THE ISOLATION, CHARACTERIZATION, AND UTILIZATION OF PLASMA MEMBRANES FROM TUMOR CELLS

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NOMENCLATURE

C	degree centrigrade
cm	centimeter
DMSO	dimethyl sulfoxide
GEM	5 mM Glycine, 1 mM EDTA, 5 mM 2-Mercaptoethanol buffer
g·min	gravity times minutes
HBS	Hepes Balanced Salts
I.P.	interperitoneal
LM	L cells (fetal mouse)
M	Molar
M-199	Medium-199
MAT	Mammary Ascites Tumor
mg	milligram
ml	milliliter
mM	millimolar
O.D.	optical density
PMSF	phenylmethylsulfonylfloride
rpm	revolutions per minute
S-180	Sarcoma-180 cultured cells
SA-180	Sarcoma-180 ascites cells
g	microgram
Vi	included volume
Vo	void volume
% (w/w)	percent weight by weight

CHAPTER I

INTRODUCTION

Cellular membranes play important roles in processes controlling cell growth and differentiation (1,2). This is particularly true for the plasma membrane, which represents the site of interaction for other cells or extracellular factors such as hormones. Although these plasma membrane functions must be dependent on particular membrane component organizations and their organizational changes, very little is known about molecular details of plasma membrane structure. Considerable progress has been made in delineating some features of erythrocyte membrane structure (3-7), but many questions still remain. For more complex cells the problem is aggravated by difficulties inherent in the isolation of plasma membranes without intracellular membrane contamination (8), which makes even an enumeration and quantitation of plasma membrane components difficult.

Plasma membranes are a complex mixture of proteins, glycoproteins, neutral lipids, phospholipids and glycolipids, possibly with some polysaccharide attached. There is mounting evidence that submembrane proteins are also associated with the plasma membrane in its native functional state and these structures should be investigated in conjunction with the plasma membrane.

Plasma Membrane Isolation and Structure

The art of plasma membrane isolation has been reviewed by Steck

and Wallach (8). Although new membrane isolation techniques have been presented since the publication of this review, they generally fall into the same two categories considered in the review. In the first case, the cell is homogenized in an appropriate medium to release the plasma membrane as large fragments, which can be separated from the intracellular components. In the second case, the cell is burst by rapid decompression of an inert gas in a bomb to reduce all of the cell membranes to semipermeable vesicles. In either case the plasma membrane must be separated from the internal membranous constituents. This is usually achieved by density gradient centrifugation of the homogenates or the microsomal vesicles on sucrose or polysaccharide gradients (9). Before homogenization membranes are often "stabilized" in order to prevent membrane disruption and to facilitate the isolation of intact envelopes. This is achieved by addition of sulhydryl agents, divalent cations such as Zn^{++} (10) or borate buffer (11). The basis for the stabilization is still uncertain. Other techniques have been developed which might be useful in plasma membrane isolation. Roizman and Spear (12) have noted the enhanced density gradient sedimentation rate of antibody-plasma membrane complexes. Isoelectric focusing has been applied to the fractionztion of cellular membranes (13). The use of affinity chromatography should also be feasible, since it has been applied successfully to the separation of whole cells (14).

Isolation of plasma membranes from the Sarcoma-180 ascites tumor was first reported by Shin and Carraway (15) in 1973. The isolation method, which yielded a reasonable preparation of plasma membranes was not, however, optimized, nor were any attempts made to understand the effects of the various isolation steps on the final isolation method.

Two different methods were used, which although not optimal in their results, yielded plasma membranes of reasonable purity. The two methods differed mainly in the presence or absence of divalent cation (Zn^{++}) which yield plasma membranes whose polypeptide distribution differs primarily by the lack of a group of high molecular weight plasma membrane proteins in the cells homogenized in the absence of divalent cation (Zn^{++}). The authors also described a method for the surface labeling of the SA-180 cell with ^{125}I using lactoperoxidase.

The major difficulty in isolating plasma membranes comes in identifying the correct fraction after separation has been achieved. Identification is usually based on morphology, cholesterol-phospholipid ratios (16), sialic acid content (17), presence or absence of specific enzyme markers such as 5'-nucleotidase (18-20), immunochemical markers (17,21) and covalent chemical labels (22,23,24). The last have seen only very limited use (25) outside the red blood cell field. More extensive use of immunochemical identification would be expected if purified cell surface antigens can be obtained for preparation of antibodies.

Considerable advances in the conceptualization of membrane structure have occurred in recent years. The most popular model is the fluid mosaic membrane of Singer and Nicolson (26), which envisions proteins associated with a lipid bilayer primarily by hydrophobic forces. These proteins may be accessible to one surface of the membrane or to both surfaces (if extended across the bilayer). Some of the proteins show a considerable degree of lateral mobility in the membrane. This has been demonstrated with rhodopsin, the immunoglobulins (27) and cell surface antigens (28). The topological distribution of lectin receptors can be

studied by electron microscope techniques. From these it has been suggested that the altered distribution of cell surface sites occurs because of crosslinking of the mobile components to which lectins (or antibodies) are bound to prevent them from distributing randomly. One difference between transformed and nontransformed cells appears to be the mobility of the components in the membrane (29). The source of these variations in mobility is still unclear. Nicolson and Painter (30) have studied the distribution of sialic acid in erythrocyte ghosts and have shown that this can be altered by antibody perturbation of components at the interior surface of the membranes. Thus protein-protein interactions within the membrane may play a role in determining the mobility of surface components. Unfortunately, the lectin techniques do not permit one to distinguish which components (or even what kind of components --- glycoproteins or glycolipid) are under observation. More specific studies, e.g. with rhodopsin or immunoglobulins, may relate to particular cellular phenomena that are not general for all membrane components. Singer (31) has recently reviewed the general aspects of membrane structure, particularly the fluid mosaic model, which relate to immunological properties of cells and to immune responses.

Cell Surface Properties

The importance of the cell surface to the control of cellular activities is widely recognized. Cell-cell interactions which are obviously important to cell aggregation and tissue formation are mediated at the cell surface. Contact inhibition or density-dependent growth is an example of control of cell growth by cell interactions. The action of some hormones on cellular activity has been shown to be

initiated at the cell surface (32). The association of cancer with changes in cell surface properties has been well established. Alterations in the adhesiveness of cancer cells have been recognized for some time (33). Electrophoretic mobility, antigenicity and permeability changes are all indicative of significant differences in the cell membrane (17). Investigations of cultured cells transformed with oncogenic viruses have been quite fruitful for the detection of membrane changes.

The presence of carbohydrates at the mammalian cell surface can be demonstrated by a number of techniques. Electron microscopists often refer to the "fuzzy coat" (34), which is rich in carbohydrates. Proteolysis of mammalian cells releases glycopeptides (35,36). The function of cell surface carbohydrates is largely obscure. Recently Pricer and Ashwell (37) have shown that removal of sialic acid from liver plasma membranes decreases their ability to bind circulating glycoproteins which are destroyed in the liver. Thus the carbohydrate of the liver cell surface may be involved in recognition of the circulating glycoproteins. Cuatrecasas and Illiano (38) have shown that sialic acid removal from fat cells results in enhanced glucose transport and loss of the effects of insulin on glucose transport and lipolysis, even though the binding of insulin is unaffected. The question must be raised whether the glycoprotein plays a direct role in these processes or whether it has a structural role of such importance that alteration of its conformation by sialic acid removal results in perturbation of the activities of other membrane components. Cell surface carbohydrate has also been implicated in cell adhesion or cell-cell aggregation (39). Changes in cell surface carbohydrates have been noted after treatment of cultured mammalian cells with oncogenic viruses. These changes are manifested as variation in

glycolipid composition (40-42) and agglutinability by plant phytohemagglutinins (43,44), which bind specifically to carbohydrates. Cells which have been released from "contact inhibition" by viral transformation show a restoration of the "contact inhibition" after treatment with monovalent plant agglutinins (45). Protease treatment of normal cells mimics viral transformation in that it also renders the cells agglutinable by plant lectins (43). Recent experiments with ferritin-labeled agglutinins have shown that the transformation or proteolysis results in an altered distribution of the agglutinin binding sites on the cell membranes rather than or as well as an increased number of agglutinin receptor sites (46). Warren, Glick and coworkers (47) have demonstrated differences in glycopeptides from normal and transformed cultured cells. The differences are manifested by molecular weight differences in the glycopeptides which appear to be related to a difference in their sialic acid contents and the presence of sialyl transferase.

High Molecular Weight Plasma Membrane Polypeptides

The high molecular weight (HMW) polypeptides will be defined for this presentation as polypeptides having <u>apparent</u> molecular weights greater than 150,000 and showing little or no carbohydrate by gross analytical or staining procedures. The latter qualification is made to exclude highly glycosylated glycoproteins which also may have high <u>apparent</u> molecular weights. The primary technique for demonstrating these species has been sodium dodecyl sulfate (SDS) electrophoresis, a method which has some drawbacks (48-50), but which has contributed enormously to progress in membrane studies. HMW polypeptides have

been noted for plasma membranes isolated from erythrocytes (48,50-53), BHK and L Cells (54), Sarcoma-180 ascites tumor cells (15) and rat kidney or liver (55). Results from other workers using different cell types (55) indicate that the HMW polypeptides are more characteristic of plasma membranes than of internal membranes of the cell. However, the association of these polypeptides with the plasma membrane is also dependent on membrane isolation conditions (15), so they might not be observed in all cases. With the exception of the erythrocyte membrane protein spectrin, which will be discussed in the next section, the functions of these proteins have not been described.

The question of whether these polypeptides are single, continuous sequences of amino acids has also not been adequately answered. The possibility of covalent crosslinking between individual amino acid residues on separate polypeptide chains cannot be ruled out. Such links occur in collagen (56), fibrin (57) and other structural molecules. The presence of such links in membrane proteins has been suggested by Brickbirkler, et al. (58). It is less likely that the HMW polypeptides result from noncovalent interactions because of their behavior in denaturing media (52), although this possibility has been suggested by Dunn and Maddy (59).

Spectrin

The only HMW membrane protein which has been characterized to a significant extent is spectrin of the erythrocyte membrane. The protein was originally isolated and named by Marchesi and Steers (53), who showed that it formed coiled filaments when incubated with ATP and Ca^{++} or Mg^{++}. The same protein was also isolated by Clarke (60), who called

it tektin A. The protein is present in erythrocyte ghosts from all species examined (61), and can be solubilized by dialysis or incubation of the membranes in low ionic strength buffers. EDTA or ATP may be included to aid in solubilization. The protein contains two polypeptide chains with molecular weights of about 200,000 and 220,000 as determined by SDS electrophoresis (62) or guanidine hydrocholoride chromatography (53). Dunn and Maddy (59) have recently suggested that these polypeptides are comprised of subunits of 40,000 based on a combination of electrophoresis and chromatography. This leaves the molecular nature of these polypeptides still somewhat uncertain until careful characterization studies are finished. Physical measurements suggest that the protein exists in a rod-like form and has limited solubility with increased ionic strength, divalent cation or decreased pH (60). Cross-linking studies indicate that the 200,000 and 220,000 units are present as a dimer in the protein (60), which then can be polymerized or can exist in the membrane in higher molecular weight forms (63).

When erythrocyte membranes are extracted with EDTA, a smaller polypeptide (MW 45,000 as determined by SDS electrophoresis) is eluted along with the spectrin (48,50). In some studies on spectrin it is difficult to determine whether this material was present in the preparations. The polypeptide sizes of the eluted materials are very similar to those observed from myosin and actin of muscle. In fact Guidotti (64) has suggested that these polypeptides represent an "erythrocyte actomyosin" which serves as an underlying network to support the structure of the membrane. Evidence to support the similarity to actomyosin is rather sparse, particularly since the physical properties of spectrin appear to be quite disparate from those of myosin. A low ATPase activity

(65) has been associated with spectrin extracts. In addition the lower MW polypeptide (200,000) is rather specifically phosphorylated by a membrane kinase (66). The question which remains in this field is whether there are actin-like or myosin-like molecules which perform purely structural roles in cells.

The association of spectrin with other molecules in the erythrocyte membrane may be of great importance to the membrane structure. Juliano, et al., (67) have demonstrated an interaction between spectrin and phospholipid monolayers or vesicles. Of even greater interest are the studies of Nicolson and Painter (30), who showed that antispectrin antibody sequestered inside ghosts will cause clustering of sialic acid residues at the cell surface. This implies an interaction between the spectrin and the membrane glycoprotein, which must span the bilayer. Such an interaction could be important as part of a transducing system for relaying signals from the exterior of the cell (glycoprotein) to the interior (spectrin).

The Effect of Proteolysis on Cells

Mild proteolysis of cultured fibroblasts causes changes that are similar, but not necessarily identical, to those which occur during cell transformation (68). Included among these are alterations in cell morphology (69), lectin agglutinability (70), cell surface distribution of lectin receptors (71), growth rate (72), and the presence of a surface protein detected by lactoperoxidase (73). The presence of plasminogen activating factors in transformed cells (74), has led to the suggestion that proteolysis may be directly involved in fibroblast transformation. Although this hypothesis may not be correct (75), the action of proteases

on fibroblasts is certainly of interest because of their multitudinous effects on cellular phenomena. In particular the effects of proteolysis on cell morphology and lectin receptors suggest that alterations in the cell cytoskeleton may be occurring (68). Shin and Carraway recently described a set of membrane associated polypeptides which may play a role in membrane stabilization and cell shape (76).

Immunology of Tumors

It is now generally accepted that most tumors in animals as well as man have tumor-specific antigens and/or tumor-associated antigens (TSA) the latter of which may also be found in small amounts on certain normal tissues (77-81). In addition, most investigators agree that the host's immune system plays a major role in the defense against neoplasms (80, 82-85). Because of the large number of expressed and potentially expressed antigenic determinants the immunogenicity of the cell surface is extremely complex (81). It is now apparent that certain tumors (virus induced) express some cross-reacting TSA's as well as possessing distinct non-crossreacting antigenic components. In contrast, tumors induced by a variety of chemical or physical agents appear to possess individually specific TSA's that do not cross-protect against similar tumors induced in other syngeneic animals with the identical carcinogens (81).

The primary goal of immunotherapy is to utilize the host's immune system to prevent manifestation of the tumor by specifically destroying all neoplastic cells even though these cells are disseminated throughout the host's body. Surgery, radiotherapy and chemotherapy are all vital techniques required to reduce tumor burden, but none of theses has

sufficient specificity to recognize and discriminately destroy widely distributed metastatic cells. Evidence indicates that tumor destruction can be accomplished by immunization with modified tumor cells, whose surfaces have been altered to enhance tumor specific immunogenicity without provoking autoimmune rejection of normal host tissue (86,87). For example, immunization has been successful in several tumor-host systems employing whole tumor cells which have been modified by repeated freeze thawing or neuraminidase treatment. Alternatively cells can be injected into the animal mixed with a "non-specific" immune stimulant such as bacillus Calmette-Guerin (88-91). More recent evidence has shown that sulfhydryl modifying agents (iodoacetamide) can enhance tumor cell immunogenicity in certain tumor-host systems (92). These findings certainly encourage the notion that efficient and effective immunizing agents do exist and can be obtained through suitable manipulation.

In conjunction with the search for superior immunizing agents one must also consider the immunological capabilities of the host prior to tumor development (normal status), during active tumor growth and at different times following anticancer therapy. Many aspects of the immunological scheme are as yet only partially resolved. The humoral and cell-mediated components, once believed to be separate and rather independent faculties of the immune system, now appear to vary in their degree of interaction from nearly independent activity against some types os stimulation to very close interdependence in reaction to other stimuli (80,81,84,85,92). In mice, once the tumor cell population reaches a mass of between 10^3 and 10^4 cells it appears to be sufficiently large to nullify those immunological responses of the host capable of inhibiting tumor growth (81,91). Many investigators now believe that

autosolubilized antigens, mostly of fetal origin, are released from the tumor cell. These antigens, free in circulation, are then able to interact with the host's lymphoid cells causing the loss of killer activity. These soluble antigens may also stimulate the production of specific immunoglobulins which apparently cover the tumor surface and protect it from immune destruction (80,81,85). Tumor burden, therefore, must be reduced below this nullifying level to allow immunotherapy to proceed. Therefore, surgery, radiotherapy or chemotherapy are required to reduce tumor burden but they must be timed correctly to allow regain of immune responsiveness of the host (94,95). Using chemotherapy, for example, the time of recovery of immunocompetence for any given host depends on the drug and its dosage schedule employed (96). At different times following administration of BCNU to mice with on going L-1210 leukemia infections, Kollmorgen found 36 hours between chemotherapy and immunotherapy optimal (91). This means that therapeutic protocols for each technique and agent employed to reduce tumor burden must be evaluated in a given tumor-host system prior to practical testing of immunotherapeutic agents and techniques. As complex as the testing program appears, the success in those few systems developed is encouraging.

CHAPTER II

METHODS

Growth and Isolation of Cells

Sarcoma-180 Ascites Tumor

Sarcoma-180 ascites tumor cells maintained at this university since 1965 were transferred by weekly intraperitoneal injection of approximately $1x10^5$ cells in 0.1 ml of Hepes balanced salts (97) (HBS) in COBS albino mice (Ham/ICR) of both sexes. After 7 days, approximately $8x10^7$ cells were recovered from the peritoneal cavity of the mouse by aspiration, and washed four times with ice cold Hepes balanced salts and centrifuged at 210g·min (750 rpm x 3 min in a Sorvall SS-34 head).

Sarcoma-180 in vitro Grown Cells

Sarcoma-180 (CCRF S-180) CCL8, purchased from the American Type Culture Collection Cell Respository in the 88th passage, was grown in milk dilution bottles with McCoy's 5A modified medium + 10% calf serum (98). Just prior to reaching confluency, the monolayer was scraped from the bottle with a rubber policeman and washed four times in ice cold HBS and centrifuged at 810g·min (2000 rpm x 3 min in a Sorvall SS-34 head).

13762 MAT

The 13762 MAT (Mammary Ascites Tumor), obtained from the Mason

Research Institute Tumor Bank (Worcester, Massachusetts) was maintained in Fischer 344 strain females 40-45 days old by intraperitoneal injection of 0.2 to 0.3 ml of ascites fluid from rats 10 days postimplantation. After 6-10 days, the cells were removed from the peritoneal cavity by aspiration and washed three times in ice cold HBS and centrifuged at 810g·min (2000 rpm x 3 min in Sorvall SS-34 head).

L-1210 Leukemia

L-1210 acute lymphoblastic leukemia, first described by Law, et al., was obtained from Dr. Kollmorgen, Oklahoma University Health Sciences Center (Oklahoma City, Oklahoma) and maintained by intraperitoneal injection of $1x10^3$ cells in 0.1 ml of HBS into BDF_1 mice of both sexes. After 5-6 days, the cells were removed from the peritoneal cavity by aspiration and washed four times in ice cold HBS and centrifuged 810g·min (2000 rpm x 3 min in a Sorvall SS-34 head).

Fetal Mouse Fibroblast in vitro Grown Cells

In vitro grown fetal mouse fibroblast cells (LM) were grown on medium-199 plus 10% calf serum (99) in milk dilution bottles. Cells were harvested using a rubber-policeman, washed four times with Hepes and 10^4 cells injected (i.p.) per animal. Fresh cells were prepared for each day of injections.

Plasma Membrane Isolation

Isolation of Plasma Membranes from the Sarcoma-180 Ascites Tumor

Isolation by a Membrane Stabilization Method

Washed ascites cells (SA-180) were suspended in 10 volumes of ice cold 40 mM Tris (pH 7.4) and the cells allowed to swell for three minutes. The swollen cells were then centrifuged 1446g·min (2000 rpm x 3 min in a SS-34 head). This procedure hemolyzes erythrocytes and leaves ghosts in the supernatant. The pellet of washed, swollen cells was suspended in 10 volumes of 1 mM $ZnCl_2$ at 25° C. for 15 minutes, then cooled on ice for an additional 15 minutes. The cells, which appeared swollen, but intact under phase contrast microscopy, were then homogenized by 15-20 strokes of a Dounce homogenizer fitted with a tight pestle until most of the cells appeared ruptured and their membranes were visible as large sheets and intact envelopes. Nuclei were not ruptured as judged by comparisons of photomicrographs and cell vs. nuclei counts made after each stroke. The homogenate was diluted with an equal volume of 40 mM Tris (pH 7.4) and centrifuged 210g·min in a swinging bucket head (750 rpm x 3 min in a Sorvall HB-4 head), a step which pellets nuclei and whole cells, but leaves plasma membranes in the supernatant. The supernatant was then decanted into a new centrifuge tube and centrifuged 4820g·min (2000 rpm x 10 min in a Sorvall HB-4 head) to pellet the plasma membranes and a few contaminating nuclei. The pellet was washed once more in 40 mM Tris (pH 7.4) and centrifuged 4820g·min (2000 rpm x 10 min in a Sorvall HB-4 head).

Purification by Sucrose Density Gradient. The washed pellet was diluted in 40 mM Tris (pH 7.4) and layered on a discontinuous sucrose gradient composed of 8 mls each of 40, 45, 50, 55% (w/w) sucrose (determined by refractive index) in 20 mM Tris (pH 7.4) and centrifuged using Model L-5 65 Beckman ultracentrifuge in a SW-27 head at 15,000 rpm for 60 minutes at 4° C. (1,788 x 10^6g·min). Bands at the 40-45% (B_2) and 45/50% (B_3) interfaces contained plasma membrane envelopes free of nuclei with little particulate material. These bands were removed either with a bent needle or ISCO Density Gradient Fractionator equipped with O.D. Monitor (O.D. 405) and syringe pump. The membranes were washed three times in 40 mM Tris (pH 7.4) and centrifuged 4820g·min. HBS could be substituted at this step with no detectable changes in the membrane.

Purification by the Two-Phase Method of Brunette and Till. The washed pellet of crude plasma membranes could alternately be purified by partition between two immiscible phases composed of dextran (Dextran T500 Pharmacia Uppsala, Sweden), and polyethylene glycol (Carbowax 6000 Applied Sciences Laboratories, Inc., State College, Pa) polymers in a phosphate buffer (pH 6.5) with 1 mM $ZnCl_2$. The pellet was suspended in four volumes of upper phase, mixed with four volumes of lower phase and centrifuged 1.2 x 10^5g·min (10,000 rpm x 10 min in a Sorvall HB-4 head) in a swinging bucket head to separate the two phases. The interface band was further purified by repeating the two-phase separation. The interface band, which appeared to be composed of large plasma membrane fragments, was washed four times in either HBS or 40 mM Tris (pH 7.4).

Isolation by a Microsomal Method

Washed ascites cells were washed additionally two times in 0.25 M sucrose-20 mM Tris (pH 7.4) and suspended in 120 ml of 0.25 M sucrose-20 mM Tris (pH 7.4). This buffer does not cause the cells to swell noticeably. The cells were disrupted on ice using a Sorvall Omni-mixer with 50 ml chamber and spinning at 11,000 rpm. Usually 6-8 30 second bursts, alternated with 60 second cooling periods were required to swell and burst the cells. The cells were observed by phase contrast microscopy for loss of plasma membranes from cells and appearance of nuclei. Cell vs. nuclei counts showed no breakage of nuclei. Homogenization was stopped when about 60% of the cells were disrupted. The homogenate was centrifuged 7540g·min (2500 rpm x 10 min in a Sorvall SS-34 head) to sediment nuclei and whole cells. The supernatant was saved on ice and the pellet resuspended in 50 ml of 0.25 M sucrose-10 mM Tris (pH 7.4), and homogenized in the Omni-mixer at 11,000 rpm for 3-4 additional bursts, until 50% of these cells were broken with the nuclei remaining intact. Use of a small aliquot to determine the point of nuclei breaking (6-8 bursts) allowed us to stop short of this event. The homogenate was centrifuged 7540g·min and the supernatant combined with that of the first homogenization and centrifuged 1.737×10^6g·min (12,000 rpm x 10 min in a Sorvall SS-34 head) to pellet mitochondria.

