EVIDENCE FOR CELL SURFACE ASSOCIATED NUCLEIC ACID: A STUDY USING WHOLE CELL ELECTROPHORESIS ON THE S-180 MOUSE TUMOR

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ABSTRACT

EVIDENCE FOR CELL SURFACE ASSOCIATED NUCLEIC ACID:
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A whole cell electrophoresis apparatus was built which was capable of measuring the average electrophoretic mobility of a suspension of cells in 3-5 minutes. This was used to measure the mobility of S-180 mouse ascitic tumor cells under various conditions. The mobility was determined as a function of the age of the tumor and treatment with cis-dichlorodiammeplatinum(II) (cisplatin). The cisplatin treatment of mice bearing the S-180 caused a reduction in mobility of the tumor cells by 10-20%. The cells, after removal from the animals, were incubated with several enzymes to see if any of them would mimic the effect of cisplatin treatment. In the S-180 tumor, RNase mimicked the effect of cisplatin by lowering the mobility of controls to the level of the cisplatin treated cells. The RNase had no effect on the platinum treated cells. In the S-180-J tumor, both RNase and DNase mimicked the effect of cisplatin treatment. Several experiments were then performed to establish the presence of nucleic acids on the cell surface, which was implied by the first enzyme studies. pH profiles, ionic strength profiles, binding of anti-DNA antibody, binding of 'platinum-thymine-blues', incubation with Sepharose immobilized DNase and Agarose

immobilized RNase, incubation with restriction endonucleases Eco-R1 and Bam I, enzyme kinetics and *in vitro* incubations with cisplatin and other antitumor drugs all gave support for cell surface nucleic acids. These nucleic acids appeared to be in the process of flux through the membrane with a turnover half-time of about 2 hours. Inhibition of cell nucleic acid metabolism resulted in the loss of their expression on the surface of the cell. The possible roles of these surface nucleic acids are discussed, particularly with regard to their possible immunological roles.

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Introduction

In the last ten years the development of the successful treatment of many cancers with cis-dichlorodiammineplatinum-II (Cisplatin) has led to many investigations of its mode of action in tumor regression. Most of this research falls into one of three catagories; studies of platinum chemistry, results of clinical trials, and interactions of platinum compounds with DNA. Examination of this new drug's interaction with cellular biochemistry is noticably lacking and cisplatin's effect on the cell membrane is virtually unknown.

The first purpose of this study is to examine a particular characteristic associated with the cell membrane as a function of platinum treatment. That characteristic is the average membrane surface charge which is derived from the total cellular electrophoretic mobility. This is measured on viable cells in physiological buffer solutions.

The average surface charge is the sum of all surface groups which are charged at physiological pH. This average charge represents very little by itself, but it can be used to determine the nature of the individual charged groups which are present on the outer surface of the cell. These charged groups are mainly negative, consisting of carboxylic groups and phosphates. Proteins in the membrane contain negatively charged carboxylic acid groups,

non-charged sulfhydral groups, and positively charged amines.[30] Sugar moieties probably contribute the majority of the negative charged carboxylic acid residues, particularly in the form of sialic acid.[24] Phosphates are present in the lipids of the membrane and also in some surface macromolecules, and sulfates are also present in small amounts. When the removal of one or another of these groups can be identified by changes in cell electrophoresis then a physiologically significant piece of information about the cell emerges.

To determine the types of groups present, the technique of whole cell electrophoresis is used in conjunction with controlled surface effectors. These surface effectors are used to selectively remove or alter known groups on the surface in order to establish their presence. The most commonly used effectors are digestive enzymes specific for saccharide, peptide, or nucleic acid bonds. Other effectors that can be used include charged macromolecules which electrostatically bind to the surface groups, antibodies specific to surface antigens, and reagents which specifically alter exposed molecules. Once groups are determined, their relative concentrations on the surface can be followed as a function of cellular treatments, such as chemotherapy, immunotherapy, radiation treatment, etc. This technique is used in this study to examine the S-180 mouse tumor system as a function of platinum chemotherapy.

When a tumor bearing animal is treated with a therapeutic dose of cisplatin the electrophoretic mobility of the tumor cells decrease by approximately 20%. This change occurs over the first 24 hours and remains depressed unless the tumor begins to grow again.

This phenomenon occurs simultaneously with an invasion of the tumor by neutrophils of the host, followed in later days by large numbers of macrophages. This host response may be due to inflammation caused by platinum therapy, by dying cells, or by an increased immune response to the tumor.

It was this last possibility, perhaps the least likely of the three, that spurred interest in examining the phenomenon of the reduced cell surface charge. This reduced charge could lower the electrostatic repulsion between cells and increase cell-cell interaction resulting in greater phagocytic activity by host cells. Since most tumors show increased surface charge[37] they could be protected from the cell-cell interaction necessary for phagocytosis.

Changes in surface charge could also represent a loss or addition of one or more specific groups, such as receptors, transport enzymes, catalytic enzymes, glycoprotein or mucopolysaccharide antigens etc. These types of changes would effect the cell's interaction with it's environment and, therefore, could alter the immune response.

Due to length of time it takes for the mobility to decrease, approximately 1 day, the possibility of cisplatin binding to the membrane and thereby altering the charge was virtually ruled out. Cisplatin is also a neutral molecule at physiological chloride ion concentration and its surface binding, therefore, would go unmeasured. The most probable route for the drug would be to enter the cell where the changes it induced resulted later in a surface alteration. The purpose of this thesis was to find what these

alterations of the membrane were and to determine, if possible, whether these alterations were functional or simply incidental.

In addition to the interest in cisplatin's effect we were intrigued by another phenomenon which was observed on the surface of tumor cells that had been stained with the 'platinum-pyrimidine-blues'[88]. Electron microscopy studies showed patches on the surfaces of tumor cells which were attributed to nucleic acids. These patches were susceptible to nucleases and to neuraminidase, but were not present at all on non-tumorigenic cells. It seemed likely that we could detect surface nucleic acids with whole cell electrophoresis due to their net negative charge at pH 7.4. If they could be detected, then it would corroborate the staining studies which implicated nucleic acids on the very periphery of the cell surface - a highly unlikely and unusual location. Therefore, the second purpose of this work is to examine cells electrophoretically for cell surface nucleic acids.

The experimental techniques of this dissertation project are mostly limited to those of whole cell electrophoresis. It appeared to be a very effective way to examine very small amounts of a surface moiety on viable cells especially after cell surface nucleic acids were implicated. This thesis presents some evidence for a new phenomenon, speculates on it's role, and suggests some further experiments. I first present some backround information on whole cell electrophoresis; the relevant areas of cancer research, particularly tumor cell surfaces and tumor antigens; platinum chemotherapy; and the work to date on extracellular RNA and DNA. This is followed by

my experimental results and then a discussion section in which I examine the possible roles of cell surface nucleic acids in light of some other reports in the literature.

Literature Review

I. Platinum Research

cis-dichlorodiammineplatinum-II, cisplatin, has proven to be one of a group of very potent platinum base anti-tumor drugs which is currently being used in human chemotherapy protocols. It's mode of action is still not known although there is a large amount of evidence for the primary lesion being an attack on DNA. Initial studies of it's binding to protein and RNA show little or no effect on their synthesis, however the number of such studies is small. The majority of the published work on platinum compounds in cancer research are concentrated in three areas; platinum chemistry, platinum interactions with DNA,and clinical results. This thesis does not address the chemical or clinical aspects but rather, the biological areas and macromolecular interactions.

A. Biological Effects

The first effect noted on biological systems, was the filamentation of *Escherichia coli* which led to the discovery of cisplatin's potential use for tumor chemotherapy.[1] The E. coli continued to metabolize and to grow, but they did not divide. This immediately led to the investigation of platinum interactions with DNA, since similar effects can be induced with ultraviolet irradiation, X-ray irradiation, or alkylating agents - all DNA effectors. Platinum interactions with ribonucleoproteins in filamentous E. coli did not

seem to interfere with protein synthesis,[2,15] so, DNA appeared to be the target molecule.

Lysogenic strains of E.coli could be activated into phage production by incubation with very low levels of platinum compounds.[3] The level of anti-tumor activity correlated well with the ability to induce phage in the lysogenic E. coli.

Cisplatin is also a mutagen. Beck and Brubaker[4] showed that reversion of mutants back to the prototrophic condition occurred at a rate 10^5 times greater than spontaneous reversions. It was later shown that the induced mutation is a base substitution rather than a frame shift.[5] In general, the platinum anti-tumor drugs exhibit a correlation between mutagenicity and cytotoxicity.[5,6]

The carcinogenic potential of platinum drugs has been recently tested[7] and shown to be positive for the production of benign tumors in mice. Lung adenomas were produced in 100% of animals treated with cisplatin and, in conjunction with croton oil, skin papillomas were induced, some of which turned malignant. These results are indicative of moderate carcinogenic activity.

These biological effects all suggest that DNA is the molecule attacked by the platinum drugs. Other effects on cells and animals are not so straightforward but suggest other concommittant roles for platinum action.

When used in animals as an anti-cancer drug, cisplatin causes tumor regression which is probably assisted by an enhanced immune response. Conran[8] demonstrated that immunostimulants can increase the efficacy of therapy, and that immunodepressants decrease efficacy.

Sodhi[9] showed that within a few hours after platinum treatment of a tumor the host macrophages attach in greater numbers to the tumor cells. This occurs despite cisplatin's apparent immunosuppressive properties as shown by its ability to reduce hemolytic plaque forming spleen cells and lymphocyte blastogensis in mice.[10]

Many of the cells after treatment turn into giant cells with several nuclei,[11,12] very reminiscent of the filamentous growth in bacteria. In Erhlich ascites cells exhibiting this giant cell growth, the synthesis of DNA is depressed, yet there are several copies per cell. Both RNA and protein synthesis is above normal,[13] which may be due to these extra copies of DNA. In general, these cells are blocked in the G2 phase of the cell cycle and are unable to continue on to mitosis.

Respiratory inhibition of chicken heart mitochondria occurs with cisplatin but only at doses far in excess of therapeutic levels. This probably means that respiration is not inhibited in normally treated tumors. Zakharova,[14] however, showed that adenosine triphosphatase can be inhibited at levels of cisplatin closer to treatment values used. This could play a role in cellular energy levels and could enhance tumor regression.

B. Macromolecular Interactions

DNA has been studied the most thoroughly of all macromolecules in its binding with platinum. Studies [2,15] indicate that DNA synthesis is inhibited, but RNA and protein synthesis is not. This was taken to mean that platinum's main effect was on the DNA and, further, that

this effect was due to platinum binding to DNA.

Pascoe and Roberts [16,17] showed that the amount of cisplatin bound to DNA, mRNA, rRNA, and tRNA was about equal by weight. Approximately 1 platinum molecule is bound per 10^5 bases. In the case of protein, there was 1 platinum per 10^6 amino acids. They make the point that since DNA is so large it contains many more platinum residues per molecule than the smaller RNA molecules and therefore poses a greater threat to the information coded on the DNA. Whereas RNA can be transcribed from short pieces of DNA, DNA synthesis would require an intact path for DNA polymerase. If this path was blocked by platinum, synthesis would be stopped or slowed down while repair occurred, if repair could occur. This, unfortunately, does not address the problem of giant, multinucleated cells which appear to be undergoing DNA synthesis but are unable to divide.

The binding of platinum to DNA is fastest at the guanosine residue,[18] with the N-7 site the most prevalent attachment point. The N-1, O-6 of guanine and the 6-NH_2 of adenine are some of the other possible binding sites. The active <u>cis</u> configuration platinum drugs have the ability to crosslink in both an inter and intra strand fashion between sites on neighboring nucleotides,[19] thereby creating a physical block.[20]

DNA repair appears to exist for some platinum induced lesions because introduction of caffeine, an excision repair inhibitor, reduces survival in chinese hamster cells after cisplatin treatment.[21] Although postreplicative repair may be involved in the removal of lesions, it appears that most repair is the caffeine sensitive

excision repair which repairs the DNA before replication occurs.[19] Differences in cells' ability to repair their DNA may explain why 'normal' tissues are not as effected by platinum as tumor tissues. The tumor cells may lack a repair process making them more vulnerable to the effect of platinum binding.

The interaction of platinum with RNA and protein is poorly understood. Early work showed that platinum does bind to both, but that it did not seem to effect the new synthesis of either. Work exploring the possibility that platinum binding reduces the functionality of RNA or protein has not been done. Hermann et al.[22] did show significant physical aterations in double stranded synthetic RNA, and Robins et al.[23] indicate binding to serum proteins and some cellular proteins. Hörer and Nicolau [102] indicated a significant binding of cisplatin to protiens of the red blood cell membrane using shifts in fluorescence, and they suspect the platinum is bound to amino acids other than the fluorochromic ones. This suggests that platinum could have a significant membrane effect before it enters the cell. Electron Spin Resonance spectroscopy studies of Sinha et al.[103] also showed tight binding of cisplatin to proteins in the membranes of red blood cells and the P-815 mastocytoma. Wolf et al.[104] further showed cisplatin exists in three fractions in the blood; bound to cells, serum proteins, and free. They showed that the binding to the red cell and serum proteins is very strong. The significance of platinum binding to protein and ribonucleic acid is not known, however, possible roles include interference with enzyme activity or functionality of newly synthesized protein, sequestering platinum in a protective manner, and specialized transport of platinum.

II. Whole Cell Electrophoresis

A. Theory

When cells are suspended in an isotonic solution in which an electric field has been established, they migrate toward the positive source of the field. This was not supprising to early investigators because it was known that the cell membrane contained phospholipids, glycolipids, glycoproteins and proteins which can have both negative and positive charges at physiologic pH.

The surfaces of cells are probably a complicated mixture of these different groups. These cell surfaces undergo turnover of their components, expand and contract with movement, growth, and cell-cell interactions, and are not flat surfaces but have depth probably more like a molecular sponge.[24] At some point at the very periphery of the cell is a surface contour called the slip plane where a physical demarcation exists between the charged surface molecules, with their tightly associated counterions, and the ions in the solution bathing the cell. The negative charges on the surface are balanced by positive charges in solution. These counterions of the extracellular space crowd up near the surface and their concentration falls off exponentially along radial directions from the cell. Most of the counterions lie beyond the slip plane (Appendix I, Figure 20) and therefore there is a potential (zeta potential) at this plane representing the unbalanced charges at the surface. When the cell is placed in an electric field the force of the field acts on the charges at this plane and the cell moves toward the positive electrode.

Its velocity depends upon the zeta potential at the slip plane and it is this velocity which is taken as the electrophoretic mobility.

The relationship used to obtain the actual surface potential or surface charge from the mobility has been discussed by many authors (see review by Abramson[25]). The Smoluchowski equation[28] relates mobility, u, to the zeta potential, ζ,

$$u = \frac{\varepsilon \zeta}{4\pi\eta}$$

where ε is the dielectric constant and η is the viscosity. The values of these two constants are taken to be those of the bathing solution. (This may be a source of error.[26]) This equation is only valid for particles or cells or a sufficient size, (approximately 10 micrometers or larger) and it assumes that their surfaces are impenetrable to ions.

The surface charge, σ, is related to the mobility by the equation

$$u = \frac{\sigma}{K\varepsilon}$$

where 1/K is the Debye-Hückel double layer thickness, and represents the distance over which the potential drops to 1/e.

These equations are only approximations and have been modified by several authors to take into account membrane penetrability,[27] microvilli and surface contours,[24] pH changes at the surface and membrane conductivity.[28] However, the inaccuracy of the relationship of electrophoretic mobility to surface potential or charge is not of major importance in the use of the technique.

Most of the information obtained through whole cell electrophoresis

comes from measuring differences in mobility which represent small differences in the amount of charged moieties on the cell surface. An average mammalian cell exhibits 10^8 - 10^9 net negative charges on it's surface an measured by electrophoresis. Changes of 10% are easily measured. This represents the gain or loss of 10^7 - 10^8 charges or approximately 10^{-16} moles. The nature of these changes are determined by specific treatment of the membrane, usually with enzymes which will cleave off known groups.

The surface charges of particles, bacteria, viruses, sperm cells, blood cells, and tissue cells both normal and transformed, have been examined with whole cell electrophoresis by many investigators over the past 40-50 years.[28,29,30] It wasn't until the discovery of an enzyme, neuraminidase, from Vibrio cholerae[31], which was shown to cleave sialic acid groups from the cell surfaces[32] that interest in the technique rose. Sialic acids were found in great amounts on the surface of some cells, [24,33] representing as much as 80% of the surface charge on red blood cells. These sialic acids were found to be bound to glycoproteins [34] and glycolipids[35,36] through glycosidic bonds. The carboxylic group on the sialic acid remained free, and at pH 7.0 contributed a negative charge to the cell surface. Cells of all types were then examined in order to see if they had specific sialic acid profiles. Of particular interest was the turnover rate, the topography of residues, the levels during phases of the cell cycle, the role in antigen-antibody interaction and possible changes in transformed cells.[37]

B. Effects of pH and Ionic Strength

The existence of a group like sialic acid with a pK between 3.0 and 4.0 had been expected by investigators because the isoelectric point of many cells is in that range. The isoelectric point was first determined by Kozawa [38] in 1914 by measuring the mobility of red blood cells in buffers of varying pH. At a particular pH the cells stopped moving and below that pH they reversed direction. The point where there was no movement, and therefore, no net charge on the cell, was their isoelectric point. On many cells this occurred between pH 3.0 and 4.0. Heard and Seaman[24] examined the red blood cell in great detail with a variety of buffers and ionic strengths. They determined ranges of pH over which the red cell was stable as a function of the ionic strength.

Altering the ionic strength of the measuring solution and making it isotonic with a nonionic molecule, like sorbitol, is another way to obtain more information from the mobility measurements.

In normal ionic strength buffers the membrane groups that lie as little as 10 Å under the surface do not contribute to the zeta potential. This is due to the abundance of counterions which can diffuse into the membrane and neutralize the charges. By lowering the ionic strength the electric potential of the membrane charges extendsfarther away from the cell due to the lack of counterions. The groups deeper in the membrane then contribute more to the surface charge and the zeta potential.[24] Mobility measurements at lower ionic strengths therefore have components which represent charges deeper than 10 Å. (For greater detail, see Appendix I.)

The red cell shows a membrane ionic strength profile which suggests that it has little thickness observable with mobility measurements, whereas the opposite is true for other mammalian cells.[39,40] (See Appendix I)

C. Effects of Enzymes and Other Surface Effectors

Enzyme studies improved the value of electrophoretic mobility measurements because specific groups could be removed and the resulting changes in surface charge noted. Neuraminidase and proteolytic enzymes showed that sialic acid and proteins or glycoproteins accounted for a large amount of the surface negativity of the red cell.[41]

The use of ribonuclease by Weiss and Mayhew[71] showed that there is probably RNA on the surfaces of some lymphocytes and tumor cells. More will be mentioned of this in a later section.

Mehrishi [30] treated cells with 6,6'-dithiodinicotinic acid which binds specifically to free -SH groups. Upon binding, the molecule is cleaved. The half remaining bound to the cell has a free carboxylic acid group which adds a net negative charge to the cell. The other half is in solution and it has a characteristic absorption peak at 344 nm. Using this method, Mehrishi[42] showed that no detectable levels of -SH groups existed on red blood cells, however, it changed the surface charge of lymphocytes by 10%, Ehrlich ascites by 15%, and platelets by 5%.

Mehrishi also showed that the platelet has a membrane susceptibility to alkaline phosphatase which removes about 8% of the cell's negative charge.[30]

In certain cells hyaluronidase also removes surface coat material and lowers the mobility. Sato et al.[40] showed that tumor cells treated with X-ray irradiation underwent a surface reorientation that moved the hyaluronidase sensitive sites deeper in the membrane. They determined this by lowering the ionic strength of the measuring buffer to probe the charges deeper in the membrane.

Other treatments of cells have been used besides those designed to discover specific groups on the surface. Mehrishi [42] has incubated cells with the synthetic polypeptides; polylysine, polyarginine, and polyornithine and found that cells can be made less negative, zero, or even positive depending on the concentration of these molecules. Cells of similar mobilities required differing amounts of polycations to reduce their charge. This may be due to varying topographical distributions of charges on the surface. Many of these polycations also have anti-tumor properties. It was suggested that the reduction in surface charge could result in increased phagocytosis by host cells.

In similar experiments, Mitchell and Cater[43] showed reduction in mobility by polycations, ptomaines, inflammatory mediators, antisera, and heparin. Heparin served to reverse the mobility lowering of polyamines and protamine binding, and to partly reverse the effect of antisera on BP8 tumor cells. Their most interesting discovery is that lymph node cells from animals immunized with BP8 tumor cells could be divided into two populations by polyamines, one negatively charged and one positively charged. In a later paper,[44] they showed that only the negatively charged cells could confer immunity to BP8 tumor cells in C3H mice and protect them from a subsequent tumor challenge

Lymphocytes in general show a heterogeneous distribution of electrophoretic mobilities. It has been shown that the T and B cell populations can be separated by preparative electrophoresis.[45] Ware et al.[46] have examined these two populations and have shown that they have different mobilities and different susceptibilities to neuraminidase. They also showed that the T-cells can have subpopulations which suggests that there is a correlation between the different functions of T-lymphocytes and their surface properties.

Conconavalin-A has been used in many membrane studes and in a few electrophoretic studies. Blume et al.[47] showed that the binding of Conconavalin-A to thymocytes resulted in an increase of mobility which they suggested was due to cellular response rather than the binding of Conconavalin-A alone. The effect occurred at levels of Conconavalin-A of 10^{-12} ug/ml which amounted to 220 molecules per 10^6 cells. Therefore Conconavalin-A probably induced a change in the thymocytes' metabolism which resulted in an alteration of the membrane organization. In this case mobility measurements serve as a very sensitive monitor of binding activity.

D. Changes in Tumor Cells

In 1956 Ambrose[48] reported a difference between the mobility of normal and tumor kidney cells. The tumor cells exhibited a higher surface charge than the normal cells. Others [37] showed that some tumor cells had the same mobility as normal cells but were more susceptible to neuraminidase. Fuhrmann[49] showed this for liver cells. The proliferating normal liver cells and hepatoma cells had higher mobilities than non-proliferating normal cells but the tumor cells

were more sensitive to neuraminidase. Other reports[37] suggested a similar correlation, but after several other cases were examined there did not seem to be a distinct correlation between malignancy and surface charge level. Vassar[50] noted differences between mesenchymal tumor cells and carcinomas in some cases, and there was a difference in the sensitivities to neuraminidase but there was no difference between the normal and malignant cells examined. Weiss[51] also examined cell surface charge as a function of the degree of malignancy and found no significant differences. In general, however, any change in mobility associated with tumorigenesis is usually an increase, which is also typical of fast growing cells.[52]

III. Tumor Membranes

It has long been hoped that there would be a simple, measureable difference between tumor cells and normal cells. The search for this key difference has been extensive, but no such difference appears to exist. The membranes of tumor cells have been examined ever since early studies indicated that contact inhibition was lost in tumor cell cultures[53], and that tumor cells were less adhesive.[54] This search has led to the examination of surface proteins, glycoproteins, glycolipids, glycosaminoglycans, lectin agglutinability, lectin receptor mobiltiy, membrane bound enzymes, membrane transport, proteolysis, tumor associated antigens, the H-2 antigen complex, antigen shedding, and the different cell-cell interactions. Each of these areas, as well as others, have volumes of reports addressing the issue of tumor cell membranes and several good reviews exist which attempt to piece it all together.[55,56,57,58] So far, however, the only safe conclusions that can be drawn are that there are a number of changes that sometimes occur in tumorigenesis but they are not substantially different from the changes normal cells undergo during rapid growth or in mitosis.

This does not mean that these findings are unimportant because the membrane is still the mediator between intracellular and extracellular activities. The interaction with the environment that normal cells undergo during mitosis and rapid growth is critical to the understanding of tumor cell interactions with the host even if the basic cause of the cancerous state lies more deeply within the cell.

The following is a brief review of the currently known changes in membranes associated with neoplastic transformation. It is a synopsis of the material in the major reviews cited earlier.

A. Changes in Cell Behavior

Malignant transformation always shows some departure from the normal pattern of contact inhibition. Each cell type has its own pattern of migration and behavior when it makes contact with neighboring cells. Malignancy often introduces subtle changes in the normal migration and growth of these cells as viewed in vitro.[53] Epithelial and glial cells, which usually form strong cell-cell adhesions, stop their locomotion and contract, show after transformation, a diminished tendency for intercellular adhesions. Fibroblasts show variable patterns after transformation, with increased overlapping, less adhesions to other cells, and continued membrane ruffling. Malignant lymphoma cells appear less mobile than normal lymphoblastoid cells. In conjunction with contact inhibition type behavior, tumor cells often lose density-dependent growth restrictions and in vitro many are capable of growing in suspension with no anchorage dependence.

These behavioral changes in vitro have not been thoroughly reproduced in vivo, so it is not known if changes in migratory patterns and contact interactions are important in the tumor-host system, particularly in metastasis.

B. Membrane Components

A variety of membrane alterations occur in the malignant state, particularly in glycolipids, glycoproteins and proteins.

1. Glycoproteins

The carbohydrate portions of glycoproteins undergo changes in size and in sialic acid content. Sialyl tranferase has been shown to be less in some cells, resulting in incomplete synthesis of certain large glycoproteins.[35] On the other hand, small molecular weight glycoproteins have more sialic acids in transformed cells and in fast growing normal cells.[55] Virus transformed, non-tumorigenic kidney fibroblasts showed longer and more highly branched oligosaccharides of the surface glycoproteins. [56] This has also been found in tumor cells after virus infection. Similar earlier work seemed to indicate larger glycopeptide fragments in tumor cells and mitotic cells when the cells were treated with trypsin and pronase. This work is comparable to that of the virus induced tumors.

