpm/ml, 1.6 mg virus/ml) was added. Non-neuraminidase treated Krebs plasma membrane and erythrocyte controls were also each mixed with 3 µl of ^{3}H labelled EMC virus. All of the samples were incubated at 4°C for 30 minutes. Unattached radioactivity was removed by washing, the membranes and erythrocytes being sedimented in a microfuge, and the unattached cell or membrane bound radioactive counts were determined.

Treatment of erythrocytes with neuraminidase reduced EMC

Sample	Unattached radioactivity (cpm)	Cell bound radioactivity (cpm)	Percentage of radioactivity bound
Neuraminidase treated Krebs plasma membranes	2,927	1,369	31.8
Untreated Krebs plasma membranes	2,631	1,945	42.5
Neuraminidase treated sheep erythrocytes	5,015	123	2.4
Untreated sheep erythrocytes	1,934	1,087	36.0

Table 14. The effect of neuraminidase treatment of Krebs plasma membranes on EMC virus attachment. Krebs plasma membranes and sheep erythrocytes were treated with 10 units of neuraminidase for 1 hour at 37°C prior to mixing with ^{3}H labelled EMC virus. Attachment of virus was measured by monitoring the amount of radioactivity bound to cells or membranes. The results are the mean values of duplicate readings.

virus attachment to about 7% of the untreated erythrocyte control, (table 14). Neuraminidase treatment of the Krebs plasma membranes reduced EMC virus attachment to 75% of the plasma membrane control. Sialic acid is, therefore, probably required for EMC virus attachment to Krebs plasma membranes, but possibly not to the same extent as in the attachment of EMC virus to erythrocytes.

ii) The effect of neuraminidase treatment of cells on the attachment of ^{3}H labelled EMC virus to Krebs cells.

To determine whether neuraminidase treatment of whole cells had the same effect on EMC virus attachment as neuraminidase treatment of plasma membranes the following experiment was performed.

A 1 ml suspension of 10^{8} Krebs cells was incubated for 1 hour at 37°C with 25 units of neuraminidase and then washed three times to remove the neuraminidase. This neuraminidase treated sample and an untreated 1 ml cell sample were each incubated at 4°C for 30 minutes with 2 µl of ^{3}H adenosine labelled EMC virus. (4.7×10^{6} cpm/ml, 1.6 mg virus/ml). Unattached radioactivity was removed by washing and was measured along with the cell bound radioactivity. The experiment was repeated using human erythrocyte samples of 20 µl of packed washed cells in 1 ml of PBS instead of Krebs cells.

Neuraminidase treatment of human erythrocytes reduced attachment to about 1% of the untreated sample. Treatment of Krebs cells with neuraminidase reduced EMC virus attachment to 60% of the untreated control (table 15). This decrease in ^{3}H labelled virus binding was greater than that seen with plasma membranes (table 14)

Sample	Unattached radioactivity (cpm)	Cell bound radioactivity (cpm)	Percentage of radioactivity bound to cells
Neuraminidase treated Krebs cells	12,403	3,617	22.6
Untreated Krebs cells	7,782	4,709	37.7
Neuraminidase treated human erythrocytes	11,890	75	0.6
Untreated human erythrocytes	2,751	2,916	51.5

Table 15. The effect of neuraminidase treatment of Krebs cells on EMC virus attachment. Krebs cell and human erythrocyte suspensions were incubated at 37°C for 1 hour with 25 units of neuraminidase. After the removal of neuraminidase by washing these cell samples and two similar untreated control samples were mixed with ^{3}H labelled EMC virus. The amount of virus which attached was measured by monitoring the unattached and cell bound radioactivity.

but more enzyme was used in this experiment.

iii) The ability of ^{3}H labelled EMC virus to attach to Krebs cells after the release of sialic acid over an extended time period.

The effect of neuraminidase treatment of Krebs cells or plasma membranes is not as great as that on erythrocytes with regards to EMC virus attachment. Since some EMC virus could still attach to Krebs cells or plasma membranes even after neuraminidase treatment the time period of exposure to neuraminidase was increased to determine if EMC virus attachment to Krebs cells would be further reduced.

Samples of Krebs cells, 0.5 ml at 10^{8} cells/ml concentration, were treated with 10 units of neuraminidase and incubated for 1, 2 and 4 hours at 37°C. A further 0.5 ml sample of Krebs cells was incubated at 37°C for 1 hour in the absence of neuraminidase. After incubation, the cells were centrifuged and the supernatant fluids retained for sialic acid determination. The cells were washed twice more to remove neuraminidase and then incubated at 4°C for 30 minutes with 20 µl of ^{3}H adenosine labelled EMC virus (4.7×10^{6} cpm/ml, 1.6 mg virus/ml). The cell samples were then washed three times to remove unattached radioactivity. The radioactivity in the cell samples and unattached radioactivity were then measured.

In attachment experiments it is possible that non-specific adsorption to the surface of test-tubes may occur. To test for this a control sample was included in this experiment. In the absence of cells, 20 µl of ^{3}H labelled EMC virus was diluted to

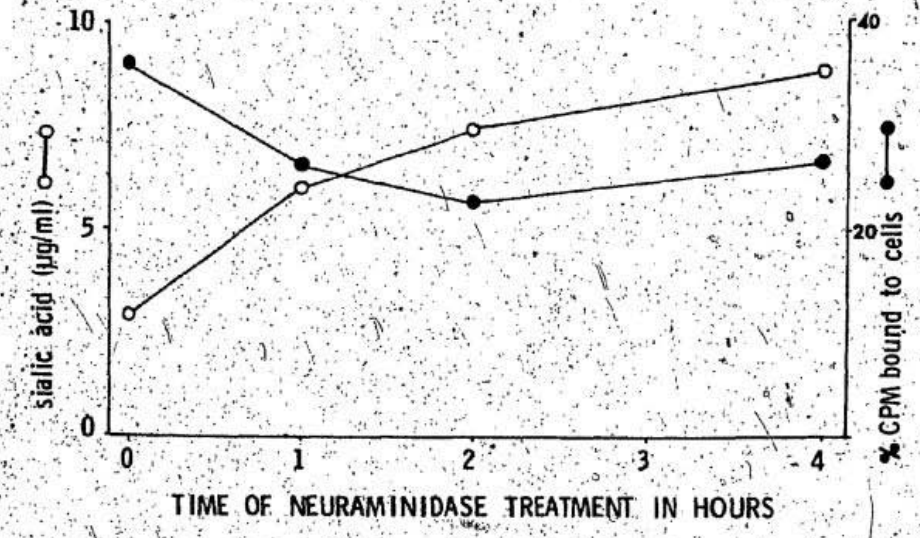

Figure 19. The ability of EMC virus to attach to cells lacking sialic acid. Samples of Krebs cells were treated with 10 units of neuraminidase for different time periods. The amount of sialic acid released into the cell suspensions was measured using the Warren assay. The cells were then exposed to radioactively labelled EMC virus, at 4°C for 30 minutes. Cell bound radioactivity was then measured after removing unattached radioactivity by washing the cells.

0.5 ml with PBS and incubated at 4°C for 30 minutes. This sample was also monitored for radioactivity.

As more sialic acid was released the percentage of radioactivity adsorbing to cells tended to decrease except after 2 hours neuraminidase treatment (fig. 19). The increase in radioactivity bound in the 4 hour sample may have been due to experimental variation or, alternatively, may have been caused by the inactivation of neuraminidase and the regeneration of sialic acid moieties on the cell surface. Since virtually 100% of the radioactivity added to the control without cells was recovered, there must have been no loss of radioactivity owing to non-specific adsorption to test tubes. This is most probably the case for all the attachment experiments performed because similar equipment was used.

iv) <u>The effect of neuraminidase treatment of Krebs cells on the infectivity of EMC virus.</u>

To determine whether neuraminidase treatment of cells affected the attachment of infectious virus particles in particular, the effect of neuraminidase treatment of cells on infectivity was investigated.