The supernatant was carefully removed and centrifuged 7.222×10^6 g·min (27,000 rpm x 75 min in a SW-27 Beckman head) at 4° C. The pellet consisting of membrane microsomes was then fractionated by discontinuous sucrose gradient flotation. A discontinuous gradient was formed by making the pellet to 40% (w/w) in sucrose with 60% sucrose and layering over the pellet 9 mls each of 36% (w/w) sucrose, 32% (w/w) sucrose and

8.3% (w/w) sucrose with all solutions made 20 mM in Tris (pH 7.4). The gradient was centrifuged to equilibrium (27,000 rpm x 4 hours) and the band at the 32-36% (w/w) sucrose interphase removed either with a bent needle or ISCO Density gradient fractionator and washed four times in 40 mM Tris (pH 7.4) by centrifuging 1.2 x 10^5g·min (10,000 rpm x 10 min in a SS-34 head).

Plasma Membrane Isolation from the L-1210 Leukemia by a Membrane Stabilization Method

Washed leukemia cells were suspended in 10 volumes of ice cold 40 mM Tris (pH 7.4) for four minutes and allowed to swell before centrifuging 1440g·min (2000 rpm x 3 min in a Sorvall SS-34 head). This step increases the size of the L-1210 leukemia cells by 1.5 to 2 fold while hemolyzing erythrocytes and leaving ghosts in the supernatant. The swelling step was repeated one time. The pellet was then suspended in 10 volumes of ice cold 1 mM $ZnCl_2$ for three minutes and homogenized 10-15 strokes with a Dounce homogenizer fitted with the tight pestle until 80-90% of the cells appeared broken by phase contrast microscopy, a step which does not break nuclei. The homogenate was then diluted with an equal volume of 40 mM Tris and centrifuged, 1.07 x 10^4g·min (3000 rpm x 10 min in a Sorvall SS-34 head). The pellet was then suspended in 10 volumes of 40 mM Tris (pH 7.4) and centrifuged 813g·min (1500 rpm x 3 min in a Sorvall HB-4 head) in a swinging bucket head. This pellets nuclei and whole cells, leaving plasma membranes in the supernatant. The plasma membrane enriched pellet was washed two times by diluting in 40 mM Tris and centrifuging 1.07 x 10^4g·min.

The pellet from the plasma membrane enriched fraction was then

purified by the two-phase method of Brunette and Till (100) consisting of Dextran-T500 and Carbowax 6000 a polyethylene glycol polymer, phosphate buffer (pH 6.5) and 1 mM $ZnCl_2$. The plasma membrane enriched fraction was suspended in 10 original cell volumes of upper phase, and 10 volumes of lower phase was added. The two phases were separated by centrifugation 1.20 x 10^3g·min (10,000 rpm x 10 min in a HB-4 head).

The interface band was removed either by a bent needle or decanted and the two-phase separation was repeated. The second two-phase pellet was judged free of nuclei and whole cells by phase contrast microscopy and was washed four times in 40 mM Tris (pH 7.4) by centrifugation of 1.07 x 10^4g·min.

Plasma Membrane Isolation from the 13762 MAT by a Membrane Stabilization Method

The washed mammary adenocarcinoma cells were suspended in 10 volumes of ice cold 40 mM Tris (pH 7.4), allowed to swell for four minutes at 4° C. and centrifuged 1928g·min (2000 rpm x 4 min in a Sorvall SS-34 head). The swelling step was repeated and the second pellet yielded swollen tumor cells while hemolyzing erthrocytes and leaving their ghosts in the supernatant. The swollen cells were suspended in 11 volumes of ice cold 1 mM $ZnCl_2$ for two minutes and homogenized by 20 strokes of a Dounce homogenizer fitted with the tight pestle, until about 85% of the cells were broken. The homogenization yields whole envelopes and large sheets of plasma membranes. The homogenate was then diluted with an equal volume of 40 mM Tris (pH 7.4) and centrifuged 4.7 x 10^4g·min (1750 rpm x 10 min in a Sorvall HB-4 head) in a swinging bucket head. Exact speed is critical here since 4700g·min leaves plasma

membranes in the supernatant whereas 4820g·min pellets them. A Power Instruments, Inc. (Skokie, Illinois) Optical Tachometer model B-891 was used to set the centrifuge speed for each run. The supernatant was decanted and recentrifuged at 2.44 x 10^5g·min (4500 rpm x 10 min in a Sorvall HB-4 head) to pellet plasma membranes. The pellet was washed two times by suspending in 40 mM Tris (pH 7.4) and centrifuging 2.44 x 10^5g·min. The pellet was then purified by layering it in 40 mM Tris (pH 7.4) on a discontinuous sucrose density gradient composed of 8 ml each of 40%, 45%, 50%, 55% (w/w) sucrose in 20 mM Tris (pH 7.4) and centrifuging it in a model L-5 65 Beckman ultracentrifuge using a Beckman SW-27 head at 15,000 rpm for 60 minutes at 4° C. (1.788 x 10^6g·min ave.). All bands were removed either with a bent needle or ISCO Density gradient fractionator and washed two times by suspending in either 40 mM Tris (pH 7.4) or HBS and centrifuged 1.20 x 10^5g·min (10,000 rpm x 10 min in a Sorvall SS-34 head).

Studies on the Structure of the S-180 Plasma Membrane

The Effect of Trypsin on the S-180 Plasma Membrane

Time-Lapse Cinematography of the Effect of Trypsin on the S-180 Cell

Cinematography studies were carried out in a Dvorak-Stotler Controlled Environment Culture System. S-180 cells suspended in M-199 + 10% calf serum were inoculated into the chamber and incubated (inverted) in a CO_2 gas phase incubator at 37° C. for 4 hours to allow the cells to

attach to the coverslip. The chamber was then placed onto the microscope stage and perfused with M-199 + 10% calf serum at 1 ml/hr. The chamber temperature was maintained at 37° C. by a Sage air curtain.

Photography was performed using phase contrast optics and a 16 mm time-lapse system. Sequences were filmed at 15 frames/minute with a 0.75 second exposure.

Cells were perfused for 12 hours prior to experimentation to allow flattening and characteristic spindle morphology to occur. The culture was then filmed for at least 1 hour to record its characteristics as a control. Trypsin (10 ug/ml in M-199 minus calf serum) was added by flushing the chamber to insure uniform trypsin exposure. Filming was continued throughout the process and 15 minutes after trypsin addition, the chamber was flushed with normal medium (M-199 + calf) and returned to perfusion at 1 ml/hour and filming continued to record changes in cell morphology. Control sequences were run in which only M-199 minus calf serum without trypsin was used in place of trypsin. These sequences showed no change in cell morphology either during or following the 15 minute M-199 minus calf serum exposure.

The Effect of Trypsin on the SA-180 Cell

Five days after implantation, SA-180 ascites cells were aseptically removed from the mouse peritoneal cavity, washed three times in Hepes balanced salts and once in culture medium-199 at 4° C. The cells were resuspended to 1 x 10^4/ml in M-199, warmed to 37° and trypsin added to give 0, 0.1, 1.0 and 5.0 ug of trypsin per ml. The cells were mixed by gentle swirling and incubation at 37° C. continued. Control samples were kept on ice without trypsin to check for proteases in the 37° C.

zero trypsin control. After 15 minutes the reaction was stopped by either: 1) adding a 20 fold excess of phenylmethylsulfonylfloride (PMSF) in DMSO, and allowing the cells to incubate at 23° C. for 30 minutes followed by four washes at 4° C. in HBS, or 2) by adding a 100 fold excess of soy trypsin inhibitor (chromagraphically purified) at 4° C., incubating for 30 minutes then washing four times at 4° C. with HBS. The membranes were then isolated by the membrane stabilization two-phase method.

The Effect of Trypsin on the S-180 Cell

Sarcoma-180 in vitro grown cells (S-180) were grown in milk dilution bottles (glass), with McCoy's 5A modified medium + 10% calf serum (A). Just prior to reaching confluency the monolayer was scraped from the bottle with a rubber policeman, pelleted at low speed and washed three times with HBS at 4° C. once in M-199, then suspended to 1 x 10^4 cell/ml in M-199 and trypsinized as described above.

The Effect of Trypsin on the Isolated SA-180 Plasma Membranes

SA-180 plasma membranes isolated by the membrane stabilization two-phase procedure were suspended in Hepes balanced salts at 3 mg/ml. One sample was kept at 4° C. to check for membrane proteolysis. The membranes were warmed at 37° C. and trypsin added to give 0.0, 0.1, 1.0, 5.0, 10.0, 20.0, 50.0 ug trypsin per ml. The membranes were mixed and incubation at 37° C. continued. After 15 minutes, the reaction was stopped by adding a 20 fold excess of PMSF in DMSO and allowing the membranes to incubate at 23° C. for 30 minutes then washing four times in HBS. The

membranes were immediately solubilized in the solubilization buffer and boiled.

Lactoperoxidase Labeling of S-180 and SA-180 Cells

S-180 *in vitro* grown cells grown and harvested as described for SA-180 cells were washed four times in Hanks balanced salts (pH 7.4) and adjusted to 4 x 10^7 cells/ml with Hanks. Two ml of the cells were added to 1 ml of labeling mixture (0.5 uM lactoperoxidase from bovine milk), 10 uM KI, 100 uCi $Na^{125}I$ in Hanks balanced salts (pH 7.4) and 5 aliquots of 2.3 mM H_2O_2 added with stirring each 30 seconds for 13 minutes. At 15 minutes the labeling was stopped by adding 0.5 ml of 100 mM KI and stirring an additional 10 minutes. The labeled cells were washed four times in Hanks balanced salts and membranes isolated by the membrane stabilization two-phase method.

Isolation of Several High Molecular Weight Proteins from the SA-180 Plasma Membrane

A Comparison of Extraction Fluids

SA-180 plasma membranes isolated by the membrane stabilization two-phase method and stored frozen were thawed and pipetted into three centrifuge tubes to a final concentration of 14 mg membrane protein per tube. The tubes were centrifuged at 4000 rpm x 10 minutes to pellet the plasma membranes. The pellets were then resuspended in 20 ml of extraction fluid: a) distilled deionized water (pH 6.0), b) 1.0 M NaCl, c) 5 mM glycine, 1 mM EDTA, 5 mM mercaptoethanol pH 9.5 (GEM), vortexed for 10 seconds and incubated at room temperature (23° C.) for 20 minutes.

At this time, the membranes were slightly fragmented as shown by phase contrast microscopy. The extracts were then incubated at 4° C. overnight and centrifuged in a SW 25.1 head at 25,000 rpm for 60 minutes at 4° C. ($5880 \times 10^3 g \cdot min$). The supernatant from each extract was concentrated by pressure dialysis using a Diaflo XM-50 membrane at 4° C. (molecular weight exclusion limit 50,000 MW). The pellets and supernatants were examined by SDS 5% acrylamide electrophoresis and stained with coomassie blue.

A Comparison of Extraction Times

SA-180 plasma membranes isolated by the membrane stabilization two-phase method and stored frozen as a pellet (30 mg of membrane protein) were extracted with 5 ml of GEM pH 9.4 at 4° C. with stirring for four hours. The extract was then centrifuged in a SW 65 head at 100,000 x g average for one hour ($6.0 \times 10^6 g \cdot min$). The pellet was resuspended in 5 ml of GEM pH 9.4 and stirred at 4° C. for an additional 20 hours and centrifuged for $6.0 \times 10^6 g \cdot min$ as before. Both supernatants were concentrated by pressure dialysis using a Diaflo XM-50 membrane. The total extracted protein was 10 mg or 33% of the original protein. The supernatants were analyzed by SDS electrophoresis and coomassie blue staining.

Isolation of the High Molecular Weight Doublet

SA-180 plasma membrane isolated by the membrane stabilization sucrose method, and stored frozen was extracted overnight with GEM pH 9.4 at 4° C. with stirring and centrifuged in a SS-34 centrifuge head at 15,000 rpm for 30 minutes ($910 \times 10^3 g \cdot min$). This yielded 22 mg of extracted soluble protein. The supernatant was dialyzed against distilled

water with four changes and the dialysis centrifuged at 15,000 rpm x 10 minutes (200 x 10^3g·min). The supernatant contained 2.0 mg of protein, the pellet had 14 mg. The pellet was solubilized in 2 ml of GEM pH 9.4, made to 10% sucrose and applied to a 90 cm x 2.5 cm G-200 column and eluted with GEM pH 9.4. The void volume (Vo) from the column which showed a large A_{280} absorbance was pooled and dialyzed against distilled water overnight and centrifuged at 18,000 rpm for 30 minutes (1200 x 10^3 g·min).

The pellet was analyzed by SDS electrophoresis and coomassie blue staining. Part of this pellet was suspended in 6.0 N HCL, evacuated, sealed and hydrolyzed at 110° C. for 22 hours (101). The tube was opened and the HCL removed by evaporation under vacum. The sample was run on a Beckman Model 120 C equipped with an autolab analyzer.

Ammonium Sulfate Fractionization

SA-180 plasma membranes isolated by the Zn-2Ph method, stored frozen were extracted with GEM pH 9.4 at 4° C. with stirring, centrifuged 6.0 x 10^6g·min at 4° C. The supernatant was concentrated using a Diaflo XM-50 pressure dialysis at 4° C. The supernatant in GEM was stirred at 4° C. and powdered ammonium sulfate added to give 0.205 gm/ml (29.4% saturation) and centrifuged at 5000 rpm x 20 minutes. The supernatant was stirred and made to 0.364 gm/ml (52.2% saturation) and centrifuged 5000 rpm x 20 minutes. The supernatant was made to 0.5 gm/ml (71.1% saturation) and centrifuged 5000 rpm x 20 minutes. The pellets and supernatant were dialyzed against distilled water and analyzed by SDS acrylamide electrophoresis.

Sepharose 4B Chromatography under
Non-Denaturing Conditions

SA-180 plasma membranes (32 mg) isolated by the membrane stabilization two-phase method, stored frozen, were thawed and extracted with 35 ml of GEM pH 9.4 overnight at 4° C. and centrifuged 6.0 x 10^6g.min. The supernatant was concentrated by pressure dialysis using a Diaflo XM-50 membrane to 2 ml and applied to a 90 x 2.5 cm Sepharose 4B column and eluted with GEM pH 9.4. Three ml fractions were collected and A_{280} determined on a Perkin-Elmer model 220 spectrometer. Void volume (Vo) was determined using 12 mg of blue dextran and included volume (Vi) using 40 mg of 2 mercaptoethanol.

Sepharose 4B Chromatography under
Denaturing Conditions

SA-180 plasma membranes isolated by the membrane stabilization two-phase method stored frozen, were thawed (46 mg membrane protein) and solubilized in 5 ml of 5% SDS, 5 mM EDTA, 50 mM phosphate pH 7.4 by placing in boiling water for 10 minutes, made to 4% in mercaptoethanol and O_2 removed by purging with N_2 gas. The solubilized membranes were covered, stirred overnight and then applied to a 90 x 2.5 cm Sepharose 4B column and eluted with 0.05 M Tris pH 7.4 + 0.1% SDS and 3 ml fractions collected. Protein was monitored as A_{280} using an ISCO 2A column monitor. The major peaks were pooled and concentrated by pressure dialysis using a Diaflo chamber using a XM-50 membrane. The pooled fractions were analyzed by SDS acrylamide electrophoresis.

The Use of S-180 Plasma Membranes as Anticancer Agents

Animals for Immunological Testing

Female Ham/ICR mice, 50 to 55 days of age, were used for each experiment following at least one week of isolation in this laboratory. The mice were maintained on Purina laboratory chow and tap water ad libitum.

Membrane Isolation

SA-180 plasma membranes were isolated by the membrane stabilization two-phase method. All isolation procedures were conducted under aseptic conditions, either in a dust hood or a laminar flow hood. Membrane preparations were used immediately or stored frozen (-30° C.) until use (one freeze-thawing had no detectable effect on immunizing potential).

Human and mouse erythrocyte membranes were prepared as described by Carraway and Shin (76). Human blood was obtained from the Dallas Community Blood Bank and used within 1 week of withdrawal date. Ham/ICR mouse blood was removed from the retroorbital sinus, stored in Alsever's solution, modified and used within two days of withdrawal.

Immunization of Mice

Membrane protein concentrations were determined by the Lowry method (102). Samples were suspended in Hepes balanced salts solution to the desired protein concentration. Animals were immunized with 0.1 ml i.p./injection of the Hepes-membrane suspension or Hepes solution alone as the control. Immunization schedules consisted of 3 injections/week for two

weeks with a total of 3.0 gm membrane protein injected or two injections per week for three weeks with a total injection of 4.4 mg of membrane protein.

Analytical Procedures

Enzyme Assays

Protein was determined by the method of Lowry, et al., (102). Inorganic phosphate released during enzyme assays was measured by the method of Lazarus (103), except in the final step, phosphomolybdate was extracted by mixing the final reaction mixture with one volume of isoamyl alcohol. After centrifugation the absorbance of the alcohol phase was measured at 740 m . The 5'-nucleotidase and succinate dehydrogenase (succinate 2-(p-indophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium reductase) were assayed as described by Morre' (104). Glucose-6-phosphatase was assayed by the method of Goldfischer (105).

SDS Acrylamide Gel Electrophoresis

Membrane samples were dissolved by boiling for 5 minutes in 4% SDS-1% mercaptoethanol-5 mM EDTA-50 mM phosphate (pH 7.4) at a protein concentration of 3-10 mg/ml, and the sample incubated overnight at 37° C. One-tenth volume of glycerol-0.01 mg ml bromphenol blue was added and a sample 50-300 ug of protein per gel applied to 1 cm gels (concentration 5%; crosslinker/monomer ratio, 0.026). After a 45 minute prerun to remove persulfate, the samples were loaded and the gels were run at 8 m amp/gel until the bromphenol blue tracking dye reached the end of the gel (5 hours total running time). Gels were stained for protein and carbohydrate by the method of Fairbanks, et al., (50). The stained gels

were scanned at A_{560} on a Gilford model 240 single beam spectrophotometer equipped with a model 2410 linear gel scanner.

The molecular weights of the membranes were estimated from a plot of the logarithm of molecular weight vs. migration distance for a series of standard proteins: ribonuclease, bovine serum albumin, β-galactosidase.

Electrophoresis of ^{125}I Labeled Membranes

Plasma membranes were solubilized and electrophoresed as described above. Three gels were run, one stained for coomassie blue, one for PAS and the other immediately sliced into 2 mm slices with a home-made gel slicer. The slices were incubated with 0.5 ml of NCS solubilizer at 50° C. for 24-48 hours, cooled and 10 ml of Brays liquid scintillation cocktail (106) added. The vials were kept at room temperature for 12 hours, then cooled to 4° C. before counting in Packard Tri-Carb liquid scintillation spectrometer (Model 3320, Packard Instrument Co., Downer Grove, Illinois).

CHAPTER III

RESULTS

Plasma Membrane Purification

The methods used to isolate plasma membranes from the 3 lines of ascites tumors studied are based on two very different approaches. The first is based on homogenization of the cell in 0.25 M sucrose with a high speed blender which reduces all of the cellular membranes to semi-permeable microsomes. The other is based on a stabilization of the cell plasma membrane by the divalent metal ion Zn^{++} followed by gentle homogenization to yield large fragments of plasma membranes. Each of the cell types required modification of the isolation scheme to yield plasma membranes of reasonable purity.

Isolation and Characterization of S-180 and SA-180 Plasma Membranes

The isolation of plasma membranes from the Sarcoma-180 ascites tumor by the Zn^{++} method, shown in Fig. 1, yielded plasma membranes of reasonable purity when either the sucrose gradient or the two-phase isolation was used. Enzymatic specific activities and relative enrichments for the various fractions are shown in Table I. The location of plasma membrane envelopes seen by phase contrast microscopy during the isolation protocol correlated very well with the location of 5'-nucleotidase, and showed a 23 fold purification over the homogenate value.

Figure 1. Membrane Isolation Scheme for the Sarcoma-180 Tumor Using the Satbilization Technique

Details of the isolation procedure are given in the methods section.

Washed Cells

40 mM Tris

Supernatant (Hemolyzed RBC)

Pellet (Swollen Cells)

1 mM $ZnCl_2$ at 25° C. for 15 min
cool on ice for 15 min.

Zn^{++} Treated Cells

Homogenize (Dounce)
Dilute with 40 mM Tris

Supernatant (Cytoplasm)

Pellet (Nuclei, Whole Cells and Plasma Membranes)

Centrifuge at 750 rpm x 3 min

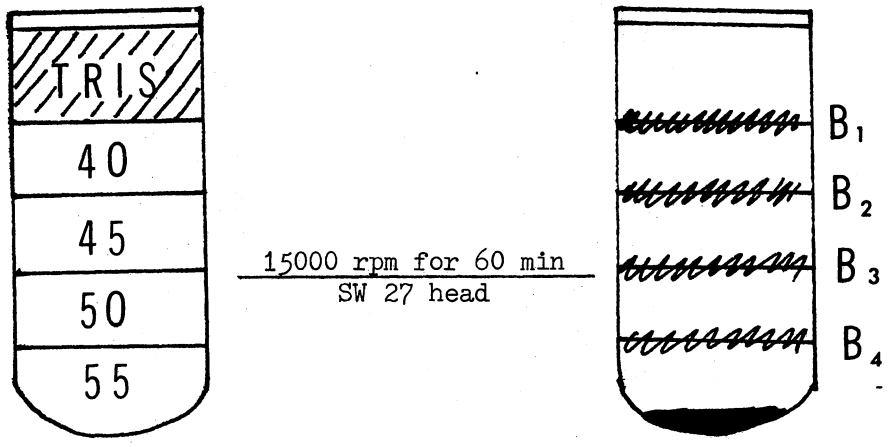
Pellet (Nuclei and Whole Cells)

Supernatant (Plasma Membrane enriched fraction)

Centrifuge at 2000 rpm x 10 min

Supernatant (Cytoplasm)

Pellet (Plasma Membrane enriched fraction)



Wash in 40 mM Tris

TABLE I

ENZYME SPECIFIC ACTIVITIES AND ENRICHMENT OF MEMBRANE FRACTIONS OF SARCOMA-180 ASCITES TUMOR AND IN VITRO SARCOMA-180

Values in parentheses are relative specific activities expressed as the ratio of the value for the particular fraction to that of the homogenate. All specific activities are in μMoles/mg/min.