Probably the most agreed upon change in glycoproteins is the loss of a 200K-250K glycoprotein[58] which goes under several names; fibronectin, LETS (large external trypsin sensitive) protein, Z protein, galactoprotein,and fibroblast surface antigen. This protein exists in high levels in membranes of normal cells but transiently disappears during mitosis. It can be removed with trypsin and its disappearance occurs simultaneously with induction of the cell into the cell cycle. This had led several investigators to suggest that this protein is involved with growth control. Tumor cells appear to be lacking in this membrane protein but they also may expel it into the medium. Lectin binding sites have been found on this protein, and the protein appears to interact with the cell's fibrillar structures. It has even been proposed that the lectin cap formation in tumor cells is due to the

lectins binding to less competive, mobile proteins which are normally not bound when the LETS protein is available.[58] Future work will probably elucidate the role of this protein.

2. Glycolipids

The glycolipids in the membrane of tumor cells seem to be less complex than those of normal cells with fewer molecules present and they are not accessible to antibodies, enzymes and lectins. This was determined on the NIL hamster cells by Hakonori.[59] Most of the virus transformed cells examined also showed similar reductions in complex glycolipids. As normal cells grow and contact each other there is an increased synthesis of these glycolipids, suggesting that they may be involved in growth control. Spontaneous tumors of the mouse do not show this reduction unless the cells are cultured for several generations,[55] which raises the question of relevance between *in vitro* and *in vivo* findings.

In general, there are measurable changes in both glycoproteins and glycolipids between normal, transformed, transformed and tumorigenic cells, and cells during development.[60] These changes are not specific to cancer, but may be more correlated to levels of differentiation and cell growth rate.

3. Proteins

The proteins that are altered in tumor cell membranes (besides glycoproteins) are transport enzymes, adenyl cyclase, $Na^+ - K^+$ - ATPase, proteases and glycosidases.[55,56]

Sugar entry increases in transformed cells as does entry of certain amino acids and phosphates.[55] Na^+ and K^+ movement is increased due to

the 4-5 fold increase in $Na^+ - K^+$ - ATPase activity. It has been suggested that malignancy is due to the increase in cell transport which also brings in molecules which stimulate growth.[55] It is perhaps easier to envision the increased transport as a secondary response to increased energy needs of malignant cells.

Elevated levels of glycosidase activity in human tumors with respect to similar normal tissues have been reported, however these measurements are made on cell homogenates. It is postulated that they can influence both the tumor cell origin and the surrounding cells. The increased levels are seen in some transformed tissue culture lines but not all. [55]

Protease changes have been reported and raise some interesting questions. Since protease treatment of normal cells can induce the mitotic cycle, remove LETS protein, alter other glycoproteins, change glucose transport, increase agglutinability with lectins, and make them more reactive to antibodies, a role of proteases in cell cycle control has been hypothesized.[57] A tumor cell could be releasing proteases at precise moments in the cell cycle which stimulates the next round of division. Tumor cells also release a plasminogen activator which normal subconfluent cells release. However, unlike the normal cells the tumor cells release it continuously. Roblin[61] has shown that tumor growth serum depleted in plasminogen results in major morphological changes in tumor cells back to their normal configuration. This indicates that the tumor activated plasmin causes surface alterations of the tumor cells, which may be important to the transformed state. The importance of protease action on the membrane

could be great. Antigen sites could be altered or their steric orientation changed, membrane permeability, membrane-cytoplasmic communication, surface receptors, etc., could all be significantly influenced by protease action. Further work in the area should be very enlightening to growth and cell-cell interactions.

4. Lectins

The agglutination of tumor cells by lectins has been known for several years. The capping phenomenon and the deduced greater mobility of binding sites has been intriquing, but no real meaning has been attributed to the phenomenon. Mannino and Burger[62] suggest that the surface change detected by agglutination commits the cell to enter the next round of the cell cycle. Since normal cells in mitosis exhibit agglutinability there does seem to be a connection. The question is whether or not it is a causal relationship. They blocked the Con-A binding sites of 3T3 cells but did not agglutinate the cells and showed that if the blocking agent was added in early mitoses the cells finished the cell cycle and entered G_o. The removal of the block allowed the cells to enter the cycle again. This suggested that a stop signal was necessary for cell control and that stop receptor was associated with lectin binding sites. This raised the question of what the stop signal molecule might be.

5. Antigens.

The antigen alterations of tumor cells is another large area of investigation, especially for the possibilities of effective anti-tumor immunotherapy.[63,64] Tumor specific antigens have been demonstrated that are capable of eliciting an immune response.[55]

Viral antigens are usually present in virus transformed cells and fetal type surface antigens are also present on some tumors.[65] The fetal antigens, carcinoembryonic antigen (CEA) and alpha-fetoprotein (αFP) are released by certain human tumors but their significance is not known. They are being used as a monitor for recurrent tumors in some cases. The release of antigens like CEA and αFP has spurred interest in the idea of antigen shedding by tumors.[66] Some results have shown that antigens released by tumors combine with antibody and form complexes that act as blocking factors which suppress the response of sensitized lymphocytes. Shed antigens alone are also claimed to be inhibitory to sensitized lymphocytes[66]. The possiblity of large numbers of released antigens simply overwhelming the immune system is yet another theory of tumor protection.[66]

The field of tumor immunology is immense[65] but it seems that the most interesting work is not on the antigen sites of tumors but in the host's response system. The possible suppression activities of tumor cells is one very important aspect which has only recently been examined extensively.[67] Another related area is the importance of the histocompatibility complex H-2 or HL-A. There is evidence[68] that they are involved in antigen recognition by the cells of the immune system. This cell-cell interaction at the membrane level, particularly the necessary conformations of antigen and H-2 complex could be an area for new breakthroughs and of importance to tumor immunology.

I have outlined briefly only the major changes in the membranes of tumor cells. Many internal differences have been cataloged. Many of them are probably the result of tumorigenesis and not the cause, as are some of these membrane alterations.

IV. Surface Nucleic Acids

A. Surface RNA

In 1952, Lansing and Rosenthal[69] showed evidence for ribonucleic acid on the surface of Arbacia eggs through light microscopy staining with toluidin blue. They also used the plant Elodea canadensis and measured calcium oxalate formation before and after ribonuclease treatment. The peripheral cell edges lost staining ability after RNase treatment in the first case and less calcium oxalate crystals formed after RNase treatment in the second case. They suggested that a membrane RNA molecule was important as a calcium repository and could possible serve as a transmembrane carrier for certain molecules.

Chaudhari and Lieberman[70] found RNA on the surface of liver nuclei by using particle electrophoresis and treatment with RNase. The RNA was only on the nuclei of liver cells of partially hepatectomized rats, where the cells are undergoing much greater DNA synthesis rates.

The next report of surface nucleic acids was made by Weiss and Mayhew[71]. They used whole cell electrophoresis to show that cultured L1210 tumors and RPMI no.41 cells exhibited ribonuclease sensitive sites on their outer surfaces. Mouse, human and chicken erythrocytes did not change after RNase treatment, whereas all cells measured were sensitive to neuraminidase, giving a reduction in mobility.[72] Further work of theirs[72] indicated that these RNase sensitive sites were able to bind Ca^{++} ions, and at low concentrations

of Ca^{++} ions, these were the only surface binding sites. They suggested that there was membrane bound RNA and that it was a structural component with one of it's functions being a reservoir of divalent cations.

They continued to study this surface RNA[39] and showed that it did not easily wash off cells, nor could it be added to cells by debris from broken cells. The rate of cell metabolism and culture division time did, however, determine the level of surface RNA. Cells deprived of serum in their tissue culture media lost a significant amount of charge due to RNA, whereas this starvation did not effect the level of neuraminidase sensitive sites. This led them to suggest that the surface RNA was a relatively labile group but it was not an adventitious group.

Mayhew and Weiss[71] also showed this RNase sensitivity on mouse lymphocytes, polymorphonuclear leukocytes, thymus cells, liver cells, and in S37 ascites cells. The tumor cells and transformed tissue culture cells exhibited the greatest amount.

Mayhew[52] also showed that the surface charge of tissue culture cells (RPMI no. 41) increased during the M phase of the cell cycle. An increased level of surface sialic acids seemed to be responsible for this change. When non-synchronized cells were examined at different growth rates, however, the sialic acid content was constant even though the surface charge increased with increasing growth rates. This, and their previously described work,suggested that fast growing cells have an increased amount of negatively charged groups which are susceptible to RNase removal but not to neuraminidase removal.

They further substantiated that hypothesis by examining normal and rapidly proliferating lymphoid cells in the mouse.[73] The rapidly growing, more immature cells contained RNA on their surface while the normal controls did not.

Weiss et al. continued this work by exploring the relative density of these RNase and neuraminidase sensitive sites on the surface of Ehrlich ascites cells.[74,75] The cells were fixed in glutaraldehyde and treated with positively charged, colloidal iron hydroxide particles. The cells were sectioned and viewed by electron microscopy. When cells were treated with RNase - leaving behind the sialic acid residues - the distribution of the particles was a uniform Poisson distribution. When the cells were treated with neuraminidase - leaving the RNA molecules - the particles were found in clusters and the clusters were distributed in a uniform Poisson distribution. When both enzymes were used, the remaining 66% of the negativity of the cell surface was not strong enough to hold any particles bound. From this they suggested that sialic acid on the cell was clustered in small patches, each patch with enough negativity, $\sim$30 electrons, to hold one particle bound. The RNA was clustered in larger patches, each patch capable of binding several particles. The remainder of the cell's negative charge was distributed evenly and its density wasn't great enough to keep a colloidal iron particle bound.

Other electrophoresis work which has demonstrated RNase susceptibility on cell surfaces was done by Woo and Cater[76]. They were examining antigens on adult liver cells, fetal liver cells, and hepatomas in the mouse. During their studies they showed that one

of the similarities between the fetal and hepatoma cells was a susceptibility to RNase treatment. RNase did not effect normal liver cells but decreased the mobility of fetal cells by 17% and hepatoma cells by 29%. They also examined the BP8 ascites tumor and lymph node cells and showed a 39% reduction in mobility with RNase in the tumor cells as compared to a 4.8% reduction in normal lymphnode cells and 13% reduction in lymph node cells from animals immunized with BP8 cells. They spent considerable effort in the attempt to wash the RNA off of the cells, with no effect, and to make sure that the RNase enzyme was not binding and causing reduced mobility. They suggested the RNA was real and hypothesized that it might simply be due to mitotically active cells or might serve a role in cell-cell recognition or information transfer.

In another electrophoresis study, Droege et al.[77] examined chicken lymphocytes from blood, spleen, bursa, and thymus. The blood lymphocytes showed a 22% reduction in mobility after RNase treatment, whereas none of the lymphocytes from other organs were sensitive to RNase. They suggesed that this RNA could serve as an endogenous adjuvant in the lymphocytes response to antigen.

Besides the electrophoresis work there have been a few other studies suggesting surface RNA. During the isolation of liver membranes investigators have found 1-2%[78] and 2-4%[79,80] RNA in their membrane preparations. The problem with this is that this quantity of RNA could easily be contamination. In fact, most researchers attributed RNA in their membrane preparations to contamination. Davidova and Shapot[81] examined more closely this RNA

in membrane preparations using rat liver and hepatoma cells. They found that the plasma membrane-bound RNA contained different size components than the endoplasmic reticulum RNA. They also added a known amount of radiolabelled contaminant RNA to their liver homogenate and determined that only 4-7% of the membrane associated RNA was contaminated by cytoplasmic RNA. They suggested from the isolation techniques that the RNA is bound to protein and that this is complexed with lipid.

Using L cells Glick[82] examined the RNA associated with membrane preparations and compared it to ribosomal RNA. She found that the base compositions and profiles on sucrose gradients were similar. However, the synthesis rate of surface RNA was greater (as measured by uptake of ^{3}H uridine) and the surface RNA was less sensitive to Actinomycin-D and to RNase digestion. She suggested that protein producing ribosomes are associated with the cell outer membrane and that they synthesized specific types of proteins. The question which was not addressed by this study was whether the membrane RNA examined originated from the inside or the outside of the membrane.

The possibility of an RNA-protein complex of some sort located on the surface was supported by Rieber and Bacalao.[83] They labelled Chinese hamster ovary cells with ^{32}P-phosphate and ^{3}H-leucine and then treated them with trypsin. The trypsin treatment released radioactive phosphate which was sensitive to RNase treatment and also contained protein. With several methods they tried to assure that the trypsin was not getting into the cell and releasing cellular

phosphate containing macromolecules. When cells were incubated with Bt_2-cAMP-testosterone they showed a greater amount of a higher molecular weight phosphate containing-RNase sensitive macromolecule. They suggested that this membrane ribonucleoprotein may be associated with cell proliferation and differentiation.

B. Surface DNA

In 1970, Singer and Reid[84] examined the mucoid coats of cervical cells under the electron microscope and found fibrous bundles and filaments. Using a method to remove mucoid coats from cells they examined rat thymus cells.[85] After using DNA extraction methods on the cell coats they found a DNA which had a higher, more abrupt melting temperature but lower hyperchromicity than intracellular DNA. This DNA was shorter and aggregated into bundles and rings and had a greater buoyant density. They suggested that it might be partially single stranded and may contain repeating sequences. Reid[86] presented an elaborate argument that this surface DNA is involved with cellular communication in an electronic network fashion.

Lerner et al.[87] then reported a plasma membrane associated DNA in diploid human lymphocytes. The DNA was isolated from membrane fractions and shown to be smaller in size than nuclear DNA. It replicated during G_1 at a rate 16% of the S-phase rate, whereas the nuclear DNA's replication rate in G_1 was only 3% of the S-phase rate. They suggested that this DNA was different from the nuclear DNA and was not a membrane contaminant. The membrane DNA was susceptible to DNase while on the membrane, indicating that it was in an exposed

position. Finally, they noted that the number of DNA molecules in the membrane corresponded about one to one with the number of bound IgG molecules. They suggested that this membrane DNA could be involved in immunoglobulin synthesis, gene amplification, or immunologically associated information transfer between cells.

In 1975, Aggarwal et al.[88] suggested the presence of DNA on the surface of tumor cells. Using platinum-pyrimidine complexes as electron dense stains for electron microscopy, they noted patches on the surface of tumor cells which could be removed by neuraminidase or DNase, but not RNase, trypsin, or hyaluronidase. Eight tumorigenic cell types showed patches, while 11 non-tumorigenic tested negative. They suggested that DNA was present on the outer surface of tumor cells, and was bound in association with sialic acids. Rosenberg[121] suggested that this surface DNA could have an immunosuppressive role by acting to mask tumor antigens on the cell surface.

Further work along this line by McAllister et al.[89] suggested that other cells besides tumors also stained on the surface, and that the technique needed refinement. She also suggested that under certain conditions other molecules than nucleic acids could be stained.

Aggarwal strengthened his argument by using an autoradiographic technique in conjunction with electron microscopy.[90] He labelled cells with ^{3}H thymidine and plotted the number of grains versus distance from the nucleus. He compared this to a similar plot of the densities of platinum staining versus distance. There were distinct peaks inside of the nucleus, at the nuclear membrane, and at the plasma membrane.

Russell and Golub[91] found a subpopulation of leukemic spleen

cells capable of suppressing anti-sheep erythrocyte antibody response of normal mouse AKR spleen cells. They showed that this subpopulation of cells had DNA located on their surface. The suppressor population could be isolated by passing the cells down a column containing bound anti-calf thymus DNA antibody. Treatment of these cells with DNase eliminated the suppressor activity. In the assay for suppressor activity cell contact was required, which could mean an physical transfer of DNA or a product was necessary for suppression.

Hollinshead and Stewart[92] have also made an observation involving membrane DNA. They found a DNase sensitive factor on the membranes of cells from human Oat cell lung cancer. This appears to be involved as an inhibitor, protecting the tumor cells from an immune response directed against the cell's tumor associated antigens.

C. Cell Released Nucleic Acid.

There have been many reports of RNA molecules that have an effect on cells. The term immune-RNA or informational-RNA or transfer-RNA is used to indicate RNA which is extracted from cells, usually sensitized cells, and given to other cells or injected into animals. Transfer of specific immunity has been demonstrated by this technique as well as induction of differention. Several reviews exist on this subject[93,94,95] but they do not discuss the release of RNA from cells. A few studies have shown a release of an RNA-DNA complex which will be discussed below.

The <u>in vitro</u> release of DNA from lymphocytes,[97,101,100,105,99] bacteria,[96] frog auricles,[97] and the water mould <u>Allomyces arbuscula</u>[98] has been demonstrated in recent years. Khandjian et al.[98] and Stroun

et al.[97] described a complex of RNA-DNA and protein as being released. Stroun et al.[97] have shown that extracellular concentrations released from lymphocytes attained a steady state level which was recognized and regulated by the cell. They showed that the RNA-DNA-protein complex was quite stable and required drastic proceedures for removal of the RNA. The complex was not susceptible to RNase degredation. Only after phenol extraction, DNase digestion, and chloroform and sodium perchlorate extraction was the remaining nucleic acid RNase sensitive.[97] They also showed that this complex was capable of extracellular DNA synthesis.

Rogers[100] described a DNA which was excreted by phytohemagglutinin-stimulated lymphocytes. He showed that the DNA excreted was homologous with only 10% of the nuclear DNA and was smaller than the nuclear DNA. He followed the course of labelled DNA from the nucleus to the supernatant on normal and stimulated lymphocytes.[101] The DNA traveled from the nucleus to the supernatant during the course of 3 days. In the stimulated cells 3-4 times the amount of the DNA was excreted. He suggested that this excretion of a particular segment of the cell DNA may be very important in the activation of lymphocytes.

The DNA released by lymphocytes has also been shown to attach to red blood cells and cause rosette formation.[105] The significance of this is not known.

Developement of Electrophoresis Apparatus

I. Backround and Overall Design

The technique of whole cell electrophoresis consists of placing a cell in a solution containing an electric field and determining its induced velocity. The cell migrates toward one of the electrodes with a velocity directly related to the value of its average surface charge. Under normal conditions cells are negatively charged and therefore migrate toward the positive electrode. The electrophoretic mobility is measured as velocity per unit electric field, u/sec/Volt/cm. The standard technique for determining this mobility was to watch a single cell and determine the time it took to travel a set distance thereby obtaining its velocity. This is a tedious proceedure because several cells had to be examined in this way to obtain a relevent representation of the population. This also exposed the cells to the electric field for prolonged times. In order to be able to collect data rapidly on a large number of cells a new technique was necessary.

A new method of measurement was developed using a technique first described by Goetz and Penniman.[106] It consisted of mechanically moving the image of the cells, which are viewed through a microscope, in a direction opposite to that induced by the electric field. When the two are equal the cells appear to stand still and the mechanical movement just equals the field induced velocity of the cell. Their apparatus consisted of using a prism oriented in the optical path of the microscope which shifted the cell image with delicately controlled minute rotations. These were

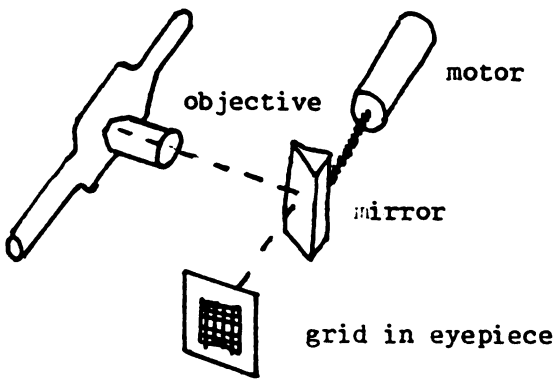
controlled with relatively elaborate electronic servo systems.

This technique was altered for the present apparatus by incorporating a sliding mirror which, as shown in Figure 1 , serves to shift the image at the reticle located in the eyepiece of the microscope. The mirror is driven by a motor through a worm gear and the speed of the motor is accurately controlled and monitored. When a measurement of cellular mobility is made a 'cloud' of cells is watched and the velocity of the mirror is adjusted until the 'cloud' appears stationary. The motor speed is then directly proportional to cell velocity. This method allows for rapid determinations of large numbers of cells thereby obtaining statistically reliable measurements with a minimum of exposure for the cells. (A five minute period is usually sufficient to obtain a mobility value with a coeficient of variation of 1-2%)

To further speed the data taking process a television camera was attached to the eyepiece so that the cells could be viewed on a TV monitor, reducing the eyestrain from prolonged microscope work.

II. Measuring Chamber and Electrodes.

The measuring chamber was obtained from Thomas Inc. and is the flat,parallel plate design as shown in Figure 2 . The distance between the inner surfaces of the plates is 400 micrometers. This distance times the width of the chamber defines the cross sectional area of the chamber and equals 0.048 cm^2. A water jacket was added to the chamber, Figure 3, and the chamber is connected to the dual purpose electrode chambers/fill and drain ports. Stopcocks allow the sample to be added and drained or the electrodes to be added to the circuit when the electric field is applied.



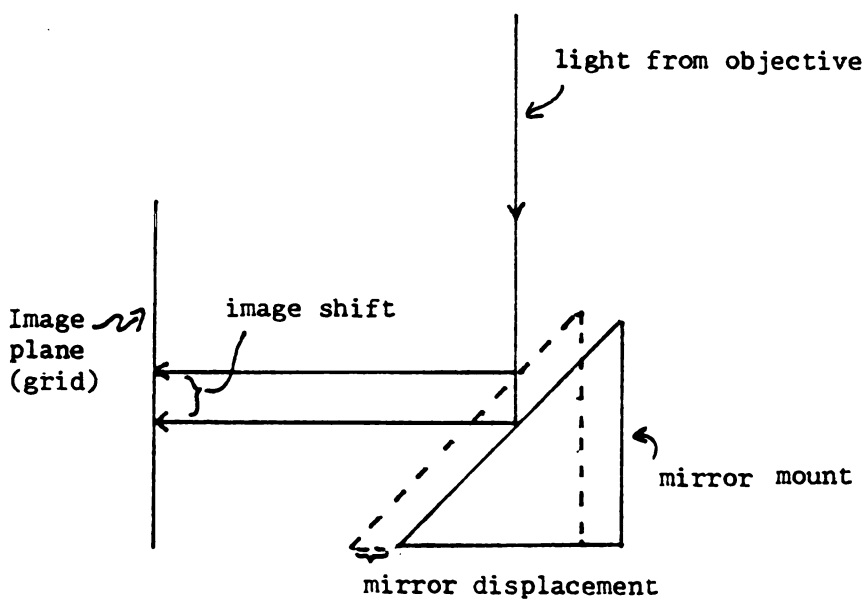


Figure 1. Diagram of mirror movement.

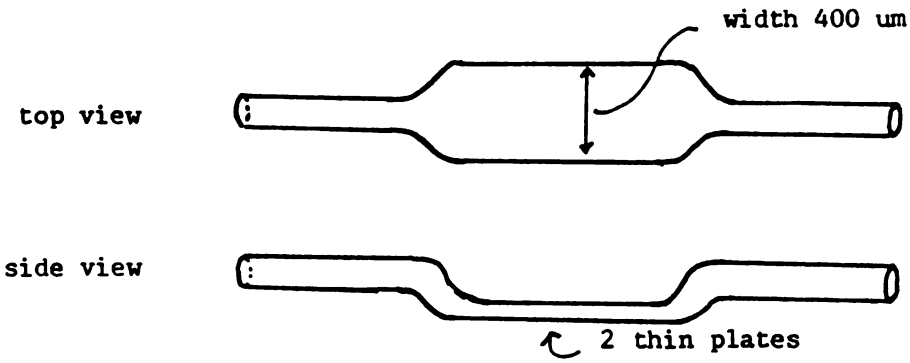


Figure 2. Diagram of measuring chamber.

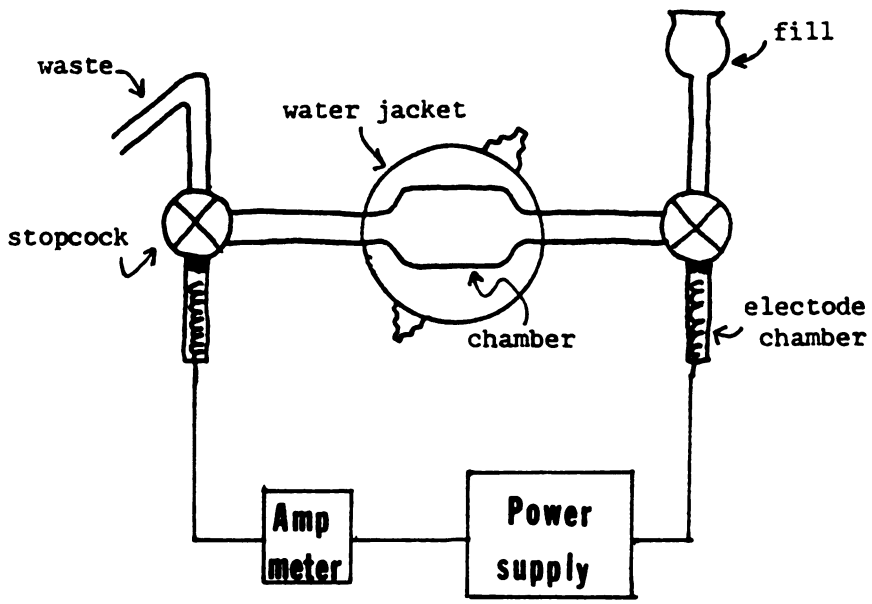


Figure 3. Diagram of chamber and electrode circuit.

The field strength inside the chamber cannot be measured directly and therefore it is derived by measuring the electric current flowing in the electrode circuit (the ammeter in Figure 3). The currents flowing in all elements of the circuit are identical therefore this represents the current flowing in the measuring chamber.