The infectious centre assay was carried out using an untreated control and a second 0.5 ml sample of 5×10^7 cells that had been incubated with 25 units of neuraminidase at 37°C for 30 minutes. The cells were washed three times to remove neuraminidase after incubation.

Neuraminidase treatment of Krebs cells was found to decrease their ability to bind infectious EMC virus to, on average, 53% of

Sample	Percentage infectivity		Percentage reduction in infectivity due to neuraminidase
	Neuraminidase treated	Untreated	
1	1.15	3.15	63.5
2	2.25	4.15	45.8
3	2.00	6.50	69.2
4	19.00	27.50	30.9
5	6.30	8.50	25.9

Table 16. The effect of neuraminidase treatment of Krebs cells on the infectivity of EMC virus. The infectious centre assay was carried out using untreated Krebs cell samples and those that had been incubated at 37°C for 30 minutes with 25 units of neuraminidase. The mean reduction in infectivity is 47.06% with a standard error of 8.58, (5 determinations). The percentage infectivity is the percentage of total infected cells at a certain cell concentration which gave rise to plaques.

the untreated control (table 16).

III Isolation and characterization of the EMC virus receptor on Krebs cells.

a) Extraction of the EMC virus receptor from Krebs cells.

The reduction in the attachment of EMC virus to cells treated with trypsin and neuraminidase is consistent with the receptor being a sialoglycoprotein. To investigate this possibility attempts were made to isolate glycoprotein from whole Krebs cells, crude Krebs plasma membranes and from purified Krebs plasma membranes so that they could be characterized and tested for biological activity. Lithium diiodosalicylate, (LIS), and phenol extraction has been used to isolate glycoproteins from other cells such as erythrocytes (75), L1210, P-388 and HeLa cells (16) and, therefore, this method was tried.

During the later stages of the extraction procedure material is partitioned between aqueous and phenol layers. Usually, the aqueous layer, containing the isolated glycoproteins is further purified whilst the phenol layer is discarded. During an extraction of purified plasma membranes material from the phenol layer, like the aqueous layer was also further extracted with alcohol, dialysed against distilled water and lyophilized so that a comparison could be made between the material in the aqueous and phenol layers.

The lyophilized phenol layer extract was resuspended in 0.01M tris-HCl buffer, pH 8.0, and examined by SDS-PAGE. Coomassie blue staining revealed one clear band but no bands were observed on the PAS stained gels. This contrasted with SDS-PAGE analysis

Sample	Number of cups of inhibition	Number of cups of HA in the virus control	Percentage inhibition
Phenol layer	2.5	60	4.2
Aqueous layer	26	57	45.6

Table 17. Extraction of the EMC virus receptor from purified Krebs plasma membranes. During the extraction of glycoproteins the phenol layer of the extraction, in addition to the aqueous layer was further extracted. Samples of both layers were tested for their ability to inhibit haemagglutination (HA). The number of cups of inhibition was counted, that is, the number of cups with no HA but which would have shown HA if the virus was present alone. The number of cups of HA in the virus control was also counted and the percentage inhibition calculated.

of material in the aqueous layer which contained PAS staining material as will be described later.

Material derived from the phenol and aqueous layers of the extraction were tested for their ability to inhibit haemagglutination. The phenol layer which as reported (16, 75) and shown by SDS-PAGE contained proteins but no glycoprotein had very little effect on EMC virus haemagglutination (table 17). In contrast material from the aqueous layer, reported to contain glycoproteins (16, 75) was able to strongly inhibit EMC virus HA, probably by competing with the erythrocytes to bind virus. Thus, the EMC virus receptor is probably a glycoprotein that became localized in the aqueous layer during the LIS-phenol extraction procedure.

b) Inhibition of haemagglutination by the isolated Krebs glycoprotein fraction.

Attempts to measure the concentrations of protein and sialic acid, if present, in the preparations were not successful because the assays used gave negative or only marginally positive results. In order to be able to compare the biological activity of the glycoprotein preparations, saturated solutions of glycoprotein were assayed for haemagglutination inhibition. Saturated solutions of glycoproteins isolated from whole cells, crude membranes and purified membranes all inhibited haemagglutination (table 18). The purified membrane derived glycoprotein was apparently a better inhibitor than crude membrane derived glycoprotein which, in turn, was better than whole cell derived glycoprotein at inhibiting haemagglutination. These differences may have arisen due to one or more causes. For

Source of glycoprotein	Percentage of inhibition of haemagglutination
whole cells	32.8 ± 3.3 (14)
crude membranes	35.3 ± 4.2 (9)
purified membranes	45.6

Table 18. Inhibition of haemagglutination by glycoprotein prepared from different sources. Glycoproteins prepared from different sources were tested for their ability to inhibit haemagglutination. The percentage inhibition of haemagglutination is a measure of the cups of HA inhibited by each glycoprotein preparation as compared to a virus control with no glycoprotein present. Standard errors are given and the figures in brackets are the numbers of determinations. The value given for purified membrane derived glycoprotein is the mean of duplicate readings.

Time of freezing in days	Cups of inhibition	Cups of HA of the virus control	Percentage of inhibition of HA
0	24.5	42	58.3
5	24.0	48	50.0
7	15.5	42	36.9

Table 19. The decline in biological activity of a crude membrane derived glycoprotein preparation after freezing. A preparation of crude membrane derived glycoprotein was tested for its ability to inhibit HA after being stored frozen. The percentage inhibition of haemagglutination is a measure of the cups of HA inhibited by each glycoprotein preparation as compared to the cups of HA due to the virus control where glycoprotein was absent.

instance, contaminants present in the whole cell or crude membrane derived preparations might have caused degradation of the biologically active material or reduced the amount of it dissolved in a saturated solution.

Glycoproteins prepared from any of these sources lost their biological activity at 4°C or -15°C. For instance, the activity of whole cell or crude membrane derived glycoproteins was reduced to zero after storing at 4°C for 48 hours. An example of the decline in activity of a frozen crude membrane derived glycoprotein preparation is shown in table 19. Glycoprotein derived from purified plasma membranes was more stable and remained active for 4 to 5 days. This loss of activity could have been caused by the presence of proteolytic enzymes as contaminants especially since trypsin has already been shown to inactivate the receptor in the Krebs cell. Glycoprotein preparations could, however, be stored successfully in a freeze dried form with no loss of activity.

c) <u>The effect of different treatments on the biological activity of Krebs glycoprotein</u>.

Krebs glycoprotein preparations contained HA inhibitors which may have been the glycoproteins per se or some other component present in the preparations. In addition, only trace amounts, if any, of sialic acid were detected in whole cell or crude membrane derived preparations but nucleic acid was detected from ultra-violet absorbance measurements. To investigate the chemical nature of the inhibitor the glycoprotein preparations were tested as follows.

1. Trypsin treatment of an inhibitor that is a protein would reduce

HA inhibition. To test this 1 ml of glycoprotein preparation was incubated at 37°C for 30 minutes with 34 BAEE units of trypsin. The reaction was stopped with 0.2 ml of 1% lima bean trypsin inhibitor.

2. Trypsin inhibitor was tested as a control of the trypsin experiment by mixing 1 ml glycoprotein preparation with 0.2 ml of 1% lima bean trypsin inhibitor.

3. Neuraminidase should affect the biological activity of sialic acid containing molecules and this was tested by incubating 0.5 ml of glycoprotein preparation with 250 units of neuraminidase. The reaction was stopped by boiling for 10 minutes.

4. Boiling was tested as a control for neuraminidase. Many proteins, particularly enzymes such as neuraminidase are destroyed by boiling. Sialoglycoproteins, however, can resist boiling, for example, glycophorin (30). To test the effect of boiling 0.5 ml of glycoprotein preparation was diluted with 0.5 ml of water and boiled for ten minutes.

5. Chloroform / methanol extraction would remove any lipid components such as gangliosides which could act as cellular receptors for viruses. Any lipids present were removed from the glycoprotein preparation by mixing 0.5 ml with an equal volume of chloroform/methanol (1:1 v/v) and extracting the aqueous layer three times. The chloroform/methanol and aqueous layers and the precipitate which formed at the interface were evaporated to dryness and resuspended in 0.5 ml water and tested for their inhibitory properties.