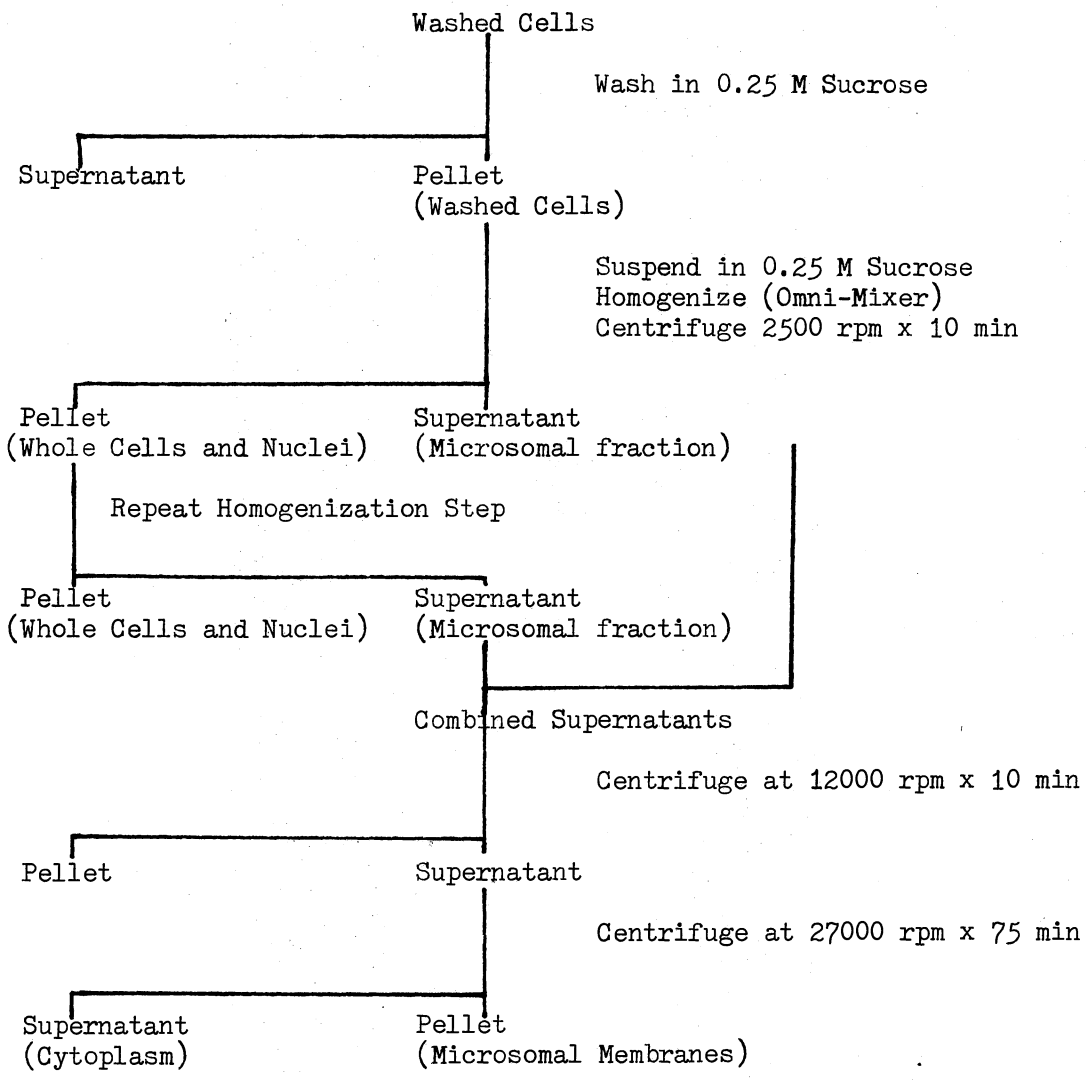
Fraction	Glucose 6 Phosphatase	Succinic INT Reductase	5'-Nucleotidase
SA-180 Membrane Stabilization Sucrose Method			
Homogenate	0.1 (1)	2.3 (1)	0.1 (1)
Plasma Membranes (Bd 2+3)	0.5 (5)	1.0 (0.4)	2.3 (23)
SA-180 Microsomal Method			
Homogenate	0.3 (1)	7.0 (1)	0.2 (1)
F_1	18.1 (60)	8.0 (1.1)	0.4 (2.0)
F_2	5.2 (17)	2.0 (0.3)	3.8 (19)
F_3	5.2 (17)	16.0 (2.3)	0.8 (4)
F_4	0.2 (0.7)	4.0 (0.6)	0.3 (1.5)
Sarcoma-180 In Vitro Line			
Homogenate	0.2 (1)	2.0 (1)	0.3 (1)
Interface band	0.5 (2.5)	0.1 (0.05)	5.3 (18)

Nuclei were not present as judged by phase contrast microscopy. Succinate dehydrogenase, a mitochondrial marker, was reduced to less than the homogenate, but there was some glucose-6-phosphatase contamination by the sucrose method, indicative of endoplasmic reticulum. The two-phase method did not have this enzyme contamination.

The microsomal preparation shown in Fig. 2, yielded a homogenate in which only the nuclei could be distinguished, and all subsequent purification steps had to be monitored by marker enzymes. The F2 fraction showed a 19 fold enrichment of 5'-nucleotidase over the homogenate value, without contamination by succinate dehydrogenase or nuclei. There was apparent contamination with glucose-6-phosphatase, however. The three ways of isolating plasma membranes did vary in their yield of membrane protein from a comparable number of cells. The two-phase separation yielded about 2.5 mg/8 x 10^7 cells, the sucrose gradient about 1.1 mg/ 8 x 10^7 cells and the microsomal method about 0.5 mg/ 8 x 10^7 cells. Because of the increased yield of membranes per cell by the two-phase method, this was the only method used to isolate plasma membranes from the S-180 in vitro grown cells. Plasma membranes isolated from these cells showed a 18 fold purification of 5'-nucleotidase with no succinate dehydrogenase or nuclei and low levels of glucose-6-phosphatase. Plasma membranes from Sarcoma-180 isolated by various methods were next subjected to electrophoresis on 5% acrylamide gels and stained for protein by coomassie blue and glycoprotein by the PAS stain.

When the membrane stabilization sucrose method was compared with the microsomal method for SA-180 membranes in Fig. 3, the following differences were noted. Membranes isolated by the microsomal method had greatly reduced amounts of the three highest molecular weight

Figure 2. Microsomal Method Isolation Scheme for the Sarcoma-180 Tumor


Washed Cells
Wash in 0.25 M Sucrose
Supernatant
Pellet
(Washed Cells)
Suspend in 0.25 M Sucrose
Homogenize (Omni-Mixer)
Centrifuge 2500 rpm x 10 min
Pellet
(Whole Cells and Nuclei)
Supernatant
(Microsomal fraction)
Repeat Homogenization Step
Pellet
(Whole Cells and Nuclei)
Supernatant
(Microsomal fraction)
Combined Supernatants
Centrifuge at 12000 rpm x 10 min
Pellet
Supernatant
Centrifuge at 27000 rpm x 75 min
Supernatant
(Cytoplasm)
Pellet
(Microsomal Membranes)

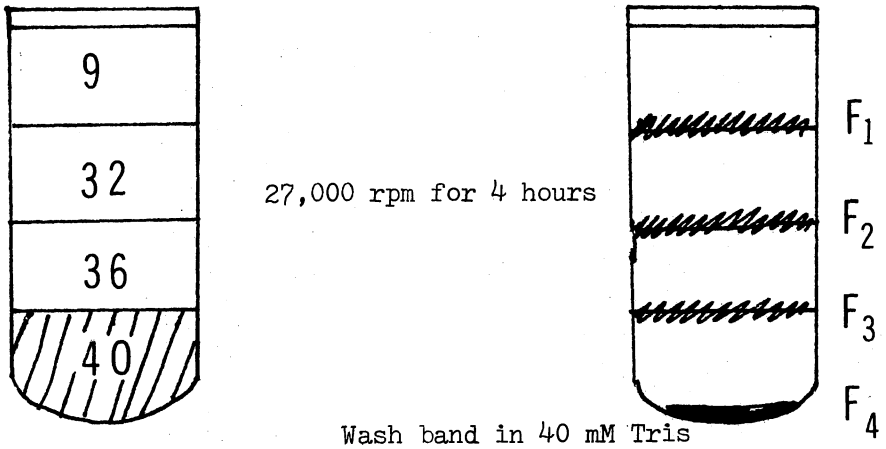
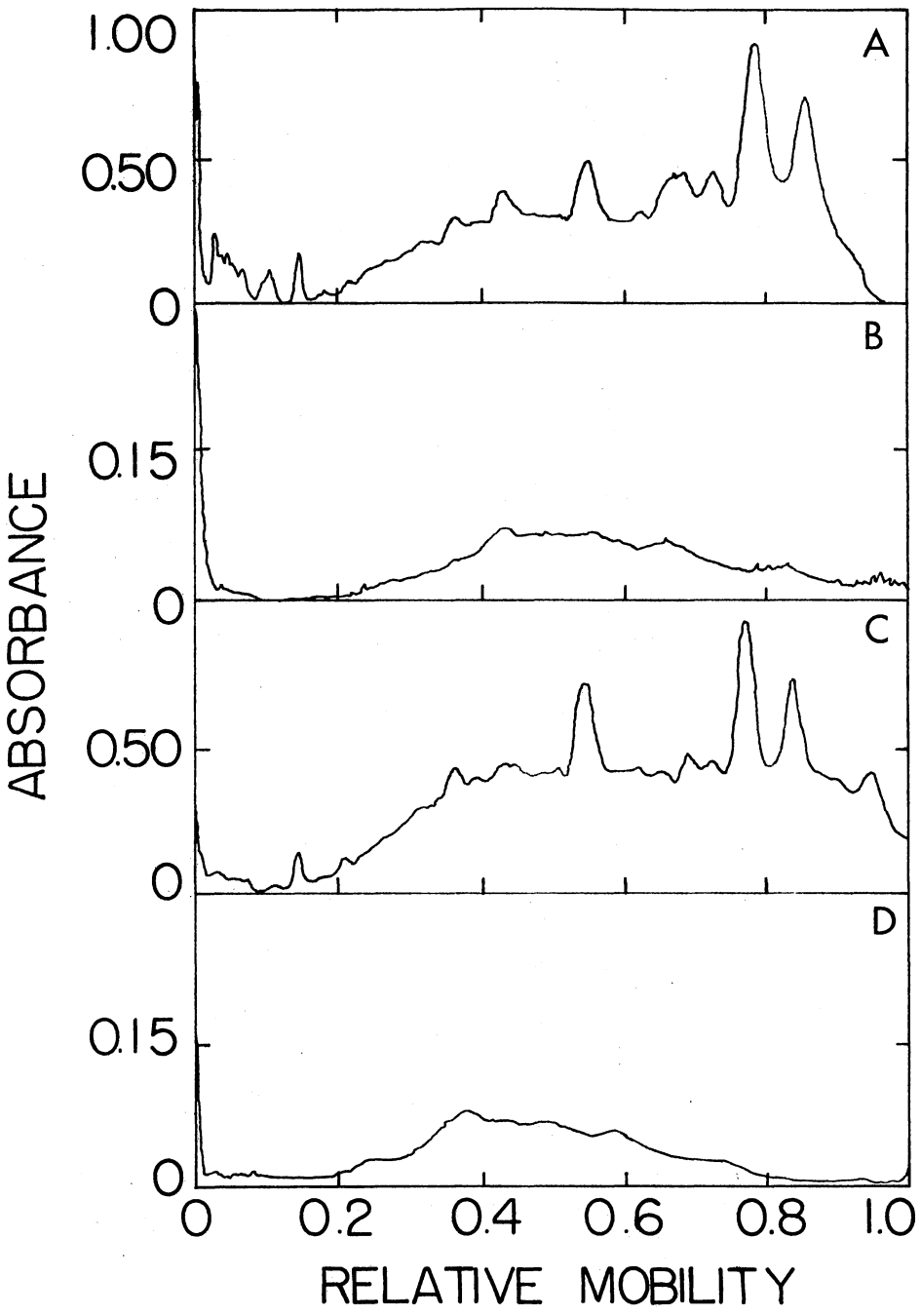

9
32
36
40
27,000 rpm for 4 hours
F1
F2
F3
F4
Wash band in 40 mM Tris

Figure 3. Gel Scans of Coomassie Blue and Periodate Acid-Schiff stained Polyacrylamide Gels of Sarcoma-180 Ascites Tumor Plasma Membranes Isolated by the Membrane Stabilization and Microsomal Methods

(A) CB and (B) PAS stains of SA-180 membrane stabilization method isolated plasma membranes. (C) CB and (D) PAS stains of SA-180 microsomal method isolated plasma membranes.


1.00
0.50
0
A
B
0.15
0
C
0.50
0
D
0.15
0
ABSORBANCE
0
0.2
0.4
0.6
0.8
1.0
RELATIVE MOBILITY

coomassie blue bands (300^{+}, 280, 240) but had almost quantitatively the same amount of protein in all other bands. When the glycoprotein patterns shown by the PAS stain of SA-180 were compared between the Zn^{++} and microsomal method, the microsomal showed a higher apparent molecular weight glycoprotein (115 vs. 100 x 10^{-3}) and a low molecular weight glycoproteins. When the gel patterns for the S-180 plasma membranes were examined the polypeptide distribution was identical to that of the SA-180 plasma membrane although there are some minor differences in staining intensity. By comparison the periodate-Schiff staining patterns show striking differences. The ascites form has a major band near the center of the gel plus smaller amounts of staining material near the top of the gel and near the bottom. The cultured form seen in Fig. 4, shows two major intense (PAS-1 and 2) sharp bands which have barely migrated into the gel plus smaller amounts of staining material near the center and bottom of the gel. Because of variation in the amount of the high molecular weight band during several isolations, plasma membranes were isolated from the cell just prior to confluency and two days after the cells reached confluency. The cells prior to confluency showed large amounts of the high apparent molecular weight glycoprotein, while those harvested after confluency were almost devoid of the glycoprotein.

Isolation and Characterization of L-1210 Plasma Membranes

The isolation scheme shown in Fig. 5, stabilizes the L-1210 plasma membrane so that whole envelopes and large fragments can be isolated. This cell type required two swelling steps and a very short treatment with ice cold 1 mM $ZnCl_2$ to allow the disruption of the cell without

Figure 4. Gel Scans of Coomassie Blue and Periodate-Acid-Schiff Stained Polyacrylamide Gels of the *in vitro* grown Sarcoma-180 Plasma Membrane Isolated by the Membrane Stabilization Method

(A) CB and (B) PAS stains of S-180 plasma membrane from 5% acrylamide gels run at 8 M Amp/gel for 5 hours. (C) PAS stain of 5% acrylamide gel run at 8 M Amp/gel for 10 hours to allow it to migrate further into the gel.

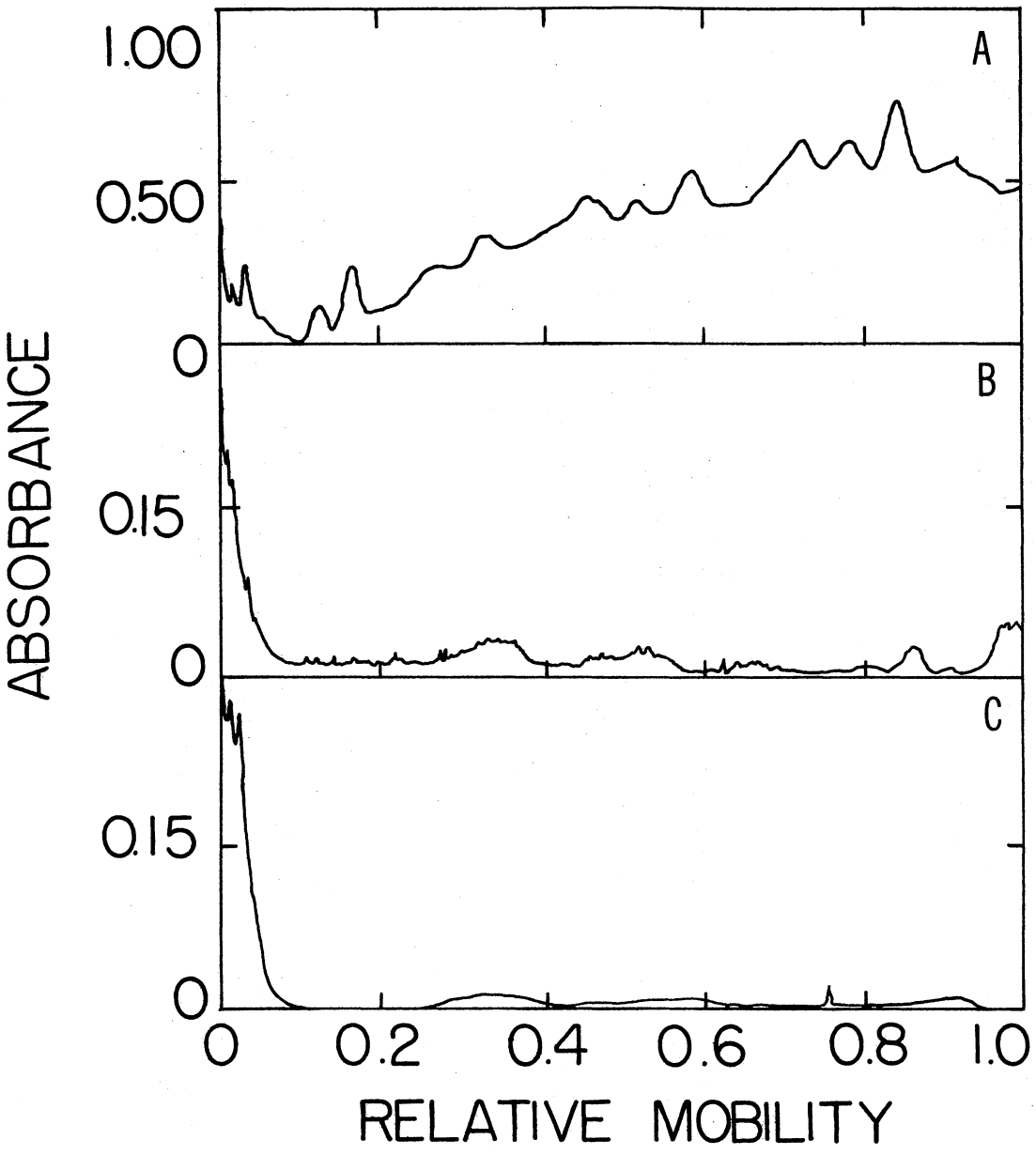
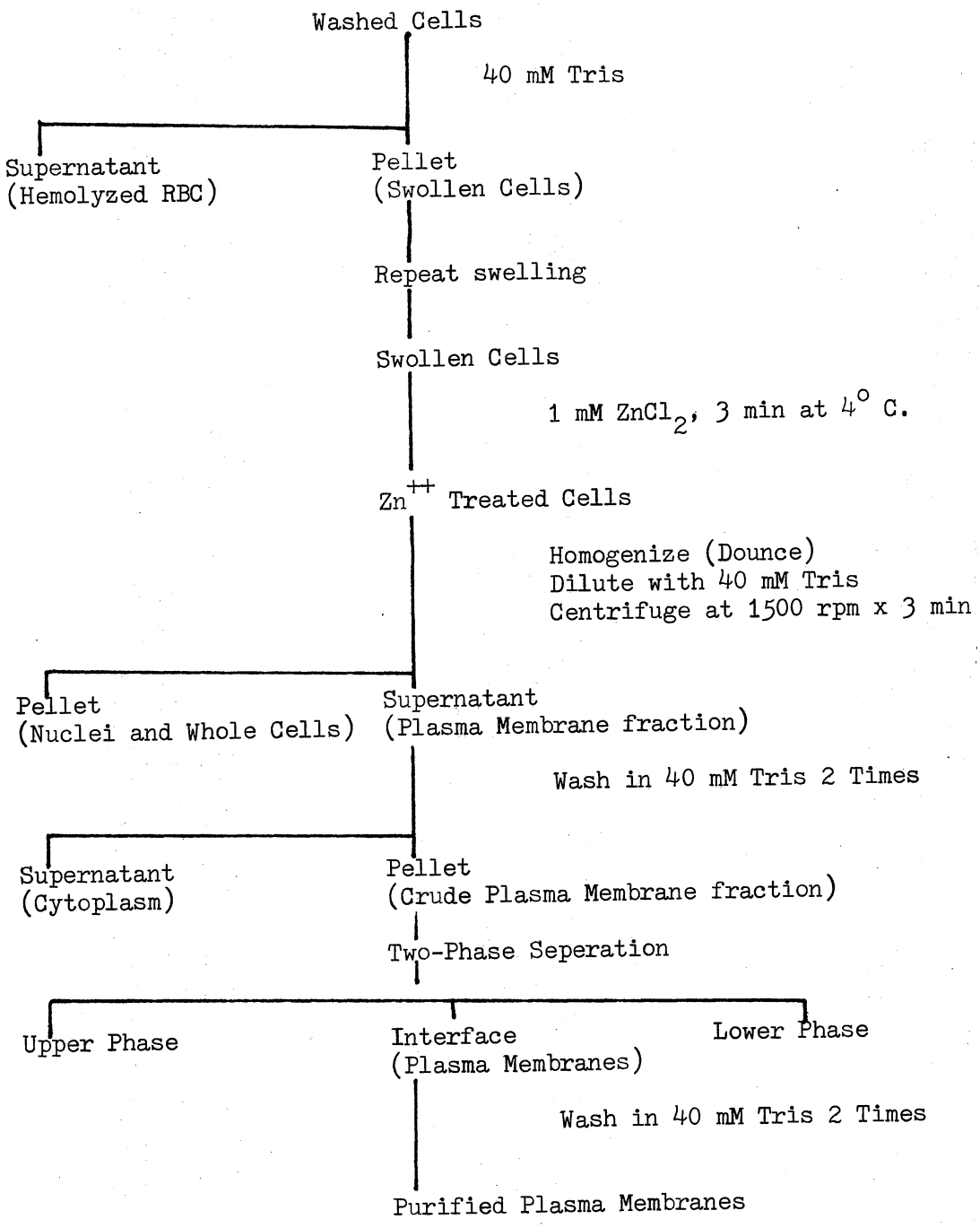

1.00
0.50
0
A
B
0.15
0
C
0.15
0
ABSORBANCE
0
0.2
0.4
0.6
0.8
1.0
RELATIVE MOBILITY

Figure 5. Membrane Isolation Scheme for the L-1210 Leukemia Using the Stabilization Technique

Details of the isolation are given in the methods section.


Washed Cells
40 mM Tris
Supernatant
(Hemolyzed RBC)
Pellet
(Swollen Cells)
Repeat swelling
Swollen Cells
1 mM $ZnCl_2$, 3 min at 4° C.
Zn^{++} Treated Cells
Homogenize (Dounce)
Dilute with 40 mM Tris
Centrifuge at 1500 rpm x 3 min
Pellet
(Nuclei and Whole Cells)
Supernatant
(Plasma Membrane fraction)
Wash in 40 mM Tris 2 Times
Supernatant
(Cytoplasm)
Pellet
(Crude Plasma Membrane fraction)
Two-Phase Seperation
Upper Phase
Interface
(Plasma Membranes)
Lower Phase
Wash in 40 mM Tris 2 Times
Purified Plasma Membranes

fragmenting the plasma membranes into vesicles. Table II shows the enzyme specific activities of the band at the interphase of the two-phase system, which was composed of folded whole envelopes and large membrane fragments and was judged free of nuclei by phase contrast microscopy. The band showed a 34 fold purification of 5'-nucleotidase without significant contamination by succinate dehydrogenase and glucose-6-phosphatase. The L-1210 membranes were solubilized and subjected to SDS acrylamide electrophoresis. The gel scans shown in Fig. 6, show 19 polypeptides ranging from 73,000 to 25,000 apparent molecular weight. Several high molecular weight proteins can be seen in the PAS scan.