The electric field is derived from the current by the following equation:

$$E = \frac{I}{K\,A} \quad ,$$

where E is the electric field, I is the current, K is the conductivity of the buffer, and A is the cross sectional area of the chamber. K is determined with the following equation:

$$K = \frac{I_{buff}}{I_{std}} \cdot K_{std} \quad ,$$

where a 0.1 N KCl of known conductivity of 1.119, at 18^{o}C is used to determine K. The standard is placed in the chamber first and the current is measured at a fixed voltage between the electrodes. The standard solution is then replaced with the buffer and the current is again measured.

The electrodes are Ag| AgCl reversible electrodes in saturated KCl solutions. They are made by plating AgCl onto silver wire in a 0.1 N HCl solution with a current of 10-15 mA for 20 minutes. The saturated KCl solution in the electrode chambers is separated from the physiological buffer solution in the measuring chamber by plugs of glass wool. Risks of cell damage due to leaking of the KCl through the plugs during measurements is reduced by 10-15 cm connecting tubes from the measuring chamber to the electrode chambers.

III. Mirror Movement

The microscope used for this apparatus was the Nikon MS inverted microscope. It had the advantage of a right angle bend design in the optical path from objective to eyepiece. This allowed for placement of the movable 45° mirror as shown in Figure 1 . The motor attached through the worm gear to the mirror contains a gear reduction unit and a built in tachometer to monitor motor speed. The motor input and tachometer output are attached to a speed control power supply. The motor and power supply were purchased from Servo-Tek Inc. The tachometer output is also monitored with a digital volt meter (DVM) and is used to determine cell velocity.

The mirror velocity is proportional to the velocity of the cells with a multiplicative constant given by the power of the lens objective. The mirror velocity, in turn, is proportional to the r.p.m. of the motor with a proportionality constant given by the pitch of the worm gear.

The mirror movement was calibrated with a stopwatch. The stopwatch timing of a stationary particle in the field of view as it was moved by the mirror gave an actual value for the velocity. The tachometer output from the mirror is also related to the velocity observed by the equation,

$$\text{Tach voltage} \times \frac{0.3545}{Y} = \text{velocity}$$

where the factor 0.3545 is a constant obtained from motor speed, the gear ratio, and conversion factors. After calibration it was determined that this factor was off by 1.69% and was therefore adjusted to the value 0.3605. (Y is the power of the objective,)

The overall schematic for the switches and interconnections is shown in figure 4 . Provisions are made to switch polarity of the electrode voltage and reverse direction of the motor rotation. A chart recorder is used to monitor electode current while a DVM is used for the tachometer voltage. Further details and operating instructions are given in Appendix III.

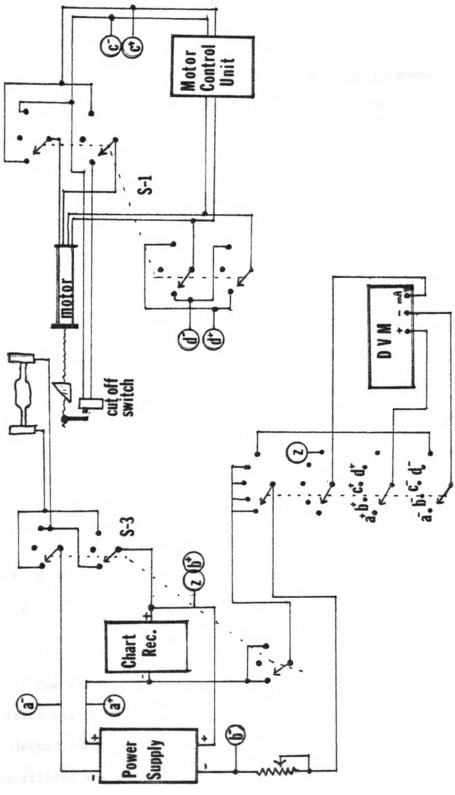


Figure 4. Circuit diagram of electrophoresis apparatus.

Materials and Methods

I. Buffers and Solutions

The buffer used for all electrophoresis measurements was a citrate-phosphate base buffer with a pH range from 2.2 to 8.0. (NaCl = 0.137M; KCl = 0.004M; Na_2HPO_4 = 0.011M; Citric acid = 0.006M for a pH = 7.4) For pH = 6, citric acid = 0.0029M and Na_2HPO_4 = 0.01M ; for pH = 4.6, citric acid = 0.0052M and Na_2HPO_4 = 0.009M; for pH = 3.0, citric acid = 0.016M, Na_2HPO_4 = 0.006M.

When the ionic strength of the solutions were changed, isotonic (5%) mannitol was added to this buffer.

All incubations with enzymes were performed in a modified Hanks solution (NaCl = 0.137M; KCl = 0.005M; $MgSO_4$ = 0.0016M; $CaCl_2$ = 0.00081M; glucose = 0.011M; NaH_2PO_4 = 0.001M; and Na_2HPO_4 = 0.0045M). The bicarbonate has been removed to eliminate the need for a CO_2 atmosphere.

When cells are removed from the animals they are diluted with a 0.9% NaCl solution.

The perfusion buffer used during the isolation of cells from the liver, fetus, thymus, spleen and solid S-180 consists of NaCl at 0.08 M, EDTA at 0.027M and sodium citrate at 0.027M.

II. Cells

The S-180 mouse tumor cells are grown in ascitic form in the peritoneal cavity of the ICR mouse. 2, 4, or 8 x 10^6 cells are injected into the animal and then at later times the animal was sacrificed and the cells washed out of the peritoneal cavity with saline. Twenty four hours after injection of the cells is denoted

as day 1 and so on from there.

The animals were injected with cisplatin on day 1 (day 3 in some experiments) with a dosage of 7 mg/Kg. This cisplatin was dissolved in saline and injected under sterile conditions with an approximate injection volume of 0.5 ml.

After the cells were removed from the animals they were washed by centrifugation to remove blood cells and were stored on ice for short periods before incubations and before electrophoretic mobility measurements.

The P388 and P815 cell lines were grown in the DBA/2 mouse strain. AKR spleen and thymus cells were obtained from leukemic (those showing physical signs of sickness) and preleukemic (showing no signs of sickness) AKR mice. The L1210 and V79 cells were obtained from tissue culture. Blood cells were obtained from mice via intra-orbital bleeding.

To obtain liver cells in suspension the animal was sacrificed via cervical dislocation and the thoracic cavity was opened. The animal was then perfused through the heart with citrate-EDTA-saline. Livers of successfully perfused animals were removed and minced finely with scissors. These pieces were then forced through a 40 mesh stainless steel screen and the resulting cell and tissue mixture was then allowed to sediment for approximately one minute at 1 g and the supernatant was removed. This supernatant contained the whole cells and the debris. The cells were cleaned by centrifugation in citrate-saline.

The same proceedure was followed for spleen, thymus, fetal, and the solid S-180 cells.

III. Enzymes

The enzymes used in the study were obtained from different sources:

RNase A	Miles Laboratories 36-511	5X crystalized -protease free
	Sigma Type I	" "
	Sigma Type XI-A best grade	"
	Worthington RAF-OFA	phosphate free "
DNase I	Sigma Type I	chromatographically purified best grade
	ICN 100575	2X crystalized
Neuraminidase	Sigma Type V	
Protease	Calbiochemical -B grade	
Trypsin	Nutritional biochemicals 1-300	
Hyaluronidase	Sigma Type IV	
Immobilized DNase	Worthington	
Immobilized RNase	Miles Laboratories	
Eco R1	Miles Laboratories	

Enzyme concentrations for RNase and DNase, unless otherwise noted, are 0.2mg/ml RNase, Sigma Type XI-A, and 0.1mg/ml DNase, Sigma Type I. The other types listed above were tried but were not found to be different in their effects, so one type was used thereafter.

IV. Tissue Culture Media for Incubations

For the long term incubations <u>in vitro</u> the cells are placed into tissue culture medium. Both of the following were used; Medium 199 with Hanks base and 10% calf serum, and NCTC 135 with Earles balanced salt + L-glutamine and 10% fetal calf serum.

V. Enzyme Treatment

Enzymes were dissolved in modified Hanks buffer and the cells added. Incubation was at 37°C for various times in closed vials containing no air with glass beads added or in open tubes. The former were placed in a 37°C hot air incubator on a rotating mixer, the latter was incubated at 37°C in a water bath. After incubation the cells were washed once by centrifugation then placed on ice.

VI. Cell Pathology

The cell population and cell morphology was primarily analyzed by using smears on glass slides stained with Wrights Stain. Peroxidase Stain specific for neutrophils was also used and in a special examination performed by Dr. Steven Stockham, veterinary pathologist, methylene blue and PAP smears were also used. Peritoneal washes were spun down and resuspended in a minimum fluid in order to obtain a cell suspension suitable for smearing onto slides. Slides were examined with light microscopy.

VII. Analysis of Raw Electrophoretic Mobility Data.

Measurements from the EPM apparatus consist of pairs of left and right readings. The cloud of cells is nulled with electric field set for migration to the left and then repeated with the electric field reversed. This proceedure of alternating the field is continued until a representative sample is taken. There are two numbers obtained for either a left or a right measurement, the current reading and the tachometer voltage from the mirror motor. These numbers are then entered into a computer program which converts the current into an electric field value and the tachometer value into cell velocity. Dividing the velocity by the electric field gives

the electrophoretic mobility in the usual form of u/sec/Volt/cm.

The left and right mobility values are averaged to obtain an overall mobility. A special kind of averaging is performed to remove a bias in measuring that would occur due to changes in the drift of cells in the chamber, to currents caused by sedimenting cells, to the motion of cells due to their own mobility or due to the electroosmotic effects. The problem arises from the serial nature in which the measurements are recorded,ie. left, right, left, right, etc. Normally the first left and right pair are measured and averaged, and the second pair and so on. The final average is taken from the average of these pair values. If all of the factors I listed above are constant during the measurement of each pair then their average is a true representation of the cell mobility. However, if these factors are changing during the measurement of any one pair the value of the mobility will be slightly in error. It is therefore obvious that the arbitrary choice of pairs in the overall left-right sequence adds an artificial constraint. To attempt to alleviate this, the 'left' should be partially averaged with the previous 'right' in the train of measurements and the 'right' with the subsequent 'left' with modifications for the first and last measurements. Therefore, a measurement is averaged with the previous and subsequent values in the train giving ½ weights to each of its neighbors. This proceedure does not alter the total average value of the mobility, which is the average of all the measurements, no matter what the conditions. It only reduces the variation caused by the extraneous forces acting in time intervals shorter than the time it takes to measure one pair of values.

VIII. Computer

The mobility measurements were computed on the CDC 6500 computer located at the Computer Center of Michigan State University. The current readings and the tachometer voltages are entered into a computer program and the mobilites are computed, the average taken, the standard deviation computed, and the deviations from the mean plotted.

Outline

In this section I will present a summary outline of the different experiments that will follow in the Results section.

Some of the early experiments were designed to observe and quantitate phenomena. The experiments following those were to elucidate the observed lowered mobility after platinum treatment and the susceptibility of the cell membranes to RNase and DNase incubation. In particular, experiments were designed to test an hypothesis of cell surface nucleic acids in several different ways using whole cell electrophoresis.

In several of the reports in the literature, which were mentionned earlier, cell surface RNA and DNA was described. Dr. Weiss' laboratory performed many experiments with cell surface RNA. They described its appearance on tumor cells and stimulated lymphocytes, its correlation to cell growth rate, and it distribution on the surface. They used electrophoresis to observe this surface RNA. Other investigators discovered DNA on the surface mostly using membrane preparations, surface staining, and radioactive labelling techniques. They made comparisons to nuclear DNA by size, homology, and melting characteristics.

In this study the first electrophoretic mobility observations were on tumor cells as a function of tumor age in the animals. Normal tumors were then compared to platinum treated tumors. There was a distinct difference in mobility, so further observations were made in an attempt to elucidate the reasons for the difference. pH profiles were run to compare their isoelectric points, different

enzyme incubations were performed to determine if a specific membrane group was effected by platinum treatment, and ionic strength profiles were obtained to probe into the membrane.

These observations led to the hypothesis that cell surface nucleic acids were present on the surfaces of the S-180 tumor cells and that treatment with cis-Pt(II) caused this group to disappear.

To help substantiate the hypothesis, enzyme kinetics were determined for RNase and DNase to assure a true enzymatic process was occuring. Enzymes bound to large Sepharose and Agarose beads were incubate with cells to insure only surface action. The restriction endonuclease Eco-R1 was incubated with cells to further strengthen the surface DNA hypothesis. Cells were exposed to anti-DNA sera to see if there was any membrane binding. Positively charged 'platinum-thymine-blues' were allowed to bind to cells. Those cells with surface nucleic acids would bind this molecule most readily and their mobility would drop.

All of these experiments supported the hypothesis of surface nucleic acids. Other experiments using neuraminidase and trypsin and low ionic strength showed that these nucleic acids are only loosely associated to the membrane. In vitro experiments further showed that the nucleic acids probably have a turnover rate on the membrane of 2-4 hours. Other in vitro work indicates that cis-Pt(II), methotrexate, mercaptopurine, and Actinomycin-D act in a similar way to cause the loss of this surface nucleic acid, probably by inhibiting its synthesis within the cell.

Next, some immunological tests were performed to see if an obvious role for the surface nucleic acids was evident.

These experiments lent little evidence one way or another.

Finally, other cell types were examined. The solid S-180, the P388 and P815 ascitic tumors, the AKR spontaneous leukemia, normal liver, fetal, spleen, and thymus, and the V79 and L1210 tissue culture cells. The normal cells showed no surface nucleic acids, the cells of lymphocyte origin seemed to have only DNA on their surface while other tumor cells contained RNA or both.

Results

The cell type used for the majority of this study was the mouse S-180 tumor. This tumor has been serially transplanted in ICR mice in our lab for several years. This study involves three different strains of the S-180 tumor. The first, called S-180, was the tumor used in our lab until the Summer of 1978. At this point the animals were diagnosed as diseased and new mice and a new tumor were procurred. This tumor was used until December 1978 and is refered to as'interim S-180'. The third tumor was obtained in January 1979. This tumor has characteristics closer to the original S-180 strain. It had been obtained from Japan, therfore it is refered to as the S-180-J. We sought this tumor because of difficulties which existed with the 'interim S-180'. The 'interim S-180' was too sensitive to cis-Pt(II) and was not a good tumor for drug screening. It also had a lower electrophoretic mobility and less sensitivity to RNase, which was the most intersting electrophoretic property of the original S-180 tumor. The S-180-J had an intermediate sensitivity and allowed me to continue my examination.

Notes on conventions of wording:

- platinum treated - means neoplastic cells removed from animals which have received a theraputic dose of cis-Pt(II).
- incubated - as in 'platinum incubated' or'RNase incubated' This means cells that have been incubated in vitro.
- mobility - is used to refer to whole cell electrophoretic mobility.

I. Cell Pathology

A. S-180

Samples of tumor cells removed from animals were placed on slides and stained with Wrights stain. These were examined at different stages of tumor growth with and without platinum treatment.

I examined these cells with light microsoopy and noted that the normal tumor cells were all uniform in size (20-40um), contained large nuclei and little cytoplasm, and constituted at least 95% of the total cell population. The platinum treated cells were different. The cells, which I believed to be neoplastic, were often somewhat smaller or very much larger with many nuclei, they contained more vacuoles, and their percentage of the total was considerably less. A large population of smaller cells were present (10-15um). These cells appeared to be leukocytes, in particular, neutrophils.

A peroxidase stain was used to determine if these cells were neutrophils. This stain specifically reacts with the peroxidase enzyme present within the cell and leaves a dark green to black residue at thelocation of large concentrations of the enzyme. The platinum treated tumor did contain a very large population of neutrophils, which increased with time after platinum treatment.

This response led me to consider the possibility of a host interaction after platinum treatment. Neutrophils are involved in inflammatory reactions but are also capable of tumor cell phagocytosis. A further examination of the cell types present after platinum treatment was necessary and I arranged with a pathologist at MSU to do this for me.

His findings, which are presented in the next section, were on the 'interim S-180' tumor strain. There were distinct differences between the S-180 and the 'interim S-180'. The S-180 cells, from the time of their injection into the animal, contained very few host cells, were more regular in size and shape, and were less sensitive to cis-Pt(II) treatment. The 'interim S-180' seemed more antigenic because of the neutrophils and macrophages that appeared immediately after injection of the tumor.

B. 'Interim S-180'

During the course of this tumor growth samples were obtained and given to Dr. Steve Stockham, a pathologist at MSU. He determined the cell populations in both the control tumor and platinum treated tumor as a function of time. Figure 5 is a compilation of the information he returned.

The control tumor begins with relatively large populations of macrophages and neutrophils but their numbers decline with respect to the tumor cells as time progresses. The platinum treated tumor, however, shows a large decline in neoplastic cells and an overwhelming amount of host cells. In the first couple of days neutrophils predominate but are then followed by macrophages.

Neutrophils have been shown to be cytotoxic to tumor cells, but only if they outnumber the tumor cells.[107,108,109] Here, then is a possible second line of defense after platinum treatment. The platinum's first effect is upon the cell itself to slow its growth and perhaps second to alter its surface making it more susceptible to host phagocytes.

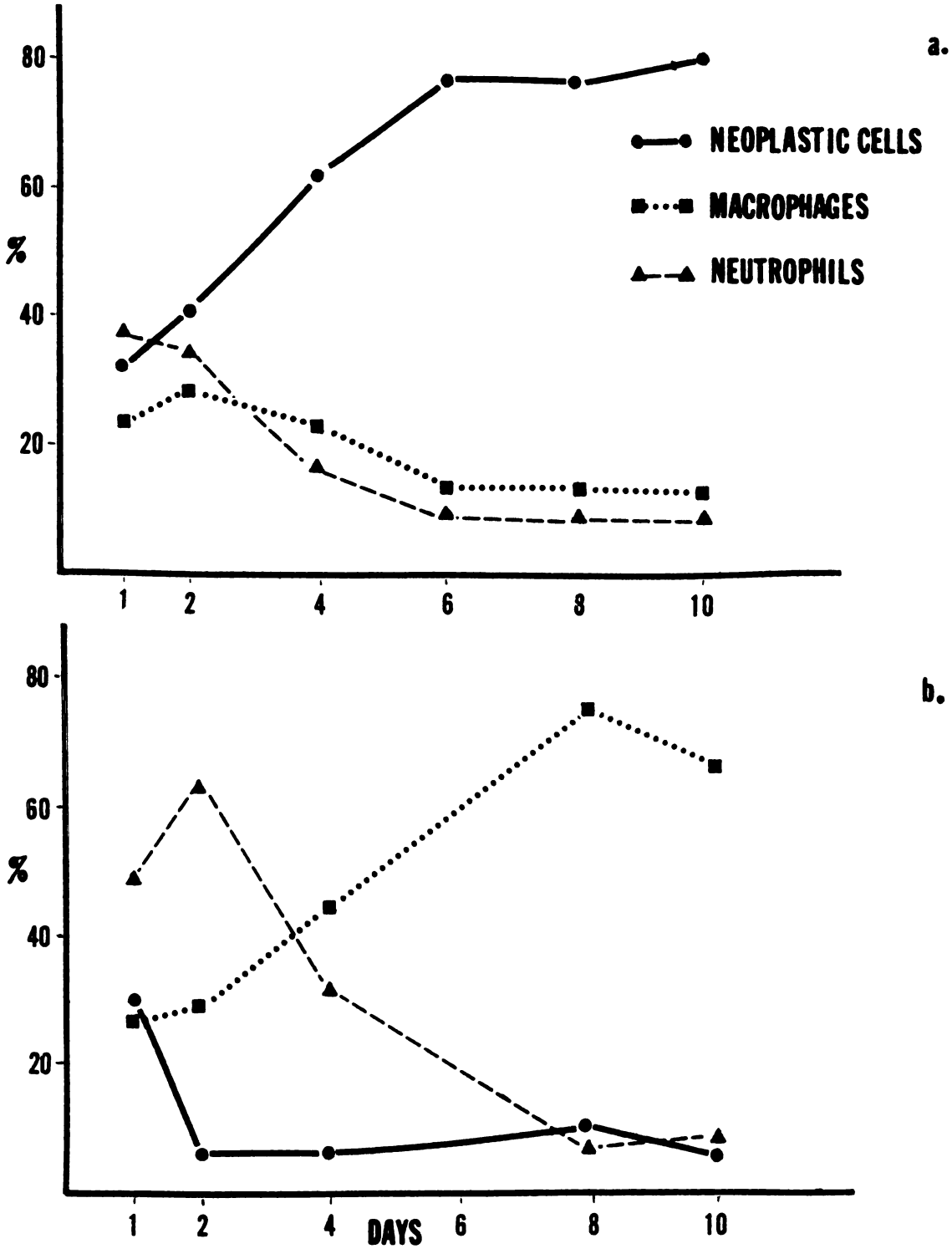


Figure 5. Percent of major cell types versus age of tumor in normal tumor, a, and platinum treated tumor, b.

Macrophages are also considered important in the host's defense against tumors. This large macrophage to tumor cell ratio in later days after platinum treatment might be the third strike against the remaining neoplastic cells.

II. Electrophoresis

A. Observations with Electrophoresis

1. Mobilities as a function of the tumor age.

One of the first studies was the examination of the electrophoretic mobilities of the S-180 tumor as a function of time after injection of the tumor into the mice. One set of animals received the tumor on day 0 and the other received the tumor on day 0 and 7mg/kg cis-Pt(II) on day 1. Figure 6 shows the mobilites up through 9 days of tumor growth. (After the 9th day the control becomes very large and bloody, while the platinum treated animals contain very few cells.) There is a striking reduction in mobility in the treated cells beginning about 7 hours after treatment. For the first 4 hours there is no change suggesting that cis-Pt(II) does not lower the mobility by simply binding to the membrane. As mentioned previously the treated cell population contains several cell types, where the neoplastic cells are in the minority. When measuring the mobility, only the large, neoplastic cells are visually chosen for measurement.

2. The distribution of mobilities

The apparatus used in this study to measure the mobilities determines the average for the population in view. In order to determine if this was a valid representation of their mobilities, individual cells were timed. Samples of control cells and platinum treated cells were measured using a stopwatch to measure their mobilities. Figure 7 shows histograms of both samples. Forty cells were measured in each case and in the platinum treated cells the size of the cell was also recorded. This resulted in two populations appearing, the small host cells and the large neoplastic cells. The spread on the neoplastic cells

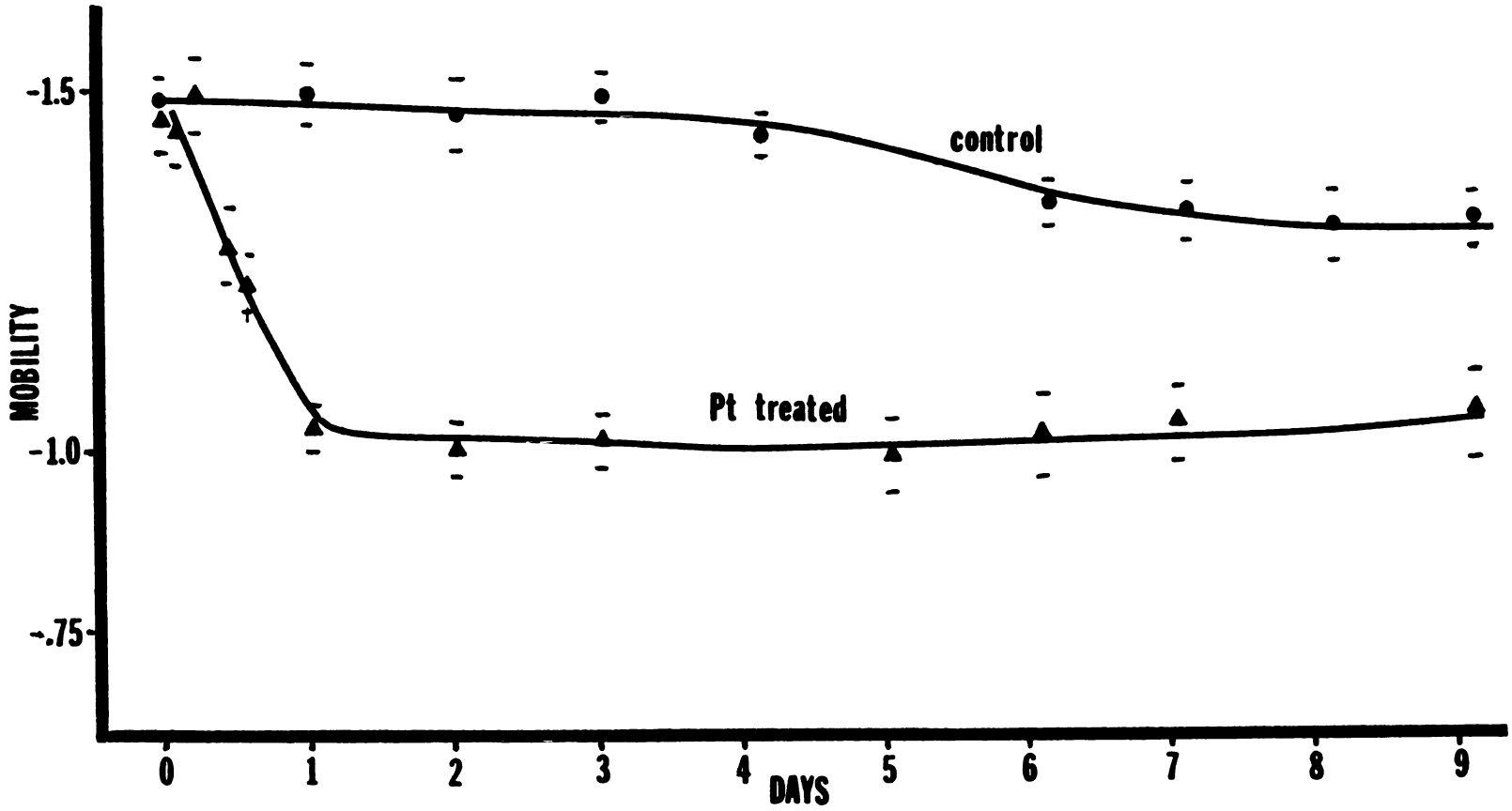


Figure 6. The electrophoretic mobility of S-180 tumor cells with and without platinum treatment as a function of tumor age. Bars represent the standard error.