6. Ribonuclease (RNAse) treatment was used to determine the importance of the nucleic acids found in the whole cell and crude membrane derived glycoprotein preparations. To test this, 1 ml of glycoprotein preparation was incubated with 54 units of RNAse at 37°C for 30 minutes. RNAse alone had no effect on HA.

7. Lithium diiodosalicylate was present as a contaminant in glycoprotein preparations. LIS, at a concentration of 0.5 mg/ml, similar to the concentration found in glycoprotein preparations had no effect on HA. Controls, without treatment, were done on all of the glycoprotein preparations tested to determine the maximum inhibition possible due to each glycoprotein preparation. The results of various treatments can then be compared by looking at the percent inhibition relative to the control (table 20).

Trypsin and neuraminidase treatment of the glycoprotein fraction reduces its inhibitory capability by about 50% suggesting that it is a sialoglycoprotein in the Krebs glycoprotein fractions that has virus receptor characteristics. Trypsin inhibitor and boiling alone had little effect on HA inhibition confirming that it was the effect of trypsin and neuraminidase that was reducing inhibition of HA by glycoprotein fractions. This also shows that the receptor is resistant to boiling. The chloroform/methanol layer was unable to inhibit HA but the precipitate and aqueous layer could inhibit HA showing that the receptor is not a lipid. Ribonuclease did not affect HA very much and thus the RNA present in some glycoprotein preparations is probably not the biologically active component.

Treatment	Mean percentage inhibition		Percentage inhibition relative to the control
	treated sample	untreated sample	
Trypsin treated	19 ± 2.0 (6)	37 ± 5.0 (6)	51
trypsin inhibitor treated	21	25	84
Neuraminidase treated	21 ± 4.0 (5)	37 ± 3.0 (5)	57
Boiling	31 ± 3.0 (5)	37 ± 3.0 (5)	84
Ribonuclease	33	37	89
Chloroform/ methanol extraction			
1) aqueous layer	14	25	56
2) chloroform/ methanol layer	0	25	0
3) precipitate at the interface	21	25	84

Table 20. The effect of various substances on the inhibition of haemagglutination by Krebs glycoprotein. Trypsin and neuraminidase and the treatments used to stop their activity, trypsin inhibitor and boiling respectively, were used to treat the Krebs glycoprotein before carrying out the HA inhibition assay. Krebs glycoprotein was extracted with chloroform/methanol and the three fractions formed, the chloroform/methanol and aqueous layers, and the precipitate were tested for their inhibitory properties. The percentage of HA inhibition compared to the HA of virus alone is shown for untreated controls and the treated samples. The percentage inhibition of treated samples relative to untreated controls is also shown. Standard errors are given followed by the number of determinations in brackets, except where a result is the mean of duplicate readings.

d) Inhibition of Wheat germ agglutinin haemagglutination by Krebs glycoprotein.

EMC virus and WGA attach to the same erythrocyte receptor (87) which is similar in its properties to Krebs cell glycoprotein in that it can be extracted with LIS and phenol (87). Since, like EMC virus, WGA attaches to and agglutinates Krebs cells it was important to determine whether both attached to the same receptor on Krebs cells. The ability of Krebs glycoproteins to inhibit WGA haemagglutination was therefore tested with WGA solutions of 50 µg/ml and 25 µg/ml instead of EMC virus. Using the same glycoprotein preparations the HA inhibition assay was carried out with EMC virus also.

WGA haemagglutination was inhibited by Krebs glycoprotein at both concentrations of WGA used (table 21). Inhibition was lower than that observed for EMC virus HA at the higher WGA concentration but approximately the same as EMC virus HA at the lower WGA concentration.

e) The effect of isolated Krebs glycoprotein on the attachment of ^{3}H labelled EMC virus to Krebs cells.

The results so far have shown that Krebs glycoprotein preparations inhibit the attachment of EMC virus to erythrocytes. It is important to determine also if the attachment of EMC virus to a host cell, the Krebs cell, is affected by the presence of Krebs glycoprotein.

To do this, Krebs cells at 10^{8} cells/ml of 2x concentration PBS were distributed in 1 ml aliquots and to two of the samples, 1 ml

WGA concentration µg 1 ml	cups of inhibition	cups of HA of WGA control	percentage inhibition of haemagglutination	percentage inhibition of EMC virus HA by these glycoprotein preparations
50	6.25 ± 0.63 (4)	29.25 ± 0.75 (4)	21.4	43.2 ± 1.9 (4)
25	12	36	33.3	25.0

Table 21. Inhibition of Wheat germ agglutinin haemagglutination by Krebs glycoprotein. Krebs glycoprotein preparations were tested to determine their ability to inhibit WGA haemagglutination at two concentrations of WGA. The percentage inhibition of haemagglutination is a measure of the cups of HA inhibited by the glycoprotein preparation as compared to the cups of HA due to the WGA control where glycoprotein was absent. Standard errors followed by the number of determinations, in brackets, are given except where the result is the mean of duplicate readings. Finally the same glycoprotein preparations were tested for their ability to inhibit EMC virus HA (far right hand column).

Glycoprotein preparation	Unattached radioactivity (cpm)	Cell bound radioactivity (cpm)	percentage of radioactivity bound to cells
absent	4,436	1,459	25.2
present	5,869	1,077	15.5

Table 22. The effect of solubilized Krebs glycoprotein on the attachment of EMC virus to Krebs cells. Samples of Krebs cells were mixed with Krebs glycoprotein and then along with a control sample of Krebs cells were exposed to ^{3}H labelled EMC virus. Attachment of virus was measured by monitoring unattached radioactivity, which was removed by washing, and cell bound radioactivity.

of distilled water was added, and to two more samples 1 ml of a saturated solution of crude membrane glycoprotein in water was added. To all of the samples 20 µl of ^{3}H amino acid labelled EMC virus (5×10^{5} cpm/ml, 1.6 mg virus/ml) was added and the samples were incubated to 4°C for 30 minutes. The cells were then washed three times to remove unattached radioactivity which was counted along with the cell samples.

In the presence of the glycoprotein, attachment was reduced to 60% of the control, suggesting that the glycoprotein and Krebs cells had been competing to bind virus (table 22).

f) The effect of the solubilized Krebs glycoprotein on the infectivity of EMC virus.

Attachment of ^{3}H labelled EMC virus to Krebs cells was inhibited by Krebs glycoprotein preparations. Only a small proportion of the ^{3}H labelled EMC virions would be infectious and in order to investigate the attachment of infectious virions in particular the effect of the Krebs glycoprotein preparations on the infectivity of EMC virus was measured.

An infectious centre assay was carried out using 0.5 ml of 10^{8} Krebs cells/ml suspended in X2 concentration PBS mixed with 0.5 ml of a saturated solution of crude membrane glycoprotein, and a similar 0.5 ml sample of Krebs cells mixed with 0.5 ml of distilled water.

The mean number of infectious centres in the control was 95 and in the presence of glycoprotein was reduced to 66 or 69% of the control. This percentage reduction is very similar to that for

the reduction of ^{3}H labelled EMC virus attachment in the presence of glycoprotein which suggests that infectious and apparently non-infectious virions attach in the same way and their attachment is inhibited by the isolated Krebs glycoprotein.

IV. Molecular weight of the Krebs cell receptor for EMC virus.

a) Protein and sialic acid content.

Proteins generally exhibit an adsorption maximum at 280 nm owing to their tyrosine, tryptophan, and phenylalanine content (41). To determine if Krebs glycoprotein could be detected in this way freshly prepared samples were scanned from 200 to 340 nm wavelengths. A peak was observed at 260 nm, indicating the presence of nucleic acid in Krebs glycoprotein derived from whole cells or crude membranes. In the pure membrane glycoprotein preparations the only peak observed was at 320 nm, the wavelength at which lithium diiodosalicylate has maximum adsorption; LIS was previously shown to have no biological activity in terms of HA inhibition. RNAse treatment of the Krebs glycoprotein fractions had no effect on biological activity either. Despite the lack of detectable nucleic acid in the pure membrane derived glycoprotein preparations the biological activity of these preparations was at least as high as those derived from whole cells or crude membranes. These results indicate that the biologically active component is present in very small quantities but is nevertheless highly active.