Isolation of Plasma Membranes from the 13762 MAT

The membrane stabilization method using Zn^{++} shown in Fig. 7, allows for the isolation of whole plasma membrane envelopes and large sheets of plasma membrane with only low levels of other organelle contamination. A series of photomicrographs seen in Fig. 8, shows the isolation procedure. In frame A, the whole cell is seen just after removal from the animal. Frame B shows the cell after swelling. The diameter of the cell has been seen to increase and some separation of the cytoplasm from the plasma membrane is seen. Frame C shows the Zn^{++} treated cell. The plasma membrane has a much more pronounced appearance and the cytoplasm more granular. Frame D shows the homogenized cells containing whole plasma membranes and large sheets. Frame E shows the plasma membrane fraction from the sucrose density gradient and Frane F shows one of the membranes at higher magnification. The enzyme specific activities and enrichment of membrane fractions is shown in Table III. The band at the

TABLE II

ENZYME SPECIFIC ACTIVITIES AND ENRICHMENT OF MEMBRANE FRACTIONS OF THE L-1210 LEUKEMIA

Values in parentheses are relative specific activities expressed as the ratio of the value for the particular fraction to that of the homogenate. All specific activities are in μMoles/mg/min.

Fraction	Glucose 6 Phosphatase	Succinic INT Reductase	5'-Nucleotidase
Membrane Stabilization Method			
Homogenate	0.4 (1)	4.3 (1)	0.2 (1)
Two-phase interface	0.9 (2)	4.3 (1)	7.9 (34)

Figure 6. Gel Scans of Coomassie Blue and Periodate-Acid-Schiff Stained Polyacrylamide Gel Scans of L-1210 Plasma Membranes

(A) CB and (B) PAS stains.

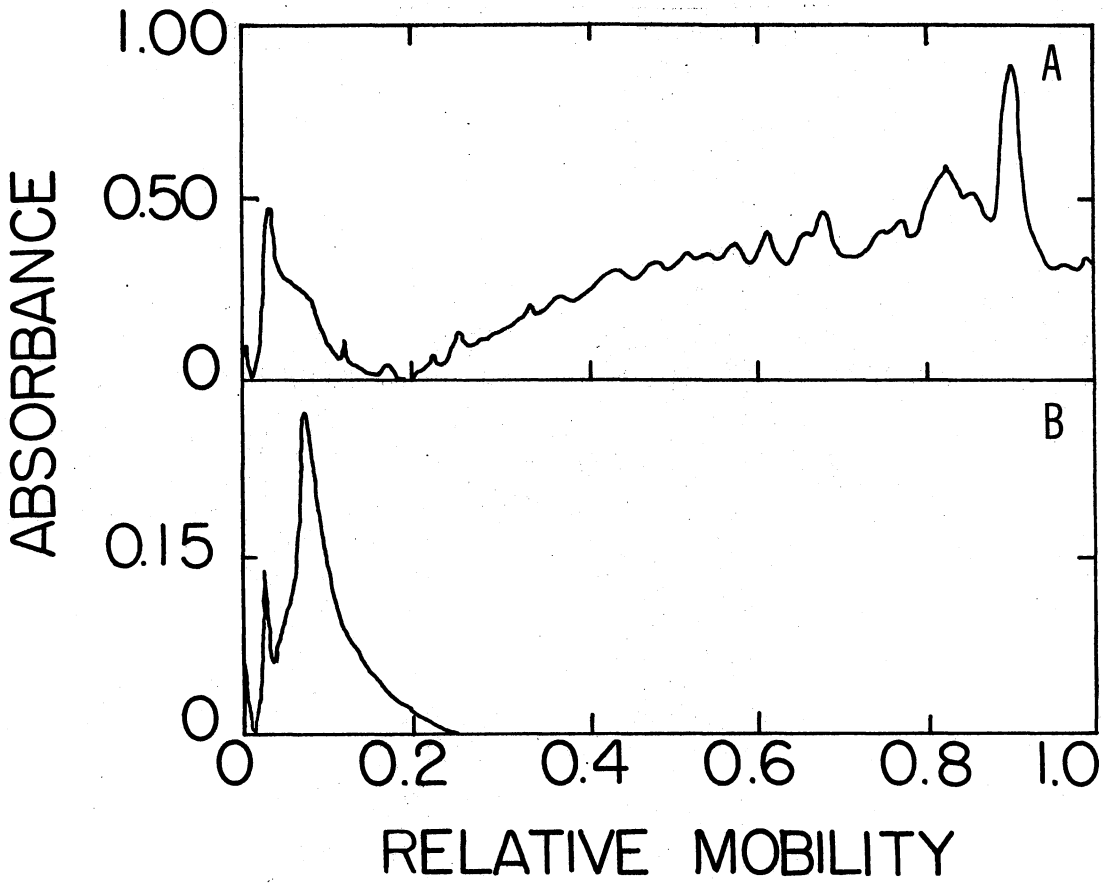

1.00
0.50
0
A
B
0.15
0
ABSORBANCE
0
0.2
0.4
0.6
0.8
1.0
RELATIVE MOBILITY

Figure 7. Membrane Stabilization Method Isolation Scheme for the 13762 MAT

Details of the isolation are given in the methods section.

Washed Cells

40 mM Tris

Supernatant (Hemolyzed RBC)

Pellet (Swollen Cells)

Repeat Swelling

Swollen Cells

1 mM $ZnCl_2$, 4 min at 4° C.

Zn^{++} Treated Cells

Homogenize (Dounce)
Dilute with 40 mM Tris
Centrifuge at 1750 rpm x 10 min

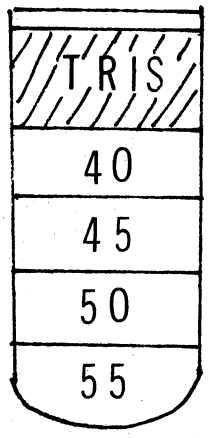
Pellet (Nuclei and Whole Cells)

Supernatant (Plasma Membranes)

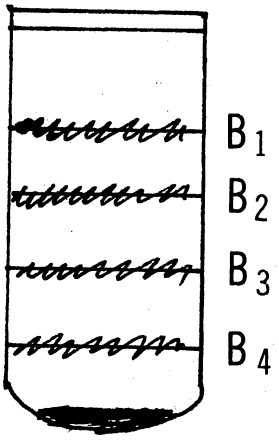
Centrifuge at 4500 rpm x 10 min

Supernatant (Cytoplasm)

Pellet (Crude Plasma Membrane Fraction)



15000 rpm for 60 min at 4° C.



Wash band in 40 mM Tris

Figure 8. Photomicrographs of the 13762 MAT during the Isolation Procedure

Frame (A) shows the whole cell in the peritoneal fluid. Frame (B) shows the cell following swelling in 40 mM Tris (pH 7.4). Frame (C) shows the cell following treatment with Zn^{++}. Frame (D) shows the cell after one stroke of homogenization. Frame (E) shows the isolated plasma membrane.

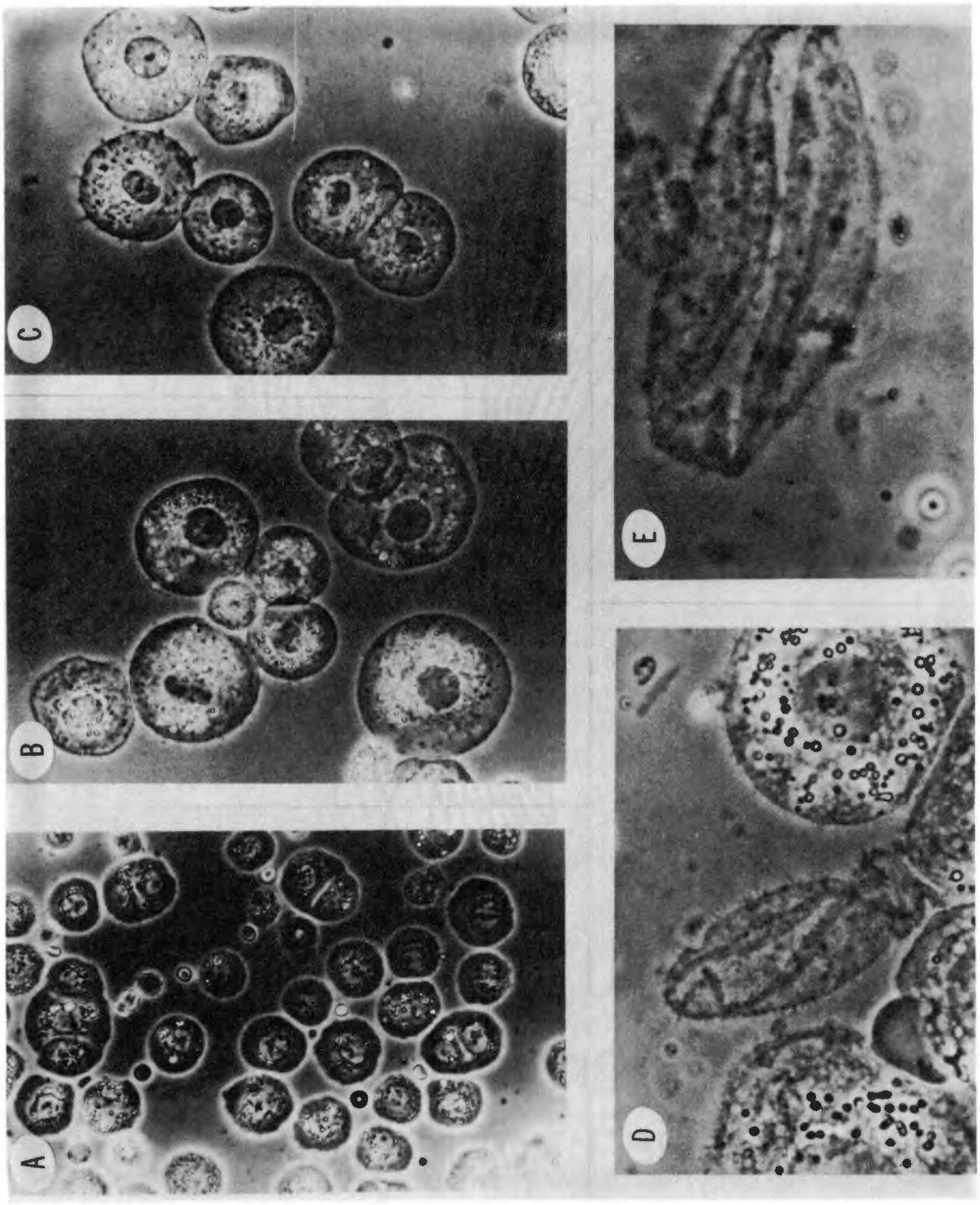

A
B
C
D
E

TABLE III

ENZYME SPECIFIC ACTIVITIES AND ENRICHMENT OF MEMBRANE FRACTIONS OF THE 13762 MAT

Values in parentheses are relative specific activities expressed as the ratio of the value for the particular fraction to that of the homogenate. All specific activities are in μMoles/mg/min.

Fraction	Glucose 6 Phosphatase	Succinic INT Reductase	5'-Nucleotidase
Membrane Stabilization Method			
Homogenate	1.0 (1)	1.5 (1)	1.95 (1)
Cytoplasm	0.9 (0.9)	2.0 (1.3)	1.5 (0.8)
Nuclear fraction	0.1 (0.1)	1.0 (0.7)	0.6 (0.3)
B_1	0.2 (0.2)	14.8 (9.9)	22.5 (11.5)
B_2	0.3 (0.3)	2.0 (1.3)	27.0 (13.5)
B_3	0.5 (0.5)	1.9 (1.3)	13.5 (6.9)
B_4	8.4 (8.3)	N. D.[1]	10.5 (5.4)

1. value not determined.

SDG 40/45% (w/w) interface showed a 13.8 fold enrichment of 5'-nucleotidase over the homogenate. The enzyme proved to correlate well with the location of whole membrane envelopes but was not stable on storage with time so the assay was run within 10 hours after isolation. The same fraction was also free of glucose-6-phosphatase and succinate dehydrogenase contamination. The B_2 fraction was free of nuclei by phase contrast microscopy. This material was next solubilized and subjected to SDS acrylamide electrophoresis. The results are shown in Fig. 9. The coomassie blue band shows 19 major bands varying from 500^{+} to 28×10^{-3} apparent molecular weight. Three major PAS positive bands of high apparent molecular weight and two minor bands were observed although molecular weight is very questionable by SDS acrylamide methods. The methods described above yield plasma membranes from three different tumors reasonably free of other cellular organelles. Table IV shows a tabulation of the molecular weights for the polypeptides for all cell types. Fig. 10, shows PAS scans for the three cell types showing no major similarities in proteins although there are groups of proteins with about the same molecular weight.

Organization of the Sarcoma-180 Plasma Membrane

The Effect of Trypsin on the Sarcoma-180 Plasma Membrane

Mild trypsinization of the cultured S-180 causes pronounced changes in cellular morphology as shown by phase contrast time-lapse cinematography. Fig. 11, shows the results of trypsin treatment on S-180 cells attached to a glass substrate. The sequences begin (A) 4 minutes after trypsin treatment in which no morphological changes have yet occurred.

Figure 9. Gel Scans of Coomassie Blue and Periodate-Acid-Schiff Stained Polyacrylamide Gels of the 13762 MAT

(A) CB and (B) PAS stains.

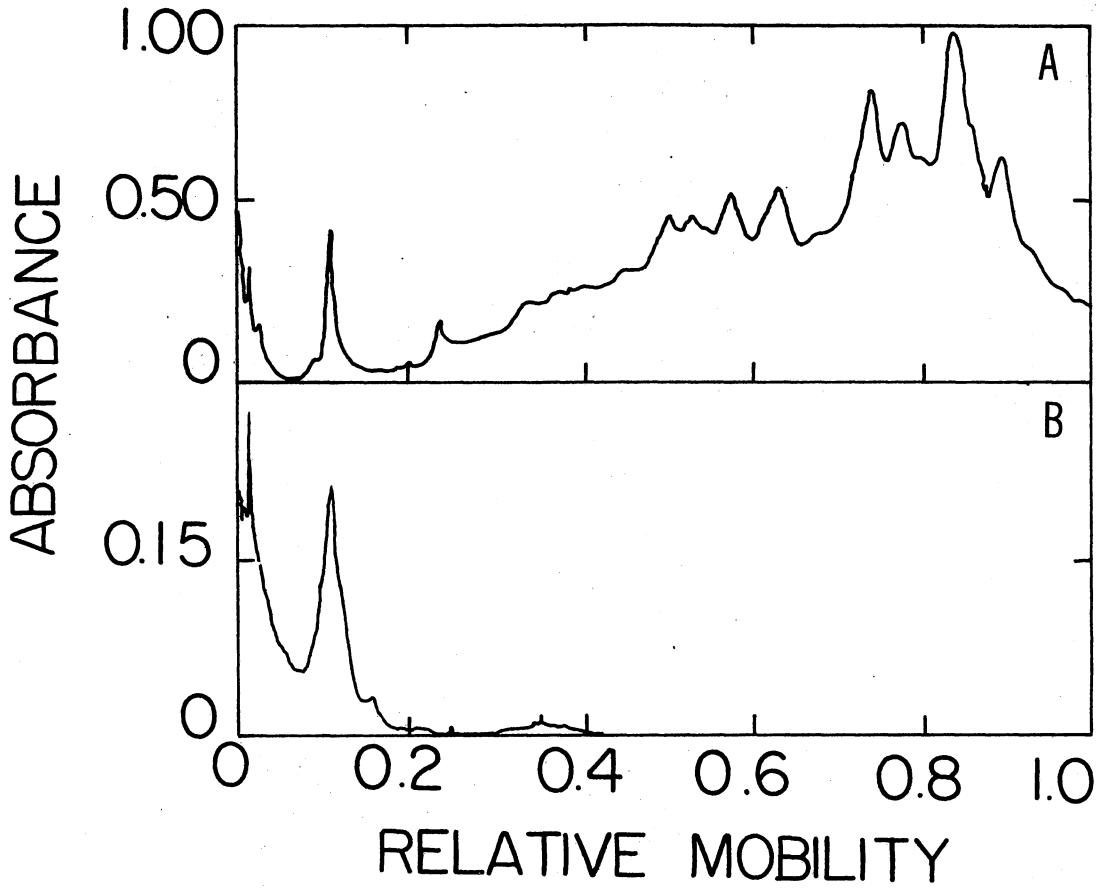

1.00
0.50
0
A
B
0.15
0
ABSORBANCE
0
0.2
0.4
0.6
0.8
1.0
RELATIVE MOBILITY

TABLE IV

COOMASSIE BLUE (CB) AND PERIODATE SCHIFF (PAS) STAINING POLYPEPTIDES
VALUES ARE GIVEN AS APPARENT MOLECULAR WEIGHTS X 10^{-3}

	13762, Zn		L-1210, Zn		SA-180, Zn		SA-180 Microsomal	
	CB	PAS	CB	PAS	CB	PAS	CB	PAS
1	500	750	300	300	300	-		115
2	300	300	265	293	280	100		-
3	240	250	235	290	237	75	237	75
4	175	210	208	268	216	55	216	55
5	125	180	182		190	36	190	
6	120		168		178		178	
7	112		135		148		148	
8	98		122		126		126	
9	88		104		112		112	
10	81		91		78		78	
11	75		84		56		56	
12	63		72		48		48	
13	56		66		42		42	
14	47		55		36		36	
15	42		47		32		32	
16	38		44					
17	33		38					
18	32		31					
19	28		25					

Figure 10. Gel Scans of Periodate-Schiff Stained Polyacrylamide Gels of Several Tumors

(A) SA-180 membrane stabilization method. (B) SA-180 microsomal method. (C) S-180 membrane stabilization method. (D) L-1210 Leukemia. (E) 13762 MAT.

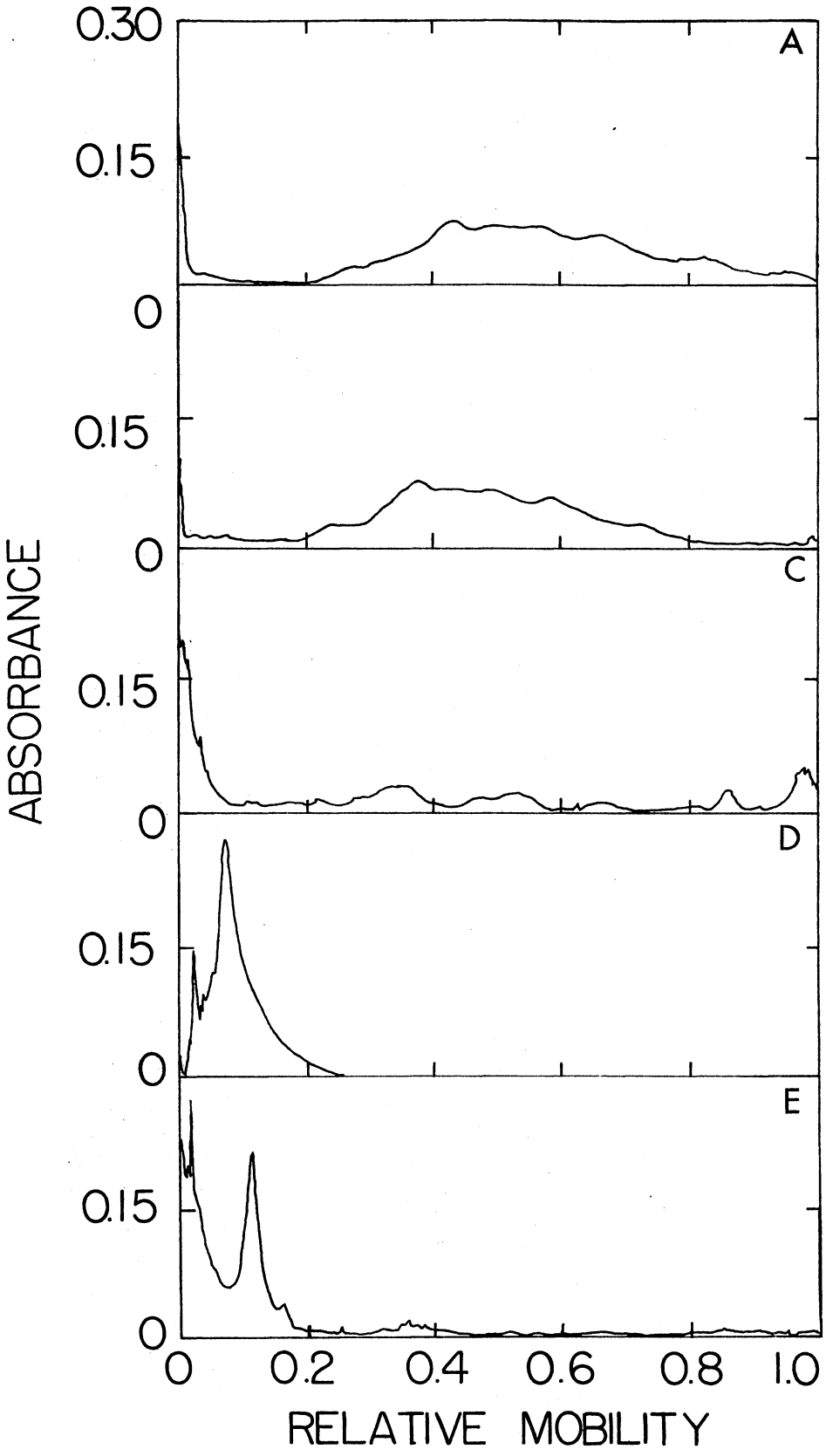
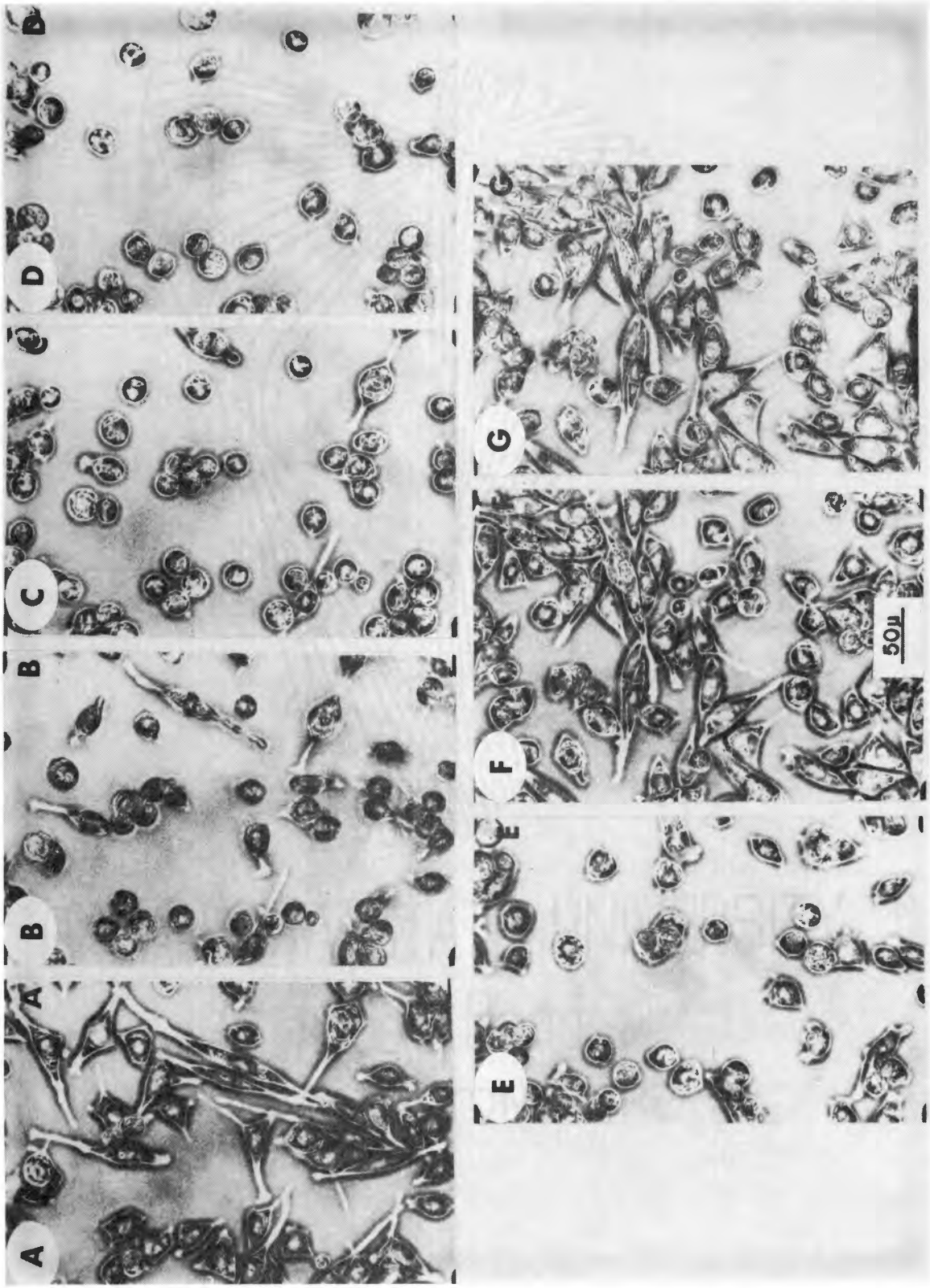

0.30
0.15
0
A
0.15
0
C
0.15
0
D
0.15
0
E
0.15
0
ABSORBANCE
0
0.2
0.4
0.6
0.8
1.0
RELATIVE MOBILITY

Figure 11. The Effect of Trypsin on the *in vitro* Grown Sarcoma-180

The frames are following trypsin addition, (A) 4 min., (B) 12.5 min., (C) 16.3 min., (D) 20 min., (E) 8 hours, (F) 20 hours, (G) 24 hours.