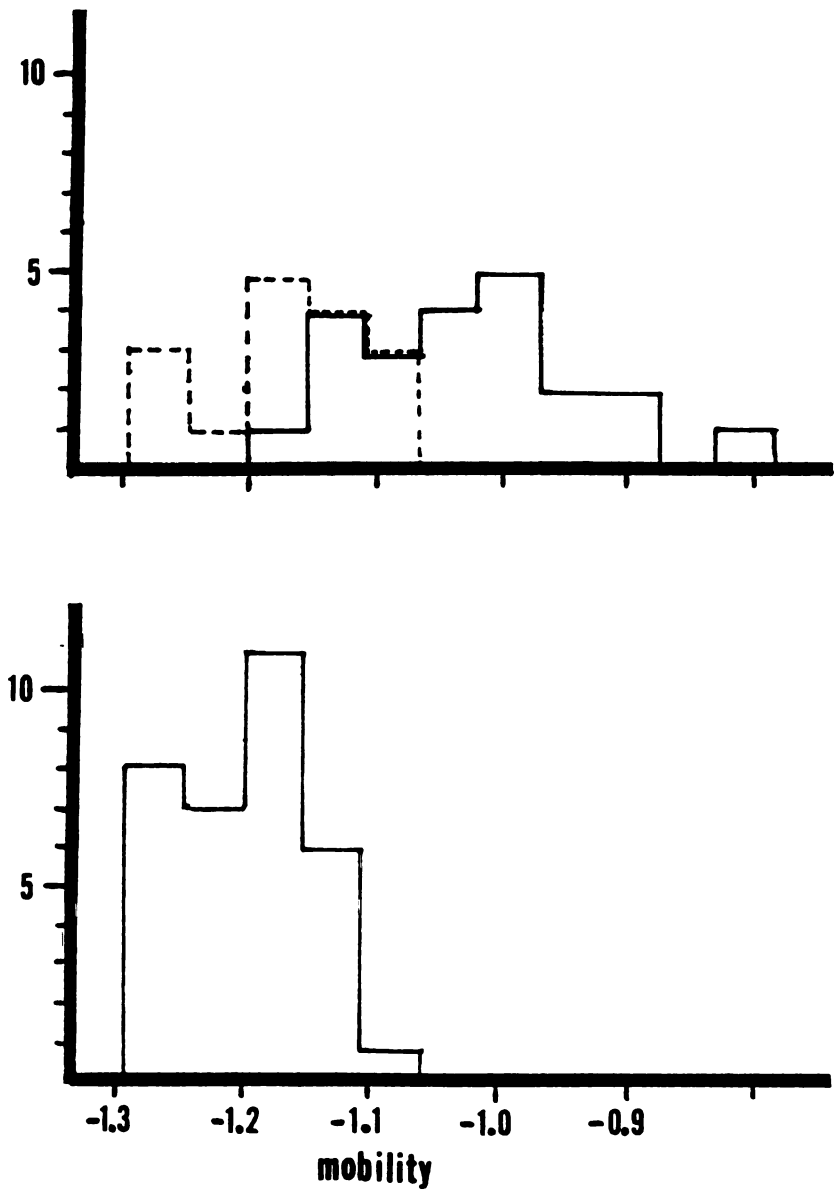


Figure 7. Distributions of mobility for the S-180-J. a) cisplatin treated tumor, hatched line is host cells, solid is tumor. b) untreated tumor, tumor cells only. The ordinate is number of cells.

is large but they appear to be unimodal so the averaged measurement obtained from the electrophoresis apparatus is meaningful.

3. Enzyme incubations.

In Table I the three S-180 strains, both control and platinum treated, are listed versus incubation with the enzymes, neuraminidase, protease, hyaluronidase, DNase, and RNase. In the S-180 and 'interim S-180' RNase was the only enzyme incubation which mimicked the effect of platinum treatment in the control and had no effect on the platinum treated cell. In the S-180-J both RNase and DNase could mimic the effect of platinum treatment on cell mobility. Both protease and neuraminidase lowered the mobilities of the controls and treated cells, but each enzyme lowered the mobilities of the control and treated cells to a common level.

4. Mobilities as a function of tumor age and enzyme treatment - S-180-J.

The time course of the mobilities of the S-180-J is shown in Figure 8 . The enzymes RNase and DNase at each day are also shown. The platinum treatment occured at day 3 instead of day 1 which meant that fewer cures were seen and the tumor resumed normal growth sonner. As can be seen in the platinum treated animals the RNase and DNase susceptibility returns with time. This is the first evidence that the cells are acquiring these groups and that the groups may be associated with growth rate.

Table I. The effects of enzyme incubations on the mobility of the S-180 tumor.

	Control	Protease	Neuramin.	RNase	DNase	Hyal.
S-180	1.32 (.011)	0.97 (.018)*	0.96 (0.86)*	1.06 (.016)*	1.36 (.04)	1.30 (.03)
S-180 +Pt	1.08 (.015)	0.93 (.025)*	0.85 (.052)*	1.05 (.024)	1.06 (.05)	-
'interim S-180'	0.99 (.013)	-	0.76 (.037)*	0.90 (.02)*	1.00 (.025)	-
'interim S-180'+Pt	0.95 (.035)	-	0.74 (.017)*	0.93 (.032)	-	-
S-180-J	1.19 (.015)	0.93(.03)	0.92 (.016)*	1.03 (.05)*	1.02 (.05)*	1.25 (.03)
S-180-J + Pt	1.06 (.07)	0.94(.02)	0.91 (.014)*	1.05 (.06)	1.05 (.06)	-

Notes: The protease used for the S-180 was pronase and for the S-180-J was trypsin.

The values listed with an astericks are significantly different from their respective controls at a significance level of .01 or better.

The cells listed as(+ Pt) are tumors from animals that have been treated with 7mg/kg cis-Pt(II).

The values listed in parenthesis are the standard errors. Mobilities will be listed with their standard errors unless otherwise noted.

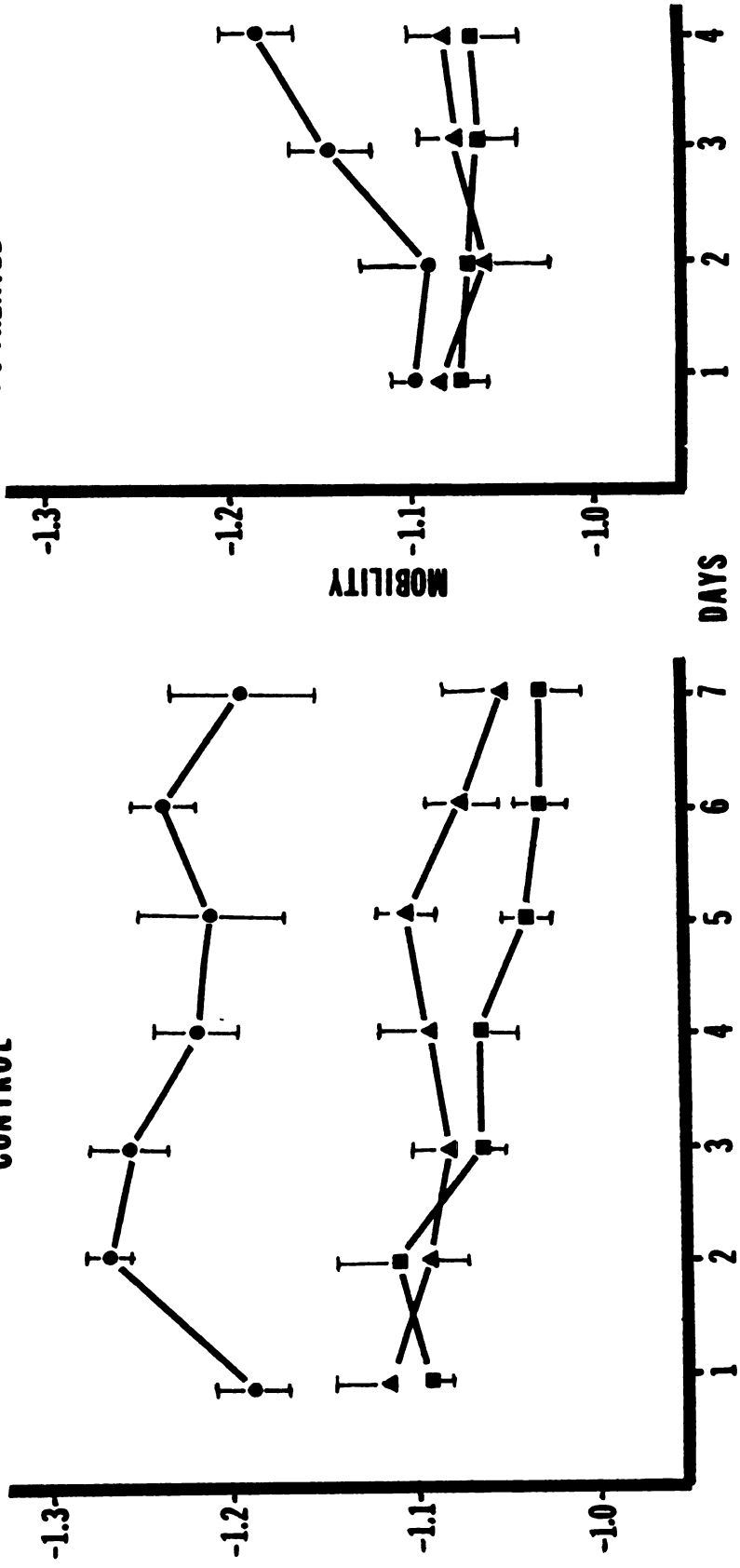


Figure 8. Mobility as a function of tumor age for control and platinum treated cells. Squares represent RNase incubation, triangles DNase incubation, and circles are incubation controls.

5. Enzyme kinetics

The enzyme RNase is positively charged at physiological pH and therefore it could bind to the cell membrane and lower the mobility. The enzyme action was examined in such a way that this possibility was eliminated. In Figure 9 a,the change in mobility as a function of time for RNase action on the S-180-J is shown. Samples were taken at increasing times after the cells were exposed to the enzyme. These samples were then placed on ice until the final sample was taken, then the enzyme was washed out of all tubes simultaneously. This proceedure rules out electrostatic binding because this kind of binding occurs at ice temperatures as well or even better than at 37^{o} C, whereas ezymatic action occurs only at higher temperatures. The curve clearly shows that RNase action is stopped by ice temperatures in the early samples.

Similar kinetics for DNase and RNase+DNase are shown in Figure 9 b,c. The RNase, DNase, and RNase+DNase kinetics are first order curves with rate constants of 0.065, 0.074, and 0.161 min^{-1} respectively. The noteworthy element of these rate constants is the additivity for the two enzymes. The rate constant for RNase+DNase is almost exactly the sum of each of their separate rate constants.

The level to which the mobility drops is about equal for both RNase and DNase and RNase+DNase. From this and the additivity of their rates it is probable that they are both acting on the same site.

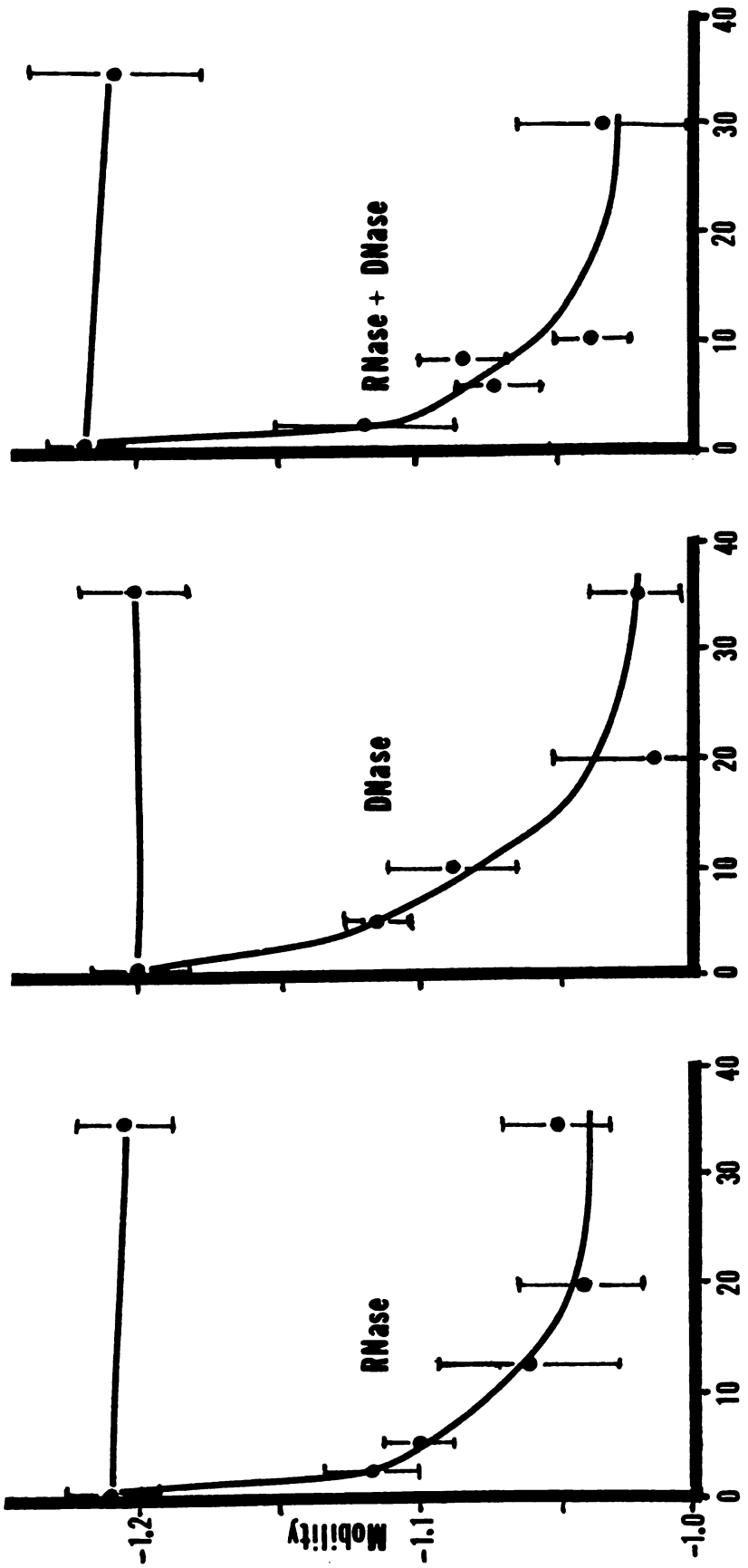


Figure 9. Kinetics of enzyme action upon mobility of the S-180-J. Top curve in each figure is the incubation control.

B. Further Experiments to Test for Nucleic Acids

1. pH profiles of the S-180 and S-180-J.

When the cells are measured in buffers of varying pH a profile is obtained which usually shows an isoelectric point where the cells have no net charge. This point is approximated by interpolating between negative and positive mobilities. In Figure 10,the pH profiles for control and platinum treated S-180 cells are shown. The iseelectric points are 3.4 and 3.9 respectively. This shift to higher pH in the platinum treated cells could be due to the loss of a group with a pK lower than 3.4. A rough calculation can be done to show the approximate pK of this group.

At pH 7.0, where the profiles are relatively flat, the platinum treated cells have about 20% lower mobility. Therefore, roughly 80% of the charge of the control has an average pK of 3.9 . (that of the treated cells). The remaining 20% of the charge must have a pK low enough such that when it is averaged with the remaining 80% a pK of 3.4 is obtained. Such a pK of the missing group computes out to be about 1.4 .

The calculation assumes that there is a group missing from the platinum treated cell surfaces rather than groups added, such as positively charge molecules. (This is a reasonable assumption - see Appendix II) This also assumes that the pK's of different groups average into one cellular pK in a linearly additive fashion.

A pK of 1-2 for a surface moiety does not allow too many possibilities. The two groups that come to mind are phosphates and sulfates. Sulfates are not commonly known in biological molecules

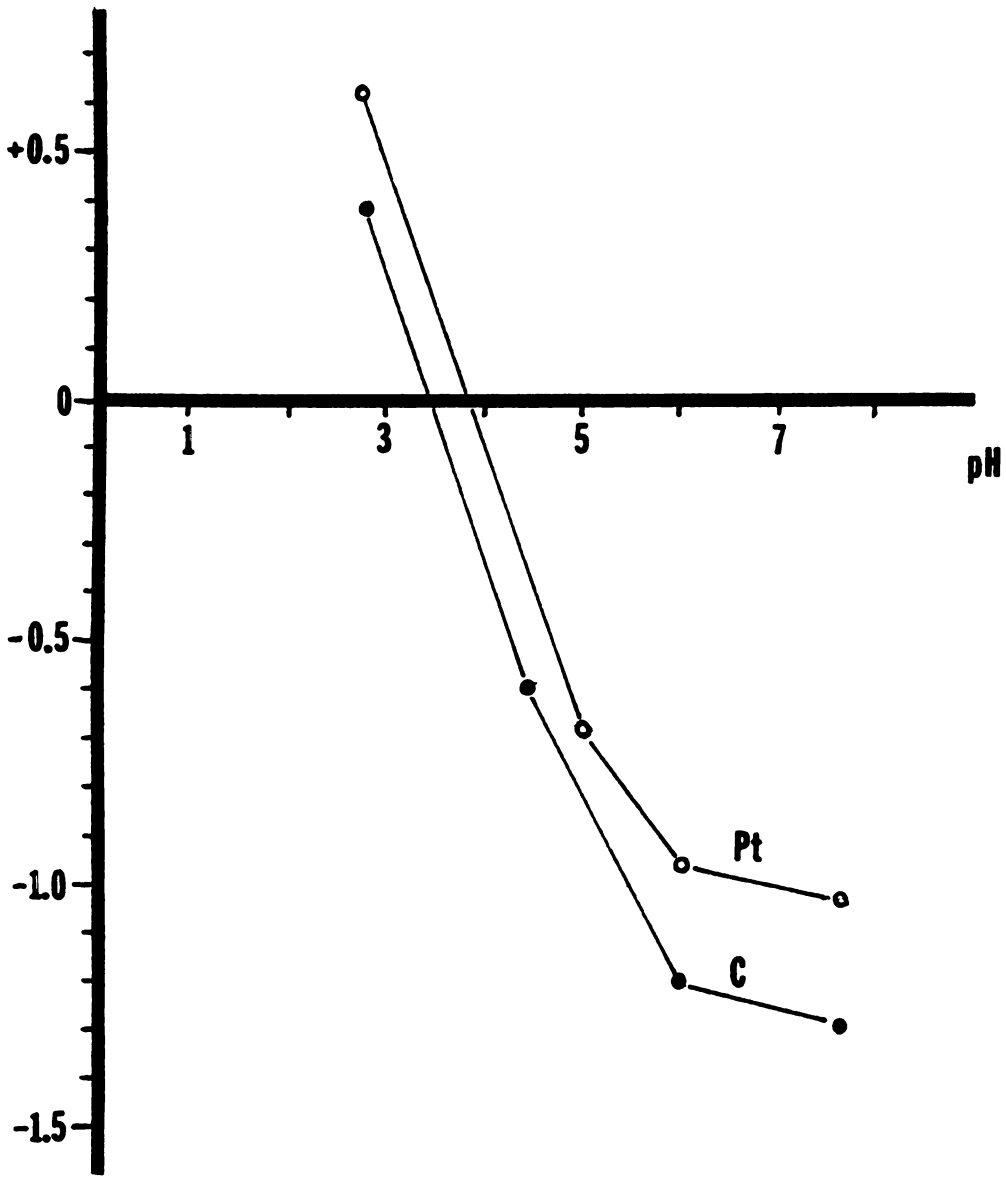


Figure 10. Plot of mobility versus pH of the measuring buffer for control(C) and platinum treated (Pt) S-180.

but phosphates are. Phosphates are located in membrane phospholipids and are part of the backbone of polynucleic acids. The pK of ribonucleic acid is around 1.0 and pK of the phospholipid phosphates is between 1.0 and 2.0.

The major phospholipids of mammalian cell membranes are phosphotidyl ethanolamine, phosphotidyl choline, and sphingomyelin. All of these are zwitterions with no net charge and with the positive group the most exterior group. The lack of an exposed negative charge would eliminate the possibility that the loss of one of one of these groups was responsible for the effect seen in Figure 10.

The possibility of surface ribonucleic acids existing on certain cell types was shown by other authors(see section IV of the Literature Review), so an examination of the S-180 using various enzymes including RNase was undertaken.

In Figure 11, the pH profile for the S-180-J tumor is give. The curve of solid circles is the untreated control, the open circles are the platinum treated cells, and the squares and triangles represent cells incubated with RNase or DNase respectively. Only the control is significantly different from the other five curves. This and other evidence which will be presented indicates a similarity between platinum treatment and incubation with nucleases.

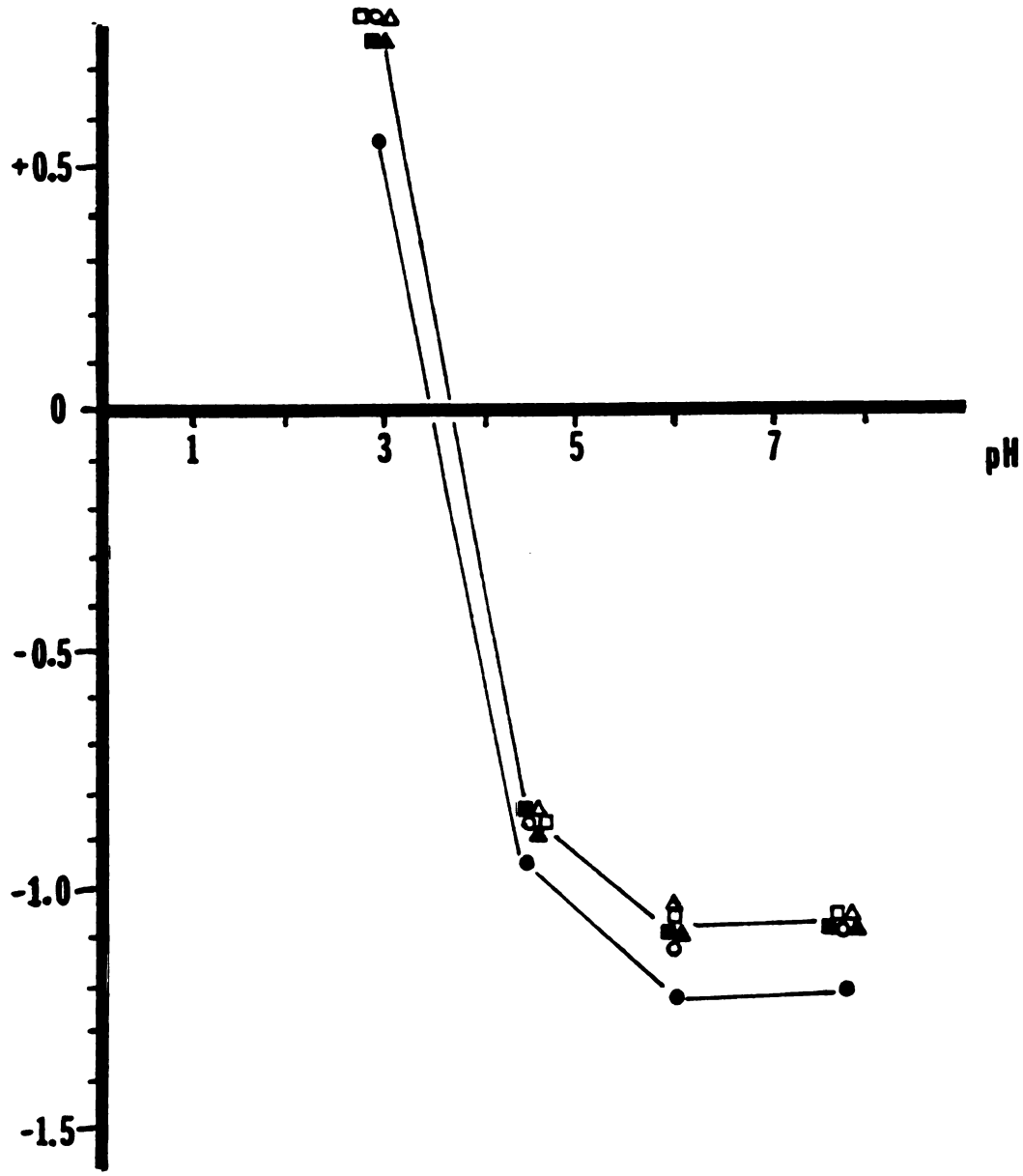


Figure 11. Plot of mobility versus pH for the S-180-J. Solid data points are control cells, hollow points are Pt treated. Squares are RNase incubated, triangles are DNase incubated and circles are incubation controls.

2. Ionic strength profiles

The Literature Review section on electrophoresis and Appendix I discuss the rationale for measuring mobilities over varying ionic strengths. Basically, it allows groups deeper within the membrane's three dimensional structure to exert their potential upon the zeta potential of the slip plane. In the Appendix, I discuss the ionic strenth profiles of the S-180 control and RNase treated cells and I conclude that it is reasonable to suggest that the RNase susceptible group is localized on the periphery of the cell surface.