Using the Lowry method trace amounts of protein were detected in crude membrane derived glycoprotein preparations but a measureable amount of about 0.3 mg/ml of protein was detected in

glycoprotein derived from purified plasma membranes containing 13.5 mg protein.

In pure membrane derived glycoprotein preparations only, 11 µg/ml sialic acid was detected, which is equivalent to 32 µg sialic acid/mg protein in the glycoprotein preparation.

b) SDS PAGE of Krebs pure membrane glycoprotein.

i) Estimate of the glycoprotein molecular weight using human erythrocyte glycoproteins of known molecular weight.

Krebs glycoprotein prepared from purified plasma membranes was analysed by SDS-PAGE. Glycoproteins from human erythrocyte membranes were run in parallel as molecular weight markers. For comparison a gel was also run with purified Krebs plasma membranes. It was found, by experience, that a lot of material was required for detection by PAS staining of gels and, therefore, to obtain maximum glycoprotein concentration freeze-dried material was dissolved directly in degradation buffer and boiled for 10 minutes before loading on to gels. Electrophoresis was carried out for 2 hours after which gels were stained with PAS reagent and scanned as soon as possible at 550 nm wavelength in a Gilford spectrophotomotor fitted with a gel scanner.

Four major bands were revealed with PAS reagent on human erythrocyte membrane gels as expected. PAS staining of Krebs plasma membranes and the isolated Krebs glycoprotein revealed very similar patterns consisting one major band close to the origin of the gel. This pink band faded within 1 or 2 days on the plasma membrane gel and overnight on the isolated glycoprotein gel. From

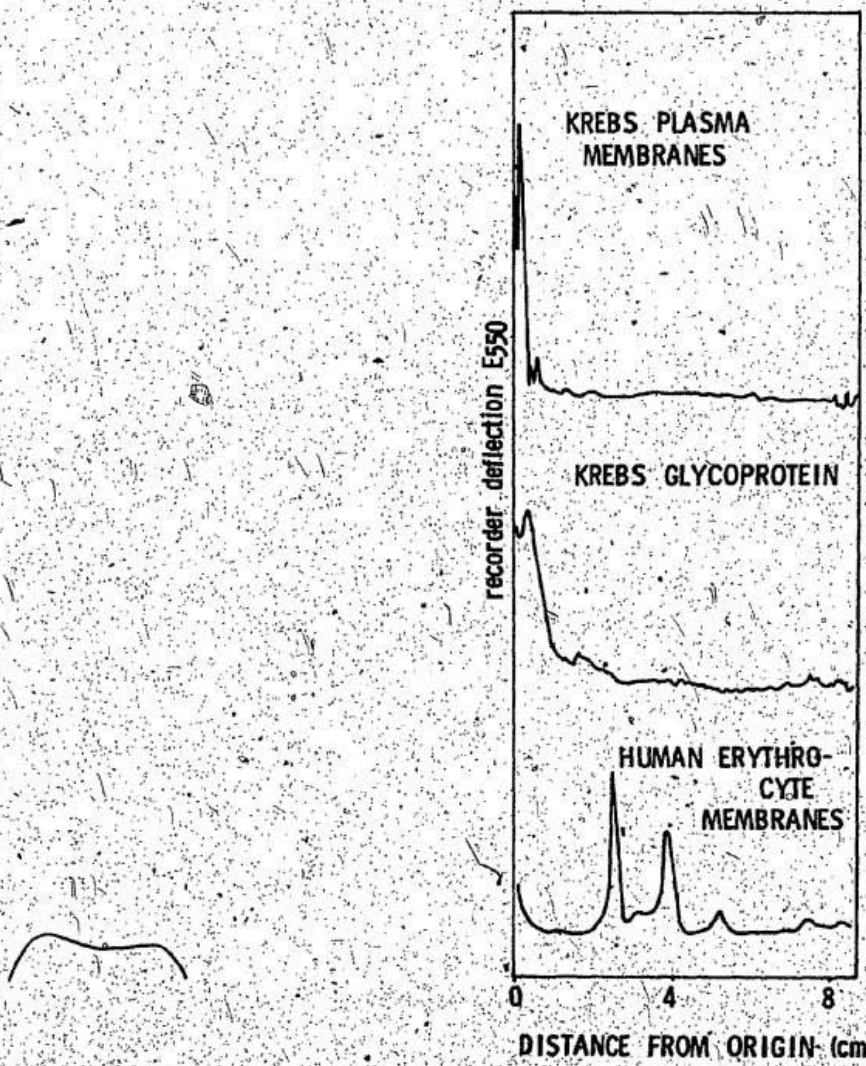

Figure 20. SDS-PAGE scans of Krebs glycoprotein stained with PAS reagent. SDS-PAGE was carried out using samples of isolated glycoprotein, Krebs plasma membranes and human erythrocyte membranes for 2 hours. The gels were stained with PAS reagent and scanned.

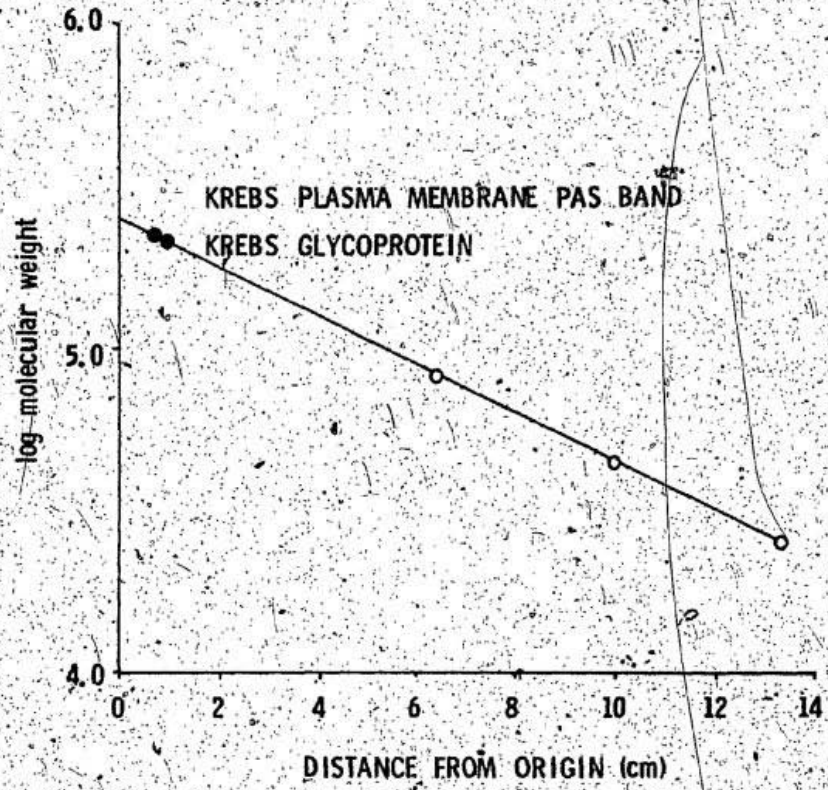

Figure 21. Estimation of the Krebs glycoprotein molecular weight. SDS-PAGE was carried out for 2 hours using human erythrocyte membranes, Krebs plasma membranes and the isolated Krebs glycoprotein. The gels were stained with PAS reagent and the distance of each band from the origin of each gel was measured and plotted against the log of molecular weight. The 3 major peaks of the erythrocyte PAS profile are known to be of molecular weights 83,500, 45,600 and 25,500.

the mobilities of the erythrocyte glycoproteins of known molecular weight (31) graphical estimations were made of the size of both the isolated glycoprotein and plasma membrane PAS staining components (Fig. 20, 21). The plasma membrane PAS band was estimated to have a molecular weight 225,000 whereas that for the isolated glycoprotein was 215,000 daltons.

ii) Estimation of the Krebs glycoprotein molecular weight using protein markers.