A
B
B
C
D
E
F
G
50µ

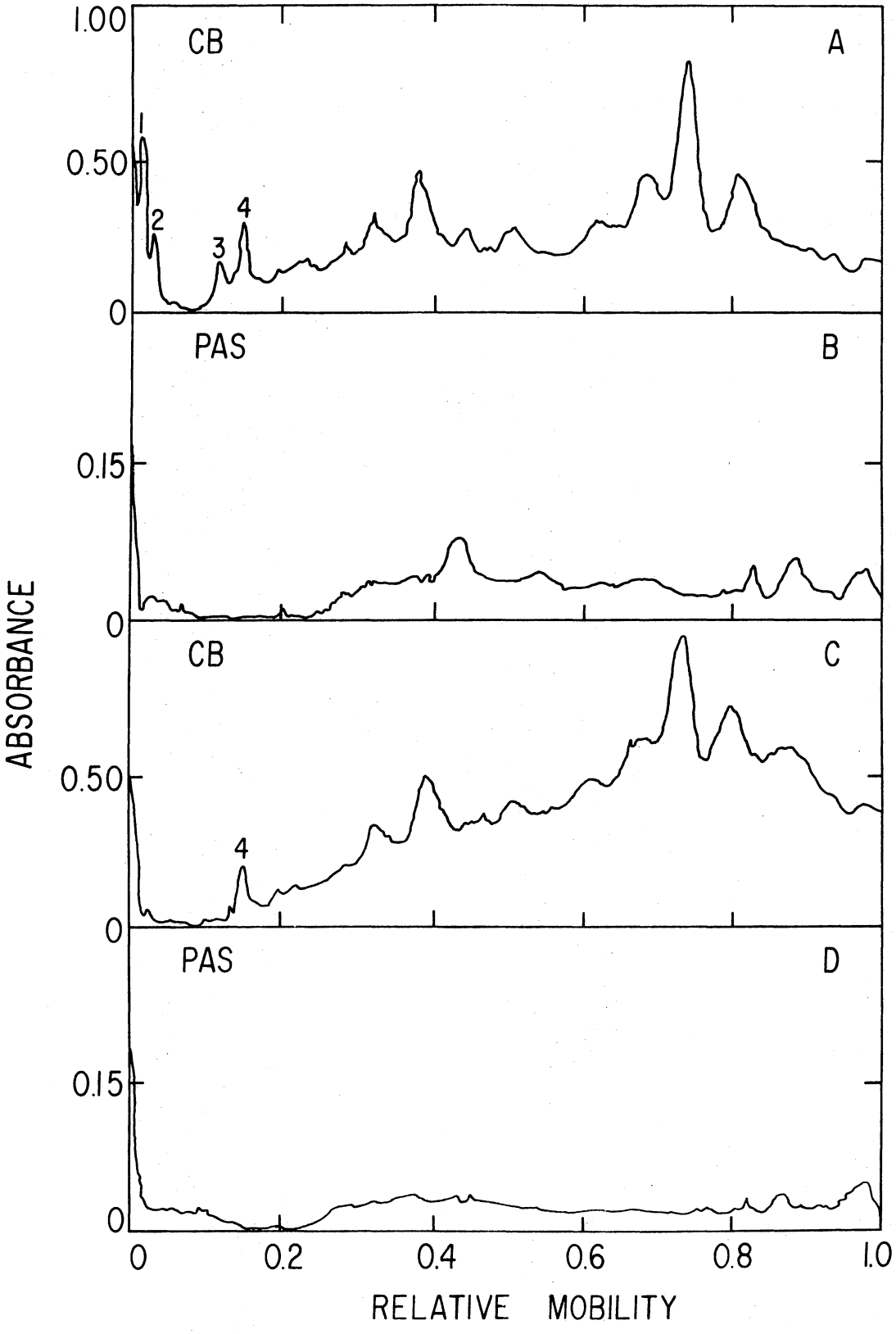
Frame (B), 12.5 minutes after trypsin addition, shows a number of cells that have begun to withdraw their appendages and become rounded. At 15 minutes the trypsin is removed and the cells are prefused with the M-199 + calf serum. Frame (C) shows the cells 16.3 minutes after trypsin addition and frame (D), taken at 20 minutes after trypsin addition, shows that all the cells are rounded with only minute projections very similar to cells undergoing mitosis.

Frame (E) shows the cells some 8 hours after trypsin treatment and some flattening of the cells has begun to take place. This flattening and spindle formation continues and in frames (F) and (G), 20 and 24 hours after trypsin treatment the cells have regained their characteristic fibroblast morphology seen in frame (A) and many cells have undergone at least one division. Only two or three of the cells in the original field became detached from the glass and swept away by the perfusion medium. Most of the cells did undergo subsequent division following trypsin treatment indicating that the tryptic action, while able to cause a dramatic change in cell morphology, does not cause loss of cell viability.

Because of this effect on cell morphology it was of interest to determine what membrane changes occur at the molecular level. The initial observations were made with the ascites form of the sarcoma-180, since it occurs as a single cell stage, facilitating trypsin treatment and membrane stabilization two-phase method and subjected to acrylamide gel electrophoresis in sodium dodecyl sulfate. The polypeptide and glycoprotein patterns for trypsinized (5 ug/ml) and untreated samples are shown in Fig. 12. As expected, the glycoproteins have been eliminated from the patterns by trypsinization. In addition, a group of high

Figure 12. The Effect of Trypsin on the Sarcoma-180 Ascites Tumor

Gel scans of Coomassie Blue and Periodate-Acid-Schiff stained polyacrylamide gels. SA-180 plasma membranes isolated from control cells stained for CB (A) and PAS (B), and (D) SA-180 plasma membranes from cells treated with trypsin and stained for CB (C) and PAS.


1.00
0.50
0
CB
A
1
2
3
4
PAS
B
0.15
0
CB
C
0.50
4
0
PAS
D
0.15
0
0
0.2
0.4
0.6
0.8
1.0
ABSORBANCE
RELATIVE MOBILITY

molecular weight polypeptides are not observed in the proteolyzed samples. There are no other significant changes in the patterns except for an increase in material in the lower molecular weight regions of the gels, probably arising from degradation products of the trypsinization. The missing polypeptides are the same species that we have suggested are involved in membrane "stabilization" during isolation by the method of Warren and Glick (10). They are present in the "stabilized" membrane sheets or envelopes but not is vesicular membrane preparations.

Separate studies were performed on the in vitro Sarcoma-180. The primary difference between these two cell lines is a difference in surface membrane glycoproteins which are present on the cultured form but not on the ascites form. Trypsinization of the cultured cells followed by membrane isolation gave similar results to those found with the ascites form, but the changes were not quite as dramatic (Fig. 13). The glycoproteins are essentially completely removed from the pattern, but the high molecular weight polypeptides are shown to be decreased only about 70% when staining densities are normalized against smaller polypeptides that are unaffected. To establish that the disappearance of the three high molecular weight proteins and the glycoproteins was not due to extreme susceptibility to cleavage by trypsin isolated SA-180 plasma membranes were trypsinized. The gels from the experiment in Fig. 14, show that the high molecular weight component is relatively insensitive and that the glycoproteins are not cleaved except at high trypsin concentration.

To determine if the S-180 PM high apparent molecular weight glycoprotein doublet was exposed to the outside of the cell, surface labeling with lactoperoxidase was performed on the S-180 and SA-180 cells. The

Figure 13. The Effect of Trypsin on the *In Vitro* Grown Sarcoma-180

Gel scans of Coomassie Blue and Periodate-Acid-Schiff stained polyacrylamide gels. S-180 plasma membranes isolated from control cells stained for CB (A) and PAS (B), and (D) S-180 plasma membranes from cells treated with trypsin and stained for CB (C) and PAS.

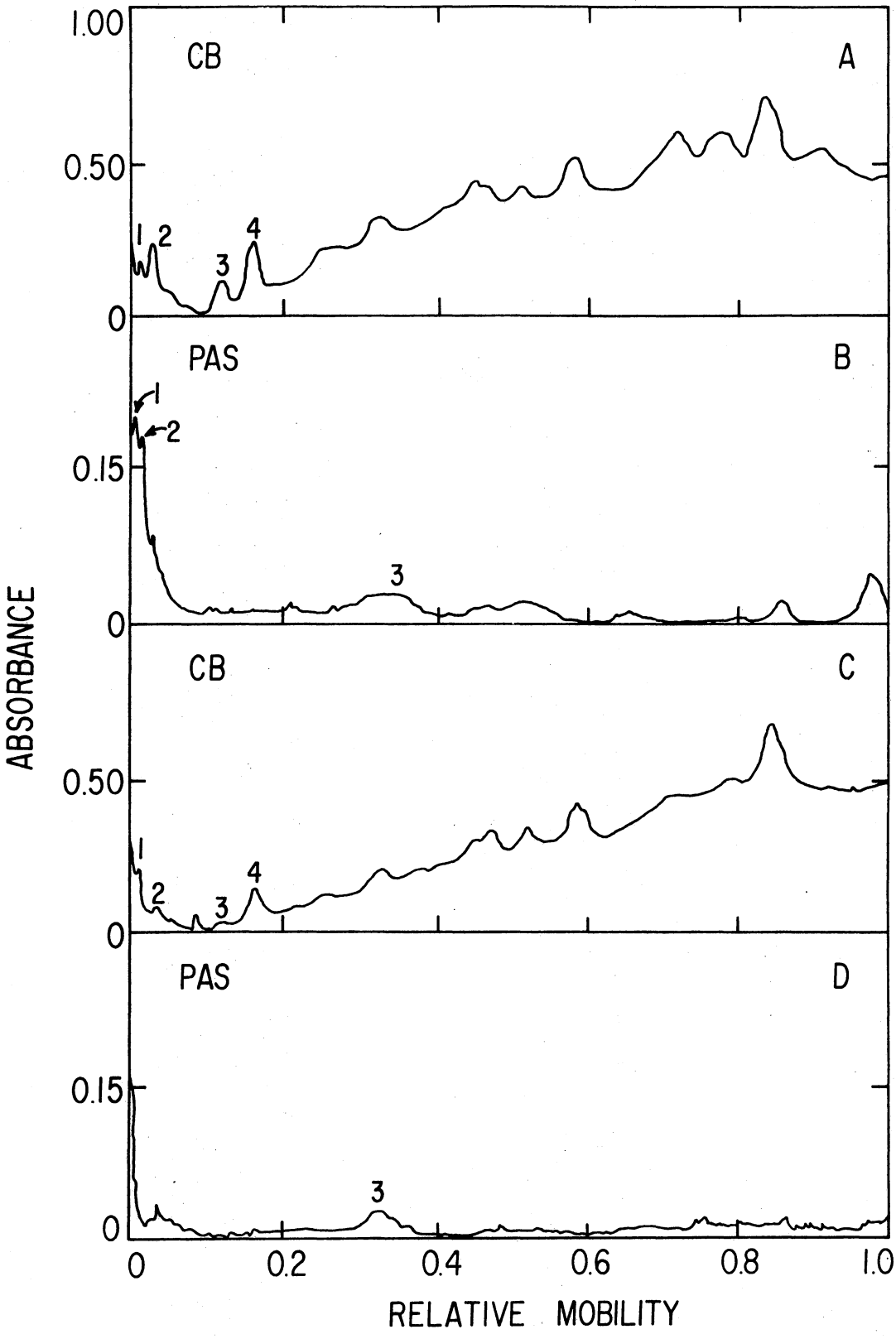
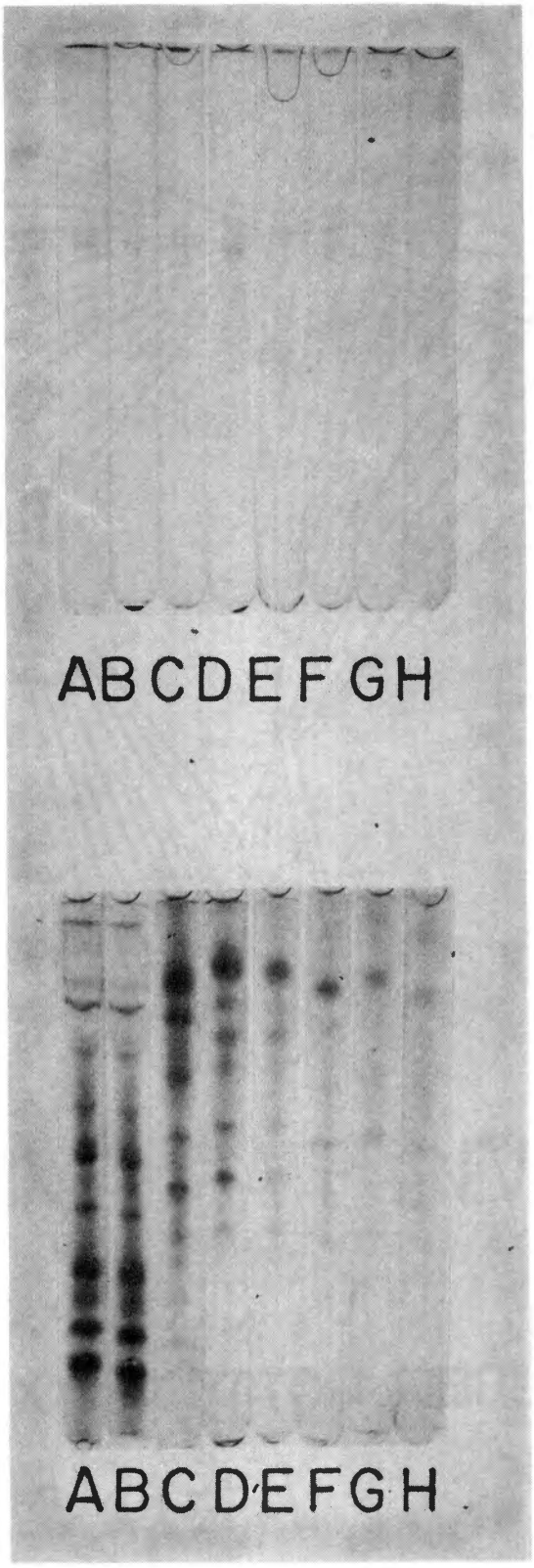

1.00
0.50
0
CB
A
1
2
3
4
PAS
B
0.15
0
CB
C
0.50
0
PAS
D
0.15
0
ABSORBANCE
0
0.2
0.4
0.6
0.8
1.0
RELATIVE MOBILITY

Figure 14. The Effect of Trypsin on the Sarcoma-180 Ascites Tumor Cell Isolated Plasma Membrane

Plasma membranes isolated by the membrane stabilization method were trypsionized as described in the methods section. The upper photograph is the PAS stained gels, and the lower photograph is the CB stain. Trypsin concentrations for each set are; (A) none, (B) 0.1, (C) 1.0, (D) 5.0, (E) 10.0, (F) 20.0, (G) Ug/ml.


A B C D E F G H
A B C D E F G H

coomassie blue and PAS patterns are the same as those shown in Fig. 3 and 4. The labeling profile in Fig. 15 shows the major peak to be in the same slice as that of the glycoprotein, there was no coomassie blue staining at the origin. The SA-180 pattern is shown also for reference.

The Isolation of the High Molecular Weight Proteins from the Sarcoma-180 Plasma Membrane

Because of the similarity in molecular weights between the 240, 220 x 10^{-3} molecular weight doublet and spectrin the structural protein of the red cell, and because the disappearance could be correlated with shape changes in the cell, attempts were made to isolate the high molecular weight proteins from the SA-180 PM. Plasma membranes were extracted overnight with 1.0 M NaCl and two low ionic strength buffers. Gel profiles for the three extracts are shown in Fig. 16. The high ionic strength extract showed very little of the high molecular weight proteins (300^{+}, 280, 240, 220 x 10^{-3}) in the supernatant of the 1.0 M NaCl extract, but several low molecular weight proteins (>72 x 10^{-3}). The pellet had 85% of the membrane protein, but was congealed into a gummy pellet, which could not be used for further extractions. The two low ionic strength extracts showed similar results. A number of high molecular weight proteins were partially extracted from the membranes (300^{+}, 280, 240, 220 x 10^{-3}) along with several lower molecular weight proteins (112, 78, 56, 48, 36 x 10^{-3}). The GEM buffer extracted less of the lower molecular weight proteins and was chosen for further studies. The GEM buffer was used to extract PM for 4 hours and the residue extracted an additional 20 hours. The two extracts are shown on Fig. 17, and show that 13 major are extractable from the membranes. The four-hour extract

Figure 15. Lactoperoxidase Labeling of Sarcoma-180 Cell

S-180 cells were labeled with ^{125}I using lactoperoxidase. The plasma membranes were isolated, electrophoresed on acrylamide gels, cut into 2 mm sections and ^{125}I counted by liquid scinilization counting. (A) is the SA-180 *in vivo* tumor, (B) is the S-180 *in vitro* grown cell.

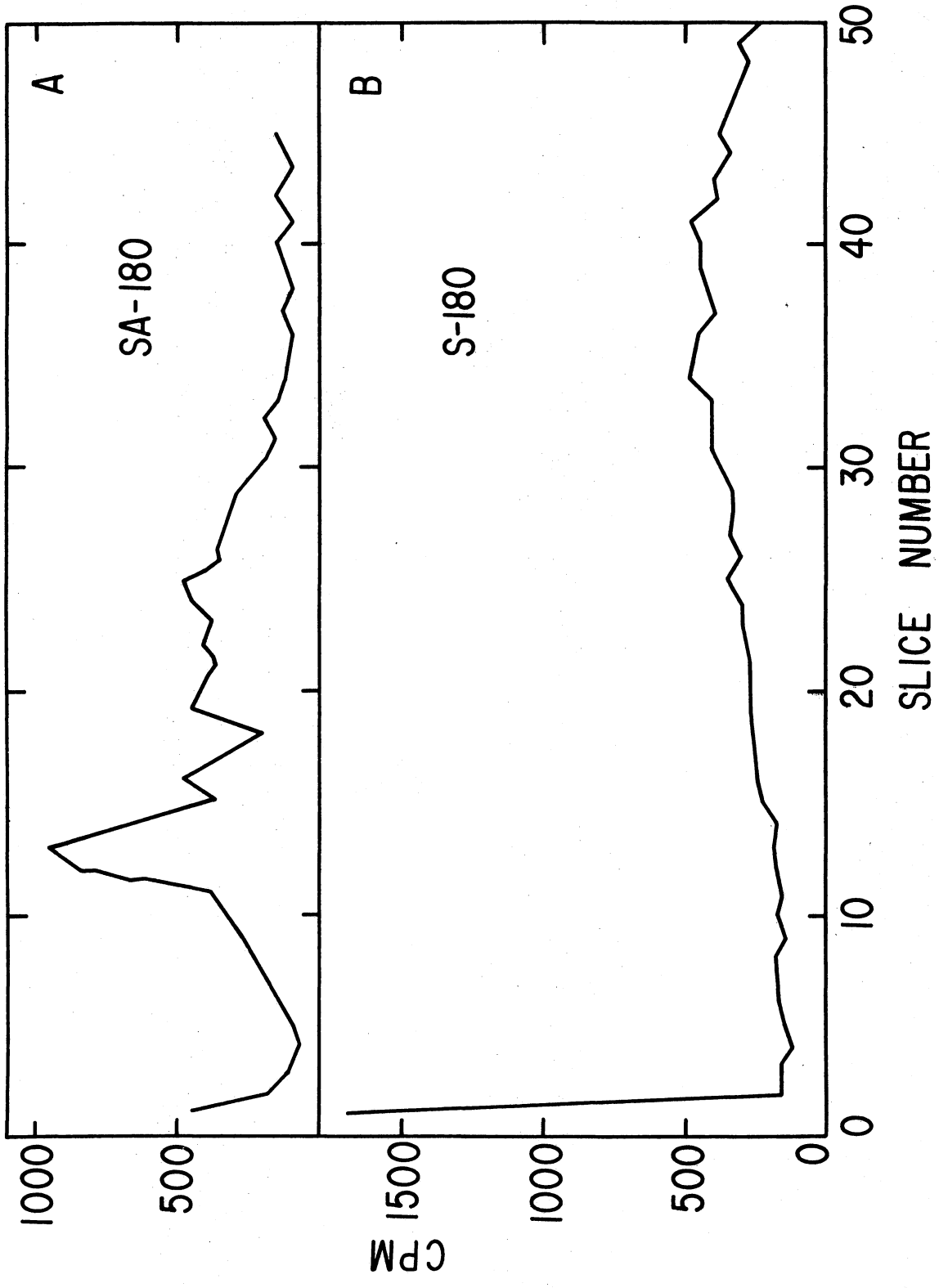

A
SA-180
B
S-180
1000
500
1500
1000
500
0
CPM
0
10
20
30
40
50
SLICE NUMBER

Figure 16. Gel Profiles of Membrane Extracts

(A) 1.0 M NaCl extract, (B) 1.0 M NaCl extracted membrane pellet, (C) distilled water extract, (D) distilled water extracted membrane pellet, (E) GEM extract, (F) GEM extracted membrane pellet.