In Figures 12 and 13 the ionic strength profiles of S-180 and S-180-J are given for control and platinum treatment with RNase, protease and neuraminidase incubation of S-180 and RNase, DNase, and neuraminidase incubation of S-180-J.

Most notably in both cell strains there is a smaller percent drop in mobility at lower ionic strengths and concommitantly an increase in slope of the curves for the nuclease treated cells. Both of these can be argued to indicate the peripheral nature of the nuclease susceptible group or its possible lability.

Again it is seen in this experiment that neuriminidase and protease lowers the control and the platinum treated cells to a common level. So these enzymes probably take the proposed nucleic acid off the control cells during their normal action on the membrane.

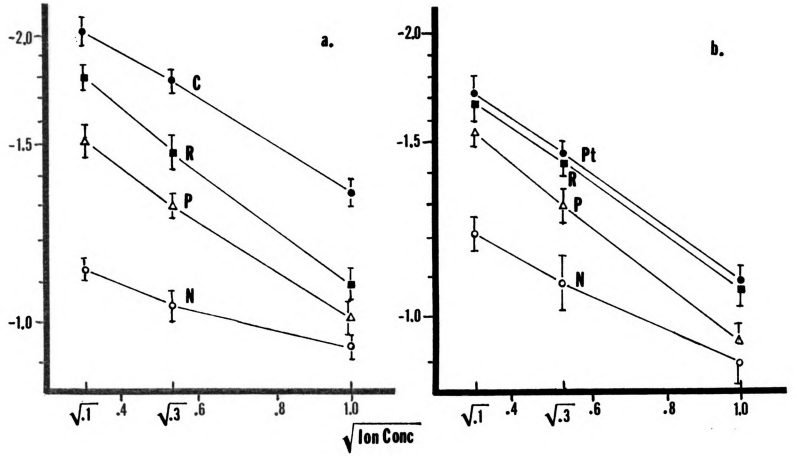


Figure 12. Log of electrophoretic mobility versus the square root of the ionic strength of the measuring media. a) Control cells, b) platinum treated tumor. R,P,N, represent RNase incubation, protease incubation, and neuraminidase incubation respectively.

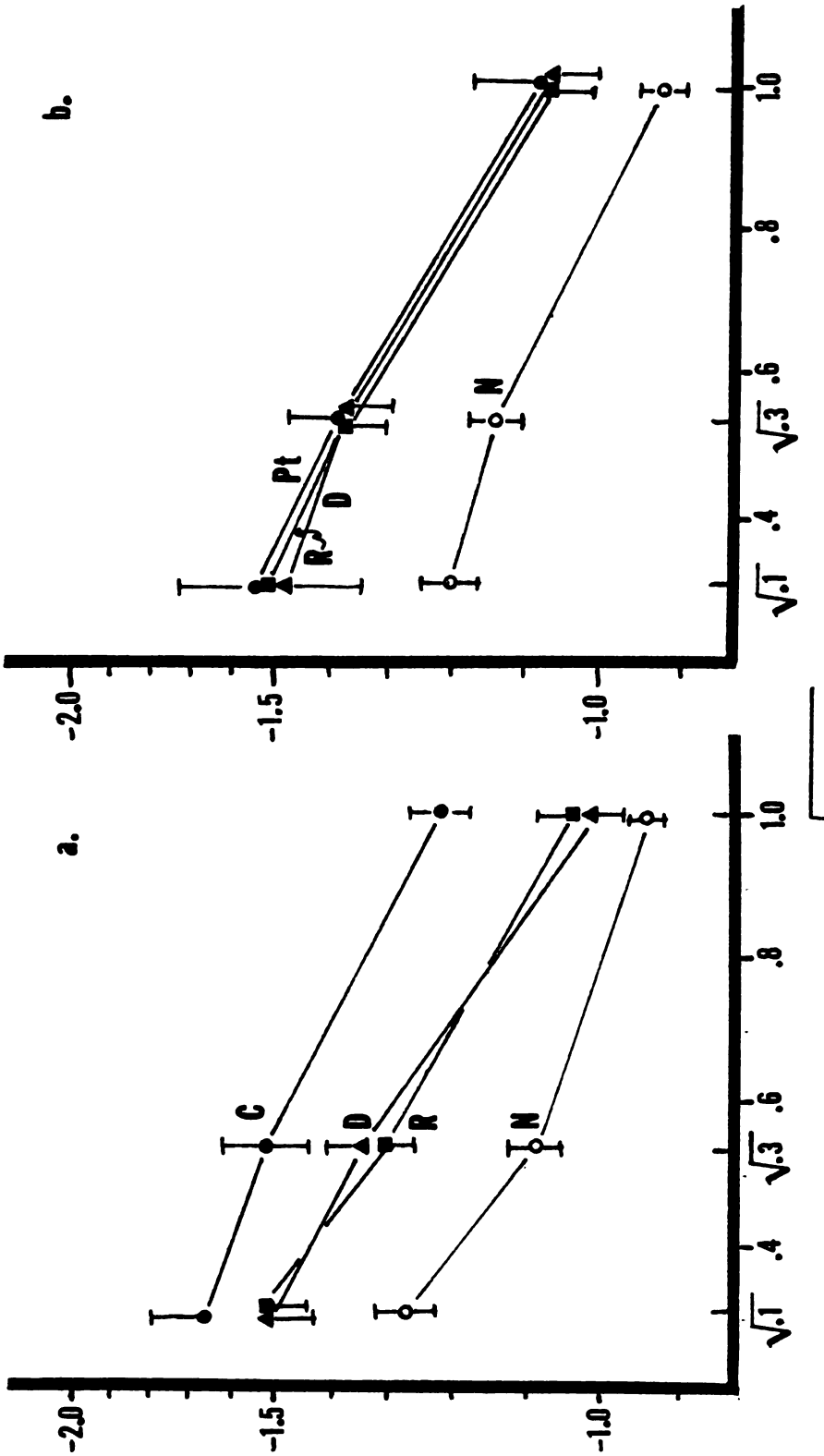


Figure 13. As in Figure 12 except this is S-180-J. R,D,N represent incubations with RNase, DNase, and neuraminidase respectively.

3. Incubation with Sepharose bead immobilized DNase and RNase

One of the arguments against using free DNase and RNase enzymes is the possibility that they can enter the cell. This could result in effects other than those directed upon the surface. In Table II the results of an incubation with Sepharose bound DNase and Agarose bound RNase are shown. After the incubation of the cells with the bound enzymes Ficoll is added to a final concentration of 15%. When the mixture of cells and beads are centrifuged in this solution, only the beads sediment. The cells in the supernatant are then washed free of the Ficoll. Mobilities are shown for Ficoll wash alone and plain Sepharose beads with Ficoll wash to show that there is no major effect due to the incubation proceedure.

The effect of bound enzyme action is evident. Following with regular unbound enzymes shows no further changes.

Table II., Incubation of cells with immobilized RNase and DNase.

	Control	RNase	DNase
S-180-J	-1.143 (.011)	-1.048(.028)*	-1.046 (.013)*
" +Ficoll wash	-1.14 (.01)	ND	ND
" +Sepharose beads and Ficoll	-1.17 (.03)	ND	ND
" +Seph-DNase	-1.07 (.02)ç	ND	-1.06 (.022)ç
" +Agar-RNase	-1.08 (.023)ç	-1.06 (.032)ç	ND

Notes: Those values with the ç are significantly different from the S-180-J control without any enzyme treatment.

Cells are incubated with the bound enzyme for 45 minutes with a Seph-DNase concentration of approx. 400 units/ml (see Wothington catalog) and a Agar-RNase concentration of approx. 6 Units/ml (see Miles Lab catalog)

ND - not determined

* - significance level of .01

4. 'Platinum-thymine-blue' attachment to the cell membrane.

'Platinum-pyrimidine-blues' bind to nucleic acids, probably due to their polycation properties and their pyrimidine base composition. 'platinum-thymine-blue' was incubated with S-180-J cells at a concentration of 10ug/ml for 30 minutes at 37°C. Table III shows the effect of 'platinum-blues' on the mobilities of control and platinum treated cells as well as these cells incubated first with RNase or DNase and then with 'platinum-blues'. Cells treated with cis-Pt(II) or any cells incubated with nucleases showed virtually no binding effects of 'platinum-thymine-blue'. This reinforces the evidence that there are surface nucleic acids whibh are removed by cis-Pt(II) as well as nucleases.

5. Incubation with anti-DNA antisera.

Dr. Edward Golub generously gave me rabbit anti-DNA antisera which was made by injecting calf thymus DNA plus bovine serum albumin into rabbits. The titre of this anti-sera is low due to the low antigenicity of nucleic acids.

When antibodies bind to specific cell surface components the mobility of the cells is reduced. This is probably due to the covering of some charges with a relatively non charged molecule. (See Appendix IV)

S-180-J control and platinum treated cells were incubated with anti-DNA antisera and the results are shown in Table IV . This basically shows that there is a small reduction in control cells at the dilutions shown but not in the platinum treated cells. Incubation with normal rabbit sera at the lowest dilution also had no effect.

Table III. The binding of 'platinum-thymine-blues' to the S-180-J.

	Enzyme Incubation		
	Control	RNase	DNase
S-180-J Control	-1.21 (.02)	-1.08 (.04)	-1.08 (.015)
'Pt-thymine-blue' added	-1.11 (.02)	-1.06 (.02)	-1.05 (.02)
S-180-J Cis-Pt(II) treated	-1.08 (.03)	-1.07 (.02)	-1.08 (.02)
'Pt-Thymine-blue added	-1.06 (.04)	-1.11 (.01)	-1.04 (.01)

Notes: Pt-thymine-blue was incubated with the cells for 10 minutes at 37°C at a concentration of 10ug/ml

Parenthetic values are one standard deviation

Table IV. The incubation of cells with anti-DNA antisera.

	Control	Dilutions of Anti-DNA antisera		
		1:10	1:5	1:2
S-180-J	-1.23 (.003)	-1.18 (.010)*	-1.17 (.003)*	-1.16 (.04)*
Pt treated S-180-J	-1.11 (.03)	-	-1.08 (.02)	-

Notes: When treated with a 1:2 dilution of normal rabbit sera the S-180-J's mobility was -1.22 (.014)

Anti-sera was incubated at 23°C for 30 minutes, then the cells were washed once by centrifugation.

* - significance level of .01

6. Incubation with the restriction enzyme Eco-Rl and Bam I.

In the S-180-J the sensitivity to RNase is accompanied by a sensitivity to DNase. The possibility of surface DNA is further examined here by use of the specific endonuclease restriction enzyme Eco-Rl. This enzyme recognizes the following double strand sequence:

G/AATT C
C TTAA/G

and cleaves the double stranded DNA at the slash marks. A demonstrable effect of this enzyme on the mobility of S-180-J cells would stranghten the implication of surface DNA and would suggest it is double stranded.

In Table V, Eco-Rl is shown to be active in control cells, but not in platinum treated cells and if EDTA is added to remove the activating ion, Mg^{++}, the effect disappears.

Also shown in Table V is the effect of Nuclease S-1 which is specific for single stranded RNA or DNA. At very large concentrations of the enzyme no effect is seen. This supports the results of Eco-Rl in indicating that the surface nucleic acid is double stranded.

In Table VI, the effect of restriction enzyme Bam I is shown. An effect is noticable at lower enzyme concentrations than seen in Eco-Rl. Both of these enzymes are used at extremely high concentrations, however, and probably do damage the cells' nuclei.

Table V. The effect of restriction endonuclease Eco-Rl and nuclease Sl on cell mobility.

		Eco-Rl Activity		
	Control	20,000units	10,000units	5,000units
S-180-J				
+10mM $MgSO_4$	-1.20 (.03)	-1.03 (.03)*	-1.08 (.01)*	-1.16 (.03)
+10mM EDTA	-1.23 (.02)	-1.21 (.03)	-1.20 (.01)	-1.21 (.04)
S-180-J Pt treated day 1				
+10mM $MgSO_4$	-1.15 (.02)	-1.15 (.02)	-1.18 (.02)	-1.19 (.01)
+10mM EDTA	-1.17 (.01)	-1.17 (.02)	-1.15 (.02)	-1.14 (.03)
S-180-J Pt treated day 2				
+10mM $MgSO_4$	-1.05 (.02)	-1.03 (.02)	-1.05 (.02)	-1.09 (.03)

Notes: the platinum treated cells on day 1 show a curious phenomena which I see occasionally. The overall mobility does not drop but the sensitivity to enzymes disappears. By day two the full effect of the platinum seems to have taken effect.

Parenthetic values are one standard deviation.

		Nuclease S1 Activity	
	Control	330,000units/ml	125,000 units/ml
S-180-J	-1.22 (.02)	-1.23 (.02)	-1.24 (.01)

Table VI. Incubation of cells with restriction endonuclease Bam I.

	Control	2000 units/ml	500 units/ml	100 units/ml
Untreated				
+Mg	-1.18(.02)	-1.0(.01)	-1.09(.01)	-1.18(.02)
+EDTA	-1.19(.03)	-1.12(.03)	-1.21(.02)	-1.23(.04)
Pt treated				
+Mg	-1.07(.02)	-1.09(.04)	-1.05(.02)	-1.05(.02)
+EDTA	-1.054(.04)	-1.09(.02)	-1.07(.01)	-1.06(.03)

7. A spectrophotometric study.

I examined the possibility of detecting nucleic acids as they were being removed from the cells in the presence of the nucleases. The procedure was to incubate the cells with enzymes and remove the supernatant, filter it, and examine the ultraviolet absorption spectra for the characteristic 260nm absorption peak for nucleic acids.

In Figure 14,curve (d) is the difference in the absorbance of the supernatants of cells incubated with RNase and without at $37^{o}C$ for 30 minutes. 2.2×10^{8} cells were incubated in each sample. The peak absorbance is 0.62 and $A_{260/280} = 1.88$, $A_{260/230} = 31.3$. Using a standard curve of RNA absorption versus concentration, the absorbance of 0.62 represents 1.7 mM nucleic acids in solution.

This particular incubation proceedure has a major problem: that RNase is only in one tube. If RNase releases material from inside the cell, that would cause an inflated reading. Therefore, the same cells were washed and both treated with RNase. In this case the cells previously treated with RNase should have no surface nucleic acids and the previous controls should. So the latter should release more than the former, while the backround should be about constant.

In Figure 14, curve(a),(b),(c) these results are shown. Curve (a) represents the previous controls, curve (b) those previously treated with RNase, and curve (c) the difference. In this case the difference is very small and one might think not even significant, but a quick calculation will show that it could be about one or two orders of magnitude too big.

From the electrophoretic mobility the number of surface charges influencing the zeta potential can be calculated.[72] For these cells that is about 10^8-10^9 charges. If we are generous and suggest that 10 times that many are undetected by electrophoresis then that implies 10^{10} charges per cell surface. RNase removes approximately 10% of these or 10^9 charges. In this study 2×10^8 cells were used which implies 2×10^{17} nucleic acid 'charges' should be released specifically from the membrane. That is only 0.001 mM, one hundred times less than observed spectrophotmetrically.

This experiment points out two things. The first is that in normal incubation many nucleic acid molecules are released either during regular metabolism (the absorbance for cells incubated without RNase was 0.29 at 260nm.) or from dead cells. The second is that

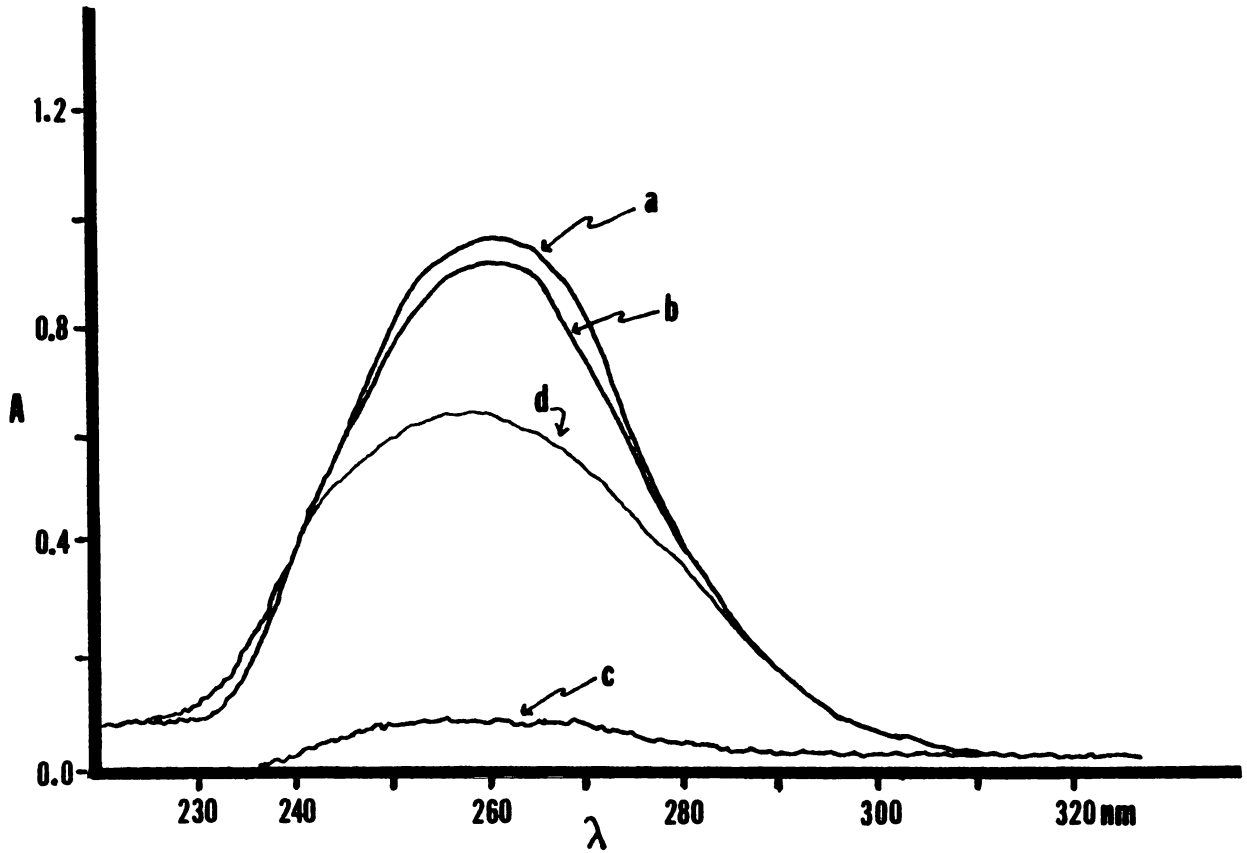


Figure 14. UV spectra of supernatant from cells (S-180-J incubated under various conditions. See text for details.

measuring released nucleic acids during membrane enzyme incubation is not sensitive enough to detect the changes.

If the 0.11 mM nucleic acid levels detected here are real it would suggest very large complexes of nucleic acids attached to the membrane and probably sticking out into the extracellular space. These large complexes would have many interior nucleic acids that would not contribute to the surface charge and would go undetected.

C. Binding and Origin

1. Cellular debris

One of the possible origins for surface nucleic acids is debris from broken cells adhering to intact cells. The normal handling of cells during the experiment argues against this. Cell breakage most likely occurs during centrifugation and when the cells are stored on ice. Yet after enzyme treatment and after they have been centrifuged and vortexed, the mobility remains depressed. There seems to be no effect due to the ever present debris.

To strengthen this argument, however, cells were exposed to homogenized S-180-J tumor cells. A hand homogenizer was used to break up about 10^7 cells. Normal cells and enzyme treated cells were incubated with this debris for 45 minutes at ice temperatures (the temperature that the cells were normally stored at in typical experiments). This temperature would also facilitate electrostatic binding. They were then washed normally and their mobilites measured. In Table VII, the results are shown. The debris did not bind to enzyme treated cells and in no case did it raise the mobility, which would result if nucleic acids bound to the surface.

Table VII. The effect of cell debris incubation on S-180-J mobility.

	control	RNase	DNase
S-180-J	-1.23 (.01)	-1.10 (.02)	-1.07 (.04)
" + incubation with cell debris	↓ -1.14 (.02)	↓ -1.08 (.03)	↓ -1.06 (.03)

Note: The arrows indicate that after the cells were treated with RNase and DNase they were then exposed tc the cell debris and remeasured.

2. Lability of nucleic acids with trypsin, neuraminidase and low ionic strength.

In Table VIII a,b the S-180-J cells are incubated with neuraminidase and trypsin then each are followed with incubations with RNase or DNase. The action of either trypsin or neuraminidase lowers the mobility to a specifed level for each enzyme, but incubation with either nuclease does not change this. This seems to indicate that nuclease susceptible groups are loosely bound and depend upon sialic acid residues and/or protein molecules to remain attached to the cell.

If the ionic strength of the cell bathing solution is lowered the electrostatic forces between surface molecules change. Attractive forces increase and repelling forces increase. Of course, in low ionic strength buffers transmembrane ionic fluxes will be drastically altered too. However, since the membrane is negatively charged and DNA and RNA are negatively charged perhaps lowered ionic strength can cause the nucleic acids to come off.

Table VIII. Multiple enzyme incubations.

Table VIII a

5 min. incubation	Hanks -1.24 (.02)	Hanks + Trypsin -1.07 (.03)	
	↓	↙	↘
Followed by 20 min. incubation	Hanks -1.24 (.01)	Hanks+RNase -1.06 (.01)	Hanks+DNase -1.08 (.03)

Table VIII b

15 min incubation	Hanks -1.27 (.02)	Hanks + Neuraminidase -0.92 (.02)	
	↓	↙	↘
Followed by 15 min incubation	Hanks -1.27 (.02)	Hanks+RNase -0.91 (.02)	Hanks+DNase -0.93 (.01)

Notes: Trypsin concentration used was 0.005mg/ml

Parenthetic values are standard deviations.

Neuraminidase concentration was 0.5mg/ml

In Table IX a,b two experiments are shown. Cells were incubated at 3 different temperatures in part (a), and it appears that 23°C is the most detrimental to the cells' mobility both for the 100% saline control and the 1% saline-99% isotonic mannitol test. At all temperatures the lower ionic strengths lowered mobility, but the least effected were cells at 37°C. This is possibly due to cell metabolism reducing membrane loses. In part (b), a 23°C experiment is shown. Here it is shown that the effect of low ionic strength saline on the cells occurs over time. Following these incubations with RNase results in bringing them all to about one common level. So the most labile group to low ionic strength is the nucleic acid moiety.

The fact that trypsin, neuraminidase, and low ionic strength can remove this nucleic acid group leads me to suggest that this group must be weakly bound to the membrane. One explanation for this could be that it is in a process of flux through the membrane and it only exists on the surface for a short time.

3. In vitro studies

One of the early questions involving the effect of platinum treatment upon the cells was whether the platinum acted directly upon the cells to cause the mobility drop or through some host response. In order to test this, in vitro experiments were performed where the cells were removed from an animal and incubated with platinum or other compounds in Hanks buffer and then placed into

Table IX. The effect of ionic strength and temperature on the mobility of the S-180-J.

Table IX a

	Temperature		
30 min. incubation	2°C	23°C	37°C
S-180-J saline	-1.21 (.01)	-1.16 (.01)	-1.21 (.04)
" in 1% saline 99% mannitol	-1.08 (.02)	-1.03 (.02)	-1.13 (.02)

Table IX b

23° incubation	Control	RNase
5 minutes		
S-180-J in saline	-1.20 (.02)	-1.04 (.04)
" in 1% sal 99% man	-1.13 (.02)	-1.03 (.01)
15 minutes		
S-180-J saline	-1.18 (.03)	-1.05 (.01)
" 1%sal/99%man	-1.09 (.02)	-1.02 (.03)
30 minutes		
S-180-J saline	-1.15 (.01)	-1.0 (.03)
" 1%sal/99%man	-1.04 (.02)	-1.01 (.01)

tissue culture media for up to 22 hours. The culture medium was changed at regular intervals of 1 to 2 hours. Samples were removed using sterile technique and the mobility was measured.

The curves in Figure 15 show the way the mobility changes with time. The control cells show a continual decline and a characteristic dip between 4 and 8 hours. The effect of cis-Pt(II) is to increase the rate of decline to an even lower value with only a slight dip. The RNase treatment of controls (open circles) is a curve similar to that of cis-Pt(II) beginning at about 4 hours. Therefore, the effect of platinum in vitro is to cause the loss of this surface nucleic acid.

An RNA inhibitor, Actinomycin-D is also shown (dotted line) in this figure. It too causes the reduction to a level similar to RNase and platinum. Its effect is slower, but this may be dose dependent.

Other inhibitors were also tried to further substantiate the nucleic acid hypothesis and its possible origin inside the cell. In Figure 16 c the effects of mercaptopurine, methotrexate are shown. They produce an effect similar to platinum by lowering the mobility to that produced by RNase. Both of these drugs are DNA synthesis inhibitors and are used in antitumor therapy. Puromycin, a protein synthesis inhibitor, is shown in Figure 16 d. It lowers the mobility much further than the other drugs but in the process also removes the RNase susceptible group. At the five hour mark the bar under the triangle indicates the mobility of that five hour sample after RNase treatment-there is no change. It was shown, before, that the loss of protein causes the loss of the nuclease susceptible groups.

Figure 15. Plot of mobility versus time in in vitro incubations. Cells were removed from animals pre-incubated with cisplatin, (Pt), or with Actinomycin-D, (Act-D), for forty minutes before time zero. Time shown is time in normal tissue culture media. R represents RNase incubation of samples taken during the course of the in vitro incubations.