Since the erythrocyte glycoproteins previously used as molecular markers were in a lower molecular weight range than the Krebs glycoprotein, a mixture of protein markers of a higher molecular weight range were used in further SDS-PAGE studies of Krebs glycoprotein derived from purified membranes. The protein mixture contained the following:

Protein	Molecular weight	Subunit molecular weight in SDS
Thyroglobulin	669,000	330,000
Ferritin	440,000	18,500 (220,000)
Phosphorylase b	188,000	94,000
Albumin	67,000	67,000
Calatase	232,000	60,000
Lactate dehydrogenase	140,000	36,000

All samples were boiled for 10 minutes in degradation buffer before loading on gels. As before, to get maximum concentration, the freeze-dried Krebs glycoprotein preparation was dissolved by boiling in the degradation buffer. The gels were run for six hours to

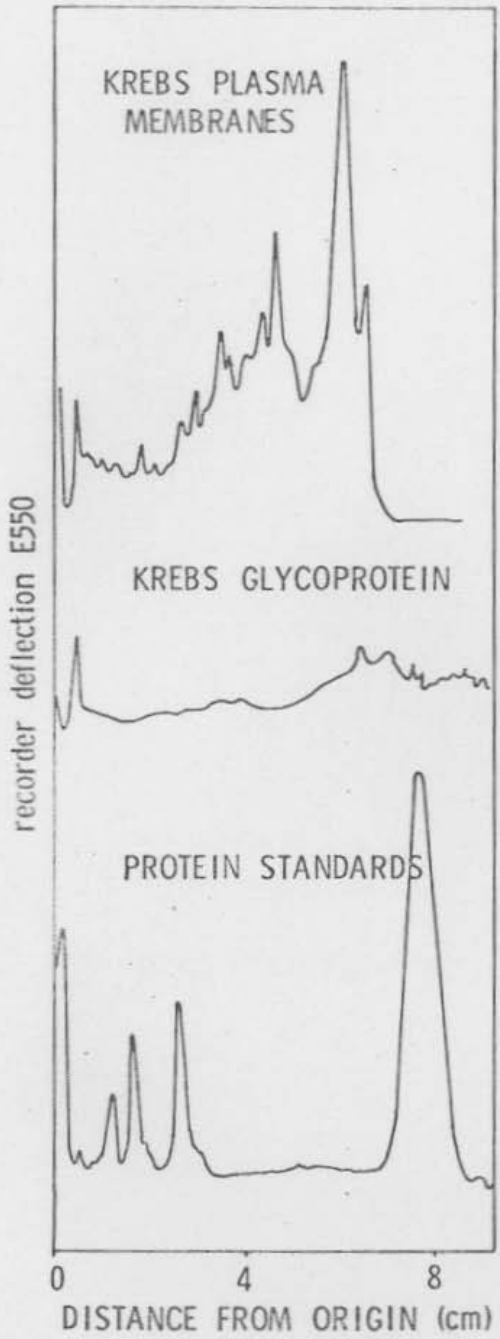

Figure 22. SDS-PAGE scans of Krebs glycoprotein. These scans are of gels of isolated Krebs glycoprotein, Krebs plasma membranes and protein standards electrophoresed for 6 hours and stained with coomassie blue. The protein standards range in molecular weight from 330,000 to 18,500 daltons.

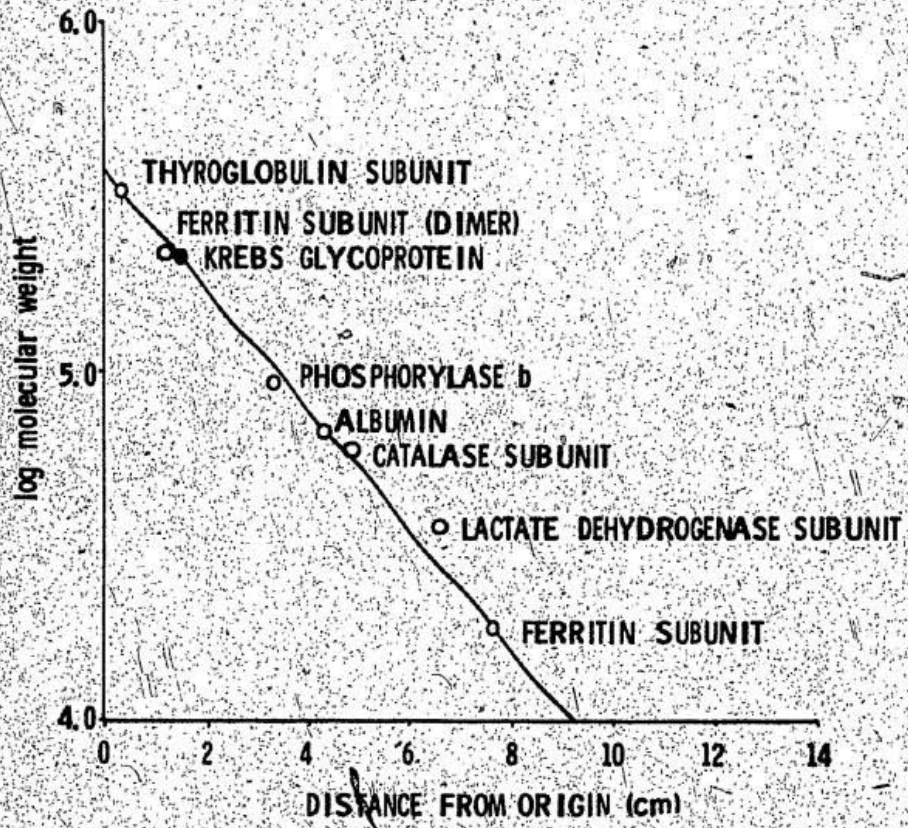

Figure 23. Estimation of the Krebs glycoprotein molecular weight. SDS-PAGE was carried out for 6 hours using a mixture of protein standards of known subunit molecular weights ranging from 330,000 to 18,500 daltons and the Krebs cell glycoprotein. The gels were stained with coomassie blue and the distance moved by each band from the origin of each gel was measured. This distance was plotted against the log of the molecular weight.

increase the migration of the glycoprotein band into the gel thus making measurements of mobilities more accurate. The gels were stained with coomassie blue and scanned as previously described.

The relationship between log of molecular weight and mobility was linear for the protein markers (Fig. 23). The coomassie blue profile of the Krebs glycoprotein (Fig. 22) was very simple compared to that of purified membranes (Fig. 22). There was one distinct peak near the origin of the gel and several small peaks in the lower molecular weight region of the gel. Using the distance moved by each band into the gels and the subunit molecular weights the molecular weight of the Krebs protein peak near the origin was estimated graphically to be 215,000 (Fig. 23). This is the same value estimated by using erythrocyte glycoproteins as markers and is probably quite accurate but can only be described as an estimate because the carbohydrate content, which may affect mobility is not known for the Krebs cell glycoprotein.

Discussion

Part I. The Krebs cell surface.

a) Krebs plasma membranes.

1) Preparation and purification of Krebs plasma membranes.

Plasma membranes were prepared from Krebs cells by hypotonic shock and differential centrifugation followed by centrifugation on sucrose gradients. This type of method has been used to prepare plasma membranes from many other cell types, for example a combination of hypotonic and ultrasonic disruption was used to prepare mouse liver plasma membranes (39). Different cells require varying degrees of severity of hypotonic disruption ranging from 50 to 100 strokes in a dounce homogenizer for Baby Hamster Kidney (BHK) cells (35), to 10 strokes in a teflon pestle homogenizer for rat liver cells (108).

To prepare Krebs plasma membranes the method of Aitkinson and Summers (1) was initially used but mostly membrane fragments were produced rather than whole membrane ghosts. By modifying this method, a gentler method of membrane preparation involving shorter times of centrifugation and different stages of hypotonic swelling was formulated. Seven strokes in a teflon pestle homogenizer produced mostly whole membrane ghosts as shown in the electron micrographs (Figs. 2, 3, 4).