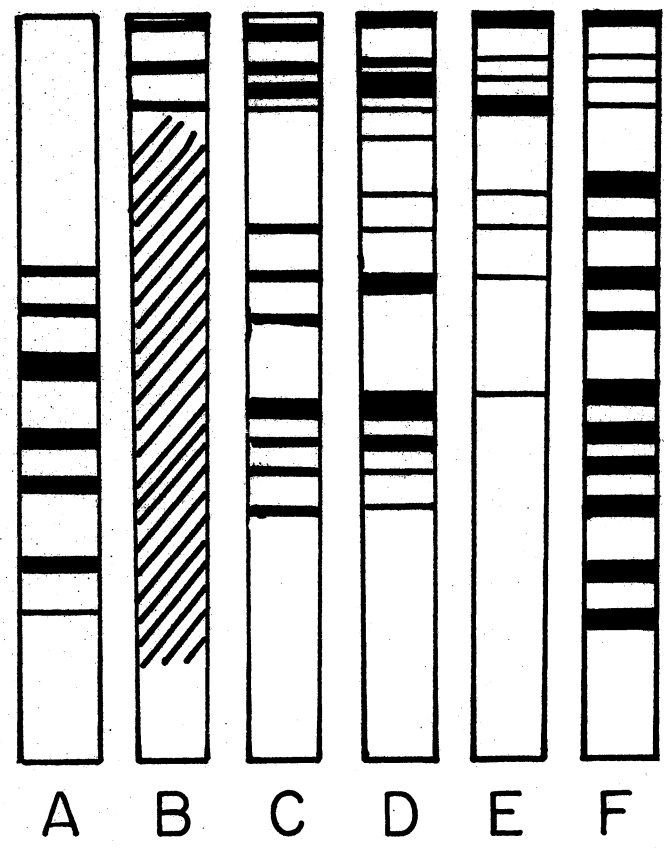
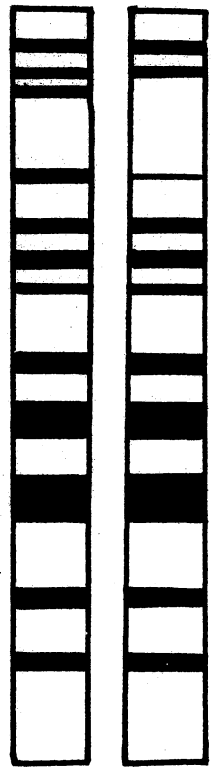

A
B
C
D
E
F

Figure 17. A comparison of Extraction Times

SA-180 plasma membranes extracted 0-4 hours (A) and 4-24 hours (B).


A
B

does not, however, remove all of the extractable high molecular weight proteins. Therefore, longer extraction periods were used to obtain enough of the extract for subsequent pruification attempts. The 0-4 hour extract had only the 280, 240 x 10^{-3} molecular weight bands, presumable because the small amount of the 220 x 10^{-3} molecular weight band that remains in the membrane is unextractable.

The G-200 column elution profile (Fig. 18) showed a large void volume peak. The gel profile for the extract and Vo peak is shown in Fig. 19. The Vo peak contained primarily (<90%) the 240, 220 x 10^{-3} molecular weight doublet. Amino acid analysis of the doublet is shown in Table V. Comparison of the amino acid composition with that of spectrin the 240, 220 x 10^{-3} molecular weight structural protein of the human red blood cell by the method of Marchalonis and Weltman (107) can be interpreted as relatedness. The extracted proteins were then separated by gel filtration. In an attempt to further purify the high molecular weight proteins, the extract was fractionated with ammonium sulfate (Fig. 20). The ammonium sulfate fractionation precipitated the high molecular weight proteins between 0 and 0.205 gm/ml while leaving a large number of the lower molecular weight proteins in solution. This increases the amount of the high molecular weight protein in the fraction from 20% to 60% of the total protein.

Gel filtration column chromatography was then investigated to see if the soluble proteins could be purified in their native state. Attempts to separate the high molecular weight proteins by non-denaturing gel filtration chromatography are shown in Fig. 21. The peaks were pooled, concentrated by pressure dialysis and electrophoresed as described. The first two fractions off of the column both contained the

Figure 18. G-200 Fractionation of the GEM Extract

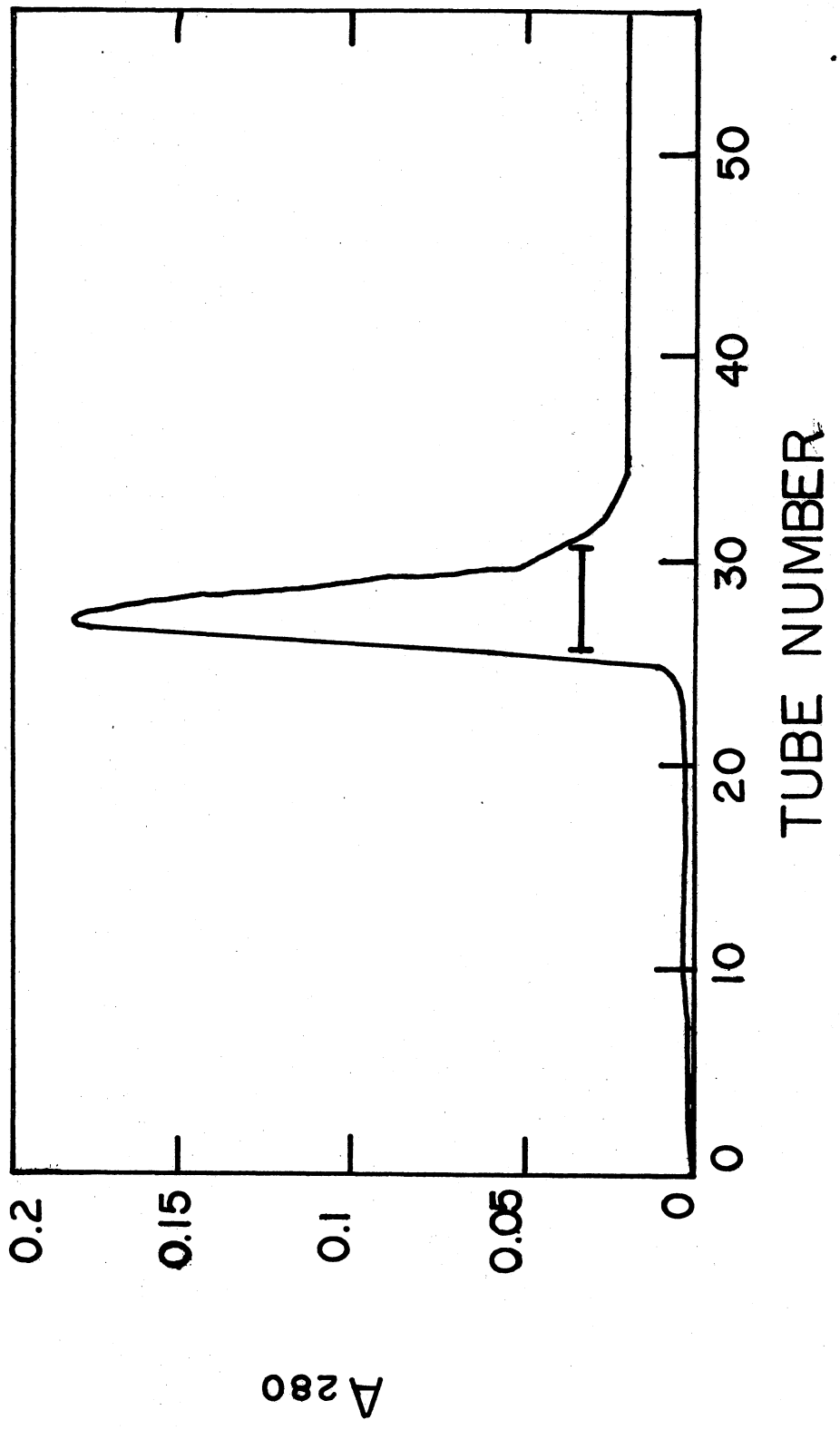

A280
0.2
0.15
0.1
0.05
0
0
10
20
30
40
50
TUBE NUMBER

Figure 19. Purification of the High Molecular Weight SA-180 Doublet

(A) shows the GEM extract, (B) the G-200 void volume peak.

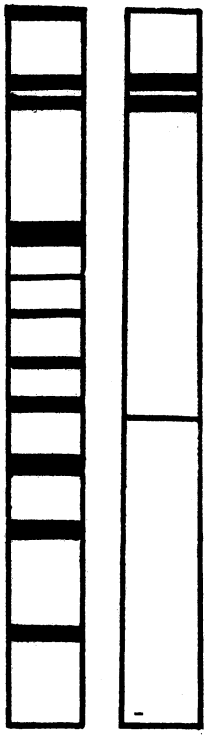

A
B

TABLE V

AMINO ACID COMPOSITION OF A HIGH MOLECULAR WEIGHT PROTEIN DOUBLET FROM THE SA-180 PLASMA MEMBRANE

	SA-180[1] Doublet	Spectrin[1] Doublet	$(X_i - X_k)^2$
Aspartic Acid + Asparagine	9.57	10.43	0.74
Threonine	5.40	4.29	1.23
Serine	8.33	5.87	6.05
Glutamic Acid + Glutamine	13.92	18.03	16.89
Proline	3.75	2.31	2.07
Glycine	8.48	4.70	14.29
Alanine	7.99	9.12	1.28
Valine	4.69	4.45	0.06
Methionine	1.59	1.82	0.05
Isoleucine	3.51	3.65	0.02
Leucine	9.66	12.01	5.52
Tyrosine	2.18	2.04	0.02
Phenylalanine	5.06	3.25	3.28
Lysine	6.84	6.66	0.03
Histidine	2.33	2.83	0.25
Arginine	6.73	5.92	0.87
½ Cystine	N. D.[3]	1.1	N. D.
$S\Delta Q$			52.65

1. Values are given as mole %
2. SA-180 (mole %) - Spectrin (mole %) ^{2} by the method of Marchalonis and Weltman (107)
3. Not determined

Figure 20. Ammonium Sulfate Fractionation of the GEM Extract (A) 30%, (B) 52%, (C) 71% saturation cuts.

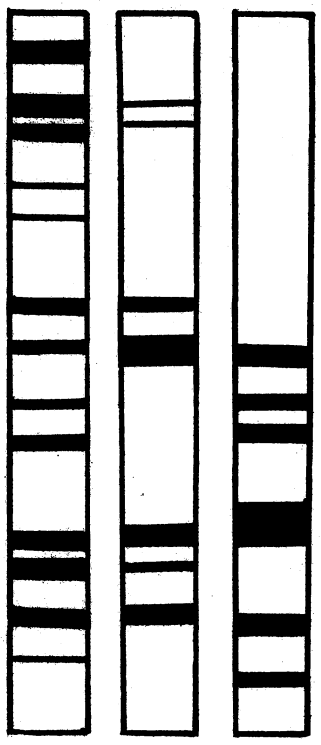
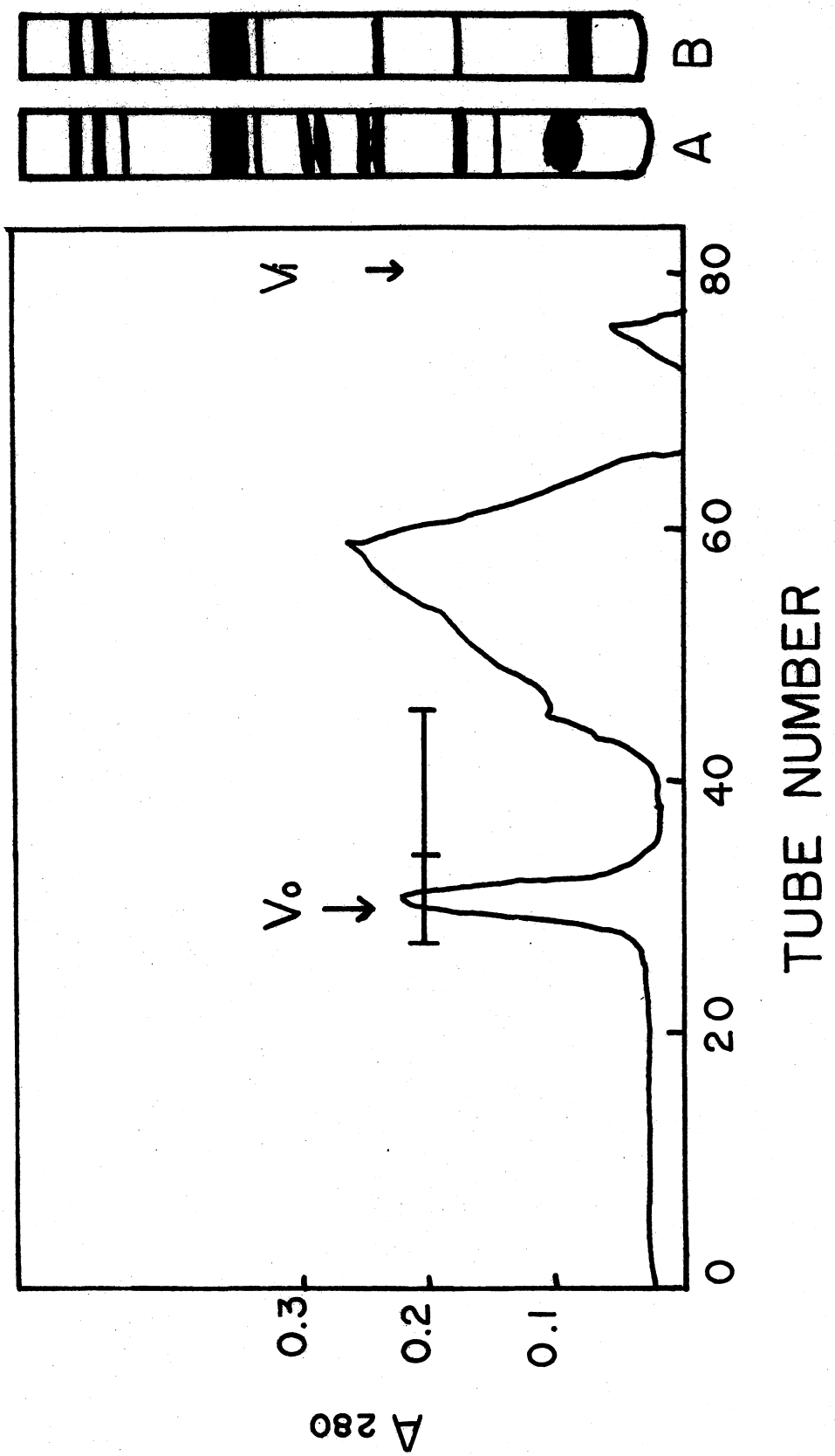

A
B
C

Figure 21. Sepharose 4B in GEM Fractionation of the GEM Extracted Proteins

Gel (A) is from fraction I, and gel (B) from fraction II.


B
A
V_t
V_o
A 280
0.3
0.2
0.1
0
20
40
60
80
TUBE NUMBER

high molecular weight proteins along with several lower molecular weight proteins. The great difference in molecular weight of the polypeptides is due to the relatively native states of the proteins fractionated by the column. The membrane extract is composed of a series of very high molecular weight proteins, probably due to the association of the small molecular weight polypeptides into complexes of large molecular weight, which, therefore, elute very near the void volume of the column. Because of the large number of polypeptides which elute with the high molecular weight proteins, isolation was undertaken in SDS under reducing conditions. The column elution profile in Fig. 22, shows that the column was separated by molecular weight as expected for a Sepharose 4B column. The column separation was run on three separate occasions with similar results; however, it was not possible to successfully resolve the 280, 240, 220 x 10^{-3} molecular weight group of proteins by this method. The use of an SDS column is very helpful in separating proteins from plasma membranes which cannot be solubilized easily in aqueous non-detergent solutions. The method, however, has the disadvantage of disassociating subunits and at least partially denaturing the polypeptides involved. Separations of this type are, however, useful in getting material for chemical analysis.

Isolated SA-180 Tumor Cell Plasma Membranes as Anticancer Immunizing Agents

The interaction of the Ham/ICR outbred mouse with the SA-180 tumor was characterized. The life span of the host was determined following a challenge with viable SA-180 at concentrations of 10^2 through 10^6 tumor cells per animal. The results (Fig. 23) show the per cent

Figure 22. Sepharose 4B in SDS Fractionation of the Solubilized SA-180 Plasma Membrane

Gels for the various fractions are shown above the peak.

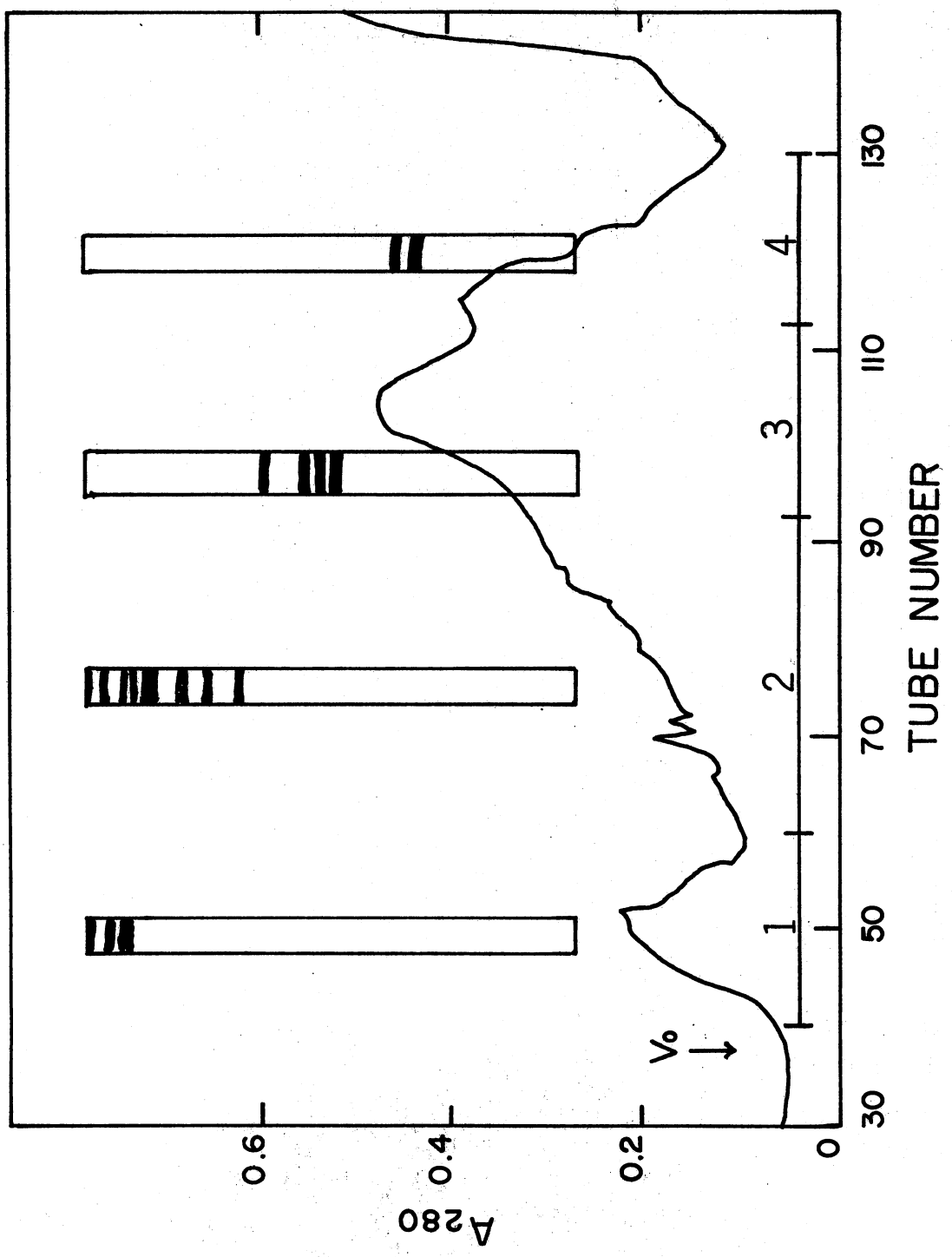
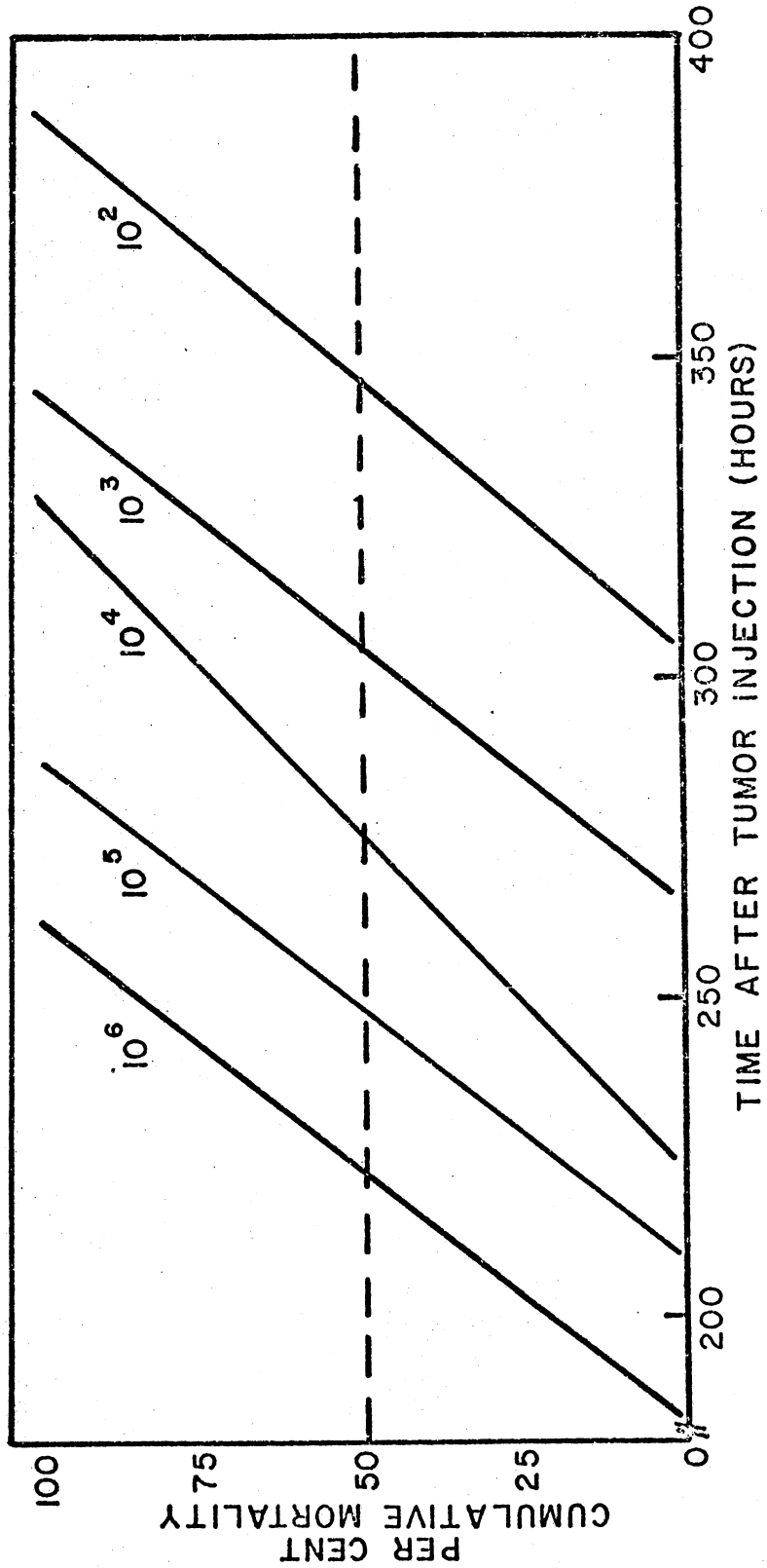

A280
0.6
0.4
0.2
0
V0
30
50
70
90
110
130
TUBE NUMBER
1
2
3
4

Figure 23. The Mortality of Ham/ICR Mice Following Injection of Different Cell Concentrations of SA-180

Groups of Ham/ICR mice (15 animals/group) were challenged with viable SA-180 at concentrations of 10^2 through 10^6 cells. The number of animals dead from each group, plotted as the per cent cumulative mortality, is shown in a function of the time (hours) following tumor challenge.