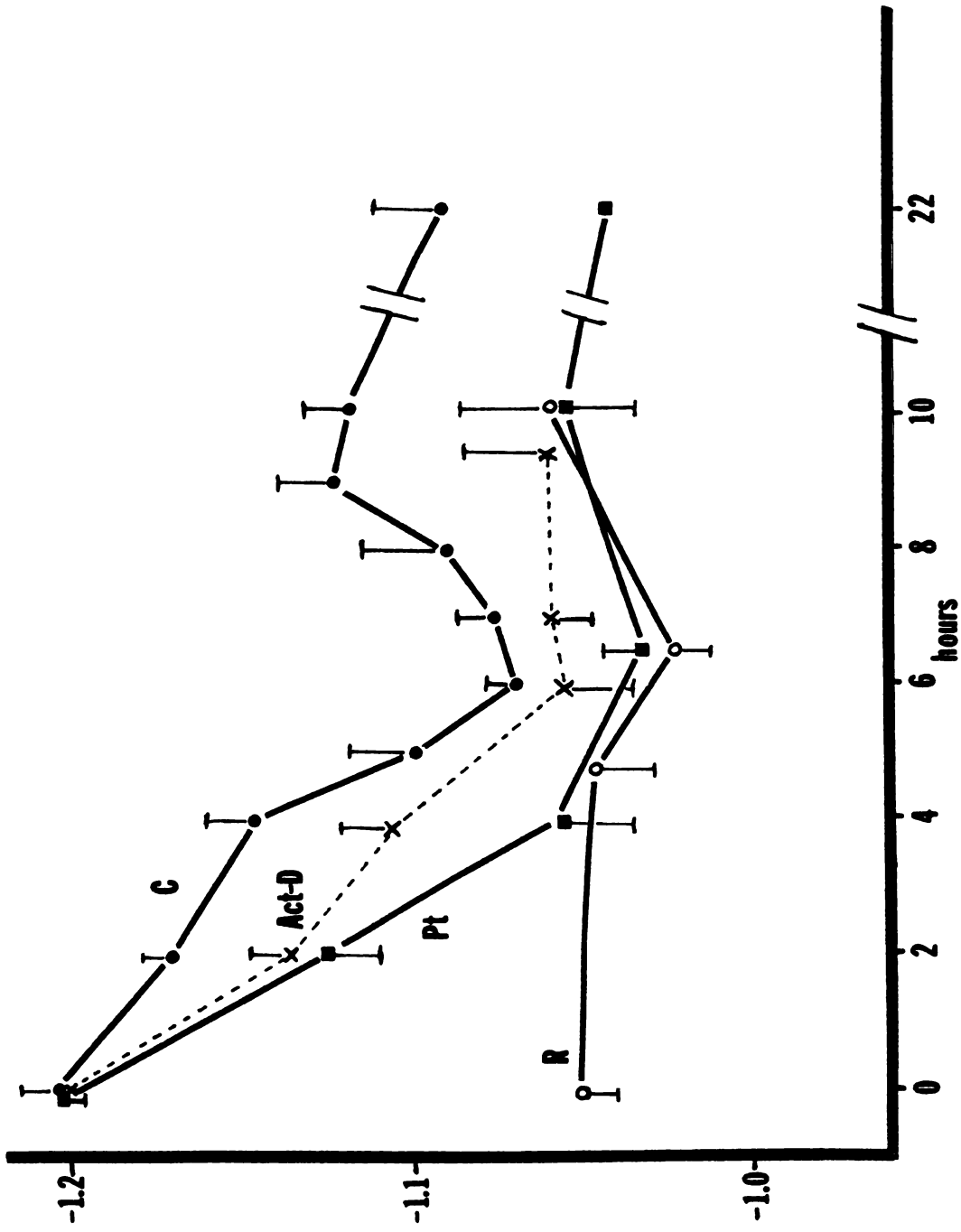


Figure 15.

Figure 16. In vitro experiments as described in Figure 15. The control curve is repeated in each figure as reference. a) Normal S-180-J were preincubated with RNase and then allowed to grow in vitro. b) similar to (a) except cells were preincubated with DNase. c) Squares; cells were preincubated with mercaptopurine 0.5mg/ml. Crosses: cells were incubated with methotrexate 0.5mg/ml. d) The RNase curve of Figure 15 is repeated. Triangles; cells were preincubated with 1mg/ml puromycin.

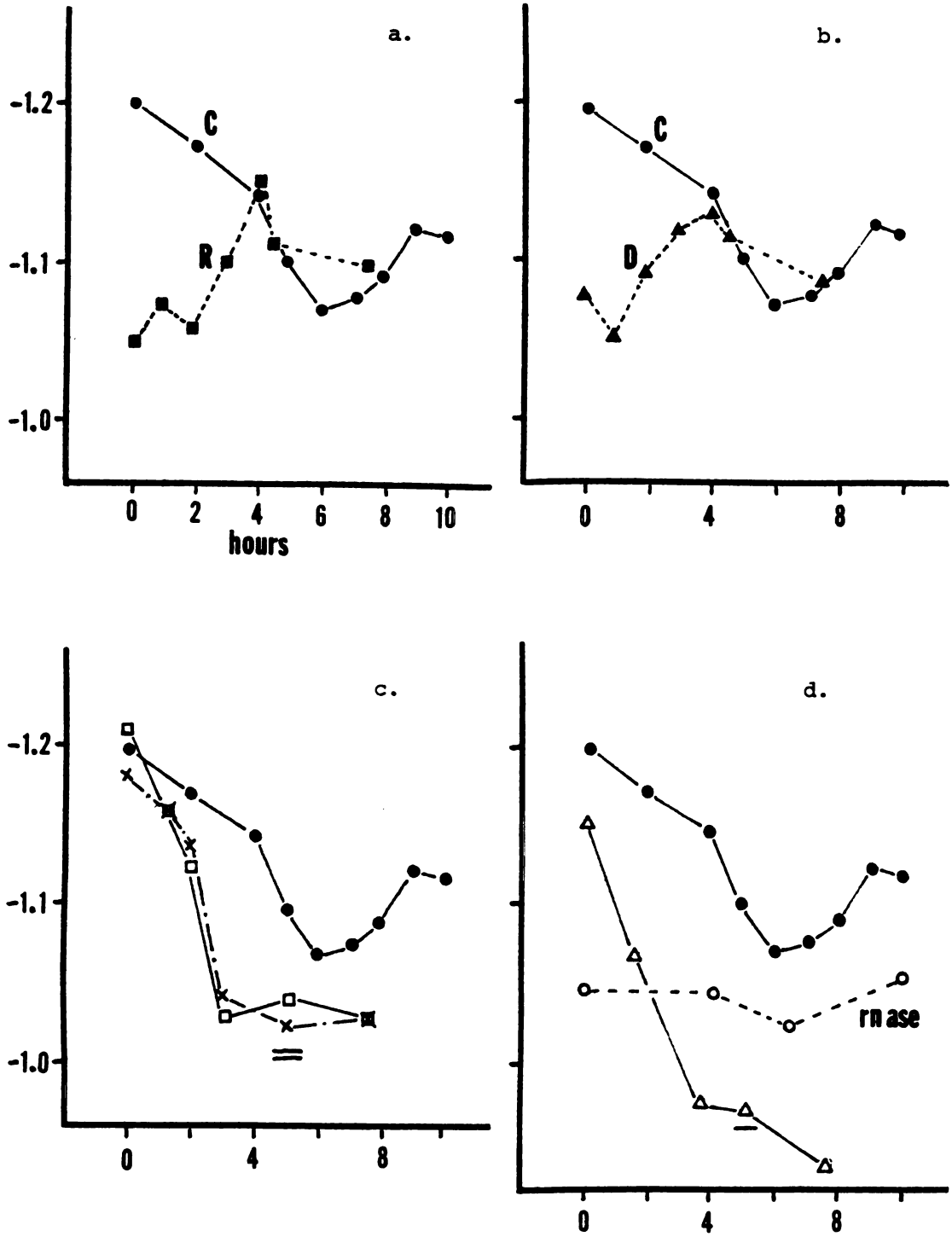


Figure 16.

In this case the main point is that puromycin causes more membrane changes than the loss of nucleic acids.

In general, the action of these drugs supports a nucleic acid origin for these nuclease susceptible groups and a link to cell metabolism.

This is further supported by Figures 16 a,b. In these experiments cells were first treated with RNase or DNase and then placed in tissue culture media. Within 4hours the mobility increases and the curves join the curve of the control cells. It appears that this group is renewed on the surface and the cell may be continually producing this material and exuding it into the media.

D. Other Experiments

The results shown up to this point allow me to suggest that there are nucleic acids on the cell surface, that they are probably loosely bound, and their presence is linked to the metabolism of the cell. There is no evidence which could be used to indicate a function or purpose to these nucleic acids. Whereas my goal was not to determine their function I did attempt a few 'quick and dirty' experiments which I will include here for completeness.

1. Binding of antitumor sera to 'interim S-180'.

Mice were given normal injections of tumor and platinum and 7 days later they were bled and their serum expressed and pooled. Two types of sera were obtained,(T-sera) from animals with tumor only and (Pt-sera) from animal with platinum treated tumor. Each sera was then incubated with control cells, platinum treated cells and the RNase incubation of each of these.

In Table X results are shown over varying concentrations of sera for the control and control + RNase, and at 1 to 5 dilutions for the platinum treated cells. The results are shown as differences from those cells incubated without sera.

Basically, the results show nothing. The only interesting thing is the difference between the RNase incubated controls treated with T-sera versus Pt-sera. It is interesting because of the hypothesis that the nucleic acids may exist as antigen masks. If this were true there should be antibody in platinum treated animals to something under the RNA since these cells contain no surface RNA after platinum therapy. There is the slightest hint of extra binding

Table X. Incubation of the S-180-J with sera from tumor bearing animals.

	Serum Dilutions		
	1:5	1:10	1:20
S-180-J			
+ T-sera	0.056 (.01)	0.06 (.01)	0.04 (.01)
+Pt-sera	0.046 (.008)	0.04 (.01)	0.03 (.01)
S-180-J / RNase incubated			
+ T-sera	0.026 (.014)	0.00 (.04)	0.00 (.01)
+ Pt sera	0.056 (.01)	0.055 (.005)	0.025 (.005)
S-180-J Pt treated			
+ T-sera	0.075 (.01)		
+ Pt-sera	0.085 (.02)		
S-180-J Pt treated /RNase incubated			
+T-sera	0.08 (.02)		
+Pt-sera	0.09 (.03)		

to the RNase treated cells by something in the Pt-sera. However, why is there a similar amount of binding of T-sera and Pt-sera to control cells? From a different point of view, in the comparison of T-sera binding to control cells and RNase + control cells there is less binding of T-sera to RNase incubated cells. This would imply that there is antibody in T-sera to this RNase susceptible group. The data is too weak to dwell on it any longer.

2.Animals injected with nuclease treated cells.

In persuing the hypothesis of antigen masking animals were injected with S-180-J tumor cells treated with RNase and DNase. The animals were then followed until day of death and any increased life span over controls was noted. The results of three experiments are shown in Table XIa,b,c. In Table XIa, S-180-J cells were preincubated with RNase and DNAse at 1mg/ml for one hour at 37°C. Their viability, as measured by trypan blue,was 80% for control, 76% for DNase, and 68% for RN ase. Cell concentrations were then adjusted to 10^6 viable cells per sample and unjected into 10 mice each. Average day of death is shown in the table and suggest that the RNase treated cells were more susceptible to a host response.

The low viabilities suggested that the incubations were too harsh on the cells so another test was run. This time the cells were incubated with the standard enzyme concentrations; 0.2 mg/ml RNase, 0.1 mg/ml DNase, for 20 minutes at 37°C. According to the kinetics of Figure 13 this is enough time to just remove the surface groups. Viabilities were all greater than 90% and as is shown in Table XIb,there is no significant increase in day of death over the control group.

Table XI. Day of death of mice injected with S-180-J incubated in RNase and DNase

Table XI a

	Average Day of death excluding survivors	Survivors
S-180-J incubated 1 hr.		
Hanks	19.6 (1.84)	1
" +Rnase 1mg/ml	15.3 (3.9)	4
" +DNase 1mg/ml	20.4 (4.43)	0

Table XI b

S-180-J incubated 20 min.		
Hanks	13.8 (2.35)	0
" +Rnase 0.2mg/ml	11.25 (3.1)	0
" +DNase 0.1mg/ml	12.4 (2.9)	0

Table XI c

S-180-J incubated 35 min.		
Hanks	14.0 (2.3)	0
" +Sepharose Dnase	16.3 (3.4) (17%ILS)	0
Hanks/Ficoll wash	12.8 (1.92) (21%ILS)	0
" + Seph-DNase/ Ficoll wash	15.5 (2.8)	0

Notes: Parenthetic values are one standard deviation
ILS - increased life span

A third experiment was run using Sepharose bound DNase. Cells were incubated for 35 minutes in DNase-Sepharose and injected into the animals. In Table XIc the results are shown. In case I the Sepharose beads with the DNase were not washed out but injected with the cells. In case II, 15% Ficoll was used to remove the beads as was described earlier. In both cases a slight increased life span (ILS) is noted but its significance is questionable.

These results would seem to indicate that if there is an antigen masking phenomena it can not be determined by this type of experiment. There is even question if the mice can mount an immune response in 7-12 days and if they could it does not preclude the cells from making more nucleic acids to mask their antigens.

3. Tumor supernatant and antibody production.

Another possible function of the surface nucleic acids is that of immune suppression. As others have suggested nucleic acids may be exuded by the cell into the extracellular space. This could transmit a suppressive message to the lymphocyte population .

To test this, sheep red blood cells were injected into mice intraperitoneally. One set of animals received RBC's alone, another RBC's plus the supernatant of S-180-J cells incubated in vitro, and a third RBC's plus the supernatant treated with RNase. These animals were older and therfore immunocompetent. After two sets of injections 7 days apart the animals were bled and the sera expressed. The mobility of sheep RBC's was then determined after incubation with different dilutions of these sera.

In Figure 17 the results are shown. After each incubation

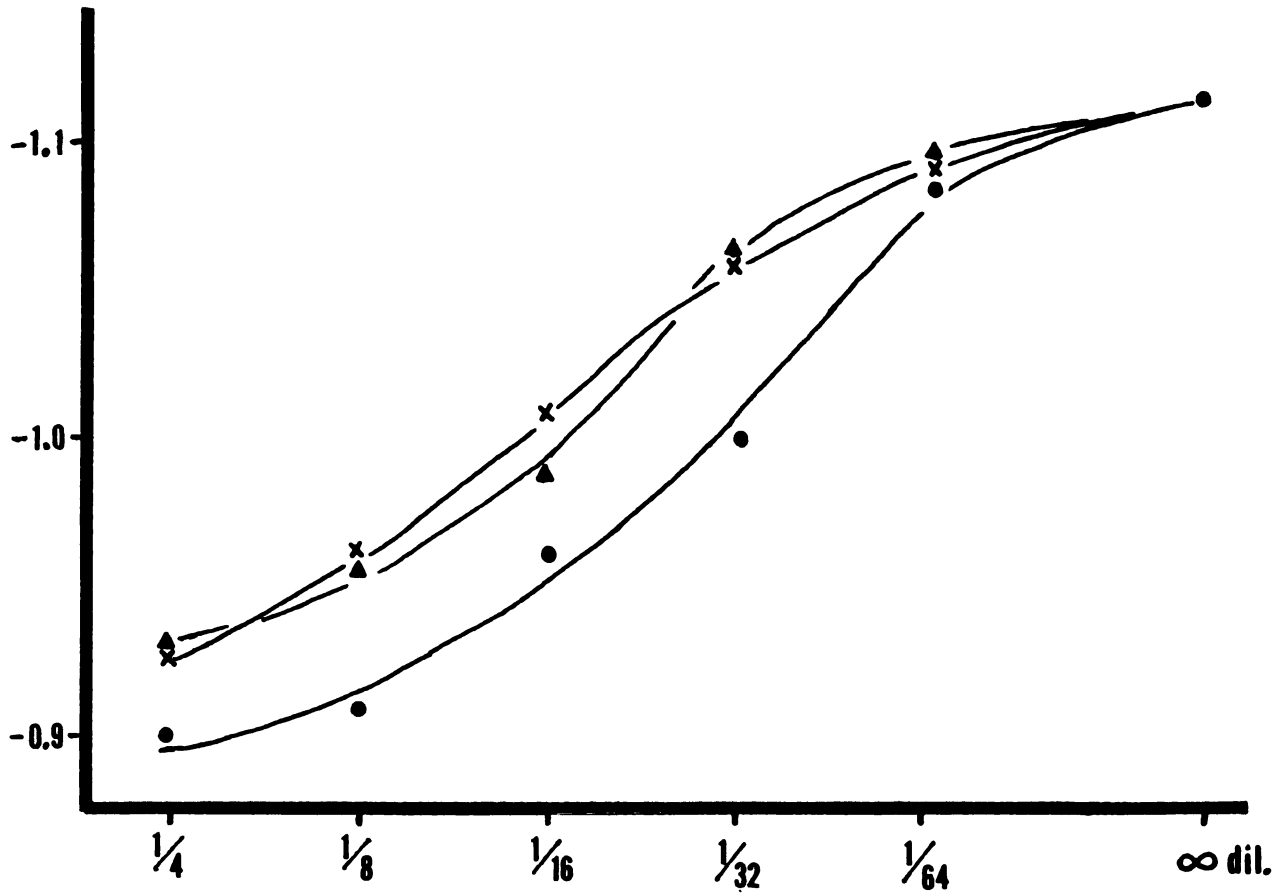


Figure 17. Mobility of sheep red blood cells as a function of sera taken from mice injected with (C) sheep RBC's only, (S) sheep RBC's + S-180-J supernatant, and (R) sheep RBC's + tumor cupernatant incubated with RNase.

another incubation occured using anti-mouse IgG antibody. This would bind to any bound antibody on the sheep RBC's and increase the reduction in the mobility.

The curve labeled C shows the amount of antibody against the sheep RBC's. When the blood cells were injected with supernatant the results of antibody production are on curve S. When the supernatant was treated with RNase, curve S-R, there is no change from curve S. This does show some reduction in antibody titre, about 1/2, due to the presence of the tumor supernatant, but RNase does not effect it.

This does not rule out a nucleic acid containing suppressor substance, however, because a nucleoprotein complex could easily be insensitive to RNase as shown by Stroun et al [97].

E. Other Cell Types

Up to this point all of the experiments were performed with one of the S-180 ascitic tumor strains. In this section I will describe some work with other tumors and some normal cells.

1. S-180 and S-180-J solid tumors.

One very interesting question is raised by using the ascitic form of the S-180: Does the surface nucleic acid require single cell suspension?

The S-180 tumor also grows very well in the solid form if it is injected subcutaneously. Tumors were, therfore, grown in the subaxillary region of the ICR mice. From one to two weeks after injection they were removed as described in materials and methods using a sodium citrate - EDTA - saline bathing solution and separated into cell suspensions and then treated with enzymes.

In Table XI the results are shown. Both the 'interim S-180' and the S-180-J show the same sensitivities as their respective ascitic forms, ie. the'interim S-180' is sensitive to RNase while the S-180-J is sensitive to both RNase and DNase. Also included in this table is an S-180-J ascitic tumor processed with the sodium citrate-EDTA-saline buffer in a similar manner as the solid to assure the buffer did not effect the cells.

2. P388 and P815 ascitic tumors.

The P388 is a lymphocytic leukemia and the P815 is a mastocytoma both of which are syngeneic to the DBA/2 mouse strain. They were grown intraperitoneally in the DBA/2 mice. Both of these were tested for RNase and DNase sensitivities and the results are shown in Table XIII

Table XII. The effect of RNase and DNase on the mobility of 'interim S-180' and S-180-j in the solid form.

	Control	RNase	DNase
Solid 'interim S-180'	-1.00 (.02)	-0.95 (.01)ç	-0.99 (.005)
Solid S-180-J	-1.16 (.013)	-1.03 (.02)*	-1.02 (.003)*
S-180-J ascitic Na citrate-EDTA-sal.	-1.20 (.005)	-1.08 (.05)*	-1.03 (.02)*

Note: ç denotes significance at the 0.05 level.in all tables on this page.

Table XIII. Nuclease effect on the mobility of various cell types.

	Control	RNase	DNase
P388	-1.048 (.01)	-1.045 (.010)	-0.978 (.012)*
P815	-1.113 (.006)	-1.05 (.008)*	-1.032 (.007)*
Fetal cells	-1.141 (.008)	-1.116 (.004)	-1.124 (.010)
Liver cells	-1.09 (.02)	-1.09 (.02)	-1.06 (.04)
AKR preleukemic			
spleen	-1.10 (.02)	-1.07 (.01)	-1.08 (.04)
Thymus	-1.11 (.03)	-1.11 (.02)	-1.08 (.05)
AKR leukemic			
Spleen	-1.07 (.04)	-1.05 (.04)	-1.03 (.03)
Thymus	-1.05 (.03)	-1.05 (.06)	-0.98 (.04)ç
V-79 tissue culture	-1.25 (.05)	-1.24 (.05)	-1.27 (.03)

	C - RNase	C - DNase
L1210	-.0036 (.006)	0.037 (.006)*
L1210 virus infected	0.0233 (.005)ç	0.051 (.010)ç
L1210 resistant to Pt	-0.021 (.002)*	-0.024 (.006)ç

Both were sensitive to DNase, but only the P815 was sensitive to RNase.

3. Liver and fetal cells.

These cells, like the solid tumors were removed and broken into single cell suspensions as described earlier. The fetal cells were from pregnant mice 10-14 days into gestation. The embryos were removed from the placenta and were dissociated into individual cells. Some of the livers were from these same pregnant mice and were noticeably larger than normal livers. The results are in Table XII. There was a lack of effect of RNase and DNase on these "normal' cells.

4. AKR spontaneous leukemia

AKR mice were obtained from Dr. Golub, Purdue University. After a certain age these mice spontaneously form a lymphoid leukemia. Both preleukemic and leukemic animals were examined. Their spleens and thymuses were removed, dissociated into individual cells, and measured to determine their mobility. Again, DNase and RNase were used to determine if there was any sensitivity. This was particularly interesting because Dr. Golub had shown that a population of suppressor cells in the leukemic mice had DNA on their surface.

In Table XIII the results support his finding by showing a DNase sensitivity in the leukemic animals.

5. Tissue culture cells V79 and L1210.

Two tissue culture cells were examined, the non-tumorigenic V79 and the leukemic L1210. Three different types of L1210 were examined, two of them are grown in 0.3 ug/ml and 1.0 ug/ml of cisplatin

respectively. The 0.3 ug/ml L1210 is a strain resistant to cisplatin. The 1.0ug/ml strain is a resistant strain infected with a virus.

The results, as given in Table XIII, show differences for these three strains. The normal L1210 has a DNase sensitivity, the virus infected cells have a slight RNase sensitivity as well as DNase and the resistant strain shows a significant increase in mobility when incubated with these enzymes. This increase is a result which I cannot interpret.

The V79 cells show no sensitivity to either enzyme. (See Table XIII)

6. Summary of cell types

In Table XIV all the cells examined are listed. A plus sign is placed by a cell type if it responded to the enzyme and the difference using the t-test was significant at the 0.01 level unless otherwise noted.

Table XIV. Summary of nuclease effects on several cell types.

	RNase	DNase
S-180	+	-
'interim S-180'	+	-
'interim S-180' solid	+	-
S-180-J	+	+
S-180-J solid	+	+
P815 ascitic	+	+
P388 ascitic	-	+
AKR leukemic		
spleen	-	-
thymus	-	+ç
AKR preleukemic		
spleen	-	-
thymus	-	-
Fetal cells	-	-
Liver	-	-
V-79 tissue culture	-	-
L1210 tissue culture	-	+
L1210 virus infected	+ç	+ç
L1210 Pt resistant	+	+ç

Notes: ç indicates significant at the .05 level.
+ indicates a significant effect of the enzymes at a level of .01
- indicates no significant effect of the enzyme treatment.

F. Summary of Conclusions

1.Treatment of tumor bearing animals with cisplatin results in a sustained lowering of the tumor cell electrophoretic mobility.

2. The pH profiles of control and cisplatin treated cells suggests the loss of a charged group with a low pK value from the platinum treated cells.

3. Incubating the S-180 with RNase or the S-180-J with RNase or DNase lowers the control mobility to the level of platinum treated cells but does not effect the mobility of the platinum treated cells.

4. RNase or DNase separately or together lower the mobility of the S-180-J to the same level. Therefore they probably act upon the same site. Enzyme kinetics support this by showing the additivity of reaction rates.

5. The enzyme kinetics also represent a true enzymatic reaction and not simple adsorption.

6. Preincubation of cells with either neuraminidase or trypsin or low ionic strength removes the nuclease susceptible groups.

7. From ionic strength profiles the nuclease susceptible groups appear to be located at the very periphery of the cells. The pre-incubation with neuraminidase and trypsin supports this.

8. Incubation with cell debris, however, does not result in increased mobility after nuclease treatment.

9.The ability of 'platinum-thymine-blue' to bind to control cells but not to cisplatin or enzyme treated cells further supports the hypothesis of cell surface nucleic acids.

10. anti-DNA antisera binding to the S-180-J again implies cell surface nucleic acids.

11. The restriction enzyme,Eco-R1,acts in a similar way as DNase, therfore offering some further evidence for surface nucleic acids.

12. Sepharose bound DNase and RNase limits their action to the cell surface thereby precluding secondary membrane charge changes due to the enzyme acting inside the cells.

13. Measuring the nucleic acids released using spectrophotometric methods could be 100 times too insensitive.

14. In vitro studies indicate that cisplatin , actinomycin-D, methotrexate, and mercaptopurine are effective in mimicking nuclease incubation. The effect is not immediate but occurs about 6 hours after exposure to the drugs. The protein inhibitor puromycin removes the nuclease sensitive groups but also has other mobility lowering effects.