Only a small amount of plasma membranes were produced from a relatively large number of whole cells. It was, however, considered better to forfeit efficiency in membrane preparation because,

in further experiments the results were more reproducible if the membrane preparation comprised mainly intact plasma membranes.

During their preparation Krebs plasma membranes were also purified. To obtain a reliable estimate of purity it was considered advisable to employ a combination of methods which included, marker enzyme assays, electron micnoscopy and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Certain enzymes, as described in the methods section, are found preferentially in specific cell organelles. A commonly found plasma membrane enzyme is 5' nucleotidase which is enriched 30 fold in purified plasma membranes from chick embryo cells (71) and 25 fold in those from mouse liver cells (89) as compared to the activity in the cell homogenates. In lymphocyte plasma membranes 5' nucleotidase is also concentrated (33) but in some plasma membranes, for example, from Ehrlich (36) and A66 hepatoma cells (83) 5' nucleotidase is low and is therefore not a good plasma membrane marker. In Krebs cell plasma membranes, 5' nucleotidase was concentrated 5 fold as compared to the cell homogenate.

Another plasma membrane specific enzyme, Na^+K^+ dependent ATPase, is enriched 13 fold in plasma membranes of A66 hepatoma cells (83), 33 fold in HeLa plasma membranes (11) and 27 fold in Ehrlich plasma membranes (36) as compared to the whole cell homogenates. Krebs cell fractions including purified plasma membranes had low Na^+K^+ dependent ATPase activity although there was about a 5 fold enrichment of enzyme activity in the plasma membrane fraction.

Alkaline phosphatase is a less often used plasma membrane marker but has been demonstrated to be enriched 20 fold in HeLa plasma membranes (11). In Krebs plasma membranes alkaline phosphatase was concentrated almost 2 fold as compared to the cell homogenate.

Marker enzymes for cell organelles other than the plasma membrane were looked for in Krebs cell fractions. NADH oxidoreductase, a microsomal marker enzyme is present in chick embryo cells (71), lymphocytes (33), A66 hepatoma (83) and Ehrlich cells (36) but is present in very low activity or absent from plasma membranes prepared from these cells. In purified Krebs plasma membranes NADH oxidoreductase activity was 10 fold less than in the crude membrane fraction indicating, slight, if any microsomal contamination.

Glucose-6-phosphatase is also a microsomal enzyme and its activity is reduced to almost zero during the preparation of plasma membranes from Ehrlich (36) and mouse liver cells (39) but is not present at all in A66 hepatoma cells (83). In Krebs cells glucose-6-phosphatase activity was very low and it was only found in the microsomal fraction where the enzyme activity would be expected to be enriched.

Succinic dehydrogenase is a mitochondrial enzyme present, for example, in HeLa (11), Ehrlich (36) and mouse liver cells (39) but its activity in plasma membranes prepared from these cells is almost zero. In the Krebs cell homogenate succinic dehydrogenase activity was low and only a small amount of activity was detected

in the plasma membrane fraction indicating possible mitochondrial contamination.

The enzyme activities present in Krebs cell fractions do not show high enrichment in different fractions although they do seem to follow the expected pattern of distribution within the cell. Problems were encountered with non-uniform turbidity in assays where cell fractions were combined with reagents in a cuvette and absorbance read directly. For this reason results for some assays are absent because the accuracy in reading those samples was reduced. It is possible that the enzyme activities were low in Krebs cell fractions because of a decline in activity during storage at -15°C.

Since enzyme assays on Krebs cell fractions have not been carried out previously so that the location and activity of the enzymes could be confirmed it is not acceptable to depend on the enzyme assays as a measure of purity. Krebs plasma membrane suspensions were examined in the electron microscope. Other workers have used electron microscopy to look for nuclei, mitochondria and ribosomes which may be present as impurities in plasma membrane preparations. Sections of membrane pellets of, for example, HeLa (1) and Ehrlich (36) plasma membranes, have been examined by electron microscopy and they resemble smooth sheets of membranes. Suspensions of purified membrane ghosts of BHK cells (35) appear as flattened sack-like structures. Suspensions of Krebs plasma membranes were examined by electron microscopy and mostly whole membrane ghosts were seen although there were some aggregated

membranes of indeterminant shape and some membrane fragmented (Fig. 2, 3 4). No contamination with other cell organelles was observed in the Krebs plasma membranes suspensions examined under the electron microscope.

Fractions from Krebs cells were examined by SDS PAGE. Some coomassie blue staining components were common to the homogenate, mitochondrial and microsomal gels. The crude membrane gel had bands in common with the homogenate gel but the pattern was distinctly different from the patterns of the mitochondrial and microsomal gels as was the pattern of bands in the purified membrane gel. The bands on the purified membrane gel were sharper than those seen on gels of the other cell fractions which indicates the components of the plasma membrane were free from contamination. The distinctly different pattern of bands of the purified membrane gel also indicates that there is little or no contamination with other cell fractions.

ii) Characterization of the Krebs plasma membrane.

Protein determinations of membrane preparations were used as a measure of concentration. Sialic acid determinations were also routinely carried out. Krebs plasma membrane contained less sialic acid than that of human erythrocytes (76) but some was detected indicating the presence of sialoglycoproteins, gangliosides or both.

SDS-PAGE was used to analyse purified plasma membrane preparations. The coomassie blue stained profile of Krebs plasma membranes analysed by 2 hour SDS-PAGE was more complex and quite

different from that of human erythrocyte membranes (Figs. 7, 8). A66 hepatoma plasma membranes (83), however, are similar to Krebs plasma membranes in that coomassie blue staining reveals many components, of which, a few are intensely stained, for both membrane types.

Only one PAS staining band was seen on gels when Krebs plasma membranes were examined by 2 hour SDS-PAGE as contrasted with the four PAS staining components detected in human erythrocyte membrane preparations (Fig. 7, 8). The Krebs plasma membrane PAS staining component migrated only a short distance into the gel and was therefore of high molecular weight, in an aggregated form or both.

Preparations electrophoresed for 18 hours and stained with coomassie blue revealed 2 components near the bottom of the gel and one faintly staining component near the top of the gel (Fig. 9). Similar gels stained with PAS reagent revealed the same faintly staining component and a more intensely stained single band with a shoulder near the bottom of the gel (Fig. 9). Since there was also PAS staining material on top of the gel it is probable that this material and the faintly stained material were aggregates of the more intensely stained material. It is therefore probable that the PAS staining component of the Krebs plasma membrane is a single component that forms aggregates but it is possible that there is more than one PAS staining component in the plasma membrane.

It is interesting to note that coomassie blue stained gels

were stable for a considerable time and began to fade after storage for several months but Krebs plasma membrane gels stained with PAS reagent faded after 1 or 2 days unlike the PAS stained erythrocyte membrane gels which were stable for several weeks. No explanation, except that the PAS reaction with the Krebs plasma membrane was unusually unstable, could be found but the phenomenon was consistently observed.

Plasma membrane components from cell types such as HeLa cells have been successfully labelled with ^{3}H fucose and the incorporation of label into the plasma membrane was used to monitor purity (1). A66 hepatoma plasma membranes have been labelled by lactoperoxidase catalysed iodination (83).

Krebs cells were radioactively labelled with fucose, glucosamine and iodine and subsequently fractionated to prepare plasma membranes. Radioactivity was found in many cell fractions with poor incorporation into the purified plasma membranes, therefore, incorporation of radioactive label could not be used as a purity monitor for Krebs plasma membranes and these results are not shown. Radioactively labelled cell components, possibly including the EMC virus receptor on Krebs cells, would nevertheless be useful in further experiments so further studies were carried out using lactoperoxidase catalysed iodination of the Krebs cell surface.

Increasing the quantity of ^{125}I and cell labelling time improved the amount of radioactivity incorporated into plasma membranes only slightly if at all (Table 2). In contrast to labelling whole cells, when Krebs plasma membranes were isolated

and purified before iodination, a considerable amount of radioactive label was incorporated. Increasing the initial amount of ^{125}I added to the purified membranes improved the efficiency of labelling although time periods of labelling longer than 1 hour did not increase the amount of label incorporated into the plasma membranes, (Table 5).