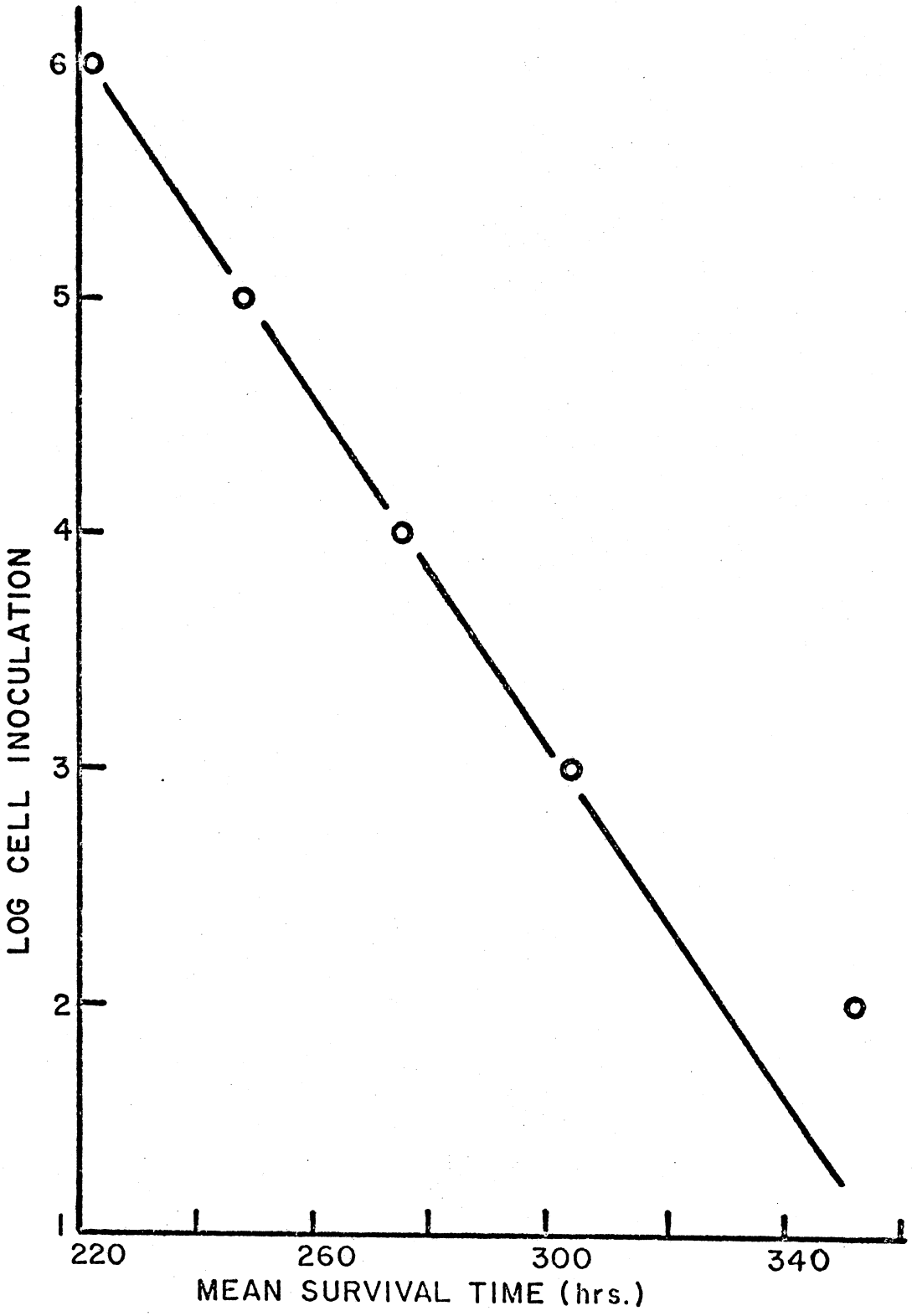

PER CENT
CUMULATIVE MORTALITY
100
75
50
25
0
200
250
300
350
400
TIME AFTER TUMOR INJECTION (HOURS)
10^2
10^3
10^4
10^5
10^6

cumulative mortality for each inoculation group (15 animals/group) plotted against the time of death after inoculation. Because the host in these studies is from outbred stock, the distribution of deaths within each challenge group is greater than that generally observed for inbred strains. From the cumulative mortality data a diagram plotting the mean survival time as a function of the cell inoculation concentration was constructed (Fig. 24). The results show that the mean survival time for the Ham/ICR host is a linear function of the inoculation density and, therefore, a good measure of the effects of immunization on tumor survival. The approximately 20 hours deviation in mean survival time resulting from a 10^2 tumor inoculation may result from the efforts of the host in combating tumor challenge by a small tumor burden. Calculations based on these data indicate that the SA-180 tumor cell has a generation time in this host of approximately 9 hours. Taking the generation time and the mean survival data at each tumor inoculation concentration, there should be about 1×10^{13} tumor cells in the host at death. Actual cell counts using animals near death from the various inoculation groups revealed that the tumor concentration at host death is about 9×10^{12} cells regardless of the inoculated tumor cell concentration.

Experiments were conducted to determine if the immunizing potential, i.e. those tumor associated antigens which are capable of eliciting an effective anti-SA-180 immune response, were retained in an active conformation on the isolated plasma membrane.

Test materials were administered i.p. at 3 injections/week for 2 weeks. Controls in group (a) included 10 animals which received only saline and 10 animals which received human erythrocyte membranes (a

Figure 24. Relationship of Tumor Inoculation Concentration to the Host Mean Survival Time

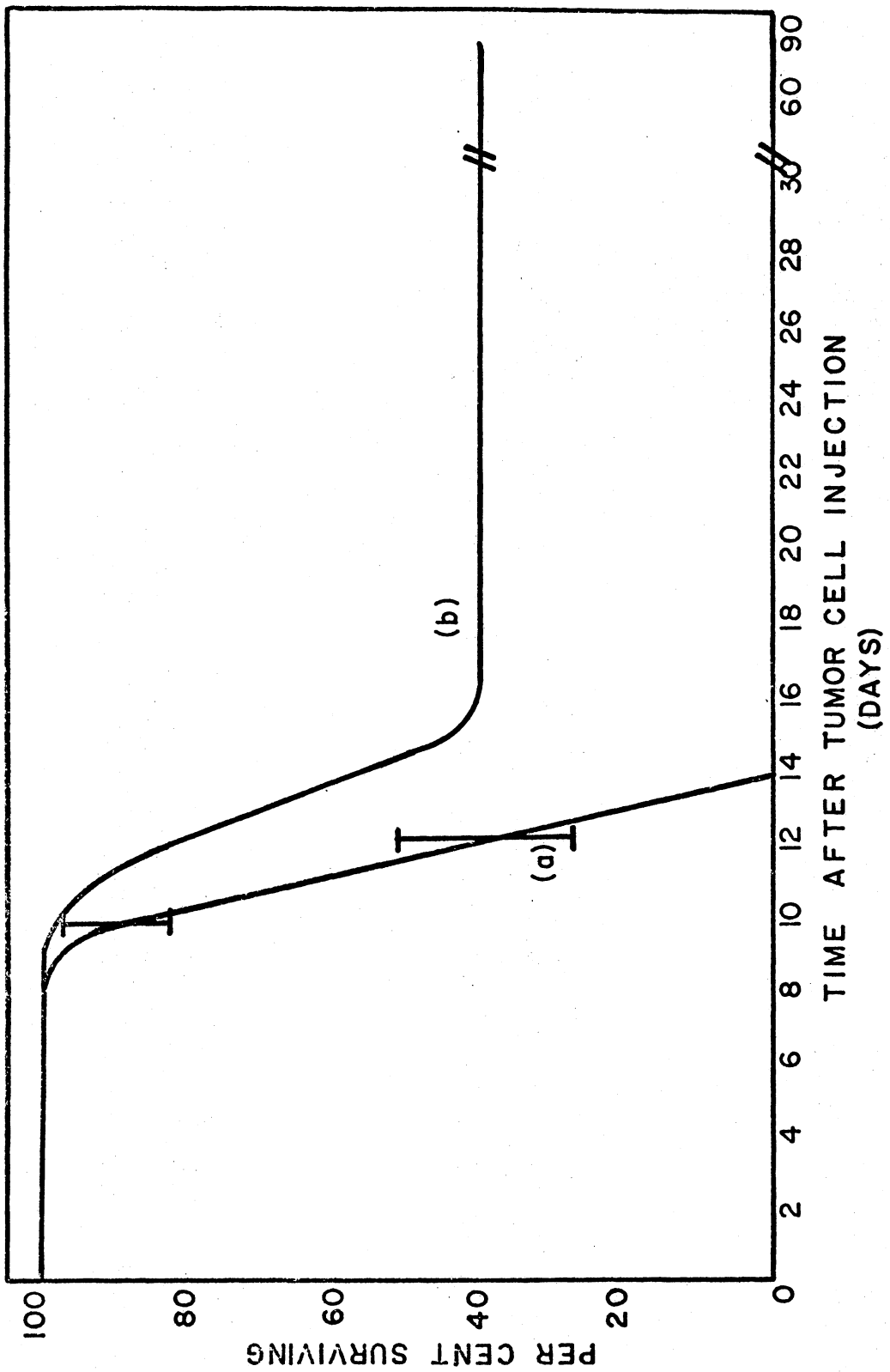

6
5
4
3
2
1
LOG CELL INOCULATION
220
260
300
340
MEAN SURVIVAL TIME (hrs.)

total of 3.0 mg membrane protein suspended in Hepes). Group (b) received a preparation of SA-180 plasma membrane (a total of 3.0 mg membrane protein suspended in Hepes). One week following the final injection of test material all animals were challenged (i.p.) with 1 x 10^4 viable SA-180 cells and the survival times, recorded for each animal, show the per cent of animals surviving in each group (Fig. 25). Control groups which had recieved Hepes or a non-tumor membrane protein (human erythrocyte ghosts) had a mean survival time of about 12 days. These results correlate well with our expected mean survival data of 11.5 days and show that the injection of a non-tumor membrane protein does not significantly stimulate the host. In group (b), (the mice which received the SA-180 membranes) the animals that died had a mean survival time of 15 days, a substantial increase. Even more important, 40% or 4 out of the 10 animals in this group completely survived the tumor challenge. Thirty days after the initial challenge, these survivors were again challenged with 10^4 viable SA-180 cells and all 4 animals have survived for approximately 8 months. Those animals in group (b) which did not survive the initial challenge and whose mean survival time was 15 days were apparently able to combat a major portion of the tumor challenge. Based on the inoculation concentration and mean survival time, these animals neutralized about 90% of the original 10^4 challenge dose.

To further substantiate the anticancer immunizing potential of the isolated SA-180 plasma membrane, a different immunizing schedule and additional types of control material were used. The tumor membrane test group consisted of 10 animals which received the SA-180 plasma membranes prepared as described above at 0.4 mg protein on the first injection and 0.8 mg on each of the remaining 5 injections. Controls were animals

Figure 25. Per Cent of Animals Surviving At Various Times Following Injection of 10^4 SA-180 Cells

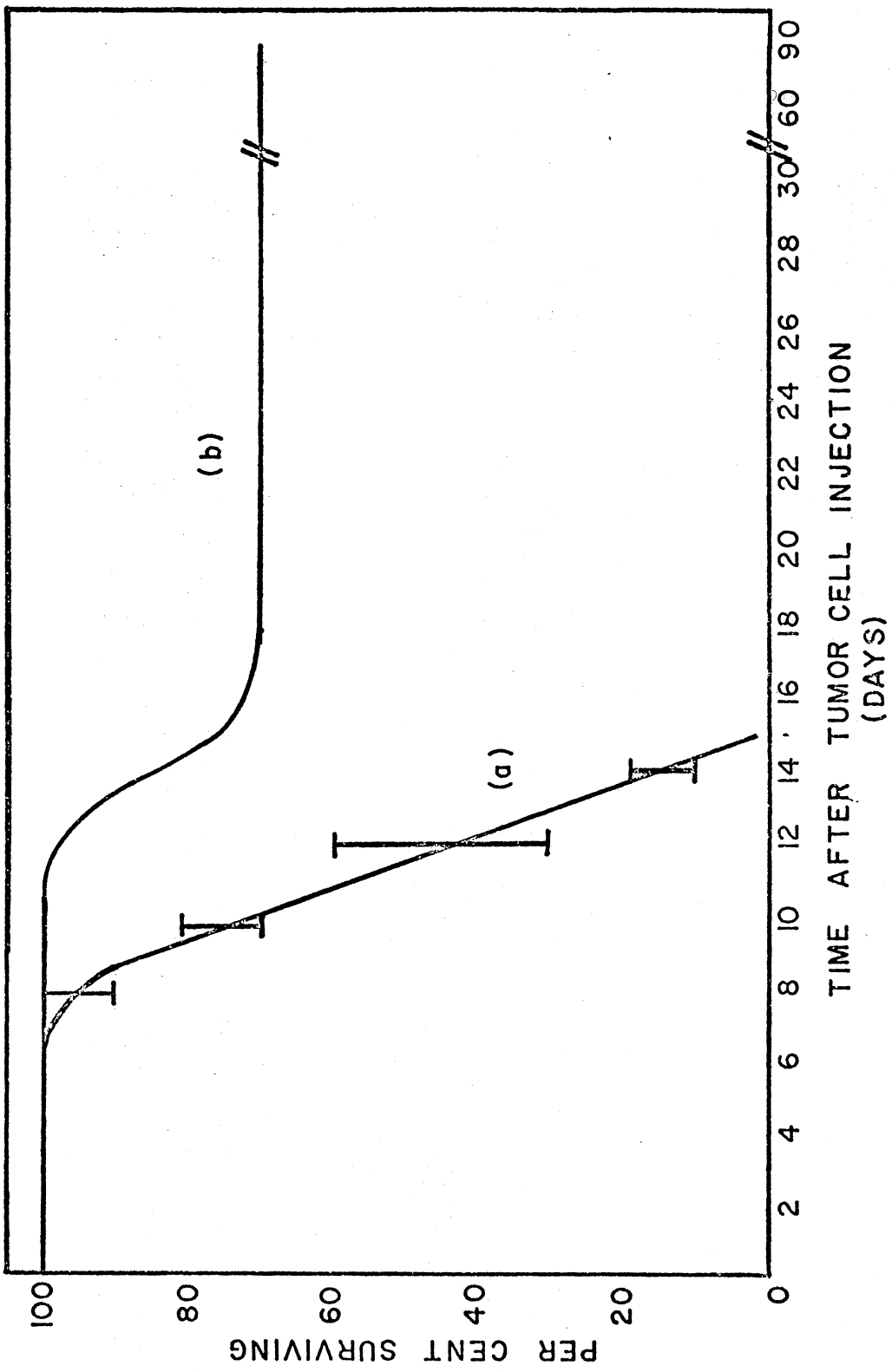
Group (a) received Hepes, human erythrocyte membranes, (3.0 mg protein total, i.p. 3 injections/week for 2 weeks). Group (b) received SA-180 membranes (3.0 mg protein total i.p. 3 injections/week for 2 weeks). One week following the final injection all animals were challenged (i.p.) with 1 x 10^4 viable SA-180 cells.


PER CENT SURVIVING
100
80
60
40
20
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
60
90
TIME AFTER TUMOR CELL INJECTION
(DAYS)
(a)
(b)

receiving Hepes, human erythrocyte membranes, types A, B or O, Ham/ICR mouse erythrocyte membranes or washed suspensions of *in vitro* grown fetal mouse cells (2 injections/week for 3 weeks). The control membranes were administered at 0.4 mg membrane protein on the first injection and 0.8 mg protein in each of the remaining 5 injections (4.4 mg total). The LM cells were administered at a concentration of 10^4 cells per injection for each of the 6 injections. This provides a total of 25 control animals receiving various types of membrane proteins as well as whole fetal mouse cells administered at one equivalent challenge dose per injection. One week following the final injection all animals were challenged with 10^4 viable SA-180 tumor cells and the host survival times recorded. Fig. 26, shows the per cent of animals surviving after tumor injection. As with the previous experiment, the control animals receiving saline, type A, B, or O human erythrocyte membranes, Ham/ICR mouse erythrocyte membranes or LM cells had a mean survival time of about 11.5 days. Those animals which died after receiving the SA-180 plasma membranes had a mean survival time of about 13.5 days. However, 70% or 7 out of the 10 tumor membrane immunized animals survived the initial challenge. Thirty days following the initial challenge these animals were again challenged with 10^4 viable SA-180 cells and all 7 lived an additional 90 days. At 90 days, the animals were sacrificed and necropsy revealed no evidence of tumor formation or gross abnormality of the major internal organs. These data indicate that the isolated SA-180 plasma membrane does retain the tumor associated antigens which are capable of eliciting an immune response in the host which was sufficient to neutralize most if not all viable S-180 cells following challenge.

Figure 26. Per Cent of Animals Surviving at Various Times Following Injection of 10^4 SA-180 Cells

Group (a) received Hepes, type A, B, or O human erythrocyte membranes, Ham/ICR erythrocyte membranes (4.4 mg protein total, i.p. 2 injections/week for 3 weeks) or *in vitro* fetal mouse cells (10^4 cells/injection, i.p. 2 injections/week for 3 weeks). Group (b) received SA-180 membranes (4.4 mg protein total, i.p. 2 injections/week for 3 weeks). One week following the final injection all animals were challenged (i.p.) with 1 x 10^4 viable SA-180 cells.


PER CENT SURVIVING
100
80
60
40
20
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
60
90
TIME AFTER TUMOR CELL INJECTION
(DAYS)
(a)
(b)

Studies were conducted to determine the effectiveness of immunization against challenges of different numbers of tumor cells. Control animals (40) were injected with Hepes (2 injections/week for 3 weeks) and 80 animals were injected with SA-180 membranes (2 injections/week for 3 weeks) with 4.4 mg total membrane protein as described above. Three days following the final membrane injection all animals including an additional control of 40 non-injected mice were divided into groups (10 animals/control group and 20 animals/membrane immunized group) and challenged with 10^2, 10^3, 10^4, or 10^5 viable SA-180 cells. Table VI shows the per cent survivors (surviving 30 days with no sign of tumor) and the mean survival time (MST) for each group. The results indicate that under these conditions the immune system is relatively successful in combating a tumor burden of 10^4 cells or smaller but unsuccessful when the tumor burden reaches 10^5 cells. These findings are in general agreement with experiments using other tumor host systems which show that the limits of tumor burden for successful immunotherapy are quite low (91,94). It should be noted that the interval from the last injection to challenge for Fig. 26, was 7 days compared to 3 days for Table VI which may explain the 20% difference in survivors at a 10^4 challenge.

TABLE VI

TITRATION OF MEMBRANE IMMUNIZATION BY CHALLENGING MICE WITH SA-180 AT CONCENTRATIONS OF 10^2 THROUGH 10^5

	Challenge Concentration							
	Number of Viable SA-180 Injected Per Mouse							
	10^2		10^3		10^4		10^5	
IMMUNIZING AGENT	%S[a]	MST[b]	%S[a]	MST[b]	%S[a]	MST[b]	%S[a]	MST[b]
SA-180 Membranes	80	400 ± 9	70	380 ± 15	50	310 ± 8	0	280 ± 8
Hepes Control	0	355 ± 13	0	323 ± 10	0	265 ± 23	0	240 ± 20
Uninjected Control	0	350 ± 13	0	310 ± 12	0	270 ± 17	0	250 ± 18

[a]%S--Per cent of animals surviving 30 days following SA-180 challenge. Control groups contained 10 animals per challenge concentration and membrane immunized groups contained 20 animals per challenge group. Animals were immunized with S-180 membrane (4.4 mg protein total, 2 injections/week for 3 weeks) or Hepes and challenged 3 days following the last injection. Uninjected controls received only the tumor challenge.

[b]MST--Mean survival time (hours) for those animals in each group which died $\pm$S.D.

CHAPTER IV

DISCUSSION

One of the more productive approaches to studying the structure of plasma membranes has been through studies of the chemical composition of isolated plasma membranes. A basic knowledge of the components and organization within the membrane is very important in any attempt at understanding the many functions which the plasma membranes performs. A prerequisite, however, to any such analysis is the isolation of plasma membranes which are free of other organelle contamination. A number of differing approaches have been used to obtain mammalian cell membranes. The tumor lines chosen for this study are all ascites lines, that is, they grow as single cells within the peritoneal cavity of the animal, although they are all capable of forming solid tumors, if injected subcutaneously. The single cells make the isolation easier since the problems created by cell-cell junctions are eliminated. The isolation procedures which were used yielded plasma membranes, in which 5'-nucleotidase (a plasma membrane marker) was purified 14-38 fold, depending on the cell type. The membranes were contaminated by low levels of other subcellular organelle marker enzymes; however, their levels are at least as low as preparations by most other investigators. The analysis of polypeptides present in the membranes of the three tumors did not show any major similarities, however, the real informative value of the membrane patterns comes from studying how they change as

cells or membranes are manipulated under various controlled conditions. One method for understanding the organization of the membrane is to study the various methods used to isolate plasma membranes. The primary difference between membrane stabilization method and the microsomal method for the Sarcoma-180 is the presence of a group of high molecular weight proteins in the stabilized membranes. Several methods have been studied to determine what function these proteins serve. Shin and Carraway (15), showed that the presence of divalent cation in the homogenization medium allowed for the isolation of recognizable membrane sheets instead of microsomes. The microsomal method seems to work in a similar manner in that no stabilization reagent was present and the membranes were in the form of vesicles. The marked similarity in all the other bands lends credence to the idea that the plasma membrane fraction is in fact composed primarily of the Sarcoma-180 plasma membrane, since the two methods are based on different isolation properties. Application of the membrane stabilization method to the Sarcoma-180 in vitro grown line yielded plasma membranes which differed by the presence of glycoproteins on the surface membranes of in vitro grown Sarcoma-180 tumor cells which are drastically reduced in the ascites form of the tumor. These well defined glycoprotein differences contrast sharply with the comparisons of membrane polypeptides, which are essentially the same for the two cell lines. A number of lines of evidence indicate that these are surface membrane associated glycoproteins. They are present predominantly on the surface membrane fraction, which is distinguished morphologically and by enzyme markers from other cell membrane fractions. They are not readily removed from the membrane by extraction with 1 M salt or dilute EDTA solutions, although other proteins can be

removed from these membranes by these methods. The proteins are cleaved with release of glycopeptides and removed from the gel patterns by treatment of the intact cells with trypsin, suggesting that they are on the surface of the cell. The glycoprotein could be iodinated by the lactoperoxidase surface labeling technique as first shown by Shin and Carraway (15) for the in vivo line.