15. After removing the surface nucleic acids with enzymes and allowing the cells to grow in vitro the surface charge returns to the control level in about 4 hours.

16. Similarly, in platinum treated tumors in vivo that begin to grow again the RNase and DNase sensitivity returns. So this sensitivity is probably linked to cell metabolism.

17. No conclusive evidence is presented regarding the role of cell surface nucleic acids.

18. Other tumors also show nucleic acids on their surface, with DNA more commonly found on cells of lymphatic origin. The non tumorigenic cells tested showed no sensitivity to nucleases.

Discussion

The work presented here adds new evidence to the slowly growing story of cell surface nucleic acids. An old tumor line, the S-180, has provided an excellent model for this study because of its relatively large amounts of apparent RNA-DNA on the surface. Perhaps this tumor will make a good model cell for examining the origins,and for physically characterizing these surface groups.

I. Proof of Surface Nucleic acids

There is no absolute proof for cell surface RNA or DNA presented here. There is a lot of evidence suggesting such molecules, however. The action of RNase and DNase is the most thoroughly examined and probably the best evidence and it is strengthened by the Sepharose-DNase and Agarose-RNase and the positive action of Eco-R1 and Bam I endonuclease restriction enzymes.

The specific binding of the 'platinum-blues' to only the cells expected to have nucleic acids and the similar binding of anti-DNA antisera to untreated cells lend further indirect evidence of the existence of membrane nucleic acids.

In the <u>in vitro</u> studies, the RNA or DNA synthesis inhibitors specifically cause the loss of surface charge equal to that which could be released by incubation with RNase. Puromycin and cycloheximide (not shown) cause lowered mobility which results in the loss of RNase susceptible groups but they also cause further reductions probably due to the loss of membrane proteins. The exact mimicking of RNase by the nucleic acid synthesis inhibitors is further evidence of surface nucleic acids.

II. Overlapping effects of RNase and DNase.

The interlocking effects of RNase and DNase raise interesting questions. Examination of these enzyme has shown no protease contamination which rules out that form of common denominator. The responses of different cell types, some DNase sensitive only, some RNase sensitive only, also tend to contradict a common contaminant as the cause of the overlapping actions of DNase and RNase.

If they each are acting only on DNA and RNA respectively then the surface of the cell must harbor a nucleic acid complex. Furthermore, this complex must be bound to the membrane in such a way that partial degradation of either RNA or DNA would release it. An electrostatic binding would fit this requirement. Once part of the negative charge is removed by RNase or DNase the remaining charges could be insufficient to keep the complex bound.

III. Lability of Surface Nucleic Acids and Their Turnover

The ease with which this nucleic acid group comes off with neuraminidase or trypsin or low ionic strength is another interesting phenomena. I suspect, therefore, that this nucleic acid is on the very periphery of the cell, weakly bound to the most exterior membrane components. Its conformation is such, though, that it allows neuraminidase and protease to act on their binding sites.

The turnover rate demonstrated in the in vitro studies and this relative weak surface binding supports the possibility of the exuded nucleoprotein complex described by Stroun et al[97], Rogers[100], and Kandjian et al.[98]. The fact that it can be detected on the surface may be due to temporary binding and the amount detected may be due to synthesis rate or to variations in membrane affinity.

The extracellular nucleoprotein found by Stroun et al.[97] was virtually insensitive to degradation by RNase or DNase. This raises two points. The first is that it could explain the negative results of the experiment in section II-D-3 of the Results. The antibody titre for sheep RBC's was lowered by tumor supernatant but did not return to normal after RNase incubation. If the complex was insensitive to RNase then that is what would be expected. This leads to the second point. If the complex is not sensitive to RNase why does the electrophoretic mobility of the cell decrease upon incubation with RNase? This forces the existance of both a susceptible and an unsusceptible RNA fraction in the complex to satisfy these observations. While this is possible there is no evidence supporting it.

The results I have shown do not directly indicate a nucleic acid molecule excreted by the S-180. The evidence does show that their presence on the surface is linked to cell metabolism, particularly nucleic acid metabolism. This is shown by incubation with methotrexate, mercaptopurine, and Actinomycin-D. After incubation with these inhibitors, the nucleic acid disappears from the surface over a time period of about 4 hours. If the surface nucleic acids are removed with enzymes, they reappear in a similar period of 2-4 hours. These times are shorter than one cell cycle which is about 24 hours for these cells. This nucleic acid flux either means these nucleic acids are being degraded and need to be replaced or that they are being released from the cell.

IV. Size of Nucleic Acid Group

In the Results section II-B-7, I calculated the amount of nucleic acids released into the supernatant by RNase treatment to be 100 times greater than that detected by whole cell electrophoresis when measured spectrophotometrically. I also said that if it was real, then the nucleic acid would have to exist in a large complex which sits on the surface. Using this ratio of 1:100 an approximation of the size of this proposed complex can be calculated. Several non trivial assumptions must be made, however.

Let us assume a nucleic acid complex in the shape of a sphere of radius R. Let us further assume that only the charges facing away from the membrane and close to the slip plane will contribute to the zeta potential. Let us assume that only those charges subtended by a solid angle of 60^{o} contribute significantly. (See Figure 18). This

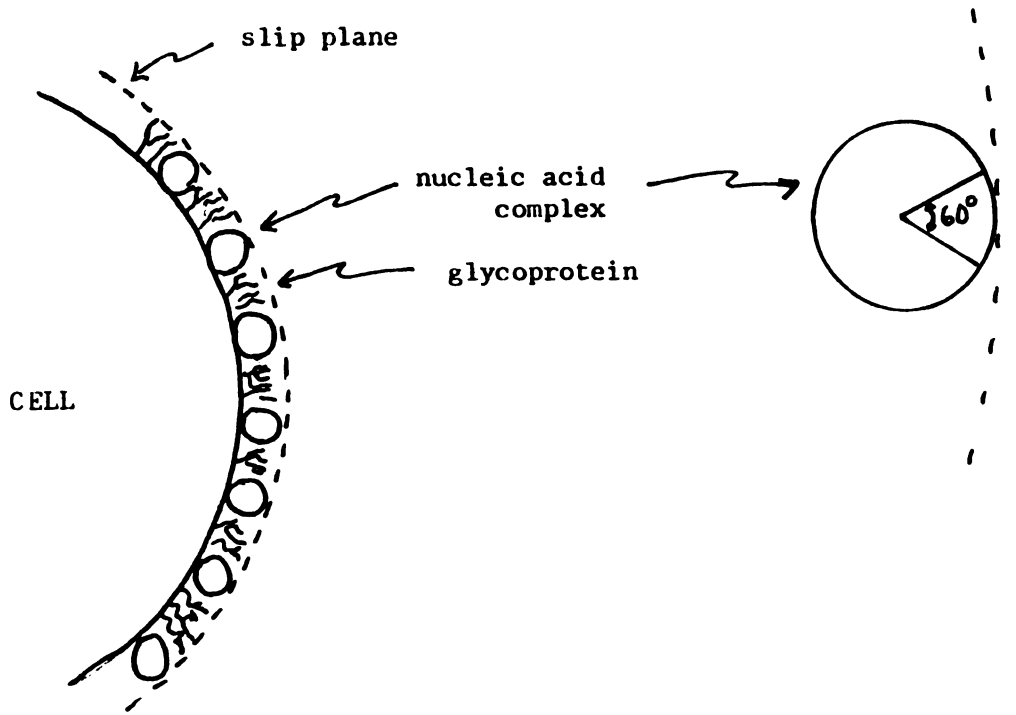


Figure 18. Model of the relative orientation of the slip plane to a hypothetical nucleic acid complex.

turns out to be only 0.047 of the total area of the sphere.

Now, let X be the total number (or volume) of nucleotides in the sphere. The volume of a sphere is $\frac{4}{3}\pi R^3$ where we can let R be the radius in 'nucleotide units'. The volume in 'nucleotide units' is equal to X, so $X = \frac{4}{3}\pi R^3$ and it follows that $R = \left[\frac{3}{4\pi} X\right]^{1/3}$. The surface area is the $4\pi R^2$ or $4\pi\left[\frac{3}{4\pi} X\right]^{2/3}$. Of this surface area only 0.047 contributes to the zeta potential or $0.047 \cdot 4\pi\left[\frac{3}{4\pi} X\right]^{2/3}$. This simplifies to $0.227\left[X\right]^{2/3}$, which represents the number of nucleotides in the sphere that contribute to the zeta potential.

Now, using this we can compute what size spherical nucleic acid complex would be necessary to contain an amount of total charges 100 times greater than the charges that contribute to the zeta potential. So, to get this we set up the simple proportion equation:

$$\frac{0.227\left[X\right]^{2/3}}{X} = \frac{1}{100} \quad .$$

Simplifying, this becomes $X = \left[22.7\right]^3$ or $X = 11{,}697$. This implies a molecular weight of 3.5×10^6. This is smaller than most viral genomes, larger than most messenger RNA molecules, but not out of the range of either an RNA or DNA molecule.

The major assumptions in this calculation are: 1.) The complex is a close packed sphere; 2.) the complex is only made up of nucleic acids; 3.) the slip plane is not bent around the complex. The assumption of a sphere is not that crucial and a relatively standard one. The assumption of only nucleic acids is not that bad either. If we assume that 25% of the complex is protein then the molecular weight of the nucleic acid would only be reduced to 1.48×10^6 daltons and the protein component would be 0.24×10^6

daltons (about the size of a LETS protein).[58] But the last assumption could make dramatic changes in the calculation if it were altered. However, the concept of the slip plane is, in itself, an assumption and several correction factors have been calculated to make adjustments for membrane topography, such as large lumps. Basically, these corrections require recognizing that the mobility is lower than would be expected when the surface has bumps.[28] This supports the model of the slip plane 'missing' some of the charges, and therefore supports the assumption of only part of the surface nucleotides contributing to the zeta potential.

Calculating a size that is within reason is somewhat satisfying and even gives some hope for simple spectrophotometric studies. The main problem is in detecting small differences in large numbers because the cells normally appear to excrete nucleic acids or nucleotides.

V. Solid Tumors

The similarity of response of S-180 solid tumors to their ascitic counterparts after RNase and DNase treatment is almost unexpected. The disruptive procedures necessary to take the tumor mass to a single cell suspension would seem strong enough to leave a membrane highly scarred. Since these surface nucleic acids are removable with a variety of enzymes and low ionic strength it is strange that they survive these isolation proceedures. The role of calcium ions here is probably very important. Weiss[72] has shown that calcium ions are closely associated with cell surface ribonucleic acids. Calcium is also important in cell to cell adhesion [110,111]

solute permeability through the hyaluronic acid matrix[112], in the interaction of antibody and compliment[113], and in cell proliferation mechanisms[114], to name a few. Calcium chelating agents are routinely used when isolating single cells from tissue because it reduces cell-cell adhesion. The chelation of calcium does not seem to destabilize the membrane nucleic acid because EDTA and sodium citrate do not effect ascitic S-180. Perhaps removing the calcium makes the membrane components less rigid and therefore more plastic and resiliant. Thus this would assure more cell survival and more intact membranes during the physical shearing of tissue separations.

VI. The P815 mastocytoma.

The P815 mastocytoma would make another good model for cell surface RNA and DNA. The effect of both nucleases exists with this cell although its not as pronounced as in the S-180 tumor. The major benefit, though,is that this tumor is syngeneic with the DBA/2 mouse. This allows much greater versatility in designing immunological experiments and removes doubt associated with non-syngeneic tumors like the S-180.

VII. Results of Other Cells.

Most of the cells tested exhibit nucleic acid on the cell surface if they are tumorigeneic and no nucleic acid of they are normal. Other investigators have shown that a cell does not have to be transformed to exhibit cell surface nucleic acids[77,83,84,87] or to excrete nucleic acids.[96,97,98] Weiss[73] has shown that rapidly growing lymphoid cells have RNA on their surface; Lerner[87] showed that stimulated lymphocytes have DNA associated with their membrane; and

Rogers[100] found that these stimulated lymphocytes excreted DNA. Cater and Woo[76] indicate, by electrophoresis, that fetal liver cells have RNA on their surface, about half as much as hepatoma cells. I did not find nuclease susceptibility on mixed fetal cells or on thymocytes or splenocytes of the preleukemic AKR mouse. These lymphocytes, though, were not specifically activated to a proliferative state which is probably crucial, and the fetal cells were not carefully divided into tissue types which may be important.

More cells will have to be examined, particularly more normal cells. The problem is obtaining normal tissue that can be dissociated into individual cells while keeping the cells intact. There are few normal cells that are in suspension like ascitic tumors. Of course, there are few spontaneous tumors that are in suspension either. The proceedure of tissue separation will also have to be examined and perhaps improved.

VIII. Roles of Surface Nucleic Acids

The evidence, both weak and strong, available at this point is as follows.

RNase or DNase susceptible groups have been shown on tumor cells,[71,72,88,91] stimulated lymphocytes,[73,87] fetal liver cells,[73,76] and sperm cells.[84]

In each case, as in this study, the investigators have concluded that these groups were nucleic acids.

In this study, the presence of these surface nucleic acids was linked to the cell's nucleic acid metabolism, and furthermore, they exhibited turnover on the membrane.

Studies have indicated a nucleoprotein complex released from cells.[83,97,98]

AKR leukemic mice have a suppressor T-cell popluation exhibiting surface DNA.[91]

Human lung cancers have an immune inhibitory substance on their membrane which has been reported to be a nucleoprotein.[92]

Could this surface nucleic acid have a role in tumor growth or immune suppression? At this point there are only conjectures, but let me outline some of the possibilites.

1. No role at all - These nucleic acids could be simply debris exuded by very rapidly growing cells. Most of the work to date could not argue againstthis because the concentration of effort has been on proving it's existance not on it's function.

2. Surface functions - Their role could be fulfilled by their location on or in the membrane.

a.) antigen masking - due to their low antigenicity nucleic acids could cover tumor antigens.

b.) immunosuppressive molecules - They could be transmitted to lymphocytes along with tumor antigens and when inside the lymphocyte suppress antibody reproduction or lymphocyte mitogenesis.

c.) The surface nucleic acids could be stimulators of the very cell they are located on. These surface groups could act as receptors which,upon activation,are incorporated into the cell where their translated products act. This could be a mitogenic action or a differentiation or dedifferentiation signal, etc.

3. Intercellular release - these nucleic acids could transmit information to other cells.

a.) immunosuppressive - analogous to 2,b., these groups could be released as generalized or specific immune suppressors which act on any of a number of cells; lymphocytes (killer, suppressor, helper,cytotoxic), macrophages, or neutrophils.

b.) mitogenic - analogous to 2,c., these nucleic acids could activate nearby tumor cells by releasing a growth factor stimulating the tumor growth . They could also act as a transforming agent to nearby normal cells.

c.) promotor of angiogenesis - these released nucleic acids could activate tumor vascularization by interacting with endothelial cells of the capillary walls and stimulating their proliferation.

d.) embryonic communication - the interrelationship betweeen embryo cells could be partly mediated by nucleoprotein complexes. The appearance of these complexes during tumor growth may be due to dedifferentiation and there may not be a target for them to act upon.

(The converse of this is also interesting. If the tumor cell is a dedifferentiated cell trying to interact with the other cells around it of a different place and time developmentally, then it would not receive the correct feedback signal for normal differentiation. It would be stuck in its current state forever.)

The problem with the science of tumor immunology is that none of these possibilities can be definitely ruled out. The factors that tumors excrete, that lymphocytes and macrophages excrete, the chemical nature of their antigens, their mode of binding and killing etc. are not well characterized. The most common technique for tumor antigen extraction, for example, presumes that it is protein.[65] They dissolve the membrane in 3M KCl then salt out the soluble fraction with ammonium sulfate. This only recovers 15-49% of the antigenic activity and makes one wonder if there is something lost because of the design of the technique, such as a nucleic acid component.

The idea of antigen masking by surface nucleic acids could be a possibility because of the low antigenicity of nucleic acids. (Note: antibodies do form to the phosphate backbone of polynucleotides, called NG-I antibodies,[115] and crossreact with most types of nucleic acids) This hypothesis requires that the cell use a potentially valuable molecule in a capacity which does not use its specialized information carrying capacity. It also requires that the cell knows what substance on its surface is antigenic and that it is in an environment such that it should cover this antigen. These are amazing assumptions

considering the nature of tumor cells. Tumor cells are aberrant growths within an organism and are not parasites which have invaded an organism. Because they do not have a life cycle outside of the organism, and because they have developed from cells within the organism, they have not been exposed to evolutionary pressures. Without this evolutionary development they would not have special defense mechanisms, nor the ability to select defenses appropriate to their environment. Therefore, the notion that the tumor cell is capable of masking antigens seems untenable on teleological grounds.

A more likely role than passive antigen masking for these nucleic acids would be active immunosuppression. This idea uses the information carrying potential of RNA and DNA. This kind of information transfer need not be limited to immune suppression but could be involved in many signals transmitted between cells. A nucleic acid excreted by a tumor could be part of an embryological communication system. Stimulated lymphocytes could excrete a signal which recruits other lymphocytes into specific stimulation. Activation of macrophages could occur through nucleic acids. Many soluble factors circulate in the blood sera and many 'growth factors', 'inhibitory factors', 'suppression factors', 'transfer factors', 'activation factors', have been postulated[55,56,58] and only partially characterized. The possibility that they contain nucleic acid should be kept in mind by investigators when examining these factors.

The blood serum is obviously accustomed to the presence of nucleic acids because RNase circulates there. Some cancer patients have elevated levels of RNase[117] perhaps to remove breakdown products,or

perhaps in response to some form of nucleic acid 'factor'. A serum protein which binds DNA is also elevated during malignancy[118,119] but its role is currently unknown. The role of excretion of cellular nucleic acids and their interactions with the organism await further investigation for clarification.

There is some evidence, however, that the nucleic acid has a function on the cell surface. Work of Russell and Golub[91] shows an immune suppression activity of AKR leukemic cells when these cells are allowed to make contact with sensitized normal spleen cells. These cells also contain a surface DNA moiety, which when removed stops suppression. In a communication from Dr. Golub, he states that the suppressor cells have a specific antigenic marker - a histocompatability marker - which is also necessary for suppression. This information along with the H-2 restriction (lymphocytes must recognize an H-2 antigen site on affector and effected cells) supports the idea of nucleic acids being localized on the cell surface for their action. When two compatible cells make intimate contact the informational nucleic acid could be transferred from the surface of one of the cells to the interior of the other.

A similar process could occur during macrophage antigen collection. The macrophage is believed to transmit antigenic material, probably with a carrier, to lymphocytes. It is conceivable that it could transmit a suppressor molecule concurrently, or, that the suppressor molecule could directly inhibit the macrophage. Currently, the only support for a surface nucleic acid having a suppressive role is the

work of Russell and Golub.[91] Whether it is an immunosuppressant; whether its action depends upon its location on the membrane or in solution; or whether it is an artifact of fast growing cells will have to be answered by future experiments.

IX. Future Experiments

It seems that a sufficient amount of data exists to support the fact that there are cell surface nucleic acids. From this point I believe the emphasis in reasearch should focus on two areas. The first is isolating and characterizing this RNA and DNA - some of this has already been started. The second is to determine it's role, if any.

Experiments to accomplish the first goal will probably be forced to use the techniques currently being used by Rogers,[100] Lerner,[87] Glick,[82] Reid,[85] and Stroun[97]. These involve membrane preparations, nucleic acid extraction and isolation, sucrose density gradient separations and characterizations, melting point determinations, and homology studies.

The techniques that I would like to see used are to make proteins from the RNA as well as RNA and proteins from the DNA. These proteins may have recognizable properties which can give important clues to the purpose of the nucleic acids. Roger's[101] work indicates that only a small portion of the total nuclear DNA shows up being excreted by lymphocytes. Such a specific piece of DNA may be translatable and transcribable into recognizable proteins. These proteins could be analyzed in a variety of ways. They could be assayed for immunosuppressive properties using a technique similar to Golub's[91]; they

could be sequenced and compared to known protein sequences, or they could be added to tissue cultures to observe growth changes that they might induce. Use of the S-180 tumor system may be useful for some of this nucleic acid isolation and characterization because of the large quantities present on this cell type.

Experiments to ascertain a role for these surface nucleic acids will probably start with immunology. Since the first indication of a role is in immunosuppression, this should be used as a starting point. While Golub's laboratory pursues the AKR leukemia system other tumor systems should be explored.

The P815 mastocytoma, syngeneic with the DBA/2 mouse offers a possible system. I have found both DNase and RNase sensitivity on this tumor, although small, so it offers a potential yet to be explored. DNA-RNA could be extracted from the membrane or from the supernatant and used in immunosuppressant experiments. Extracted nucleic acid could be used in vitro in hemolytic plaque assays with sensitized spleen cells, in macrophage or lymphocyte versus tumor cytotoxicity assays or in vivo to determine it's suppressive effects on foreign antigens. If extracted nucleic acids do not produce a response in the in vitro assays, whole tumor cells could be used. This would be necessary of H-2 restriction was involved or if other surface components were important.

The use of the S-180 cell for the examination of nucleic acid role may be limited due to the unknown character of this tumor. Although, since it is a non-syngeneic tumor to all mice, it may represent a system which requires tremendous immunosuppressive qualities to overcome major histocompatibility-transplantation antigens.

X. The Effect of cisplatin.

No new modes of action for cisplatin were derived from this study. The drug did have a major effect upon the cell surface nucleic acid but so did other antitumor drugs specific for nucleic acid synthesis.

The apparent connection between cell surface nucleic acids and tumor cells, and their removal by the action of antitumor drugs, and the possible immunosuppressive role of cell surface DNA, is all very interesting. These findings suggest that the antitumor drugs, like cisplatin, may have other effects than the direct interference with nuclear DNA synthesis.

APPENDICES

Appendix I

The Helmholtz-Smoluchowski equation relates mobility, u, to the potential at the surface of a slip plane, ζ, with delectric constant, ϵ, and viscosity, η.

$$u = \frac{\zeta \epsilon}{4\pi\eta} \tag{1}$$

The surface charge that produces the potential is given by the Debye-Huckel equation for large, smooth particles.

$$\sigma = \frac{\epsilon \zeta k}{4\pi} \tag{2}$$

where σ is the surface charge and k is the Debye-Huckel constant which can be defined by the equation,

$$\zeta = \psi e^{-kx} \tag{3}$$

which gives the potential, ζ, at any point X away from the surface. 1/K is in units of distance and is often called the thickness of the ionic double layer which surrounds the particle. For large smooth particles equations (1) and (2) can be combined to give[120],

$$u = \frac{\sigma}{\eta K} \tag{4}$$

and 1/K can be related to the ionic strength of the bathing solution:

$$1/K = 96/z\sqrt{c} \tag{5}$$

where z is the ionic valency and c is the concentration in millimolar. 1/K is then given in Ångstroms.

Substituting (5) into (4) we can say that the mobility is proportional to the reciprocal of the square root of the ionic

concentration.

$$u \propto \sigma \cdot 1/\sqrt{c} \qquad (6)$$

When the mobility is plotted versus $1/\sqrt{c}$ a straight line should occur, if σ remains constant.

In Figure 19 the mobilities of red blood cells (from Heard and Seaman[24]) and the S-180 are plotted. The red blood cells give a good straight line with an intercept of zero (infinite ionic concentration - no mobility). The tumor cells, however, do not give a good straight line but their mobilities are lower than expected, indicative of increasing σ. Therefore they do not qualify as smooth particles.

Another equation can be derived which can take this into account. In Figure 20, a hypothetical membrane is diagrammed. This membrane has thickness with charges inbedded within it and counterions from solution which can diffuse into it. The charges that lie on any of a series of imaginary planes within the membrane contribute to the zeta potential depending on their distance in and the ionic strength of the bathing solution. At low ionic strength there are fewer counterions and the inner charges exert more influence (figure 20,b). Haydan [27] has developed a model to simplify the contribution of the inner charges. He defines a potential, ψ_0, due to the charges at the surface and another potential of the same magnitude located a distance d beneath the surface. This arbitrary internal potential is an average of the potentials of all the imaginary planes within the membrane. Its distance from the surface is such that it equals the surface potential. With these assumptions he

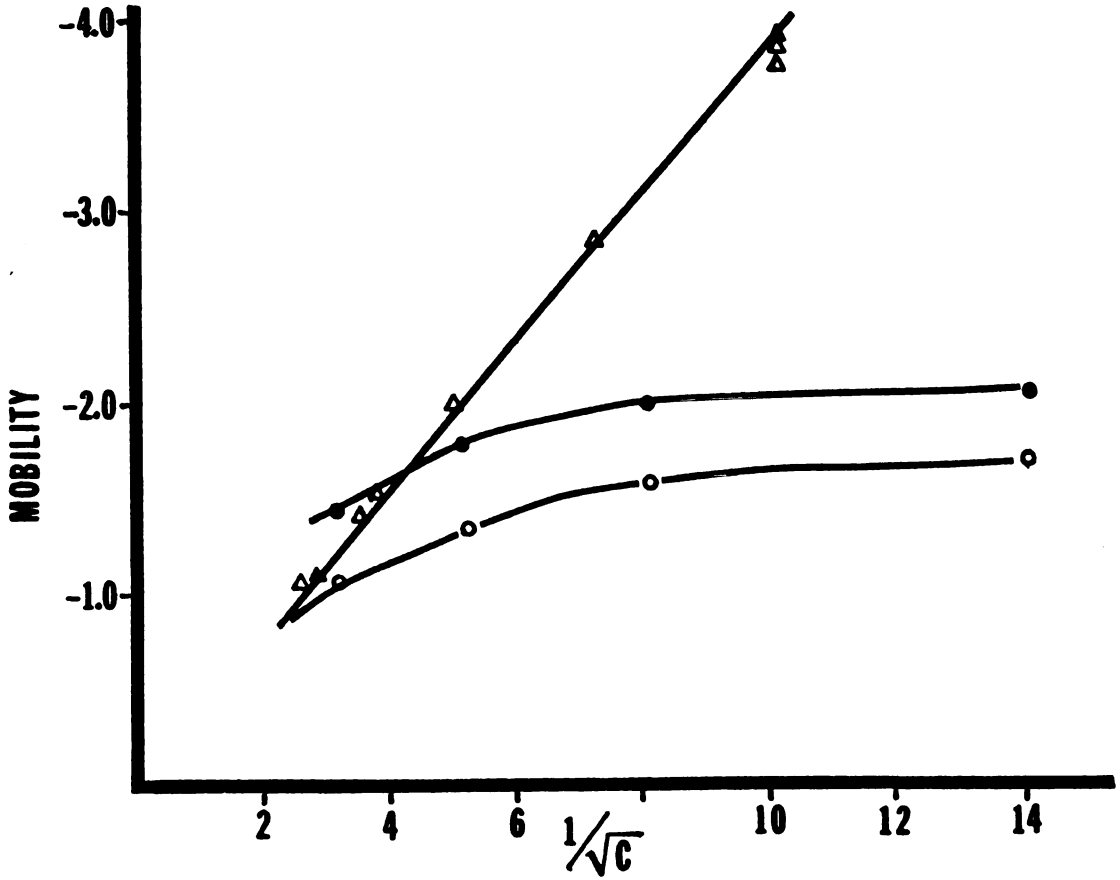


Figure 19. Plot of mobility versus the reciprocol of the square root of the ionic strength of the measuring buffer. Solid circles are the S-180 controls, open circles are the S-180 RNase incubated, and triangles are red blood cell data from Heard and Seaman[24].