SDS-PAGE was carried out on iodinated plasma membranes and 1 small and 3 major radioactive peaks were observed (Fig. 10). ^{125}I associated with plasma membranes, in the absence of lactoperoxidase was probably unreacted iodine because no peaks were observed on a gel of this sample and the radioactivity of a similar sample of membranes was reduced to background level by 8 or 9 further washes.

The receptor for EMC virus on human erythrocytes also serves as the WGA receptor (87). Chromatography of the iodinated Krebs plasma membranes showed that the WGA receptor on Krebs cells had not become labelled with iodine (Fig. 11). If the WGA receptor was also the EMC virus receptor then the EMC virus receptor was not labelled either. The unbound fraction containing nearly 100% of the recovered ^{125}I was examined by SDS-PAGE and found to contain one major peak and several smaller peaks (Fig. 12). This suggests that probably two of the major peaks of the original profile had been degraded during solubilization and chromatography, or that one or more of the original components were aggregates of several molecules which had become separated.

b) Krebs cell glycocalyx.

Many cells, perhaps all cells, have a cell coat or glycocalyx (7).

Krebs cells have a glycocalyx which was released by stirring cells gently at 4°C as described by Rittenhouse et al (95) for Ehrlich cells. Most of the material was released within the first 45 minutes but there was a further release of proteins between 2 and 4 hours of stirring possibly because the glycocalyx had been regenerated and was again being released.

Krebs glycocalyx release was monitored by protein determinations. No sialic acid was detected in the Krebs glycocalyx and neither has any been reported in the Ehrlich cell glycocalyx (86). Krebs cell glycocalyx fractions were examined by SDS-PAGE. No PAS stained bands were observed on the gels despite the presence of carbohydrates being characteristic of the glycocalyx of different cell types (7). It is possible that free sugars, other than sialic acid, were present loosely bound to cell surface components in the glycocalyx so that they were not apparent in SDS-PAGE.

The pattern of coomassie blue stained bands of glycocalyx gels was quite complex, with many components. The cell coat fraction contained predominantly low molecular weight species, whereas the cell coat particle contained mostly higher molecular weight species (Fig. 14, 15). The crude glycocalyx contained both low and high molecular weight components some of which corresponded in mobility to the Krebs homogenate and microsomal supernatant fluid components (Figs. 5, 6), but they did not have the same mobility as any of the purified membrane components, (Fig. 7, 8). Glycocalyx components are thus removed during plasma membrane preparation and they are probably responsible for blocking the

iodination of the plasma membrane on whole cells since only isolated plasma membranes could be successfully iodinated.

Neuraminidase released more sialic acid from Krebs cells lacking a glycocalyx compared with complete cells. This indicates that sialic acid residues are protected from neuraminidase action by the glycocalyx. Sialic acid was not spontaneously released from membranes by removing the glycocalyx confirming that it was the enzyme that was prevented from attacking sialic acid residues on the cell surface that caused the difference in sialic acid release. After 2 to 4 hours of digestion with neuraminidase the amount of sialic acid released from the cell surface with the glycocalyx removed declined, possibly due to the regeneration of the glycocalyx and a decline in the efficiency of the neuraminidase.

Part 2. The attachment of EMC virus to Krebs cells.

a) The location of the EMC virus receptor on the Krebs cell surface.

There are two parts to the Krebs cell surface: the plasma membrane and the glycocalyx. An initially important stage in understanding the nature of EMC virus attachment was to determine the location of the virus receptor on the cell surface.

Rittenhouse et al found that a high molecular weight aggregate containing IgG was released from Ehrlich cells with the glycocalyx (95). Similarly surface antigens are shed from the surface of human melanoma cells (111) and could constitute part of the glycocalyx. It was, therefore, feasible that the receptor for EMC virus on Krebs cells might have been shed as part of the glycocalyx rather than be present in the plasma membrane. Several experiments were carried

out and demonstrated that the EMC virus receptor was not a glycocalyx component.

Attachment of radioactively labelled EMC virus was not impaired by the removal of the glycocalyx from Krebs cells. Cells harvested from mice without washing bound slightly more radioactivity than cells washed three times. Viral receptors may have been lost during washing but a more feasible explanation is that there were probably some erythrocytes present in the Krebs cell suspension to which the radioactive virus also bound. Even over longer periods of stirring to release the glycocalyx the same amount of radioactive virus was still able to attach to glycocalyx depleted cells as to cells with a glycocalyx.

Growth of EMC virus was not impaired by the removal of the glycocalyx from Krebs cells which shows that the attachment of infectious virions in particular was not affected by the absence or presence of the glycocalyx. Confirmation that the EMC virus receptor is not a glycocalyx component was obtained by testing preparations of glycocalyx fractions for their ability to inhibit EMC virus haemagglutination. There was little or no inhibition of haemagglutination by any of these preparations indicating that glycocalyx components had not prevented virus from binding to erythrocytes and, therefore, probably did not contain the EMC virus receptor. If EMC virus does attach to a specific cell surface component, that receptor is probably present in the plasma membrane.

b) Characterization of the EMC virus-Krebs cell attachment process.

1) The attachment of EMC virus to Krebs cells and isolated plasma membranes.

Having shown that the EMC virus receptor on Krebs cells is not located in the glycocalyx and, therefore, is probably a plasma membrane component attention was turned to looking at the attachment of EMC virus to whole cells and isolated plasma membranes. In many cases parallel experiments were done using human or sheep erythrocytes for comparison.

Exposing Krebs cells to EMC virus for different time periods showed that most of the virus which could attach had done so within fifteen minutes. The maximum attachment, at a cell concentration of 2.5×10^7 Krebs cells/ml, lower than that normally used, occurred between 15 and 30 minutes of exposure to virus. This could have been due to experimental variation. Cells and virions in suspension are, however, constantly coming into contact and moving apart except when the virion, by chance, comes into contact with the cellular receptor for the virus and then remains attached. If there are fewer cells it would take longer for the virions to come into contact with and attach to their cellular receptors.

At two different cell concentrations the same amount of virus was able to attach, that is 30 to 40% of that added. Some of the virions were, therefore, unable to attach even though there must have been available receptor sites, at least in the more concentrated cell suspension. There were thus some virions present in the suspension which had either broken down or were unable to attach

for some other reason.

Radioactively labelled EMC virus attachment to Krebs cells and erythrocytes was compared. During the first cycle of attachment only slightly fewer ^{3}H labelled virions bound to Krebs cells than erythrocytes. Only a very low percentage of the unattached radioactive virus from the previous incubation with Krebs cells was able to attach to fresh Krebs cells. Virions may have become inactivated by contact with the cell surface although attachment did not occur or a certain proportion of the virions originally added may have been broken down and thus be unable to attach. The same phenomenon occurred when the unattached radioactive virus, after mixing with the second Krebs cell samples was incubated with human erythrocytes. If the poor attachment in the second and third incubation periods was caused by previous contact with host cells this would suggest a similarity between attachment to erythrocytes and Krebs cells because attachment was poor with both cell types.

Radioactively labelled EMC virus can also attach to isolated Krebs plasma membranes. Plasma membranes must, therefore, possess receptors which bind EMC virus, unlike the glycocalyx. The amount of virus which bound to plasma membranes was similar to that bound to whole cells although this is a tentative comparison since the numbers of membranes could not be determined, as was explained in the results. Of the radioactively labelled virus which did not attach to Krebs plasma membranes, very little was subsequently able to attach to erythrocytes. This result was similar to that obtained

when whole Krebs cells were used which suggests that the interaction of EMC virus with plasma membrane was similar or identical in nature to the interaction with whole cells.