The function of these glycoproteins is unknown. Their preferential association with in vitro grown cells suggests that they may be involved in the association of the cells with the substrate on which they grow. This is further supported by the observation that their appearance in the isolated plasma membrane, and presumably, also in the cell is a function of the growth state of the cell, since they seem to disappear from the cell after it reaches confluency. The fact that cells are released from the substrate by trypsinization which cleaves the glycoproteins lends some credence to this idea. Further studies on conditions which promote attachment or release of the cells are needed in order to evaluate the role of the glycoproteins in this phenomenen.

Mild trypsinization of Sarcoma-180 tumor cells causes the loss of two different types of components from membrane electrophoretic profiles. One of these is the glycoproteins of the surface membranes and the other is a group of high molecular weight polypeptides associated with surface membranes of cells treated with zinc and other membrane "stabilizing" agents. Proteolytic cleavage of glycoprotein at cell surfaces was expected, since this has been demonstrated for a number of mammalian cell types by either release of glycopeptides or loss of the glycoproteins from electrophoretic profiles. The loss of the high molecular weight polypeptides was not expected. There are three explanations

that we consider most likely to explain this result: 1) The polypeptides are present at the exterior surface of the cell and cleaved by trypsin acting on the cell surface. 2) The polypeptides are associated with the interior surface of the membrane and cleaved by trypsin which passes into the cell. 3) The polypeptides are associated with the interior surface of the membrane and are released from this association as a result of membrane structural alterations that occur during proteolysis of cell surface components. We consider the last of these explanations most likely, since our circumstantial evidence (lactoperoxidase labeling) indicates that these components are not present on the cell exterior and since specific cleavage of these components by trypsin inside the cell would indicate a substrate specificity for the trypsin which appears unlikely (although not impossible), and which it does not exhibit with the isolated membranes. The third hypothesis is also attractive because it relates elements which may be involved in maintaining membrane shape to events which occur at the cell surface.

Similar interactions have been proposed in the erythrocyte membrane between the surface glycoproteins and spectrin, which apparently plays a role in cell shape and membrane stabilization. There are noteworthy similarities between the properties of the polypeptides studied in this report and spectrin. Investigations of receptor mobility in membranes have suggested the interaction of surface receptors with submembrane cytoskeletal elements. It is not necessary that these interactions be direct as has been proposed with the erythrocyte. Instead they could involve cooperative effects such as have been observed for hormone-membrane interactions. Since the strength and stability of the interactions may vary with the cell system and membrane

isolation procedure, the loss of these polypeptides with trypsinization may not necessarily be observed with other cell types. Further characterization of the trypsinization process and of these polypeptides will be necessary to relate these observations to other cellular changes which occur with proteolysis.

The correlation between the disappearance of the high molecular weight proteins and the shape change of the Sarcoma-180 lends support to the possibility that the proteins were involved in maintaining the shape of the cell while it was attached to glass and while it was moving around the coverslip, as was seen when the cells were growing 12-24 hours after trypsinization was observed. Studies were, therefore, undertaken to gain an understanding of organization of these proteins with the plasma membrane. As shown earlier, these proteins are only found associated with the plasma membrane under certain conditions, which would yield whole envelopes or very large sheets of plasma membranes. The use of Zn^{++} allowed isolation of the proteins with the membrane in a form where a large percentage could be extracted by low ionic strength buffers under conditions which remove spectrin, a 240, 220 x 10^3 molecular weight doublet of the same apparent molecular weight as bands 3-4 from the Sarcoma-180. The extraction, however, also removed a large number of other proteins from the membrane at the same time. In one experiment it was possible to purify a small amount of bands 3 and 4 and subject it to amino acid analysis. The lack of hydroxyproline and hydroxylsine, which our amino acid system could detect shows that the band was not composed of collagen. When it was compared to spectrin, a marginal relatedness could be determined. Further attempts to isolate large enough quantities for structural studies have been plagued by diffi-

culties in removing the lower molecular weight contamination since under non-denaturing conditions these proteins have a very large molecular weight. Ammonium sulfate fractionation was successful in spearating a large amount of the lower molecular weight proteins from bands 1-4. Chromatography in SDS was also shown to separate the various proteins by molecular weight and will be helpful in obtaining proteins for chemical analysis of the type which was first performed on spectrin during the earlier stages of its study.

Several investigators have achieved some success in using whole cell preparations as anticancer immunizing agents (86,91,92,108). We, therefore, became interested in whether the outside of the cell, i.e. the plasma membrane, could be used in a similar manner. The use of whole cells, even though inactivated, may pose many problems when applied to treatment protocols. The use of isolated plasma membranes eliminates many of these problems.

Our findings established that isolated SA-180 plasma membranes can be effective immunizing agents against homologous tumor. The administration of non-tumor membranes or <u>in vitro</u> grown fetal mouse cells showed no stimulation of the host's immune system in combating tumor challenge. Tunor cell membranes did enhance the survival time of the host and in many cases caused complete elimination of the challenge dose. This indicates that the reaction of the host to the tumor material results in a specific immune stimulation and not a non-specific response attributed to agents like BCG.

The immunization of mice with SA-180 membranes was relatively successful with 50 to 70% of the animals surviving a 10^4 challenge. Use of combination immunotherapy, i.e. modified and unmodified membranes or

groups of membranes containing different modifications, may facilitate the rejection of even greater numbers of tumor cells. The use of membranes as immunizing agents is highly desirable and represents a logical extension of current research efforts for the following reasons: a) The cell surface is that portion of the tumor cell which interacts with the host's immune system. b) Only homologous material contains the specificity required for effective tumor rejection with the human situation probably requiring autochthonous material. c) Since the host's immune system has a finite response capability which has already been diminished by tumor burden, it should be presented with the maximum quantity of surface immunogen (cell membrane) while minimizing the addition of non-surface antigen (DNA, RNA, enzymes, mitochondria, etc.). d) Administration of membranes minimizes the risk of infecting the host with viruses or other foreign agents which reside in cells both <u>in vivo</u> and <u>in vitro</u> and often remain active after the cells become non-viable. The findings described above indicate that the isolated plasma membrane may represent an important initial step toward the development of a more effective tumor specific immunotherapy agent.

CHAPTER V

SUMMARY

Plasma membrane-enriched fractions were isolated from four cell types by homogenization in zinc solutions, followed by sucrose gradient centrifugation, or two-phase partition, and from the Sarcoma-180 ascites cell also by microsomal homogenization and separation on sucrose gradients. The Sarcoma-180 tumor cells grown in vitro in cell culture or in vivo as an ascites form in mice were isolated by the Zn^{++} homogenization method. 5'-Nucleotidase was assayed as a plasma membrane marker and was found to be 18-23 fold increased compared to homogenates. Membranes from both cell lines appeared as ruptured envelopes or large fragments. Membranes were also isolated from the ascites form by a microsomal homogenization followed by sucrose density gradient centrifugation. The plasma membrane enriched fraction showed a 19 fold increase in 5'-nucleotidase with some glucose 6-phosphatase contamination, an endoplasmic reticulum marker.

Application of the Zn^{++} homogenization prodecure to the mouse L-1210 acute lymphoblastic leukemia yielded a plasma membrane fraction with a 34 fold increase in 5'-nucleotidase. Application to the 13762 MAT, derived from the 13762 rat mammary adenocarcinoma yielded a fraction with a 13.8 fold increase in 5'-nucleotidase. The protein and glycoprotein components of the plasma membranes are shown by coomassie blue and periodate-Schiff (PAS) staining of dodecyl sulfate acrylamide

gels of isolated plasma membrane fractions from the cell lines. Patterns for the 13762 MAT and L-1210 show 19 major proteins and 5 (13762 MAT) and 4 (L-1210) glycoproteins for the cell plasma membranes, ranging from 500,000^{+} to 25,000 molecular weight. The Sarcoma-180 lines show 15 major proteins and 3 glycoproteins. There are significant differences between membranes isolated by the stabilization and microsomal methods in only three protein bands. The 500,000^{+}, 280,000 and 237,000 molecular weight bands are missing in the microsomal isolation technique which yields very small vesicles.

A comparison of the plasma membranes from the two Sarcoma lines reveals no major differences in membrane proteins, but the cells grown in culture show the presence of a set of surface membrane glycoproteins which are absent or markedly decreased in the ascites form. Conversely, the ascites form has a glycoprotein which is not evident in the cells in culture. These glycoproteins can be demonstrated both by PAS staining and lactoperoxidase labeling. The glycoprotein differences may be related to the ability of the cells to interact with each other or a substratum.

The effect of mild proteolysis in the Sarcoma-180 cells was investigated using low concentrations of trypsin (5mg/ml). Morphological changes occuring in the cultured form attached to glass show a change from a fibroblastic shape to a rounded form over a 30 minute period during and following a 15 minute exposure to trypsin. To study the molecular basis for this change, plasma membranes were isolated from control and 15 minute trypsinized cells and subjected to electrophoresis. As expected, the surface glycoproteins were removed from the profiles. However, a set of high molecular weight proteins were also lost from

the profile. The same results were obtained from both cell lines. These proteins appear to be important in maintaining cell shape and may represent a cell cytoskeleton. Because the high molecular weight proteins affected by trypsin were similar to spectrin, a protein doublet of the red blood cell membrane believed to be important in maintaining shape in that cell, comparisons were made between the cells. The Sarcoma-180 ascites tumor plasma membrane high molecular weight doublet protein can be extracted by low ionic strength and partially purified by column chromatography in dodecyl sulfate, or partially purified in its native form on a G-200 column. The protein appears to be similar to spectrin in its behavior.

Isolated Sarcoma-180 ascites plasma membranes have been investigated as potential anticancer agents. Mice were immunized with Sarcoma-180 plasma membranes, and then challenged with 10^2-10^5 viable tumor cells. Up to 70% of the mice were able to combat 10^4 cells and survive 30 days, at which time they were rechallenged with 1×10^4 cells and all survived an additional 60 days. Control animals immunized with saline, mouse or human red blood cell membranes, or fetal mouse cells were afforded no protection. This method may represent a starting point for a possible treatment method.

A SELECTED BIBLIOGRAPHY

(1) Burger, M. M. (1973) Fed. Proc. 32, 91.

(2) Loewenstein, W. R. (1973) Fed. Proc. 32, 60.

(3) Carraway, K. L., Kobylka, D. and Triplett, R. B. (1971) Biochim. Biophys. Acta 241, 934.

(4) Bender, W. W., Garan, H. and Berg, H. C. (1971) J. Mol. Biol. 58, 783.

(5) Bretscher, M. S. (1971) J. Mol. Biol. 58, 775.

(6) Phillips, D. R. and Morrison, M. (1971) FEBS Letters 18, 95.

(7) Triplett, R. B. and Carraway, K. L. (1972) Biochemistry 11, 2897.

(8) Steck, T. L. and Wallach, D. F. H. (1970) Methods in Cancer Res. 5, 93.

(9) Wallach, D. F. H., and Kamat, V. B. (1966) Methods in Enzym. 8, 164.

(10) Warren, L., Glick, M. C., and Nass, M. K. (1966) J. Cell. Physiol. 68, 269.

(11) McCollester, D. L. (1970) Cancer Res. 30, 2832.

(12) Roizman, B., and Spear, P. G. (1971) Science 171, 298.

(13) Jamieson, G. A., and Groh, N. (1971) Anal. Biochem. 43, 259.

(14) Truffa-Bachi, P., and Wofsy, L. (1970) Proc. Natl. Acad. Sci. 66, 685.

(15) Shin, B. C., and Carraway, K. L. (1973) Biochem, Biophys. Acta 330.

(16) Korn, E. D. (1969) Fed. Proc. 28, 6.

(17) Wallach, D. F. H. (1967) in "The Specificity of Cell Surfaces", D. B. Davis and L. Warren, Eds., Prentice-Hall Englewood Cliffs, New Jersey.

(18) Emmelot, P., Bos, C. J., Benedetti, E. L., and Rumke, P. (1964) Biochem. Biophys. Acta 90, 126.

(19) Hagopian, A., Bosmann, H. B., and Eylar, E. H. (1968) Arch Biochem. Biophys. 128, 387.

(20) Graham, J. M., Higgins, J. A., and Green, C. (1968) Biochim. Biophys. Acta 150, 303.

(21) Boone, C. W., Ford, L. E., Bond, H. E., Stuart, D. C., and Lorenz, D. (1969) J. Cell. Biol. 41, 378.

(22) Maddy, A. H. (1964) Biochim Biophys. Acta 88, 448.

(23) Berg, H. C. (1969) Biochim. Biophys. Acta 183, 65.

(24) Phillips, D. R., and Morrison, M. (1970) Biochem. Biophys. Res. Commun. 40, 284.

(25) Marinetti, G. V., and Gray, G. M. (1967) Biochim. Biophys. Acta 135, 580.

(26) Singer, S. J., and Nicolson, G. L. (1974) Science 175, 720.

(27) Taylor, R. B., Duffus, W. P. H., Raff, M. C., and de Petris, S. (1971) Nature New Biol. 233, 225.

(28) Frye, C. D., and Edidin, M. (1970) J. Cell. Sci. 8, 313.

(29) Rosenblith, J. Z., Ukena, T. E. H. H., Berlin, R. D., and Karnovsky, M. J. (1973) Proc. Nat. Acad. Sci., U.S.A. 70, 1625.

(30) Nicolson, G. L., and Painter, R. G. (1973) J. Cell. Biol. 59, 395.

(31) Singer, S. J. (1974) Adv. in Immunol. 19, 1.

(32) Cuatrecasas, P. (1969) Proc. Natl. Acad. Sci., U.S.A. 63, 450.

(33) Abercrombie, M., and Ambrose, E. J. (1962) Cancer Res. 22, 525.

(34) Revel, J. P. and Ito, S. (1967) in "The Specificity of Cell Surfaces", B. D. Davis and J. Warren, eds., Prentice-Hall, Englewood Cliffs, New Jersey, p. 211.

(35) Winzler, R. J., Harris, E. D., Pekas, D. J., Johnson, C. A., and Weber, P. (1967) Biochemistry 6, 2195.

(36) Langley, O. K., and Ambrose, E. J. (1967) Biochem J. 102, 367.

(37) Pricer, W. E., Jr. and Ashwell, G. (1971) J. Biol. Chem. 246, 4825.

(38) Cuatrecasas, P., and Illiano, G. (1971) J. Biol. Chem. 246, 4938.

(39) Lilien, J. E., Chiller, J. M., and Weigle, W. O. (1973) J. Exp. Med., 138, 148.

(40) Hakamori, S. I., and Murakami, W. T. (1968) Proc. Natl. Acad. Sci. U.S.A. 60, 459.

(41) Bosmann, H. B. (1969) Expl. Cell. Res. 54, 217.

(42) Grimes, W. J. (1970) Biochemistry 9, 5083.

(43) Burger, M. M. (1969) Proc. Natl. Acad. Sci., U.S.A. 62, 994.

(44) Inbar, M., Ben-Bassat, H., and Sachs, L. (1971) J. Membranes Biol. 6, 195.

(45) Burger, M. M., and Nooman, K. D. (1970) Nature 228, 512.

(46) Nicolson, G. L. (1971) Nature 233, 244.

(47) Warren, L., Fuber, J. P., and Buck, C. A. (1972) Proc. Nat. Acad. Sci., U.S.A. 69, 1838.

(48) Kobylka, D., Khettry, A., Shin, B. C., and Carraway, K. L. (1972) Arch. Biochem. Biophys. 148, 475.

(49) Marton, L. S. G., and Garvin, J. E. (1973) Biochem. Biophys. Res. Commun. 52, 1457.

(50) Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971) Biochemistry 10, 2606.

(51) Rosenberg, S. A., and Guidotti, C. (1969) J. Biol. Chem. 244, 5118.

(52) Gwynne, J. T., and Tanford, C. (1970) J. Biol. Chem. 245, 3259.

(53) Marchesi, V. T., and Steers, E., Jr. (1968) Science 159, 203.

(54) Greenberg, C. S., and Glick, M. C. (1972) Biochemistry 11, 3680.

(55) Naville, D. M., Jr., and Clossmann, H. (1971) J. Biol. Chem. 246, 6335.

(56) Bailey, A. J., Peach, C. M., and Fowler, L. J. (1970) Biochem. J. 117, 815.

(57) Lorand, L., Chenoweth, D., and Domanik, R. A. (1969) Biochem. Biophy. Res. Commun. 37, 219.

(58) Birckbichler, P. J., Dowben, R. M., Matacic, S., and Loewy, A. G. (1973) Biochim. Biophys. Acta 291, 149.

(59) Dunn, M. J., and Maddy, A. H. (1973) FEBS Letters 36, 79.

(60) Clarke, M. (1971) Biochem. Biophys. Res. Commun. 45, 1063.

(61) Tillack, T. W., Marchesi, S. L., Marchesi, V. T., and Steers, E., Jr. (1970) Biochim. Biophys. Acta 200, 125.

(62) Trayer, H. R., Nezaki, Y., Reynolds, J. A., and Tanford, C. (1917) J. Biol. Chem. 246, 4485.

(63) Hulla, F. W., and Gratzer, W. B. (1972) FEBS Letters 25, 275.

(64) Guidotti, G. (1972) Arch. Interm. Med. 129, 194.

(65) Rosenthal, A. S., Kregenow, F. M. and Moses, H. L. (1970) Biochim. Biophys. Acta 196, 254.

(66) Guthrow, C. E., Jr., Allen, J. E., and Rasmussen, H. (1972) J. Biol. Chem. 247, 8145.

(67) Juliano, R. L., Kinelberg, H. K., and Papahadjopoulos, D. (1971) Biochim. Biophys. Acta 241, 894.

(68) Hughes, R. C. (1973) Prog. in Biophys. and Molec. Biol. 26, 191.

(69) Hynes, R. O. (1974) Cell 1, 147.

(70) Burger, M. M. (1973) Fed. Proc. 32, 91.

(71) Buck, C. A., Glick, M. C., and Warren, L. (1970) Biochemistry 9, 4567.

(72) Vaheri, A., and Ruoslahti, E. (1974) Int. J. Cancer 13, 579.

(73) Hogg, N. M. (1974) Proc. Nat. Acad. Sci., U.S.A. 71, 489.

(74) Hynes, R. O. (1973) Proc. Nat. Acad. Sci., U.S.A. 70, 3170.

(75) Gahmberg, J., and Hakomori, S.--I. (1973) Proc. Nat. Acad. Sci. U.S.A. 70, 3327.

(76) Shin, B. C., and Carraway, K. L. (1974) Biochim. Biophys. Acta 345, 141.

(77) Hellstrom, K. E., and Moller, G. (1965) Progr. Allergy 9, 158.

(78) Klein, G. (1971) Advance Immunol. 14, 187.

(79) Alexander, P. (1968) Progs. Exp. Tumor Res. 10, 23.

(80) Hellstrom, K. E., and Hellstrom, I. (1971) in "Advances in immunology", F. J. Dixon and H. G. Kunkel, eds., Academic Press, New York, p. 209.

(81) Coggin, J. H., Jr., and Anderson, N. G. (1974) in "Advances in Cancer Res.", G. Klein and S. Weinhouse, eds., Academic Press, New York, p. 105.

(82) Klein, G., Sjogren, J. O., Klein E. and Hellstrom, K. E. (1960) Cancer Res. 20, 1561.

(83) Smith, R. T., and Landy, M. (1971) "Immuno Surveillance", Academic Press, New York.

(84) Cerottine, J., and Brunner, K. T. (1974) in "Advances in Immunology", F. J. Dixon and H. G. Kumkel, eds., Academic Press, New York, p. 67.

(85) Herberman, R. B. (1974) in "Advances in Cancer Res.", G. Klein and S. Weinhouse, eds., Academic Press, New York, p. 207.

(86) Simmons, R. L., Rios, A. (1972) Ann. Surg. 176, 188.

(87) Newcomb, E. W., Weksler, M. E. (1974) Cancer Res. 34, 100.

(88) Faraci, R. P. and Schour, L. (1974) Science 185, 68.

(89) Atkinson, P., and Davies, I. (1974) Experientia 30, 427.

(90) Bekesi, J. G., Arneault, G., Walter, L., and Holland, J. G. (1972) J. Nat. Can. Inst. 49, 107.

(91) Kollmorgen, G. M., Erwin, D. N., Killion, J. J., and Sansing, W. A. (1973) Ann. Oklahoma Acad. Sci. pp. 25.

(92) Prager, M. D., Derr, I., Swann, A., and Cotropia, J. (1971) Cancer Res. 31, 1488.

(93) Hakala, T. R., and Lange, P. H. (1974) Science 184, 795.

(94) Hersh, E. M., Gutterman, T. U., and Maulight, G. (1973) "Immunology of Cancer in Man", C. C. Thomas ed., Springfield, Illinois.

(95) Mathe, C., and Weiner, R. (1974) "Recent Results in Cancer Research", Springer-Verlag, New York.

(96) Abramoff, P. and La Via, M. F. (1970) "Biology of the Immune Response", McGraw-Hill, New York, p. 272.

(97) Shipman, C. (1969) P.S.E.B.M. 130, 305.

(98) Iwakata, S. and Grace, J. T., Jr. (1964) N. Y. Jour. of Med. 64, 2279.

(99) Morgan, J. F., Martin, H. J., and Parker, R. C. (1950) Proc. Soc. Exp. Biol. and Med. 73, 1.

(100) Brunette, D. M. and Till, J. E. (1971) J. Membrane Biol. 5, 215.

(101) Spackman, D. H., Stein, W. H., and Moore, S. (1958) Anal. Chem. 30, 119.

(102) Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265.

(103) Lazarus, L. H. and Chou, S.--C. (1972) Anal. Biochem. 45, 557.

(104) Morre', D. J. (1971) Methods in Enzymol. 22, 130.

(105) Goldfischer, S., Essener, E., and Nivikoff, A. B. (1964) J. Histochem. Cytochem. 12, 72.

(106) Bray, G. A. (1960) Anal. Biochem. 1, 279.

(107) Marchalonis, J. J., and Weltman, J. K. (1971) Comp. Biochem. Physiol. 38B, 609.

(108) Bekesi, J. G., St-Arneault, G., and Holland, J. F. (1971) Cancer Res. 31, 2130.

VITA 2

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