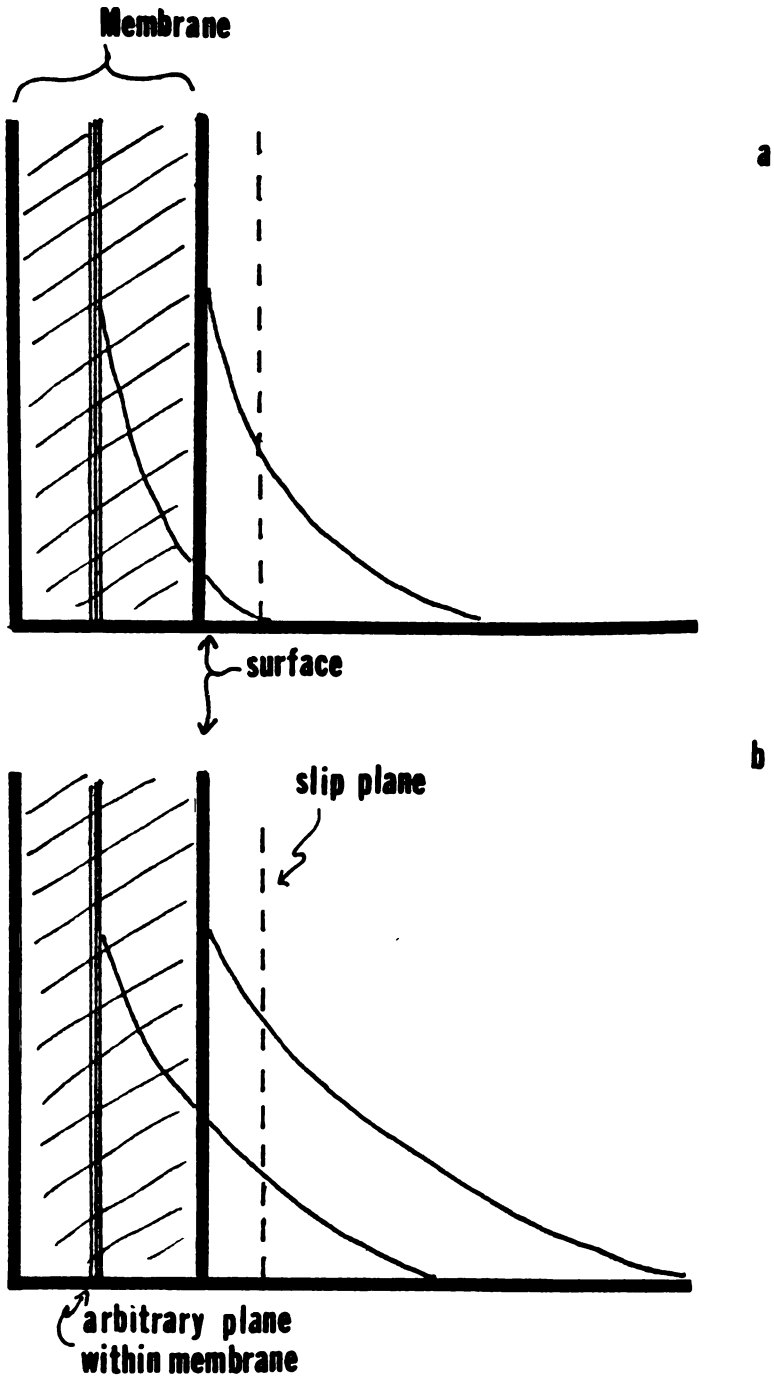


Figure 20. Model of cell surface with thickness and the potential curves from charges in the membrane in a) normal ionic strength and b) low ionic strength.

arrives at an equation relating the potential ψ_o, the distance d, and the Debye-Huckel constant K to the measured surface charge σ. This equation can be simplified to a first order approximation which gives

$$\sigma \propto \psi_o e^{-Kd} \qquad (7)$$

If this is substituted into equation (4) and rearranged and the proportionalbity between K and $\sqrt{c}$ substituted it gives

$$\ln u - \ln \psi_o \propto -\sqrt{c}\, d - \ln \sqrt{c} \qquad (8)$$

If $\ln \sqrt{c}$ is ignored an error of less than 10% is added but for out purposes this is acceptable. ψ_o is taken as a constant so a relation appears which is linear between ln u and $\sqrt{c}$ with a slope of d. In Figures 11 and 12 of Results section, the mobilities versus ionic strength are plotted as ln u versus $\sqrt{c}$. The slopes of these curves represent d, the distance between the surface and an interior plane which represents the interior charges. For Figure 11a, d, was calculated for the control and RNase incubated cells. The value for the control is 4.1Å and for the RNase treated cells 5.8Å.

Even though the value ,d, is an arbitrary construct representing a vaguely defined depth in the membrane it can be used for comparison purposes. The RNase incubated d value is 40% greater than the control. This suggests a greater influence of deeper charges in the RNase incubated cells, perhaps due to the loss of charges that predominate near the surface.

Appendix II

The pH profile curves shown in Figures 8 and 9 show a shift in isoelectric point to higher pH and an overall uplifting of the curve due to platinum treatment. Besides the loss of a group of pK 1-2 as argued in the Results section, the shift in isoelectric point could be attributed to the addition of a positively charged group with a pK of 6.3 . This, however, does not follow from the profiles shown. A pK of 6.3 would indicate a group positively charged below pH6.3 but neutral above pH 6.3 and while this would explain the differences in the curves at low pH's it does not explain the much larger differences at pH 7.4 .

Appendix III

Operating Proceedure

1. Turn on Dual power supply, chart recorder, DVM(set on DC Volts) TV, TV camera, microscope light, water bath pump(cooling water from tap should have water bath cold- about 16°C), and turn motor speed control from Standby to ON. Make sure that the water bath settles to an operating temperature of 18°C.

2. Turn stopcocks to drain and pass distilled water through system.

3. Fill chamber with 0.1 M KCl and turn stopcocks to connect chamber with electrodes.

4. Set electrode power supply to about 200V and turn S-3 left or right. The chart recorder pen should move towards one side or the other. (S-2 should be in position D). Turn voltage control on reference power supply until pen of chart recorder zeros. (chart recorder zero should be set in the middle for convenience). Turn S-3 to center position, turn S-2 to E, and place DVM on DC amperes and read milliamps off meter. Turn S-2 back to D.

5. Flush out KCl, add measuring buffer, and turn stopcocks to electrodes. Repeat proceedure in step 4 to obtain amperage through buffer.

6. Set reference power supply until DVM reads desired reference current, usually 6mA. Turn S-2 to D, and DVM to DC voltage setting. The chart recorder will now read from 5.5 to 6.5 mA with 6mA at the the zero point in the center.

7. Flush out buffer, add new buffer and cells to be measured, close stopcocks. Make sure that the microscope is focused at 1/5 of the distance between the two inner surfaces of the measuring chamber. the chamber is 400 um thick, 0 is considered at the inner glass surface closest to the observer.(The inner surface is easy to find because it is usually slightly dirty.)

8. Turn S-3 to the left and S-1 to the left. Adjust motor speed control until the cells appear stationary. Put S-3 switch at the center point and record tachometer voltage from the DVM and the chamber current from the chart recorder. Then turn S-1 to the center position. Do the same for the right hand settings of S-3 and S-1. Repeat these right and left pairs of meaurements until satisfied a representative

sample is obtained - about 6-10 times.

9. Flush out sample and put in new sample or clean chamber with distilled water or detergent.

Note: Settings A,B,C on S-2 are designed to allow monitoring of electrode voltage, reference voltage, and motor control voltage respectively.

Appendix IV

In this discussion I will show the mobility lowering ability of antisera when it binds to antigens on the cell surface. Sheep red blood cells, in Alsevers solution and Rabbit anti-sheep RBC antisera were obtained from the Colorado Serum Co. Varying concentrations of this sera were incubated with washed red cells and the mobilites measured. Incubation was performed in a barbitone buffered saline for 30 minutes at 23^{o}C. Normal rabbit sera was used as a control. In Figure 21 the results are shown. There is a large drop in mobility with increasing binding of immunoglobulin. At the highest concentrations of sera the cells lysed,(sera was heat inactivated to remove compliment activity) probably due to residual compliment.

The onset of a definitive change in mobility occurs at a higher concentration of antisera than would be necessary in a compliment fixation test, so it is not as sensitive as compliment fixation. However, for those cells that are not susceptible to compliment induced lysis, this is one way to determine antibody binding.

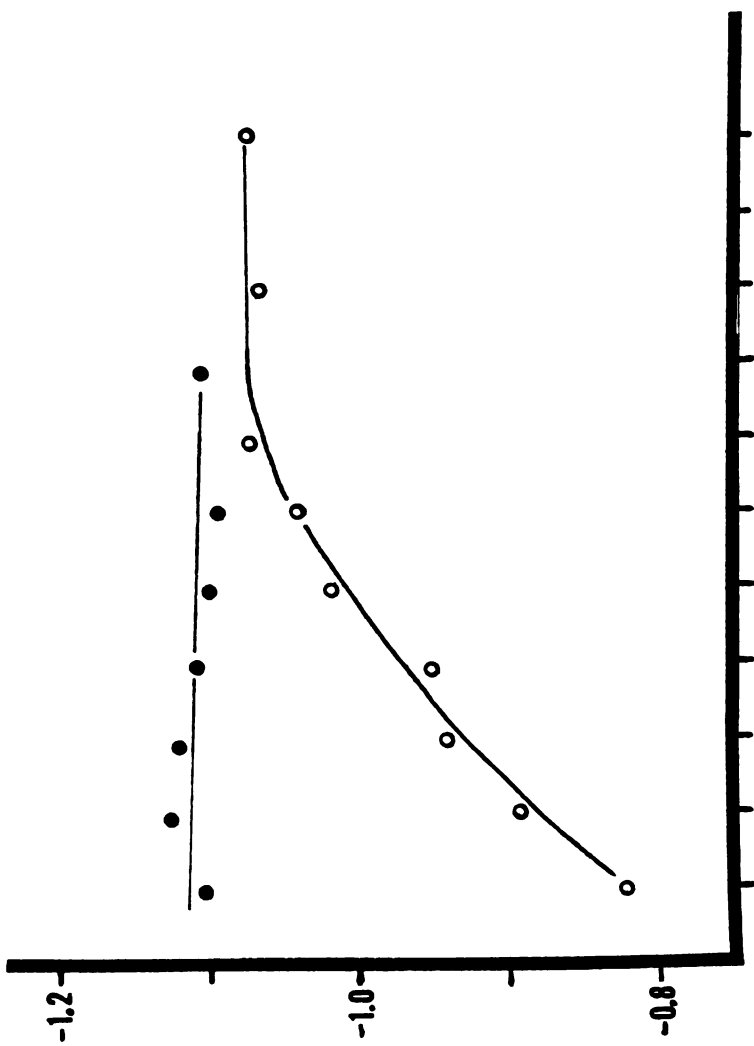


Figure 21. Plot of mobilities of sheep RBC's as a function of sera dilutions. Closed circles are normal rabbit sera and open circles are anti-sheep RBC antisera.

BIBLIOGRAPHY

Bibliography

1. B. Rosenberg, L. Van Camp, and T. Krigas, Nature, 205(1965)698.

2. J.A. Howle and G.R. Gale J. Bact. 103(1970)259.

3. S. Reslova, Chem.-Biol. Interact. 4(1971-1972)66.

4. D.J. Beck and R.R. Brubaker, Mutat. Res 27(1975)181.

5. D.J. Beck and J.E. Fisch, submitted for publication (1978), referrred to in Biochimie 19(1978)233.

6. R. Lecointe, J.P.Macquet, J.L. Butour and C.Paoletti, Mutation Res. 48(1977)139.

7. W.R. Leoposd, E.C. Miller, and J.A. Miller, Cancer Res.,39(1979)913.

8. P.B. Conran, and B. Rosenberg, "Advances in Antimicrobial and Antineoplastic Chemotherapy", Vol II, Univ. Park Press, Balt., p.235, 1972.

9. A. Khan, in:"Platinum coordination Complexes in Cancer Chemotherapy" (Ed. by T.A. Connors and J.J. Roberts) Springer-Verlag, Heidelberg, p 124,1974.

10. A. Sodhi Abs. of the Toulouse Mtg on Coord. Chem and Cancer Chemo. July 1978.

11. A. Sodhi, J. of Clin. Onc. and Hema., 7(1977)569.

12. E. Heinen, see ref #10.

13. M. Gosalvez, M. Blanco, J. Hunter, M. Miko, B. Chance, Eur. J. of Cancer, 19(1974)567.

14. Kahkarova, Tatžanenko, Moshkovsky, Raykhman, Kondratyeva, published in 1977 in Russian - english abstract.

15. H.C. Harder, B.Rosenberg, Int. J. of Cancer 6(1970)207.

16. J.M. Pascoe and J.J. Roberts,Biochem. Pharmacol 23(1974)1345.

17. J.M. Pascoe and J.J. Roberts, Biochem. Pharmocol., 23(1974)1359.

18. S, Mansey, B. Rosenberg and A.J. Thomson, Journal of the Amer. Chem. Soc., 95(1973)1633.

19. J.J. Roberts and A.J. Thomson, In;"Nucleic Acid Research and Molecular Biology", p. 71, 1979.

20. H.W.van den Berg and J.J. Roberts, Mutation Res. 33(1978)111.

21. J. Froehlich, R. Srivastava, and G. Eickhorn, Abs. see ref 10.

22. D. Herman, W. Guschlbauer, Abs. see ref 10.

23. A.B. Robins, Abs. see ref 10.

24. D.H. Heard and G.V.G. Seaman, J. Gen. Physiol., 43(1960)635.

25. H.A. Abramson, "Electrokinetic Phenomena and Their Application to Biology and Medicine". The Chemical Catalog Co. New York, 1934.

26. J. Henniker, Rev. Mod. Phys. 21(1949)322.

27. D.A. Haydon, Biochim. Biophys. Acta, 50(1961)450.

28. C.C. Briton,Jr. and M.A.Lauffer, In: " Electrophoresis, Theory, Methods, and Applications.", Ed. M.Bier, 1959. p.427.

29. "Cell Electrophoresis", Ed., E.J. Ambrose, J.A. Churchill Ltd., London, 1965.

30. J.N. Mehrishi, In: Prog. in Bioph. and Mol. Biol. 25(1972)1.

31. F.M. Burnet, J.F. McCrea, and J.D. Stone, Brit. J. Exptl. Pathol., 27(1946)228.

32. G.L. Ada and J.D. Stone, Brit. J. Expt. Pathol., 31(1950)263.

33. E. Klenk, "Ciba Found. Symp. Cheme. Biol. Mucopolysaccharides." p 311, 1958.

34. H.C. Wu, E. Merxan, P.H.Black, Biochem. J., 8(1969)2509.

35. C.C. Sweeley, G. Dawson, In: "Red Cell Membrane", Eds., G.A. Jamieson and T.J. Greenwalt, 1969, p172.

36. S. Hakomori, Proc. Natl. Acad. Sci.,USA, 67(1970)1741.

37. L. Weiss, J. Natl. Cancer Inst. 50(1973)3.

38. S. Kozawa, Biochem Z. 60(1974)146.

39. E. Mayhew and L. Weiss, Expt. Cell Res. 50(1968)441.

40. C. Sato, K. Kojima, K. Nishizawa, Biochim. Biophys. Acta,470(1977)446

41. G.V.G. Seaman and G.M.W. Cook, In: Cell elctrophoresis, see ref 29. p. 48.

42. J.N. Mehrishi, D.R. Grassetti, Nature, 224(1969)563.

43. D.M. Mitchel and D.B. Cater, Br. J. Exp. Path. 52(1971)152.

44. C.B. Thoman and D.B. Cater, Br. J. Exp Path. 53(1972)111.

45. A.L.Griffich, N.Catsimpoolas, N.H.Wortis, life Sciences 16:1693.

46. S.A. Smith and B.R.Ware Biophysical J. 21(1978)179a.

47. P.Blume A. Mallen, R.Knox, G.V.F. Seaman, Nature 271(1978)378.

48. E.J.Ambeose, A.M. Jones, J.H.B. Lowick, Nature 177(1956)576.

49. C.F. Fuhrmann, In: "Cell Elctrophoresis," see ref 29, p. 92.

50. P.S. Vassar, G.V.F. Seaman, D.E. Brooks, In: Proc. 7th Canadian Cancer Research Conf. 1967, p265.

51. L.Weiss, T.S. Hauschika, Int. J. Cancer 6(1970)270.

52. E.Mayhew, J.Gen. Physiol 49(1966)717.

54. D.R. Coman, Cancer Res., 4(1944)625.

53. J. Ponten, Contact Inhibition, in: Cancer: a Comprehensive Treatise Vol III, Plenum Press, 1975. p55.

55. J.C. Robbins and G.L. Nicolson, Surfaces of Normal and Transformed Cells, in: Cancer, A Comprehensive Treatise. p. 3.

56. M.C. Glick, Cell Surfaces Changes Associated with Malignancy, In: Fundamental Aspects of Metastasis, Ed. L.Weiss, 1976.

57. L. Weiss, In: "Fundamental Aspects of Metastasis", p. 25.

58. C.G. Gahmberg, In: Cell Surface Reviews, Vol.3, Eds., G. Post and G.L. Nicolson, 1977.

59. S. Hakomori, Adv. Cancer Res. 18(1973)265.

60. T.Muramatsu, G. Gachelin, J.F. Nicolas, H.Condamine, H.Jakob, F.Jacob, Proc. Natl. Acad. Sci. USA, 75(1978)2315.

61. R. Roblin, I.-N.Chou, P.H. Black, Adv. Cancer Res. 22(1975)

62. R.J. Mannino, M.M. Burger,In: Regulation of Growth and Differentiated Function in Eukaryote Cells, Ed. G.P. Talwar, 1975.

63. E.M. Hersh, J.U. Gutterman, G.M. Mavligit., Adv. Int. Med. 22(1977)145.

64. C.W. Parker, Pharm. Rev. 25(1973)325.

65. J.E. Castro, In: Immunological Aspects of Cancer, Ed. J.E. Castro, 1978, p. 1.

67. D.Noar, Adv. Cancer Res. 29(1979)45.

66. M.R. Price and R.W. Baldwin, In: Cell Surface Reviews,; 423.

68. J.F.A.P. Miller, Adv. Cancer Res, 29(1979)45.

69. A.I. Lansing, T.R. Rosenthal J.Cell. Comp. Physiol. 40(1952)337.

70. S. Chaudhare and I. Lieberman, Expt. Cell Res. 40(1965)527.

71. L. Weiss and E. Mayhew, J. Cell. Physiol. 68(1966)345.

72. L. Weiss and E. Mayhew, J. Cell Physiol. 69(1968)281.

73. M. Bennett, E. Mayhew, L. Weiss, J. Cell Phys. 74(1969)183

74. L. Weiss, O.S. Jung, R. Zeigel, Int. J. Cancer, 9(1972)48.

75. L. Weiss, and R. Zeigel, J. Theor. Biol., 34(1972)21.

76. J. Woo and D.B. Cater, Biochem J. 128(1972)1273.

77. W. Droege, D. Malchow, J.L. Strominger, Eur. J. Immunol., 2(1972)156.

78. D.F. Wallach, P.H.Zahler, Proc.Natl.Acad.Sci.USA,56(1967)1552.

79. T. Takeuchi and H.Terryama, Expt. Cell Res. 40(1965)32.

80. M.C. Glick, L.Warren, Proc.Natl.Acad.Sci.USA,63(1969)563.

81. S.Ya. Davidova, V.S. Shapot, FEBS letters 6(1970)349.

82. M.C. Glick, In: Biogenesis and Turnover of Membrane Macromolecules, Ed. J.S.Cook, 1976.

83. M. Rieber, J. Bacalao, Proc.Natl.Acad.Sci.USA,71(1974)4960.

84. A. Singer, B.L. Reid. J. Reprod. Fertil. 23(1970)249.

85. B.L. Reid, P.M. Blackwell, Aust.J. Med. Technol.,3(1972)121.

86. B.L. Ried, Biosystems, 5(1974)207.

87. R.A. Lerner, W. Meinke, D.A. Goldstein, PNAS, 68(1971)1212.

88. S.K. Aggarwal, R.W.Wagner, P.K.McAllister, B. Rosenberg, Proc.Natl.Acad.Sci.USA,72(1975)928.

89. P.K. McAllister, B. Rosenberg, S.K. Aggarwal, R.W. Wagner. J. of Clin Hema. Oncol., 7(2)(1977)717.

90. S.K. Aggarwal, J. Histochem. Cytochem., 25(1977)359.

91. J.L. Russell, E.S. Golub, Proc.Natl.Acad.Sci.USA,75(1978)6211.

92. A.C.Hollinshead, Proceedings, IX International Symp. Comp. Leukemia and Related Diseases.Oct. 1979. preprint.

93. "Immune RNA in Neoplasia"

94. S. Sell, J. Mendelsohn, Arch. Pathol. Lab. Med. 102(1978)217.

95. A.A. Gottlieb, R.H.Schwartz, Cell. Immunology, 5(1972)341.

96. A. Hollinshead, T.Stewart, In: International Cancer Congress, Symp.XII,Ed. Chacon, Pergamon Press, Ltd. In Press.1978.

97. M. Stroun, P. Anker, M.Beljanski, J.Henri, C. Lederrey, M. Ojha, P.A. Maurice, Cancer Res. 38(1978)3546.

98. E. Khandjian, G. Turian, Differentiation 5(1976)171.

99. P. Anker, M. Stroun, P.A. Maurice, Canc. Res. 36(1976)2832.

100. J.C. Rogers, J. Expt Med., 143(1976)1249.

101. J.C. Rogers, Proc. Natl.Acad.Sci.USA,73(1976)3211.

102. O.L. Horer, C.Nicolau, FEBS Letters 14(1971)262.

103. B.K. Sinha, C.F. Chingnell,Biochem.Biophys.Res.Com.,86(1979)1051.

104. W. Wolf, W.Cole, R.C. Manaka, B. Odenheimer, Chem-Biol. Inter. 22(1978)353.

105. G. Politis, M.G.Plassara, H.Thomou-Politi, Nature, 257(1975)485.

106. P.J. Goetz, J.G. Penniman,Jr., Amer. Lab. Oct 1976,p21.

107. D. Williams, V.Esquenazi, R.cirocca, J.Jensen, J. Immunol, 116(1976)554.

108. R.P. Gale, J. Zighelboim, J. Immunol., 114(1975)1047.

109. A.H. Pickaver, N.A.Ratcliffe, A.E. Williams, H. Smith, Nature(new Biol)235(1972)186.

110. I. Vlodavski, L.Sachs, Exp. Cell Res. 105(1977)179.

111. N. Zakai, G.Kulka, A.Loyter, Proc.Natl.Acad.Sci.USA, 74(1977)2417.

112. M.A. Napier, N.M. Hadler, Proc.Natl.Acad.Sci.USA.,75(1978)2261.

113. W.T. Shearer, J.P. Atkinson, C.W. Parker, J. Immunol. 117(1976)973

114. S.H.H. Swierenga, J.A. Whitefield, S.Narasaki, Proc.Natl. Acad. Sci.USA, 75(1978)6069.

115. J. Panijel, M.C. Delaunez, Biochem.Biophys.Res.Com. 129(1967)158.

116. I. Kamo, He. Friedman, Adv, Canc. Res., 25(1977)271.

117. R. H. Kottel, S.O. Hock, R.G. Parsons, J.A. Hoch, Br. J. Cancer. 38(1978)280.

118. R.G. Parson. P.H. Aldenderfer, R.Kowal, J. Natl. Canc. Insts. 63(1979)43.

119. R.G. Parsons, R.L. Longmire, S.O. Hoch, J.A. Hoch, Canc. Res. 37(1977)692.

120. J. Th.G. Overbeek, and J. Lijklema, In: "Electrophoresis, Theory, Method, Applications." 1959.p.1.

121. B. Rosenberg, Cancer Chemotherapy Rep.59(1975)589.