Virus which did not attach during initial exposure to Krebs cells or plasma membranes was subsequently unable to attach to other cells or membranes. Analysis of radioactive virus, which had been mixed with plasma membranes but did not attach, on sucrose gradients showed that there was no degradation of the virus. The single peak observed in the radioactive profile of control virus was very similar to that observed using virus exposed to Krebs plasma membranes.

ii) <u>The effect of enzyme treatment of cells on the attachment of EMC virus to Krebs cells.</u>

Very few cellular receptors for viruses have been isolated but the receptor for adenovirus is a protein (84) and the receptor for influenza virus is a glycoprotein (37). The possibility that the EMC virus receptor on Krebs cells is also a protein was suggested by experiments with trypsin.

Firstly, the attachment of radioactive EMC virus to Krebs cells was reduced by 90% after treatment of the cells with trypsin. Secondly, infectious centre formation was reduced by 90%, as compared to an untreated control, using trypsin treated cells. The EMC virus receptor on Krebs cells is, therefore, probably a protein. It should be remembered, however, that trypsin can dramatically alter the membrane structure as a whole possibly causing receptors to become masked or changing their conformation. Glycopeptides may

be released by trypsin but the carbohydrate rather than the protein part of the molecule may be of importance in attachment. It is, thus, not necessarily valid to state that the EMC virus receptor on cells is a protein from these experiments with trypsin alone.

Some host cell receptors for cardioviruses, including EMC virus are sensitive to neuraminidase (57). The effect of neuraminidase treatment of Krebs cells on EMC virus attachment was tested. Treatment of Krebs plasma membranes with neuraminidase reduced the attachment of radioactive virus by 10% whereas treatment of whole Krebs cells with neuraminidase reduced attachment by 15%. Attachment to erythrocytes in control experiments was reduced by 35-50%.

When the time period of exposure of Krebs cells to neuraminidase was increased the amount of radioactive virus attaching decreased except after 4 hours. It is possible that during this time sialic acid residues on the cell surface were being regenerated but it was also possible that neuraminidase activity declined because in the first hour 3.1 µg sialic acid/ml were released and then 2.3 µg sialic acid/ml/hour and 1.3 µg sialic/ml/hour during 2 and 4 hours of treatment respectively.

Finally, the effect of neuraminidase treatment of cells on the attachment of infectious virions in particular was tested. The infectivity of EMC virus was reduced by 45% when cells were pretreated with neuraminidase. Sialic acid is thus required for attachment, either as part of the receptor site or to maintain the charge or conformation of the receptor.

In summary, EMC virus can attach to and infect Krebs cells with both attachment and infectivity being reduced by trypsin and neuraminidase treatment of Krebs cells. These results are consistent with the receptor on Krebs for EMC virus being a sialoglycoprotein. Other workers have shown that attachment of cardioviruses is inhibited by Francis inhibitors (74) and of EMC virus is inhibited by glycophorin (30). The experimental evidence combined with evidence from other workers strongly implicates a sialoglycoprotein as the Krebs cell receptor for EMC virus. The next stage of this project was the isolation of the receptor.

c) Isolation and characterization of the Krebs cell receptor for EMC virus.

Biologically active material was isolated from Krebs cells or from plasma membranes in the aqueous fraction of LIS-phenol extraction, which is the fraction reported to contain glycoproteins from other similarly treated cell types (16, 75). Biological activity was demonstrated by EMC virus haemagglutination inhibition and this assay was used to examine the properties of this biologically active fraction which probably contained Krebs cell membrane glycoproteins.

Glycoprotein fractions isolated from purified Krebs plasma membranes were better at inhibiting haemagglutination than crude membrane derived glycoprotein fractions which, in turn, were slightly better inhibitors than whole cell derived glycoprotein fractions. The activity of the glycoprotein preparations decreased with time of storage even when frozen but purified membrane derived

glycoprotein preparations were the most stable. The greater biological activity and stability of purified membrane derived glycoprotein preparations could be explained by the presence of a contaminant in crude membrane or whole cell derived membrane fractions which degraded the biologically active component.

Freshly prepared glycoprotein fractions scanned in the ultra-violet absorbance range revealed that lithium diiodosalicylate was present in glycoprotein fractions and nucleic acids were present in crude membrane and whole cell derived glycoprotein fractions but not in purified membrane derived glycoprotein fractions. It is also possible that some lipids may have been present as contaminants in glycoprotein preparations. Experiments were carried out using the haemagglutination inhibition assay which demonstrated that the above substances, if present, were contaminants and not biologically active. LIS alone, had no inhibitory effect on haemagglutination. RNAse treatment of glycoprotein fractions did not decrease their inhibitory properties suggesting that the RNA present was a contaminant. Corroborating with this result was the absence of nucleic acid in purified membrane derived glycoprotein preparations which were nevertheless very good inhibitors of haemagglutination. The biological activity of glycoprotein preparations extracted with chloroform/methanol was found mostly in the aqueous layer but also in the precipitate at the interface with the chloroform/methanol layer which, itself, showed no activity but would have contained any lipids present as contaminants in the glycoprotein preparations.

Having become assured that none of the possible contaminants

were responsible for the biological activity of the glycoprotein preparations it was necessary to determine the nature of the biologically active component. Trace amounts of protein were found in the crude membrane derived glycoprotein preparations but measurable amounts were found in purified membrane derived glycoprotein preparations. Sialic acid was detected only in purified membrane derived glycoprotein preparations.

Experiments with trypsin and neuraminidase strongly suggested that it was a sialoglycoprotein in the preparations that was the biologically active component. Trypsin and neuraminidase treatment of glycoprotein fractions reduced their capacity to inhibit haemagglutination by almost 50%.

The glycoprotein fractions were also able to inhibit WGA haemagglutination which suggests that the EMC virus and WGA receptors may be parts of the same molecule or that the preparations contained two similar molecular species, the WGA and EMC virus receptors. If the WGA and EMC virus receptors are on the same molecule then none of the iodinated membrane proteins could be the receptor since they did not bind to the WGA sepharose column.

The haemagglutination inhibition assay is convenient but measures the attachment of virus to erythrocytes rather than host cells. Attachment of radioactively labelled EMC virus was, however, reduced to 60% of the control value in the presence of the Krebs glycoprotein fraction. Infectivity of EMC virus was reduced to 69% of the control when the Krebs glycoprotein fraction was present.

The evidence from the previous experiments suggests that a

component of the Krebs glycoprotein fraction competes with Krebs cells and erythrocytes to bind virus. Whilst the glycoprotein preparations, particularly the whole cell and crude membrane derived preparations, may be contaminated the biologically active molecule with receptor properties is a sialoglycoprotein. Further investigations were carried out using the purified membrane derived glycoprotein preparations to determine the molecular weight of the active component and try and match it with a plasma membrane component.

d) The molecular weight of the Krebs cell receptor for EMC virus.

SDS-PAGE of purified membrane derived glycoprotein revealed a single PAS staining band near the origin of the gel. The isolated Krebs glycoprotein was estimated as being of molecular weight 215,000 whilst the PAS component of purified Krebs plasma membranes was of molecular weight 225,000, which is reasonably close. The molecular weight cannot be calculated accurately because the erythrocyte PAS staining components used as markers are in a lower molecular weight range and it is not known how much of the Krebs glycoprotein comprises carbohydrate which might influence the mobility of the glycoprotein.

The molecular weight of the Krebs glycoprotein was estimated a second time using protein markers with a wider molecular weight range. The molecular weight of the Krebs glycoprotein was estimated to be 215,000 using these standards but again the oligosaccharides present in the glycoprotein may affect its mobility.

Both estimates of the Krebs glycoprotein molecular weight were

215,000 daltons which is close to the estimate of 225,000 daltons for the Krebs plasma membrane glycoprotein. As described previously the Krebs plasma membrane, PAS band was stained pink and was unstable, fading within one or two days. This same phenomenon of instability was observed with isolated Krebs glycoprotein except that the PAS band, which was the same pink colour as the membrane glycoprotein PAS band, faded overnight. All of these results together strongly imply that the PAS staining component of Krebs plasma membranes had been successfully isolated in Krebs glycoprotein fractions and is the receptor for EMC virus on the Krebs cell surface.

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